Mitochondria, neurosteroids and biological rhythms: implications in health and disease states.

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À mes parents

À mes frères et sœur

À tous ceux que j'aime et qui m'ont soutenu tout au long du chemin

PREFACE

The following dissertation was written by the author.

The INTRODUCTION is partly based on updated versions of previous reviews and book chapters **(see APPENDICES)**.

The RESULTS section of this dissertation consists of one published manuscript and two manuscripts 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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Appendix 3: Grimm A, Schmitt K, Eckert A, Advanced mitochondrial respiration assay for evaluation of mitochondrial dysfunction in Alzheimer's disease, Systems Biology of Alzheimer's Disease: Methods and Protocols, edited by Walker JM, Humana Press, Springer publishing group (Submitted).

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SUMMARY

Mitochondria are considered as the “powerhouses” of the cell. They provide the main source of cellular energy via the production of adenosine triphosphate (ATP) molecules that is accomplished through oxidative phosphorylation (OXPHOS) from nutritional sources. Mitochondria are highly dynamic organelles that continuously fuse and divide to mix and exchange their material, including proteins, DNA and metabolites, according to cellular energy requirements. The brain is especially dependent on ATP due to its high energy demand. Mitochondria produce the energy required for almost all cellular processes, from cell survival and death, to the regulation of intracellular calcium homeostasis, synaptic plasticity and neurotransmitter synthesis. Thus, impaired mitochondrial bioenergetics and dynamics lead inevitably to disease, ranging from subtle alterations in neuronal function to cell death and neurodegeneration. For instance, early stages of Alzheimer’s disease (AD) are associated with impaired mitochondrial bioenergetics and dynamics (fusion/fission) in the brain, which is paralleled by oxidative stress, particularly in mitochondria themselves, and ultimately lead to neuronal death.

In this context, the understanding of the intrinsic mechanisms regulating mitochondrial activity and dynamics is becoming of major importance, especially for the search of new drugs for the therapy and prevention of neurodegenerative diseases, such as AD.

The purpose of the joint-PhD thesis was therefore to deepen our understanding of the regulation of mitochondrial function, and to identify key factors (endogenous and / or exogenous) that are critical in the control of mitochondrial bioenergetics and dynamics. Hence, these factors could be used as tools to develop strategies against diseases involving mitochondrial dysfunction. To achieve this goal, the thesis was divided into two main parts.

1) Since a growing body of evidence suggests that neurosteroids have a strong neuroprotective potential, the first part is based on the hypothesis that neurosteroids may exert a determinant action against neurodegeneration by improving mitochondrial bioenergetics, ***(A)*** under “healthy” physiological conditions as well as ***(B)*** under pathological conditions (AD).

2) In the second part ***(C)***, we determined whether the biological clock, which coordinates a whole range of daily behaviors and physiological processes, is involved in the endogenous regulation of mitochondrial dynamics and bioenergetics.

(A) In the first part of this thesis, the ability of different neurosteroids to regulate mitochondrial bioenergetics and redox homeostasis in neuronal cells was evaluated *in vitro*.

SUMMARY

Neurosteroids constitute a category of steroids that are synthetized within the nervous system, independently of peripheral endocrine glands, and act on the nervous system in an auto/paracrine configuration. Neurosteroids are involved in a broad range of brain-specific functions and the gradual decline of neurosteroid levels with increasing age may be associated with the appearance of age-related neuronal dysfunction, cognitive impairments, and with neurodegenerative diseases, such as AD. Indeed, a growing body of evidence attests that neurosteroids possess strong neuroprotective properties. In particular, many studies are focused on estradiol which is also known to boost bioenergetic metabolism in cells. However, no studies sought to test the effects of other neurosteroids on mitochondrial bioenergetics and redox homeostasis in neuronal cells.

Therefore, to gain insights into the underlying mechanism of neuroprotective action of neurosteroids, we selected a panel of neurosteroids (progesterone, estradiol, estrone, testosterone, 3α-androstanediol, dehydroepiandrosterone (DHEA) and allopregnanolone) as potential candidates able to modulate mitochondrial function. Before characterizing the mode of action of neurosteroids on bioenergetics under pathological conditions, we first aimed to understand their effects *per se* using human SH-SY5Y neuroblastoma cells. We showed that most of the neurosteroids we tested were able to improve the bioenergetic metabolism in neuronal cells by increasing ATP levels, mitochondrial membrane potential and basal mitochondrial respiration. Each neurosteroid appeared to have a distinct bioenergetic profile, possibly because different receptors were involved in their effects on mitochondria. Indeed, neurosteroids seemed to act via their corresponding receptor since the effects on ATP levels were abolished in the presence of specific steroid receptor antagonists. In parallel to an increased mitochondrial respiration, we observed a rise in reactive oxygen species (ROS) levels after treatment with neurosteroids. In response to this slight enhancement of ROS which may result from the rise in oxygen consumption, antioxidant activity was up-regulated, suggesting that neurosteroids may directly or indirectly modulate redox homeostasis in neuronal cells.

Together, these first data indicated that neurosteroids were indeed able to boost mitochondrial function in a delicate balance. We can speculate that neurosteroids are acting, at least in part, via their corresponding receptors to regulate the expression of genes involved in glycolysis and oxidative phosphorylation, but also the content and activity of mitochondrial respiratory complexes. Further investigations are required to determine the underlying molecular mechanisms.

(B) Based on these findings, we investigated in the next step whether neurosteroids were able to alleviate AD-related bioenergetic deficits. For that purpose, we treated different AD cell culture models with neurosteroids: i) human neuroblastoma cells (SH-SY5Y)

overexpressing the human amyloid precursor protein (APP) and amyloid-β peptide (Aβ); ii) human neuroblastoma cells stably transfected with the wild-type tau protein (wtTau) or; iii) the mutant tau protein (P301L) inducting abnormal tau hyperphosphorylation and; iv) their respective vector control cells. We first demonstrated that the presence of APP/Aβ and abnormally hyperphosphorylated tau protein differentially impacted mitochondrial respiration in cellular models of AD. In line with previous studies, both AD pathogenic features induced a decrease in ATP level. However, abnormal tau protein only impaired the maximal mitochondrial respiration and spare respiratory capacity, whereas the overexpression of APP/Aβ induced in addition a decrease in basal respiration, ATP turnover and glycolytic reserve. All the neurosteroids tested showed beneficial effects on ATP production and mitochondrial membrane potential in APP/Aβ overexpressing cells, while only progesterone and estradiol increased ATP levels in mutant tau cells. In addition, we showed that testosterone, that is also the main male steroid hormone, was more efficient to alleviate mitochondrial deficits induced by APP/Aβ, whereas neurosteroids belonging to the family of female steroid hormones, progesterone and estrogens, were more efficient with productivity rising bioenergetic outcomes in our cellular model of AD-related tauopathies.

Together, our findings lend further evidence to the neuroprotective effects of neurosteroids in AD pathology and indicate that these molecules represent promising tools able to increase mitochondrial bioenergetics via enhanced mitochondrial respiration, in healthy and pathological conditions, respectively. Our results provide a potential molecular basis for the beneficial and neuroprotective effects of neurosteroids, which may open new avenues for drug development with regard to targeting mitochondria in neurodegeneration.

(C) The aim of the second part of this thesis was to investigate more closely how mitochondrial function is endogenously regulated within the cells. More specifically, we aimed to determine whether and how mitochondrial dynamics as well as bioenergetics were modulated by the biological clock in a circadian manner. The circadian clock is a hierarchical network of oscillators which coordinate a variety of daily behavioural and physiological processes to the optimal time of day, anticipating the periodical changes of the external environment for all living organisms. Since there are increasing evidences that metabolic homeostasis and the circadian clock are connected in numerous ways through reciprocal regulation, we asked whether mitochondrial bioenergetics and dynamics may exhibit circadian oscillations and whether mitochondria themselves may be able to influence the circadian clock. Using human primary fibroblasts as a peripheral clock model, we showed that the mitochondrial shape and OXPHOS metabolism are under the control of the clock and oscillate within a period length of about 24 hrs. More precisely, we found that ATP levels exhibit a circadian rhythmicity that is consistent with the rhythm of mitochondrial

fusion/fission activity we observed *in vitro* and *in vivo* in mouse brains. This rhythmicity appeared to be dependent of Drp1 activity, a protein involved in mitochondrial fission, since inhibiting or knocking down Drp1 abolished the ATP oscillation *in vitro*. In addition, the mitochondrial respiration pattern was in line with the observed mitochondrial network-dependent ATP oscillations together with rhythmic oscillations of by-products of mitochondrial activity, ROS and NAD+ levels, both indicators of the redox environment. Furthermore, we also found that mitochondria may themselves influence the circadian clock through several retrograde signals, such as the activation of AMP-activated protein kinases (AMPK) which can regulate clock gene expression depending on the ratio AMP/ATP.

Thus, in this second part, we established a detailed molecular link between circadian control of mitochondrial dynamics and bioenergetics, suggesting a key role of the clock-controlled mitochondrial network to anticipate energetic requirements of diverse cellular functions in response to environmental constraints.

Overall, the studies performed in the present thesis importantly helped to deepen our knowledge about the modulation of mitochondrial function in health and disease states. First, our work identifies neurosteroids as very promising molecules able to counteract the bioenergetic deficits in neurodegenerative diseases. Second, we show that mitochondrial dynamics and bioenergetics are controlled by the biological clock, and vice versa. Our findings could have multiple implications with regard to the regulation of metabolic homeostasis in health and disease states associated with mitochondrial impairments and / or circadian disruption.

I. INTRODUCTION

A. Mitochondria

Mitochondria play a central role in eukaryotic cell survival and death because they are orchestrating both energy metabolism and apoptotic pathways. They are considered as the "powerhouses of cells", providing the main source of cellular energy under the form of adenosine triphosphate (ATP) molecules that is accomplished through oxidative phosphorylation (OXPHOX) from nutritional sources. Thus, they contribute to plenty of cellular functions, including apoptosis, cell growth and differentiation, regulation of intracellular calcium homeostasis, alteration of the cellular reduction–oxidation (redox) state and synaptic plasticity. In this context, mitochondria are particularly important in the nervous system that has high energy demand and requires for about 20% of the body's total basal oxygen consumption ***(1)***. As a result, mitochondrial dysfunctions have been associated with neurodegenerative diseases, such as Alzheimer's disease (AD), that are characterized by a cerebral hypometabolism and an impaired homeostasis of the redox status ***(2)***.

1. Mitochondrial structure and pivotal roles within cells

Today, it is widely accepted that mitochondria come from the endocytosis of an α-proteobacteria by a precursor of eukaryotic cells and that this endosymbiosis provoked a move forward in the evolution by permitting the aerobic life ***(3)***. As a consequence of this endocytosis, mitochondria carry a residual genome (approximately 16 kilobase) coding for 13 proteins essential for mitochondrial respiratory chain function ***(4)***. In addition, mitochondria comprise an inner and outer membrane, the intermembrane space and the matrix **(Fig 1).** The size, aspect and organization of mitochondrial membranes vary between species, tissues and physiological conditions and largely differ from the classical representation of mitochondrial ultrastructure.

In humans, mitochondrial DNA (mtDNA) is transmitted in a non-Mendelian manner via the maternal oocyte (uniparental inheritance) whereas normally the paternal mitochondria are destroyed directly after fertilization. The replication and segregation of mtDNA are not coupled to cell cycle and the underlying mechanisms remain unclear ***(4)***. However, it appears that the quality control of mtDNA replication is not as efficient as nuclear DNA (nDNA), resulting in an increased risk of mtDNA mutations ***(5)***. Thus, to avoid the accumulation of such mutations, mitochondria are remarkably dynamic organelles that divide and fuse to ensure the mixing of mtDNA but also mitochondrial metabolites and proteins.

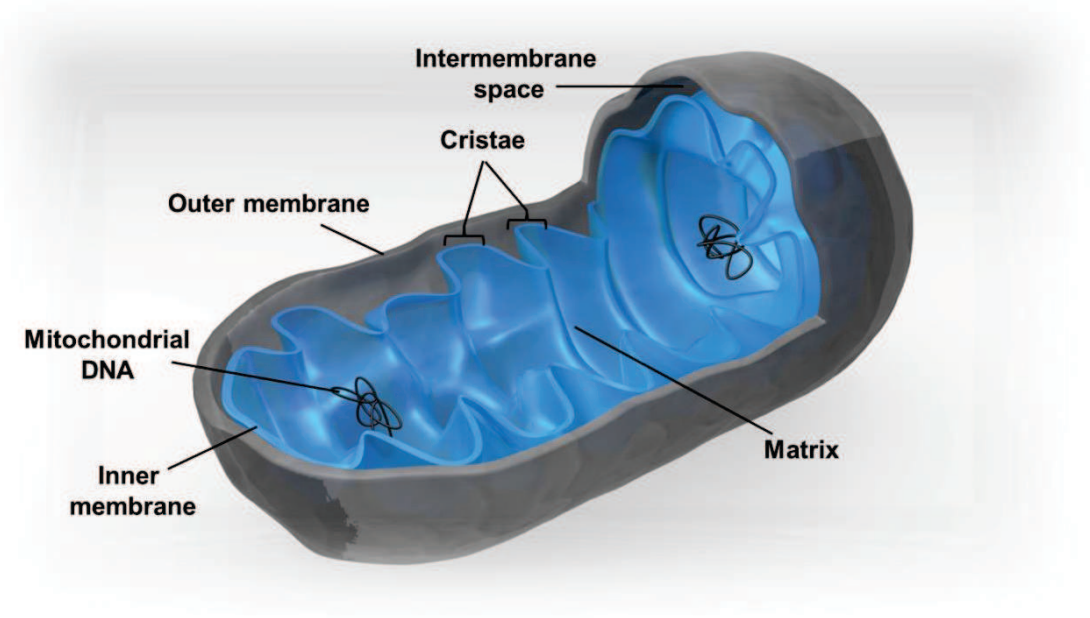

Fig. 1: Classical representation of mitochondrion ultrastructure (courtesy of M. Wanner Fabio).

a) Mitochondria: versatile organelles

Since mitochondria are really small organelles (from 0.5 to 1.0 µm in diameter), the characterization and understanding of mitochondrial ultrastructure became possible only when techniques for electron and confocal microscopy were perfected ***(3)***.

Mitochondria possess a heterogeneous and complex morphology resulting from the continuous fusion and fission of mitochondrial membranes ***(6; 7)***. Thus, mitochondrial network is often compared to a syncytium that constantly cycle between a fused, intermediate and fragmented state **(Fig 2).**

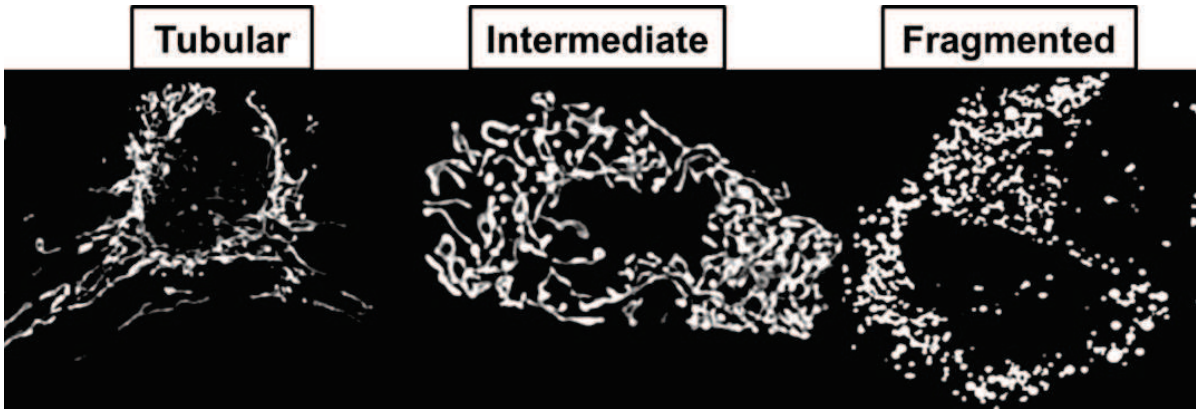

Fig.2: Different mitochondrial shapes (Adapted from ***(6)***)

As mentioned above, mitochondrial dynamics allow the mixing and exchange of material between those organelles but also their redistribution within the cells. This phenomenon is of major importance, especially in neurons, that are long, excitable and, highly compartmentalized cells ***(4)***. The proper distribution of mitochondria is paramount to sustain the spatial and temporal demand of energy in neurons, that differs within the axons and synapses compared to dendrites and cell body. Thus, on one hand, mitochondrial fission enables the renewal, redistribution and proliferation of mitochondria into synapses, where they are important for calcium buffering and cycling of reserve pool of synaptic vesicles. On the other hand, mitochondrial fusion allows mitochondria to interact and communicate with each other, and facilitating mitochondrial movement and distribution across long distances and to the synapses **(Fig.3)** ***(5)***.

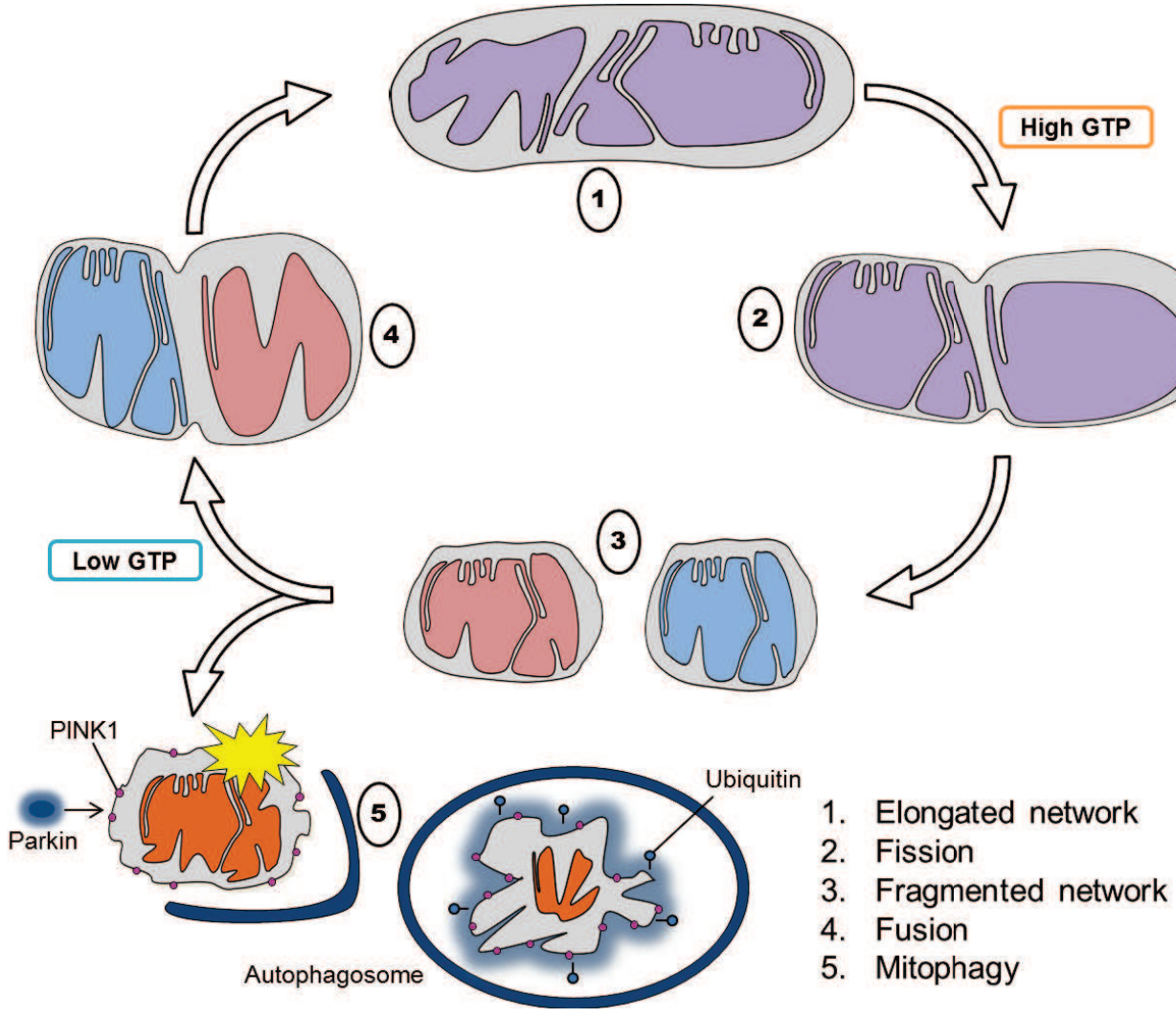

Fig 3: Mitochondrial dynamics. (A) Mitochondria cyclically shift between elongated (tubular) and fragmented state. Following the fission event, the mitochondrion can either be transported, or enter in fusion again. Defective mitochondrion accumulates PINK1 kinase (PTEN-induced putative kinase 1), recruiting the E3 ubiquitin ligase parkin, which ubiquitylates mitochondrial proteins and triggers mitophagy (adapted from ***(8)***).

Besides, mitochondrial fusion/fission activity is also integrated with mitochondrial quality control pathways that detect and respond to cellular and mitochondrial dysfunction, which leads to mitophagy **(Fig 3)**.

(i) Mitochondrial fission

Mitochondrial division involves dynamin-related GTPases such as fission protein 1 (FIS1) and dynamin-related protein 1 (DRP1), two proteins that are conserved through evolution **(Fig. 4)**. The activity of DRP1 is highly regulated by post-translational modifications. The inactive form of DRP1 is dispersed in the cytosol and the activation by dephosphorylation is required to target mitochondrial membrane ***(9)***. FIS1 was proposed to be a DRP1 receptor located at the outer mitochondrial membrane but the exact mechanism remains unresolved ***(8)***. FIS1 fixes DRP1 that wraps around the mitochondrial surface, assembles on the outer membrane in multimeric ringlike structures to facilitate scission of the double membrane. This process requires the hydrolysis of GTP. Mutations in genes coding for DRP1 and FIS1 result in aberrant mitochondria morphology (hyperfused network), heterogeneous population of mitochondria with non-uniform mtDNA distribution, varied ability to produce ATP, increased capacity to generate reactive oxygen species and increased susceptibility of cells to undergo apoptosis ***(10)***.

(ii) Mitochondrial fusion

Fusion events are a two step process and also require the action of two evolutionarily distinct dynamin-related GTPases **(Fig. 4)**. The fusion of mitochondrial outer membranes is controlled by mitofusin 1 and 2 (MFN1/2), whereas optic atrophy 1 (OPA1) controls inner membrane fusion ***(9; 4)***. The mechanisms underlying fusion machinery is still poorly understood. However, mitochondrial fusion is essential to maintain a homogenous mitochondrial population and ensures inter-complementation of mtDNA ***(5)***. Mutations in MFN2 or OPA1 cause the autosomal dominant peripheral neuropathy, Charcot Marie-Tooth disease 2A, and autosomal dominant optic atrophy respectively, and result in an extensive fragmentation of the mitochondrial network ***(10)***.

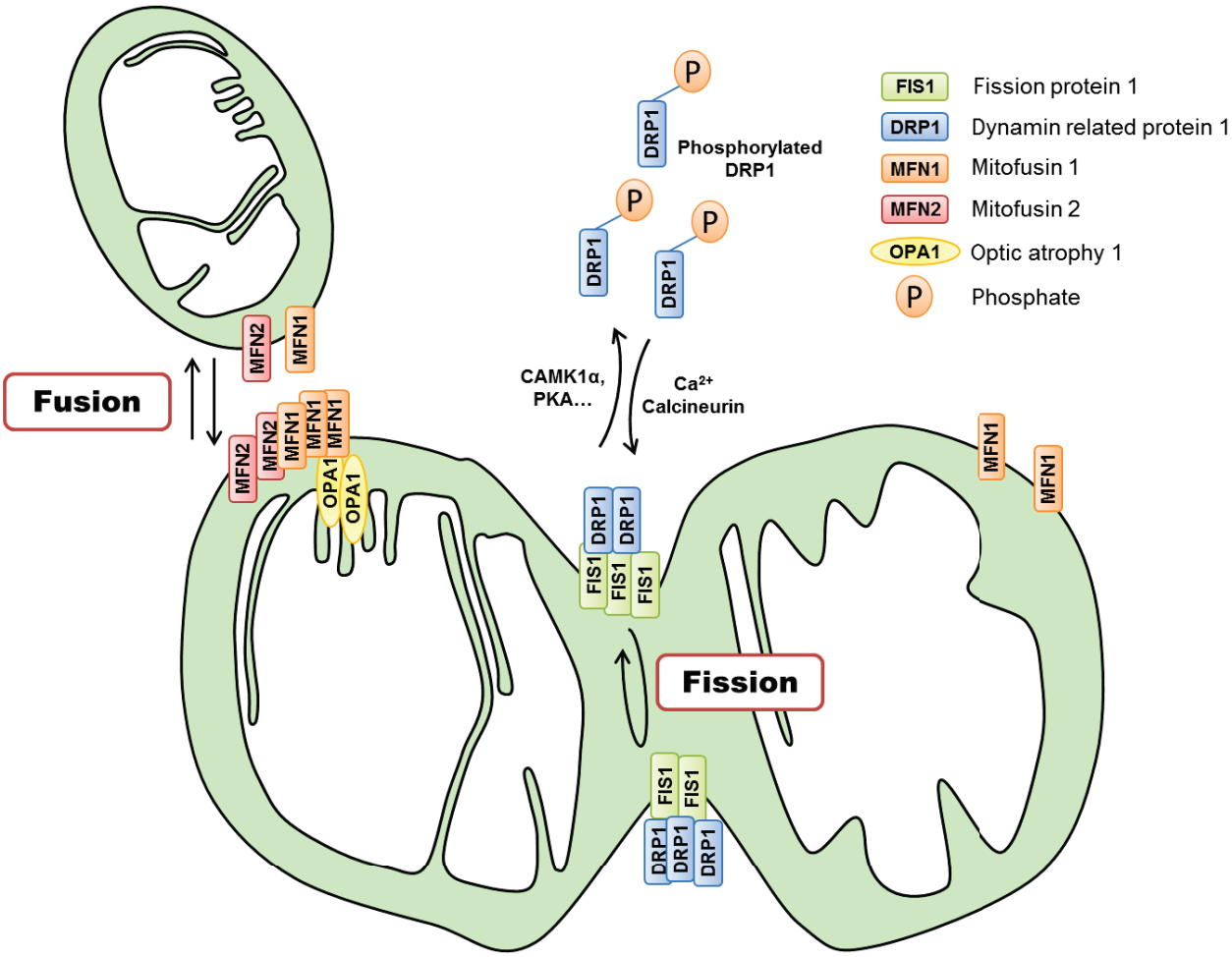

Fig.4: Schematic mechanisms of mitochondrial fission and fusion. The localization, as well as some interactions and modifications of the principal proteins involved in the two processes are shown. Once dephosphorylated, DRP1 is recruited to the outer membrane by FIS1. The oligomerization of DRP1 is followed by constriction of the membrane and mitochondrial fission. The pro-fusion proteins (MFNs on the outer membrane and OPA1 on the inner membrane) oligomerize to induce fusion of the membranes (adapted from ***(9)***).

(iii) Mitophagy

Fusion/fission activity is also important for the removal of aged or damaged mitochondria through a specific form of autophagy, termed mitophagy ***(11)***. The exact mechanism underlying mitophagy, more specifically what triggers mitophagy, remains to be elucidated. In healthy conditions, mitochondria generate an electrochemical gradient, which powers the oxidative phosphorylation (OXPHOS) system **(see section I.A.1.b.)**. It has been proposed that damages can lead to a loss of mitochondrial membrane potential. Uncoupled mitochondria accumulate the protein PTEN-induced putative kinase 1 (PINK1) at the surface of the mitochondrial outer membrane ***(11; 12)*** recruiting the E3 ubiquitin ligase parkin specifically to the damaged mitochondrion **(Fig.3)**. Then, parkin ubiquitylates mitochondrial proteins leading to the formation of an autophagosomes and the digestion of the mitochondrion. This process mediates mitochondrial quality control.

b) Orchestrating cellular energy production

ATP molecules are the universal fuel of living cells. These molecules are composed of an adenosine (nitrogen base), a ribose sugar, and three phosphate groups **(Fig. 5)**. Breaking the bond between the second and third phosphates, which is a high-energy bond, will release the energy necessary for numberless cellular processes, including the synthesis of macromolecules (DNA, RNA, proteins…), transport of macromolecules (e. g. endocytosis and exocytosis), intra/extracellular cell signaling or locomotion (muscle contraction). In eukaryotic cells, ATP is mainly produced via two pathways: the glycolysis and the oxidative phosphorylation.

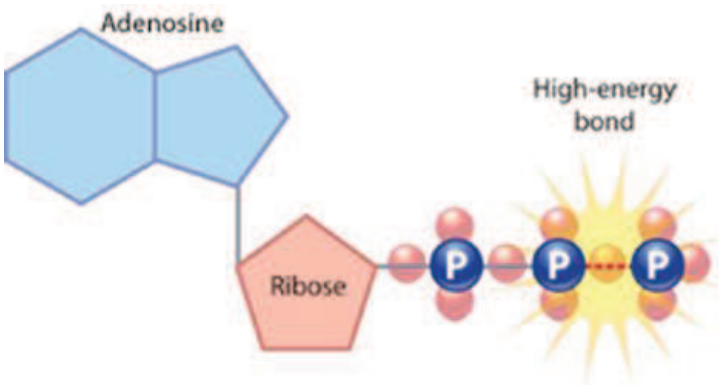

Fig.5: A molecule of ATP (http://www.nature.com/scitable/topicpage/cell-energy-and-cell-functions-14024533).

(i) Cellular glycolysis

Glycolysis is the first energy pathway which consists of converting molecules of glucose (coming from nutritional sources) into two molecules of pyruvates **(Fig. 6)**. This requires a series of ten chemical reactions giving a net gain of two ATP molecules. Two NADH (nicotine adenine dinucleotide) molecules are also produced and serve as electron carriers for other biochemical reactions in the cell ***(13)***. Next, pyruvate enters in mitochondria and is converted into acetyl-CoA, a two-carbon energy carrier, that reaches the Krebs cycle, also known as the tricarboxylic acid cycle (TCA cycle), taking place within the mitochondrial matrix.

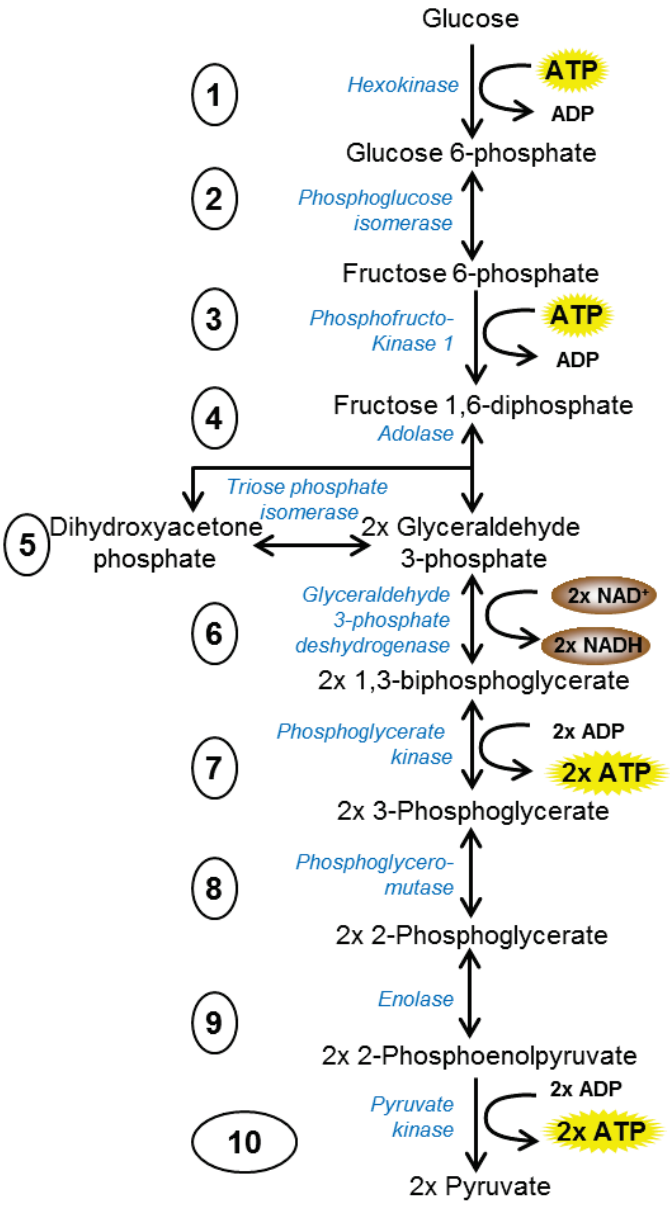

Fig. 6: Cellular glycolysis. The glycolysis involves a series of enzymatic reactions (from 1 – 10) that consume two ATP molecules, produce 4 ATP molecules (net gain= 2 ATP) and two molecules of NADH. Molecules of pyruvate, end product of glycolysis, reach the Krebs cycle within mitochondria.

(i) Mitochondrial respiration

The enzymatic reaction involved in the Krebs cycle aims to convert: i) three NAD+ (nicotine adenine dinucleotide) into three reduced equivalent molecules (NADH); ii) one molecule of FAD+ (flavin adenine dinucleotide) into one equivalent of $FADH_2$; iii) one molecule of GDP (guanosine diphosphate) into one molecule of GTP (guanosine triphosphate) **(Fig. 7)**. The NADH and $FADH_2$ generated by the Krebs cycle will be used by the oxidative phosphorylation (OXPHOS) pathway to generate ATP.

The second main pathway for the synthesis of ATP is the mitochondrial respiration, a process usually called oxidative phosphorylation (OXPHOS) ***(8; 3)***. Here, the electrons, carried by the electron donors NADH and $FADH_2$, are used to generate a difference of potential across the inner mitochondrial membrane, by pumping protons from the matrix to the intermembrane space. The gradient of protons, which is generated during electron transfer, is ultimately used to power the synthesis of three additional ATP molecules for every electron that travels along the chain.

The transfer of electrons is carried out by four multisubunit protein complexes (encoded by mitochondrial or nuclear DNA) embedded in the inner mitochondrial membrane and known as the electron transport chain (ETC) **(Fig. 7)**. First, NADH donates two electrons to the largest of the respiratory complexes, NADH dehydrogenase or complex I. These electrons are then passed to coenzyme Q (UQ), a lipid soluble redox carrier. The reaction is accompanied by the transfer of four protons from the matrix to the intermembrane space. In parallel, complex II, or succinate dehydrogenase that is also part of the Krebs cycle, catalyzes the reduction of FAD to $FADH_2$, giving additional electrons into the quinone pool (UQ). Unlike complex I, no protons are pumped from the matrix by complex II that is also the only complex composed of proteins exclusively encoded by nuclear DNA.

Next, the reduced coenzyme Q freely diffuses through the inner membrane and gives its electrons to the complex III or ubiquinol cytochrome c oxidoreductase. The enzyme oxidizes coenzyme Q and transfers the liberated electrons to two molecules of another soluble redox protein, cytochrome c. The reaction is coupled by the translocation of four protons toward the intermembrane space. Finally, electrons are removed from cytochrome c and transferred to molecular oxygen (O_2), producing two molecules of water. Again, four protons are pumped from the matrix into the intermembrane space.

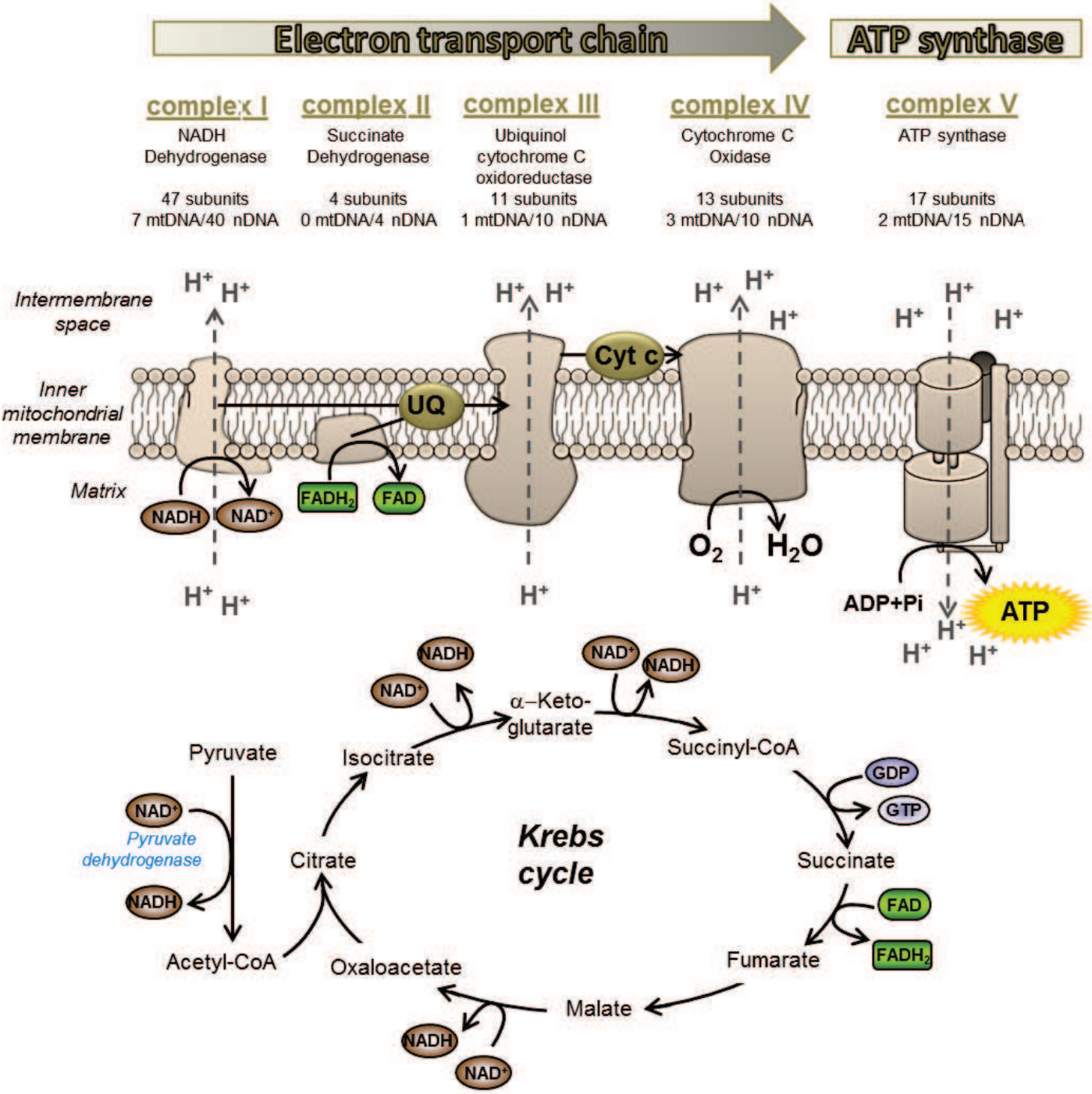

Fig. 7: Bioenergetic of the electron transport chain and the Krebs cycle. Pyruvate is transferred within mitochondria and is converted into acetyl-coA that enters into the Krebs cycle. NADH, $FADH_2$ and GTP molecules are generated in each cycle. NADH generated is shuttled to complex I and is converted to NAD+ driving OXPHOS. Transfer of electrons through the chain maintains the membrane potential via proton pumping into the intermembrane space. In this final step, ADP is phosphorylated to form ATP via complex V (ATP synthase).The number of subunits encoded either by nuclear or mitochondrial DNA (nDNA/mtDNA respectively) are indicated. UQ; coenzyme Q, Cyt c; cytochrome c. (adapted from ***(8)*** and ***(14)***).

The enzyme responsible for the OXPHOS final step is ATP synthase (Complex V), alternatively named F_0F_1 ATPase, which consists of two domains: F_0, the transmembrane component acting as a proton channel that provides a proton flux back into the mitochondrial matrix; and F_1, the catalytic component that uses the free energy produced during the generation of the oxidized forms of the electron carriers (NAD^+ and Q) to drive ATP synthesis from ADP and inorganic phosphate (Pi). ATP synthase acts as a rotary molecular motor powered by the proton gradient and the mitochondrial membrane potential (MMP) generated by the ETC, with each turn of the rotor producing three molecules of ATP. Of note, MMP is estimated to be about 150–180 mV (negative in the matrix), and is a key parameter indicating the bioenergetic competence of mitochondria. The net gain of OXPHOS is about 30-32 ATP molecules per molecule of glucose.

(ii) Bioenergetic state and mitochondrial shape

Energy homeostasis requires a constant coordination between cellular energy demand (ATP consumption) and energy supply (ATP production). Thus, molecular sensors are available in cells, such as kinases and transcription factors, to regulate bioenergetic activity in answer to nutrient deprivation or to cellular stress ***(14)***. Notably, the alteration in the cellular AMP/ATP ratio activates the AMP-activated protein kinase (AMPK) which triggers an intracellular cascade of phosphorylation to activate glycolysis and OXPHOS ***(15)***. As mentioned in **section I.A.1.a**, mitochondria are dynamic organelles that cyclically shift between tubular and fragmented states. Increasing evidences suggest that alterations in energy production, notably in pathological conditions such as in AD **(see section I.A.2)**, are paralleled by a disturbed mitochondrial dynamics ***(16-19)***. The causal relationship between both is not clear. For instance, studies showed that ATP levels and OXPHOS activity are affected by mitochondrial dynamics, with a decreased respiration and ATP production when mitochondria are in a fragmented state ***(20)***. On one hand, evidences suggested that mitochondrial dynamics can control energy metabolism, since a down regulation of OPA1 and Mfn1 (involved in mitochondrial fusion) led to a decrease in OXPHOS and glucose utilization ***(20)***. On the other hand, studies showed that the collapse of MMP leads to fragmentation of the mitochondria and inhibits the fusion activity ***(21; 22)***.

Understanding the link between mitochondrial shape and function is a recent research field that aims to increase our knowledge about the underlying mechanism involved in the regulation of bioenergetics **(see section II.C)** and may offer opportunities to discover new drugs that target mitochondrial fusion or fission with a stimulatory effect on energy metabolism ***(14)***.

c) Mitochondrial: paradoxical organelles

As described above, mitochondria are the "powerhouses of cells", providing energy via ATP generation. However, when mitochondria fulfill their physiological functions, they can also be compared to a double-edged sword that, on one hand, produces the energy necessary for cell survival, and on the other hand, induces the formation of reactive oxygen species (ROS) that can be harmful for cells when produced in excess with mitochondria as the first target of toxicity.

(i) Mitochondria: source and target of reactive oxygen and nitrogen species

The production of ATP molecules by mitochondria requires about 85% of O_2 used by cells. The fate of most electrons from NADH or $FADH_2$ driven in the respiratory chain is the reduction of O_2 into H_2O by the mitochondrial complex IV. However, an inevitable by-product of ETC activity is the formation of superoxide anion radicals ($O_2^{\bullet-}$), mostly by complexes I and III **(Fig. 8)**. Indeed, a small portion of electrons (up to 2%), escaping from ETC, can react with O_2 to form $O_2^{\bullet-}$ that can be converted into other ROS such as hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($OH^{\bullet}$) through enzymatic and non-enzymatic reactions ***(23-25)***.

The exact mechanisms underlying mitochondrial ROS production requires further clarification to identify the precise site of $O_2^{\bullet-}$ generation. Thus, $O_2^{\bullet-}$ production by the mitochondrial complex I is still discussed. It was suggested that $O_2^{\bullet-}$ was generated by reverse electron transfer in the absence of NAD+-linked substrates (in hypoxic conditions) ***(25; 26)***. The contribution of complex III in ROS-forming mechanisms is more understood and involves the autooxidation of ubisemiquinone anion radicals ($UQ^{\bullet-}$). Indeed, $UQ^{\bullet-}$ can react with the O_2 dissolved in the IMM, yielding to $O_2^{\bullet-}$ formation, especially when the MMP is high ***(25; 27; 26)***. Besides the ETC, ROS can be produced by the Krebs cycle enzyme α-KGDH (α-ketoglutarate dehydrogenase) and by monoamine oxidases (MAO) located at the OMM ***(23)***. Nitric oxide (•NO) is another diffusible free radical that is synthesized by three main isoforms of the nitric oxide synthase (NOS) **(Fig. 8)**. •NO can combine with $O_2^{\bullet-}$ to form reactive nitrogen species (RNS) peroxynitrite ($OONO^-$) which can further react to yield hydroxyl radical •OH ***(25)***.

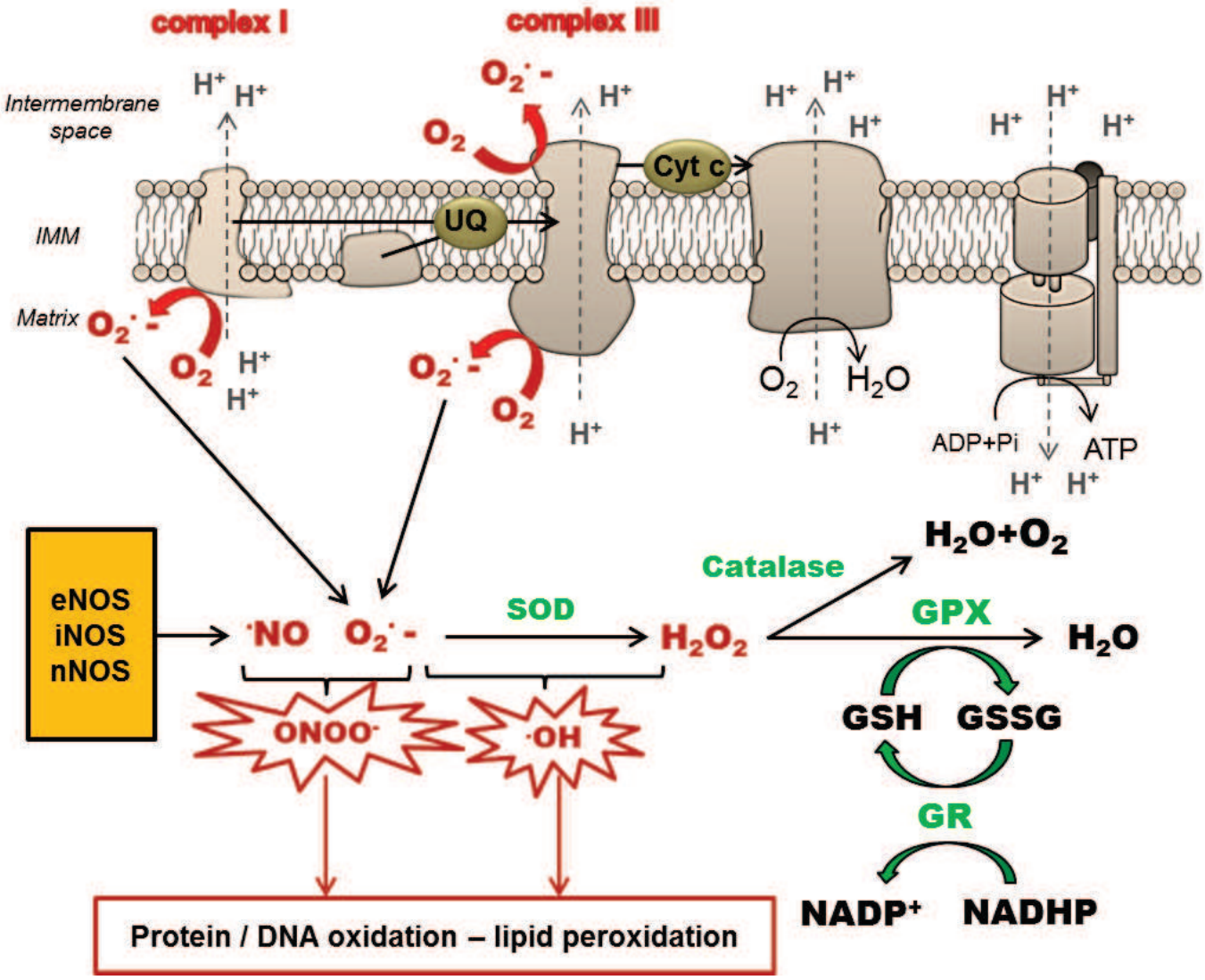

Fig.8: Pathways of reactive oxygen species (ROS) formation and detoxification. Superoxide anion radicals ($O_2^{\cdot-}$) are generated by complexes I and III during electron transfer through ETC. $O_2^{\cdot-}$ can interact with •NO, produced by nitric oxide synthase (NOS), to generate peroxynitrite (ONOO-) or can also react to form •OH (hydroxyl radical). Detoxification involves the enzymatic activity of mitochondrial manganese superoxide dismutase (SOD) that converts $O_2^{\cdot-}$ to H_2O_2 (hydrogen peroxide) and may diffuse to the cytoplasmic compartment where glutathione peroxidase (GPX) and catalase convert H_2O_2 to H_2O. Of note, •NO and its derivate (reactive nitrogen species or RNS) belong also to the group of ROS. eNOS/iNOS/nNOS; endothelial/inducible/neuronal nitric oxide synthase, GSH/GSSG; reduced/oxidized glutathione, GPX; glutathione peroxidase, GR; glutathione reductase.

In physiological conditions, ROS can play a role of signaling molecules and are involved in processes such as immune response, inflammation, as well as synaptic plasticity, learning and memory ***(28-30)***. However, when produced in excess, those highly reactive species can induce an oxidative stress, damaging proteins and DNA, and inducing lipid peroxidation, with the corresponding mitochondrial structures as the first targets of toxicity. Especially, long polyunsaturated fatty acid chains of mitochondrial membranes are very susceptible to oxidation and may lead to the membrane depolarization and consecutively to mitochondrial impairments ***(31)***. $ONOO^-$ can induce nitration of proteins and impair their function ***(32)***, and •NO has been shown to inhibit the complex IV activity by competitive binding on its oxygen site ***(33)***. Furthermore, since mtDNA is localized close to ROS production sites, it is directly in contact with those harmful molecules and can exhibit oxidative damages. Taken together, oxidative stress, caused by ROS and RNS, can trigger cell death and has been implicated in the pathogenesis of many neurodegenerative diseases, such as AD.

(ii) Antioxidant defenses: weapons against oxidative stress

Fortunately, cells are armed against oxidative stress and are endowed with robust antioxidant defenses to counteract excessive ROS production. First, $O_2^{\bullet -}$ can be detoxified by manganese superoxide dismutase (MnSOD) in the mitochondrial matrix or copper/zinc superoxide dismutase (Cu/Zn SOD) in the IMS and the cytosol, giving hydrogen peroxide (H_2O_2) ***(25)***. H_2O_2 is then detoxified to H_2O by catalase or is supported by detoxification mechanisms involving glutathione (GSH) system **(Fig.8)**. GSH constitute the most important redox buffer and mitochondrial antioxidant, due to its relatively high concentrations and inducible metabolism ***(25; 34)***. Glutathione peroxidase (GPX) degrades H_2O_2 and other ROS to water by converting GSH to its oxidized form GSSG. This latter is then reduced again to GSH by glutathione reductase (GR) using NADPH as a cofactor. The ratio GSH/GSSG is often used as a measure of cellular toxicity and regulates overall ROS levels to maintain physiological homeostasis ***(35)***.

However, it can happen that ROS production overwhelms endogenous antioxidant systems and can lead to harmful effects on cellular compounds. As mentioned above, oxidative stress can lead to a shutdown of energy production ***(36)***, possibly leading to a decrease of antioxidant defense (e.g. GSH). The enhancement of ROS triggers the “vicious cycle” of oxidative stress, mitochondrial dysfunction and apoptosis. Since neurons are post-mitotic and excitable cells with high energy requirements, they are more sensitive to stress ***(37)***. Thus, this “vicious cycle” of oxidative stress has been implicated in many neurodegenerative diseases, notably AD but also in normal aging ***(38; 5; 39)***. However, the

exact cause and effect relationship between ROS production and detoxification remains unsolved and one can ask the question whether increased ROS production is a primary consequence of mitochondrial dysfunction or whether a primary defect in the ROS scavenging activity is responsible for an abnormal respiratory function. This ambiguous point is particularly challenging in many pathological cases involving mitochondrial dysfunction.

(iii) Mitochondria and cell suicide

Another paradoxical aspect with regard to mitochondrial function is the role of this small organelle in both cell survival and cell death. Indeed, if the mitochondria are indispensable for cell survival by providing energy, they are also a starting point leading to cell suicide, a mechanism commonly known as apoptosis or programmed cell death. There are two main apoptotic pathways: i) the extrinsic pathway triggered by extracellular signals acting via plasma membrane receptor (e.g. death receptors, not described here); and ii) the intrinsic (or mitochondrial) pathway triggered by intracellular stimuli such as Ca^{2+} overload and over-generation of ROS ***(37)***. Mitochondria are central components of the apoptotic death machinery, integrating death signals through Bcl-2 family members and coordinating caspase activation through the release of cytochrome c (Cyt c).

Once the lethal signals are detected, mitochondrial permeability transition pores (mPTP) are opened and mitochondrial membranes become permeable **(Fig. 9)**. This opening disrupts mitochondrial function and apoptotic signals are released from IMS into the cytosol, namely Cyt c and apoptosis-inducing factor (AIF) ***(40)*** that are regulated by pro- (bax, bak, bad, bim, bid) and anti-apoptotic proteins (bcl-2 and bcl-xl) of the Bcl-family ***(41)***. Here, Cyt c binds to Apaf-1 (apoptotic protease activating factor 1) which recruits procaspase 9 in the presence of deoxyadenosine triphosphate (dATP) to form the apoptosome. This results in the sequential activation of caspase 9 which in turn activates other caspases, such as caspase 3, that are in charge of the cell's execution ***(42; 37)***.

In summary, mitochondria are paramount for proper cell function, especially in neurons that are postmitotic, excitable cells with high energy requirements. Thus, impaired mitochondrial function ineluctably leads to cell death and is involved in pathophysiological mechanisms of neurodegenerative diseases, including Alzheimer's disease (AD).

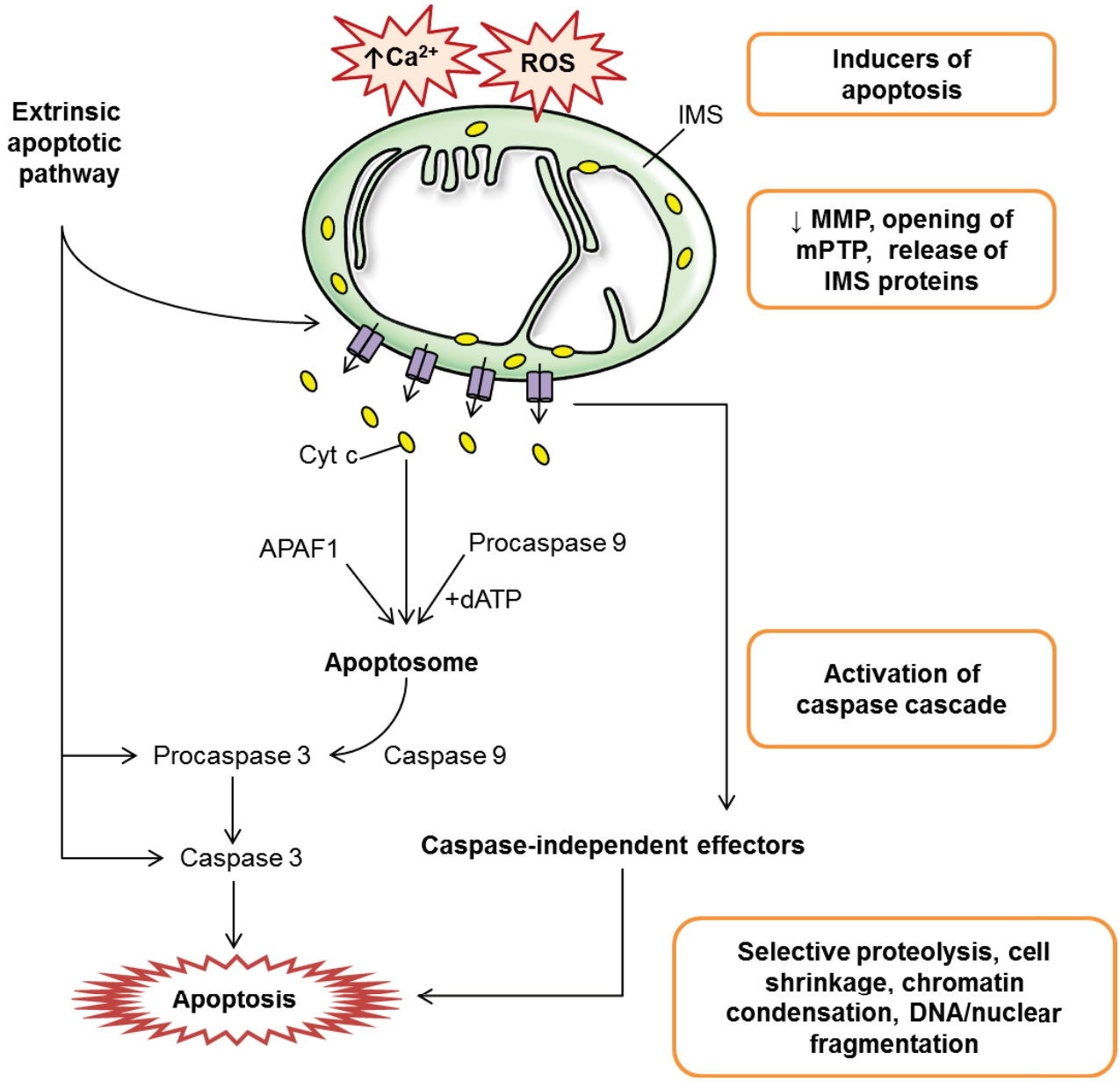

Fig.9: Simplified mitochondrial pathways of apoptosis. Apoptosis can result from the activation of two biochemical cascades, which are known as the extrinsic and the intrinsic (or mitochondrial, detailed here) pathways. The release of cytochrome c (Cyt c) from the intermembrane space (IMS) triggers the caspase cascade (see details in the text) yielding to the cell death by apoptosis. MMP; mitochondrial membrane potential, mPTP; mitochondrial permeability transition pore (adapted from ***(37)***).

2. Mitochondrial dysfunction in Alzheimer's disease

AD is an age-related neurodegenerative disorder that currently affects about 2% of the population in industrialized countries and accounts for more than 60% of all dementia cases ***(43)***. The disease is marked by a progressive physical and cognitive decline due to a reduced size of brain regions involved in learning and memory, such as temporal/frontal lobes and hippocampus, as the result of neuronal death and synaptic degenerations **(Fig.10)**. In addition, positron emission tomography (Pet) studies performed in AD patients revealed a large hypometabolism and decrease of glucose uptake in the frontal cortex and temporal lobes, which correlates with the neuronal loss observed in these regions ***(44; 45)***.

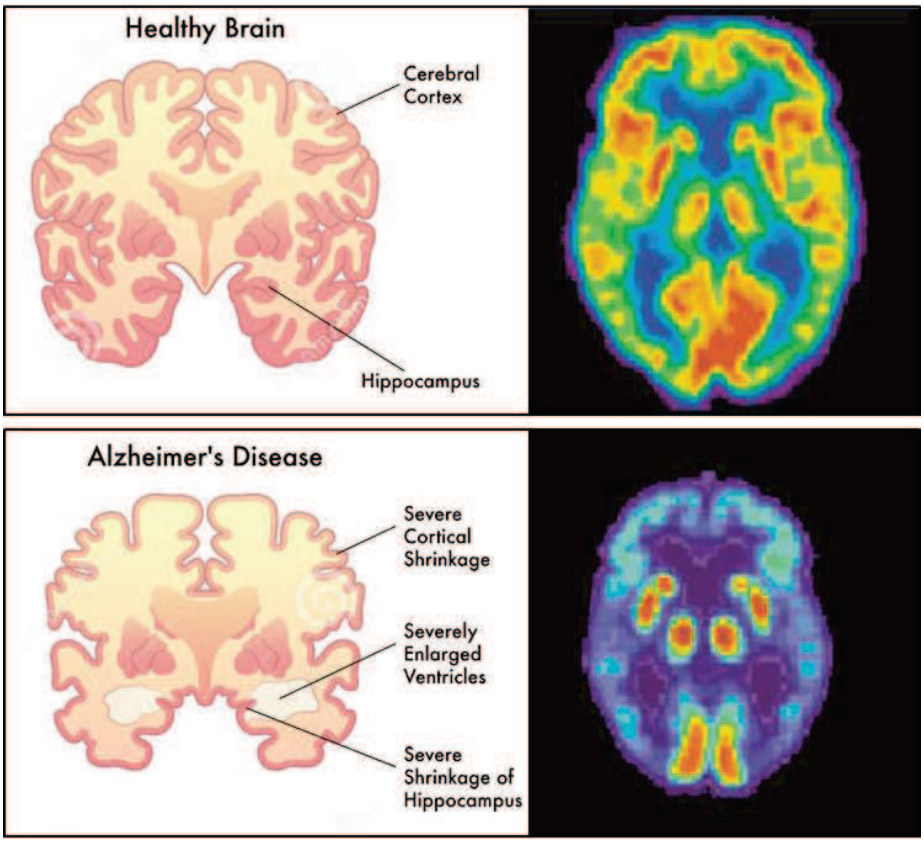

Fig. 10: AD results in shrinkage of brain regions involved in learning and memory. Positron emission tomography (Pet) images show glucose uptake (red and yellow=high levels of uptake) in a healthy person (top) and an AD patient (bottom). The AD patient exhibits a drop in energy metabolism in the frontal cortex and the temporal lobes (adapted from http://www.google.fr/images/Alzheimerdisease and ***(44)***).

This neuropathology will become increasingly burdensome and costly in the coming years as AD prevalence is expected to double within the next two decades ***(43)***. A post-mortem examination is required to clearly diagnose AD with the detection of “plaques” (amyloid-β (Aβ) deposits) and “neurofibrillary tangles” (NFT, aggregated tau protein) in the brain parenchyma, especially in the entorhinal cortex, hippocampus, basal forebrain and amygdala, that are regions involved in learning and memory processes ***(44)***.

a) Clinical symptoms and etiological factors

AD is a progressive neurodegenerative disease that usually begins in late life (onset ± 65 years old) and is first marked by episodic memory deficits with preserved alertness and motor functions ***(46)***. Over time, progressive deterioration of other cognitive abilities appears, leading to profound impairments in language, abstraction and orientation. In addition, other neuropsychiatric symptoms can be detected such as mood disturbances, delusions and hallucinations, personality changes and behavior disorders (aggressiveness, depression, circadian disturbances) but may vary from one patient to another ***(46; 47)***. On average, death occurs a mere seven years following diagnosis, typically from medical complications (bronchitis or pneumonia) ***(43)***. Until now, there are no treatments to cure, prevent or slow the disease ***(47)***.

The complete etiological picture of AD remains unknown. However, the disease can be classified into two different forms: i) The sporadic AD (SAD) which represents the vast majority of AD case with an onset occurring at an age over 60 years; ii) The rare familial forms (FAD) which represent less than 1% of the total number of AD case and are characterized by an early disease onset at an age younger than 60 years ***(48; 49)***. Both forms of AD show the same clinical symptoms and neuropathology, as memory loss, amyloid deposits in the brain and neurofibrillary tangles. However, FAD is marked by a more rapid disease progression and can be transmitted to offspring in a Mendelian manner ***(50)***.

Genetic studies in FAD patients have identified autosomal dominant mutations in three different genes: the amyloid precursor protein (APP, more than 20 pathogenic mutations identified) and the presenilins 1 and 2 (PS1 and PS2, over 130 mutations identified) ***(51; 50)***. These genes are directly linked to the accumulation of Aβ deposits in the brain due to the increased production of Aβ peptide from its precursor protein APP **(Fig. 11A)**.

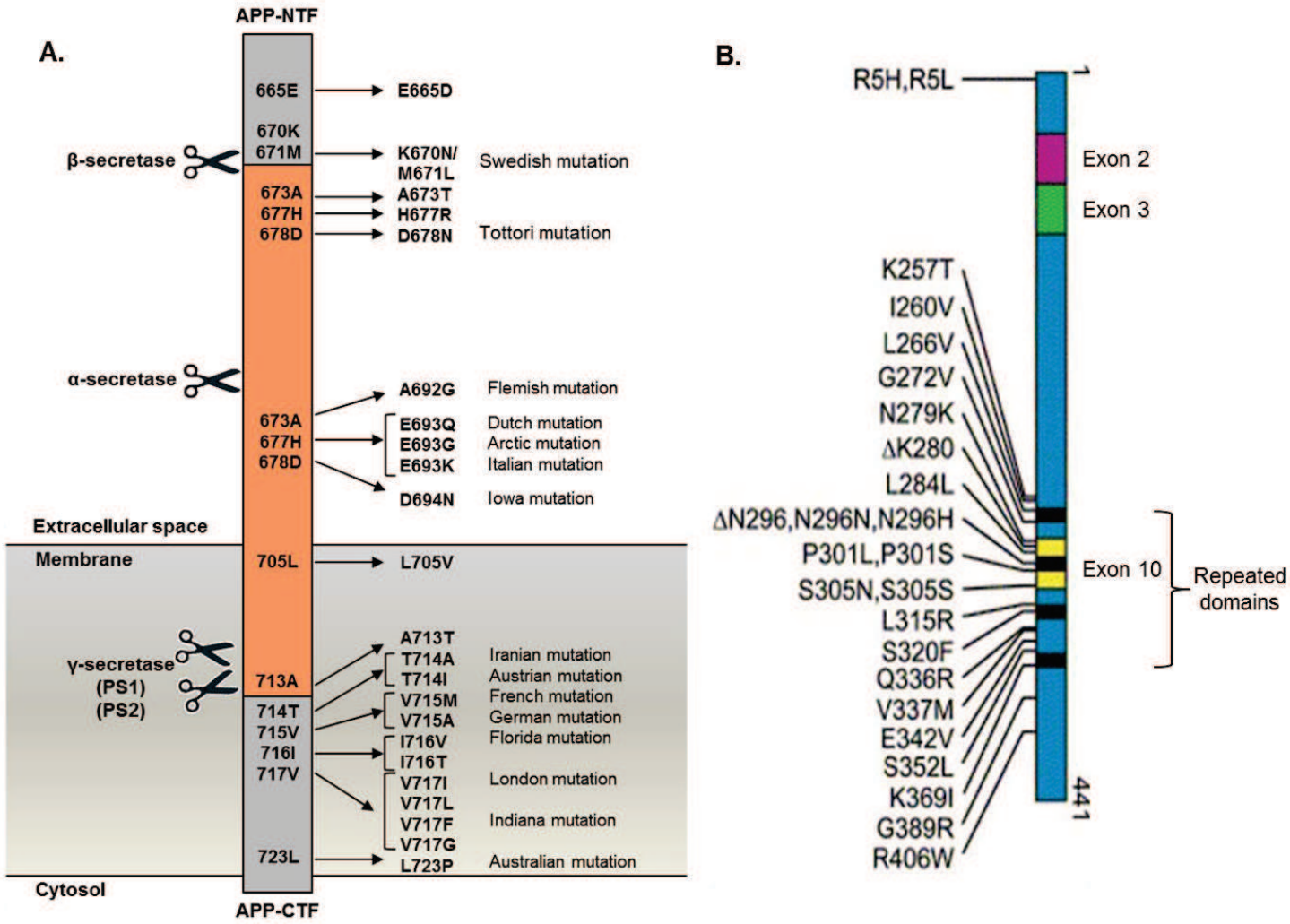

Fig.11: Mutations in APP and tau associated with FAD and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) respectively. (A) Part of the APP amino-acid sequence where mutations associated with early-onset Alzheimer's disease have been highlighted. Most mutations are clustered in the close vicinity of secretase-cleavage sites, thereby influencing APP processing, and are named after the nationality or location of the first family in which that specific mutation was demonstrated. (B) Mutations in the tau gene in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Schematic diagram of the longest tau isoform (441 amino acids) with mutations in the coding region. Twenty missense mutations, two deletion mutations and three silent mutations are shown (adapted from ***(50)*** and ***(52)***).

Surprisingly, no mutations in microtubules associated protein tau (MAPT), the protein responsible for NFT formation, has been so far detected in FAD. However, such mutations have been identified in familial frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) ***(52)*** **(Fig. 11B)** and lead to NFT formation. The identification of these genes enabled, among others, the generation of simple, double and even triple transgenic animal models bearing these mutations. Transgenic models have now become the most used tools to study AD pathogenic mechanism *in vivo* ***(51)***.

Regarding SAD cases, no clear etiological factors have been established until now. The main risk factor remains age since the incidence rate for AD increases exponentially after 60 years, especially during the 7th and 8th decades of life ***(48)***. Epidemiological studies revealed that two thirds of AD patients are women and the sudden drop in estrogen levels after the menopause has been proposed to be one risk factor in AD ***(53; 54)*** **(see section I.B.4)**. Susceptibility genes have also been identified, in particular gene coding for the E4 isoform of apolipoprotein E (apoE4). Evidences showed that ApoE4 reduces Aβ clearance and increase Aβ aggregation, and individuals producing this isoform have an increased risk to develop AD ***(44; 48)***. Besides, the recent development of powerful and sophisticated genotyping approaches used in genome-wide association studies (GWAS) enabled to highlight others genes of "susceptibility" belonging to four main pathways: immune response, APP processing, lipid metabolism and endocytosis (reviewed in ***(48)***).

Epidemiological findings include other risk factors such as behavioral (sedentary lifestyle), diets (high-calories, high-fat diets) and diverse environmental factors (low education level, history of head trauma). However, despite accumulating data, clear causative factors of AD have not been established.

b) Histopathological hallmarks

The two key histopathological features of AD (amyloid deposits and neurofibrillary tangles) were already described in 1907 by Alois Alzheimer after post-mortem examination of the brain of a 51-year-old woman (Auguste D) who initially was diagnosed to suffer from a delusional disorder followed by a rapid loss of short term memory ***(55)***. Today, we know that those extracellular plaques are composed of the amyloid-beta (Aβ) peptide and the tangles (or intracellular neurofibrillary tangles, NFT) are formed by the aggregation of hyperphosphorylated tau, a microtubule-associated protein. These two histopathological hallmarks are frequently coupled with additional pathological changes including reactive microgliosis and, as mentioned above, neuronal loss and synaptic degeneration ***(47)***.

(i) Neurofibrillary tangles and hyperphosphorylated tau

Tau belongs to the family of microtubule-associated proteins (MAP) that stabilizes microtubule function and assembly, and maintains appropriate functions of motor proteins within cells ***(56)***. Tau, which is expressed in most neurons, is mainly concentrated in axons and almost totally absent in dendrites ***(57)***. Since microtubules are important structures for axonal transport, cell polarity and shape, alterations in tau may seriously impair normal cellular physiology. AD and other tauopathies, like the FTDP-17, are similarly characterized

by an aberrant intracellular accumulation of tau within neurons, which results in tau hyperphosphorylation and its assembly into abnormal filaments **(Fig.12)** ***(52; 51)***.

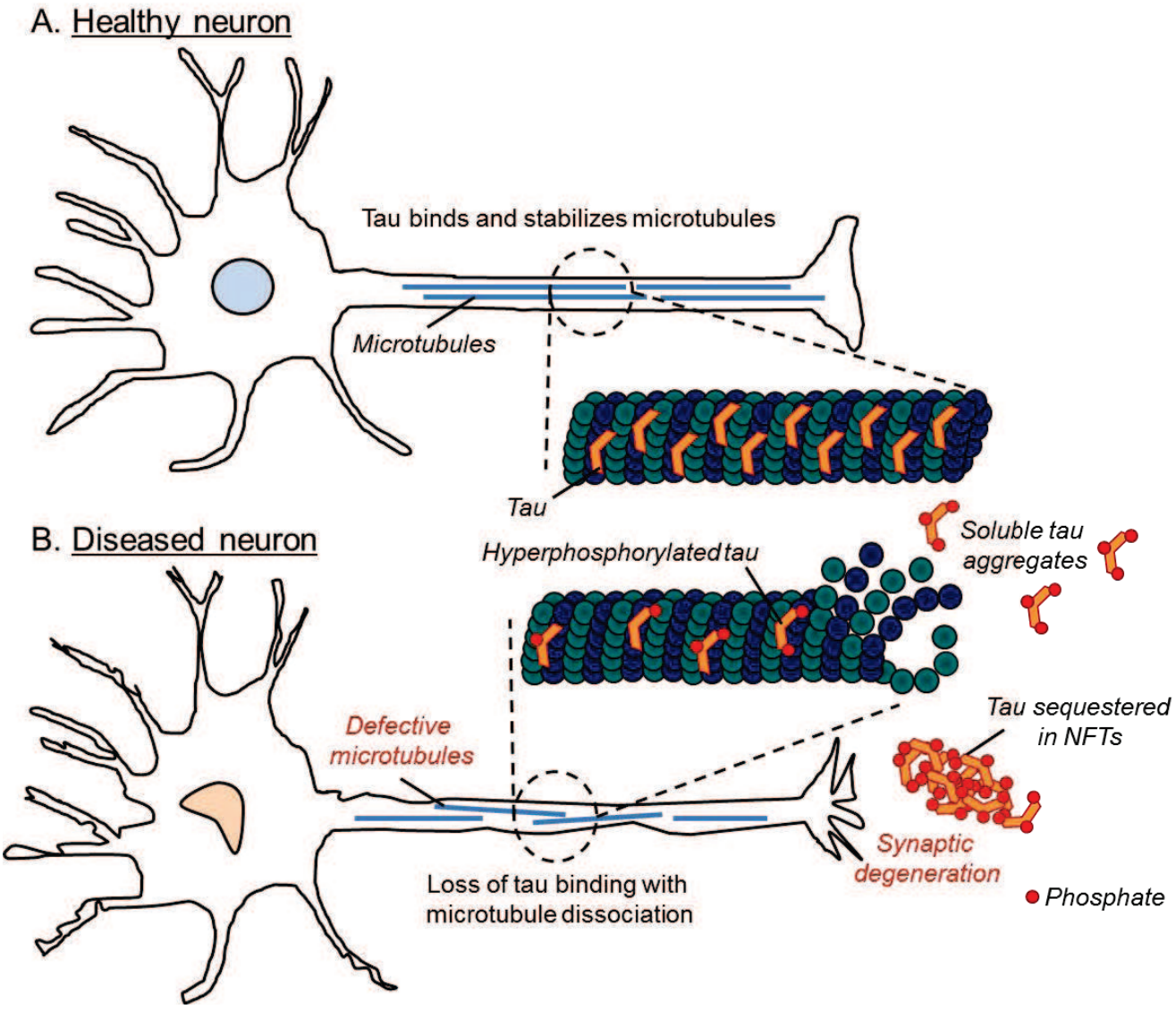

Fig.12: Hyperphosphorylated tau and neurofibrillary tangles (NFT). (A) Tau stabilizes microtubules that are essential for the trafficking of cellular cargo along the axons of neurons. (B) In AD, tau is abnormally hyperphosphorylated, which reduces its binding on microtubules and leads to tau sequestration into NFT. The loss of tau binding induces microtubules instability, reduces axonal transport and contributes to synaptic degeneration (adapted from ***(60)***).

The causes of tau aggregation in AD are not fully understood and no tau mutation has been found in FAD. Since tau is a phosphoprotein containing 5 tyrosine residues and 80 serine/threonine residues, it can be potentially phosphorylated by many kinases, including glycogen synthase kinase-3β (GSK-3β), cyclin-dependent kinase 5 (cdk5), extracellular signal-regulated kinase 2 (ERK2), protein kinase A and C, calcium-calmodulin dependent

protein kinase II (CaMKII), and mitogen-associated protein affinity-regulating kinases (MARK) ***(58)***. Studies have shown that tau is three to four times more phosphorylated in AD brain than in normal brain, especially at the microtubules binding site ***(59; 58)***. This induces conformational changes which result in the detachment of tau from microtubules, causing them to depolymerize, as well as tau aggregation in NFT **(Fig.12)**. This histopathological hallmark of AD generates problems with synaptic integration that inexorably leads to neurodegeneration ***(60; 61)***.

(ii) Amyloidogenic pathway and Aβ deposits

Although the causative factors of AD remain unknown, the leading hypothesis is still that Aβ accumulation, a product of amyloid precursor protein (APP) processing, is an underlying pathological component of the disease. The discovery of mutations in genes that code for APP, PS1 or PS2 and responsible for FAD cases, have led Hardy and Higgins to propose the "Amyloid cascade hypothesis" in 1992 ***(62)***. This hypothesis posits that Aβ deposition is the causative agent of AD pathology and the direct results of this deposition are the formation of NFT, neuronal dysfunction and death, leading to dementia ***(62; 63)***. APP is a type 1 integral glycoprotein (110-130 kDa) that is ubiquitously expressed in human tissues ***(64; 65)***. The physiological functions of APP remain unclear but previous studies highlighted its role in cell adhesion, synaptic plasticity, regulation of neuronal survival and neuritis outgrowth (reviewed in ***(66)***). APP is located at the plasma membrane (or the luminal side of the endoplasmic reticulum (ER) and Golgi apparatus) and contains a 40 or 42 amino acid sequence, respectively, called $A\beta_{40}$ and $A\beta_{42}$ ***(67)***. APP undergoes two proteolysis pathways by secretases: a non-amyloidogenic pathway and an amyloidogenic pathway **(Fig. 13A)**. In the first one (non-amyloidogenic), APP is cleaved by the α-secretase, an enzyme that belongs to the ADAM family (a disintegrin and metalloproteinase family enzymes), which produces the α-APPs and the membrane-anchored C83 fragments ***(65)***. The latter is subsequently catalyzed by γ-secretase (for which presenilin 1 and 2 (PS1 and PS2) act as catalytic subunits) to form the nontoxic or neurotrophic products, the APP intracellular domain (AICD) and P3 fragment **(Fig. 13A)**.

In the amyloidogenic pathway, APP is first cleaved by β-secretase (mostly β-site of APP cleaving enzyme or BACE) that cut APP at the N-terminus to form β-APPs and C99 fragments. The latter is subsequently cleaved by γ-secretase producing Aβ and AICD. Of note, the released Aβ can vary in length, depending on the site of cleavage, of those $A\beta_{42}$ that is more hydrophobic and has a higher propensity to form fibril aggregates found in the brain of AD patients, whereas $A\beta_{40}$ is more common but less fibrillogenic **(Fig. 13B)** ***(65; 50)***.

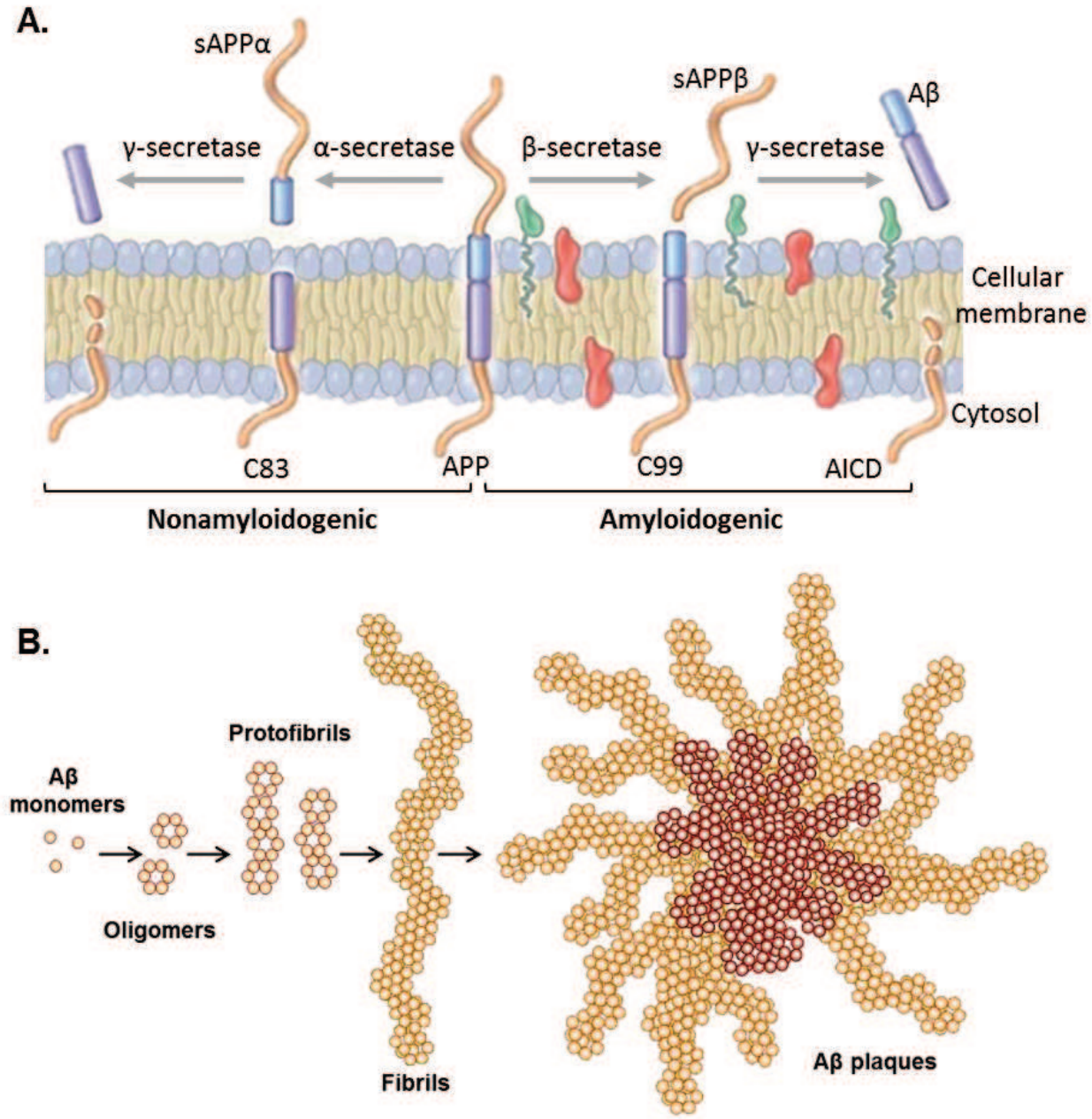

Fig. 13: APP processing and Aβ assembly stages. (A) APP is cleaved by α-, β- and γ-secretase to produce various APP products, including Aβ peptide (see details in the text). (B) Aβ peptide exists in multiple assembly states, including monomers, oligomers, protofibrils and fibrils which can accumulate in amyloid plaques in the brain of AD patients (adapted from ***(65)***).

Aβ is produced as a monomer and does not appear to be toxic under this form ***(65)***. Nevertheless, Aβ readily aggregates to form oligomers, protofibrils and fibrils, and the oligomeric species of Aβ have been found to be the earliest pathological and toxic form, inducing oxidative stress, disrupting Ca^{2+} homeostasis, impairing synaptic plasticity and leading to neuronal death **(Fig.14)** ***(65; 44)***. However, the "Amyloid cascade hypothesis" has more recently been challenged by the "Alzheimer mitochondrial cascade hypothesis", which place mitochondria in the center of the pathophysiological events taking place in AD.

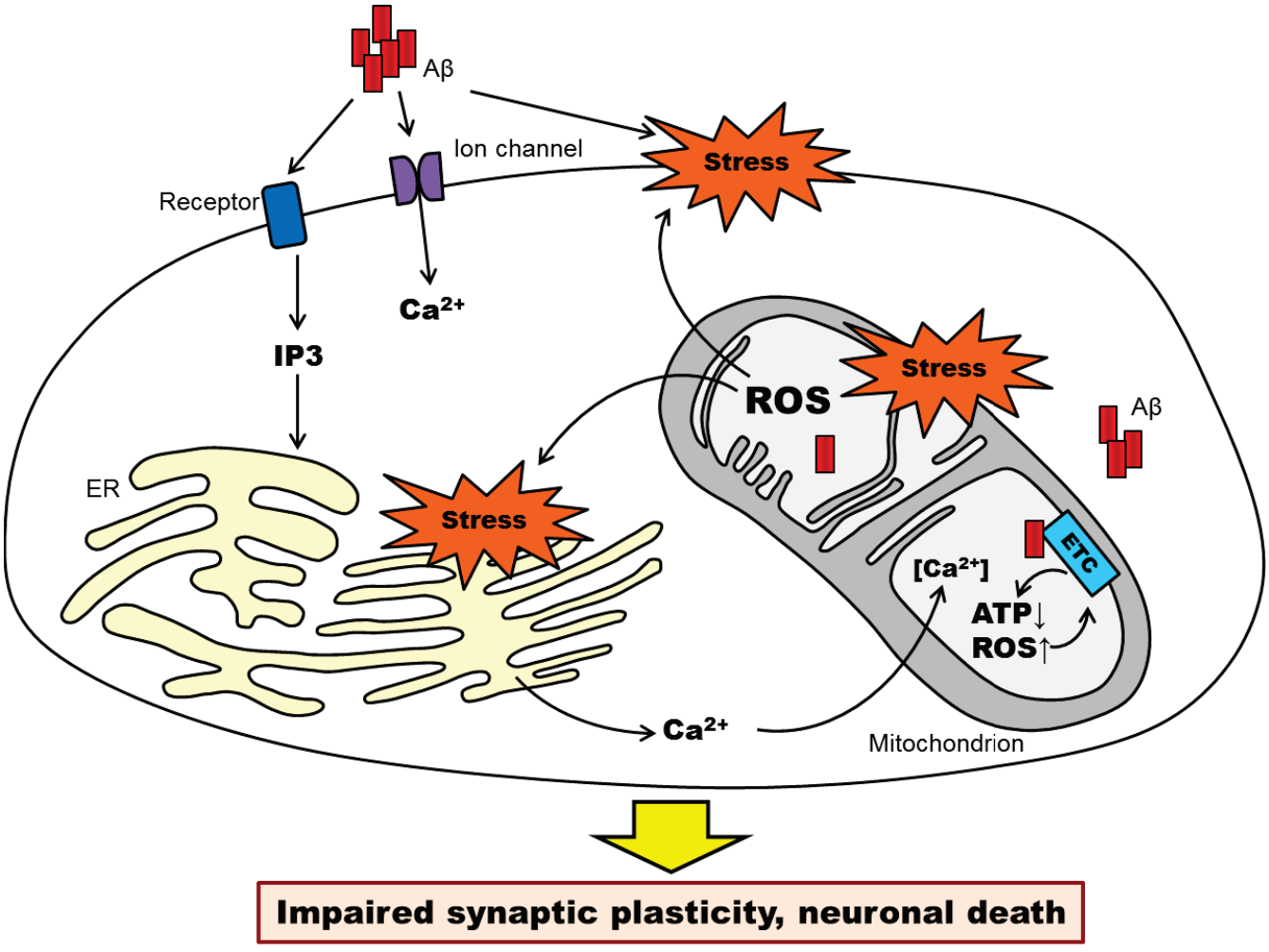

Fig.14: Toxic effects of Aβ in neurons. Aβ peptide-induced oxidative stress leads to lipid peroxidation (at the plasma and mitochondrial membranes) and to oxidation of proteins including membrane transporters, receptors (GTP-binding proteins…) or ion channels (voltage-dependent chloride channel, N-methyl-D-aspartate receptor). Aβ can also alter calcium homeostasis and impair endoplasmic reticulum (ER) and mitochondrial functions, leading to an increased ROS production, decreased ATP production and, finally, cell death (adapted from ***(44)***).

c) Mitochondria: a common target of Aβ and tau

There is increasing evidence supporting that mitochondrial dysfunction is a prominent and early event of AD, since energy deficiency is a fundamental characteristic of AD brains ***(68)*** as well as of peripheral cells derived from AD patients ***(69)***. Indeed, brain glucose hypometabolism has been observed in living AD patients even before the onset of clinical symptoms ***(70)***. This characteristic is also observed in AD mouse models in which mitochondrial dysfunctions can be detected before the appearance of Aβ deposits, NFT and cognitive impairments (reviewed in ***(71)***, see **APPENDIX 1**). In addition, a decreased activity of enzymes involved in glycolysis, Krebs cycle and ETC, such as α-ketoglutarate dehydrogenase(α-KGDH), pyruvate dehydrogenase (PDH), and cytochrome c oxidase (COX, complex IV) ***(72; 73; 69)***, have been reported in AD post-mortem brains as well as in platelets and fibroblasts coming from AD patients. In parallel, post-mortem tissues revealed an increased level of protein oxidation/nitration and lipid peroxidation in brain areas containing Aβ deposits and NFT ***(74)***. Taken together, these data support the hypothesis that mitochondrial dysfunction is a highly relevant event in AD and has been proposed as a possible link between Aβ and tau pathology.

(i) Aβ toxicity and mitochondria

Aβ peptide has been shown to interact with mitochondria in numerous ways **(Fig. 15)** ***(67)***. Evidence demonstrated that APP harbors a mitochondrial targeting signal and, specifically in human AD brain, accumulates in the protein import channels of mitochondria and may interact with translocases of the outer and inner mitochondrial membranes (TOM and TIM respectively) ***(75)***. Additional findings using cellular models overexpressing APP showed the presence of this protein in mitochondria, which led to the hypothesis that Aβ peptide may be produced within this organelle and contribute to mitochondrial dysfunction ***(76; 77)***. In agreement, studies performed in AD patients and animal models of AD showed an accumulation of Aβ in mitochondria, a phenomenon occurring early in the disease development, well before the formation of plaques ***(78-80)***. Within mitochondria, Aβ may directly interact with proteins, such as cyclophilin D (Cyp D) which is involved in the mitochondrial permeability transition pore (mPTP) and potentiate free radical production, or can directly bind the Aβ-binding alcohol dehydrogenase (ABAD), a protein up-regulated in the brain of AD patients and involved in mitochondrial estradiol metabolism **(see section I.B.4.b)**.

Metabolic effects of Aβ and its precursor APP in cellular models of AD are in agreement with observations made in postmortem brains of AD patients. Indeed, studies performed on mice, PC12 cells, or human SH-SY5Y neuroblastoma cells overexpressing APP indicated a decrease in ATP production, an impaired MMP, as well as a decrease in mitochondrial complex IV activity, paralleled by an increase in ROS levels ***(76; 81; 82; 19)***. Of note, in the presence of a γ-secretase inhibitor that prevent Aβ production, ATP and ROS levels were normalized, indicating that Aβ is directly involved in these mechanisms ***(76)***. In a recent study, quantitative iTRAQ proteomics was used to quantify the amount of protein deregulated after the treatment of native SH-SY5Y cells with $A\beta_{42}$ (0.5 μM, 5 days). Data showed that 69 proteins were deregulated by $A\beta_{42}$, and more than 25% of those proteins were involved in mitochondrial function and energy metabolism, again supporting the notion that mitochondrial dysfunction is a target of Aβ toxicity ***(83)***. This assumption was confirmed by *in vivo* data coming from studies performed on various transgenic mouse models. Indeed, mitochondrial dysfunction was detected early in the disease progression in simple transgenic mice bearing a mutation in APP or in double transgenic mice bearing mutations in APP and PS1 or APP and PS2. These mice presented a decrease in ATP level, decreased MMP, decrease complex IV activity, decreased mitochondrial respiration and increased oxidative stress in their brains ***(84-89)***. Of note, mitochondrial deficits were paralleled by soluble Aβ accumulation and were already detected before the presence of Aβ deposits and cognitive impairments (reviewed in ***(71)***).

Finally, in addition to its metabolic impact, Aβ seems also to impair mitochondrial dynamics. For instance, in APP overexpressing cells, mitochondria appeared to be highly fragmented and abnormally distributed within cells compared to control cells **(19)**. Quantification of proteins involved in fusion/fission activity revealed that DRP1 and OPA1 were reduced, whereas FIS1 was significantly increased in APP cells **(17-19)**. The disturbed fusion/fission activity observed in cells overexpressing APP leads mitochondria to a fragmented state that might, vice versa, have an effect on bioenergetic functions of these organelles.

