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Characterization of novel mitochondrial modulators for the development of neuroprotective strategies

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PREFACE

This present thesis was a part of an international research program, INTERREG IV Upper Rhine. This PhD work was performed within the framework of the Offensive Sciences on neuroprotection and neurogenesis led by the consortium Neuro-Rhine, which includes laboratories from three neighbouring countries (France, Switzerland and Germany) of the Upper Rhine Valley. This trinational research program was funded by the European Union (*Fonds Européen de Développement Régional*), Offensive Sciences, Région Alsace and INTERREG IV Rhin Supérieur.

The following dissertation was written by the author. The INTRODUCTION is based on the literature.

The RESULTS section of this dissertation consists of one revised manuscript, additional experiments, and two other manuscipts that will shortly be submitted for publication. Please refer to the author contributions section of the manuscripts, where the contribution of each co-author to this work is listed.

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toujours donné de la force pour persévérer dans la vie.
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Imane

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SUMMARY

Mitochondria, known as the powerhouses of the cell, play a critical role in the neuronal survival and cell death, because they regulate both energy metabolism and apoptotic pathways. Mitochondria provide the majority of cellular energy in the form of adenosine triphosphate (ATP) and regulate the production of reactive oxygen species (ROS). Evidence shows that mitochondrial abnormalities are involved in the pathophysiology of Alzheimer's disease (AD). An increase in oxidative damage and decreased energy metabolism were observed at early stages of the disease before the generation of amyloid plaques and neurofibrillary tangles, representing the two main histopathological characteristics of AD. Therefore, current pharmacological concepts for the development of therapeutic strategies against AD have a particular interest in the track of the regulation of mitochondrial functions in neurons, including the control of ATP synthesis, mitochondrial respiration, and ROS production. The decreased neurosteroidogenesis and low brain concentrations of allopregnanolone have also been correlated with a cognitive decline seen in AD patients. These data have generated a strong interest in the development of therapeutic strategies based on the use of allopregnanolone. However, the pleiotropic nature of the effects of allopregnanolone does not facilitate the development of targeted therapy, particular focused on one specific indication, such as neuroprotection, while avoiding stimulation of cell proliferation that possibly could facilitate the emergence of glioma. Though, in cases of functional deficits related to a decline in neurogenesis, it might be interesting to produce chemically modified analogs that have a greater and more specific neurogenic action than that of allopregnanolone.

Regarding the data mentioned above, the main purpose of this thesis was to evaluate the ability of two families of new patented compounds that exert neuroprotective and / or neurogenic effects via the modulation of mitochondrial functions in nerve cells. These two families of compounds are the allopregnanolone analogs (ANS) and the ligands of the mitochondrial translocation of protein or translocator protein (LTSPO) which transfers cholesterol from the outer membrane to the inner mitochondrial membrane, where it is

converted to pregnenolone by the cytochrome P450side-chain-cleavage, a major precursor in the biosynthesis of various neurosteroids. LTSPO are important modulators of neurosteroidogenesis.

To achieve our goal, the thesis was divided into two main parts:

1) The first part is divided into three subprojects (A), (B) and (C).Taking into account bibliographic data suggesting that allopregnanolone has a great neuroprotective potential, we decided to evaluate the ability of allopregnanolone and four of its analogs to improve mitochondrial functions in an *in vitro* cellular model of AD mimicking the accumulation of β-amyloid peptide (Aß) in human SH-SY5Y neuroblastoma, overexpressing the amyloid precursor protein or APP (APP cells) (A) and *in vivo* in a transgenic mouse model (B) as well as in an mouse model of aging (C).

2) In the second part, we compared the effects of novel TSPO ligands to reference ligands with regard to improve the energy balance in APP and in control cells.

(A) Previous work from our team showed a decrease in ATP production in APP cells, which are more sensitive to oxidative stress than the control cells. In the first part of this thesis, the ability of allopregnanolone and its analogs (ANS) to modulate the bioenergetics in APP and control cells was investigated. Our results showed that at the concentration of 500 nM, allopregnanolone (AP) and its analog O-allyl-epiAP (BR297) stimulated the ATP production in APP and control cells. In contrast, the analogs O-allyl-AP (BR351), 12-oxo-epiAP (BR053) and 12-oxo-AP (BR338) did not alter the ATP synthesis. The powerful stimulatory effect on the ATP production exerted by BR297 has encouraged us to compare its capacity to protect against cell death, induced by oxidative stress generated by hydrogen peroxide (H_2O_2) with that of allopregnanolone. Both compounds, allopregnanolone and BR297 were able to effectively protect APP cells against death induced by H_2O_2, while in control cells only BR297 treatment was efficient. Our results also showed that BR297 and allopregnanolone, which significantly decrease the concentrations of cytosolic and mitochondrial ROS, strongly reduced the Aβ-related elevated anion superoxide levels in APP

cells. Furthermore, our work shows that allopregnanolone and BR297 improved both oxygen consumption (OCR) and glycolysis (ECAR), increasing the bioenergetic activity in APP and control cells. In particular, our work showed that BR297 modulated the respiratory control ratio (RCR), an indicator of the respiratory capacity of mitochondria in both cell types, but allopregnanolone had no significant effect on APP cells.

Our results identify BR297 as an analog which is capable of inducing beneficial effects on mitochondrial bioenergetics without stimulating cell proliferation, as does allopregnanolone. These data give BR297 an interesting profile to develop neuroprotective strategies without the induction of proliferative effects.

(B) Notably, additional *in vivo* experiments confirmed the beneficial effects of BR297 in an AD transgenic mouse model (Tg2576) by ameliorating the cellular energy level as well as the mitochondrial complexes activities in the cortex and the hippocampus of Tg2576 up to the mitochondrial activity level of the wild-type (WT) group at a dose of 8 mg/kg of BR297. In line with our *in vitro* and *in vivo* findings, collaborators showed that BR297 had no effect on neurogenesis but that this analogue induced a positive trend to ameliorate the behavioral deficits in Tg2576 mice.

(C) Given that age is a risk factor for dementia, the protective effect of two analogs, O-allyl-epiallopregnanolone (BR297) and O-allyl-allopregnanolone (BR351), was investigated on age-related disturbances of energy maintenance in the brain. 7-month and 21-month old mice were subjected to a treatment with one of the analogs or the vehicle (control group). The treatment regimen involved three injections per week during four weeks at the doses of 2, 4, 8 mg/kg for BR297 and 1, 2, 4 mg/kg for BR351. Following the behavioural test of pattern separation, brains were collected for evaluation of the mitochondrial activity. Analysis of ATP levels as well as mitochondrial activity of complex I and complex IV were used to evaluate the effect on age-induced mitochondrial impairments in the cortex and hippocampus. Aged mice exhibited significant deficits in cellular ATP levels in the cortex and hippocampus and in mitochondrial ATP levels, especially in the hippocampus. Brains from

21-month old mice only showed a trend to reduced complex activities. Notably, BR297 ameliorated cellular and mitochondrial ATP levels and complex I and IV activity in both brain regions. The beneficial effect of the BR351 treatment was more specific to the cortex by enhancing cellular and mitochondrial ATP levels. Concerning complex activities, BR351 increased only the activity of complex I but not that of complex IV. In total, BR297 exhibited a higher efficacy in the hippocampus with respect to the alleviation of age-related mitochondrial deficiency compared to BR351. These findings suggest that allopregnanolone analogs are promising compounds for the treatment of age-related neurodegenerative diseases.

The aim of the second part was to study the effects of new LTSPO analogs on mitochondrial functions. Few studies have suggested that LTSPO could exert neuroprotective action by stimulating the biosynthesis of neurosteroids as neuroprotective AP. However, most of the currently available LTSPO exhibit anxiolytic effects, low dissolution under physiological conditions and a lack of selectivity or specificity. Moreover, the involvement of LTSPO in neuroprotection processes, particularly their effects on mitochondrial bioenergetics in nerve cells, was not studied until now. Pre-tests by members of our group have shown that different new ligands induced the production of pregnenolone in glial cells. We therefore determined the effects of eleven new compounds and compared those to that of four LTSPO reference molecules, namely diazepam, XBD-173, SSR-180,575, and Ro5-4864 in APP cells. Out of eleven new TSPO ligands, two were very promising: 6a and 6b.The TSPO ligand 6b has a structure similar to 6a, but differs by a lateral chain dipropylamide instead of diethylamide. This structural difference gives the compound 6b a higher affinity for TSPO. Our results have demonstrated a non-toxic effect of the new analogs at 10 nM on cell survival. The higher affinity of 6b for TSPO is correlated to a greater increase in ATP levels than after a treatment with 6a in control and APP cells. In control cells, ATP levels were increased by both compounds but with a higher effect after 6b treatment. In APP cells, both compounds ameliorated the production of ATP to a similar extent. Generally, the new TSPO ligands 6a and 6b are more efficient in compensating the energy loss caused by an overexpression of Aβ peptide than the LTSPO described in the literature. More precisely, the lateral chain

dipropylamide structure of the best ligand 6b allowed the highest amelioration of the energy production as well as an increase of affinity and selectivity for the TSPO receptor

Taking together, all the results showed that the imidazoquinazolinone structure seems to be importantl to alleviate the bioenergetic deficit observed in AD pathology.

In conclusion, this PhD work has identified new molecules that regulate mitochondrial functions and are promising for the development of optimized and better targeted neuroprotective strategies. The new analog of allopregnanolone, O-allyl-epiAP or BR297, which is able to exercise effective neuroprotective effects involving a strong upregulation of mitochondrial activity devoid of cell proliferation, seems to be a promising compound. Another new analog of allopregnanolone, BR351 or O-allyl-AP, has been identified to be also effective in neuroprotection, but its effect mainly results from neurogenic and / or proliferative action that does not seem to depend on a strong mobilization of mitochondrial functions. First *in vitro* results already identified 6a and 6b as effective in ameliorating the cellular energy homeostasis in APP cells. Ultimately, the results of this thesis will provide valuable preclinical data for the development of new therapeutic strategies against AD or neurodegenerative diseases.

I. Introduction

A. Neurodegenerative diseases

Neurodegenerative diseases are amongst the major causes of disability, decreased quality of life and death worldwide [1]. They are defined as incurable, sporadic and hereditary conditions which are characterized by gradual nervous system dysfunction. Aging, a challenge to every living organism, is linked to accumulation of metabolism impairments and cell damage, causing disease development [2]. Neurodegenerative diseases often correlate with atrophy of the central or peripheral nervous system, where the nerve cells progressively degenerate or die. These diseases included disorders as Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, Motor Neurone disease, Prion diseases, Amyotrophic Lateral Sclerosis, Multiple Sclerosis and many other dementias. They all lead to progressive brain damage and neurodegeneration [3]. Parkinson's disease and AD exhibit different clinical features but appear to be similar at the cellular level in the disease process. For example, Parkinson's disease depletes the basal ganglia of the brain of dopamine, which leads to bradykinesia, stiffness, rigidity, and tremors in the major muscles of the body [4]. In Alzheimer's disease, deposits of protein plaques and hyperphosphorylation of protein damage different parts of the brain and lead to progressive loss of memory.

Medication can only help to ameliorate the patients' quality of life and reduce symptoms. For example, Levodopa can increase the brain's dopamine levels to help alleviate symptoms of Parkinson's disease with still many side effects and memantine or donepezil can sometimes slow the progression of dementia symptoms in some people with Alzheimer's disease. The incidence of these age-related disorders is on rising and they are the attract of great deal of attention due to their irreversibility, accompanied with economic and social burden and the lack of effective treatment [2].

B. Alzheimer's disease

1. Clinical symptoms, risks and etiological factors

The first form of dementia was described by the German neuropathologist and psychiatrist, Alois Alzheimer in 1906 and later became known as Alzheimer's disease (AD) at the suggestion of Emil Kraepelin [5]. Alzheimer examined a woman from Frankfurt, Auguste Deter, who had shown a striking cluster of symptoms that included psychosocial incompetence, hallucinations, delusions, progressive cognitive deficit and focal symptoms. At necropsy, Alzheimer reported the histopathological finding of this disease: tangles of fibrils within the cytoplasm of neurons, widespread outside plaques pathology [6].

According to Alzheimer's Association in the report 2015 *Alzheimer's Disease Facts and Figures,* AD is the most common cause of dementia. It account for 60 percent to 80 percent of dementia cases and is the first leading cause of death. By 2050, there will be over 115 million people diagnosed with AD worldwide [7]. The triad of risk factors for AD are age, the apolipoprotein E ε4 (ApoE) and chromosomal sex [8]. The most important risk factor for Alzheimer's disease is age above 65 years (National Institute of Aging 2015). People younger than 65 can also develop the disease but it is rare. The female prevalence of AD is correlated to a greater life span relative to men [9]. ApoE is a lipid binding protein that transports and coordinates the mobilization of cholesterol between and within the cells. ApoE is expressed mainly in the liver and brain [10]. Among AD patients, 60% carry at least one ε4 allele [11]. In AD, there is still a variability that is not explained by sex difference or ApoE genotype suggesting that the biologic system of aging is more likely the driving factor [8].

AD progresses generally slowly in three different stages: preclinical (early stage), mild cognitive impairment (middle-stage) and dementia (severe stage) [12]. In the preclinical stage there are no reliable and valid signs and symptoms for an early diagnosis. In the mild cognitive impairment, deficits in declarative memory are usually important and depressive symptoms are uncommon, but patients manage to live alone. In the severe stage, patients are completely dependent, develop neurological disturbances and need supervision, if

perception and behaviour are affected [13]. In most people with Alzheimer's disease which is caused in different ways, the experienced symptoms and the progress of the disease's stages will be different.

2. Neuropathological hallmarks of Alzheimer's disease:

By creating a continuum between the normal aging and AD, clinicopathological correlation studies generate hypotheses on the pathophysiology of AD that amyloid plaque development appears initially before the emergence of cognitive deficits, whereas neuronal loss, neurofibrillary tangles, and specifically synaptic loss appear at the same time as the evolution of cognitive decline. The neuropathological hallmarks of AD incorporate positive lesions like glial responses, neurofibrillary tangles, and amyloid plaques, and negative lesions such as synaptic and neuronal loss [14].

Neurofibrillary tangles formed by intraneuronal aggregation of hyperphosphorylated and misfolded tau protein, and amyloid plaques composed of the abnormal amyloid beta protein deposit as senile plaques, are considered to be the two major hallmarks of AD (**Fig. 1**) [15, 16].

A. **B.**

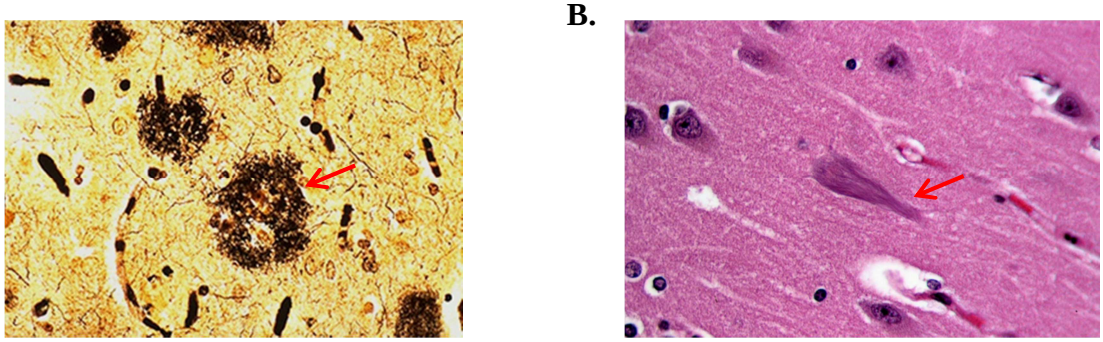

Figure 1: Amyloid plaques and neurofibrillary tangles in the cerebral cortex and hippocampus of an Alzheimer's disease patient: (A) The plaques of Alzheimer's disease are seen here with a bielchowski silver stain. (B) Neurofibrillary tangles of Alzheimer's disease appear as long pink filaments in the cytoplasm with a hematoxylin and eosin staining. Adapted from Neuro patho Flashcards by ProProfs and knowing neurones.

a) Neurofibrillary tangles (NFTs)

Neurofibrillary tangles, described as intraneuronal filamentous inclusions within pyramidal neurons, occur in AD and neurodegenerative tauopathies [17]. Recently, the presence of twisted ribbon-like assemblies of tau fibrils was revealed thanks to modern molecular microscopy techniques. The major component of NFTs is the microtubule-associated protein tau, which is abnormally misfolded and hyperphosphorylated (**Fig. 2**) [18]. Normally, Tau is a microtubule-associated protein (MAPs) present in a soluble form in axons and promotes microtubule assembly and stability. Tau is implicated in the axonal transport of vesicles by binding and stabilizing the microtubules [14]. In pathological condition, tau is translocated in a cell soma and is hyperphosphorylated ~3-fold more than the protein tau in the normal brain [19]. This protein becomes insoluble and misfolded, self-aggregates into paired helical filaments (PHFs), destabilizes the microtubules and gives rise to neurofibrillary tangles (**Fig. 2**). Intermediate aggregations of insoluble protein tau are cytotoxic. This form induces axonal transport impairments and cognitive deficit [20]. The density of neurofibrillary tangles in the neocortex correlates with dementia, cognitive impairments [19]. Contrary to dementia with Parkinsonism where more than 30 mutations of tau have been detected, in AD tau mutations do not occur. The reductions in score of cognitive evaluation are correlated with the increase of total and phosphorylated tau in the cerebrospinal fluid [21, 22].

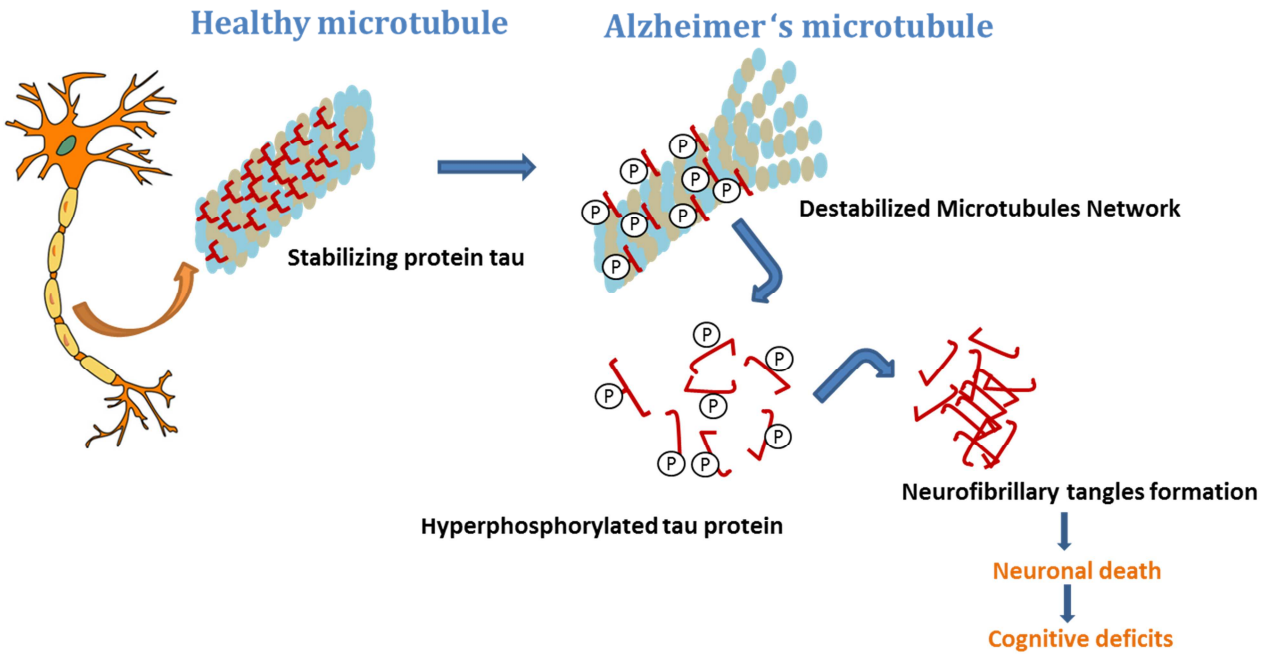

Figure 2: In AD, hyperphosphorylated tau may destabilize microtubule networks, impair axonal transport, and ultimately trigger neurofibrillary tangle (NFT) formation and neuronal death. Adapted from [23]

b) Amyloid beta plaques

i) *The amyloid precursor protein*

APP is the most abundant transmembrane protein (106-130kDa) in the central nervous system (CNS) and it is also expressed in peripheral tissues such as blood cells and epithelium [24]. Through alternative splicing, APP presents isoforms ranging from 665 up to 770 amino acids, whereof APP_{695} is the most dominant form in the CNS. The physiological role of APP is still unclear but implications in synaptic plasticity, cell adhesion, neurite outgrowth, and neuronal migration have been shown [25-28]. APP is metabolized by two distinct routes: (i) the non-amyloidogenic α-secretase pathway and, and (ii) the amyloidogenic β-secretase pathway **(Fig. 3).**

In the non-amyloidogenic pathway, APP is cleaved in the middle of the Aβ region by α-secretase releasing a soluble N-terminal APPsα ectodomain and a membrane bound carboxyl-terminal fragment (APP-CFT83). The C83 fragment is cleaved by γ-secretase

releasing a C-terminal fragment C3 and an APP intracellular fragment called AICD [29]. The action of the APPSα has been considered beneficial by protecting neurons, exerting neurotrophic effects and promoting neurogenesis [30, 31]. The P3 fragment is not toxic but showed neuroprotective effects [32, 33].

In the amyloidogenic pathway, APP is cleaved by β-secretase, also named β-site APP cleaving enzyme 1 (BACE1), releasing a soluble N-terminal APPsβ fragment and a membrane bound carboxyl-terminal fragment (APP-CFT99), which is cleaved by γ-secretase, producing the full length β-amyloid peptides (Aβ) and an AICD fragment. AICD domain is shown to be involved in neuroprotection. Elevated levels of AICD are correlated with enhancement of memory and synaptic plasticity [34].

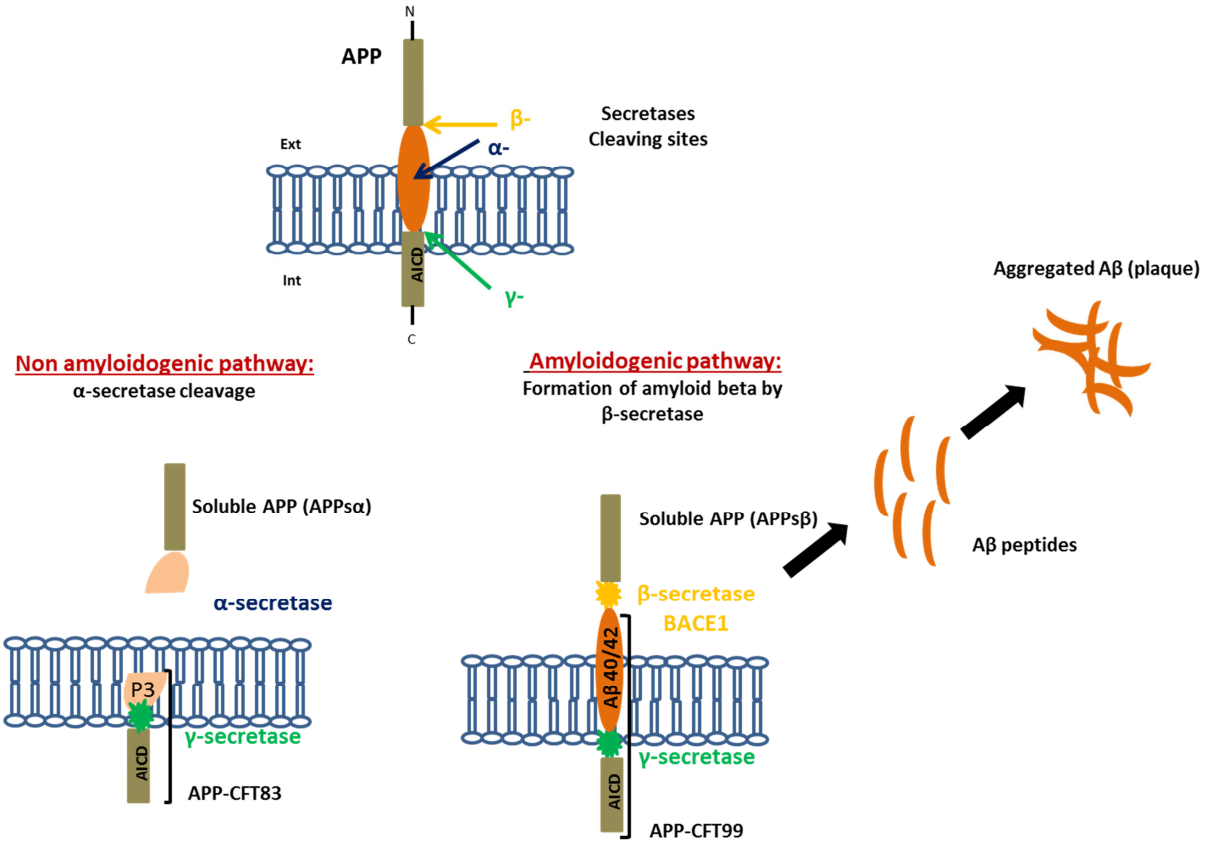

Figure 3: The amyloid precursor protein (APP) is a transmembrane protein cleaved by secretase enzymes. In the non-amyloidogenic pathway, APP is cleaved by α-secretase and then by the γ-secretase. In the amyloidogenic pathway, Aβ peptides are released after sequential cleavage of APP by β- and γ-secretases, and accumulate into oligomeric aggregates to form plaques. Adapted from [23, 35].

ii) Enzymes involved in APP proteolysis

The production of Aβ peptide has been at the center of Alzheimer's disease research by successive enzymatic cleavages of APP. The α-secretase activity is mediated by enzymes from the family of desintegrin and metalloproteinase domain proteins (ADAM), ADAM 9, 10, 17 and 19 to process four functions: proteolysis, cell adhesion, cell fusion and cell signalling [36]. The overexpression of ADAM 10 has been shown to reduce Aβ production and plaques deposition as well as decrease the cognitive impairments in a mouse model of AD [37, 38]. ADAM 17 has been shown to specifically cleave the precursor of tumor necrosis-α (TNF-α) [36]. APP is sequentially cleaved in the luminal and extracellular compartments by β-secretase and within the transmembrane domain by γ-secretase [39]. The BACE1 is a transmembrane aspartic protease and has been shown to be the major neuronal β-secretase [40]. The subcellular localization of BACE1 is within the endoplasmic reticulum and the trans-Golgi network [41]. The expression of BACE1 is increased in cellular stress situations such as energy deprivation but also during oxidative stress [42]. Recently, a study that supports the fact that Aβ causes microtubule disruption, showed that impaired axonal transport and the accumulation of BACE1 in peri-plaque dystrophies induces BACE1 cleavage of APP and exacerbation of amyloid pathology in AD [43]. γ-Secretase, a membrane-embedded protease, cleaves the transmembrane region of APP to generate Aβ [39]. γ-Secretase is a multi-subunit aspartyl protease complex. The γ-secretase activity interacts with four proteins: presenilin 1 or 2 (PSEN1 or 2) forming the catalytic subunit and having a protease activity, nicastrin (Nct), anterior pharynx-defective 1 (Aph-1) and presenilin enhancer 2 (Pen2) [39]. The three last partners are required for the assembly and the stabilization of the complex and for the presenilin endoproteolysis. PSEN1 is the dominant PSEN in the brain [38]. In AD, mutations in the PSEN gene have been shown to be a major risk factor [44]. In humans, at least six different possible γ-secretase complexes may have cell- or tissue specificity [45]. γ-Secretase complexes have been localized whithin the mitochondria by promoting apoptosis [46].

iii) Amyloid-β peptide

Amyloid beta (Aβ) is a 40 or 42 amino acid peptide, a normal by product derived from sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase. Aβ is found in the cerebrospinal fluid (CSF) and secreted to the extracellular environment of the human brain. The physiological role of Aβ is related to modulate the synaptic activity, but it is still controversial. At picomolar range in normal physiological level, Aβ peptides positively regulate the synaptic function and learning [47]. Other suggested physiological roles of Aβ include protection as antioxidant molecule against metal-induced oxidative damage, regulation of cholesterol transport, kinase activation, and transcriptional regulation of AD-associated genes [48-51]. Insufficient Aβ levels may induce a loss of normal function but excess Aβ could give rise to dysfunction [52]. The increase of Aβ peptide concentration induces pathological effects under pathological conditions such as stress, increased neuronal activity, or the presence of familial Alzheimer's disease (FAD) mutations [47].

iv) Amyloid beta plaque formation and consequences

The protein aggregation (fibrillization) involves different conformations of the native protein, oligomers formation creating to protofibrils and finally mature fibrils (**Fig. 4**) [53]. While the aggregation features of Aβ42 and Aβ40 are equal, Aβ42 is more important than Aβ40 within the plaques. The additional amino acids confer an important rate of insolubility and fibrillization to Aβ42 [54]. A common conserved molecular architecture of Aβ (1-40) peptide fold is present for all the Aβ fibrils [55]. Diffuse plaques and dense-core plaques are the two types of amyloid plaques staining distinguishable by the β-sheet pleated conformation with specific dyes such as Thioflavin-S. The dense-core plaques are positive to Thioflavin-S staining and are associated with deleterious effects such as synaptic and neuronal loss [56].

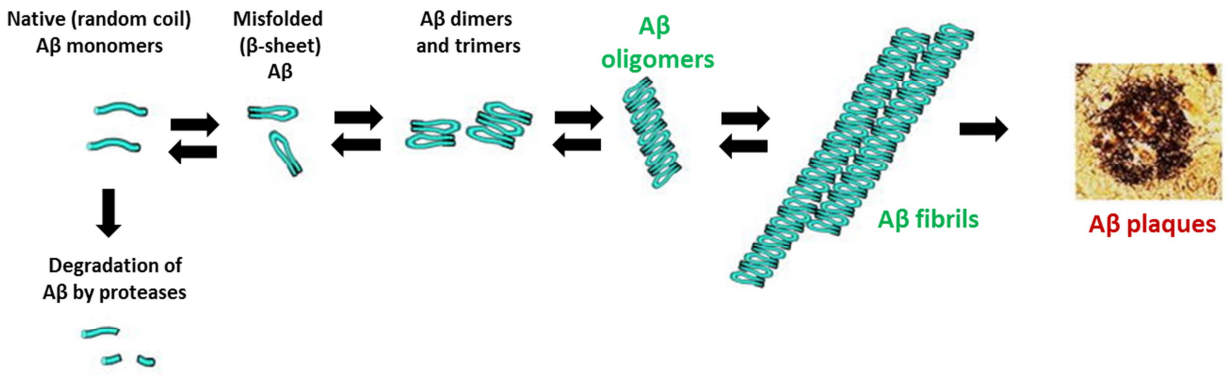

Figure 4: Protein fibrillization: schematic model for amyloid β (Aβ) misfolding and aggregation. Soluble native protein is misfolded and associates in the form of oligomers and other intermediates that eventually give rise to fibrils. Adapted from [57]

v) Toxicity of amyloid β oligomers

Concerning oligomers and fibrils, a difference of toxicity is suggested by their distinct structure and morphology [58]. A*β* oligomers and its further conversion to fibrils induced toxic mechanisms formation such as disruption of membrane receptors, adsorption on membrane surface which alters the property of the membrane, formation of pore which causes the leakage of Ca^{2+}, and the accumulation of intraneuronal A*β* (**Fig. 5**) [59]. In AD, accumulation of Aβ forming intermediate soluble oligomers is synaptotoxic as well as cerebral angiopathy (primarly Aβ40) and insoluble β-sheet pleated amyloid fibrils [14]. After peptide solubilization, Aβ42 hexamers and dodecamers rapidly become the dominant oligomers, even at low (1 μM) concentrations. Aβ40 produces smaller oligomers [60]. The phosphorylation of Aβ peptide by protein kinase A promotes the aggregation of this peptide. In AD pathogenesis, the stabilization of Aβ by phosphorylation might be a trigger for formation of toxic aggregates by increasing the concentration of Aβ in the brain [61].

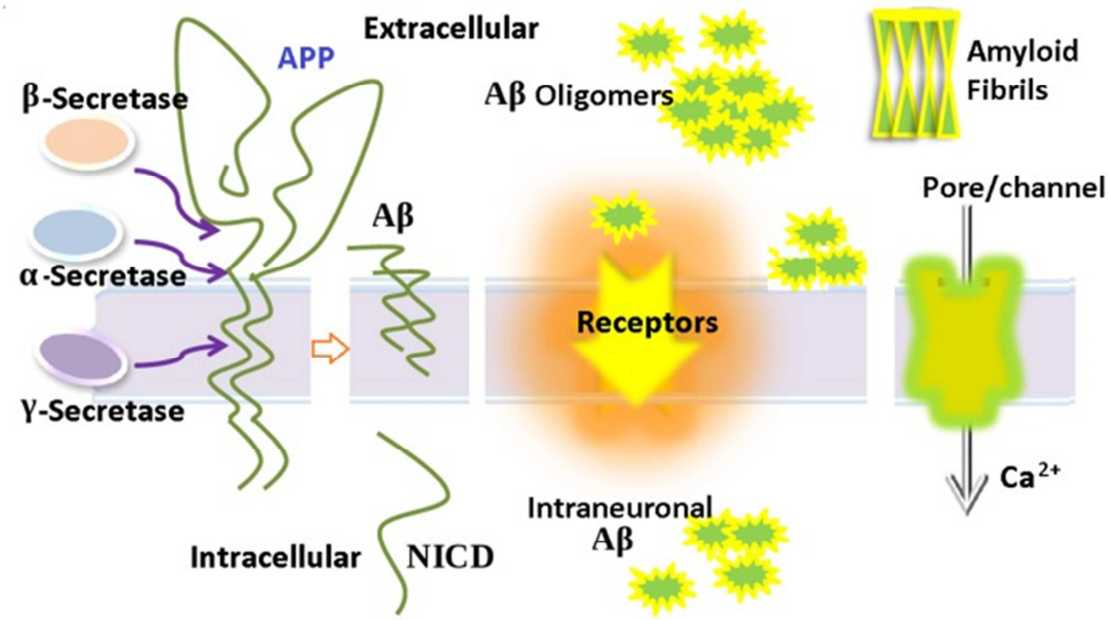

Figure 5: APP cleavages are shown on the left side of the figure by *α*-, *β*- and *γ*-secretase and the production of A*β* peptides. The following toxic mechanisms are illustrated: formation of A*β* oligomers and its further conversion to fibrils; disruption of membrane receptors; adsorption on membrane surface which alters the property of the membrane; formation of pore which causes the leakage of Ca^{2+}; and the accumulation of intraneuronal A*β* [59].

3. The amyloid cascade hypothesis

The mutation in the gene coding for APP produces the Aβ peptide via sequential scission by the β-APP cleaving enzyme (BACE) and γ-secretase. In the amyloid cascade hypothesis, accumulation of Aβ peptide is considered as the primary influence driving AD pathogenesis. Glenner and Wong were the first to suggest this hypothesis after the isolation and identification of Aβ peptide in the brain of AD patients [15]. Several studies supported this hypothesis, and in 1992, Hardy and Higgins called it the *amyloid cascade hypothesis* [62]. The hypothesis combines genetic and histopathological information. This hypothesis was supported by the discovery that AD could also be induced by autosomal dominant mutations in three genes: presenilin (PSEN1), PSEN2, and APP, causing the early onset familial Alzheimer's disease (FAD). The concept of soluble toxic oligomers has been proposed to explain the neurotoxicity of the amyloid-β peptide [63, 64]. The potential mechanism of

toxicity called "aggregate stress" may lead to Aβ aggregation due to an imbalance between Aβ production and Aβ clearance, and having as consequences the formation of paired helical filaments (PHFs) of tau aggregates and finally neuronal loss, and dementia [65, 66]. This imbalance between Aβ production and Aβ clearance induces several pathogenic events including activation of microglia and astrocytes, impaired cell communication and synaptic function, oxidative injury and neuronal ionic homeostasis, altered kinase/ phosphatase activities leading to formation of neurofibrillary tangles and mitochondrial dysfunction [65, 67-70].

Over the years, this hypothesis has been modified as the correlation between dementia or other cognitive alterations and Aβ accumulation within the amyloid plaque in the brain is not linear, neither in mice nor in humans [71].

In contrast to the amyloid cascade hypothesis, other studies correlate NFTs pathology with cognitive deficit or dementia in AD patients [72, 73]. In another study also, the severity of the dementia was positively related to the NFTs in the neocortex and not to the degree of senile plaque deposition [74]. The tau hypothesis suggests that tau tangles pathology occurs prior to the Aβ plaque formation and is more correlated with the severity and progression of the disease [72].

4. Mitochondrial cascade hypothesis: attractive target for AD treatment strategy

Most AD researches were focused on the "amyloid-β cascade hypothesis" supposing that Aβ plaques were the only cause of AD. However, this hypothesis was based on AD rare familial autosomal dominant cases, which explained only about 1% of the AD cases [75-77]. "Mitochondrial cascade hypothesis" was published by Prof Swerdlow and Prof Khan from the University of Virginia (USA) could explain the biggest part of common sporadic late-onset AD forms. This hypothesis present explanation for AD forms (sporadic, age-related) by considering more aspects of the disease [78, 79]. In the hypothesis, mitochondrial function is placed in the center of pathological events and correlated with the reactive oxygen species (ROS) coming principally from the electron transport chain. ROS are the cause of acquired

age-related mitochondrial damage. The theory present that mutation in mtDNA accumulate all along a person's life causing a slowly mitochondrial decline leading to a decrease in energy production and increase in oxidative stress. These considerations could be able to explain more AD related problems: damaged mitochondria may affect APP expression causing an accumulation of Aβ plaques. This causes an elevation of hyperphosphorylated tau protein and thus, together with Aβ plaques, disturbs mitochondria trafficking and function. The result is a negative vicious cycle, which implicates mtDNA mutation, oxidative stress, synaptic dysfunction and neuronal death by apoptosis [78, 80, 81]. Aging is marked by a gradual increase in brain oxidative stress and consequent damages (**Fig. 6**). The causes and/or consequences of mitochondrial deficit may be exacerbated by environmental and genetic factors and may lead to impaired mitochondrial bioenergetics and dynamics that, in turn, increase ROS production. This vicious cycle may be additionally fed by further vicious cycles, involving Aβ generation, microglial activation and tau hyperphosphorylation. The accumulation of damages initiates the collapse in cellular bioenergetics, cellular dysfunction, and ultimately, neuronal death and dementia by exceeding an individually different threshold (**Fig. 6**, dotted line) [82].

Based on these considerations, mitochondria represent an interesting target for AD treatment strategy and/or prevention.

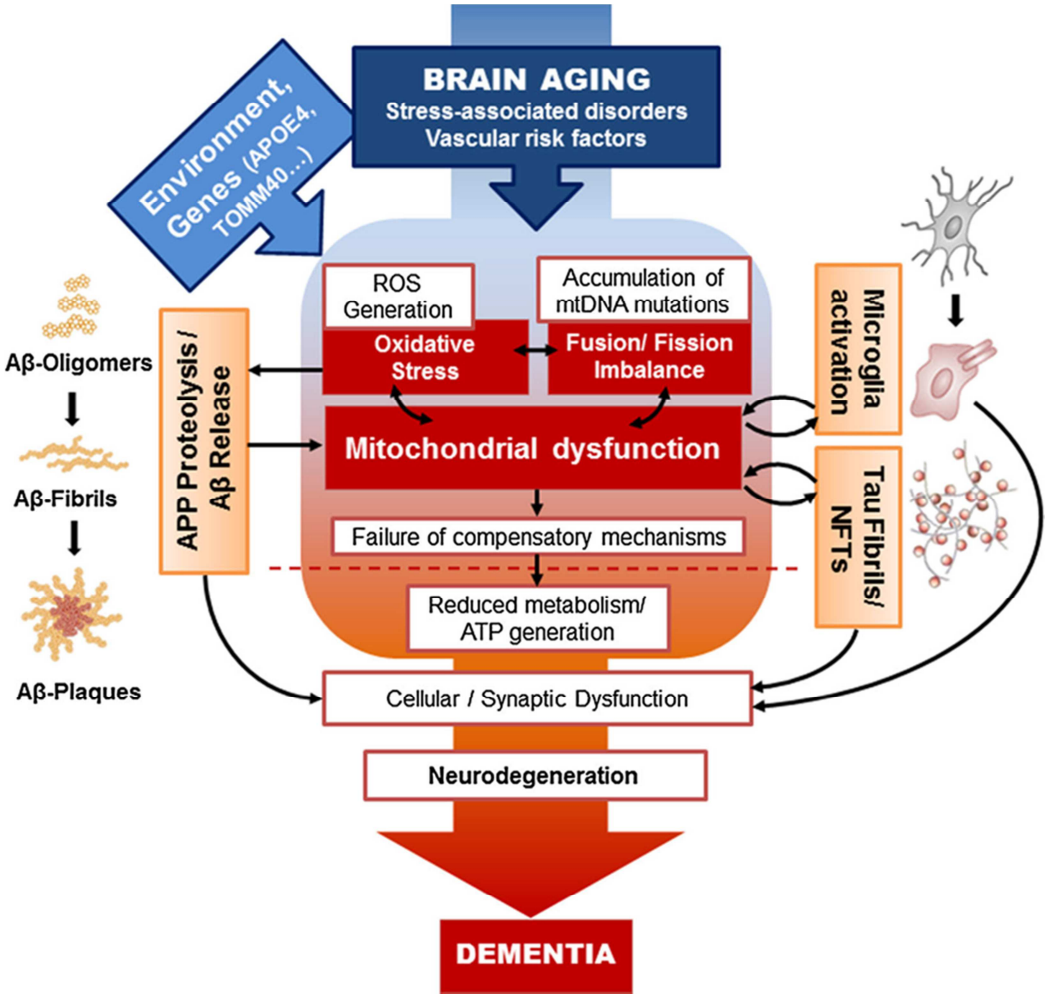

Figure 6: Conceptual representation of sequential events linking brain aging and sporadic Alzheimer's disease. APP amyloid precursor protein; NFTs neurofibrillary tangles; ROS reactive oxygen species

C. Mitochondria

Mitochondria are described as “the powerhouses of the cell”, producing a major part of the cellular energy via the adenosine triphosphate (ATP) generation through oxidative phosphorylation (OXPHOS) [83].

1. Structure of mitochondria

Mitochondria appear to originate 1.5 billion years ago of an endosymbiosis of an aerobic prokaryote into a eukaryotic cell which allowed essential aerobic life [84, 85]. The size, shape, and number of mitochondria in a cell vary from one tissue to another as well as to the physiological state of the cells. Mitochondria are ovoid bodies with a length of 1-2 µm (**Fig. 7**) [86]. The basic morphology of mitochondria is defined by the inner membrane, an inter-membrane space (IMS) and the outer membrane [83]. The inner membrane is divided into two domains: one juxtaposed closely to the outer membrane (“inner boundary membrane”), the second forms the cristae that are interpreted to form tubular and lamellar structures and to be connected to the inner boundary membrane by small tubular structures named *crista junctions [87]*. Concerning the permeability of mitochondria, the outer mitochondrial membrane (OMM) is easily permeable while the inner mitochondrial membrane (IMM) has a low permeability and contains cristae which have the role to increase the IMM surface. The big surface area of mitochondria induces their capacity to produce ATP. Mitochondria have a special feature which is the presence of their own mitochondrial DNA (mtDNA) composed of 37 genes, all of which are crucial for normal mitochondrial function. The matrix contains mtDNA and also a host of enzymes, as well as ribosomes for protein synthesis. These enzymes catalyze many critical steps of cellular respiration. In respiration, other proteins, including the enzyme that generates ATP, are embedded in the mitochondrial inner membrane. Folding of the cristae increases the surface area for hosting enzymes involved in cellular respiration.

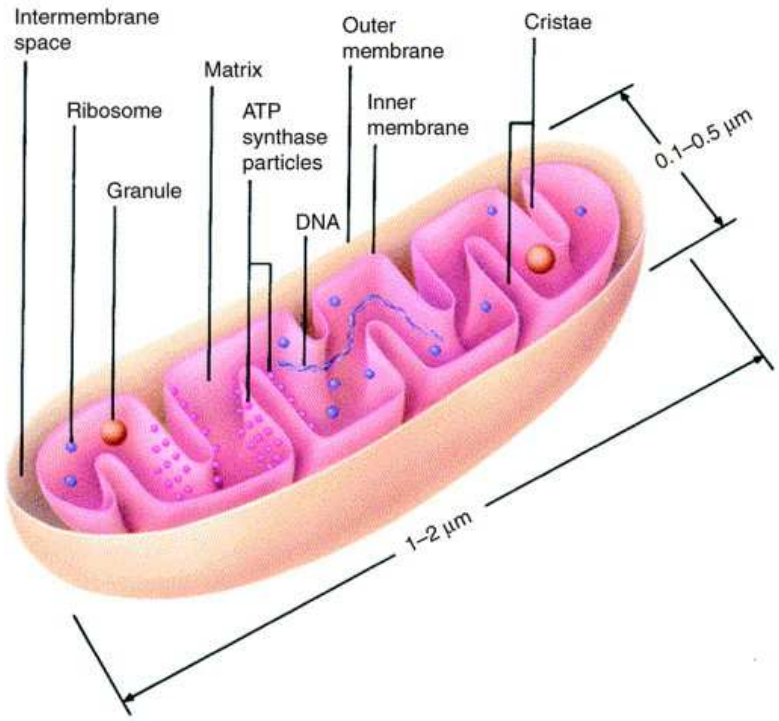

Figure 7: Structure of a mitochondrion from [88]

2. Mitochondria functions within the cells

Mitochondria are special organelles present in every living cell. Their principal function is to supply the energy necessary to the cells to survive.

a) Mitochondrial DNA

In total, 37 genes compose the mitochondrial DNA and are essential for normal mitochondrial function. Thirteen of these genes have directive roles for the production of enzymes involved in oxidative phosphorylation. The OXPHOS process uses oxygen and simple sugars to create ATP, the cell's main energy source. The remaining genes provide the instructions to produce Transfer RNA (tRNA) and ribosomal RNA (rRNA). These RNA help piece together protein building blocks (amino acids) into functioning proteins [89]. One ATP molecule contains one adenosine, a ribose sugar and three phosphates, reach bonded by oxygen

(**Fig. 8**). This bond between the phosphates is highly energetic and release energy when it breaks [86].