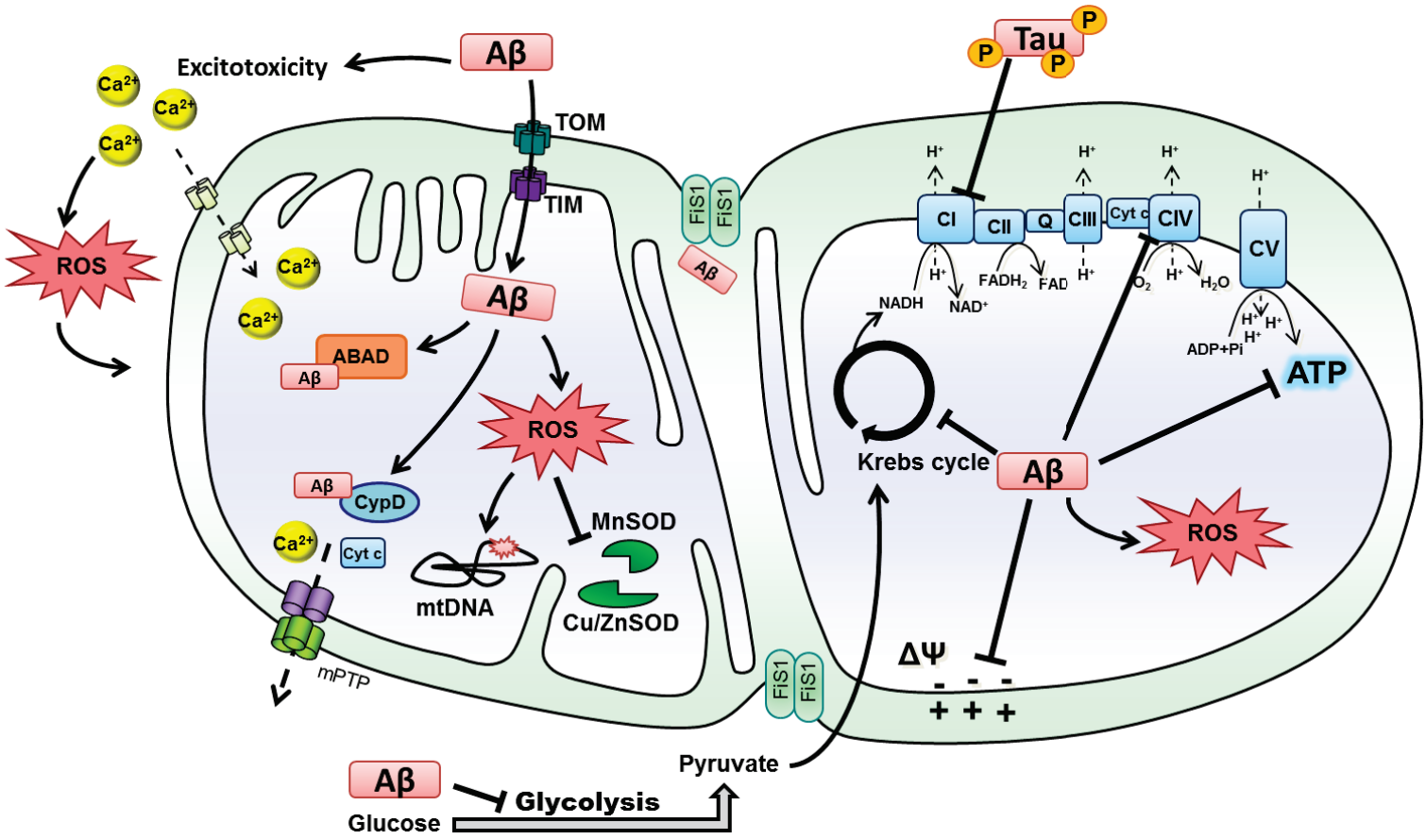

Fig.15: Pathogenic convergence of Aβ and hyperphosphorylated tau on mitochondria. Aβ and tau are able to impair mitochondrial respiration by inhibiting the ETC (more precisely, complex IV and complex I respectively) inducing a decrease in oxygen consumption, ATP production and an increase in ROS level. This oxidative stress induced by ETC dysfunction can surpass cellular and mitochondrial scavenger (MnSOD, Cu/ ZnSOD) and impacts on MMP as well as mitochondrial DNA (mtDNA). Within mitochondria, Aβ can bind ABAD and CypD, leading to an enhanced ROS production and the opening of the mitochondrial permeability transition pore (mPTP). In parallel, Aβ can also be responsible of metabolic impairments, by inhibiting enzymes involved in glycolysis and the Krebs cycle, as well as the calcium-induced excitotoxicity in neurons. Finally, Aβ and tau can disturb mitochondrial fusion/fission activity, leading to abnormal mitochondrial network. CI complex I, CII complex II, CIII complex III, CIV complex IV, CV complex V, cyt c cytochrome c, Cu/Zn SOD copper/zinc superoxide dismutase, MnSOD manganese superoxide dismutase, ROS reactive oxygen species, mtDNA mitochondrial DNA, ΔΨ mitochondrial membrane potential, TOM/TIM translocases of the outer/inner mitochondrial membranes, ABAD Aβ-binding alcohol dehydrogenase, FIS, fission protein 1, CypD cyclophilin D (adapted from ***(67)*** and ***(90)***).

(ii) Role of hyperphosphorylated tau in mitochondrial dysfunction

As mentioned above **(section I.A.2.b)**, when tau is abnormally hyperphosphorylated, the binding of tau on microtubules is reduced which leads to microtubule dissociation and impairs the transport of distinct cargoes along the axons, including mitochondria. Indeed, animal studies using transgenic mice overexpressing mutant tau protein revealed an impaired anterograde mitochondrial transport, reducing the number of mitochondria at the synapse (reviewed in ***(59)***, see **APPENDIX 2**). In consequence, energy deprivation was observed at the synapse, which led to synaptic degeneration and neuronal death ***(91)***. Proteomic studies analyzing the brain proteins of mutant tau transgenic mice (P301L tau mice) showed a deregulation of metabolism-related proteins, mainly components of mitochondrial respiratory chain complexes (including complex V), antioxidant enzymes and synaptic proteins ***(92)***. Of note, a decrease in complex V levels was also observed in the brain of FTDP-17 patients ***(92)***. Moreover, functional analysis performed on P301L tau mice showed that these animals presented an age-related mitochondrial dysfunction with a decrease of complex I activity, a decrease in mitochondrial respiration and ATP levels, and an increase of ROS production ***(92; 89)***. More recently, studies using human-derived neuroblastoma cells (SH-SY5Y) overexpressing either the wild type tau (wtTau) or P301L mutant tau showed that mutant cells had a decrease in ATP levels and an increased vulnerability to oxidative stress ***(16)***. In addition, compared to wtTau cells, P301L-transfected cells displayed smaller mitochondria with globular cristae and extensive branching of cristae membranes, and an impaired mitochondrial motility together with a down-regulation of both mitochondrial fusion and fission ***(16)***. Morphological signs were consistent with reductions in ATP turnover in mutant cells. Little is known about the role of tau in the impairment of mitochondrial dynamics. However, further evidences coming from animal studies, showed that the overexpression of human tau induced mitochondrial elongation in both Drosophila and mouse neurons ***(93)***. In this study, the authors shed new lights on tau's role in mitochondrial dynamics and hypothesized that disruption of mitochondrial dynamics could be a direct mechanism of tau toxicity in neurons *in vivo.* However, since AD is marked by both Aβ and tau pathologies, both molecules have to be taken into account to dissect the underlying mechanisms of mitochondria dysfunction.

(iii) Pathogenic convergence of Aβ and tau on mitochondria

Recent evidences suggest that Aβ peptide and abnormally hyperphosphorylated tau protein may act synergistically to trigger mitochondrial dysfunction in AD ***(73; 94; 71)***. Firstly,

in vivo studies revealed that injection of $A\beta_{42}$ fibrils in the brain of mutant tau transgenic mice (P301L mice) led to a 5-fold increase in NFT pathology ***(95)***. These results were confirmed by *in vitro* studies which showed that mitochondria coming from P301L mouse brains displayed an enhanced vulnerability in the presence of $A\beta_{42}$ compared to wild-type control brains ***(84; 85)***. Indeed, isolated mitochondria from P301L mice showed a decreased MMP and an impaired OXPHOS activity in the presence of oligomeric or fibrillar Aβ. Of note, the oligomeric Aβ was more toxic than the fibrillar Aβ, suggesting that they both exert different degrees of toxicity.

Secondly, crossing APP mice with mutant tau mice enabled to understand more precisely AD-related neurodegenerative mechanisms mediated by Aβ and tau. Indeed, APPxTau double transgenic mice exhibited NFT pathology in spinal cord and pons already at 3 months of age, compared to 6 months of age in simple tau transgenic mice ***(96; 97)***. Aβ plaques were also detected earlier (6 months of age compared to 12 months in APP transgenic mice), suggesting the existence of interplays between the two key proteins ***(86; 97)***. Together, these findings indicated that Aβ pathology may triggers, or at least aggravates tau pathology.

Other studies investigated more precisely mitochondrial deficits in mouse models with deficiencies in both Aβ and tau proteins. In a triple transgenic mouse model of AD (^{triple}AD, APPxPS2xP301L), proteomic mass spectrometry analysis revealed that more than a third of dysfunctional proteins were mitochondrial proteins with major deficiencies found in subunits of complex I and IV of the ETC ***(89)***. The investigation of OXPHOS function revealed that deregulation of complex I activity was related to tau, whereas deregulation of complex IV activity was dependent of Aβ. Interestingly, a decrease in MMP was already detected at 8 months of age in ^{triple}AD mice compared to 12 months of age in theirs double transgenic littermates (APPxPS2). Moreover ^{triple}AD mice exhibited stronger defects on OXPHOS, synthesis of ATP, and reactive oxygen species, emphasizing synergistic, age-associated effects of Aβ and tau upon mitochondria (***(89)*** and see also **APPENDIX 3**). Of note, as in ^{triple}AD mice, mitochondrial dysfunction preceded AD symptoms in another triple transgenic mouse model (3xTgAD), and impairments were further associated with higher ROS levels in aged transgenic mice ***(98)***. Together, these findings highlighted the key role of mitochondria in AD pathogenesis and consolidate the notion that a synergistic effect of tau and Aβ enhances the pathological mitochondria dysfunction at an early stage of AD.

(iv) Alzheimer mitochondrial cascade hypothesis

Placing mitochondria in the center of the degenerative processes is the basis of the “Alzheimer mitochondrial cascade hypothesis”. This hypothesis was first stated in 2004 by

Swerdlow and colleagues ***(99)***, and postulated that mitochondrial function may affect APP expression and processing as well as Aβ accumulation which triggers the amyloid cascade. In fact, the amyloid cascade hypothesis has been postulated with regard to FAD cases with mutations in APP or PS1/PS2 genes that represent less than 1% of total AD patients and cannot explain the etiology of sporadic AD (SAD) cases. As mentioned in **section I.A.2.a**, aging is the first "risk factor" of SAD. The role of mitochondria in aging has been widely investigated for decades ***(100; 101; 36)*** and a growing body of evidence championed the idea that somatic mtDNA mutations, accumulating over a person's lifespan, may influence aging and may cause neurodegeneration, such as in AD (reviewed in ***(102)***). In this way, the Alzheimer mitochondrial cascade hypothesis maintains that the decline of mitochondrial function observed during aging, namely the decreased in energy production and the increase in ROS production and oxidative stress, eventually surpasses a threshold and triggers the amyloidogenic pathway leading to Aβ accumulation **(Fig. 16)**.

Aβ can perturb mitochondrial function by influencing OXPHOS activity, impairing mitochondrial fusion/fission activity and disturbing calcium homeostasis. The improper mitochondrial function generates a decrease in MMP and ATP production - paralleled by an increase in ROS production which, again, influences APP processing - induces mtDNA mutation, augments tau hyperphosphorylation and NFT formation which in turn disturbs mitochondrial trafficking and function. Thus, several vicious cycles are triggered, each accelerating the other, which finally leads to synaptic dysfunction and neuronal death by apoptosis **(Fig.16).**

In this context, with regard to their critical roles in the early pathogenesis of AD, mitochondria represent attractive targets for treatment strategies.

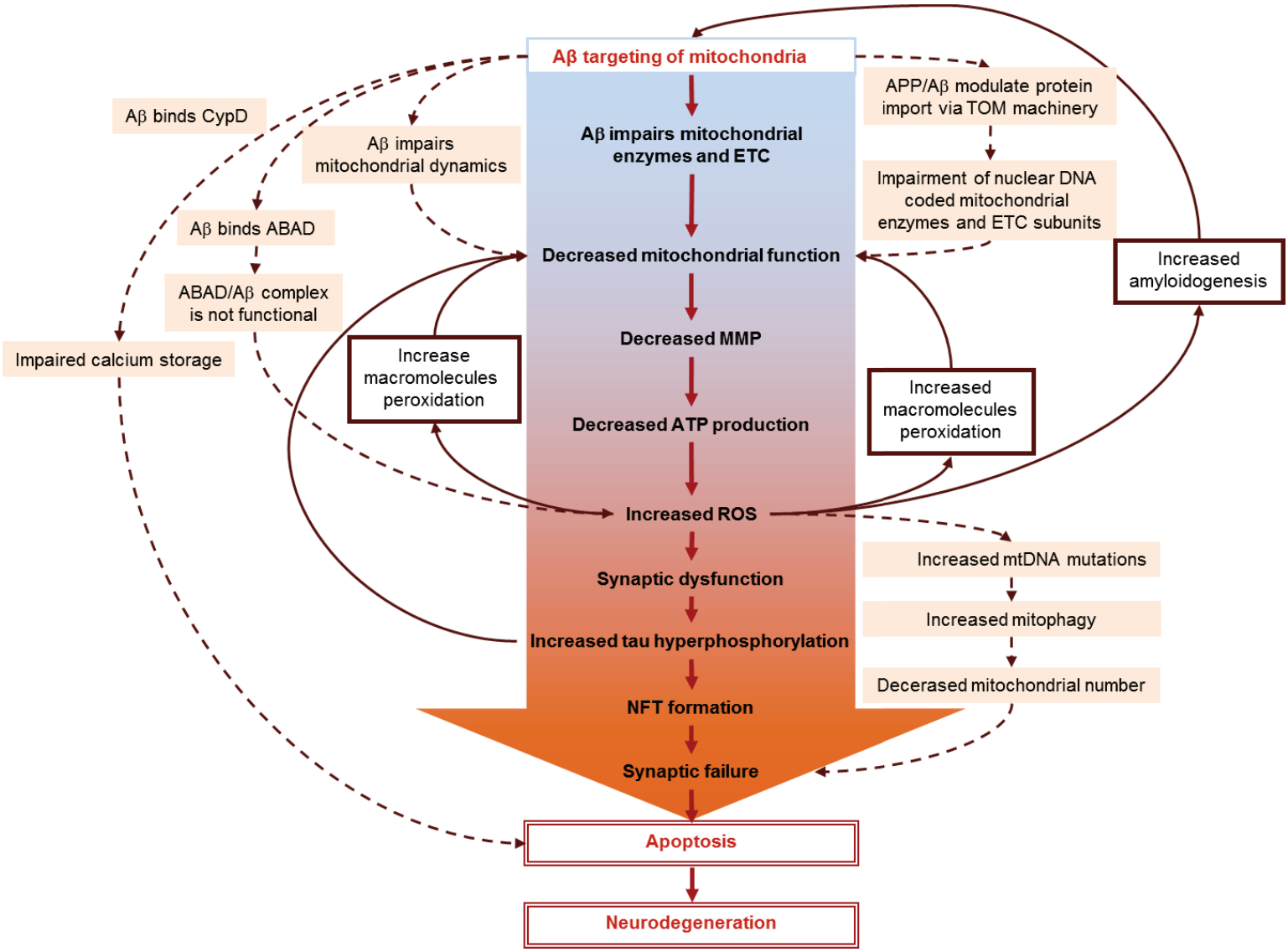

Fig.16: A hypothetical sequence of the pathogenic steps of the Alzheimer mitochondrial cascade hypothesis. The main cytotoxic pathway of Aβ (red arrows) involves Aβ-induced mitochondrial function, increased ROS production, activation of neurofibrillary tangles (NFT) formation, synaptic failure, and neurodegeneration. Several other pathways feed this cascade via feeding back (dark red arrows) or forward (dashed arrows) revealing several vicious cycles within a larger vicious cycle. All of them, once set in motion, amplify their own processes, thus accelerating the development of AD. ROS reactive oxygen species, mtDNA mitochondrial DNA, MMP mitochondrial membrane potential, TOM translocases of the outer mitochondrial membranes, ABAD Aβ-binding alcohol dehydrogenase, CypD cyclophilin D, ETC electron transport chain (adapted from ***(67)***).

(v) Mitochondria as therapeutic target in neurodegeneration

The role of mitochondrial dysfunction in neurodegeneration is not restricted to AD but is becoming increasingly apparent in a broad range of degenerative diseases, including Parkinson's and Huntington's disease and amyotrophic lateral sclerosis ***(103)***. Accordingly, many studies are focused on the search of drug candidates that preferentially target mitochondria and current pharmacological concepts aim to: increase mitochondrial respiration and ATP production, reduce mitochondrial ROS production, stabilize the mPTP, and induce mitochondrial biogenesis. For instance, a study using APP/Aβ overexpressing cells showed that a treatment with Ginkgo Biloba extract attenuated Aβ-induced mitochondrial dysfunction by decreasing Aβ secretion and ROS levels, improving mitochondrial respiration, as well as increasing ATP synthesis and mitochondrial biogenesis ***(82)***.

Since oxidative stress is a redundant mechanism in neurodegeneration, various antioxidants, including creatine, vitamin E and C, β-carotenes or co-enzyme Q, have been tested *in vitro*, *in vivo* and even in human with anecdotal but no generally recognized degree of success ***(104; 103; 105; 106)***. More recently, new mitochondria-targeted antioxidants and peptides that selectively block mitochondrial oxidative damage have been developed ***(107; 108)***. These compounds use the MMP to accumulate within mitochondria, reducing side effects by avoiding non-specific interaction with other targets. One of these compounds, MitoQ, has been shown to decrease oxidative stress, Aβ accumulation, astrogliosis, synaptic loss, and apoptosis in the brain of 3xTgAD mice, which prevented cognitive decline ***(107)***.

Other studies aimed to increase energy metabolism by boosting mitochondria or inducing mitochondrial biogenesis. Notably, resveratrol and thiazolidinedione represents drug candidates that are able to induce mitochondrial biogenesis and it was recently proposed that AD patients might benefit thiazolidinedione mitochondrial effects ***(106)***.

Further investigations and larger clinical trials are needed to judge the therapeutic efficacy of these compounds. Here, we propose neurosteroids as new candidates to boost mitochondrial function in health and disease states **(see sections II.A and B)**.

B. Neurosteroids

1. Definition

The term “neurosteroid” appears for the first time in the 80s and defines those steroids that are synthesized *de novo* in the nervous system. They accumulate within the nervous system, independent from peripheral steroidogenic glands ***(109; 110)***. Indeed, in 1981, the pioneering studies of Baulieu and coworkers have demonstrated the production of steroids within the brain itself ***(111)***. They showed that the level of some steroids, such as dehydroepiandrosterone (DHEA), was even four times higher in the anterior brain of rats than in plasma and nearly 18 times higher than in the posterior brain with regard to its sulfated form (DHEAS). Later on, a number of studies showed that other steroids were synthesized in the brain, and enzymatic activities of proteins involved in steroidogenesis were shown in neurons as well as in glial cells ***(112; 109; 113; 110; 114)***. Unlike steroidal sex hormones that are released in the blood and act at a distance from their glands of origin in an endocrine way, neurosteroids were identified to be synthesized within the nervous system and act on the nervous system in an auto/paracrine configuration.

The family of neurosteroids includes different categories of steroids: i) non-exclusive neurosteroids such as estradiol, testosterone, pregnenolone (PREG), progesterone (PROG) or dehydroepiandrosterone (DHEA) that are steroidal hormones synthesized in neurons, glial cells or per endocrine glands, ii) semi-exclusive neurosteroids such as allopregnanolone that is mainly synthesized in the nervous system but also produced in substantial amounts within endocrine glands; iii) exclusive neurosteroids such as epiallopregnanolone that are steroids only produced in nerve cells ***(115; 116)***. All neurosteroids derive from cholesterol and other blood borne steroidal precursors, and have closely related structures based on the classic cyclopentanophenanthrene 4-ring structure **(Fig.17)**. The first step of steroidogenesis takes place within mitochondria.

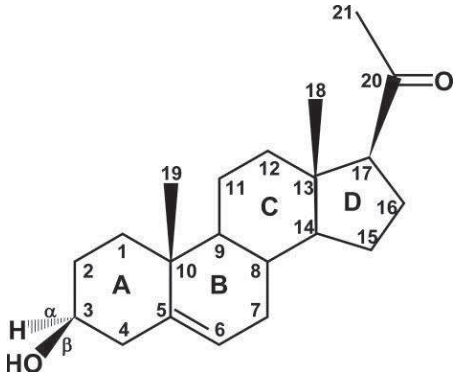

Fig. 17: Structure of pregnenolone, illustrating the cycloperhydropentano-phenanthrene structure common to all steroids. The carbon atoms are indicated by *numbers*, and the rings are designated by *letters* according to standard conventions. Substituents and hydrogens are labeled as α or β if they are positioned behind or in front of the plane of the page, respectively (adapted from ***(115)***).

2. Neurosteroidogenesis

The transfer of cholesterol from the cytosol to the mitochondrial matrix constitutes the rate-limiting step of steroidogenesis. Cholesterol is essentially insoluble (critical micellar concentration, ~25–40 nM) ***(117)*** and its transfer requires the involvement of a multiprotein complex that is composed of protein located in the cytosol and at the outer and inner mitochondrial membrane ***(118)***. Once in the mitochondrial matrix, cholesterol is converted into pregnenolone that is further metabolized into subsequent neurosteroids either in mitochondria or in the endoplasmic reticulum (ER) major pathway.

a) Cholesterol transfer to mitochondria

Intracellular cholesterol required for the subsequent steroid synthesis is known to come from three different sources ***(119)***: i) cholesterol is synthesized *de novo* in the endoplasmic reticulum (ER) from acetate. Then, it is trafficked to the Golgi apparatus where it can be targeted to the mitochondria by binding the protein acyl-CoA binding domain-containing 3 (ACBD3). Passive diffusion from the ER to the mitochondria is also possible; ii) cholesterol comes from the plasma membrane, more specifically, in human, from plasma low-density lipoproteins (LDLs) derived from dietary cholesterol and is trafficked through the endosomal pathway ***(120)***; iii) Cholesterol can be mobilized from the lipid droplets where, irrespective of source, it can be esterified by acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT) and stored as cholesterol esters.

Once at the mitochondrial membrane, the regulation of the cholesterol flow into mitochondria is accomplished by a multiprotein complex, also called “transduceosome” ***(118)***. This multicomplex is composed of: ACBD3, the protein kinase A regulatory subunit I alpha (PKA-RIα), the steroidogenesis acute regulatory protein (STAR), the translocator protein (18 kDa, TSPO) and the voltage-dependent anion channel (VDAC) **(Fig. 18)**. The main actor of this transduceosome is the translocator protein (TSPO), previously known as the peripheral-type benzodiazepine receptor (PBR), which is a ubiquitous mitochondrial protein enriched at the outer/inner mitochondrial membrane contact sites ***(121)***. Briefly, free cholesterol accumulates outside of mitochondria and binds to StAR protein, a hormone-induced mitochondrial-targeted protein that initiates cholesterol transfer into mitochondria. Then, cholesterol is transported inside mitochondria by TSPO that forms a channel-like structure from its five transmembrane helices and is associated with VDAC and the adenine nucleotide transporter (ANT). TSPO is fundamental in neurosteroid production since the translocation of cholesterol from the outer membrane to the inner membrane of mitochondria

is the rate-limiting step of steroid synthesis ***(119)***. In fact, the ability of cholesterol to enter into mitochondria to be available to cytochrome P450 cholesterol side chain cleavage enzyme (P450scc), located in the inner side of the mitochondrial membrane and responsible for the conversion of cholesterol to pregnenolone (PREG), will determine the efficiency of steroidogenesis. Of note, a recent study showed that mitochondrial fusion was coupled with an increased steroidogenesis, suggesting that mitochondrial dynamics could play an essential role in steroid synthesis ***(122)***.

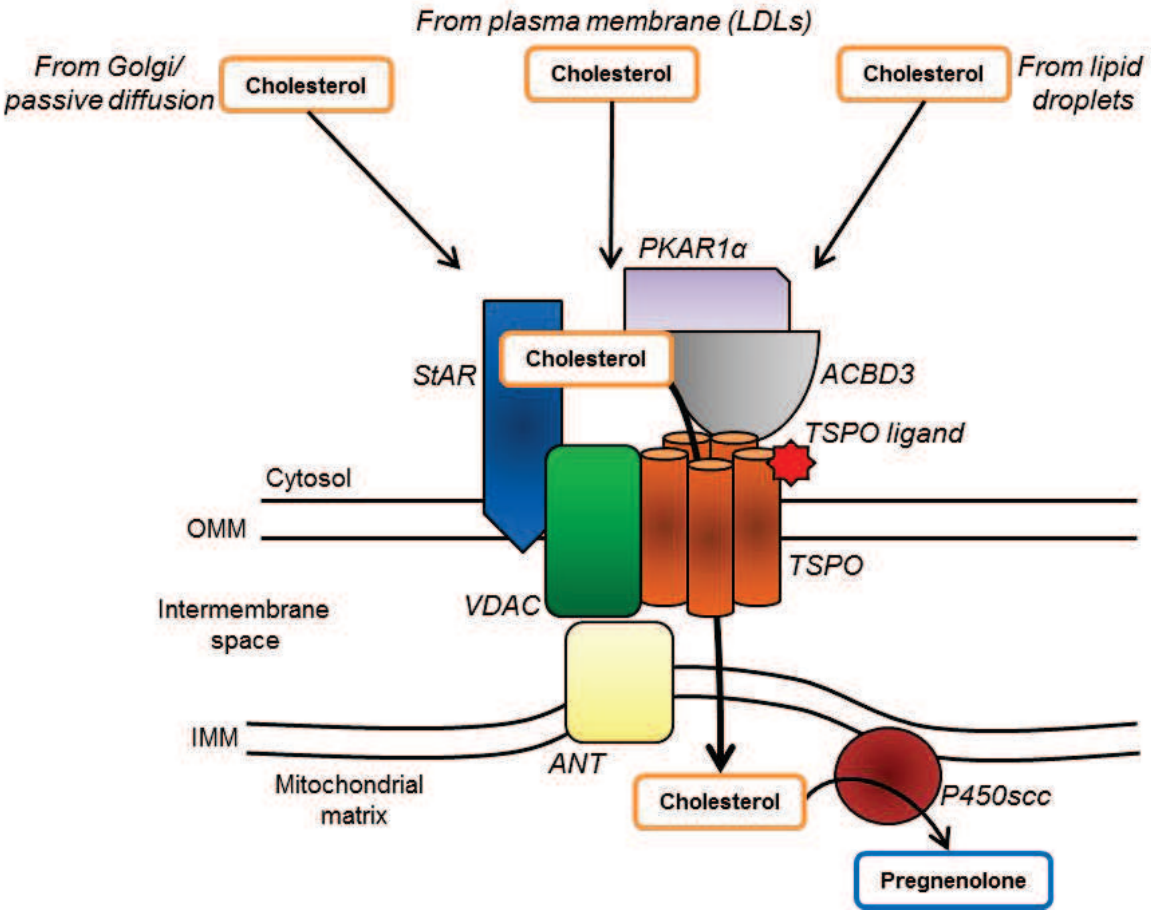

Fig. 18: Transfer of cholesterol in mitochondria. Cholesterol, coming from different sources, accumulates outside of mitochondria and binds to the steroidogenic acute regulatory protein (StAR). Upon ligand activation, cholesterol is transported inside mitochondria by the transduceosome composed of translocator protein (TSPO), located in the outer mitochondrial membrane (OMM), voltage-dependent anion channel (VDAC), the protein acyl-CoA binding domain-containing 3 (ACBD3), protein kinase A regulatory subunit I alpha (PKA-RIa), StAR and the adenine nucleotide transporter (ANT). After transfer in the mitochondrial matrix, cholesterol is converted in pregnenolone by the cytochrome P450 side chain cleavage (P450scc) located in the inner mitochondrial membrane (IMM). LDLs; low density lipoproteins (adapted from ***(119)***).

b) Enzymatic pathways of steroidogenesis

The enzymes involved in neurosteroidogenesis belong mainly to two families: i) cytochrome P450 enzymes and ii) hydroxysteroid dehydrogenase (reviewed in ***(123; 115)***). The first family represents a group of oxidative enzymes containing about 500 amino acids and a single heme group, and can be found either in mitochondria (P450 type 1) or in the endoplasmic reticulum (P450 type 2). The name cytochrome P450 (pigment 450) comes from the fact that these enzymes absorb the light at 450 nm in their reduced states. The enzymes of the second family, the hydroxysteroid dehydrogenase (HSD), have molecular masses of about 35 to 45 kDa. They do not possess heme groups, and nicotinamide adenine dinucleotides (phosphates) ($NADH/NAD^+$ or $NADPH/NADP^+$) are the cofactors necessary to either reduce or oxidize a steroid by two electrons via a hydride transfer mechanism. Each enzyme of both families is able to catalyze several reactions involved in neurosteroidogenesis and can form different neurosteroids **(Fig. 19)**.

Thus, once within the mitochondrial matrix, cholesterol is directly converted to PREG by P450scc. This process involves three distinct chemical reactions: the 22-hydroxylation of cholesterol, 20-hydroxylation of 22(R)-hydroxycholesterol, and oxidative scission of the C20–22 bond of 20(R),22(R)-dihydroxycholesterol (the side-chain cleavage event) ***(115)***. These three reactions occur on a single active site that is in contact with the IMM and lead to the formation of PREG, the precursor of all steroids. After its synthesis within mitochondria, PREG can follow several pathways to form different neurosteroids ***(112)*** **(Fig 19)**.

PREG can either be catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) to form progesterone (PROG) or by the cytochrome P450c17 enzyme (P450c17), also called 17α- hydroxylase/17, 20-lyase, to form dehydroepiandrosterone (DHEA). P450c17 catalyzes the 17α-hydroxylation of PREG in a two step reaction giving, first, 17-hydroxyPREG (17OH-PREG) and, then the final product, DHEA. Each step requires the molecules NADPH and O_2. The same enzyme metabolizes PROG that is converted into androstenedione with the 17-hydroxyPROG as an intermediate product of the reaction. The enzymes 3β-HSD is also involved in other reactions and uses NAD^+ as cofactor to oxidize hydroxysteroids, such as 17OH-PREG and DHEA, into their respective ketosteroids, 17OH-PROG and androstenedione. Then, androstenedione is either converted into testosterone by another hydroxysteroid dehydrogenase called 17β-HSD or in estrone by P450aro, also called aromatase. Of note, 17β-HSD possesses several isoforms, and one of them (17β-HSD- 10) is located in the mitochondrial matrix and is also known under the name of Aβ binding

alcohol dehydrogenase (ABAD). ABAD was recently linked to AD because of its ability to bind Aβ peptide, thus inducing mitochondrial dysfunction ***(124; 79)*** **(see section I.B.4.b)**. 17β-HSD and P450aro are involved in the synthesis of estradiol from estrone and testosterone respectively. At this level, the 5α-reductase enzyme (5α-R), a microsomal NADPH-dependent protein, intervenes to catalyze the transfer of two atoms of hydrogen from NADH to form the 5α-reduced metabolite of testosterone, dihydrotestosterone (DHT). Finally, the enzyme 3α-hydroxysteroid oxido-reductase (3α-HSOR), also called 3α-hydroxysteroid dehydrogenase, catalyzed the reversible conversion of DHT in the neuroactive steroid 3α-androstanediol. The latter enzymes intervene also at another level, in the second main steroidogenic pathway that derives from PROG. In fact, PROG is successively metabolized by the 5α-R and the 3α-HSOR to form dihydroprogesterone (DHP) and 3α/5α-tetrahydroprogesterone (3α/5α-THP), also known under the name allopregnanolone, another neuroactive steroid. PREG and DHEA can also be sulfated and de-sulfated by the hydroxysteroid sulfotransferase (HST) and sulfatase respectively, to form PREG sulfate and DHEA sulfate.

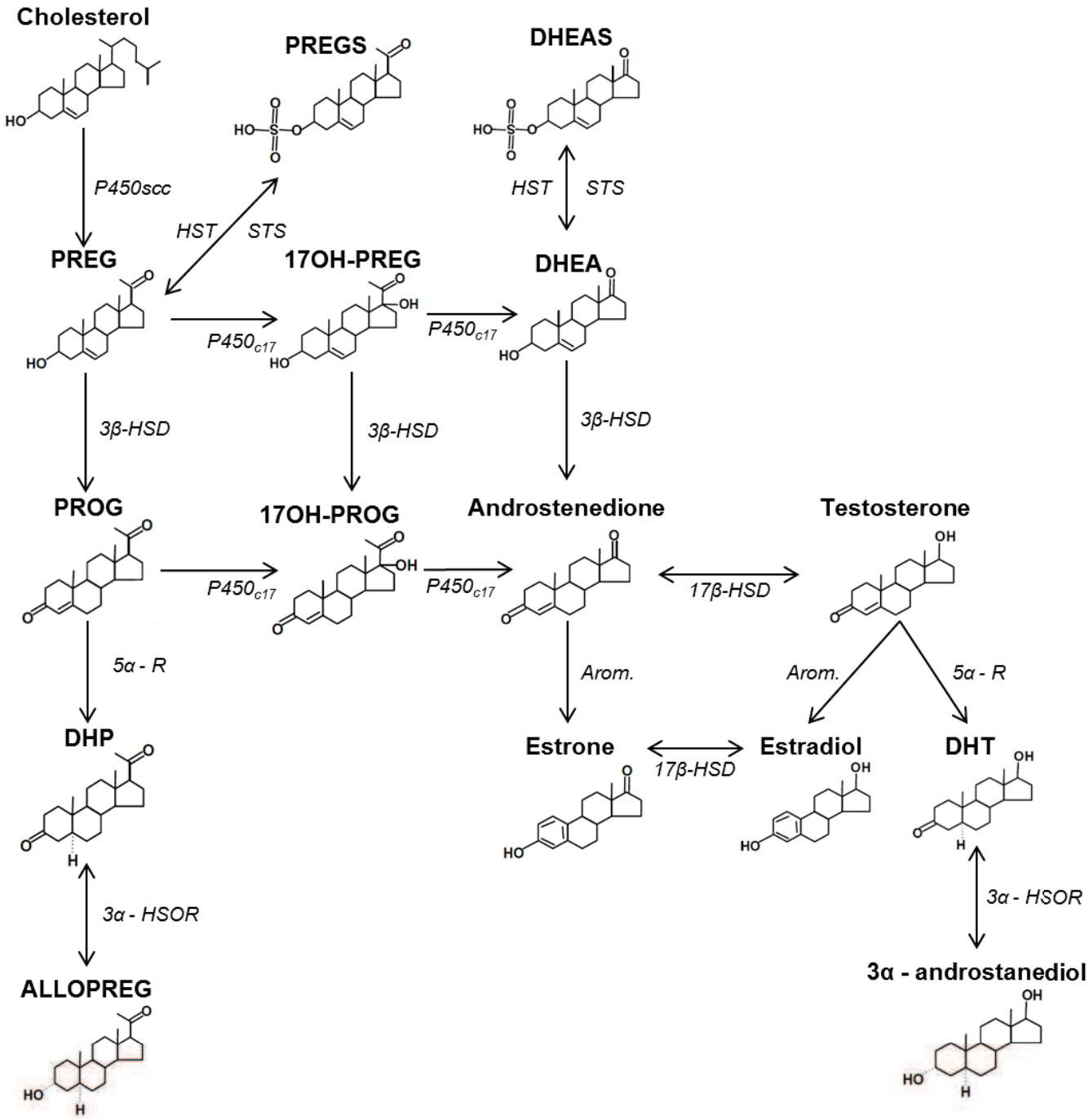

Fig.19: Schematic representation of main biochemical pathways for neurosteroids biosynthesis in the vertebrate brain (see details in the text). 17OH-PREG; 17-hydroxypregnenolone, 17OH-PROG; 17-hydroxyprogesterone, DHEA; dehydroepiandrosterone, DHP; dihydroprogesterone, ALLOPREG; allopregnenolone, DHT; dihydrotestosterone, P450scc; cytochrome P450 cholesterol side chain cleavage, $P450_{c17}$;cytochrome P450c17, 3β-HSD; 3β-hydroxysteroid dehydrogenase, 5α-R; 5α-reductase, Arom.; aromatase, 21-OHase; 21-hydroxylase, 3α-HSOR; 3α-hydroxysteroid oxydoreductase,17β-HSD; 17β-hydroxysteroid dehydrogenase, HST; hydroxysteroid sulfotransferase, STS; steroid sulfatase.

The expression and activity of enzymes involved in neurosteroid biosynthesis were demonstrated in different regions of the brain, and also in the peripheral nervous system ***(112; 109; 113)***. Studies mainly performed in amphibian and bird models showed that the activity of those enzymes could be regulated by neurotransmitters and neuropeptides **(Table 1**), but little is known regarding the neuronal mechanisms regulating neurosteroid synthesis in the mammalian brain (reviewed in ***(112)***). The fact that the ability to produce neurosteroids was conserved during vertebrate evolution suggests that this category of molecules plays an important role in living things.

Table 1: Effects of neurotransmitters and neuropeptides on steroidogenic enzyme activity in the brain.

	P450scc	3β-HSD	$P450_{c17}$	3α-HSOR	$P450_{7\alpha}$	Aromatase	HST
GABA	↓	↓	↓	nd	nd	→	nd
Dopamine	nd	↓	nd	↓	nd	↓	nd
Glutamate	nd	nd	nd	nd	nd	↓	nd
Melatonin	nd	nd	nd	nd	↓	nd	nd
Endozepines	↑	↑	↑	nd	nd	nd	nd
Vasotocin/mesotocin	nd	↑	↑	nd	nd	nd	nd
Neuropeptide Y (NPY)	nd	nd	nd	nd	nd	nd	↓

↑; stimulatory, ↓; inhibitory, →; no effect, nd; not determined (adapted from ***(112)***).

3. Mechanism of action of neurosteroids and physiological roles

The lipophilic nature of neurosteroids allows them to act both via membrane receptor or to cross cellular membranes and to act through a conventional genomic pathway via nuclear receptors **(Fig.20)**. The genomic action of steroids seems to be important during neonatal life where it has been shown that neurosteroids, as PROG or estradiol, are able to promote dendritic growth, spinogenesis, synaptogenesis and cell survival, particularly in the cerebellum ***(114)***. Among classical nuclear steroid receptor, we found estrogen receptors (ER), androgen receptors (AR) and progestin receptors (PR). The most studied steroid nuclear receptors are the estrogen receptors α and β (ERα/β) that are expressed in metabolic tissue such as adipose tissue, skeletal muscle, liver and pancreas, as well as in the central nervous system. Studies demonstrated that these receptors play a role in the regulation of glucose homeostasis and lipid metabolism ***(125)*** while other studies showed that they were also implicated in neuroprotection **(see section I.B.5)**. More recently, the pregnane X receptor was identified as a new nuclear receptor activated by progesterone and

allopregnanolone, and its activation seemed to be involved in cholesterol homeostasis and neurosteroid synthesis ***(126-128)***.

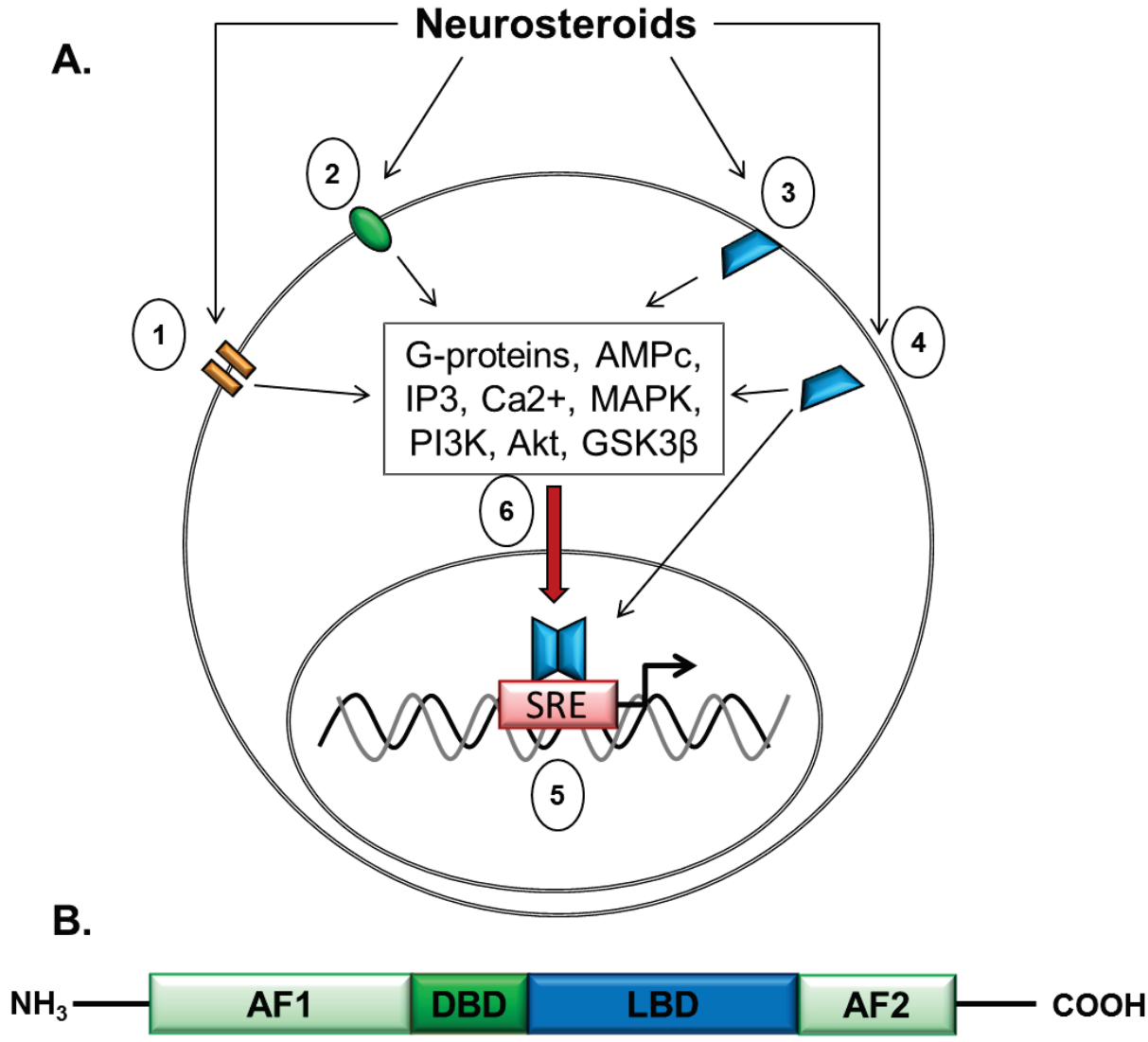

Fig. 20: Different potential mechanisms of action of neuroactive steroids, including hormonal steroids, in the nervous system. (A) Neuroactive steroids may bind to ion channels associated to neurotransmitter receptors (1), to putative steroid receptors in the plasma membrane (2), to classical nuclear steroid receptors associated with the plasma membrane (3) or to classical nuclear steroid receptors located in the cytoplasm (4). Intracellular signaling pathways activated by neuroactive steroids are depicted. The activation of classical nuclear receptors (4) results in their dimerization and binding to steroid responsive elements (SRE) in the promoters of specific genes and the consequent regulation of transcription (5). In addition, membrane and cytoplasmic signaling modulated by neuroactive steroids can also impact transcriptional activity (6) (adapted from ***(132)***). (B) Schematic representation of structural domains of steroid nuclear receptors. AF1/2; activation function 1/2, DBD; DNA binding domain, LBD; ligand binding domain (adapted from ***(133)***).

Neurosteroids can also act via membrane receptors as allosteric modulators of neurotransmitter receptors. They have been found to act as allosteric modulators of the $GABA_A$/central-type benzodiazepine receptor complex, NMDA receptors, kainate receptors, AMPA receptors, sigma receptors and glycine receptors ***(129-131; 110)***. More precisely, DHEA and its sulfate ester DHEAS are known to be excitatory neurosteroids and can act as antagonists at $GABA_A$ receptors or as agonists at sigma receptors ***(115)***. Pregnenolone sulfate is also known as a negative regulator of $GABA_A$, kainite, and AMPA receptors, and as positive regulator of NMDA receptors, thus acting as an excitatory neurosteroid ***(115; 110)***. In contrary, allopregnanolone is a positive $GABA_A$-R allosteric modulator that strengthens the effects of GABA. Allopregnanolone acts on $GABA_A$-R at nanomolar concentrations at sites distinct from those bound by GABA, benzodiazepines, and barbiturates, functioning as an allosteric modulator to open the channel and increase chloride flux **(Fig 21)**.

Furthermore, it is known that neurosteroids modulate neurotransmitter binding sites or receptors including calcium channels and P2X receptors in the brain, spinal cord, as well as the dorsal root ganglia (DRG) ***(134)***. Of note, recent clinical and pharmaceutical studies showed that estrogens can interact with several neurotransmitter systems, as cholinergic and serotoninergic system, to influence cognitive performance in animals and humans ***(135)***.

Thus, neurosteroids seem to play an important role in the nervous system during development as well as in adult brain, by regulating gene transcription and different neurotransmitter systems. Their implication was already demonstrated in several pathologies, especially in AD.

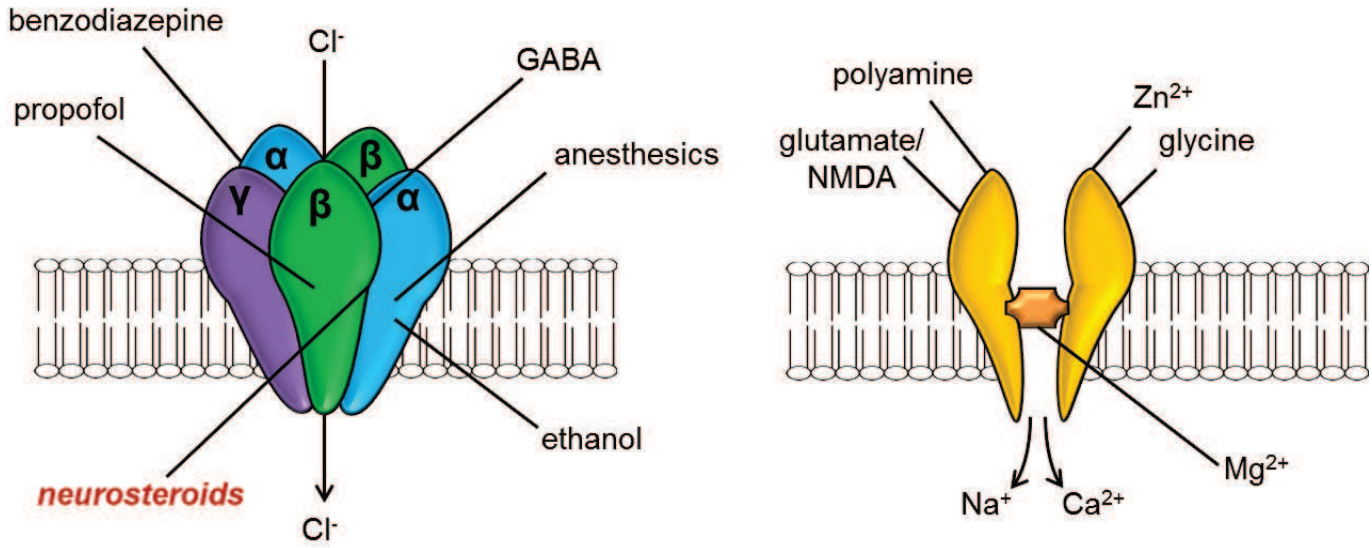

Fig. 21: Structure of the $GABA_A$ (A) and the NMDA (B) receptors. Both can be modulated by neurosteroids (adapted from ***(136)***).

4. Neurosteroids, Aging and Alzheimer's disease

a) Age-related changes in brain neurosteroid levels

Because steroid hormones are lipophilic molecules, those that are synthesized by the peripheral steroidogenic glands can easily cross the blood-brain barrier and act on the nervous system in an endocrine way. The blood levels of these neuroactive steroids are known to decrease with age ***(137)***. In women, estrogen levels drop after the menopause, whereas men present a gradual reduction in testosterone over the life course eliminating approximately 2% of circulating testosterone every year ***(138; 53)***. Of note, the age-related decrease of steroid hormones has been presented as a risk factor to develop neurodegenerative diseases, including AD (***(90)***, see **APPENDIX 4**). However, since neurosteroids are locally synthesized within the nervous system, steroid blood levels do not necessarily correspond to steroid brain concentrations ***(137)***. Thus, the age-related changes in neurosteroid levels are challenging to investigate in humans due to the presence of blood-derived steroid hormones.

Studies performed in rats showed that the synthesis of PREG sulfate was decreased in the hippocampus of aged rats (2 years old) compared to young rats (3 months old) ***(139)***. The hippocampus is a brain region involved in learning and memory, and the decrease of PREG sulfate in this region correlated with cognitive impairments in old rats. Of note, these cognitive deficits were transiently reduced after intrahippocampal injection of PREG sulfate, suggesting that this neurosteroid plays a role in the maintenance of cognitive function during aging. More recently, Caruso and colleagues (2013) compared neurosteroid levels in the limbic region of young (7 months old) and old mice (24 months old) ***(140)***. They showed an alteration in neurosteroid levels with a general trend toward lower steroid levels in the brain of aged mice compared to young mice.

Together, these data indicate that neurosteroid levels decline gradually with advancing age which may induce a range of age-related neuronal dysfunction, cognitive impairments and neurodegeneration due to the loss of neurosteroid protective effects **(see section I.B.5)**.

b) Disturbed neurosteroidogenesis in Alzheimer's disease

Studies performed in AD patients as well as in animals and cellular models of AD showed some alterations in the synthesis of neurosteroids that declined during brain aging paralleled by a loss of important nervous functions, such as memory ***(141; 140; 142)***. TSPO,

which regulates the first step of steroidogenesis, was over-expressed in post-mortem brains from AD patients, resulting in an increased level of PREG in the hippocampal region of those brains ***(119)***. Interestingly, the level of 22(R)-hydroxycholesterol, a steroid intermediate in the conversion of cholesterol to PREG, was found at lower levels in AD brain compared to control, which suggest that TSPO does not function normally in Alzheimer patients ***(141; 121)***. Another study showed that DHEA was significantly elevated in AD brain and cerebro-spinal fluid when compared to control subjects ***(141)***. Similar results were obtained *in vitro* in oligodendrocytes, where DHEA production was up-regulated under oxidative stress condition induced by treatment with Aβ peptide ***(143)***. In a study using 3xTg-AD mice, modified levels of specific neurosteroids, in particular in the levels of progesterone and testosterone metabolites, were measured in aged mice (24 months) compared to young (7 months), and were associated with age-related neuropathological changes in the brain, such as Aβ accumulation and gliosis ***(140)***. In accordance, several neurosteroids were quantified post-mortem in various brain regions of aged AD patients and aged non-demented controls. Results showed a general trend towards lower steroid levels in AD patients compared to controls, associated with a negative correlation between neurosteroid levels and Aβ as well as hyperphosphorylated tau protein in some brain regions ***(137)***. Finally, several reports propose the role of allopregnanolone as a plasmatic biomarker for AD, since it was shown that the level of this neurosteroid is decreased by 25 % in the plasma of demented patients compared with control subjects ***(144; 142)***.

In accordance, recent findings corroborated that AD key proteins - Aβ and hyperphosphorylated tau - distinctly impacted neurosteroidogenesis in a cellular AD model **(Fig. 22)** (***(145; 146)*** and see also **APPENDIX 5**).

Overexpression of wtTau protein induced an increase in the production of PROG, 3α-androstanediol and 17OH-PROG, in contrast to overexpression of the abnormally hyperphosphorylated tau bearing the P301L mutation which led to a decrease in the production of these neurosteroids. In parallel, a decrease of PROG and 17OH-PROG production was observed in APP overexpressing cells, whereas 3α-androstanediol and estradiol levels were increased. These results provided the first evidence that AD key proteins are able to modulate, directly or indirectly, the biological activity of the enzymatic machinery producing neurosteroids. Other *in vitro* experiments using native SH-SY5Y cells treated with aggregated $A\beta_{42}$ fibrils for 24 h were in line with these findings ***(145)***. A treatment with “non-toxic” Aβ concentrations (within the nanomolar range, non-cell death inducing $A\beta_{42}$ concentrations) revealed an increase in estradiol production, whereas toxic Aβ concentrations (within the micromolar range, leading to cell death) showed the opposite effect ***(145)***.

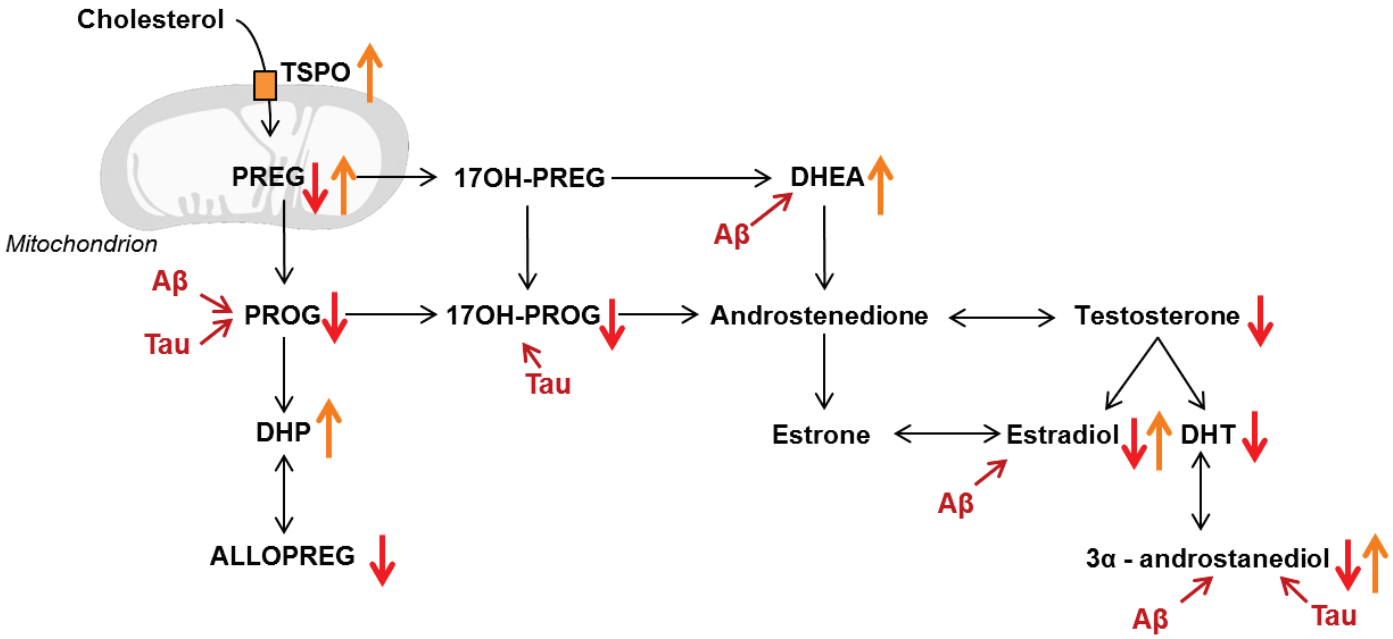

Fig. 22: Disturbed neurosteroidogenesis in AD. PREG; pregnenolone, 17OH-PREG; 17-hydroxypregnenolone; PROG; progesterone, 17OH-PROG; 17-hydroxyprogesterone, DHEA; dehydroepiandrosterone, DHP; dihydroprogesterone, ALLOPREG; allopregnanolone, DHT; dihydrotestosterone, Tau; abnormally hyperphosphorylated tau protein, Aβ; amyloid-β protein.

As mentioned in **section I.A.2.c**, recent reports indicate that Aβ may also interact directly with intracellular proteins such as the mitochondrial enzyme ABAD (Aβ binding alcohol dehydrogenase) in executing its toxic effects ***(79)***. ABAD belongs to the family of 17β-hydroxysteroid dehydrogenase and is able to metabolize estradiol within the mitochondrial compartment. ABAD is up-regulated in AD brain areas affected by Aβ pathology such as the cortex and hippocampus, as well as in AD transgenic mouse models ***(79; 147)***. Studies performed in double transgenic mice overexpressing mutant APP and ABAD showed that the binding of Aβ on ABAD exacerbates mitochondrial dysfunction induced by Aβ, namely a decrease of mitochondrial complex IV activity, diminution of O_2 consumption and increase of ROS ***(147)***. Furthermore, these mice presented an early onset of cognitive impairment and histopathological changes compared to APP mice, suggesting that Aβ-ABAD interaction is an important mechanism underlying Aβ toxicity. The inhibition of Aβ-ABAD interaction, using a decoy peptide, was able to restore mitochondrial deficits induced by Aβ *in vivo* and improve neuronal and cognitive function ***(147)***.

To gain insights into the pathological mechanisms of Aβ upon mitochondria, we investigated the role of a novel small ABAD-specific compound inhibitor (AG18051) on Aβ-induced mitochondrial toxicity and estradiol metabolism in SH-SY5Y cells (***(124)***, see

APPENDIX 6). We found that AG18051 partially blocked Aβ-ABAD interaction and prevented the Aβ-induced down-regulation of ABAD activity by normalizing estradiol levels. Furthermore, AG18051 was protective against Aβ and reduced Aβ-induced impairment of mitochondrial respiration, oxidative stress and cell death. Our results emphasized the inhibition of ABAD by compounds such as AG18051 as a promising therapeutic strategy for the prevention and treatment of AD, and suggest that the endogenous modulation of mitochondrial estradiol metabolism is important for mitochondrial activity ***(90)***.

Taken together, these data suggest that disturbances in neurosteroid metabolism may be an underlying mechanism in AD. Neurosteroids may act in a delicate balance on the brain and mitochondrial function, and offer interesting therapeutic opportunities because of their pleiotropic effects in the nervous system.

5. Evidence of neuroprotective action of neurosteroids against Alzheimer's disease

The exact mechanisms underlying neurosteroids action in the nervous system are still unclear. However, studies based on behavioral responses evoked in animals by steroid injections suggested neurosteroid involvement in various neurophysiological processes including the development of the nervous system, adaptive responses of neuronal and glial cells under pathological conditions, and neuronal plasticity.

For instance, *in vitro* studies demonstrated that nanomolar concentration of DHEA and DHEAS stimulated the outgrowth of axons and dendrites, respectively, in primary cultures of embryonic mouse neurons ***(148)***. In accordance, subcutaneous injection of DHEA enabled an increased neurogenesis in the dentate gyrus of rats ***(149)***. Progesterone and estradiol also appears to stimulate cerebellar development since they were shown to promote dendritic growth, spinogenesis and synaptogenesis in developing Purkinje cells ***(114)***. Indeed, those neurosteroids have the ability to bind to microtubule-associated protein 2 (MAP2), known to promote tubulin polymerization or microtubule stability, in cultured neurons ***(112)***. Furthermore, *in vivo* studies showed that testosterone, estradiol and PROG are able to regulate the phosphorylation of tau protein, which is essential for its association with axonal microtubules and the regulation of axonal growth ***(132)***. In addition, progesterone is able to stimulate myelin synthesis in the peripheral nervous system by acting on neuronal gene expression via its classic nuclear receptor ***(150; 151)***. Other studies demonstrated that neurosteroids, such as pregnenolone sulfate, PROG or allopregnanolone enhances memory in rodents and exerts behavioral effects, probably via their non-genomic action ***(115)***.

Taken together, these findings suggest that neurosteroids may represent promising therapeutics for the treatment of neurodegenerative disorders by their ability to regulate brain

function, from the cellular level to the modulation of high cognitive functions. Indeed, a growing body of evidence highlighted the role of neurosteroids in neuroprotection. Studies showed that these molecules were protective against excitotoxicity, brain oedema, inflammatory processes and oxidative stress in a wide range of diseases including brain and spinal cord injury, stroke, Parkinson's disease, epilepsy or AD (reviewed in ***(152)***). The following evidences are focused on the neuroprotective effects of neurosteroids in AD.

The potential neuroprotective role of neurosteroids has been widely investigated, especially those of sex hormone-related neuroactive steroids (non-exclusive neurosteroids). Indeed, epidemiological studies showed that cognitive decline and the risk to develop neurodegenerative diseases, such as AD, could be associated with an age-related loss of estrogens (estrone (E1), estradiol (E2), and estriol (E3)), testosterone as well as progesterone in both, women and men ***(153; 53; 54)***. Studies demonstrated that estrogen depletion in postmenopausal women represents a significant risk factor for the development of AD and that a hormonal replacement therapy (HRT) might decrease this risk and even delay disease progression ***(154; 155)***. But, beneficial effects of HRT are still under debate since results from the "Woman's health initiative memory study" (WHIMS) showed negative effects of long-term HRT with, however, synthetic estradiol and medroxyprogesterone instead of using natural hormones, in older women ***(156; 157)*** (see **APPENDIX 4**). In contrast, animal studies showed that treatment with estradiol was able to protect the brain against excitotoxicity, Aβ peptide-induced toxicity, free radical generators and ischemia ***(158)***. Estrogens are able to enhance cerebral blood flow, to prevent atrophy of cholinergic neurons, and to modulate the effects of trophic factors in the brain ***(159)***.

Studies using animal models of AD have shown that a treatment with estradiol had an impact on APP processing, decreasing Aβ levels and its aggregation into plaques in mice expressing mutations in human APP (Swedish and Indiana mutation) compared to wild-type mice ***(160)***. The underlying mechanisms are still poorly understood but it has been proposed that estrogens are able to trigger the α-secretase pathway (non-amyloidogenic) via activation of extracellular-regulated kinase 1 and 2 (ERK 1 and 2) and through protein kinase C (PKC) signalling pathway ***(153)***. In accordance, in female AD triple transgenic mice (3xTgAD), the depletion of sex steroid hormones induced by ovariectomy, enhanced significantly Aβ accumulation and had a negative impact on cognitive performance ***(161-163)***. Those effects were prevented by a treatment with estradiol.

At the cellular level, estradiol can act as transcription factor by binding nuclear receptors, such as ER α and β. It has been shown that estradiol enhanced the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and down-regulated the expression of Bim, a pro-apoptotic factor, preventing the initialisation of the cell death program by mitochondria

(153; 164). Estradiol can also exert direct and indirect antioxidant effects by: i) up-regulating the expression of manganese superoxide dismutase (Mn-SOD) and gluthatione peroxidase ***(165)***; ii) increasing glutathione (GSH) levels and decreasing oxidative DNA damage in mitochondria, as observed in a study using ovariectomized female rats ***(166)***; iii) modulating the redox state of cells by acting on several signalling pathways, such as MAPK (mitogen-activated protein kinase), G protein regulated signalling, NFκB, c-fos, CREB, phosphatidylinositol-3-kinase, PKC and Ca^{2+} influx ***(167; 164)***. On the basis of this complex mode of action, estradiol seem to be able to decrease oxidative stress markers, including lipid peroxidation, protein oxidation and DNA damage, but can also directly regulate mitochondrial function (reviewed in ***(90)***) **(Fig 23)**.

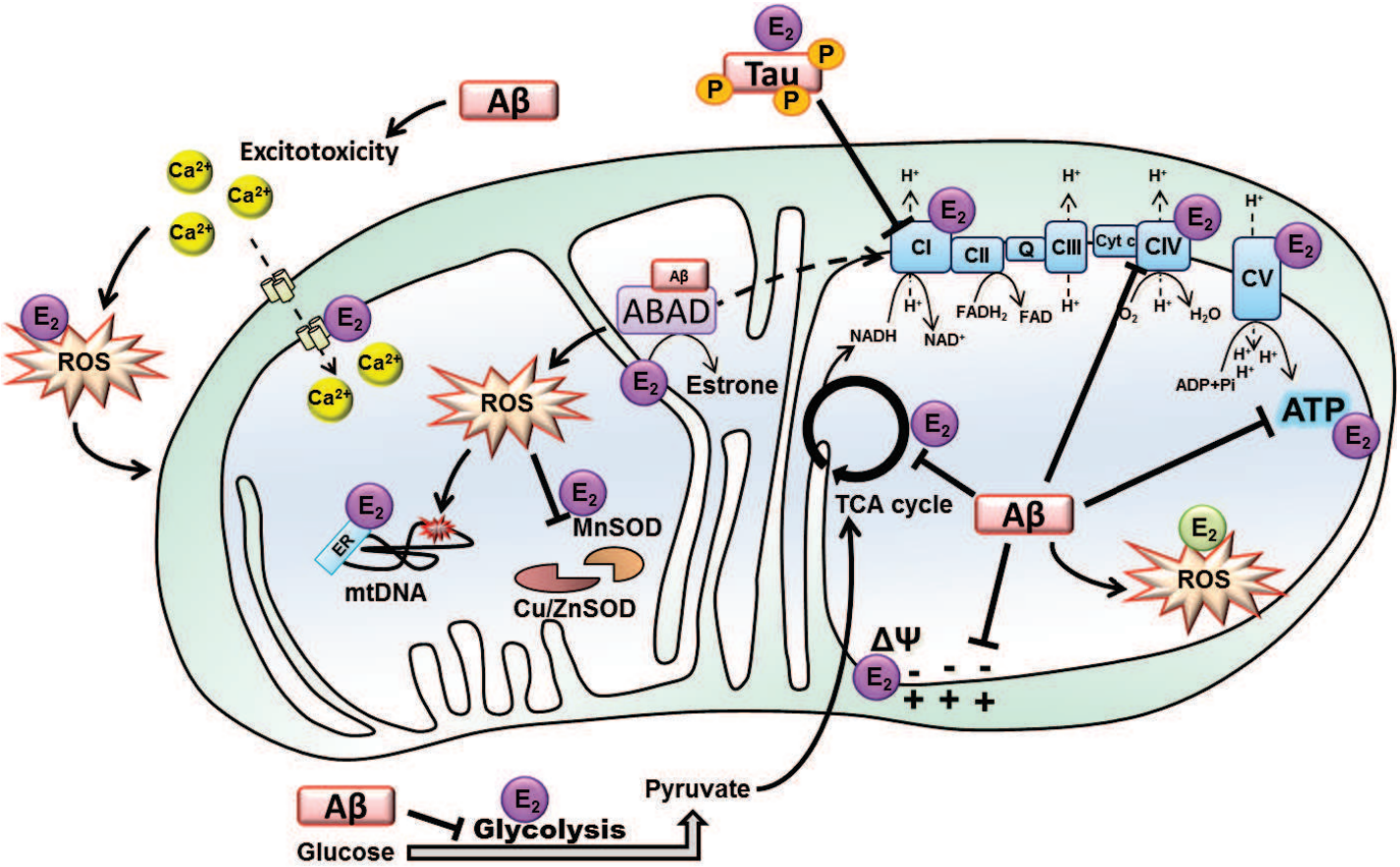

Fig.23: Estradiol and mitochondrial dysfunction in AD. In AD, mitochondrial dysfunction was found to be a central pathological mechanism which occurs already in early stages of the disease *(see details in **section I.A.2**)*. It has been shown that estradiol can increase glucose utilization by cells as well as ETC activity, stabilize the MMP and prevent ROS production and calcium-induced excitotoxicity. In the graph, E2 designates where estradiol potentially acts on the mitochondria to compensate Aβ-induced toxicity. ABAD Aβ-binding alcohol dehydrogenase, CI complex I, CII complex II, CIII complex III, CIV complex IV, CV complex V, cyt c cytochrome c, Cu/Zn SOD copper/zinc superoxide dismutase, MnSOD manganese superoxide dismutase, TCA tricyclic acid, E2 estradiol, ROS reactive oxygen species, mtDNA mitochondrial DNA, ER estrogen receptor (adapted from ***(90)***).

Human and animal studies suggested that androgen deprivation represents a risk factor for AD pathogenesis in men ***(153; 168)***. Notably, in a triple transgenic mouse model of AD (3xTgAD), it has been shown that orchiectomized males presented an increased Aβ accumulation in the brain, coupled with impaired cognitive performances compared to sham operated mice ***(169)***. Treatment with androgens significantly attenuated the increase in AD pathology ***(168; 169)***. Testosterone appears to prevent tau hyperphosphorylation in a model of heat shock-induced phosphorylation through GSK signalling inhibition ***(170)***. Rosario and colleagues ***(168)*** demonstrated less abnormal tau accumulation in gonadectimized male 3xTgAD mice treated with testosterone. Estradiol is aromatized from testosterone and other androgens, and this implies that testosterone may exert indirect effects on mitochondria, though some studies have shown that testosterone has anti-apoptotic effects mediated through the androgen receptor ***(137)***. Overk and colleagues ***(171)*** examined basal levels of serum and brain testosterone in male 3xTgAD mice and found that testosterone levels rise with disease progression. This increase in testosterone in aged male 3xTgAD mice was correlated with reduced Aβ plaque pathology. This suggests that testosterone may have some neuroprotective benefits against the AD disease course, but that testosterone administration is associated more with lesser Aβ protein burden rather than a reduction in abnormal tau protein. In fact, testosterone alters processing of amyloid precursor protein and enhances expression of neprilysin, an enzyme responsible for Aβ degradation ***(172)***.

The effects of PROG in AD mice have also been investigated. For instance, PROG was able to improve the cognitive performance and reduced tau hyperphosphorylation in mice bearing a double mutation in APP^{sw}xPS1 compared to wild-type mice ***(162)***. Those mice were characterized by decreased hippocampally-mediated cognitive performances and presented decreased levels of allopregnanolone in the hippocampus, suggesting that a part of the deficit in hippocampal function may be due to reduced capacity to form allopregnanolone in the hippocampus. Interestingly, recent studies demonstrated a protective role of allopregnanolone in AD triple transgenic mice (3xTgAD), showing reduced Aβ generation in hippocampus, cortex and amygdala, increased proliferation of neuronal progenitor cells and reversed neurogenic and cognitive deficits compared to non-transgenic littermates ***(173; 174)***.

Together, these data suggest that neurosteroids represent interesting tools for the therapy and prevention of neurodegenerative diseases, especially AD. With regard to AD-related mitochondrial dysfunction, extensive studies only focused on beneficial effects of estradiol, while the effects of other neurosteroids on mitochondria have not been investigated up to now (see **section II.A and II.B**).

C. Circadian rhythms

1. Concept of circadian rhythm

Circadian rhythms are believed to be an evolutionary adaptation to daily environmental cycles that are synchronized by the 24 hours patterns of light and temperature produced by the earth's rotation around its axis ***(175)***. They coordinate our physiology at a fundamental level and govern a wide variety of physiological and metabolic functions in most organisms, from cyanobacteria and fungi to insects and mammals ***(176)***. Circadian oscillations (from the Latin "circa diem" = about a day) occur with a period length of about 24 hours and play a key role in the adaptation of living organisms to the environmental changes, such as light/dark cycles which are associated with food availability. These oscillations are defined by their period length (τ), amplitude (A) and phase (ϕ) **(Fig. 24A)**.

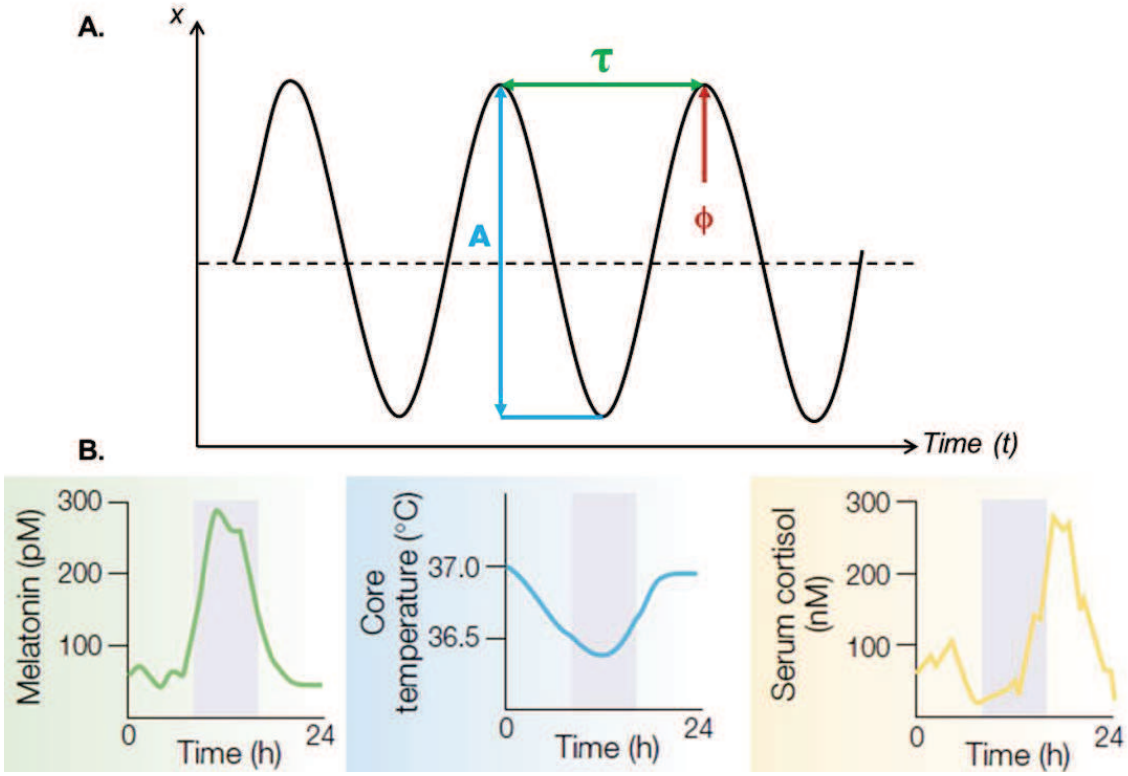

Fig. 24: (A) Parameters of a hypothetical rhythm. Given a variation of an element "X" in units of time "t", several parameters of a rhythm can be measured, such as amplitude (**A**, in blue, defined as the maximum absolute value of a periodically varying quantity), period length (**τ**, in green, the time spent between two peaks) and phase (ϕ, in red, that represents the position of a peak in function of time). (B) Examples of circadian rhythms in human. Melatonin (left) is secreted during the night (gray shading) with a peak in anti-phase with the body temperature (middle). Cortisol secretion (right) starts during the night, with a peak at the beginning of the light phase (adapted from ***(177)***).

A biological rhythm (or oscillation) must meet three general criteria to be called "circadian". First, the rhythm has to be innate, endogenous, with a free-running period length of about 24 hours, and self-sustained in constant condition (e.g. in constant darkness). This criterion is important to distinguish circadian oscillations from daily oscillations that are responses to external and environmental cues. A rhythm is considered as endogenous only if it persists in constant conditions. Second, the rhythm has to be entrainable (synchronized) by external stimuli, also called "Zeitgebers" (time giver), such as light/dark cycles or feeding/fasting cycles. Finally, temperature compensation is the third criterion that defines circadian rhythms, meaning that the period length of these rhythms are unaffected by temperature changes within physiological permissible limits ***(176)***.

Overall, this allows the organism to coordinate a variety of daily behavioral and physiological processes to the optimal time of day by anticipating the periodic changes of the external environment. The most obvious circadian rhythm observed in humans (and other animals) is the sleep-wake cycle. During the light phase (or circadian day), catabolic processes are predominant to facilitate engagement with the external world. In contrast, the dark phase (or circadian night) promotes anabolic functions of growth, repair and consolidation ***(177)***. For example, body temperature drops during the night and melatonin is secreted, facilitating sleep during which growth hormones are released **(Fig. 24B)**. Cortisol levels increase during the night, with a peak at the beginning of the activity period, preparing the body for physical and mental demands of awakening.

Molecular mechanisms underlying circadian rhythms have been unraveled during the last decade and involve interconnected feedback loops of gene transcription and translation ***(178)***.

2. Clock genes and circadian machinery

The maintenance of a rhythm with a period length of about 24 hours is made possible by self-sustained transcriptional-translational feedback loops. In these loops, it is possible to distinguish positive and negative components. The first loop begins with the heterodimerization of transactivating (positive) components CLOCK and BMAL1 **(Fig. 25)**, two transcription factors that reach the nucleus and initiate the transcription of clock-controlled genes (CCG) containing E-box (5′-CACGTG-3′) or E′-box (5′-CACGTT-3′) cis-regulatory elements ***(178)***. Notably, the CLOCK/BMAL1 heterodimer triggers the transcription/translation of the clock genes Period (isoforms Per1-3) and Cryptochrome (isoforms Cry1 and 2) that constitute negative (transinhibiting) components of the molecular feedback loop ***(179)*** **(Fig. 25)**. In the cytoplasm, PER and CRY proteins dimerize and are

translocated into the nucleus where they repress transcription of their own genes by directly inhibiting CLOCK/BMAL1 ***(178)***. Degradation of the negative limb proteins PER and CRY is required to terminate the repression phase and restart a new cycle of transcription.

Additional components contribute to the robustness of this molecular clockwork circuitry. For instance, REV-ERBα (reverse orientation c-erb α) and ROR (orphan nuclear-receptor genes) interconnect the circadian transcription of the positive and negative "limbs" of the molecular clock. REV-ERBα transcription is activated by CLOCK/BMAL1 complex through the binding to E-box sequences present in its promoter, resulting in its circadian accumulation. REV-ERBα protein leads to periodic repression of Bmal1 transcription. This repression leads to a rhythmic expression of Bmal1 in antiphase with Rev-Erbα expression ***(180)***. Posttranslational mechanisms such as protein phosphorylation also play important roles in generating oscillations of approximately 24 hours. For example, casein kinase 1ε (CK1ε) phosphorylates PER, CRY, and BMAL1 proteins ***(178)***. Hypophosphorylated PER proteins have a higher metabolic stability than their hyperphosphorylated counterparts and this may lead to an increased accumulation of PER proteins.

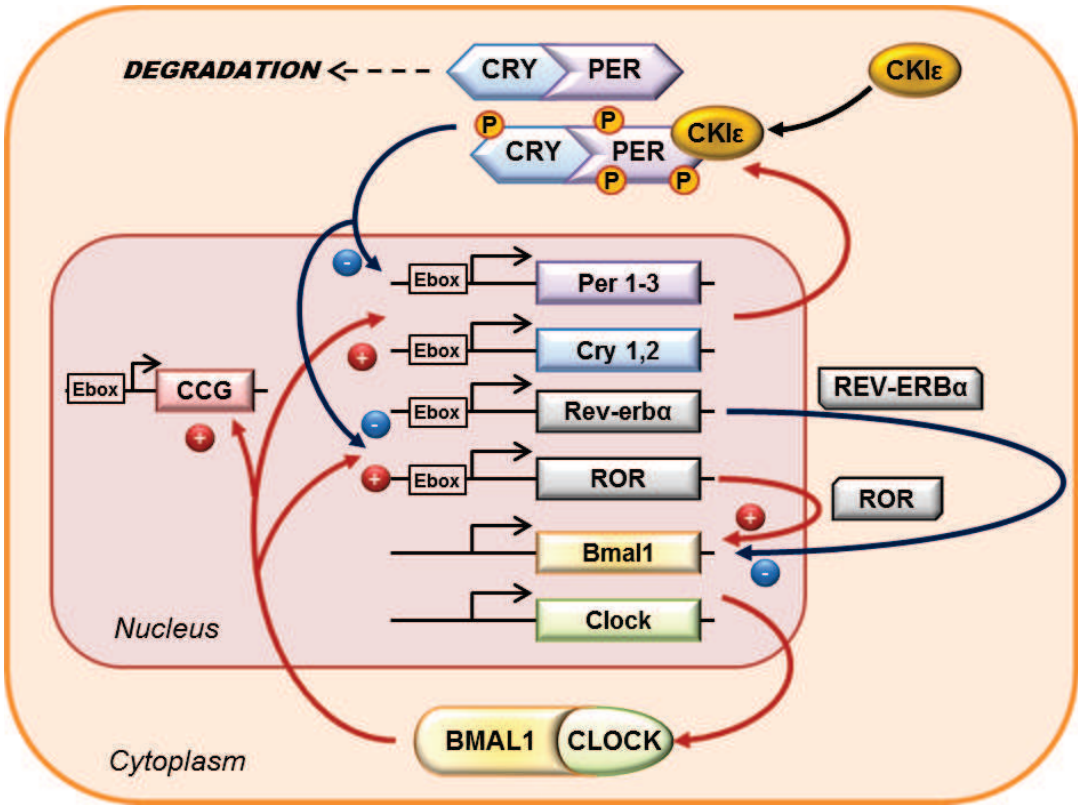

Fig. 25: Schematic representation of the molecular circadian clock machinery. The system consists of two feedback loops: CLOCK/BMAL1 heterodimer forms the positive component in this loop, while the PER/CRY complex acts as the negative component. Details are described in the text. CCG; clock controlled genes, P; phosphate (adapted from ***(178)***).

Mutations in clock genes are accompanied by a spectrum of behavioral abnormalities, including mania, hyperactivity or increased alcohol consumption, and disturb neurochemical systems, such as dopaminergic and glutamatergic neurotransmission ***(176)***. Identification of clock genes enabled the development of transgenic models that help to understand in more details the underlying mechanisms of the molecular clock machinery and its impact on behavioral outputs (reviewed in ***(181)***).

In mammals, the circadian molecular clock is virtually present in all cells of the body ***(179)***. To synchronize all these clocks, the circadian timekeeping system possesses a complex hierarchical architecture, with a central pacemaker in the brain and subsidiary clocks in the rest of the body.

3. Organization of the circadian clock

One of the main properties of the circadian system is its ability to synchronize the individual circadian clock at all levels. Thus, clocks contained in peripheral tissues (peripheral clocks) are kept in a stable phase-relationship to maintain a coherent function of the entire organism. In turn, these individual oscillators can send back information to the master clock present in the brain's suprachiasmatic nuclei (SCN) that adapts organism's physiology in response to environmental parameters.

a) Master clock

The SCN of the anterior hypothalamus is the site of the master circadian clock in the mammalian brain ***(182)***. Indeed, lesion experiments in the brain of rodents enabled the identification of this structure located above the optic chiasma. SCN-lesioned animals were arrhythmic in entrained condition and presented a disruption of locomotor activity and a loss of rhythmicity in corticosterone secretion ***(183; 184)***. Circadian locomotor activity was restored in SCN-lesioned animal by transplantation of fetal SCN tissue into the third ventricle ***(185)***. A determinant demonstration of the clock function of the SCN was made possible using the *Tau* (τ) mutant hamster ***(182)***. The *Tau* mutation shortens circadian period length from 24 hours in the wild type to 20 hours in homozygote mutant animals. This shortened period length results from a missense mutation within the substrate recognition site of the enzyme CK1ε (see **section I.C.2**) ***(186)***. Again, transplantation studies revealed that SCN grafts from wild-type animals restores the circadian period length of *Tau* mutant hamsters suggesting that circadian parameters are determined by the genotype of the donor, not the host ***(182)***. In addition, *in vitro* studies revealed that electrical activity of SCN neurons follows a circadian pattern. This rhythmic firing is maintained even after three weeks in culture ***(187)***.

This property is the direct consequence of the tight coupling between SCN neurons via conventional synapses (likely GABAergic), electrical synapses (i.e. gap junctions) and neuropeptidergic coupling (e.g. vasoactive intestinal peptide (VIP)) to avoid the damping of the oscillations ***(176)***.

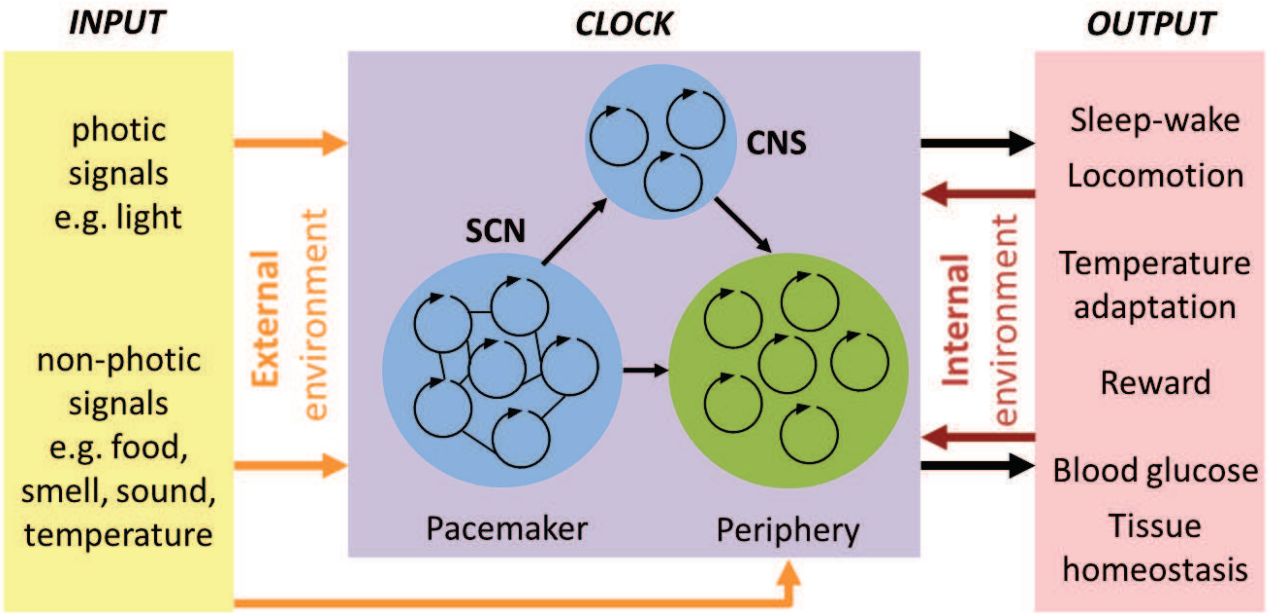

Fig. 26: Subdivision of the circadian system: input to the clock, clock mechanism, and clock output. This division can be made at the cellular level as well as at the systemic level. SCN suprachiasmatic nuclei, CNS central nervous system (adapted from ***(179)***).

The role of SCN in the regulation of circadian system has been investigated for many years (reviewed in ***(188)*** and ***(189)***). The key function of this structure is to serve as relay between external environment and the body by: i) perceiving environmental inputs; ii) integrating time-related information; iii) transmitting adjusted timing information to other tissues and organs that subsequently send feedback information to the SCN **(Fig. 26)**.

For example, when light is detected by the retina, signals are transmitted to the SCN (=input from the external environment) **(Fig.27)**. There, signals are integrated to adjust the information about the time of the day (light onset: dawn or light offset: dusk). The SCN can send various output signals (hormones, metabolites, neuronal signals…) through which the generated rhythms are manifested via control of various metabolic, physiological, and behavioral processes.

Thus, SCN is on the top of the hierarchical organization of the circadian system and serve as a central conductor orchestrating the peripheral clocks.

b) Peripheral clocks

When the molecular components of the circadian clock were identified, it has been shown that clock genes are also expressed rhythmically outside the SCN and even outside the brain in many peripheral tissues ***(190)***. The mRNA expression of main clock genes, such as Bmal1, Reverbα or Per1-3, has been found in cells coming from different peripheral systems (heart, lung, liver, stomach, spleen, kidney…) ***(182)***. Of note, in the peripheral tissues, mRNA peaks of clock genes occurred approximately 4 hours after those in the SCN ***(191; 180; 192; 193)***. In addition, genome-wide transcriptome profiling studies showed that gene expression exhibit robust circadian oscillation in the above mentioned organs, suggesting that many cellular functions are under the control of the circadian clock (reviewed in ***(182)*** and ***(190)***). For instance, depending on the tissue, it has been shown that between 2% and 10% of all analyzed genes were rhythmically expressed, especially in the liver which contains about 1000 circadian transcripts ***(194)***. Many of these genes encode key enzymes involved in metabolic pathways including food processing, carbohydrate and lipid metabolism, cholesterol utilization and xenobiotic detoxification ***(190)***. Indeed, it makes sense that the production or sequestration of chemically incompatible processes may be potentially harmful if they take place during the same time window. For instance, if glycogen synthase and phosphatase were expressed at the same time, it would be incompatible with the conversion of glucose in glycogen, and vice versa ***(182)***. Similarly, xenobiotic detoxification starts slightly before feeding time, anticipating the absorption of toxins present in food (e.g. plant alkaloids, coumarin…) ***(190)***. Daily feeding-fasting cycles represent the main synchronizer (Zeitgeber) of the peripheral organs, including the liver, kidney, pancreas and heart muscle **(Fig. 27)**.

This observation led Schiebler and colleagues ***(190)*** to propose a new hypothesis postulating that the most important roles of peripheral clock can be summarized in three points: i) the anticipation of metabolic pathways to optimize food processing; ii) the limitation of metabolic processes with adverse side effects to time periods when they are needed; iii) the sequestration of chemically incompatible reactions to different time windows. Even if peripheral clocks are self-sustained, autonomous oscillators, a functional SCN is required to maintain phase coherence between them **(Fig. 27)**. The properties of peripheral clocks are similar to the master clock, which make them preferential models to understand in more details the circadian machinery and the implications in health and disease.

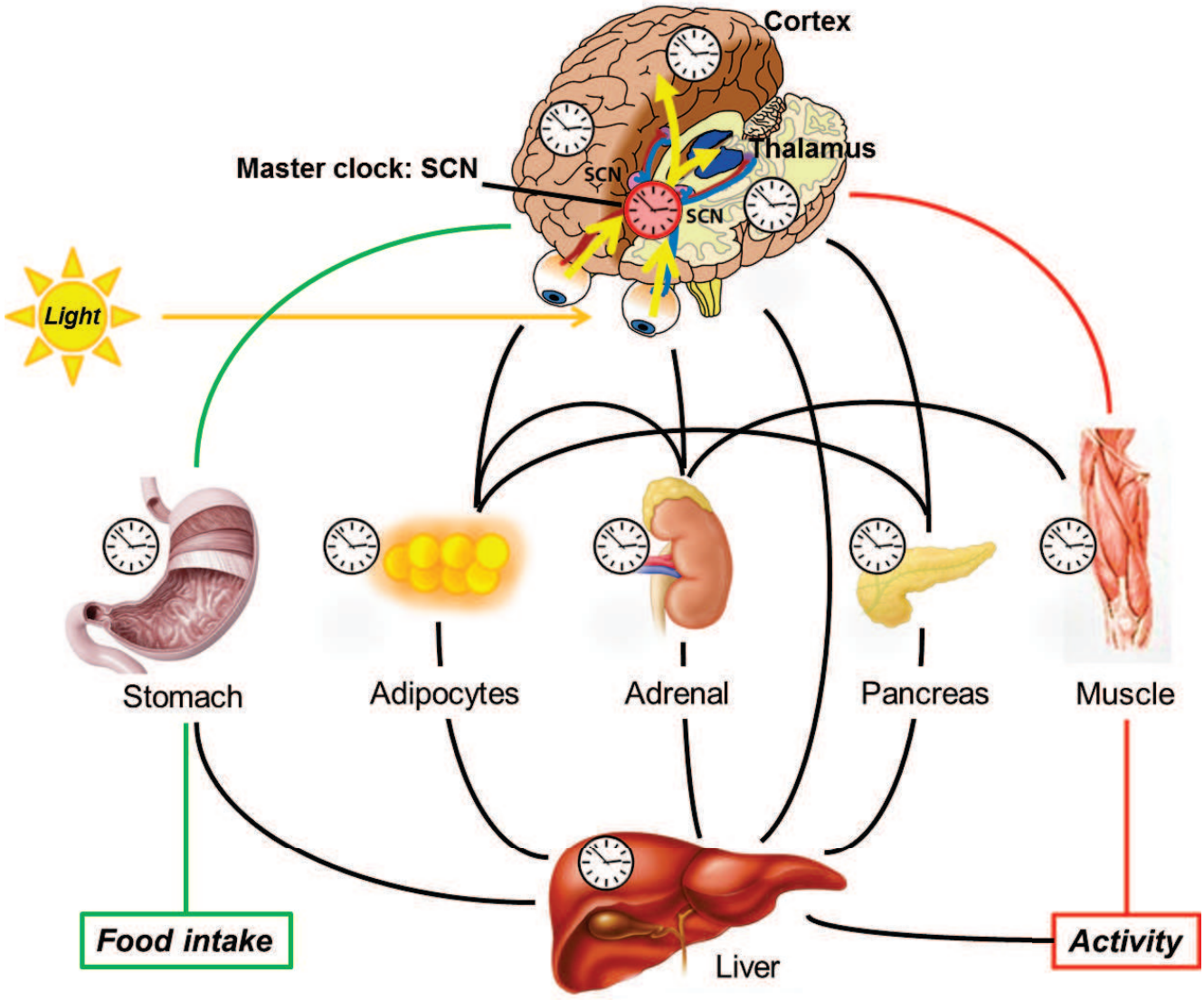

Fig. 27: Schematic organization of the circadian system. Circadian clocks are found in all cells of various organs. The master clock is located in the SCN (red clock) and synchronizes the other central clocks that are located in different part of the brain (cortex, thalamus…) to regulate metabolic integration and motor coordination. The main synchronizers are the light (yellow), food intake (green) and locomotor activity (red). SCN and other organs communicate via hormonal signaling (melatonin, ghrelin, leptin, insulin/glucagon, adrenaline…), metabolic signaling (carbohydrate, fatty acids, amino acids) or neuronal connections (adapted from ***(179)***).

c) Studying circadian systems using peripheral cells

Circadian systems can be studied from cellular level and up to systemic and behavioral levels. In human, several protocols were developed to reveal the endogenous circadian component of rhythms ***(195)***. In these protocols one or more circadian markers are usually measured, namely melatonin (in blood or saliva), cortisol (in plasma) or core body temperature (see also **Fig. 24B**). The main issues in human is that environmental components can influence these markers such as light, temperature, body position, food intake, and many other factors. These environmental components can easily be controlled in animal studies. Since rodents voluntary use a running wheel in their home cage, locomotor activity served as a particularly reliable and convenient measure of the output of their circadian system ***(196)***. A panel of protocols is available to study circadian parameters in rodents. Usually, animals are placed in constant conditions (e.g. constant darkness: dark-dark (DD)) with the subjective time-of-day referred to as the circadian time (CT), in contrast to entrainable conditions (e.g. light-dark cycle (LD): 12 hours light – 12 hours dark) where the time of the day is given by the zeitgeber time (ZT). Besides, the identification of the molecular clock machinery allowed a move forward in the understanding of the circadian system and promoted the development of transgenic models that are widely used in the circadian research fields ***(197; 181; 198-200)***.

In 1998, Balsalobre and colleagues demonstrated that immortalized rat fibroblasts in culture possess robust autonomous and self-sustained circadian rhythm ***(201)***. Since then, the identification of the molecular clock machinery in isolated human fibroblasts has been widely demonstrated. The cultivation and genetic manipulation of fibroblasts are easy to perform and these are some of the main reasons why fibroblasts are a valuable model of peripheral oscillator ***(202; 203)***. Indeed, protocols have been developed to measure clock function in skin cells using lentivirally-delivered circadian luciferase reporter vectors **(Fig. 28)**. For example, fibroblast period length can be measured via cyclical expression of the circadian reporter Bmal1: luciferase ***(202-204)***.

As mentioned in **section I.C.3.a**, SCN neurons are tightly coupled one with another, which results in a robust circadian rhythmicity even *in vitro*. In contrast, rhythmic patterns of peripheral cells, including fibroblast, have the tendency to damp after several cycles due to phase desynchronization of individually oscillating cells ***(205; 204)***. Thus, fibroblasts need to be resynchronized after few days in culture to be able to continue to detect a circadian rhythmicity. Two classical methods used to synchronize peripheral oscillators are: (i) serum

shock, that consists in the incubation of the cells in a high percentage horse serum medium for 1-2 hours ***(201)***; (ii) activation of the glucocorticoid receptor by the use of dexamethasone (an analog of glucocorticoids) ***(206; 207)***.

More recently, a study performed on human primary fibroblasts cultivated from skin biopsies showed that the period length of human circadian behavior could be approximated by measurement of the circadian period length in fibroblasts ***(203)***. These data indicated for the first time that it may be possible to study circadian parameters of an individual using those of its fibroblasts and may enable to unravel relationships between circadian clock defects and associated diseases, using peripheral cells.

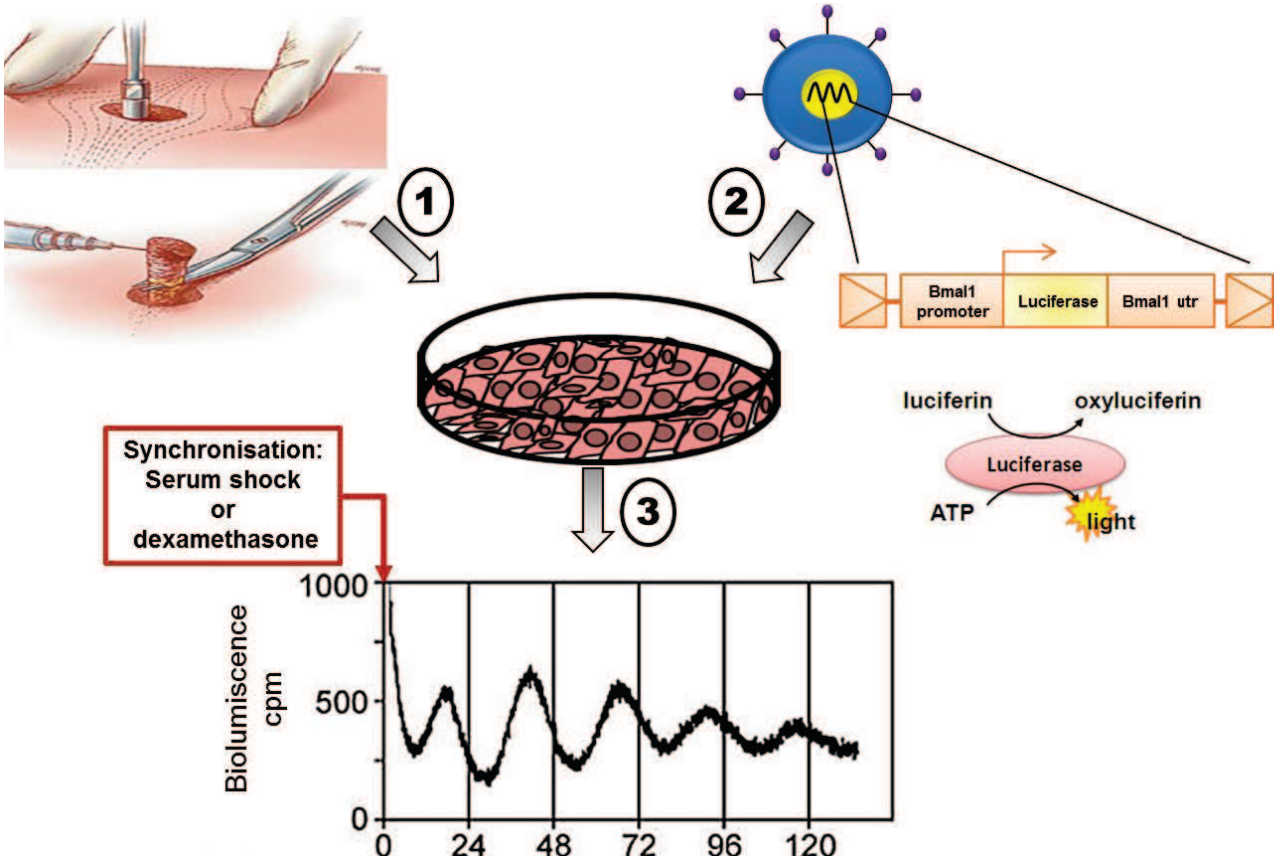

Fig. 28: Protocol to study the circadian rhythms of human skin fibroblasts. A punch skin biopsy is harvested (1) and cultivated under sterile conditions. Fibroblasts that grow around the biopsy are isolated, amplified and infected with a circadian reporter (2) coding for firefly luciferase under a clock gene promoter, i.e. Bmal1. The infected cells are selected and then, after synchronization of circadian rhythms, the light emitted by the cells is measured in the Lumicycle (Actimetrics) (3).

4. Clock control of cellular metabolism and vice versa

Circadian clock may represent an evolutionary advantage, by enhancing metabolic efficiency through temporal separation of anabolic and catabolic reactions **(Fig. 29)**. Understanding the relationship between metabolic system and the molecular clock represents an emerging field of research with the aim to increase our knowledge about metabolic impairments linked to circadian disruption, such as sleep disorders, cardiometabolic diseases or type 2 diabetes ***(208)***.

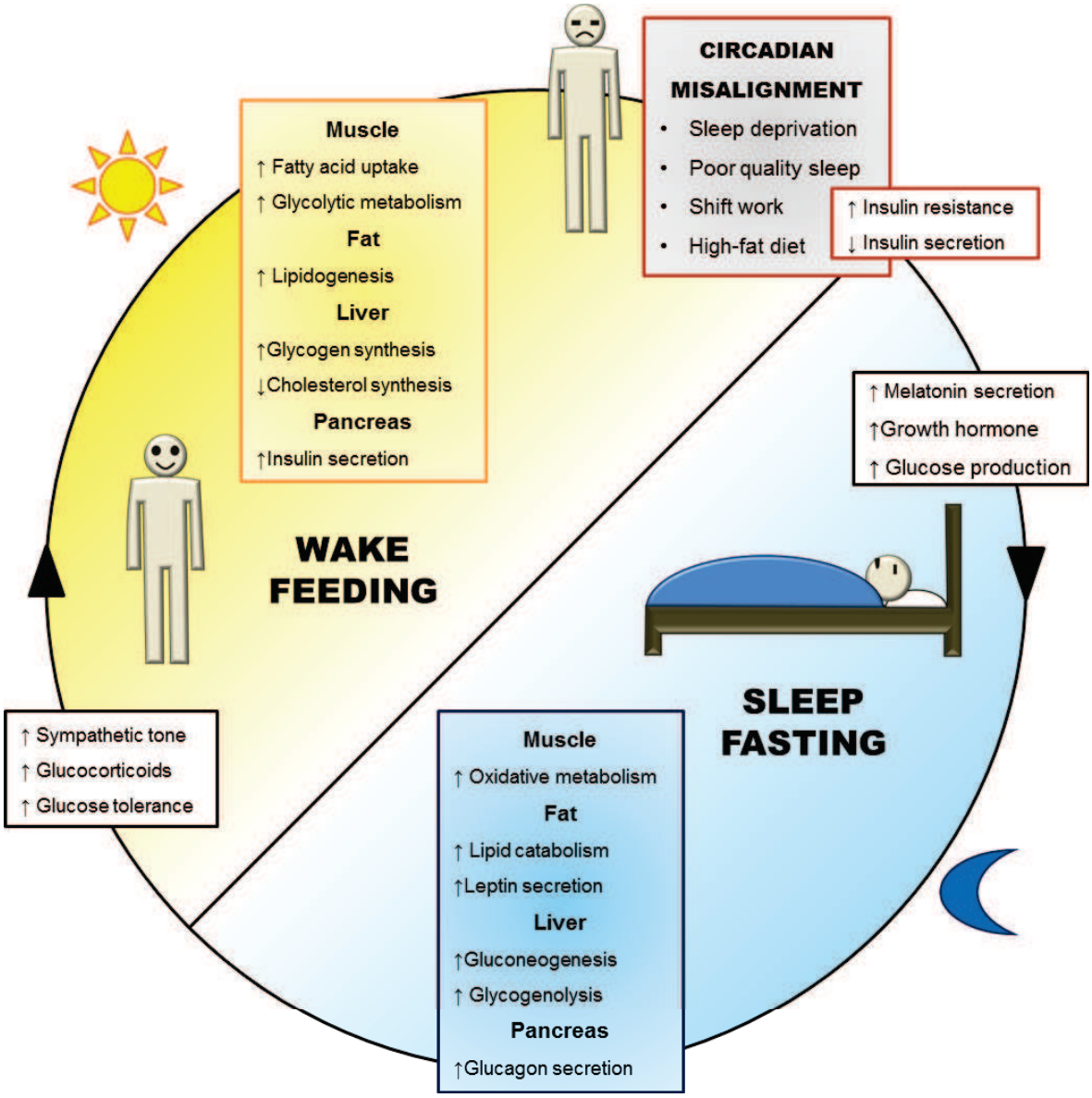

Fig.29: Rhythmicity of metabolic processes according to time of day. The clock coordinates appropriate metabolic responses with the light/dark cycle and enhances energetic efficiency through temporal separation of anabolic and catabolic reactions in peripheral tissues. Circadian misalignment, which occurs during sleep disruption, shift work, and dietary alterations, disrupts the integration of circadian and metabolic systems, leading to adverse metabolic health effects (adapted from ***(209)***)

Recent studies have highlighted the role of some molecules coming from metabolic activity in the regulation of the circadian clock ***(210-212)***. One potential candidate is NAD+ that is, among others, involved in cellular redox reactions. Indeed, *in vivo* studies showed that the expression of the key rate-limiting enzyme in the NAD+ salvage pathway, the NAMPT (nicotinamide phosphoribosyltransferase), is directly regulated by CLOCK/BMAL1 **(Fig.30)** ***(213)***. Other findings revealed that NAD+ display circadian oscillations in peripheral tissues (e.g. in the liver or in adipocytes), even when the mice are kept in DD conditions (constant darkness) ***(214)***. NAD+ is also a co-factor for several enzymes, including members of the sirtuin family. It was recently shown that the circadian clock modulates the activity of SIRT1 (sirtuin 1), a NAD+-dependent protein deacetylase involved in the deacetylation of histones and several transcription factors ***(215)***. SIRT1 (and other sirtuins) has emerged as a key metabolic sensor that regulates gluconeogenesis, lipid metabolism, insulin sensitivity and other metabolic processes (reviewed in ***(216)***). The circadian oscillation of NAD+ levels appears to regulate SIRT1 activity, which then coordinates the daily pattern of metabolic processes mentioned above ***(215)***. In turn, SIRT1 modulates the circadian clock via a negative feedback loop by interacting with CLOCK, BMAL1 and PER2 **(Fig.30)** ***(217; 211)***.

In addition, the cellular redox state, translated by the ratios NAD+/NADH and NADP+/NADPH, seems to affect the circadian system. McKnight and colleagues ***(218)*** were the firsts to show that high levels of NAD+ and NADP+ (oxidized state) decrease the binding of CLOCK/BMAL1 to DNA, whereas the reduced forms (NADH and NADPH) increase this binding. Interestingly, more recent studies showed a 24 hours redox cycle in red blood cells that are cells devoid of nuclei, suggesting that the cellular redox state is self-sustained even in the absence of the transcriptional control of circadian gene expression ***(219; 220)***.

Finally, another measure of cellular metabolic state is the ratio AMP/ATP (ATP consuming pathways/ATP generating pathways). A major sensor of this ratio is the enzyme AMPK (adenosine monophosphate-dependent protein kinase) which is activated by phosphorylation when the ratio AMP/ATP increases. Studies showed that AMPK can directly modulate the circadian clock by phosphorylating CRY1 and PER2 ***(221; 222)***. This led to the degradation of both clock components and consequently affected the negative limb of the molecular circadian machinery.

Together, these data underpin the emerging relationship between the molecular clock and metabolic system. Of note, even if mitochondria play a central role in both metabolism and redox homeostasis, its implication in the regulation of the circadian system (and vice versa) is only hypothetical ***(223)*** and no clear link has been established until now (see **section II.C**).

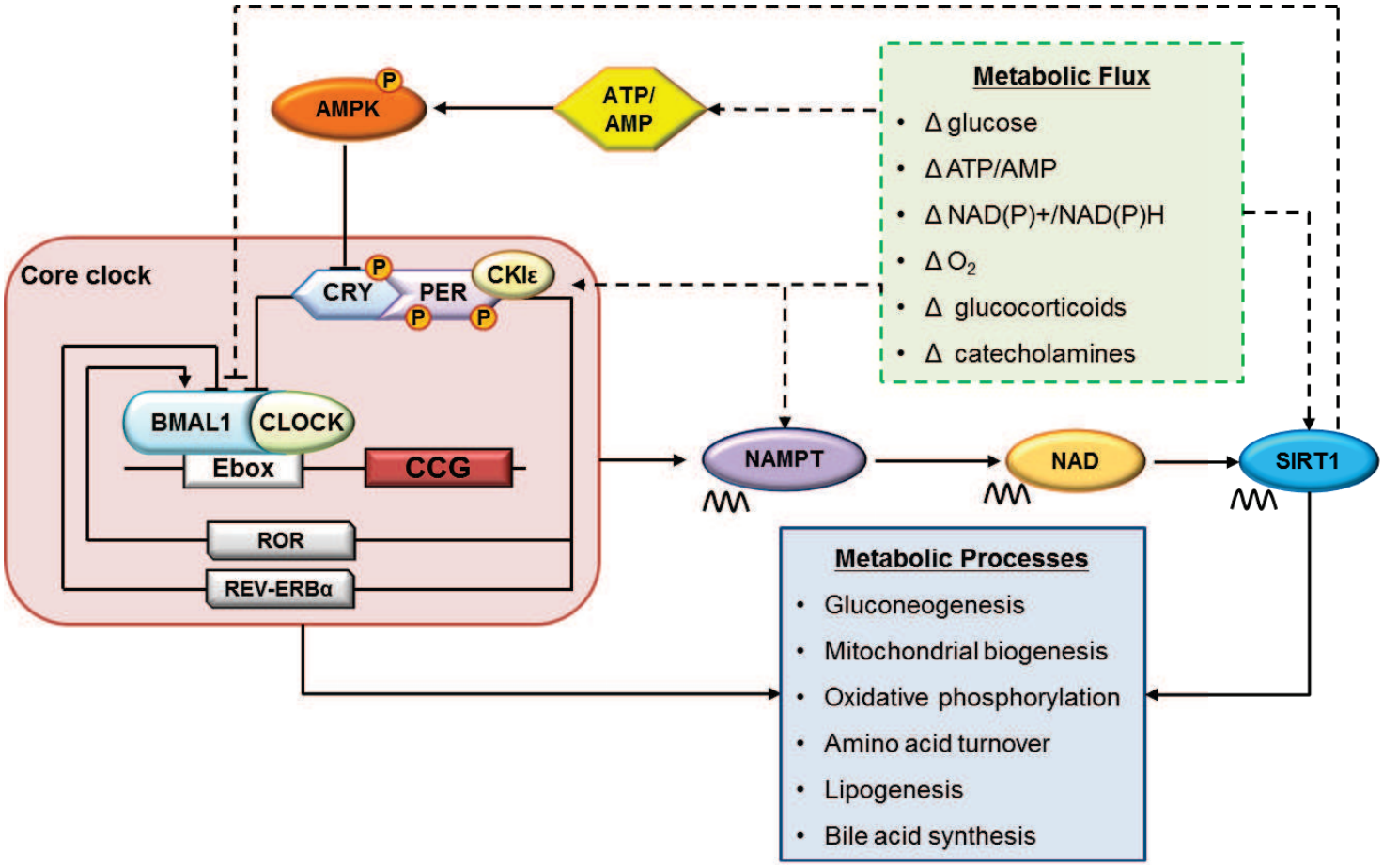

Fig. 30: Cross talk between the core clock mechanism and metabolic pathways. The core clock can directly or indirectly synchronize diverse metabolic processes. The clock receives reciprocal input from nutrient signaling pathways (including SIRT1 and AMPK), which function as rheostats to coordinate metabolic processes with daily cycles of sleep/wakefulness and fasting/feeding. NAMPT; nicotinamide phosphoribosyltransferase, SIRT1; sirtuin 1, AMPK; adenosine monophosphate-dependent protein kinase (adapted from ***(211)***).

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II. RESULTS

A. Improvement of neuronal bioenergetics by neurosteroids: Implications for age-related neurodegenerative disorders.

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Abstract

The brain has high energy requirements to maintain neuronal activity. Consequently impaired mitochondrial function will lead to disease. Normal aging is associated with several alterations in neurosteroid production and secretion. Decreases in neurosteroid levels might contribute to brain aging and loss of important nervous functions, such as memory. Up to now, extensive studies only focused on estradiol as a promising neurosteroid compound that is able to ameliorate cellular bioenergetics, while the effects of other steroids on brain mitochondria are poorly understood or not investigated at all. Thus, we aimed to characterize the bioenergetic modulating profile of a panel of seven structurally diverse neurosteroids (progesterone, estradiol, estrone, testosterone, 3α-androstanediol, DHEA and allopregnanolone), known to be involved in brain function regulation. Of note, most of the steroids tested were able to improve bioenergetic activity in neuronal cells by increasing ATP levels, mitochondrial membrane potential and basal mitochondrial respiration. In parallel, they modulated redox homeostasis by increasing antioxidant activity, probably as a compensatory mechanism to a slight enhancement of ROS which might result from the rise in oxygen consumption. Thereby, neurosteroids appeared to act via their corresponding receptors and exhibited specific bioenergetic profiles. Taken together, our results indicate that the ability to boost mitochondria is not unique to estradiol, but seems to be a rather common mechanism of different steroids in the brain. Thus, neurosteroids may act upon neuronal bioenergetics in a delicate balance and an age-related steroid disturbance might be involved in mitochondrial dysfunction underlying neurodegenerative disorders.

Keywords: Mitochondria, Neurosteroid, Bioenergetics, Amyloid-β peptide, tau protein.

Abbreviations:

3α-A, 3α-androstanediol; AD, Alzheimer's disease; APP, amyloid-β precursor protein; AP, allopregnanolone; D, DHEA (dihydroepiandrosterone); DHR, dihydrorhodamine 123; DMSO, dimethylsulfoxide; E1, estrone; E2, 17β-estradiol; E3, estriol; ECAR, extracellular acidification rate; ETC, electron transport chain; MAS, mitochondrial assay solution; MPP, mitochondrial membrane potential; mtROS, mitochondrial reactive oxygen species; OCR, oxygen consumption rate; OXPHOS, Oxidative phosphorylation; P, progesterone; PD, Parkinson's disease; PMP, plasma membrane permeabilizer; RCR, respiratory control ratio; roGFP, redox sensitive green fluorescent protein; ROS, reactive oxygen species; SRA, steroid receptor antagonist; T, testosterone; TCA, tricyclic acid.

1. Introduction

The brain is a highly differentiated organ with high energy requirements, mainly in the form of adenosine triphosphate (ATP) molecules. Despite its small size, it accounts for about 20% of the body's total basal oxygen consumption ***(1)***. As a result, the brain is more sensitive to neuronal damage during hypometabolic states and impaired redox homeostasis, as observed in normal aging and in neurodegenerative diseases associated with a decline in energy production and changes in the redox status ***(2)***. In this context, mitochondria, small organelles that are present in almost all cell types playing a predominant role in cellular bioenergetics, are particularly important in the nervous system because of its high energy demand. Mitochondria are not only the "powerhouses of the cell", providing the main source of cellular energy via ATP generation through oxidative phosphorylation, but they also contribute to plenty of cellular functions, including apoptosis, intracellular calcium homeostasis, alteration of the cellular reduction–oxidation (redox) state and synaptic plasticity ***(3; 4)***. Thus, it is more and more recognized that mitochondrial dysfunction is a significant and early event of neurodegeneration, and that the pathophysiological mechanisms of a range of neurodegenerative diseases, including Alzheimer's (AD) and Parkinson's disease (PD), are associated with a decline in bioenergetic activity and an increase in oxidative stress, particularly in mitochondria themselves ***(5-10)***.

Steroid hormones are molecules involved in the control of many physiological processes in the periphery, from reproductive behavior to the stress response. They are mainly produced by endocrine glands, such as the adrenal glands, gonads and placenta, but in 1981 Baulieu and co-workers were the first to demonstrate the production of steroids within the nervous system itself ***(11)***. This last category of molecules is now called "neurosteroids" and is defined as steroids that are synthetized within the nervous system independently of peripheral endocrine glands. Neurosteroid levels remain elevated even after adrenalectomy and castration ***(12; 13)*** and are involved in brain-specific functions. Since the ability to produce neurosteroids is conserved during vertebrate evolution, one can suggest that this family of molecules is important for living things and that the modulation of their biosynthesis plays an important role in the pathophysiology of neurodegenerative disorders.

Studies performed in humans, animals, and cellular models have shown alterations in the synthesis of neurosteroids that declined during brain aging paralleled by a loss of important nervous functions, such as memory, and were further associated with PD and AD ***(14-16)***. Thus, several neurosteroids have been quantified in various brain regions of aged AD patients and aged non-demented controls. This showed a general trend toward lower steroid levels in AD patients compared to controls, associated with a negative correlation

between neurosteroid levels and amyloid-β (Aβ) and phospho-tau in some brain regions ***(17)***. In accordance with these observations, previous data from our groups provided first evidence that, vice versa, Aβ and hyperphosphorylated tau differentially impacted neurosteroidogenesis **(Fig. 1)** ***(18-20)***. Indeed, a decrease of progesterone and 17-hydroxyprogesterone production was observed in amyloid precursor protein (APP)/Aβ-overexpressing cells, while 3α-androstanediol and estradiol levels were increased ***(19)***. Moreover, *in vitro* treatment of human neuroblastoma cells with "non-toxic" Aβ concentrations (within the nanomolar range) revealed an increase in estradiol production, whereas toxic Aβ concentrations (within the micromolar range) showed the opposite effect ***(18)***. Overexpression of human wild type tau (hTau40) protein induced an increase in production of progesterone, 3α-androstanediol, and 17-hydroxyprogesterone, in contrast to the abnormally hyperphosphorylated tau bearing the P301L mutation that led to decreased production of these neurosteroids ***(19)***.

Moreover, a growing body of evidence has highlighted neuroprotective effects of steroids, particularly estradiol, against AD-related injury (reviewed in ***(21)***). Because the drop of estrogen in a post-menopausal woman is considered as a risk factor in AD (two-thirds of AD patients are women), the neuroprotective action of estrogen has been widely investigated (reviewed in ***(22)***). One in vivo study showed that estradiol treatment of ovariectomized female rats up-regulated enzymes involved in glycolysis and oxidative phosphorylation, and increased ATP synthase expression which was translated into an increased mitochondrial respiration ***(23)***. These findings were additionally confirmed in an AD mouse model by Yao and coworkers ***(24)***.

However, there is little evidence that other steroids are also able to act on mitochondrial function, and to our knowledge, no study has aimed to compare the effects of neurosteroids besides estradiol on cellular bioenergetics and redox environment in neuronal cells. Thus, the objective of our study was to investigate the effects of different neurosteroids on bioenergetic activity *in vitro*. For this purpose, we selected seven neurosteroids - progesterone, estradiol and estrone, belonging to the estrogen family; testosterone and 3α-androstanediol, belonging to androgen family; and DHEA and allopregnanolone - known to be involved in brain function regulation ***(12; 13; 25; 18; 19; 17)***. Neurosteroid effects on ATP production, mitochondrial membrane potential (MMP), mitochondrial respiration, glycolysis and the consequences on the modulation of the redox environment were investigated in neuronal cells.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's-modified Eagle's medium (DMEM), RPMI-1640 medium, fetal calf serum (FCS), penicillin/streptomycin, progesterone, 17β-estradiol, estrone, 3α-androstanediol, DHR, TMRM, ADP, pyruvate, succinate and malate were from Sigma-Aldrich (St. Louis, MO USA). Glutamax, MitoSOX, DPBS, Neurobasal medium and B27 were from Gibco Invitrogen (Waltham, MA, USA). DHEA and allopregnanolone were from Calbiochem (Billerica, MA, USA). PMP and XF Cell Mitostress kit were from Seahorse Bioscience (North Billerica, MA, USA). Testosterone was from AppliChem (Darmstadt, Germany). Horse serum (HS) was from Amimed, Bioconcept (Allschwil, Switzerland). RU-486, ICI-187.780, and 2-hydroxy flutamide were from Cayman Chemical (Ann Arbor, MI, USA).

2.2. 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 mM Glutamax and 1% (v/v) penicillin/streptomycin. Cells were passaged 1-2 times per week, and plated for treatment when they reached 80–90% confluence.

2.3. Primary neuronal cultures

Mouse cortical neurons were prepared from E15 embryos according to the French guidelines, as previously described ***(26)***. Cells were plated in poly-L-lysine-coated plates at a density of 1.5×10^4 cells/well for ATP measurement (white 96-well plate) or 5×10^4 cells/well for measurement with the Seahorse XF24 Analyser (XF24 cell culture microplate). After 7 days at 37 °C, 50% of the medium was replaced with fresh medium every third day. ATP level, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were investigated in this primary neuronal culture after a 24 h treatment with the different neurosteroids.

2.4. Treatment paradigm

Assessment of cell viability was performed on SH-SY5Y neuroblastoma cells to determine the potential toxic concentration range of neurosteroids (from 10 nM to 1000 nM, data not shown) and steroid receptor antagonists (SRA, from 1 nM to 1 μM, data not shown) using a MTT reduction assay (Roche, Basel, Switzerland). On the basis of the MTT results, the concentrations 10 nM and 100 nM of steroids were then selected and used in all assays. SH-SY5Y cells were treated one day after plating either with DMEM (untreated control

condition) or with a final concentration of 10 nM and 100 nM of progesterone, 17β-estradiol, estrone, testosterone, 3α-androstanediol, DHEA or allopregnanolone made from a stock solution in DMSO for 24 h (final concentration of DMSO < 0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition). In the experiment using SRA, cells were pre-treated for 1 h. with 100 nM of RU-486 and ICI-187.780, and 1 µM of 2-hydroxy flutamide (2OH-flutamide), and then treated for 24 h with the corresponding neurosteroids. To limit cell growth and to optimize mitochondrial respiration, treatment medium contained only a low amount of fetal calf serum (5% FCS) as well as glucose (1 g/l) and was supplemented with 4 mM pyruvate. Each assay was repeated at least 3 times.

2.5. 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 ***(27)***. SH-SY5Y cells were plated in 5 replicates into a white 96-wells cell culture plate at a density of 1.5×10^4 cells/well. The bioluminescent method measures the formation of light from ATP and luciferin by luciferase. The emitted light was linearly related to the ATP concentration and was measured using the multilabel plate reader VictorX5 (Perkin Elmer).

2.6. Cell proliferation assay

To verify if our treatment had an impact on cell cycle and induced proliferation, the BrdU Cell Proliferation Assay (Calbiochem, Darmstadt, Germany) was used following the instructions of the manufacturer. Briefly, SH-SY5Y cells were plated in 6 replicates into a 96-wells cell culture plate at a density of 1×10^4 cells/well. During the final 12 h of neurosteroid treatment, BrdU was added to the wells and incorporated into the DNA of dividing cells. The detection of BrdU was performed using an anti-BrdU antibody recognized by a horseradish peroxidase-conjugated anti-mouse. After addition of the substrate (TMB), the color reaction was quantified using the multilabel plate reader VictorX5 at 450nm. Values are proportional to the number of dividing cells.

2.7. Determination of mitochondrial membrane potential

The MMP was measured using the fluorescent dye tetramethylrhodamine, methyl ester, and perchlorate (TMRM). SH-SY5Y cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5×10^4 cells/well. Cells were loaded with the dye at a

concentration of 0.4 µM for 15 min. After washing twice with HBSS, the fluorescence was detected using the multilabel plate reader VictorX5 (PerkinElmer) at 530 nm (excitation)/590 nm (emission). Transmembrane distribution of the dye was dependent on MMP.

2.8. Oxygen consumption rate and extracellular acidification rate

The Seahorse Bioscience XF24 Analyser was used to perform a simultaneous real-time measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). XF24 cell culture microplates (Seahorse Bioscience) were coated with 0.1% gelatine and SH-SY5Y cells were plated at a density of 2.5×10^4 cells/well in 100 µl of the treatment medium containing 5% FCS, 1 g/l glucose and 4 mM pyruvate. After neurosteroid 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 Analyser and basal OCR and ECAR were recorded during 30 min. For primary neuronal culture, the same conditions were kept, except the medium; here DMEM was replaced by RPMI-1640 medium.

2.9. Mitochondrial respiration

The investigation of mitochondrial respiration was performed using the Seahorse Bioscience XF24 analyser. XF24 cell culture microplates were coated with 0.1% gelatine and cells were plated at a density of 2.5×10^4 cells/well in 100 µl of treatment medium containing 5% FCS, 1 g/l glucose and 4 mM pyruvate. After neurosteroid treatment, cells were washed with 1× 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; iii) FCCP (2 µM) to induce state 3 uncoupled (3u); and iv) Antimycin A/rotenone (0.5 µM and 1 µM respectively) to shut down mitochondrial respiration. 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.

2.10. $GABA_A$ receptor expression

Cells were lysed and total RNA was extracted using the RNeasy Mini Kit from Qiagen (Venlo, Netherlands), according to the instructions of the manufacturer to measure $GABA_A$ receptor (subunits α1 and β2) mRNA levels. The first cDNA strand was synthesized using all RNA extracted by reverse transcription in a final volume of 30 µl using the Ready-to-Go You-Prime First-Strand Bead cDNA synthesis kit (GE Healthcare, Little Chalfont, UK) according to the supplied protocol. After reverse transcription, the cDNA was diluted 1:3 and 3 µl were amplified by real-time PCR (StepOne™ System) in 20 µl using DyNAmy Flash Probe qPCR Kit (Thermo Scientific, Waltham, MA, USA) with conventional Applied Biosystems cycling parameters (40 cycles of 95°C, 5 s, and 60°C, 1 min). Primers for human and mouse $GABA_A$ receptor subunit α1 and β2 were obtained from Life Technologies (Waltham, MA, USA). References of the primers are: GABRA1: Hs00971228_m1; GABRB2: Hs00241451_m1; gabra1: Mm00439046_m1; and gabrb2: Mm00433467_m1. After amplification, the size of the quantitative real-time PCR products was verified by electrophoresis on 2% (wt/vol) ethidium bromide-stained agarose gel. CDK4 was used as control housekeeping gene to assess the validity of the cDNA mixture and the PCR reaction. The gene expression of CDK4 was clearly detected in SH-SY5Y (data not shown), but not that of $GABA_A$ receptor.

2.11. Reactive oxygen species detection

Total level of mitochondrial reactive oxygen species (mtROS) and specific level of mitochondrial superoxide anion radicals were assessed using the fluorescent dye dihydrorhodamine 123 (DHR) 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 1.5×10^4 cells/well. After neurosteroid treatment, cells were loaded with 10 µM of DHR for 15 min or 5 µM of MitoSOX for 90 min at room temperature in the dark on an orbital shaker. After washing twice with HBSS (Sigma), DHR, which is oxidized to cationic rhodamine 123 localized within the mitochondria, exhibits a green fluorescence that was 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 or superoxide anion radicals in mitochondria.

2.12. MnSOD activity

The DetectX Superoxide Dismutase (SOD) Activity Kit (Ann Arbor, MI, USA) was used to quantitatively measure manganese SOD (MnSOD) activity following the instructions of the manufacturer. Briefly, 1 × 10^6 SH-SY5Y cells were collected for protein extraction. After a short sonication in PBS, the cellular homogenate was centrifuged at 1.500 × g for 10 min at 4 °C. The supernatant was then centrifuged at 10,000 × g for 15 min and the obtained cell pellet was treated with 2 mM potassium cyanide, and assayed for MnSOD activity.

2.13. Mitochondrial redox environment

To investigate changes in mitochondrial redox environment, SH-SY5Y cells were transfected with a plasmid coding for a redox sensitive green fluorescent protein with a mitochondrial targeting sequence (pRA305 in pEGFP-N1). In an oxidized environment the absorption increases at short wavelengths (390 nm) at the expense of absorption at longer wavelengths (485 nm). The fluorescence ratio indicates oxidation/reduction, i.e., the redox environment in the mitochondria ***(28)***. Cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5 × 10^4 cells/well. After neurosteroid treatment, cells were washed twice with PBS and placed in a HEPES buffer (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose, and 20 mM HEPES). The ratio 390 nm/485 nm was measured using the multilabel plate reader VictorX5 detecting fluorescence at 390 nm or 485 nm (excitation)/510 nm (emission). An increase of the ratio indicates a more oxidized environment.

2.14. 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 a Dunnett's multiple comparison test versus the control. For statistical comparisons of two groups, Student unpaired t-test was used. P values < 0.05 were considered statistically significant. Statistical correlations were determined using Pearson’s correlation coefficients.

3. Results

3.1. Neurosteroids modulate mitochondrial bioenergetics

To investigate the effects of neurosteroids on cellular bioenergetic activity, we first studied the SH-SY5Y cell line, a commonly used neuronal culture *in vitro* model that

expresses a variety of neuronal receptors, including steroid receptors (progesterone, estrogen and androgen receptors) ***(29; 30)***. Cells were treated with different neurosteroids: progesterone (P), estradiol (E2), estrone (E1), testosterone (T), 3α-androstanediol (3α-A), DHEA (D) or allopregnanolone (AP), at two physiologically relevant concentrations, 10 nM and 100 nM ***(31-35)*** , and ATP level was measured after 24 h of treatment. All neurosteroids, except allopregnanolone, were able to significantly increase ATP level **(Fig. 2A)**, ranging from a 10% increase after 3α-A treatment (10 nM) up to a 22% increase induced by progesterone (100 nM) compared to the untreated control.

A pre-treatment for 1 h with different steroid receptor antagonists (SRA) including the progesterone receptor antagonist RU-486 (assay concentration 100 nM), the estrogen receptor antagonist ICI-182.780 (assay concentration 100 nM), and the androgen receptor antagonist 2OH-flutamide (assay concentration 1 µM) completely abolished the action of P, E2 and E1, as well as T and 3α-A, respectively **(Fig. 2B)**. The SRAs alone were devoid of the effects of ATP production. These results indicate that the action of neurosteroids may be mediated by nuclear receptors via gene regulation, at least for those neurosteroids that act via these receptors (progesterone, estrogens, and androgens).

To exclude that this rise in ATP was due to enhanced cell proliferation, we investigated the effects of the different neurosteroids. Of note, only allopregnanolone at 100 nM induced a significant increase of cell division by about 6% compared to the control (Table 1). Thus, our results indicate that the neurosteroid-induced up-regulation of cellular energy levels was independent of cell proliferation demands.

To verify whether the increase of ATP levels was directly linked to mitochondrial activity, we investigated the effects of neurosteroids on MMP, an indicator of the proton motive force necessary for ATP synthesis by the mitochondrial ATP synthase ***(36)***. Our results show that, at least for one of the two concentrations tested, neurosteroids induced a significant increase in MMP **(Fig. 2C)**. The low concentration of 10 nM was particularly effective, ranging from an 18% increase after estradiol treatment up to a 32% increase induced by DHEA. Again, allopregnanolone was not able to significantly modulate the MMP. Thus, the observed increase in ATP is consistent with the finding of a slight hyperpolarization of the MMP.

Because molecules of ATP are produced by two main pathways, the cellular glycolysis and oxidative phosphorylation (OXPHOS) in mitochondria, we determined whether and which of those neurosteroids were able to modulate one or both pathways. For this purpose, we simultaneously monitored in real-time the extracellular acidification rate (ECAR), an indicator of glycolysis, and the oxygen consumption rate (OCR), an indicator of basal respiration, using a Seahorse Bioscience XF24 Analyser **(Fig. 3A-C)**. On the one hand, despite a slight general increase, only estradiol and DHEA were able to significantly

modulate the ECAR after 24 h of treatment (about 16.4% and 19.4% respectively) **(Fig. 3A)**. On the other hand, our findings demonstrate that estradiol, estrone, testosterone, 3α-A and DHEA significantly increased the OCR, with the most pronounced effect detected after a testosterone treatment at 100 nM (+26.5% compared to the control) **(Fig. 3B)**. To compare the action of neurosteroids on glycolysis and basal respiration, we characterized the bioenergetic profile of SH-SY5Y neuroblastoma cells, representing OCR versus ECAR under the different treatment conditions **(Fig. 3C)**. Notably, after treatment with the neurosteroid panel cells were switched to a metabolically more active state, with a tendency to increase both, glycolytic activity (ECAR) and basal respiration (OCR).

A Pearson correlation was performed to study whether the ATP levels correlated with OCR, ECAR or MMP **(Fig. 4)**. A positive linear correlation between ATP level and OCR **(Fig. 4A)**, but not between ATP and ECAR **(Fig. 4B)** or MMP **(Fig. 4C)** was detected, suggesting that the improvement in ATP production was preferentially linked to an increase of mitochondrial respiration (oxygen consumption).

To investigate more deeply the effects of neurosteroids on mitochondrial OXPHOS, OCR was measured using permeabilized SH-SY5Y cells, which allows the evaluation of different respiratory states and the respiratory control ratio (RCR=state 3/state 4) **(Fig. 5)**. Especially testosterone significantly up-regulated the mitochondrial respiratory capacity by increasing the respiratory state 3 (ADP-dependent), state 3 uncoupled (in the absence of proton gradient after injection of FCCP) and the RCR **(Fig. 5A-C)**. The treatment with DHEA (10 nM) showed a similar effect on the RCR under these experimental conditions (Table 1), while the rest of the tested steroid compounds had no beneficial effect on RCR. Thus, our findings suggest that neurosteroids primarily act on basal respiration in neuroblastoma cells, and that testosterone and DHEA are additionally able to increase the capacity for substrate oxidation (high RCR), which is important when cells have specific or high energy demands.

Since SH-SY5Y cells and other cell lines are not as highly dependent on OXPHOS as primary cell cultures to produce ATP ***(37)***, we investigated the action of neurosteroids on primary cell cultures from mouse brain cortex. Data demonstrate that, except for the treatment with progesterone, the level of ATP was significantly increased with at least one of the two concentrations tested, ranging from a 27% increase after treatment with estrone (100 nM) up to a 59% induced increase by DHEA (10 nM) **(Fig 6A)**. Compared to the data obtained with SH-SY5Y neuroblastoma cells **(Fig. 2A)**, the magnitude of the rise in ATP concentration was higher in the primary cortical cell culture (maximal increase of 22.6% in SH-SH5Y cells versus 59.2% in primary neurons). This result implies that primary cell cultures have a greater capacity to produce ATP than neuroblastoma cells. Moreover, both concentrations of allopregnanolone were able to increase ATP level in primary cells, which

was not the case in SH-SY5Y cells. Allopregnanolone mainly acts as an allosteric positive modulator of $GABA_A$ receptor ($GABA_A$-R). To verify the implication of this receptor, we first investigated whether it was expressed in both cell types. We found that SH-SY5Y cells do not express the $GABA_A$-R subunits α1 and β2 that are involved in the allopregnanolone binding site ***(38)***, in contrast to primary cortical neurons **(Suppl. Fig. 1)**, indicating that $GABA_A$-R may be involved in the modulation of bioenergetics by allopregnanolone in neurons.

To determine whether the increase in ATP level was due to an improvement of glycolytic activity or mitochondrial respiration in this cellular model, we again performed a simultaneous real-time monitoring of the ECAR and the OCR **(Fig. 6B-D)**. We measured a significant effect on the OCR for most of the neurosteroids tested, starting with a 59% increase after treatment with testosterone (10 nM) up to a 128% increase induced by 3α-A (10 nM) **(Fig. 6B)**. Again, the magnitude of change was higher compared to the neuroblastoma cell line (maximal increase of 26.5%). In parallel, we measured a slight, but not significant, decrease in the glycolytic activity, except for the treatment with progesterone at 100 nM which in contrast induced a huge increase in the ECAR (+51.% compared to the control condition) **(Fig. 6C)**. The bioenergetic profile (OCR versus ECAR) revealed that after treatment with neurosteroids, the primary cortical neurons had the general tendency to switch to a more aerobic state **(Fig. 6D)** by increasing their oxygen consumption (OCR) and decreasing their glycolytic activity (ECAR), especially at the low concentration of 10 nM.

Taken together, these data indicate that in primary mouse neurons, most of the neurosteroids from the tested panel were able to increase ATP production via improvement of mitochondrial respiration.

3.2. Neurosteroids modulate the redox homeostasis

The increase of OXPHOS is often coupled with an increase in mitochondrial reactive oxygen species (mtROS) production ***(39; 40)***. Since neurosteroids were able to significantly increase mitochondrial respiration, we investigated whether ROS levels were also increased within mitochondria (mtROS) by measuring the oxidation of the fluorescent dye dihydrorhodamine 123 (DHR). All neurosteroids induced a significant dose-dependent increase in mtROS levels, ranging from a 43% increase after DHEA treatment (10 nM) up to a 111.3% increase induced by testosterone (100 nM) **(Fig. 7A)**. Moreover, the specific measure of mitochondrial superoxide anion radicals revealed that some of the ROS produced were indeed superoxide anions **(Table 1)**. However, the extent of mtROS production, which in excess can lead to massive oxidative stress, and finally cell death, did

not seem to be sufficient to trigger cell death under those experimental conditions (data not shown).

Therefore, we next tested the antioxidant defense system in mitochondria. We quantitatively measured the activity of the manganese superoxide dismutase activity (MnSOD), which is present within the mitochondrial matrix. Indeed, MnSOD activity was significantly increased **(Fig. 7B)** after treatment with the whole panel of neurosteroids, ranging from a 28.6% (progesterone, 100 nM) up to a 49.3% increase (testosterone, 100 nM). The increase in mtROS was paralleled by an increase of antioxidant activity. In addition, mtROS level and MnSOD activity correlated with one another **(Fig. 7C)**, suggesting that the increase of MnSOD activity was substrate-dependent.

Finally, to verify whether the mitochondrial redox environment was impacted by this increase of ROS versus increase of antioxidant defenses, SH-SY5Y cells stably transfected with a reporter gene coding for a redox sensitive green fluorescent protein (AR305 roGFP) located within mitochondria were treated with our panel of neurosteroids ***(28)***. Figure 7D displays the oxidation/reduction state in mitochondria, and indicates that, despite a slight switch toward a more oxidized state, only testosterone (100 nM) and DHEA (100 nM) significantly modified the redox environment in mitochondria.

Taken together, our data indicate that neurosteroids increased mitochondrial activity, which was paralleled by an enhancement in mtROS levels. However, cell viability was still unchanged and the raise of mtROS appeared to be at least in part compensated by an increase in antioxidant activity, which in turn led to a slight switch to an oxidized state within mitochondria.

4. Discussion

The aim of our study was to investigate the effects of seven neurosteroids on cellular bioenergetics and redox homeostasis in neuronal cells. The key findings were that: i) the majority of these steroids increased energy metabolism, mainly via an up-regulation of the mitochondrial activity and at least in part via receptor activation, and ii) neurosteroids regulated redox homeostasis by increasing the antioxidant activity as a compensatory mechanism to the ROS level enhancement which might result from the acceleration in oxygen consumption accompanied by a greater electron leakage from the electron transport chain. Additionally, each neurosteroid seems to have a specific bioenergetic profile. The single profiles are delineated as pie charts for SH-SY5Y **(Fig. 8A)** and mouse primary cortical neurons **(Fig. 8B)**.

Remarkably, each steroid doesn't seem to act in the same way on both cell types. For example, allopregnanolone, which had no effects on ATP level and basal respiration in SH-

SY5Y cells, appeared to increase those two parameters in primary neuronal cells. On the contrary, progesterone was able to increase ATP production in SH-SY5Y cells, but showed a significant effect only on glycolysis in primary cells. One explanation could be that SH-SY5Y cells and primary neuronal culture may exhibit steroid receptor expression profiles that are slightly different. Steroid receptor expression, such as that of progesterone, estrogen and androgen receptors, has already been demonstrated in both SH-SY5Y cells ***(29; 30)*** and in mouse neurons ***(41-43)***, respectively. It is known that allopregnanolone doesn't bind to a conventional steroid receptor but mainly acts as a positive $GABA_A$ receptor ($GABA_A$-R) allosteric modulator that strengthens the effects of GABA. We found that SH-SY5Y cells do not express $GABA_A$-R unlike in primary neurons **(Suppl. Fig. 1)**. This indicates that allopregnanolone may act via $GABA_A$-R to increase ATP level especially in primary neurons and explains the lack of effect on ATP in SH-SY5Y cells. Furthermore, other signaling pathways and receptors may be involved in the effects of allopregnanolone upon bioenergetics in primary cortical neurons, such as the newly characterized pregnane xenobiotic receptor ***(44)***.

Moreover, it is known that proliferative cells and tumors have a net tendency to use the cellular glycolysis to produce ATP instead of the OXPHOS system. This phenomenon is called "Warburg effect" ***(37)***. On the contrary, primary neurons, which are differentiated cells, rely almost exclusively on the OXPHOS system to produce ATP and glycolysis is really low (raw data not shown). Indeed, in the latter model, ATP level appeared to be strictly coupled with the basal respiration. The bioenergetic profile of primary cortical cells revealed that neurosteroids preferentially increased mitochondrial respiration and not the glycolytic pathway, while both pathways were increased in SH-SY5Ycells **(Fig. 3C, Fig. 6D)**.

In the recent years, neurosteroids have emerged as new potential therapeutic tools against neurodegeneration ***(45)***. Among the steroids, the family of sex steroid hormones is the most widely studied. They are in the focus of research on neurodegenerative diseases since cognitive decline and the risk to develop AD appear to be associated with an age-related loss of sex hormones (e.g. estradiol, testosterone but also progesterone) in both, women and men ***(46; 25)***, a hypothesis largely supported by epidemiological evidence ***(47)***. *In vitro* and *in vivo* studies demonstrated neuroprotective effects of sex hormones, particularly with mitochondria proposed as the primary site of action of estradiol ***(48; 32; 49; 24)***. Indeed, estrone (E1), estradiol (E2), and estriol (E3), are known to play a fundamental role in the regulation of the female metabolic system ***(50)***. It has been reported that estrogens can regulate mitochondrial metabolism by increasing the expression of glucose transporter subunits and by regulating some enzymes involved in the tricarboxylic acid cycle (TCA cycle) and glycolysis, which leads to an improvement in glucose utilization by cells

(reviewed in ***(21)***). Estrogens seem also able to up-regulate genes coding for some electron transport chain components such as subunits of mitochondrial complex I (CI), cytochrome c oxidase (complex IV), and the F1 subunit of ATP synthase. In line with these findings, our data demonstrated that both female sex hormones, estradiol (E2) and estrone (E1), were able to increase ATP levels, basal respiration, and MMP in neuroblastoma cells **(Fig. 8A)**. Of note, the increase of ATP levels induced by E2 and E1 was abolished in the presence of ICI-182.780, an estrogen receptor (α and β) antagonist **(Fig. 2B)** suggesting that estrogens, such as E2 and E1, may act via these receptors to up-regulate genes involved in cellular bioenergetics, as mentioned above. Estradiol seemed to be more potent that estrone, because both concentrations (10 nM and 100 nM) were effective to increase ATP levels and mitochondrial respiration. In addition, estradiol was able to regulate glycolysis. This difference can be explained by the observation that, despite estrone's capability as an estrogenic compound, it is about 10 times less estrogenic than estradiol ***(21)***. The same finding was observed in primary neurons **(Fig. 8B)**.

Regarding the predominantly male hormone testosterone, we witnessed an increase in ATP levels, basal respiration and mitochondrial membrane potential in neuroblastoma cells **(Fig. 8A)**. Moreover, testosterone was also the only steroid besides DHEA inducing an acceleration of the respiratory control ratio (RCR), an indicator of the capacity for substrate oxidation (high RCR), which is important when cells have specific or high energy demands. The role of androgens on mitochondrial function, especially testosterone, has received little attention up to now, compared to the estrogens. Only one study demonstrated a similar effect of testosterone on MMP ***(51)***. Furthermore, it has been proposed that estradiol and testosterone can regulate energy production by inducing nuclear and mitochondrial OXPHOS genes, since the subunits of mitochondrial chain complexes are encoded by the nuclear and the mitochondrial genome, respectively, and both contain hormone responsive elements ***(52)***. Again, those findings are in line with our results, since we have shown that the increase of ATP levels was blocked in the presence of estrogen and androgen receptor antagonists **(Fig. 2B)**.

Progesterone is the second main female sex hormone but it is also a precursor for estrogens and androgens. Progesterone, and its 3α-5α-derivate allopregnanolone (or 3α, 5α-tetrahydroprogesterone) as well as 3α-androstanediol, seem to play a role in mood modulation. Their therapeutic potential for the treatment of depression, anxiety ***(53-55)*** and more recently AD is currently under investigation ***(35)***. In the present study, we demonstrated that progesterone increased ATP levels and MMP without significant effects on basal respiration in neuroblastoma cells **(Fig. 8A)**. An increase in glycolysis was also observed

after treatment in the primary neurons **(Fig. 8B)**. Again, the up-regulatory effect of progesterone on ATP levels was shut down in the presence of the progesterone receptor antagonist RU-486 **(Fig. 2B)**, suggesting that progesterone also modulates cellular bioenergetics by regulating gene expression via a progesterone nuclear receptor. Studies performed on ovariectomized rats revealed that a 24 h treatment with progesterone (subcutaneous injection, 30 µg/kg) increased OXPHOS capacity in isolated mitochondria, in part by enhancing cytochrome c oxidase activity and expression ***(32)***. Interestingly, the increase of OXPHOS capacity was suppressed by a co-treatment with estradiol and progesterone, suggesting a competitive mode of action between both steroids. Another study using wobbler ALS (amyotrophic lateral sclerosis) model mice showed that progesterone was able to normalize the deficits in mitochondrial complex I activity observed in motor neurons of the cervical spinal cord ***(56)***. Because progesterone seems to have different functional effects, one can speculate that its action on mitochondrial respiration may be distinct to specific nerve cell populations.

Allopregnanolone and 3α-androstanediol have a distinct mode of action compared to sex hormones because they mainly act on membrane receptors (allosteric modulator of $GABA_A$-R) and not nuclear receptors ***(57)***. Their effects on mitochondrial bioenergetics cannot be explained by a direct regulation of genes involved in the OXPHOS system as previously proposed for sex hormones. In our study, 3α-androstanediol showed a similar effect compared to progesterone in the neuronal cell line, but was also able to significantly increase the basal respiration (at 100 nM) **(Fig. 8A)**. Both concentrations were effective to increase ATP and respiration in primary cells **(Fig. 8B)**. Allopregnanolone significantly regulated ATP levels and basal respiration only in primary neurons, whereas no effect was detected in the neuroblastoma cell line. Based on those observations, we can speculate that: i) $GABA_A$-R is involved in the up-regulatory effect of allopregnanolone on ATP levels in primary cells because no increase was observed in SH-SY5Y cells lacking of this receptor **(Suppl. Fig. 1)**; and ii) 3α-androstanediol could act via androgen receptor because its effect on ATP levels was abolished in the presence of an androgen receptor antagonist **(Fig. 2B)**. However, further investigations will be required to understand the exact underlying mechanisms. Besides, due to the high complexity of the neurosteroid pathway synthesis, it is difficult to conclude that the effect which we observed on bioenergetics is due to the tested neurosteroid itself, or to one of its metabolites, because they all belong to crisscross pathways **(Fig. 1)**. However, since blocking progesterone, estrogen and androgen receptors abolishes the effects of their respective agonists, we have good evidence that the neurosteroids themselves exhibit the mode of action. In the same way, we can exclude that progesterone is acting via its metabolite allopregnanolone because the latter has no effects

on bioenergetics in SH-SY5Y cells. Nevertheless, it is also possible that 3α-androstanediol doesn't act directly on androgen receptors but is converted in dihydrotestosterone, another testosterone metabolite which has high affinity for this receptor. In a similar way, DHEA can be converted in androgens and estrogens and may act via the corresponding steroid nuclear receptor.

DHEA (dehydroepiandrosterone) was the first neurosteroid identified in 1981 ***(11)***, and its physiological action involves both genomic and non-genomic mechanisms, in part via activation of androgen/estrogen receptors and allosteric modulation of NMDA receptors, respectively ***(58)***. Human studies showed an age-related decrease in DHEA levels in the brain and in the blood in relation to the age-associated cognitive decline ***(59; 17)***. *In vitro*, we showed that DHEA enhanced ATP levels and basal respiration in primary neurons **(Fig. 8B)**. A similar effect was observed in the neuronal cell line with an additional increase in MMP, glycolysis and RCR **(Fig. 8A, Table 1)**. In agreement with our findings, DHEA was able to improve mitochondrial respiration in the brain of old rats (18-24 months) which exhibited a decline in mitochondrial function when compared to young rats (8-10 weeks) ***(60)***. More specifically, DHEA stimulated the respiratory state 3 in old rats which consequently was similar to that of untreated young rats. Furthermore, DHEA increased cytochrome c content in young and old mouse brains and enhanced mitochondrial dehydrogenase activities.

Thus, the different bioenergetic profiles we observed after treatment with our panel of steroids could be explained by their distinct abilities to directly or indirectly regulate the transcription of genes involved in glycolysis and oxidative phosphorylation (probably, via steroid nuclear receptors), but also the content and activity of mitochondrial respiratory complexes. Further investigations are required to determine in more detail which genes are involved in these processes.

Mitochondria are known to be paradoxical organelles. They can be compared to a double-edged sword that, on one hand, produces the energy necessary for cell survival, and on the other hand, induces the formation of ROS that can be harmful for cells when produced in excess with mitochondria as the first target of toxicity ***(39; 40)***. In our study, the increase in ATP appeared to be coupled to an increase in MMP and improved basal respiration **(Fig. 8A)**. In parallel, we detected higher mitochondrial ROS levels, supporting the hypothesis that increased mitochondrial activity generates more ROS. The only exception was observed after treatment with allopregnanolone where we detected more ROS but no increase in ATP level, MMP, or basal respiration. We can speculate that, in this model, allopregnanolone might be able to increase ROS-producing metabolic functions via other mechanisms. But with regard to the other neurosteroids, the increase of mitochondrial ROS was paralleled by

an increase in MnSOD activity. The MnSOD is located in the mitochondrial matrix and represents one of the first antioxidant defenses against ROS produced by OXPHOS ***(61)***. Its improved activity could be in part explained by an up-regulation of gene expression and protein level of MnSOD. Indeed, in studies which focused on antioxidant effects of steroids in ovariectomized female rats, an increase of MnSOD protein level has been observed after treatment with estradiol or progesterone ***(32)***, whereas DHEA preferentially up-regulated the expression of Cu/ZnSOD ***(31)***. In orchiectomized male rats, testosterone was also able to increase MnSOD protein level compared to the control (sham operated) ***(62)***. A similar observation was made in the wobbler ALS mouse model, where MnSOD expression was elevated after treatment with progesterone ***(56)***.

In our study, the correlation between mitochondrial ROS level and MnSOD activity implies that the increase of enzymatic SOD activity might be preferentially substrate-dependent, but can be explained, at least in part, by an up-regulation of gene expression.

Based on our observation, one can speculate that pre-treatment with neurosteroids may exert a protective action against oxidative stress, possibly through a preconditioning mechanism via their ability to increase antioxidant defenses (i.e. MnSOD activity). However, in an already oxidized environment, the presence of neurosteroids may be deleterious for cells because they also appear to further increase ROS production. This observation reinforces the “critical window hypothesis” of the therapeutic use of steroids as debated recently with regard to the hormone replacement therapy in post-menopausal women ***(63)*** and implies that this kind of therapy should begin at an age when the redox system is still balanced, thus favoring the reference postulating early onset administration.

It is known that some neurosteroid levels decline during aging and are further modified in neurodegenerative conditions (i.e. AD and PD). In addition, mitochondrial dysfunction has been well-documented in aging and age-related neurodegenerative diseases ***(64)***. Steroids offer interesting therapeutic opportunities for promoting successful aging because of their pleiotropic effects in the nervous system. Our findings highlight, for the first time, up-regulatory effects of neurosteroids upon the neuronal bioenergetic activity via up-regulation of the mitochondrial oxygen consumption as a common mechanism underlying neurosteroid action. In addition, these steroids can modulate the redox homeostasis, by balancing the increase of ROS production via improved mitochondrial antioxidant activity **(Fig. 9)**. Thus, our results provide new insights in re-defining the biological model of how neurosteroids control neuronal functions. Because each steroid appeared to have a specific profile in bioenergetic outcome and redox homeostasis, the underlying mechanisms have to

be elucidated in more details in the future, as well as those in models of neurodegenerative diseases, such as AD.

Table 1: Effects of neurosteroids on cellular bioenergetics in neuroblastoma cells.

		Progesterone		Estradiol		Estrone		Testosterone		3α-androstanediol		DHEA		Allo-pregnanolone	
		10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM	10 nM	100 nM
ATP level		113.9*	122.6*	113.4*	114.6*	116*	120.4*	110.7*	118*	110.1*	111.4*	107.6*	112.4*	95.8	100.3
Cell proliferation		98.7	100.9	98.2	100.3	97.2	99.2	97.9	102.1	100.1	103.8	97.6	103.7	101.7	106.2*
MMP		120*	108.2	118.5*	118.8*	120*	119.2*	128.1*	119.9*	123.2*	109.8	132.2*	119.9*	111.5	114.2
Glycolysis		115.4	102.7	119.4*	116.1*	113.8	111.1	108.1	105.5	105.7	106.6	106.2	116.4*	102.2	101.6
Mitochondrial respiration	Basal	111	105.9	118.3*	123.2*	110.9	118.1*	115.1*	126.5*	106.9	114.1*	110.8*	106.7	99.6	109.6
	RCR	97.9	98.7	93.7	89.6	100.6	102.8	132.7*	116.5*	95.3	104	120.3*	97.81	105.7	102.1
Mitochondrial ROS	Total	108.4	151.7*	122.9	160.8*	121.8	171.3*	145.2*	211.3*	148.4*	182.8*	143.9*	200.6*	151*	207.2*
	Superoxide	100.7	104*	103	106.2*	102.8	108*	103.6*	105.8*	103.8*	105.9*	105*	106.4*	103.6*	107.4*
MnSOD activity		110.6	128.6*	120.7	136.6*	128.7	141.9*	137.4*	149.3*	134.7*	127.6	135.3*	128.1*	128.7*	147.4*
Mitochondrial redox state		105.7	110.6	108.1	108.9	104.1	109.2	109.9	113.6*	109.8	109.1	104.2	113.3*	91.7	102.5

Values represent the mean normalized on 100% of the control group (untreated). * indicates when the percentage is significantly different from the control group. MMP; mitochondrial membrane potential, RCR; respiratory control ration, ROS; reactive oxygen species, MnSOD; manganese superoxide dismutase.

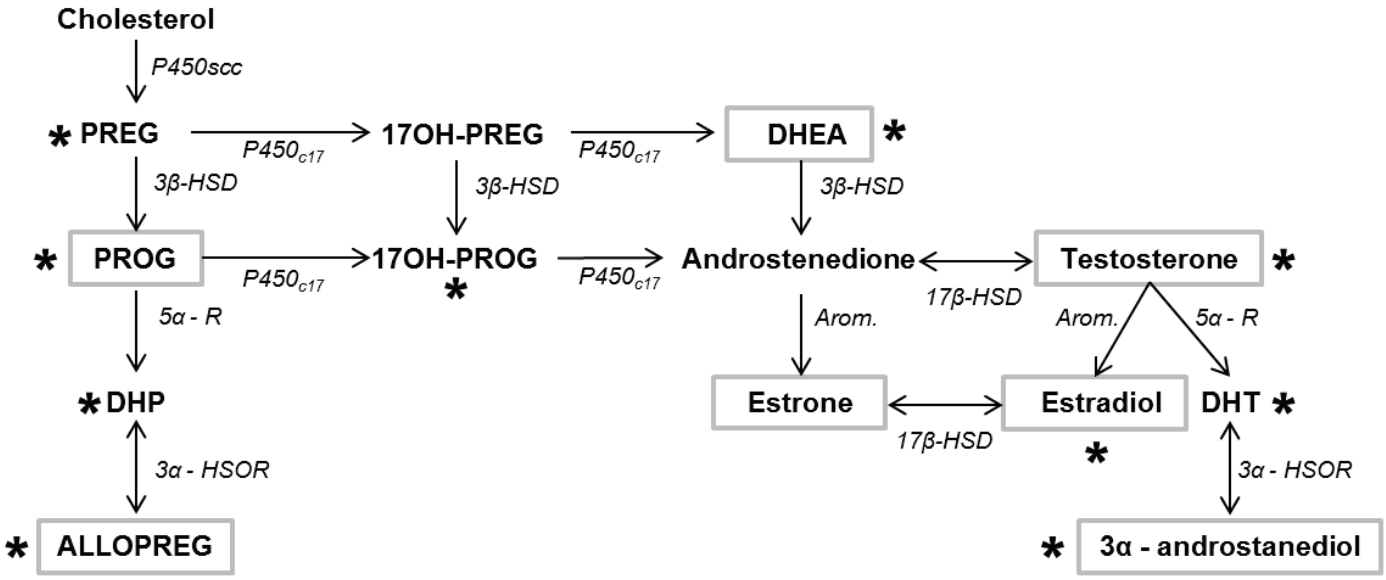


Fig. 1: Schematic representation of the main biochemical pathways for neurosteroidogenesis in the vertebrate brain. Boxes represent neurosteroids tested in our study. * indicates neurosteroids whose synthesis is impacted in AD. PREG; pregnenolone, PROG; progesterone, 17OH-PREG; 17-hydroxypregnenolone, 17OH-PROG; 17-hydroxyprogesterone, DHEA; dehydroepiandrosterone, DHP; dihydroprogesterone, ALLOPREG; allopregnanolone, DHT; dihydrotestosterone, P450scc; cytochrome P450 cholesterol side chain cleavage, P450c17; cytochrome P450c17, 3β-HSD; 3β-hydroxysteroid dehydrogenase, 5α-R; 5α-reductase, Arom.; aromatase, 21-OHase; 21-hydroxylase, 3α-HSOR; 3α-hydroxysteroid oxydoreductase, 17β-HSD; 17β-hydroxysteroid dehydrogenase

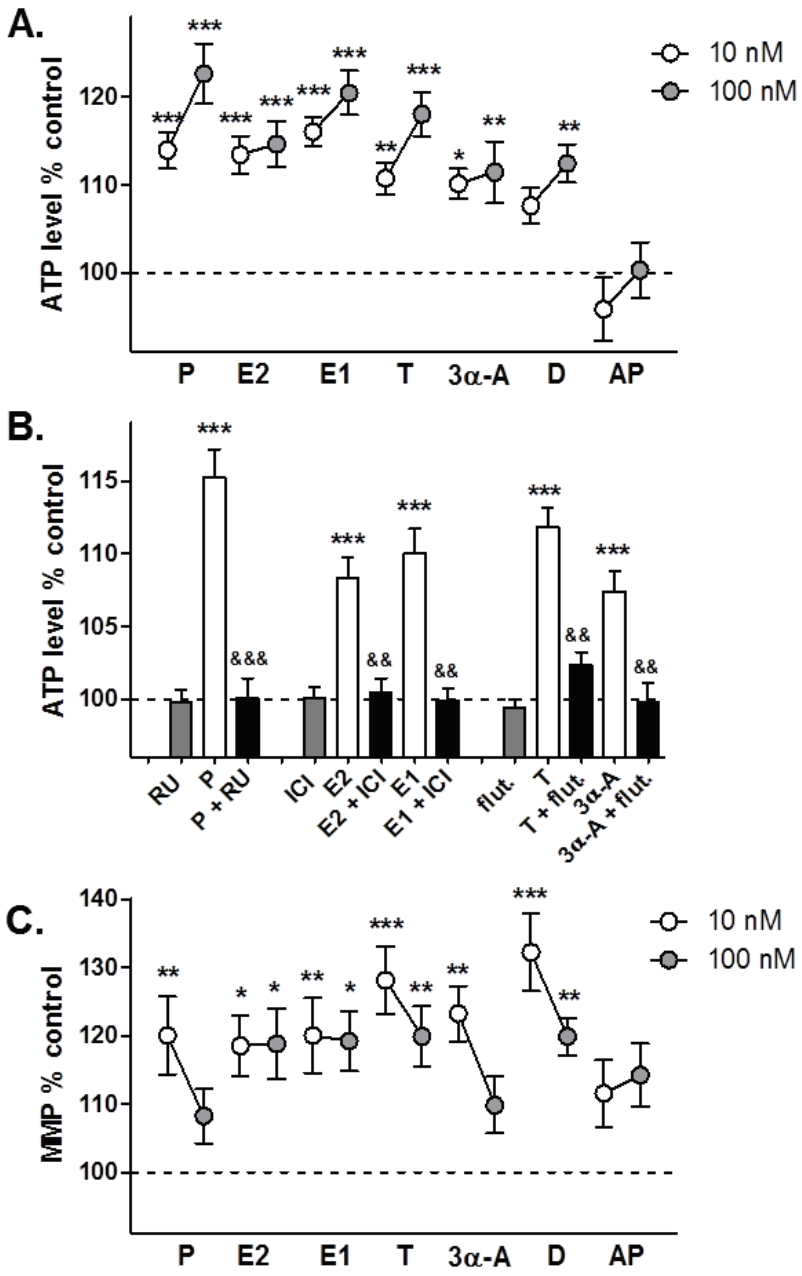


Fig. 2. Neurosteroids increase ATP level in SH-SY5Y neuroblastoma cells. (A) ATP level was significantly increased after neurosteroid treatment for 24 h at a concentration of 10 nM (white boxes) or 100 nM (gray boxes).(B) ATP level was measured after pre-treatment of cells for 1 h with either progesterone receptor antagonist RU-486 (100 nM), or estrogen receptor antagonist ICI-182.780 (100 nM), or androgen receptor antagonist 2OH-flutamide (1 μM) and then treated for 24 h with the corresponding steroid agonist. (C) Mitochondrial membrane potential (MMP) was significantly increased after neurosteroid treatment for 24 h at a concentration of 10 nM (white boxes) or 100 nM (gray boxes). (A-C) Values represent the mean ± SEM; n=12-18 replicates of three independent experiments. One-way ANOVA and post hoc Dunnetts' multiple comparison test versus control (untreated), *P<0.05; **P<0.01; ***P<0.001. Student unpaired t-test, && P<0.01; &&&P<0.001. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone, RU; RU-486, ICI; ICI-182.780, flut.; 2OH-flutamide.

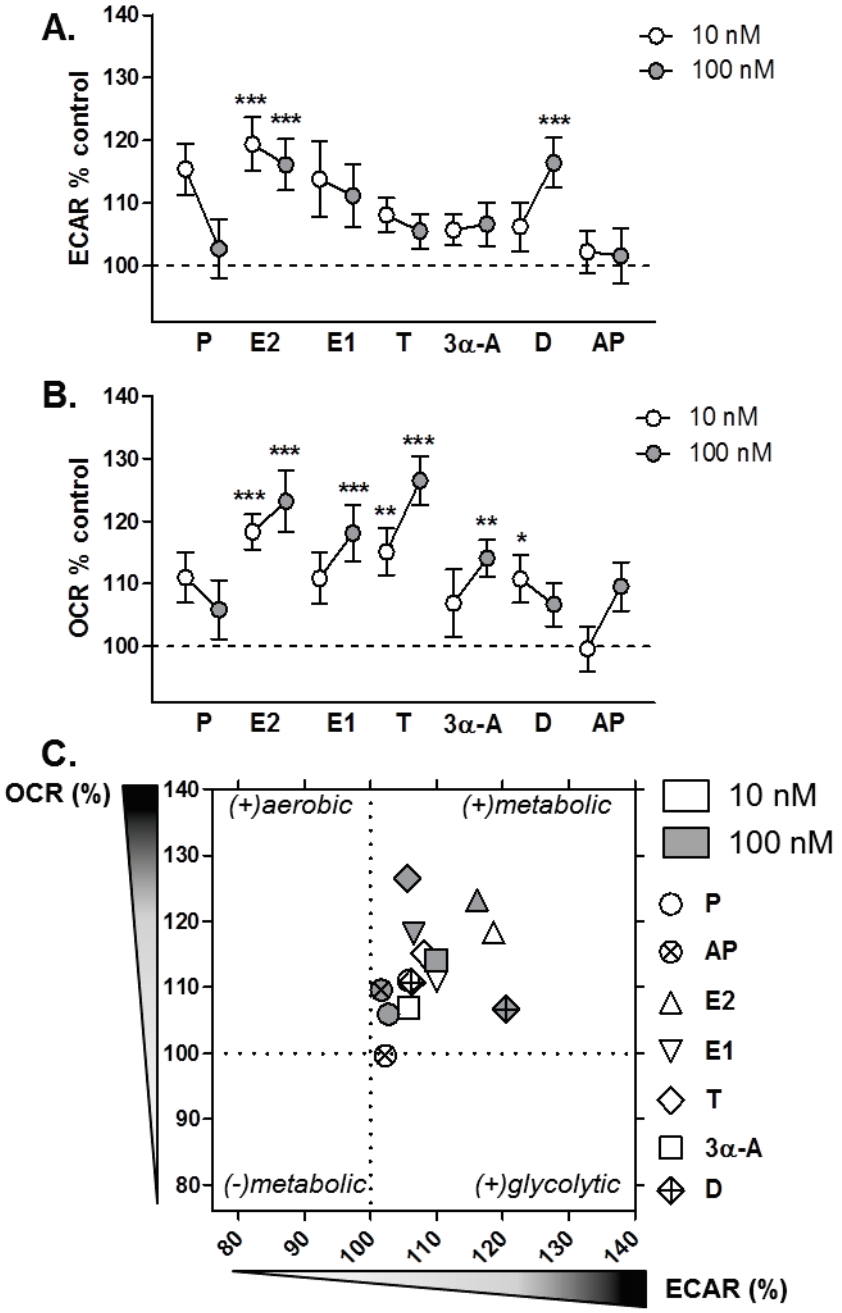


Fig. 3. Neurosteroids positively regulate bioenergetic activity in SH-SY5Y neuroblastoma cells. (A) Extracellular acidification rate (ECAR) and (B) oxygen consumption rate (OCR) were measured simultaneously using a Seahorse Biosciences XF24 Analyser in the same experimental conditions. (C) Bioenergetic profiling of SH-SY5Y cells (OCR versus ECAR) revealed increased metabolic activity after treatment with neurosteroids. Values represent the mean of each group (mean of the ECAR in abscissa/mean of the OCR in ordinate) normalized to the untreated control group (=100%). (A-C) Values represent the mean ± SEM; n=12-18 replicates of three independent experiments. One-way ANOVA and post hoc Dunnetts' multiple comparison test versus control (untreated), *P<0.05; **P<0.01; ***P<0.001; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone.

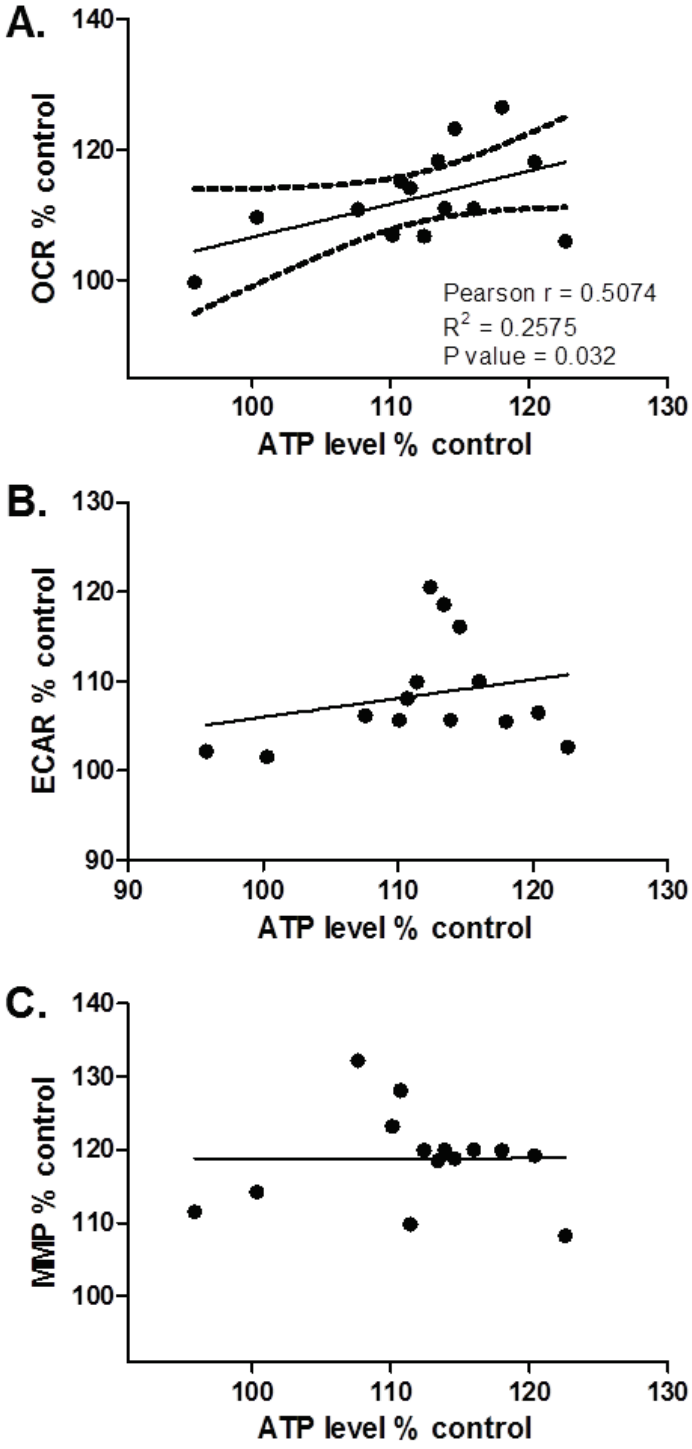


Fig. 4. ATP levels did correlate with basal mitochondrial respiration. Graph representing ATP levels in abscissa versus (A) OCR or (B) ECAR or (C) MMP in ordinate. Values represent the mean of each treatment group normalized to the control group (=100%). Pearson correlation r=0.5074, R2=0.2575, P=0.0032. OCR; oxygen consumption rate, ECAR; extracellular acidification rate; MMP; mitochondrial membrane potential.

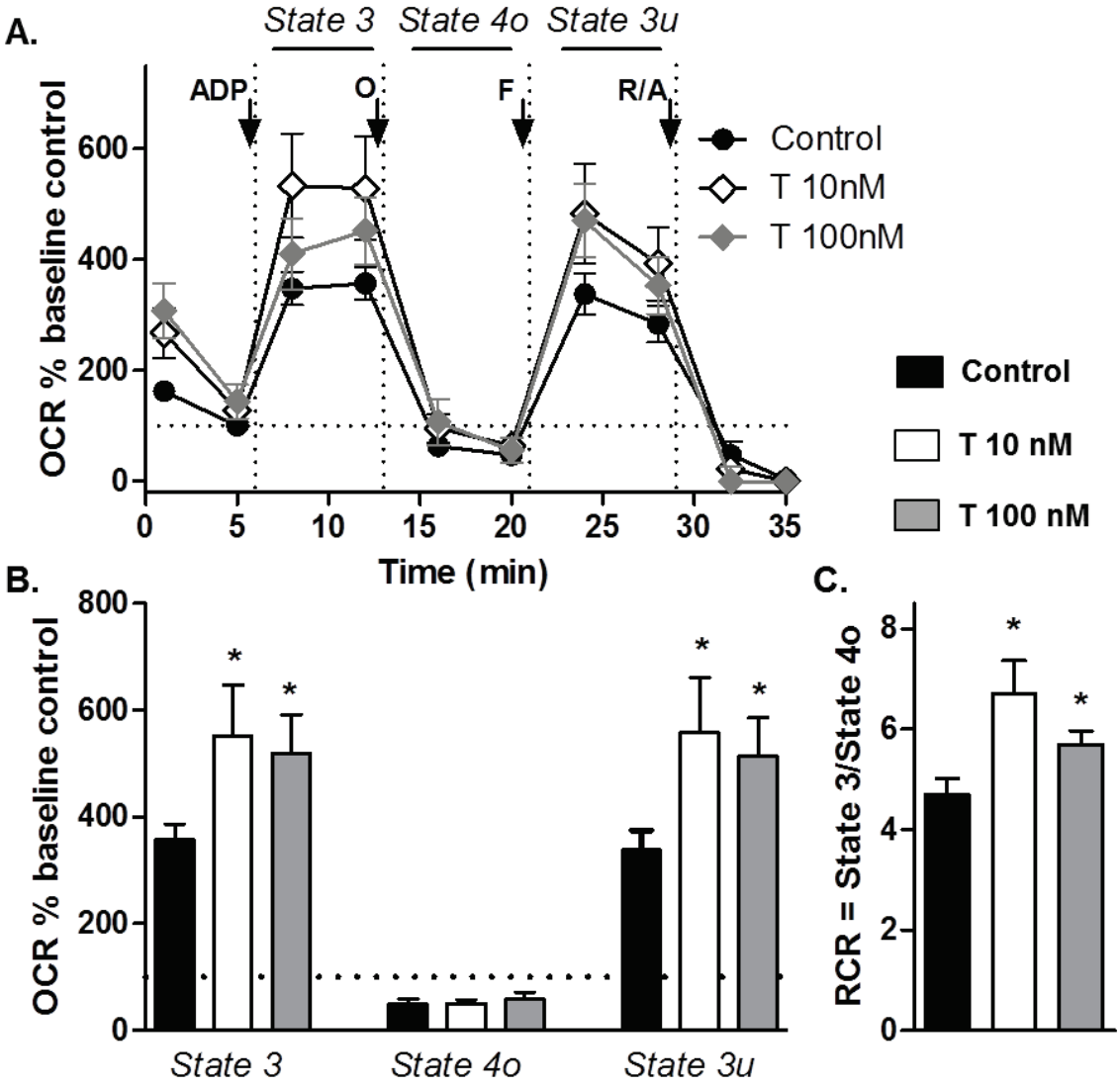


Fig. 5. Testosterone increased mitochondrial respiratory capacity. (A) Oxygen consumption rate (OCR), was measured on permeabilized SH-SY5Y cells after treatment with testosterone for 24 h, using a XF24 Analyser (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors is indicated by arrows (see details in the Materials and Methods section). (B) Values corresponding to the different respiratory states are represented as mean ± SEM (n= 15-18 replicate of three independent experiments/group) and were normalized to the basal respiration of the control group (=100%). (C) The respiratory control ratio (RCR= State 3/State 4o), which reflects the mitochondrial respiratory capacity, was increased by testosterone. Student unpaired t-test, *P<0.05. T 10 nM; testosterone at a concentration of 10 nM, T 100 nM; testosterone at a concentration of 100 nM, O; oligomycin, F; FCCP, R/A; rotenone/antimycin A.

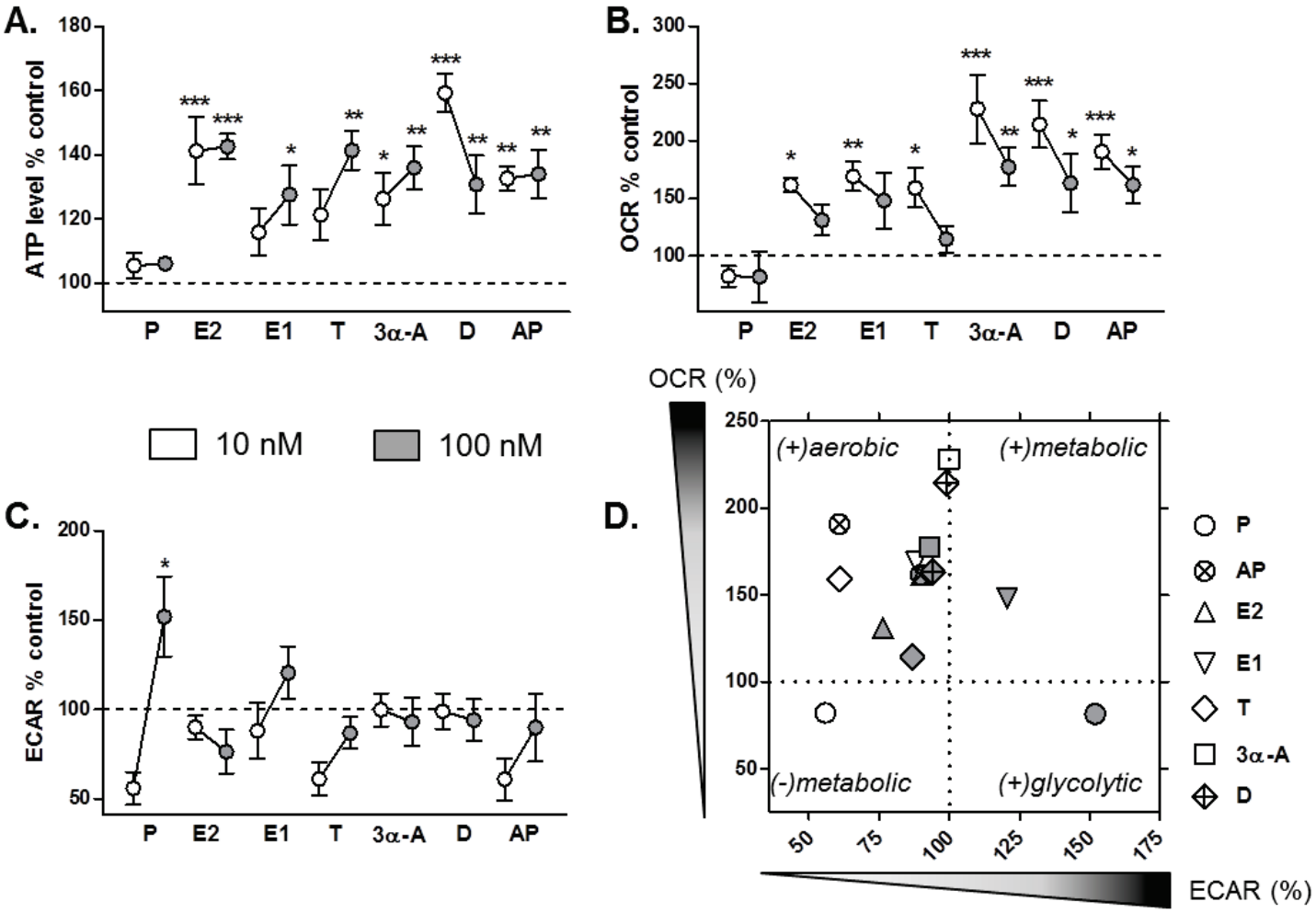


Fig. 6. Neurosteroids up-regulated the bioenergetic activity in primary cortical cells. (A) ATP level was significantly increased after neurosteroid treatment (24 h) at a concentration of 10nM (white boxes) and 100nM (gray boxes). (B) Oxygen consumption rate (OCR) and (C) extracellular acidification rate (ECAR) were measured simultaneously using a Seahorse Biosciences XF24 Analyser under the same experimental conditions. (D) Bioenergetic profile of primary cortical cells (OCR versus ECAR) revealed an increased aerobic activity (O2 consumption) after treatment with neurosteroids. 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%). (A-C) Values represent the mean ± SEM, n=4-6 replicates of three independent experiments / group, and were normalized to the control group (=100%). One-way ANOVA and post hoc Dunnetts' multiple comparison test versus control (untreated), *P<0.05; **P<0.01; ***P<0.001; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α-A; 3α-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone.

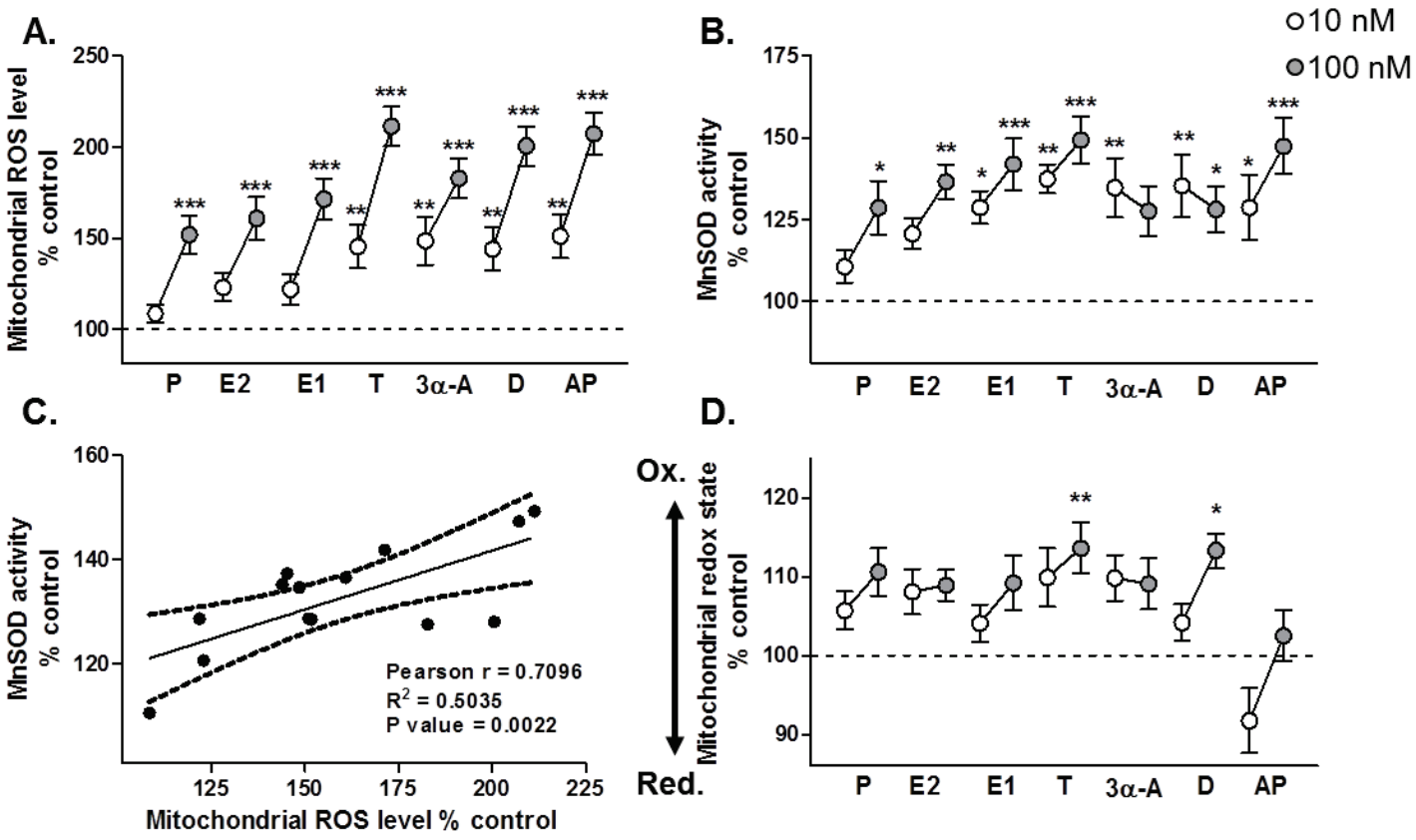


Fig. 7. Neurosteroids modulate the mitochondrial redox environment in SH-SY5Y neuroblastoma cells. (A) Mitochondrial reactive oxygen species (mtROS) levels were significantly increased after neurosteroid treatment (24 h) at a concentration of 10 nM (white boxes) and 100 nM (gray boxes). (B) This increase was accompanied by an up-regulation of manganese superoxide dismutase activity (MnSOD). (C) A positive correlation was observed between ROS levels and MnSOD activity. (D) Using a reporter gene coding for a redox sensitive green fluorescent protein (AR305 roGFP) located within mitochondria, the mitochondrial redox state underwent a switch to a more oxidized state after neurosteroid treatment compared to the untreated control. (A, B) Values represent the mean ± SEM and were normalized to the corresponding untreated control group (=100%). (C) Values represent the mean of each group (mean of the mitochondrial ROS level in abscissa / mean of the MnSOD activity in ordinate) normalized to the untreated control group (=100%). Pearson correlation r=0.7096, R2=0.5035, P=0.0022. (D) Values represent the mean ± SEM of the ratio “oxidized state/reduced state”, n=8-15 replicates of three independent experiments/group. Values were normalized to the control 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; P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3a-A; 3a-androstanediol, D; dihydroepiandrostanedione (DHEA), AP; allopregnanolone, Ox.; oxidized environment, Red.; reduced environment.

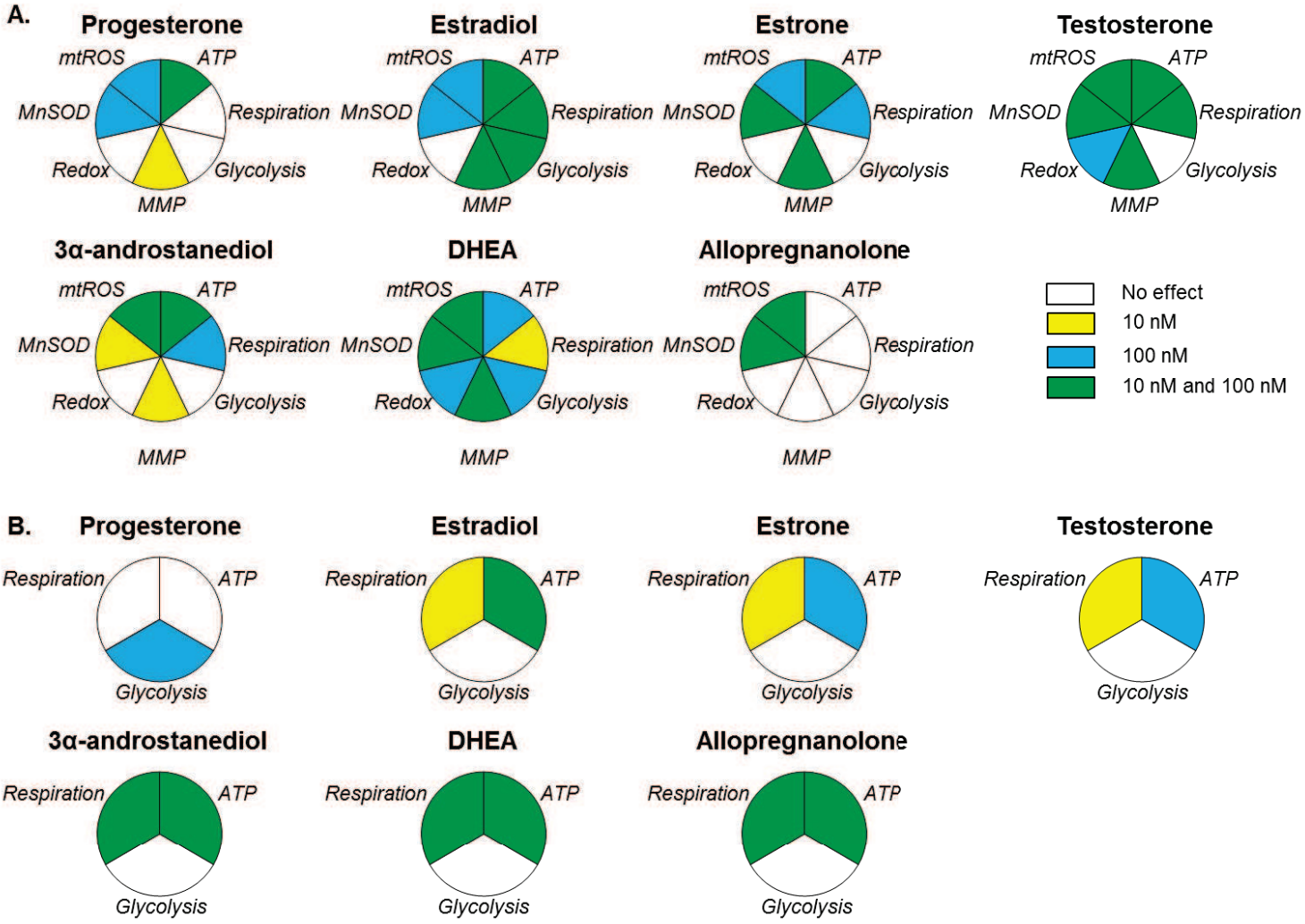


Fig. 8. Different action profile of neurosteroids on cellular bioenergetics. Representative diagrams of the effects of neurosteroids on the bioenergetic activity (ATP level, basal respiration, glycolysis, MMP) and the modulation of mitochondrial redox environment (mtROS levels, MnSOD activity, redox state) in SH-SH5Y neuroblastoma cells (A), and the bioenergetic activity in primary cortical cells (B). No effect is represented in white color. A significant increase of the respective parameter is marked either in yellow (significant only at 10 nM), blue (significant only at 100 nM), or green (significant at both concentrations). mtROS; mitochondrial reactive oxygen species, MMP; mitochondrial membrane potential, MnSOD; manganese superoxide dismutase activity.