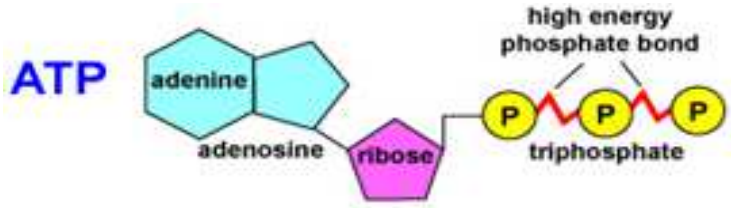

Figure 8: A molecule of ATP (http://schoolworkhelper.net/wp-content/uploads/2010/07/atp-adp-structure.jpg)

ATP is essential for life and is generated in mitochondria via two pathways: glycolysis and oxidative phosphorylation.

b) Cellular energy production

i) *Cellular glycolysis*

Glycolysis, the first energy pathway, is a metabolic pathway that happens in the cytoplasm of cells that convert glucose molecules (coming from nutritional sources) in ten enzymatic steps into two pyruvates. In the process there is a release of free energy used to form the high-energy compounds nicotinamide adenine dinucleotide (NADH) and ATP. This energy is necessary for several cellular processes such as the synthesis of macromolecules (DNA, RNA, and proteins), their transport (endocytosis, exocytosis), cell signalling, and locomotion. Pyruvate molecules enter the mitochondria and are converted to an Acetyl coenzymeA. This molecule is transported to the mitochondrial matrix and enters in the citric acid cycle (**Fig. 9**) [86].

The Krebs cycle, also known as tricarboxylic acid cycle, is a cycle of enzymatic reactions, whose net gain is three NADH and one flavin adenosine dinucleotide ($FADH_2$), one guanosine triphosphate (GTP). The newly produced molecules NADH and $FADH_2$ will be used in the oxidative phosphorylation (OXPHOS) pathway to generate ATP (**Fig. 9**) [86].

ii) Mitochondrial respiration

The second main pathway to meet the massive demand of ATP synthesis is called mitochondrial respiration or oxidative phosphorylation [85, 90]. The electrons carried by NADH and $FADH_2$, coupled with protons pumped from the matrix to the intermembrane space, are used to generate a chemical potential across the inner mitochondrial membrane (**Fig. 9**). During electron transfer, the gradient of protons generated is used to drive the synthesis of three additional ATP molecules for every electron that travels along the chain. The mitochondrial respiration takes place at the IMM mediated by a complex of proteins (encoded by mitochondrial or nuclear DNA) called electron transport chain (ETC). The ETC consists of five multi-subunit protein complexes connected by the mobile electron acceptor cytochrome c and electron donor ubiquinone. First, two electrons are donated by NADH to the respiratory complexes, complex I (CI) and NADH dehydrogenase. Next, they are passed to a lipid soluble redox carrier, coenzyme Q (Q). These transports are accompanied by the transfer of four protons from the matrix to the intermembrane space. Complex II (CII) and succinate dehydrogenase, also involved in the Krebs cycle, catalyse the reduction of FAD to $FADH_2$. This reaction gives additional electrons into the quinine pool (Q). Compared to complex I, complex II, the only complex that contains proteins exclusively encoded by nuclear DNA, does not pump protonfrom the matrix. Then the reduced coenzyme Q diffuses through the inner membrane and its electrons are transferred to the complex III (CIII or ubiquinol cytochrome c oxidoreductase). Coenzyme Q is oxidized by the enzyme and the liberated electrons are transferred to two molecules of cytochrome c, another soluble redox protein. Parallelly, the reaction is coupled by the translocation of protons toward the intermembrane space. Finally, cytochrome c removes electrons and transferred them to

molecular oxygen (O_2), forming two molecules of water (H_2O). Four protons are again pumped from the matrix into the intermembrane space. ATP synthase, also called complex V (CV) is the last step of OXPHOS. Between IMS and the matrix, the proton gradient and the mitochondrial membrane potential generated by ETC ensure the rotation of the ATP synthase. This reaction creates ATP from adenosine diphosphate (ADP) and phosphate (Pi). The whole process allows the formation of 32 molecules of ATP.

In addition to their fundamental role in orchestrating energy production, mitochondria play also important roles including the amino acid metabolism, fatty acid and steroid metabolism, calcium homeostasis, and a particular function in ROS production.

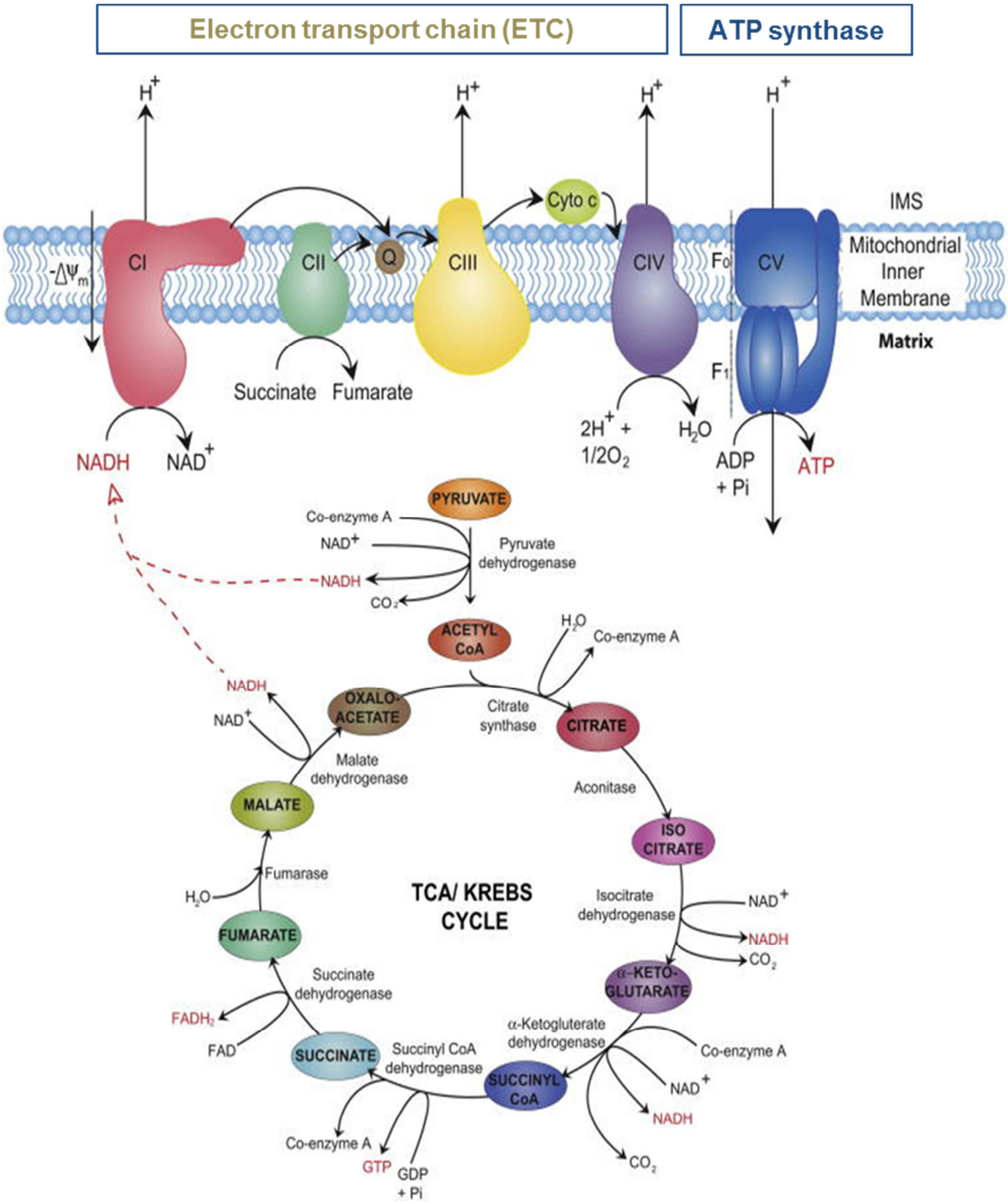

Figure 9: Bioenergetics of the electron transport chain and the Kerbs cycle. Pyruvate is converted to high-energy molecules such as NADH, GTP and $FADH_2$ by TCA/Kerbs cycle enzymes through catalyzation. NADH generated is shuttled to complex I and is converted to NAD^+ driving oxidative phosphorylation. Transfer of electrons by the chain maintains the membrane potential via proton pumping into the IMS. ADP is phosphorylated to form ATP via complex V (ATP synthase). Adapted from [90]

c) Mitochondria as sources and targets of reactive oxygen species

Paradoxically, mitochondria are the major producers of ROS but at the same time the most susceptible targets of ROS toxicity (**Fig. 10**) [91]. Cell respiration requires oxygen and the majority of O_2 is reduced into H_2O by the ETC at the mitochondrial complex IV [92]. Nevertheless, the ETC lets escape a small amountof electron that react with oxygen producing superoxide radical ($O_2^{\cdot-}$). $O_2^{\cdot-}$ can be easily transfomed into ROS, such as hydroxyle radical (OH) and hydrogen peroxide (H_2O_2) through enzymatic reactions (**Fig. 10**) [93, 94]. There is other sources of ROS, a small part derives from other enzyme systems [95].

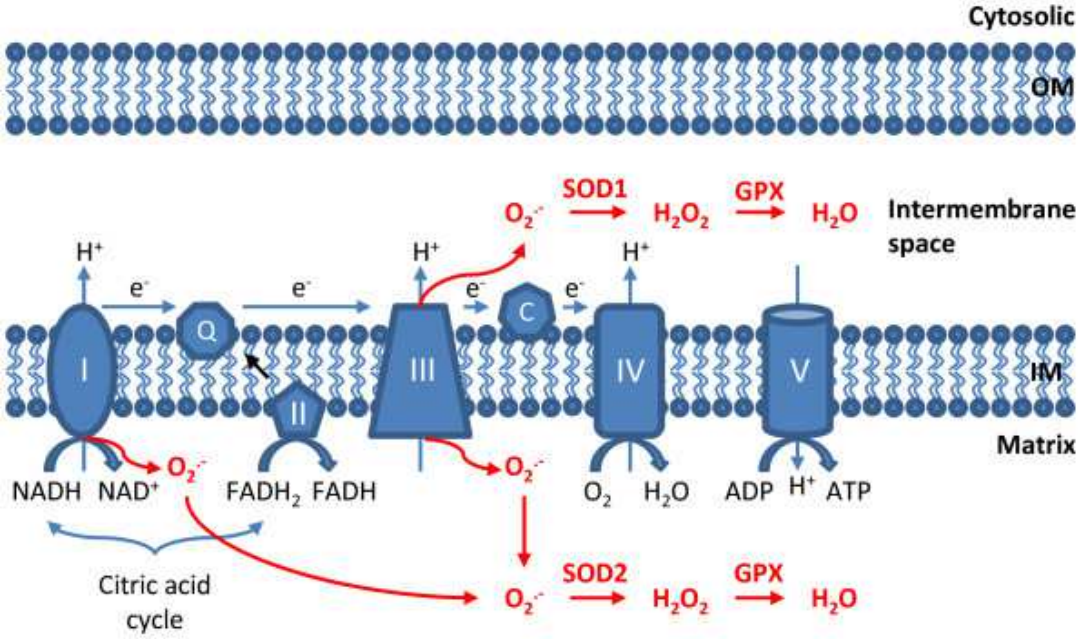

Figure 10: Production and disposal of mtROS. Electrons (e^-) donated from NADH and $FADH_2$ pass through the ETC and reduce O_2 to form H_2O at complex IV. MtROS are provided from the leakage of $e^-$to form superoxide ($O_2^{\cdot-}$) at complex I and complex III. $O_2^{\cdot-}$ is produced within matrix at complex I, whereas at complex III $O_2^{\cdot-}$ is released towards the matrix and the intermembrane space. Next, $O_2^{\cdot-}$ is dismutated to H_2O_2 by superoxide dismutase 1 (SOD1) in the intermembrane space and by SOD2 in the matrix. H_2O_2 is fully reduced to water by glutathione peroxidase (GPX). Both $O_2^{\cdot-}$ and H_2O_2 are considered as mtROS. OM: outer membrane; IM: inner membrane [96]

ROS can be produced in response to environmental stimuli such as growth factors, inflammatory cytokines, chemicals oxidants, chemotherapeutics, toxins hyperoxia, ionizing

radiation, and transition metals [97-100]. At low levels, mtROS are involved in the process of hypoxia adaptation by regulating the stability of hypoxia-inducible factor 1α (HIF-1α). Moderate levels of mtROS participate in regulation of the production of proinflammatory cytokines. High levels of mtROS are capable of inducing apoptosis and autophagy (**Fig.11**).

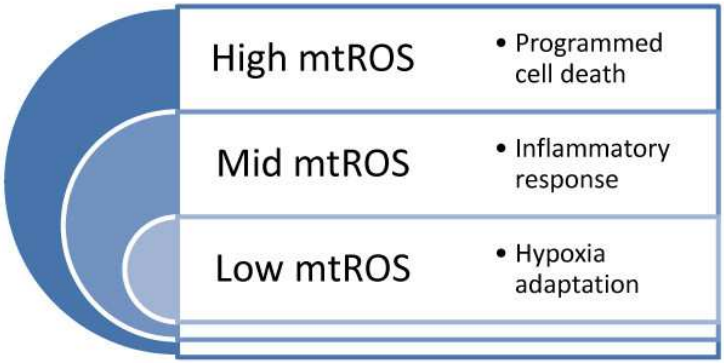

Figure 11: Signaling of mtROS [96]

Produced ROS could react with proteins, lipids, and nucleic acid causing oxidative damage to these macromolecules [101]. Mammalian cells have evolved a number of defense mechanisms to limit the cellular damage caused by ROS. In addition to the DNA lesions repairs (excision, double-strand break, and mismatch repairs), the damaging effects of ROS can be mostly neutralized via elevated antioxidant defense [102]. Indeed, to compensate or regulate the high ROS production, mitochondria possess essential endogenous antioxidants to face oxidative stress. The most important are superoxide dismutase, which can convert $O_2^{\cdot-}$in O_2, gluthation peroxidase, and catalase, which converts hydrogen peroxide into water and oxygen [103].

Antioxidants should be in balance with the reactive species. Unbalance could induce harmful dysfunctions connected to oxidative stress, apoptosis, aging processes, mitochondrial abnormalities and Alzheimer's disease can rise.

3. Age-associated changes of mitochondria:

The mitochondrial energy-transduction ability is crucial for the maintenance of neuronal activity. The hallmark of brain aging is the damage of energy metabolism and redox homeostasis, which is more important in early stages of neurodegenerative diseases. The communication between mitochondria and the rest of the cell is established by controlling cellular energy levels and the redox environment [104].

Aging is characterized by a progressive drop in physiological functions and an increase in mortality, often accompanied by pathological diseases [102]. A multitude of factors is involved in the complex process of aging. Mitochondrial dysfunction and oxidative stress are two important factors contributing to the aging process [102]. Neurodegeneration and aging present common impairments with declines in energy production in the brain and parallel changes in redox status with a pro-oxidant shift, partly due to the mitochondrial generation of $O_2^{\cdot-}$and H_2O_2 (**Fig. 12**) [104]. In aging, the mitochondrial energy metabolism is tissue specific and more prominent in tissues that contain mostly postmitotic cells, such as brain, heart, and skeletal muscle [104].

Mitochondria isolated from aged animals showed a partial loss of the energy transducing capacity. As has been described above mitochondrial OXPHOS is a process that includes the respiratory chain, and electron transfer through the complexes I, II, III and IV. The electron transport chain across the membrane, coupled to a proton gradient, drive the ATP synthesis through the complex V. In aged rat brains, electron transfer is decreased, which leads to a decreased mitochondrial inner membrane potential [105, 106]. Components of complex I and III are transported to generate $O_2^{\cdot-}$. Elevated oxidant production is observed since the activities of complex I, III, and IV decrease during aging: The rates of $O_2^{\cdot-}$and H_2O_2 are higher with age [104]. Age induces a decrease of the F_1-ATPase activity of complex V due to the nitration of Tyr^{269} close to the Mg^{++} binding site of the $F_1\beta$ subunit [107]. State 3 (active respiration) and the related respiratory control ratio decreased in aging and the state 4 respiration increased, indicating lower energy-transducting efficiency [107, 108].

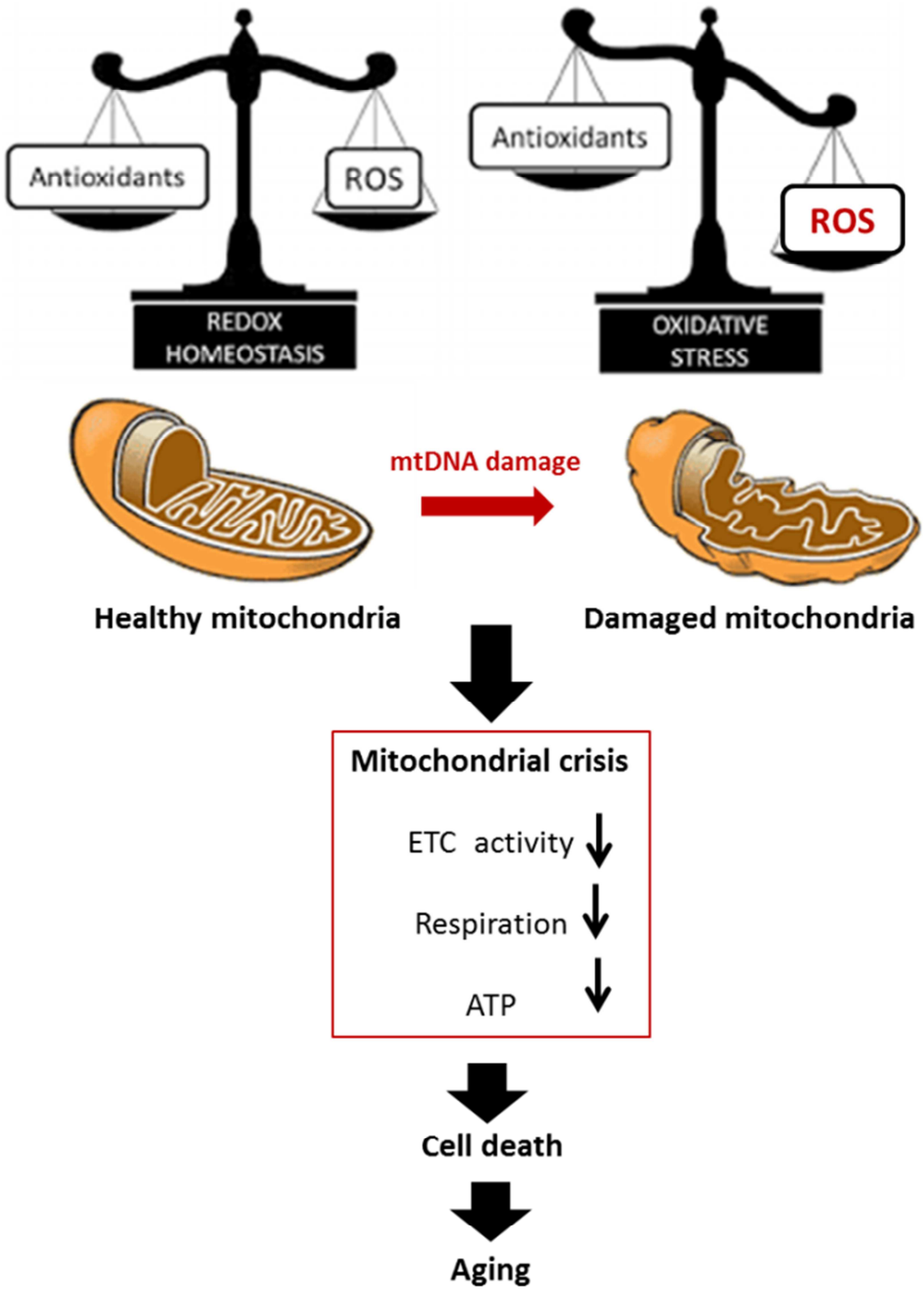

Figure 12: Model of mitochondrial dysfunction in aging. Toxic ROS generated during normal biological activity impair cellular homeostatic pathways including the electron transfer chain (ETC) and mtDNA. Oxidative insults to mitochondria, in turn, impair energy transduction, biogenesis of metabolites, and regulation of redox biology with age, thereby contributing to a vicious cycle of accumulating mitochondrial damage that culminates in a mitochondrial functional crisis. This results in cell death and aging. Adapted from [109]

4. Mitochondrial impairment in age-associated Alzheimer's disease

Mitochondria play an important role in the pathogenesis of neurodegenerative disease such as Alzheimer's disease. Because of their high energy demand, neurons are especially vulnerable and sensitive to any abnormal mitochondrial activity.

The nervous system (NS) requires about 20 % of the body's total basal oxygen consumption, showing the vital importance of mitochondria for this system. Mitochondrial dysfunction in the NS induces risk for neurones because of the energy deprivation. Analysis of brain positron emission tomography (PET) of an AD patient showed reduced glucose uptake and metabolism compared to a healthy brain (**Fig. 13**).

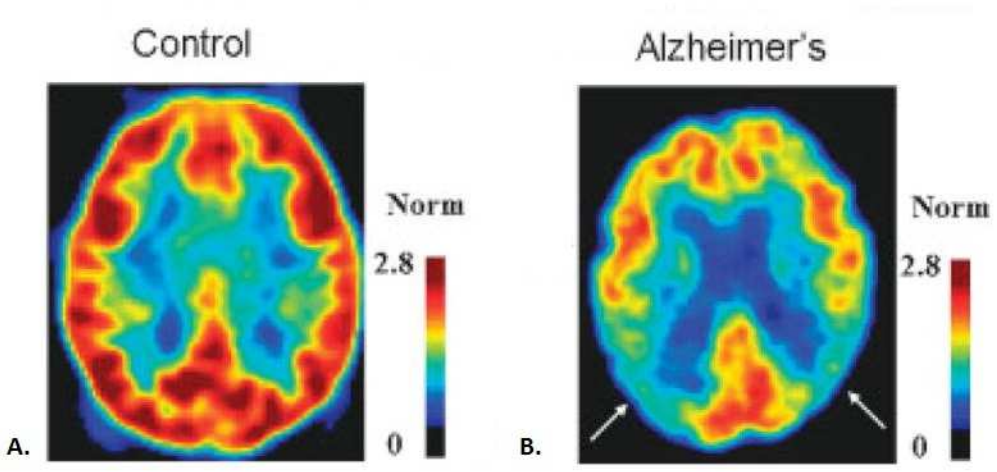

Figure 13: Positron emission tomography (PET) of a healthy subject (A), and of an AD patient (B). Red and yellow colors show high level of glucose uptake of the brain. The AD-related decrease in energy metabolism is evident. Adapted from [110]

Second to oxidative stress, the impairment of brain resident energy metabolism is an essential event found to be altered already in early stages of AD. Decreased ATP production conducts to impairment of ATP-dependent processes, on which all cellular functions depend [111]. Tramutola and collaborators suggest that reduced glucose utilization, mitochondrial deficit, and decreased ATP production are early nitrosative stress (OS)-events (**Fig.14**). They contribute to the neurodegenerative process and its progression, culminating in AD pathology.

A growing body of evidence supports mitochondrial dysfunction as a prominent and early chronic oxidative stress-associated event that contributes to synaptic abnormalities and neuronal degeneration in AD [112, 113]. Grimm and collaborators highlighted the critical key role of mitochondria and the close inter-relationship of this organelle with the two main pathological features in the pathogenic process underlying AD: showing independent as well as synergistic effects of Aβ peptide and hyperphosphorylated tau on mitochondrial function by using a high-resolution respirometry system (Oxygraph-2k). The two hallmarks of AD, Aβ and NFT in cells, have been shown to lead to mitochondrial dysfunction. The consequences of abnormal elevation of Aβ are diverse, including an increase in ROS production, alterations of OXPHOS, and interactions with mitochondrial dynamics and proteins leading to synaptic loss [81, 114]. Numerous studies have demonstrated mitochondrial deficit through the generation of ROS as an important factor involved in the pathogenesis of AD [115]. Aβ peptide and the presence of trace metal ions have been identified as a potential source of OS [116, 117]. In AD, mitochondria are a target of Aβ toxicity, which may act directly or indirectly on several proteins, leading to mitochondrial deficit. The deregulation of the ETC leads to a decreased complex V activity and a lower ATP synthesis, in addition to an increased ROS generation [91].

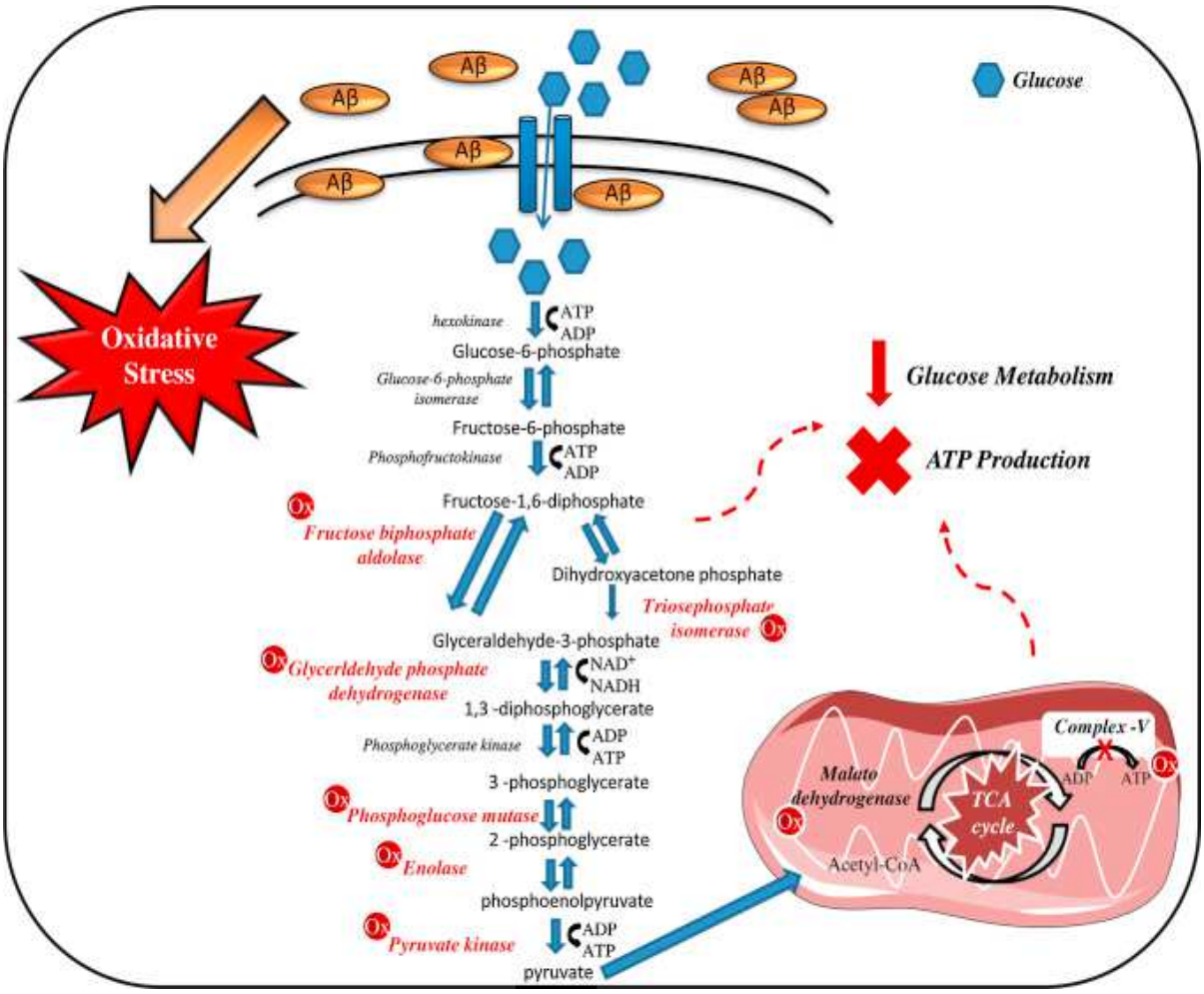

Figure 14: Protein oxidation of enzymes in energy metabolism. Increased production of Aβ induces oxidative stress that causes the oxidation of glycolytic enzymes, highlighted in Red, and TCA enzymes, highlighted in Black. The oxidative modifications of these targeted enzymes culminate in reduced glucose metabolism and decreased synthesis of ATP in AD brain. From [111]

In mitochondria, NFT consequences are similar: mitochondrial transport is blocked, and a loss of energy and an increase in oxidative stress occurs. All these phenomena lead to neuronal death [118-120]. Concerning the neurites plaque, positive dense-cores plaques to thioflavin-S are correlated with impairments such as dystrophic neurites that contain abnormal mitochondria and dense bodies of probable mitochondrial and lysosomal origin [14, 121]. Deregulation of complex I is mainly tau dependent, while dysfunction of complex IV is Aβ dependent, at protein and activity level [91].

D. Neurosteroids

1. Definition

In the 80s, the term neurosteroid was defined as a sub-category of steroids which are synthesized de novo in the nervous system. Independent of peripheral steroidogenic glands, neurosteroids accumulate in the nervous system [122-124]. Baulieu and coworkers showed that the production of steroids in the brain [125]. They demonstrated that the concentration of diverse steroids such as pregnenolone, dehydroepiandrosterone (DHEA) and their sulfated derivatives is higher in the brain and peripheral nerves than in the plasma of rats and still high even after gonadectomy or adrenalectomy. Some studies showed that other steroids were synthesized within the brain. In neurons as well as glial cells enzymatic activities of proteins involved in steroidogenesis have been showed [124, 126-128]. To distinguish steroids synthesized within the nervous system from steroids derived from the periphery, such as gonads, adrenals, and placenta, they were named “neurosteroids”. Thus, the definition of neurosteroids considers them as endogenous steroidal molecules synthesized in glial cells or neurons of the CNS and PNS. Neurosteroids include steroids that still persist in substantial levels in the nervous system after removal of peripheral steroidogenic glands [124, 129]. This term refers to their site of production within the nervous system. Neurosteroids act in an autocrine or paracrine configuration and strongly influence system functions and reactions to injury or disease. Neurosteroids are composed of different groups of steroids. The first category is the non-exclusive neurosteroids that are steroidal hormones synthesized in glial cells, neurons or endocrine glands, such as pregnenolone (PREG), progesterone (PROG), or DHEA. The second is the semi-exculsive neurosteroids such as allopregnanolone that are mainly produced in the nervous system and in substantial amounts in the endocrine gland. The last is the exclusive neurosteroids that are steroids exclusively synthesized in nerve cells such as epiallopregnanolone [130, 131]. Neurosteroids derive from cholesterol that is translocated within mitochondria via the translocator protein (TSPO) and

has a common structure, which is the classic cyclopentanophenanthrene 4-ring structure (**Figure 15**) [130].

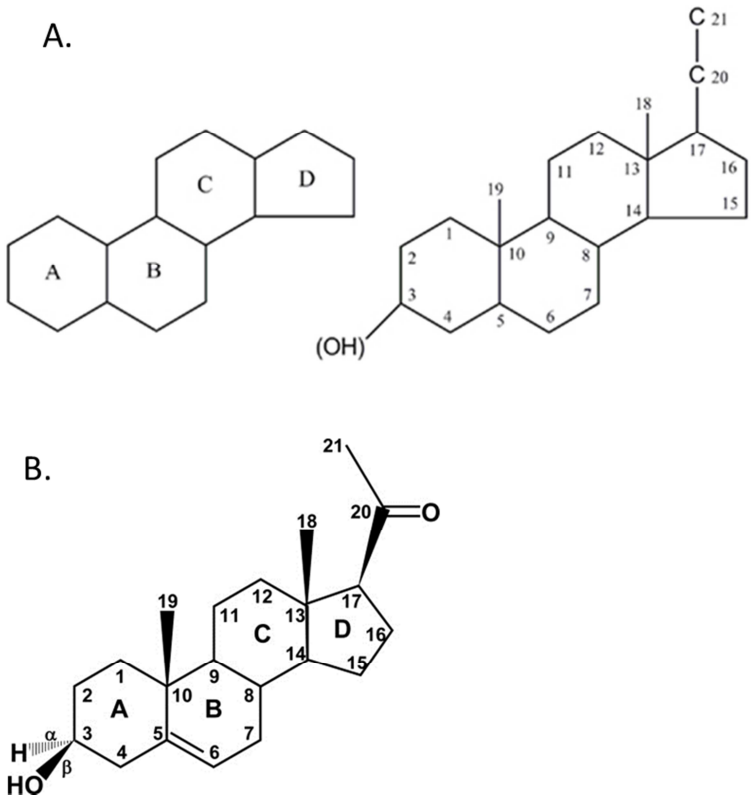

Figure 15: (A) Features of general structure of the neurosteroid. Figure shows intact four ring system that often contains hydroxyl side chains as sterols. Hydroxyl groups are denoted α if they are oriented above the plane (solid line), and α if they are oriented below the plane (dashed line). Stereoisomerism and structural modifications were discussed at carbon 3, 5, 6, 7, 17, 20, and 21 positions (Adapted from [132]. **(B) Structure of pregnenolone, showing the cycloperhydropentano-phenanthrene structure common to all steroids.** The rings are designated by *letters* according to standard convention, and the carbon atoms are indicated by *numbers*. Substituents and hydrogens are labeled as α or β if they are positioned behind or in front of the plane of the page, respectively (adapted from [130]).

2. Neurosteroids biosynthesized

In the nervous system, enzymes taking part in steroid hormone synthesis have been localized. [133, 134]. Hormones can be catalyzed in two steps to neurosteroids by the sequential action of the enzymes 5α-reductase and 3α- hydroxysteroid dehydrogenase (3α-HSD). Both enzymes are expressed by neurons and glial cells [135].

The rate-limiting step of steroidogenesis is the transfer of cholesterol from the cytosol to the mitochondrial matrix. Cholesterol is insoluble and its transfer requires the involvement of a multiprotein complex (see section TSPO complex) that is composed of a protein located in the cytosol and at the outer and inner mitochondrial membrane [136, 137]. Once cholesterol is in the mitochondria, the first step to convert it into a neurosteroid occurs through the enzyme cytochrome P450 side chain cleavage (P450scc), which is able to convert cholesterol in PREG via the following three chemical reactions: i) 22-hydroxylation of cholesterol; ii) 20-hydroxycholesterol of 22(R)-hydroxycholesterol; iii) oxidative scission of the C20-C22 bond of 20 (R)-22(R)-dihydroxycholesterol [130].

Inside the mitochondrial matrix, cholesterol is converted into the precursor of neurosteroids, pregnenolone, which is metabolized into neurosteroids either in mitochondria or the endoplasmic reticulum (ER) major pathway. Once synthesized whitin mitochondria, PREG can be involved in several pathways to produce different neurosteroids [126]. PREG can be metabolized by 3β-hydroxysteroid dehydrogenase (3β-HSD) to form PROG or by the cytochrome P450c17 enzyme (P450c17), also named 17α- hydroxylase/17, 20-lyase, to form DHEA. For this last conversion, the 17α-hydroxylation of PREG by P450c17 happens in two steps, firstly resulting in reaction giving, 17-hydroxyPREG (17OHPREG) and requiring molecules of NADPH and O_2. P450c17 converts PROG into an intermediate product, 17-hydroxyPROG, and then into the final metabolite androstenedione. Next, androstenedione is either metabolized into testosterone or estrone by another hydroxysteroid dehydrogenase called 17β-HSD or by P450aro (aromatase) respectively. The synthesis of estradiol from estrone and testosterone is performed by 17β-HSD or P450aro, respectively. The 5α-reductase enzyme (5α-R) catalyzes the transfer of two atoms of hydrogen from NADH to produce dihydrotestosterone (DHT), a 5α-reduced metabolite of testosterone. Finally, the 3α-hydroxysteroid oxido-reductase (3α-HSOR) enzyme, also named 3α-hydroxysteroid dehydrogenase, catalyses the reversible conversion of DHT into the neuroactive steroid 3α-androstanediol.

In the second main steroidogenic pathway, PREG is reduced to PROG by 3β-hydroxysteroid dehydrogenase in mitochondria (**Fig. 16**). PROG is catalysed to 5-α dihydroprogesterone (5-α-DHP) by the enzyme 5α-reductase. PROG and 5α-DHP bind to the classical intracellular progesterone receptor (PR). They regulate gene transcription within the cell nucleus or interact with kinases and components of intracellular signalling pathways within extra-nuclear compartments. Next, 3α-hydroxysteroid dehydrogenase converts 5α-DHP into allopregnanolone (AP or 3α/5α-tetrahydroprogesterone or 3α/5α-THP) [138]. Because of its hydroxyl group at the position carbon 3, AP cannot bind to PR. AP is a potent positive allosteric modulator of $GABA_A$ receptors [139]. At the last step of AP synthesis, AP can be converted back into 5α-DHP, which is a potent agonist at the nuclear PR, where AP could regulate gene transcription.

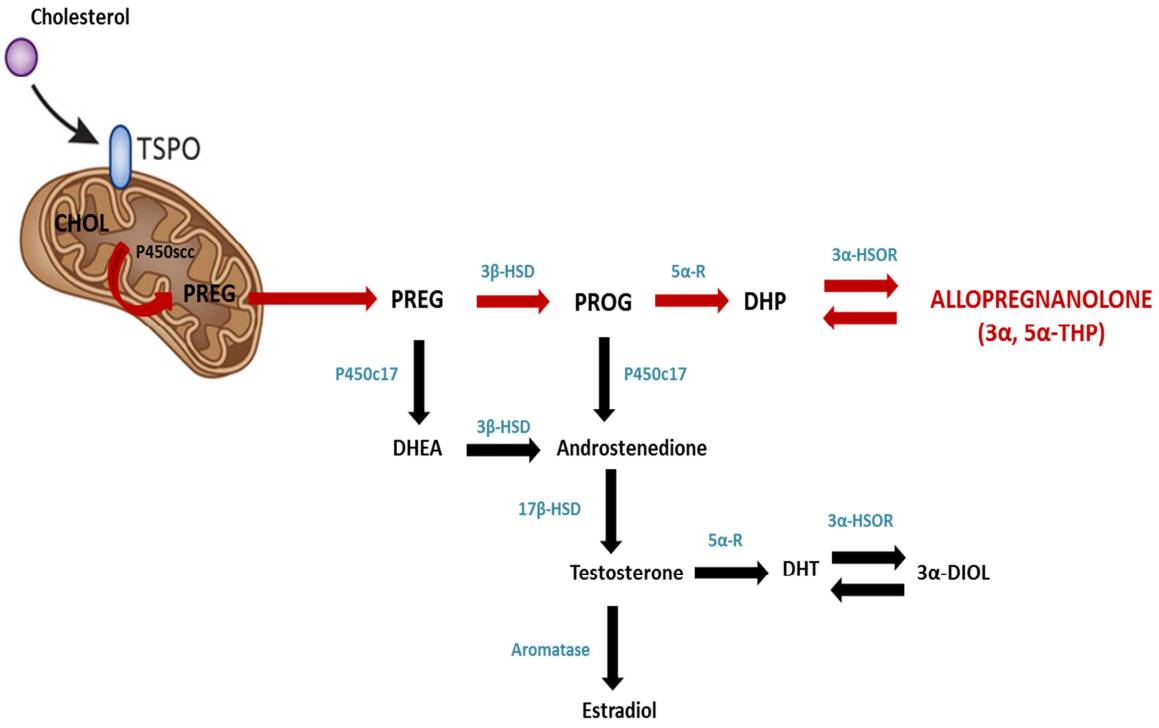

Figure 16: Biochemical pathways leading to neurosteroid biosynthesis. The second main steroidogenic pathway is highlighted by red arrow. P450scc, cytochrome P450side-chain-cleavage; P450c17, cytochrome P450c17 or 17ahydroxylase/ 17,20lyase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5αreductase; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; 17β-HSD, 17βhydroxysteroid dehydrogenase; PREG, pregnenolone; PROG, progesterone; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; 3α,5α-THP, 3α,5αtetrahydroprogesterone (allopregnanolone); DHT, dihydrotestosterone; 3α-DIOL, 3α-androstanediol. Adapted from [140]

3. Mechanism of action

Due to their high lipophilic nature, neurosteroids can easily cross cell membranes and act on nuclear receptors, regulating the gene transcription [141]. Furthermore, neurosteroids play an important role in the development, maturation and differentiation of the central and peripheral nervous system thanks to their essential nervous system position [142]. Steroid hormones exert biological actions in the nervous system as neurosteroids via nuclear steroid receptors (nSRs). nSRs regulate gene expression and are categorized in five groups: androgen receptors, estrogen receptors, glucocorticoid receptors, mineralocorticoid receptors and progestin receptors [143]. Recently, a new nRS, pregnane xenobiotic receptor (PXR), was identified. PXR is activated by progesterone and allopregnanolone and seems to act on cholesterol homeostasis as well as on the neurosteroidogenesis of allopregnanolone in the brain [144-146]. Steroid receptors are composed of three main functional domains: a hormone-independent activation function 1 domain (AF-1 domain), a DNA-binding function (DBD domain) and a hormone-dependent activation function 2 domain (AF-2 domain) (Fig. 17) [147].

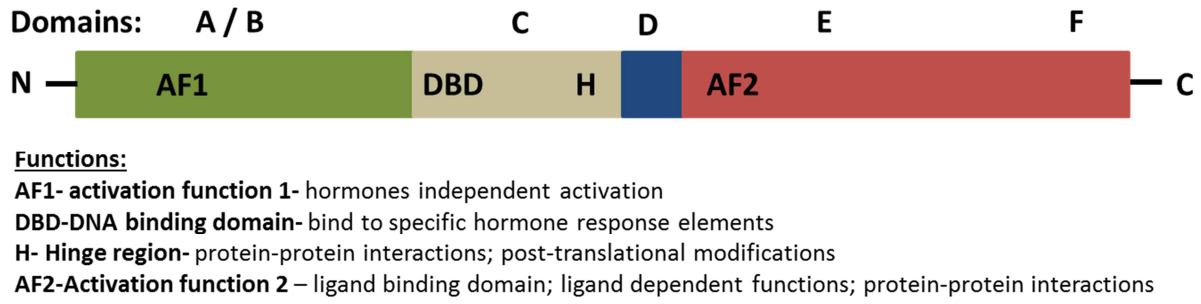

Figure 17: General structure of nuclear hormone receptors. Steroid receptors are generally composed of multiple (5–6) structural domains (A–F), and functional domains (in colors) but differ in details. Adapted from [147]

In the cytoplasm, there is an inactive receptor protein that dimerizes when ligands bind to it. Itis translocated to the nucleus and binds to specific DNA sequences and regulates the transcription of genes which are sensitive to steroids.

Besides, neurosteroids can act as allosteric modulators of neurotransmitter receptors of the $GABA_A$ central-type benzodiazepine receptor complex, NMDA receptors (**Fig. 18**), kainate receptors, AMPA receptors, sigma receptors and glycine receptors via membrane receptors [129, 138, 139, 148]. Reduced metabolites of hormone steroids like allopregnanolone or sulfated steroids like pregnenolone sulfate do not directly influence gene transcription, because they do not bind to the cytoplasmic steroid receptor. Genomic steroid action could be developed relatively slowly (minutes to days), while the non-genomic steroid effects are insensitive to inhibitors of transcription and protein synthesis, and have a rapid onset of action (seconds to minutes). PREG can also be sulfated by the hydroxysteroid sulfotransferase (HST) to form PREG sulfate. Pregnenolone sulfate is known to act as an excitatory neurosteroid, a negative regulator of $GABA_A$, kainite and AMPA receptors and as a positive regulator of NMDA receptors [129, 130]. Allopregnanolone is a positive $GABA_A$-acting at nanomolar concentration as an allosteric modulator by opening the channel and improve chloride flux.

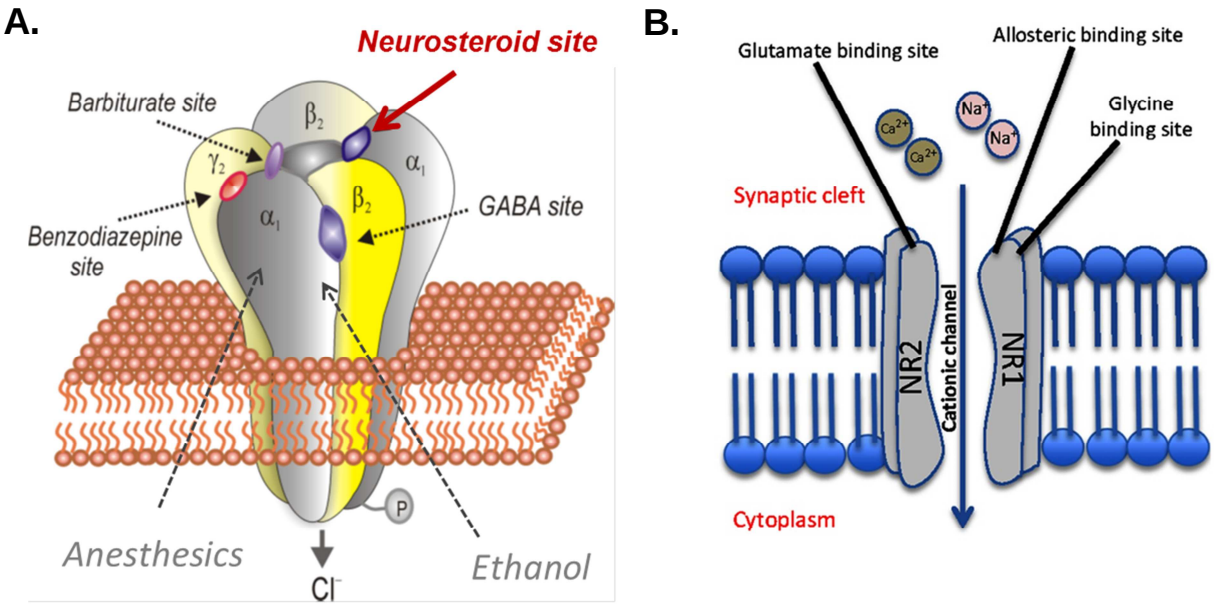

Figure 18: Structure of $GABA_A$ (A) and NMDA (B) receptors. NMDA receptor contains four subunits, two glycine binding NR1 subunits and two glutamate binding NR2 subunits, and allows for cationic influx from the synaptic cleft into the cell. Adapted from [149, 150]

4. Age-related changes in neurosteroid levels

Steroid hormones synthesized by steroidogenic glands are lipophilic compounds that can cross the blood-brain barrier and act in the nervous system. Age-related changes in brain neurosteroid levels have been demonstrated in several studies. Schumacher and collaborators reported that the blood level of neuroactive steroids decreased with age [151]. Men showed a gradual reduction in testosterone of approximatively two percent per year and women estrogen level drop after the menopause [152, 153]. Neurosteroids are locally synthesized within the nervous system. Steroid blood levels do not necessarily correlate with steroid concentrations in the brain [151]. In the hippocampus of aged rats (2 years old), the synthesis of PREG sulfate was decreased compared to young rats (3 months old) [154]. Cognitive impairments in old rats are correlated with the reduction of PREG sulfate in the hippocampus, a brain region involved in learning and memory. Injection of PREG sulfate decreased the cognitive deficits, suggesting the importance of neurosteroids for the maintenance of cognitive function during aging. Caruso and colleagues showed an alteration in neurosteroid levels with a general trend toward lower steroid levels in the brain of aged mice (24 months old) compared to young mice (7 months old) [155]. 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 [156]. The unprotected brain could be predisposed to several age-related illnesses such as Alzheimer's disease [155]. Several studies investigated neurosteroid administration to reverse aging processes including mood, memory, energy level, and overall quality of life [157, 158].