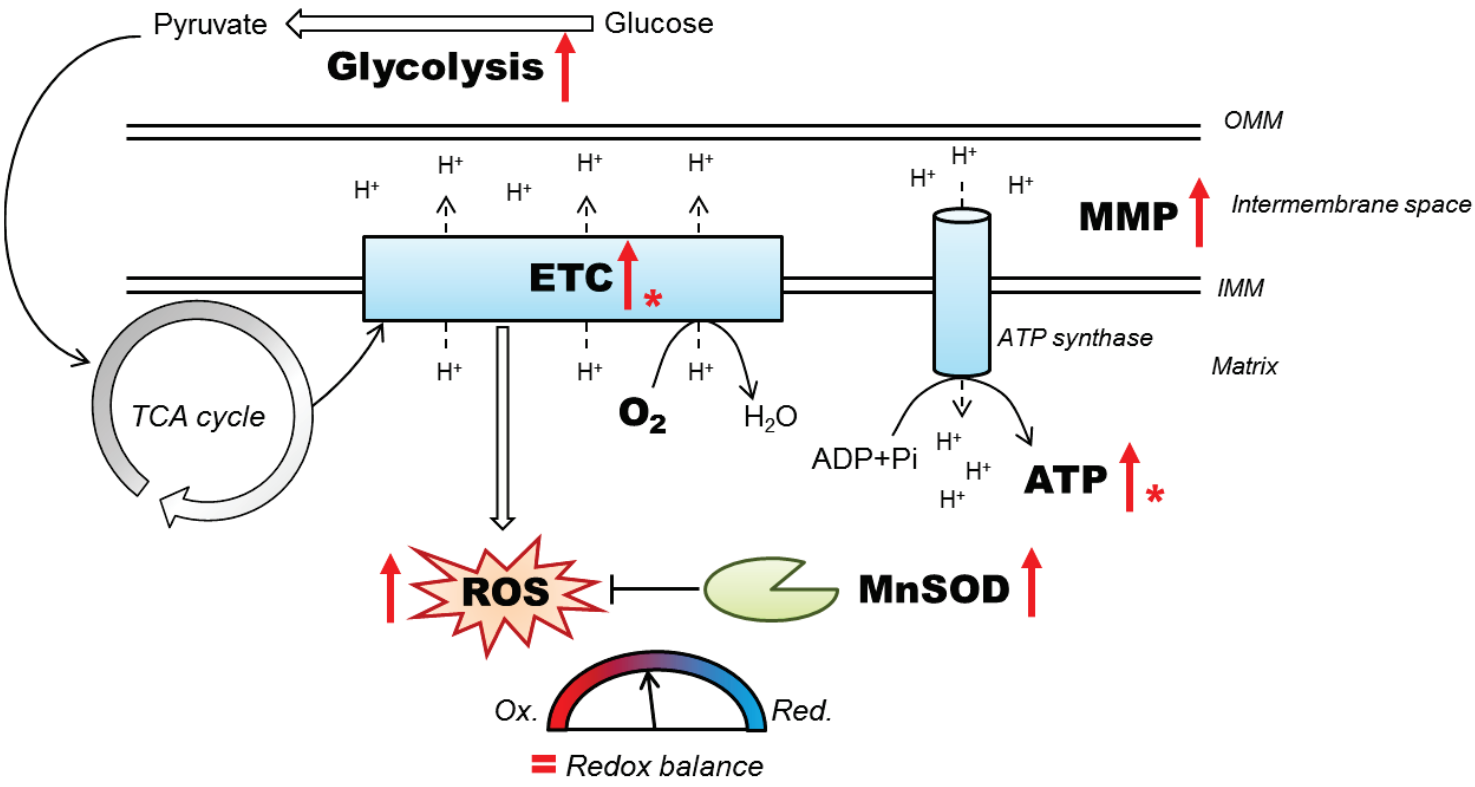
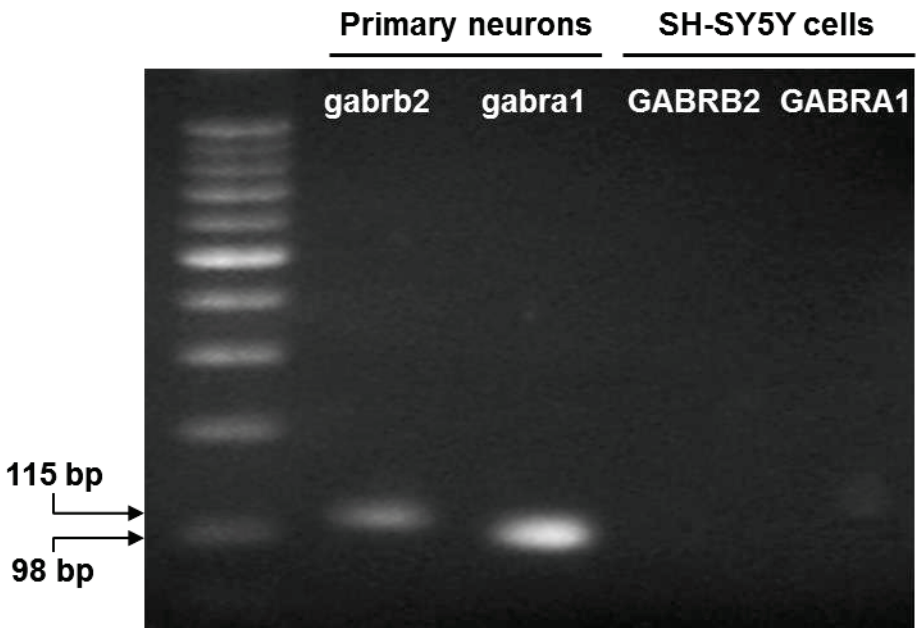


Fig. 9. Schematic representation of the effects of neurosteroids on mitochondrial bioenergetics and redox environment in SH-SH5Y neuroblastoma cells. * indicates that the effect was similar to that observed in primary cortical cells. ETC; electron transport chain, TCA; tricyclic acid, MnSOD; manganese superoxide dismutase, ROS; reactive oxygen species, MMP; mitochondrial membrane potential.



Suppl. Fig. 1. PCR analysis of GABAA receptor in SH-SY5Y human neuroblastoma cells and mouse primary cortical cells. Total RNA obtained from both cell types was amplified with the corresponding primers for human and mouse GABAA receptor subunits α1 and β2. GABRA1; human subunit α1 (82 bp), GABRB2; human subunit β2 (143 bp), gabra1; mouse subunit α1 (98 bp), mouse; gabrb2; mouse subunit β2 (115 bp).

Contributions

AG and KS performed experiments. UEL, AGMN and AE conceived the project, coordinated and supervised research. AG, AGMN and AE wrote the manuscript.

Acknowledgments

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B. Sex hormone-related neurosteroids differentially rescue bioenergetic deficits induced by Amyloid-β or hyperphosphorylated tau protein.

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(In preparation)

Abstract:

Alzheimer's disease (AD) is an age-related neurodegenerative disease marked by a progressive physical and cognitive decline. Metabolic and mitochondrial impairments are common hallmarks of AD, and amyloid-β (Aβ) peptide and hyperphosphorylated tau protein – the two foremost histopathological signs of AD - have been implicated in mitochondrial dysfunction. Neurosteroids have recently shown promise in alleviating cognitive and neuronal sequelae of AD. The present study evaluates the impact of neurosteroids belonging to the sex hormone family (progesterone, estradiol, estrone, testosterone, 3α-androstanediol) on mitochondrial dysfunction in cellular models of AD. For that purpose, we treated human neuroblastoma cells (SH-SY5Y) stably transfected with the human amyloid precursor protein (APP) that overexpress APP and Aβ, wild-type tau protein (wtTau), or mutant tau (P301L), that induces abnormal tau hyperphosphorylation for 24 hrs with neurosteroids. We showed that APP and P301L cells both display a drop in ATP levels but they present distinct mitochondrial impairments with regard to their bioenergetic profiles. The P301L cells presented a decreased maximal respiration and spare respiratory capacity, while APP cells exhibited, in addition, a decrease in basal respiration, ATP turnover and glycolytic reserve. All the neurosteroids tested showed beneficial effects on ATP production and mitochondrial membrane potential in APP/Aβ overexpressing cells while only progesterone and estradiol increased ATP levels in mutant tau cells. Of note, testosterone was more efficient to alleviate Aβ-induced mitochondrial deficits, and progesterone and estrogen were more effective in rising bioenergetic outcomes in our model of AD-related tauopathy. Our findings lend further evidence to the neuroprotective effects of neurosteroids in AD pathology and may open new avenues for the development of gender-based therapeutic approaches in AD.

Key words: Mitochondria, Neurosteroid, Bioenergetics, Amyloid-β peptide, tau protein.

Abbreviations:

3α-A, 3α-androstanediol; Aβ, Amyloid-β peptide; AD, Alzheimer's disease; APP, amyloid-β precursor protein; DMSO, dimethylsulfoxide; E1, estrone; E2, 17β-estradiol; ECAR, extracellular acidification rate; ETC, electron transport chain; MMP, mitochondrial membrane potential; OCR, oxygen consumption rate; OXPHOS, Oxidative phosphorylation; P, progesterone; P301L, tau mutation; PD, Parkinson's disease; ROS, reactive oxygen species; T, testosterone; wtTau, wild-type tau.

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease that accounts for more than 60% of all dementia cases. AD will become increasingly burdensome and costly in the coming years as prevalence is expected to double within the next two decades ***(1)***. The disease is characterized by cognitive deficits and memory loss and, from a histopathological point of view, by the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles (NFT) composed of abnormally hyperphosphorylated tau protein in the brain. Genetic studies link mutations in the amyloid-β protein precursor (APP) to familial AD (FAD) cases. These mutations lead to an increased Aβ production in the brain of AD patients ***(2)***. Interestingly no mutations in the tau coding gene have been identified so far in FAD. However, such mutations were detected in familial frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) leading to NFT formation ***(3)***. Both histopathological hallmarks of AD, Aβ and abnormal tau protein, generate problems with synaptic integration, oxidative stress, disrupting Ca^{2+} homeostasis that inexorably lead to neurodegeneration ***(4-6)***.

Mitochondria are essential components of metabolic function and serve as the "powerhouses" of cells, providing energy in the form of adenosine triphosphate (ATP) that is used by cells to power a variety of cellular processes including apoptosis, intracellular calcium homeostasis, alteration of the cellular reduction–oxidation (redox) state and synaptic plasticity ***(7; 8)***. Mounting evidence suggests that mitochondrial dysfunction serves as a catalyst in AD, since the disease is associated with a decline in bioenergetic activity and an increase in oxidative stress that can already be detected at early stages of the disease ***(9-11)***. Indeed, brain glucose hypometabolism could be observed in living AD patients even before the onset of clinical symptoms ***(12)***. This disease characteristic was also observed in AD mouse models in which mitochondrial dysfunction is already obvious before the appearance of Aβ deposits, NFT and cognitive impairments (reviewed in ***(13)***). With regard to their critical role in the early pathogenesis of AD, mitochondria therefore represent interesting targets for the development of novel treatment avenues.

Based on recent breakthroughs, interventions that concentrate on mitochondrial deficits, such as neurosteroids, may serve as potential strategies for the treatment of AD ***(14-16)***. In 1981, Baulieu and colleagues characterized a new category of steroids that are synthesized within the nervous system and are still present in substantial amounts after removal of the peripheral steroidogenic glands ***(17)***. This category of molecules was dubbed "neurosteroids". In a recent study, we characterized the bioenergetic modulating profile of seven structurally diverse neurosteroids that are known to be involved in the regulation of

brain functions ***(18-21)***, namely progesterone, estradiol, estrone, testosterone, 3α-androstanediol, DHEA and allopregnanolone. We found that most of the steroids we tested were able to improve bioenergetic activity in neuronal cells by increasing ATP levels, mitochondrial membrane potential (MMP) and mitochondrial respiration ***(16)***. Our results provide new insights in re-defining the biological model of how neurosteroids control neuronal functions and further emphasize the role of neurosteroids in neuroprotection.

In line with our study, a growing body of evidence attests to the neuroprotective effects of neurosteroids, especially estrogenic compounds, against AD-related cellular injury (reviewed in ***(15)***). However, little is known about the influence of neurosteroids on AD-related mitochondrial dysfunction. Additionally, the primary focus of neurosteroid treatment in AD has centered in the past on Aβ plaques rather than tau-related NFT.

Thus, the aim of our study was to assess whether neurosteroids of the sex hormone family could attenuate the toxic effects of Aβ and/or abnormal tau on mitochondria and also to differentiate the influence of neurosteroids on tau-related deficits independent of Aβ. For this purpose, we investigated the effects of progesterone, estradiol, estrone, testosterone and 3α-androstanediol on bioenergetics in SH-SY5Y neuroblastoma cells stably transfected with wild type human APP (APP cells) or the empty vector (Mock cells), and wild type human tau (wtTau cells) or mutated tau (P301L cells), respectively. Of note, both AD cell culture models, APP and P301L cells, exhibit the characteristics of a mitochondrial malfunction when compared to their respective controls. They had in common decreased ATP levels as well as impaired mitochondrial respiration ***(22; 23)***, but differed in underlying mechanisms. Thus, APP cells presented a defect in complex IV activity ***(22)***, whereas complex I activity was impacted by mutant tau in P301L cells ***(23)***. On the basis of our previous findings, it was therefore of utmost interest to examine whether neurosteroids are able to alleviate mitochondrial deficits manifested in these AD cellular models. In particular, their impact on ATP production, mitochondrial membrane potential (MMP), mitochondrial respiration and glycolysis was investigated.

Materials and methods

Chemicals and reagents

Dulbecco's-modified Eagle medium (DMEM), foetal calf serum (FCS), penicillin/streptomycin, progesterone, 17β-estradiol, estrone, 3α-androstanediol and pyruvate were from Sigma-Aldrich (St. Louis, MO USA). Glutamax was from Gibco Invitrogen (Waltham, MA USA). XF Cell Mitostress kit was from Seahorse Bioscience (North Billerica,

MA USA). Testosterone was from AppliChem (Darmstadt, Germany). Horse serum (HS) was from Amimed, Bioconcept (Allschwil, Switzerland).

Cell culture

APP cells were obtained by transfecting the human neuroblastoma SH-SY5Y with cDNAs (pCEP4 vector) containing either vector alone (Mock cells) or the entire coding region of human APP (APP695, APP cells) as previously described ***(22; 24)***. Stably transfected human SH-SY5Y neuroblastoma cells with wild-type (wtTau cells) and P301L mutant tau (P301L cells) were kindly provided by Jürgen Götz, Queensland Brain Institute (QBI), Brisbane, Queensland, Australia ***(25)***. 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 mM Glutamax and 1% (v/v) penicillin/streptomycin. Cells were passaged 1-2 times per week, and plated for treatment when they reached 80–90% confluence.

Treatment paradigm

Assessment of cell viability was performed on SH-SY5Y neuroblastoma cells (Mock, APP, wtTau and P301L cells) to determine the potential toxic concentration range of neurosteroids (from 10 nM to 1000 nM, data not shown) using a MTT reduction assay (Roche, Basel, Switzerland) on the basis of the MTT results, and according to previous data obtained in untransfected SH-SY5Y neuroblastoma cells ***(16)*** the concentration 100 nM was then selected and used in all assays. Cells were treated one day after plating either with DMEM (untreated control condition) or with a final concentration of 100 nM of progesterone, 17β-estradiol, estrone, testosterone or 3α-androstanediol made from a stock solution in DMSO for 24 hrs (final concentration of DMSO < 0.002%, no effect of the vehicle solution (DMSO) alone compared to the untreated condition). To limit cell growth and to optimize mitochondrial respiration, treatment medium contained only a low amount of fetal calf serum (5% FCS) as well as glucose (1 g/l) and was supplemented with 4 mM pyruvate. Each assay was repeated at least 3 times.

ATP levels

Total ATP content was determined using a bioluminescence assay (ViaLighTM HT; Cambrex Bio Science) according to the instructions of the manufacturer, as previously described ***(22)***. Cells were plated in 5 replicates into a white 96-well cell culture plate at a

density of 1.5 x 10^4 cells/well. The bioluminescent method measures the formation of light from ATP and luciferin by luciferase. The emitted light was linearly related to the ATP concentration and was measured using multilabel plate reader VictorX5 (Perkin Elmer).

Determination of mitochondrial membrane potential

The MMP was measured using the fluorescent dye tetramethylrhodamine, methyl ester, perchlorate (TMRM) ***(16)***. Cells were plated in 6 replicates into a black 96-well cell culture plate at a density of 1.5 x 10^4 cells/well. Cells were loaded with the dye at a concentration of 0.4 µM for 15 min. After washing twice with HBSS, the fluorescence was detected using the multilabel plate reader VictorX5 (PerkinElmer) at 530 nm (excitation)/590 nm (emission). Transmembrane distribution of the dye was dependent on MMP.

Mitochondrial respiration

The investigation of mitochondrial respiration and cellular glycolysis was performed using the Seahorse Bioscience XF24 analyser. XF24 cell culture microplates were coated with 0.1% gelatin and cells were plated at a density of 2.5 x 10^4 cells/well in 100 µl of treatment medium containing 5% FCS, 1 g/l glucose and 4 mM pyruvate. After neurosteroid treatment, cells were washed with 1X pre-warmed PBS and 500 µl of DMEM containing 1 g/l of glucose and 4 mM of pyruvate were added in each well. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded simultaneously before and after the sequential injection of i) Oligomycin (0.5 µM), ii) FCCP (2 µM), iii) Antimycin A and rotenone (0.5 µM and 1 µM respectively). Data were extracted from the Seahorse XF-24 software and bioenergetic parameters (basal respiration, ATP turn over, maximal respiration, spare respiratory capacity and glycolytic reserve) were calculated according to the guideline of the company.

Statistical Analysis

Data are given as the mean ± S.E.M. Statistical analyses were performed using Graph Pad Prism software. For statistical comparisons of more than two groups, one-way ANOVA was used, followed by a Dunnett's multiple comparison tests versus the control. For statistical comparisons of two groups, student unpaired t-tests were used. P values < 0.05 were considered statistically significant.

Results

APP and hyperphosphorylated tau differentially impair mitochondrial bioenergetics

To measure efficiency of mitochondrial respiration and cellular bioenergetics in APP/Aβ overexpressing cells, we simultaneously monitored in real-time the oxygen consumption rate (OCR) **(Fig. 1A)**, an indicator of mitochondrial respiration, as well as the extracellular acidification rate (ECAR) **(Fig. 1B)**, an indicator of glycolysis, using a Seahorse Bioscience XF24 Analyzer. We first performed experiments on untreated control and APP cells to record AD-related differences in OCR and ECAR readouts. A significant decrease (about 50 %) in basal respiration, ATP turnover, maximal respiration, as well as glycolytic reserve was observed in APP cells when compared to control cells **(Fig. 1C)**, paralleled by a reduction in ATP levels (-20% compared to control cells) **(Fig. 1D)**. Surprisingly, a slight increase in MMP was observed in APP cells **(Fig. 1E)**, translating to a hyperpolarization of the mitochondrial membrane potential.

The same experiments were conducted to characterize wtTau cells and P301L cells **(Fig. 2)**. No significant difference in basal respiration, ATP turnover and glycolytic reserve was found between the two cell lines **(Fig. 2A-C)**. However, wtTau cells had higher maximal respiration and spare respiratory capacity than P301L-transfected cells, indicating that mutant cells have some level of metabolic impairment, especially with regard to their mitochondrial reserve capacity **(Fig. 2C)**. ATP levels were also significantly reduced in P301L cells (-27% compared to wtTau cells) **(Fig. 2D)**, paralleled by a depolarization of mitochondrial membrane potential (decreased MMP, -10% compared to wtTau cells) **(Fig. 2E)**.

Taken together, these results confirm that APP/Aβ and hyperphosphorylated tau exhibit negative impacts on mitochondrial function leading to mitochondrial respiration deficiency and diminished ATP outcome. Since different bioenergetic parameters are impaired between APP and P301L cells, Aβ and abnormal tau appear to exert a different degree of toxicity on mitochondrial function.

Sex steroid hormones distinctively increase mitochondrial bioenergetics in APP/Aβ and tau-overexpressing cells

To address whether treatment with neurosteroids can improve mitochondrial function in AD cell culture models, ATP levels and MMP were analyzed in APP/Aβ and tau-overexpressing cells after 24 hrs of treatment (concentration 100 nM) with different steroids: progesterone (P), estradiol (E2), estrone (E1), testosterone (T) and 3α-androstanediol (3α)

(Fig. 3). In APP cells, all steroids tested were able to significantly increase ATP levels **(Fig. 3A)** as well as MMP **(Fig. 3B)**. However, in P301L cells, only P and E2 induced a significant increase in ATP levels **(Fig. 3C)**. P was particularly efficient since ATP levels in P301L were even higher (+6%) compared to those of the untreated wtTau cells. In addition, all the tested steroids, except testosterone, significantly increased MMP in P301L cells **(Fig. 3D)** with estrogens (E2 and E1) having the highest effect (+8% compared to untreated P301L cells).

To characterize the bioenergetic modulating profile of sex steroids on APP/Aβ and tau overexpressing cells, measurements with a Seahorse Bioscience XF24 analyzer were performed after 24 hrs of treatments (concentration 100 nM) **(Fig. 4-5)**. In APP/Aβ overexpressing cells, only the testosterone treated group exhibited a higher basal respiration **(Fig. 4A)**, ATP turnover **(Fig. 4B)**, maximal respiration **(Fig. 4C)**, spare respiratory capacity **(Fig. 4D)** and glycolytic reserve **(Fig. 4E)** compared to the untreated control group. In addition, 3α-androstanediol induced an improvement in the spare respiratory capacity **(Fig. 4D)** and progesterone significantly increased glycolytic reserve **(Fig. 4E)**. These data suggest that especially testosterone, the main male sex hormone, exhibits a beneficial impact on mitochondrial malfunction in AD cells modeling Aβ pathology.

Regarding the bioenergetic modulating profile of sex steroids on P301L cells, no significant changes were present in basal respiration **(Fig. 5A)**. Nevertheless, the two main female sex hormones, P and E2, significantly enhanced ATP turnover **(Fig. 5B)**, maximal respiration **(Fig. 5C)** as well as spare respiratory capacity **(Fig. 5D)** compared to the untreated P301L cells. Estrone (E1), another estrogen, was also able to significantly increase spare respiratory capacity **(Fig. 5D)** and 3α-androstanediol was the only steroid able to grow the glycolytic reserve **(Fig. 5E)** in P301L cells. Together, these data suggests that mainly female sex steroid hormones, P and E2/E1, improve mitochondrial bioenergetics in cells modeling tau pathology.

A full analysis of preference for oxidative phosphorylation as indicated by percent of OCR dedicated to ATP turnover **(Fig. 6A, C)** or spare respiratory capacity **(Fig. 6B, D)**, versus the use of glycolytic reserves was performed in both APP/Aβ and tau-overexpressing cells. Tendency for greater or lesser metabolic activity is displayed on a second axis. Overall, APP cells were switched to a metabolically more active state after treatment with androgenic compounds (T and 3α), with a tendency to increase both glycolytic reserve (ECAR) and ATP turnover/spare respiratory capacity (OCR) **(Fig. 6A-B)**. In P301L cells, ATP turnover and spare respiratory capacity were enhanced by progesterone (P) and estrogenic compounds (E1 and E2), leading to a more aerobic state **(Fig. 6C-D)**.

Taken together, these results indicate that distinct sex steroid hormones are able to improve mitochondrial bioenergetics in APP/Aβ and tau-overexpressing cells by increasing

ATP levels, MMP and mitochondrial respiration which contribute to the alleviation of mitochondrial deficits observed in those cell lines.

Discussion

In this study, we distinguished the effects of several neurosteroids on ATP synthesis, MMP, mitochondrial respiration and glycolysis in two AD cellular models. Key findings were that: i) APP/Aβ and mutant tau-overexpressing cells present distinct bioenergetic impairments, with APP/Aβ having the strongest deleterious effect on mitochondrial function; ii) the male steroid hormone, testosterone, was more efficient to alleviate mitochondrial deficits in a model of AD-related amyloidopathy, whereas female steroid hormones, progesterone and estrogen, were more efficient to increase bioenergetic outcomes in a model of AD-related tauopathies.

Remarkably, bioenergetic profiles were differentially impacted in APP/Aβ-overexpressing cells and abnormally hyperphosphorylated tau-overexpressing cells. Only the maximal respiration and spare respiratory capacity were reduced in P301L cells, while APP cells presented, in addition, a decrease in basal respiration, ATP turnover and glycolytic reserve. A drop in ATP levels was observed in both cell lines as well as a decreased MMP in P301L. Interestingly, APP cells presented a slight hyperpolarized mitochondrial membrane compared to the control Mock cells. This characteristic was previously observed in PC12 cells overexpressing APP bearing the Swedish mutation (APPsw), another cellular model mimicking Aβ pathology ***(26)***. The authors hypothesized that this hyperpolarization may be due to the increased nitric oxide levels present in this cell line where Aβ production is enhanced. Of note, Aβ secretion was similar in wild type APP-overexpressing human SH-SY5Y cells used in the present study and in APPsw-overexpressing PC12 cells within the low nanomolar range ***(26; 24)***, whereas higher Aβ levels obviously lead to MMP depolarization ***(26)***.

The different bioenergetic output observed between APP/Aβ and abnormal tau overexpressing cells can be explained by the fact that Aβ and tau differentially target mitochondria. Indeed, previous data of our group showed that APP cells present a decreased mitochondrial complex IV activity ***(22)***, whereas complex I activity was impacted in P301L cells ***(23)***. Moreover, we showed that Aβ peptide and abnormally hyperphosphorylated tau protein may act synergistically to trigger mitochondrial dysfunction in a triple transgenic mouse model of AD (^{triple}AD) obtained after crossing P301L tau transgenic mice with APPswPS2 double-transgenic mice ***(27)***. The investigation of oxidative phosphorylation

(OXPHOS) activity revealed that deregulation of complex I activity was related to tau, whereas deregulation of complex IV activity was dependent on Aβ.

Thus, on one hand, the lower complex I activity observed in P301L cells may lead to a decreased ability to reach maximal respiration, which reduces the spare respiratory capacity of the cells. On the other hand, the reduced complex IV activity, which is directly involved in oxygen consumption, may decrease additional respiratory parameters, including ATP turnover in APP cells.

A treatment with sex steroid hormones was able to alleviate bioenergetic impairments observed in APP and P301L cells in general. More precisely, the male hormone testosterone was able to enhance all the bioenergetic parameters that were impaired in APP/Aβ overexpressing cells, namely basal respiration, ATP turnover, maximal respiration and cellular glycolysis. In contrast, treatment with female hormones improved maximal respiration and spare respiratory capacity, two bioenergetic parameters that were disturbed in P301L cells. ATP turnover is an indication of the coupling efficiency that is directly linked to ATP production in mitochondria. Bioenergetic profile revealed that male and female steroid hormones were able to differentially increase ATP synthesis in APP and P301L cells respectively. The spare respiratory capacity and glycolytic reserve give an indication of the ability of cells to respond to stress under conditions of increased energy demand ***(28)***. Spare respiratory capacity was increased by estrogens and progesterone in P301L cells, whereas both parameters were enhance after treatment with androgen in APP cells. Together, data indicated that the cells were switched to a metabolically more active state, with a tendency to increase both ATP synthesis and metabolic reserves.

The ability of neurosteroids to modulate cellular bioenergetics was the focus of a recent study in our group. In particular, we showed that neurosteroids, including steroids that were investigated in the present study, were able to increase ATP levels and mitochondrial respiration in native SH-SY5Y neuroblastoma cells and mouse cortical neurons ***(16)***. Moreover, we showed that the effects we observed were, at least in part, mediated by steroid (progesterone, estrogen and androgen) receptor activation since the inhibition of those receptors by specific antagonists shut down the effects of the corresponding steroid ligand on ATP production.

Steroid receptors are nuclear receptors involved in the regulation of gene expression. With regards to bioenergetics, estrogens have been shown to up-regulate genes coding for some electron transport chain components such as subunits of mitochondrial complex I (CI), cytochrome c oxidase (complex IV), and the F1 subunit of ATP synthase, but also glucose transporter subunits, enzymes involved in the tricarboxylic acid cycle (TCA cycle) and glycolysis, leading to increased glucose utilization and mitochondrial respiration (reviewed in

(15)). Of note, since mitochondrial genome itself contains hormone responsive elements, it has been proposed that estradiol and testosterone can regulate energy production by inducing mitochondrial oxidative phosphorylation (OXPHOS) genes encoded in the mitochondrial DNA ***(29)***. In a similar way, progesterone has been shown to increase complex IV and V (ATP synthase) expression, paralleled by enhanced mitochondrial respiratory activity ***(30)***. With regards to the results obtained in the present study, we can speculate that the underlying mechanisms are similar and that the effects we observed are mediated, at least in part, by an increased expression of genes involved in OXPHOS and glycolysis. Further investigations will be needed to identify in detail which genes are concerned.

It is interesting to observe that sex steroid hormones didn't have the same modulating profile upon bioenergetic in presence of Aβ-related or tau-related mitochondrial dysfunction. Estrogens and progesterone seem to confer beneficial effects on mitochondrial-related dysfunction in tau pathology, whereas testosterone was more efficient alleviating mitochondrial deficits in APP/Aβ overexpressing cells. These findings may imply that women and men differentially answer to mitochondrial insults mediated either by Aβ or by abnormal tau. Epidemiological studies revealed that two thirds of AD patients are women and the sudden drop of estrogen levels after the menopause has been proposed to be one risk factor in Alzheimer's disease (AD) ***(31; 32)***. Indeed, estradiol is the major product of estrogen biosynthesis and it remains the most abundant estrogen in a woman's pre-menopausal life. After menopause, women have comparable levels of estradiol compared to men, and it is at this time that women become more susceptible to AD. Thus, the precipitous decline of estrogens during menopause may contribute to AD onset as well as a greater vulnerability to the disease in women ***(15)***. Men, in contrast, present with a gradual reduction in testosterone over the life course eliminating approximately 2% of circulating testosterone every year ***(33)***. However, human and animal studies also suggested that androgen deprivation represents a risk factor for AD pathogenesis ***(34-36)***. Notably, in a triple transgenic mouse model of AD (3xTgAD), it has been shown that orchiectomized males presented an increased Aβ accumulation in the brain, coupled with impaired cognitive performances compared to sham operated mice ***(37)***. Treatment with androgens significantly attenuated the increase in AD pathology ***(37; 35)***. Further studies have indicated that advancing age in men enhances tau hyperphosphorylation consistent with AD pathology ***(38)***. These findings confirm that steroid influence on tau remains a promising, but understudied research avenue in AD. Of note, no sex predilection has been identified in patients with FTDP-17, a disease characterized by a strictly tau-dependent pathology ***(39; 40)***. This might suggest that the relationship between hormonal loss, in both women and men, and the risk to develop AD may be preferentially linked to Aβ pathology and not tau.

A few studies have focused on the impact of steroids on abnormal tau. Liu and colleagues ***(41)*** discovered that protein kinase A may initiate phosphorylation of tau, and estradiol treatment of human embryonic kidney cells attenuated protein kinase A activity and reduced tau phosphorylation. Estradiol also exhibited rescue of aberrant tau in primary rat cortical neurons and SH-SY5Y neuroblastoma cells ***(42)***. Additional studies showed that both estrogen and progesterone were able to modulate activities of kinases and phosphatases involved in the regulation of tau phosphorylation, possibly by modulating glycogen synthase kinase (GSK) pathway ***(34)***. Specifically, estrogen appeared to reduce GSK-3β activity and progesterone decreased the expression of both GSK-3β ***(42; 34)***. These results imply that estrogen and progesterone can function along the same or disparate molecular pathways to modify abnormal tau protein. Regarding AD-linked Aβ pathology, studies have focused on the impact of estrogen on the deposition and clearance of Aβ ***(43; 44)***. Both estrone and estradiol decreased polymerization and stabilization of Aβ ***(45; 44)***. Other studies indicate that deficiencies in estrogen-related steroids can exacerbate Aβ plaques in AD mouse models and that treatment with estradiol was able to reduce Aβ burden, possibly via the increase of non-amyloidogenic pathways of APP processing ***(43; 46)***. The effects of progesterone on Aβ deposition and clearance are less investigated, but a recent study showed that progesterone and estradiol encouraged an increase in the expression of Aβ clearance factors *in vitro* and *in vivo* ***(47)***.

In the present study, testosterone ameliorated the effects of mitochondrial dysfunction caused by APP/Aβ but not abnormal tau. In our previous study investigating neurosteroid effects on bioenergetics in physiological conditions, the testosterone metabolite, 3α-androstanediol, presented an effect similar to its precursor and was able to increase MMP, ATP levels and mitochondrial respiration in untransfected neuroblastoma cells and primary cortical cells ***(16)***. Here, 3α-androstanediol was less efficient to alleviate bioenergetic deficits in APP cells, suggesting a distinct mode of action compared to testosterone. Neuroprotective effects of testosterone on hyperphosphorylated tau are less well-characterized in the literature. Testosterone appears to prevent tau hyperphosphorylation in an *in vivo* model of heat shock induced phosphorylation through GSK-3β signaling inhibition ***(38)***. Interestingly, Rosario and colleagues (2010) ***(36)*** revealed less abnormal tau accumulation in gonadectomized male 3xTgAD mice treated with testosterone or estradiol, but not the testosterone metabolite dihydrotestosterone (DHT) ***(36)***. This implies that testosterone may exert indirect effects on tau hyperphosphorylation via its conversion to estradiol by the enzyme aromatase and by acting on estrogen receptors. In the same model, testosterone and DHT were able to decrease Aβ deposits with a higher efficiency than estradiol, suggesting an androgen receptor-dependent mechanism. Overk and colleagues (2013) ***(48)***

examined basal levels of serum and brain testosterone in male 3xTgAD mice and found that testosterone levels rise with disease progression. This increase in testosterone in aged male 3xTgAD mice was correlated with reduced Aβ plaque pathology. This suggests that testosterone may have some neuroprotective benefits on the AD disease course, but that testosterone administration is associated more with lesser Aβ protein burden rather than abnormal tau protein. In fact, testosterone has been shown to alter processing of amyloid precursor protein and enhances expression of neprilysin, an enzyme responsible for Aβ degradation ***(49)***.

The decrease in sex steroid hormones was proposed to be one risk factor of AD in both men and women. However, there is little information concerning changes of steroid levels in the human brain during ageing and under dementia conditions. Steroid hormone originate from the endocrine glands can freely pass through the blood brain barrier and act on nervous tissues. Since steroids can also be synthesized within the nervous system, changes in blood levels of steroids with increasing age do not necessarily reflect changes in brain levels. Schumacher and colleagues (2003) have quantified the level of different neurosteroids in various brain regions of aged AD patients and aged non-demented controls ***(21)***. They showed a general trend towards lower neurosteroid levels in AD patients. Additionally, neurosteroid levels were negatively correlated with Aβ and abnormal tau in some brain regions, suggesting a link between neurosteroid homeostasis and AD pathogenesis. Our experiments have shown a rescue of metabolic dysfunction in models of AD-linked tauopathies and amyloidopathies with neurosteroids belonging to the sex hormone family. Taking into account the data available in the literature and in our previous study, this rescue may possibly occur at two levels: i) neurosteroids can directly boost mitochondrial function via gene regulation; ii) neurosteroids can act to decrease Aβ accumulation and NFT formation, which alleviates mitochondrial impairments induced by Aβ and tau. An interaction between these mechanisms cannot be excluded. Experiments dissecting the mechanistic pathways of neurosteroid function underlying the gender differences in AD are further potential research paths for the better understanding of how neurosteroids impact mitochondrial function in AD. Ultimately, our research will potentially open new avenues for the development of gender-based therapeutic approaches in AD.

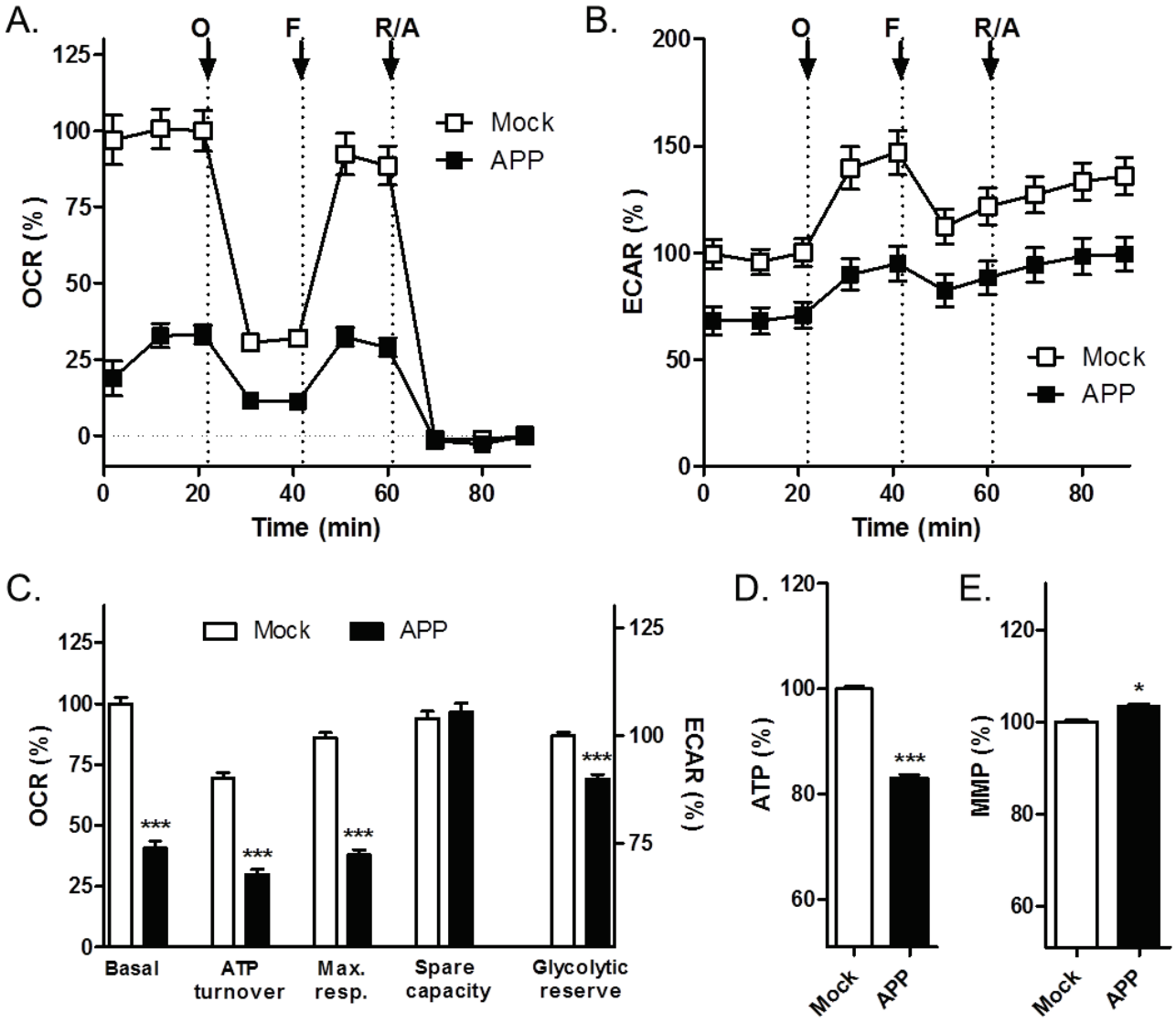

Fig. 1: Characterization of bioenergetic deficits in APP cells. (A) Oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) of Mock and APP cells were simultaneously measured using a XF24 Analyser (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors is indicated by arrows (see details in the Materials and Methods section). (C) Values corresponding to the different bioenergetic parameters are represented as mean ± SEM (n= 8-10 replicates). (A-C) Values were normalized to the basal OCR and ECAR of Mock cells (=100%). (D) ATP levels and (E) mitochondrial membrane potential (MMP) in Mock and APP cells. Values represent the mean ± SEM (n=12-18 replicates of three independent experiments) and were normalized to 100% of Mock cells. Student unpaired t-test, *P<0.05; ***P<0.001. O; oligomycin, F; FCCP, R/A; rotenone/antimycin A.

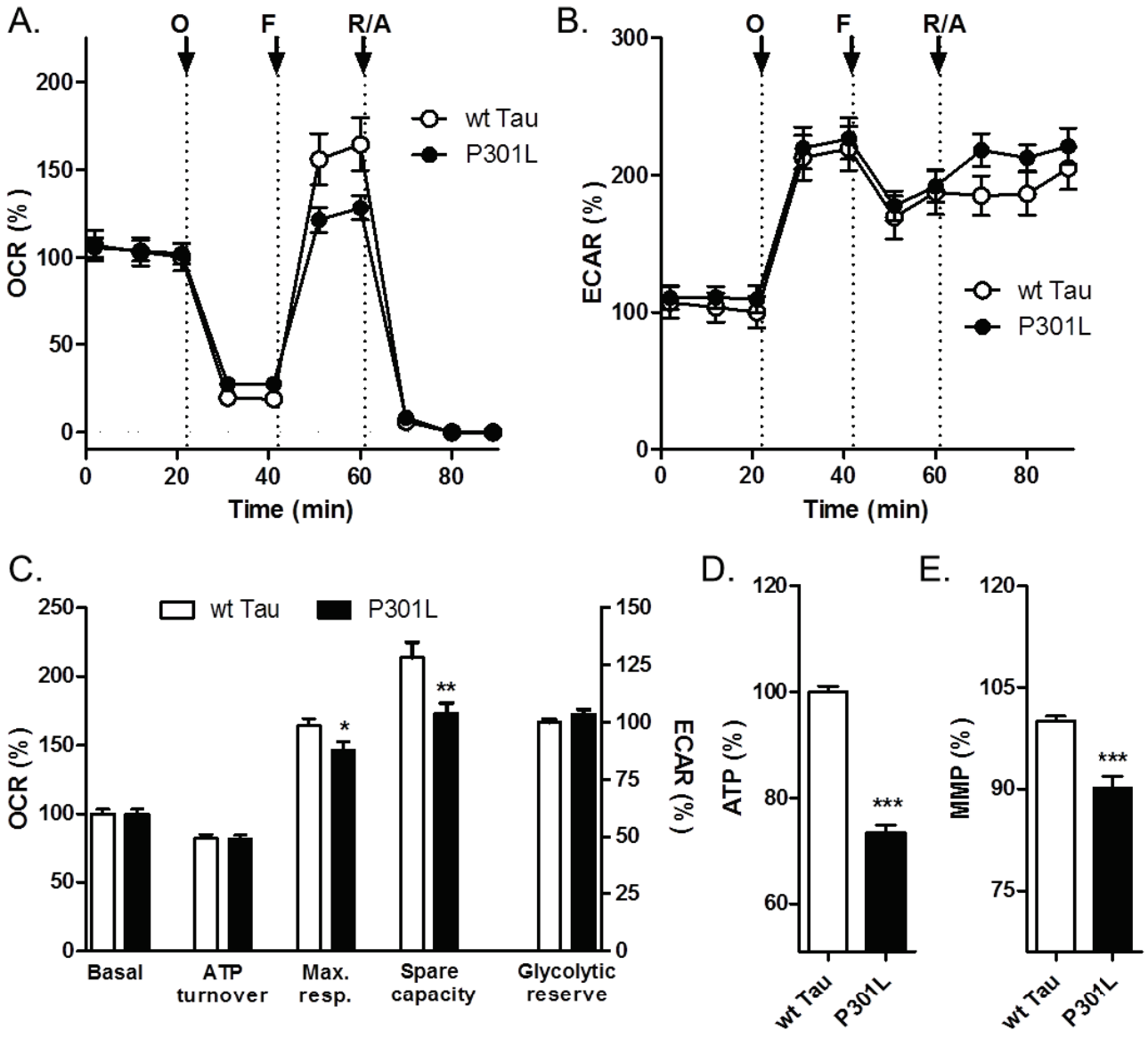

Fig. 2: Characterization of bioenergetic deficits in P301L cells. (A) Oxygen consumption rate (OCR) and (B) extracellular acidification rate (ECAR) of wtTau and P301L cells were simultaneously measured using a XF24 Analyser (Seahorse Bioscience). The sequential injection of mitochondrial inhibitors is indicated by arrows (see details in the Materials and Methods section). (C) Values corresponding to the different bioenergetic parameters are represented as mean ± SEM (n= 8-10 replicates). (A-C) Values were normalized to the basal OCR and ECAR of wtTau cells (=100%). (D) ATP levels and (E) mitochondrial membrane potential (MMP) in Mock and APP cells. Values represent the mean ± SEM (n=12-18 replicates of three independent experiments) and were normalized to 100% of wtTau cells. Student unpaired t-test, *P<0.05; ***P<0.001. O; oligomycin, F; FCCP, R/A; rotenone/antimycin A.

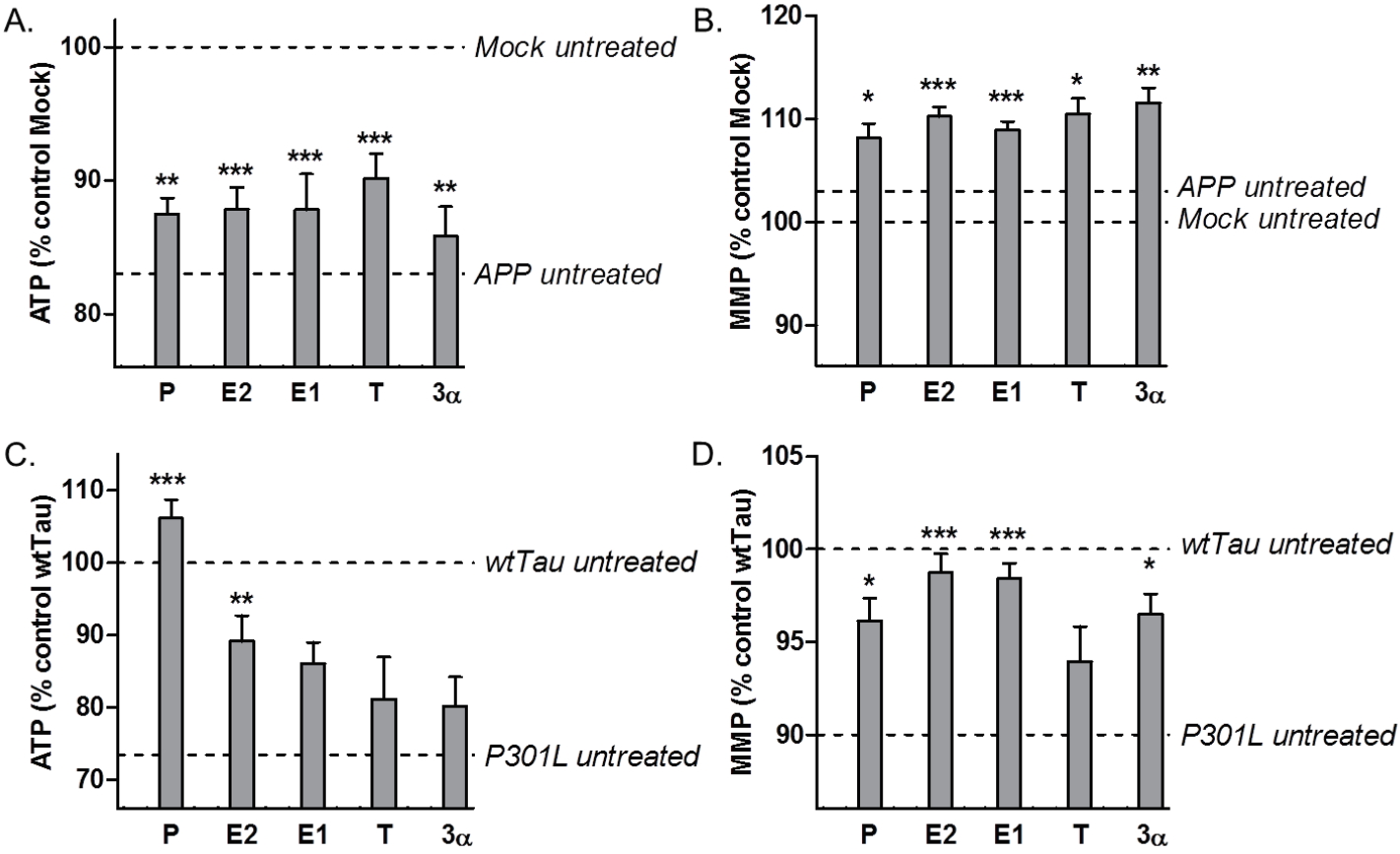

Fig. 3: Neurosteroids increase ATP level and MMP in APP and P301L cells. ATP levels and MMP were measured after neurosteroid treatment for 24 hrs at a concentration of 100 nM in APP cells (A-B) and P301L cells (C-D) respectively. Values represent the mean ± SEM (n=12-18 replicates of three independent experiments) and were normalized to 100% of untreated Mock cells (A-B) or untreated wtTau cells (C-D). One-way ANOVA and post hoc Dunnett's multiple comparison test versus untreated Mock or wtTau, *P<0.05; **P<0.01; ***P<0.001. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α; 3α-androstanediol.

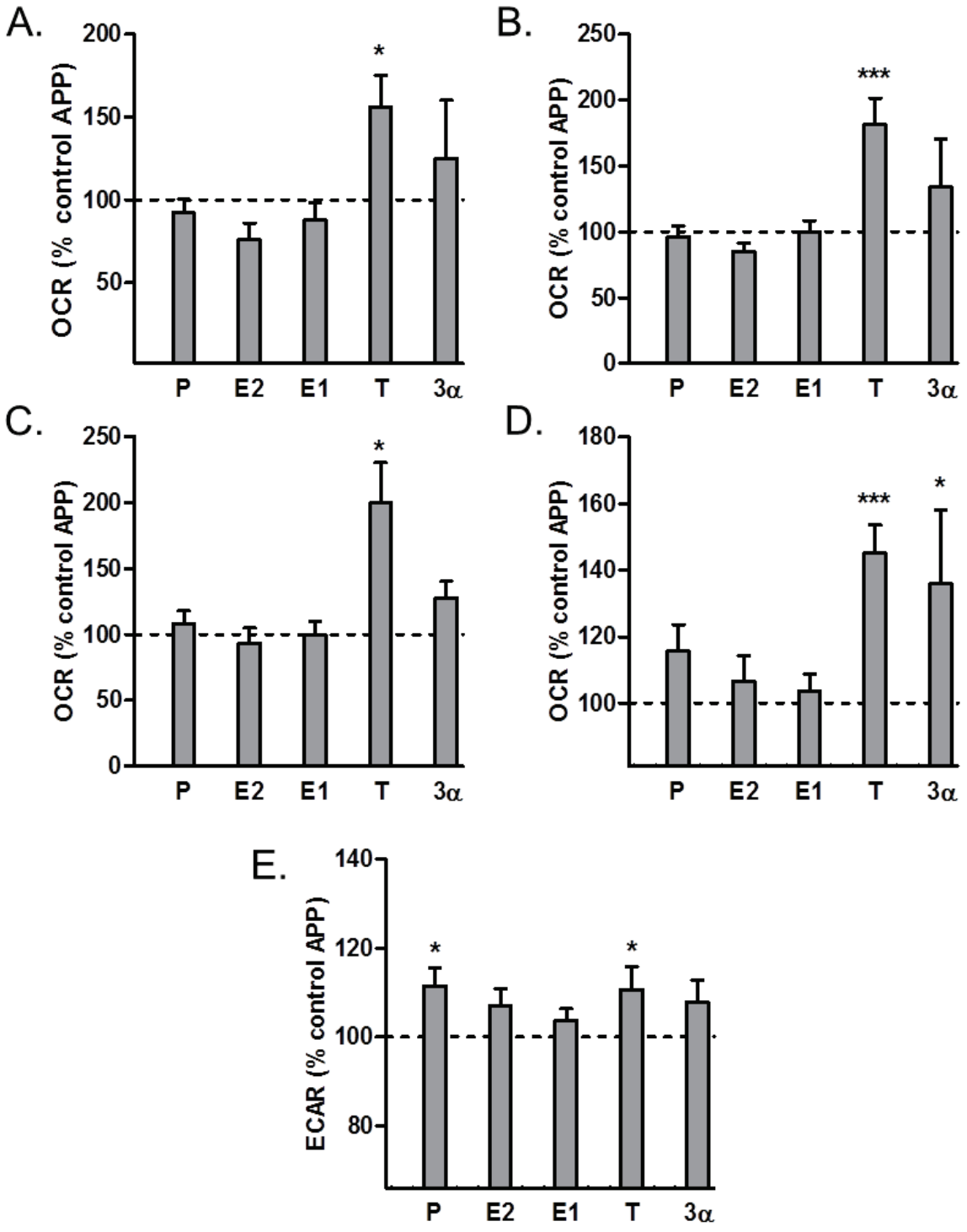

Fig. 4: Effects of neurosteroids on bioenergetic parameters in APP cells. (A) Basal respiration, (B) ATP turnover, (C) maximal respiration, (D) spare respiratory capacity and (E) glycolytic reserve were measured after neurosteroid treatment for 24 hrs at a concentration of 100 nM in APP cells, using a XF24 Analyzer (Seahorse Bioscience). Values represent the mean ± SEM (n=8-10 replicates) and were normalized to 100% of the control group (untreated APP cells). One-way ANOVA and post hoc Dunnett's multiple comparison test versus control, *P<0.05; ***P<0.001. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α; 3α-androstanediol.

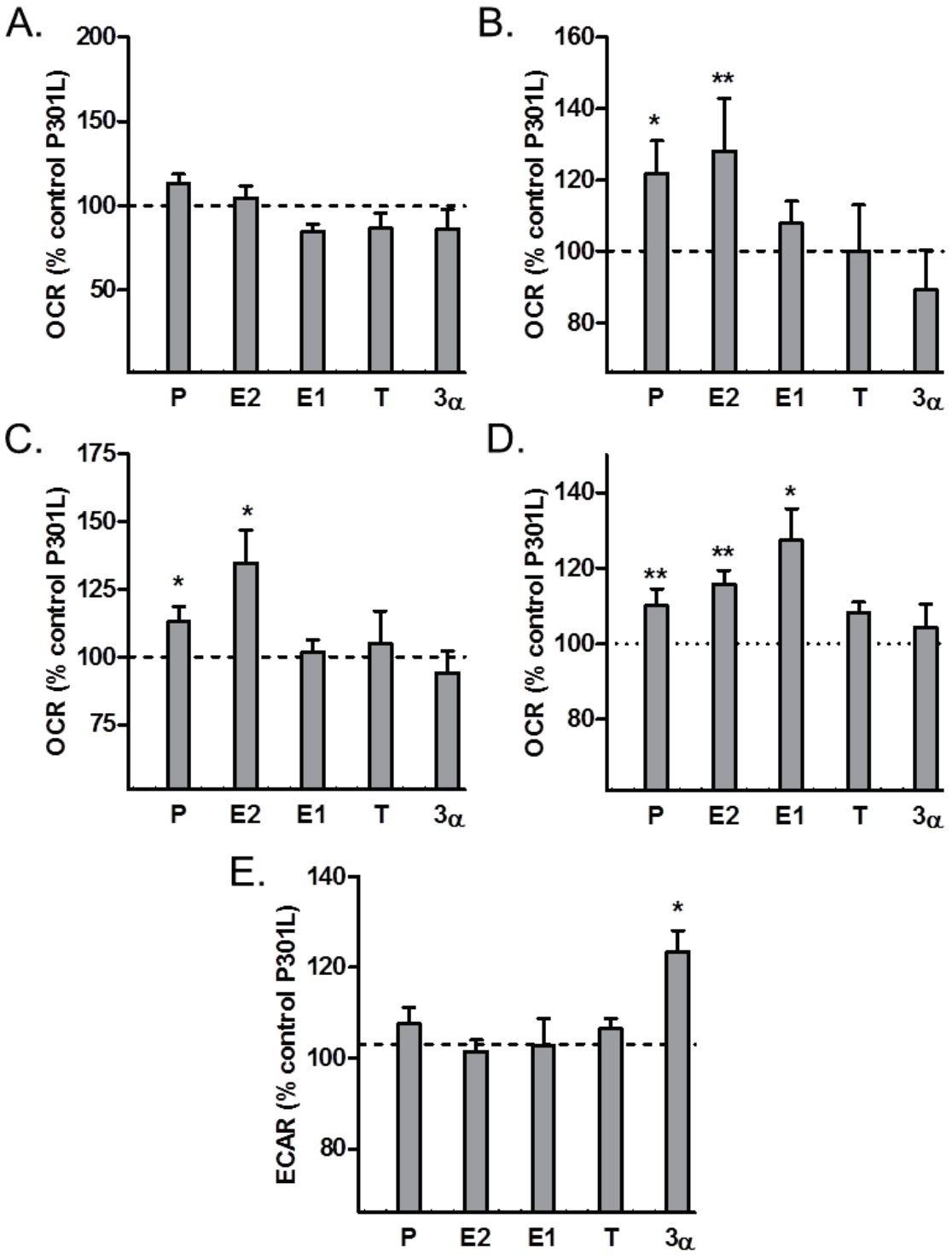

Fig. 5: Effects of neurosteroids on bioenergetic parameters in P301L cells. (A) Basal respiration, (B) ATP turnover, (C) maximal respiration, (D) spare respiratory capacity and (E) glycolytic reserve were measured after neurosteroid treatment for 24 hrs at a concentration of 100 nM in P301L cells, using a XF24 Analyzer (Seahorse Bioscience). Values represent the mean ± SEM (n=8-10 replicates) and were normalized to 100% of the control group (untreated P301L cells). One-way ANOVA and post hoc Dunnett's multiple comparison test versus control, *P<0.05; ***P<0.001. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α; 3α-androstanediol.

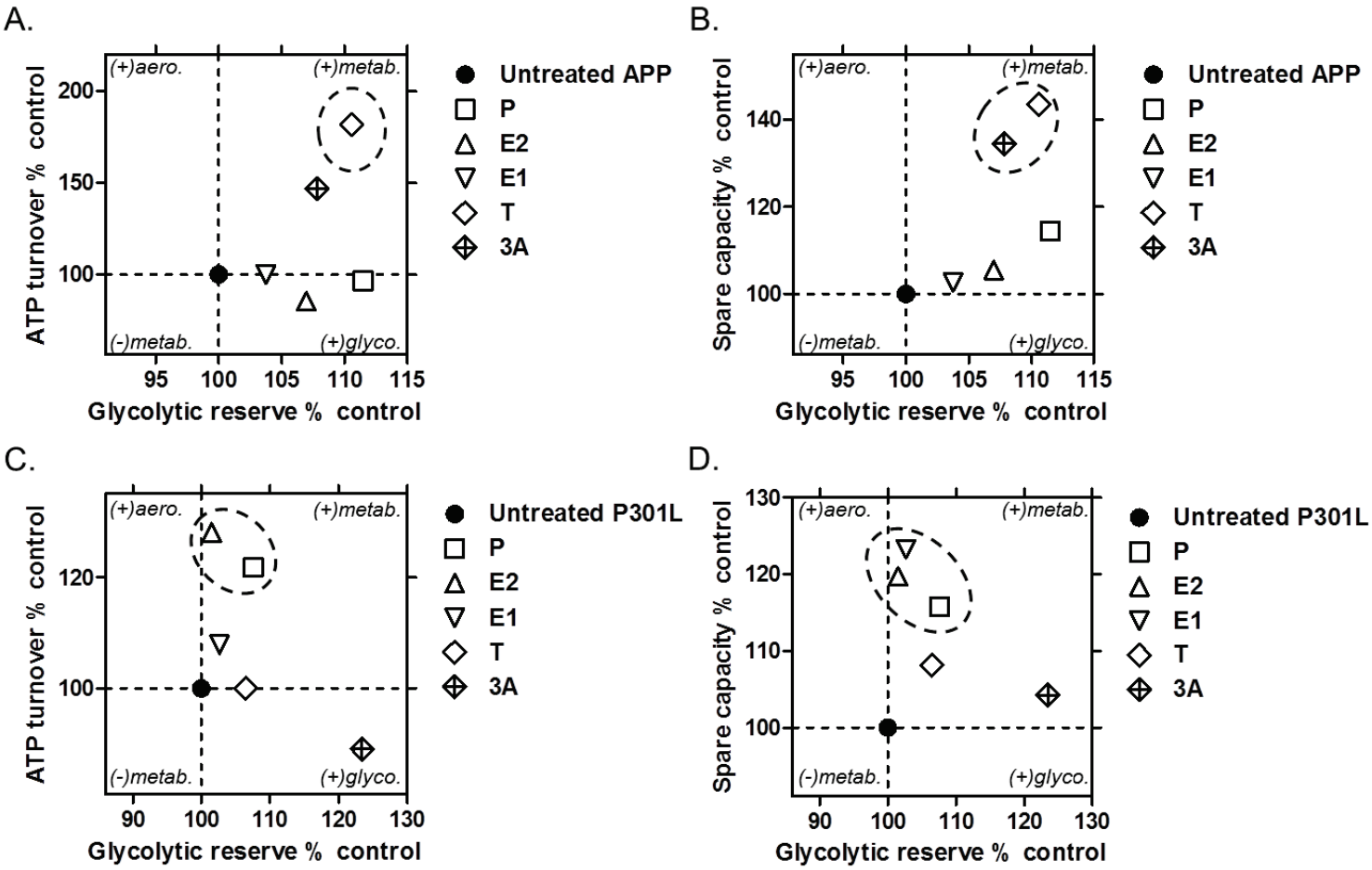

Fig. 6: Neurosteroids differentially regulate bioenergetic profile in APP/Aβ and abnormal tau-overexpressing cells. (A-B) Characterization of bioenergetic profiles of APP cells after neurosteroid treatment along two axes. Degree of (A) ATP turnover or (B) spare respiratory capacity is shown (in ordinate) in function of glycolytic reserve (in abscissa). The same parameters are displayed for P301L (C-D) respectively. Values represent the mean of each group normalized to the control group (untreated APP or P301L cells =100%). Significant changes upon respiratory parameters are highlighted by dashed circles. P; progesterone, E2; estradiol, E1; estrone, T; testosterone, 3α; 3α-androstanediol.

Contributions

AG and EB performed experiments. UEL, AGMN and AE conceived the project, coordinated and supervised research. AG, EB, AGMN and AE wrote the manuscript.

Acknowledgments

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C. Circadian control of Drp1 activity regulates mitochondrial dynamics and bioenergetics

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(In preparation)

Abstract

Circadian clocks are self-sustained cellular oscillators that are tightly connected to metabolic processes through reciprocal regulation from metabolites to transcription factors. Within the cell, metabolism is a highly dynamic process where mitochondria network is a prominent actor in regulation of both energy metabolism and apoptotic pathways. We found that mitochondrial bioenergetic homeostasis, including mitochondrial respiration and consequently generation of its own byproducts as adenosine triphosphate (ATP) and reactive oxygen species (ROS), is directly coupled to mitochondrial network which is, at least in part, regulated by clock-controlled phosphorylation of Drp1, the main factor involved in mitochondrial fission. The time-dependent reorganization of mitochondrial architecture in turn regulates the clock through circadian oscillation of mitochondrial ATP which can act as input signal through activation of AMP-activated protein kinase (AMPK). Our findings highlight new insights in the understanding of the reciprocal temporal crosstalk that governs the molecular interplay between the coupling of mitochondrial dynamics and metabolism and circadian rhythms.

Keywords: mitochondria, bioenergetics, dynamics, Drp1, circadian clock

Abbreviations:

2DG, 2 deoxy-D-glucose; AMPK, AMP-activated protein kinase; AraC, Cytosine β-D-arabinofuranoside; ATP, adenosine tri-phosphate; cROS, cytosolic reactive oxygen species; DRP1, dynamin-related protein 1; ECAR, extracellular acidification rate; hFIS1, human fission protein 1; MEF, mouse embryonic fibroblast; MFN1/2, mitofusin 1/ 2; mROS, mitochondrial reactive oxygen species; NAD+(H), nicotinamide adenine dinucleotide (oxidized/reduced form); OCR, oxygen consumption rate; OXPHOS, Oxidative phosphorylation; OPA 1, optic atrophy 1; Per, clock gene Period; ROS, reactive oxygen species; SCN, suprachiasmatic nuclei; Ser637, residue serine 637; VDAC, voltage-dependent anionic channels.

Introduction

The circadian clock is a hierarchical network of oscillators which coordinate a wide variety of endocrine, physiological and metabolic functions to the optimal time of day anticipating the periodical changes of the external environment for all living organisms, from cyanobacteria and fungi ***(1)*** to insects ***(2)*** and mammals ***(3)***.

Over the years, a growing body of evidences suggested particularly that energy metabolism (i.e. ATP release, reactive oxygen species (ROS)) and cellular defense mechanisms are coordinated by circadian clock ***(4; 5)***. The disruption of the clock impairs metabolic homeostasis among the living organisms ***(6; 7)***. This close relationship between circadian and metabolic cycles has been described in studies showing that the circadian clock exerts its control over metabolism by (i) controlling the expression of ascertained genes and enzymes involved in metabolic processes, (ii) intertwining nuclear receptors and nutrient sensors (e.g. SIRT1 and CLOCK, AMPK and CRY1) with the clock machinery, and/ or (iii) regulating metabolite levels (e.g. NAD+, cAMP) ***(8-12)***. While the regulation of metabolic pathways is well known to be achieved by the circadian clock, it has also been suggested that various hormones, nutrient sensors, redox sensors and metabolites are not only clock output but can also regulate in turn the biological clock by acting as an input signal ***(13; 14)***. While several studies showed diurnal oscillations in key bioenergetic parameters including expression of genes involved in mitochondrial respiration, mitochondrial membrane potential, cytochrome c oxidase activity in the SCN ***(15; 16)***, the whole mechanisms behind the relationship between the clock and the mitochondrial network remains mostly elusive.

Mitochondria are highly dynamic cellular organelles known to play a major role in cellular energy metabolism and maintenance of cell steadiness. These organelles are, among others, the place of synthesis of the main source of energy from nutritional sources in cells via ATP generation which is mainly accomplished through oxidative phosphorylation ***(17)***. To achieve the integrity of a healthy mitochondrial population within the cells but also the integrity of the cell itself, mitochondrial shape has to be modified to meet changing requirements in energy production and other mitochondrial functions. This requires a tightly regulated equilibrium between opposing mitochondrial fusion and fission activities ***(18; 19)***.

Thus, we have addressed the question of whether mitochondrial dynamics and metabolism are coupled events that may exhibit circadian oscillations and whether the mitochondrial network and the mitochondrial metabolism themelves may be able to influence the circadian clock by acting as retrograde signaling. For this purpose, the latest state-of-art approaches have been engaged in order to dissect the multilevel relationship between the

clock and mitochondrial function. Our findings are consistent with the hypothesis of the existence of crosstalk between the clock and mitochondrial network in order to maintain the bioenergetic homeostasis through the day in response to metabolic changes.

Materials and Methods

Mice

Drp1$^{flx/flx}$ mice ***(20)*** were crossed with mice expressing an inducible Cre recombinase transgene under the control of the CamKIIα promoter (Cre+) which is active in the hippocampus and the cortex of adult mice (from the European Mouse Mutant Archive EMMA strain 02125) ***(21)***. At 8 weeks of age the resulting *Drp1*$^{flx/flx}$ *Cre*+ mice were injected i.p. with 1 mg tamoxifen (Sigma; 10 mg/ml tamoxifen dissolved in a 9:1 ratio of sunflower seed oil to ethanol) twice daily on five consecutive days to induce recombination of the Drp1 locus (Oettinghaus et al, *submitted*).

For the constant darkness (DD) experiments, all the mice were maintained for one week on a 12:12 light dark cycle prior to placement in DD 5 days before the beginning of the experiment with free access to food and water.

Brain Homogenate Preparation

Brain homogenate preparations were obtained to determine nucleotides levels and AMPK activation. For that, brains were quickly dissected on ice and washed in an ice-cold buffer (210 mM mannitol, 70 mM sucrose, 10 mM Hepes, 1 mM EDTA, 0.45% BSA, 0.5 mM DTT, and Complete Protease Inhibitor mixture tablets (RocheDiagnostics). After removing the cerebellum, the tissue sample was homogenized in 2 ml of buffer with a glass homogenizer (10–15 strokes, 400 rpm). We used 10µl of the suspension for protein determination. All brain homogenate samples were normalized on 5 mg/ml of protein before ATP content, on 1 mg/ml for NAD^+ and NADH level and for activated AMPK determination.

Human Skin Fibroblast

Fibroblasts were isolated from biopsies, infected with the lentiviral circadian reporter mice Bmal1::luciferase and cultured in DMEM/1% penicillin streptomycin (Sigma)/1% Glutamax (Sigma) (DMEMc)/20% FBS (Sigma) as described previously (Brown, Fleury-Olela et al. 2005, Pagani PNAS 2009). For nucleotides level, ROS content, activated AMPK determination, oxygen consumption rate and mitochondrial morphology in human skin

fibroblasts, circadian rhythms were synchronized by serum shock treatment (50% horse serum supplemented DMEMc) for 2 hours at 37 °C.For the period length determination, circadian rhythms were synchronized by 100 nM dexamethasone (Sigma) in DMEMc + 20% FBS. DMEMc without phenol red was supplemented with 0.1 mM luciferin (Molecular Probes) and 10% FBS to obtain the counting medium (CM).

Mouse Embryonic Fibroblast (MEF)

Drp1 lox/lox were prepared from E13.5 Drp1 lox/lox embryos as previously described ***(20)***. Drp1 -/- MEFs were subsequently generated by the expression of Cre recombinase in Drp1 lox/lox MEFs. The MEFs were cultured in DMEM/1% penicillin streptomycin (Sigma)/1% Glutamine (Sigma) (DMEMc)/10% FBS (Sigma)/1% non-essential amino acids. MEFs lacking mPer1$^{-/-}$; mPer2$^{-/-}$ isolated from double knockout mice were generously provided by U. Albrecht (University of Fribourg) ***(22)***. MEF cultures were established from day 12 embryos as previously described ***(23)*** and the dissociated cells were plated in DMEM containing 10% FCS.

Mitochondrial Morphology

For determination of mitochondrial dynamics in synchronized fibroblasts, cells were seeded on collagen - coated coverslip (0.05 mg/ml) at cell density sufficient to reach 50% of confluence the next day. After serum shock treatment, the medium was exchanged to DMEMc without phenol red + 2% FBS containing 75 nM Mitotracker® Red CMX ROS (579/599, Life technologies) which is a red-fluorescent dye that stains mitochondria in live cells and its accumulation is dependent upon membrane potential. From 8 hours post-synchronization time point and at 8 hours intervals over the course of 24 hours, the stained cells were fixed with 4% paraformaldehyde/PBS for 30 minutes at room temperature. After extensive wash, the fixed cells were permeabilized with 0.15% Triton 100x prior staining nuclei with Sytox Green (Invitrogen) according to the manufacturer's recommendation. After mounting the coverslips, images were processed by using a confocal microscope (60x oil objective, Leica).

To assess structure of the mitochondrial network in brain and liver slices from wild-type mice at CT0 (onset of subjective day or rest period) and CT12 (onset of subjective night or activity period), samples were subjected to immunohistochemistry against Voltage-Dependent Anionic Channels (VDAC, Cell Signaling), an outer mitochondrial membrane porin, followed by addition of anti-rabbit IgG, FITC (Sigma, 490/525). Prior to mounting the coverslips, brain and liver slices were nuclei-stained using TO-PRO-3iode (1uM, Invitrogen

642/661) according to the manufacturer's recommendation. Images were obtained with confocal microscope (60x oil objective, Leica).

Protein Gel Electrophoresis and Immunoblotting

After DC protein Assay (Biorad) quantification, equal amounts (50 µg from human skin fibroblasts protein lysates and 20 µg from mice brain lysates) were loaded on a 4-12% acrylamide gel (Invitrogen) to perform SDS-PAGE at 100V for 90 min and then transferred to PVDF membrane (Amersham Biosciences). We used primary antibodies to DRP1 (Cell Signaling), ser637-phosphorylated DRP1 (Cell Signaling) and VDAC (Cell Signaling). PVDF membranes were then treated with anti-IgG, horseradish-coupled secondary antibody. The bands were specifically detected by enhanced chemiluminescence reaction using SuperSignal™ West Dura Chemiluminescent Substrate (Thermo Scientific). Experiments were performed starting from 12 hours post-synchronization time point and measured at 4 hours intervals for 6 time points.

Nucleotides Measurements

Total ATP content from synchronized human skin fibroblasts, Drp1 lox/lox and Drp1 -/- MEFs, Per1/2$^{+/+}$ and Per1/2$^{-/-}$ MEFs and mice brain homogenates was determined using bioluminescence assay (ViaLighTM HT; Cambrex Bio Science) according to the instruction of the manufacturer. The enzyme luciferase, which catalyzes the formation of light from ATP and luciferin was used. The emitted light is linearly related to the ATP concentration and is measured using a luminometer (VictorX5, Perkin Elmer). To define the origin of ATP oscillation, DMEMc without phenol red + 2% FBS supplemented with 2-Deoxy-D-glucose (4.5 g/l) a glycolysis inhibitor or oligomycin (2 µM), an ATP synthase inhibitor was added on synchronized human skin fibroblasts. To test the possibility that rhythmic ATP is a byproduct of rhythmic cell division, pharmacological disruption of the cell cycle was accomplished with the inhibitor AraC, an anticancer drug that prevents cell division (100 µm; Sigma). AraC was added to human skin fibroblasts cultures 3 hours after the seeding, directly after the medium exchange following the synchronization and then every 24 hours to assure continued block of cell division. To investigate the role of clock- regulated Drp1 in mitochondrial bioenergenetics, mitochondrial fission was abolished by selectively inhibition of mitochondrial division dynamin, Drp1, in presence of mdivi-1 (50 µM, Sigma).

For measurement of NAD+ and NADH from synchronized fibroblasts and mice brain homogenates, NAD+ and NADH were separately extracted using an acid-base extraction (HCL 0.1 mol/l – NAOH 0.1 mol/l). The determination of both NAD+ and NADH was

performed using an enzyme cycling assay based on passing the electron from ethanol trough reduced pyridine nucleotides to MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in a PES (phenazine ethosulfate) coupled reaction resulting in a purple formazan product that can be quantitatively measured at a wavelength of 595 nm (VictorX5, Perkin Elmer). Experiments were performed starting from 12 hours post-synchronization time point and measured at 4 hours intervals for 6 time points.

Oxygen Consumption Rate (OCR) Measurements

OCR was measured in synchronized fibroblasts as recommended as previously described ***(24)***. Briefly, human skin fibroblasts were seeded at the density of $3x10^4$ cells/100 µl per well on Seahorse Biosciences 24-well culture plates one day prior to the beginning of assay. After serum shock synchronization, medium was exchanged to 500 µl of assay medium (glucose-free RPMI-1640 medium containing 2% FBS, 2 mM sodium pyruvate, pH ~ 7.4). Prior to measurements the microplates were equilibrated in a CO_2 free incubator at 37 °C for 60 minutes. The drug injections ports of the XF Assay Cartridge were loaded with the assay reagents at 10X in assay medium. 55 µl of oligomycin (10 µM), 62 µl of FCCP (7 µM), 68µl of a mix of antimycin A (40 µM) and rotenone (20 µM) were added to ports A, B and C respectively. Experiments were performed at 16 hours post-shock and 28 hours post-shock.

U2OS Cells Metabolome

Metabolic profiles also were obtained from dexamethasone-synchronized U2OS cells beginning at the 12 hour post synchronization time point and sampled at 4 hour intervals for 6 time points. For metabolic profiles dexamethasone-synchronized U2OS cells beginning at the 12hr post synchronization time point and sampled at 4 hour intervals for 6 time points. Small-Molecule Determination. Metabolon analyzed metabolites in dexamethasone-synchronized U2OS cells, as described previously ***(25; 26)***.

Circadian period length determination

In transfected fibroblast, light output was measured in homemade light-tight atmosphere-controlled boxes for at least 5 days. To measure the fibroblast basal circadian rhythms, CM was supplemented with 10% FBS; to determine the influence of ATP on circadian period length, CM was supplemented with mitochondrial respiration inhibitors: rotenone, an complex I inhibitor (1 µM), oligomycin, ATP synthase inhibitor (2 µM) or carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of proton

gradient (4 µM). To determine the role of Drp1-regulated mitochondrial bioenergenetics, CM was supplemented with mdivi-1 (50 µM, Sigma), a mitochondrial division inhibitor.

Activated AMPK Quantification

The quantification of endogenous activated-AMPK was determined in lysates from synchronized fibroblasts and from brain homogenates by using PathScan® phospho-AMPKα (Thr172) sandwich ELISA (Cell Signaling) according to the instruction of the manufacturer. Briefly, AMPKα (phospho and nonphospho) is captured by the coated AMPKα rabbit antibody in the microplate. Then, a phospho-AMPKα (Thr172) mouse detection antibody is added to detect phosphorylation of Thr172 on the captured AMPKα protein. Anti-mouse IgG, HRP-linked antibody is then used to recognize the bound detection antibody. HRP substrate, TMB, is added to develop color. The absorbance is measured at 450 nm within 30 minutes using a luminometer (VictorX5, Perkin Elmer) and is proportional to the quantity of AMPKα phosphorylated at Thr172. Experiments were performed starting from 12 hours post-synchronization time point and measured at 4 hours intervals for 6 time points in fibroblasts and every 4 hours over the course of 24 hours in wild-type mice brain.

ROS level

The formation of cytosolic (cROS) and mitochondrial (mROS) reactive oxygen species in synchronized fibroblasts were measured using, respectively, the fluorescent probe H2DCF-DA (DCF) and the non-fluorescent dihydrorhodamine-123 (DHR). Synchronized fibroblasts were loaded for 15 min with 10 µM DCF or 15 min with 10 µM DHR at 37 °C. After washing twice with HBSS, the formation of the reduced fluorescent product dichlorofluorescein was detected using the VictorX5 multilabel reader (PerkinElmer Life Sciences) at 485 nm (excitation)/535 nm (emission). DHR, which is oxidized to cationic rhodamine 123 which localizes in the mitochondria and exhibits green fluorescence, was detected using the VictorX5 multilabel reader at 490 nm (excitation)/590 nm (emission). The levels of superoxide anion radical were also assessed using the Red Mitochondrial Superoxide Indicator (MitoSOX, 5 µM, 30 min). 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 mROS levels or superoxide anion radicals in mitochondria. Experiments were performed starting from 12 hours post-synchronization time point and measured at 4 hours intervals for 6 time points.

Quantitative Real-Time PCR

Total RNA was extracted from lysates of synchronized fibroblasts using RNeasy Mini KIT (Qiagen). cDNA was generated using Ready-to-Go You-Prime First-Strand Beads (GE Healthcare). For data analysis, human Cdk4 was used as an endogenous control. Data are expressed as relative expression for each individual gene normalized to their corresponding controls using the comparative CT method. The primers used were purchased from Applied Biosystems (Probe ID: see Primers section for details). Data are expressed as relative expression for each individual gene normalized to their corresponding controls.

Primer sequences

	Primer	Probe ID (Applied Biosystems)
Fusion	*MFN1*	Hs00250475_m1
	MFN2	Hs00208382_m1
	OPA1	Hs00323399_m1
Fission	*DRP1*	Hs00247147_m1
	FIS1	Hs00211420_m1
OXPHOS	*NDUFA2* (complex I)	Hs00159575_m1
	NDUFB5 (complex I)	Hs00159582_m1
	NDUFC1 (complex I)	Hs00159587_m1
	NDUFV2 (complex I)	Hs00221478_m1
	COX4I1 (complex IV)	Hs00971639_m1
	COX6A1 (complex IV)	Hs01924685_g1
	COX7A2 (complex IV)	Hs01652418_m1
	COX7B (complex IV)	Hs00371307_m1
	ATP5G2 (ATP synthase)	Hs01096582_m1
	ATP5C1 (ATP synthase)	Hs01101219_g1
	ATP5L (ATP synthase)	Hs00758883_s1

Statistical Analysis

Data were presented as mean ± S.E.M. For statistical comparisons, unpaired and paired Student's *t*-test, respectively, or Two-way ANOVA was used. Rhythmicity of metabolites was assessed using an algorithm previously described for rhythmic transcripts.

The JTK-cycle algorithm was used as implemented in R by Kronauer as previously described ***(26)***. P values less than 0.05 were considered statistically significant.

Results

Mitochondrial oxidative metabolism is driven by circadian clock.

To investigate whether the clock influences the cellular bioenergetic activity, we first monitored whole cell ATP content in synchronized human skin fibroblasts (**Fig. 1A**). Total ATP content exhibited a circadian rhythmicity with a peak occurring at 16 hours post-shock and a trough at 28 hours post- shock in synchronized fibroblasts (**Fig. 1A**). Similar observations were made in brain homogenates from wild-type mice kept in constant darkness where total ATP displayed ~24hours oscillations with a peak occurring at CT4, near the beginning of the rest period and a trough occurring at CT16, the beginning of the activity period (**Fig. 1B**).

As ATP molecules are produced by two main pathways, the cellular glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), we monitored ATP content in synchronized fibroblasts in the presence of an ATP synthase inhibitor (oligomycin) or glycolysis inhibitor (2-deoxy-glucose) in order to determine which pathway is causative of ATP oscillation (**Fig. 1A**). We observed that the circadian oscillations in ATP were significantly dampened in the presence of oligomycin. In contrast, only the amplitude was decreased in the presence of 2-deoxy-glucose, suggesting that the circadian rhythm of ATP is primarily generated from mitochondrial oxidative phosphorylation.

In order to confirm that the observed ATP oscillations are not associated with synchronous cell cycle ***(27)***, we evaluated ATP content in Cytosine β-D-arabinofuranoside (AraC)-treated fibroblasts to induce disruption of cell cycle (**Fig 1C**). While AraC treatment decreased significantly cell proliferation (**data not shown**), pharmacological disruption of cell division with AraC led to a decreased level in rhythmic ATP content compared to untreated cells but no alteration in the period was observed in parallel as the ATP peak was occurring at 16 hours post-shock in AraC-treated cultures (**Fig. 1C**) which is consistent with observations made in astrocytes ***(28; 29)***. Together, these results demonstrate that circadian fluctuations in ATP levels mainly originated from mitochondrial oxidative metabolism (**Fig. 1A**), independently of the cell cycle (**Fig.1C**).

To further investigate whether expression of the core molecular clock transcription factors impact mitochondrial ATP generation, we evaluated ATP content in mouse embryonic fibroblasts (MEFs) isolated from mice deficient in the clock transcriptional repressors (PER1 and PER2) (**Fig. 1D**). MEFs lacking the clock repressor genes *Per1* and *Per2* showed complete abolished ATP rhythm compared to control MEFs suggesting that circadian clock transcription repressors appear to influence, at least in part, oxidative metabolism.

To further investigate the clock-related regulation of mitochondrial oxidative metabolism, we monitored in real–time oxygen consumption rate (OCR) in synchronized fibroblast at 16 hours post-shock corresponding to total ATP peak and at 28 hours post-shock related to total ATP trough **(Fig. 2)**. For this purpose, OCR was assessed by using a Seahorse Bioscience XF24 Flux Analyzer by sequential injection of (i) the ATP synthase inhibitor oligomycin (1µM) to determine the OCR devoted to ATP synthesis counterbalancing against ATP consumption (also called ATP turnover), (ii) an uncoupling agent, FCCP [carbonyl cyanide p (trifluoromethoxy) phenylhydrazone] (0.7µM) to measure the maximal respiration in the absence of a proton gradient and (iii) inhibitors of activities of complexes I-III, respectively rotenone (2µM) and antimycin A (4µM) to evaluate the OCR of non-mitochondrial respiration (**Fig. 2A**). Consistent with the aforementioned ATP rhythms, we observed lower OCR in the basal respiration (~19%) as well as in ATP turnover (~24%) and maximal respiration (~14%) at 28 hours post-shock compared to OCR measured at 16 hours post- shock supporting the hypothesis of the clock-driven mitochondrial oxidative metabolism (**Fig. 2B**). The spare respiratory capacity corresponding to the ability of the cell to respond to an energetic demand as well as how closely the cell is to respiring to its theoretical maximum was higher (~48%) at 28 hours post-shock compared to OCR measured at 16 hours post-shock (**Fig.2C**). The rate of oxygen consumption due to non-mitochondrial sources (**Fig. 2B)**, not coupled to ATP production but required to overcome the natural proton leak across the inner mitochondrial membrane (**Fig. 2D**) and related to glycolysis indicated by the extracellular acidification rate (ECAR) (**Fig. 2E**) was unchanged confirming the role of the oxidative phosphorylation in the generation and maintenance of ATP rhythm. Finally, we characterized the bioenergetic profile of synchronized human skin fibroblasts, representing OCR (basal respiration) versus ECAR (glycolysis) at 16 hours and 28 hours post-shock (**Fig. 2F**). Remarkably, cells were switched between a metabolically active state corresponding to 16 hours post-shock and a metabolically resting state corresponding to 28 hours post-shock, with only a variation of basal respiration (OCR).

To better understand the impact of the clock on mitochondrial respiration, we then investigated mitochondrial biogenesis around the clock by determining the gene expression

of subunits involved in oxidative phosphorylation, particularly in the electron transport chain (complex I and complex IV) and in ATP generation (ATP synthase or complex V) (**Fig. S1**) ***(15)***. RNA samples were isolated from synchronized human skin fibroblasts and relative quantification was performed by quantitative PCR (**Fig. S1**). Relative mRNA expression of complex I subunits exhibited a circadian rhythmicity with a peak occurring at 16 hours post-shock and a trough at 28 hours post-shock (**Fig. S1**), while relative mRNA expression of complex IV subunits exhibited a circadian rhythmicity with a trough occurring at 16 hours post-shock and a peak at 28 hours post-shock (**Fig. S1**). Relative gene expression of ATP synthase (complex V) subunits displayed a circadian rhythmicity with a peak occurring at 20 hours post-shock and a trough at 32 hours post-shock (**Fig. S1**).

Although oxidative phosphorylation (OXPHOS) is a vital part of metabolism, it produces in parallel reactive oxygen species (ROS). Since circadian ATP oscillation was correlated to mitochondrial respiration, we examined whether ROS levels within the cell followed a circadian pattern by measuring the fluoresecent probe H2DCF-DA and dihydrorhodamine 123 used respectively, for cytosolic (cROS) and mitochondrial (mROS) ROS in synchronized human skin fibroblasts (**Fig. SI2**). Remarkably, mROS levels displayed a circadian oscillation with a peak at 16 hours post-shock and a trough at 28 hours post-shock while cROS level remained unchanged over the time. Moreover, the specific measurement of mitochondrial superoxide anion radicals revealed that a part of rhythmic ROS were indeed superoxide anions.

As important substrate in mitochondrial bioenergetics, we monitored NAD^+ content in synchronized fibroblast (**FIG S3**) and in brain homogenate from wild-type non-fasted mice kept in constant darkness (**FIG S3**). We observed ~24hours oscillations of NAD^+ with peak occurring at 16 hours post-shock in fibroblast culture (**FIG S3**) and near the beginning of the rest period (CT4) in mice brain homogenate (**FIG S3**). Both observations coincided with the rhythm of total ATP and thereby can be correlated to clock-driven mitochondrial metabolism.

To gain further insights into the signaling pathway between the clock and the mitochondrial network, we examined the metabolome *in vitro* to determine the key players of the metabolome in mitochondrial functions (**FIG S4**). Wide metabolic profiles were obtained from dexamethasone-synchronized U2OS cells over the course of 24 hours. All time points were subsequently analyzed by GC/LC-MS ***(26)*** (**FIG S4**). Overall, 228 metabolites were identified and ~29% of the identified metabolites (67 of 228) related amino acids, carbohydrates, cofactors, vitamins, energy, lipids, nucleotides and peptides metabolism displayed a circadian profile (**FIG S4**). Among the metabolites identified as rhythmic, the

largest group of compounds was represented by amino acids (33 of 67 rhythmic metabolites), which exhibited a restricted phase distribution around the clock (**Fig S4**). Remarkably, all others groups identified as rhythmic displayed an identical phase distribution (**FIG S4**). Interestingly, the majority of the rhythmic metabolites are likely related to numerous metabolomic pathways closely or remotely implicated in mitochondrial functions including energy metabolism and redox state (**FIG S4**).

Overall, these findings indicate a central role of the clock in the regulation of mitochondrial bioenergetic homeostasis, in part through mitochondrial biogenesis and in other part through the circadian modulation of metabolites connected to mitochondrial metabolism.

Clock control of Drp1- dependent mitochondrial metabolism

Because mitochondria are known to be highly dynamic organelles, we investigated whether the circadian clock intervenes in the control and the maintenance of the mitochondrial network architecture (**Fig. 3**). Hence, we examined mitochondrial morphology using the red-fluorescent dye Mitotracker CMXROS in synchronized human primary skin fibroblasts and images were processed by confocal microscopy (**Fig. 3A + SI5**). We observed that mitochondrial network morphology displayed a circadian rhythmicity with three distinct states (tubular at 8 hours post-shock, intermediate at 16 hours post-shock and fragmented at 24 hours post-shock) in synchronized fibroblast culture. In parallel, we investigated the relationship between the clock and the mitochondrial architecture in brain and liver sections from wild-type mice at CT0 (onset of subjective day or rest period) and CT12 (onset of subjective night or activity period) by immunohistochemistry labeling VDAC (Voltage-Dependent Anionic Channels), an outer mitochondrial membrane porin (**Fig 3B**). Consistent with our in vitro observations, the mitochondrial network in both central and peripheral tissues exhibited a tubular morphology at CT0 corresponding to the beginning at the rest period while the shape network revealed a fragmented state at CT12 matching with the beginning of the activity period.

To further assess the relationship between the clock and mitochondrial shaping events, we analyzed the gene expression of the fusion proteins (mitofusins 1 and 2, MFN1 and 2; optic atrophy 1, OPA1) and fission proteins (dynamin-related protein1, DRP1; human fission protein 1, hFIS1) required in the maintenance and regulation of mitochondrial system ***(30; 31)*** (**Fig. 3C+ SI5**). For this purpose, mRNA samples were isolated in synchronized human skin fibroblasts and relative quantification was performed by quantitative PCR (**Fig.**

3C+ SI5). Interestingly, none of the genes displayed a circadian pattern in their expression (**Fig. 3C+ SI5**), suggesting that 24-hour oscillations observed in the mitochondrial network architecture *in vitro* and *in vivo* may be controlled by post-transcriptional modifications of the mitochondrial shaping proteins.

To investigate whether regulation of the mitochondrial shaping trough activation/ inactivation of DRP1 protein, the main factor involved in mitochondrial fission, is under the control of the clock (**Fig. 3D**), we considered the potential contribution of the biological clock in the posttranslational modification of DRP1 by phosphorylation at a serine residue (Ser637) using immunoblotting against successively DRP1 and phosphorylated DRP1 at serine 637 in lysates (**i**) from synchronized human primary skin fibroblasts and (**ii**) from mouse brain of non-fasted wild-type mice maintained in constant darkness (**Fig. 3D**). While total DRP1 protein did not display circadian oscillation in both (**i**) cell lysates and (**ii**) brain lysates, phosphorylated DRP1 at serine 637 exhibited ~24 hours rhythms with a peak occurring respectively, at (**i**) 16 hours post-shock and at (**ii**) CT12 (onset of the subjective night) (**Fig. 3D**). Taken together, these results highlight that the remodeling events of the mitochondrial network *in vitro* and *in vivo* can be integrated into the biological clock network, partly through posttranslational modulation of DRP1 protein, suggesting a key role for the clock in mitochondrial network tasks to anticipate energetic requirements depending on the time of the day.

Because a growing body of evidence suggests a tight connection between mitochondrial shape changes and mitochondrial bioenergetic homeostasis, we investigated whether the circadian control of DRP1 is directly involved in the circadian ATP oscillation (**Fig. 4**). A treatment with a mitochondrial division inhibitor, mdivi-1, completely abolished ATP oscillation compared to untreated human skin fibroblasts (**Fig. 4A**). To further confirm whether inhibition or absence of DRP1 is able to impact ATP oscillation, we evaluated ATP content in mouse embryonic fibroblasts (MEFs) isolated from mice deficient in Drp1 as well as in Drp1-ablated hippocampus of adult mice kept in constant darkness (**Fig.4B-C**). MEFs lacking of Drp1 did not display ATP oscillation compared to control MEFs (**Fig 4B**). In addition, ATP content was evaluated at CT4, near the beginning of the rest period and at CT16, the beginning of the activity period accordingly to the aforementioned observation (**Fig.1C**). Consistent with our in vitro observations, ATP measured in Drp1 -/- hippocampi mice did not display a significant change between CT4 and CT16 compared to control hippocampi while the cerebrum from Drp1 -/- mice exhibited a circadian ATP changes equivalent to control cerebrum (**Fig. 4C**). Together, these findings provide new insights into

the control and maintenance of the mitochondrial metabolism connected to mitochondrial network changes through the clock modulation of DRP1.

Crosstalk between Drp1- dependent mitochondrial metabolism and clock

To highlight the status of mitochondrial network and its signaling molecules in the regulation of the clock, we characterized the circadian period length of dexamethasone-synchronized fibroblasts infected with a lentivirus that harbored a circadian reporter construct (the Bmal1 promoter driving expression of the firefly luciferase gene) by disturbing either mitochondrial respiration or mitochondrial network (**Fig.5A-B**). Rotenone, oligomycin or FCCP were used to inhibit mitochondrial respiration leading to a depletion in ATP content (**FIG 5A**). Notably, cells had a significantly longer period length in the presence of mitochondrial respiration inhibitors compared to the control cells. Moreover, disruption of mitochondrial division by mdivi-1 treatment significantly increased period length (**Fig.5B**). These findings suggest that Drp1-mediated mitochondrial ATP oscillations may play a key role in the retrograde signaling to the clock.

To better understand the underlying mechanisms in this retrograde signaling, we evaluated the activation of AMP-activated protein kinase (AMPK) via threonine phosphorylation, which is reported to transmit energy-dependent signals to the mammalian clock (**FIG 5C-D**) ***(32; 11)***. Activation of AMPK by phosphorylation was measured in synchronized human skin fibroblasts (**FIG 5C**) and in brain of non-fasted wild-type mice kept in constant darkness (**FIG 5D**). Relative phosphorylation on threonine 172 of AMPK displayed ~24 hours oscillations in both fibroblast culture and brain homogenate with a trough occurring respectively at 20 hours post- shock (**FIG 5C**) and a trough at CT 12 (**FIG 5D**).

Together with the observations of the circadian ATP fluctuations, these data establish new insights in the multilevel regulation of the mitochondrial structure–function relationship by the biological clock, indicating a timekeeping system not only cyclic, but also outlining prior and upcoming events, in order to maintain energetic homeostasis in response to cellular metabolic change over the time.

DISCUSSION

In our study, we aimed to investigate the clock-control on the coupling between mitochondrial network and bioenergetics to anticipate energetic requirements of diverse

cellular functions in response to cellular and environmental constraints. The major breakthroughs were that, (i) along rhythmic mitochondrial respiration, ATP, ROS and NAD^+ as readouts of normal mitochondrial metabolism displayed circadian oscillation; (ii) these rhythmic byproducts were directly coupled with mitochondrial dynamics through the clock-controlled Drp1 activity and (iii) both mitochondrial dynamics and bioenergetics might feed input signals back to the circadian clock.