5. Neurosteroid level disturbance in Alzheimer's disease

Neurosteroids including pregnenolone and allopregnanolone play an important role in the performance of memory, aging conditions, and physiopathology. Indeed, some studies performed in human and animal models have shown that age-related drop of neurosteroid levels give rise to neuronal dysfunction and degeneration owing to the loss of neurosteroid protective and neuroregenerative effect [155, 159].

Neurosteroids are known as regenerative agents in the brain. In particular AP presents several beneficial effects in neurodegenerative disease and CNS or PNS disorders [160, 161]. AP is also known to ensure the recovery of memory and learning function and reduce age-related neurodegenerative diseases such as AD pathology in preclinical studies of efficacy [161].

In post-mortem brains from AD patients, TSPO has been shown to regulate the first step of steroidogenesis by improving the level of PREG in the hippocampal region [162]. In AD brains the level of a steroid intermediate in the conversion of PREG, the 22(R)-hydroxycholesterol, is found at lower levels compared to controls, suggesting that TSPO presents abnormal functions in AD patients [163, 164]. DHEA is increased in AD brains and the cerebrospinal fluid when compared to controls [163]. After treating oligodendrocytes with Aβ peptide under stress condition, DHEA is up-regulated [165]. Aβ and hyperphosphorylated tau distinctly impacted on neurosteroidogenesis in a cellular AD model [166]. PROG and 17OH-PROG production was reduced in APP overexpressing cells, whereas 3α-androstanediol and estradiol levels were increased. AD key proteins suggest a modulation, directly or indirectly, of the biological activity of the enzymatic machinery producing neurosteroids. Caruso and collaborators showed that modified levels of progesterone and testosterone metabolites in aged mice (24 months) compared to young (7 months), were associated with Aβ accumulation and gliosis [155]. In post mortem brains of aged AD patients and aged non-demented controls, several neurosteroids quantified were reduced and associated with Aβ as well as hyperphosphorylated tau protein [151]. Allopregnanolone is proposed in several studies as a plasmatic biomarker for AD because of its reduced level in plasma of demented patients [159]. Interestingly, previous data from our groups provided first evidence that the presence of Aβ and abnormal tau protein have a direct impact on neurosteroidogenesis in *in vitro* models of AD [166, 167]. In a transgenic mouse model of AD (APPswe+PSEN1Δ9 mice), animals displayed a decreased ability to form AP in the hippocampus compared to their wildtype littermates [168].

Disturbances in neurosteroid metabolism may be an underlying mechanism in AD. Neurosteroids offer interesting therapeutic opportunities because of their pleiotropic effects in the nervous system.

E. Allopregnanolone

1. Allopregnanolone in psychiatric disorders

AP plays an important role in the CNS in psychiatric disorders as anesthesic, analgesic, anxiolytic as well as antidepressant and sedatives characteristics via activation of $GABA_AR$s [169, 170]. Some studies suggested that a low level of allopregnanolone in the brain could be a risk factor for the development of psychiatric disorders. Several affective disorders such as depression may be correlated with a reduction of AP in cerebrospinal fluid, plasma and serum [171, 172]. Other affective disorders such as anxiety disorders [173], post-traumatic stress disorders [174], negative symptom in schizophrenia [175] or impulsive aggression [176] may be linked to low levels of AP. Elevated plasma AP levels in patients with panic disorders were observed [177], while AP levels are reduced in bipolar patients during depressive episodes [178], suggesting a protective stabilizing role of AP. In a posttraumatic stress disorder study (PTSD), decreased levels of AP have been correlated with the aggressive behavior of socially isolated mice in corticolimbic regions. Then, Pibiri and collaborators demonstrated that AP administration improved PTSD and anxiety [179]. AP brain levels were restored by administration of selective serotonin reuptake inhibitors (SSRIs) such as Fluoxetine, an antidepressant agent in affective disorders like depression. Fluoxetine attenuates behavioral deficits exhibited by socially isolated mice correlated with an increase of AP levels [180, 181].

Nevertheless, AP presents some restrictions for its therapeutic use by having a low bioavailability and a rapid inactivation (glucuronidation and sulfate conjugation), but also being inactivated by oxidation of the ketone at the 3α-hydroxyl group (**Fig. 19**).

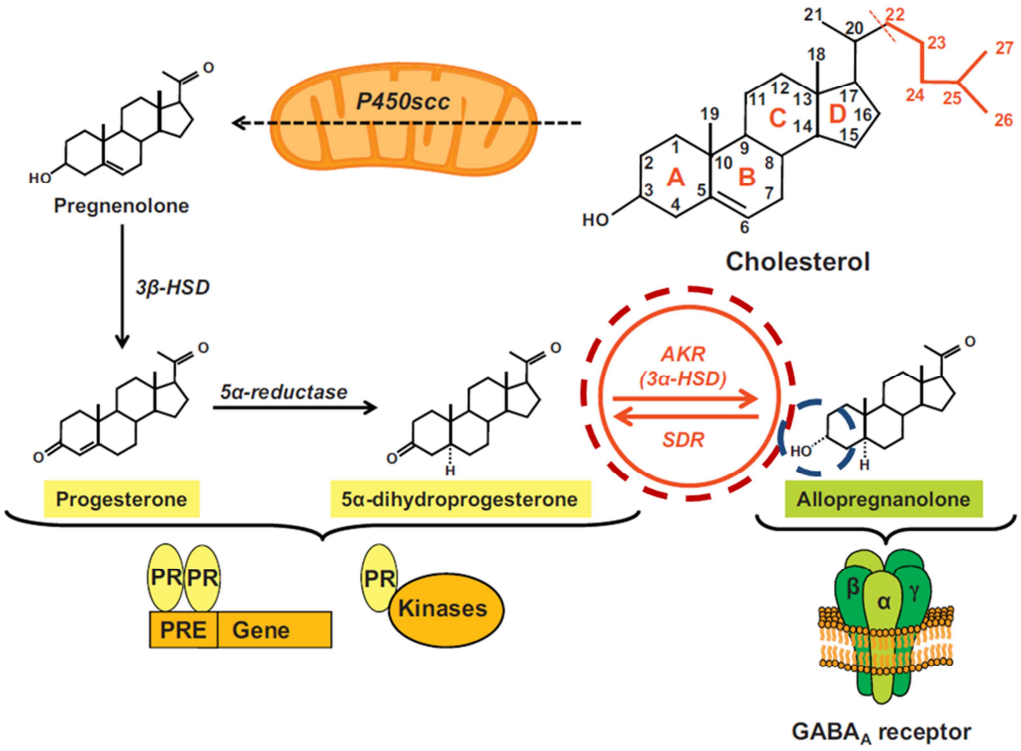

Figure 19: **Biosynthetic pathway of allopregnanolone.** 5a-DHP can be metabolized to allopregnanolone (3a, 5a-tetrahydroprogesterone, AP) by aldo-keto reductases (ARK; 3a-HSD = 3a-hydroxysteroid dehydrogenase). Because of its hydroxyl group at C3 (bleu circle), AP has no affinity for the intracellular PR, but is a potent allosteric modulator of GABAA receptors. However, AP can activate gene transcription via PR after being converted back to 5a-dihydroprogesterone by short-chain dehydrogenases/reductases (SDR), (red circle). Adapted from [182]

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 [183]. Ganaxolone appears to improve dysfunctional emotional behavior associated with deficits in AP in mice and provides an alternative treatment for PTSD patients [184]. Another synthetic analog of AP, Co 2-6749 is known for its anxiolytic effect by modulating the receptor GABAA [185]. Other synthetic neurosteroids have been investigated with respect to their therapeutic potential such as alphaxalone, and minaxolone.

2. Allopregnanolone in neuropathic pain

In the literature, AP is proposed as a therapeutic candidate for the treatment of anti-cancer drug-induced painful neuropathy. Indeed, in our laboratory some studies demonstrated which monitored nociception the presence and the activity of enzymes implicated in AP synthesis in nervous structures [186-190]. They also showed that binding sites of AP are present in the spinal cord dorsal horn, which controls pain transmission, and that endogenous AP is involved in the control of sciatic nerve injury-induced neuropathic pain [127, 140, 189, 191]. The inhibition of the enzyme 3α-HSD by using siRNA induced an increase in pain perception [188, 192]. Oxaliplatin and vincristine are antineoplastic drugs used in cancer chemotherapy and however also induce neuropathic pain, such as hyperalgesia and allodynia, as a side-effect [193]. Data from our lab showed that AP supressed hyperalgesia and allodynia, when applied either before (prophylactic) or after (corrective) treatment with vincristine or oxaliplatin [194, 195]. Zheng and collaborators reported the presence of functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy, particularly deficits in mitochondrial respiration and ATP production [196].

Taking together, all these studies suggested AP as a promising therapeutic candidate for the treatment of mitochondrial deficits involved in anti-cancer drug-induced painful neuropathy.

3. Neuroprotective effect of allopregnanolone in neurodegenerative disorders

Allopregnanolone is known to ensure the recovery of learning and memory function and reduced aged-related neurodegenerative disease [161]. AP was also shown to reduce cognitive deficits and protect against cell death after a contusion of the rat pre-frontal cortex by decreasing the expression of the pro-apoptotic proteins caspace-3 and Bax, reduced mitochondrial cytochrome c release and apoptotic DNA fragmentation [197, 198]. AP enhanced Purkinje and granule cell survival in the cerebellum, retarded the beginning of neurological symptoms, reduced cholesterol accumulation, decreased inflammation and promotes myelination in Niemann-Pick type C disease [199, 200]. Allopregnanolone, is more

effective than progesterone to decrease cerebral infarction volume, improved blood brain barrier integrity as well as memory and learning in animal models of acute ischemic stroke [201]. In an animal model of excitotoxicity, ovariectomized female rat injected with kainic acid showed a reduction of reactive gliosis in the hippocampus after AP administration [202]. In a model of multiple sclerosis (EAE), AP reduced the immunoreactivity of several markers in the lumbar spinal cord, thus blocking aggravation of the immune response [203]. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a chemical toxin that produces parkinsonian pathology in mice.Aedosun and collaborators showed that AP promotes the restoration of tyrosine hydroxylase immunoreactive neurons and total cells in the nigrostriatal tract, improves the motor performance in MPTP-treated mice, and may serve as a therapeutic strategy for Parkinson's disease [204]. AP appears as a potential therapeutic candidate against AD in the CNS as detailed below, but also in the PNS. Diabetic neuropathic pain is induced by hyperglycemia causing oxidative stress in neurons, resulting in neuronal cell apoptosis and dysfunction. AP has been shown to possess protective effects against hyperglycemic-induced cellular damage and prevention of cell apoptosis is involved in its mechanisms *in vitro* and *in vivo* studies [205]. As detailed above, Patte-Mensah and group showed the ability of AP to reduced chemotherapy-induced peripheral neuropathy [160, 194, 195]. Recently, allopregnanolone shows neuroprotective effect by attenuating neuronal injury induced by oxygen-glucose deprivation/reoxygenation in organotypic hippocampal slice cultures [206].

4. Allopregnanolone, a therapeutic candidate for the treatment of Alzheimer's disease pathology

Data obtained by the group of Roberta Brinton report 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 [207]. Further, acute single administration of AP promotes neurogenegesis 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 [208]. Next, Chen and colleagues demonstrated that AP administrated 1/week for 6 months promotes survival of newly generated neurons and decreased Aβ generation in hippocampus, cortex and amygdala in 3xTgAD at 3 months of age [209]. They showed also that AP reduces microglia activation, increases oligodendrocyte myelin markers and ameliorates expression of protein regulating cholesterol homeostasis and clearance from brain such as liver X receptor (LXR) and 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 memory and learning [210]. AP promotes regeneration and repair in the brain, recovery of learning and memory function and reduces AD pathology [161]. Therapeutic windows of AP efficacy in preclinical studies are defined by age and AD burden. Brinton analysis there are three aspects of regulation of neurogenesis by AP that are important to consider: i) the APα restores the regenerative potential of the brain to a normal level, not above normal, ii) the effect of regenerating the AP is dose-dependent with a classical dose-response curve for a growth factor by which the overrun neurogenic doses does not lead to higher response, iii) regenerating the AP effect varies with the age of the subject and the level of progress of the disease [161].

The intermittently administration of AP promoted renewal and repair while continuous infusions of AP were antiregenerative in mouse models of AD [161]. All these studies showed that AP ensured the neuroprotection against the Aβ toxicity in 3xTgAD mouse model but also the stimulation of rodent and human neural progenitor cell proliferation to compensate the cell loss [207, 209].

F. Implication of TSPO

1. TSPO definition

The receptor translocator protein, commonly named TSPO (18Kda) plays a fundamental role in steroidogenesis. TSPO was previously called peripheral-type benzodiazepine receptor (PBR) due to its high affinity to benzodiazepines and to be distinguished from the central benzodiazepines receptor. PBR nomenclature defines certain characteristics of the protein but does not reflect its nature and function. In fact, other ligands of other structures such as cholesterol could bind to this receptor and the term receptor and peripheral type are imprecise [164]. Then, Papadopoulos and his team renamed the receptor TSPO. The ligand cholesterol is needed by the cells to overcome the IMM and the OMM through TSPO in order to continue its transformation into neurosteroids. In order to satisfy their cholesterol demand, cells have several sources: (i) they can synthesize it *de novo* in the endoplasmic reticulum (ER) from acetate. Next, it is transported to the Golgi apparatus to be transferred to the mitochondria by binding the protein acyl-CoA binding domain containing 3 (ACBD3), (ii) cells can mobilize cholesterol from the plasma membrane (in humans from plasma low-density lipoproteins, LDLs), which is rich in cholesterol, or (iii) they can mobilize it from lipid droplets [142, 162].

2. Localization and structure of TSPO

TSPO ismainly located in the OMM [211]. Its presence is also confirmed in nuclear fractions of normal and cancerous human liver tissues, as well as in the plasma membrane and other organelle membranes of diverse cell types [212]. In the central nervous system (CNS), the localization of TSPO is in ependymal and glial cells and also expressed throughout the body, particularly in tissues which produce steroids such as adrenocortical cells [164].

The TSPO is part of a 240kDa [213] protein complex that is located in the contact zones between the OMM and IMM mitochondria. In 1989, Sprengel and collaborators were able to identify the DNA sequence encoding the TSPO and to deduce the protein sequence of TSPO

[214]. This protein consists of 169 amino acids and has a molecular weight of about 18 kDa. After decoding the sequence, a hydropathic analysis was used to highlight the presence of 5 transmembrane regions. The model described in this time has since been confirmed and slightly modified, in particular as regarding the size of the areas transmembrane [215]. The TSPO structure is composed of five alpha helices forming a pore that allows the passage of cholesterol and possesses a ligand binding site [85].

3. Structure and function of the complex TSPO

TSPO is a single protein, but to perform the function of the complex, it needs the help of other mitochondrial proteins. The other proteins are StAR (30 kDa steroidogenic acute regulatory protein), VDAC (32kDa voltage dependent anion-channel), ANT (30 kDa adenine nucleotide translocase), PKARIα (54 kDa cAMP dependent protein kinase regulatory subunit Iα), ACBD3 (Acyl-coezyme A binding domain containing 3), and PAP7 (60 kDa PBR-associated protein 7). This multiprotein complex of 240kDa is called “transduceosome” (**Fig. 20**).

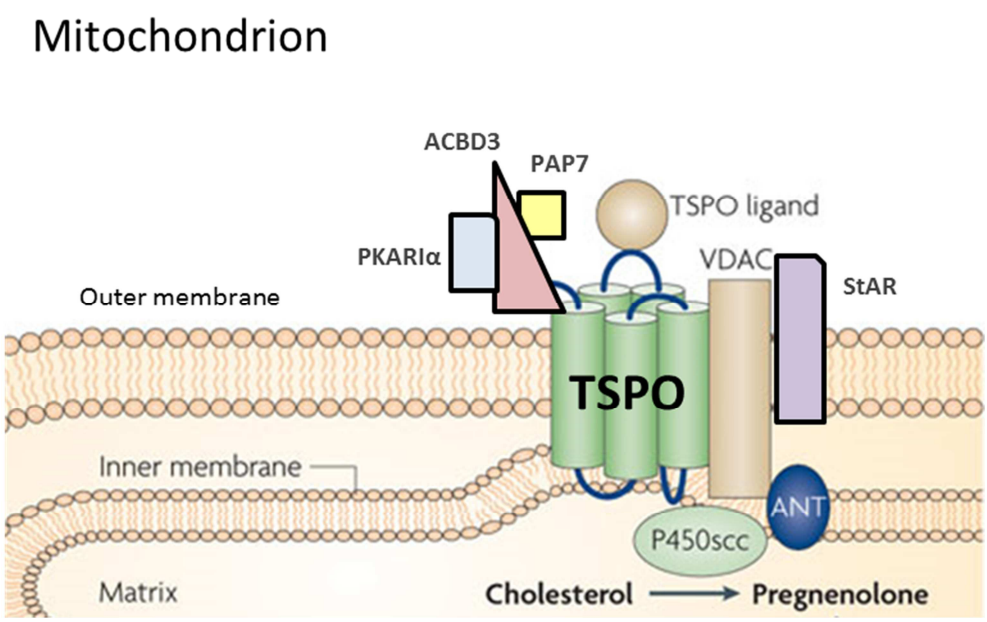

Figure 20: Complex of proteins called transduceosome, necessary for the passage of cholesterol in mitochondria. It is composed of TSPO (18kDa), VDAC, StAR, ANT, PAP7, ACBD3, PKARIα, P450scc. Adapted from [216]. (VDAC) 32kDa voltage dependent anion-channel; (StAR) steroidogenic acute regulatory protein; (ANT) 30 kDa adenine nucleotide translocase), (PKARIα) cAMP dependent protein kinase regulatory subunit Iα, (ACBD3) Acyl-coezyme A binding domain containing 3, (PAP7) PBR-associated protein 7; (P450scc) cytrochrome P450 side chain cleavage.

Cholesterol binds to the StAR protein and is then translocated by TSPO into the mitochondria. TSPO plays a crucial role by representing the main actor of the transduceosome and is the only rate-determining step of the steroidogenesis [85]. The annexed proteins are structured to support the passage of cholesterol. Furthermore, VDAC interacts with TSPO creating a channel protein that regulates the passage of small ions and molecules through the OMM and determines the mitochondrial membrane potential. The mitochondrial permeability pore (MPTP) is formed by the interaction of ANT and VDAC, and regulates the cell apoptosis [162]. Cholesterol in the IMM is converted by cytochrome P450scc to PREG, which is the precursor of all the neurosteroids.

4. TSPO ligands:

TSPO ligands are molecules with high affinity for the TSPO binding site. The presence of ligand can activate TSPO, increasing the cholesterol flux into mitochondria and stimulating the pregnenolone production which is correlated with an increase of neurosteroids synthesis such as allopregnanolone (**Fig. 21**).

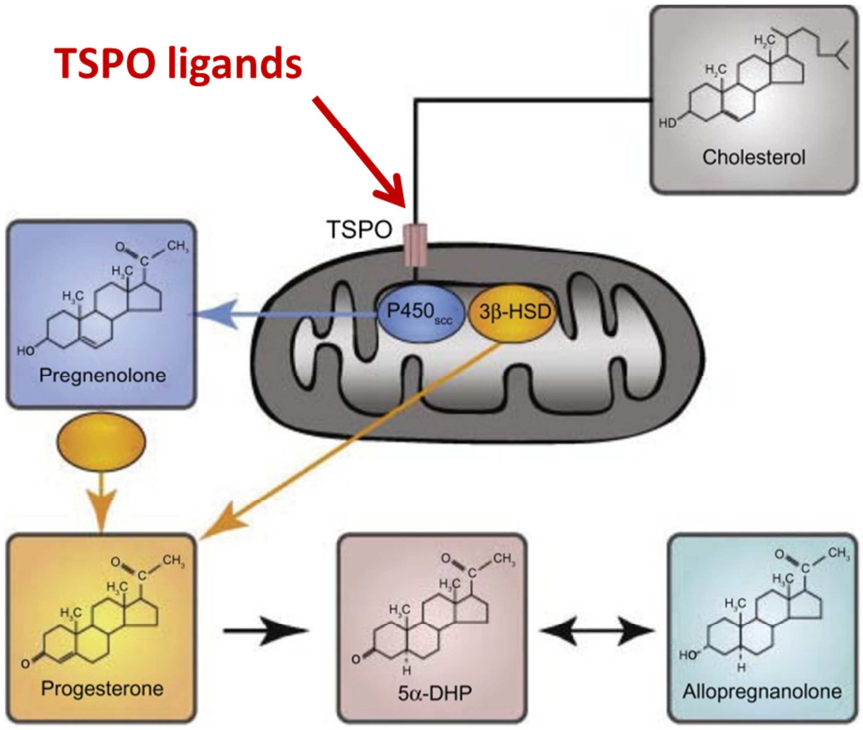

Figure 21: Ligands TSPO can stimulate the passage of cholesterol into the mitochondria and the synthesis of pregnenolone , the precursor of neurosteroid biosynthesis. Adapted from [217].

a) Endogenous TSPO ligands:

Few endogenous TSPO ligands have been described in the literature. Cholesterol and porphyrins are endogenous ligands that bind at nanomolar affinity to the C-terminal into the cytosol at the CRAC domain and the N-terminal area respectively. Another TSPO ligand, endozepine was isolated from rat brain extracts neuropeptides and showed an ability to displace the binding of benzodiazepines to their binding site on the GABA. These

compounds are derived from endogenous proteolysis DBI (Diazepam Binding Inhibitor) [218]. The two fragments octadecaneuropeptide DBI33-50 (ODN) and triakontatetraneuropeptide DBI17-50 (TTN) are the most active and they stimulate the synthesis of steroids by mitochondria [219]. The endozepines are synthesized by Schwann cells in peripheral tissues. The expression of TSPO and production of endozepines are increased in damaged tissue [220]. In the CNS, the gene encoding DBI is mainly expressed in glial cells. In AD, the expression of endozepine seems to be involved in the autocrine and paracrine response of glial cells. The synthesis of endozepine is stimulated by the Aβ peptide, which has a pathogenic role in AD. High levels of DBI were identified in CSF of AD patients [221].

All these observations demonstrated the therapeutic potential of TSPO ligands in the treatment of neurodegenerative diseases.

b) Synthetic TSPO ligands:

Some studies indicated that synthetic ligands of TSPO have been developed for three purposes: i) increasing the understanding of the mechanisms of TSPO, ii) the use of medical imaging as markers of inflammation, and iii) discovering new ways to treat diseases affecting the CNS. TSPO ligands have been developed as neuroimaging agents and as diagnostic tools for *in vivo* imaging of TSPO to visualise affected areas in disease brains [222].

c) Therapeutic use of TSPO ligands

i) *LTSPO in psychiatric disorders*

Neurosteroids from the conversion of cholesterol to pregnenolone are allosteric modulators of the GABA. Stimulation of TSPO increases the concentration of neurosteroids in the central level and modulates synaptic transmission. Several ligands TSPO such as etifoxine, the XBD-173, series imidazopyridines and acetamides phenoxyphenyl have shown anxiolytic and anti-panic properties in experimental animal models. The main advantage of TSPO ligands in these indications is the rapid anxiolytic effect, similar to benzodiazepines without

their deleterious effects of tolerance and withdrawal / dependence. These properties have been demonstrated for the XBD-173 in humans and animal [173, 223]. XBD-173 has a long residence time (RT, 127 min) leading to an efficacious stimulation of the neurosteroidogenesis, in terms of pregnenolone production [224]. TSPO ligand RT is important to predict their effective neurosteroidogenic activity and promising anxiolytic action. More generally, the effects of allopregnanolone on anxiety and depression are relatively well described, we can speculate a beneficial effect of the ligands in this type of disease. It is important nevertheless to conduct longer clinical trials to study if hepatic toxicity alpidem or etifoxine is related to their chemical structures or their action on the TSPO [216].

ii) LTSPO in Alzheimer's disease

A consortium of researchers from several countries studied the effect of Ro5-4864 on the development of Alzheimer's disease in transgenic mice triple 3xTgAD [216]. They studied the effects on neurosteroidogenesis and neuropathology associated with AD including the accumulation of Aß, gliosis and behavioral deterioration in elderly 7 or 24 months mice to compare the therapeutic intervention at different stages of disease development. They observed that the administration of Ro5-4864 attenuated accumulation of Aβ in the hippocampus, decreased the gliosis, altered the secretion of testosterone and progesterone in the brain and improved cognitive measures 3xTgAD mouse. The anxiolytic effects of Ro5-4864 are also responsible for these results, because anxiety is involved in cognitive deterioration of Alzheimer's patients or 3xTgAD mouse. Simultaneous targeting of both components (neurodegeneration and anxiety) improves cognitive outcomes among 3xTgAD mouse.

This is the first study showing the beneficial effect of TSPO ligands on Alzheimer's disease by acting on several clinical targets as memory function and anxiety behaviors. TSPO Ligands seem to offer an alternative therapeutic strategies focused on reducing the accumulation of Aβ as they simultaneously target multiple facets of neurodegenerative

cascade as neuroinflammation, oxidative stress, mitochondrial dysfunction and neuronal loss.

G. PhD project

1. Hypothesis

Evidence shows that mitochondrial abnormalities are involved in the pathophysiology of Alzheimer's disease (AD). A decreased energy metabolism and an increase in oxidative damage were demonstrated at early stages of the disease before the generation of amyloid plaques and neurofibrillary tangles, representing the two main histopathological characteristics of AD. Therefore, current pharmacological concepts for the development of therapeutic strategies against AD have a particular interest in the track of the regulation of mitochondrial functions in neurons, including the control of ATP synthesis, mitochondrial respiration, and ROS production. Interestingly, several studies showed a decreased age-related production of certain neuroprotective neurosteroids like allopregnanolone (AP) that also exhibits neurogenic, and neuroregenerative effectsand stimulates cell proliferation. The decrease of neurosteroïdogenesis and brain concentrations of AP has also been correlated with a cognitive decline seen in AD. These data have generated strong interest in the development of therapeutic strategies based on the use of the AP. However, the pleiotropic nature of the effects of the allopregnanolone does not facilitate the development of targeted therapy with regard to a specific indication, such neuroprotection while avoiding stimulating cell proliferation that may facilitate the emergence of glioma. However, in cases of functional deficits that are related to a decline in neurogenesis, it might be interesting to identify analogs having a greater neurogenic action and show a more specific mode of action than the AP. In view of the data mentioned above, the main purpose of the thesis was to evaluate the ability of two great families of new patented compounds exerting neuroprotective effects via the modulation of mitochondrial function in nerve cells. These two families of compounds are the novel allopregnanolone analogs (ANS) and the ligands of the mitochondrial translocation of protein or translocator protein (LTSPO) which transfers the outer membrane cholesterol to the inner mitochondrial membrane where is located the cytochrome P450side-

chain-cleavage catalyzing the conversion of cholesterol to pregnenolone, a major precursor in the biosynthesis of various neurosteroids.

The novel ANS were developed and synthetized by the laboratory of Dr. Miesch (University of Strasbourg) (Patent number *PCT/FR2012/05616*; *WO 2012/127176 A1*; *US 2014/0058079 A1*). The ANS compounds tested included four analogs: pregnane-12,20 dione 3-hydroxy (3α, 5α) or **12 oxo-AP**; pregnane-12,20 dione 3-hydroxy (3β, 5α) or **12 oxo-epiAP**; pregnan-20 one 3 (2-propen-1-yloxy) (3α, 5α) or **O-allyl-AP**; pregnan-20 one 3-(2-propen-1-yloxy) (3β, 5α) or **O-allyl-epiAP**. Allopregnanolone possess a rather a low bioavailability which is due to its rapid inactivation through sulfatation or glucuronidation at the 3α-hydroxyl group. On the one hand, derivative analogs of allopregnanolone at the hydroxyl group should theoretically exhibit a higher biological efficacy by preventing its inactivation and elimination. On the other hand, certain analogs of allopregananolone were developed with the absence of a free hydroxyl group at position 3 in order to prevent the re-oxidation of allopregnanolone to 5α-dihydroprogesterone which may enable additional non-allopregnanolone specific genomic effects via binding to the nuclear steroid receptor.

TSPO ligands seem to offer alternative therapeutic strategies focused on reducing the accumulation of Aβ, as they simultaneously target multiple facets of the neurodegenerative cascade, such as oxidative stress, mitochondrial dysfunction and neuronal loss. Moreover, the LTSPO are important modulators of neurosteroïdogenesis. Although many TSPO ligands were already described in the literature, they suffer from a common problem of solubility and bioavailibility. Based on detailed analyses of structure-activity relationships associating TSPO with its ligands (endogenous or synthetic), several ligands of different chemical structures were developed and tested: imidazo[1,2-c]quinazolinone family with nanomolar affinity and a good selectivity for TSPO. The novel TSPO ligands 6a and 6b effects were compared to TSPO ligands described in the literature (diazepam, Ro5-4864, XBD-173 and SSR-180,575). The TSPO ligand 6b has a structure similar to 6a, but differs by a lateral chain dipropylamide instead of diethylamide.

2. Objectives

The main objectives of my thesis work were:

1- to evaluate the ability of allopregnanolone and its analogs to improve mitochondrial functions in an *in vitro* cellular model of AD mimicking the enhanced generation of β-amyloid peptide (Aß) in human SH-SY5Y neuroblastoma, overexpressing the amyloid precursor protein or APP (APP cells)

2- To test the ability of ANS to attenuate the decrease of mitochondrial activity *in vivo* in a transgenic mouse model (Tg2576) reproducing symptoms of AD.

3- To examine the effects of ANS against the age-dependent decline of mitochondrial activity *in vivo*.

4- To investigate the effects of novel TSPO ligands compared to reference ligands with regard to improve the energy balance in APP and in control cells.

3. Experimental models

a) <u>In vitro model</u>

To reach our objectives, the human neuroblastoma cells SH-SY5Y cells, a cellular model that is widely used in neuroscience research and possesses all the neuronal features, were used [113, 225-227]. The human neuroblastoma cells stably transfected with the wild type form of human APP represents a cellular AD model that is well established in the Eckert laboratory. These cells present characteristics found in AD pathology including an increased Aβ production and ROS generation as well as impaired mitochondrial function such as decrease in ATP production, mitochondrial respiration and mitochondrial complex IV activity [113, 228, 229]. SH-SY5Y cells are described also to express several steroidogenic enzymes implicated in the neurosteroidogenesis [135, 166, 167]. Previous studies from our laboratory have shown that APP cells exhibited a reduction of neurosteroid production compared to their respective control cells [167]. All bioenergetics experiments were carried out in the Eckert

laboratory following a multilevel assay approach developed and established by the Eckert group with the aim to screen and characterize drugs with regard to their potential to ameliorate mitochondrial activity and function in control and APP cells [228, 229]. MTT reduction assays (for the evaluation of cell viability/ neurotrophic potential), ATP bioluminescence assay (as a measure of energy homeostasis), oxygen consumption by mitochondrial respiration (using an innovative protocol enabling the determination of oxygen consumption in whole cells), reactive oxygen production (using fluorescent dye markers), as well as pregnenolone production of SH-SY5Y cells by using a newly established pregnenolone direct ELISA blocking specifically the pregnenolone metabolism with trilostane and abiraterone).

i) *ANS*

The effects of ANS and allopregnanolone were evaluated under physiological condition on the amelioration of the bioenergetics by measuring the ATP production, the activation of the metabolic activity and the mitochondrial respiration. Previous studies in our laboratory showed that compared to control cells, APP cells are more sensitive to oxidative stress using the hydrogen peroxide (H_2O_2) which is well established in the literature to generate relevant experimental models [230, 231]. The neuroprotective effects of the best analog and allopragnanolone were also investigated under oxidative stress using (H_2O_2) against the bioenergetic deficits leading to a neuronal death.

ii) LTSPO

The effect of a set of novel synthetic TSPO ligands was investigated on the mitochondrial bioenergetics in APP cells, compared to TSPO reference ligands described in the literature: diazepam, Ro5-4864, XBD-173 and SSR-180,575 [173, 232-234].

b) In vivo models

The ability of the selected analog was examined to ameliorate the mitochondrial deficits seen in the Tg2576 mice, used as an AD mouse model and in 21-month-old C57BL/6J male mice, used as normal brain aging mouse model. The treatment design was developed by the principal investigator of the Neuro-Rhine project, Professor Mensah-Nyagan, and the Mathis and Cassel group. Tg2576 exhibit an overexpression of APP and develop amyloid plaques and cognitive deficits [235]. On both experimental models, analysis of ATP levels as well as mitochondrial complex I and complex IV activity were used to evaluate the effect of the analogs on age or AD-induced mitochondrial impairments in the cortex and hippocampus, following already established protocols of the Eckert group with the regard to evaluate mitochondrial dysfunction in the brain of transgenic AD as well as the neuroprotective potential of drugs/new compounds *in vivo* [236, 237].

H. References

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II. Results

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

B Neuroprotective effect of BR297, analog of allopregnanolone, on Tg2576 Alzheimer's disease mouse model-related mitochondrial dysfunction

C Neuroprotective effect of analogs of allopregnanolone on age-related mitochondrial dysfunctions

D The future of TSPO ligands in Alzheimer's disease

A. 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 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 cells 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 H_2O_2-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 (Aβ) 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 Aβ 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 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 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 Aβ 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 (5α-pregnane-3α-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 synthetize from progesterone by the enzyme 5α-reductase and then 3α-

hydroxysteroid oxidoreductase, with the 5α-dihydroprogesterone (5α-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 acide 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 (APPswe+PSEN1Δ9 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 Aβ 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 5α-DHP by NAD^+- dependent membrane-associated short-chain dehydrogenases/ reductases via its 3α-hydroxyl group. This reconversion can induce undesirable side effects by the binding of 5α-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/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), 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 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 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 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_2that 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 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/Aβ 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 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.

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 neurogenegesis 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 Aβ 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 Aβ 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 3α-hydroxyl group which decrease its bioavailability. Furthermore, AP can be converted back to 5α-dihydroprogesterone (5α-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 weredeveloped 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 Aβ 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 3β-O-allyl-Epi-Allopregnanolone, BR 297, which has no hydroxyl group, cannot be converted back to other metabolite such as 5α-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 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).

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. H_2O_2, a major ROS, is known as a mediator of brain damages caused by the abnormal elevation of β-amyloid peptides (Aβ) in AD (Behl, Davis et al. 1994, Citron 2010). First, we demonstrated that APP cells are more sensitive to H_2O_2 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 H_2O_2.

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 H_2O_2 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 gluthatione system).

In line with a recent study, the compound BR 297 (3β -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 H_2O_2-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.

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Material 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, Hambsch et al. 2001). 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_2alone.

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_2DCF-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

Figures caption

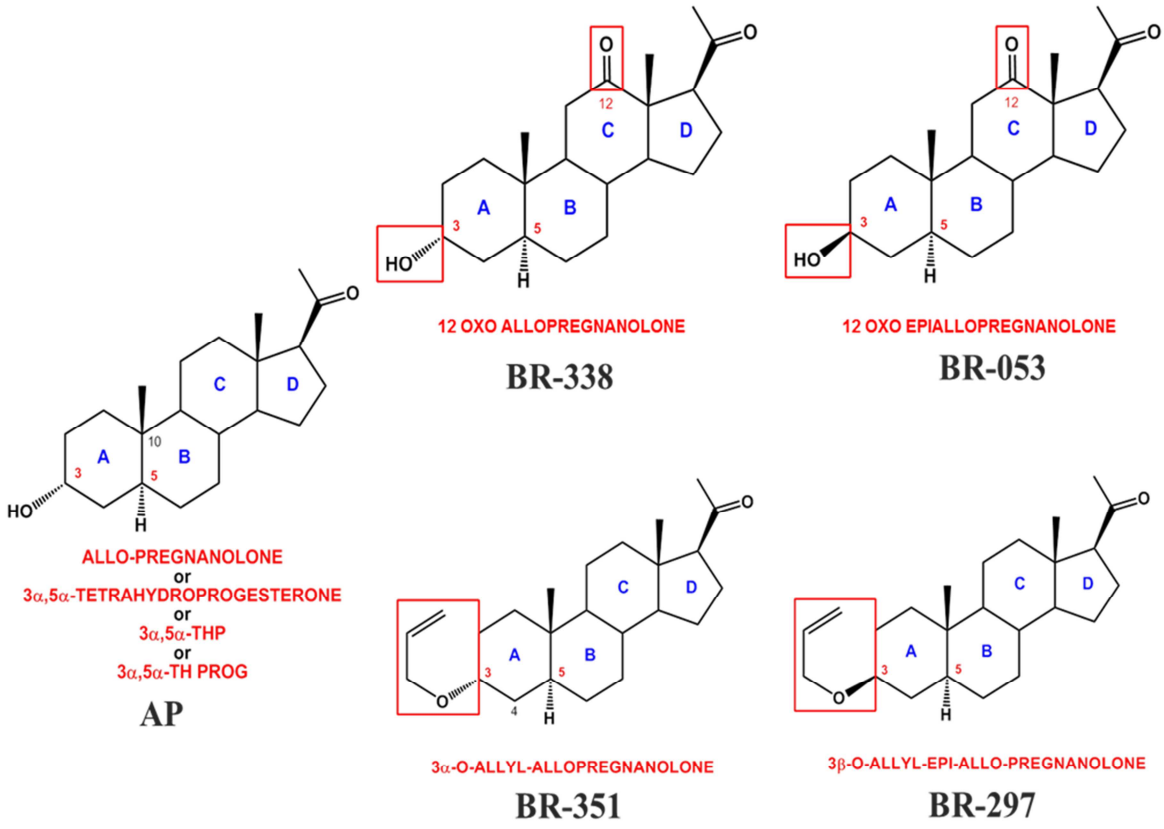


Figure 1: Structure of the synthetic analogs (ANS) of the natural neurosteroid allopregnanolone. Chemical structures derived from allopregnanolone or epiallopregnanolone: 12-oxo-allopregnanolone or BR 338 (12 oxo-AP), 3α-O-allyl-allopregnanolone or BR 351 (O-allyl-AP), 12-oxo-epiallopregnanolone or BR 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 hydroxyle group preventing enzymatic oxidation of the hydroxyle group and thus re-conversion.

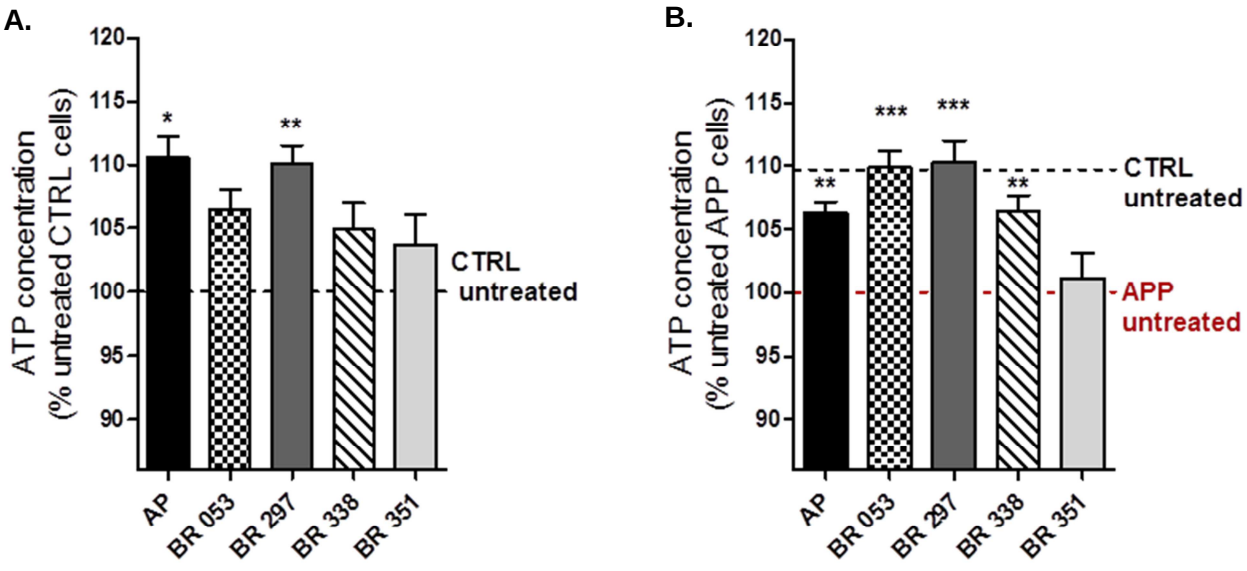


Figure 2: AP and its analogs increase ATP level in CTRL and APP transfected cells. ATP level was measured after a treatment of 24 h with AP, BR 053, BR 297, BR 338 and BR 351 at a concentration of 500 nM in (A) CTRL and (B) APP cells. Values represent the mean ± SEM (n=12-18 replicates of three independent 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.

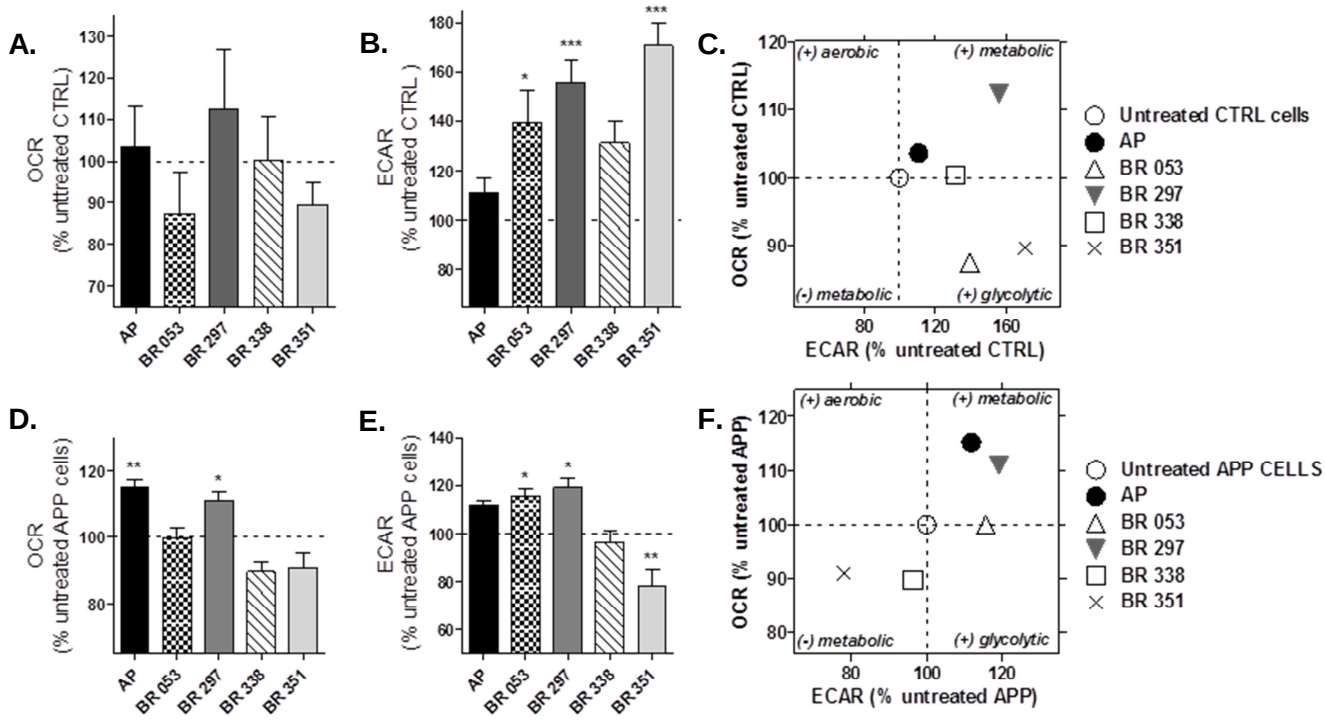


Figure 3: Modulation of the bioenergetic phenotype by AP and its analogs. (A, D) Oxygen consumption rate (OCR) and (B, E) extracellular acidification rate (ECAR) were measured simultaneously using a Seahorse XF24 Analyzer in the same experimental conditions in CTRL cells (A-B) and APP cells (D-E). Values 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%); OCR: Oxygen Consumption Rate (mitochondrial respiration), ECAR: Extracellular Acidification Rate (Glycolysis), AP: allopregnanolone.

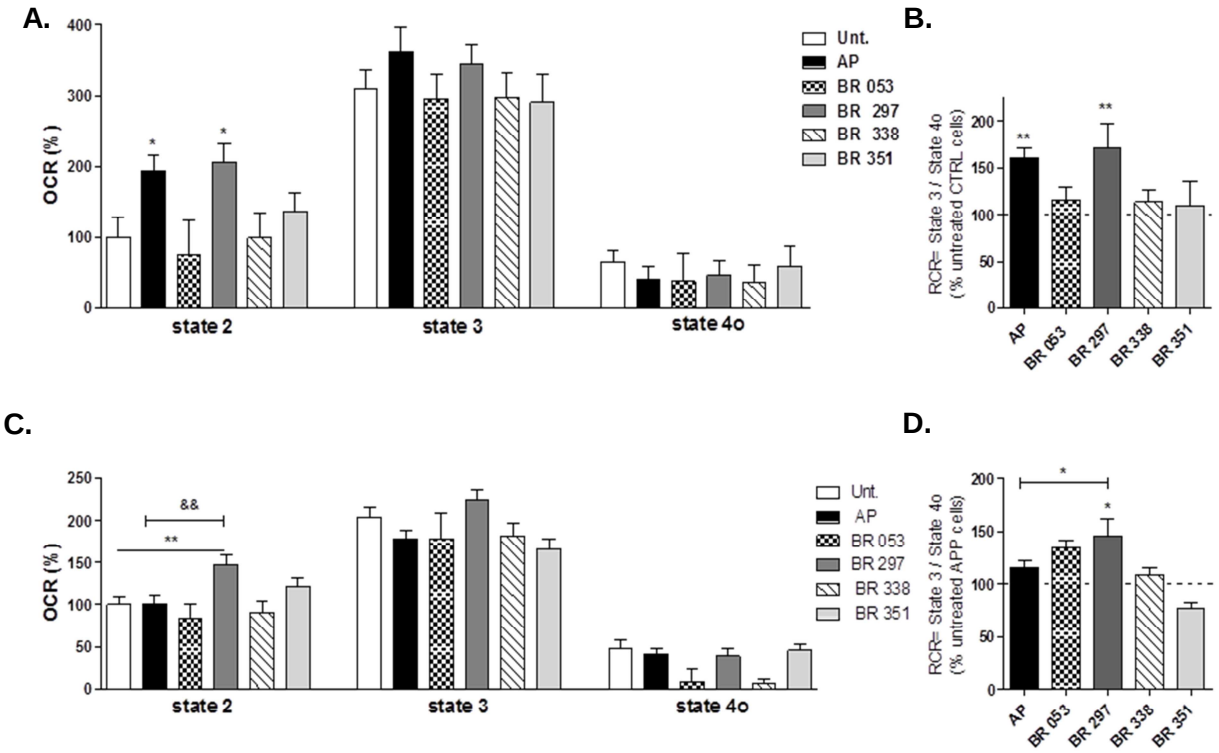


Figure 4: BR 297 increases mitochondrial respiratory capacity in CTRL and APP cells. (A, C) Oxygen consumption rate (OCR), was measured on permeabilized CTRL (A) or APP cells (C) after treatment with AP or its analogs for 24 h, using a XF24 Analyser (Seahorse Bioscience). The sequential injection of 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, $^{\&\&}$ P<0.01; $^{\&\&\&}$ P<0.001.

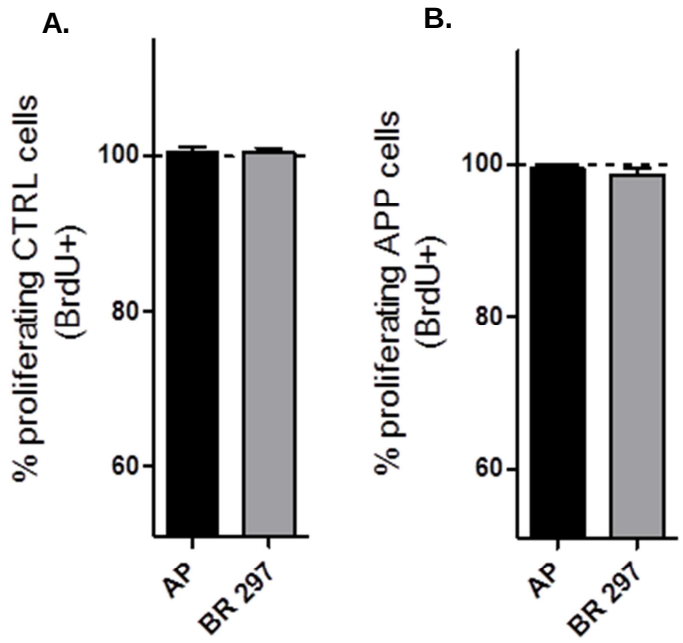


Figure 5: No proliferative effect of AP and ANS on CTRL cells (A) and APP transfected cells (B). Cell proliferation was assessed using the BrdU cell proliferation assay after 24 h of treatment with AP or BR297 at a concentration of 500nM. Values represent the mean ± SEM (n=8-16 replicates) of three independent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

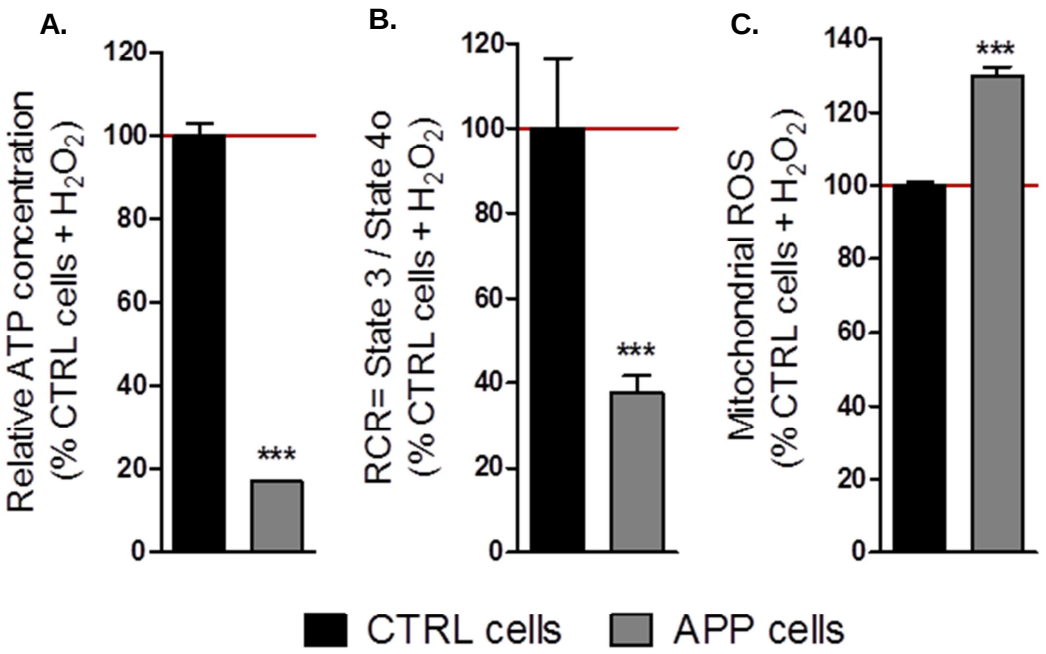


Figure 6: Oxidative stress exacerbates mitochondrial dysfunction in APP cells. In APP transfected cells, H_2O_2 (500 µM for 3h) decreases the ATP level (A) and RCR (B), paralleled by an increase in mitochondrial ROS levels (C) compared to the CTRL cell. Values represent the mean ± SEM; n= 3-6 replicates of three independent experiments. Student unpaired t test ***P<0.001 CTRL vs APP cells under oxidative stress conditions.

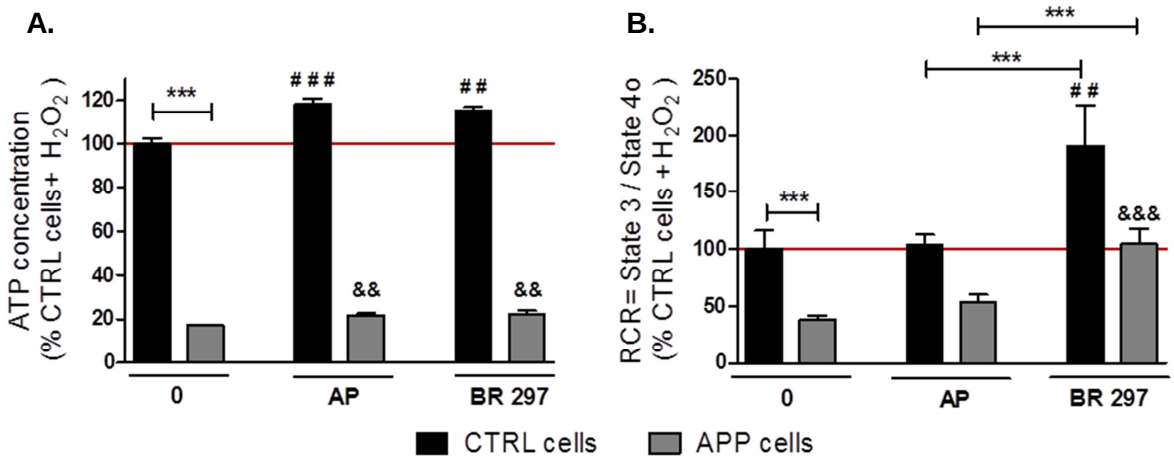


Figure 7: AP and BR 297 improve mitochondrial bioenergetics in oxidative stress conditions. AP and BR 297 increase cellular ATP production (A) and mitochondrial respiration (B), protecting CTRL and APP transfected cells against H_2O_2-induced drop of bioenergetics. CTRL and APP transfected cells were 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.

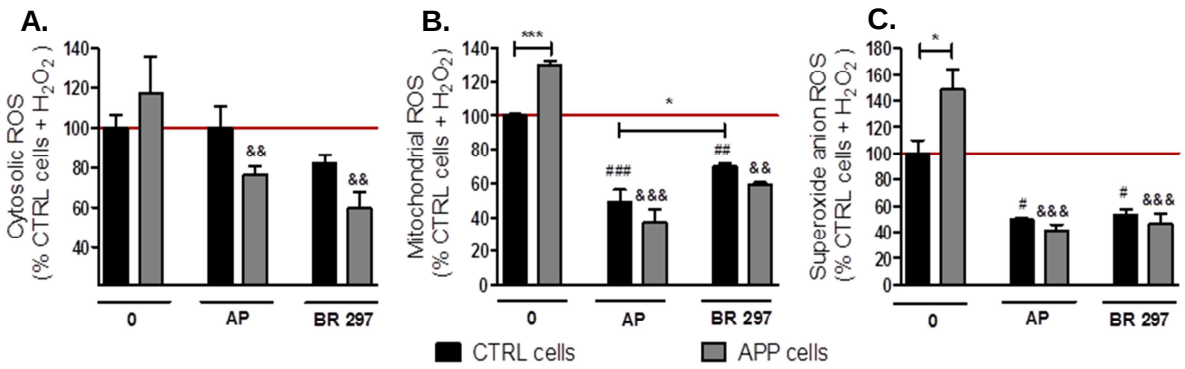


Figure 8: AP and BR 297 protect against H_2O_2-induced raise of ROS. H_2O_2 treatment induces an increase of the cytosolic ROS (A), mitochondrial ROS (B) and superoxide anion (C) in APP transfected cells compared to CTRL cells. Pre-treatment with AP or BR 297 (500 nM) reduced significantly the ROS generation 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.

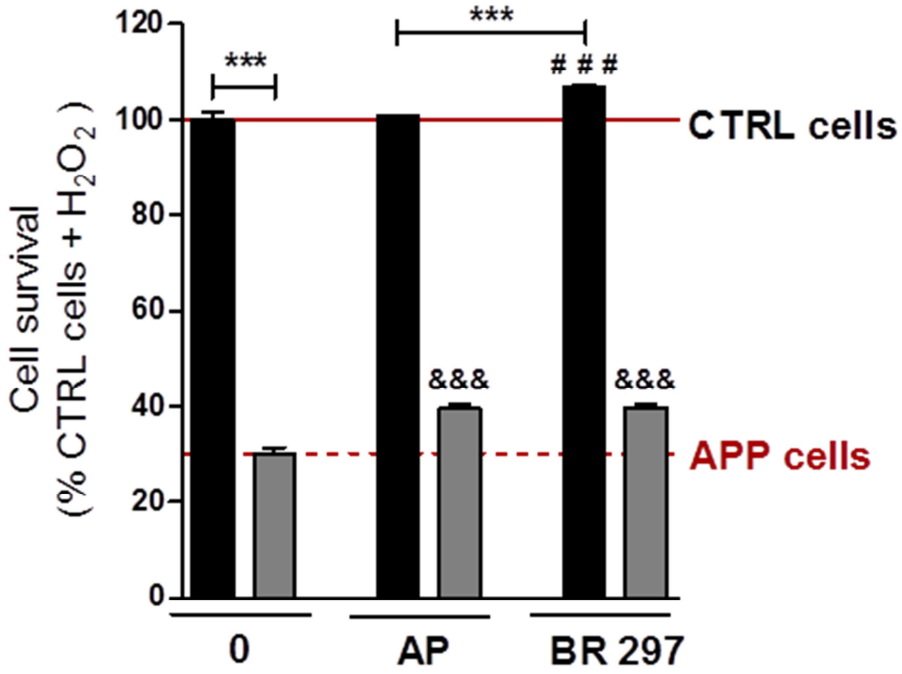


Figure 9: Protective effect of AP and BR 297 against H_2O_2-induced cell death. CTRL and APP transfected cells were pre-treated with 500 nM of AP and BR 297 for 24 h and then exposed to H_2O_2 (500 µM for 3h). Pre-treatment with BR 297 and AP ameliorate significantly the cell viability in oxidative stress 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.

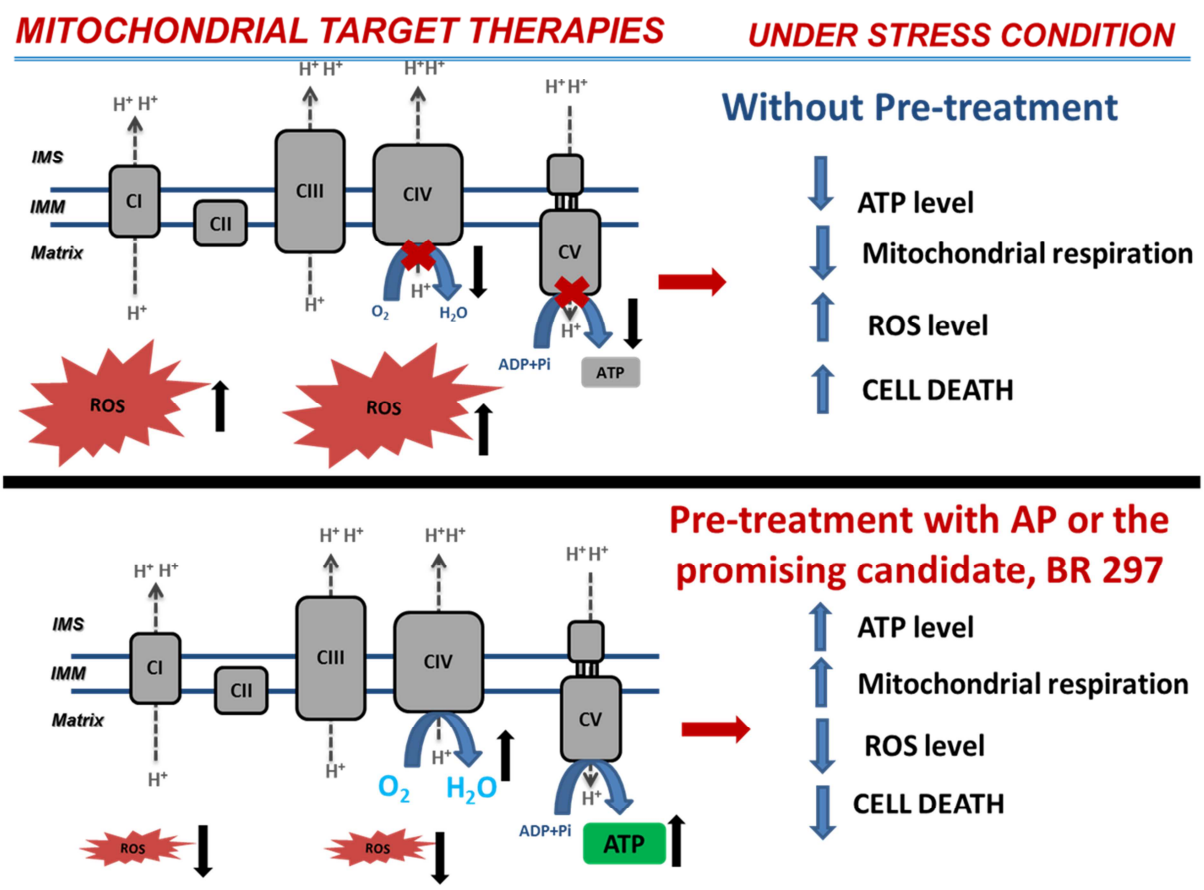
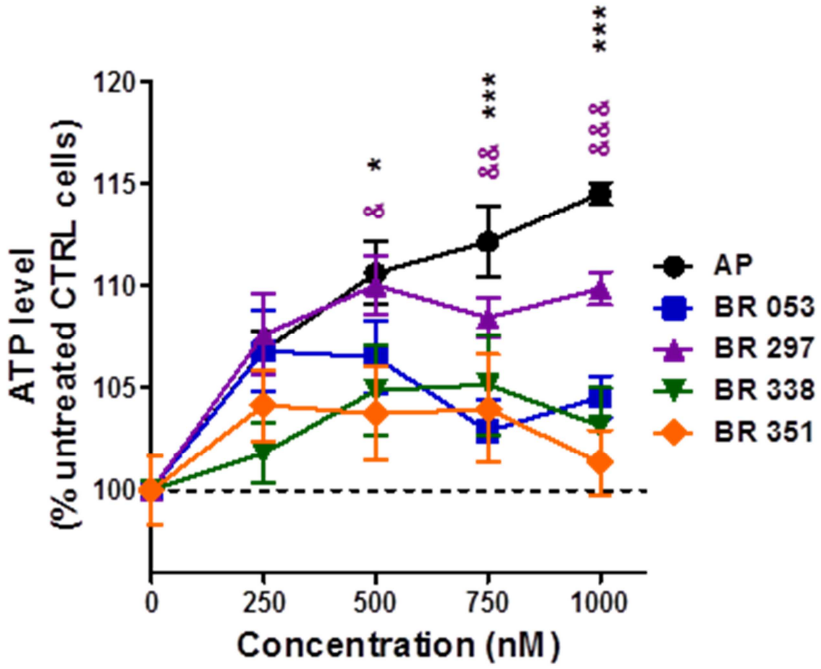
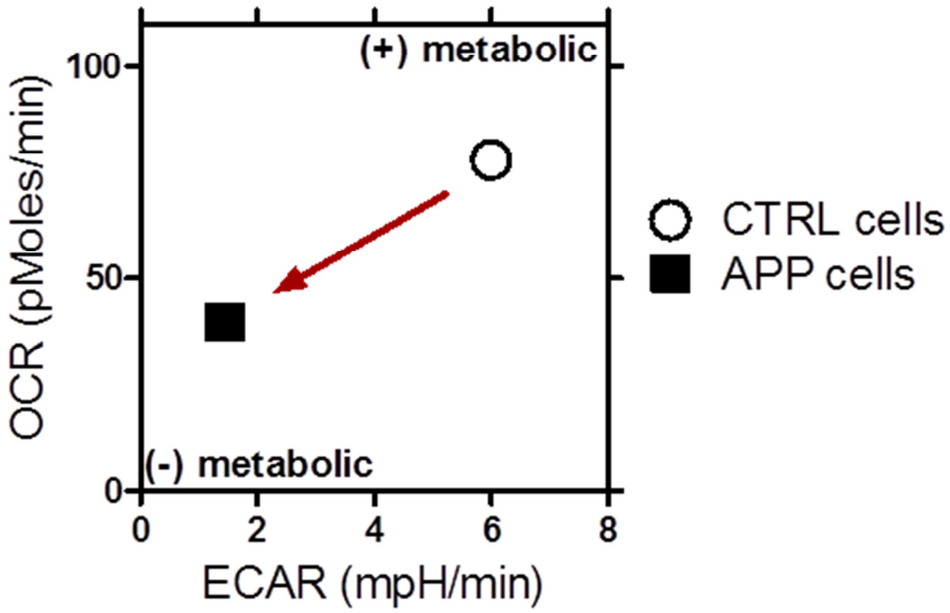


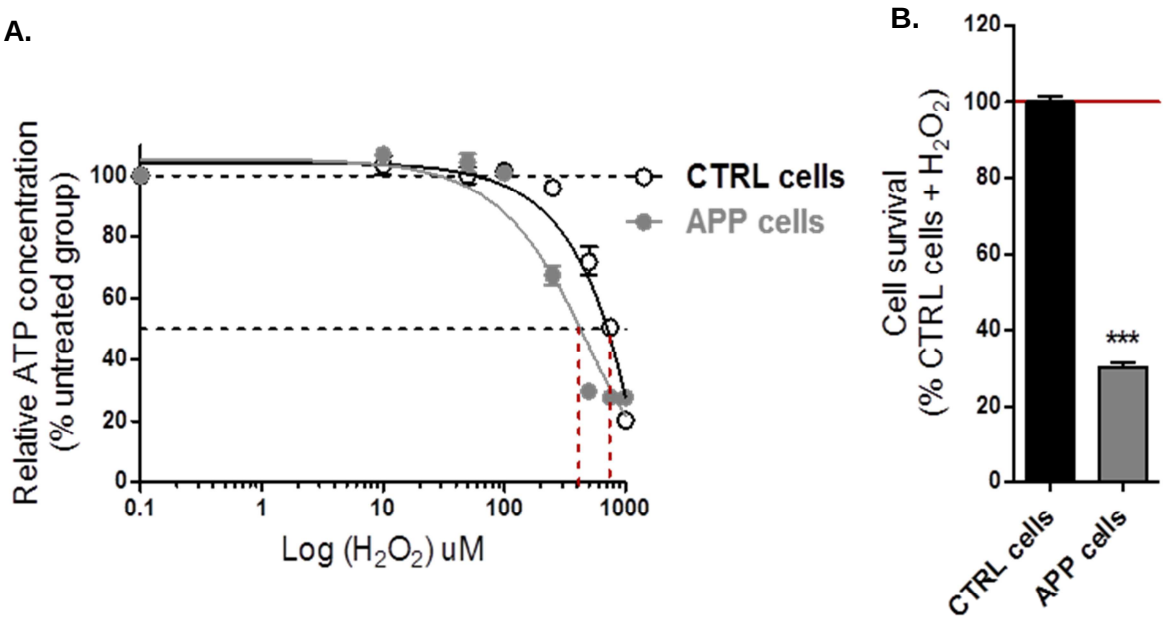
Figure 10: Schematic representation of the protective effects AP or its analog BR 297 against oxidative stress. H_2O_2-induced oxidative stress decreased ATP level and mitochondrial respiration, and increase the level of ROS leading to cell death (upper panel). AP and BR 297 are able to ameliorate mitochondrial 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.



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 H_2O_2 (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 H_2O_2) is arbitrarily set at 100%. H_2O_2 concentration evoking 50% of decrease of ATP production corresponds to the EC_{50} (500 µM and 750µM) for each cell type is indicated by a dotted line. (B) H_2O_2 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 H_2O_2. Student unpaired t test ***P<0.001 CTRL vs APP cells under oxidative stress condition.

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B. Additional experiments

Neuroprotective effect of BR297, analog of allopregnanolone, on Tg2576 Alzheimer's disease mouse model-related mitochondrial dysfunction

Introduction

In a transgenic mouse model of AD (APPswe+PSEN1Δ9 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 concentrations and improved cognitive performances in treated animals. In addition, *in vivo* studies conducted on 3xTgAD mice, triple transgenic AD mice, revealed that AP reduced Aβ 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).

In our previous study, the effect of analogs of the natural neurosteroid allopregnanolone (ANS) on bioenergetics and oxidative stress in APP/Aβ overexpressing neuroblastoma cells were investigated to select compounds with higher efficacy and specific neuroprotective properties (Lejri et al. *submitted*). First, under physiological conditions, AP and the analog BR297 showed an improvement of the bioenergetics by ameliorating the cellular ATP production, activating the bioenergetic metabolism and increasing the mitochondrial respiration without effect on the cell proliferation. Then under stress conditions induced with 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. The protective pattern of AP and BR297 was evident under physiological and oxidative stress conditions in a cellular model of AD-related amyloidopathy.

Based on these results, we aimed *in vivo* to evaluate the ability of BR297 to ameliorate or attenuate the mitochondrial deficit in a transgenic mouse model (Tg2576) that reproduce symptoms of AD.

Materials and Methods

Animals

In vivo studies including animal housing, treatments, training, toxics, and behavioral tests were performed under the supervision of Dr. Chantal Mathis in the Laboratoire de Neurosciences Cognitives et Adaptatives (LNCA), University of Strasbourg, UMR 7237 Centre National de la Recherche Scientifique, Strasbourg (France), and the principal investigator of the Neuro-Rhine project, Professor Mensah-Nyagan. All the experiments were directed in conformity with the institutional guidelines (council directive 87/848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la protection animal ; National Institutes of Health publication, 86–23, revised 1985). The project was approved by the local ethics committee CREMEAS (AL/14/21/02/13).

Tg2576 (Tg) female mice were used as the Alzheimer's disease mouse model and wild type (WT) female mice were their littermates (Taconic Europe A/S, Danemark). The transgenic mice carry a transgene coding for the 695-aminoacid isoform of human Alzheimer β-amyloid precursor protein APP (double mutation Lys670-Asn, Met671-Leu [K670N, M671L] of human APP (1-695)) under the control of a promoter of the hamster prion protein (Hsiao, Chapman et al. 1996). In Makrolon cages (16 x 32 x 14 cm) under controlled temperature (23 ± 1°C), mice were housed individually with 12/12 hours light/dark cycle (lights on at 7:00 am). Food and water were available at libitum. Each animal was regularly weighed.

Treatments

A cohort of Tg2576 mouse model was created and consisted of 6 WT (10 month old) mice and 24 Tg (10 month old) mice. 6 WT and 6 Tg mice were injected intraperitoneally with 0.3 % hydroxypropyl cellulose, used as vehicle. In addition, three group of this cohort were injected with three doses of O-allyl-epiAP, named BR297, in 0.3% hydroxypropyl cellulose (2 mg/kg, 4 mg/kg and 8 mg/kg) three times per week for 4 weeks (0,1 ml/ 10g). The range of

doses was selected after acute and chronic toxicological tests. The cohort was composed of 5 groups with 6 animals per group:

- WT 10 month old vehicle-treated mice
- Tg 10 month old vehicle-treated mice
- Tg 10 month old 2 mg/kg O-allyl-epiAP-treated mice (BR 297)
- Tg 10 month old 4 mg/kg O-allyl-epiAP-treated mice (BR 297)
- Tg 10 month old 8 mg/kg O-allyl-epiAP-treated mice (BR 297)

After four weeks of treatment, the behavioural test object location (memory performance, moving objects test) was performed (Yassine, Lazaris et al. 2013). Afterwards, analyses of brain tissue samples for the evaluation of mitochondrial function were performed.

Brain tissue preparation

The mice were killed, decapitated, and their brains were removed and washed in medium 1 buffer (138 mM NaCl, 5.4 mM KCL, 0.17 mM Na_2HPO_4, 5.5 mM Glucose, 58.4 mM Saccharose, pH 7.35). Cortex and hippocampus regions were rapidly frozen in liquid nitrogen and stored at -80°C until used or freshly homogenated to obtain dissociated cortical and hippocampal brain cells.

Cellular analysis

Cortical and hippocampal brain cells were dissociated to determine mitochondrial functions such as the cellular ATP level. To determine the concentration of proteins in the homogenate, we used bovine serum albumin standard.

Cellular ATP Levels

The ATP content of cortical and hippocampal dissociated cells was determined with a bioluminescence photometer (ViaLight™ HT, Cambrex Bio Science) according to the instruction of the manufacturer. The enzyme luciferase, which catalyses the formation of light from ATP, and luciferin, was utilized. The dissociated brain cells were lysed before the

addition of the reagent. The emitted light is linearly related to the ATP concentration and was measured using a luminometer (Victor™X5 Perkin).

Assays using isolated mitochondria

Mitochondria were isolated from frozen samples to investigate complex activities as well as mitochondrial ATP levels. Several mitochondrial enzyme activities (complex I and complex IV) were examined.

Preparation of isolated mitochondria

Mitochondria were isolated from mice brains as described previously. Briefly, the cortex sample was homogenized in 2 ml, and the hippocampi sample in 1 ml of ice-cold buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, 0.45% bovine serum albumin, 0.5 mM dithiothreitol, and complete protease inhibitor mixture tablets (Roche Diagnostics)) with a glass homogenizer (10–15 strokes, 400 rpm potter). The resulting homogenate was centrifuged at 1450 rcf for 7 min at 4°C to remove nuclei and tissue particles. The low-speed centrifugation step was repeated once with the supernatant for 3 min. Then, the supernatant fraction was centrifuged at 10000 rcf for 5 min at 4°C to pellet mitochondria. The resulting pellet was resuspended in 1 ml of ice-cold buffer and centrifuged at 1450 rcf for 3 min at 4°C to obtain the mitochondria enriched supernatant which was centrifuged at 10000 rcf for 5 min at 4°C to obtain the mitochondrial fraction. This fraction was suspended in 300 µl of ice-cold buffer, followed by determination of protein content. To determine the concentration of protein in isolated mitochondria, we used bovine serum albumin standard.

Activity of complex I

100 µg of isolated mitochondria were solubilized in n-dodecyl β-D-maltoside (DTT; 0.1 mg). NADH: hexaammineruthenium(III)-chloride (HAR) activity was measured at 30°C in a buffer containing 2 mM Na+/MOPS, 50 mM NaCl, and 2 mM KCN, pH 7.2, using 2 mM HAR and

200 µM NADH as substrates to estimate the complex I content. 100 µM *n*-decylubiquinone (DBQ) and 100 µM NADH were used as substrates and 5 µM rotenone as an inhibitor to determine NADH-ubiquinone oxidoreductase activity. Oxidation rates of NADH were recorded with a Shimadzu Multi Spec-1501 diode array spectrophotometer (ε340-400 nm = 6.1 mM-1 cm-1). We determined the DBQ/HAR ratio using the normalization of Complex I activity to the complex I content of the mitochondrial preparation.

Activity of complex IV

Cytochrome *c* oxidase activity was determined in intact isolated mitochondria (1 µg mitochondria/well or 0.2 mg/ml) using the Cytochrome *c* Oxidase Assay Kit. The colorimetric assay is based on the fact that a decrease in absorbance at 550 nm of ferrocytochrome c is due to its oxidation to ferricytochrome c by cytochrome c oxidase.

Statistical analysis

Data are represented as means ± S.E.M. For statistical comparison, One-way ANOVA followed by Dunnett's post hoc test or Student's t-test were used.

Results

Strong mitochondrial deficits in the Tg2576 AD mouse model

The difference in AD-related mitochondrial dysfunctions between WT (10-month old) mice and Tg (10-month old) mice, both treated with the vehicle hydroxypropylcellullose (HPC), were first investigated. Vehicle-treated WT mice (100%) were compared to Tg mice treated with the vehicle. Therefore, ATP levels and complex I and IV activities were used to evaluate energetic dysfunction in AD in two specific brain regions, the cortex and hippocampus, whereof the latter is particularly involved in learning. Tg2576 mice exhibited significant deficits in cellular ATP levels in the cortex and hippocampus when compared to WT mice (**Figure 1**). Tg2576 mice showed lower cellular ATP levels of about -13% in the cortex and -29% in the hippocampus than WT mice. The complex I activity was significantly reduced in Tg2576 mice as compared to WT mice at rates of about -30% in the cortex and -50% in the hippocampus (**Figure 2**). The deficit is significantly higher in the latest brain region. Tg2576 mice presented significant decrease in the complex IV activity of about -54% in the cortex and -50% in the hippocampus compared to the WT mice (**Figure 3**).

BR297 alleviated cellular energy deficit in the Tg2576 brain

Previous *in vitro* studies from our group indicated that BR297 was able to counteract the bioenergetic deficits observed in neuroblastoma cells (SH-SY5Y) overexpressing the amyloid precursor protein (APP) (Lejri et al 2016 *submitted*). BR297 stimulated neuroprotective effects in adult neural stem cells (Karout, Miesch et al. 2016). Based on these promising results, we investigated if the AP analog BR297 had beneficial effects on mitochondrial deficits observed in AD pathology by measuring cellular ATP levels as well as complex activities. Vehicle-treated Tg mice were compared to Tg mice treated with three different concentrations of BR297. 8 mg/kg BR297 increased significantly the cellular ATP level with +11% of improvement in the cortex (**Figure 1**) compared to vehicle-treated Tg mice. No

effect was observed at lower doses. 2, 4, and 8 mg/kg doses of BR 297 enhanced cellular ATP levels in the hippocampus of Tg2576 mice by ameliorating the cellular ATP levels about +10%, +21% and +28%, respectively, compared to vehicle-treated Tg mice (**Figure 1**). Interestingly, BR297 presented a higher efficacy in the hippocampus, the same region where the drop of the cellular ATP level was stronger in Tg mice. At a dose of 8 mg/kg, BR297 was able to equalize the ATP level in Tg mice to the level of WT mice.

BR297 enhanced complex I activity in the Tg2576 mice

To explore more deeply the effect of the AP analog BR297, the complex I activity was assessed. A significant effect of treatment was observed in the cortex of Tg2576 mice at all three doses of 2, 4 and 8 mg/kg BR297 by increasing the complex I activity by about +104%, +165% and +123%, respectively, compared to vehicle-treated Tg mice (**Figure 2**). In the hippocampus, 4 and 8 mg/kg BR297 significantly ameliorated the complex I activity in Tg2576 mice up to an equalization to the complex I activity of WT mice, and up to a +9% increase compared to WT mice, respectively (**Figure 2**). In the hippocampus, 2 mg/kg of BR297 showed a trend ($p=0.0167$) to increase the complex I activity in the hippocampus of Tg2576 mice. BR297 was able to restore and ameliorate the complex I activity in Tg2576 with an increase of efficacy in the cortex region of up to +135% compared to WT mice (**Figure 2**).

BR297 improved complex IV activity in the Tg2576 mice

BR297 was able to improve significantly the complex IV activity in the cortex of Tg2576 mice at a 8 mg/kg dose by about +37% and in hippocampus at 2 and 8 mg/kg doses by about +40% and +31%, respectively, compared to vehicle-treated Tg mice (**Figure 3**). A similar but non-significant trend ($p=0.0204$ and $p=0.0026$) was observed for 2 and 4 mg/kg BR297 treatments, respectively, on the complex IV activity in the cortex (**Figure 3**).

Discussion

The contributing factor of the cognitive decline observed in AD is attributed to the dysfunctional mitochondrial bioenergetics. Tg2576 mice exhibited from the age of 6 months Aβ accumulation, cognitive impairment and revealed an impaired state 3 respiration in brain mitochondria (Gillardon, Rist et al. 2007). Amyloid plaques depositions are found in the cortex and the hippocampus at an age of 9 to 13 months (Kawarabayashi, Younkin et al. 2001, Yassine, Lazaris et al. 2013). Aβ dependent mitochondrial dysfunctions start already at 3 months of age in AD transgenic mice by reducing ATP levels when elevated intracellular but not Aβ extracellular deposits present (Hauptmann, Scherping et al. 2009). Our results are reported first that the vehicle-treated 10-month old Tg2576 mice showed a strong drop of mitochondrial function due to decreased ATP levels and complexes I and IV activities compared to the vehicle-treated 10-month old WT mice. We demonstrated that the BR297 analog treatment has attenuated mitochondrial dysfunctions observed in Tg2576 mice and has restored and increased the mitochondrial activity compared to their nontransgenic littermates. These results are in agreement with *in vitro* studies, in which the best ANS, BR297, displayed protective effects on the mitochondrial function in the cellular model of AD. For instance, 8 mg/kg of BR297 significantly increased the ATP levels, complexes I and IV activities in cortex and hippocampi regions of 10-months old Tg2576 mice, suggesting protective effects of BR297 against AD-induced mitochondrial deficits. The improvement of the mitochondrial function by the BR297 treatment seems to be correlated with a positive trend in the behavioral test, object location. Further investigations are required to clearly determine other potential mechanisms of the protective effect of BR297 to act on the Aβ levels in the cortex and the hippocampi of the Tg2576 mice.

Our results demonstrated that BR297 could modulate brain energetics to preserve mitochondrial functional protein activities, and improve cognitive functions in AD mice (Tg2576).

Figures caption

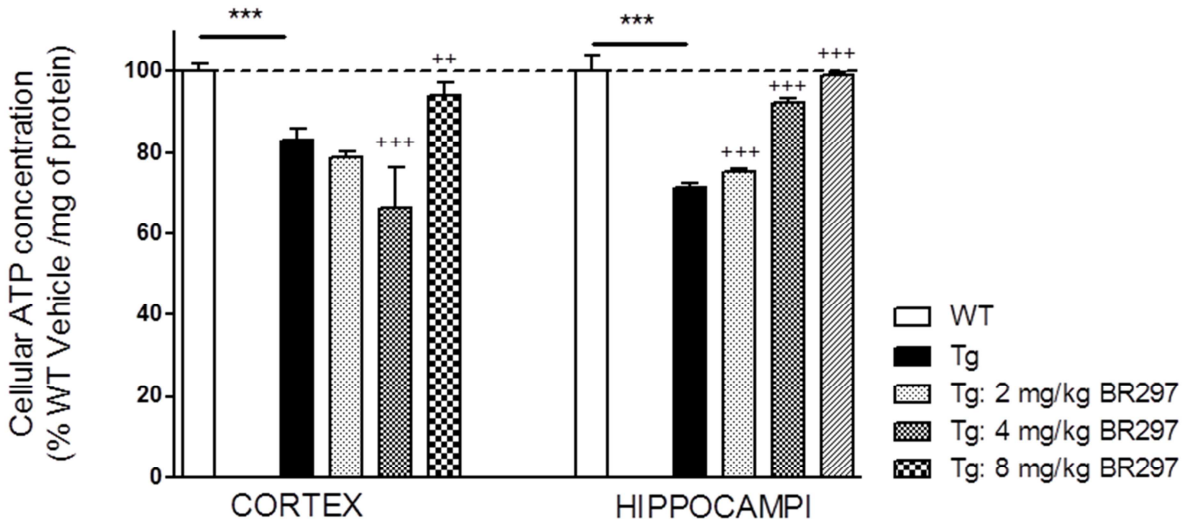

Figure 1: Effect of BR297 on cellular ATP levels in the cortex and hippocampus of Tg2576 mice. The cellular ATP level was significantly decreased in the cortex and the hippocampus of Tg2576 mice compared to WT mice. Tg2576 mice treated with BR297 showed an increase of the cellular ATP levels at a 8 mg/kg dose in the cortex and at doses of 2, 4 and 8 mg/kg in the hippocampus. 8 mg/kg of BR297 is able to normalize the ATP level of the Tg2576 to the WT mice ATP level. Values represent the mean ± SEM (n=5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 10 month WT mice. Student t test ***P<0.001 versus vehicle-treated 10 month WT mice. One way ANOVA and post hoc testing revealed significant differences $^{++}$P<0.01, $^{+++}$ P<0.001 versus vehicle-treated 10 month Tg2576 mice.

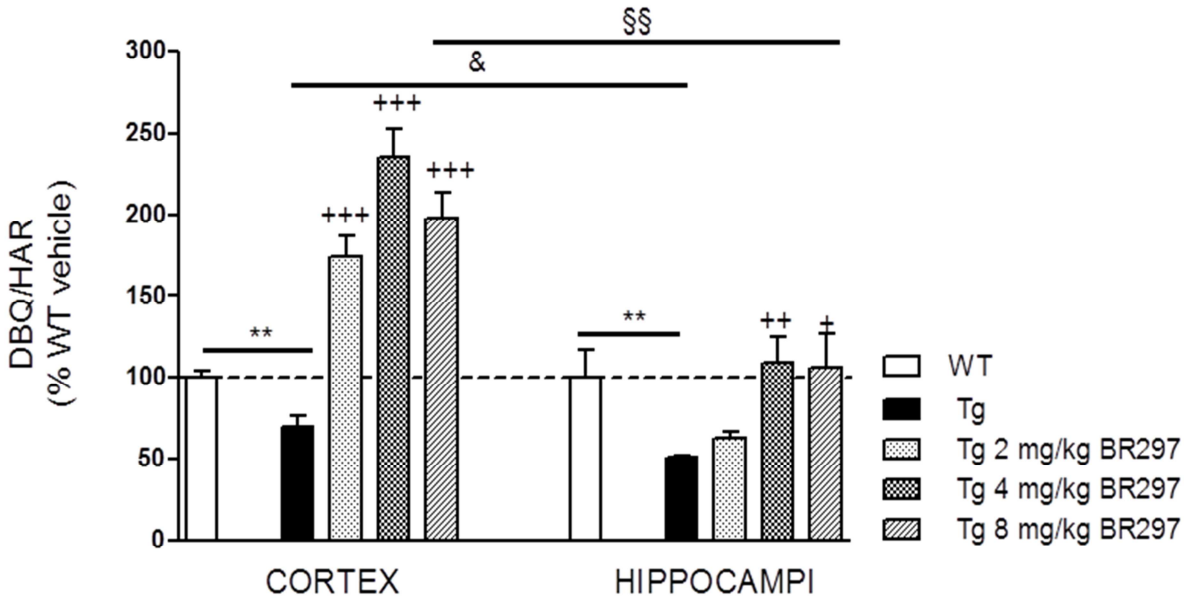

Figure 2: Effect of BR297 on the complex I activity in the cortex and hippocampus of Tg2576 mice. The complex I activity (DBQ/HAR ratio) is significantly decreased in the cortex and the hippocampus of Tg2576 mice compared to WT mice. The deficit on the complex I activity is significantly higher in the hippocampus than in the cortex of Tg2576. BR297 strongly improved the complex I activity in the cortex at all three doses of 2, 4, and 8 mg/kg while in the hippocampus only at the doses of 4 and 8 mg/kg when compared with the vehicle-treated Tg2576 mice. BR297 treatment was more efficient in the cortex of Tg2576 mice than in the hippocampus, significantly at the 8 mg/kg dose. In the hippocampus, 2 mg/kg of BR297 showed a trend (p=0.0167) to ameliorate the complex I activity in Tg2576 mice. Student t test **P<0.01 versus vehicle-treated 10 month WT mice, $^{\&}$P<0.05 vehicle-treated 10 month Tg2576 cortex vs hippocampus, §§P<0.01 8 mg/kg BR297 vehicle-treated 10 month Tg2576 cortex vs hippocampus. One way ANOVA and post hoc testing revealed significant differences $^{+}$P<0.05, $^{++}$P<0.01, $^{+++}$ P<0.001 versus vehicle-treated 10 month Tg2576 mice.

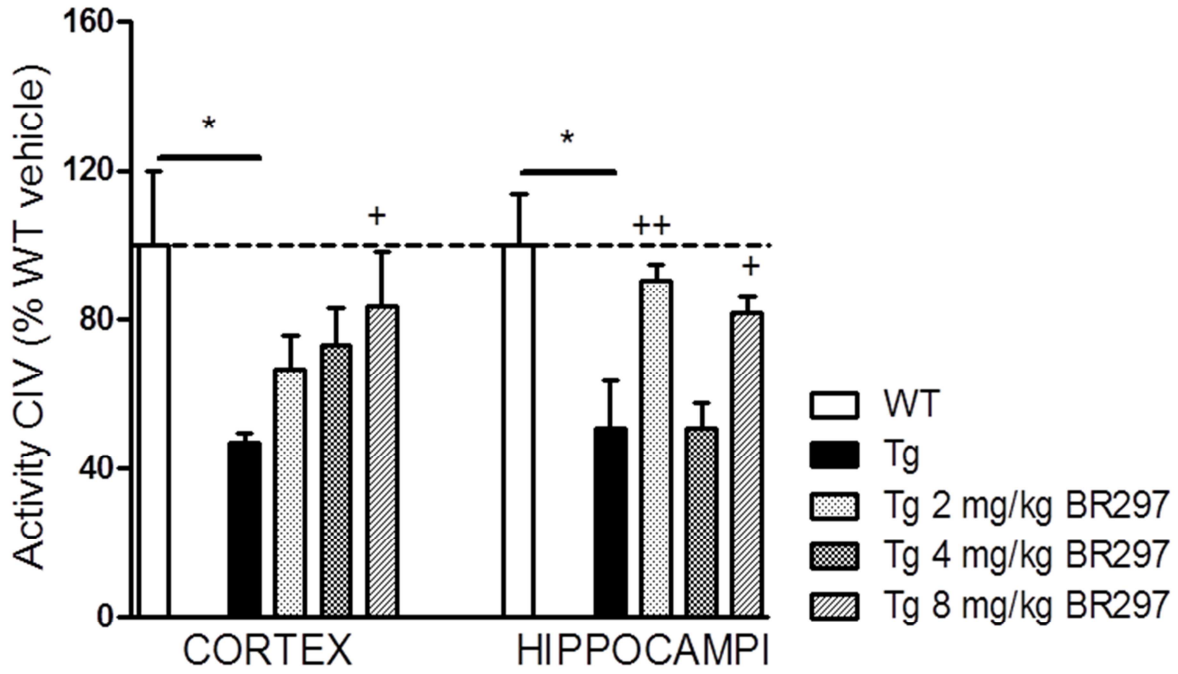

Figure 3: Effect of BR297 on the complex IV activity in the cortex and the hippocampus in Tg2576 mice. The complex IV activity was significantly reduced in the cortex and the hippocampus of Tg2576 compared to WT mice. BR297 ameliorated significantly the complex IV activity at 8 mg/kg in the cortex and at 2 and 8 mg/kg in the hippocampus when compared with vehicle-treated Tg2576 mice. In the cortex, 2 and 4 mg/kg doses of BR297 showed a trend (p=0.0204 and p=0.0026, respectively) to ameliorate the complex IV activity. Student t test *P<0.05 versus vehicle-treated 10 month WT mice. One way ANOVA and post hoc testing revealed significant differences $^{+}$ P<0.05, $^{++}$P<0.01, versus vehicle-treated 10 month Tg2576 mice.

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C. Neuroprotective effect of analogs of allopregnanolone on age-related mitochondrial dysfunctions

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In Preparation

Abstract

This study investigated the protective effect of two analogs of the natural neurosteroid allopregnanolone O-allyl-epiallopregnanolone (BR297) and O-allyl-allopregnanolone (BR351) on age-related disturbances of energy maintenance in the brain. 7-month and 21-month old mice were subjected to a treatment with one of the analogs or the vehicle. The treatment regimen involved three injections per week during four weeks at doses of 2, 4, 8 mg/kg for BR297 and 1, 2, 4 mg/kg for BR351. Following behavioural testing, brains were collected for evaluation of the mitochondrial activity. Analysis of ATP levels as well as mitochondrial complex I and complex IV activity were used to evaluate the effect of the analogs on age-induced mitochondrial impairments in the cortex and hippocampus. Aged mice exhibited significant deficits in cellular as well as mitochondrial ATP levels in the cortex and especially in the hippocampus compared to young mice. 21- month old mice brains only showed a trend of reduced complex activities. Notably, BR297 ameliorated cellular and mitochondrial ATP levels, complex I and IV activity in both brain regions. The beneficial effect of BR351 treatment was more specific to the cortex by enhancing cellular and mitochondrial ATP levels. Concerning complex activities, BR351 increased only the activity of complex I but not that of complex IV. In total, BR297 exhibited a higher efficacy in alleviating the age-related mitochondrial deficiency in the hippocampus compared to BR351. These findings suggest that allopregnanolone analogs are promising compounds for the treatment of age-related neurodegenerative diseases.