Examination of the ATP content revealed that human skin fibroblasts and MEFs displayed circadian oscillations in ATP levels entirely coupled with rhythmic mitochondrial oxygen consumption in vitro. Remarkably, the bioenergetic profile showed that only mitochondrial respiration varied in a circadian manner over the time whereas the glycolysis remained unchanged. It indicated that this event is significantly dependent on mitochondrial respiration, consistent with the notion of mitochondria as major producers of ATP within the cell. Furthermore, when in resting state (low ATP), the cells exhibited a higher spare respiratory capacity indicating that the cells are more flexible to response to fluctuations in cellular energy demands than those cells which are already in high energy production state. The importance of a functional molecular clock in rhythmic ATP generation was confirmed in MEfs deficient in core clock repressors *Per1* and *Per2* consistent with the findings on depletion of ATP in astrocytes from both *Per1* and *Per2* -/-, as well as *Cry1* and *Cry2* -/- knock-out mice ***(28)***. Nevertheless, the opposite effect was observed in MEFs lacking of clock repressors in galactose-containing medium, where increased mitochondrial ATP production was consistent with increased mitochondrial oxidative metabolism ***(33)***. Similarly, mouse brain exhibited circadian oscillations in ATP levels in vivo according to the period of activity and rest. These findings can be explained, at least, with results from transcriptional profiling studies. Consistent with previous data in SCN2.2 cells and the SCN in vivo ***(15; 34; 35)***, the expression of several subunits involved in the electron transport chain as well as in the ATP synthase appeared to be clock-controlled in human skin fibroblasts. In addition, numerous key bioenergetic parameters, including mitochondrial membrane potential and cytochrome c oxidase activity ***(16)*** as well as extracellular ATP accumulation ***(29)***, seem to follow circadian oscillations in SCN as well as in astrocytes. Moreover, the ATP breakdown product adenosine is known as crucial for the transition from wakefulness to sleep ***(36)***. Accumulation of adenosine in the basal forebrain induces the release of GABA, one of the inhibitory neurotransmitters involved in this transition, which eventually initiates the sleep phase. Overall, these findings support the concept of temporal organization of mitochondrial bioenergetic metabolism generating rhythmic mitochondrial ATP which in turn, along its metabolites, represents a physiological output of the mammalian cellular clock, especially in the brain, one of the highest energy demanding tissues of the human body.

Along the rhythmic ATP coupled with rhythmic mitochondrial respiration, we also observed that several metabolites, including ROS and NAD^+, remotely or closely connected to mitochondrial functions exhibited circadian oscillation in vitro and in vivo, suggesting a direct effect of the endogenous circadian clock on multiple metabolic pathways. In addition, our global circadian metabolomic analysis demonstrated that half of all rhythmic compounds were amino acids and associated metabolites known to be engaged in mitochondrial energetic homeostasis through branched-chain amino acids metabolism, glycolysis and TCA cycle to provide the energetic substrates such as pyruvate for the generation of ATP through oxidative phosphorylation, exhibited circadian profile in vitro. Furthermore, numerous rhythmic metabolites are also engaged in redox homeostasis through glutathione metabolism, which is one major endogenous defense system to combat cellular oxidative stress. Overall, the major circadian pathway signatures of the cellular metabolome that we have identified are consistent with those previously described in rodents ***(37)*** and humans ***(26)***. Moreover, it was recently suggested that clock control over mitochondrial activity is mediated, in part, by cycling metabolites (e.g: NAD^+) and protein acetylation ***(33; 38)***. Taken together, these findings support the hypothesis that the circadian clock synchronizes mitochondrial ATP production in a time-of-day-dependent manner in response to daily fluctuations in cellular energy demands. The regulation of mitochondrial function appears to be achieved by the clock, among others potential pathways, through several rhythmic metabolites acting as upstream signals from the clock to mitochondria.

Once perceived as solitary structures, mitochondria are now recognized as highly mobile along cytoskeletal tracks and dynamic organelles that continually fuse and divide in response of cellular energy requirements ***(39)***. Our findings highlight, on the one hand, that mitochondrial bioenergetics are coupled to mitochondrial network changes in physiological conditions in vivo and in vitro, and, on the other hand, this coupling is under the control of the circadian clock. Interestingly, expression of genes involved in fusion and fission did not exhibit any oscillations whereas, in accordance with the circadian variations of ATP, the mitochondrial network showed significant circadian changes in its architecture in vitro and in vivo, suggesting that clock-controlled post-transcriptional modifications might be involved in the regulation of the mitochondrial network. Drp1, the only known mammalian mediator of mitochondrial fission, is one of the proteins that ensures the integrity of a healthy mitochondrial population within the cell but also the integrity of the cell itself. Analysis of regulation of Drp1 by the couple PKA/ Calcineurin at the phosphorylation site serine 637 revealed that this process followed a circadian pattern. Moreover, it has been described that the activity of calcineurin, but not its protein expression, is under circadian regulation ***(40)***

suggesting that the oscillation of Drp1 phosphorylation may be dependent of the dephosphorylation activity of calcineurin. Likewise, when Drp1 activity is pharmacologically or genetically impaired, we revealed that the circadian fluctuations in ATP content were abolished in vitro and in vivo confirming the clock control of Drp1-dependent mitochondrial metabolism. Altogether, the data showed for the first time that the regulation of mitochondrial function is achieved by the clock-controlled mitochondrial network, in part, through the post-transcriptional regulation of Drp1.

While the extensive roles of mitochondria in maintaining cellular homeostasis and activity have created the need for diverse means of communication to and from the mitochondrial network, the underlying signaling mechanisms between the biological clock and mitochondrial network remain mostly unclear. When both mitochondrial respiration and fission are pharmacologically inhibited, we observed the dampening of mitochondrial ATP rhythm as well as the increase of circadian period in human skin fibroblasts. Interestingly, these findings can be explained by the well-established nutrient sensors such as AMPK involved in the crosstalk between metabolism and the biological clock ***(11)***. According to this study, the activation of AMPK followed a circadian pattern in vitro and in vivo, partially in antiphase to cycling ATP confirming that rhythmic mitochondrial ATP has a key role in retrograde signaling to the clock via the activation of AMPK, which in turn mediates the phosphorylation of CRY leading to proteolytic degradation of the negative arm of the central oscillator ***(32)***.

Overall, along the growing body of evidence stating the importance to integrate the cellular metabolism to circadian clocks ***(41)***, our findings establish a detailed molecular link among circadian control of the mitochondrial architecture and mitochondrial oxidative metabolism suggesting the key role of the clock-controlled mitochondrial network to anticipate energetic requirements of diverse cellular functions in response to cellular and environmental constraints (**FIG S6**). The events of mitochondrial morphological transitions as central actor in coupling circadian and mitochondrial metabolic cycles through metabolic retrograde signaling strengthen the still elusive understanding of the crosstalk between cellular systems and the biological clock. Thus, our findings could have multiple implications in the context of metabolic homeostasis in human health and diseases linked to impairment in circadian clock and/ or mitochondrial function. Perturbations in the clock may be a key initiating factor in diseases linked to mitochondrial weakening including neurodegenerative diseases as Alzheimer's disease.

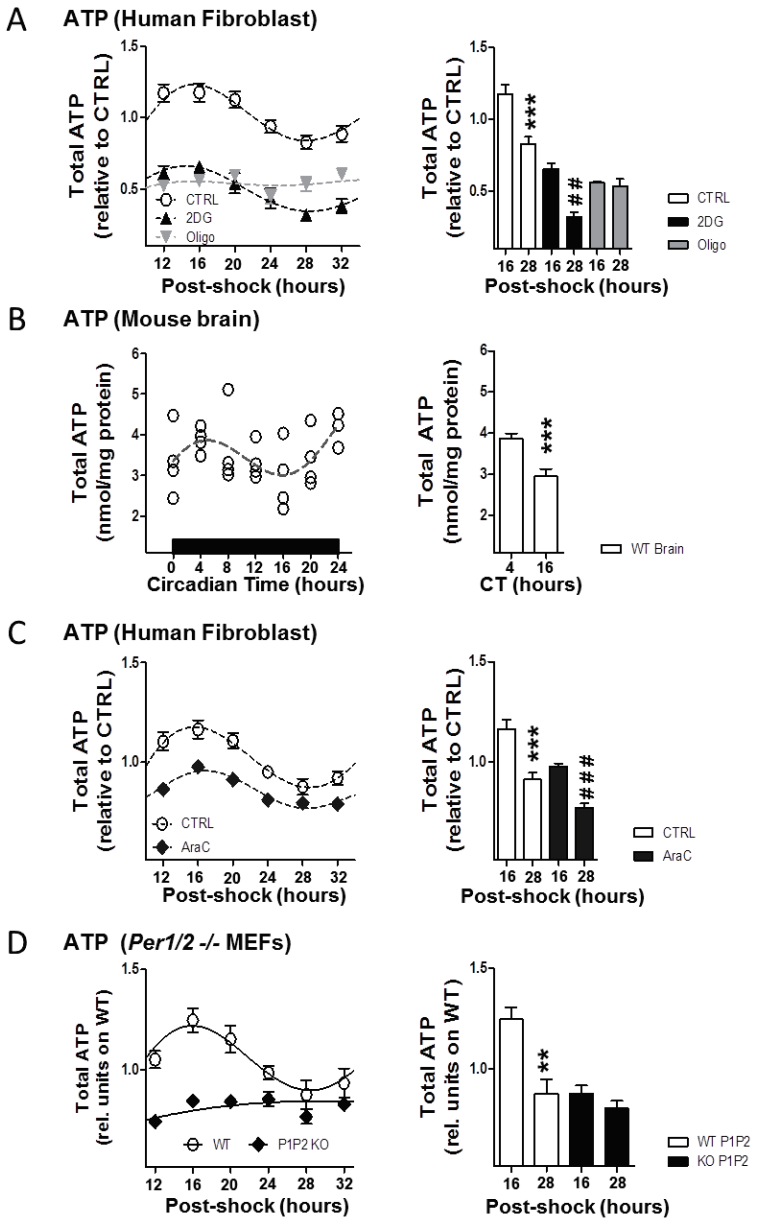

Fig. 1. Circadian clock control of mitochondrial ATP. (A) Relative total ATP level measured from 12 hours post-synchronization time point every 4 hours for 6 time points in human skin fibroblasts treated with 2 - deoxy-D-glucose (2DG, 4.5g/L) or oligomycin (OLIGO, 2 µM) compared to non-treated cells (CTRL) (n=4). Left panel displays relative total ATP level at 16 hours post-shock (peak of ATP content) and at 28 hours (trough of ATP content) in control and treated conditions. **(B)** Relative total ATP level measured in brain of non-fasted wild-type mice kept in constant darkness every 4 hours for 24 hours (n= 4). Left panel displays relative total ATP level at CT4 (peak of ATP content) and at CT16 (trough of ATP content) in control and treated conditions. **(C)** Relative total ATP content measured from 12 hours post-synchronization time point with 4 hours intervals for 6 time points in AraC- treated fibroblasts (100 µM) compared to non-treated cells (CTRL) (n= 8). Left panel displays relative total ATP level at 16 hours post-shock (peak of ATP content) and at 28 hours (trough of ATP content) in control and treated conditions. **(D)** Relative total ATP level measured from 12 hours post-synchronization time point every 4 hours for 6 time points in *Per1/2 −/−* MEFs compared to controls. Left panel displays relative total ATP level at 16 hours post-shock (peak of ATP content) and at 28 hours (trough of ATP content) in control and *Per1−/− and Per2−/−* MEFs**.** $^{**/\#\#}$ P < 0.01, $^{***/\#\#\#}$ P < 0.001 for Student's two-tailed t test comparing single time points of 16 hours versus 28 hours. Data are represented as average ± SEM.

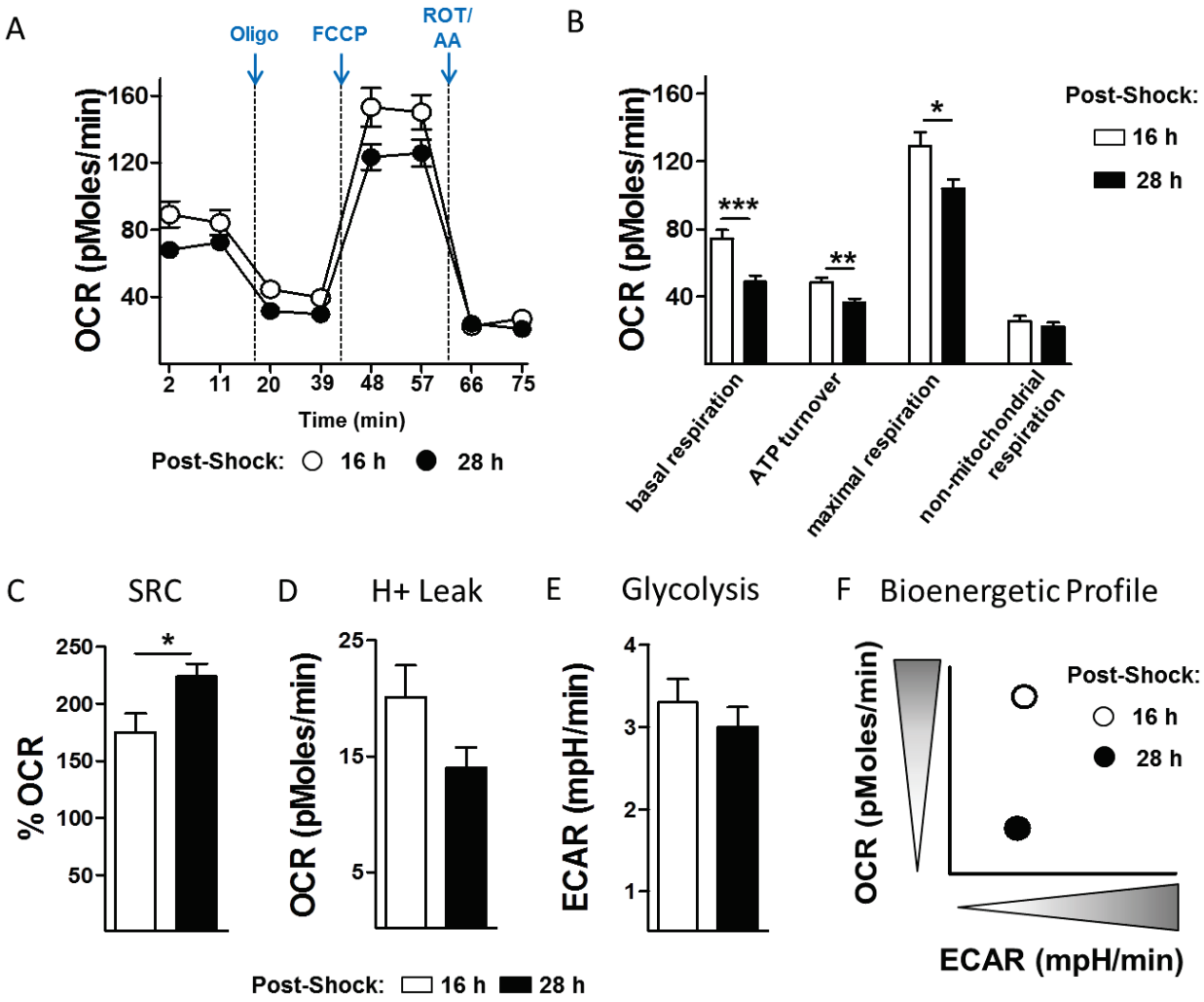

Fig. 2. Circadian modulation of mitochondrial respiration. (**A**) Oxygen Consumption Rate (OCR) evaluated in synchronized fibroblasts treated sequentially with oligomycin (Oligo, 2 µM), FCCP (0.7 µM), rotenone (ROT, 2 µM) and antimycin A (AA, 4 µM) at 16 hours post-shock and at 28 hours post-shock (n= 2). (**B**) Bioenergetic profile of synchronized fibroblasts at 16 hours post-shock and at 28 hours post-shock (n= 2). Basal respiration, ATP turnover and maximal respiration are determined after normalization to non-mitochondrial respiration. (**C**) Percentage of Oxygen Consumption Rate (OCR) linked to spare respiratory capacity (SRC) at 16 hours post-shock and 28 hours post-shock in human skin fibroblast (n= 2). (**D**) OCR related to the proton leak (independent to ATP production) at 16 hours post-shock and 28 hours post-shock in human skin fibroblast (n= 2). (**E**) Extracellular Acidification Rate (ECAR) corresponding to glycolytic rate at 16 hours post-shock and 28 hours post-shock in human skin fibroblast (n= 2). (**F**) Cellular bioenergetic profile of human skin fibroblast at 16 hours post-shock and 28 hours post-shock (n= 2). *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

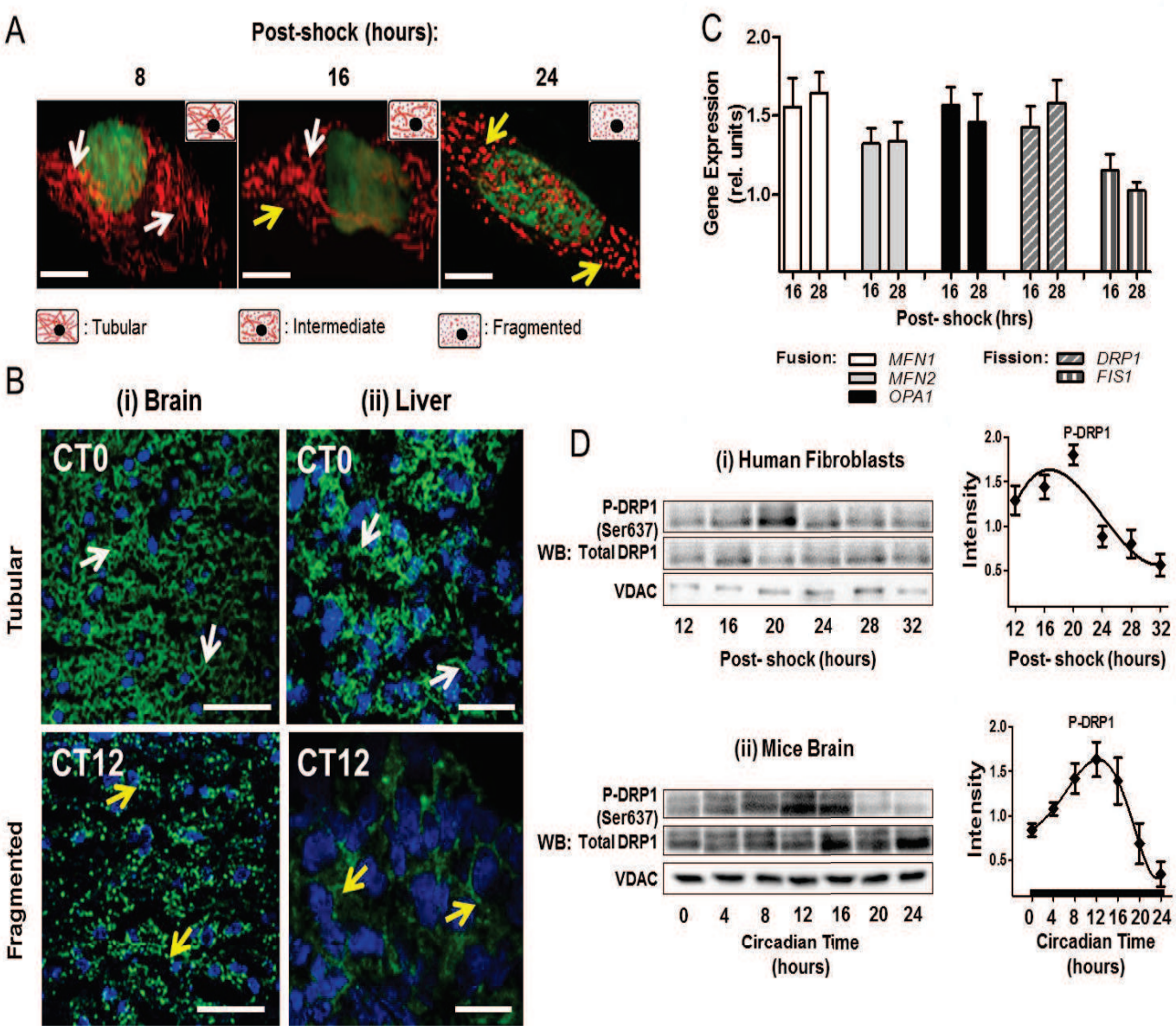

Fig. 3. Circadian clock regulation of mitochondrial dynamics. (**A** and **B**) Mitochondrial network morphology assessed in (**A**) synchronized human skin fibroblasts at 8 hours intervals for 3 time points and in (**B**) (i) brain and (ii) liver from non-fasted wild-type mice kept in darkness condition at CT0 and CT12. White arrows indicate the tubular network and yellow arrows designate the fragmented network. Scale bars, (**A**) fibroblast, 7.5 µm, (**B**) brain, 50 µm; liver, 25 µm. (**C**) Relative mRNA expression of nuclearly-encoded genes related to mitochondrial fusion (*MFN1*, *MFN2* and *OPA1*) and fission (*DRP1* and *hFIS1*) measured at 16 hours and 28 hours post-synchronization in human skin fibroblasts (n = 5 per time points). (**D**) Right: Phosphorylation of DRP1 (P-DRP1) on serine 637 and total DRP1 evaluated in (**i**) human skin fibroblasts 12 hours post-synchronization time point at 4 hours intervals for 6 time points and in (**ii**) brain homogenate of non-fasted wild-type mice kept in constant darkness every 4 hours over the course of 24 hours. Left: Relative DRP1 phosphorylation (ser637) measured in (i) human skin fibroblasts and in (**ii**) brain homogenate of non-fasted wild-type mice kept in constant darkness (n = 3). Data are represented as average ± SEM.

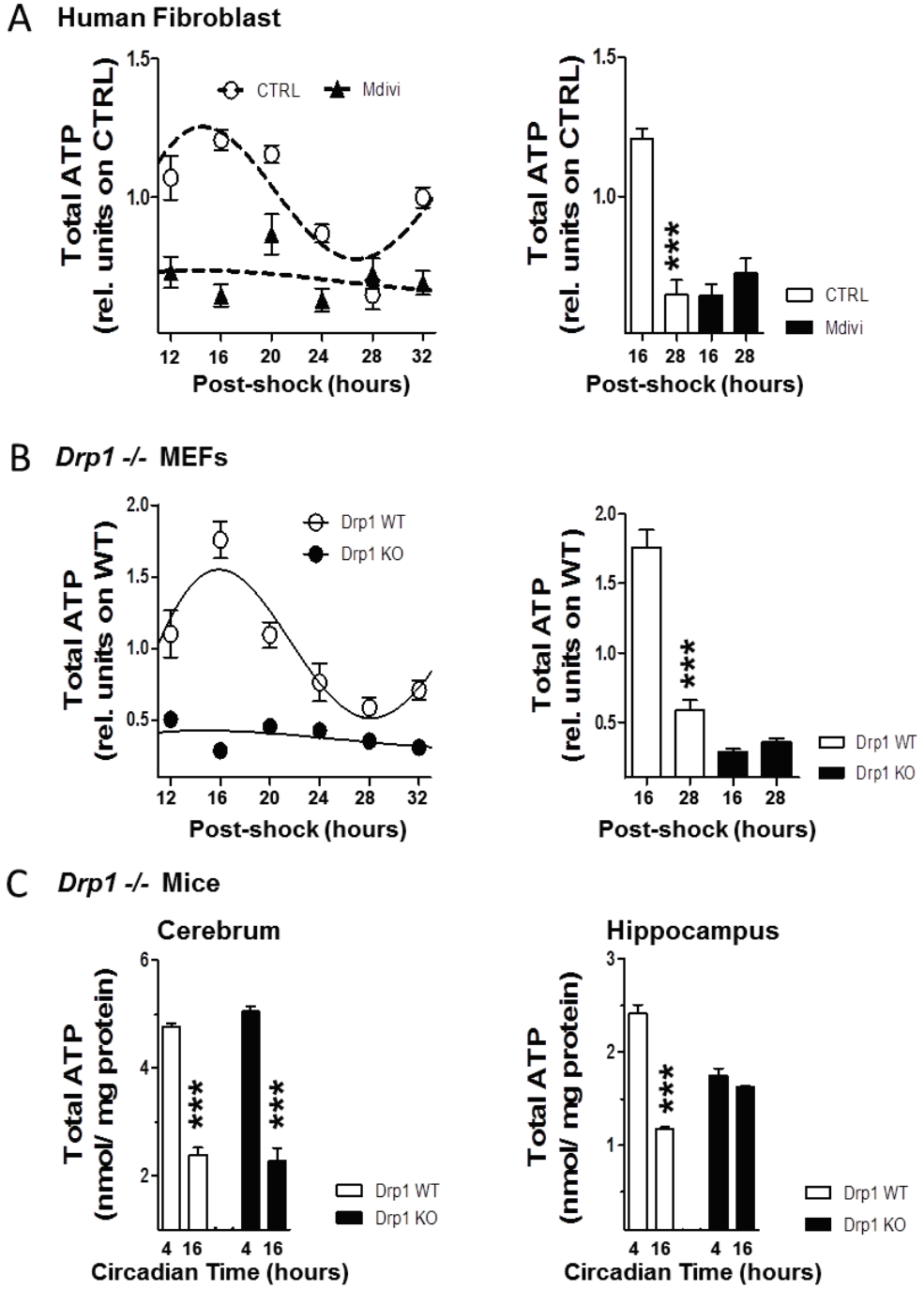

Fig. 4. Clock control of Drp1-dependent mitochondrial metabolism. (A and B) Relative total ATP level measured from 12 hours post-synchronization time point every 4 hours for 6 time points in (**A**) human skin fibroblasts treated with Mdivi-1 (50 µM) compared to non-treated cells (CTRL) and (**B**) in *Drp1 -/-* MEFs (n=4). Left panel displays relative total ATP level at 16 hours post-shock (peak of ATP content) and at 28 hours (trough of ATP content) in control and treated conditions. **(C)** Total ATP level measured in cortex and hippocampi of non-fasted *Drp1 -/-* mice kept in constant darkness at CT4 (peak of ATP content) and at CT16 (trough of ATP content) (n= 4). *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

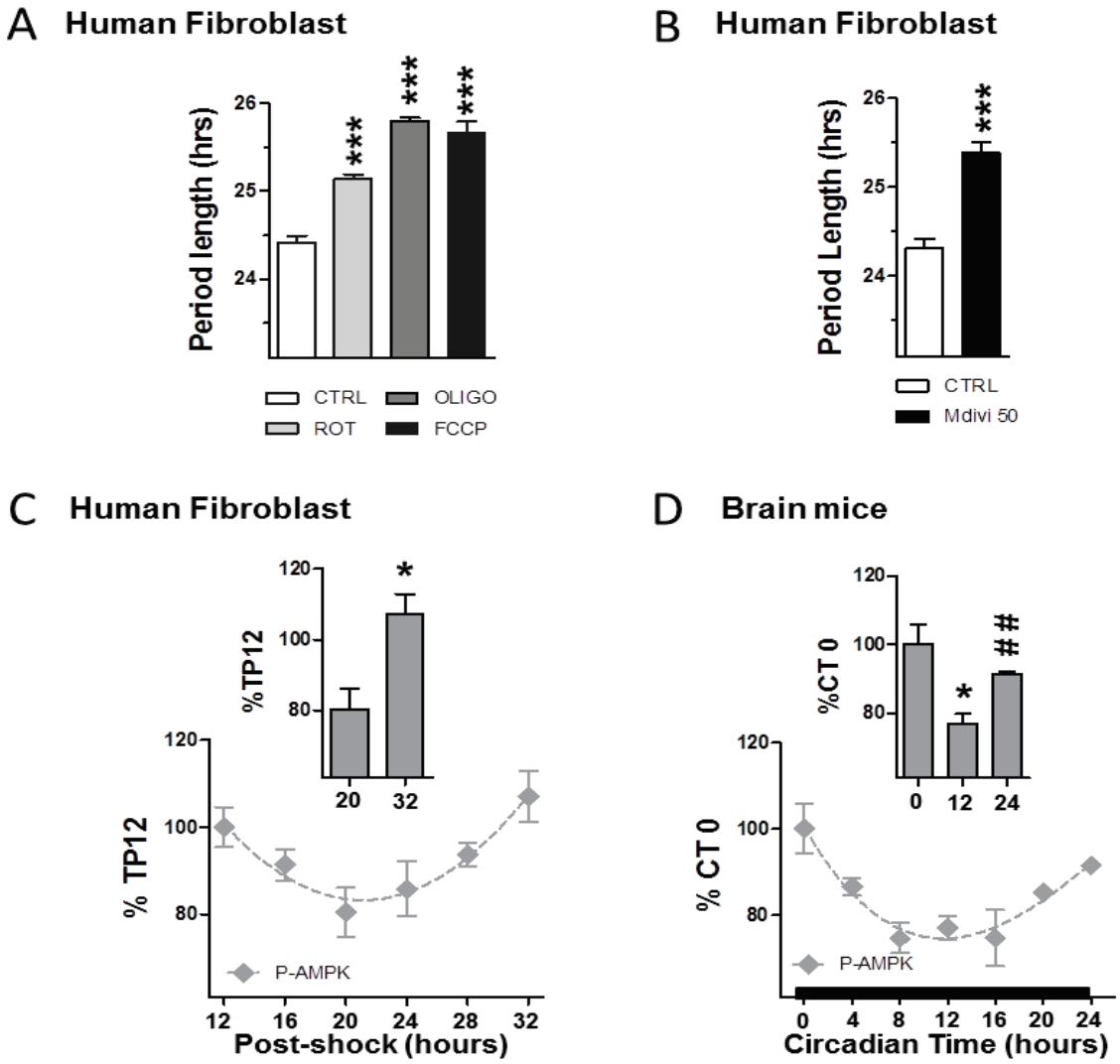

Figure 5. Retrograde signaling from mitochondrial network to the clock. (A and B) Circadian period length was determined in dexamethasone - synchronized human skin fibroblasts transfected with Bmal1::luciferase reporter in presence of (**A**) rotenone (ROT, 1 µM), oligomycin (OLIGO, 2 µM) or FCCP (4 µM) and (**B**) Mdiv-1 (50 µM) compared to control (CRTL). Bars represent the mean of three independent measurements ± SEM. **(C-D)** Activation of AMPK by phosphorylation was assessed from 12 hours post-synchronization time point at 4 hours intervals for 6 time points in **(C)** human skin fibroblasts (n = 4) and from **(D)** brain of non-fasted wild-type mice kept in constant darkness every 4 hours for 24 hours (n = 4). Quantification of activated AMPK is normalized on (**C**) values at 12 hours post-shock and on (**D**) value at CT 0. Insets in (**C-D**) represent the amount of phosphorylated AMPK at (**C**) 20 hours post-shock (trough) and at 32 hours (peak) and at (**D**) CT 0, 12 and 24. *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

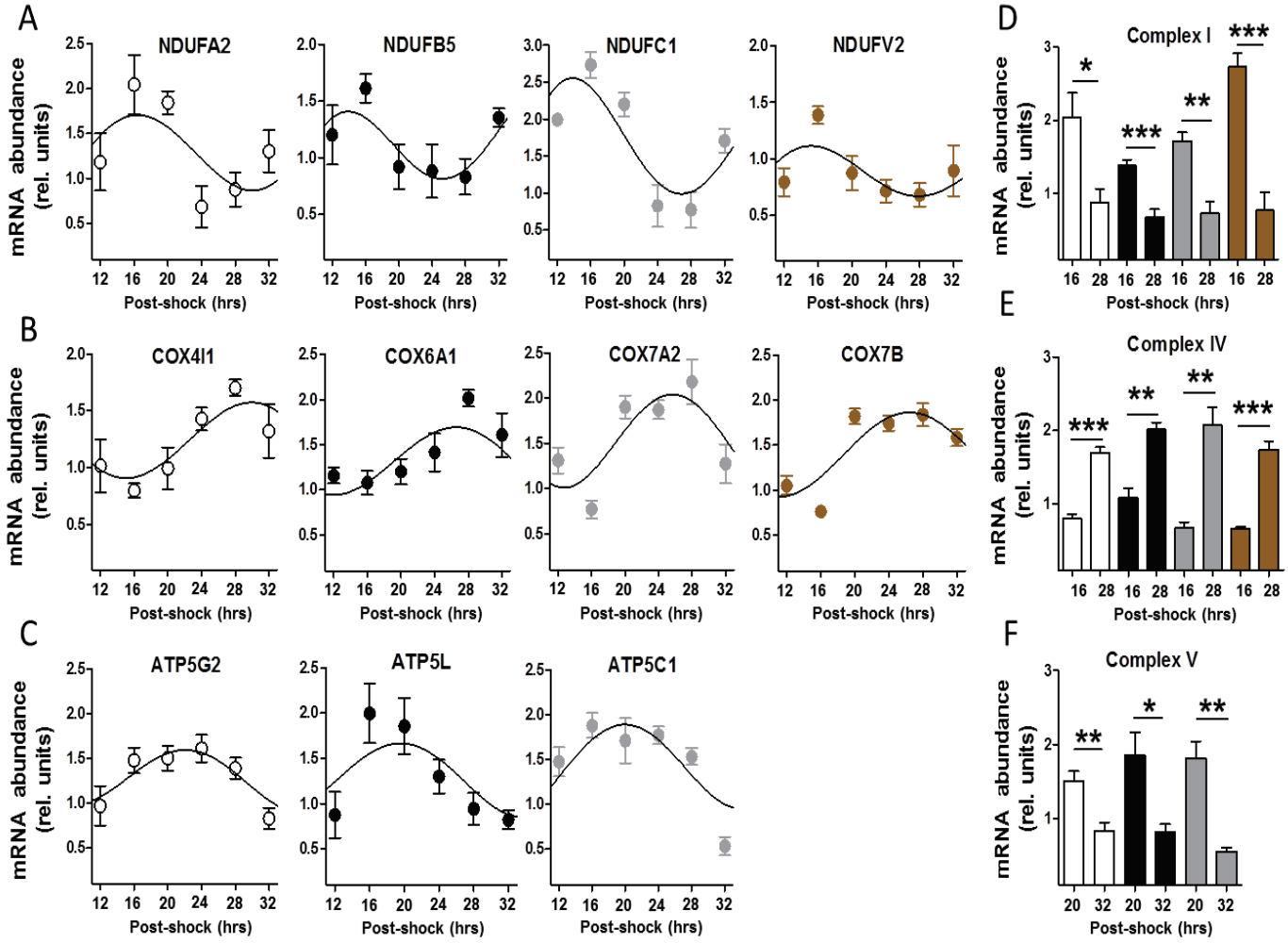

Figure S1. Circadian control of gene expression related to mitochondrial oxidative metabolism. (A-C) Circadian profiles of relative mRNA expression of (**A**) complex I, (**B**) complex IV and (**C**) complex V subunits involved in the electron transport chain and oxidative phosphorylation from 12 hours post-synchronization time point every 4 hours for 6 time points in human skin fibroblasts. (**D-F**) Relative mRNA expression of (**D**) complex I subunits, (**E**) complex IV subunits at 16 hours post-shock (corresponding respectively to the peak and trough) and 28 hours post-shock (corresponding respectively to the trough and peak) and (**F**) complex V subunits at 20 hours post-shock and 32 hours post-shock (corresponding respectively to the peak and trough) in synchronized fibroblast. *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

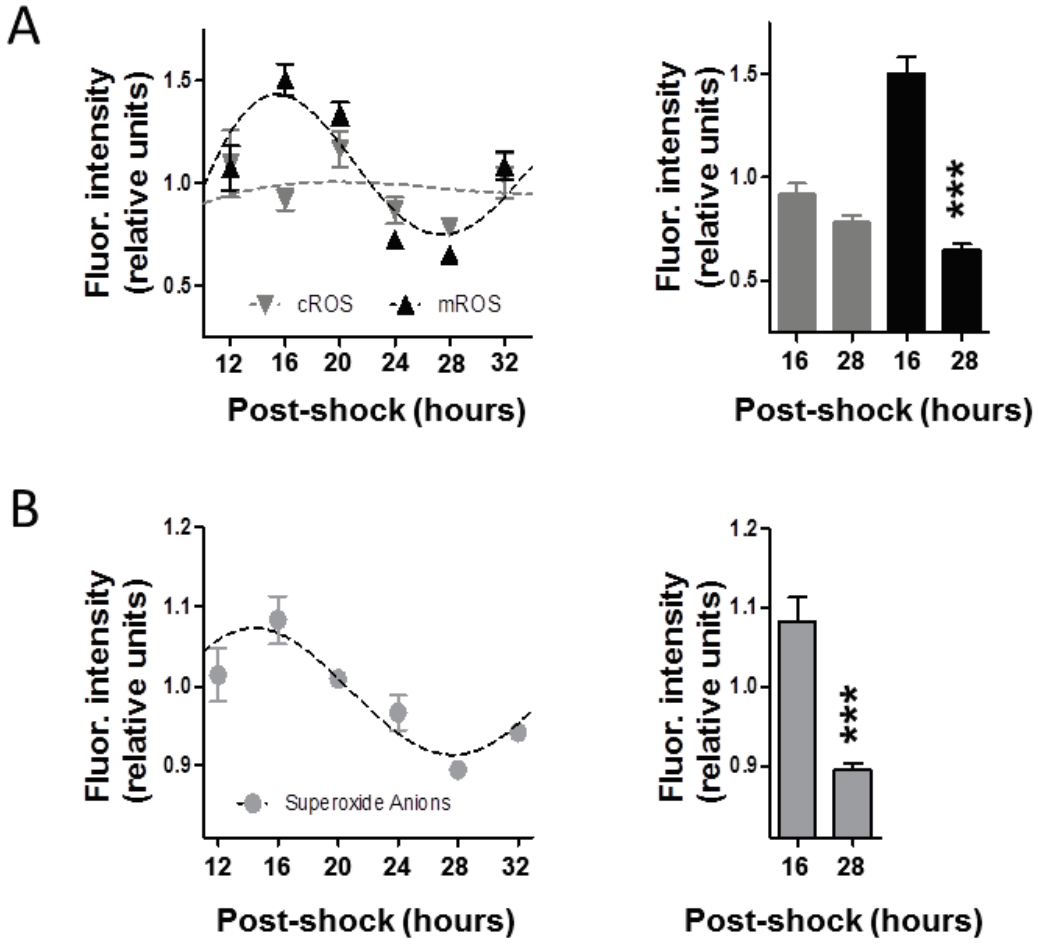

Figure S2. Mitochondrial ROS are driven by the clock. (A) Cytosolic (cROS) and mitochondrial (mROS) reactive oxygen species levels were evaluated from 12 hours post-synchronization time point with 4 hours intervals for 6 time points in human skin fibroblasts (n= 4). Left panel displays cROS and mROS levels at 16 hours post-shock (peak) and at 28 hours (trough). **(B)** Superoxide anions level was evaluated from 12 hours post-synchronization time point with 4 hours intervals for 6 time points in human skin fibroblasts (n= 4). Left panel displays superoxide anions level at 16 hours post-shock (peak) and at 28 hours (trough). *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

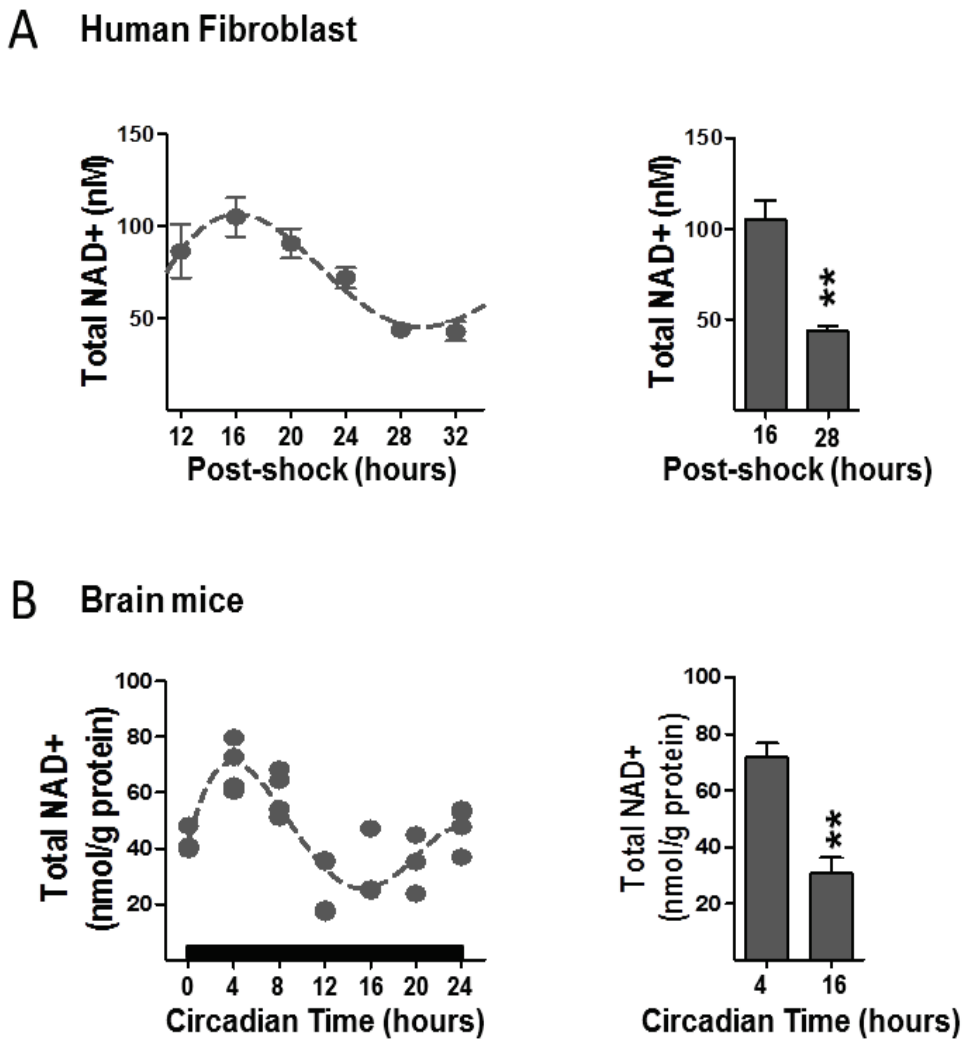

Figure S3. NAD+ content is clock-driven in vitro and vivo. (**A**) Total NAD+ measured 12 hours post-synchronization time point with 4 hours intervals for 6 time points in human skin fibroblasts (n=4 per time points). Inset in (D): Relative total NAD+ level at 16 hours post-shock (peak) and at 28 hours (trough). **(B)** Total NAD+ content measured every 4 hours for 24 hours from brain of non-fasted wild-type mice kept in constant darkness (n= 4). Inset in (E): Relative total NAD+ level at CT4 (peak) and at CT16 (trough). *P < 0.05, **P < 0.01, ***P < 0.001 for Student's two-tailed t test comparing single time points. Data are represented as average ± SEM.

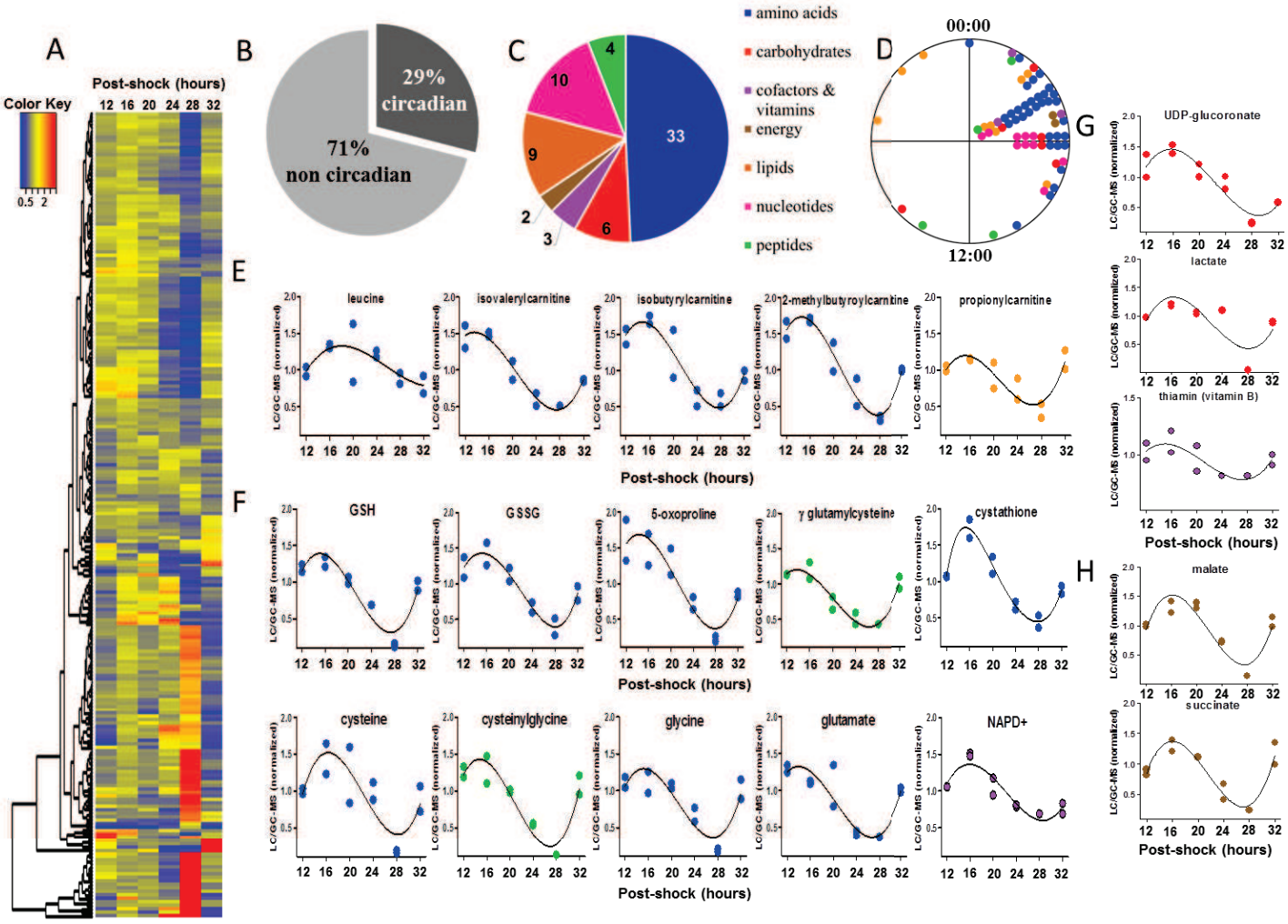

Figure S3. Rhythmic metabolites involved in mitochondrial metabolism. (A) Heat plots for all identified metabolites in synchronized human U2OS cells. High levels of metabolites are shown in red and low levels are shown in blue. **(B)** Percentage of non-circadian and circadian metabolites in synchronized U2OS cells. **(C-D)** Pathway analyses **(C)** and time-of-day distribution **(D)** of peak phases of rhythmic metabolites in synchronized human U2OS cells. Pathways are color-coded as follows: amino acids, blue; carbohydrates, red; cofactors & vitamins, violet; energy, brown; lipids, orange; nucleotides, pink; peptides, green. **(E-H)** Rhythmic profiles of ascertained metabolites previously described as engaged in (**E**) branched-chain amino acids metabolism, in (**F**) GSH/GSSG metabolism, in (**G**) glycolysis and (**H**) TCA cycle (n= 2).

A

	Tubular	Intermediate	Fragmented
8hrs/32hrs	85	10	5
16hrs/40hrs	17	73	10
24hrs/48hrs	2	6	92

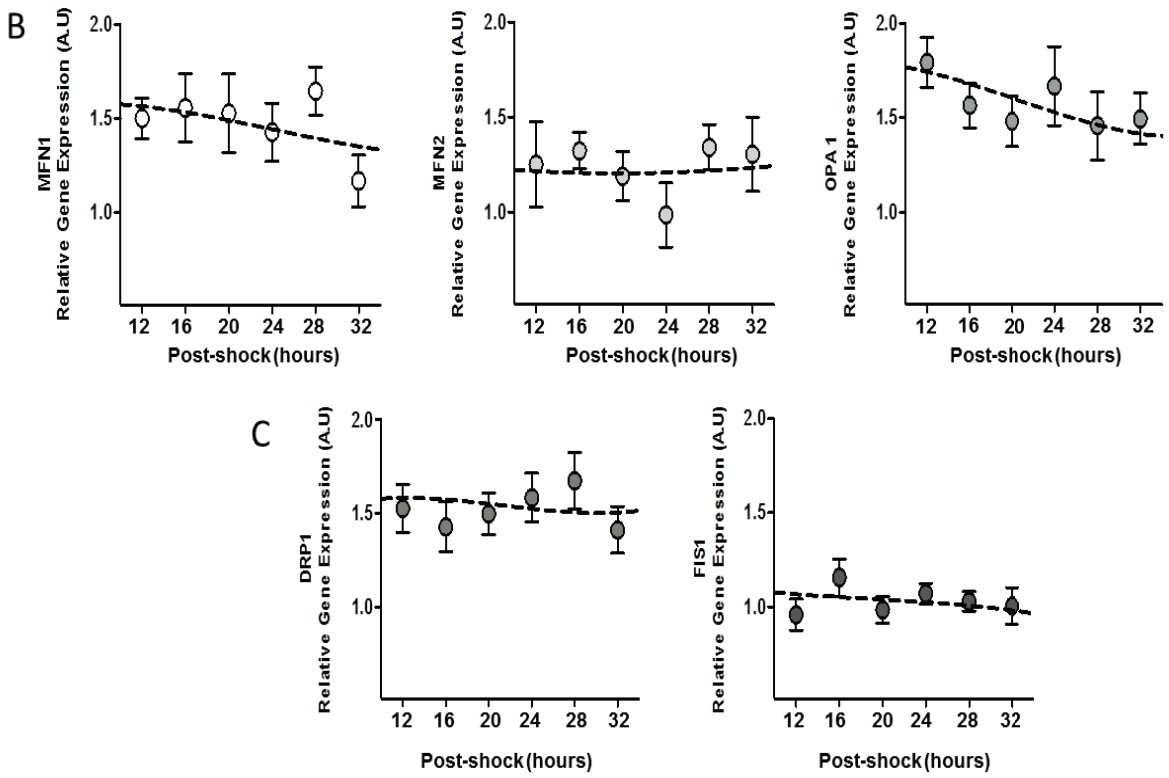

Figure S5. Clock- controlled mitochondrial structural network organization. (**A**) Number of cells displaying one of the mitochondrial network states (tubular, intermediate and fragmented) determined from 8 hours post-shock every 8 hours over the course of 48 hours (mean of 3 independent experiments, 100 cells counted per conditions). (**B, C**) Profile of relative mRNA expression of nuclearly-encoded genes related to (**B**) mitochondrial fusion (*MFN1*, *MFN2* and *OPA1*) and (**C**) fission (*DRP1* and h*FIS1*) was evaluated from 12 hours post-shock every 4 hours for 6 time points in human skin fibroblasts without displaying circadian oscillations. Data are represented as average ± SEM.

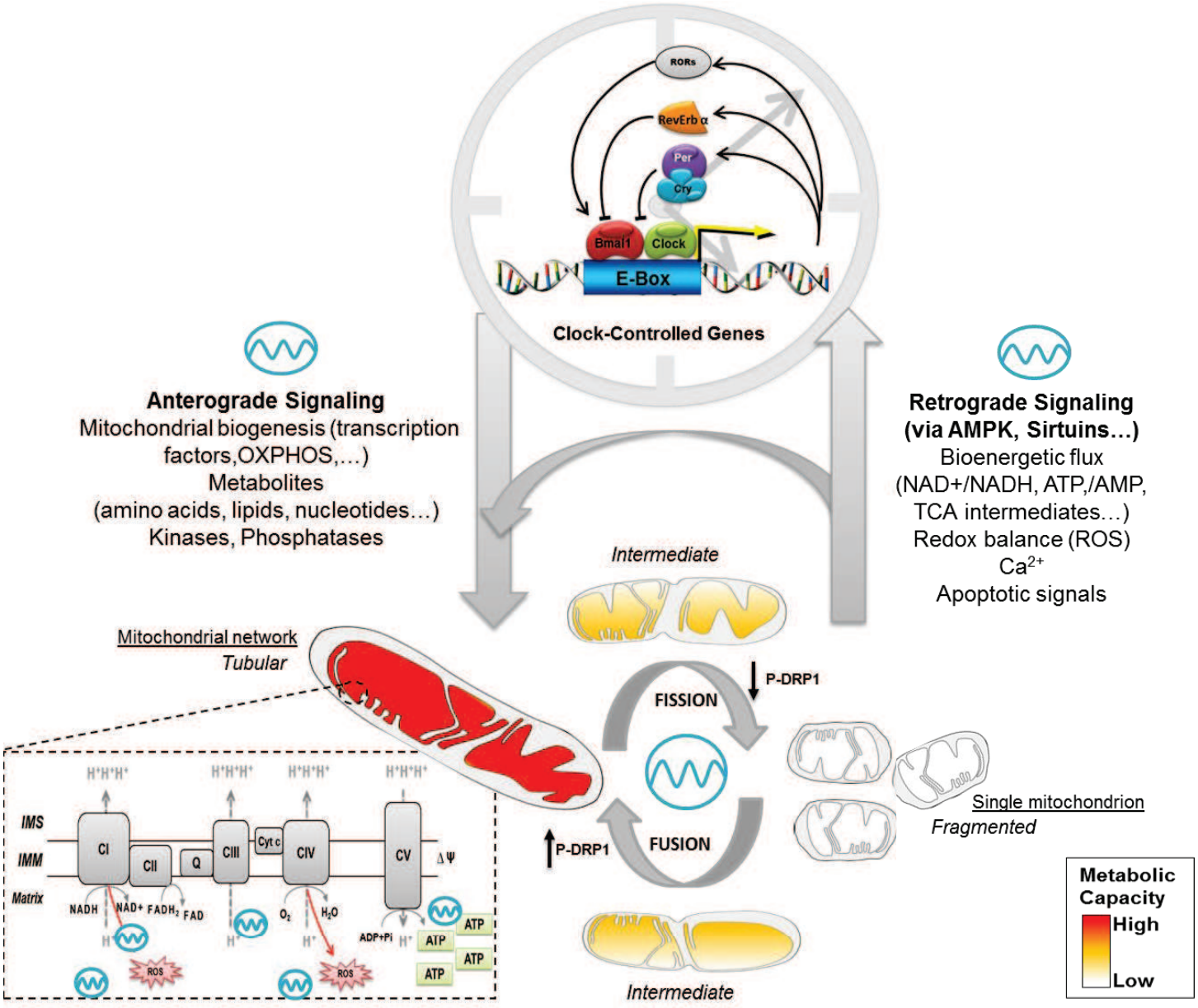

Figure S6. Schematic representation of the relationship between the core molecular clock and mitochondrial dynamics.

Contributions

KS, AG, BO, RD performed experiments. JAR and UA provided Per1/2+/+ and Per1/2-/- mice. SF provided the *Drp1*$^{flx/flx}$ *Cre*+ mice and Drp1 lox/lox and Drp1 -/- MEFs. SBB provided Per1/2+/+ and Per1/2-/- MEFs. UEL, SF, SBB and AE conceived the project, coordinated and supervised research. KS, SF, SBB and AE wrote the manuscript.

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III. CONCLUSION

CONCLUSION

The fine understanding of how mitochondria function is a major field of research since it is becoming increasingly apparent that mitochondrial dysfunction is involved in a broad range of metabolic and degenerative diseases, and aging ***(1)***. Mitochondria are particularly important in the brain which has high energy requirements since neurons need a lot of energy in form of ATP molecules to fulfill their roles ***(2)***. Increasing evidences showed that reduced energy metabolism together with mitochondrial dysfunction, oxidative stress, and impaired mitochondrial fusion/fission activity, constitutes a prominent and early event in neurodegenerative diseases, including AD ***(3; 1)***. Thus, current pharmacological concepts aim to boost mitochondrial bioenergetics, especially by improving cellular respiration and by increasing ATP production.

In the last decade, neurosteroids have emerged as new potential therapeutic tools in degenerative diseases as peripheral neuropathies and AD ***(4; 5)***. For instance, neurosteroids, such as estradiol and allopregnanolone, were able to alleviate deficits manifested in AD by inducing neurogenesis, reducing Aβ generation and neuroinflammation, and decreasing cognitive impairments in AD transgenic mice ***(6-9)***. However, except for estradiol, the effects of neurosteroids on mitochondrial function remained unresolved.

In the present PhD work, we demonstrated that not only estradiol but also other neurosteroids were able to boost mitochondrial function in neuronal cells. In physiological (healthy) conditions, most of the neurosteroids were able to improve mitochondrial bioenergetics by increasing ATP levels, MMP and basal respiration (see **section II.A**). Each of them appeared to have a specific action profile upon bioenergetic outcomes, possibly because they are acting via different steroid receptors. These observations are in line with other studies showing that some neurosteroids regulated the expression of different genes coding for proteins involved in the bioenergetic metabolism, such as enzymes involved in glycolysis, the Krebs cycle, and mitochondrial complexes (reviewed in ***(10)***, see **APPENDIX 4**).

In pathological conditions, more specifically in cellular models mimicking certain histopathological aspects of AD - namely the presence of Aβ peptide and abnormally hyperphosphorylated tau – we showed that neurosteroids belonging also to the family of sex hormones were able to alleviate bioenergetic deficits occurring in these cellular models (see **section II.B**). Interestingly, female hormones (estradiol and progesterone) were more efficient to alleviate mitochondrial deficits induced by abnormal tau, whereas testosterone, the male hormone, increased bioenergetic output in cell overexpressing APP/Aβ. In agreement with these findings, previous studies showed that neurosteroids may exert their neuroprotective action, at least in part by modulating Aβ clearance/accumulation and tau hyperphosphorylation, but also by protecting neurons against oxidative stress and apoptosis

(11). Here, we showed that their protective action may be mediated by an improvement of mitochondrial function that is impacted by Aβ and abnormal tau, possibly via the regulation of genes involved in the bioenergetic metabolism, as hypothesized in **section II.A**.

The decrease of blood steroid levels (e.g. the drop of estradiol in women after the menopause) and brain neurosteroid levels with increasing age – a phenomenon even more pronounced in AD – are believed to impact neuronal and cognitive functions ***(12-14)***. Indeed, studies showed that the decrease in brain steroid levels was paralleled by Aβ accumulation and tau hyperphosphorylation in some brain regions, as well as cognitive deficits ***(12; 14)***, suggesting that neurosteroid protective effects play a role in maintaining proper brain function during aging. In line with these observations, numerous studies attest the protective effects of neurosteroids *in vitro* and *in vivo*, with a major focus on sex steroid hormones, especially estradiol, the main female hormone, due to the fact that one-third of AD patients are woman (see **APPENDICES 4 and 5**). However, clinical trials showed contradictory results: on the one hand, hormonal replacement therapy (HRT) was shown to have beneficial effects in postmenopausal women by decreasing the risk to develop AD ***(15; 16)***. On the other hand, results from the "Woman's health initiative memory study" (WHIMS) showed that long term HRT aggravate cognitive symptoms with, in addition, diverse side effects, including increased risks for breast cancer, pulmonary embolism and stroke ***(17; 18)***. The synthetic nature of the tested hormones has been implicated in the failure of the WHIMS trial but also the "critical time windows" in which steroid hormones, like estrogens, might exert a neuroprotective effect. Indeed, a recent study performed on ovariectomized rats shows that only early onset estrogen replacement therapy was able to prevent oxidative stress and metabolic alteration induced by the loss of sex steroid hormones after ovariectomy ***(19)***. In our study (**section II.A**), we showed that the improvement of mitochondrial respiration induced by neurosteroids in neuronal cells was coupled with an increase of ROS levels, probably resulting from a greater electron leakage by the electron transport chain. Nevertheless, neurosteroids were able to regulated redox homeostasis by increasing the antioxidant activity to compensate this rise of ROS. Based on these observations, we can hypothesize that, to be protective, a neurosteroid-based therapy has to be administrated at an age when the redox system is still balanced, because one can assume that if the treatment starts too late, the presence of neurosteroids might be potentially deleterious due to increased ROS production when antioxidant defense mechanisms function insufficiently.

Taken together, our results provide a potential molecular basis for the beneficial and neuroprotective effects of neurosteroids, which may open new avenues for drug development with regard to targeting mitochondria in prevention of neurodegeneration.

Because each neurosteroid appeared to have a specific action profile on bioenergetic outcomes in healthy condition or in the presence of Aβ or tau, the underlying molecular mechanisms still need to be elucidated in more detail, such as their ability to regulate the expression and activity of mitochondrial complexes involved in oxidative phosphorylation.

To improve our understanding of the mechanisms regulating mitochondrial function, we additionally focused our research on the relationship between mitochondrial bioenergetics and dynamics, and their potential regulation by the circadian system. A growing body of evidences shows that the metabolic homeostasis and the circadian clock are connected in numerous ways through reciprocal regulation ***(20-22)***. In addition, circadian disruptions, which can occur during shift work, disturbed sleep/wake cycle, and dietary alterations were shown to impair metabolic homeostasis, leading to adverse health effects (reviewed in ***(23)***). Although mitochondria play a central role in cellular metabolic processes, the relationship between the circadian clock and mitochondrial function (dynamics and bioenergetics) remains mostly elusive.

In our work, we showed that mitochondrial bioenergetics, namely mitochondrial respiration and its byproducts (ATP, NAD+ and ROS), oscillate with a period length of about 24 hours in fibroblasts, an *in vitro* model of peripheral clock (**see section II.C**). These rhythmic oscillations in bioenergetics were consistent with the rhythms of mitochondrial changes in fusion/fission that are under the clock-controlled activity of the fission protein Drp1. The latter results could be confirmed also in vivo in mouse brains. A recent study is in line with our findings showing that the circadian clock controls mitochondrial oxidative function through rhythmic regulation of NAD+ biosynthesis in mice ***(24)***. Other studies addressed the question of circadian regulation of ATP production ***(25; 26)*** but no underlying mechanisms were described. Here, we established a detailed molecular link among the circadian control of mitochondrial bioenergetics and dynamics. More precisely, our findings suggest that the circadian clock is able to regulate mitochondrial ATP production in a time-dependent manner in order to anticipate the energy demand in response to cellular and environmental constraints. In addition, we showed that mitochondria may, directly or indirectly, send signals back to the circadian clock (e.g. NAD+ or activation of AMPK), which might, in turn, regulate the expression/activity of molecular clock components (e.g. PER, CRY).

Together, these findings shed new light on the circadian regulation of mitochondrial function, and vice versa, and could have multiple implications in the context of metabolic

homeostasis in human health and disease states linked to impairment in biological rhythm and/or mitochondrial function.

Altogether, our findings improved the knowledge about the modulation of mitochondrial bioenergetics in heath and disease states by: i) neurosteroids which constitute new therapeutic options to overcome bioenergetic deficits in neurodegenerative diseases, and ii) the biological clock which anticipates daily fluctuations in cellular energy demands **(Fig. 31).** Further investigations need to be performed to enlighten the underlying mechanisms in more details.

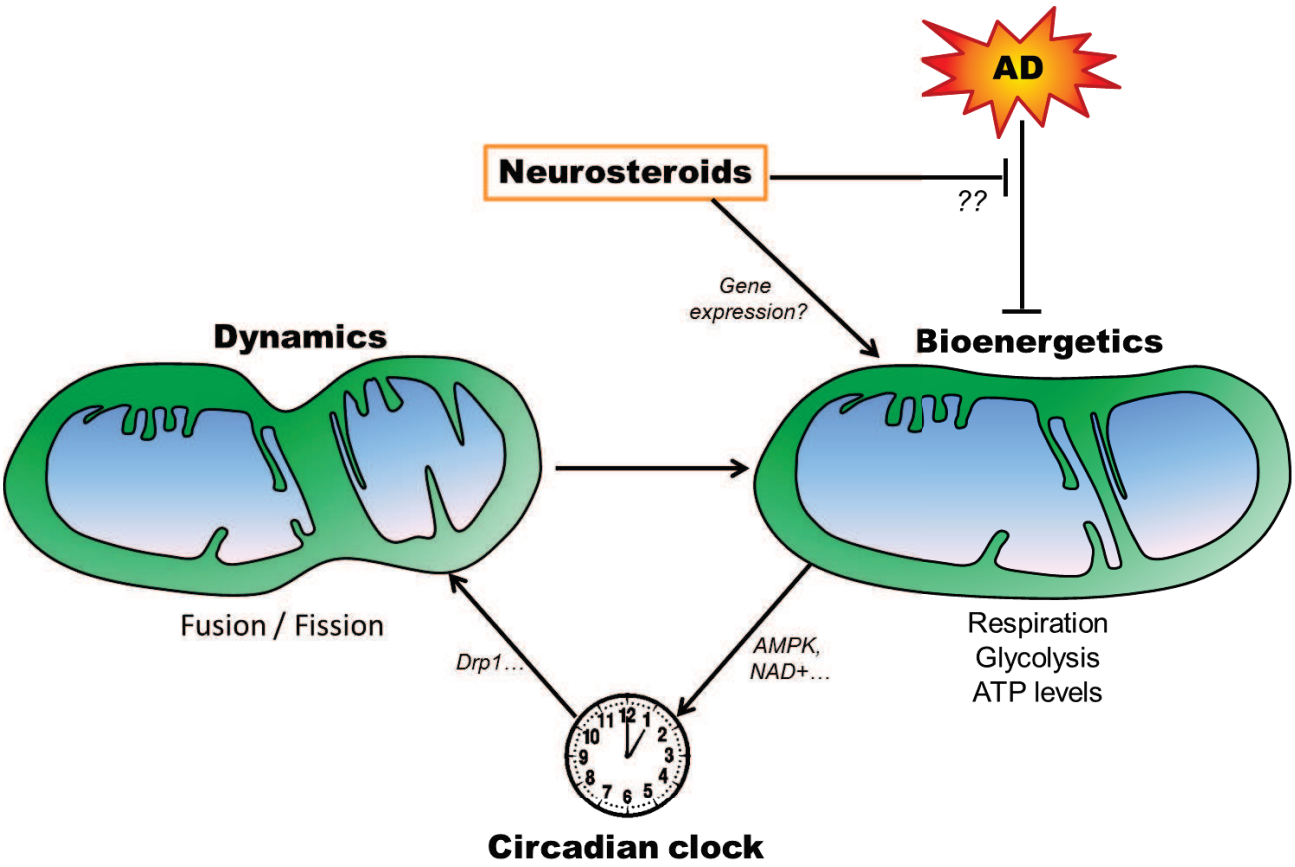

Fig. 31: Hypothetical links between mitochondrial function and the circadian clock and their regulation by neurosteroids. On the one hand, we showed that neurosteroids can increase mitochondrial bioenergetics which can alleviate mitochondrial dysfunction manifested in Alzheimer's disease (AD). On the other hand we showed that the circadian clock controls mitochondrial dynamics, which in turn modulate bioenergetic activity. In addition, mitochondria can send signals back to the clock that regulates mitochondrial dynamics and bioenergetics.

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IV. DESCRIPTIF SYNTHETIQUE EN FRANCAIS DES TRAVAUX DE LA THESE

Les mitochondries sont de minuscules organelles qui génèrent néanmoins la quasi-totalité de l'énergie cellulaire sous la forme de molécules d'adénosine triphosphate (ATP). Elles exercent un rôle fondamental au sein des cellules eucaryotes car elles orchestrent à la fois le métabolisme énergétique et les voies apoptotiques qui contrôlent la survie et la mort cellulaire. Il est maintenant largement admis que les mitochondries sont le produit de l'endosymbiose d'une α-proteobactérie avec l'ancêtre des cellules eucaryotes ***(1)***. De ce fait, elles possèdent encore un génome résiduel ainsi qu'une double membrane **(Fig. 1)**.

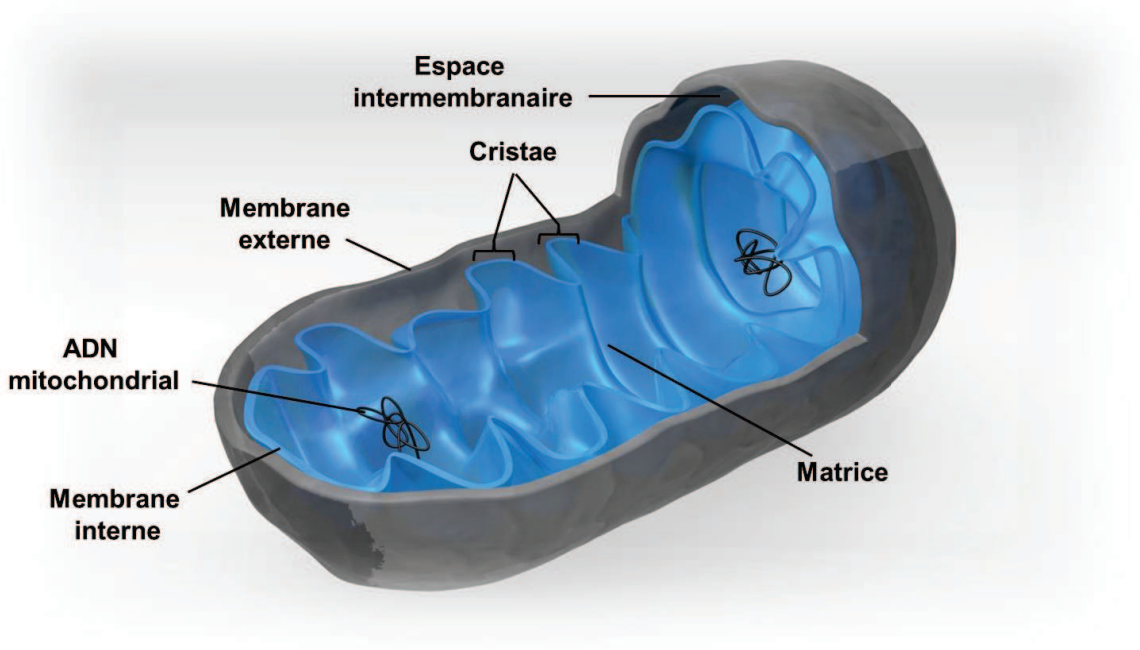

Fig. 1: Représentation classique de l'ultrastructure mitochondrial (courtoisie de M. Wanner Fabio).

Les mitochondries sont des organelles extrêmement dynamiques dont la taille et l'aspect varient selon les espèces et organes, mais aussi en fonction de l'environnement cellulaire. Afin de maintenir une population mitochondriale homogène, les mitochondries se divisent et fusionnent de manière cyclique pour mélanger leur contenu en métabolites et protéines, mais aussi pour éviter l'apparition de mutation dans l'ADN mitochondrial (mtDNA). Ce phénomène est appelé « fusion / fission » mitochondriale et permet la redistribution des organelles au sein des cellules en fonction des besoins énergétiques ***(2; 3)***.

Les mitochondries remplissent plusieurs rôles qui incluent notamment la régulation du calcium intracellulaire et l'homéostasie de l'état réduit/oxydé (redox) des cellules, la plasticité synaptique et la neurotransmission ***(1)***. Elles sont surtout considérées comme les "usines énergétiques" des cellules et une de leur fonction essentielle est la production de molécules d'ATP, qui est la source universelle d'énergie cellulaire, synthétisées pendant la phosphorylation oxydative (OXPHOS) à partir des sources nutritionnelles. En effet, l'ATP est

principalement produite via deux voies métaboliques : la glycolyse et l'OXPHOS (ou respiration cellulaire). La première voie fournie un gain net de deux molécules d'ATP, ainsi que deux molécules de nicotinamide adénine dinucleotides (NADH) et deux molécules de pyruvate. Ce dernier est ensuite transféré dans la matrice mitochondriale et rejoint le cycle de Krebs qui permet de produire les principaux donneurs d'électrons (NADH et flavine adénine dinucléotides ($FADH_2$)) utilisés pendant l'OXPHOS.

La deuxième voie de synthèse d'ATP, l'OXPHOS, utilise les donneurs d'électrons synthétisés par le cycle de Krebs pour générer une différence de potentiel au niveau de la membrane mitochondriale interne **(Fig. 2)**. En effet, les électrons apportés par NADH et $FADH_2$ sont transportés au sein de la chaîne de transport d'électrons (*electron transport chain* : ETC) composés de quatre complexes protéiques (complexe I à IV) responsables du transfert de protons (H^+) de la matrice vers l'espace intermembranaire. La différence de potentiel ainsi générée servira de moteur à l'ATP synthase (ou complexe V), l'enzyme responsable de la synthèse d'ATP. Comparé à la glycolyse qui ne produit que deux molécules d'ATP, l'OXPHOS génère entre 30 et 32 ATP par molécules de glucose ***(4)***.

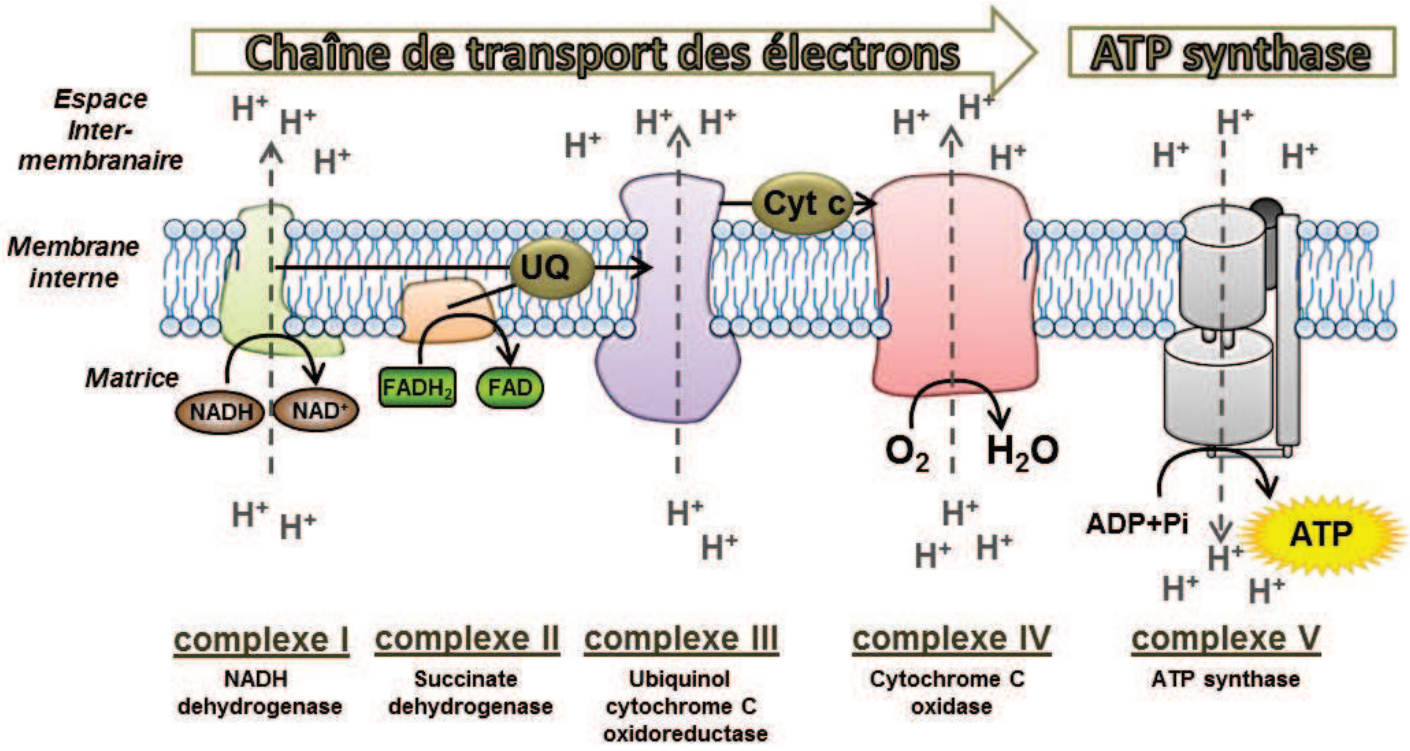

Fig. 2: Bioénergétique de la chaîne de transport d'électrons. Après le cycle de Krebs, le NADH généré est transféré au complexe I et converti en NAD+. Le transfert d'électrons à travers la chaîne maintient le potentiel de membrane mitochondriale en pompant des protons de la matrice vers l'espace intermembranaire. Au final, l'ADP est phosphorylée pour former l'ATP par le complexe V (ATP synthase). UQ; coenzyme Q, Cyt c; cytochrome c (adapté de ***(5)*** et ***(6)***).

Paradoxalement, alors qu'elles produisent la principale énergie vitale des cellules (ATP), les mitochondries produisent aussi des molécules toxiques dérivées de l'oxygène

(*reactive oxygen species* : ROS). Ces ROS sont en partie produites au cours de l'OXPHOS et, en temps normal, sont détoxifiées par le système de défenses antioxydant présent dans les cellules, comme par exemple les superoxydes dismutases (SOD) ***(7)***. Il arrive cependant que ce système de défense antioxydants soit dépassé, et l'accumulation des ROS peut engendrer un stress oxydant qui va perturber le bon fonctionnement des cellules et, au final, conduit à la mort cellulaire. Les neurones sont particulièrement sensibles face au stress oxydant et, étant des cellules postmitotiques et différenciées, leur mort engendrera des dysfonctionnements cérébraux et des troubles cognitifs, tels que ceux observé dans les maladies neurodégénératives.

En effet, le cerveau est un organe particulièrement énergivore qui, malgré sa petite taille, a besoin d'environ 20% de l'oxygène présent dans l'organisme pour fonctionner normalement ***(8)***. Ceci s'explique par le fait que les neurones sont des cellules excitables qui requièrent beaucoup d'ATP pour permettre la fusion et le recyclage des vésicules nécessaires à la sécrétion de neurotransmetteurs, mais aussi pour maintenir le potentiel de membrane des cellules ***(9)***. Ainsi, une perturbation de la fonction mitochondriale mènera inévitablement à un état pathologique pouvant aller d'une subtile altération des activités neuronales jusqu'à la mort cellulaire et à la neurodégénérescence, comme c'est le cas par exemple dans la maladie d'Alzheimer (MA).

Dans ce contexte, la compréhension des mécanismes intrinsèques qui contrôlent la dynamique et le métabolisme énergétique mitochondrial (ou bioénergétique) représente un enjeu majeur pour lutter efficacement contre les déficits métaboliques et les maladies neurodégénératives, telles que la MA.

Dans le cadre de ce travail de thèse, notre principal objectif a été d'approfondir nos connaissances sur la régulation de la fonction mitochondriale et d'identifier des facteurs clés (endogènes et/ou exogènes) qui sont déterminants dans le contrôle de l'activité mitochondriale. Ces facteurs pourraient alors servir d'outils pour élaborer des stratégies contre les pathologies impliquant des dysfonctionnements mitochondriaux. Pour atteindre cet objectif, nous avons organisé notre travail en deux parties:

1) En tenant compte des données bibliographiques qui suggèrent que les neurostéroïdes possèdent un fort potentiel neuroprotecteur, nous avons décidé d'évaluer leur capacité à améliorer la fonction mitochondriale en situation normale (physiologique) et dans des modèles cellulaires mimant les caractéristiques de la MA, telles que l'accumulation du peptide amyloïde-β (Aβ) et l'hyperphosphorylation de la protéine tau.

2) Dans la deuxième partie, nous avons cherché à déterminer si l'horloge biologique, qui coordonne tout un panel de comportements journaliers et de processus physiologiques, est impliquée dans la régulation de la fonction mitochondriale.

Ainsi, dans la première partie de cette thèse, nous avons étudié *in vitro* la capacité de différents neurostéroïdes à réguler la bioénergétique mitochondriale. Plus particulièrement, nous avons voulu savoir s'ils étaient capables de compenser ou d'alléger les déficits bioénergétiques observés dans des modèles cellulaires présentant l'une ou l'autre des protéines impliquées dans la physiopathologie de la MA, à savoir le peptide amyloïde-β (Aβ) et la protéine tau hyperphosphorylée.

La MA est une maladie neurodégénérative liée à l'âge qui affecte près de 2% de la population dans les pays industrialisés et représente environ 60% des cas de démence à travers le monde ***(10)***. En 2006, près de 26.6 millions de cas ont été diagnostiqués, et les études prédisent que le nombre de patients devrait quadrupler d'ici 2050, ce qui en fait une des maladies les plus coûteuses pour la société dans les pays développés ***(11)***. La maladie touche en général les individus de plus de 65 ans, excepté dans les rares cas familiaux (<1%) ***(12)***, et se traduit par un progressif déclin physique et cognitif, en particulier pour les tâches sollicitant la mémoire, le langage ou l'orientation spatiale ***(13; 14)***. À ce jour, aucun traitement n'est en mesure de prévenir, guérir ou même de ralentir la progression de la maladie ***(14)***.

D'un point de vue histopathologique, le MA est caractérisée par la présence de plaques extracellulaires et d'une dégénérescence neurofibrillaire intracellulaire (*DNF*) qui apparaissent dans certaines zones du cerveau, notamment dans l'hippocampe, une structure qui joue un rôle central dans la mémoire et l'apprentissage ***(15; 16; 14)***. Ces plaques extracellulaires (aussi appelées plaques séniles) sont formées par l'accumulation du peptide Aβ issue de la protéolyse de la protéine précurseur de l'amyloïde β (APP), par la β et la γ sécrétase ***(17)***. La DNF est quant à elle générée par l'hyperphosphorylation anormale de tau, une protéine associée aux microtubules, qui se détache et forme des agrégats intracellulaires ***(18)***. Bien que ces deux phénomènes soient au cœur des recherches contre la MA depuis de nombreuses années, les causes de la formation des plaques séniles et de la DNF n'ont pas encore été élucidés.

Des études plus récentes ont montré que les stades précoces de la MA sont associés à un déclin de l'activité bioénergétique cérébrale et une augmentation du stress oxydant (en particulier dans les mitochondries) ***(16; 19)***. Plus précisément, il a été montré que des patients atteints de la MA présentaient un hypométabolisme cérébral (baisse de consommation de glucose) et ceci avant même l'apparition des symptômes cliniques ***(19)***. Cette caractéristique est retrouvée chez l'animal, dans des modèles de souris transgéniques

de la MA, où un dysfonctionnement mitochondrial a pu être détecté avant les plaques séniles, la DNF et les troubles cognitifs (***(20)*** voir **ANNEXE 1**).

Les recherches ciblant les dysfonctionnements mitochondriaux dans la MA ont révélé que le peptide Aβ et la protéine tau hyperphosphorylée avaient tous deux un impact négatif sur la fonction mitochondriale (métabolisme bioénergétique et dynamique mitochondrial) et pouvait agir soit de manière séparé soit de manière synergétique pour induire leurs effets toxiques (***(21; 20)*** voir **ANNEXE 1 et 2**). Pris séparément, Aβ et tau hyperphosphorylée engendrent tous deux une baisse de la production d'ATP, de la respiration cellulaire, augmentent la production de ROS et perturbent le potentiel de membrane mitochondriale ***(21; 22; 20)*** (voir **Fig. 3**). Notamment, il a été montré que l'Aβ pouvait réduire l'activité du complexe IV mitochondrial ***(23; 24)***, alors que la présence de tau hyperphosphorylée diminuait l'activité du complexe I ***(25)***.

Les deux protéines ont également un impact négatif sur la dynamique mitochondriale (activité de fusion/fission). En effet, les cellules surexprimant l'APP présentent un réseau mitochondrial fragmenté ***(27; 28)***, alors que la surexpression de tau engendre une élongation anormale des mitochondries dans des neurones de drosophiles et de souris ***(29)***.

Des études effectuées sur les souris triples transgéniques (^{triple}AD : APPxPS2xpR5) ont permis de montrer que Aβ et tau hyperphosphorylée peuvent agir de manière synergétique sur la mitochondrie (***(30)*** voir aussi **ANNEXE 3**). En effet, les dysfonctionnements mitochondriaux étaient déjà apparents à 8 mois chez les souris ^{triple}AD alors qu'ils n'apparaissaient qu'à 12 mois chez les souris doubles transgéniques (APPxPS2). De plus, ces souris présentaient des déficits exacerbés au niveau de l'OXPHOS et la synthèse d'ATP, ainsi qu'une augmentation de la production de ROS.

En résumé, ces données indiquent fortement que les dysfonctionnements mitochondriaux jouent un rôle clef dans la pathogenèse de la MA. La mitochondrie représente donc une cible intéressante pour élaborer des stratégies thérapeutiques dans les stades encore précoces de la maladie. C'est pourquoi, les concepts pharmacologiques actuels visent à : stimuler la respiration mitochondriale, augmenter la production d'ATP et réduire le stress oxydant (niveaux de ROS).

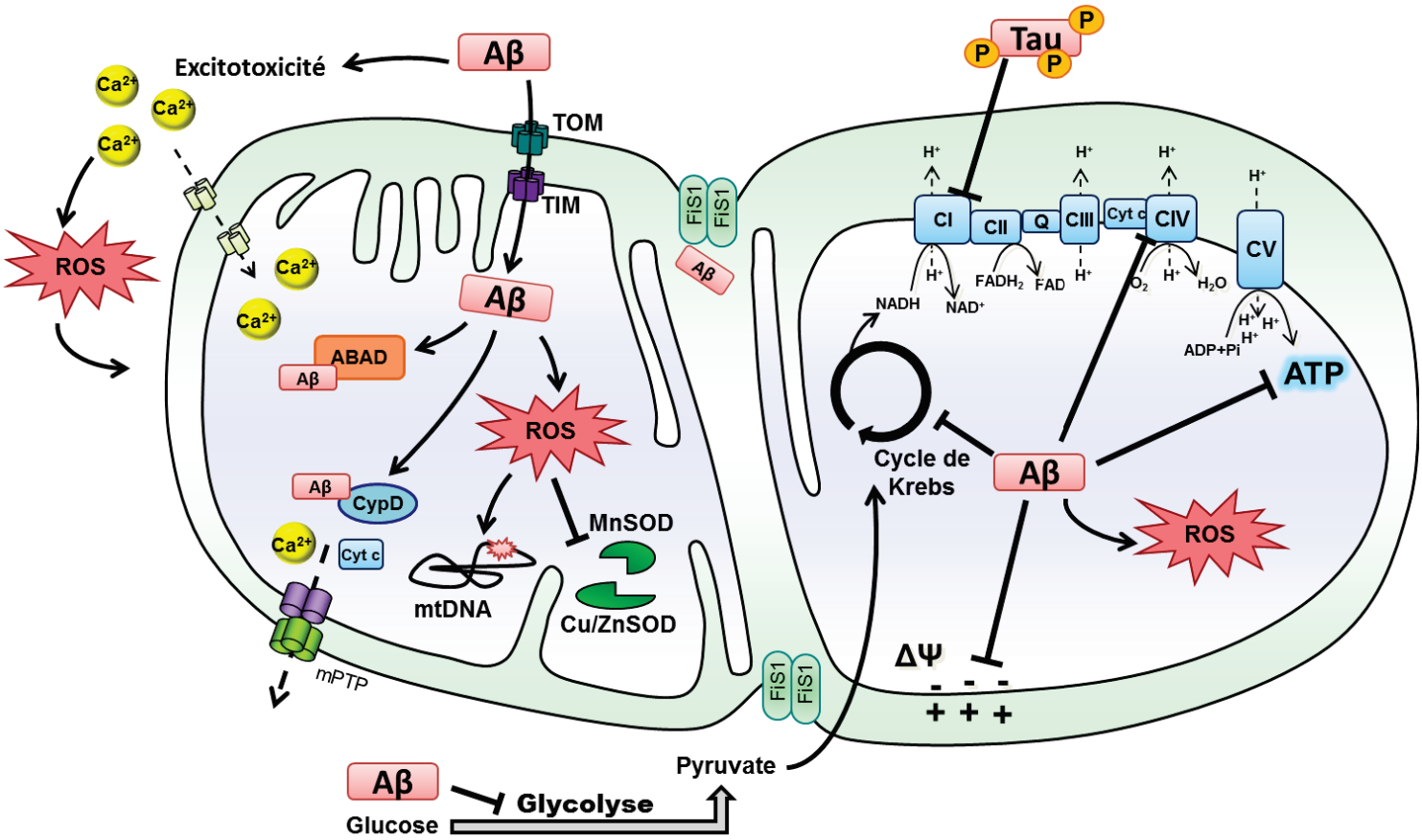

Fig. 3 : Convergence pathogène de la protéine tau hyperphosphorylée et le peptide Aß sur les mitochondries. Aß et la protéine tau sont capables d'altérer la respiration mitochondriale en inhibant le complexe IV et complexe I respectivement. Ceci induit une diminution de la consommation d'oxygène, de la production d'ATP et une augmentation des niveaux de ROS. Le stress oxydant induit par le dysfonctionnement de la chaîne de transport des électrons peut surpasser les défenses antioxydantes des cellules (MnSOD, Cu/ZnSOD) et impacter le potentiel de membrane (ΔΨ) ainsi que l'ADN mitochondrial (mtDNA). Dans les mitochondries, le peptide Aß peut se lier à des protéines comme l'ABAD et CypD, ce qui génère plus de ROS et mène à l'ouverture des pores de transition de perméabilité mitochondriale (mPTP), déclenchant les voies apoptotiques par la libération de Cyt C dans le cytosol. En parallèle, le peptide Aß peut également être responsable des altérations métaboliques en inhibant les enzymes impliquées dans la glycolyse et du cycle de Krebs, de même que l'excitotoxicité induite par le calcium dans les neurones. Enfin, l'Aß et la protéine tau peuvent perturber l'activité de fusion / fission mitochondriale, conduisant à une mauvaise répartition des mitochondries dans les neurones. CI complexe I, CII complexe II , CIII complexe III, CIV complexe IV, CV complexe V, cyt c cytochrome c, Cu/Zn SOD cuivre/zinc superoxyde dismutase, MnSOD manganèse superoxyde dismutase, ROS espèces réactives de l'oxygène, mtDNA ADN mitochondrial, TOM / TIM translocases des membranes mitochondriales interne / externe, ABAD Aß binding alcohol dehydrogenase, FIS, protéine fission 1, CypD cyclophiline D (adapté de ***(26)*** et ***(22)***).

Dans les travaux de thèse présentés ici, nous avons fait l'hypothèse que les neurostéroïdes pourraient être de nouveaux candidats capables de stimuler la respiration mitochondriale.

Les neurostéroïdes sont des stéroïdes synthétisés par les neurones et/ou les cellules gliales du système nerveux central ou périphérique. Pour être considéré comme un neurostéroïde, une concentration substantielle du stéroïde doit persister dans le système nerveux après ablation des glandes endocrines périphériques qui sécrètent les hormones stéroïdiennes ***(31; 32)***. Il est possible de distinguer 3 catégories de neurostéroïdes: (i) les neurostéroïdes non-exclusifs tels que l'estradiol, la testostérone et la progestérone, qui sont des hormones stéroïdiennes synthétisées par les glandes endocrines mais également par les neurones et les cellules gliales; ii) les neurostéroïdes semi-exclusifs comme l'alloprégnanolone qui est principalement synthétisée dans le système nerveux mais aussi en faible quantité par les glandes endocrines; iii) les neurostéroïdes exclusifs comme l'épialloprégnanolone qui ne sont produits que dans le système nerveux ***(35)***.

La mitochondrie est un organite qui joue un rôle crucial dans la biosynthèse des stéroïdes périphériques ou hormones stéroïdiennes (stéroïdogenèse) et des neurostéroïdes (neurostéroïdogenèse). En effet, la mitochondrie est le siège de la première étape des voies enzymatiques de la stéroïdogenèse et de la neurostéroïdogenèse ***(31; 33; 34)*** **(Fig. 4)**.

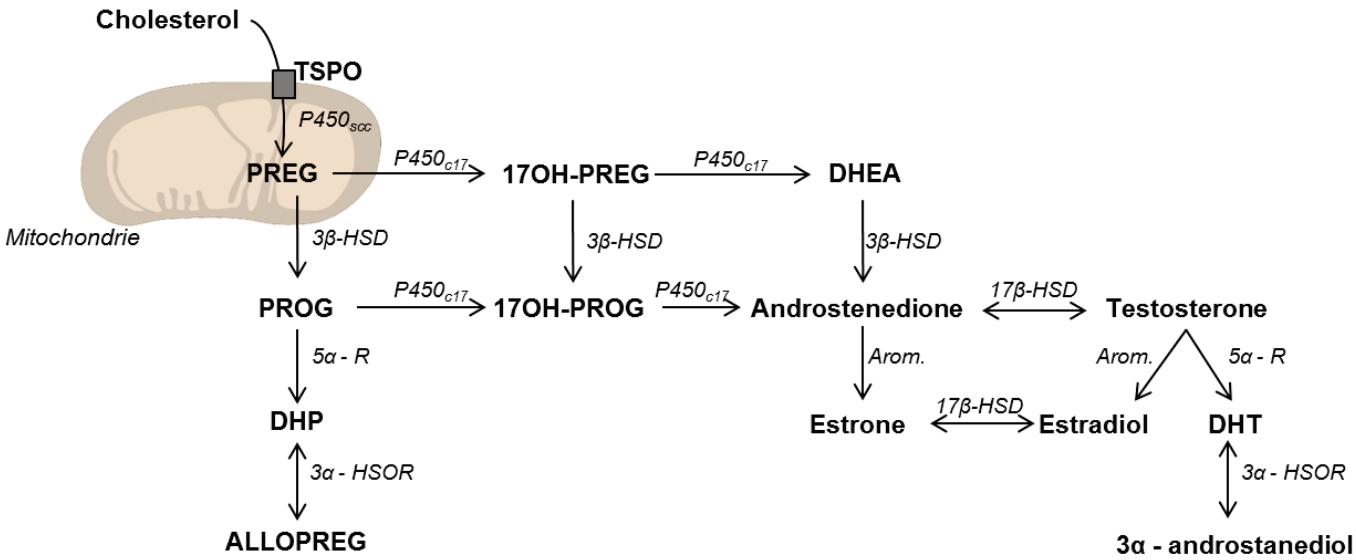

Fig. 4: Représentation schématique des principales voies de biosynthèse des neurostéroïdes dans le cerveau des vertébrés. TSPO ; translocator protein, 17OH-PREG; 17-hydroxypregnenolone, 17OH-PROG; 17-hydroxyprogesterone, DHEA; dehydroepiandrosterone, DHP; dihydroprogesterone, ALLOPREG; allopregnenolone, DHT; dihydrotestosterone, P450scc; cytochrome P450 cholesterol side chain cleavage, P450c17;cytochrome P450c17, 3β-HSD; 3β-hydroxysteroid dehydrogenase, 5α-R; 5α-reductase, Arom.; aromatase, 21-OHase; 21-hydroxylase, 3α-HSOR; 3α-hydroxysteroid oxydoreductase,17β-HSD; 17β-hydroxysteroid dehydrogenase.

Contrairement aux hormones stéroïdiennes qui agissent par un mode d'action endocrine (effet sur des cibles lointaines par rapport aux glandes endocrines sécrétrices), les neurostéroïdes ont une action autocrine et paracrine. Ils modulent plusieurs processus physiologiques tels que: la neurotransmission, la viabilité neuronale, la myélinisation, l'apprentissage, la cognition et la mémorisation ***(36; 37; 32)***.