Keywords: Mitochondrial dysfunction, Aging, Analogs of allopregnanolone, Bioenergetics, Neuroprotection**.**

Abbreviations

AD: Alzheimer's disease

ANS: Analogue of neurosteroid

AP: Allopregnanolone

APP: Amyloid precursor protein

ATP: Adenosine tri-phosphate

Aβ: beta-amyloid

DBQ: n-decylubquinone

DTT: n-dodecy β-D maltoside

EDTA: Ethylenediaminetetraacetic acid

GSH/GSSG: Glutathione/glutathione disulfide

HAR: Hexaammineruthenium (III)-chloride

HPC: Hydroxypropylcellulose

KCl: Potassium chloride

KCN: Potassium cyanide

Min: Minute

Na^+/MOPS: Sodium/ 3-(N-morpholino)propanesulfonic acid

Na_2HPO_4: Disodium hydrogen phosphate

NaCl: Sodium chloride

NADH: Nicotinamide adenine dinucleotide (nad) + hydrogen (h)

nm: Nanomolar

OXPHOS: Oxidative phosphorylation

rcf: Relative *centrifugal* force

SH-SY5Y: Neuroblastoma cells

Introduction

Dysfunction of mitochondria is one of the characteristic features of aging. Energetic deficits and mitochondrial impairment are common pathological mechanisms in healthy aging and age-related neurodegenerative diseases (Lam, Yin et al. 2009). Aging is one of the major factors that stands out for having an influence on the onset of the most common neurodegenerative disease, Alzheimer's disease (AD). The link between AD and aging is not completely understood, but both genetic and environmental factors are likely involved. The decreased electron transfer rates in complex I and complex IV are the main cause for the impairment of mitochondrial function in aging brains (Navarro and Boveris 2004). Accordingly, the accelerated senescence-prone 8 (SAMP-8) mouse model displays a phenotype of accelerated aging and presents a decrease of the complex IV activity and ATP levels (Xu, Shi et al. 2007, Shi, Xiao et al. 2010).The Hippocampus and cerebral cortex, considered the most affected brain areas by aging processes, are marked by changes in reduction of mitochondrial respiration in the aged rat brain (Navarro and Boveris 2008). A decreased GSH/GSSG ratio (gluthathione reduced/oxidized form) in the cortex and hippocampi regions, linked to age-related loss of cognitive function, was detected in 21-month old mice compared to 3-month old mice (Rebrin, Forster et al. 2007). During normal aging there is no strong loss of neurons and the capacity of regeneration is maintained, but rather a loss of neuronal function is evident. Pharmacological treatments, such as neurosteroids, may reverse the changes in the nervous system of the aging brain and also maintain or improve the cognitive performance (Schumacher, Weill-Engerer et al. 2003). Previous results showed that an O-allyl compound, BR297 (ANS, analog of the neurosteroid allopregnanolone), exhibits notable advantages over AP regarding both protection of mitochondrial function and reduction of oxidative stress in a cellular model of Alzheimer's disease (Lejri et al. *submitted*). Data from other collaborators also showed that the compound BR297 exerted neuroprotective effects on adult neural stem cells (Karout, Miesch et al. 2016). They also highlighted another O-allyl analog, BR351 (ANS) which is able to

utilize neuroprotective properties of neurosteroids and simultaneously stimulate neurogenesis. In addition to the evaluations of the effects of BR297, a compound showing a neuroprotective effect without affecting proliferation, we also studied the effects of BR351, an analog exhibiting neurogenic and neuroprotective, as well as proliferative effects, on cognitive performance and mitochondrial functions in brains from 21-month old mice. These mice were treated with three different doses (2, 4 and 8 mg/kg) of the compound BR297 (O-allyl-epi-AP) or (1, 2 and 4 mg/kg) BR351 (O-allyl-AP) for one month at a rate of 3 injections per week. The spatial memory of mice was evaluated using the behavioural test of Pattern Separation, which is known to be sensitive to the neurogenic activity in the dentate gyrus/CA3 region of the adult hippocampus in rodents and humans (Holden and Gilbert 2012). The brain samples were analyzed for evaluation of mitochondrial function.

Material and methods

Animals

In vivo studies including animal housing, treatments, training, toxic and behavioral tests were performed under the supervision of Dr. Chantal Mathis in the Laboratoire de Neurosciences Cognitives et Adaptatives (LNCA), University of Strasbourg, UMR 7237 Centre National de la Recherche Scientifique, Strasbourg (France). All the experiments were directed in conformity with the institutional guidelines (council directive 87/848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la protection animal ; National Institutes of Health publication, 86–23, revised 1985). The project was approved by the local ethics committee CREMEAS (AL/14/21/02/13).

C57BL/6J male mice were used at the age of 7 and 21 months (Janvier, Le Genest Saint Isle, France). In Makrolon cages (16 x 32 x 14 cm), under controlled temperature (23 ± 1 °C), mice were housed individually with a 12/12 hours light/dark cycle (lights on at 7:00 am). Food and water were available ad libitum. Each animal was regularly weighed.

Treatment regimen

Two cohorts of mice were created and each consisted of 6 young (7 month old) and 24 aged mice (21 month old). 6 young and 6 old mice of each cohort were injected intraperitoneally with 0.3 % hydroxypropylcellulose, used as vehicle. In addition, the first cohort was divided in three subgroups and injected with three increasing doses of O-allyl-epiAP (BR297) in 0.3% hydroxypropylcellulose (2 mg/kg, 4 mg/kg and 8 mg/kg) and the second cohort with O-allyl-AP (BR351)in 0.3% hydroxypropylcellulose (1 mg/kg, 2 mg/kg and 4 mg/kg) three times per week for four weeks (0,1 ml/10g). The range of doses was selected after acute and chronic toxicological tests.

For each cohort, there were 5 groups with 6 animals per group:

- Young 7-month old vehicle-treated mice

  Aged 21-month old vehicle-treated mice

  Aged 21-month old 2 mg/kg O-allyl-epiAP-treated mice (BR 297)

  Aged 21-month old 4 mg/kg O-allyl-epiAP-treated mice (BR 297)

  Aged 21-month old 8 mg/kg O-allyl-epiAP-treated mice (BR 297)

- Young 7-month old vehicle-treated mice

  Aged 21-month old vehicle-treated mice

  Aged 21-month old 1 mg/kg O-allyl-AP-treated mice (BR 351)

  Aged 21-month old 2 mg/kg O-allyl-AP-treated mice (BR 351)

  Aged 21-month old 4 mg/kg O-allyl-AP-treated mice (BR 351)

After the four weeks of treatment, the behavioural test of pattern separation (episodic memory) was performed (Yassine, Lazaris et al. 2013). Afterwards, analyses of brain tissue samples for the evaluation of mitochondrial function were performed.

Brain tissue preparation

The mice were killed, decapitated, and their brains were removed and washed in medium 1 buffer (138 mM NaCl, 5.4 mM KCL, 0.17 mM Na_2HPO_4, 5.5 mM Glucose, 58.4 mM Saccharose, pH 7.35). Cortex and hippocampus regions were rapidly frozen in liquid nitrogen and stored at -80°C until used or freshly homogenated to obtain dissociated cortical and hippocampal brain cells. The brain harvesting was performed in the Mensah Laboratory.

Cellular analysis

Cortical and hippocampal brain cells were dissociated to determine mitochondrial functions such as the cellular ATP level. To determine the concentration of proteins in the homogenate, we used bovin serum albumin standard.

Cellular ATP levels

The ATP content of cortical and hippocampal dissociated cells was determined with a bioluminescence photometer (ViaLight™ HT, Cambrex Bio Science) according to the instruction of the manufacturer. The enzyme luciferase, which catalyses the formation of light from ATP, and luciferin, were utilized. The dissociated brain cells were lysed before the addition of the reagent. The emitted light is linearly related to the ATP concentration and was measured using a luminometer (Victor™X5 Perkin).

Assays using isolated mitochondria

Mitochondria were isolated from frozen samples to investigate complex activities as well as mitochondrial ATP levels. Several mitochondrial enzyme activities (complex I and complex IV) were examined.

Preparation of isolated mitochondria

Mitochondria were isolated from mice brains as described previously. Briefly, the cortex sample was homogenized in 2 ml, and the hippocampi sample in 1 ml of ice-cold buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, 0.45% bovine serum albumin, 0.5 mM dithiothreitol, and complete protease inhibitor mixture tablets (Roche Diagnostics)) with a glass homogenizer (10–15 strokes, 400 rpm potter). The resulting homogenate was centrifuged at 1450 rcf for 7 min at 4°C to remove nuclei and tissue particles. The low-speed centrifugation step was repeated once with the supernatant for 3 min. Then, the supernatant fraction was centrifuged at 10,000 rcf for 5 min at 4°C to pellet mitochondria. The resulting

pellet was resuspended in 1 ml of ice-cold buffer and centrifuged at 1450 rcf for 3 min at 4°C to obtain the mitochondria enriched supernatant which was centrifuged at 10000 rcf for 5 min at 4°C to obtain the mitochondrial fraction. This fraction was suspended in 300 µl of ice-cold buffer, followed by determination of protein content. To determine the concentration of protein in isolated mitochondria, we used bovin serum albumin standard.

Mitochondrial ATP levels

The ATP content of isolated cortical and hippocampal mitochondria was determined with a bioluminescence photometer (ViaLight™ HT, Cambrex Bio Science) according to the instruction of the manufacturer. The enzyme luciferase and luciferin is utilized.The emitted light is linearly related to the ATP concentration and was measured using a luminometer (Victor™X5 Perkin).

Activity of complex I

100 µg of isolated mitochondria were solubilized in n-dodecyl β-D-maltoside (DTT; 0.1 mg). NADH: hexaammineruthenium(III)-chloride (HAR) activity was measured at 30 °C in a buffer containing 2 mM Na+/MOPS, 50 mM NaCl, and 2 mM KCN, pH 7.2, using 2 mM HAR and 200 µM NADH as substrates to estimate the complex I content. 100 µM *n*-decylubiquinone (DBQ) and 100 µM NADH were used as substrates and 5 µM rotenone as an inhibitor to determine NADH-ubiquinone oxidoreductase activity. Oxidation rates of NADH were recorded with a Shimadzu Multi Spec-1501 diode array spectrophotometer (ε340-400 nm = 6.1 mM-1 cm-1). We determined the DBQ/HAR ratio using the normalization of Complex I activity to the complex I content of the mitochondrial preparation.

Activity of Complex IV

Cytochrome *c* oxidase activity was determined in intact isolated mitochondria (1 µg mitochondria/well or 0.2 mg/ml) using the Cytochrome *c* Oxidase Assay Kit. The colorimetric

assay is based on the fact that a decrease in absorbance at 550 nm of ferrocytochrome c is due to its oxidation to ferricytochrome c by cytochrome c oxidase.

Statistical analysis

Data are represented as means ± S.E.M. For statistical comparison, One-way ANOVA followed by Dunnett's post hoc test or Student's t-test were used.

Results

Deficit in cellular and mitochondrial energy in the adult mouse brain during normal aging

As characteristics of brain aging in mice, a deficit in mitochondrial OXPHOS activity, affecting in particular complex I and IV activities and ATP synthase, was described (Leuner, Muller et al. 2012) . In line with these previous results, we first investigated age-related mitochondrial dysfunctions between aged (21-month old) mice and young (7-month old) mice, both treated with the vehicle hydroxypropylcellullose (HPC). Respectively, ATP level and complex I and IV activities were used to evaluate energetic dysfunction during aging in two specific brain regions, the cortex and hippocampus, whereof the latter is particularly involved in learning. Aged mice exhibited significant deficits in cellular ATP levels in the cortex and hippocampus when compared to young mice (**Figure 1 A, B**). The mice at the age of7 months showed higher cellular ATP levels of about 21% in the cortex and 40% in the hippocampus than 21-month old mice. Mitochondrial ATP levels were reduced (-6%) in hippocampus of 21-month old mice when compared to young mice while levels were unchanged in the cortex region (**Figure 1 C, D**). Complex I and complex IV activities showed no change in 21-month old mice as compared to 7-month old mice (**Figure 1 E-H**). The mitochondrial deficits were strongly marked in the hippocampus brain region of aged mice compared to young mice due to reduced cellular and mitochondrial ATP levels

BR297 and BR351 alleviated cellular and mitochondrial energy deficits in the aged brain

Previous *in vitro* studies from our group indicated that BR297 was able to counteract the bioenergetic deficits observed in neuroblastoma cells (SH-SY5Y) overexpressing the amyloid precursor protein (APP) (Lejri et al 2016 **submitted**). O-allyl-AP (BR351) simultaneously

stimulated neuroprotective and neurogenic effects in adult neural stem cells (Karout, Miesch et al. 2016). Based on these promising results, we investigated if the AP analogs BR297 and BR351 had beneficial effects on mitochondrial deficits observed during aging by measuring cellular and mitochondrial ATP levels as well as complex activities. Vehicle-treated old mice (100%) were compared to old mice treated with three different concentrations of the two compounds.

First, cellular ATP level measurements showed that only 8 mg/kg BR297 had beneficial effects (+13% of increase) in the cortex (**Figure 2A**). No effect was observed at lower doses. 2, 4, and 8 mg/kg BR 297 enhanced cellular ATP levels in the hippocampus (+6%, +29% and +40% respectively) (**Figure 2B**). Depending on the brain region, BR351 indicated a reverse pattern of efficiency on cellular ATP levels compared to BR297. Indeed, BR351 increased cellular ATP levels at 1, 2, and 4 mg/kg (+64%, +32% and +17% respectively) in the cortex and only at 1 mg/kg in hippocampus (+4%) (**Figure 2**). Interestingly, BR297 presented a higher efficacy in the hippocampus, the same region where the drop of the cellular ATP level was stronger in aged mice, while BR 351 was vice versa more efficient in the cortex.

BR297 and BR351 induced an increase of mitochondrial ATP levels in the cortex at the three selected doses up to 34% with the highest efficiency at 8 mg/kg for BR297 (34%) and 1 mg/kg for BR351 with (33%)(**Figure 3A**). In the hippocampus, only BR297 at 2, 4, and 8 mg/kg enhanced mitochondrial ATP levels up to +38%. BR351 had no beneficial effect (**Figure 3B**). BR297 presented a higher increase on mitochondrial ATP levels in the cortex and hippocampus, while BR351 was only efficient in the cortex..

BR297 and BR351 enhanced complex I activity in the aged brain

To explore more deeply the effect of the AP analogs, the complex I activity was assessed. A significant effect of treatment was observed on complex I activity in the cortex at 4 and 8 mg/kg BR297 (+127% and 44% of increase, respectively), and 1 and 2 mg/kg BR351 (+92% and 48% of increase respectively) (**Figure 4A**). In the hippocampus, 2 and 4 mg/kg BR297

(up to +49% of increase) and 2 mg/kg BR351 (+41% of increase) were associated with a significant amelioration of complex I activity (**Figure 4B**).

BR297 ameliorated complex IV activity in the aged brain

Only BR297 was able to improve the complex IV activity in the cortex of old mice at a 2 mg/kg dose (+73%) and in hippocampus at 2 and 4 mg/kg doses (+51% and +47%, respectively) (**Figure 5A**). A similar but nonsignificant trend (p=0.0558) was observed for 8 mg/kg BR297 treatments on the complex IV activity in the hippocampus. BR351 had no significant effect on the complex IV activity in the cortex and hippocampus (**Figure 5B**).

Reproduction trial to confirm the beneficial effect of AP analogs on mitochondrial functions in the aged brain

The cellular energy deficit was again confirmed in the cortex and hippocampi of old mice (**Figure 6 A, B versus Figure 1**). The selected dose of 8 mg/kg of BR297 ameliorated significantly cellular energy levels in the cortex (+25%) and showed a trend to improve it in the hippocampus region (+6%) of old mice (**Figure 6 C, D**). BR351 also increased significantly the cellular ATP levels in the cortex (+31%) and hippocampus (+23%) of old mice (**Figure 6 C, D**). The reproduction trial allowed us to affirm the beneficial effect of BR297 and BR351 on mitochondrial activity of 21-month old mice. Taking together, these results consistently demonstrate a protective-promoting action of AP analogs in the aged mouse brain.

Discussion

The mitochondrial energy metabolism in aging is tissue specific and more prominent in tissues such as brain, heart, and skeletal muscle (Yin, Boveris et al. 2014). Mitochondrial OXPHOS is a process that includes the respiratory chain and electron transfer through the complexes I, II, III and IV. The electron transport chain across the membrane is coupled to a proton gradient that drives the ATP synthesis through the complex V. Aging and neurodegeneration exhibit declines in energy production in the brain and parallel changes in redox status with a pro-oxidant shift, partly due to the mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2. Brain Mitochondria isolated from aged animals showed a partial loss of energy transducing capacity (Yin, Boveris et al. 2014). In the aged brain, electron transfer and membrane enzyme activities such as those of complexes I and IV decrease in mitochondria (Beckman and Ames 1998, Navarro and Boveris 2007). Age causes a decline in regenerative factors in the neurogenic niche resulting in a neural stem cell transition from an active dividing state to a quiescent state within the subgranular zone of the dentate gyrus. This leads to a decline of the regenerative potential of the aging brain (Encinas, Michurina et al. 2011). Episodic memory (EM) is sensible to the effects of age and disturbed in Alzheimer's disease. The first memory system known to decline in both normal and pathological aging is EM (Tromp, Dufour et al. 2015).

Given the involvement of certain mitochondrial enzymes in the synthesis of steroids and the crucial role played by neurosteroids in the regulation of differentiation, growth, and survival of nerve cells, various works have also sought changes in biosynthesis of neurosteroids (neurosteroidogenesis) in the nervous system during aging and in the pathophysiology of AD. Interestingly, this work revealed a decline linked to age in the production of certain neuroprotective neurosteroids like allopregnanolone. Further, allopregnanolone also mediates neurogenic effects and neuroregeneration, and stimulates cell proliferation. The decreased neurosteroidogenesis and brain concentration of AP was also correlated to cognitive decline seen in AD. Neurosteroids including pregnenolone and allopregnanolone

play an essential role in the performance of memory, aging conditions, and the physiopathology. Indeed, studies performed in human and animal models have shown that age-related drops of neurosteroid levels give rise to neuronal dysfunction and degeneration due to the loss of protective and neuroregenerative effects (Smith, Wekstein et al. 2006, Caruso, Barron et al. 2013). The unprotected vulnerable brain could then be predisposed to several age-related illnesses such as AD (Caruso, Barron et al. 2013). Several studies investigated neurosteroid administration to reverse aging processes including mood, memory, energy level, and overall quality of life (Grimm, Schmitt et al. 2014). Diverse neurosteroids tested *in vitro* by Grimm and her coworkers were able to improve bioenergetic activity by increasing ATP levels, mitochondrial membrane potential, and basal mitochondrial respiration, and modulated redox homeostasis by ameliorating the antioxidant activity in neural cells (Grimm, Schmitt et al. 2014).

Our particular focus is the natural neurosteroid allopregnanolone and its capacity to promote neurogenesis and neuroprotection in aging models. Allopregnanolone is known for its beneficial action on neurogenesis and neuroprotection, but also for its anxiolytic and analgesic effects (Grimm, Schmitt et al. 2014, Irwin, Solinsky et al. 2014, Melcangi and Panzica 2014). Some studies have generated strong interest in the development of therapeutic strategies based on the use of AP. However, the pleiotropic nature of the effects exercised by AP does not facilitate the development of targeted therapy particularly focused on a specific indication, such as neuroprotection, while avoiding stimulating cell proliferation, which may facilitate the occurrence of gliomas. In contrast, in the case of decline in neurogenesis related to functional deficits, it might be interesting to produce analogs that have greater and more specific neurogenic action than AP. The development of pharmaceutical compounds as therapeutic tools remains important against aging conditions and is based on neurosteroid activity with safer and more efficacious compounds. We have successfully identified from *in vitro* studies two derivatives of AP, BR297 and BR351, with different effects on neuroprotection and proliferation (Karout, Miesch et al. 2016) (Lejri et al.

submitted). Taking together these results drive us to further investigate the protective effect of both analogs BR297 and BR351 on the mitochondrial dysfunction in adult brain mice.

Wang and collaborators showed that AP regulates the cell-cycling gene and protein expression and induces the proliferation of rodent and human neural progenitor cells (Wang, Johnston et al. 2005). AP induced a neurogenic effect in 12-month old mice that is statistically significant at 15 months of age (Wang, Singh et al. 2010, Singh, Liu et al. 2012). AP is also known to ensure the recovery of learning and memory function and to reduce age-related neurodegenerative diseases such as AD in preclinical studies of efficacy (Brinton 2013). Karout and collaborators showed through *in vitro* studies also that BR351 stimulated proliferation in adult ventricular zone stem cell cultures and in primary hippocampal cultures whereas BR297 was ineffective (Karout, Miesch et al. 2016). BR351 promoted differentiation of doublecortin-positive neurons. In neural stem cells, BR297 and BR351 induced an increase of neuroprotective activity as compared to allopregnanolone and prevented amyloid-induced caspase 3/7 activation (Karout, Miesch et al. 2016). The decrease of adult neurogenesis is also identified as one of the main factors contributing to the genesis of the cognitive deficits associated with neurodegenerative diseases. Unpublished data from our group show that BR351 (1 mg/kg) allows an improved performance of aged mice in the Pattern Separation test (specific test for hippocampal learning and episodic memory), which is positively correlated with an increase in the number of neural stem cells and progenitor cells in the dentate gyrus of the hippocampus (unpublished data). BR297 does not affect the performance of aged mice in the Pattern Separation test, probably due to the fact that BR297 does not exercise proliferative or neurogenic effects (unpublished data).

Moreover, a boost of mitochondria is a rather common mechanism of different neurosteroids (Grimm, Schmitt et al. 2014). Previous *in vitro* results from our laboratory, showed that AP and its analog BR297 were able to: i) alleviate bioenergetic dysfunctions observed in APP SH-SY5Y cells compared to control and ii) protect these cells against oxidative stress-induced cell death (Lejri et al. submitted). We investigated the ability of BR297 and BR351 to improve mitochondrial functions in a model of non-pathological brain aging. Our results

demonstrated that BR297 significantly corrected age-related mitochondrial deficits in the cortex and hippocampus. BR297 ameliorated cellular and mitochondrial ATP levels, and complex I and IV activities in both brain regions. The beneficial effect of BR351 treatment is that it is more specific to the cortex by enhancing cellular and mitochondrial ATP levels. Concerning the effect of BR351 on complex activities, this analog increased only the activity of complex I but not that of complex IV. BR297 exhibits a higher efficacy on alleviation of mitochondrial deficits observed in aging in comparison to BR351. It therefore appears that in old mice, neurogenic action is essential to restore a performance similar to that of young mice in the Pattern Separation test and a slight improvement in mitochondrial function may suffice to support this neurogenesis (**Figure 7**). This effect profile corresponds to the biological activities of the BR351 profile. However, in the Tg2576 mouse model, improved memory performance (moving objects test) requires effective neuroprotectants against beta-amyloid toxicity and oxidative stress. This neuroprotective action, which involves a strong positive modulation of mitochondrial functions without necessarily inducing cell proliferation, could correspond better to the biological activity profile of the compound BR297.

BR297 and BR351 showed protective effects in aged mice and may be a promising multitarget therapy to reduce or prevent age-related illness processes.

Figure captions

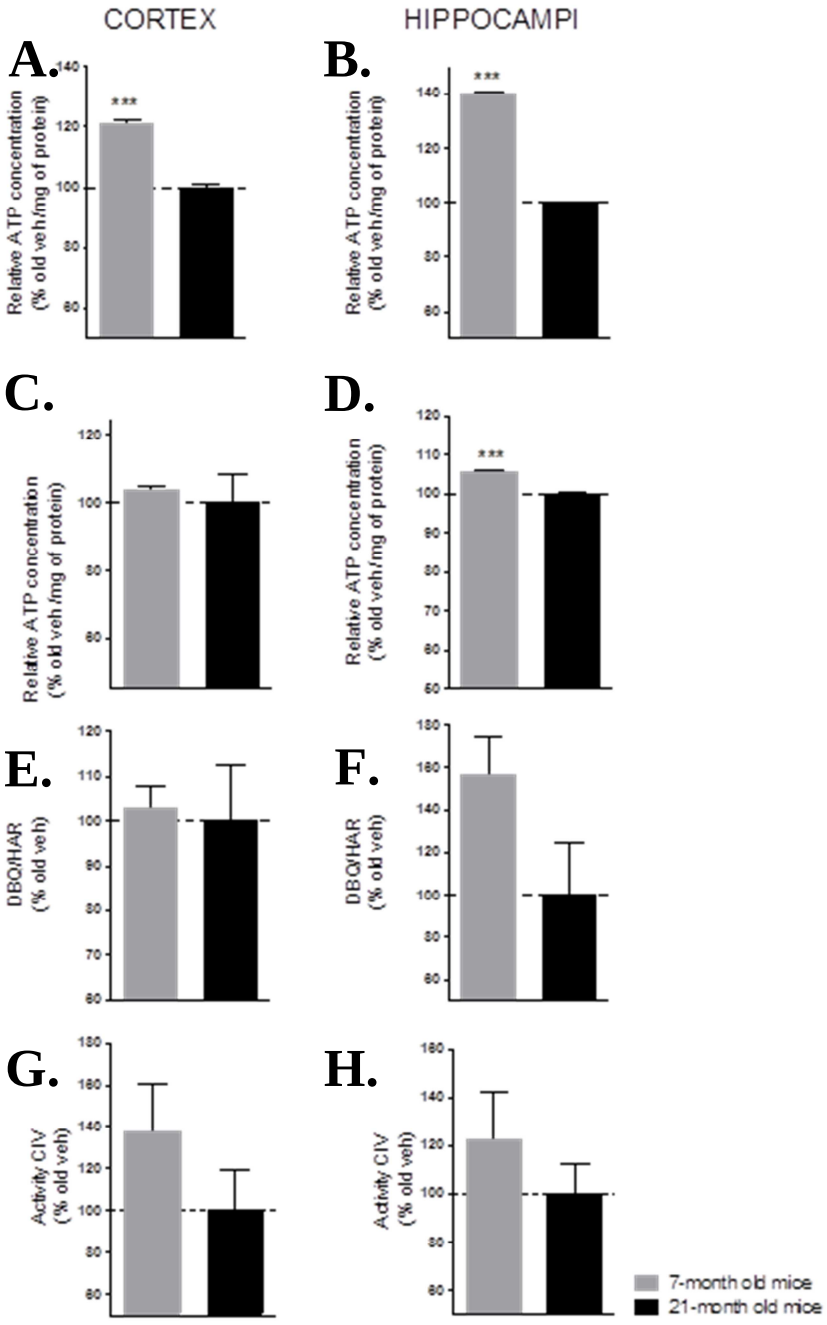

Figure 1: Mitochondrial dysfunctions in the brain during normal brain aging. The Cellular ATP level was significantly decreased in the cortex (A) and hippocampus (B) of old mice compared to young mice. A significant deficit in mitochondrial ATP levels was observed in the hippocampus of old compared to young mice while there was no change in the cortex (C). Complex I activity (DBQ/ HAR ratio) and complex IV activity showed no variation in 21-month old mice compared to 7-month old mice in the cortex (E,G) and hippocampus (F,H). Values represent the mean ± SEM (n=5-6 animals per condition) and were normalized to 100 % of 21-month old mice. Student t test *** P<0.001 vs 21-month old mice.

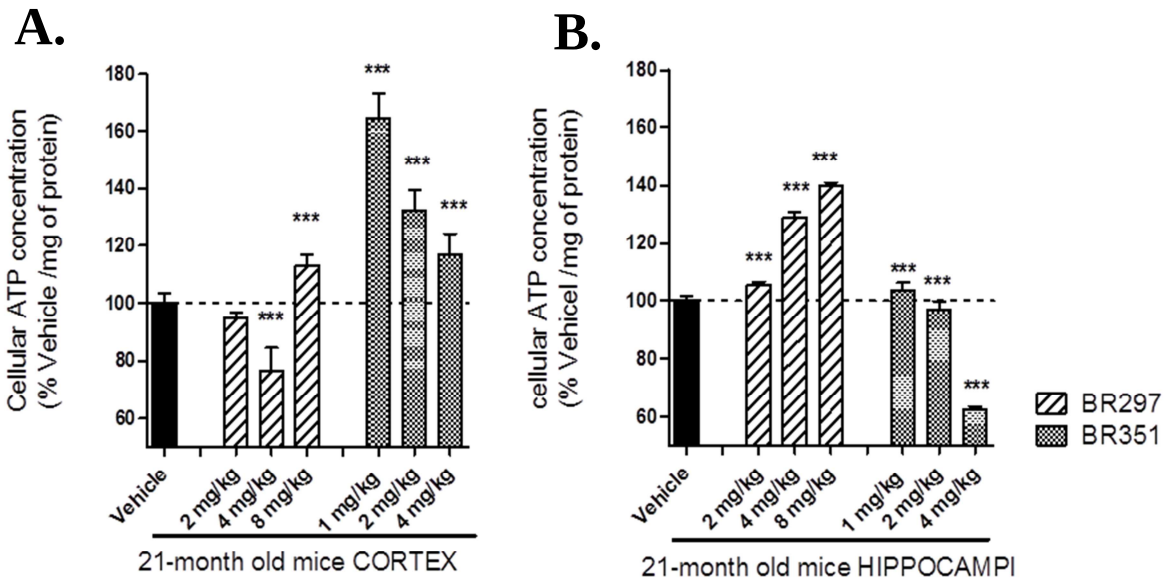

Figure 2: Effect of BR297 and BR351 on cellular ATP levels in the cortex and hippocampus of adult mice. Old mice treated with BR297 showed an increase of the cellular ATP levels at a 8 mg/kg dose in the cortex (A) and at doses of 2, 4, and 8 mg/kg in the hippocampus (B). BR351 presented an inverse pattern of efficiency by improving the cellular ATP level at all three doses in the cortex and only at a 1 mg/kg dose in the hippocampus (B). Values represent the mean ± SEM (n= 5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 21-month old mice. One way ANOVA and post hoc testing revealed significant differences $^{***}P<0.001$ versus vehicle-treated control mice.

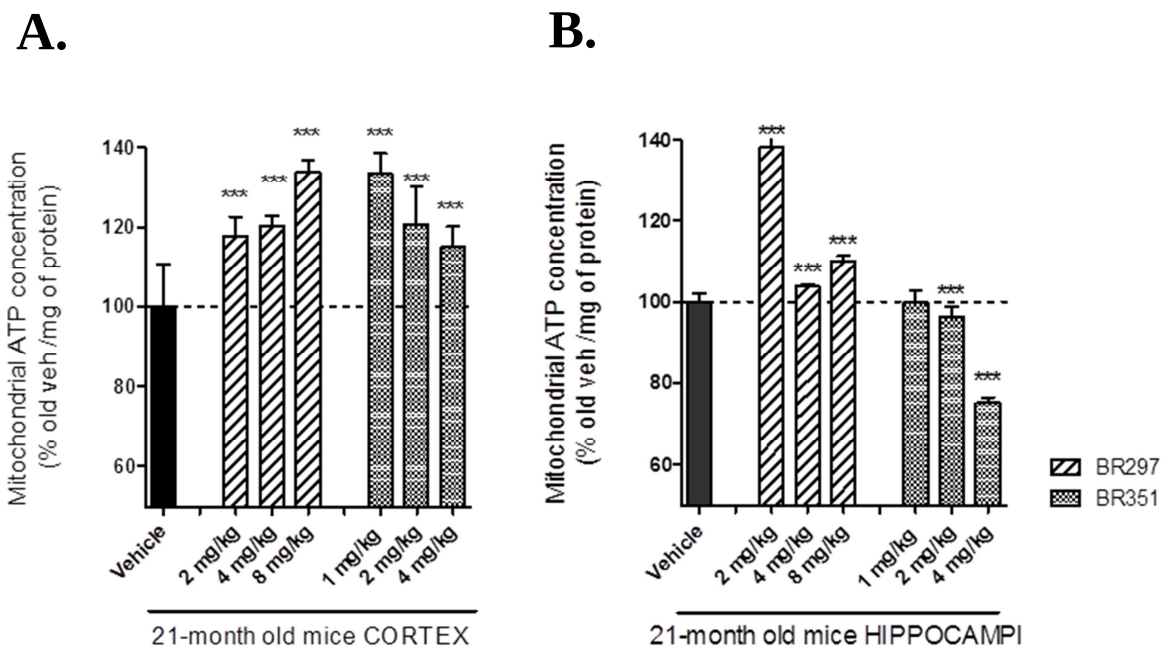

Figure 3: Effect of BR297 and BR351 on ATP levels of isolated mitochondria in the cortex and hippocampus of adult mice. A treatment with BR297 induced an amelioration of the mitochondrial ATP levels in the cortex (A) and hippocampus (B) of old mice at all three doses with the highest efficacy for the dose of 8 mg/kg. BR351 improved the mitochondrial ATP levels only in the cortex (A) at all three doses of 1, 2, and 4 mg/kg, but no ATP-enhancing effect was detectable in the hippocampus (B) when compared with the vehicle-treated mice. Values represent the mean ± SEM (n= 5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 21-month old mice. One way ANOVA and post hoc testing revealed significant differences $^{***}P<0.001$ versus vehicle-treated control mice.

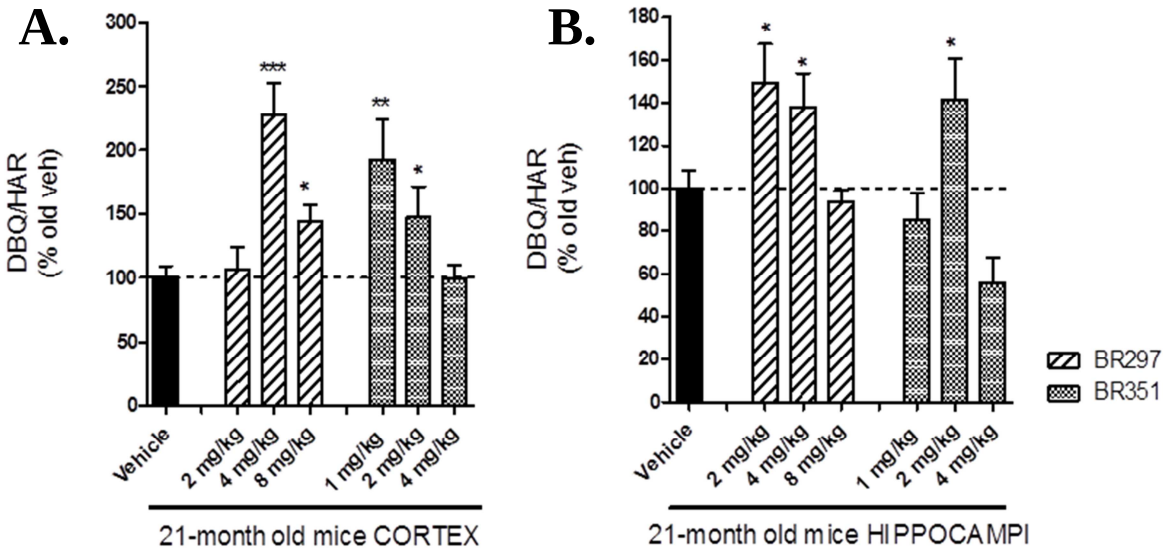

Figure 4: Effect of BR297 and BR351 on the complex I activity in the cortex and hippocampus of adult mice. Complex I activity (DBQ/HAR ratio) was significantly increased in the cortex (A) of old mice treated with 4 and 8 mg/kg of BR297 and in the hippocampus of old mice treated with 2 and 4 mg/kg of BR297. BR351 ameliorated the complex I activity at 1 and 2 mg/kg of BR351 in the cortex (A) and at 2 mg/kg in the hippocampus (B) when compared with vehicle-treated mice. Values represent the mean ± SEM (n= 5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 21-month old mice. One way ANOVA and post hoc testing revealed significant differences*P<0.05, **P<0.01, ***P<0.001***P<0.001 versus vehicle-treated control mice.

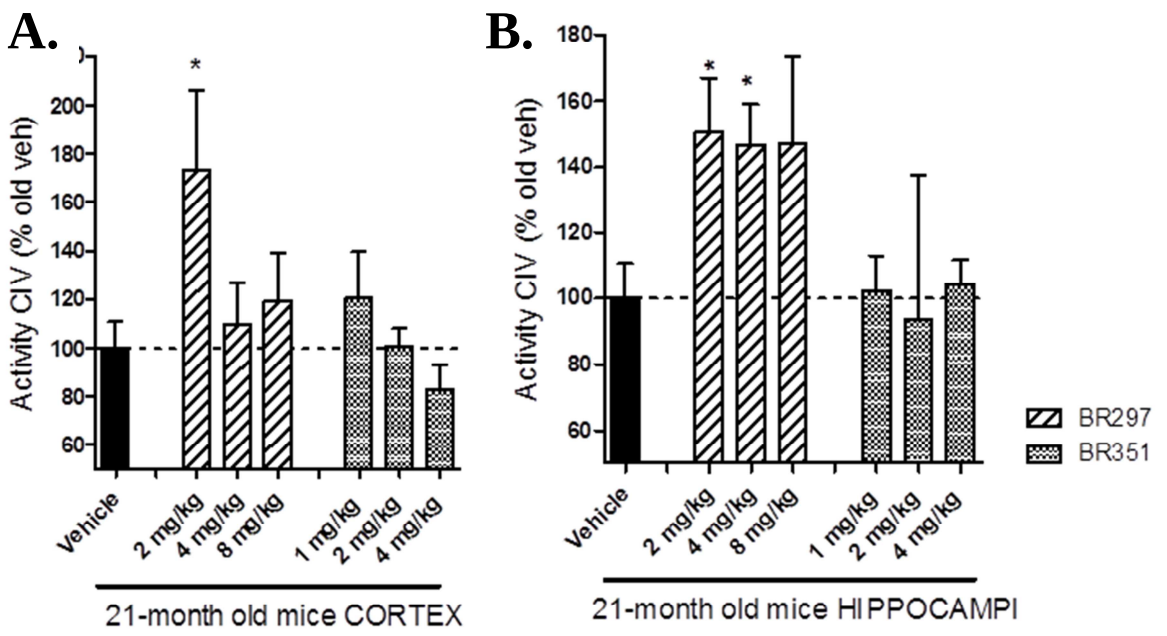

Figure 5: Effect of BR297 and BR351 on the complex IV activity in the cortex and hippocampus of adult mice. 2 mg/kg of BR297 induced a significant amelioration of complex IV activity in the cortex (A) and hippocampus (B) at doses of 2 and 4 mg/kg. In the hippocampus, 8 mg/kg of BR297 showed a trend (p=0.0558) to ameliorate the complex IV activity. BR351 had no significant effect on the complex IV activity in both brain regions. Values represent the mean ± SEM (n= 5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 21-month old mice. One way ANOVA and post hoc testing revealed significant differences*P<0.05 versus vehicle-treated control mice.

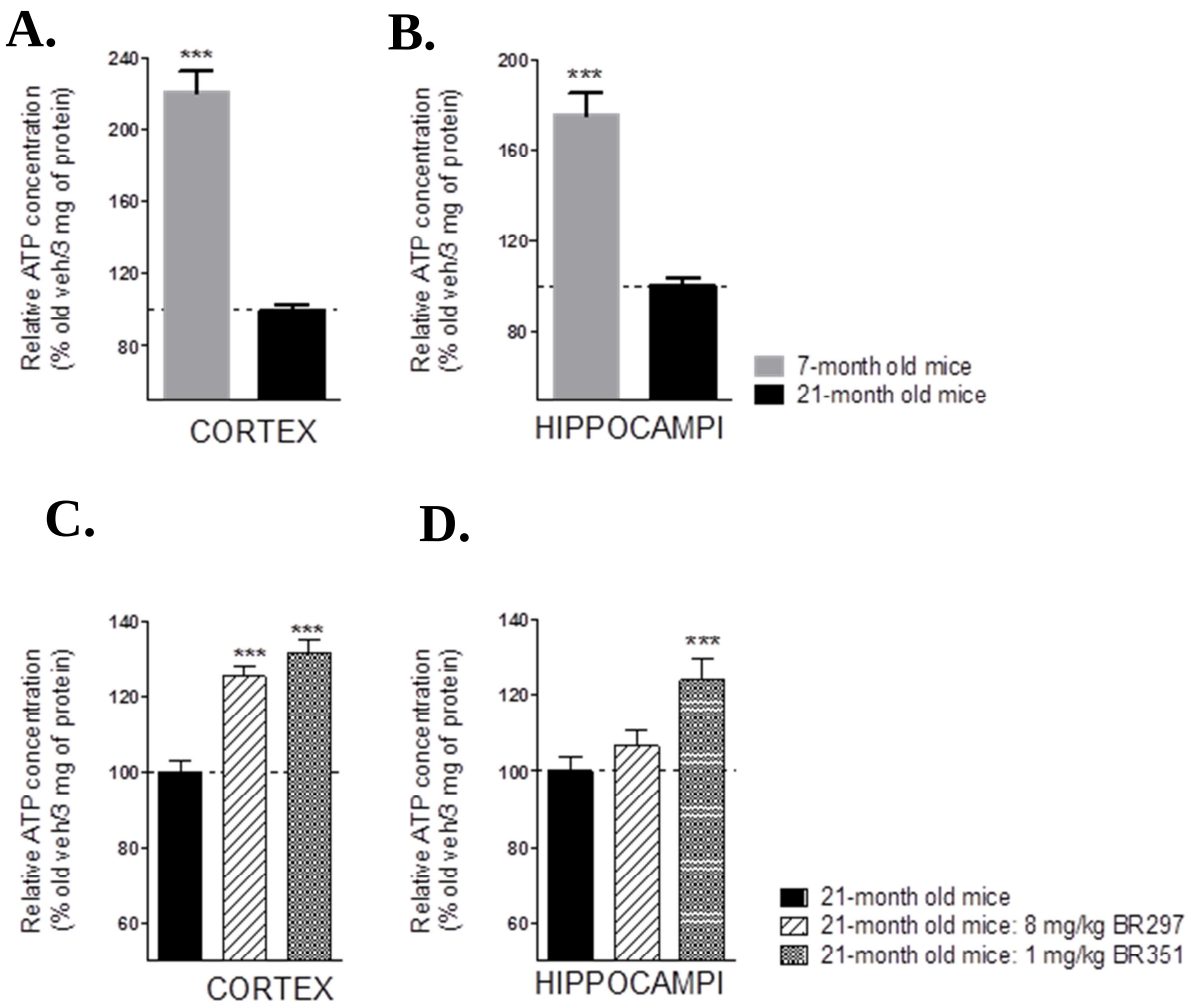

Figure 6: Reproduction trial confirmed the beneficial effects of BR297 and BR351 to protect the mitochondrial function in the cortex and hippocampus against the age-related drop of energy. Cellular ATP level deficit in the cortex (A) and hippocampus (B) of old mice compared with young mice was confirmed. The amelioration of cellular ATP levels in the cortex (C) and hippocampus (D) has been confirmed in old mice treated with the selected doses of 8 mg/kg of BR297 and 1mg/kg of BR351 compared with vehicle-treated old mice after 3 injections per week during 4 weeks. Values represent the mean ± SEM (n= 5-6 animals per treatment condition) and were normalized to 100 % of vehicle-treated 21-month old mice.One way ANOVA and post hoc or Student t-test testing revealed significant differences ***P<0.001 versus vehicle-treated control mice.

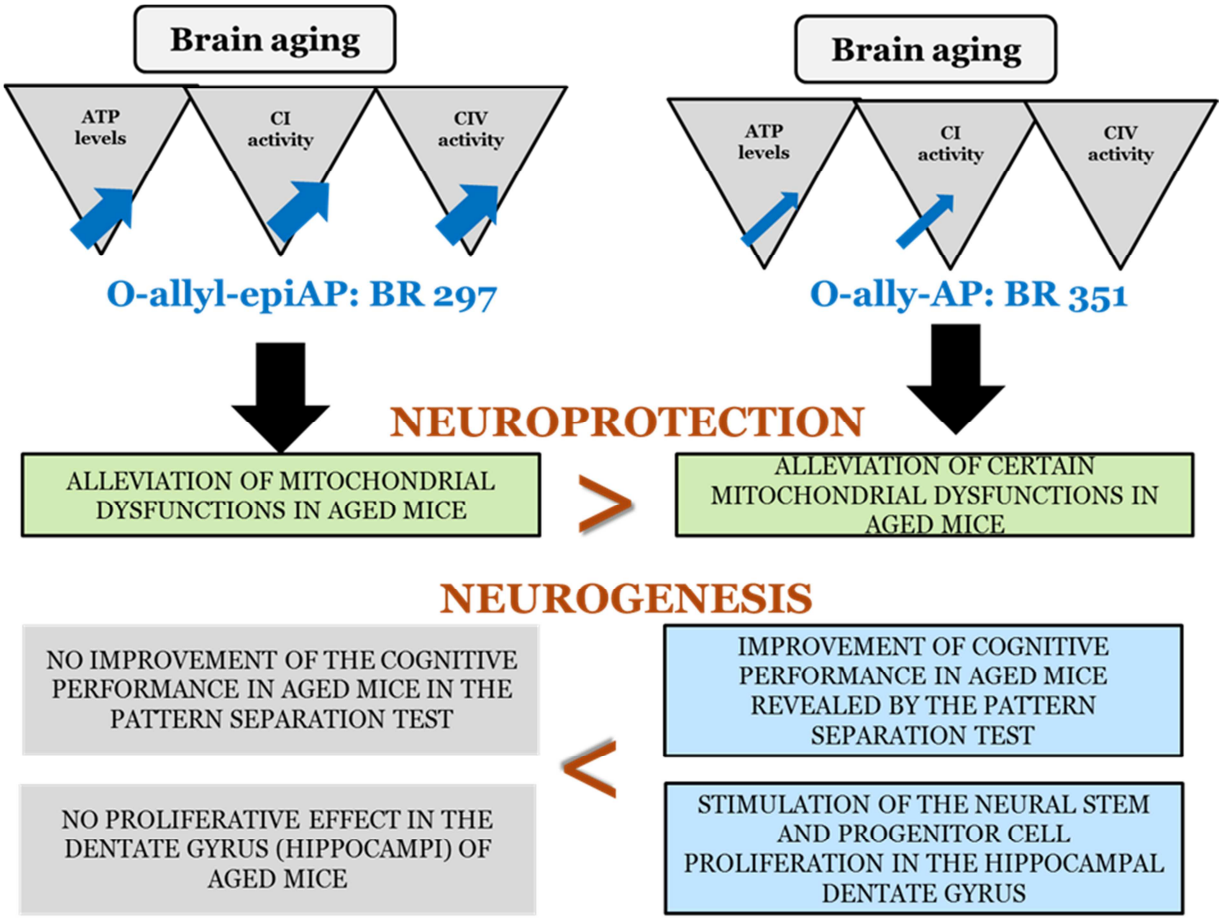

Figure 7: Effect profile corresponds to the biological activities of BR297 and BR351. BR297 exhibits a higher efficacy on alleviation of mitochondrial deficit observed in aging in comparison to BR351. BR297 is able to exercise effective neuroprotective effects involving a strong upregulation of mitochondrial activity and reminiscent cell proliferation. BR351, is also effective in neuroprotection, but its effect mainly results from neurogenic and/or proliferative action does not seem primarily depend on a strong mobilization of mitochondrial functions. BR297 and BR351 showed protective effects in aged mice and may be a promising target to reduce or prevent age-related illness processes.