Divers travaux réalisés dans des modèles expérimentaux de maladies neurodégénératives et de neuropathies périphériques suggèrent que les neurostéroïdes ont un fort potentiel neuroprotecteur ***(38-45)***. Par ailleurs, des études post-mortem ont révélé une baisse des concentrations endogènes de neurostéroïdes dans le cerveau des patients Alzheimer comparés aux sujets contrôles ***(46)***. Cette baisse est négativement corrélée à l'accumulation du peptide Aβ et à l'hyperphosphorylation de la protéine tau dans les structures cérébrales limbiques. Des données similaires ont été mises en évidence dans le cerveau de souris transgéniques modélisant la MA ***(47)***. Collectivement, ces résultats suggèrent que la neurodégénérescence et/ou les dysfonctionnements neuronaux évoqués par la toxicité Aβ et l'hyperphosphorylation de tau pourraient résulter, au moins en partie, de la diminution des concentrations cérébrales de neurostéroïdes neuroprotecteurs.

En ce qui concerne les déficits mitochondriaux observés dans la MA, de nombreuses études sont axées sur l'estradiol (voir **ANNEXE 4**) qui est également connu pour stimuler le métabolisme bioénergétique dans les cellules. Cependant, aucune étude n'a testé les effets d'autres neurostéroïdes sur la bioénergétique mitochondriale et l'homéostasie redox dans les cellules neuronales.

Par conséquent, dans le but d'élargir nos connaissances sur les effets neuroprotecteurs des neurostéroïdes et leurs mécanismes sous-jacents, nous avons sélectionné un panel de neurostéroïdes (progestérone, estradiol, l'estrone, testostérone, 3α-androstanediol, DHEA et alloprégnanolone) comme candidats potentiels capables de moduler la fonction mitochondriale.

Pour choisir le panel à tester, nous nous sommes appuyés sur des travaux antérieurs qui ont révélé, d'une part, que l'Aβ et tau hyperphosphorylée modulent la production endogène de neurostéroïdes ***(48; 49)***, et d'autre part, que l'homéostasie de l'estradiol dans les mitochondries est perturbée par les peptides Aβ qui inhibent l'*amyloid-β binding alcohol dehydrogenase* (ABAD) connue également sous l'appellation de 17β-hydroxystéroïde déshydrogénase **(Fig. 3-4)** (***(50)*** voir aussi **ANNEXE 5 et 6**). Avant de caractériser le mode d'action des neurostéroïdes sur la bioénergétique mitochondriale dans des conditions pathologiques (MA), nous avons d'abord cherché à comprendre leurs effets *per se* en utilisant des cellules de neuroblastome humains, SH-SY5Y, traitées pendant 24 heures. Les

effets des 7 neurostéroïdes sélectionnés ont été testés sur la production d'ATP, le potentiel de membrane mitochondriale (MMP), la respiration mitochondriale, la glycolyse et l'environnement redox des cellules.

Nos résultats montrent que la progestérone, l'estradiol, l'estrone, la testostérone, la 3α-androstanediol et la DHEA stimulent l'activité bioénergétique cellulaire après 24 heures de traitement (10 nM et 100 nM), en augmentant les niveaux d'ATP, le MMP et la respiration basale, sans effets significatifs sur la glycolyse. La testostérone et la DHEA améliorent en plus le *respiratory control ratio* (RCR), un indicateur de la capacité respiratoire des mitochondries. Il apparaît donc que les neurostéroïdes agissent principalement sur la respiration basale en augmentant les niveaux énergétiques des cellules déjà à l'état de repos, et lorsque les cellules ont une forte demande énergétique, la testostérone et la DHEA sont capables d'améliorer le RCR pour répondre à cette demande.

Les neurostéroïdes sont des molécules lipophiles et peuvent donc traverser les membranes plasmiques des cellules pour activer des récepteurs nucléaires, régulant ainsi la transcription de gènes. Afin de voir si les récepteurs nucléaires des stéroïdes étaient impliqués dans la modulation de la bioénergétique mitochondriale, nous avons utilisé des antagonistes spécifiques (RU 486 : antagoniste des récepteurs à la progestérone, ICI 182.780 : antagoniste des récepteurs aux estrogènes, dihydroxy-flutamide : antagoniste des récepteurs aux androgènes) et évalués leurs effets sur la production d'ATP en présence de l'agoniste stéroïdien correspondant. Les résultats montrent qu'en présence de la molécule antagoniste, l'effet des neurostéroïdes sur la production d'ATP est complètement aboli, suggérant que la modulation de la bioénergétique se fait, au moins en partie, par l'activation de la transcription de gènes codant pour des protéines impliquées dans la glycolyse ou l'OXPHOS.

D'autre part, nous avons observé que l'augmentation de l'activité mitochondriale induite par les neurostéroïdes était parallèle à une élévation des niveaux de ROS dans les mitochondries, sans pour autant affecter la survie des cellules. Cette élévation résulte probablement d'une fuite d'électrons par la chaîne de transport d'électrons car, comme expliqué précédemment, si les mitochondries produisent de l'ATP, elles produisent également des ROS. Les neurostéroïdes sont cependant capables de compenser cette augmentation des ROS mitochondriaux par une hausse de l'activité antioxydante (ici, l'activité de la manganèse superoxydes dismutase), ce qui fait que l'état redox intramitochondrial n'en ressort que légèrement oxydé.

Ainsi, ces premières données indiquent que les neurostéroïdes sont en effet en mesure de stimuler la fonction mitochondriale en condition physiologique. Nos principales conclusions sont que: i) la majorité de ces stéroïdes induisent une augmentation du

métabolisme énergétique, principalement par l'intermédiaire d'une hausse de l'activité mitochondriale; ii) les neurostéroïdes testés sont capables de réguler l'homéostasie redox en augmentant l'activité antioxydante pour compenser l'élévation des niveaux de ROS produit lors de l'OXPHOS. De plus, chaque neurostéroïde semble avoir un profil bioénergétique spécifique. Les résultats obtenus après l'utilisation d'antagonistes des récepteurs nucléaires aux stéroïdes suggèrent qu'ils agissent, au moins en partie, par l'intermédiaire de leur récepteur correspondant pour réguler l'expression de gènes impliqués dans la glycolyse et l'OXPHOS, mais peut-être aussi sur l'activité des complexes respiratoires mitochondriaux. D'autres études sont maintenant nécessaires pour déterminer plus en détail les mécanismes moléculaires qui sont impliqués dans ces processus.(mettreplus haut)

En nous appuyant sur ces premiers résultats, nous avons cherché à savoir si les neurostéroïdes avaient un effet bénéfique pour contrecarrer les déficits mitochondriaux observés dans la MA.

Nous avons donc traité des cellules de neuroblastome humain (SH-SY5Y) transfectées de manière stable, soit avec la protéine précurseur de l'amyloïde humaine (APP) qui surexpriment l'APP et l'Aß ***(23; 24)***, soit avec la protéine tau « normale » (wild-type tau : wtTau), ou la protéine tau mutante (P301L), qui induit une hyperphosphorylation anormale de tau ***(25)***, pendant 24 heures avec différents neurostéroïdes appartenant à la famille des hormones sexuelles (progestérones, estradiol, estrone, testostérone, 3α-androstanediol). Plusieurs paramètres bioénergétiques ont ensuite été mesurés, à savoir : les niveaux d'ATP, le MMP, la respiration basale, le renouvellement de l'ATP (ATP turnover), la respiration mitochondriale maximale, la capacité respiratoire de réserve ainsi que la réserve glycolytique.

En accord avec les données bibliographiques mentionnées précédemment, nous avons d'abord démontré que la présence de l'APP/Aß ou la protéine tau mutante avait une incidence différente sur la bioénergétique mitochondriale dans ces modèles cellulaires ***(23; 25)***. La surexpression de l'une ou l'autre de ces protéines induisait une diminution similaire des niveaux d'ATP. Cependant, alors que la protéine tau mutante n'affectait que la respiration mitochondriale maximale et la capacité respiratoire de réserve, la surexpression de l'APP/Aß induisait en plus une diminution de la respiration basale, du renouvellement de l'ATP (ATP turnover) et de la réserve glycolytique. Tous les neurostéroïdes que nous avons testés étaient en mesure d'augmenter les niveaux d'ATP et le MMP dans les cellules surexprimant l'APP/Aß. En revanche, seules la progestérone et l'estradiol induisaient une augmentation de l'ATP dans les cellules exprimant la protéine tau mutante.

De plus, nous avons pu montrer que l'hormone stéroïdienne mâle, la testostérone, était plus efficace pour réduire les déficits mitochondriaux induits l'APP/Aß. En effet, elle était

la seule molécule capable d'augmenter la respiration basale, le renouvellement d'ATP, la respiration mitochondriale maximale et la capacité respiratoire de réserve dans les cellules surexprimant l'APP/Aß. Les hormones stéroïdiennes femelles, la progestérone et les estrogènes (l'estradiol et l'estrone), étaient quant à elles plus efficaces pour augmenter ces différents paramètres bioénergétiques dans le modèle cellulaire de tauopathies liés à la MA.

En résumé, nos résultats apportent de nouvelles évidences attestant des effets neuroprotecteurs des neurostéroïdes dans la MA. Ces molécules représentent donc des candidats prometteurs capables d'augmenter la bioénergétique cellulaire en conditions pathologiques.

L'ensemble des résultats de la première partie de cette thèse fournit une base moléculaire potentielle pour les effets bénéfiques et neuroprotecteurs des neurostéroïdes, et ouvrent de nouvelles voies pour le développement de médicaments ciblant les mitochondries dans la neurodégénérescence. Nos résultats indiquent fortement que les neurostéroïdes représentent des molécules prometteuses capables d'augmenter l'activité bioénergétique via l'amélioration de la respiration mitochondriale, dans des conditions saines et pathologiques. Comme chaque neurostéroïde semble avoir un profil d'action spécifique sur la bioénergétique cellulaire, que ce soit en conditions normales ou en présence de Aß ou tau, il est maintenant nécessaire d'élucider plus en détail quels sont les mécanismes moléculaires sous-jacents, comme par exemple leurs capacités individuelles à réguler l'expression et l'activité des complexes mitochondriaux impliquée dans l'OXPHOS.

Dans la deuxième partie de cette thèse, nous avons cherché à comprendre comment la fonction mitochondriale est régulée de manière endogène au sein des cellules. Plus précisément, nous avons cherché à déterminer si, et surtout comment, la dynamique mitochondriale (activité de fusion/fission) ainsi que la bioénergétique était modulées par l'horloge biologique de manière circadienne.

L'horloge circadienne (du latin « circa diem» : environ un jour) représente un réseau hiérarchique d'oscillateurs qui coordonnent toute une variété de comportements journaliers et de processus physiologiques à un moment optimal de la journée, dans le but d'anticiper les changements de l'environnement extérieur chez tous les êtres vivants ***(51)***.

Pour être considéré comme circadien, un rythme (ou oscillation) doit remplir trois critères essentiels ***(52)***. Tout d'abord, il doit être endogène, avec une période d'environ 24 heures, et persister en condition constante, par exemple quand l'individu ou l'animal sont placés dans l'obscurité, sans stimuli lumineux. Deuxièmement, le rythme doit être entrainable (ou synchronisable) par des stimuli externes appelés « Zeitgebers » comme par

exemple les cycles jour/nuit ou les prises alimentaires. Enfin, la période du rythme (le temps que met une oscillation pour se répéter) ne doit pas être affectée par les changements de température dans des limites physiologiquement acceptables.

Dans l'ensemble, les rythmes circadiens permettent à l'organisme de coordonner divers processus physiologiques et comportementaux au cours de la journée. Le rythme circadien le plus évident observé chez les humains, et d'autres animaux, est le cycle veille-sommeil.

L'une des principales propriétés du système circadien est sa capacité à synchroniser l'horloge circadienne à tous les niveaux (de l'horloge moléculaire à l'organisme entier). Virtuellement, toutes les cellules de l'organisme sont dotées d'horloges endogènes qui fonctionnent en phase les unes avec les autres afin de maintenir un fonctionnement cohérent entre les différents organes du corps ***(53)***. Pour orchestrer tous ces oscillateurs individuels, le système circadien possède une horloge centrale, située dans les noyaux suprachiasmatiques (SCN) du cerveau ***(54; 55)***, qui remplit un rôle clef et permet de: i) percevoir les signaux environnementaux (ex : la lumière) ; ii) intégrer les informations temporelles (ex : le moment de la journée (jour/nuit)) ; iii) transmettre des signaux pour synchroniser les horloges périphériques situées dans différents tissus ou organes (foie, estomac, muscles, pancréas…) qui à leur tour transmettant des signaux rétroactifs au SCN **(Fig. 5)**.

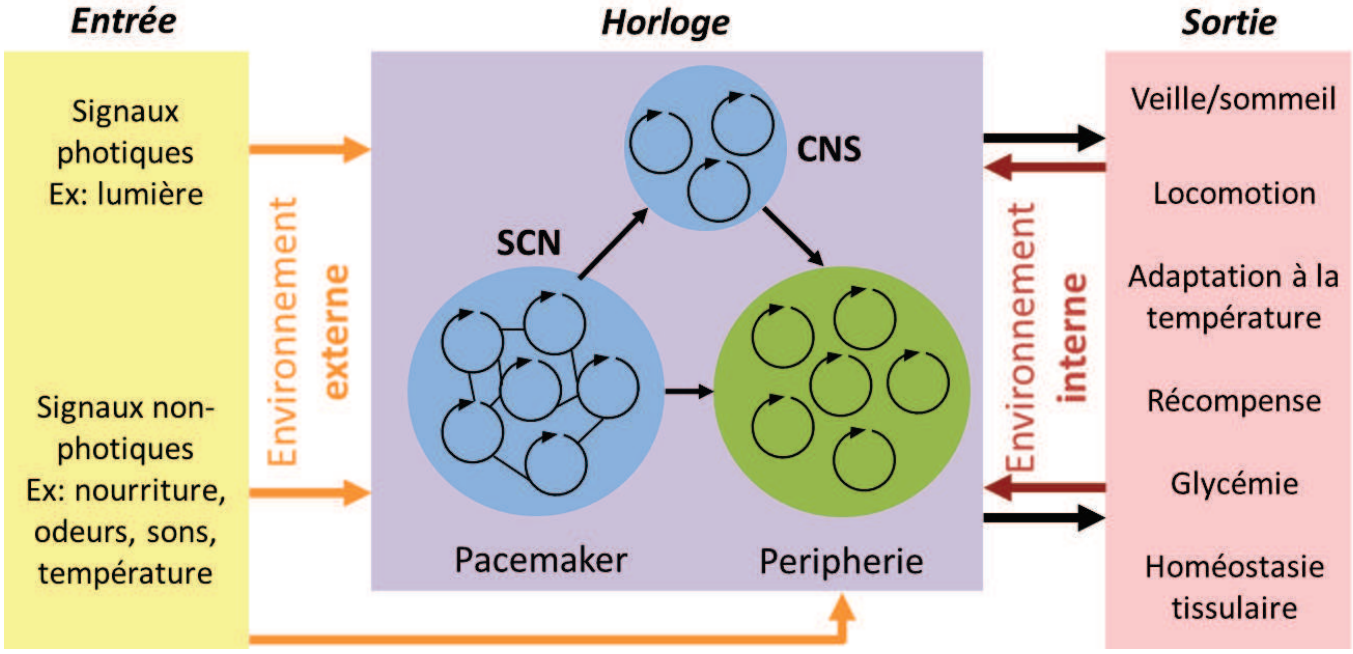

Fig. 5: Subdivisions du système circadien: entrée, effecteurs d'horloge et sortie d'horloge. Cette division peut être faite au niveau cellulaire ainsi qu'au niveau systémique. SCN noyaux suprachiasmatiques, CNS système nerveux central (adapté de ***(53)***).

Au niveau moléculaire, le maintien d'un rythme avec une période de 24 heures est rendu possible grâce à des boucles rétroactives de transcription/traduction composées d'éléments trans-activateurs (positifs) ou trans-inhibiteurs (négatifs) **(Fig.6)**. BMAL1 et CLOCK font partie des éléments activateurs de transcription qui régulent les gènes sous le contrôle de l'horloge (CCG : *Clock-controlled genes*) ainsi que la transcription des éléments inhibiteurs : Cryptochrome (CRY) et Period (PER) ***(56)***. Ces derniers inhibent la transcription de BMAL1, et donc leur propre transcription, et la boucle peut recommencer. D'autres éléments peuvent venir réguler l'expression ou la stabilité des composants de cette l'horloge moléculaire, comme par exemple REV-ERBα (*reverse orientation c-erb α*) ou ROR (*orphan nuclear receptor genes*).

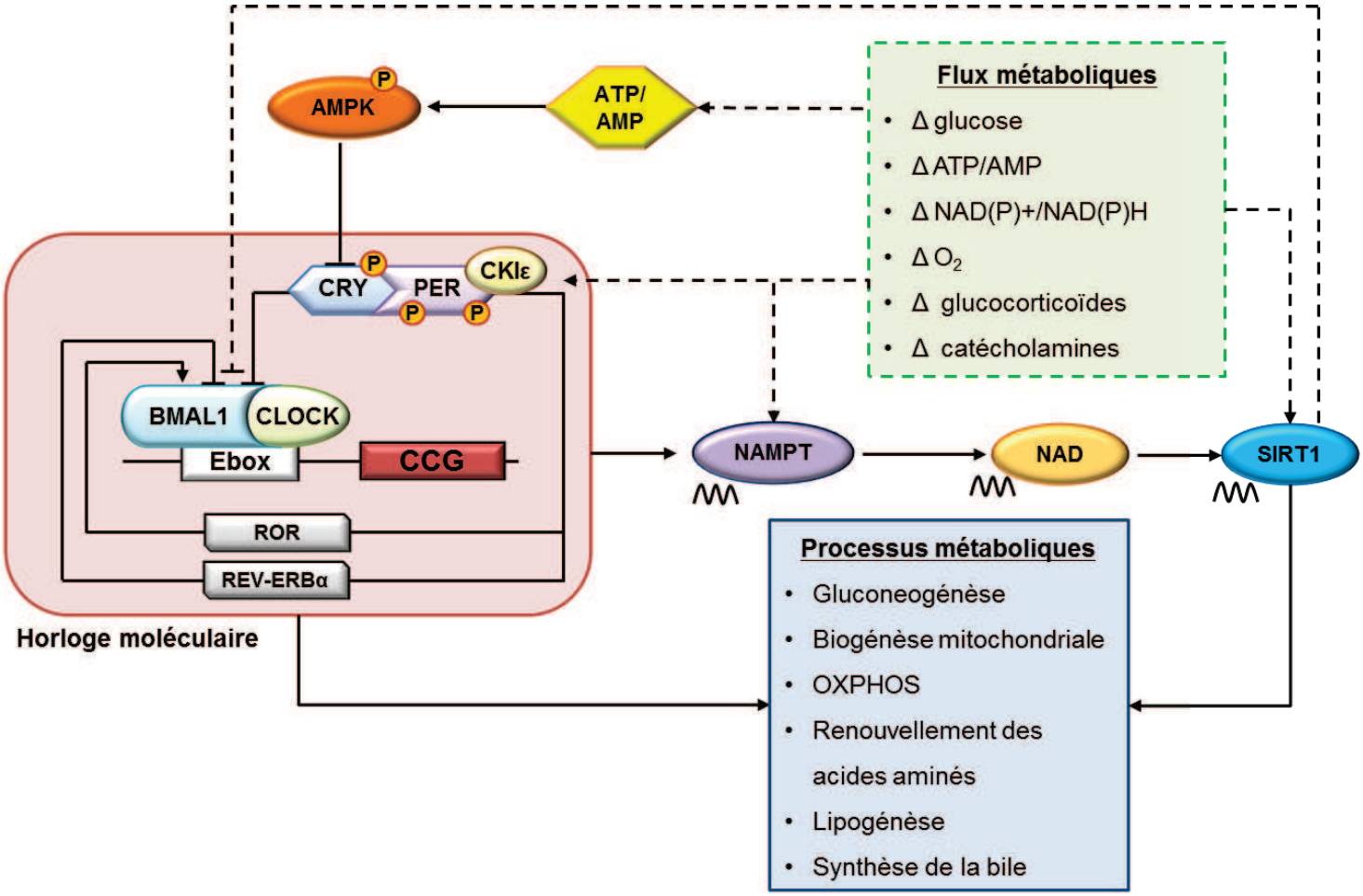

Fig. 6: Liens entre l'horloge moléculaire et le métabolisme. L'horloge moléculaire peut directement ou indirectement synchroniser divers processus métaboliques en activant la transcription de gènes qui sont sous le contrôle de l'horloge (CCG : clock-controlled genes). Vice versa, les flux métaboliques ainsi générés peuvent renvoyer des signaux à l'horloge moléculaire (via SIRT1 ou l'AMPK) à la manière d'un rhéostat afin de coordonner les processus métaboliques avec les cycles jour/nuit ou les périodes de jeûne et de prise alimentaire. NAMPT; nicotinamide phosphoribosyltransferase, SIRT1; sirtuines 1, AMPK; protéine kinase dépendante de l'adénosine monophosphate (adapté de ***(57)***).

Descriptif synthétique en français des travaux de la thèse

Les rythmes circadiens apparaissent de plus en plus comme une adaptation évolutive, notamment au niveau métabolique, qui permet la séparation temporelle des réactions cataboliques et anaboliques pour les aligner avec les rythmes jour/nuit ou les périodes de jeûne ou de prise alimentaire ***(58)***. Bien qu'il soit de plus en plus admis que l'horloge moléculaire est étroitement liée au métabolisme ***(59; 53)***, les mécanismes sous-jacents n'ont pas encore été clairement élucidés. Certaines pistes sont actuellement à l'étude et impliquent des molécules telles que le NAD^+, NAMPT (nicotinamide phosphoribosyltransferase), SIRT1 (sirtuines 1) ou encore l'AMPK (protéine kinase dépendante de l'adénosine monophosphate) ***(60-64)*** **(Fig. 6)**.

De nouvelles évidences suggèrent que le métabolisme énergétique (libération d'ATP, ROS et mécanismes de défense cellulaire) serait lui aussi coordonné par l'horloge circadienne ***(65; 66)***. Bien qu''il semble probable que la mitochondrie, véritable machinerie métabolique des cellules, soit étroitement liée de plusieurs façons à travers une régulation réciproque avec l'horloge circadienne, la relation entre le système circadien et la fonction mitochondriale reste encore très floue.

Ainsi, nous avons voulu savoir, d'une part, si l'horloge circadienne exerçait un contrôle sur la fonction mitochondriale, notamment sur la dynamique et la bioénergétique, et d'autre part, si la mitochondrie pouvait elle-même influencer des paramètres de l'horloge.

Pour ce faire, nous avons utilisé des cultures primaires de fibroblastes humains comme modèles d'horloge périphérique ***(67; 68)***. Après synchronisation des cellules, nous avons étudié la dynamique (morphologie mitochondriale, activité de fusion/fission) ainsi que la bioénergétique mitochondriale (production d'ATP et respiration).

Nos données ont révélé que la forme des mitochondries et le métabolisme bioénergétique (OXPHOS) sont sous le contrôle de l'horloge et oscillent avec une période de 24 heures environ. Plus précisément, nous avons constaté que les niveaux d'ATP présentent une rythmicité circadienne avec un pic et un creux survenant respectivement 16 heures et 28 heures après synchronisation. Ces données ont pu être confirmées *in vivo,* dans des cerveaux de souris maintenues en obscurité constante, où les taux d'ATP présentaient également une oscillation de 24 heures, avec un pic survenant au début de la période de repos. De plus, l'étude de la respiration mitochondriale *in vitro* a également fourni des résultats en corrélation avec les oscillations d'ATP. Nous avons observé que les taux de consommation d'oxygène (OCR) sont plus bas 28 heures postsynchronisation comparés à 16 heures, supportant l'hypothèse que le métabolisme oxydatif de la mitochondrie est contrôlé par l'horloge biologique.

L'étude de la dynamique mitochondriale a quant à elle révélé que l'activité de fusion/fission des mitochondries présente aussi un rythme d'environ 24 heures en corrélation avec la synthèse d'ATP. En effet, le réseau mitochondrial était dans un état : i) tubulaire (ou fusionné) 8 heures après synchronisation, quand les taux d'ATP augmentent; ii) intermédiaire 16 heures après synchronisation, au pic d'ATP ; iii) fragmenté 24 heures après synchronisation, au début du creux d'ATP. Cette rythmicité semble être dépendante de l'activité de DRP1, une protéine impliquée dans la fission mitochondriale. En effet, l'utilisation de fibroblastes DRP1 K.O ou le traitement des cellules avec un inhibiteur de fission a complètement aboli l'oscillation de l'ATP *in vitro*. De plus, la forme phosphorylée de DRP1 (correspondant ici à la forme inactive de cette protéine) présente une oscillation circadienne au niveau protéique, suggérant que la fission mitochondrial, dépendant de DRP1, est sous le contrôle de l'horloge biologique. À nouveau, ces derniers résultats ont pu être confirmés *in vivo* dans les cerveaux de souris.

Nous avons également montré que des sous-produits dérivés de l'activité mitochondriale, comme les ROS et les niveaux NAD+, tous deux indicateurs de l'environnement redox, présentent aussi une oscillation circadienne en phase avec les niveaux d'ATP et la respiration mitochondriale.

Pour finir, nous avons également constaté que les mitochondries peuvent eux-mêmes agir sur le rythme circadien via plusieurs signaux rétrogrades, dont l'activation de l'AMPK par phosphorylation (dépendant du ratio AMP/ATP) qui peuvent réguler l'activité des composants de l'horloge moléculaire tels que PER et CRY.

L'ensemble des résultats de la seconde partie de cette thèse nous ont permis d'établir un lien moléculaire entre le contrôle circadien de la dynamique mitochondriale et la bioénergétique, et vice versa. Ces données suggèrent que l'horloge circadienne joue un rôle clé dans le contrôle du métabolisme mitochondrial pour anticiper les besoins énergétiques nécessaires à la réalisation de diverses fonctions cellulaires en réponse aux exigences environnementales et/ou intracellulaires.

En conclusion, les résultats de ces travaux de thèse contribuent fortement à accroître nos connaissances sur la modulation de la fonction mitochondriale en conditions physiologiques et pathologiques **(Fig. 7)**. Notre travail permet d'identifier les neurostéroïdes comme étant des molécules très prometteuses à exploiter pour contrecarrer les déficits bioénergétiques dans les maladies neurodégénératives, en particulier dans la MA. De plus, nous démontrons que la dynamique et la bioénergétique mitochondriales sont contrôlées par l'horloge biologique, ce qui pourrait avoir de multiples implications dans la régulation de

l'homéostasie métabolique chez le sujet sain ou chez les patients atteints de pathologies liées à une altération des rythmes biologiques et/ou des fonctions mitochondriales.

Des recherches complémentaires doivent maintenant être effectuées pour élucider plus en détail quels sont les mécanismes sous-jacents.

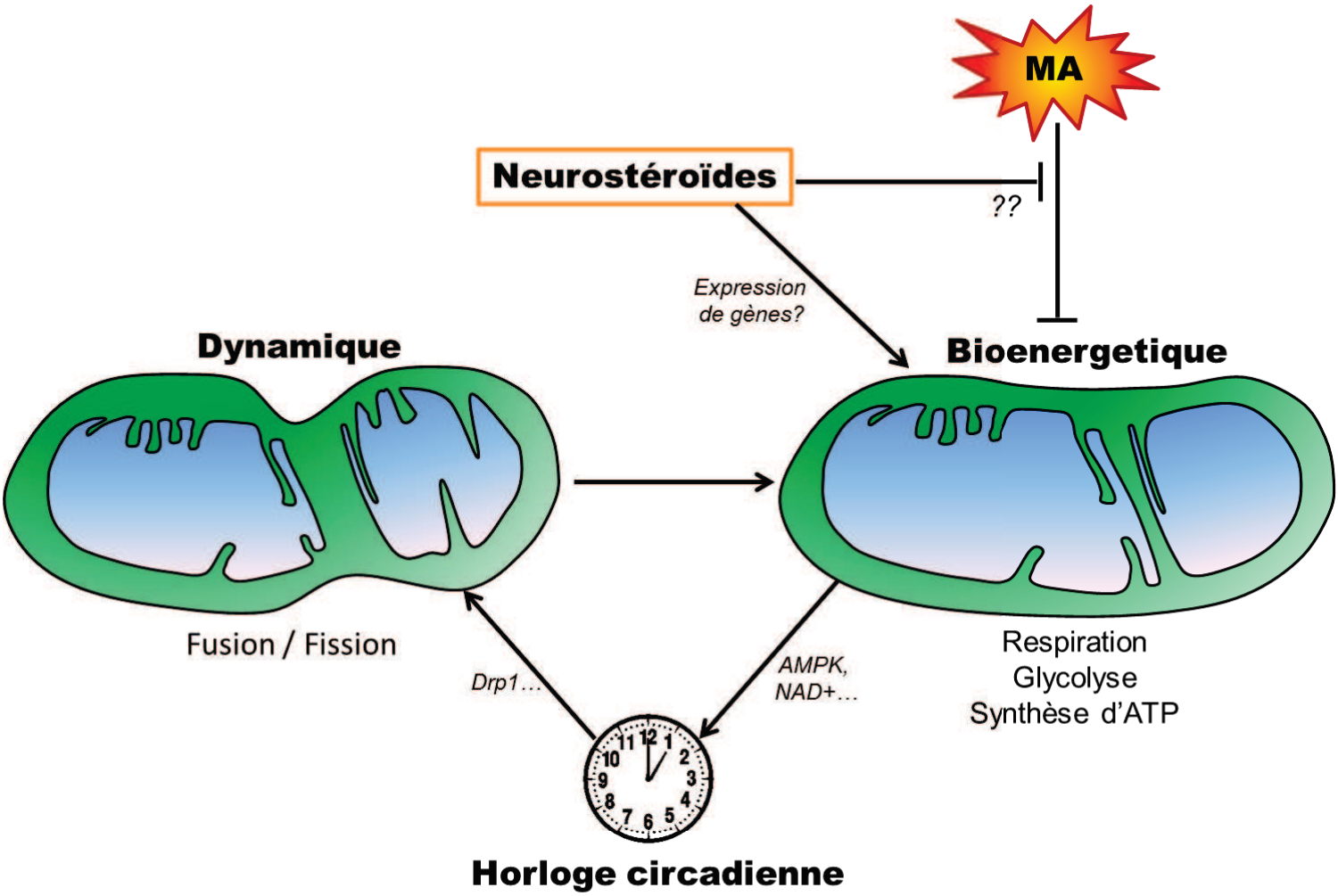

Fig. 7 : Liens hypothétiques entre la fonction mitochondriale et l'horloge circadienne, et leur régulation par les neurostéroïdes. D'une part, nous avons montré que les neurostéroïdes peuvent augmenter la bioénergétique mitochondriale, ce qui peut partiellement pallier la dysfonction mitochondriale observée dans la maladie d'Alzheimer (MA). D'autre part nous avons montré que l'horloge circadienne contrôle la dynamique mitochondriale, qui à son tour module l'activité bioénergétique. De plus, la mitochondrie peut envoyer des signaux rétrogrades à l'horloge, ce qui influence à nouveau la dynamique et la bioénergétique mitochondriale.

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ABBREVIATIONS

3α-HSOR 3α-hydroxysteroid oxydoreductase
3β-HSD 3β-hydroxysteroid dehydrogenase
5α-R 5α-reductase
17β-HSD 17β-hydroxysteroid dehydrogenase
17OH-PREG 17-hydroxypregnenolone
17OH-PROG 17-hydroxyprogesterone
21-OHase 21-hydroxylase
α-KGDH α-ketoglutarate dehydrogenase
Aβ Amyloid-beta peptide
ABAD Aβ binding protein alcohol dehydrogenase
ACBD3 Acyl-CoA binding domain-containing 3
AD Alzheimer's disease
ADAM A disintegrin and metalloproteinase
AICD Aβ intracellular cytoplasmic domain
AIF Apoptosis-inducing factor
ALLOPREG Allopregnenolone
AMPK AMP-activated protein kinases
ANT Adenine nucleotide transporter
APOE Apolipoprotein E
APP Amyloid precursor protein
AR Androgen receptor
Arom. Aromatase
ATP Adenosine triphosphate
BACE β-site of APP cleaving enzyme
C83 83-amino-acid Ct APP fragment
C99 99-amino-acid Ct APP fragment
CaMKII Calcium-calmodulin dependent protein kinase II
CCG Clock-controlled gene
cdk5 Cyclin-dependent kinase 5
CK1ε casein kinase 1ε
COX Cytochrome c oxidase
Cry 1-2 Cryptochrome 1-2
CT Circadian time
Cu/Zn SOD Copper/zinc superoxide dismutase
Cyp D cyclophilin D
DD Dark/dark
DHEA(S) Dehydroepiandrosterone (sulphate)
DHP Dihydroprogesterone

DHT Dihydrotestosterone

Drp1 Dynamin-related protein 1

ER Endoplasmic reticulum

ERα/β Estrogen receptors α and β

ERK2 Extracellular signal-regulated kinase 2

ETC Electron transport chain

FAD Familial Alzheimer's disease form

FAD+ Flavin adenine dinucleotide

FIS1 Fission protein 1

FTDP-17 Fronto-temporal dementia with Parkinsonism linked to chromosome 17

GDP Guanosine diphosphate

GPX Glutathione peroxidase

GR Glutathione reductase

GSH Glutathione

GSK-3β Glycogen synthase kinase-3β

GTP Guanosine triphosphate

H2O2 Hydrogen peroxide

HST Hydroxysteroid sulfotransferase

IMM Inner mitochondrial membrane

IMS Intermembrane space

iTRAQ Isobaric tags for relative and absolute quantitation

LD Light/dark

MAO Monoamine oxidase A

MAP Microtubule-associated protein

MFN1/2 Mitofusin 1 and 2

MnSOD Manganese superoxide dismutase

MMP Mitochondrial membrane potential

mPTP mitochondrial permeability transition pore

mtDNA Mitochondrial DNA

NADH Nicotine adenine dinucleotide

nDNA Nuclear DNA

NFT Neurofibrillay tangles

NMDA N-methyl-D-aspartate

NO. Nitric oxide

NOS Nitric oxide synthase

eNOS/iNOS/nNOS endothelial/inducible/neuronal nitric oxide synthase

O2-. Superoxide anion

OH. Hydroxyl radical

OMM Outer mitochondrial membrane

ONOO- Peroxynitrite

OPA1 Optic atrophy 1

OXPHOS Oxidative phosphorylation system

P301L mice/cells mutant tau mice/cells

P450scc Cytochrome P450 cholesterol side chain cleavage

P450c17 Cytochrome P450c17

PDH Pyruvate dehydrogenase

PER 1-3 Period 1-3

PET Positron emission tomography

PINK1 PTEN-induced putative kinase 1

PKA-RIα protein kinase A regulatory subunit I alpha

PKC Protein kinase C

PR progestins receptors

PROG Progesterone

PS1/2 Presenelin 1 and 2

REV-ERBα Reverse orientation c-erb α

RNS Reactive nitrogen species

ROR Orphan nuclear-receptor

ROS Reactive oxygen species

SAD Sporadic Alzheimer's disease form

SCN Suprachiasmatic nucleus

SIRT1 Sirtuin 1

SRE Steroid responsive elements

STAR Steroidogenesis acute regulatory protein

STS Steroid sulfatase

TIM Translocator inner membrane

TOM Translocator outer membrane

^{triple}**AD** mutated Tau (P301L), PS2 (N141I) and APPSwe (KM670/671NL) triple transgenic mouse model

TSPO Translocator protein

UQ˙- Ubisemiquinone anion radicals

UQ Coenzyme Q

VDAC Voltage-dependant anion channels

wtTau Wild type tau

ZT Zeitgeber time

CURRICULUM VITAE AND LIST OF PUBLICATIONS

CURRICULUM VITAE

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June 2008: Training course, University of Strasbourg, Laboratory of Neurobiology of Rhythms, Institut des Neurosciences Cellulaires et Intégratives (INCI), Strasbourg (France). Head: Pr. David Hicks.

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- **Grimm A**, Mensah-Nyagan AG, Eckert A (2014) *Effects of neuroactive steroids on cellular bioenergetics.* European College of Neuropsychopharmacology (ECNP) Workshop for Junior Scientists in Europe, (Poster session # P0.1.015), Nice (France), March 6-3, 2014.

- **Grimm A**, Mensah-Nyagan AG, Eckert A (2014) *Effects of neuroactive steroids on cellular bioenergetics.* Second International Meeting of the Neuro-Rhine Consortium (Data blitz and poster session), Strasbourg (France), March 28, 2014.

TRAVEL FELLOSHIPS / HONORS

- 13th Biennial Meeting of Society for Research on Biological Rhythms), Destin, Florida, USA, May 19-24, 2012.

  Society for Research on Biological Rhythms Research Merit Award: *Mitochondrial Activity: From genes to function.*

- 11th International Conference on Alzheimer´s and Parkinson´s Diseases, Florence, Italy, March 6-10, 2013.

  Seahorse Travel Award (Seahorse Bioscience): *Amyloid-β Binding Alcohol Dehydrogenase (ABAD), estradiol and mitochondrial dysfunction in Alzheimer's disease.*

- 1st Scientific meeting of the «Fédération de Médecine Translationnelle de Strasbourg» (FMTS), June 13-14, 2013.

  Award of the «Fédération de Médecine Translationnelle de Strasbourg» for the best short communication, section Neuroscience: *Estradiol metabolism and mitochondrial function in Alzheimer's disease.*

- European College of Neuropsychopharmacology (ECNP) Workshop for Junior Scientists in Europe, March 6-3, 2014.

  ECNP Travel Award: *Effects of neuroactive steroids on cellular bioenergetics.*

APPENDICES

Appendix 1

Insights into mitochondrial dysfunction: Aging, amyloid-beta, and tau-A deleterious trio.

Schmitt K, **Grimm A,** Kazmierczak A, Strosznajder JB, Gotz J, Eckert A.

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FORUM REVIEW ARTICLE

Insights into Mitochondrial Dysfunction: Aging, Amyloid-β, and Tau–A Deleterious Trio

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Abstract

Significance: Alzheimer's disease (AD) is an age-related progressive neurodegenerative disorder mainly affecting elderly individuals. The pathology of AD is characterized by amyloid plaques (aggregates of amyloid-β [Aβ]) and neurofibrillary tangles (aggregates of tau), but the mechanisms underlying this dysfunction are still partially unclear. ***Recent Advances:*** A growing body of evidence supports mitochondrial dysfunction as a prominent and early, chronic oxidative stress-associated event that contributes to synaptic abnormalities and, ultimately, selective neuronal degeneration in AD. ***Critical Issues:*** In this review, we discuss on the one hand whether mitochondrial decline observed in brain aging is a determinant event in the onset of AD and on the other hand the close interrelationship of this organelle with Aβ and tau in the pathogenic process underlying AD. Moreover, we summarize evidence from aging and Alzheimer models showing that the harmful trio "aging, Aβ, and tau protein" triggers mitochondrial dysfunction through a number of pathways, such as impairment of oxidative phosphorylation (OXPHOS), elevation of reactive oxygen species production, and interaction with mitochondrial proteins, contributing to the development and progression of the disease. ***Future Directions:*** The aging process may weaken the mitochondrial OXPHOS system in a more general way over many years providing a basis for the specific and destructive effects of Aβ and tau. Establishing strategies involving efforts to protect cells at the mitochondrial level by stabilizing or restoring mitochondrial function and energy homeostasis appears to be challenging, but very promising route on the horizon. *Antioxid. Redox Signal*. 16, 1456–1466.

Introduction

Aging is an inevitable biological process that results in a progressive structural and functional decline, as well as biochemical alterations that altogether lead to reduced ability to adapt to environmental changes. Although aging is almost universally conserved among all organisms, the molecular mechanisms underlying this phenomenon still remain unclear. There are several theories of aging, in which free radical (oxidative stress), DNA, or protein modifications are suggested to play the major causative role (54, 72). A growing body of evidence supports mitochondrial dysfunction as a prominent and early, chronic oxidative stress-associated event that contributes to synaptic abnormalities in aging and, ultimately, increased susceptibility to age-related disorders including Alzheimer's disease (AD) (58). AD is the most common neurodegenerative disorder among elderly individuals. It accounts for up to 80% of all dementia cases and ranks as the fourth leading cause of death among those above 65 years of age. With the increasing average life span of humans, it is highly probable that the number of AD cases will dangerously raise. The pathology of AD characterized by abnormal formation of amyloid plaques (aggregates of amyloid-β [Aβ]) and neurofibrillary tangles (NFT; aggregates of tau) was shown to be accompanied by mitochondrial dysfunction. However, the mechanisms underlying this dysfunction are poorly understood. There remain several open questions: Is age-related oxidative stress accelerating the NFT and Aβ pathologies? Are these lesions causing oxidative stress themselves? Or are there other mechanisms involved? Within the past years, several mouse models have been developed that reproduce the aging process and diverse aspects of AD. These models help in understanding the age-related pathogenic mechanisms that lead to mitochondrial failure in AD, and in particular the interplay of AD-related cellular modifications within this process (17, 18).

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Mitochondrial Aging—the Beginning of the End in AD?

Mitochondria play a pivotal role in cell survival and death by regulating both energy metabolism and apoptotic pathways; they contribute to many cellular functions, including intracellular calcium homeostasis, the alteration of the cellular reduction-oxidation (redox) potential, cell cycle regulation, and synaptic plasticity (47). They are the "powerhouses of cells," providing energy from nutritional sources *via* ATP generation, which is accomplished through oxidative phosphorylation (OXPHOS) (65). However, when mitochondria fulfill their physiological function, it is as if Pandora's box has been opened, as this vital organelle contains potentially harmful proteins and biochemical reaction centers; mitochondria are the major producers of reactive oxygen species (ROS) at the same time being susceptible targets of ROS toxicity. Unstable ROS are capable of damaging many types of mitochondrial components; this includes oxidative deterioration of mitochondrial DNA (mtDNA), lipids of the mitochondrial membrane, and mitochondrial proteins, and it is thought that this damage that may accumulate over time from ROS generated from aerobic respiration may play a significant role in aging (Fig. 1). Moreover, it was previously demonstrated that nitrosative stress evoked by increased nitric oxide synthesis also leads to protein oxidation as well as mitochondrial and DNA damage, which are common mechanisms occurred in elderly (13, 34, 70).

Although most mitochondrial proteins are encoded by the nuclear genome, the mitochondrial genome encodes proteins required for 13 polypeptide complexes of the respiratory chain involved in ATP synthesis. Given that mtDNA exists in the inner matrix and this is in close proximity to the inner membrane where electrons can form unstable compounds, mtDNA, unlike nuclear DNA (nDNA), is not protected by histones (4) making it more vulnerable to oxidative stress and its mutation rate is about 10-fold higher than that of nDNA, especially in tissues with a high ATP demand like the brain (54). These mtDNA mutations occur in genes encoding electron transport chain (ETC) subunits including NADH dehydrogenase, cytochrome c oxidase (COX), and ATP synthase (83). Eventually, ROS-related mtDNA mutations can result in the synthesis of mutant ETC proteins that, in turn, can lead to the leakage of more electrons and increased ROS production. This so-called "vicious cycle" is hypothesized to play a critical role in the aging process according to the mitochondrial theory of aging. In addition to age-associated increase in mtDNA mutations, the amount of mtDNA also declines with age in various human and rodent tissues (2, 68). Furthermore, abundance of mtDNA correlates with the rate of mitochondrial ATP production (68), suggesting that age-related mitochondrial dysfunction in muscle is related to reduced mtDNA abundance. However, age-associated change in mtDNA abundance seems to be tissue specific, as several studies have reported no change in mtDNA abundance with age in other than muscular tissues in both man and mouse (20, 46).

How does the somatic mtDNA involved in aging phenotypes contribute to AD development? As only a small fraction of AD is caused by autosomal dominant mutations, this comes down to the question of what is causing the prevalent sporadic cases in the first place. Somatic mutations in mtDNA could cause energy deficiency, increased oxidative stress, and accumulation of Aβ, which act in a vicious cycle reinforcing mtDNA damage and oxidative stress (45). Indeed, defects in mtDNA associated with decreased cytochrome oxidase activity have been found in AD patients (9). Although a similarly impaired mitochondrial function and subsequent compensatory response have been observed in both nondemented aged and AD subjects, no clear causative mutations in the mtDNA have been correlated to AD; although some variations have functional consequences, including changes in enzymatic activity (40). Perhaps the main differences are that, in AD brains, defects are more profound due to Aβ and tau accumulation, because of decreased compensatory response machinery (Fig. 1).

Many investigators have developed models for studying mitochondrial-related aging (36). Among them senescence-accelerated mice (SAM) strains are especially useful models to understand the mechanisms of the age-related mitochondrial decline. Behavioral studies showed that learning and memory deficits already started as early as 6 months and worsened with aging in SAMP8 mice (accelerated senescence-prone 8) (53, 77). Moreover, Omata and collaborators showed age-related changes in cerebral energy production in the 2-month-old SAMP8 followed by a decrease in mitochondrial function compared with SAMR1 mice (accelerated senescence-resistant 1) (51). Aging is not only connected with increased mitochondrial ROS production due to ETC impairment but also with a dysbalance of the protective antioxidant machinery inside mitochondria. For instance, age-related changes in levels of antioxidant enzymes, such as copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese SOD (Mn-SOD), have been found in liver and cortex of SAMP8 mice when compared with age-matched SAMR1 mice, supporting increased oxidative stress as a key mechanism involved in the aging process (37). More recently, Yew and collaborators have shown an impairment of mitochondrial functions including a decrease of COX activity, mitochondrial ATP content, and mitochondrial glutathione (GSH) level at a relatively early age in SAMP8 mice compared with SAMR1 mice (67, 78). Furthermore, the biochemical consequences of aging have been investigated using proteomic analysis in the brain of SAMP8 and SAMR1 mice at presymptomatic (5-month old) and symptomatic (15-month old) stages (84), revealing differentially expressed proteins with age in both mouse strains, such as Cu/Zn-SOD. Besides the progressive mitochondrial decline and increased oxidative stress, tau hyperphosphorylation was also observed at an early age in the brain of SAMP8 mice (1, 71). In addition, SAMP8 mice showed an age-related increase in mRNA and protein levels of amyloid-β precursor protein (APP). The cleavage product Aβ was significantly increased at 9 months in SAMP8 and amyloid plaques started to form at around 16 months of age (48, 73). Altogether, these data indicate that mitochondrial dysfunction is a highly relevant event in the aging process, which is also known as the primary risk factor for AD and other prevalent neurodegenerative disorders.

Age-Related Aβ and Tau Effects on Mitochondria in AD

AD is a progressive, neurodegenerative disorder, characterized by an age-dependent loss of memory and an impairment of multiple cognitive functions. From a genetic point of view, AD can be classified into two different forms: rare familial forms (FAD) where the disease onset is at an age below

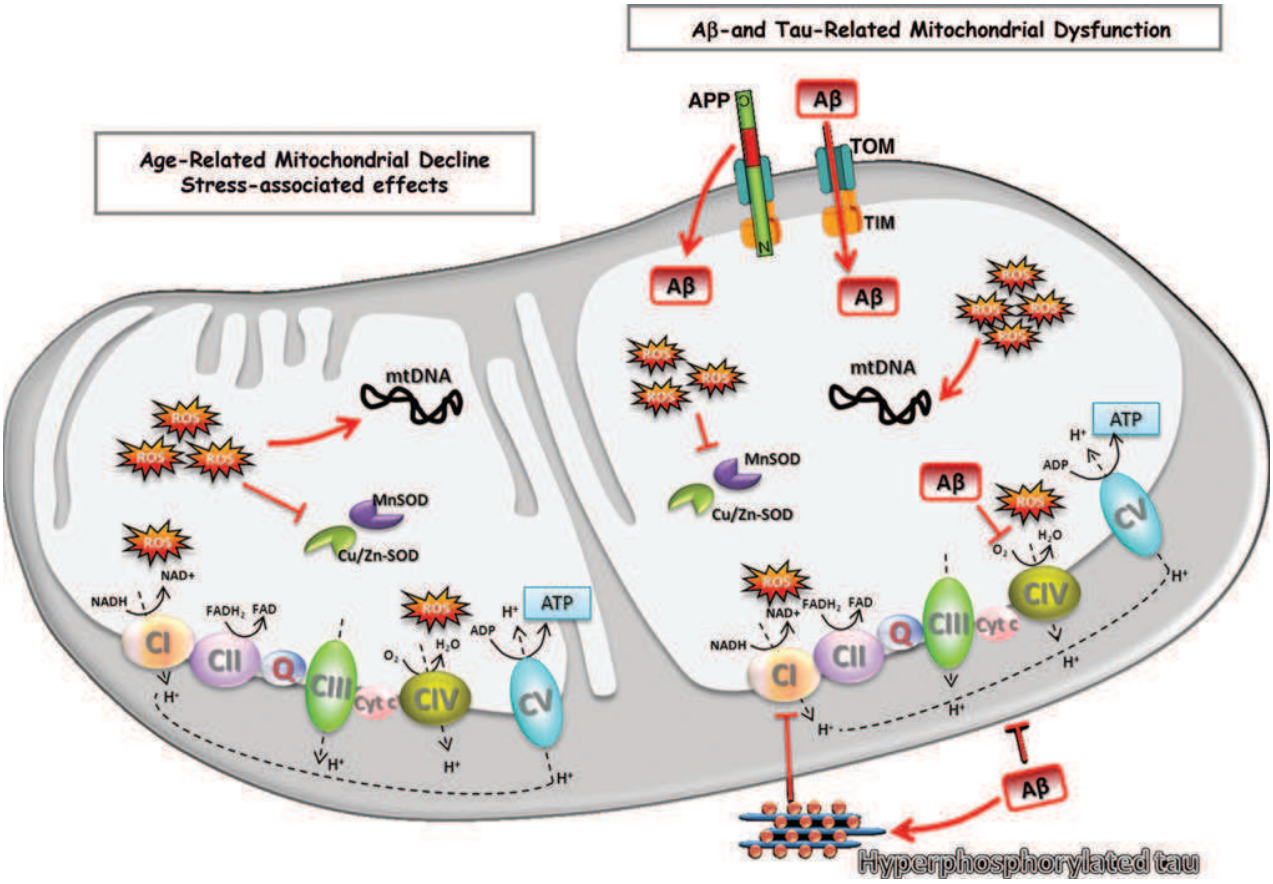

FIG. 1. Aging, Aβ, and tau: toxic consequence on mitochondria. The aging process may weaken the mitochondrial OXPHOS (oxidative phosphorylation) system in a more general way by the accumulation of ROS-induced damage over many years thereby sowing the seeds for specific and destructive effects of A*β* and tau. ROS induce peroxidation of several mitochondrial macromolecules, such as mtDNA and mitochondrial lipids, contributing to mitochondrial impairment in the mitochondrial matrix. In AD, mitochondria were found to be a target of A*β* toxicity, which may act directly or indirectly on several proteins, leading to mitochondrial dysfunction. Indeed, A*β* was found in the OMM and IMM as well as in the matrix. The interaction of A*β* with the OMM might affect the transport of nuclear-encoded mitochondrial proteins, such as subunits of the ETC CIV, into the organelle *via* the TOM import machinery. A*β* seems to be able to enter into the mitochondrial matrix through TOM and TIM or could be derived from mitochondria-associated APP metabolism. The interaction of A*β* with the IMM would bring it into contact with respiratory chain complexes with the potential for myriad effects on cellular metabolism. It may be that A*β* by these interactions affects the activity of several enzymes decreasing the ETC enzyme CIV, reducing the amount of hydrogen that is translocated from the matrix to the intermembrane space, thus impairing the MMP. The dysfunction of the ETC leads to a decreased CV activity and so to a lower ATP synthesis, in addition to an increased ROS production. Interestingly, deregulation of CI is mainly tau dependent, while deregulation of CIV is A*β* dependent, at both the protein and activity level. A*β*, amyloid-*β*; AD, Alzheimer's disease; APP, amyloid precursor protein; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V, Cu/Zn-SOD, copper/zinc superoxide dismutase; cyt c, cytochrome c; ETC, electron transport chain; IMM, inner mitochondrial membrane; MMP, mitochondrial membrane potential; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane. (To see this illustration in color the reader is referred to the Web version of this article at www.liebertonline.com/ars).

60 years (<1% of the total number of AD case) and the vast majority of sporadic AD cases where onset occurs at an age over 60 years. Genetic studies in FAD patients have identified autosomal dominant mutations in three different genes, encoding the *APP* (over 20 pathogenic mutations identified) and the presenilins *PS1* and *PS2* (more than 130 mutations identified) (26). These mutations are directly linked to the increased production of A*β* from its precursor protein APP, suggesting a direct and pathological role for A*β* accumulation in the development of AD.

Mitochondrial dysfunction has been proposed as an underlying mechanism in the early stages of AD, since energy deficiency is a fundamental characteristic feature of AD brains (44) as well as of peripheral cells derived from AD patients (22). Understanding the molecular pathways by which the various pathological alterations including A*β* and tau compromise neuronal integrity, leading to clinical symptoms, has been a long-standing goal of AD research. The successful development of mouse models that mimic diverse aspects of the AD process has facilitated this effort and assisted in

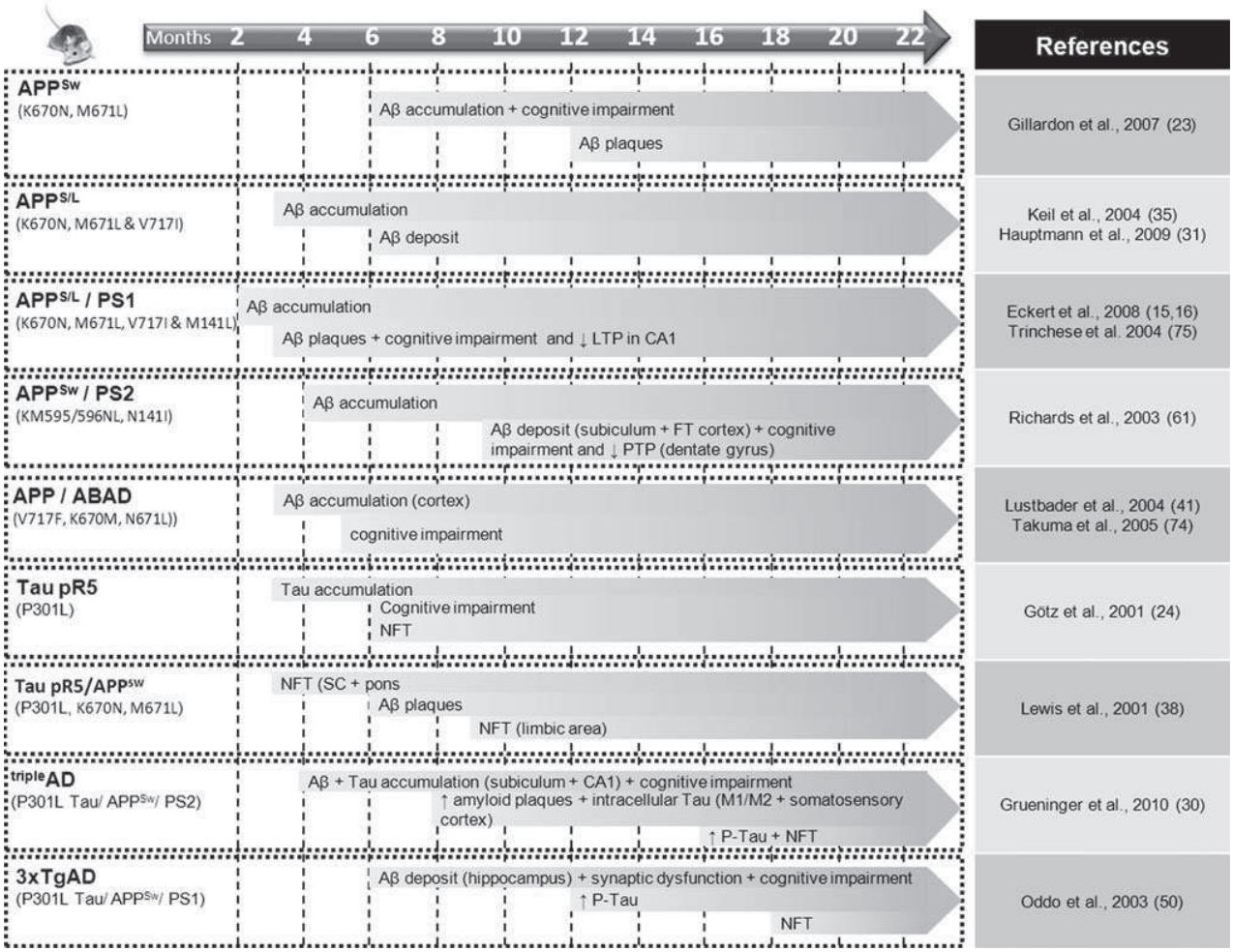

FIG. 2. Age-dependent appearance of histopathological hallmarks in transgenic AD mouse model.

understanding of the age-dependent interplay of Aβ and tau on bioenergetics processes *in vivo* (Figs. 2 and 3).

Separate modes of Aβ and tau toxicity on mitochondria

Mitochondria were found to be a target for APP toxicity as both the full-length protein and Aβ accumulate in the mitochondrial import channels, and both lead to mitochondrial dysfunction (7, 42, 55, 56). Several evidences from cellular and animal AD models indicate that Aβ triggers mitochondrial dysfunction through a number of pathways such as impairment of OXPHOS, elevation of ROS production, interaction with mitochondrial proteins, and alteration of mitochondrial dynamics (52). Indeed, abnormal mitochondrial dynamics have been identified in sporadic and familial AD cases (43, 76) as well as in AD mouse model (6); a distortion probably mediated by altered expression of dynamin-like protein 1 (DLP1), a regulator of mitochondrial fission and distribution, due to elevated oxidative and/or Aβ-induced stress. This modification can disturb the balance between fission and fusion of mitochondria in favor of mitochondrial fission followed by mitochondrial depletion from axons and dendrites and, subsequently, synaptic loss.

Success in developing mouse models that mimic diverse facets of the disease process has greatly facilitated the understanding of physiopathological mechanisms underlying AD. Thus, in 1995, Games and collaborators established the first APP mice model (called PDAPP) bearing the human "Indiana" mutation of the APP gene (V171F). They observed the accumulation of Aβ in the brain and subsequent amyloid plaque formation as well as astrocytosis and neuritic dystrophy (21). Interestingly, in this model cognitive deficits, such as spatial learning impairment, occur before the formation of Aβ plaques and increase with age (8). This phenomenon was also observed in Tg2576 transgenic mice bearing the human Swedish mutation of the *APP* gene (K670N, M671L). In fact, in most of the APP mouse models, the cognitive impairment begins concomitantly with Aβ oligomer formation in the brain (around 6 months of age), while neuritic amyloid deposits become visible only between 12 and 23 months and then the amount of deposits increases (23, 31, 35). Thus, memory deficits seem to directly correlate with the accumulation of intracellular Aβ oligomers and not with amyloid plaque formation. Crossing APP transgenic mice with those bearing a mutation in presenilin 1 gene enabled an earlier onset of amyloid plaques compared with APP mice. In one of the most

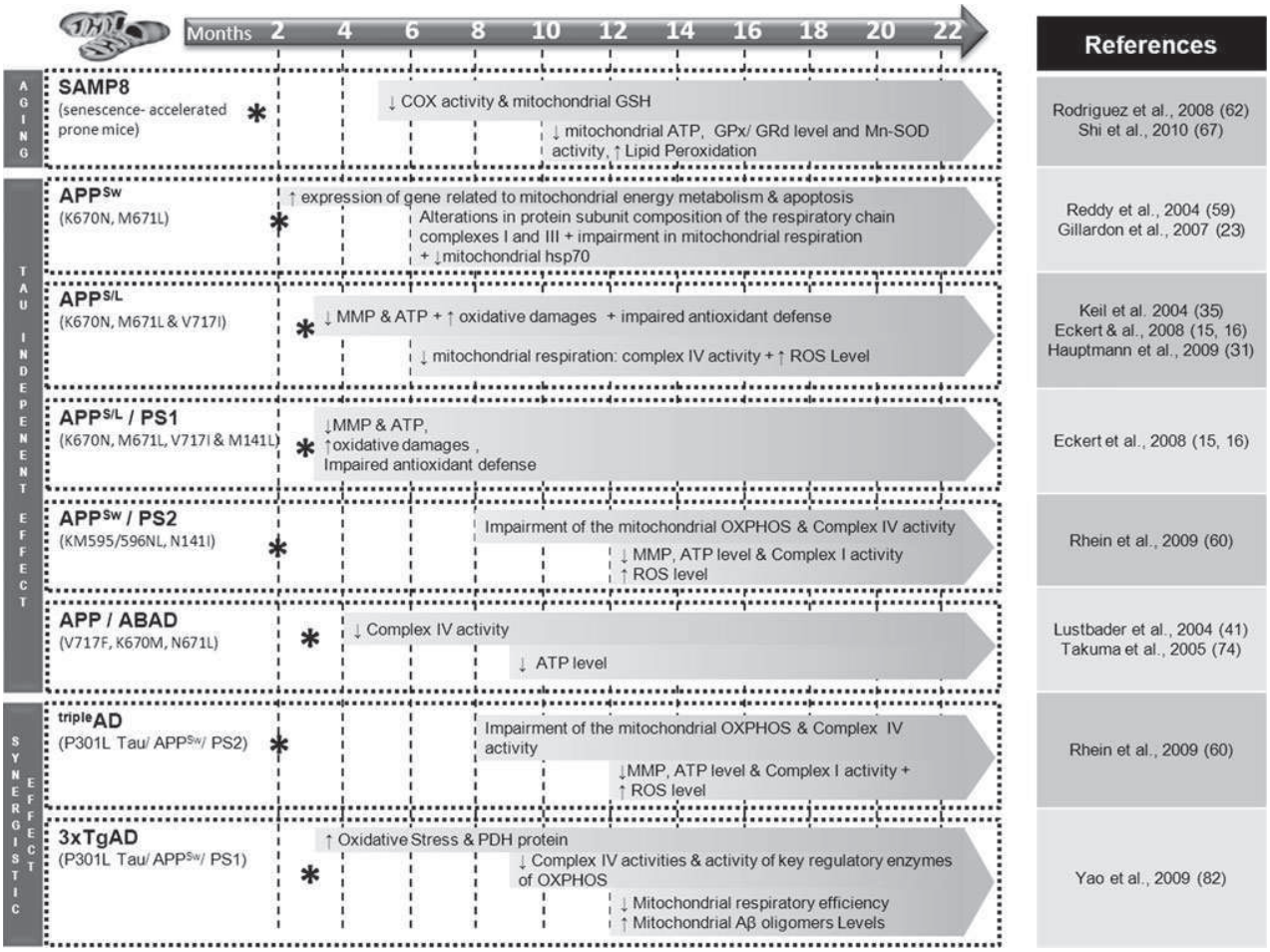

FIG. 3. Age-dependent mitochondrial dysfunction in senescence-accelerated and transgenic AD mouse models. (Star: start of the experiments).

aggressive models, double-transgenic $APP^{S/L}$/PS1 ($APP^{Swedish/London}$/$PS1^{M141L}$) mice, Aβ accumulation begins very soon at 1–2 months of age while cognitive deficits and amyloid plaque formation are already observed at 3 months (3, 16). A stronger decrease of mitochondrial membrane potential as well as ATP level was also found in these mice.

Mitochondrial dysfunctions also appear to a very early stage in these transgenic mouse models. For example, in the APP^{Sw} transgenic strain Tg2576, an upregulation of genes related to mitochondrial energy metabolism and apoptosis was observed already at 2 months of age. Alterations in composition of the mitochondrial respiratory chain complexes I and III protein subunit as well as impairment of mitochondrial respiration were detected around 6 months, when soluble Aβ accumulated in the brain without plaque formation (10, 23, 59). To test the hypothesis that oxidative stress can underlie the deleterious effects of PS mutations, Schuessel and collaborators analyzed lipid peroxidation products (4-hydroxynonenal [HNE] and malondialdehyde) and antioxidant defense mechanisms in brain tissue and ROS levels in splenic lymphocytes from transgenic mice bearing the human PS1 M146L mutation (PS1M146L) compared with those from mice transgenic for wild-type human PS1 (PS1wt) and non-transgenic littermate control mice (66). In brain tissue, HNE levels were increased only in aged (19–22 months) PS1M146L transgenic animals compared with PS1wt mice and not in young (3–4 months) or middle-aged mice (13–15 months). Similarly, in splenic lymphocytes expressing the transgenic PS1 proteins, mitochondrial and cytosolic ROS levels were significantly elevated compared with controls only in cells from aged PS1M146L animals. Antioxidant defense mechanisms (activities of antioxidant enzymes including Cu/Zn-SOD, GSH peroxidase, and GSH reductase) as well as susceptibility to oxidative stress *in vitro* were unaltered. In summary, these results demonstrate that the PS1M146L mutation increases mitochondrial ROS formation and oxidative damage selectively in aged mice. Consistent with this observation, in Swedish amyloid precursor protein (APP^{Sw})/PS2 double-transgenic mice, mitochondrial impairment was first detected at 8 months of age, before amyloid plaque deposition, but after soluble Aβ accumulation (60, 61). Taken together, these findings are consistent with the recently proposed hypothesis of the age-related Aβ toxicity cascade that suggests that the most toxic Aβ species that cause majority of molecular and biochemical abnormalities are in fact intracellular soluble oligomeric

aggregates rather than the extracellular, insoluble plaques that may comprise the form of cellular defense against toxicity of oligomers (19). Interestingly, human amylin that aggregates in type 2 diabetic pancreas and shares with Aβ its amyloidogenic properties also causes an impaired complex IV activity, whereas nonamyloidogenic rat amylin did not (39).

How does tau interfere with mitochondrial function? In its hyperphosphorylated form, tau, which forms the NFTs, the second hallmark lesion in AD, has been shown to block mitochondrial transport, which results in energy deprivation and oxidative stress at the synapse and, hence, neurodegeneration (27, 33, 57). Till now, no mutations in microtubule-associated protein tau (MAPT) coding genes have been detected in relation to familial forms of AD. However, in familial frontotemporal dementia (FTD) with parkinsonism, mutations in the MAPT gene were identified on chromosome 17. This was the basis for creating a robust mouse model for tau pathology in 2001. These P301L tau–expressing pR5 mice (longest four-repeat 4R2N) show an accumulation of tau as soon as 3 months of age and develop NFTs around 6 months of age (24). A mass spectrometric analysis of the brain proteins from these mice revealed mainly a deregulation of mitochondrial respiratory chain complex components (including complex V), antioxidant enzymes, and synaptic protein space (11). The reduction in mitochondrial complex V levels in the P301L tau mice that was revealed using proteomics was also confirmed as decreased in human P301L FTDP-17 (FTD with parkinsonism linked to chromosome 17) brains. The functional analysis demonstrated age-related mitochondrial dysfunction, together with reduced NADH-ubiquinone oxidoreductase (complex I) activity as well as age-related impaired mitochondrial respiration and ATP synthesis in pR5 mice model. Mitochondrial dysfunction was also associated with higher levels of ROS in aged transgenic mice. Increased tau pathology resulted in modification of lipid peroxidation levels and the upregulation of antioxidant enzymes in response to oxidative stress (11). Thus, this evidence demonstrated for the first time that not only Aβ but also tau pathology leads to metabolic impairment and oxidative stress by distinct mechanisms from that caused by Aβ in AD.

Synergistic modes of Aβ and tau toxicity on mitochondria

Although Aβ and tau pathologies are both known hallmarks of AD, the mechanisms underlying the interplay between plaques and NFTs (or Aβ and tau, respectively) have remained unresolved. However, a close relationship between mitochondrial impairment and Aβ on the one hand and tau on the other hand has been already established. How do both AD features relate to each other? Is it possible that these two molecules synergistically affect mitochondrial integrity? Several studies suggest that Aβ aggregates and hyperphosphorylated tau may block the mitochondrial carriage to the synapse leading to energy deficiency and neurodegeneration (28). Moreover, the enhanced tau levels may inhibit the transport of APP into axons and dendrites, which suggests a direct link between tau and APP in axonal failure (14, 69). Remarkably, intracerebral Aβ injections amplify a preexisting tau pathology in several transgenic mouse models (5, 25, 29), whereas lack of tau abrogates Aβ toxicity (32, 33). Our findings indicate that in tau transgenic pR5 mice, mitochondria display an enhanced vulnerability toward an Aβ insult *in vitro* (12, 15, 16), suggesting a synergistic action of tau and Aβ pathology on this organelle (Figs. 2 and 3). The Aβ caused a significant reduction of mitochondrial membrane potential in cerebral cells from pR5 mice (11). Furthermore, incubation of isolated mitochondria from pR5 mice with either oligomeric or fibrillar Aβ species resulted in an impairment of the mitochondrial membrane potential and respiration. Interestingly, aging particularly increased the sensitivity of mitochondria to oligomeric Aβ insult compared with that of fibrillar Aβ (15). This suggests that while both oligomeric and fibrillar Aβ species are toxic, they exert different degrees of toxicity. Crossing P301L mutant tau transgenic JNPL3 mice (shortest four-repeat [4R0N] tau together with the P301L mutation) with APP^{Sw} transgenic Tg2576 mice revealed the presence of NFT pathology in spinal cord and pons already at 3 months of age (38). Aβ plaques were detected at the age of 6 months and had the same morphology and distribution than in the 1-year-old Tg2576 mice. Taken together, these studies illustrate the existence of a complex interplay between the two key proteins in AD.

Additionally, in recent years triple-transgenic mouse models have been established that combine Aβ and tau pathologies (Figs. 2 and 3). In these models the contribution of both AD-related proteins on the mitochondrial respiratory machinery and energy homeostasis has been investigated *in vivo*. Indeed, our group demonstrated a mitochondrial dysfunction in a novel triple-transgenic mouse model ($pR5/APP^{Sw}/PS2^{N141I}$)—^{triple}AD mice—using proteomics followed by functional validation (60). Particularly, deregulation of activity of complex I was found to be tau dependent, whereas deregulation of complex IV was Aβ dependent, in 10-month-old ^{triple}AD mice. The convergent effects of Aβ and tau led already at the age of 8 months to a depolarization of mitochondrial membrane potential in ^{triple}AD mice. Additionally, we found that age-related oxidative stress also plays a significant part in the deleterious vicious cycle by exaggerating Aβ- and tau-induced disturbances in the respiratory system and ATP synthesis, finally leading to synaptic failure.

Our data complement those obtained in another triple-transgenic mouse model 3xTg-AD ($P301Ltau/APP^{Sw}$/PS1 M146L) (50). In these studies, mitochondrial dysfunction was evidenced by an age-related decrease in the activity of regulatory enzymes of OXPHOS such as COX, or of the Krebs cycle such as pyruvate dehydrogenase, analyzing 3xTg-AD mice aged from 3 to 12 months (82). Besides, these mice also exhibited increased oxidative stress and lipid peroxidation. Most of the effects on mitochondria were seen at the age of 9 months, whereas mitochondrial respiration was significantly decreased at 12 months of age. Importantly, mitochondrial bioenergetics deficits were found to precede the development of AD pathology in the 3xTg-AD mice. Figure 4 nicely shows that AD-specific changes including cognitive impairments, Aβ accumulation, Aβ plaques, and mitochondrial dysfunction seem to occur at an earlier onset from single, double up to triple AD transgenic mice models. Together, our studies highlight the key role of mitochondria in AD pathogenesis and consolidate the notion that a synergistic effect of tau and Aβ enhances the pathological weakening of mitochondria at an early stage of AD.

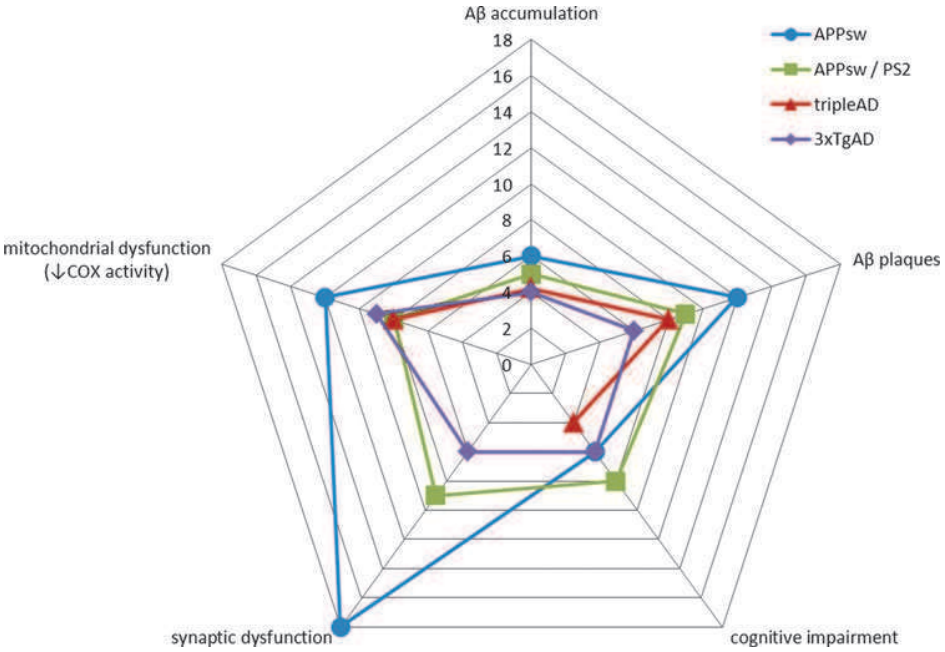

FIG. 4. Age-dependent onset of AD-associated pathological changes in different AD mouse models (age in months). In both triple Tg mouse models [^{Triple}AD, (60); 3xTgAD, (50–82)] an earlier onset in the appearance of AD-related changes in the brain can be detected when compared with double transgenic [APP^{Sw}/PS2 (60, 61)] and to mice bearing only APP mutations [APP^{Sw}, (24)], suggesting again a synergistic effect of Aβ and tau in the pathogenesis of AD. Age of the mice is given in months. APP^{Sw}, APP Swedish transgenic mice; APP^{Sw}/PS2, APP Swedish/presenilin 2 transgenic mice; tripleAD, APP Swedish/presenilin 2/P301L tau transgenic mice; 3xTgAD, APP Swedish/presenilin 1/P301L tau transgenic mice. (To see this illustration in color the reader is referred to the Web version of this article at www.liebertonline.com/ars).

Aβ-Binding Alcohol Dehydrogenase: A New Lead to Decode the Mechanisms of Aβ-Induced Mitochondrial Dysfunction

A few years ago, Yan and collaborators showed that the Aβ peptide can directly bind a mitochondrial enzyme called Aβ-binding alcohol dehydrogenase (ABAD) that is overexpressed in the brains of Alzheimer's patients and AD mouse models (79). The interaction of Aβ with this enzyme exacerbates mitochondrial dysfunction induced by Aβ (decrease of mitochondrial complex IV activity, diminution of O_2 consumption, and increase of ROS), as shown in double-transgenic mice overexpressing mutant APP and ABAD (81). Furthermore, these mice presented an earlier onset of cognitive impairment and histopathological changes when compared with APP mice, suggesting that the Aβ–ABAD interaction is an important mechanism underlying Aβ toxicity. The Aβ–ABAD complex could have a direct effect on the ETC because ABAD was found to be one of three proteins that comprise the fully functional mammalian mitochondrial RNAse P (63), a function that may not require dehydrogenase activity and that links ABAD directly to the production of mitochondrial ETC proteins and ROS generation.

Recently, it has been shown that inhibition of Aβ–ABAD interaction by a decoy peptide can restore mitochondrial deficits and improve neuronal and cognitive function (81). Our findings, using SH-SY5Y neuroblastoma cells treated with A$\beta_{1\text{-}42}$, a cellular model of AD, seem to confirm these observations (Lim *et al.*, unpublished observations). We employed a novel small ABAD-specific inhibitor to investigate the role of this enzyme in Aβ toxicity. The inhibitor significantly improved metabolic functions impaired by Aβ, and specifically reduced Aβ-induced oxidative stress and cell death. Furthermore, we have shown previously that the production of estradiol, a well-known neuroprotective neurosteroid and ABAD substrate, is increased after 24 h in the presence of a "nontoxic" concentration of Aβ and is decreased when using a toxic concentration of this peptide (64), suggesting that Aβ is able to modulate (directly or indirectly) neurosteroid levels. Accordingly, new findings from our group demonstrate that the levels of estradiol in the cytosol and in mitochondria can differently be influenced by Aβ peptide (500 nM, 5 days of treatment) (Fig. 5A, B). We observed that cytosolic estradiol is reduced in the presence of Aβ, but at the same time mitochondrial estradiol load was significantly increased. We suggest that this increase is due to an Aβ-induced decrease of ABAD activity, thus limiting the conversion of estradiol in estrone within mitochondria (Fig. 5C). Inhibition of ABAD activity by Aβ peptide was already demonstrated by Yan and collaborators (80) using 17β-estradiol as substrate of the enzyme. One mechanism that could explain this inhibition is the fact that Aβ–ABAD interaction changes the conformation of the enzyme, avoiding the binding of the cofactor NAD+, and this reduces the metabolic activity of ABAD (41). However, the total amount of estradiol is about 500-fold higher than in the mitochondrial fraction. Even if Aβ induced an increase in estradiol within mitochondria, the reduction of total estradiol level by other enzymes of the complex steroidogenic pathway may therefore be more relevant for cellular dysfunction. Besides,

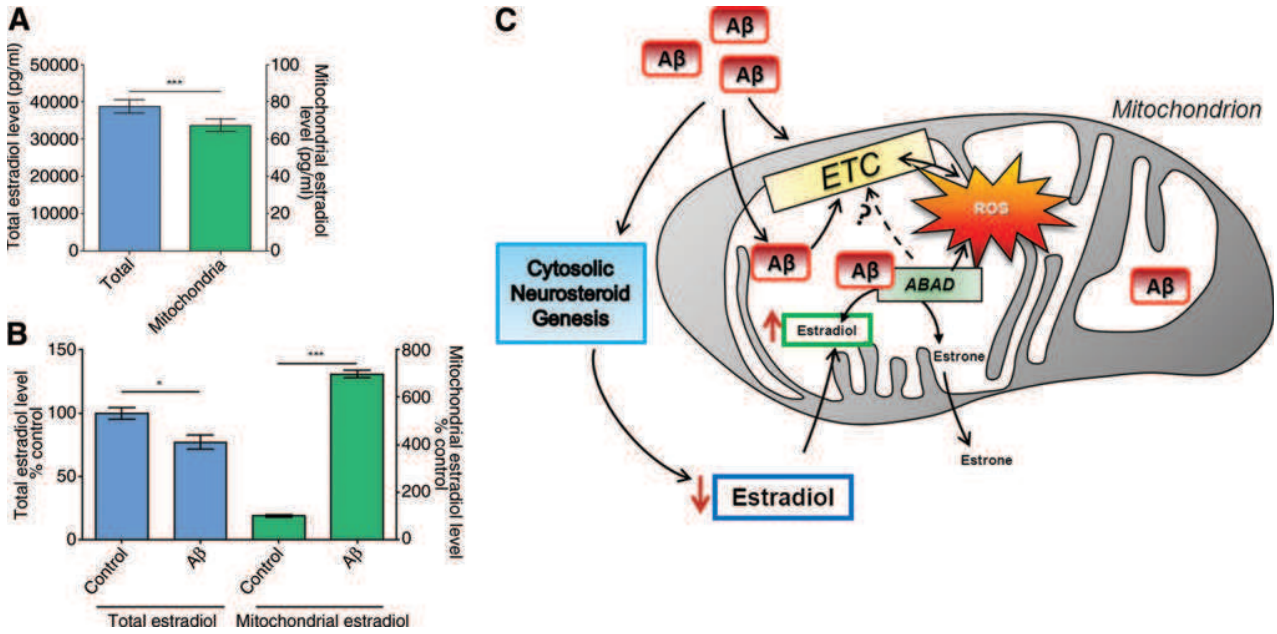

FIG. 5. Aβ–ABAD interaction and estradiol level in mitochondria. (A) Mitochondrial estradiol is very low compared with total estradiol level in SH-SY5Y neuroblastoma cells. Unpaired *t*-test, ***$p < 0.001$. **(B)** Estradiol level is differently influenced by Aβ peptide in the cytosol and in mitochondria. Paired *t*-test, *$p < 0.05$, ***$p < 0.0001$. **(C)** Aβ peptide is able to modulate ABAD function by binding directly to this mitochondrial enzyme. This results in the decrease of ABAD-induced conversion of estradiol into estrone with a concomitant increase of ROS levels and an impairment of the ETC in mitochondria. ABAD, Aβ-binding alcohol dehydrogenase. (To see this illustration in color the reader is referred to the Web version of this article at www.liebertonline.com/ars).

it was also speculated that estradiol exhibits a "prooxidant effect" in the presence of ongoing oxidative stress (49). Thereby, estradiol is hydroxylated to catecholestrogens that can enter a redox cycle generating superoxide radicals, leading to a continuous formation of ROS that amplifies oxidative stress.

Thus, inhibition of the Aβ–ABAD interaction seems to be an interesting therapeutic target to block or prevent Aβ-induced mitochondrial toxicity because it could normalize the imbalance between ROS and estradiol levels in mitochondria and thereby help in improving mitochondrial and neuronal function.

Conclusion

We discuss here the recent findings regarding the possible shared mechanisms involving mitochondrial decline driven by brain aging and the close interrelationship of this organelle with the two main pathological features in the pathogenic process underlying AD.

According to the mitochondrial aging theory, ROS-induced damage and mtDNA mutations accumulate over time inducing ETC impairment and weaken mitochondria function in a rather unspecific way; thus, laying the ground for the two common hallmarks of AD, plaques and NFTs, or Aβ and tau, respectively, which destruct independently as well as synergistically this vital organelle *via* specific mode of actions on complexes I and IV.

Given the complexities of AD, the key role of mitochondrial dysfunction in the early pathogenic pathways by which Aβ leads to neuronal dysfunction in AD is particularly challenging with respect to establishing therapeutic treatments. Besides the modulation and/or removal of both Aβ and tau pathology, strategies involving efforts to protect cells at the mitochondrial level by stabilizing or restoring mitochondrial function or by interfering with energy metabolism appear to be promising. Transgenic AD mice, and particularly triple-transgenic models that combine both pathologies in an age-dependent manner (Fig. 4), are valuable tools in monitoring therapeutic interventions at the mitochondrial level. Eventually, this may lead to therapies that prevent the progression of the age-related mitochondrial decline thereby reducing the vulnerability to Aβ and/or tau at an early stage of the disease.

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Abbreviations Used

Aβ = amyloid-β
ABAD = Aβ-binding alcohol dehydrogenase
AD = Alzheimer's disease
APP = amyloid precursor protein
APP^{Sw} = Swedish amyloid precursor protein
APP^{wt} = wild-type amyloid precursor protein
COX = cytochrome c oxidase
DLP1 = dynamin-like protein 1
ETC = electron transport chain
FTD = frontotemporal dementia
GSH = glutathione
HA = human amylin
HNE = 4-hydroxynonenal
IMM = inner mitochondrial membrane
MAPT = microtubule-associated protein tau
MMP = mitochondrial membrane potential
mtDNA = mitochondrial DNA
nDNA = nuclear DNA
NFT = neurofibrillary tangle
NO = nitric oxide
OMM = outer mitochondrial membrane
OXPHOS = oxidative phosphorylation
PDH = pyruvate dehydrogenase
PS = presenilin
ROS = reactive oxygen species
SAM = senescence-accelerated mice
SOD = superoxide dismutase
TIM = translocase of the inner membrane
TOM = translocase of the outer membrane

Appendix 2

March separate, strike together - Role of phosphorylated TAU in mitochondrial dysfunction in Alzheimer's disease.

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Review

March separate, strike together — Role of phosphorylated TAU in mitochondrial dysfunction in Alzheimer's disease ☆

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ABSTRACT

The energy demand and calcium buffering requirements of the brain are met by the high number of mitochondria in neurons and in these, especially at the synapses. Mitochondria are the major producer of reactive oxygen species (ROS); at the same time, they are damaged by ROS that are induced by abnormal protein aggregates that characterize human neurodegenerative diseases such as Alzheimer's disease (AD). Because synaptic mitochondria are long-lived, any damage exerted by these aggregates impacts severely on neuronal function. Here we review how increased TAU, a defining feature of AD and related tauopathies, impairs mitochondrial function by following the principle: 'March separate, strike together!' In the presence of amyloid-β, TAU's toxicity is augmented suggesting synergistic pathomechanisms. In order to restore mitochondrial functions in neurodegeneration as a means of therapeutic intervention it will be important to integrate the various aspects of dysfunction and get a handle on targeting distinct cell types and subcellular compartments. This article is part of a Special Issue entitled: Misfolded Proteins, Mitochondrial Dysfunction, and Neurodegenerative Diseases.

1. Introduction

The family of microtubule-associated proteins (MAPs) comprises three major classes of polypeptides: MAP1 (>250 kDa), MAP2 (~200 kDa), and TAU (50–70 kDa) [1,2]. MAP2 and TAU are expressed in most neurons, where they localize to separate subcellular compartments. While MAP2 is largely found in dendrites, TAU is concentrated in axons [3]. In the roundworm *Caenorhabditis elegans*, protein with TAU-like repeats (PTL-1) is the sole MAP homolog [4,5], allowing for studies into shared functions of TAU and MAP2 [6]. In the human brain, TAU exists as six isoforms that have either three or four microtubule-binding domains. In the adult mouse brain, there are only isoforms with four microtubule-binding domains expressed. TAU has been localized to cell-types other than neurons such as astrocytes and oligodendrocytes although under physiological conditions expression levels are relatively low [7]. When TAU was discovered in 1975 [8], the subsequent years focused mainly on its tissue distribution and the role TAU has in microtubule assembly and stabilization. With the identification of aggregates of TAU in the Alzheimer's disease (AD) brain, the focus shifted to addressing pathological functions. Histopathologically, AD is characterized by reduced synaptic density, neuronal loss in selected brain areas, as well as amyloid-β (Aβ)-containing plaques and neurofibrillary tangles (NFTs). It is the filamentous core of NFTs that is composed of highly phosphorylated forms of TAU [9,10].

1.1. TAU phosphorylation

What is the role of TAU phosphorylation in disease? TAU is a remarkable protein inasmuch as it contains 80 serine and threonine residues and 5 tyrosine residues that can be potentially phosphorylated [11]. In the normal brain there are 2–3 mol of phosphate per mole of TAU. In the AD brain, TAU is hyperphosphorylated to a stoichiometry of at least three-fold greater than normal supporting the notion that phosphorylation is a critical step in the aggregation process [12]. Whether phosphorylation at distinct sites is required or whether a generally elevated level of phosphorylation is sufficient is not known although the latter possibility is suggested by work in *Drosophila* [13]. With the entering of a formulation of the TAU dye methylene blue (Rember) into clinical trials a discussion has been initiated whether this putative drug is truly an aggregation inhibitor [14] and more specifically whether TAU in NFTs is massively phosphorylated (http://www.alzforum.org/new/detail.asp?id=3410).

While one study claims that the filaments in the NFTs are entirely composed of hyperphosphorylated TAU [15], another claims that hyperphosphorylated TAU accounts for less than 10% of total TAU that is moreover localized to the proteolytically susceptible fuzzy outer coat of the filaments, and not to their structural core [16,17].

☆ This article is part of a Special Issue entitled: Misfolded Proteins, Mitochondrial Dysfunction, and Neurodegenerative Diseases.

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1.2. MAPT mutations and TAU transgenic mice

The TAU field has received a major boost with the identification of pathogenic mutations in the *MAPT* gene that encodes TAU in familial cases (FTDP-17, frontotemporal dementia with Parkinsonism linked to chromosome 17) of frontotemporal lobar degeneration (FTLD-TAU), a disease that shares with AD the aggregation of TAU and NFT formation, but lacks an overt Aβ pathology [18–20]. This enabled us and others to express FTLD mutant forms of TAU in mice and thereby reproduce TAU aggregation and NFT formation, and also to achieve a concomitant behavioural impairment in transgenic mouse models [21]. One such mouse strain generated in our laboratory is pR5 that expresses P301L mutant TAU and because of the pattern of TAU aggregation in the brain, displays amygdala- and hippocampus-dependent memory impairments [22,23]. This and other mouse models were instrumental in determining that manipulating phosphorylation by either inhibiting kinases or activating phosphatases causes an amelioration of the TAU pathology, including a restoration of behavioural impairments and prevention of neuronal cell loss [24–26]. When TAU mutant pR5 mice that progressively develop NFTs were crossed with phosphatase (PP2A)-impaired mice, this caused 7-fold increased numbers of hippocampal neurons that specifically phosphorylated the pathological Ser422 epitope of TAU, and 8-fold increased numbers of NFTs [27]. Another mouse strain generated by us expresses K369I mutant TAU and because of a unique expression pattern that includes the substantia nigra it is characterized by Parkinsonism [28]. Again, in these mice, TAU is highly phosphorylated; however, different from the pR5 strain, the 12E8 phospho-epitope Ser262/Ser356 is spared. Together this underscores the importance of phosphorylation in disease. Importantly and relevant to the topic of this article, TAU transgenic mouse models have proven instrumental in highlighting mitochondrial dysfunction as a central mechanism in neurodegeneration.

2. Mitochondrial dysfunction — cause or consequence

Mitochondria play a pivotal role in cell survival and death by regulating both energy metabolism and apoptotic pathways. They contribute to many cellular processes including intracellular calcium homeostasis and synaptic plasticity [29]. Maternally inherited, mitochondria are compartmentalized organelles consisting of a matrix and two membranes, an outer and an inner membrane with folded cristae, separated by an intermembrane space. These organelles are the powerhouses of all nucleated cells. They produce adenosine triphosphate (ATP) via the combined efforts of the tricarboxylic cycle (TCA) and the oxidative phosphorylation (OxPhos) system of the electron transport chain (ETC). The respiratory chain comprises four biochemically linked multi-subunit complexes I, II, III and IV, as well as two electron carriers, ubiquinone/coenzyme Q and cytochrome C, that are localized at the inner mitochondrial membrane (Fig. 1). By using the energy that is stored in nutritional sources the respiratory chain generates a proton gradient across the inner membrane to drive ATP synthesis via ATP synthase (complex V), while at the same time transferring electrons to oxygen and producing water [30]. Mitochondria are the major producer of reactive oxygen species (ROS) and at the same time a target of ROS toxicity [31]. The organelle has at its disposal a powerful quality control system to deal with these challenges: firstly intra-mitochondrial proteases and molecular chaperones that maintain mitochondrial proteostasis; secondly a dynamic network maintained by membrane fission and fusion, a process termed mitochondrial dynamics, by which damaged or defective mitochondrial components are isolated and targeted for autophagy (mitophagy) [32]. Mitochondrial dynamics cannot be discussed in isolation, as mitochondrial fission (i.e. biogenesis), fusion, motility/transport and turnover (by mitophagy) are highly inter-dependent processes [33]. This is especially critical for highly polarized cells such as neurons. The role of mitochondria in ageing and in pathophysiological processes such as AD is constantly being unravelled. Concomitant to ROS production an inefficient mitochondrial base excision repair (BER) machinery has been postulated, with oxidative damage to mitochondrial DNA (mtDNA) being a determining event that occurs during ageing [34].

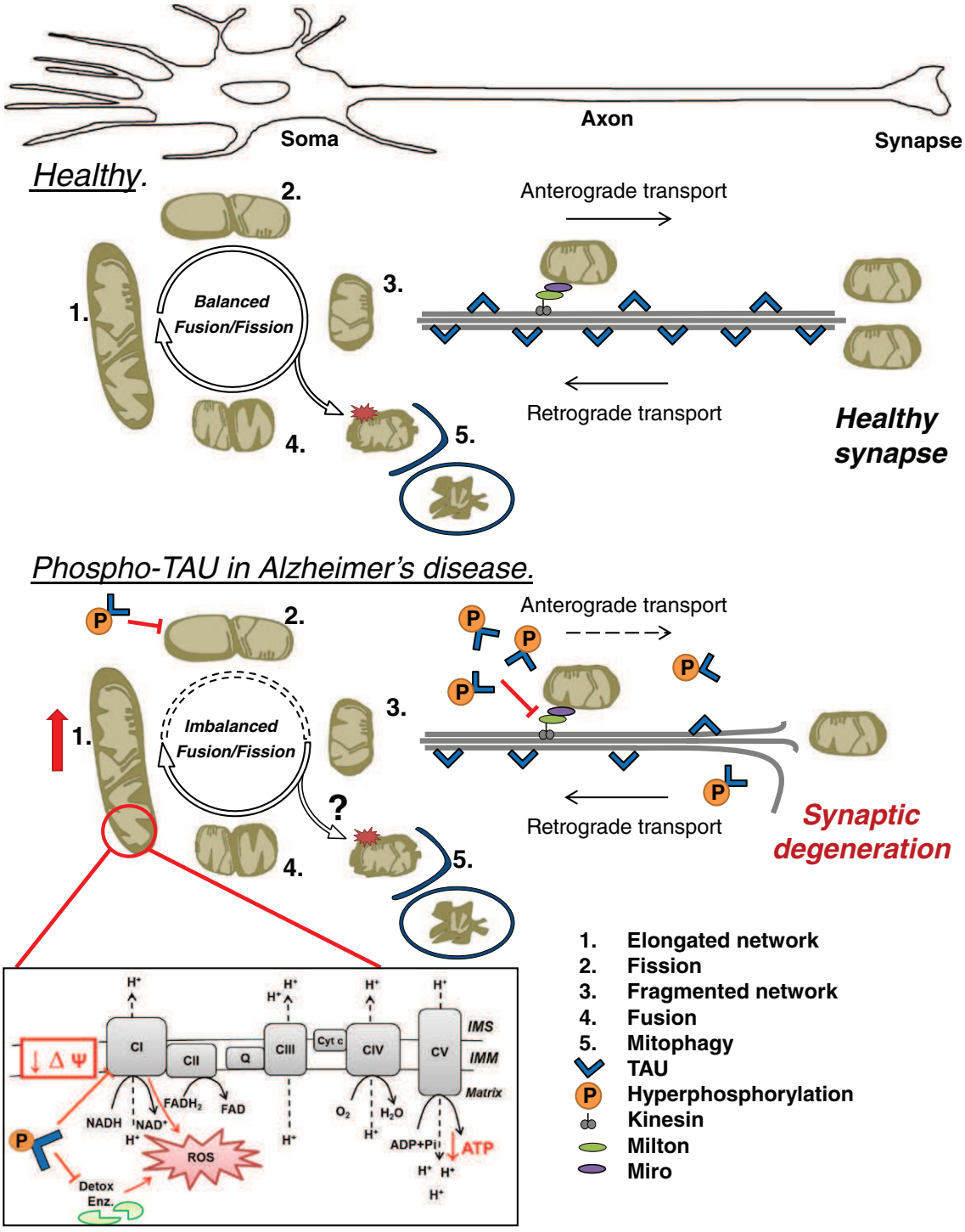
2.1. Manipulating fission and fusion genes

The question arises whether any form of chronic oxidative stress-associated event would at an early stage contribute to the synaptic abnormalities and, ultimately, selective neuronal degeneration that characterizes AD, as a growing body of evidence would suggest [35]. The bulk of AD cases are sporadic, and only a small fraction is caused by autosomal dominant mutations. Regarding the role of mitochondrial dysfunction in AD one can envisage several scenarios. Firstly, oxidative stress could be a down-stream consequence of another pathogenic event; secondly, it could be the cause of neurodegeneration; thirdly, it might accelerate and/or augment the damage elicited by TAU and/or Aβ [31]. By manipulating mitochondrial in vivo genes with a role in fusion and fission, a process discussed in more detail below, neither an AD-like TAU nor an Aβ pathology has been encountered [36–41], which would place mitochondrial dysfunction down-stream of TAU and Aβ toxicity. However, as most mouse strains that lack fission or fusion genes show early lethality it has not been possible to study consequences for TAU and Aβ and in particular the role ageing has in such an impairment.