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D. The future of TSPO ligands in Alzheimer's disease

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In Preparation

Abstract

The Translocator protein 18 kDa (TSPO) is mainly located in the mitochondrial outer membrane, and plays an important role in the steroidogenesis and cell survival. In the central nervous system (CNS), its expression is upregulated in neurodegeneration, e.g occurring in Alzheimer's disease (AD). In this study, we investigated the effect of a set of TSPO ligands on mitochondrial bioenergetics in a cellular model of AD overexpressing the amyloid beta protein. We evaluated the effect of these compounds compared to TSPO reference ligands described in the literature: diazepam, Ro5-4864, XBD-173 and SSR-180,575. Our findings showed that the compounds 6a and 6b from the imidazoquinazolinone family exerted beneficial effects on a cell model of AD by alleviating the drop of ATP levels. The TSPO ligand 6b has a structure similar to 6a, but differs by a lateral chain of dipropylamide instead of diethylamide. Indeed, both compounds, 6a and 6b, showed similar functional effects as diazepam and a significantly higher effect than those obtained with the reference products Ro5-4864, XBD-173 and SSR-180,575. More precisely, the lateral chain dipropylamide structure of the ligand 6b allowed an amelioration of the energy production as well as an increase of affinity and selectivity for the TSPO receptor. These findings indicate that the imidazoquinazolinone structure of TSPO ligands seems to be relevant to alleviate the bioenergetic deficit observed in AD pathology, suggesting that these compounds could potentially be new therapeutic tools for the treatment of bioenergetic deficit-related neurodegenerative diseases.

Keywords: Mitochondria, TSPO ligands, Bioenergetics, Neuroprotection, Alzheimer's disease

Abbreviations

[H^3]: hydronium ion

3xTgAD: triple transgenic mice of alzheimer's disease

AD: alzheimer's disease

APP: amyloid precursor protein

ATP: adenosine triphosphate

Aβ: amyloid-beta

BSA: bovine Serum Albumin

$CaCl_2$: calcium chloride

CNS: central nervous system

CO_2: carbon dioxide

DBI: diazepam binding inhibitor

DMSO: dimethyl sulfoxide

FCS: fetal calf serum

H_2O_2: hydrogen peroxide

IC_{50}: half maximal inhibitory concentration

KCl: potassium chloride

Ki: affinity

$MgSO_4$: magnesium sulfate

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or tetrazolium

NaCl: sodium chloride

NaOH: sodium hydroxide

nM: nanomolar

OXPHOS: oxidative phosphorylation

ROS: reactive oxygen species

SH-SY5Y: neuroblastoma cells

TSPO: translocator Protein

v/v: volume/volume

Introduction

About 20% of the body's total basal oxygen consumption is required by the nervous system (NS). Mitochondria are described as the powerhouse of the cell, producing a major part of the cellular energy via ATP generation through oxidative phosphorylation (OXPHOS) (Scheffler 2001). Mitochondria play an important role in the pathogenesis of neurodegenerative diseases such as AD. Because of their high energy demand, neurons are vulnerable and sensitive to any abnormal mitochondrial activity. A growing body of evidence supports mitochondrial dysfunction as a prominent and early chronic oxidative stress-associated event that contributes to synaptic abnormalities and neuronal degeneration in AD (Eckert, Hauptmann et al. 2008, Rhein, Baysang et al. 2009). The presence of beta-amyloid (Aβ) and abnormal tau protein in neuronal cells, the two hallmarks of AD, has been discovered to lead to mitochondrial dysfunction. The consequences of an abnormal elevation of Aβ are an increase in reactive oxygen species (ROS) production, alterations of OXPHOS, and interactions with mitochondrial dynamics and proteins leading to synaptic loss (Eckert, Schmitt et al. 2011, Pagani and Eckert 2011). Second to oxidative stress, the impairment of brain resident energy metabolism is an essential event found to be altered already at early stages of AD. Decreased ATP production leads to an impairment of ATP-dependent processes, on which all cellular functions depend (Tramutola, Lanzillotta et al. 2016).

Neurosteroids including pregnenolone and allopregnanolone play an important role in the performance of memory during aging. Indeed, some studies performed in human and animal models have shown that the age-related drops of neurosteroid levels give rise to neuronal dysfunction and degeneration owing to the loss of neurosteroid protection and neurodegenerative effects of neurosteroids (Smith, Wekstein et al. 2006, Caruso, Barron et al. 2013). Allopregnanolone is also known to ensure the recovery of memory and learning functions and to reduce age-related neurodegenerative diseases, such as AD pathology, in preclinical studies of efficacy (Brinton 2013). Neurosteroids improved cellular bioenergetics by increasing mitochondrial respiration and ATP generation, as well as regulated redox homeostasis in neuronal cells.

The translocator Protein 18kDa (TSPO) is known to facilitate the transport of cholesterol from the cytosol to the mitochondrial matrix, where it is metabolized into pregnenolone by cytochrome P450scc (Rupprecht, Papadopoulos et al. 2010). Pregnenolone is the main precursor in the steroid hormone and neurosteroid biosynthesis. There are molecules with high affinity for the TSPO binding site, and these molecules are called TSPO ligands (LTSPO). The most important endogenous ligands of TSPO are cholesterol and porphyrins, showing nanomolar and micromolar affinity for TSPO, respectively (Verma, Nye et al. 1987, Owen, Howell et al. 2010). Endozepines, other endogenous LTSPO, are a family of neuropeptides which are derived from a polypeptide precursor, the diazepam-binding inhibitor (DBI) that allosterically modulates GABAergic transmission in various neurodegenerative diseases (Mocchetti and Santi 1991). Biologically active peptide fragments of DBI have been shown to induce the mitochondrial steroid synthesis (Papadopoulos, Berkovich et al. 1991). In cerebrospinal fluid of AD patients, elevated levels of endozepines were measured (Ferrarese, Appollonio et al. 1990). Notably, Aβ is able to stimulate the synthesis of endozepines by astrocytes in AD (Tokay, Hachem et al. 2008).

In the literature, synthetic ligands of TSPO have been developed for three purposes: i) improving the understanding of the mechanisms TSPO, ii) the use of medical imaging as markers of inflammation, and iii) discovering new ways to treat diseases affecting the CNS (Yasuno, Ota et al. 2008). TSPO ligands have been developed as neuroimaging agents and as diagnostic tools for in vivo imaging of TSPO to visualise affected areas in the brain in disease conditions (Yasuno, Ota et al. 2008).

Several studies showed that the presence of ligands can activate TSPO by increasing the cholesterol flux into mitochondria and stimulating the pregnenolone production, which is translated into an increase of neurosteroid synthesis (Rone, Fan et al. 2009) However, the metabolism of cholesterol to pregnenolone is the limiting step of the synthesis of steroid hormones and neurosteroids.

Diazepam and Ro5-4864 are benzodiazepines and historical TSPO ligands. Diazepam (Valium®) is a drug commonly used as an anxiolytic and anticonvulsant. Studies have shown

that the administration of benzodiazepines such as diazepam had effects on the release of growth hormones, the Adrenocorticotropic hormone (ACTH), prolactin, Luteinizing hormone (LH) and steroid hormones such as testosterone (Francis, Bennett et al. 1991, Da Settimo, Simorini et al. 2008, Scarf, Auman et al. 2012, Jaremko, Jaremko et al. 2014). These results indicate a direct action of benzodiazepines on the adrenal function mediated by TSPO. Regarding the production of endogenous steroids, TSPO is a ubiquitous protein, especially enriched in tissues involved in the synthesis of steroids. This distribution seems consistent with its important role in the synthesis of endogenous steroids. TSPO is also found in the CNS, where its expression is restricted to glial cells (Primofiore, Da Settimo et al. 2004).

During the last decades, TSPO ligands were found to increase the level of neurosteroids as pregnenolone and allopregnanolone and therefore studied for their neuroprotective and anxiolytic properties (Campiani, Nacci et al. 1996, Selleri, Bruni et al. 2001, Ferzaz, Brault et al. 2002, Primofiore, Da Settimo et al. 2004, Rupprecht, Rammes et al. 2009, Karlstetter, Nothdurfter et al. 2014). PK11195 and Ro5-4864 are historical ligands with a low activity on TSPO functions, whereas XBD-173 and SSR-180,575 were tested in clinical trials against anxiety and promoting nerve regeneration, respectively (Ferzaz, Brault et al. 2002, Rupprecht, Rammes et al. 2009). The selective phenylpurine TSPO ligand XBD-173 (AC-5216, emapunil) counteracted induced panic attacks in rodents, exerted antipanic activity in humans and did not cause sedation or withdrawal symptoms (Rupprecht, Rammes et al. 2009). In intact rodent brains, XBD173 ameliorated brain allopregnanolone levels (Rupprecht, Rammes et al. 2009). SSR-180,575 promotes neuronal survival and repair by increasing brain and sciatic nerve pregnenolone levels in models of central and peripheral neurodegeneration (Ferzaz, Brault et al. 2002). The first study showing the beneficial effect of TSPO ligands on AD by acting on several clinical targets, such as memory function and anxiety behaviors, is about Ro5-4864 treatment, which attenuated hippocampal Aβ accumulation, gliosis, altered brain testosterone and progesterone levels, and behavioural impairment in the triple transgenic mouse model of AD (3xTgAD) (Barron, Garcia-Segura et

al. 2013). The benzodiazepine Ro5-4864 binds selectively to TSPO with nanomolar affinity (Rupprecht, Papadopoulos et al. 2010).

TSPO ligands seem to offer alternative therapeutic strategies focused on reducing the accumulation of Aβ, as they simultaneously target multiple facets of the neurodegenerative cascade, such as neuroinflammation, oxidative stress, mitochondrial dysfunction and neuronal loss. Although many TSPO ligands were already described in the literature, they suffer from a common problem of solubility and bioavailibility.

Based on detailed analyses of structure-activity relationships associating TSPO with its ligands (endogenous or synthetic), several ligands of different chemical structures were developed (**Figure 1**). We herein describe several ligands of TSPO based on an imidazo[1,2-c]quinazolinone scaffold with nanomolar affinity and a good selectivity for TSPO. In our study, we aimed to evaluate the effect of TSPO ligands on AD-related mitochondrial energy deficits by: i) determining the optimal concentration range of TSPO ligands on metabolic activity and bioenergetics in human neuroblastoma control cells, ii) improving the bioenergetic deficit in the cellular model of AD, SH-SY5Y cells stably transfected with wild-type human APP (APP cells), without modulating the metabolic activity.

Material 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). Glutamax and MitoSOX were from Gibco Invitrogen (Waltham, MA, USA). Horse serum (HS) was from Amimed, Bioconcept (Allschwil, Switzerland). Ligands of the receptor TSPO called LTSPO were synthetized by CNRS, University of Strasbourg, UMR 7200, Faculty of Pharmacology (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 nM 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 APP_{695} (APP cells) or the expression vector pCEP4 (Invitrogen, Saint Aubin, France) alone (control cells) using lipofectamineplus (Invitrogen, Saint Aubin, France) (Scheuermann, Hambsch et al. 2001). Transfected APP 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 a series of LTSPO and molecules of reference (from 10 nM to 1000 nM, data shown figure 2A) using a MTT reduction assay (Roche, Basel, Switzerland). On the basis of the MTT results as well as preliminary ATP data (see Figure 2 and Figure 3) the concentration of 10 nM of the best TSPO ligand was then selected. SH-SY5Y cells were treated in DMEM + 10% FCS one day after plating for 24 hours either with

DMEM alone (untreated control condition) or with a final concentration of 10 nM of XBD-173, SSR-180,575, Ro5-4864, the selected LTSPO 6b (LTSPO 2) and 6a (LTSPO 11), made from a stock solution in DMSO, (final concentration of DMSO <0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition). Each assay was repeated at least 3 times.

ATP levels

The 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 is linearly related to the ATP concentration and was measured using the multilabel plate reader VictorX5 (Perkin Elmer).

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: in order to determine the effective doses of the TSPO ligands to induce a significant increase in ATP production without an effect on both APP and vector-pCEP4-transfected SH-SY5Y control cells, cells were treated with the molecules of reference and the ligands 6b and 6a in a broad range of concentrations (10 to 1000 nM) for 24 h. The MTT signal detected for each cell type in basal conditions was arbitrarily set at 100%.

Pregnenolone direct ELISA

The evaluation of the production of pregnenolone was performed with a direct ELISA test (DRG diagnostics ©, Germany), an enzyme immunoassay for the quantitative determination of pregnenolone in C6 glioma cells (*Unpublished data from Dr. Christian Klein*). Each cell line was plated in 4-8 replicates into a white 96-well cell culture plate at a density of $2x10^4$ cells/well overnight. Cells were washed with a saline buffer (140 mM NaCl, 5 mM KCl, 1,8 mM $CaCl_2$, 1 mM $MgSO_4$+$7H_2O$, 10 mM glucose, 10 mM HEPES/NaOH, 0,1% BSA, pH 7.4), treated with molecules of reference or TSPO ligands (40 µM), and incubated for 2 h. In order to measure the production of pregnenolone in the middle, metabolism is blocked by the addition of trilostane (25 µM) and ketoconazole (25 µM). The addition of 40 uM final compounds is by complete change of saline medium. The plate is read at 450 nm using the plate reader.

Results

TSPO ligands at 10 nM showed no toxic effect on metabolic activity in human neuroblastoma cells

A set of LTSPO compounds have been tested in a broad range of concentrations from 0 to 1000 nM to evaluate the non-toxic concentration range after 24 h of treatment on metabolic activity in human neuroblastoma control cells. The first screening set included the molecules of reference Ro5-4864, diazepam and XBD-173, which are known for their anxiolytic and neuroprotective effect (Girard, Liu et al. 2008). 10 nM is a common concentration used in the literature to study effects of TSPO ligands *in vivo (Rupprecht, Papadopoulos et al. 2010, Choi, Ifuku et al. 2011).*

As an outcome of the screening test (**Figure 2A**), 10 nM was highlighted as the optimal treatment condition. In fact, all TSPO ligands tested, including the synthetic ligands 6b and 6a, had no significant effect after a 24h treatment at this concentration. Figure 2B shows that the two TSPO ligands belonging to the imidazoquinazolinone family, 6b and 6a, showing a similar structure with a difference on the lateral chain, exhibit a comparable non-toxicity at a concentration of 10 nM as diazepam, Ro5-4864, XBD-173 and SSR-180,575.

TSPO ligands improved the ATP level in human neuroblastoma cells

The ATP level was measured in cells after a 24 h treatment to investigate the effects of our set of TSPO ligands on cellular bioenergetics. A dose-response screening on ATP was conducted to evaluate the effect of LTSPO in a wide concentration range (0 to 1000 nM) in control cells. Results showed even at a low concentration (10 nM) that all TSPO ligands increased the ATP production with the highest efficacy for the compounds 6b and diazepam. The optimal treatment condition of 10 nM concentration was confirmed (**Figure 3A**). As 6b with 17 % of amelioration, the second compound 6a of the same imidazoquinazolinone family improved the cellular energy up to 10% in control cells (**Figure 3B**). Both compounds exhibited similar beneficial effects as the reference ligands diazepam (+17%), Ro5-4864

(+16%) and SSR-180,575 (+13%) and a higher significant (***P<0.001 for 6b and **p<0.01 for 6a) efficacy compared to XBD-173 that had no significant effect on the ATP levels.

Imidazoquinazolinone LTSPO 6b and 6a alleviated the drop of energy in a cellular model of Alzheimer's disease without an effect on metabolic activity

The ATP level and metabolic activity were measured in amyloid-precursor-protein (APP) transfected human neuroblastoma cells (APP cells), reproducing some parameters of Alzheimer's disease. Previous studies in our team (Grimm, Biliouris et al. 2016) reported a 10% decrease in ATP content in APP cells compared to control cells. Thereby, TSPO ligands were evaluated for their capacity to reduce bioenergetic deficits observed in AD pathology. 6b and 6a were compared to reference ligands at 10 nM after 24 h of treatment.

Both imidazoquinazolinone LTSPO compounds 6b and 6a improved cellular ATP levels up 16%, to a similar extent as diazepam (+15%) and with a higher significant efficiency than XBD-173 (+7%) and SSR-180,575 (+9%) in APP transfected cells (**Figure 4A**). Ro5-4864 had no effect on the ATP production in APP transfected cells.

Parallelly, the effect on cell proliferation of APP cells was tested under the same conditions. In APP cells, LTSPO compounds had no effect on the metabolic activity at 10 nM (**Figure 4B**). These results indicated that both imidazoquinazolinone and reference ligands up-regulated cellular energy independently of an effect on cell proliferation in the cellular model of AD.

Discussion

In the present study, we show that a pharmacological treatment with our novel TSPO ligands 6a and 6b confers protective benefit against AD-induced mitochondrial dysfunctions. Our findings were that under physiological condition, the new synthetic TSPO ligands 6a and 6b: i) provided ATP-enhancing without an effect on metabolic activity of control cells; and ii) alleviated bioenergetic deficits APP/Aβ due to overproduction in APP cells, by ameliorating the ATP level without an effect on the cell viability. Thus, the protective pattern of the imidazoquinazolinone compounds 6a and 6b are evident in a cellular model of AD-related amyloidopathy and with similar or higher effectiveness compared to TSPO ligands described in the literature (diazepam, Ro5-4864, XBD-173 and SSR-180,575).

Pre-tests established by collaborators demonstrated that some new TSPO ligands are able to increase the production of pregnenolone in human glioma cells. This production of pregnenolone suggests that the beneficial effect on bioenergetics of these TSPO ligands could be mediated by their ability to stimulate the steroid biosynthesis via TSPO agonism (Girard, Liu et al. 2008). It is an attractive hypothesis since a treatment with progesterone or allopregnanolone has provided beneficial effect on neurodegenerative conditions in animal models including transgenic AD mice. The TSPO ligands 6a and 6b present beneficial effects possibly comparable to steroids but potentially avoid the negative side effects of long term therapies with steroid hormones.

Based on the results of pregnenolone production in glioma cells, the aim of the present study was to identify TSPO ligands with different chemical structure that are able to induce ATP-enhancing to compensate the energy loss caused by an overexpression of Aβ peptide in APP-transfected SH-SY5Y cells.

The structure, affinity, and concentration of the treatment with LTSPO play a crucial role in the efficiency of these compounds. Indeed, the structure of LTSPO has properties that are indispensable to allow the bond with the TSPO binding site. James and coworkers showed that LTSPO should contain an electron-rich site and lipophilic region (James, Selleri et al.

2006). The affinity is improved by the insertion of a N,N-diethylamide chain on the free azote of urea in the quinazolinone cycle of the majority of TSPO ligands (Francois Hallé thesis)

Diazepam and Ro5-4864 are benzodiazepine molecules bearing similar structures and differentiate only by a chloride atom added in the para position in Ro5-4864 (also named 4'-chlorodiazepam). The affinity of Ro5-4864 for TSPO is given by the capacity to replace radiomarked [H^3]diazepam from the TSPO binding site. The affinity varies in different tissues: Ro5-4864 has very high IC_{50} values in the brain (163000 nM) and a low IC_{50} (nM) in peripheral tissues (4.7 nM on kidney; 4.1 nM on liver; 4.8 nM on lungs). Diazepam also binds at GABA receptors and is not only selective for TSPO, while Ro5-4864 is the most selective benzodiazepine for TSPO. The selectivity and the difference in IC_{50} between peripheral and central tissues of Ro5-4874 are explained by the presence of chloride. However, the elevated affinity for TSPO and the presence of chloride could explain the increase in ATP levels in control cells (10 nM) but not in APP-transfected cells. The presence of the chloride in Ro5-4864 is not sufficient for the ATP-enhancing in APP-transfected cells overexpressing Aβ peptide, even if it was shown in the literature that Ro5-4864 attenuated the hippocampal accumulation of Aβ (Barron, Garcia-Segura et al. 2013). Barron and collaborators highlighted *in vivo* effects of Ro5-4864 on young adult 3xTgAD mice (4 months) by increasing the levels of allopregnanolone, progesterone, dihydrotestosterone, and testosterone as well as improving the memory deficits. However, aged 3xTgAD mice (23 months) showed no regulation of neurosteroids (Barron, Garcia-Segura et al. 2013). Therefore, primary protective mechanisms of TSPO ligands are different between young and aged mice (Mukhin, Papadopoulos et al. 1989).

SSR-180,575 is a TSPO ligand with nanomolar affinity developed by the pharmaceutical company SANOFI. SSR-180,575 is lipophilic and can easily pass the blood brain barrier (BBB), but problems of solubility and pharmacokinetics may appear in *in vivo*. The IC_{50} given for the structure of SSR-180,575 in relation to the radiomarked [H^3]Ro5-4864 is 2.5±0.6 nM. The results of SSR-180,575 treatments showed an increase in ATP levels in control and APP cells.

XBD-173 has been developed in the 1990s by the pharmaceutical company Dianippon as an anxiolytic drug without the typical drug-related problem of benzodiazepines. Its affinity for TSPO varies depending on the tissues (Ki = 0.3 – 3 nM) with a solubility of 3.7 µg/ml. Clinical studies investigated this drug for the first time in patients treating anxiety (Rupprecht, Papadopoulos et al. 2010). XBD-173 ameliorated only the ATP-enhancing in APP cells. It had no effect on control cells.

The presence of the N,N-diethylacetamide chain in the TSPO ligand 6a allows to reduce the affinity for the central benzodiazepine receptor and increase the affinity for TSPO. The insertion of the imidazole cycle also ameliorated this selectivity. 6a has no affinity for the central benzodiazepine receptor (IC_{50}> 10µM), while it exhibits an IC_{50} of 80 nM for TSPO. The affinity for 6a is given in relation to its capacity to replace radiomarked [H^3]Ro5-4864 (Francois Hallé thesis). The TSPO ligand 6b has a structure similar to 6a, but differs by a lateral chain dipropylamide instead of diethylamide. This structural difference allows the compound 6b to have a better affinity for TSPO. In fact, in regard to the radiomarked [H^3]Ro5-486, the affinity of 6b is eight times higher than that one of 6a (IC_{50} 6b: 12nM) (Francois Hallé). The high affinity of 6b is correlated to a greater increase in ATP levels than in 6a treatment control and APP cells. In control cells ATP levels were increased by both compounds, but with a higher effect with a 6b treatment. In APP cells, both compounds ameliorated the production of ATP to a similar extent. Generally, the new TSPO ligands 6a and 6b are more efficient to compensate the energy loss caused by an overexpression of Aβ compared to the LTSPO described in the literature.

Taking together, our results showed that the imidazoquinazolinone structure seems to be essential to alleviate the bioenergetic deficit observed in AD pathology (**Figure 5**). More precisely, the lateral chain dipropylamide structure of the ligand 6b allowed the amelioration of the energy as well as the affinity and selectivity for the receptor TSPO. Moreover, the MTT assays confirm that the cell viability is not impacted and the non-toxic concentration is 10 nM, a concentration also widely used in the literature. Indeed, PK-11195 (alpidem), another

TSPO ligand, and Ro5-4864 have been shown to protect cells against apoptosis at a nanomolar concentration (Strohmeier, Roller et al. 2002, Kugler, Veenman et al. 2008). To study more deeply the effect of LTSPO on bioenergetics, additional investigations are needed such as mitochondrial respiration and bioenergetic phenotype to complete the profile of TSPO ligands. The neuroprotective actions of TSPO ligands may be due to the modulation of endogenous production of neurosteroids in the nervous system (Rupprecht, Rammes et al. 2009, Rupprecht, Papadopoulos et al. 2010). The stimulation of the neurosteroid synthesis may be a beneficial strategy in AD showing a drop of neurosteroid levels. In fact, endogenous allopregnanolone levels are reduced in the cerebral cortex of the brain of the 3xTgAD (Wang, Singh et al. 2010). In the brains of AD patients, post-mortem allopregnanolone concentrations were reduced and this decrease was correlated with the extent of AD pathology (Naylor, Kilts et al. 2010). Thus, further studies on the production of pregnenolone in control and APP cells could be essential to understand the mechanism underlying their mode of action on mitochondria. Neurosteroids can regulate both regeneration and repair mechanisms in the brain and studies showed the ability of allopregnanolone to promote the regenerative processes in both central and peripheral nervous system (Wang, Johnston et al. 2005, Naylor, Kilts et al. 2010, Schumacher, Hussain et al. 2012, Sun, Ou et al. 2012, Brinton 2013).

Considering the beneficial effect of TSPO ligands on neuronal viability, regeneration processes, and neuroinflammatory response, one can imagine many therapeutic uses of TSPO ligands for directions at the central and peripheral nervous system.

Figures caption

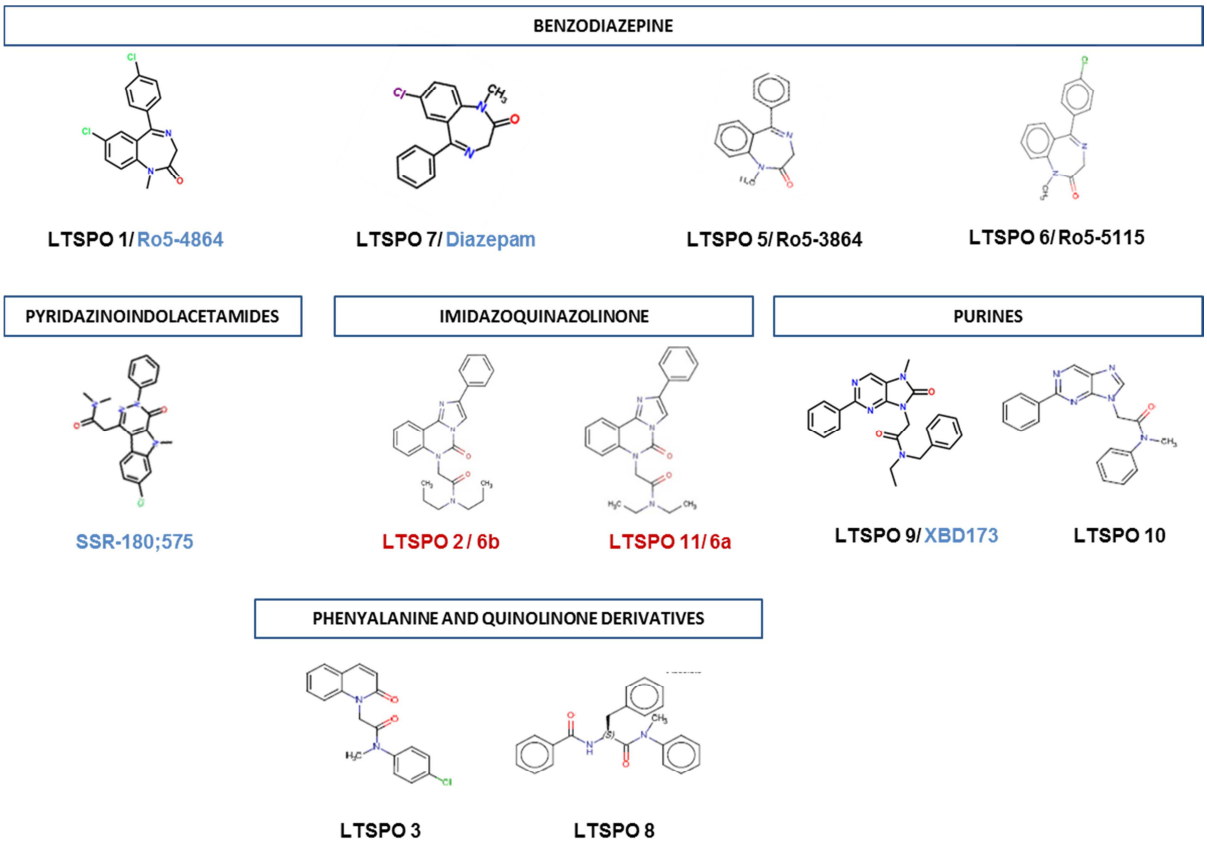


Figure 1: Structures of a set of TSPO ligands including four molecules of reference Ro5-4864, Diazepam, SSR-180,575 and XBD-173. Novel synthetic TSPO ligands LTSPO 2, 3, 5, 6, 8, 10, and 11 were developed and synthetized by the Laboratoire d'Innovation Therapeutic, University of Strasbourg (France) and tested on mitochondrial function. Reference ligands are highlighted in blue. LTSPO 11 (6a) and LTSPO 2 (6b), highlighted in red, differ only by a diethylamide or dipropylamide lateral chain, respectively. The structural modification of these TSPO ligands may enhance the solubility of the ligands, which is often very low.

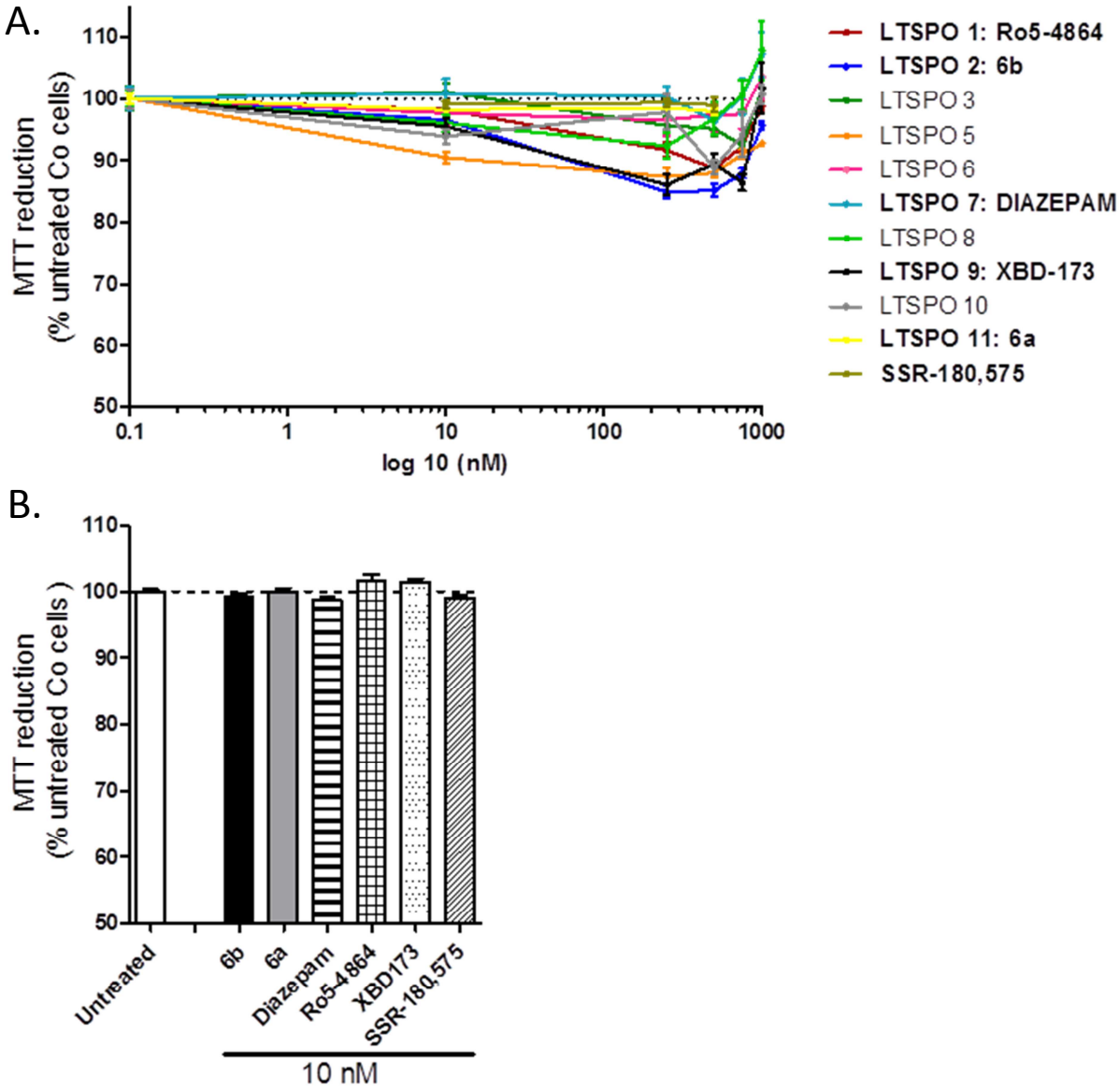


Figure 2: Dose-response screening of TSPO ligands on metabolic activity. The non-toxic concentration range was investigated using the MTT reduction test. The screening (A) was performed from 10 to 1000 nM and for 24 h. At a concentration of 10 nM the metabolic activity of the control SH-SY5Y cells was not affected by most of the ligands. MTT reduction tended to increase at higher concentrations (250 and 500nM). (B) Comparison of the effect of the reference ligands versus the selected novel ligands 6a and 6b on metabolic activity at 10 nM. Similarly, all ligands did not change cell viability. Values represent the mean ± SEM (n=8-12 replicates of at least three independent experiments) and were normalized to 100 % of untreated control cells (B). One way ANOVA and post hoc testing confirmed no effect.

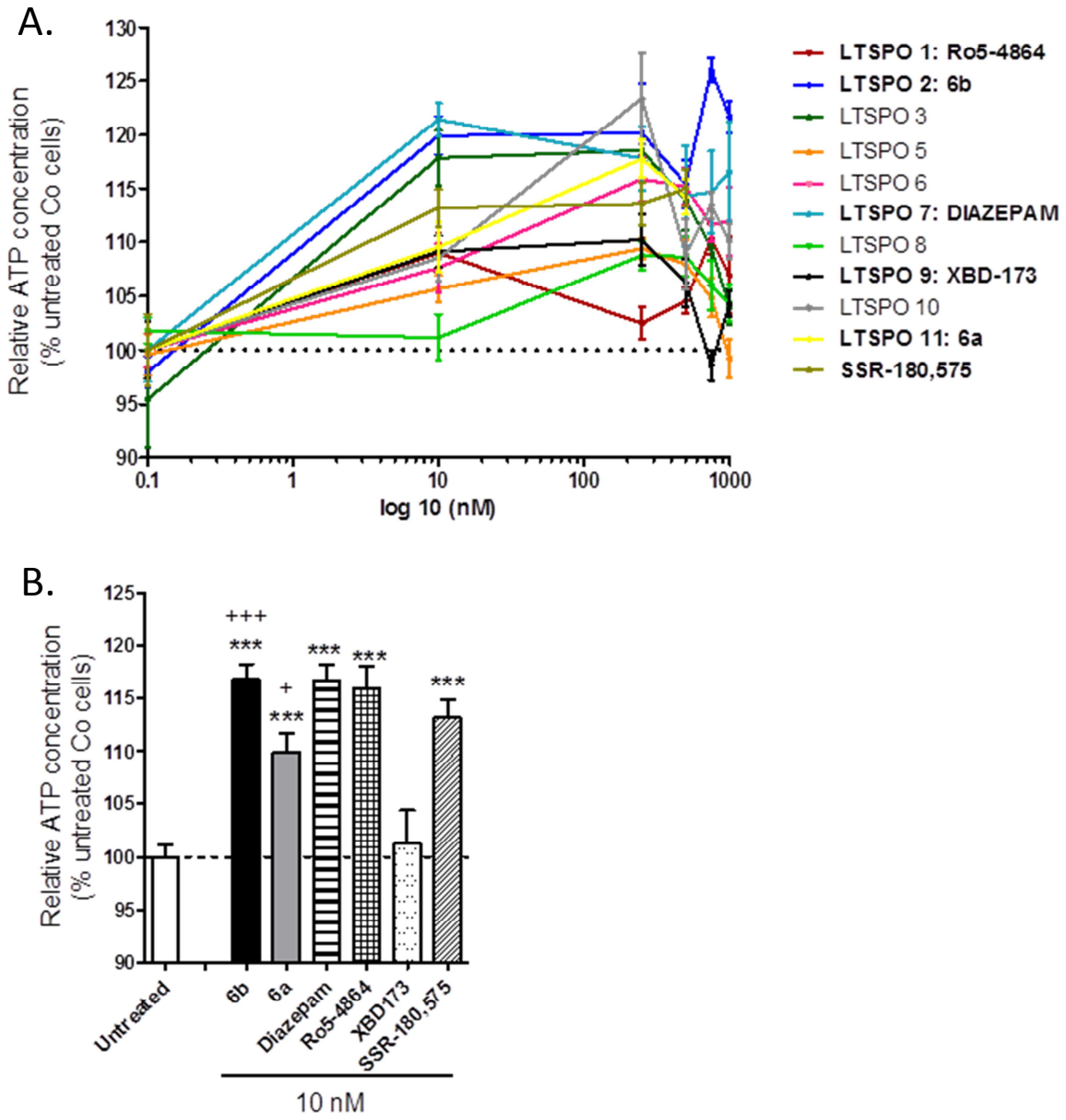
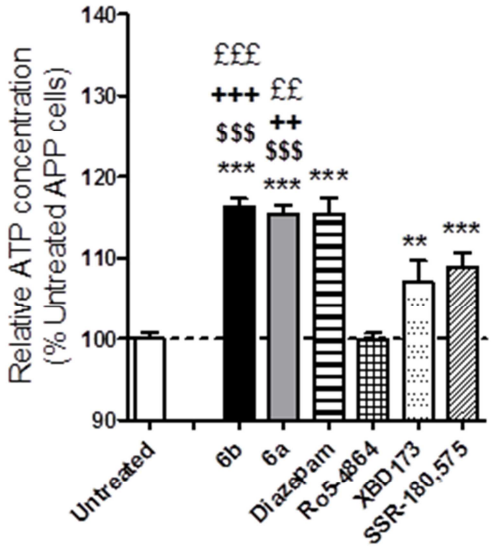


Figure 3: Dose-response screening of LTSPO on ATP production. ATP levels are expressed as percent of control (untreated control cells) after 24 h LTSPO treatment from 10 to 1000 nM (A). 6a and 6b were selected as the most effective LTSPO on ATP levels in control cells. At 10 nM (B), 6b and 6a improved ATP levels to a similar extend as diazepam, Ro5-4864, and SSR-180,575, and had a higher significant effect compared to XBD-173 in control cells. Values represent the mean ± SEM (n=8-18 replicates of three independent experiments) and were normalized to 100 % of untreated control cells (B). One way ANOVA and post hoc testing revealed significant differences ***P<0.001 versus untreated control, $^{+}$P<0.05,$^{+++}$P<0.001 versus XBD-173 treated cells.

A.



B.

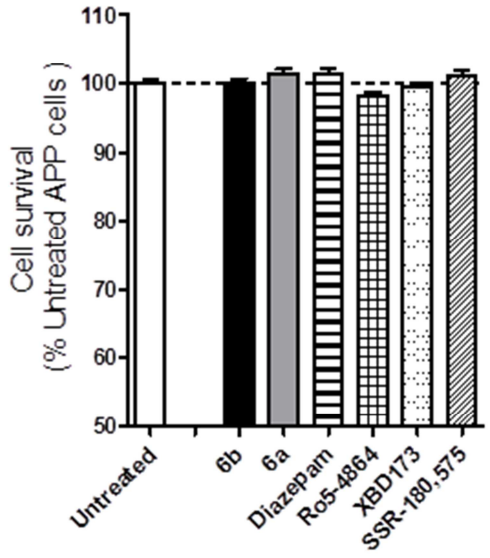


Figure 4: Effect of 6b and 6a on ATP levels and metabolic activity in APP transfected human neuroblastoma cells. The ATP test (A) and MTT reduction (B) were assessed in APP cells after 24 h LTSPO treatment at 10 nM. 6b and 6a had similar ATP-enhancing effects as diazepam and higher significant effects than Ro5-4864, XBD-173 and SSR-180,575. All ligands tested had no effect on metabolic activity in the cellular model of Alzheimer's disease. Values represent the mean ± SEM (n=8-18 replicates of three independent experiments) and were normalized to 100 % of untreated APP cells. One way ANOVA and post hoc testing, *P<0.05, **P<0.01, ***P<0.001. * versus untreated APP cells, $ versus Ro5-4864, + versus XBD-173 treated cells and £ versus SSR-180,575 treated cells

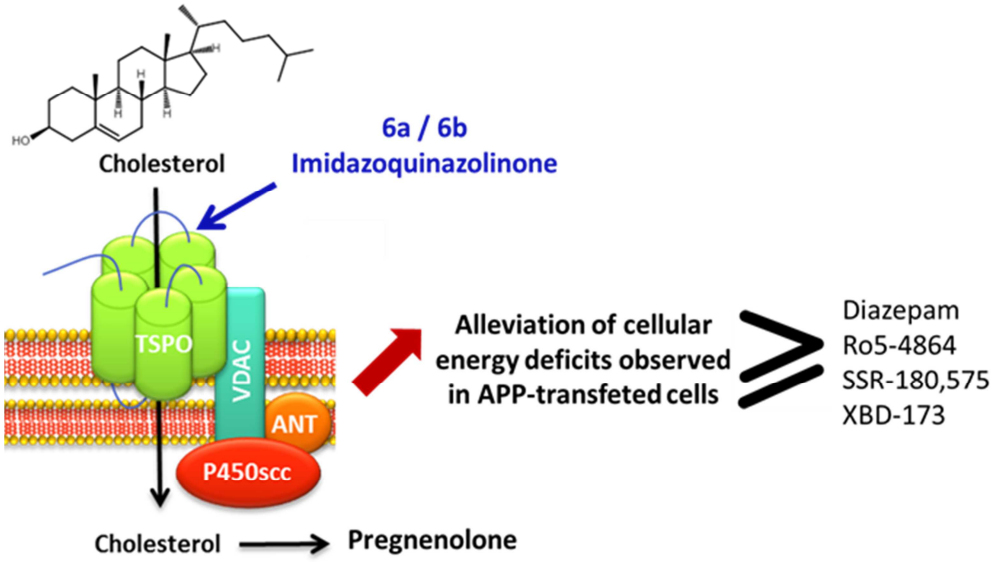


Figure 5: The imidazoquinazolinone compounds, 6a and 6b, reduce bioenergetic deficits observed in the cell model of Alzheimer's disease, with similar functional effects as diazepam and a significantly higher effect than those obtained with the reference products Ro5-4864, XBD-173 and SSR-180,575

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III. Discussion

Discussion

The main purpose of the thesis was to investigate the ability of two great families of new patented compounds to exert neuroprotective effects via the modulation of the mitochondrial activity in nerve cells. These two families of molecules are the ANS, different structural analogs of the endogenous neurosteroid allopregnanolone and ligands of the mitochondrial translocator protein (LTSPO) which transfers cholesterol from the outer membrane to the inner mitochondrial membrane and converts it to pregnenolone, a major precursor in the biosynthesis of various neurosteroids.

The major results of this study are as follows: First, BR297, an analog of allopregnanolone, was identified, which was similar or superior to the natural neurosteroid in ameliorating bioenergetics and protecting the APP cells (cellular model of Alzheimer's disease) against oxidative stress. Second, the protective effect of BR297 was confirmed through *in vivo* analysis in transgenic Alzheimer's disease mouse model suggesting that BR297 enhances bioenergetics, protects against oxidative stress *in vitro,* and can also attenuate the AD-related decline of mitochondrial function *in vivo.* Third, *in vivo* analysis in aged mice showed that BR297 exhibited a higher efficacy than BR351 in alleviating the age-related mitochondrial deficiency in the hippocampus. The last compound is another analog of allopregnanolone known for its neurogenic and neuroprotective effect in adult neural stem cells, results provided by our collaborators. These observations could be correlated with the results of cognitive tests and morphological observations obtained by our collaborators. Fourth, TSPO ligand 6a and 6b were identified to ameliorate the bioenergetics in APP cells with a comparable or a higher efficacy compared to LTSPO of reference.

1. Effect of allopregnanolone and its analog BR297 to rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement

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 Aβ production and ROS generation as well as impaired mitochondrial function (decrease of ATP production, mitochondrial respiration and mitochondrial complex IV activity), were used (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 the 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, a higher concentration of AP as well as BR297 was able to increase the ATP level in control (CTRL) and APP transfected SH-SY5Y cells. Our data showed that a concentration of 500 nM of AP and the analog BR297 were able to improve both basal respiration and glycolysis, increasing the bioenergetic activity and ATP production in the two cell lines. In particular, an upregulation of the respiratory control ratio (RCR) induced by BR297 was witnessed in both cell lines, but AP had no significant effect in APP cells. A high RCR is an indicator of the capacity of cells to oxidize substrates, when they have high energy demands. Interestingly, no effect on the cell proliferation was observed after a treatment of 24 hours with AP or BR297 at 500 nM in CTRL and APP transfected cells. Thus, the upregulatory effect of AP and BR297 on bioenergetics was not due to an increase of cell growth.

The presence of nuclear steroid receptors, 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 an allosteric modulator of $GABA_A$-R (Carver and Reddy 2013) and recent studies showed that AP can

also bind to the PXR, a nuclear steroid receptors (nSR) playing a role in xenobiotic metabolism and transporter gene expression (Ekins, Chang et al. 2007, Frye, Koonce et al. 2014). $GABA_A$-R was not detected in native SH-SY5Y cells (Grimm, Schmitt et al. 2014). AP can act via other metabolites whereas the analog, BR297, which has no hydroxyl group, cannot be converted back to other metabolites such as 5α-DHP. To understand more deeply the mechanisms underlying AP and BR297 action, further investigations are now required using antagonists of steroid receptors such as PXR or progesterone receptors.

To investigate in more detail the neuroprotective potential of AP and BR297, their effects on mitochondrial bioenergetics were tested 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).

The beneficial effect on bioenergetics of AP and BR297 that we observed under physiological condition was investigated whether it may also protect the cells against cell death under oxidative stress conditions. H_2O_2, a major ROS, is known as a mediator of brain damages caused by the abnormal elevation of β-amyloid peptides (Aβ) in AD (Behl, Davis et al. 1994, Citron 2010). First, APP cells were more sensitive to H_2O_2 compared to CTRL cells and presented a prominent rise 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 BR297 pretreated CTRL and APP cells in oxidative stress conditions, suggesting that energy supply was in part preserved. A pre-treatment with BR297 was able to induce an upregulatory effect of the respiratory capacity (RCR) of both cell lines under stress conditions. Additionally, BR297 was more efficient to improve the mitochondrial respiration in CTRL and APP cells under physiological condition 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 H_2O_2.

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 findings 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, and (2) the amelioration of the cellular and mitochondrial energy. Further investigations are required to determine in more detail the possible mechanisms underlying the protective action of BR297, especially the ability to modulate the antioxidant defences (e.g ROS scavenging activity, activation of superoxide dismutases and gluthatione system).

In line with a recent study, the compound BR297 (3β -O-allyl-AP) demonstrated an increase of the neuroprotective activity compared to AP in neural stem cell cultures treated with amyloid beta 42 (Karout, Miesch et al. 2016). Our findings showed that BR297 exhibits notable advantages over AP with regards to both protection of mitochondrial function and reduction of oxidative stress. Under physiological conditions, BR297 had no effect on cell survival but it ameliorated the bioenergetics by increasing the cellular ATP level and mitochondrial respiration. BR297 has no effect on the proliferation but seems to block the cell death mechanism, improving the cell viability. Indeed, a pre-treatment with BR297 increases the survival of CTRL and APP cells under stress conditions, protecting the cells against H_2O_2-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

2. Effect of BR297 on mitochondrial function in Tg2576

The contributing factor of the cognitive decline observed in AD is attributed to the dysfunctional mitochondrial bioenergetics. Tg2576 mice exhibited from an age of 6 months Aβ accumulation, cognitive impairment and revealed an impaired state 3 respiration in brain mitochondria (Gillardon, Rist et al. 2007). Aβ dependent mitochondrial dysfunction starts already at 3 months of age in AD transgenic mice by reducing ATP levels when elevated intracellular but not Aβ extracellular deposits are present (Hauptmann, Scherping et al. 2009). Amyloid plaques depositions are found in the cortex and the hippocampus at 9 to 13 months of age (Kawarabayashi, Younkin et al. 2001, Yassine, Lazaris et al. 2013). Since BR297 has been shown to efficiently ameliorate the bioenergetics and protect the mitochondrial function against oxidative stress *in vitro*, in a cellular model of AD, we initially focused our efforts on this compound and investigated whether it would counteract the AD-dependent decline of mitochondrial activity *in vivo*.

The following, three doses of BR297 were chosen based on data from chronic and acute toxicology tests: 2, 4 and 8 mg/kg. First we reported the vehicle-treated 10-month-old Tg2576 mice showed a strong drop of mitochondrial function by decreasing the ATP levels and complexes I and IV activities compared to the vehicle-treated 10-month-old wild-type (WT) mice. Namely, our results demonstrated that BR297 analog treatment has attenuated mitochondrial dysfunction observed in Tg2576 mice until restore and ameliorate to that mitochondrial activity of their nontransgenic littermates. These results are in agreement with *in vitro* studies, in which the best ANS BR297 displayed protective effects on the mitochondrial function in the cellular model of AD. For instance, 8 mg/kg of BR297 significantly increased the ATP levels, complex I and IV activities in cortex and hippocampi regions of 10-month-old Tg2576 mice, suggesting protective effect of BR297 against AD-induced mitochondrial deficits. Further investigations are required to clearly determine other

potential mechanism of the protective effects of BR297 to act on the Aβ levels in the cortex and the hippocampi of Tg2576 mice.

Our results demonstrated that BR297 could modulate brain energetics to preserve mitochondrial functional protein activities, and improve cognitive functions in AD mice (Tg2576).

3. Effect of analogs of allopregnanolone on age-related mitochondrial deficits

Aging and neurodegeneration exhibit declines in energy production in the brain and parallel changes in redox status with a pro-oxidant shift, partly due to the mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2. Brain Mitochondria isolated from aged animals showed a partial loss of energy transducing capacity (Yin, Boveris et al. 2014). In the aged brain, electron transfer and membrane enzyme activities such as those of complexes I and IV decrease in mitochondria (Beckman and Ames 1998, Navarro and Boveris 2007). Age causes a decline in regenerative factors in the neurogenic niche resulting in a neural stem cell transition from an active dividing state to a quiescent state within the subgranular zone of the dentate gyrus. This leads to a decline of the regenerative potential of the aging brain (Encinas, Michurina et al. 2011). Episodic memory (EM) is sensible to the effects of age and disturbed in Alzheimer's disease. The first memory system known to decline in both normal and pathological aging is EM (Tromp, Dufour et al. 2015). Neurosteroids including pregnenolone and allopregnanolone play an essential role in the performance of memory, aging conditions, and the physiopathology of AD. Indeed, studies performed in human and animal models have shown that age-related drops of neurosteroid levels give rise to neuronal dysfunction and degeneration due to the loss of protective and neuroregenerative effects (Smith, Wekstein et al. 2006, Caruso, Barron et al. 2013). Diverse neurosteroids tested *in vitro* by Grimm and her coworkers were able to improve the bioenergetic activity by increasing ATP levels, mitochondrial membrane potential, and basal mitochondrial respiration, and modulated redox homeostasis by ameliorating the antioxidant activity in neural cells (Grimm, Schmitt et al. 2014). Moreover, a boost of mitochondria is a rather common mechanism of

different neurosteroids (Grimm, Schmitt et al. 2014). Collaborators showed that, another O-allyl-analog, BR351 (ANS) exerted neuroprotective and neurogenic effects on adult neural stem cells (Karout, Miesch et al. 2016).

The ability of BR297 and BR351 to improve mitochondrial functions in a model of non-pathological brain aging was investigated in this study. Our results demonstrated that BR297 significantly corrected age-related mitochondrial deficits in the cortex and hippocampus. BR297 ameliorated cellular and mitochondrial ATP levels, and complex I and IV activities in both brain regions. The beneficial effect of a BR351 treatment is that it is more specific to the cortex by enhancing cellular and mitochondrial ATP levels. Concerning the effect of BR351 on complex activities, this analogue increased only the activity of complex I but not that of complex IV. BR297 exhibits a higher efficacy on alleviation of mitochondrial deficits observed in aging in comparison to BR351. It appears that in old mice, neurogenic action is essential to restore a performance similar to that of young mice in the Pattern Separation test and a slight improvement in mitochondrial function may suffice to support this neurogenesis. This effect profile corresponds to the biological activities of the BR351 profile. However, in the Tg2576 mouse model, improved memory performance (moving objects test) requires effective neuroprotectants against beta-amyloid toxicity and oxidative stress. This neuroprotective action, which involves a strong positive modulation of mitochondrial functions without necessarily inducing cell proliferation, could correspond better to the biological activity profile of the compound BR297.

BR297 and BR351 showed protective effects in aged mice and may be a promising multitarget therapy to reduce or prevent age-related illness processes.

Taking together this thesis work showed that allopregnanolone-analogs synthesis is revealed to be a promising therapeutic tool for the development of neuroprotective and/or neurogenic strategies, but several points have to be taken into account such as treatment regimen,

dosing regimen, solubility, bioavailability, route of administration and the sex differences. The group of Brinton highlighted the importance of the optimization of the treatment regimen. The intermittently administration of AP promoted renewal and repair whereas continuous infusions of AP were antiregenerative in mouse models of AD (Brinton 2013). Chen and collaborators showed that injecting animals with AP three times per week during three months was significantly less efficient compared to a once per week injection that showed anti-amyloidogenic effects as well as an efficiently stimulation of neurogenesis (Chen, Wang et al. 2011). AP had beneficial effects on cell survival and on learning and memory in the state of intraneuronal Aβ accumulation in 6-month-old mice (Wang, Singh et al. 2010, Chen, Wang et al. 2011).

Concerning the optimal treatment dose, allopregnanolone was shown to exert inhibitory effects at higher concentrations due to a conversion to allopregnanediol (reduction of the keto group at C20) and to stimulate proliferation of neural progenitors at lower concentrations (nM) (Wiebe and Lewis 2003). Allopregnanolone exhibits a biphasic effect dose response profil (Wang and Brinton 2008). However, Aβ accumulation and impairment of learning and memory are the consequence of chronically elevated AP-level (Bengtsson, Johansson et al. 2013). BR297 showed protective effects on mitochondrial functions at the highest dose (8 mg/kg) whereas BR351 at the lowest dose (1 mg/kg). Lower doses for BR351 and higher doses for BR297 should be tested in the future.

The bioavailability may be limited by an oral route (metabolization in the digestive tract and the liver). This is why several formulations of allopregnanolone were developed for parenteral administration (Irwin and Brinton 2014). No studies are available yet for the analogs of allopregnanolone. AP showed different effects between males and females in clinical human trials, so the question about the sex differences for our analogs is important (Kask, Backstrom et al. 2008, Kask, Backstrom et al. 2009).

4. Effect of TSPO ligand on cellular energy in Alzheimer's disease

The structure, affinity, and concentration of the treatment with LTSPO play a crucial role in the efficiency of these compounds. James and coworkers showed that LTSPO should contain an electron-rich site and lipophilic region (James, Selleri et al. 2006). The affinity is improved by the insertion of a N,N-diethylamide chain on the free azote of urea in the quinazolinone cycle of the majority of TSPO ligands.

Diazepam and Ro5-4864 are benzodiazepine molecules bearing similar structures and differentiate only by a chloride atom added in the para position in Ro5-4864 (also named 4'-chlorodiazepam). The affinity of Ro5-4864 for TSPO is given by the capacity to replace radiomarked [H^3]diazepam from the TSPO binding site. The affinity varies in different tissues: Ro5-4864 has very high IC_{50} values in the brain (163000 nM) and a low IC_{50} (nM) in peripheral tissues (4.7 nM on kidney; 4.1 nM on liver; 4.8 nM on lungs). Diazepam also binds at GABA receptors and is not only selective for TSPO, while Ro5-4864 is the most selective benzodiazepine for TSPO. The selectivity and the difference in IC_{50} between peripheral and central tissues of Ro5-4874 are explained by the presence of chloride. However, the elevated affinity for TSPO and the presence of chloride could explain the increase in ATP levels in control cells (10 nM) but not in APP-transfected cells. The presence of the chloride in Ro5-4864 is not sufficient for the ATP-enhancing in APP-transfected cells overexpressing the Aβ peptide, even if it was shown in the literature that Ro5-4864 attenuated the hippocampal accumulation of Aβ (Barron, Garcia-Segura et al. 2013). Barron and collaborators highlighted *in vivo* effects of Ro5-4864 on young adult 3xTgAD mice (4 months) by increasing the levels of allopregnanolone, progesterone, dihydrotestosterone, and testosterone as well as improving the memory deficits. However, aged 3xTgAD mice (23 months) showed no regulation of neurosteroids (Barron, Garcia-Segura et al. 2013). Therefore, primary protective mechanisms of TSPO ligands are different between young and aged mice (Mukhin, Papadopoulos et al. 1989).

SSR-180,575 is a TSPO ligand with nanomolar affinity developed by the pharmaceutical company SANOFI. SSR-180,575 is lipophilic and can easily pass the blood brain barrier

(BBB), but problems of solubility and pharmacokinetics may appear in *in vivo*. The IC_{50} given for the structure of SSR-180,575 in relation to the radiomarked [H^3]Ro5-4864 is 2.5±0.6 nM. The results of SSR-180,575 treatments showed an increase in ATP levels in control and APP cells.

XBD-173 has been developed in the 1990s by the pharmaceutical company Dianippon as an anxiolytic drug without the typical drug-related problem of benzodiazepines. Its affinity for TSPO varies depending on the tissues (Ki = 0.3 – 3 nM) with a solubility of 3.7 µg/ml. Clinical studies investigated this drug for the first time in patients treating anxiety (Rupprecht, Papadopoulos et al. 2010). XBD-173 ameliorated only the ATP-enhancing in APP cells. It had no effect on control cells.

The presence of the N,N-diethylacetamide chain in the TSPO ligand 6a allows to reduce the affinity for the central benzodiazepine receptor and increase the affinity for TSPO. The insertion of the imidazole cycle also ameliorated this selectivity. 6a has no affinity for the central benzodiazepine receptor (IC_{50}> 10µM), while it exhibits an IC_{50} of 80 nM for TSPO. The affinity for 6a is given in relation to its capacity to replace radiomarked [H^3]Ro5-4864. The TSPO ligand 6b has a structure similar to 6a, but differs by a lateral chain dipropylamide instead of diethylamide. This structural difference allows the compound 6b to have a better affinity for TSPO. In fact, in regard to the radiomarked [H^3]Ro5-486, the affinity of 6b is eight times higher than the one of 6a (IC_{50} 6b: 12nM). The high affinity of 6b is correlated to a greater increase in ATP levels than in 6a treatment control and APP cells. In control cells, ATP levels were increased by both compounds, but with a higher effect with a 6b treatment. In APP cells, both compounds ameliorated the production of ATP to a similar extent. Generally, the new TSPO ligands 6a and 6b are more efficient to compensate the energy loss caused by an overexpression of Aβ compared to the LTSPO described in the literature.

Taking together, our results showed that the imidazoquinazolinone structure seems to be essential to alleviate the bioenergetic deficit observed in AD pathology. More precisely, the

lateral chain dipropylamide structure of the ligand 6b allowed the amelioration of the energy as well as the affinity and selectivity for the receptor TSPO.

IV. Conclusion and perspectives

Conclusions and perspectives

Neurodegenerative diseases are amongst the major causes of disability, decreased quality of life, and death worldwide. They are characterized by gradual nervous system dysfunctions (Winter, Korchounov et al. 2011). Alzheimer's disease (AD), the most common cause of dementia, is characterized by the presence of two main histopathological signs in the brain: intracellular neurofibrillary tangles (NFTs) composed of aggregations of abnormally hyperphosphorylated tau protein, and extracellular amyloid-β (Aβ) plaques (Berchtold and Cotman 1998). Aging is the most important risk factor for AD and a challenge to every living organism. It is linked to successive metabolism impairments and cell damage, leading to disease development (Hung, Chen et al. 2010). Mitochondrial dysfunctions represent a pathophysiological mechanism of AD, since an elevation in oxidative damages and a reduction of energy metabolism are already described at early stages of the disease, even before the appearance of NFTs and Aβ 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). Thus, current pharmacological concepts for the development of therapeutic strategies against AD have a particular interest in the track of the regulation of mitochondrial functions in neurons, including the control of ATP synthesis, mitochondrial respiration, and ROS production.

In the last decade, neurosteroids and the modulation of their biosynthesis have been shown to play a major role in the pathophysiology of neurodegenerative disorders such as AD. In particular allopregnanolone presents several beneficial effects in neurodegenerative diseases and CNS or PNS disorders (Brinton 2013, Patte-Mensah, Meyer et al. 2014). Allopregnanolone is known to ensure the recovery of learning and memory functions, and promote regeneration in the brain (Brinton 2013). Allopregnanolone ensured neuroprotection against Aβ toxicity, but also stimulated rodent and human neural progenitor cell proliferation to compensate the AD-related cell loss in a triple transgenic (3xTgAD) mouse model of AD, (Wang, Johnston et al. 2005, Chen, Wang et al. 2011). Together, these findings show that allopregnanolone 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 allopregnanolone, nothing is known about its effects on AD-induced mitochondrial deficits. Furthermore, the pleiotropic nature of the effects of allopregnanolone does not facilitate the development of targeted therapy, specifically focused on a specific indication, such as neuroprotection, while avoiding stimulation of cell proliferation.

In line with these observations, the main purpose of this thesis was to evaluate the ability of two families of new patented compounds to exert neuroprotective effects via the modulation of mitochondrial functions in nerve cells. The two families of compounds are the allopregnanolone analogs (ANS) and the ligands of the translocator protein (LTSPO). TSPO allows the transfer of cholesterol from the outer membrane to the inner mitochondrial membrane, where it is converted by the cytochrome P450side-chain-cleavage to pregnenolone, a major precursor in the biosynthesis of various neurosteroids. TSPO ligands represent important modulators of the neurosteroidogenesis.

In the present PhD thesis work, our study revealed that allopregnanolone (AP) and its analog O-allyl-epiAP (BR297) were able to boost the ATP production *in vitro,* in a cellular model of AD, human SH-SY5Y neuroblastoma cells mimicking the enhanced generation of β-amyloid peptide (Aß), overexpressing the human wild-type amyloid precursor protein or APP (APP cells), and their respective control cells. In contrast, the analogs O-allyl-AP (BR351), 12-oxo-epiAP (BR053) and 12-oxo-AP (BR338) do not alter significantly the ATP synthesis. These observations are in line with other studies showing that neurosteroids improved bioenergetic activity by increasing ATP levels, mitochondrial membrane potential, basal mitochondrial respiration, and modulated redox homeostasis by ameliorating the antioxidant activity in neural cells (Grimm, Schmitt et al. 2014).

A growing body of evidence has highlighted neuroprotective effects of neurosteroids against AD injury (Grimm, Lim et al. 2012, Brinton 2013). This thesis showed that only BR297 (500

nM) is effective in ameliorating the survival of control cells suffering from oxidative stress, while both allopregnanolone and BR297 are able to effectively protect APP cells against death induced by oxidative stress generated by hydrogen peroxide (H_2O_2). Oxidative stress plays a determinant role in the pathogenesis of AD by exacerbating mitochondrial deficits (Grimm, Friedland et al. 2016). Commonly used as an indicator of oxidative stress, intracellular ROS formation was reduced in allopregnanolone and BR297 pre-treated control and APP cells, suggesting that BR297 also exerts its protective action by reducing ROS levels during H_2O_2 exposure. These data are in line with the compound BR297 (3β -O-allyl-AP) that demonstrated an increase of the neuroprotective activity as compared to allopregnanolone in neural stem cell cultures treated with $A\beta_{42}$ (Karout, Miesch et al. 2016). Grimm and coworkers showed that a selection of neurosteroids, including allopregnanolone, were able to modulate the cellular redox state in native SH-SY5Y cells by increasing antioxidant activity (Grimm, Schmitt et al. 2014). Furthermore, the present thesis showed that allopregnanolone and BR297 improved both oxygen consumption (OCR) and glycolysis (ECAR), increasing the bioenergetic activity in APP and control cells. In particular, BR297 modulated the respiratory control ratio (RCR), an indicator of respiratory capacity of mitochondria in both cell types, but allopregnanolone had no significant effect on APP cells. Thus, allopregnanolone and its analog BR297 were able to: i) alleviate bioenergetic dysfunctions observed in APP cells compared to control and ii) protect these cells against oxidative stress-induced cell death.

Together, our findings suggest that a pre-treatment with BR297 and allopregnanolone could exhibit a protective action against oxidative stress through the reduction of the ROS generation, and the amelioration of the cellular and mitochondrial energy.

BR297 is identified as an analog, which is capable of inducing beneficial effects on mitochondrial bioenergetics without stimulating cell proliferation, as does allopregnanolone. BR297 presents an interesting profile to develop neuroprotective strategies without the induction of proliferative effects.

Because of its strong protective effect observed in *in vitro* studies in a cellular model of AD, the effect of BR297 (O-allyl-epiAP) was tested with regard to its ability to reduce mitochondrial dysfunctions observed in an experimental animal model of AD with the transgenic Tg2576 AD mice. ATP production and complex activity analyses demonstrated the protective effect of BR297 by an amelioration of these mitochondrial functions in the cortex and the hippocampus of Tg2576 mice up to the level of the vehicle-treated wild-type (WT) mice. Data from collaborators confirmed that BR297 had no effect on the neurogenesis but induced a positive trend to behavioral improvements.

One of the characteristic features of aging is the dysfunction of mitochondria. Energetic deficits and mitochondrial impairment are common pathological mechanisms in healthy aging and age-related neurodegenerative diseases (Lam, Yin et al. 2009). Data from other collaborators also showed that the compound BR297 exerted neuroprotective effects on adult neural stem cells (Karout, Miesch et al. 2016). They also highlighted another O-allyl analog, BR351 (ANS), which is able to utilize neuroprotective properties of neurosteroids and simultaneously stimulate neurogenesis. Given that age is a risk factor for dementia, the protective effect of the two analogs of the natural neurosteroid allopregnanolone O-allyl-epiAP (BR297) and O-allyl-AP (BR351) was investigated on age-related disturbances of energy maintenance in the brain. The decreased electron transfer rates in complex I and complex IV are the main cause for the impairment of mitochondrial functions in aging brain (Navarro and Boveris 2004). In line with this study, the present thesis work showed that aged mice exhibited significant deficits in cellular ATP levels in the cortex and hippocampus and in mitochondrial ATP levels, especially in the hippocampus. 21-month old mice brains only showed a trend to reduced complex activities. BR297 significantly corrected age-related mitochondrial deficits in the cortex and hippocampus. BR297 ameliorated cellular and mitochondrial ATP levels, and complex I and IV activities in both brain regions. The beneficial effect of BR351 treatment is more specific to the cortex by enhancing cellular and mitochondrial ATP levels. BR351 increased only the activity of complex I but not that of the

complex IV. A higher efficacy on alleviation of mitochondrial deficit observed in aging was exhibited by BR297 in comparison to BR351.

A slight amelioration in mitochondrial activity exhibited by BR351 in old mice could suffice to support the neurogenic action which is important to restore a performance similar to that of young mice in the Pattern Separation test. In the Tg2576 mouse model, the neuroprotective action of BR297 is required to fight against beta-amyloid toxicity and oxidative stress as well as for the memory performance. This neuroprotective action could correspond better to the biological activity profile of the compound BR297, which involves a strong positive modulation of mitochondrial functions without necessarily inducing cell proliferation. BR297 and BR351, O-allyl-substituted allopregnanolone analogs, showed protective effects in aged mice and may be a promising targeted therapy to reduce or prevent age-related illness processes, a major risk factor for Alzheimer's disease.

The protective effect of these analogs revealed that the correlation structure-activity seems to be independent to the α- and the β-conformation. BR351 (O-allyl-AP), in addition to a protective effect, a neurogenic effect was reported by collaborators *in vivo* in behavioural observation using the pattern separation test and an increase of numbers of stem cells as well as proliferating progenitors in aged mice. The substitution of the hydroxyl group by an O-allyl group at the position 3, preventing the re-oxidation and the glucuronisation of the neurosteroid, allowed an increase of the bioavailability leading to a higher efficacy of the analogs compared to allopregnanolone.

In conclusion, the *in vitro* and *in vivo parts* of our studies on the ANS effects identified the O-allyl-derivatives of allopregnanolone, BR297 and BR351 as promising candidates for the treatment of neurodegenerative disease such as AD. Future experiments should aim at more understanding of the molecular mechanisms underlying the activities of the AP analogs.

TSPO ligands, studied for their neuroprotective and anxiolytic properties, were found to increase the level of neurosteroids as pregnenolone and allopregnanolone (Campiani, Nacci

et al. 1996, Selleri, Bruni et al. 2001, Ferzaz, Brault et al. 2002, Primofiore, Da Settimo et al. 2004, Rupprecht, Rammes et al. 2009, Karlstetter, Nothdurfter et al. 2014). Ro5-4864 treatment is the first study showing the beneficial effect of TSPO ligands on AD by acting on several clinical targets, such as memory function and anxiety behaviors, attenuating hippocampal Aβ accumulation, gliosis, altering brain testosterone and progesterone levels, and behavioural impairment in the 3xTgAD model of AD (Barron, Garcia-Segura et al. 2013). LTSPO seem to offer alternative therapeutic strategies focused on reducing the accumulation of Aβ, as they simultaneously target multiple facets of the neurodegenerative cascade, such as neuroinflammation, oxidative stress, mitochondrial dysfunction, and neuronal loss. Although, many TSPO ligands suffer from a common problem of solubility and bioavailibility. The implication of LTSPO in neuroprotection processes, particularly their effects on mitochondrial bioenergetics in nerve cells, has been of great interest. Pre-tests have shown that the new TSPO ligands 6a and 6b, developped and synthetized by collaborators, induced the production of pregnenolone in glial cells.

In this thesis, the effects of these two new compounds, 6a and 6b, compared with four LTSPO references molecules, namely diazepam, XBD-173, SSR-180,575, and Ro5-4864, were determined in APP cells. 6b has a structure similar to 6a, but differs by a lateral chain dipropylamide instead of diethylamide. This structural difference gives the compound 6b a higher affinity for TSPO. A non-toxic effect was demonstrated by the new LTSPO at 10 nM on cell survival. Indeed, at a nanomolar concentration PK-11195 (alpidem), another TSPO ligand, and Ro5-4864 have been shown to protect cells against apoptosis (Strohmeier, Roller et al. 2002, Kugler, Veenman et al. 2008). The higher affinity of 6b for TSPO is correlated to a greater amelioration in ATP levels than in a 6a treatment in control and APP cells. In control cells, ATP levels were improved by both compounds but with a higher effect for the 6b treatment. In APP cells, both compounds increased the production of ATP to a similar extent. Thus, 6a and 6b are more efficient in compensating the energy loss caused by an overexpression of Aβ peptide than the LTSPO described in the literature.

The neuroprotective actions of the TSPO ligands may be due to the modulation of endogenous production of neurosteroids in the nervous system (Rupprecht, Rammes et al. 2009, Rupprecht, Papadopoulos et al. 2010). This has to be confirmed for 6a and 6b in further studies.

Taking together, all the results showed that the imidazoquinazolinone structure seems to be important to counteract the bioenergetic deficit observed in AD pathology. Particularly, the lateral dipropylamide chain of the ligand 6b allowed a higher amelioration of the energy as well as of the affinity and the selectivity for the TSPO.

Altogether, this PhD work leads to the identification of new modulators of mitochondrial bioenergetics in aging and AD for the development of optimized and better targeted neuroprotective strategies. *In vitro* and *in vivo* studies highlight the new analogs of allopregnanolone with neuroprotective and/or proliferative effects and TSPO ligands with the imidazoquinazolinone structure as promising candidates for the treatment of neurodegenerative diseases such as AD.

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V. Descriptif synthétique des travaux de la thèse

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Les mitochondries, véritables centrales énergétiques des cellules, jouent un rôle essentiel dans la survie et la mort des cellules neuronales car elles sont régulatrices à la fois du métabolisme énergétique et des voies apoptotiques. Les mitochondries fournissent la majorité de l'énergie cellulaire sous la forme d'adénosine triphosphate (ATP) et régulent la production des espèces réactives oxygénées (ROS) (Schmitt, Grimm et al. 2012). De nombreuses évidences révèlent que les anomalies mitochondriales sont impliquées dans les mécanismes physiopathologiques de la maladie d'Alzheimer (MA) car une augmentation des dommages oxydatifs et une diminution du métabolisme énergétique sont observées à des stades précoces de la maladie avant l'apparition des plaques amyloïdes et des dégénérescences neurofibrillaires représentant les deux principales caractéristiques histopathologiques de la MA (Eckert, Hauptmann et al. 2008, Rhein, Baysang et al. 2009). Par conséquent, les concepts pharmacologiques actuels visant le développement de stratégies thérapeutiques contre la MA portent un intérêt particulier à la piste de la régulation des fonctions mitochondriales dans les neurones, notamment le contrôle de la synthèse d'ATP, de la respiration mitochondriale et de la production de ROS. Par ailleurs, compte tenu de l'implication de certaines enzymes mitochondriales dans la synthèse des stéroïdes et plus particulièrement du rôle crucial joué par les neurostéroïdes dans la régulation de la différenciation, de la croissance et de la survie des cellules nerveuses, divers travaux ont également recherché les modifications de la biosynthèse des neurostéroïdes (neurostéroïdogenèse) dans le système nerveux au cours du vieillissement et dans la physiopathologie de la MA (Mensah-Nyagan, Do-Rego et al. 1999, Patte-Mensah, Meyer et al. 2010, Brinton 2013, Caruso, Barron et al. 2013). De façon intéressante, ces travaux ont révélé une baisse liée à l'âge de la production de certains neurostéroïdes neuroprotecteurs comme l'alloprégnanolone (AP) qui exerce également des effets neurogéniques, neurorégénérateurs et stimule la prolifération cellulaire (Schumacher, Weill-Engerer et al. 2003). La diminution de la neurostéroïdogenèse et des concentrations cérébrales d'AP a également été corrélée au déclin cognitif observé dans la MA (Frye and Walf 2008, Caruso,

Barron et al. 2013). Ces données ont suscité un fort intérêt pour le développement de stratégies thérapeutiques basées sur l'utilisation de l'AP. Toutefois, le caractère pléiotrope des effets exercés par l'AP ne facilite pas l'élaboration de thérapie ciblée permettant de se focaliser spécifiquement sur une indication bien précise, par exemple la neuroprotection, tout en évitant de stimuler la prolifération cellulaire pouvant faciliter l'apparition de gliomes. En revanche, dans les cas de déficits fonctionnels corrélés au déclin de la neurogenèse, il pourrait être intéressant de produire des analogues ayant une action neurogénique plus importante et plus spécifique que celle de l'AP.

Au regard des données rappelées ci-dessus, le principal but du travail de thèse a été d'évaluer la capacité de deux grandes familles de nouveaux composés brevetés à exercer des effets neuroprotecteurs et/ou neurogéniques via la modulation des fonctions mitochondriales dans les cellules nerveuses. Ces deux familles de composés sont les analogues de l'AP (ANS) et les ligands de la protéine de translocation mitochondriale ou *translocator protein* (LTSPO) qui assure le transfert du cholestérol de la membrane externe vers la membrane interne des mitochondries où est localisé le cytochrome P450*side-chain-cleavage* catalysant la transformation du cholestérol en prégnènolone, un précurseur majeur de la biosynthèse de divers neurostéroïdes. Les LTSPO sont alors des modulateurs importants de la neurostéroïdogenèse. Le travail de thèse (co-tutelle Strasbourg-Bâle) a été réalisé dans le cadre du programme collaboratif de recherche du Consortium Trinational NeuroRhine qui a été lauréat de l'appel 2012 "Offensives Sciences et INTERREG IV Rhin Supérieur".

Pour atteindre notre objectif, nous avons dans un premier temps utilisé les neuroblastomes humains SH-SY5Y sur-exprimant l'*amyloid precursor protein* ou APP (SH-SY5YAPPwt) car ces cellules constituent un modèle expérimental bien connu et pertinent pour l'étude des mécanismes moléculaires et cellulaires impliqués dans la physiopathologie de la MA (Scheuermann, Hambsch et al. 2001, Schaeffer, Patte-Mensah et al. 2006, Rhein, Baysang

et al. 2009, Grimm, Biliouris et al. 2016). Des travaux antérieurs de notre équipe ont montré une baisse de la production d'ATP dans les SH-SY5YAPPwt qui sont aussi plus sensibles et vulnérables au stress oxydant que les cellules témoins (transfectées avec le vecteur vide) (Schaeffer, Patte-Mensah et al. 2008, Rhein, Baysang et al. 2009, Schulz, Eckert et al. 2012, Wendt, Kemmel et al. 2014).

Nous avons alors évalué la capacité de l'AP et de ses analogues ANS à moduler la production d'ATP dans les SH-SY5YAPPwt et les cellules témoins. Nos résultats montrent qu'à la concentration de 500 nM, l'AP et son analogue O-allyl-epiAP (BR297) stimulent la production d'ATP dans les cellules SH-SY5YAPPwt et témoins. En revanche, les analogues O-allyl-AP (BR351), 12 oxo epiAP (BR053) and 12 oxo-AP (BR331) ne modifient pas la synthèse d'ATP. Le puissant effet stimulateur de la production d'ATP exercé par le BR297 nous a encouragés à comparer sa capacité à celle de l'AP dans la protection contre la mort cellulaire induite par le stress oxydant généré par le peroxyde d'hydrogène (H_2O_2) (**Figure 1**). Nous avons observé que seul le BR297 (500 nM) est efficace dans l'amélioration de la survie des cellules témoins en situation de stress oxydant alors que les deux composés AP et BR297 sont capables de protéger efficacement les cellules SH-SY5YAPPwt contre la mort induite par l'H_2O_2 (**Figure 1**). Nos résultats montrent aussi que le BR297 et l'AP, qui diminuent significativement les concentrations de ROS cytosoliques et mitochondriales, réduisent fortement les niveaux élevés d'anions superoxydés dans les SH-SY5YAPPwt (**Figure 1**). Par ailleurs, nos travaux montrent que l'AP et le BR 297 améliorent à la fois la consommation d'oxygène (OCR) et la glycolyse (ECAR) augmentant ainsi l'activité bioénergétique dans les cellules SH-SY5YAPPwt et témoins. En particulier, nous avons observé que le BR297 module le *respiratory control ratio* (RCR), un indicateur de la capacité respiratoire des mitochondries, dans les deux catégories cellulaires, mais l'AP n'exerce aucun effet significatif sur les cellules SH-SY5YAPPwt. Un RCR élevé est un indicateur de la capacité d'oxydation de substrats lorsque les cellules ont des exigences élevées en énergie. Nos résultats permettent donc d'identifier le BR297 comme un analogue qui est capable d'induire des effets bénéfiques sur la bioénergétique mitochondriale sans stimuler la

prolifération cellulaire comme le fait l'AP. Ces données confèrent au BR297 un profil très intéressant pour l'élaboration de stratégies neuroprotectrices sans induction d'effet proliférateur.

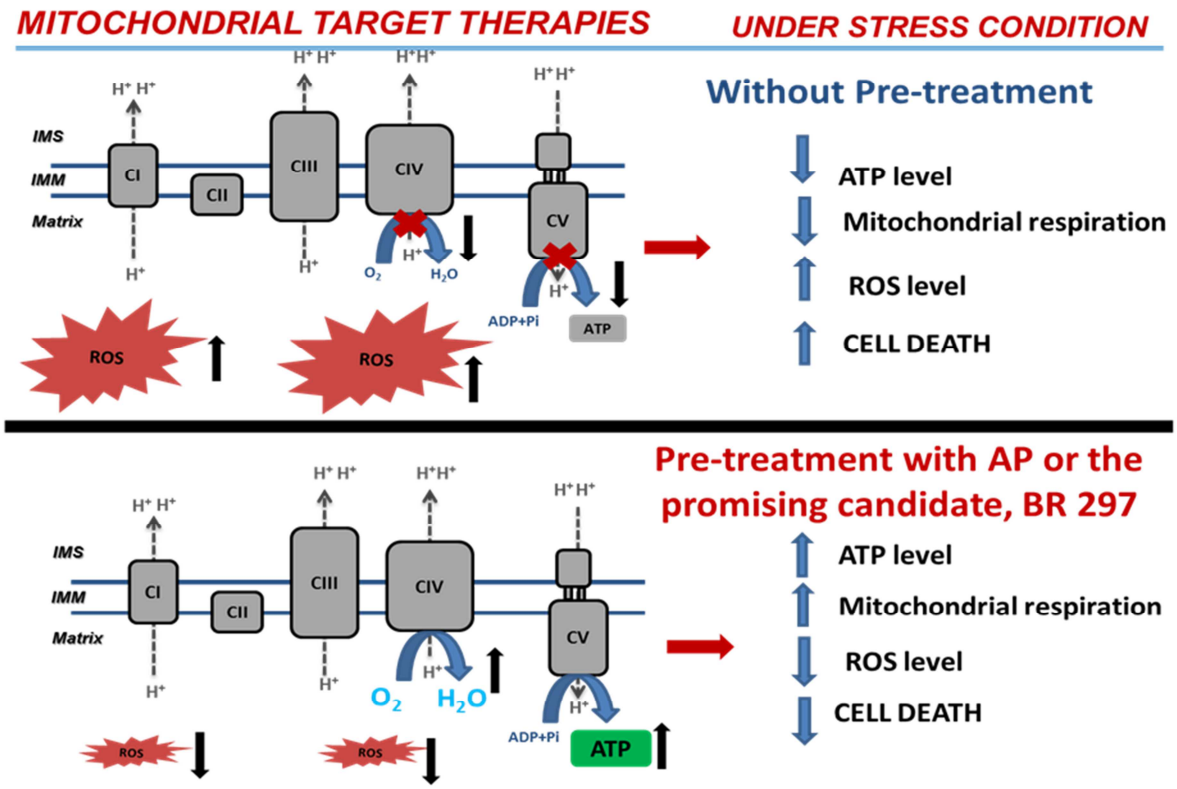

Figure 1 : Schéma récapitulatif de l'effet protecteur de l'AP et du BR297 contre le stress oxydant évoqué par le peroxyde d'hydrogène ou H_2O_2. L'exposition des cellules au H_2O_2, qui provoque une diminution du niveau d'ATP et de la respiration mitochondriale, augmente parallèlement la concentration de ROS conduisant à la mort cellulaire (partie supérieure). L'administration d'AP ou de BR 297 stimule la production d'ATP et la respiration mitochondriale tout en réduisant, les niveaux de ROS, ce qui permet d'améliorer la survie cellulaire malgré le stress oxydant (panneau inférieur). ROS: espèces réactives de l'oxygène, AP: alloprégnanolone, ATP: adénosine triphosphate.

La deuxième partie de notre travail de thèse avait pour objectif de vérifier si les données obtenues sur le BR297 *in vitro* pouvaient se reproduire chez des modèles animaux de la MA *in vivo*, notamment si l'administration du BR297 *in vivo* permet de corriger les déficits mitochondriaux qui sous-tendent les dysfonctionnements neuronaux, la perte neuronale et/ou le déclin cognitif dans la MA. Nous avons alors utilisé le modèle de souris transgéniques Tg2576 qui sont caractérisées par l'accumulation cérébrales des peptides

beta-amyloïdes toxiques et reproduisent les principaux symptômes mnésiques/cognitifs de la MA. Ces souris Tg2576 constituent un modèle *in vivo* qui complète de façon intéressante le modèle cellulaire SH-SY5YAPPwt qui nous a permis d'explorer *in vitro* les effets neuroprotecteurs des ANS car les cellules SH-SY5YAPPwt accumulent aussi les peptides beta-amyloïdes à cause de la sur-expression de l'APP.

Les souris Tg2576 âgées de 9 mois ont été traitées avec le véhicule (hydroxypropyl cellulose 0.3%) ou le BR297 (2, 4 and 8 mg/kg dissous dans le véhicule) à raison de 3 injections (ip) par semaine pendant 4 semaines. Grâce à la collaboration du Dr Chantal Mathis (LNCA, Strasbourg), la performance cognitive des souris a été évaluée dans un test de déplacement d'objets. La performance des souris témoins (wild-type ou WT) traitées au véhicule diffère significativement de celle des souris Tg2576 traitées au véhicule et au BR297 2mg/kg. Les groupes WT-véhicule et Tg2576 4 mg/kg détectent le changement spatial des objets.

Après les tests comportementaux, les cerveaux des souris ont été prélevés et les mitochondries extraites pour une analyse des activités mitochondriales dans le cortex cérébral et l'hippocampe. Nos résultats ont révélé des déficits mitochondriaux notables dans le cerveau des souris Tg2576 comparées au WT. En particulier, une diminution drastique de la concentration d'ATP ainsi qu'une baisse de l'activité des complexes mitochondriaux I et IV ont été détectées dans le cortex cérébral et l'hippocampe des souris Tg2576. De façon intéressante, les déficits mitochondriaux sont nettement réduits ou corrigés dans le cerveau des souris Tg2576 traitées par le BR297.

Etant donné que l'âge est un facteur de risque des maladies neurodégénératives, nous avons également testé la capacité du BR297 à améliorer les fonctions mitochondriales dans un modèle de vieillissement cérébral non pathologique. Les principales causes de dysfonctionnements mitochondriaux liés à l'âge sont la réduction de l'activité des complexes I et IV se traduisant par une diminution de transfert d'électron dans la chaine respiratoire (Navarro and Boveris 2004). La baisse de la neurogenèse adulte est aussi identifiée comme étant un des principaux facteurs qui contribuent à la genèse des déficits cognitifs associés aux maladies neurodégénératives (Leuner, Kozorovitskiy et al. 2007, Amrein, Isler et al.

2011, Winner and Winkler 2015). Ainsi, en plus de l'évaluation des effets du BR297 (neuroprotecteur sans effet prolifératrice), nous avons aussi étudié les effets du BR351 ou O-allyl-AP (neuroprotecteur ayant une action neurogénique et proliférative) (Karout, Miesch et al. 2016), sur les performances cognitives et les fonctions mitochondriales dans le cerveau de souris normales âgées de 21 mois. Ces souris ont été traitées avec trois doses différentes de BR297 [O-allyl-épi-AP] (2, 4 et 8 mg/kg) ou de BR351 [O-allyl-AP] (1, 2 et 4 mg/kg) -pendant un mois à raison de 3 injections par semaine. La mémoire spatiale des souris a été évaluée grâce au test comportemental du *Pattern Separation* (collaboration avec le Dr Mathis, LNCA, Strasbourg) qui est connu comme étant sensible à l'activité neurogénique dans le gyrus denté de l'hippocampe adulte (Holden and Gilbert 2012, Yassine, Lazaris et al. 2013). Cette étude comportementale a été complétée par des analyses effectuées sur des prélèvements de cerveaux à savoir des extractions tissulaires pour l'évaluation des fonctions mitochondriales (notre équipe) ou des études histologiques (collaboration avec une autre équipe du Consortium NeuroRhine, Prof. Hofmann, Freiburg) utilisant des marqueurs spécifiques des cellules souches et des cellules en prolifération ou en différenciation dans la zone sous-granulaire du gyrus denté de l'hippocampe. Nos résultats montrent que le BR351 ou O-allyl-AP (1 mg/kg) permet une amélioration des performances des souris âgées dans le *Pattern Separation test* qui est positivement corrélée à une augmentation du nombre de cellules souches neurales et de cellules progénitrices dans le gyrus denté de l'hippocampe. Le BR297 (O-allyl-epi-AP) ne modifie pas la performance des souris âgées dans le *Pattern Separation test* puisque le BR297 n'exerce pas d'effet prolifératrice ou neurogénique. En revanche, il corrige significativement les déficits mitochondriaux, notamment la baisse de la production d'ATP et la diminution des activités des complexes I et IV qui sont détectées dans le cortex et l'hippocampe des souris âgées (Leuner, Muller et al. 2012) **(Figure 2)**. Le BR351 montre également quelques effets bénéfiques mais son action sur l'activité mitochondriale dans le cerveau âgé est nettement inférieure à celle du BR297 **(Figure 2)**.

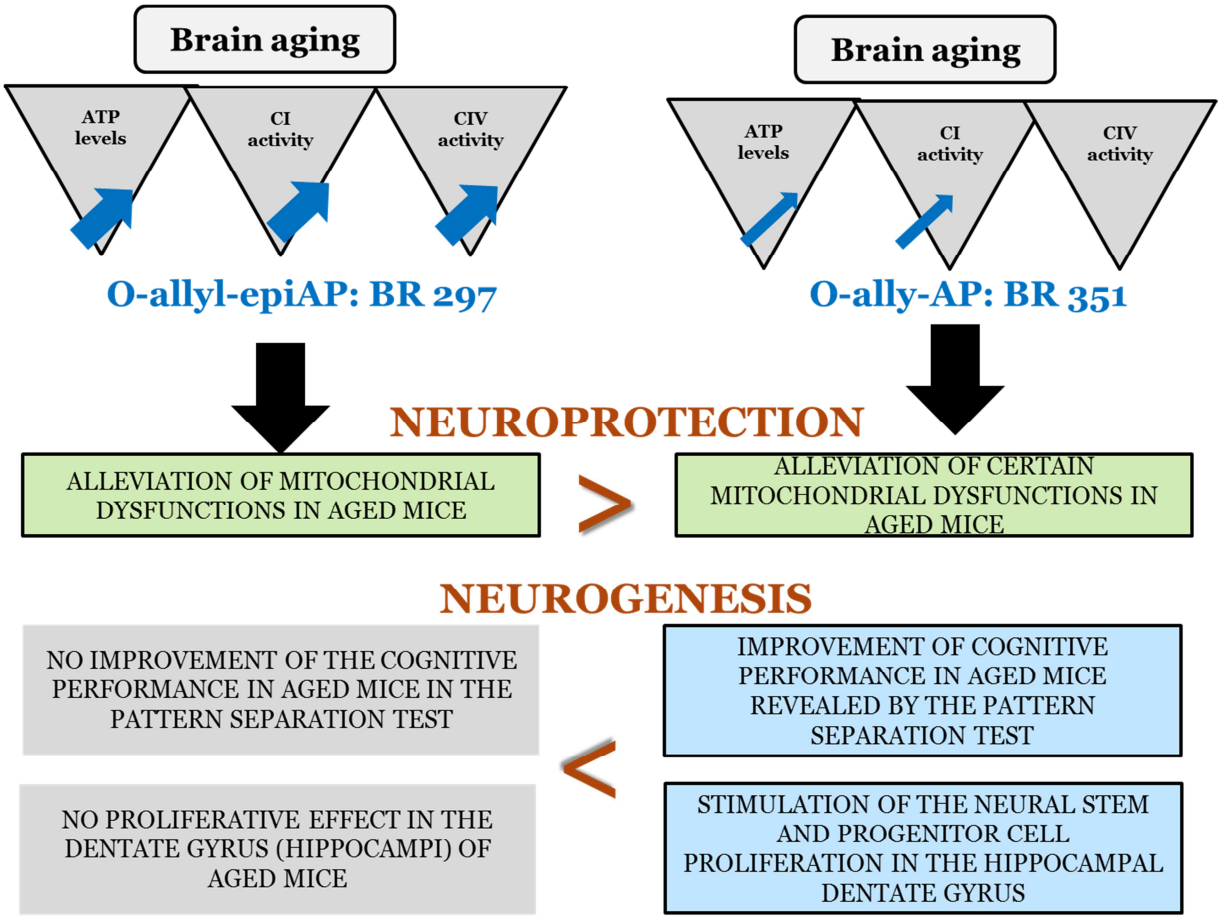

Figure 2: Illustration du profil hypothétique d'activité biologique du BR297 et du BR351 dans un modèle de vieillissement cérébral non pathologique. Le BR297, qui présente une efficacité supérieure à celle du BR351 dans l'amélioration des dysfonctionnements mitochondriaux, exerce un effet neuroprotecteur sans induire de prolifération cellulaire. En revanche, l'action neuroprotectrice évoquée par le BR351 résulte principalement d'une stimulation de la neurogenèse et/ou de la neuroprolifération qui semble ne pas requérir une forte mobilisation des fonctions mitochondriales. ATP : adenosine triphosphate, CI : complex I, CIV : complex IV.

Il apparaît donc que dans le modèle de souris âgée, l'action neurogénique est indispensable pour restaurer une performance similaire à celle des souris jeunes dans le *Pattern Separation test* et une légère amélioration des fonctions mitochondriales peut suffire pour soutenir cette neurogenèse. Ce profil d'effets correspond bien au profil d'activités biologiques du BR351. En revanche, dans le modèle de souris Tg2576, l'amélioration des performances mnésiques (test de déplacement d'objets) requiert une action neuroprotectrice efficace contre la toxicité beta-amyloïde et le stress oxydant. Cette action neuroprotectrice, qui implique une forte modulation positive des fonctions mitochondriales sans nécessairement

induire de prolifération cellulaire, correspondrait mieux au profil d'activités biologiques du composé BR297.