2.2. Senescence-accelerated mice

In studying mitochondrial functions and age-related mitochondrial decline, senescence-accelerated mouse strains such as SAMP8 (senescence accelerated prone 8) are useful, as the mice display many features known to occur early in the pathogenesis of AD, such as increased oxidative stress and memory impairment [42]. Together with a series of related senescence-accelerated mice, the SAMP8 strain was established in the mid 1970s by conventional inbreeding of AKR/J-derived mice that displayed features of accelerated ageing such as hair loss, reduced activity, shortened life expectancy, lordokyphosis (increased curvature of the spine), and periophthalmic (around the eye) problems [43]. Littermates that did not show a senescence-associated phenotype were also inbred and senescence-resistant, longer-lived SAMR strains were obtained such as SAMR1 (senescence accelerated mouse resistant 1). To better delineate the role of specific single nucleotide variants (SNVs) of these multigenic strains with distinct phenotypes, two recent independent studies used whole exome sequencing to make the strains more useful for studies into ageing and neurodegeneration [44,45].

In SAMP8 mice learning and memory deficits were already evident at 6 months of age and became more pronounced with advanced age [46]. TAU was found to be hyperphosphorylated using a small set of phosphorylation site-specific antibodies, but filament and NFT formation has not been reported indicating that the SAMP8 mice present with an early rather than a more advanced TAU pathology [47]. Staining with Aβ-specific antibodies suggested Aβ deposition [48,49], although, because the murine protein differs from the human Aβ-precursor protein (APP) and lacks the amino acids that are required to generate Aβ, these deposits have been termed 'Aβ-like' [48]. Compared with SAMR1 mice, age-related changes in cerebral energy production were found in SAMP8 at 2 months followed by a decrease in mitochondrial function [50,51]. More recent studies extended this finding by revealing decreases in cytochrome C oxidase (COX) activity, mitochondrial ATP content, and mitochondrial glutathione (GSH) levels in young SAMP8 compared with SAMR1 mice [52,53]. Ageing is accompanied not only by increased mitochondrial ROS production due to ETC impairment but also by an imbalance of the protective mitochondrial antioxidant machinery. For instance, age-related changes in levels of antioxidant enzymes, such as copper/zinc superoxide dismutase (Cu/Zn-SOD) and manganese SOD (Mn-SOD) were found in the liver and cortex of



IMS, inter-membrane space; IMM, inner mitochondrial membrane; ROS, reactive oxygen species; ΔΨ; mitochondrial membrane potential; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V.

Fig. 1. The mitochondrion under physiological and pathological conditions. Under healthy conditions, fusion and fission are balanced, and damaged mitochondria are removed by a process termed mitophagy. Both antero- and retrograde axonal transports are unimpaired providing the synapses with sufficient numbers of functional mitochondria to meet the special energy requirement and calcium buffering of this cellular compartment. Under conditions of elevated TAU, the delicate balance of fission and fusion is deregulated. Fission is impaired and an elongated mitochondrial network results. This may or may not affect mitophagy. Phosphorylated TAU also impairs anterograde transport of distinct cargoes, which include mitochondria. As a consequence, less mitochondria are provisioned to the synapses, which leads to synaptic degeneration. In the scheme, key molecules with roles in mitochondrial transport are indicated, with kinesin being the actual motor and Miro and Milton being adaptor proteins. Inset: Elevated levels of phosphorylated TAU selectively impair complex I of the electron transfer chain, ultimately resulting in reduced levels of ATP and hence, energy deprivation of affected neurons. Hyperphosphorylated forms of TAU also cause an increased production of reactive oxygen species (ROS) and reduced activities of detoxifying enzymes (detox. enz.) including superoxide dismutase (SOD).

SAMP8 mice compared with age-matched SAMR1 mice [54–56]. Increased lipid peroxidation and carbonyl damage is present as early as two months of age [57]. Furthermore, SAMP8 mice reveal age-dependent reductions of various receptors including the NMDA receptor that has a role in excitotoxicity as discussed below [58]. Whereas SAMP mice are certainly a better model for accelerated ageing rather than neurodegeneration, they nonetheless support increased oxidative stress as a key mechanism in the ageing process. Crossing TAU transgenic mice

with mitochondrial impairment onto a SAMP8 genetic background might establish a more advanced model of neurodegeneration compared to one where the TAU transgene is expressed on a conventional inbred background such as C57Bl/6.

2.3. The Harlequin model

Another, although less frequently, employed strain with mitochondrial dysfunction and oxidative stress is the Harlequin (Hq) mutant mouse strain. Hq mice show an 80–90% depletion of the mitochondrial apoptosis-inducing factor (AIF) resulting in reduced levels of complex I of the ETC and increased oxidative stress [59]. Interestingly, although AIF is reduced throughout the brain, neuronal loss is largely restricted to distinct brain regions (cerebellum and retina), indicating selective vulnerability. Because a mitochondrial dysfunction specifically of complex I characterizes the P301L TAU transgenic pR5 mice, the two strains were crossed. This caused an increased TAU pathology (higher degree of phosphorylation and more NFTs) and age-dependent cerebellar neurodegeneration that was preceded by decreased activities of the ETC and depletion of ATP levels [60]. Interestingly, low levels of TAU in the cerebellar granule cell layer significantly increased cerebellar apoptosis and led to an aggravation of motor deficits even though only a very small number of cerebellar granule neurons were positive for TAU phospho-epitopes. This implies that in particularly vulnerable neuronal populations such as the Hq cerebellar granule cells, even low levels of non-hyperphosphorylated TAU may be sufficient to induce apoptosis and cause functional neurological impairment [60]. Analysis of the activities of complexes I–V revealed a complex picture: In the TAU/Hq mice an additive effect of the two mutations was observed, i.e. both a reduced complex I protein content caused by the Hq mouse mutation and a functional reduction of the remaining complex I activity caused by additional TAU expression. For complex III, for example, early decreases in activity were observed in 1-month-old TAU transgenic and TAU/Hq mice. However, while TAU single mutant mice were able to compensate for this deficiency, complex III activity remained reduced in the TAU/Hq double mutant mice at 7 months. Together this study demonstrates a mutual reinforcement of the TAU pathology and mitochondrial dysfunction in vivo, proposing TAU/Hq double mutant mice as a valuable model to study TAU-related neurodegenerative changes in a setting of impaired mitochondrial function.

3. TAU specifically impairs complex I and Aβ complex IV

3.1. Proteomic and functional studies in P301L TAU transgenic mice

An unbiased approach to address TAU-mediated impairment in model systems is by functional genomics that generally generates long lists of deregulated transcripts and proteins, which can then be grouped by category analysis [61]. P301L TAU expressing pR5 mice reveal a significant aggregation of TAU at an early age, with NFTs developing around the age of 6 months, and hence they represent a model suited for the proteomic investigation of TAU-related changes in AD [62,63]. A mass spectrometric analysis of fractionated brain proteins derived from these mice revealed mainly a deregulation of mitochondrial respiratory chain complex components (including complex V), antioxidant enzymes, and synaptic proteins [64]. The reduction in mitochondrial complex V levels in the pR5 mice that was revealed using proteomics was also confirmed as decreased in brains from human carriers of the P301L FTDP-17 mutation. The functional analysis demonstrated age-related mitochondrial dysfunction, together with reduced NADH ubiquinone oxidoreductase (complex I) activity as well as age-related impaired mitochondrial respiration and ATP synthesis in the pR5 mouse model. Mitochondrial dysfunction was further associated with higher ROS levels in aged transgenic mice, concomitant with the upregulation of antioxidant enzymes in response to oxidative stress (Fig. 1). Increased TAU pathology resulted also in lipid peroxidation [64]. Because prior studies had shown that Aβ mainly impairs complex IV [65,66], the finding that TAU mainly impairs complex I of the ETC demonstrated for the first time that TAU pathology also leads to metabolic impairment and oxidative stress, by mechanisms that are distinct from those exerted by Aβ. How TAU affects complex activities is not understood and TAU may well do so indirectly. For Aβ, interaction with and binding to mitochondrial proteins (such as Aβ-binding alcohol dehydrogenase, ABAD; or the voltage-dependent anion channel 1 protein, VDAC) has been postulated [67,68]. Intracellular localization of Aβ has been questioned by a recent study, however, which highlights the use of antibodies in these studies that bind not only to Aβ but also to its precursor protein, APP [69]. Because VDAC was found to interact with phosphorylated TAU it has been proposed that phosphorylated TAU may block mitochondrial pores and that one of TAU's functions is to maintain normal mitochondrial pore opening and closure [68].

3.2. P301L TAU transgenic mice crossed with Aβ-forming mice

While the focus of this review article is on TAU and not on Aβ, it is still worthwhile looking into models that combine both pathologies. When the triple transgenic ^{triple}AD mouse model (pR5/APP^{sw}/PS2 N141I) was subjected to quantitative proteomics, this revealed that one-third of the proteins had functions in mitochondria, specifically complexes I and IV. Therefore, mitochondrial functions were assessed [70]. Again, deregulation of the activity of complex I was found to be TAU-dependent, and deregulation of complex IV Aβ-dependent, when analyzing 10-month-old ^{triple}AD mice. The convergent effects of Aβ and TAU led to a depolarization of the mitochondrial membrane potential in ^{triple}AD mice already at the age of 8 months. Additionally, we found that age-related oxidative stress played a significant part in the deleterious vicious cycle by exaggerating Aβ- and TAU-induced disturbances in the respiratory system and ATP synthesis, finally leading to synaptic failure. Furthermore, synergistic effects of TAU and Aβ on mitochondrial impairment were revealed.

These data complement those obtained in another triple transgenic mouse model, 3xTg-AD (P301Ltau/APPSw/PS1 M146L) [71]. Mitochondrial dysfunction was evidenced by an age-related decrease in the activity of regulatory enzymes of the oxidative phosphorylation system such as COX, or of the TCA cycle such as pyruvate dehydrogenase, analyzing 3xTg-AD mice between 3 and 12 months of age [72]. In addition, these mice also exhibited increased oxidative stress and lipid peroxidation. Most of the effects on mitochondria were seen at the age of 9 months, whereas mitochondrial respiration was significantly decreased at 12 months of age. Importantly, mitochondrial bioenergetic deficits were found to precede the development of AD pathology in these mice. In a follow-up study, the 3xTg-AD mice were analyzed by 2D-DIGE, a quantitative proteomic profiling method [73]. Proteins that were dysregulated in 3xTg-AD cortices functioned in a wide variety of metabolic pathways, including the TCA cycle, oxidative phosphorylation, pyruvate metabolism, glycolysis, oxidative stress, fatty acid oxidation, ketone body metabolism, ion transport, apoptosis, and mitochondrial protein synthesis. These alterations in the mitochondrial proteome of the cerebral cortices of 3xTg-AD mice occurred well before the development of significant Aβ plaques and NFTs, supporting the notion that mitochondrial dysregulation is an early event in AD pathogenesis.

4. TAU impairs mitochondrial transport

Mitochondria can move in both anterograde and retrograde directions in one axon [74]. Early studies in wild-type TAU overexpressing mice using pulse-chase experiments had already revealed that TAU mediates impaired anterograde transport [75]. In K369I mutant TAU transgenic K3 mice, it was then demonstrated that elevated TAU impairs transport of distinct cargoes including mitochondria, both in the nigrostriatal pathway and in the sciatic nerve [28]. More specifically, by ligating the sciatic nerve proteins whose transport was impaired

could be discriminated from those, whose was not, indicating selectively impaired axonal transport. It was found that complex V accumulated proximally and distally of the ligation in wild-type nerves, representing bidirectional transport of mitochondria. In ligated transgenic nerves, however, complex V accumulated only in the distal part, suggesting impaired anterograde and unaffected retrograde transports of mitochondria in K3 mice (Fig. 1). As an underlying pathomechanism, trapping of the kinesin adaptor molecule JIP1 by phosphorylated forms of TAU in the soma was identified. This trapping prevented JIP1 from loading distinct cargoes (including mitochondria) onto the kinesin machinery for transport down the axon. Relocalization of JIP1 from the axon to the soma was also found in the AD brain underscoring the validity of the finding in the transgenic model [76]. Another pathomechanism was identified in the squid axon, where filamentous, but not soluble, forms of wild-type TAU were found to inhibit anterograde transport by activating axonal protein phosphatase 1 (PP1) and glycogen synthase kinase 3 (GSK3), independent of microtubule binding [77]. In a related study, increased expression of GSK3β and the p25 activator of cyclin dependent kinase 5 (cdk5) in neurons was shown to cause an increased pausing of mitochondria rather than changes to their velocities [78]. Competition for binding to kinesin has been suggested by co-immunoprecipitation experiments: The data indicate that TAU being a cargo of kinesin itself may displace other kinesin-based cargo, including cytoskeletal proteins and organelles such as mitochondria [79]. Also in *C. elegans*, perturbed axonal transport of mitochondria was reported when so-called pro-aggregant tau was expressed which causes TAU aggregation [80].

While TAU is often treated as if it were one protein, it is in fact several proteins. Differential effects of three-repeat (3R) and four-repeat (4R) TAU on mitochondrial axonal transport have been reported [81]. As 3R TAU is believed to be less tightly associated with microtubules than 4R TAU [82,83], it was postulated that 4R TAU may lead to greater alterations of organelle transport than 3R TAU. Indeed, while both 3R and 4R TAU changed the normal mitochondrial distribution within the cell body and reduced mitochondrial localization to axons, the effects of 4R TAU were more pronounced. Furthermore, 3R and 4R TAU caused different alterations in retrograde and anterograde transport dynamics; however, 3R TAU had a slightly stronger effect on axon transport dynamics. TAU over-expression in general increased the net movement of axonal mitochondria towards the neuronal cell body [81]. Multiple studies have shown that Aβ, the second key player in AD, impairs mitochondrial transport [84], while a recent study using oligomeric Aβ did not find changes to mitochondrial motility [85]. However, reducing TAU levels prevents Aβ toxicity as discussed below, and more specifically the defects in axonal transport induced by Aβ in APP mutant mice [86].

More recently, RNAi-mediated knockdown of *Milton* or *Miro*, which encodes adaptor proteins essential for axonal transport of mitochondria, in human TAU transgenic flies was found to enhance the TAU-induced neurodegeneration [87] (Fig. 1). Phosphorylation of TAU at the 12E8 phospho-epitope Ser262 was increased when Milton or Miro was reduced. Partitioning defective-1 (PAR-1), the *Drosophila* homolog of mammalian microtubule affinity-regulating kinase (MARK) mediated this increase. Mutagenesis studies suggested that increased phosphorylation of the 12E8 epitope through PAR-1 contributes to TAU-mediated neurodegeneration in a pathological context when axonal mitochondria are depleted. Mitochondrial movement in the neuritic processes of PC12 cells was inhibited when another phospho-epitope of TAU, AT8 (comprised of three sites, Ser199, Ser202, and Thr205) was changed to phosphomimetic aspartates [88]. These mutations also caused an expansion of the space between microtubules in cultured cells when membrane tension was reduced by disrupting actin filaments. Thus, the authors concluded that TAU phosphorylation at the AT8 sites may affect mitochondrial movement by controlling microtubule spacing [88]. In human embryonic stem cell-derived neural stem cells the consequences of an overexpression of the longest human tau isoform, 2N4R tau versus pseudohyperphosphorylated tau (p-tau) was studied. Interestingly, p-tau, but not 2N4R tau, readily leads to TAU aggregation and impaired mitochondrial transport in human neurons. Although these alterations did not induce cell death, p-tau-expressing neurons cultured under non-redox-protected conditions underwent a pronounced degeneration with the formation of axonal varicosities sequestering transported proteins and progressive neuronal cell death [89]. That tau can impair axonal transport of mitochondria in the absence of hyperphosphorylation has been shown in P301L tau knock-in mice that did not develop a TAU pathology. In fact, the overall phosphorylation of tau in these mice was reduced (e.g., at epitopes PHF-1 or AT270), perhaps due to a reduced microtubule binding [90]. The impact of impaired transport on TAU pathology has also been studied. In mice lacking the kinesin light chain 1 (KLC1) subunit of the anterograde motor kinesin-1, this caused an axonopathy, with dystrophic axons exhibiting abnormal tau hyperphosphorylation and accumulation [91]. Together these studies illustrate that phosphorylated forms of TAU alter mitochondrial transport.

5. TAU impairs mitochondrial dynamics

Mitochondria differ remarkably from each other in size depending on cell-type and subcellular compartment [92,93]. Because axons and dendrites have differential energy demands, the mitochondrial network is generally more elongated in the cell body and dendrites of a neuron, and more fragmented in the axon [94,95] (Fig. 1). Moreover, a distinction is made between nonsynaptic and synaptic mitochondria [96–98]. To meet the specific subcellular demands, the mitochondrial network is shaped by a set of proteins that regulates fusion and fission [99]. In mammalian cells three large GTPases govern fusion: Mitofusins 1 and 2 (MFN1 and MFN2) dimerize on the outer membranes of adjacent mitochondria to induce outer membrane fusion. This is followed by fusion of the inner membranes, a process mediated by OPA1 (optic atrophy 1) that resides in the intermembrane space [100,101]. Fission is under control of yet another GTPase, DRP1 (dynamin-related protein 1, also known as DLP1 and DNM1) [102]. A set of additional proteins, including FIS1, mitochondrial fission factor (MFF), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51), have a role in recruiting and assembling DRP1 at the outer membrane [103]. Fission and fusion need to be balanced. For example, knockdown of *Drp1* leads to mitochondrial elongation, respiratory dysfunction and, ultimately, apoptosis [104], whereas elevated mitochondrial fusion is also a stress response in certain situations, enhancing ATP production and resistance to apoptosis [105], as reviewed recently [99].

Not surprisingly, an impaired balance of fission and fusion has been reported in AD, both at the transcript and protein levels [106,107]. Reduced levels of DRP1 and increased mitochondrial length were found in one study [108], whereas another revealed reduced cytoplasmic levels but (although not statistically significant) increased mitochondrial levels of DRP1, indicative of increased fission [106,109]. This and other recent studies suggest some degree of variability in the regulation of mitochondrial dynamics. For a conclusive picture it will be crucial to analyze the subcellular localization and post-translational modifications of DRP1, rather than global changes.

Mitochondrial dynamics has also been studied in mouse models of AD. Here, a significant body of data is available for the role of Aβ, which overall suggests that its net effect is towards increased fragmentation [106,108,110,111]. However, as discussed above, in addition to Aβ, TAU also forms aggregates in the AD brain and it would not be surprising if the observed differences in the impairment of complex activities would also extend to differences in the rates of fission and fusion. While hyperphosphorylation of TAU is believed to be a precipitating event in disease, recent data suggest that carboxy-terminal cleavage of TAU, which impairs mitochondrial function may also be critical [112,113]. Full-length TAU induced an increase in mitochondrial size, whereas truncated TAU induced mitochondrial fragmentation [114]. In neuroblastoma cells, P301L TAU impaired mitochondrial motility, together with a down-regulation of both fission and fusion [115]. When

combined with Aβ, truncated TAU impaired mitochondrial transport, enhanced oxidative stress, and caused a depletion of the mitochondrial membrane potential in cortical neurons. These effects were either modest or absent when Aβ was combined with full-length TAU, suggesting a specific synergistic cooperation of cleaved TAU with Aβ in disrupting mitochondria [112,113]. An altered distribution of mitochondria, without a change in size, was found in transgenic mice with high levels of P301L mutant TAU expression [116]. In these mice, mitochondrial distribution was progressively disrupted with age, particularly in somata and neurites that contained TAU aggregates. Apparently, the effects of TAU on mitochondria, both independently and in cooperation with Aβ, vary widely between TAU species, although the reasons for these divergent effects remain to be determined.

New light on TAU's role in mitochondrial dynamics was shed by a complementary study in TAU transgenic worms and flies. It has previously been found that TAU induces the stabilization and bundling of filamentous (F)-actin [117]. Because localization of the fission protein DRP1 to mitochondria is an actin-dependent process, whereby DRP1 and mitochondria (via myosin II) must interact with filamentous (F)-actin prior to their colocalization, increased F-actin in human TAU transgenic mice and flies disrupts the physical association of mitochondria and DRP1, leading to mitochondrial elongation [118,119] (Fig. 1). This causes neurotoxicity that can be rescued by reducing mitochondrial fusion, enhancing fission, or reversing actin stabilization. The study further found that elongation is not a secondary effect of impaired axonal transport [118]. Despite those new highlights on the role of TAU in the impairment of mitochondrial dynamics, there is no clear evidence of its role in mitochondrial turnover. In fact, as mentioned above, fusion/fission activity plays an important role in mitochondrial quality control. It allows the exchange of materials such as lipids, proteins, metabolites and mtDNA throughout the mitochondrial network, avoiding energetic deficiencies. However, when mitochondria are extensively damaged, they exit the fusion/fission cycle and are selectively eliminated by mitophagy (Fig. 1). This process occurs when mitochondria are in a fragmented state and when the mitochondrial membrane is depolarized after stress [120]. Mitochondria are degraded by engulfment into autophagosomes, which fuse with lysosomes and break down the organelles. In the case where the process of mitophagy is disturbed, a decreased cellular respiration has been observed, parallel to an accumulation of oxidized proteins [33]. Nothing seems to be known about the effect of TAU on mitochondrial turn-over but since new evidence shows that TAU may lead to mitochondrial elongation (fused state), we can speculate that it might decrease the elimination of damaged mitochondria via the process of mitophagy (which requires mitochondrial fragmentation). However, because AD is characterized by both TAU and Aβ pathology, future studies into mitochondrial dynamics/mitophagy need to take both molecules into consideration. More specifically, it will be necessary to firstly analyze synaptosomal as well as total mitochondria, and secondly, human tissue that represents the full spectrum of TAU and/or Aβ pathology, to dissect effects of Aβ on mitochondria dynamics from those of TAU.

6. TAU mediates excitotoxicity

TAU affects mitochondrial dysfunction also because of its crucial role in mediating excitotoxicity, a pathomechanism that has been implicated in AD [121]. Under basal conditions, mild activation of the NMDA receptor (NMDAR) results in physiological ROS production, while under neurodegenerative conditions triggered by Aβ, over-activation of NMDARs causes excessive calcium influx, nitric oxide (NO) activation, mitochondrial depolarization and superoxide formation that result in neuronal damage and death [122–124]. Aβ is believed to exert excitotoxicity either directly or indirectly, by over-activating the NMDAR [125]. NO exerts the majority of its effects by reacting with a cysteine thiol on target proteins, a process termed S-nitrosylation. This modifies enzymes with a role in glycolysis, gluconeogenesis and oxidative phosphorylation, indicating that this type of posttranslational modification may regulate metabolism and mitochondrial bioenergetics [126]. In a recent study, inhibition of Drp1 was found to prevent excitotoxic cell death in a hippocampal cell culture system [127]. Calcium influx also stimulates kinases, causing TAU to detach from microtubules and relocalize to the somatodendritic domain, where it aberrantly interacts with proteins including JIP1, thereby impairing mitochondrial transport [28].

Is there a more direct role for TAU in excitotoxicity? In a pathocascade, Aβ has been placed upstream of TAU [128]. This concept has been proven in P301L mutant TAU transgenic mice that develop an increased number of NFTs, either by crossing them with Aβ plaque-forming transgenic mice [129], or by intracerebral injections of Aβ [130]. While Aβ causes TAU aggregation, its toxicity is also dependent on TAU as has been first shown in vitro [131] and subsequently in vivo [132]. Removing TAU largely abrogates the pathological features that characterize Aβ plaque-forming mice, namely premature mortality, high susceptibility to experimentally induced excitotoxic seizures and memory deficits [132]. Mechanistically, this protection appeared to be conferred by a reduced susceptibility to excitotoxicity either when TAU was absent or when its levels were reduced [132,133]. Even under physiological conditions, TAU was found to be present in the dendrite (although at low quantities compared with the axon), where it is critically involved in postsynaptic NMDAR downstream signalling by localizing the SRC kinase FYN to the dendrite. FYN phosphorylates the NMDAR that then recruits the postsynaptic scaffolding protein PSD-95 to form a complex [133]. The TAU axis hypothesis claims that as TAU accumulates in a phosphorylated form in the dendrite, it mediates the toxic effects of Aβ by causing increased concentrations of FYN which is then available to phosphorylate the excitotoxic NMDAR signalling complex [134]. Aβ, Fyn and TAU therefore seem to orchestrate neuronal damage [133,135,136]. It has been shown that synaptic NMDAR signalling and extrasynaptic NMDAR signalling have opposite effects on cell survival and that differentially located NMDARs are coupled to different intracellular cascades. A recent study found that Aβ induces dendritic spine loss via a pathway involving synaptic NR2A-containing NMDARs whereas activation of extrasynaptic NR2B-containing NMDARs is required for neurodegeneration that is TAU-dependent [137]. Together this suggests that manipulating components of the NMDAR or the interaction of TAU and FYN may be therapeutically beneficial. In fact, disrupting the complex between NMDAR and PSD-95 pharmacologically was found to protect Aβ plaque-forming mice from premature death, memory impairment and the susceptibility to excitotoxic seizures [133]. In conclusion, TAU affects mitochondrial dysfunction also because of its crucial role in mediating excitotoxicity, a pathomechanism that has been implicated in AD.

7. Integration — mitochondria are key targets of Aβ and TAU toxicity in AD

Mitochondria are key targets of Aβ and TAU toxicity in AD (Fig. 1). A picture is emerging whereby these two molecules damage mitochondria in multiple ways, by marching separately and striking together. While the causes of sporadic AD are not known it is evident that Aβ and TAU levels are elevated at an early stage. An impaired homeostasis because of increased Aβ production and decreased clearance causes increased levels, which acts on mitochondria by impairing complex IV function, and by facilitating the fragmentation of mitochondria. Both insults cause increases in ROS levels, decreased activities of detoxifying enzymes such as superoxide dismutase (SOD), and an impaired mitochondrial membrane potential that results in reduced ATP levels. Elevated Aβ levels also result in the overexcitation of neurons, which leads to an influx of calcium ions, with the consequence of increased COX levels, which then damages mitochondria. When Aβ levels are increased this also activates distinct TAU kinases and/or inactivates TAU phosphatases resulting in a massive hyperphosphorylation of TAU. Hyperphosphorylated TAU specifically impairs complex I of the

mitochondrial respiratory chain, again leading to increased ROS levels, lipid peroxidation, decreased activities of detoxifying enzymes such as superoxide dismutase (SOD), and an impaired mitochondrial membrane potential. It also impairs anterograde transport of mitochondria and other cargoes by trapping the kinesin adaptor molecule JIP1 in the neuronal cell body, preventing it from executing its normal function. In AD, hyperphosphorylated TAU not only accumulates in the axon, but also relocalizes and accumulates in the cell body and dendrites of affected neurons. In the dendritic compartment, it facilitates the toxic effects of Aβ that are mediated by the NMDAR. Elevated levels of TAU have also functional consequences on mitochondrial dynamics. TAU causes actin stress fibres to form. This blocks the proper execution of DRP1-mediated fission with a net result of mitochondrial elongation. This, similar to an augmented fragmentation caused by Aβ, causes increased ROS levels, decreased activities of detoxifying enzymes and an impaired mitochondrial membrane potential. In light of the apparently opposing effects Aβ has on mitochondrial size it seems that either too little or too much fission/fusion is detrimental for neurons. Obviously, with TAU impairing axonal transport of mitochondria, the calcium buffering requirements at the synapse are undermined. Together, TAU and Aβ establish a vicious cycle of misregulated dynamics and transport of the mitochondria, which together with alterations in mitochondrial components such as complex proteins or mtDNA, causes mitochondrial impairment in AD.

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Appendix 3

Advanced mitochondrial respiration assay for evaluation of mitochondrial dysfunction in Alzheimer's disease.

Grimm A, Schmitt K, Eckert A.

Systems Biology of Alzheimer's Disease: Methods and Protocols, edited by Walker JM, Humana Press, Springer publishing group (Submitted)

Advanced mitochondrial respiration assay for evaluation of mitochondrial dysfunction in Alzheimer's disease

Running head:
Mitochondrial dysfunction in Alzheimer's disease

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Abstract:

Alzheimer's disease (AD) is characterized by the presence of amyloid plaques (aggregates of amyloid-β [Aβ]) and neurofibrillary tangles (aggregates of tau) in the brain, but the underlying mechanisms of the disease are still partially unclear. A growing body of evidence supports mitochondrial dysfunction as a prominent and early, chronic oxidative stress-associated event that contributes to synaptic abnormalities, and, ultimately, selective neuronal degeneration in AD. Using a high-resolution respirometry system, we shed new light on the close interrelationship of this organelle with Aβ and tau in the pathogenic process underlying AD by showing a synergistic effect of these two hallmark proteins on the oxidative phosphorylation capacity of mitochondria isolated from the brain of transgenic AD mice.

In the present chapter, we first introduce the principle of the Aβ and tau interaction on mitochondrial respiration, and secondly, we describe in detail the used respiratory protocol.

Key words: mitochondria, Alzheimer's disease, amyloid-β, tau, Oxygraph, oxidative phosphorylation.

1. Introduction

With the increasing average life span of humans, Alzheimer's disease (AD) is the most common neurodegenerative disorder among elderly individuals. It accounts for up to 80% of all dementia cases and ranks as the fourth leading cause of death amongst those above 65 years of age ***(1)***. Although the hallmark lesions of the disease were already described by Alois Alzheimer in 1906 - amyloid-β (Aβ) containing plaques and microtubule-associated protein tau-containing neurofibrillary tangles (NFTs) –, the underlying molecular mechanisms that cause the formation of these end-stage lesions are still poorly understood. However, a growing body of evidence supports mitochondrial dysfunction as a prominent and early chronic oxidative stress-associated event that contributes to synaptic abnormalities and, ultimately, selective neuronal degeneration in AD ***(2; 3)***. Within the last few years, several cell culture models as well as single, double, and more recently, triple transgenic mouse models have been developed to reproduce diverse aspects of AD. These models help in understanding the pathogenic mechanisms that lead to mitochondrial failure in AD, and in particular the interplay of AD-related cellular modifications within this process ***(4)***.

In this chapter, we will highlight 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. Particularly, we will emphasise on the recent insights 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).

1.1 Aβ and tau induce mitochondrial toxicity

Mitochondria play a pivotal role in cell survival and death by regulating both energy metabolism and apoptotic pathways. They are the "powerhouses of cells" providing energy via ATP generation which is accomplished through oxidative phosphorylation (OXPHOS) from nutritional sources ***(5)*** [Fig 1]. Neurons have particularly high numbers of mitochondria which are especially enriched in synapses. Due to the limited glycolytic capacity of neurons, those cells are highly dependent on mitochondrial function for energy production ***(6)***. Thus, deregulation of mitochondrial function leads to synaptic stress, disruption of synaptic transmission, apoptosis and ultimately, neurodegeneration ***(7; 8)***.

Evidences from cellular and animal AD models indicate that Aβ triggers mitochondrial dysfunction through a number of pathways such as impairment of OXPHOS, elevation of ROS production, interaction with mitochondrial proteins, and alteration of mitochondrial dynamics ***(9; 10)***. Success in developing mouse models that mimic diverse facets of the disease process has greatly facilitated the understanding of pathophysiological mechanisms

underlying AD. In 1995, Games and collaborators established the first amyloid precursor protein (APP) mouse model (called PDAPP) bearing the human ‘‘Indiana’’ mutation of the APP gene (V171F). They observed the accumulation of Aβ in the brain and subsequent amyloid plaque formation, as well as astrocytosis and neuritic dystrophy ***(4)***. Interestingly, in most of the APP mouse models, the cognitive impairment begins concomitantly with Aβ oligomer formation in the brain (around 6 months of age), while neuritic amyloid deposits become visible only between 12 and 23 months and the amount of deposits increases in parallel ***(11)***. Thus, memory deficits seem to correlate directly with the accumulation of intracellular Aβ oligomers and not with amyloid plaque formation. When those mice were crossed with those bearing a mutation in presenilin 1 gene (PS1), coding for a gene involved in APP processing, an earlier onset of amyloid plaques was observed, alongside a stronger decrease of mitochondrial membrane potential as well as ATP level ***(12)***.

Mitochondrial dysfunctions occur at a very early disease stage in AD transgenic mouse models. For example, in the APPsw transgenic strain Tg2576 (Swedish mutation), an upregulation of genes related to mitochondrial energy metabolism and apoptosis was observed already at 2 months of age. Alterations in composition of the mitochondrial respiratory chain complexes I and III protein subunit as well as impairment of mitochondrial respiration were detected around 6 months, when soluble Aβ accumulated in the brain without plaque formation ***(13; 14)***.

Consistent with this observation, in APPsw / presenilin 2 (PS2) double-transgenic mice, mitochondrial impairment was first detected at 8 months of age, before amyloid plaque deposition, but after soluble Aβ accumulation ***(15)***. Taken together, these findings are consistent with the recently proposed hypothesis of an age-related Aβ toxicity cascade that suggests that the most toxic Aβ species that cause majority of molecular and biochemical abnormalities are in fact intracellular soluble oligomeric aggregates rather than the extracellular, insoluble plaques ***(16)***.

How does tau, the second hallmark lesion in AD, interfere with mitochondrial function? In its abnormally hyperphosphorylated form, which forms the NFTs, tau has been shown to block mitochondrial transport. This results in energy deprivation and oxidative stress at the synapse, and, consequently, neurodegeneration ***(17; 18)***. Untill now, no mutations in microtubule-associated protein tau (MAPT) coding genes have been detected in relation to familial forms of AD. However, in familial frontotemporal dementia (FTD) with parkinsonism, mutations in the microtubule-associated protein tau gene (MAPT) were identified on chromosome 17. This was the basis for creating a robust mouse model for tau pathology in 2001. These P301L tau–expressing pR5 mice show an accumulation of tau as soon as 3 months of age and develop NFTs around 6 months of age ***(19)***. A mass spectrometric analysis of the brain proteins from these mice (aged from 8.5-10 months)

revealed mainly a deregulation of mitochondrial respiratory chain complex components (including complex V), antioxidant enzymes, and synaptic protein space ***(20)***. The reduction in mitochondrial complex V levels in the P301L tau mice was also confirmed in human P301L FTDP-17 (FTD with parkinsonism linked to chromosome 17) brains. The functional analysis demonstrated age-related mitochondrial dysfunction, together with reduced NADH ubiquinone oxidoreductase (complex I) activity as well as age-related impaired mitochondrial respiration and ATP synthesis in a pR5 mouse model. Mitochondrial dysfunction was also associated with higher levels of ROS in aged transgenic mice. Increased tau pathology resulted in modification of lipid peroxidation levels and the upregulation of antioxidant enzymes in response to oxidative stress ***(20)***. Thus, this evidence demonstrated for the first time that not only Aβ but also tau pathology weakens gradually mitochondrial function in a rather specific way leading to metabolic impairment and oxidative stress in AD.

1.2 Synergistic mode of action of Aβ and Tau

Although Aβ and tau pathologies are both known hallmarks of AD, the mechanisms underlying the interplay between plaques and NFTs (or Aβ and tau, respectively) have remained unclear. However, a close relationship between mitochondrial impairment and Aβ on the one hand and tau on the other hand has been already established. How do both AD features relate to each other? Several studies suggest that Aβ aggregates and hyperphosphorylated tau may block the mitochondrial transport to the synapse leading to energy deficiency and neurodegeneration ***(21)***.

Remarkably, intracerebral Aβ injections amplify a pre-existing tau pathology in several transgenic mouse models ***(22; 23)***, whereas lack of tau abrogates Aβ toxicity ***(24; 18)***. Our findings indicate that in tau transgenic pR5 mice, mitochondria display an enhanced vulnerability toward Aβ insult in vitro ***(25; 2)***, suggesting a synergistic action of tau and Aβ pathology on this organelle. Thus, these studies provide the first evidence for the existence of a complex interplay between Aβ and Tau in AD whereby these two molecules damage mitochondria in multiple ways, but what about their specific effects on mitochondrial respiration?

1.3 Using high-resolution respirometry system in isolated mitochondria to evaluate OXPHOS capacity.

To address this question, we used a high-resolution respiratory system to evaluate the capacity of the entire oxidative phosphorylation system (OXPHOS) of cerebral mitochondria from mice bearing either an APP/PS2 mutation, P301L mutation (pR5 mice), or the triple

mutation APP/PS2/P301L (^{triple}AD mice) compared to wild-type mice ***(26)***. Measurement of oxygen (O_2) flux and consumption was performed at 37 °C using an Oroboros Oxygraph-2k system on freshly isolated mitochondria from cortical brains of age-matched wild-type, APP/PS2, pR5 and ^{triple}AD mice as follows. After detection of endogenous respiration, glutamate and malate were added to induce state 4 respiration [Fig 1 and 2A], then ADP was added to stimulate state 3 respiration. After determining coupled respiration, FCCP was added and the maximal respiratory capacity measured in the absence of a proton gradient. Cytochrome c (cyt c) injection was used to demonstrate mitochondrial membrane integrity. To inhibit activities of complexes I–III, rotenone (rot) and antimycine A (AA) were added. Complex IV activity was stimulated by ascorbate/TMPD (A/T) before terminating mitochondrial respiration by adding sodium azide (azide).O_2 consumption was normalised to the corresponding citrate synthase activity.

We determined flux control ratios to obtain information on metabolic states of respiration. The respiratory control ratio (RCR3/4) is an indicator of the state of coupling of mitochondria. State 3 is the rate of phosphorylating respiration in the presence of exogenous ADP, and state 4 is associated with proton leakage across the inner mitochondrial membrane in the absence of ADP. Our findings suggest a pronounced decrease of RCR3/4 in mitochondria from APP/PS2 and ^{triple}AD compared with age-matched wild-type mice already at 8 months of age. This decrease was also found in the oldest mice (12 months of age). When we examined the ETS/ROX (electron transport system/residual oxygen consumption) ratio, which yields an index of the maximum oxygen consumption capacity relative to the magnitude of residual oxygen consumption, we found that it was also decreased in APP/PS2 and ^{triple}AD compared with age-matched wild-type mice at 8 and 12 months of age. Interestingly, in a previous study, the decreased respiration of mitochondria from pR5 mice compared with wild-type controls was not visible before the age of 24 months ***(20)***. In contrast, APP/PS2 mitochondria showed a decrease in OXPHOS compared with wild-type already at the age of 8 months. At this age, OXPHOS of brain mitochondria from ^{triple}AD mice did not differ compared with that of age-matched APP/PS2 mitochondria , but it was significantly decreased in ^{triple}AD mice at the age of 12 months [Fig 2B]. Taken together, with increasing age, the global failure of the mitochondrial respiratory capacity deteriorated the strongest in mitochondria from ^{triple}AD mice, suggesting a synergistic destructive effect of tau and Aβ on mitochondria.

In conclusion, our studies highlight the key role of mitochondria in AD pathogenesis and the close interrelationship of this organelle and the two main pathological features of the disease. We showed that disturbances in the respiratory and energy system of ^{triple}AD mice seem to be due to a convergence of Aβ and tau on mitochondria, accelerating defects in

respiratory capacity, which consolidates the idea that a synergistic effect of tau and Aβ increase the pathological deterioration of mitochondria.

Now we will describe in detail the protocol which we followed previously ***(26)***. After listing the material needed, we will describe the isolation of mitochondria from mouse brains and the steps required to measure the mitochondrial respiration. It is important to note that this protocol assumes that the Oroboros Oxygraph-2k system is routinely used in the laboratory and does not include technical details about oxygraph maintenance or calibration, but only experimental procedure regarding the assessment of mitochondrial respiration.

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25°C).

2.1 Solutions for the Isolated Mitochondria Preparation

1. Medium 1: NaCl 138 mM, KCl 5.4 mM, Na_2HPO_4 0.17 mM, KH_2PO_4 0.22 mM, glucose·H_2O 5.5 mM, saccharose 58.4 mM, pH 7.35. To prepare 1 litre of medium 1, weigh 8 g NaCl, 0.4 g KCl, 0.024 g Na_2HPO_4, 0.03 g KH_2PO_4, 1.1 g glucose·H_2O, 20 g saccharose. Add water to a volume of 900 ml (using a graduated cylinder) and mix with magnetic stir bar at room temperature until all powders are dissolved. Adjust pH and make up to 1 l with water. Store at 4 ˚C.

2. Isolated Mitochondria Buffer : mannitol 210 mM, saccharose (70 mM), HEPES 10 mM, EDTA (tritriplex III) 1 mM, BSA 0.45 %, pH 7.4. To prepare 200 ml buffer, weigh 7.65 g mannitol, 4.79 g saccharose, 477 mg HEPES, 74.4 mg EDTA (tritriplex III) and 0.9 g BSA. Add water to a volume of 190 ml (using a graduated cylinder) and mix with magnetic stir bar at room temperature until all powders are dissolved. Adjust pH and make up to 200 ml with water. Prepare aliquots of 10 ml and keep at -20˚C (*see* **Note 1**).

2.2 Solutions for the Mitochondrial Respiration

1. Mitochondrial Respiration Buffer: saccharose 65mM, potassium dihydrogen phosphate 10mM, Tris HCl 10mM, $MgSO_4$. 7 H_2O 10mM, EDTA (tritriplex III). 2 H_2O 2 mM, pH 7. To prepare 200 ml, weigh 4.45 g saccharose, 0.272 g

potassium dihydrogen phosphate, 0.315 g Tris HCl, 0.493 g $MgSO_4$. 7 H_2O, 0.149 g EDTA (tritriplex III). 2 H_2O. Add water to a volume of about 190 ml (using a graduated cylinder) and mix with magnetic stir bar at room temperature until all powders are dissolved. Adjust pH and make up to 200 ml with water. Prepare aliquots of 20 ml and keep at -20˚C.

2. Mitochondrial Respiration Medium (MiR05): EGTA 0.5 mM, $MgCl_2.6H_2O$ 3 mM, K-lactobionate 60 mM, taurine 20 mM, KH_2P0_4 10 mM, HEPES 20 mM, saccharose 110 mM, BSA 1 g/l, pH = 7.1. Start to prepare the K-lactobionate stock solution (0.5 M) by dissolving 35.83 g lactobionic acid in 100 ml H_2O and adjust the pH to 7.0 with KOH and bring the volume to 200 ml. To prepare 1 litre of MiR05, weigh 0.190 g EGTA, 0.610 g $MgCl_2.6H_2O$, 2.502 g taurine, 1.361 g KH_2P0_4, 4.77 g HEPES, 37.65 g saccharose, 1 g BSA. Add about 750 ml water and 120 ml K-lactobionate stock solution (0.5 M). Mix with magnetic stir bar at room temperature, adjust the pH to 7.1 with KOH (5 N**),** and make up to 1 litre with water. Divide into 20 ml aliquots and store frozen at -20°C (see **Note 2**).

3. Substrates: The substrates employed and details of preparation are summarised in Table 1. (*see* **Notes 3-13**)

2.3 Oxygraph

Mitochondrial oxygen consumption was measured at 37 °C using an Oroboros Oxygraph-2k system following the Gnaiger method ***(27)***.

3. Method

3.1 Isolated mitochondria preparation

Before experiment: perform an instrumental and chemical background with the oxygraph. Prepare isolated mitochondria buffer (*see* **Note 1**) and keep on ice. Turn on the centrifuge (4°C).

1. Kill the mice by decapitation and dissect one brain hemisphere on ice. Wash in 10 ml of ice-cold medium 1.
2. Put the preparation in the Potter-tube to homogenise in 1 ml isolated mitochondria buffer. Pipette 10 to 15 times to homogenise the preparation (*see* **Note 14**).

3. Wash the Potter's plug 3 times with 150 ul isolated mitochondria buffer and put the preparation in a 2 ml tube. Wash the Potter's tube 3 times with 150 ul isolated mitochondria buffer and put the preparation in the same 2 ml tube. Vortex (*see* **Note 15**).
4. Centrifuge 7 min, 1450 rcf, at 4°C and recover the supernatant in a new 2 ml tube. This step removes nuclei and tissue particles.
5. Centrifuge 3 min, 1450 rcf, at 4°C and recover the supernatant again in a new 2 ml tube.
6. Centrifuge 5 min, 10 000 rcf, at 4°C, throw away the supernatant and recover the pellet.
7. Put the pellet (mitochondria) in 1ml isolated mitotochondria buffer and mix 15 times using the pipette.
8. Repeat step 7 and 8 to obtain the mitochondrial fraction and put the pellet in 100 ul isolated mitotochondria buffer. Keep on ice until the measurement (*see* **Note 16**).

3.2 Mitochondrial respiration measurement

Before experiment prepare the substrates (Table 1) and the oxygraph (*see* **Note 17**)

1. Add 50 ul isolated mitochondria preparation to each chamber and close the chamber (see **Note 18)**. Mark it as (01-state 1).
2. Add 10 ul glutamate / 5 ul malate (stock concentration = 2 M / 0.8 M respectively, assay concentration = 10 mM / 2 mM). Mark it as (02-GM2).
3. Add 8 ul ADP / chamber (stock concentration = 0.5M, assay concentration = 2 mM). Mark it as (03-GM3).
4. Add 2.5 ul FCCP / chamber (stock concentration = 0.32 mM, assay concentration = 0.4 uM). Mark it as (04-GP3u).
5. Add 5 ul Cytochrome c / chamber (stock concentration = 4 mM, assay concentration = 10 uM). Mark it as (05-GM3c).
6. Add 5 ul rotenone / chamber (stock concentration = 0.2 mM, assay concentration = 0.5 uM). Mark it as (06-rot).
7. Add 5 ul antimycine A / chamber (stock concentration = 1 mM, assay concentration = 2.5 uM). Mark it as (07-AA).
8. Add 5 ul ascorbate / chamber (stock concentration = 0.8 M, assay concentration = 2 mM) and 5 ul TMPD / chamber (stock concentration = 0.2 M, assay concentration = 0.5 mM). Mark it as (08-AT).

9. Add 20 ul sodium azide / chamber (stock concentration = 1 M, assay concentration = 10 mM). Mark it as (09-azide).

4. Analysis

After the measurement, extract the raw data from the oxygraph software (DatLab) to an Excel file. Normalise the data on citrate synthase activity, which correlates with mitochondrial content **(see Note 19)**. Perform the statistical analysis using GraphPad Prim software and a two-way ANOVA followed by Bonferroni post hoc tests to compare the different groups. Consider statistically significant only P values < 0.05. Represent data as means ± SEM.

5. Notes

1. Just before starting the experiment, warm up the isolated mitochondria buffer. For 2 brain hemispheres, add 1 tablet of Complete^{R} Mini (protease inhibitor cocktail tablet) and 5 ul dithiothréitol (DTT: Stock solution = 1M in water, Final concentration in the buffer = 0.5mM) to 10 ml buffer. Prepare maximum 3h before use.
2. The MiR05 is stable for about 2-3 months. The K-lactobionate must be prepared fresh.
3. Solution stored at low temperature: mix carefully after rewarming, since phase separation may occur and compounds may precipitate in cold solutions. During the course of the experiment keep stock solutions on ice. Solutions which contain ethanol may have a problem of evaporation and subsequent increase of concentration of stock solutions.
4. Adjust pH to 7.0 with 37% HCl and divide into 0.5 ml aliquots.Store frozen at -20˚C.
5. Neutralize with 10N KOH and divide into 0.5 ml aliquots. Store frozen at -20˚C.
6. Neutralize (pH 7.0) with 5 N KOH and divide into 100 ul aliquots. Store at -80˚C.
7. Divide into 0.2 ml aliquots. Store frozen at -20˚C.
8. To prevent autooxidation, prepare a solution of ascorbic acid (137.6 mg/ml, pH ca 2). Adjust the pH to ca 6 of the solution containing the ascorbate sodium salt with ascorbic acid. Divide into 0.2 ml aliquots. Store frozen at -20˚C. Light sensitive!
9. To prevent autooxidation, neutralize with the solution containing 0.8 M of ascorbic salt (dilution 1:80, final concentration ascorbate: 10mM). Divide into 0.2 ml aliquots. Store frozen at -20˚C.
10. Difficult to dissolve, divide into 0.2 ml aliquots, store at -20˚C. Light sensitive! Very toxic!
11. Divide into 0.2 ml aliquots, store at -20˚C. Very toxic!

12. Divide into 0.2 ml aliquots, store frozen at -20˚C. Very toxic!
13. Divide into 0.5 ml aliquots, store frozen at -20˚C.
14. Pipette gently up and down to avoid bubble formation and strong oxygenation of the sample.
15. If you have several mice, stop the process at this step, put the preparation on ice and use the next mouse to perform the centrifugation steps with all the samples at the same time. Since the Oxygraph contains 2 chambers, it is possible to investigate the mitochondrial respiration for only a few animals per day (6-8 mice / day).
16. 50 ul of the preparation will be used for the Oxygraph measurement. For protein determination, dilute 3ul of isolated mitochondria in PBS (dilution 1:5) and perform the protein assay (here, we use the Biorad DC™ Protein Assay and the bovine serum albumin (BSA) for the standard curve).
17. The experiment requires an instrumental and chemical background following the protocol of the company. Calibration will determined the “air saturation” (R1) and the “zero saturation” (R0).
18. When the oxygraph chambers are closed, check that there are no air bubbles left inside.
19. Citrate synthase is an enzyme of the tricarboxylic acid cycle. Its activity is frequently used to normalise other mitochondrial enzymatic activities and mitochondrial respiration because it correlates to mitochondrial content. To measure citrate synthase activity, follow the reduction of 5, 5’-dithiobis (2-nitrobenzoic acid) (DTNB) by citrate synthase at 412 nm (extinction coefficient of 13.6 $mM^{-1} \cdot cm^{-1}$) in a coupled reaction with coenzyme A (CoA) and oxaloacetate. Briefly, incubate a reaction mixture of 0.2 M Tris-HCl (pH 8.0), 0.1 mM acetyl-CoA, 0.1 mM DTNB, n-dodecyl-β-D-maltoside (20%), and 10 ug of mitochondrial protein at 30 °C for 5 min. Initiate the reaction by the addition of 0.5 mM oxaloacetate and monitor the absorbance change for 5 min with a Shimadzu MultiSpec-1501 diode array spectrophotometer. Calculate the enzymatic activity of citrate synthase using the slope of absorption by minute.

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Table 1: Preparation and function of the substrates used to investigate mitochondrial respiration using the Oroboros Oxygraph-2k system.

		Name / Formula	MW (g/mol)	Stock solution		Function	Note #
Mitochondrial substrates	glutamate	L-glutanic acide $C_5H_8NO_4Na$	187.1	2 M	3.742 g/10 ml H_2O	Induce state 4 respiration	4
	malate	L-malic acid $C_4H_6O_5$	134.1	0.8 M	1.073 g/10 ml H_2O	Induce state 4 respiration	5
	ADP	adenosine 5'-diphosphate $C_{10}H_{15}N_5O_{10}P_2K$	491.2	0.5 M	0.491 g/2 ml H_2O	Induce state 3 respiration	6
	cytC	cytochrome C	12500	4 mM	50 mg/1 ml H_2O	Demonstrate mitochondrial membrane integrity	7
	ascorbate	ascorbate sodium salt $C_6H_7O_6Na$	198.1	0.8 M	1.584 g/10 ml H_2O	Stimulates complex IV activity	8
	TMPD	N,N,N',N'-tetramethyl-p-phenylenediaminedichloride $C_{10}H_{16}N_2 \cdot HCl$	237.2	0.2 M	47.4 mg/1 ml H_2O	Stimulates complex IV activity	9
Mitochondrial inhibitors	rotenone	$C_{23}H_{22}O_6$	394.4	1 mM	3.94 mg/10 ml Ethanol	Inhibits complex I activity	10
	AA	antimycin A	540	5 mM	11 mg/4 ml Ethanol	Inhibits complex III activity	11
	azide	sodium azide NaN_3	65.01	1 M	65 mg/1 ml H_2O	Inhibits oxygen consumption	12
Mitochondrial uncouplers	FCCP	carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone $C_{10}H_5F_3N_4O$	254.2	0.32 mM	2.54 mg/10 ml Ethanol to have 1mM. Dilute 1: 3.125 in Ethanol	Determine uncoupled respiration in absence of a proton gradient.	13

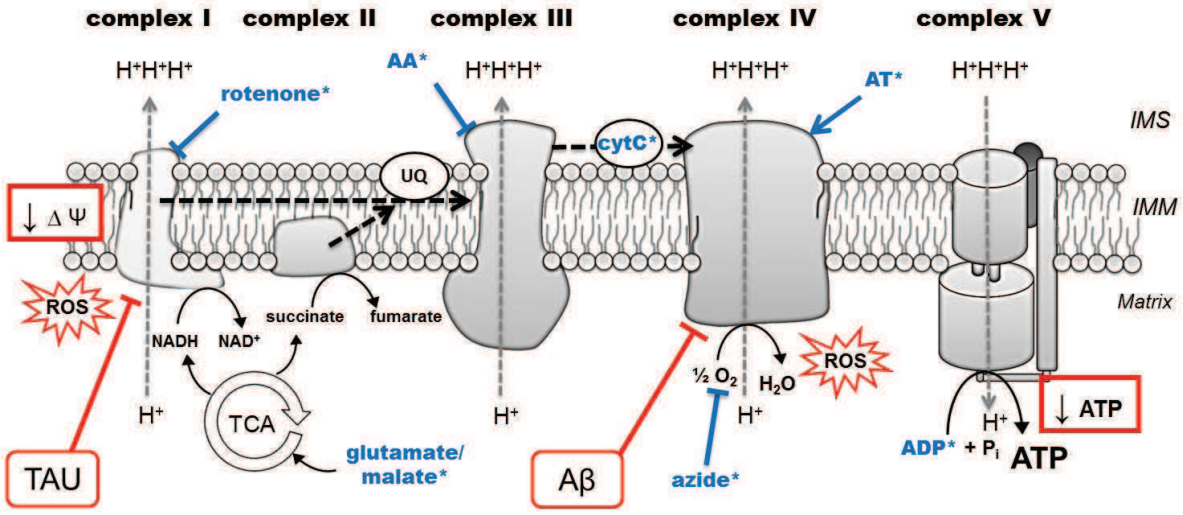

Fig. 1: The mitochondrial electron transport chain: impact of Aβ peptide, tau protein, and effects of mitochondrial substrates used during the measurement protocol with Oxygraph. Complexes I (NADH: ubiquinone oxidoreductase) and II (succinate dehydrogenase, belongs to the tricarboxylic acid (TCA) cycle) receive electrons from NADH and $FADH_2$, respectively. Electrons are then driven from complexes by the mobile carrier molecules coenzyme Q/ubiquinone (UQ) and cytochrome c (Cyt c) to the final acceptor, molecular oxygen (O2). Electron flow is coupled to proton movement across the inner mitochondrial membrane (IMM) in complexes I, III and IV. The resulting proton gradient is harvested by complex V to generate ATP. In Alzheimer's disease, abnormal mitochondrial electron activities have been observed, predominantly in complexes I and IV, leading to impaired mitochondrial membrane potential, decreased production of ATP (complex V), and enhanced reactive oxygen species (ROS) levels. Interestingly, deregulation of complex I is mainly tau-dependent, while deregulation of complex IV is amyloid-β (Aβ)-dependent, at both the protein and activity level. The targets of the different substrates used during the Oxygraph measurement are marked with * on the figure and their specific actions are summarized in Table 1. AA, antimycin A; AT, ascorbate/ TMPD; IMS, intermembrane space.

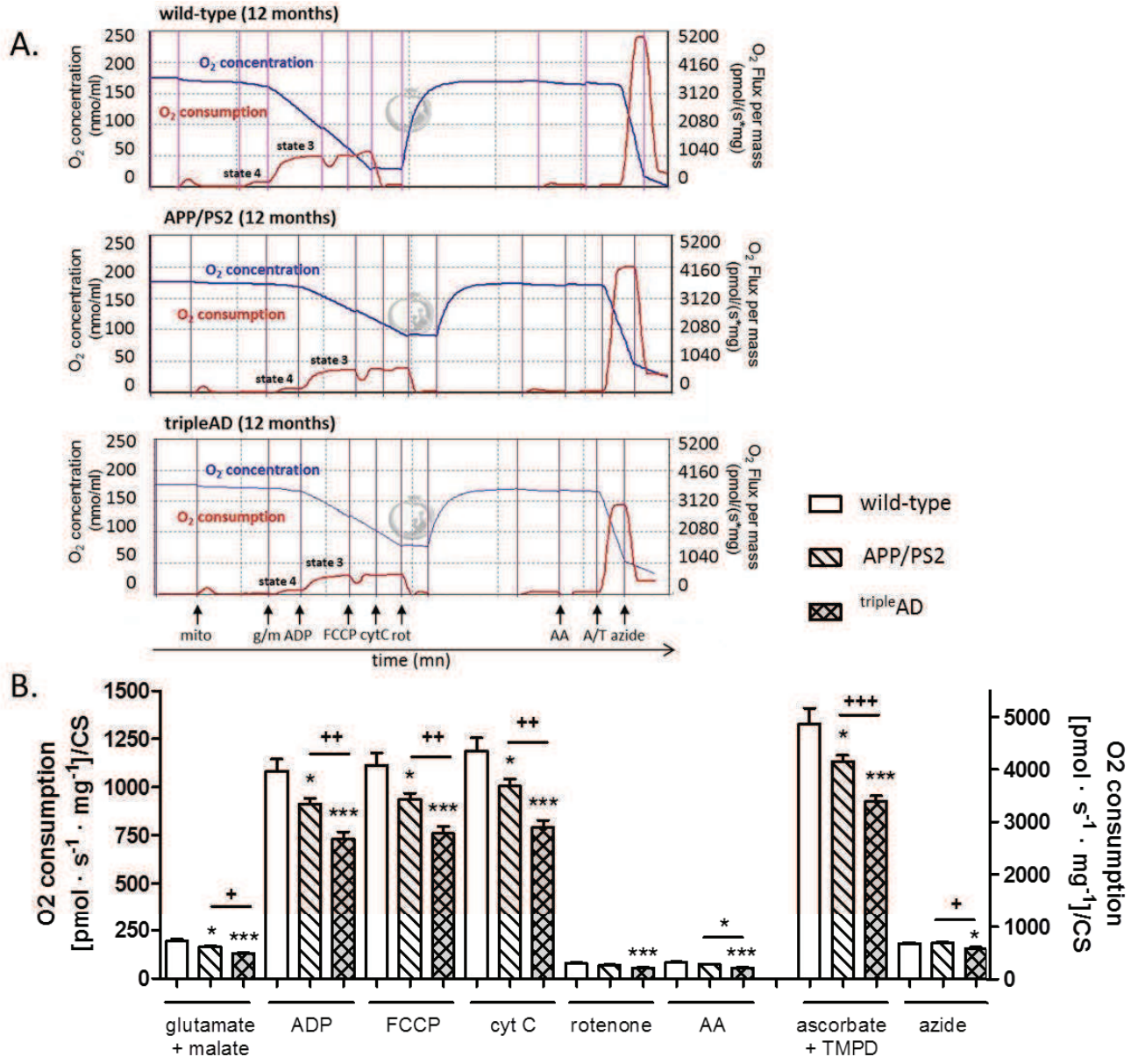

Fig. 2: Synergistic effects of Aβ and Tau on mitochondrial respiration. (A) Representative diagrams of O_2 flux and consumption in mitochondria from 12-month-old wild-type, APP/PS2, and ^{triple}AD mice in response to titrated substrates and inhibitors of mitochondrial complexes. (B) Two-way ANOVA revealed a significant effect of the transgene on the respiratory rates of mitochondria between 12-month-old wild-type and APP/PS2 mice and this impaired respiration was even more pronounced in ^{triple}AD mice. Two-way ANOVA post-hoc Bonferroni. *, P<0.05; **, P<0.01; ***, P<0.001 vs. wild-type; $^{+}$, P<0.05; $^{++}$; P<0.01; $^{+++}$; P<0.001 vs. APP/PS2 (n=7–12 animals/group). Modified from Rhein et al., PNAS 2009 ***(26)*** with permission.

Appendix 4

Alzheimer's Disease, Oestrogen and Mitochondria: An Ambiguous Relationship.

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Alzheimer's Disease, Oestrogen and Mitochondria: an Ambiguous Relationship

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Abstract Hormonal deficit in post-menopausal women has been proposed to be one risk factor in Alzheimer's disease (AD) since two thirds of AD patients are women. However, large treatment trials showed negative effects of long-term treatment with oestrogens in older women. Thus, oestrogen treatment after menopause is still under debate, and several hypotheses trying to explain the failure in outcome are under discussion. Concurrently, it was shown that amyloid-beta (Aβ) peptide, the main constituent of senile plaques, as well as abnormally hyperphosphorylated tau protein, the main component of neurofibrillary tangles, can modulate the level of neurosteroids which notably represent neuroactive steroids synthetized within the nervous system, independently of peripheral endocrine glands. In this review, we summarize the role of neurosteroids especially that of oestrogen in AD and discuss their potentially neuroprotective effects with specific regard to the role of oestrogens on the maintenance and function of mitochondria, important organelles which are highly vulnerable to Aβ- and tau-induced toxicity. We also discuss the role of Aβ-binding alcohol dehydrogenase (ABAD), a mitochondrial enzyme able to bind Aβ peptide thereby modifying mitochondrial function as well as oestradiol levels suggesting possible modes of interaction between the three, and the potential therapeutic implication of inhibiting Aβ–ABAD interaction.

Keywords Alzheimer's disease · Neurosteroids · Oestrogen · Mitochondria · ABAD

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Introduction

Steroid hormones are molecules, mainly produced by endocrine glands such as the adrenal gland, gonads and placenta, involved in the control of many physiological processes mainly in the periphery, from reproductive behaviour to stress response. In 1981, Baulieu and co-workers were the first to demonstrate steroid production within the nervous system itself [1]. They showed that the level of some steroids, such as dehydroepiandrosterone (DHEA), was even four times higher in the anterior brain of rats than in plasma and nearly 18 times higher than in the posterior brain with regard to its sulphated form (DHEAS). Of note, the level of this steroid remained elevated in the brain even after adrenalectomy and castration. In the following decades, other steroids were identified to be synthetized in situ in the brain, and enzymatic activities of proteins involved in steroidogenesis have been shown in many regions of the central and peripheral nervous system, in neurons as well as in glial cells [2–5]. Thus, this category of molecules is now called "neurosteroids" and defines neuroactive steroids that are synthetized within the nervous system, independently of peripheral endocrine glands. While steroid hormones act at a distance from their glands of origin in an endocrine way, neurosteroids are synthetized by the nervous system and act on the nervous system in an auto/paracrine configuration.

Because of their lipophilic nature, peripheral steroid hormones can freely cross cell membranes, including the blood–brain barrier, and play an important role in the development, maturation and differentiation of the central and peripheral nervous system. However, since some steroids are also synthetized within the nervous system, their blood levels do not necessarily correspond to their brain concentrations [6]. Intra-cerebral steroid synthesis seems to play a role in cognition, anxiety, depression, neuroprotection and even nociception [7].

The ability to cross cellular membranes allows them to act on nuclear receptors exhibiting genomic action by regulating gene transcription. This action seems to be important during neonatal life where it has been shown that neurosteroids, such as progesterone (PROG) or oestradiol, are able to promote dendritic growth, spinogenesis, synaptogenesis and cell survival, particularly in the cerebellum [5]. Some studies already demonstrated the role of neurosteroids, particularly oestrogens, in the regulation of glucose homeostasis and lipid metabolism [8] as well as in neuroprotection [9]. Risk for Alzheimer's disease (AD) is associated with age-related loss of sex steroid hormones in both women and men [10, 11]. On the one hand, in postmenopausal women, the precipitous depletion of oestrogens and progestogens is hypothesized to increase susceptibility to AD pathogenesis, a concept largely supported by epidemiological evidence but refuted by some clinical findings, above all, by results from the "Woman's health initiative memory study" (WHIMS) (please see detailed discussion in the "Conclusion" section). On the other hand, a growing body of evidence indicates a more gradual age-related decline in testosterone in men similarly associated with increased risk to several diseases including AD. Since testosterone is at least in part aromatized in the brain to 17β-oestradiol, a loss of it may also affect oestrogen-mediated neuroprotective pathways. But also, the difference between how rapidly and significantly the female versus male primary sex hormones decline might partially contribute to higher AD incidences in women than in men [10].

Alzheimer's Disease, Oxidative Stress, Effect of Gender and Neogenesis of Neurosteroids

AD is a neurodegenerative brain disorder and the most common form of dementia among the elderly as shown by the worldwide prevalence of the disease which was 26.6 million people in 2006 [12]. Clinical symptoms are characterized by severe and progressive loss of memory, language skills as well as spatial and temporal orientation. From a cellular point of view, the pathological hallmark of AD is the presence of extracellular senile plaques—composed of aggregated amyloid-β peptide (Aβ)—and intracellular neurofibrillary tangles (NFT)—consisting of aggregates of abnormally hyperphosphorylated tau protein. A lot of efforts have been made during the last years to understand the pathogenesis of the disease, particularly the role of AD key proteins, Aβ and tau, in oxidative stress and mitochondrial dysfunction [13].

Epidemiological and observational studies demonstrated a higher prevalence and incidence of AD in women even after adjusting for age—about two thirds of AD patients are female—as well as a greater vulnerability to the disease [14]. Thus, at early stages of neurofibrillary tangle development, women exhibit greater senile plaque deposition than men [15], and AD pathology is more strongly associated with clinical dementia in female patients than in male [16]. The drop of oestrogen levels after menopause was proposed to be one explanation to this phenomenon. However, there is little information concerning changes of steroid levels in the human brain during ageing and under dementia conditions. As steroids present in nervous tissues originate from the endocrine glands (steroid hormones) and from local synthesis (neurosteroids), changes in blood levels of steroids with age do not necessarily reflect changes in their brain levels. The concentrations of a range of neurosteroids have recently been measured in various brain regions of aged AD patients and aged non-demented controls including both genders by the very sensitive GC/MS methods [6]. Schumacher and colleagues showed a general trend towards lower level of steroids including oestrogen in AD patients compared to controls. Notably, neurosteroid levels were negatively correlated with Aβ and phospho-tau in some brain regions [6]. Another study using radioimmunoassay for steroid quantification demonstrated a decrease in oestrogen level in post-mortem brain from female AD patients aged 80 years and older but no significant difference in the 60–79-year age range compared to non-demented women [17]. However in men, an age-dependent decrease of androgen level was observed in the brain of non-demented subjects, which was even more pronounced in the brain of male AD patients [17]. Whereas large studies investigating systematically gender differences with respect to Aβ and or tau pathology in post-mortem brain tissue from AD patients are missing, broad evidence emerged from transgenic mice models of AD indicating an increased Aβ load burden and plaque number in the female brain compared to age-matched male mouse brain [11, 18]. Of note, consistent findings on greater Aβ burden in females were found in different animal AD models: Tg2576 (APPSWE) mice [19], APP/PS1 [20], APP23 [21], as well as in triple transgenic mice, like 3xTg-AD mice [18, 22] and ^{triple}AD mice ([23], with respect to gender differences: unpublished observations). On the basis that the estrous cycle in female mice is constantly repeated until approximately 11 months of age and becomes irregular between 12 and 14 months, the data demonstrating

a significant enhancement of Aβ load in important brain regions like the hippocampus from the female after the age of 11 months are striking. Regarding tau pathology, no gender differences have been observed in the latter triple AD models. In agreement, NFT formation in Aβ-injected tau transgenic mice (P301L) did not vary with gender [24]. Even though one single publication reported an enhanced neurofibrillary pathology in female TAPP mice [25], all together, these results point to the involvement of the Aβ pathway, rather than the tau pathway, in the higher risk of AD in women.

Interestingly, further supporting evidence comes from oxidative stress studies. Previous research of our group [26] demonstrated a gender-specific partial up-regulation of antioxidant defence in post-mortem brain regions from female compared to male AD patients further indicating that oxidative damage is caused rather by overproduction from reactive oxygen species (ROS) than by insufficient detoxification of ROS. Since mitochondria represent the major source of ROS, the findings from Lloret and co-workers are of specific interest showing that brain mitochondria from old female rats produce higher levels of ROS after exposure to Aβ than age-matched brain mitochondria from male rats [27].

A selection of studies attested neuroprotective effects of neurosteroids against AD-related cellular and mitochondrial injury, but the underlying mechanisms are still poorly understood.

Findings of our group corroborated that AD key proteins and oxidative stress are themselves able to modify neogenesis of neurosteroids in a cellular AD model [28, 29] (Fig. 1). In fact, treatment of human SH-SY5Y neuroblastoma cells with H_2O_2 for 24 or 48 h led to a decrease of oestradiol synthesis. This was paralleled by an increased cell death compared to untreated controls and a down-regulation of the expression of aromatase, an enzyme responsible for oestradiol formation from testosterone. Interestingly, cell death was also observed after inhibition of aromatase by treatment with letrozole, suggesting that endogenous oestradiol formation plays a critical role in cell survival. Furthermore, if cells were pre-treated with oestradiol, it was possible to protect them against H_2O_2 and letrozole-induced cell death. In agreement, a similar protective effect of oestradiol was observed in stress condition experiments treating the same cell line with heavy metals, such as cobalt and mercury [30].

In addition, modulation of neurosteroid production was observed in SH-SY5Y cells overexpressing the human amyloid processor protein (APP) or human tau protein [28]. Indeed, overexpression of human wild-type Tau (hTau 40) protein induced an increase in the production of PROG, 3α-androstanediol and 17-hydroxyprogesterone, in contrast to overexpression of the abnormally hyperphosphorylated tau bearing the P301L mutation which led to a decrease in the production of these neurosteroids. In parallel, a decrease of PROG and 17-hydroxyprogesterone production was observed in cells expressing human wild-type APP (wtAPP), whereas 3α-androstanediol and oestradiol levels were increased. These results provided first evidence that AD key proteins are able to modulate, directly or indirectly, the biological activity of the enzymatic machinery producing neurosteroids. These findings were further confirmed by in vitro experiments using native SH-SY5Y cells treated with aggregated $A\beta_{1\text{-}42}$ peptide for 24 h [31]. Since APPwt SH-

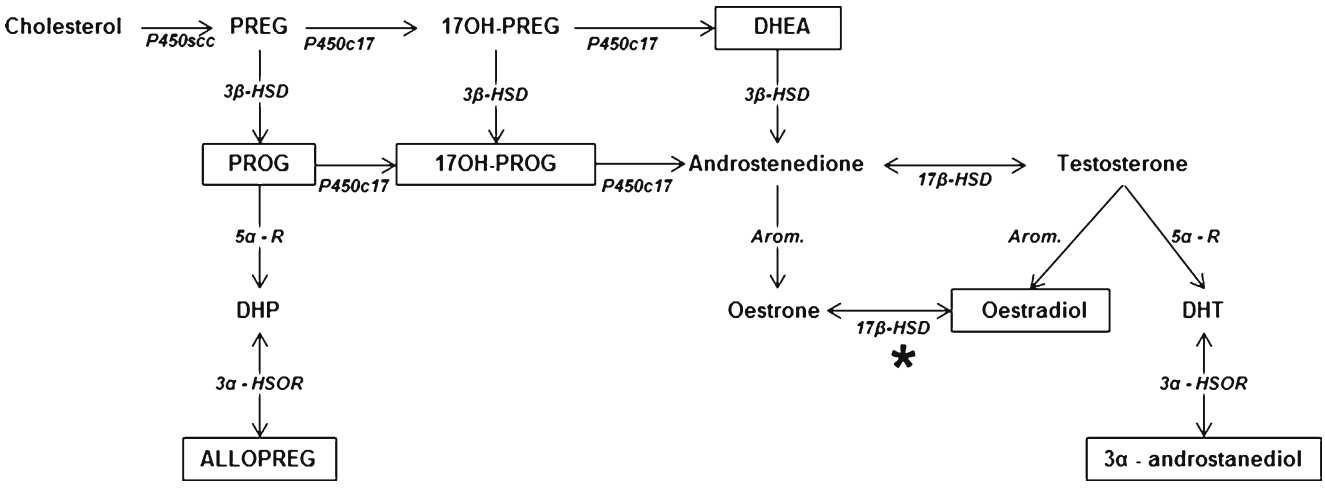

Fig. 1 Main biochemical pathways for neurosteroidogenesis in the vertebrate brain. *Boxes* represent neurosteroids which are sensitive to modulation by AD key proteins, Aβ and/or tau. Mitochondrial 17β-HSD (marked by *) is equivalent to the ABAD in mitochondria. *PREG* pregnenolone, *PROG* progesterone, *17OH-PREG* 17-hydroxypregnenolone, *17OH-PROG* 17-hydroxyprogesterone, *DHEA* dehydroepiandrosterone, *DHP* dihydroprogesterone, *ALLOPREG* allopregnanolone, *DHT* dihydrotestosterone, *P450scc* cytochrome P450 cholesterol side chain cleavage, *P450c17* cytochrome P450c17, *3β-HSD* 3β-hydroxysteroid dehydrogenase, *5α-R* 5α-reductase, *Arom.* aromatase, *21-OHase* 21-hydroxylase, *3α-HSOR* 3α-hydroxysteroid oxydoreductase, *17β-HSD* 17β-hydroxysteroid dehydrogenase

SY5Y cells secrete Aβ levels within nanomolar concentration range, treatment of native SH-SY5Y cells using a "non-toxic" concentration range (100–1,000 nM, non-cell death-inducing $A\beta_{1\text{-}42}$ concentrations) revealed an increase in oestradiol production, whereas toxic $A\beta_{1\text{–}42}$ concentrations within the micromolar range, leading to cell death, strongly reduced oestradiol levels.

Modulation of steroid production was also shown in other cell lines, for example in oligodendrocytes, where DHEA production is up-regulated under oxidative stress condition induced by treatment with Aβ peptide or Fe^{2+} [32]. Interestingly, similar results were found in Alzheimer patients where DHEA was significantly elevated in brain and cerebrospinal fluid when compared to control subjects [33]. Finally, several reports propose the role of allopregnanolone (3α, 5α-THP) as a plasmatic biomarker for AD, since it was shown that the level of this neurosteroid is decreased by 25 % in the plasma of demented patients compared with control subjects [34, 35].

The fact that the ability to produce neurosteroids is conserved in the vertebrates' evolution suggests that this category of molecules is important for living beings. Thus, we could speculate that the modulation of their biosynthesis plays an important role in the pathophysiology of neurodegenerative disorders, such as AD.

Neurosteroids, Especially Oestrogens, and Neuroprotection in AD

Evidence of Neuroprotective Action of Steroids in Cellular and Animal Studies

Neuroprotective effects of neurosteroids against a variety of brain injuries have already been described for many years. Numerous studies with the focus on oestrogens showed that these molecules are able to enhance cerebral blood flow, prevent atrophy of cholinergic neurons, and modulate the effects of trophic factors in the brain [36]. Oestrogens are a group of compounds known for their importance in the estrous cycle including oestrone (E1), oestradiol (E2), and oestriol (E3). Oestradiol is about ten times as potent as oestrone and about 80 times as potent as oestriol in its oestrogenic effect. Oestradiol is also present in males, being produced as an active metabolic product of testosterone. The serum levels of oestradiol in males (14–55 pg/mL) are roughly comparable to those of post-menopausal women (<35 pg/mL). Oestradiol in vivo is interconvertible with oestrone, oestradiol to oestrone conversion being favoured; however, evidence of metabolism is mainly derived from the periphery.

Animal studies, especially in rodents and transgenic mice models for AD, seem to confirm positive effects of oestrogen treatment. It has been shown that a treatment with oestrogen in mice expressing mutations in human APP (Swedish and Indiana mutation) had an impact on APP processing decreasing Aβ levels and so its aggregation into plaques [37]. Mechanisms underlying this action of oestrogen are still poorly understood, but as discussed by Pike et al. [11], it seems that oestrogen amongst others is able to promote the α-secretase pathway (non-amyloidogenic, meaning non-Aβ producing) via activation of extracellular-regulated kinase 1 and 2 (ERK 1 and 2) and through the protein kinase C (PKC) signalling pathway.

In triple transgenic AD mice, depletion in sex steroid hormones induced by ovariectomy in adult females increased significantly Aβ accumulation and had a negative impact on cognitive performance [18, 38]. Treatment of these ovariectomized mice with oestrogens was able to prevent these effects vice versa. Of note, when PROG was administrated in combination with oestrogens, the beneficial effects on Aβ accumulation were blocked but not on cognitive performance. However, oestrogen and PROG both can modulate kinase and phosphatase activity involved in tau phosphorylation, especially the glycogen synthase kinase-3β (GSK-3β). Thus, oestrogen can induce the phosphorylation of GSK-3β which inactivates the enzyme and reduces tau phosphorylation, whereas PROG can decrease the expression of tau and GSK-3β [11, 39]. This suggests that oestrogen and PROG not only can interact to regulate APP processing and tau phosphorylation but can also act independently on different AD pathways.

Cognitive effects of PROG were confirmed in mice bearing the Swedish double mutation of APP and mutant preseniline 1 (APPswe+PSEN1Δ9 mutant mice) which showed decreased hippocampally mediated cognitive performances compared to non-transgenic littermates [38]. In this AD mouse model, PROG was able to improve the cognitive performance in tasks involving the cortex but not in those involving the hippocampus. Besides, APPswe+PSEN1Δ9 mice presented decreased 3α, 5α-THP levels (metabolite of PROG) in the hippocampus, compared to wild-type mice, suggesting that deficits in hippocampal function may be due, at least in part, to reduced capacity to form 3α, 5α-THP in the hippocampus. Furthermore, a more recent study supported the role of 3α, 5α-THP in triple transgenic mice model of AD (3xTgAD) by showing reduced Aβ generation in the hippocampus, cortex and amygdala, coupled with an increased cellular regeneration after treatment with 3α, 5α-THP [40].

At the cellular level, oestrogen binds to nuclear receptors, such as oestrogen receptor α and β (ER α/β), and acts as transcription factor. It enhanced the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, and down-regulated the expression of Bim, a pro-apoptotic factor, preventing the initialisation of cell death programme by

mitochondria [11, 41]. Another way that oestrogen can protect cells from apoptosis is the activation of antioxidant defence systems by up-regulating the expression of manganese superoxide dismutase (MnSOD) and glutathione peroxidase [42]. Thus, oestrogen can have direct antioxidant effects by increasing reduced glutathione levels and decreasing oxidative DNA damage in mitochondria, as observed in a study using ovariectomized female rats [43]. Of note, oestrogen can also modulate the redox state of cells by intervening with several signalling pathways, such as mitogen-activated protein kinase (MAPK), G protein-regulated signalling, NFκB, c-fos, CREB, phosphatidylinositol-3-kinase, PKC and Ca^{2+} influx [41, 44]. On the basis of this complex mode of action, oestrogens not only seem to be able to decrease oxidative stress markers, including lipid peroxidation, protein oxidation and DNA damage, but can also directly act on the regulation of mitochondrial function [42].

Neurosteroids and Mitochondria: Focus on Potential Protective Effects of Oestrogen Against Aβ-Induced Toxicity

Mitochondria are the "powerhouses of the cell", providing the main part of cellular energy via ATP generation, which is accomplished through oxidative phosphorylation from nutritional sources [45]. They control cell survival and death by regulating both energy metabolism and apoptotic pathways and contribute to many cellular functions, including intracellular calcium homeostasis, alteration of the cellular reduction–oxidation potential, cell cycle regulation and synaptic plasticity [46]. Mitochondrial dysfunction has been proposed as an underlying mechanism in the early stages of AD [47, 48]. We recently summarized evidence from ageing and Alzheimer models showing that the harmful trio "ageing, Aβ and tau protein" triggers mitochondrial dysfunction through a number of pathways, such as impairment of oxidative phosphorylation, elevation of reactive oxygen species production and interaction with mitochondrial proteins, contributing to the development and progression of the disease [13, 49].

Mitochondria and neurosteroidogenesis are also closely linked since mitochondria contain the first enzyme involved in steroidogenesis, the cytochrome P450 cholesterol side chain cleavage enzyme (P450scc) located at the inner side of the mitochondrial membrane which is responsible for the conversion of cholesterol to pregnenolone (PREG). The first step of neurosteroidogenesis is the transfer of cholesterol from the outer to the inner mitochondrial membrane. It is also the rate-limiting step in the production of neurosteroids because the ability of cholesterol to enter into mitochondria to be available to the P450scc will determine the efficiency of steroidogenesis [50]. Free cholesterol accumulates outside of mitochondria and binds to the steroidogenic acute regulatory protein, a hormone-induced mitochondria-targeted protein that initiates cholesterol transfer into mitochondria. Then, molecules are transported inside mitochondria by a protein complex including translocator proteins (TSPO), a cholesterol-binding mitochondrial protein also known under the name of peripheral-type benzodiazepine receptor, which permits cholesterol transfer into mitochondria and subsequent steroid formation.

It has been shown that TSPO is up-regulated in the post-mortem brain of AD patients, resulting in an increased level of PREG in the hippocampal region of those brains [50]. Interestingly, the level of 22R-hydroxycholesterol, a steroid intermediate in the conversion of cholesterol to PREG, was found at lower levels in the AD brain compared to the control, which suggests that TSPO does not function normally in Alzheimer patients [33, 51].

From an energetic point of view, it is known that steroids such as oestrogen can regulate mitochondrial metabolism by increasing the expression of glucose transporter subunits and by regulating some enzymes involved in the tricarboxylic acid cycle (TCA cycle) as well as glycolysis, such as the hexokinase, phosphofructokinase, pyruvate and malate dehydrogenase [41, 52], which leads to improved glucose utilization by cells [11, 44] (Fig. 2).

Oestrogens seem to be able to up-regulate genes coding for some electron transport chain components present in nuclear and in mitochondrial DNA [53, 54]. In fact, an oestrogen-induced increased expression of some subunits of mitochondrial complex I (CI), cytochrome c oxidase (complex IV or CIV) and F1 subunit of ATP synthase was observed [41, 42, 52]. Furthermore, treatment of ovariectomized female rats with oestradiol induced an increase of mitochondrial respiratory function in the brain with regard to an enhancement of O_2 consumption coupled to an increased activity of cytochrome c oxidase [53]. Thus, oestrogen seems to enhance the general metabolism in cells, but besides, it seems also able to directly protect mitochondria against oxidative stress-induced injury [52]. Thus, incubation of isolated mitochondria from the rat brain with oestradiol leads to a decrease of H_2O_2 production by this organelle coupled with an increase of the mitochondrial membrane potential (MMP). Furthermore, it has been proposed that its phenolic A ring could allow oestradiol to intercalate into the mitochondrial membrane and to avoid lipid peroxidation occurring in stress condition [54], which could be responsible for the stabilization of the MMP. Moreover, oestradiol seems to prevent the release of cytochrome c by mitochondria (a mechanism known to induce apoptosis of cells by activating the caspase cascade in the cytoplasm), a mechanism increasing the efficiency of the respiratory chain [52].

Finally, another oestrogen signalling pathway avoiding the negative effects of oxidative stress is the one regulating

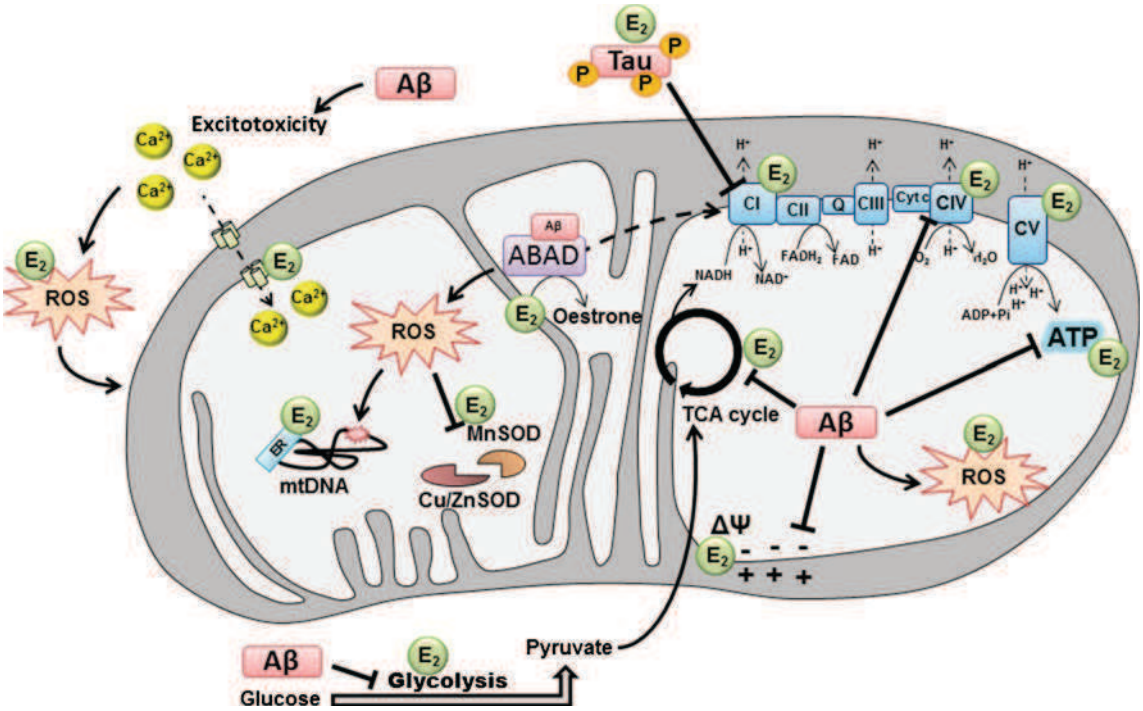

Fig. 2 Modulation of mitochondrial function by Aβ, hyperphosphorylated tau and oestradiol. In AD, mitochondrial dysfunction was found to be a central pathological mechanism which occurs already at early stages of the disease. On one hand, studies showed that amyloid-β peptide (Aβ) can be responsible of metabolic impairments, such as the decrease of glucose consumption observed in the AD brain as well as the calcium-induced excitotoxicity in neurons. It has been found that hyperphosphorylated tau and Aβ are able to impair mitochondrial respiration by inhibiting the ETC CI and CIV, respectively, inducing decreased oxygen consumption, decreased ATP production and increased ROS level. This oxidative stress induced by ETC dysfunction can surpass cellular and mitochondrial scavenger (MnSOD, Cu/ZnSOD) and impacts on MMP as well as mitochondrial DNA (mtDNA). On the other hand, it has been shown that oestradiol can increase glucose utilization by cells as well as ETC activity, stabilize the MMP and prevent ROS production and calcium-induced excitotoxicity. In the graph, E_2 designates where oestradiol potentially acts on mitochondria to compensate Aβ-induced toxicity. In turn, Aβ seems to be able to impact oestradiol metabolism in mitochondria, since it can be directly linked to the mitochondrial enzyme ABAD and possibly modulates its enzymatic activity (such as the reversible conversion of oestradiol to oestrone) and non-enzymatic activity (mitochondrial RNAse P). *ABAD* Aβ-binding alcohol dehydrogenase, *CI* complex I, *CII* complex II, *CIII* complex III, *CIV* complex IV, *CV* complex V, *cyt c* cytochrome c, *Cu/Zn SOD* copper/zinc superoxide dismutase, *MnSOD* manganese superoxide dismutase, *TCA* tricyclic acid, E_2 oestradiol, *ROS* reactive oxygen species, *mtDNA* mitochondrial DNA, *ER* oestrogen receptor

calcium homeostasis by inducing mitochondrial sequestration of cytosolic calcium [42, 54]. In fact, an imbalance of calcium regulation can lead to an increase of ROS production by activating the enzyme nitric oxide synthase, which can in turn sensitize neural cells to oxidative damage. It has been shown that an oestradiol treatment of primary hippocampal neurons was able to potentiate glutamatergic response via NMDA receptor which resulted in an increased influx of calcium in cells. This effect was coupled to an induction of mitochondrial sequestration of cytosolic calcium and an increase of the mitochondrial calcium load tolerability thereby avoiding calcium-induced excitotoxicity as well as promoting cell survival.

Taken together, all those different findings indicate that oestrogen might be able to compensate deficits and injuries that occur in AD, namely mitochondrial respiration impairments, enhanced ROS production, excitotoxicity and, more generally, metabolic deficits (Fig. 2). More recently, new light has been shed on a mitochondrial enzyme that is able to directly bind Aβ peptide and in which one of the main substrate is 17β-oestradiol [55]. This enzyme is known under the name of 17β-hydroxysteroid dehydrogenase type 10 (17β-HSD) or Aβ-binding alcohol dehydrogenase (ABAD).

ABAD, Oestradiol and Aβ-Induced Mitochondrial Impairment

ABAD belongs to the alcohol dehydrogenase family, and it is responsible for the reversible oxido/reduction of several substrates including linear alcohols and steroids, such as 17β-oestradiol, using NAD^+ as cofactor [56]. Under normal conditions (without Aβ), this enzyme plays a role in the regulation of metabolic homeostasis, and its overexpression improved cell viability and ATP content [57]. It has been shown that ABAD is up-regulated in brains of AD mice as well as AD patients [57, 58], and it has been suggested that the binding of Aβ changes the conformation of the enzyme, which seems to exacerbate mitochondrial dysfunction induced by Aβ.

More recently, studies performed in transgenic mice models of AD showed that behavioural stress or depletion of ovarian hormones by ovariectomy exacerbated mitochondrial dysfunction, aggravated plaque pathology and increased ABAD expression in the brain [59, 60]. Furthermore, double transgenic mice overexpressing mutant APP and ABAD present an earlier onset of cognitive impairment and histopathological changes when compared to APP mice [49], suggesting that Aβ–ABAD interaction is an important mechanism underlying Aβ toxicity. This hypothesis is supported by a study from Yao and collaborators who recently showed that inhibition of Aβ–ABAD interaction by a decoy peptide can restore mitochondrial deficits (activity of mitochondrial respiratory complexes, ROS level) and improve neuronal and cognitive function [60].

New interesting findings of our group seem to go in the same way with regard to the use of a novel small ABAD-specific compound inhibitor (AG18051) by investigating the role of this enzyme in Aβ toxicity in human SH-SY5Y cells treated for 5 days with $A\beta_{1-42}$ 0.5 uM [61]. The crystal structure of human ABAD in presence of AG18051 showed that the inhibitor formed a covalent link with the NAD^+ cofactor and occupied the substrate-binding site of the enzyme [62]. Thus, the inhibitor was able to prevent Aβ-induced cell death and significantly normalized metabolic functions impaired by Aβ, such as cytosolic and mitochondrial ROS as well as mitochondrial respiration. Furthermore, it was able to restore oestradiol levels which were reduced after treatment with Aβ [31, 61]. What is interesting to note is that the apparent protective effects of the ABAD inhibitor seem to be independent on its interaction with Aβ. In fact, a 24-h pre-treatment with AG18051, before the incubation of cells with $A\beta_{1-42}$, was sufficient to prevent cell death, normalize ROS production and restore mitochondrial respiration. Regarding oestradiol level, we previously showed that it decreased in the cytosol and increased in isolated mitochondria of SH-SY5Y cells after 5 days of treatment with Aβ [49]. The ABAD inhibitor normalized the oestradiol level in the cytosol [61], and preliminary data of our group suggest a similar effect in isolated mitochondria (unpublished data). Thus, we propose the following model of mode of action: ABAD inhibitor is able to block Aβ toxicity by changing ABAD configuration, which disables the binding of Aβ thus preventing its toxic effects (Fig. 3). The action of ABAD on the electron transport chain (ETC) is still unclear, but the potential role of ABAD as mitochondrial RNAse P directly links ABAD to the production of mitochondrial ETC proteins and ROS generation [63]. Notably, AG18051 was able to normalize also this function of ABAD since mitochondrial respiration was restored, but the underlying mechanisms still remain unclear [61].

Thus, the interplay between ABAD, oestradiol and mitochondria may be a very interesting lead to follow in the future to decode Aβ-induced mitochondrial toxicity and explore therapeutic strategies of ABAD inhibition.