Comme mentionné dans la partie introductive de ce descriptif synthétique, nous avons également étudié les effets de nouveaux composés analogues LTSPO sur les fonctions mitochondriales. Les LTSPO sont connus comme modulateurs de la translocation du cholestérol de la membrane externe vers la membrane interne des mitochondries où est localisé le cytochrome P450scc qui est l'enzyme catalysant la conversion du cholestérol en prégnènolone, une étape clé de la neurostéroïdogenèse. Des études antérieures ont suggéré que les LTSPO pourraient exercer une action neuroprotectrice en stimulant la biosynthèse des neurostéroïdes neuroprotecteurs comme l'allopregnanolone (Ferzaz, Brault et al. 2002, Rupprecht, Rammes et al. 2009, Barron, Garcia-Segura et al. 2013). Toutefois, les LTSPO disponibles actuellement sont connus pour leurs effets anxiolytiques, leur difficulté de solubilisation dans les milieux physiologiques et leur manque de sélectivité ou de spécificité d'action. De plus, l'implication des LTSPO dans les processus de neuroprotection, en particulier leurs effets sur la bioénergetique mitochondriale dans les cellules nerveuses restent des questions non élucidées. Nous avons donc étudié la capacité de nouveaux analogues LTSPO synthétisés par les Drs Bihel et Bouguignon (UMR 7200, Strasbourg, partenaire du Consortium NeuroRhine) à améliorer la bioénergétique cellulaire.

Des pré-tests effectués par des membres du consortium ont montré que les nouveaux ligands, appelés 6a et 6b, induisent la production de prégnénolone dans les cellules gliales. Les neuroblastomes sur-exprimant l'APP ont encore servi de modèles cellulaires pour déterminer les effets de deux nouveaux composés LTSPO. Leurs effets ont été comparés à quatre molécules LTSPO de références à savoir diazepam, XBD-173, SSR-180,575 et Ro5-4864. Nos résultats ont d'abord démontré un effet non toxique des nouveaux ligands à 10 nM sur la survie cellulaire. Parmi les nombreux LTSPO testés, les composés 6a et 6b de la famille imidazoquinazolinone ont montré une meilleure performance sur l'augmentation des niveaux d'ATP dans les cellules contrôles. En effet, à 10 nM, les molécules 6a et 6b

permettent une amélioration du niveau d'ATP à une même efficacité que les composés diazepam, Ro5-4864, SSR-180,575 et de manière significative comparée au XBD-173 dans les cellules contrôles. En particulier, nous avons observé que les molécules 6a et 6b module la production cellulaire d'ATP à 10 nM dans les cellules APP avec un effet comparable à celui du diazepam et significativement supérieur à celui des composés Ro5-4864, XBD-173 et SSR-180,575 **(Figure 3)**. Tous les ligands testés n'ont eu aucun effet sur l'activité métabolique dans le modèle cellulaire de la maladie d'Alzheimer. Les composés 6a et 6b apparaissent plus efficaces que les molécules de références dans l'induction de l'énergie cellulaire **(Figure 3)**.

A l'appui de ces données encourageantes obtenues *in vitro* sur les cellules SH-SY5YAPPwt, nous envisageons comme perspective de tester, *in vivo* les effets neuroprotecteurs des nouveaux composés LTSPO dans les modèles animaux de la MA, notamment les souris Tg2576.

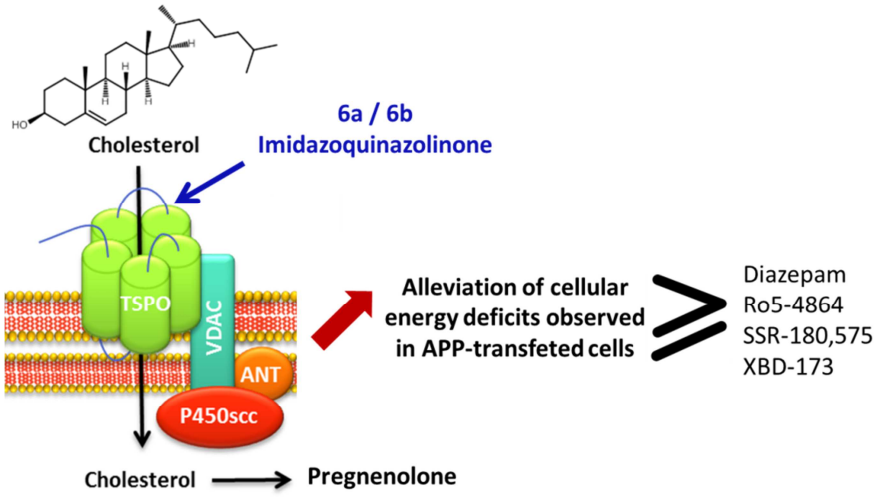

Figure 3 : Les nouveaux LTSPO composé 6a et 6b, montrent une meilleur efficacité que divers LTSPO anciennement connus (Diazepam, Ro5-4864, SSR-180,575, XBD-173) dans l'amélioration de la bioénergétique mitochondriale des cellules SH-SY5YAPPwt qui représentent un modèle pertinent pour l'étude de la MA

En conclusion, notre travail de thèse a permis d'identifier de nouvelles molécules régulatrices des fonctions mitochondriales et prometteuses pour l'élaboration de stratégies neuroprotectrices optimisées ou mieux ciblées. Nous avons caractérisé un nouvel analogue

de l'AP, l'O-allyl-epi-AP ou BR297, qui est capable d'exercer un effet neuroprotecteur efficace impliquant une forte régulation positive des activités mitochondriales sans évoquer une prolifération cellulaire. Un autre nouvel analogue de l'AP, le BR351 ou O-allyl-AP, est aussi efficace dans la neuroprotection mais son effet résulte essentiellement d'une action neurogénique et/ou prolifératrice qui ne semble pas dépendre prioritairement d'une forte mobilisation des fonctions mitochondriales. Par ailleurs, les futures investigations prévues pour la suite du travail de thèse, permettront de vérifier *in vitro* et *in vivo*, le potentiel neuroprotecteur de deux nouvelles molécules LTSPO 6a et 6b que nous avons déjà identifiées *in vitro* comme efficace dans la régulation de l'énergie cellulaire et dans la protection des cellules SH-SY5YAPPwt contre la cytotoxicité liée au stress oxydant. A terme, les résultats issus de cette thèse ainsi que ceux attendus des travaux complémentaires prévus dans les perspectives, pourront fournir des données précliniques intéressantes pour le développement de nouvelles stratégies thérapeutiques contre la MA ou les pathologies neurodégénératives associées à des dysfonctionnements mitochondriaux dans les cellules nerveuses.

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VI. Zusammenfassung der Doktorarbeit

Zusammenfassung der Doktorarbeit

Neurodegenerative Krankheiten sind häufige Leiden des Nervensystems und Hauptursache von Demenz, verminderter Lebensqualität, und Mortalität (Winter, Korchounov et al. 2011). Die Alzheimer Demenz (AD) ist die Hauptform der Demenz, die auf klinischer Ebene durch kognitive Defizite, Gedächtnisstörungen, Gemütsschwankungen, beeinträchtigtes Urteilsvermögen und Sprachfähigkeit, und auf pathophysiologischer Ebene durch eine Degeneration des Gehirns charakterisiert wird (West, Coleman et al. 1994). Von AD betroffene Gehirne weisen zwei kennzeichnende Arten von Läsionen auf: i) Amyloide Plaques, die extrazelluläre Ablagerungen des β-Amyloid-Proteins sind, (Selkoe, Abraham et al. 1986, Miller, Papayannopoulos et al. 1993); und ii) Neurofibrillenbündel, die intrazelluläre hyperphosphorylierte Aggregate des Tau-Proteins darstellen (Goedert, Wischik et al. 1988).

Zahlreiche Beweise enthüllten, dass mitochondriale Anomalien in die pathophysiologischen Mechanismen der AD involviert sind. Mitochondrien, die Kraftwerke der Zellen, spielen eine essentielle Rolle im Überlebens- und Sterbeprozess neuronaler Zellen, da sie gleichzeitig den Energiestoffwechsel sowie den Zelltod regulieren (Scheffler 2001). Sie liefern den grössten Teil der zellulären Energie in Form von Adenosintriphosphat (ATP) und regulieren die Produktion reaktiver Sauerstoffspezies (ROS) (Schmitt, Grimm et al. 2012). Eine Zunahme oxidativer Schäden und eine Abnahme des Energiestoffwechsels können in frühen Stadien der AD noch vor der Ausbildung amyloider Plaques und Neurofibrillenbündel beobachtet werden (Eckert, Hauptmann et al. 2008, Rhein, Baysang et al. 2009). Insbesondere eine abnormale Erhöhung der Aβ-Peptide induziert oxidativen Stress, der zu einer Veränderung der glykolytischen Enzyme und des Krebs-Zyklus führt. Diese oxidativen Veränderungen haben eine Reduktion des Glukosemetabolismus und eine Verminderung der ATP-Synthese in von der Alzheimer-Krankheit betroffenen Gehirnen zur Folge (Tramutola, Lanzillotta et al. 2016).

Folglich haben aktuelle pharmakologische Strategien, mit dem Ziel Therapien gegen die AD zu entwickeln, gezieltes Interesse an der Regulation mitochondrialer Funktionen in Neuronen, namentlich der ATP-Synthese, der mitochondrialen Atmung, und der Produktion von ROS.

In Hinsicht auf die Involvierung bestimmter mitochondrialer Enzyme auf die Steroidsynthese und die entscheidende Rolle von Neurostreroiden in der Regulierung der Differenzierung, des Wachstums und des Überlebens von Nervenzellen, zeigten viele Arbeiten ausserdem eine Modifizierung der Biosynthese von Neurosteroiden im ZNS bei Alterungsprozessen und der AD (Mensah-Nyagan, Do-Rego et al. 1999, Patte-Mensah, Meyer et al. 2010, Brinton 2013, Caruso, Barron et al. 2013). Interessanterweise haben diese Arbeiten eine Abnahme der Produktion von neuroprotektiven Neurosteroiden wie Allopregnanolon, das neurogene sowie neuroregenerative Effekte und Stimulation der Proliferation vermittelt, mit dem Alter in Zusammenhang gebracht (Schumacher, Weill-Engerer et al. 2003). Die Verminderung der Neurosteroidgenese sowie der zerebralen Allopregnanolon-Konzentration korrelieren gleichermassen mit der bei der AD beobachteten Abnahme der Kognition (Frye and Walf 2008, Caruso, Barron et al. 2013). Eine Allopregnanolon-Injektion förderte interessanterweise die Neurogenese in der subgranulären Zone des Hippocampus (SGZ) und behob Gedächnis- und Lernstörungen in einem dreifach transgenen Alzheimer-Mausmodell (3xTgAD) (Wang, Singh et al. 2010). Ausserdem begünstigte einmal wöchentlich verabreichtes Allopregnanolon über eine Behandlungsdauer von 6 Monaten das Überleben neu gebildeter Neuronen und verminderte die Produktion von Aβ im Hippocampus, dem Cortex und der Amygdala in 3xTgAD Mäusen (Chen, Wang et al. 2011). Diese Studie zeigte auch, dass Allopregnanolon die Aktivierung von Microglia reduzieren, Myelinmarker von Oligodendrozyten erhöhen, und die Expression der Proteine hepatischer X Rezeptor (LXR) und Pregnan-X-Rezeptor (PXR) verbessern kann. Diese Proteine regulieren die Cholesterinhomöostase und -ausscheidung im Gehirn. Singh und Mitarbeiter haben gezeigt, dass eine frühe Behandlung vor der Bildung von Plaques die Kognition, Gedächtnis, und Lernfähigkeit in 3xTgAD Mäusen verbesserte (Singh, Liu et al. 2012). Folglich scheint Allopregnanolon die Regeneration und Reparatur von

Nervenzellen, sowie die Wiederherstellung des Gedächtnisses und der Lernfähigkeit bei AD zu begünstigen (Brinton 2013). Diese Daten haben ein grosses Interesse an der Entwicklung von auf Allopregnanolon basierenden, therapeutischen Strategien ausgelöst. Die pleiortropen Effekte von Allopregnanolon beinhalten neben einer Stimulation der Neurogenese und -regeneration auch eine Stimulation der Zellproliferation. Es kann nicht ausgeschlossen werden, dass letzteres zur unerwünschten Bildung von Gliomen führt. Deshalb ist es von Interesse, Substanzen bzw. Allopregnanolon-Analoga zu entwickeln mit einem spezifischen Wirkungsprofil in Hinblick auf Neurogenese und -regeneration ohne Erhöhung der Zellproliferation.

In Anbetracht der oben genannten Gegebenheiten war es das Hauptziel dieser PhD-Thesis die Fähigkeit zwei neuer, kürzlich patentierter Familien von Verbindungen, im Hinblick auf ihr Potential im ZNS mitochondriale Funktionen zu verbessern und neuroprotektive Effekte auszuüben, zu evaluieren. Diese zwei Familien von Verbindungen sind Allopregnanolon-Analoga (ANS) und Liganden des Translocator Proteins (LTSPO), das den Transport von Cholesterin von der äusseren Membran zur inneren Membran der Mitochondrien sicherstellt, wo das Cytochrom p450 Side-Chain Cleavage die Transformation von Cholesterin zu Pregnelonon, dem Vorläufer diverser Neurosteroide in der Biosynthese, katalysiert. LTSPO sind also wichtige Modulatoren der Neurosteroidgenese. Diese als Cotutelle de thèse der Universität Strassburg und Universität Basel durchgeführte PhD-Arbeit wurde im Rahmen des kollaborativen Forschungsprograms „Offensives Sciences INTERREG IV Rhin Supérieur“ des Consortium Trinational NeuroRhine umgesetzt.

Die PhD-Thesis wurde in zwei Haupteile gegliedert, wobei der erste Teil die Subprojekte A, B, und C und der zweite Teil das Subprojekt D beinhaltet:

A) In einem zellulären in vitro AD-Model mit humanen Amyloid Precursor Protein (APP) überexprimierenden SH-SY5Y Neuroblastomazellen (APPwt Zellen), die eine erhöhte

Produktion des β-Amyloid-Proteins aufweisen, wurde die Fähigkeit von Allopregnanolon und seinen Analoga (ANS), die mitochondrialen Funktionen zu verbessern, evaluiert.

B) In einem transgenemn Alzheimer-Mausmodell (Tg2576 Mäuse, die die schwedische Mutation des humanen APP exprimieren), das die Aβ-Symptome der AD nachamt, wurde die Fähigkeit der ANS, die AD-assoziierte mitochondriale Funktionsabnahme abzumildern, getestet.

C) In einem in vivo Tiermodel der Alterung wurden die Effekte der ANS gegen die altersabhängige Abnahme der mitochondrialen Aktivität getestet.

D) In SH-SY5Y APPwt Neuroblastomazellen und Kontrollzellen wurden die Effekte neuer TSPO Liganden und Referenzliganden auf die Energiehomöostase miteinander verglichen.

(A) In SH-SY5Y APPwt Zellen und Kontrollzellen haben wir die Fähigkeit von Allopregnanolon und folgenden ANS, die zelluläre Energiehomöostase zu modulieren, evaluiert:

- Pregnane-12,20-dion-3-hydroxy (3α, 5α), auch 12 oxo-AP = BR338
- Pregnane-12,20-dion-3-hydroxy (3β, 5α), auch 12 oxo-epiAP = BR053
- Pregnan-20-on-3 (2-propen-1-yloxy) (3α, 5α), auch O-allyl-AP = BR351
- Pregnan-20-on-3 (2-propen-1-yloxy) (3β, 5α), auch O-allyl-epiAP = BR297

Die SH-SY5Y APPwt Zellen wurden verwendet, weil sie ein international anerkanntes experimentelles Model in der neurowissenschaftlichen AD Forschung darstellen. (Scheuermann, Hambsch et al. 2001, Schaeffer, Patte-Mensah et al. 2006, Rhein, Baysang et al. 2009, Grimm, Biliouris et al. 2016). Weiterhin sind SH-SY5Y APPwt Zellen sensibler gegenüber oxidativem Stress als die (mit dem leeren Vektor transfizierten) Kontrollzellen (Schaeffer, Patte-Mensah et al. 2008, Rhein, Baysang et al. 2009, Schulz, Eckert et al. 2012, Wendt, Kemmel et al. 2014). Unsere neuen Befunde zeigen, dass bei einer Konzentration von 500 nM und nach einer Behandlungszeit von 24 Stunden Allopregnanolon und sein Analog O-allyl-epiAP (BR297) in SH-SY5Y APPwt Zellen und Kontrollzellen die ATP Produktion stimulieren. Hingegen verändern die Analoge O-allyl-AP (BR351), 12 oxo-epiAP (BR053), und 12 oxo-AP (BR338) die ATP Produktion nicht. Aufgrund des potenten Effekts von BR297 auf

die ATP Produktion wurde seine Effizienz im Schutz gegen den durch oxidativen Stress (H_2O_2) hervorgerufenen Zelltod mit der von Allopregnanolon verglichen (**Abbildung 1**). Wir haben beobachtet, dass in Kontrollzellen nur BR297 (500 nM) in der Lage ist, das Überleben unter Stressbedingungen zu verbessern. Dagegen reduzieren beide Verbindungen, Allopregnanolon und BR297, in SH-SY5Y APPwt Zellen effizient den durch Hydrogenperoxid induzierten Zelltod (**Abbildung 1**). Unsere Resultate zeigen auch, dass BR297 und Allopregnanolon signifikant mitochondriale und cytosoloische ROS, sowie die erhöhten Superoxidanion-Konzentrationen in SH-SY5Y APPwt Zellen reduzieren (**Abbildung 1**). Ausserdem zeigen unsere Arbeiten, dass Allopregnanolon und BR297 den Sauerstoffkonsum (OCR) und die Glykolyse (ECAR) verbessern, was in SH-SY5Y APPwt Zellen und Kontrollzellen zu einer erhöhten bioenergetischen Aktivität führt. Insbesondere haben wir beobachtet, dass BR297 die *respiratory control ratio* (RCR), ein Indikator für die mitochondriale Atmungskapazität, in beiden Zelltypen moduliert. Allopregnanolon hat keinen signifikanten Effekt auf diese Parameter in SH-SY5Y APPwt Zellen.

(B) Basierend auf den *in vitro*-Resultaten wurden die Effekte von BR297 gegen die AD-bedingte Abnahme der mitochondrialen Aktivität in einem AD-Tiermodel (Tg2576 Mäuse) getestet. In mit Trägerflüssigkeit behandelten Tg Mäusen wurden im Vergleich zu wildtyp (WT) Mäusen erniedrigte ATP Levels und Komplex I und IV Aktivitäten im Hippocampus und Cortex festgestellt. BR297 war in bestimmten Konzentrationen in der Lage das Energiedefizit in Tg Mäusen im Cortex, sowie im Hippocampus zu vermindern. Auch konnten die Aktivitäten der Komplexe I und IV signifikant verbessert werden.

Unsere Resultate erlauben BR297 als ein Analog zu identifizieren, das in der Lage ist, vorteilhafte Effekte auf die mitochondriale Bioenergetik auszuüben, ohne die Proliferation zu stimulieren, wie dies bei Allopregnanolon der Fall ist. Diese Gegebenheiten verleihen BR297 ein sehr interessantes Profil für die Ausarbeitung neuroprotektiver Strategien ohne proliferative Nebeneffekte.

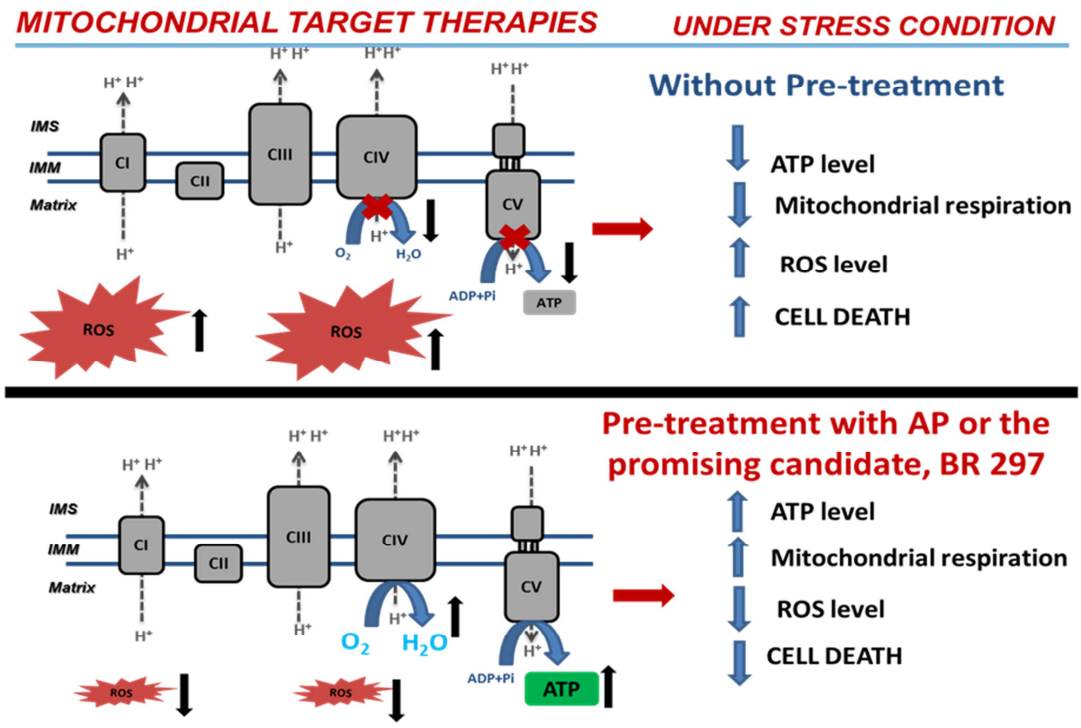

Abbildung 1: Schematische Darstellung des protektiven Effekts von Allopregnanolon und seinem Analog BR297 gegen oxidativen. Der durch H_2O_2 induzierte oxidative Stress hat das ATP Level und die mitochondriale Atmung gesenkt, und das ROS Level, das zum Zelltod führt (oberer Teil), erhöht. AP und BR297 sind in der Lage ATP Konzentrationen und die mitochondriale Atmung zu verbessern, sowie ROS Levels zu senken, was zu einem verbesserten Zellüberleben unter oxidativem Stress führt (unterer Teil). ROS: reaktive Sauerstoffspezies, AP: Allopregnanolone, ATP: Adenosintriphosphat

(C) Angesichts der Tatsache, dass fortschreitendes Alter ein Risikofaktor für neurodegenerative Krankheiten ist, ist es ein weiteres Ziel dieser PhD-Arbeit die Kapazität der neuen ANS zur Verbesserung mitochondrialer Funktionen in einem nicht-pathologischen Alterungsmodel zu testen. Die Hauptursachen für mitochondriale Fehlfunktionen sind die reduzierten Aktivitäten der Komplexe I und V, die zu einem verminderten Elektronentransfer in der Atmungskette führen, (Navarro and Boveris 2004). Die Abnahme der Neurogenese in adulten Gehirnen ist auch ein Hauptfaktor, der zum Auftreten von mit neurodegenerativen Krankheiten assoziierter kognitiver Defizite beiträgt (Leuner, Kozorovitskiy et al. 2007, Amrein, Isler et al. 2011, Winner and Winkler 2015). Folglich haben wir zusätzlich zur Evaluation der Effekte von BR297 (Neuroprotektor ohne proliferativen Effekt) auch die Effekte von BR351 (Neuroprotektor mit neurogener und proliferativer Wirkung) (Karout, Miesch et al. 2016), auf

die kognitiven Leistungen und mitochondrialen Funktionen normaler, 21 Monate alter Mäuse untersucht. Diese Mäuse wurden drei Mal pro Woche während einem Monat mit drei unterschiedlichen Dosen der Verbindung BR297 (2, 4, und 8 mg/kg) oder BR351 (1, 2, und 4 mg/kg) behandelt. Das räumliche Erinnerungsvermögen wurde mittels dem *Pattern Separation* Verhaltenstests, bekannt für seine Sensibilität für neurogene Aktivität im Gyrus dentatus des erwachsenen Hippocampus, evaluiert (Kollaboration mit Dr Mathis, LNCA, Strassburg) (Holden and Gilbert 2012, Yassine, Lazaris et al. 2013). Diese Verhaltensstudie wurde durch die Probenentnahme von Gehirnen ergänzt, um mitochondriale Funktionen (unsere Gruppe) und histologische Studien, mit Hilfe spezifischer Marker von Stammzellen, differenzierenden oder proliferierender Zellen aus der subgranulären Zone des Gyrus Dentatus des Hippocampus (Kollaboration mit einer anderen Gruppe aus dem Consortium NeuroRhine, Prof. Hofmann, Freiburg), zu evaluieren. Ihre Resultate zeigen, dass BR351 (1 mg/kg) eine Verbesserung der Leistung von älteren Mäusen im *Pattern Separation Test*, der positiv mit der Anzahl neuronaler Stammzellen und Progenitorzellen im Gyrus Dentatus des Hippocampus assoziiert ist, ermöglicht. Dagegen verändert BR297 die Leistung älterer Mäuse im *Pattern Separation Test* nicht, möglicherweise aufgrund der fehlenden proliferativen bzw. neurogenen Effekte. Hingegen konnte BR297 signifikant mitochondriale Defizite korrigieren, insbesondere die erniedrigte ATP Produktion und die verminderten Aktivitäten der Komplexe I und IV, die im Cortex und Hippocampus alter Mäuse festgestellt wurden (Leuner, Muller et al. 2012) (**Abbildung 2**). BR351 zeigt ebenfalls förderliche Effekte, aber seine Wirkung auf die mitochondriale Aktivität im gealterten Gehirn ist deutlich geringer als diejenige von BR297. (**Abbildung 2**)

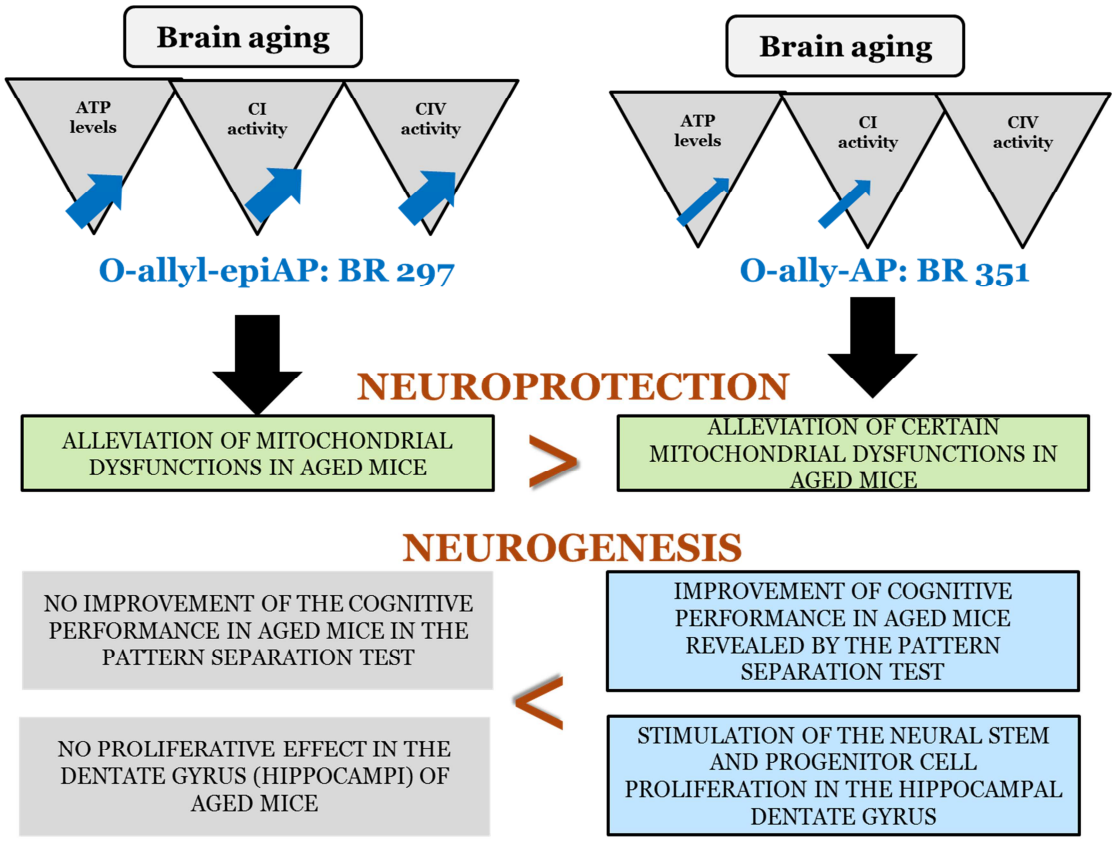

Abbildung 2: Effektprofil biologischer Aktivitäten der Verbindungen BR 297 und BR351 in einem zerebralen, nicht-pathologischem Alterungsmodel. BR 297 weist im Vergleich zu BR351 eine überlegene Effektivität in Alterungsprozessen beobachteter Reduktion mitochondrialer Defizite auf. BR297 ist in der Lage effiziente neuroprotektive Effekte zu vermitteln ohne die Zellproliferation anzutreiben, was eine positive Regulation der zellulären Proliferation impliziert. BR351 ist gleich effizient in der Neuroprotektion, aber sein Effekt resultiert hauptsächlich aus neurogenen proliferativen Eigenschaft, die nicht von der Mobilisation mitochondrialer Aktivität abhängig zu sein scheint.

Daraus lässt sich schliessen, dass zur Verbesserung altersbedingter kognitiver Defizite eine neurogene Wirkung unabdingbar zu sein scheint. Bereits eine geringe Verbesserung der mitochondrialen Funktionen kann genügen, um die Neurogenese zu unterstützen. Dieses Effektprofil stimmt gut mit dem biologischen Aktivitätsprofil von BR351 überein. Dagegen benötigt eine Verbesserung der Gedächtnisleistung (Objektverschiebungstest) im Tg2576 Mäusemodel eine deutlich effizientere neuroprotektive Wirkung, um gegen die Toxizität des Amyloid Peptids und des oxidativen Stresses anzukommen, die besser mit dem biologischen Aktivitätsprofil von BR297 übereinstimmt.

(D) Wie bereits in der Einleitung dieser Zusammenfassung erwähnt, haben wir auch die Effekte der neuen Verbindungen, den LTSPO, auf die mitochondrialen Funktionen untersucht.

LTSPO sind bekannt als Modulatoren der Cholesterintranslokation von der äusseren zur inneren Mitochondrienmembran. Dort befindet sich das Enzym Cytochrom P450scc, das die Umwandlung von Cholesterin zu Pregnenolon katalysiert, ein wichtiger Schritt in der Neurosteroidsynthese. Vorgängige Studien haben gezeigt, dass LTSPO eine neuroprotektive Wirkung durch die Stimulation von Neurosteroiden wie AP ausüben können (Ferzaz, Brault et al. 2002, Rupprecht, Rammes et al. 2009, Barron, Garcia-Segura et al. 2013). Jedoch sind aktuell verfügbare LTSPO für ihre anxiolytische Wirkung, ihre schwere Löslichkeit unter physiologischen Bedingungen, und ihre fehlende Selektivität und Spezifizität bekannt. Zudem sind die neuroprotektiven Mechanismen, die der Wirkung von den LTSPO zu Grunde liegen, insbesondere die Effekte auf die mitochondriale Bioenergetik in Nervenzellen, noch unbekannt. Wir haben daher die Kapazität neuer LTSPO Analoge, synthetisiert durch Dr Bihel und Dr Bougignon (UMR 7200, Strassburg, Partner des Konsortiums NeuroRhine), auf ihre Kapazität die zelluläre Bioenergetik zu verbessern untersucht. Aus einem Set von 11 neu synthetisierten TSPO Liganden mit unterschiedlichen Grundstrukturen (aus den Familien der Benzodiazepine, Pyridazinoindolacetamide, Imidazoquinazolinone, Purine, und Phenylalanin- und Quinolinonderivate) zeigten v.a. die neuen Liganden 6a und 6b der Imidazoquinazolinonfamilie die vielversprechendsten Ergebnisse. APP überexprimierende Neuroblastomazellen (SH-SY5Y APPwt) haben als Zellmodel gedient, um die Effekte der neuen LTSPO Verbindungen zu bestimmen. Ihre Wirkungen wurden mit den Wirkungen von vier Referenzmolekülen, namentlich Diazepam, XBD173, SSR-180,575, und Ro5-4864, verglichen. Unsere Resultate haben zunächst einen nicht-toxischen Effekt bei 10 nM auf das Zellüberleben aufgezeigt. Unter den getesteten LTSPO haben die Verbindungen 6a und 6b, Mitglieder der Familie der Imidazoquinazoline, die beste Leistung in Bezug auf die Erhöhung der ATP- und Pregnenolon-Spiegel in Kontrollzellen aufgewiesen. In der Tat ermöglichen die Verbindungen 6a und 6b bei 10 nM eine gleich effiziente Verbesserung der ATP Konzentration in Kontrollzellen verglichen mit Diazepam, Ro5-4864, SSR-180,575 und in signifikanter Weise XBD-173. Insbesondere haben wir beobachtet, dass die Moleküle 6b und 6a bei 10 nM, in vergleichbarer Weise wie Diazepam und in signifikant überlegener Weise gegenüber den

Verbindungen Ro5-4864, XBD-173, und SSR-180,575 die zelluläre ATP Produktion in SH-SY5Y APPwt Zellen modulieren (**Abbildung 3**).

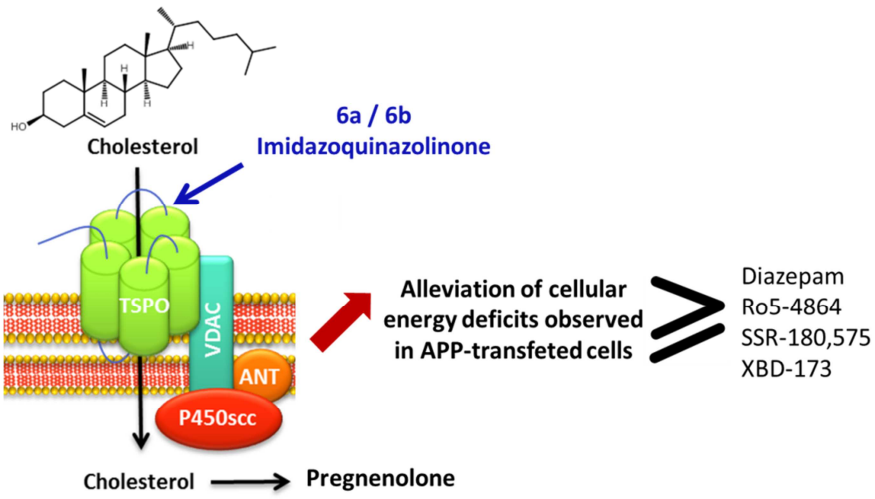

Abbildung 3: Die Imidazoquinazolinonverbindungen 6a und 6b reduzieren, in vergleichbarer Weise oder effizienter als gewisse in der Literatur beschriebene TSPO Liganden, die bioenergetischen Defizite, die im AK Zellmodel, das die Akkumulation des Peptids β-Amyloid nachahmt, beobachtet werden.

Schlussfolgernd hat diese Thesis erlaubt, neue Moleküle zu identifizieren, die mitochondriale Funktionen regulieren sowie verbessern, und vielversprechend für die Ausarbeitung optimierter und gezielter neuroprotektiver Therapien sind. Wir haben ein neues Analog von AP, O-allyl-epi-AP oder BR297, charakterisiert, das in der Lage ist neuroprotektive Effekte zu vermitteln, ohne die Zellproliferation auszulösen. Diese Effekte deuten auf eine positive Regulation der mitochondrialen Aktivitäten. Ein weiteres neues Analog von AP, O-Allyl-AP oder BR351, hat einen gleich effizienten neuroprotektiven Effekt. Diese Wirkung beruht aber hauptsächlich auf neurogenen und/oder proliferativen Eigenschaften, die nicht von der Mobilisation mitochondrialer Funktionen abhängen. Ebenfalls wurden zwei neue TSPO Liganden aus der Familie der Imidazoquinazoline (6a und 6b) identifizert, die parallel den Energiespiegel und die Pregnenolonproduktion erhöhen. Letztendlich könnten die Resultate aus dieser Thesis und komplementären Arbeiten interessante präklinische Grundlagen liefern, um neue therapeutische Strategien gegen die AD oder andere pathologische

Neurodegenerationen zu entwickeln, die in Verbindung mit mitochondrialen Fehlfunktionen im Nervensystem stehen.

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Abbreviations

17OH-PREG 17-hydroxypregnenolone

17OH-PROG 17-hydroxyprogesterone

17β-HSD 17β-hydroxysteroid dehydrogenase

21-OHase 21-hydroxylase

3xTgAD: triple AD mutated Tau (P301L), PS2 (N141I) and APPSwe (KM670/671NL) triple transgenic mouse model

3α-HSOR 3α-hydroxysteroid oxydoreductase

3β-HSD 3β-hydroxysteroid dehydrogenase

5α-R 5α-reductase

ACBD3 Acyl-CoA binding domain-containing 3

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or tetrazolium

AD Alzheimer's disease

ADAM A disintegrin and metalloproteinase

AICD Aβ intracellular cytoplasmic domain

ANT Adenine nucleotide transporter

AP Allopregnenolone

APOE Apolipoprotein E

APP Amyloid precursor protein

ATP Adenosine triphosphate

Aβ Amyloid-beta peptide

BACE β-site of APP cleaving enzyme

C83 83-amino-acid Ct APP fragment

C99 99-amino-acid Ct APP fragment

CSF Cerebrospinal fluid

DBI Diazepam binding inhibitor

DHP Dihydroprogesterone

DHT Dihydrotestosterone

ER Endoplasmic reticulum

ETC Electron transport chain

FAD Familial Alzheimer's disease form

GSH Glutathione

H_2O_2 Hydrogen peroxide

HST Hydroxysteroid sulfotransferase

IMM Inner mitochondrial membrane

IMS Intermembrane space

LXR Liver X receptor

MAP Microtubule-associated protein

MMP Mitochondrial membrane potential

MnSOD Manganese superoxide dismutase

mPTP mitochondrial permeability transition pore

mtDNA Mitochondrial DNA

NADH Nicotine adenine dinucleotide

NFT Neurofibrillay tangles

NMDA N-methyl-D-aspartate

NS Nervous system

nSR Nuclear steroid receptor

$O_2^{-\cdot}$ Superoxide anion

OH Hydroxyl radical

OMM Outer mitochondrial membrane

OXPHOS Oxidative phosphorylation system

P450c17 Cytochrome P450c17

P450scc Cytochrome P450 cholesterol side chain cleavage

PBR Peripheral benzodiazepine receptor

PET Positron emission tomography

PKA-RIα protein kinase A regulatory subunit I alpha

PR progestins receptors

PROG Progesterone

PTSD Posttraumatic stress disorder

PXR Pregnane X receptor

ROS Reactive oxygen species

SAD Sporadic Alzheimer's disease form

SGZ Subgranular zone

STAR Steroidogenesis acute regulatory protein

TSPO Translocator protein

UQ Coenzyme Q

VDAC Voltage-dependant anion channels

Curriculum Vitae

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PROFILE

Motivated, efficient, and responsible scientist with 4 years research experience in molecular biology, cell biology, and neuroscience. Adept in protocol development, research methodology, and statistical analysis. Technical and clinical expertise ensures excellent organizational, interpersonal, and managerial skills, while advanced study in biology and neuroscience grounds ideas in scientific theory and practice.

PROFESSIONAL EXPERIENCE

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 Authored paper currently being submitted for an upcoming publication.

EDUCATION

- **July 2012-June 2016:** Joint PhD student, University of Strasbourg (UDS, leading University, Professor Doctor AG Mensah-Nyagan), (Joint-PhD with the University of Basel, Professor Doctor. A. Eckert, UPK Basel)
- **July 2011**: Pre-selected Graduate school of Strasbourg
- **2010-2011 :** Obtaining Master's degree 2 Cellular and Molecular Physiopathology, University of Strasbourg
- **2009-2010:** Master's degree 1 Cellular and Molecular Physiopathology, UDS
- **2006-2009:** Obtaining License in Cell Biology and Physiology, UDS
- **2005-2006 :** PCEM1, Faculty of Medicine, Strasbourg
- **2004-2005 :** Obtaining Bachelor Science, High school Laurent Lavoisier, Mulhouse

SCIENTIFIC EXPERIENCE_____

Tissue Culture

- Maintenance of human, and mouse cell lines (e.g. neuroblastoma)
- Cell passaging
- Cultivation of frozen cells
- Isolation of cortical and hippocampal cells from brain mice
- Cell counting
- Stimulation of cells using compounds (e.g. neurosteroids, TSPO ligands)

Molecular Biology, Cell Biology, and Biochemistry Techniques

- RNA isolation using phenol/chloroform and RNeasy columns
- Identification by mass spectrometry
- RT-PCR: extraction and purification
- Western blotting
- ELISA
- Analysis of cell populations by Flow Cytometry (FACS)
- Seahorse Bioscience metabolic assays
- Bioluminescence assay for ATP level measurements
- Fluorescence assays (e.g. reactive oxygen species, complex I and complex IV activities)
- Cell viability assays (MTT test, BrdU test)

Animal Techniques

- Intraperitoneal injection mouse
- Brain harvesting
- Cortex and hippocampi sampling
- Isolation of mitochondria

LIST OF PUBLICATIONS

Articles

- **Lejri I**, Grimm A, Geoffroy P, Miesch M, Eckert A, Mensah-Nyagan AG. *Allopregnanolone and its analog BR 297 rescue neuronal cells from oxidative stress-induced death through bioenergetic improvement* (Submitted)
- **Lejri I,** Mathis C, Cès A, **,** Geoffroy P, Miesch M, Eckert A, Mensah-Nyagan AG. *Neuroprotective effect of analogs of allopregnanolone on aging* (In preparation)
- **Lejri I**, Hallé F, Abarghaz M, Klein C, Martine Schmitt, Michel Maitre, Jean-Jacques Bourguignon, Guy Mensah-Nyagan AG, *Eckert A,* Bihel F. *The future of ligand TSPO in Alzheimer's disease* (In preparation)

Abstracts

- **Lejri I**, Grimm A, Mensah-Nyagan AG; Eckert A (2013) *Effects of neurosteroids on bioenergectics.* Neurex Annual meeting (Poster session), Basel (Switzerland), June 10, 2013.
- **Lejri I,** Geoffroy P, Miesch M, Eckert A, Mensah-Nyagan AG (2014) *Characterization of novel mitochondrial modulators of the development of neuroprotective strategies.* Second International Meeting of the Neuro-Rhine Consortium (Data blitz and poster session), Strasbourg (France), March 28, 2014.
- **Lejri I,** Geoffroy P, Miesch M, Eckert A, Mensah-Nyagan AG (2014) *Allopregnanolone and mitochondrial dysfunction in a cellular model of Alzheimer's disease.* Second International Meeting of the Neuro-Rhine Consortium (Data blitz and poster session), Basel (Switzerland), March 27, 2015
- **Lejri I,** Geoffroy P, Miesch M, Eckert A, Mensah-Nyagan AG (2015) *Modulatory action of allopregnanolone and its analogs on mitochondrial ATP production and oxidative stress induced neuroblastoma cell death.* 8th Internationnal Meeting Steroids and Nervous System, (poster session # 20) Torino- Orbassano, Italy February 14-18, 2015.
- **Lejri I**, Geoffroy P, Miesch M, Mensah-Nyagan AG; Eckert A (2015) *Effects of the neurosteroid allopregnanolone on mitochondrial dysfunction in Alzheimer's disease.* 12th International Conference on Alzheimer's and Parkinson's diseases European (AD/ PD) (Poster session # 556), Nice (France), March 18-22, 2015.

Travel fellowships / honors

- 12th International Conference on Alzheimer's and Parkinson's diseases European (AD/ PD) (Poster session # 556), Nice (France), March 18-22, 2015.

Seahorse Travel Award (Seahorse Bioscience): *Effects of the neurosteroid allopregnanolone on mitochondrial dysfunction in Alzheimer's disease.*

- 8th International Meeting Steroids and Nervous System, Torino- Orbassano, Italy February 14-18, 2015.

Poster award (exhibition # 20), section: Neurosteroids, gender, neurological and psychiatric disorders, *Modulatory action of allopregnanolone and its analogs on mitochondrial ATP production and oxidative stress induced neuroblastoma cell death.*

Imane LEJRI

Characterization of novel mitochondrial modulators for the development of neuroprotective strategies

Résumé

Ce travail de thèse a permis la caractérisation de deux familles de modulateurs mitochondriaux: les analogues de l'alloprégnanolone et les nouveaux ligands synthétiques de la protéine de translocation mitochondriale ou translocator protein (TSPO) impliquée dans la neurostéroidogenèse. Nos résultats clés ont montré que: i) in vitro, BR297, un analogue de l'alloprégnanolone atténue les déficits bioénergétiques liés à la maladie d'Alzheimer et présente un effet protecteur contre le stress oxydatif, en réduisant les espèces réactives de l'oxygène et la mort dans un modèle cellulaire de la maladie d'Alzheimer, avec une plus grande efficacité comparé à l'allopregnanolone; ii) in vivo, premièrement l'effet protecteur de BR297 a été confirmé dans un modèle de souris transgénique de la maladie d'Alzheimer en atténuant les déficits mitochondriaux, puis deuxièmement, BR297 et BR351 ont démontré des effets neuroprotecteurs sur les dysfonctionnements mitochondriaux liés à l'âge par l'amélioration de bioénergétique cellulaire et des activités des complexes mitochondriaux; et iii) in vitro, les ligands TSPO représentent des molécules prometteuses qui sont capables d'augmenter bioénergétique cellulaire avec des effets comparable ou plus important que des molécules de référence dans un modèle cellulaire de la maladie d'Alzheimer.

Mots clés: Allopregnanolone, ligands TSPO, Mitochondrie, Neuroprotection, Maladie d'Alzheimer, Vieillissement

Abstract

This PhD work allowed the characterization of two families of mitochondrial modulators: novel analogues of allopregnanolone, and novel synthetic ligands of translocator protein (TSPO) implicated in the neurosteroidogenesis. Our key findings showed that: i) in vitro, BR297, an analog of allopregnanolone alleviated Alzheimer's disease-related bioenergetics deficits and exhibited protective effects against oxidative stress by reducing reactive oxygen species and decreasing death in a cellular model of Alzheimer's disease, with a higher effectiveness compared to allopregnanolone; ii) in vivo, firstly the protective effect of BR297 was confirmed in the transgenic Tg2576 mouse model by alleviating the mitochondrial deficits, secondly BR297 and another analog BR351 demonstrated neuroprotective effects on age-related mitochondrial dysfunctions via enhancement of cellular bioenergetics and complex activities; and iii) in vitro, TSPO ligands represent promising molecules which are able to increase cellular bioenergetics with similar/ or higher effects compared to different reference molecules in a cellular model of Alzheimer's disease.

Key words: Allopregnanolone, TSPO ligands, Mitochondria, Neuroprotection, Alzheimer's disease, Aging