Conclusion

It is still debated whether oestrogen treatment after menopause could result in improved cognitive function in women. This debate is based on many animal and cell culture data showing that oestrogens can positively affect the ageing and AD brain. It was recognized from former studies that oestrogen depletion in post-menopausal women represents a significant risk factor for the development of AD and that an oestrogen replacement therapy may decrease this risk and even delay disease progression [64, 65]. However, large treatment trials showed negative effects of long-term

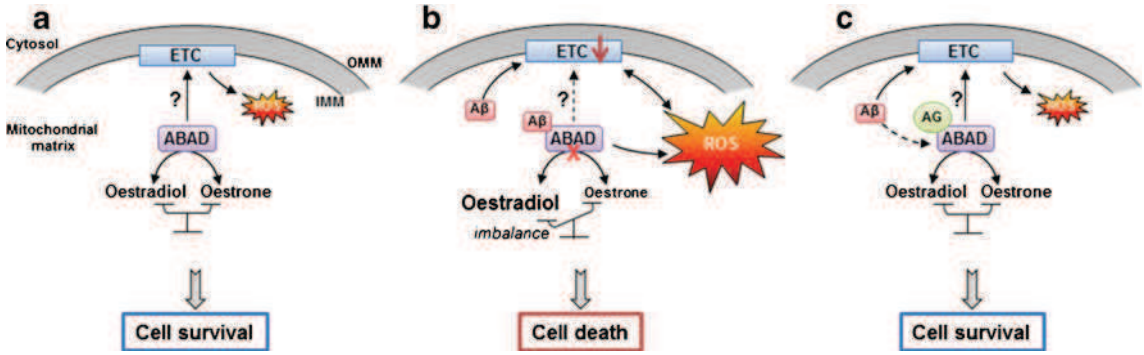

Fig. 3 Aβ, ABAD and mitochondria: modes of interactions. **a** Under normal conditions, ABAD is responsible of the reversible oxido/reduction of linear alcohols and steroids, such as the reversible conversion from oestradiol to oestrone. Its potential function as an RNAse P could also be important for the good functioning of the mitochondrial ETC. **b** Under AD-relevant pathological conditions, Aβ can directly bind the mitochondrial enzyme ABAD, changing the configuration of the enzyme which seems to inhibit its activity and creates an imbalance between oestradiol and oestrone. Aβ-induced ABAD misfolding can impact ETC functioning and increase, directly or indirectly, ROS production, which lead to cell death. **c** In the presence of AG18051 (AG), the binding of Aβ to ABAD is inhibited, normalizing oestradiol level, ROS production, ETC activity, and improves cell survival. *ABAD* Aβ-binding alcohol dehydrogenase, *IMM* inner mitochondrial membrane, *OMM* outer mitochondrial membrane

treatment with oestrogens in older women. Above all, results from the WHIMS including 4,532 post-menopausal woman aged over 68 years indicated a twofold increase in dementia after 4.2 years of hormonal treatment (p.o. treatment with premaxin plus medroxyprogesterone). In addition, the study indicated potential risks for breast cancer, pulmonary embolism and stroke [66, 67]. Some attribute this failure to the synthetic nature of the hormones used in the WHIMS trial, since in vitro studies support a beneficial role of oestradiol and progesterone, but not of medroxyprogesterone used in the WHIMS [68, 69]. Of note, medroxyprogesterone is not metabolized to 3α, 5α-THP and can inhibit conversion of PROG to 3α, 5α-THP [70]. Similarly, oestradiol, PROG or 3α, 5α-THP, but not medroxyprogesterone, showed beneficial effects in ageing, seizure, cortical contusion, ischaemia and diabetic neuropathy models [38]. Another theory which tries to explain trial failure is the "critical window hypothesis", asking for the critical period where oestrogen might exert a neuroprotective effect [71]. This hypothesis is substantiated by animal research, e.g. mice which have undergone ovariectomy, but in which oestrogen treatment was delayed substantially by months (the equivalent of years in human terms), did not benefit by this, as the animals did which received treatment immediately after ovariectomy [72]. However, a recent meta-analysis [73] indicated, contrary to expectations, that age of women and duration of time relapsed when treatment was initiated since menopause did not significantly affect treatment outcome. Thus, natural oestradiol (E2) without a progestagen should represent the preferred treatment [73]. Furthermore, the oral route of drug delivery, being non-invasive in nature, is by far the most convenient and preferred route of administration in any acute or chronic treatment. Though oestradiol itself from conventional oral oestradiol formulations has the ability to cross the blood–brain barrier (BBB) and reach the brain, but a large oral dose is required to achieve therapeutic levels of oestradiol due to its non-specificity for the brain. This non-specificity increases the peripheral drug burden and subsequently potentiates the risk of peripheral adverse effects. Furthermore, with specific regard to the brain-specific action of oestradiol as a neurosteroid, independently of its action in the periphery, other modes of administration (cyclical, nasal, polymer nanoparticles for oral delivery) need to be sought and investigated [74]. Alternatively, the true potential of phyto-oestrogens, like the soy isoflavones genistein, daidzein and glycitein, which activate the same neuroprotective pathways than oestrogens but with weak oestrogenic cellular effects that might be responsible for the lower prevalence of AD in Japanese living in their ethnic homeland compared to Japanese living in the USA [75], to beneficially modify disease processes should be studied in clinical trials [27]. In addition, the field could strongly benefit from the successful development of oestrogen derivates that have no unfavourable oestrogenic side effects. The successful use of oestrogen or oestrogen-analogue therapies to delay, prevent and/or treat AD will require additional research to optimize key parameters of therapy.

In this context, the interplay between ABAD, oestradiol and mitochondria and accordingly ABAD inhibition might represent a further interesting lead to follow in the future. Knowledge acquired from these studies will eventually be applied to unravel the pathophysiology and to inform prevention and intervention strategies of AD.

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

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Appendix 5

Neurosteroids in oxidative stress-mediated injury in Alzheimer disease.

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8

NEUROSTEROIDS IN OXIDATIVE STRESS-MEDIATED INJURY IN ALZHEIMER DISEASE

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8.1 INTRODUCTION

The brain is considered to be especially vulnerable to oxidative stress due to high levels of prooxidant factors and relatively low antioxidant defence. Putative prooxidant factors consist of a high metabolic rate, high levels of unsaturated fatty acids that readily undergo lipid peroxidation reactions, and relatively high levels of iron in some brain regions that facilitate hydroxyl radical formation from Fenton reactions [1]. Furthermore, neuronal activity results in high levels of intracellular calcium ions after depolarization that are linked to activation of phospholipase A_2, release of arachidonic acid, and subsequent formation of reactive oxygen species (ROS) from cyclooxygenase and lipoxygenase reactions (Fig. 8.1). Calcium ions also facilitate mitochondrial depolarization with release of mitochondrial factors that promote ROS formation. Furthermore, calcium ions are required for nitric oxide synthesis via endothelial and neuronal nitric oxide synthases (eNOS and nNOS). The brain contains relatively high levels of nitric oxide that can give rise to formation of highly reactive peroxynitrite. Also, catecholamine metabolism involves increased ROS formation: Superoxide can be generated from semiquinone formation, and hydrogen peroxide is released as a by-product of catecholamine synthesis by tyrosine hydroxylase and degradation by monoamine oxidases.

Despite these prooxidant factors, the brain possesses only relatively low levels of antioxidant defenses. Catalase activity is extremely low in brain tissue, and glutathione peroxidase as well as superoxide dismutase show low activity compared with other organs such as liver, heart, and kidney [2]. As a consequence, increased levels of ROS can be especially detrimental to brain tissue. Oxidative stress has accordingly been suggested to be a primary factor in the pathogenesis of several chronic neurodegenerative disorders, most prominently Alzheimer disease (AD), Parkinson disease (PD), and Huntington disease (HD).

Several studies, mainly in animals, suggest neurosteroid involvement in neuroprotection [3]. However in humans, the role of neurosteroidogenesis in the regulation of degenerative mechanisms is unknown. Since the process of neurosteroid biosynthesis is a pivotal mechanism intervening in the protection or viability of nerve cells, it might be regulated or significantly affected under oxidative stress conditions. However, the key factors interacting with neurosteroid biosynthesis under pathophysiological conditions are poorly understood. New findings demonstrate an amino acid sequence-dependent action of amyloid-β (Aβ) on neurosteroidogenic pathways [4]. The data also indicate that, unlike progesterone neosynthesis, regulation of endogenous estradiol formation by pathogenic factors may be a deciding process controlling cell death mechanisms. Targeting estradiol

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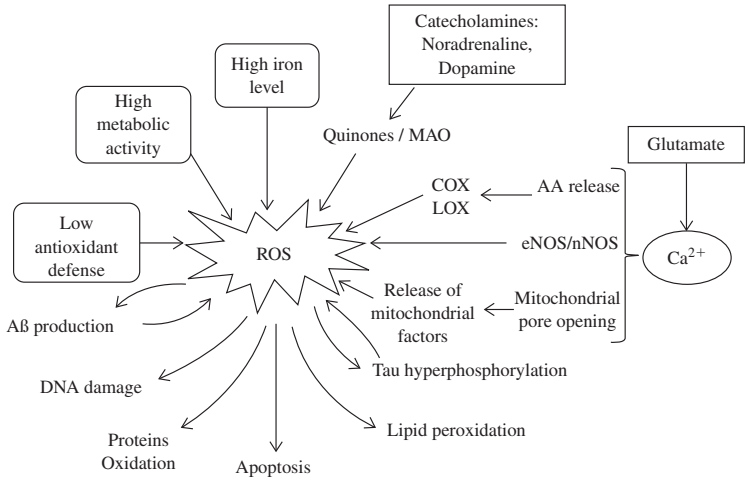

Fig. 8.1 Factors contributing to the accumulation of reactive oxygen species (ROS) in brain tissue and their implications in Alzheimer disease. See text for details. MAO, monoamine oxidase; COX, cyclooxygenase; LOX, lipoxygenase; AA, arachidonic acid; eNOS/nNOS, endothelial/neuronal nitric oxide synthase; Aβ, amyloid-β peptide

biosynthetic pathways in nerve cells may therefore be interesting in development of neuroprotective strategies.

8.2 EVIDENCE FOR A PATHOLOGICAL ROLE FOR OXIDATIVE STRESS IN AD

AD is a neurodegenerative brain disease and the most common form of dementia among the elderly. It is characterized by clinical symptoms of severe and progressive loss of memory, language skills, as well as spatial and temporal orientation. The pathology of AD is characterized by extracellular senile plaques, composed of aggregated amyloid-β peptide, and intracellular neurofibrillary tangles, consisting of aggregates of abnormally hyperphosphorylated Tau protein, and is accompanied by mitochondrial dysfunction, but the mechanisms underlying AD-related dysfunction and neurodegeneration are poorly understood.

Identification of factors that contribute to the pathology of AD comes from epidemiological as well as genetic studies: AD can be classified into two different forms, rare familial forms (FAD), in which the disease onset is at an age younger than 60 years, and the vast majority of sporadic AD cases, in which onset occurs at an age over 60. Both forms of AD show the same clinical symptoms and neuropathology. Genetic studies in FAD patients have identified mutations in the genes encoding for the amyloid precursor protein (APP) and for the presenilins PS1 and PS2 that cause an autosomal inherited form of AD with 100% penetrance. These FAD mutations consistently lead to increased production of Aβ from its precursor protein APP, which prompted Hardy and Higgins [5] to suggest a direct and pathological role for Aβ accumulation in the development of AD. In the sporadic form of the disease, several risk factors have been found that increase the risk to develop the disease but—unlike FAD mutations—do not necessarily lead to development of AD. Aging is by far the most important risk factor for AD, but the apolipoprotein E4 allele and female sex also predispose to the development of AD.

Immunohistochemical studies of postmortem AD brains have established that neurons undergo apoptotic cell death. Since ROS can elicit apoptotic signaling, the hypothesis that oxidative stress is involved in the pathogenic steps that lead to the development of AD was proposed in the 1990s by several groups [6—9]. There is a large body of evidence in support of this hypothesis: Oxidative stress has been repeatedly shown to be associated with Aβ toxicity and with risk factors for sporadic AD—mostly aging and the apolipoprotein E4 genotype.

8.2.1 Evidence for a Role for Oxidative Stress in Sporadic AD

Oxidative stress has been associated with the risk factors for sporadic AD, most prominently with aging [10], suggesting that an age-associated rise in accumulation of ROS can render the brain more vulnerable to the development of AD. Consequently, increased markers of oxidative stress have been found in AD patients: Several studies have reported elevated levels of lipid peroxidation products, oxidatively modified proteins, and oxidized DNA and RNA bases in brains and cerebrospinal

fluid from AD patients compared to age-matched nondemented control subjects [11–13]. Furthermore, tissue samples from AD brains display a higher susceptibility to in vitro oxidation [14], suggesting an impairment of antioxidant defense in AD patients. Reports on antioxidant parameters in AD brains have, however, been contradictory so far. Several antioxidant enzymes have been studied in AD brains with inconsistent results, but the majority of reports found elevations in antioxidant enzymes, suggesting an upregulation of antioxidant defense in response to increased ROS levels [15]. Interestingly, upregulation of antioxidant defence was more pronounced in female patients, and levels of 4-hydroxynonenal (HNE), a neurotoxic aldehyde derived from lipid peroxidation reactions, were elevated in female compared to male patients. These findings suggest that brains from female AD patients are under higher oxidative pressure [15], consistent with epidemiological findings that AD is more frequent in postmenopausal women compared to age-matched men. This observation can possibly be linked to the lack of sexual hormones, especially estrogens, that can modulate cognitive function and nonreproductive behaviours in humans and other mammalian species [16]. Potential sources of ROS in AD brains include ROS derived from impaired mitochondrial function [17, 18] and secondary ROS formation due to inflammatory reactions. Furthermore, increased monoamine oxidase B activity and increased levels of potentially pro-oxidative heavy metals like iron have been identified in AD brains [19, 20] and in patients with mild cognitive impairment (MCI), the "clinical precursor of AD," suggesting that oxidative stress is an early event of the disease [21].

Apart from aging, the apolipoprotein E4 allele is the second most important risk factor for the development of AD. Apolipoprotein E seems to play a role in brain lipid metabolism and neuronal and glial development. It can exist in three different alleles, E2, E3, and E4, which differ in only two amino acids: The E2 isoform contains two and the E3 isoform one cysteine residue, while the E4 isoform contains none. Carriers of the apolipoprotein E4 are at increased risk to develop sporadic AD, especially when they are homozygous carriers. The apolipoprotein E4 allele has been associated with increased oxidative damage in AD brains, with the greatest impact in homozygotic carriers [22] and an increased susceptibility to cell death in lymphocytes from carriers bearing at least one E4 allele [23]. In vitro studies have evidenced that apolipoprotein E4 is less efficient in binding HNE, a cytotoxic lipid peroxidation product. These findings suggest that the Apo E4 isoform increases susceptibility to oxidative damage, thereby possibly predisposing to the development of AD.

8.2.2 Oxidative Stress and Toxicity of Mutant APP, Presenilins, and Tau

Since the proposal of the amyloid hypothesis of AD, toxic mechanisms caused by mutant APP and presenilins related to an increased production of Aβ have been extensively studied. Cells exposed to Aβ undergo apoptotic cell death, and the toxicity of Aβ has been shown to be related to the production of ROS [24, 25]. Furthermore, toxicity of Aβ depends on its aggregation state, which can be influenced by oxidation. Thus oxidative stress can cause formation of toxic Aβ species, which in turn can further exacerbate accumulation of ROS in a vicious cycle (Fig. 8.1). This could also explain why the prevalence of AD increases with advancing age—due to rising oxidative stress levels with aging favoring Aβ toxicity.

Toxicity of Aβ is also evident in cell cultures overexpressing APP/Aβ. PC12 cells transfected with mutant APP Swedish showed higher sensitivity to ROS-induced cell death and increased mitochondrial impairment after challenge with hydrogen peroxide [26]. Similar observations were obtained in human neuroblastoma cells (SH-SY5Y) overexpressing human wild-type APP (wtAPP) [27]. The study demonstrated that chronic exposure to Aβ protein resulted in activity changes of complexes III and IV of the oxidative phosphorylation system (OXPHOS) in mitochondria coupled with a drop of ATP levels and an increase of ROS production, which may finally instigate loss of synapses and neuronal cell death in AD. Furthermore, treatments of untransfected SH-SY5H cells with Aβ or human amylin aggregates induced an increase of ROS production and had a negative impact on mitochondrial respiration by their action on OXPHOS system [25].

Toxicity of Aβ has also been evidenced in animal models of the disease. Mice transgenic for mutant APP have high levels of Aβ in their brains and show an age-dependent formation of Aβ plaques similar to the plaques found in AD patients. Increased markers of oxidative stress have been detected in brains of transgenic mice bearing mutant APP, accompanied by markers for mitochondrial damage [28]. Furthermore, mutant APP transgenic mice show reduced levels of the antioxidant enzyme copper/zinc superoxide dismutase [29]. In agreement, increased markers of oxidative stress and reduced antioxidant defense by catalase as well as a trend toward reduced activity of SOD were found in brains from FAD patients [30]. The results provide an important link of studies on toxicity of mutant APP in cell culture and animal models mimicking the pathogenesis of the disease in FAD patients, all of them bearing mutations finally causing an increased generation of Aβ.

Mutations in the presenilins PS1 and PS2 account for the majority of FAD cases and have similarly been linked with oxidative stress. Oxidative toxicity of mutant

presenilins can be either (i) due to increased formation of toxic Aβ, especially the $A\beta_{1-42}$ isoform, or (ii) due to direct toxic effects of mutant presenilins. Several mutations in the presenilins have been found that consistently lead to increased production of the long $A\beta_{1-42}$ from its precursor protein APP [31], resulting in increased Aβ levels and toxicity via the above-mentioned mechanisms. Expression of mutant presenilins in cell culture and transgenic mice sensitizes cells to apoptotic stimuli by increasing ROS production and mitochondrial damage [32, 33]. Furthermore, brains from PS1 mutant transgenic mice display reduced activities of antioxidant enzymes [34], and lymphocytes from these mice display increased sensitivity to apoptosis accompanied by high intracellular ROS and calcium levels [35]. Interestingly, increased ROS accumulation, disturbed calcium homeostasis, and diminished levels of antioxidants have also been identified in peripheral cells from FAD patients bearing APP or PS mutations as well as in cells from sporadic AD patients [36]. These results suggest that the oxidative toxicity observed in transgenic animal models of the disease can indeed play an important role to the pathogenesis of sporadic as well as familial AD in humans.

The second main hallmark lesion of AD is intracellular neurofibrillary tangles (NFTs) built up of hyperphosphorylated Tau. This protein may block the transport of mitochondria, leading to energy deprivation and oxidative stress at the synapse as well as to neurodegeneration [37]. Functional analysis showed mitochondrial dysfunction in transgenic mice (pR5 mice) expressing P301L mutation of Tau, with a reduced complex I activity and, with age, impaired mitochondrial respiration and ATP synthesis. Mitochondrial dysfunction was associated with higher levels of ROS in aged pR5 mice. Increased Tau pathology as in aged homozygous pR5 mice revealed modified lipid peroxidation levels and upregulation of antioxidant enzymes in response to oxidative stress. These findings demonstrated for the first time that not only the Aβ but also the Tau pathology acts on the enzyme metabolism of the brain and the oxidative conditions in AD. However, more recently, the successful development of double, and even triple, transgenic mouse models has facilitated the investigation of pathogenic mechanisms in AD and assisted in an understanding of the interplay of Aβ and Tau on bioenergetics processes in vivo [37]. These findings support the idea that Aβ and Tau act synergistically in amplifying mitochondrial respiratory deficits, mainly of complex I and IV activities [18].

8.2.3 Is Oxidative Stress an Early Event in the Pathogenesis of AD?

From the above evidence it can be concluded that oxidative stress is a feature of sporadic as well as familial forms of AD. However, it remains to be elucidated whether oxidative stress is a primary factor in the pathogenesis of the disease or only a secondary contributing mechanism. The fact that oxidative damage and mitochondrial dysfunction can be detected at early stages in animal models [28]—even before the presence of Aβ plaques [38]—and that oxidative stress parameters have been detected at highest levels in early stages of the disease in AD patients [39] suggest that oxidative stress is a primary event in the course of the disease. This is supported by studies that reported a reduced risk of AD in users of antioxidant vitamin supplements [40]. Although further clinical trials are needed, antioxidant therapeutic approaches seem to be most effective at very early stages of AD and are even better utilized to modulate disease risk.

8.3 NEUROSTEROIDS

Steroid hormones are now well-defined molecules that are mainly produced by endocrine glands, such as adrenal gland, gonads, and placenta. They are involved in the control of a lot of physiological processes, from reproductive behavior to stress responsiveness. With their ability to cross cellular membranes, and thus the blood-brain barrier, steroid hormones have also an important role in the development, maturation, and differentiation of the central and peripheral nervous systems.

Three decades ago, Baulieu and co-workers were the first to show a steroid production within the nervous system itself. They discovered that some steroids, such as pregnenolone (PREG) and dehydroepiandrosterone (DHEA), were more concentrated in the brain than in the plasma [41]. In addition, they could show that the level of these steroids remained elevated in the brain even after adrenalectomy and castration. These molecules are now called "neurosteroids" and are defined as neuroactive steroids that are synthesized within the nervous system, independently of peripheral endocrine glands. Enzymatic activities of proteins involved in steroidogenesis have been shown in many regions of the central and peripheral nervous systems, in neurons as well as in glial cells [42]. Pharmacological and behavioral studies showed that neurosteroids were implicated in several physiological mechanisms, for example, cognition, anxiety, depression, neuroprotection, and even nociception [43]. Thus the conservation of the ability to produce neurosteroids during vertebrates' evolution suggests that this category of molecules is important for living beings.

8.3.1 Biosynthesis of Neurosteroids

Neurosteroids derive from cholesterol and other blood-borne steroidal precursors. The first step of neurosteroidogenesis is the transfer of molecules of cholesterol from

the outer to the inner mitochondrial membrane. Free cholesterol accumulates outside of mitochondria and binds to the steroidogenic acute regulatory protein (StAR), a hormone-induced mitochondria-targeted protein that initiates cholesterol transfer into mitochondria. Then, molecules are transported inside mitochondria by a protein complex including translocator protein (TSPO), a cholesterol-binding mitochondrial protein also known under the name of peripheral-type benzodiazepine receptor (PTBR), which permits cholesterol transfer into mitochondria and subsequent steroid formation [44]. This translocation from the outer membrane to the inner membrane of mitochondria is the rate-limiting step in the production of neurosteroids. In fact, the ability of cholesterol to enter into mitochondria to be available to cytochrome *P*450 cholesterol side chain cleavage enzyme (P450scc), located in the inner side of the mitochondrial membrane and responsible for the conversion of cholesterol to PREG, will determine the efficiency of steroidogenesis.

PREG, precursor of all steroid hormones, is then transported to the endoplasmic reticulum, where it is metabolized to form neuroactive steroids (Fig. 8.2). The next enzymatic step in neurosteroidogenesis is the conversion of PREG into DHEA by the cytochrome *P*450c17 enzyme (P450c17), also called 17α-hydroxylase/17,20 lyase. This enzyme catalyzes the 17α-hydroxylation of PREG in a two-step reaction that gives first 17-hydroxyPREG (17OH-PREG) and then the final product, DHEA. Each step requires the molecules NADPH and O_2.

PREG can also be catalyzed by another enzyme called 3β-hydroxysteroid dehydrogenase (3β-HSD) into progesterone (PROG). In general, 3β-HSD uses NAD^+ as a cofactor to oxidize hydroxysteroids, such as PREG, 17OH-PREG, and DHEA, into their respective ketosteroids, PROG, 17OH-PROG, and androstenedione. Then, neurosteroidogenesis follows two main pathways with PROG as precursor: the androgen/estrogen pathway and the corticoid pathway.

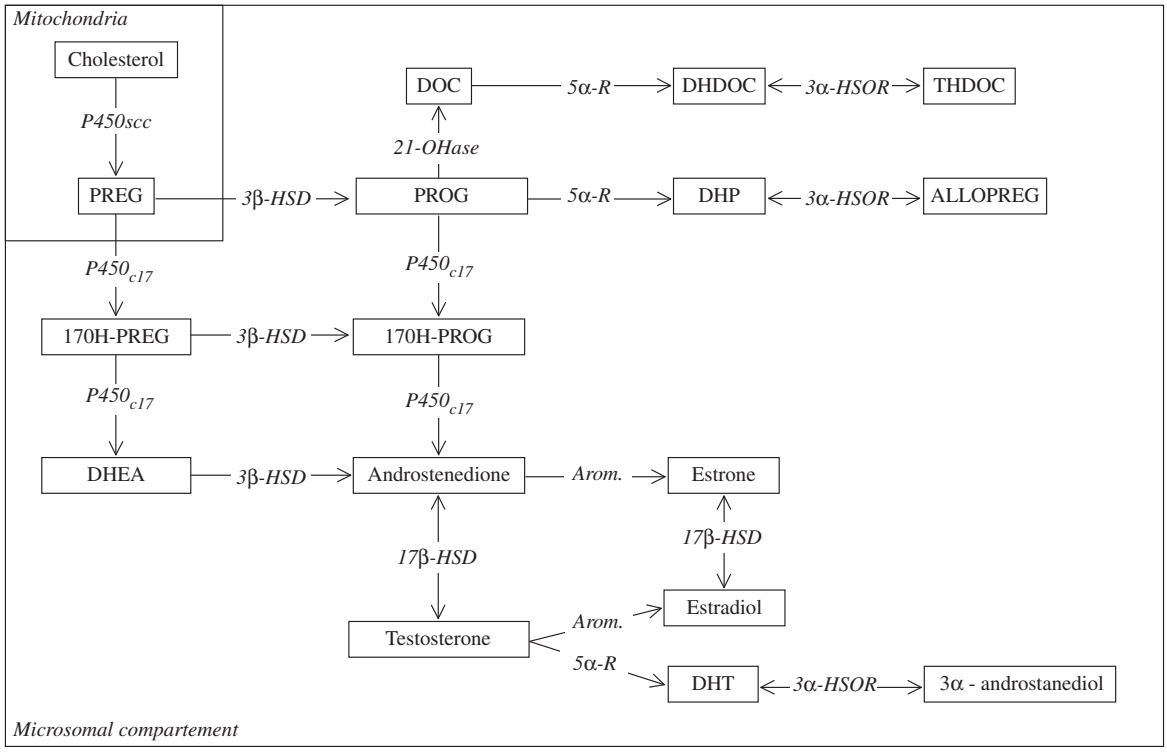

Fig. 8.2 Main biochemical pathways for neurosteroid biosynthesis and metabolism in the vertebrate brain. 17OH-PREG, 17-hydroxypregnenolone; 17OH-PROG, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; DOC, deoxycorticosterone; DHDOC, dihydroxydeoxycorticosterone; THDOC, tetrahydroxydeoxycorticosterone; DHP, dihydroprogesterone; ALLOPREG, allopregnenolone; DHT, dihydrotestosterone; P450scc, cytochrome *P*450 cholesterol side chain cleavage; $P450_{c17}$, cytochrome P450c17; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 5α-R, 5α-reductase; Arom., aromatase; 21-OHase, 21-hydroxylase; 3α-HSOR, 3α-hydroxysteroid oxydoreductase;17β-HSD, 17β-hydroxysteroid dehydrogenase

In the first pathway, PROG is metabolized by the same enzyme as PREG, the cytochrome *P*450c17, which converts PROG into androstenedione with the 17-hydroxyPROG as an intermediate product of reaction. Androstenedione is then converted in a reversible manner into testosterone by another hydroxysteroid dehydrogenase called 17β-HSD. Of note, this enzyme possesses several isoforms, and one of them, 17β-HSD-10, also called ABAD (Aβ binding alcohol dehydrogenase) or ERAB (endoplasmic reticulum-associated amyloid β-peptide binding protein), is in mitochondrial matrix. This isoform was recently linked to AD because of its ability to bind Aβ peptide, thus inducing mitochondrial dysfunction [45]. 17β-HSD is also responsible for the reversible conversion of estrone, an estrogen stemming from aromatization of androstenedione by the enzyme aromatase into estradiol. The second way to synthesize estrogens is via testosterone molecules, which can, in turn, be metabolized into estradiol by aromatase or continued metabolism via the androgen pathway. The 5α-reductase enzyme (5α-R), a microsomal NADPH-dependent protein, intervenes at this level and catalyzes the transfer of two atoms of hydrogen from NADH to form the 5α-reduced metabolite of testosterone, dihydrotestosterone (DHT) [42]. Finally, the enzyme 3α-hydroxysteroid oxido-reductase (3α-HSOR), also called 3α-hydroxysteroid dehydrogenase, catalyzes the reversible conversion of DHT into the neuroactive steroid 3α-androstanediol.

The latter enzymes also intervene at another level, in the second main steroidogenic pathway which starts with PROG. In fact, PROG is successively metabolized by the 5α-R and the 3α-HSOR to form dihydroprogesterone (DHP) and 3α/5α-tetrahydroprogesterone (3α/5α-THP), also known under the name allopregnenolone, another neuroactive steroid.

To finish by the corticoid pathway, molecules of deoxycorticosterone (DOC), stemming from the transformation of PROG by the enzyme 21-hydroxylase (21-OHase), are in turn successively converted into dihydroxydeoxycorticosterone (DHDOC) and tetrahydroxydeoxycorticosterone (THDOC) by the 5α-R and the 3α-HSOR, respectively.

8.3.2 Mechanisms of Action of Neurosteroids

The main role of steroid hormones produced by gonads or adrenal glands is now well defined and consists of a feedback loop on the hypothalamus-pituitary axis, to inhibit or activate their own synthesis. Thus they act at a distance from their glands of origin in an endocrine way. In contrast, neurosteroids are synthesized by the nervous system and act on the nervous system in an autocrine/paracrine configuration [46]. The ability of neurosteroids to cross cellular membranes allows them to act on nuclear receptors and to have a genomic action by regulating gene transcription. This action seems to be important during neonatal life, when it has been shown that neurosteroids, such as PROG or estradiol, are able to promote dendritic growth, spinogenesis, synaptogenesis, and cell survival, particularly in the cerebellum [47]. The most studied steroid nuclear receptors are the estrogen receptors α and β, which are expressed in metabolic tissue such as adipose tissue, skeletal muscle, liver, and pancreas, as well as in the central nervous system. Some studies have demonstrated that these receptors play a role in the regulation of glucose homeostasis and lipid metabolism [48], whereas other studies showed that they were also implicated in neuroprotection [49].

Neurosteroids can also act via membrane receptors and play a role in general as allosteric modulators of neurotransmitter receptors. For example, sulfate esters of DHEA and PREG are known to be excitatory neurosteroids and can inhibit the effect of GABA, an inhibitor neurotransmitter, at physiological concentration by acting via the $GABA_A$ receptor [46]. On the contrary, allopregnenolone is a positive allosteric modulator of $GABA_A$ receptors, strengthening the effects of GABA. PREG sulfate can also potentiate the effect of the main excitatory neurotransmitter glutamate by binding to *N*-methyl-D-aspartate (NMDA) receptors. On the other hand, it is well known that neurosteroids modulate neurotransmitter binding sites or receptors including calcium channels and P2X receptors in the brain, spinal cord, as well as dorsal root ganglia (DRG) [50].

Furthermore, recent clinical and pharmaceutical studies showed that estrogens can interact with several neurotransmitter systems, such as the cholinergic and serotoninergic systems, to influence cognitive performance in animals and humans [51]. Thus neurosteroids seem to play an important role in the nervous system during development as well as in adult brain, by regulating gene transcription and different neurotransmitter systems. Their implication was already demonstrated in several pathologies, such as AD or neuropathic pain [42, 52]. Thus it can be speculated that they might be an important therapeutic target to develop in the next years.

8.4 NEUROSTEROIDS AND OXIDATIVE STRESS

During recent years, a growing body of evidence has shown that neurosteroids, in particular estrogens, are implicated in the regulation of oxidative stress by acting on mitochondria [53]. However, on one hand, depending

on the level of oxidative stress within cells estrogens can have a protective effect or, on the contrary, show a negative action on cell survival. On the other hand, oxidative stress itself can have an effect on neurosteroid production within nerve cells.

8.4.1 Regulation of Neurosteroidogenesis by Oxidative Stress and Aβ Peptide

It is established that steroids can be synthesized by nonglandular tissue within the nervous system. But the regulation of their biosynthesis is still poorly understood. Recent findings showed that several glial cells, in particular oligodendrocytes, upregulated their production of DHEA under oxidative stress conditions induced by treatment with Aβ peptide or Fe^{2+} [54]. Modulation of neurosteroid production was also observed in neuroblastoma (SH-SY5Y) cells overexpressing the key AD proteins, APP/Aβ or Tau (Fig. 8.3) [52]. Indeed, overexpression of human wild-type Tau (hTau 40) protein induced an increase in production of progesterone, 3α-androstanediol, and 17-hydroxyprogesterone, in contrast to overexpression of the abnormally hyperphosphorylated Tau bearing the P301L mutation and leading to a decrease in the production of these neurosteroids. In parallel, a decrease of progesterone and 17-hydroxyprogesterone production was observed in cells expressing human wild-type APP (wtAPP), whereas

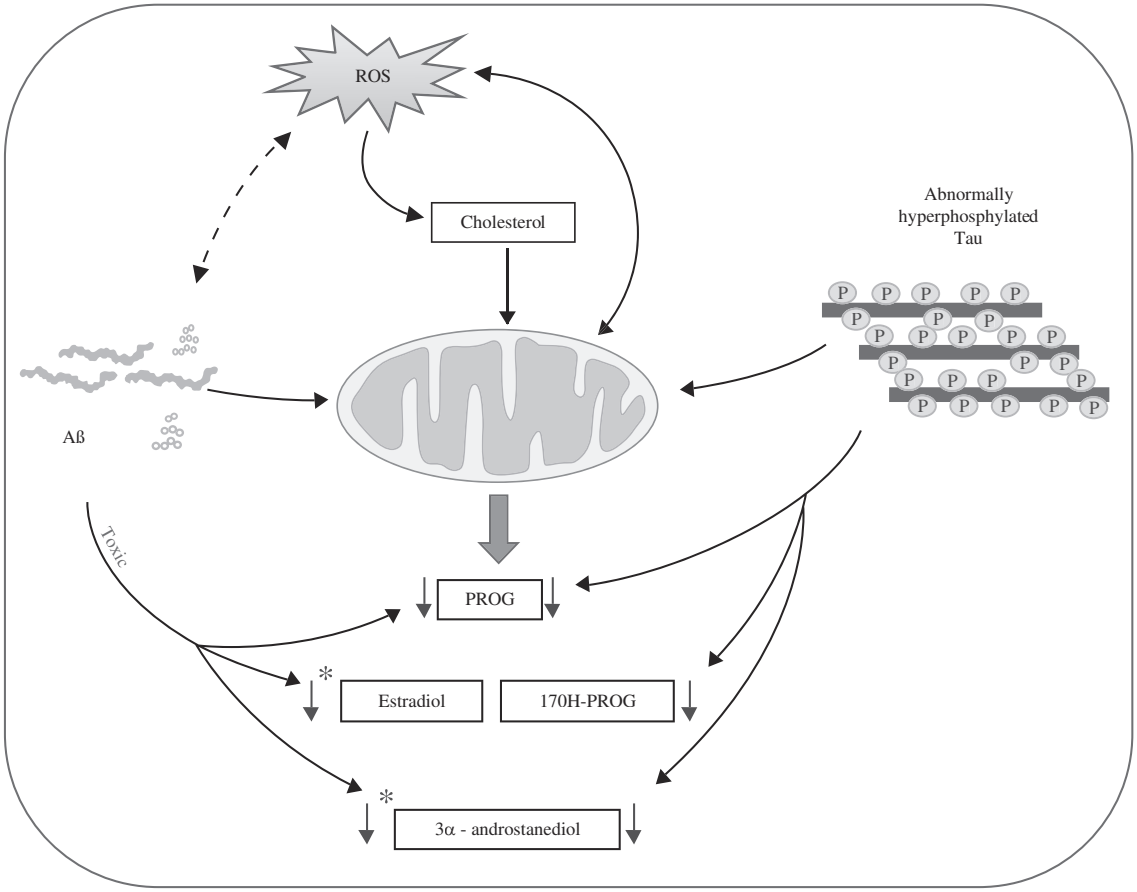

Fig. 8.3 Effect of toxic concentrations of Aβ peptides and abnormally hyperphosphorylated Tau protein on neurosteroid biosynthesis. Aβ induced a drop of the level of progesterone (PROG), estradiol, and 3α-androstanediol by acting on reactive oxygen species (ROS) formation and mitochondrial function and/or directly on steroidogenesis. The presence of abnormally hyperphosphorylated Tau protein had the same effect by inducing a decrease of progesterone, 17-hydroxyprogesterone (17OH-PROG), and 3α-androstanediol. On the other hand, it has been shown that nontoxic concentrations of Aβ induced an increase in estradiol and 3α-androstanediol levels (this pathway is marked by *)

3α-androstanediol and estradiol level were increased. The latter finding was additionally confirmed with in vitro treatment experiments [4]. APPwt SH-SY5Y cells secrete Aβ levels within the nanomolar concentration range. Consistently, treatment of native SH-SY5Y cells with "nontoxic," that is, non-cell death-inducing, $A\beta_{1\text{-}42}$ concentrations in vitro revealed an increase in estradiol production, whereas toxic $A\beta_{1\text{-}42}$ concentrations within the micromolar range strongly reduced estradiol levels revealing the exact opposite effect. Of note, oxidative stress was able to modify neogenesis of neurosteroids in a similar pattern [55]. In fact, treatment with H_2O_2 for 24 h or 48 h induced a decrease of estradiol synthesis that was correlated to a downregulation of the aromatase, the enzyme responsible for estradiol formation from testosterone. Furthermore, an increase of cell death was observed in the presence of letrozole, an inhibitor of aromatase. This suggests that endogenous estradiol formation is very important for human neuroblastoma cells and plays a critical role in cell survival. Interestingly, when cells were pretreated with estradiol, it was possible to rescue neuroblastoma cells from H_2O_2 as well as from letrozole-evoked death. In agreement, similar results were also found in stress condition experiments using heavy metals, such as cobalt and mercury, and once again estradiol was able to reverse their deleterious effect by reducing oxidative stress and β-amyloid secretion [56].

8.4.2 Estrogens and Neuroprotection

Neuroprotective effects of estrogens against a variety of brain injuries have been described for many years. Treatment with 17β-estradiol was able to protect the brain against excitotoxicity, Aβ peptide-induced toxicity, free radical generators, and ischemia in animal studies [53], but the basis of these effects is still poorly understood. It was recognized from former studies that estrogen depletion in postmenopausal women represents a significant risk factor for the development of AD and that an estrogen replacement therapy may decrease this risk and even delay disease progression [57, 58].

However, results from the "Woman's Health Initiative Memory Study" (WHIMS) including 4532 postmenopausal woman aged over 68 years indicated a twofold increase in dementia after 4.2 years of treatment (p.o. treatment with premaxin plus medroxyprogesterone). In addition, it indicated potential risks for breast cancer, pulmonary embolism, and stroke [59, 60]. Besides warrantable criticism with regard to the synthetic hormones used in the WHIMS trial, the outcome results were unexpected and disappointing. One can ask the question, "How could it be that so many scientific studies before the WHIMS trial were wrong?" Thus the currently prevailing view points about the "critical window hypothesis" [16] are asking about the critical period in which one might expect a neuroprotective effect to occur. The results of the WHIMS study also initiated a discussion about a two-edged effect of estradiol. Thus estradiol can possibly also exhibit a "prooxidant effect" in the presence of ongoing oxidative stress [53]. Thereby, estradiol can be hydroxylated to give catecholestrogens that can enter a redox cycle generating superoxide radical. In an oxidative environment, this redox cycling can lead to a continuous formation of ROS that amplifies even more oxidative stress and increases neuronal loss.

On the contrary, animal studies, especially in rodents and transgenic mice models for AD, seem to confirm positive effects of estrogen treatment on the pathophysiology of the disease. It has been shown that treatment with estrogen in mice expressing mutations in human APP (Swedish and Indiana) had an impact on APP processing, decreasing levels of Aβ and so its aggregation into plaques [61]. In triple transgenic AD mice, depletion of sex steroid hormones induced by ovariectomy in adult females significantly increased Aβ accumulation and had a negative impact on cognitive performance [62]. Treatment of these ovariectomized mice with estrogens was able to prevent these effects. Of note, when PROG was administered in combination with estrogens, the beneficial effects on Aβ accumulation were blocked but not effects on cognitive performance. Furthermore, PROG reduced Tau hyperphosphorylation when administered alone. This suggests that estrogen and PROG can interact to regulate APP processing but can also act independently on different AD pathways.

At the cellular level, estrogen was able to activate antioxidant defense systems by reducing ROS production, limiting mitochondrial protein and DNA damage, and improving the activity of the electron chain transport during oxidative phosphorylation [53]. Thus estrogen can have direct antioxidant effects by increasing reduced glutathione (GSH) levels and decreasing oxidative DNA damage in mitochondria of ovariectomized female rats [63]. This is correlated with an upregulation of the expression of two enzymes: manganese superoxide dismutase (Mn-SOD) and glutathione peroxidase, both of them implicated in the antioxidant defense system. Of note, estrogen can modulate the redox state of cells by intervening in several signaling pathways, such as MAPK, G protein-regulated signaling, NF-κB, c-fos, CREB, phosphatidylinositol-3-kinase, PKC, and Ca^{2+} influx [3, 64]. On the basis of this complex mode of action, estrogen seems to be able to decrease oxidative stress markers, including lipid peroxidation, protein oxidation, and DNA damage.

Recently, it has been proposed that estrogens exert their beneficial effects by acting directly on mitochondria

via estrogen receptor β (ERβ) [65]. In fact, incubation of isolated mitochondria from rat brain with estradiol leads to a decrease of H_2O_2 production by this organelle coupled with an increase of the mitochondrial membrane potential. Moreover, estradiol seems to prevent the release of cytochrome *c* by mitochondria (a mechanism known to induce apoptosis of cells by activating the caspase cascade in the cytoplasm), which increases the efficiency of the respiratory chain. In addition, estrogens are able to bind to nuclear receptors, such as estrogen receptor α and β (ER α/β), and to act as transcription factors. Thus estrogens enhanced the expression of the antiapoptotic proteins, Bcl-2 and Bcl-xL, preventing the initialization of the cell death program by mitochondria [3]. They were also able to increase the expression of F1 subunits of ATP synthase and glucose transporter subunits and regulate enzymes involved in the tricarboxylic acid (TCA) cycle, which has the effect of improving glucose utilization by cells.

As described recently, estrogens can have an effect on the transcription of mitochondrial genes, especially on the electron transport chain components [66]. Treatment of ovariectomized female rats with estradiol induced an increase of mitochondrial respiratory function translated into an enhancement of O_2 consumption and coupled to an increased expression and activity of cytochrome *c* oxidase (electron transport chain complex IV).

Finally, another means for estrogens to avoid negative effects of oxidative stress is to regulate calcium homeostasis by inducing mitochondrial sequestration of cytosolic calcium [53]. In fact, an imbalance of calcium handling can lead to an increase of ROS production by activating the enzyme nitric oxide synthase, which can sensitize neural cells to oxidative damage. It has been shown that estradiol treatment of primary hippocampal neurons was able to potentiate glutamatergic response via NMDA receptor, which resulted in an increased influx of calcium in cells. This effect was coupled with an induction of mitochondrial sequestration of cytosolic calcium and an increase of the mitochondrial calcium load tolerability, to avoid calcium-induced excitotoxicity and to promote cell survival.

8.5 CONCLUSION

In summary, it is now clear that oxidative stress is an important actor involved in AD pathophysiology and intervenes already at an early disease stage. Furthermore, good evidence is provided that neurosteroids, such as estrogens, are able to limit oxidative damage by reducing lipid peroxidation, protein oxidation, Ca^{2+} overload in cytosol, and DNA damage in mitochondria as well as in the nucleus. These effects are mediated by several mechanisms, from transcription of genes coding for antioxidant enzymes to the regulation of antiapoptotic pathways, by way of improvement of mitochondrial respiratory chain efficiency and glucose metabolism. Thus, with their abilities to counter excess oxidative stress, estrogens seem to be able to prevent AD-related toxic mechanisms, such as Aβ peptide aggregation, Tau hyperphosphorylation, and neuronal loss. Better human studies taking into account the critical window hypothesis are essential before drawing a final conclusion on efficacy of neurosteroids in prevention of AD.

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Appendix 6

Inhibition of the mitochondrial enzyme ABAD restores the amyloid-β-mediated deregulation of estradiol.

Lim YA, **Grimm A.** Giese M, Mensah-Nyagan AG, Villafranca JE, Ittner LM, Eckert A, Götz J.

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Inhibition of the Mitochondrial Enzyme ABAD Restores the Amyloid-β-Mediated Deregulation of Estradiol

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Abstract

Alzheimer's disease (AD) is a conformational disease that is characterized by amyloid-β (Aβ) deposition in the brain. Aβ exerts its toxicity in part by receptor-mediated interactions that cause down-stream protein misfolding and aggregation, as well as mitochondrial dysfunction. Recent reports indicate that Aβ may also interact directly with intracellular proteins such as the mitochondrial enzyme ABAD (Aβ binding alcohol dehydrogenase) in executing its toxic effects. Mitochondrial dysfunction occurs early in AD, and Aβ's toxicity is in part mediated by inhibition of ABAD as shown previously with an ABAD decoy peptide. Here, we employed AG18051, a novel small ABAD-specific compound inhibitor, to investigate the role of ABAD in Aβ toxicity. Using SH-SY5Y neuroblastoma cells, we found that AG18051 partially blocked the Aβ-ABAD interaction in a pull-down assay while it also prevented the Aβ42-induced down-regulation of ABAD activity, as measured by levels of estradiol, a known hormone and product of ABAD activity. Furthermore, AG18051 is protective against Aβ42 toxicity, as measured by LDH release and MTT absorbance. Specifically, AG18051 reduced Aβ42-induced impairment of mitochondrial respiration and oxidative stress as shown by reduced ROS (reactive oxygen species) levels. Guided by our previous finding of shared aspects of the toxicity of Aβ and human amylin (HA), with the latter forming aggregates in Type 2 diabetes mellitus (T2DM) pancreas, we determined whether AG18051 would also confer protection from HA toxicity. We found that the inhibitor conferred only partial protection from HA toxicity indicating distinct pathomechanisms of the two amyloidogenic agents. Taken together, our results present the inhibition of ABAD by compounds such as AG18051 as a promising therapeutic strategy for the prevention and treatment of AD, and suggest levels of estradiol as a suitable read-out.

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Introduction

In the Alzheimer's disease (AD) brain, amyloid-β (Aβ) has a central yet only partly understood role in the neurodegenerative process [1]. Apart from constituting the amyloid plaque as a classical hallmark lesion of AD, Aβ acts via a plethora of pathways to induce synaptic and neuronal degeneration [2–4]. Many studies reveal that in exerting its toxicity, Aβ binds to specific receptors and/or lipids at the neuronal cell membrane, and some studies even suggest a disruption of ion homeostasis by forming channels or pores [5,6]. To better understand what the prerequisites are for Aβ toxicity, we and others used transgenic mouse models and found that Aβ mediates its toxicity in part through the NMDA receptor, with an essential role for the microtubule-associated protein tau [7–9], that similar to Aβ, also forms insoluble aggregates in the AD brain. Over-activation of the NMDA receptor complex results in excessive nitric oxide (NO) levels, causing down-stream protein misfolding and aggregation, as well as mitochondrial dysfunction. The toxic signaling pathway further involves the release of mitochondrial cytochrome c and the activation of down-stream caspases as well as the formation of ROS (reactive oxygen species) [10–12], highlighting mitochondria as a prime down-stream target of Aβ [13–15].

Interestingly, mitochondria represent not only an indirect target; instead, in several studies Aβ has been localized to [16] and shown to act directly on mitochondria [17,18] whose function it impairs [19–22]. Among the mitochondrial proteins to which Aβ has been shown to bind is the enzyme amyloid-binding alcohol dehydrogenase (ABAD) [23,24]. ABAD interacts with Aβ and is a major determinant of Aβ toxicity [17,25,26]. Specifically, in mice doubly transgenic for ABAD and the Aβ-precursor APP, the toxic effects of Aβ are aggravated compared to what is found in APP single transgenic mice [17].

ABAD is the Type 10 member of a protein family, known as 17β-hydroxysteroid dehydrogenases (HSD17B) [27]. The enzyme is found in mitochondria, while the other known fourteen family members are confined to the endoplasmic reticulum (ER) suggesting that ABAD has a specialized function within mitochondria [28]. ABAD converts estradiol to estrone [29], and its levels are critical as optimal estradiol levels are an important determinant of

neuronal survival [29]. In post-menopausal women, the estrogen replacement therapy has been shown to delay the onset of AD [30]. In the placenta and in ovaries, ABAD inactivates estradiol by oxidizing it to estrone [31,32], and this may also occurs in testis [33]. Interestingly, ABAD levels themselves are sensitive to estradiol levels suggesting a feedback loop in the regulation of its activity [34].

The many reports of ABAD's enzymatic action on various substrates *in vivo* have been challenged, however, by strong evidence that a catalytically inactive mutant of ABAD as identified in a young boy had no ill effects on his health [35]. In addition, ABAD was found to be one of only three proteins that comprise the fully functional mammalian mitochondrial RNAse P [36], a function that may not require enzymatic activity and that links ABAD directly to the production of mitochondrial electron transport chain proteins and reactive oxygen species (ROS) generation [37].

Binding of Aβ to ABAD induces a conformational change that is inhibited by NAD^+ (nicotinamide adenine dinucleotide), with binding of Aβ and NAD^+ being mutually exclusive [38]. Aβ binding results in the loss of ABAD function and ultimately, neuronal apoptosis [39,40]. To directly determine whether Aβ-induced toxicity is mediated by ABAD inhibition and to establish estradiol levels as a suitable readout, we here employed the use of AG18051, a novel ABAD inhibitor [41].

Materials and Methods

Cell culture and incubation with amyloid peptides

SH-SY5Y neuroblastoma cells (DSMZ, Braunschweig, Germany; DSMZ No. ACC 209) were grown in Dulbecco's Modified Eagle Medium: F-12 (DMEM: F-12) supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) (GIBCO, Basel, Switzerland) [42,43]. Aβ42, human amylin (HA), biotinylated Aβ42 and biotinylated HA were purchased from Bachem (Germany) (H-1368, H-7905, H-5642 and 3004028, respectively). The negative control, biotin, was purchased from Sigma (B4639). Biotinylated and unmodified Aβ42 were dissolved in DMSO to make stocks of 5 mM and stored at −80°C until use. Biotinylated and unmodified HA were dissolved in 0.01 M acetic acid (AA) to make stocks of 5 mM and also stored at −80°C until use. Biotin was dissolved in DMSO to make stocks of 5 mM and kept at −80°C until use. Aging of the peptides was induced by shaking at 1000 rpm for 4 days at 37°C. 0.5 μM Aβ42 or human amylin (HA) was used for all experiments in this study, while the treatment duration was always 5 days. Pre-treatment experiments were performed by incubating SH-SY5Y cells with 0.05 μM AG18051 for 24 hours, washing 3 times with warm PBS, and then treating the cells with 0.5 μM Aβ42 or HA for 5 days. Co-treatment experiments were done by incubating SH-SY5Y cells for 5 days with 0.05 μM AG18051 and 0.5 μM Aβ42 or HA, respectively.

LDH and MTT assays

LDH and MTT assays were chosen to provide indications for cell viability after treatments. Assays were obtained from Roche and were performed according to the manufacturer's protocols. Briefly, cells were exposed to the various treatments, after which the medium was retrieved for LDH analysis, while the remaining cells were washed 3 times with sterile PBS and the MTT assay performed with the cells.

Pull-down assay

SH-SY5Y cells were grown to 70% confluency and treated with vehicle, biotinylated Aβ42, biotinylated HA, or biotin, respectively, at a final concentration of 0.5 μM for 5 days. In addition, 0.5 μM biotin and 0.5 μM biotinylated Aβ42, respectively, was co-incubated with 0.05 μM AG18051 for 5 days. After 5 days, cells were once washed with pre-warmed PBS and immediately scraped with 500 μL ice-cold IP buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA), supplemented with the Complete EDTA-free protease inhibitor cocktail (1:25) (Roche, Basel, Switzerland), and spun at 14 000× g for 10 minutes at 4°C. 20 μl of magnetic Dynabeads MyOne^{TM} Streptavidin T1 (Invitrogen) were added to each tube and tubes were rotated for 30 minutes at room temperature. Beads were subsequently washed 3 times with 1× PBS, followed by boiling in loading buffer containing β-mercaptoethanol at 95°C for 5 minutes at 1000 rpm to release bound peptides. Samples were then briefly spun down at room temperature and supernatants loaded onto 12% glycine gels for electrophoresis and Western blotting.

Quantification of estradiol as a functional read-out of ABAD activity

After treatment with amyloid peptides and controls, the cell culture medium was collected and cells were resuspended in cell lysis buffer consisting of 150 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA and Complete protease inhibitor (1:50) (Roche Diagnostics). The estradiol assay was performed according to the manufacturer's guidelines (Estradiol EIA Kit, Cayman). In brief, the plate was loaded with samples, along with the estradiol tracer and the specific antiserum to estradiol and incubated for one hour at room temperature. After five washing steps, Ellman's Reagent was added and the plate developed for 60 minutes with gentle shaking at room temperature. The calculated estradiol concentration was normalized to the total protein content of the samples.

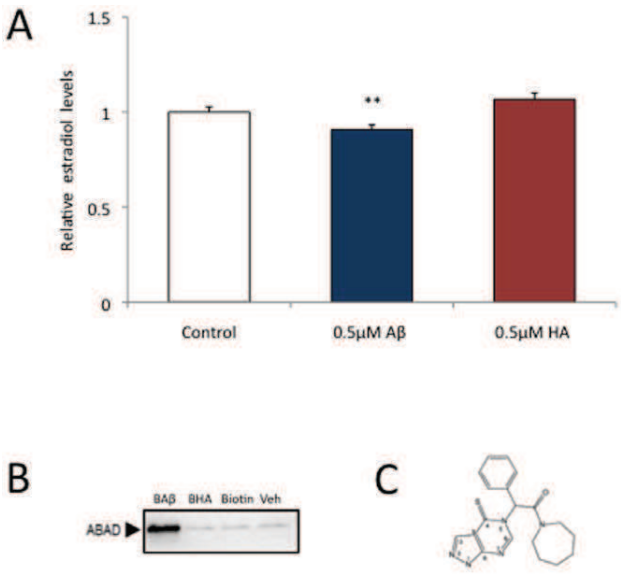

Figure 1. Aβ42 binds to and impairs ABAD activity, while HA (human amylin) does not. (A) Treatment of SH-SY5Y human neuroblastoma cells with Aβ42 causes decreased levels of estradiol, indicative of an impairment of ABAD activity, while HA does not. Results are means ± SE, (n = 5 to 6 per group), **, $P<0.01$ (B) Pull-down of ABAD from SH-SY5Y cells shows that different from HA, Aβ42 can bind to ABAD *in vitro*. (C) Structure of the ABAD inhibitor, AG18051 (adapted from Kissinger et al., JMB 2004).
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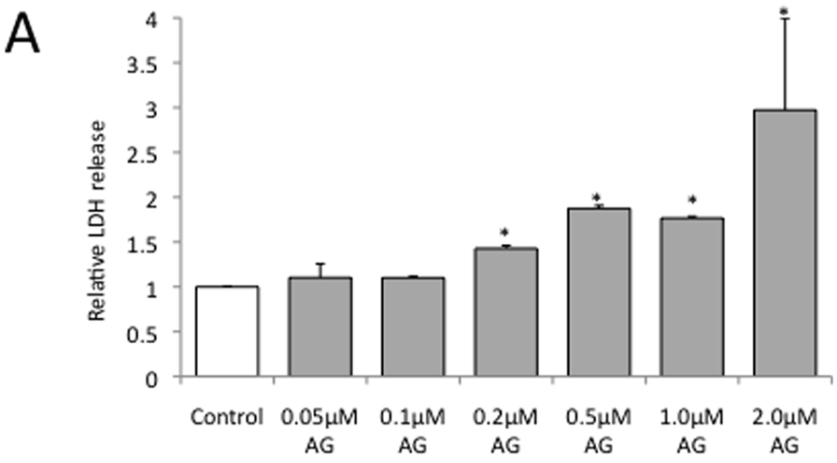
Mitochondrial respiration in vital cells

After treatment with amyloid peptides and controls, mitochondrial oxygen consumption was measured at 37°C using an Oroboros Oxygraph-2k system. Five million cells were added to 2 ml of a mitochondrial respiration medium containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2P0_4, 20 mM HEPES, 110 mM sucrose and 1 g/l BSA (pH 7.1). To measure the state 4 respiration of complex I, 5 mM pyruvate and 2 mM malate were added and cells permeabilised with 15 µg/ml digitonin. Afterwards, 2 mM ADP was added to measure state 3 respiration. The integrity of the mitochondrial membrane was checked by the addition of 10 µM cytochrome c. After determining coupled respiration, 0.4 µM FCCP (Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone) was added and respiration was measured in the absence of a proton gradient. To inhibit complex I activity, 0.5 µM rotenone was added.

Determining ROS levels

Levels of ROS were measured using different fluorescent probes that allow detection at different cellular sites. The non-fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) was used to measure cytosolic ROS, most notably hydrogen peroxide [44]. To determine levels of superoxide anion radicals, DHE was used, which is oxidized to the fluorescent ethidium cation by $O2^{\cdot -}$ [22]. For detection of mitochondria-associated ROS we used the probe dihydrorhodamine (DHR), which localizes to mitochondria and when oxidized by ROS, particularly peroxinitrite, fluoresces to the positively charged rhodamine 123 derivate. Treated cells (Aβ42, HA, and vehicle) were loaded with 10 µM H2DCF-DA, 10 µM DHR or 10 µM DHE, respectively, for 15 min. After washing twice with Hank's balanced salt (HBS) solution, the formation of fluorescent products was measured by detecting the emitted fluorescent units per 5×10^5 cells using the Fluoraskan Ascent FL multiplate reader (Labsystems, Helsinki, Finland) (i) at 485 nm (excitation)/538 nm (emission) for both



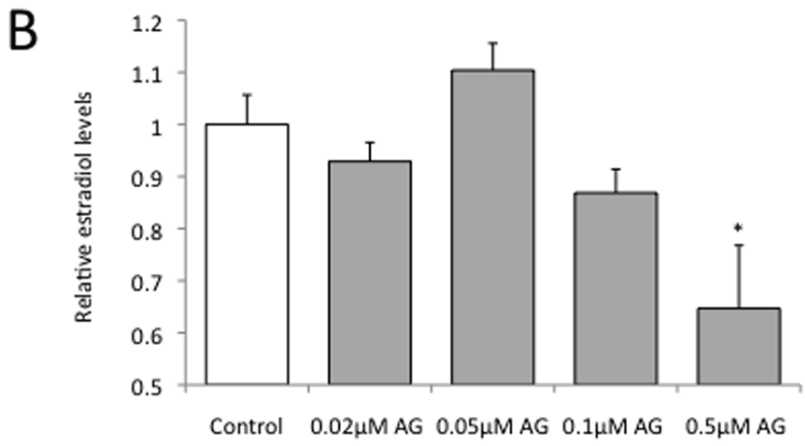


Figure 2. Effects of the ABAD inhibitor AG18051 on cell viability and estradiol levels. (A) LDH assay of SH-SY5Y human neuroblastoma cells incubated with increasing concentrations of AG18051 (normalized to 1 for control) shows that the ABAD inhibitor is not toxic at concentrations of 0.1 µM and below. (B) Treatment of SH-SY5Y cells with increasing concentrations of AG18051 causes reduced levels of estradiol. *, $P<0.05$.
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dichlorofluorescein (DCF) generated from H2DCF-DA via oxidation, and DHR, and (ii) at 530 nm (excitation)/590 nm (emission) for DHE, as previously described [45].

Results

Aβ42 binds to and down-regulates ABAD activity, different from human amylin

Aβ exerts its toxicity in part by impairing ABAD [17], an enzyme known to convert estrone to estradiol. To determine the toxicity of the major fibrillogenic form of Aβ and the role ABAD has in this process, we incubated human SH-SY5Y neuroblastoma cells for 5 days with Aβ42, followed by measuring estradiol levels in the cell lysate. We found that its levels were significantly decreased after Aβ42 exposure ($p<0.0001$) (Fig. 1A). To determine if the deregulation of ABAD activity is a common phenomenon shared by Aβ with other amyloidogenic proteins, we treated the cells also with human amylin (HA), a protein twice the size of Aβ and known to form aggregates in another disease with protein aggregation, Type 2 diabetes mellitus (T2DM). However,

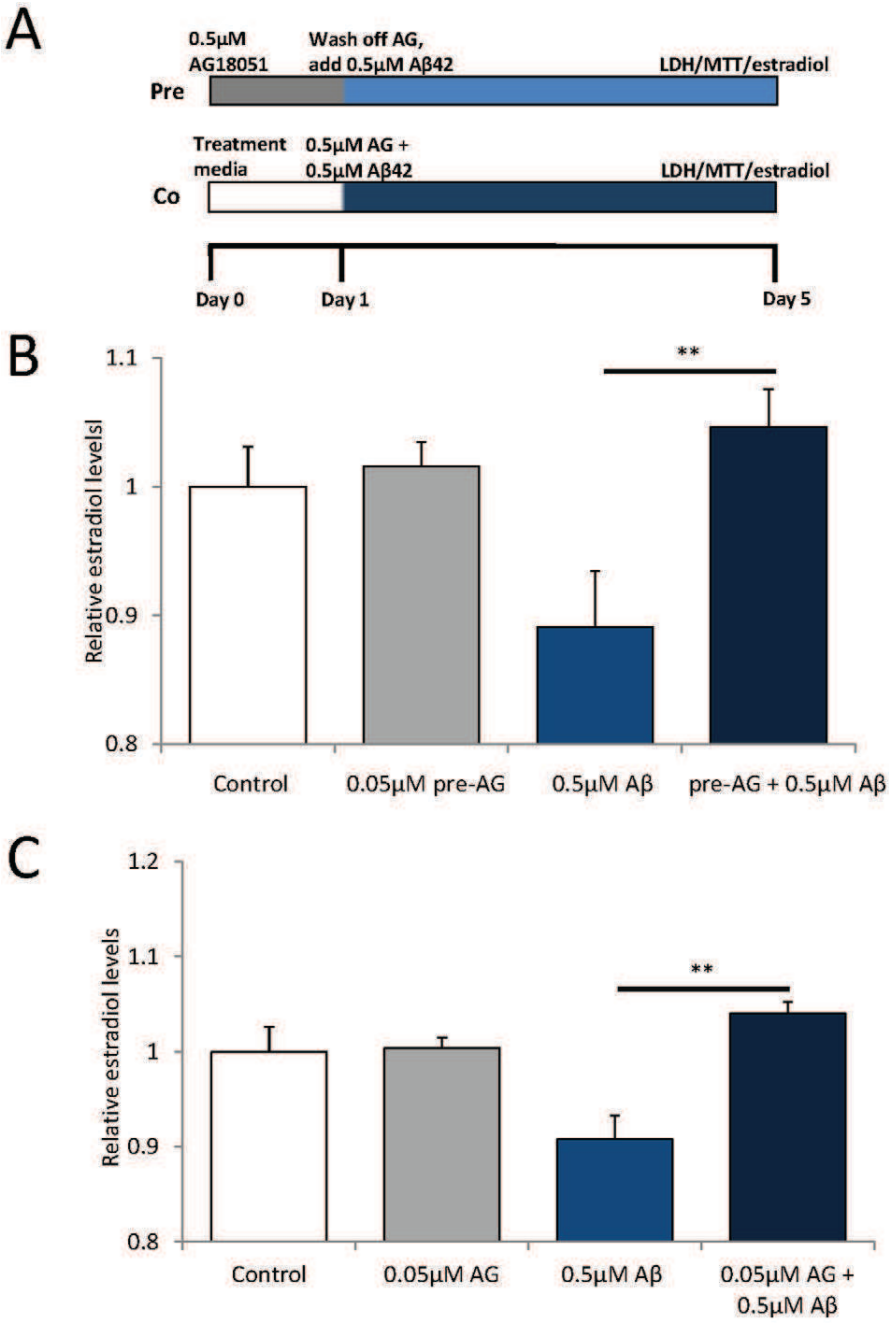

Figure 3. The Aβ-mediated decrease in estradiol levels is prevented by AG18051. (A) Scheme of pre- and co-incubation treatment. (B) Pre-treatment of cells with AG18051 for 24 hours prior to adding Aβ42 maintains estradiol levels compared to the vehicle control, (C) as does co-treatment. **, $P<0.01$.
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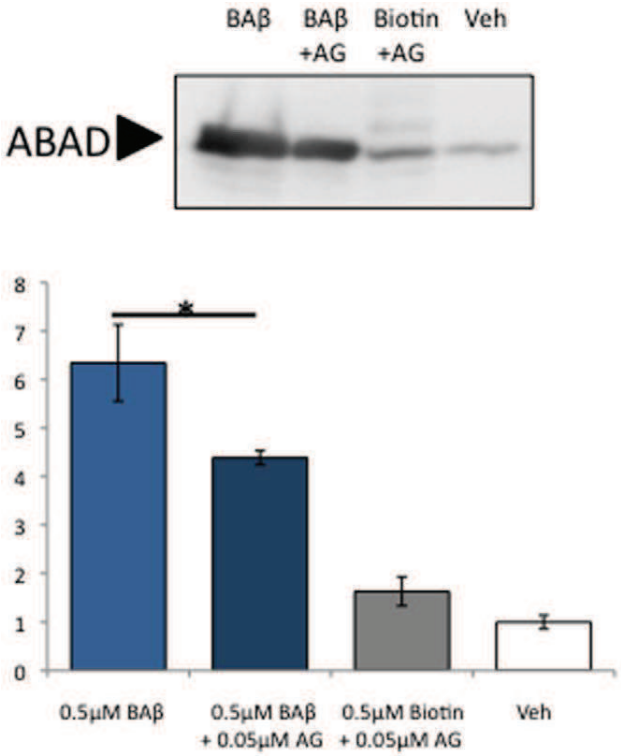

Figure 4. Pull-down of ABAD from SH-SY5Y cells shows that different from HA, Aβ42 can bind to ABAD *in vitro*. The presence of AG18051 significantly decreases the amount of ABAD pulled down by biotinylated Aβ42 (BAβ). *, $p<0.05$.
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different from Aβ42, HA did not cause reductions in estradiol levels ($p<0.0001$) (Fig. 1A).

Data obtained by x-ray crystallography of Aβ42-ABAD complexes indicate that it may be the direct association of Aβ42 and ABAD that inhibits ABAD activity, as an association of Aβ with ABAD prevents it from binding to its physiological substrate, NAD^+ [17]. Since HA, different from Aβ42, did not down-regulate ABAD activity as measured by estradiol levels, and as it is known that Aβ42 is capable of inhibiting ABAD activity by direct interaction [38], we sought to determine whether HA, similar to Aβ, would interact with ABAD. Therefore, SH-SY5Y cells were incubated with 0.5 μM biotinylated Aβ42 (BAβ), biotinylated HA (BHA), biotin or vehicle for 5 days. We found by pull-down that while biotinylated Aβ42 was bound to ABAD, biotinylated HA, along with the negative control, was not (Fig. 1B). Together with the estradiol activity assay, this suggests that the reductions in estradiol levels caused by Aβ42 may be mediated by a direct interaction of Aβ with ABAD.

The ABAD inhibitor AG18051 causes reduced estradiol levels

To better understand the effect of Aβ on estradiol levels we used AG18051, a small molecule inhibitor of ABAD with high affinity [41,46] (Fig. 1C). AG18051 exerts its inhibitory effect by occupying the substrate-binding site of ABAD, which results in the formation of a covalent adduct with the NAD^+ cofactor. As the interaction of ABAD with NAD^+ is necessary for its activity, disrupting ABAD/NAD^+ complex formation obliterates ABAD activity, resulting in decreased estradiol levels. We first determined the toxicity profile of AG18051 in our SH-SY5Y cell culture system. Concentrations of AG18051 up to 0.1 μM were not overtly toxic and therefore, concentrations within the 0.05–0.1 μM range were chosen for subsequent studies (Fig. 2A). The toxicity at higher concentrations argues for a proper dosing. The concentration range applied by us is consistent with the IC_{50} determined for AG18051 in a previous study [47,48]. With increasing concentrations of the inhibitor, as expected, estradiol levels were reduced (Fig. 2B). At 0.05 μM however, there was a slight, but non-significant increase in estradiol levels suggesting compensatory mechanisms.

AG18051 prevents Aβ42-induced reductions in estradiol levels

To determine whether AG18051 has an effect on the Aβ42-induced reduction of estradiol levels (Fig. 1A) and whether the putative neuroprotective effect is via a 'priming effect' of the cells, we both pre- and co-incubated SH-SY5Y cells with Aβ42 and 0.05–0.1 μM AG18051 as outlined in the scheme (Fig. 3A). For both treatment conditions, we found that this maintained estradiol levels in the lysate, compared to a significant reduction in the Aβ42-only treatment (Fig. 3B,C). This suggests that AG18051 is neuroprotective, and that it may exert its neuroprotective effect either by priming cells to become resistant to the effects of Aβ42, or via a direct inhibition of the Aβ42-ABAD interaction as previously suggested [41].

AG18051 partially prevents ABAD-Aβ42 interaction

To determine whether AG18051 treatment reverses the Aβ42-induced toxicity because AG18051 directly blocks the physical interaction between Aβ42 and ABAD, SH-SY5Y cells were treated with 0.5 μM of biotinylated Aβ42 (BAβ) in the presence of 0.05 μM AG18051. We found that co-incubation of AG18051 significantly decreased the amount of ABAD pulled down by biotinylated Aβ42 (BAβ) (**Fig. 4**). This suggests that AG18051 may prevent Aβ42 toxicity by directly inhibiting the association of Aβ42 with ABAD. In addition, this also suggests that AG18051 may exert its neuroprotective effects via additional pathways other than a direct inhibition of the Aβ42-ABAD interaction.

Aβ42-mediated reduction in cell viability prevented by ABAD inhibitor AG18051

Having determined 0.05–0.1 μM as a suitable concentration range for AG18051, we tested its putative neuroprotective effect in Aβ42 toxicity. SH-SY5Y cells were incubated with 0.5 μM Aβ42 with or without AG18051, and toxicity was determined as increased levels of LDH compared to vehicle control. Co-incubation of Aβ42 with either 0.05 or 0.1 μM AG18051, respectively, resulted in a significant decrease in LDH levels back to control levels (Fig. 5A). This indicates that inhibiting ABAD activity protects from Aβ toxicity.

We next investigated the effect of Aβ on metabolic functions and found that besides significantly increasing LDH levels, 0.5 μM Aβ42 also caused a significant decrease in MTT absorbance, suggesting impaired metabolic functions caused by this amyloidogenic peptide (Fig. 5B). Co-incubation of Aβ42 with either 0.05 or 0.1 μM AG18051, respectively, resulted in a smaller decrease of MTT absorbance (Fig. 5B,C). Since MTT measurements are an indicator of mitochondrial health, the results suggest that Aβ42 induces cell toxicity, as reflected by LDH levels, at least in parts via impaired mitochondrial functions, with inhibition of the mitochondrial enzyme ABAD providing partial protection.

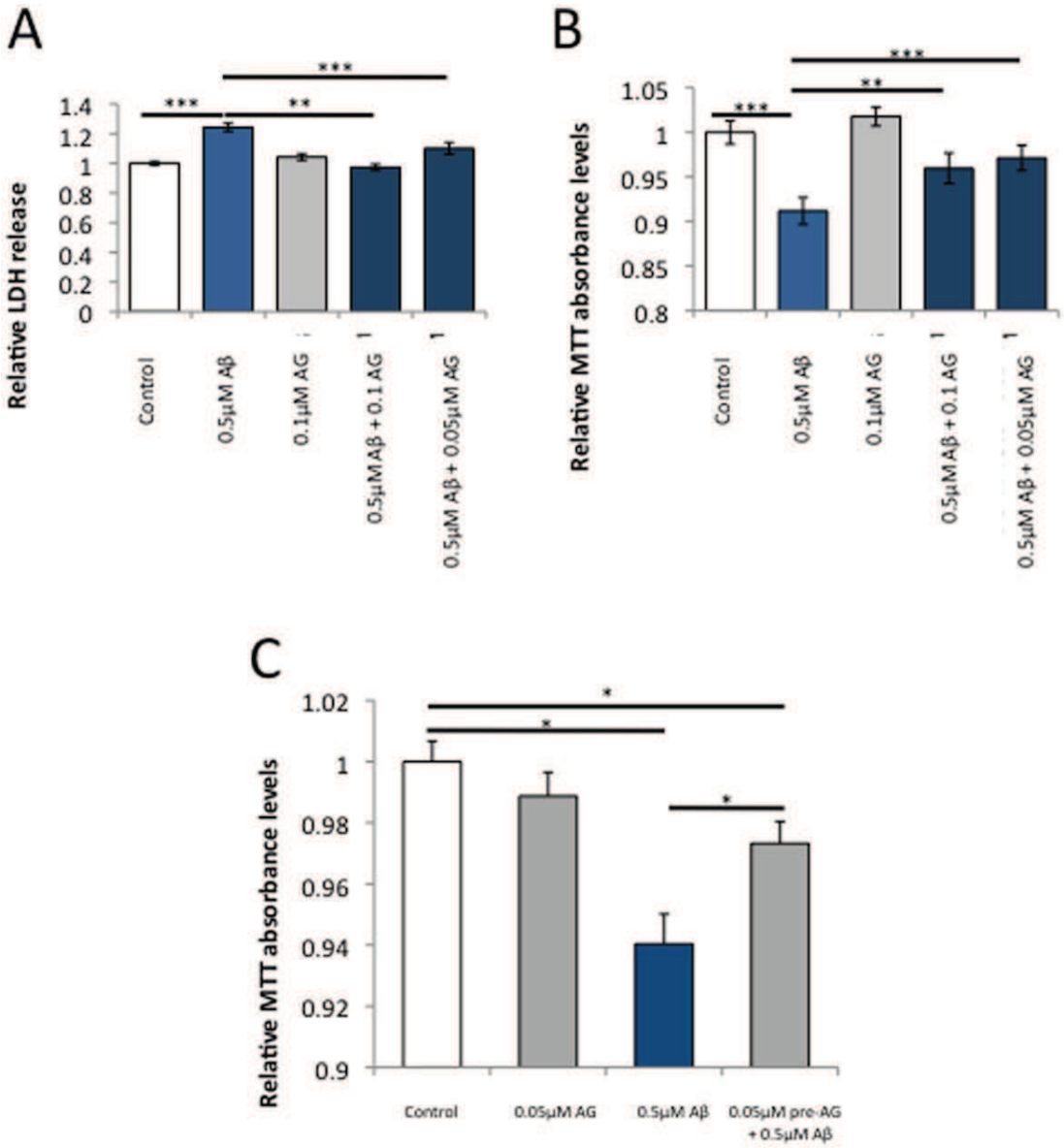

Figure 5. The ABAD inhibitor AG18051 prevents the toxicity and metabolic impairment caused by Aβ. (A) Co-incubation of AG18051 and Aβ42 maintains the Aβ42-induced change in LDH levels at baseline levels. (B) The metabolic impairment as determined with the MTT assay is also prevented by co-incubation of Aβ42 with AG18051. (C) Pre-incubation of the cells with AG18051 for 24 hours prior to adding Aβ42 is similarly protective to Aβ's toxicity as measured with the MTT assay. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.
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AG18051 restored Aβ42-induced deficits on oxidative phosphorylation (OXPHOS) capacity

To investigate the protective effect of AG18051 against Aβ42 toxicity at the mitochondrial level, we used a high-resolution respiratory protocol that we have established previously [49]. Specifically, physiological substrate combinations were used to investigate mitochondrial function in SH-SY5Y cells (Fig. 6). We compared OXPHOS, i.e. the entire electron transport system (ETS) that is composed of the four mitochondrial enzymes (complex I–IV) and the F_1F_0ATP synthase, in cells treated with either vehicle, Aβ42, AG18051, as well as in cells that were pre-treated with AG18051 followed by exposure to Aβ42 (see scheme, Fig. 3A). We used the NADH generating substrates pyruvate and malate to determine state 4 respiration (Fig. 6). State 3 respiration measures the capacity of mitochondria to metabolize oxygen and the selected substrate in the presence of a defined amount of ADP, which is a substrate for the ATP synthase (complex V). State 4 respiration represents a "basal-coupled" rate of respiratory chain activity and reflects activities of respiratory chain complexes and proton leakage across the inner mitochondrial membrane. We observed significantly reduced state 3 and state 4 respirations in Aβ42-treated cells (Fig. 6). After uncoupling with FCCP, the respiratory rate increased in the absence of a proton gradient, which indicates the maximum capacity of electron transport chain. This maximum OXPHOS capacity was again significantly impaired in Aβ42-treated (Fig. 6).

Of note, AG18051 was able to significantly ameliorate the Aβ42-induced global failure of mitochondrial respiration, but by

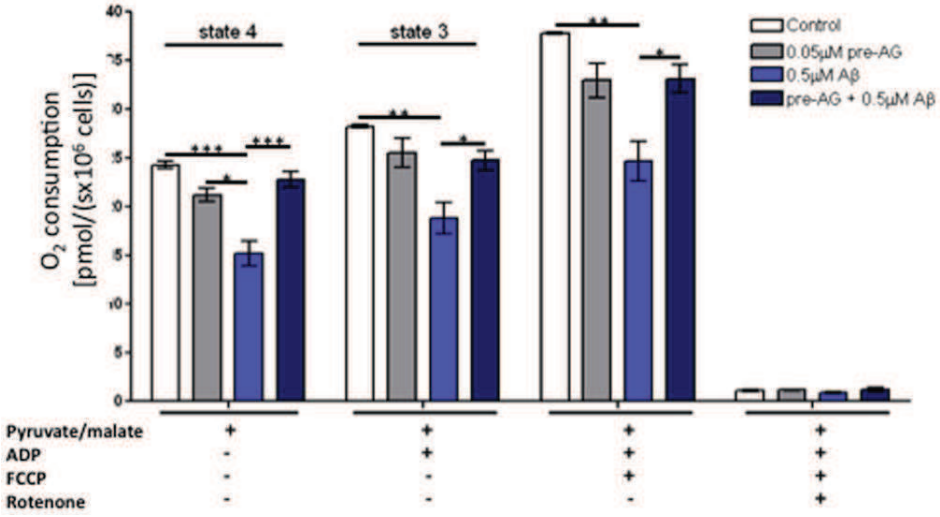

Figure 6. High-resolution respirometry revealed a reduction of oxygen consumption in Aβ42-treated cells that was restored after pre-treatment with AG18051. O_2 flux and consumption by vital cells was measured after addition of different agents: pyruvate/glutamate, ADP, FCCP, rotenone. Two-way ANOVA revealed a significant difference between the cellular respiration of the cells treated either with vehicle, Aβ42 or AG18051 alone, or AG18051 plus Aβ42 ($p<0.0001$) (see scheme Fig. 3A). The respiratory rates of mitochondria were significantly reduced in Aβ42-treated cells compared to control (vehicle treated) cells and cells pre-treated with AG18051 (24 h) before exposure to Aβ42. Values represent the means ± S.E. from n = 3–5 independent measurements. Post-hoc Bonferroni's Multiple Comparison Test analysis for single experimental respiratory conditions: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.
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itself had no effect *per se* on oxygen consumption attaining levels comparable to vehicle treatment (Fig. 6).

AG18051 prevents the increase in ROS levels caused by Aβ42

Increased ROS levels have been implicated in AD [50,51]. We had previously found that treatment of SH-SY5Y cells with Aβ42 caused significantly increased ROS (reactive oxygen species) levels, reflecting mitochondrial dysfunction and cell toxicity [52]. As pre-incubation with AG18051 was capable of preventing the toxicity induced by Aβ42, we pre-treated SH-SY5Y cells with AG18051 and exposed them then to Aβ42. Interestingly, cells pre-treated with 0.05–0.1 μM AG18051 were effectively protected from any Aβ42-induced ROS production, attaining levels comparable to vehicle treatment (Fig. 7). This suggests that AG18051 may prevent cell toxicity induced by Aβ42 in part by preventing the generation of ROS. In addition, the results also suggest that ABAD dysfunction may be upstream of ROS production since AG18051 can prevent ROS generation. Alternatively, AG18051 may trigger high estradiol levels, thereby counteracting ROS.

AG18051 only partially protects against reductions in cell viability and estradiol levels in SH-SY5Y cells when deregulated by human amylin (HA)

Previously, we had found that Aβ42 and HA share toxicity pathways via deregulation of mitochondrial proteins [52]. However, as shown above, Aβ42 binds to and down-regulates ABAD activity, while HA fails to do so (Fig. 1). We nonetheless sought to determine whether AG18051 would have an effect in the toxicity assays used above to measure Aβ toxicity. We found that HA induced an increase in LDH levels revealing its toxicity (Fig. 8A), and a corresponding decrease in MTT absorbance revealing its effect on metabolic functions (Fig. 8B). Co-incubation of AG18051 with HA significantly prevented the increase in LDH levels when compared with HA alone, but did not fully maintain LDH to the levels of vehicle treatment indicating only a partial protection from HA-induced toxicity (Fig. 8A). Co-incubation of AG18051 with HA did not have any significant impact on MTT levels (Fig. 8B).

AG18051 prevents the increase in ROS levels caused by Aβ42 and HA

Increased ROS levels have not only been implicated in AD but also T2DM [50,51]. We had previously found that ROS levels in SH-SY5Y cells are significantly increased upon exposure to either Aβ42 or HA [52]. To determine if the limited protective effect seen by AG18051 on HA-induced toxicity may be due to an inhibition of ROS generation, we pre-incubated SH-SY5Y cells with AG18051 and then exposed them to HA. Similar to Aβ42 (Fig. 7), pre-treatment with 0.05 μM AG18051 completely prevented ROS generation by 0.5 μM HA (Fig. 9). Taken together with the ROS data obtained for Aβ42 (Fig. 7), this suggests that AG18051 may be neuroprotective by preventing ROS generation induced by either Aβ42 or HA.

Discussion

Mitochondrial dysfunction has been recognized as a prominent, early event in AD, but the underlying mechanisms are only partly understood [53]. As a mediator of Aβ toxicity in AD, a role has been proposed for the mitochondrial protein ABAD, with evidence for a direct interaction of Aβ and ABAD [17]. Whether in the AD brain, intracellular Aβ - either in mitochondria or in the cytoplasm - is present at sufficiently high quantities to have a decisive role in disease is a matter to debate that has been revived by the recent analysis of Aβ plaque-forming 3xTg-AD using a panel of Aβ- and APP-specific antibodies [54]. Our current study however adds to the body of data revealing a role for ABAD in mediating Aβ, irrespective of its mode of interaction.

By using the novel compound inhibitor of ABAD, AG18051 [41], we revealed that Aβ-mediated toxicity, metabolic impairment and

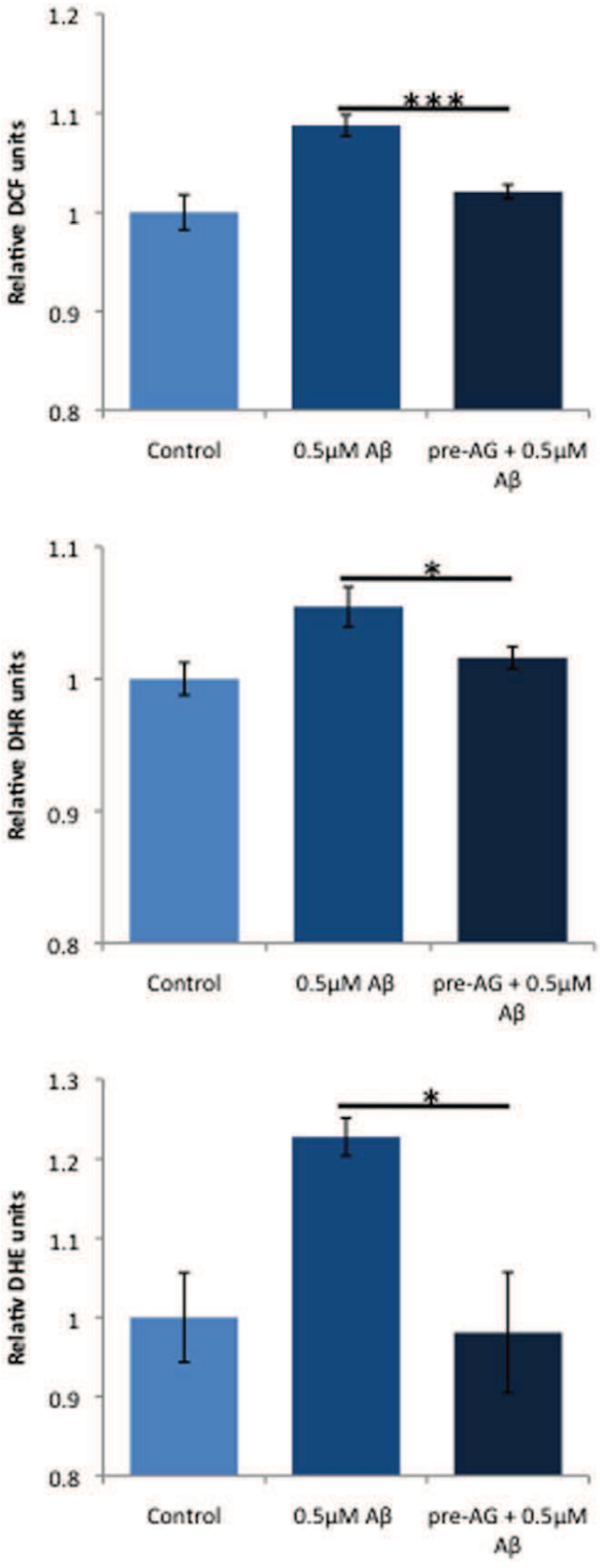

Figure 7. AG18051 pre-treatment prevents ROS generation induced by Aβ. Aβ causes reduced cellular (DCF) as well as mitochondrial ROS (DHR), e.g. reduced mitochondrial superoxide anion radicals (DHE). Levels are restored to vehicle upon pre-treatment with AG18051, irrespective of whether the SH-SY5Y cells have been incubated with Aβ or HA. *, $P<0.05$; ***, $P<0.001$.
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reductions in estradiol levels could be abrogated. However different from Aβ, the inhibitor only partially restored the toxicity of HA, that shares with Aβ its amyloidogenic properties, and it had no effect at all on the impaired metabolic activities or the reduced estradiol levels caused by HA. The compound though abolished both the Aβ42- and HA-induced increases in ROS levels. These effects may or may not require a direct interaction of Aβ and ABAD. Provided that Aβ enters mitochondria and binds ABAD, it is believed to gain access to the mitochondrial matrix via intracellular trafficking. Our pull-down experiments using a biotinylated preparation of Aβ reveal that this peptide binds to ABAD in a lysate, while in contrast, HA does not.

One of the established functions of ABAD is to convert in an equilibrium reaction estrone to estradiol, a known anti-oxidant in neuronal survival [31–33,55]. Additional substrates are known for ABAD, such as the mitochondrial 2-methyl-3-hydroxybutyryl-CoA [40,56].

ABAD is up-regulated in AD brain areas affected by Aβ pathology such as the cortex and hippocampus, as well as in Aβ-producing mouse models [17,23,26,57]. In neuroblastoma cells, the cytotoxic effects of Aβ are enhanced by ABAD over-expression, and blocked with anti-ABAD antibodies [58]. Moreover, synthetic Aβ fragments have been shown to bind and inhibit ABAD *in vitro* [39]. As cells expressing catalytically inactive mutants of ABAD failed to show an enhanced sensitivity to Aβ, it has been suggested that it is the enzymatic activity that is required for mediating Aβ toxicity [38]. *In vivo*, ABAD over-expression potentiates the toxic effects of Aβ, and obliteration of Aβ-ABAD complexes restores cell viability and memory deficits in transgenic mice [17,26]. This inhibition was achieved using a truncated version of ABAD as a decoy peptide ($ABAD^{DP}$) [17,26]. The authors of the study concluded that segregating ABAD from Aβ protects both mitochondria and neurons from Aβ toxicity by restoring ABAD's physiological functions.

Rather than employing a decoy peptide, we decided to use the small compound, AG18051, a novel ABAD inhibitor to investigate its putative protective effects. We performed co- and pre-incubation experiments to determine whether the restoration of estradiol levels by AG18051 is due to a direct inhibition of the Aβ42-ABAD interaction, or an indirect mechanism. By determining estradiol levels as a functional read-out of ABAD activity, we found that exposure to Aβ42 significantly decreased estradiol levels (Fig. 3B, C). We have been using SH-SY5Y cells, a well-established neuroblastoma cell line [43], to determine the effects of AG18051 on ABAD. We found that pre-treatment of SH-SY5Y cells with AG18051 before Aβ42 exposure (Fig. 5C) was sufficient to prevent the decrease in cell viability. This suggests that AG18051 is capable of blocking Aβ42 toxicity, possibly in part by directly binding to ABAD and preventing the Aβ42 toxicity mediated by ABAD.

To determine whether these changes are due to ABAD specifically, we performed co-incubation treatments of a novel ABAD inhibitor, AG18051, with Aβ42. We found that AG18051 effectively obliterated the toxic effects of Aβ42 as demonstrated by the restoration of LDH release and MTT absorbance to levels indistinguishable from the vehicle control. Furthermore, AG18051 restored estradiol levels upon down-regulation by Aβ42. This effect is seen both in the cell lysate and the medium (data not shown), suggesting that Aβ42 exerts its toxicity by interfering with intracellular levels of estradiol while also decreasing its secretion

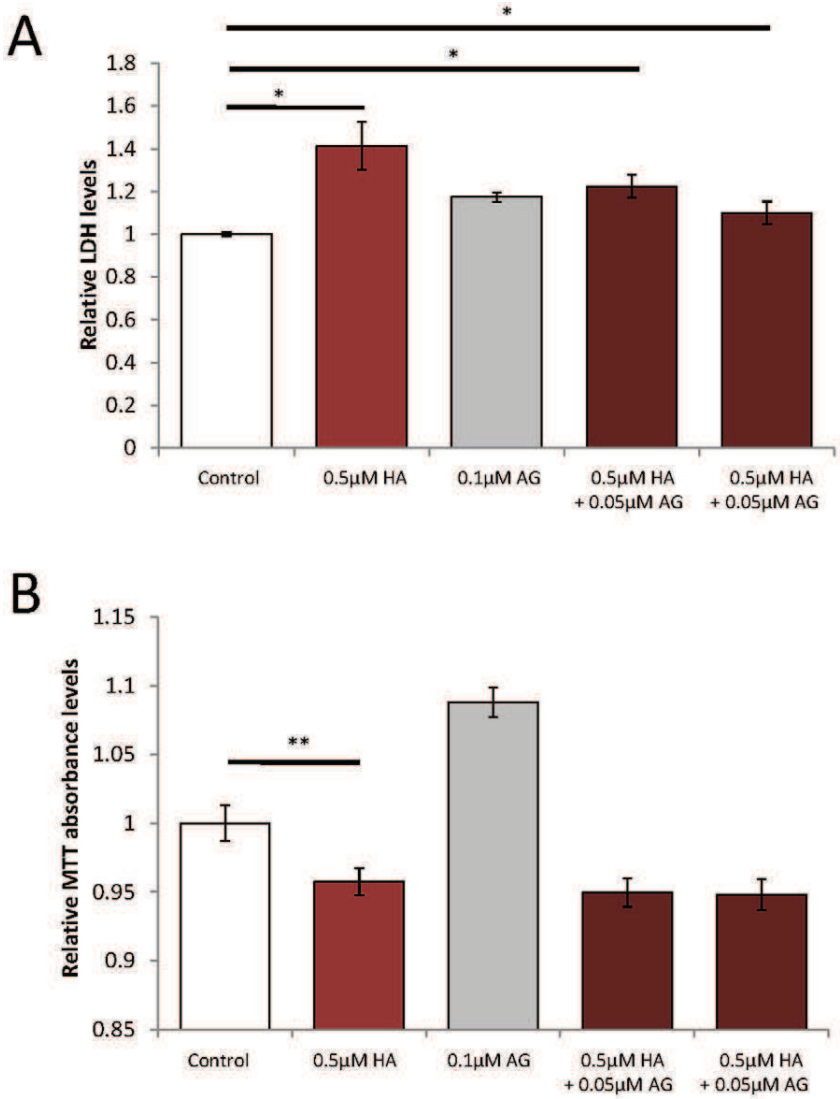

Figure 8. The ABAD inhibitor AG18051 partially prevents the toxicity of HA, but not its metabolic impairment. (A) Co-incubation of HA with AG18051 partially maintains levels of LDH release in SH-SY5Y cells suggesting that the toxicity of HA is partially mediated by ABAD. (B) Treatment with 0.5 μM HA significantly decreases metabolic activity as shown with the MTT assay, which is not prevented with co-incubation with AG18051. *, $P<0.05$; **, $P<0.01$.
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into the extracellular environment. Our data indicate that Aβ42-induced toxicity is mediated by ABAD in part via the deregulation of estradiol, which may contribute to cell toxicity.

To determine whether the change in estradiol levels is due to a direct blockage of Aβ42 by ABAD, we performed a pull-down using biotinylated Aβ42 in the presence of AG18051 (Fig. 4). We found that AG18051 significantly decreased ABAD binding to biotinylated Aβ42, suggesting that AG18051 may be neuroprotective, in part by disrupting the physical interaction of ABAD with Aβ42. However, as binding was not completely abolished, our results suggest that AG18051 may also act indirectly to prevent cell toxicity. This is supported by the fact that AG18051 is capable of preventing ROS production by HA (Fig. 9) even though HA does not seem to bind to ABAD (Fig. 1B).

To unravel the effects of Aβ42 and the involvement of ABAD on the mitochondrial respiratory capacity, we performed whole cell recording of total cellular respiration in SH-SY5Y cells (Fig. 6). Consistent with previous findings investigating the effect of a stable APP over-expression in SH-SY5Y cells gaining a chronic overproduction of Aβ within the low nanomolar range [49], we observed a comparable impairment of oxygen consumption rates in cells treated with Aβ42 species for 5 days. Of note, we present for the first time clear evidence that pre-treatment with AG18051 prevented SH-SY5Y cells from a decline in metabolic energy pathways induced by Aβ42. The capacity of mitochondria to re-phosphorylate ADP in state 3 is dependent on the degree of coupling. Thus, pre-treatment with AG18051 prevented the ETC (electronic transport chain) from Aβ42 toxicity and rescued the coupling state of mitochondria. Importantly, the comparison of the mitochondrial energetic capacity in cells treated with vehicle or AG18051 revealed a similar bioenergetic homeostasis indicating that the inhibitor, by itself, had no significant effect on respiration. These results corroborate our findings demonstrating an AG18051-induced

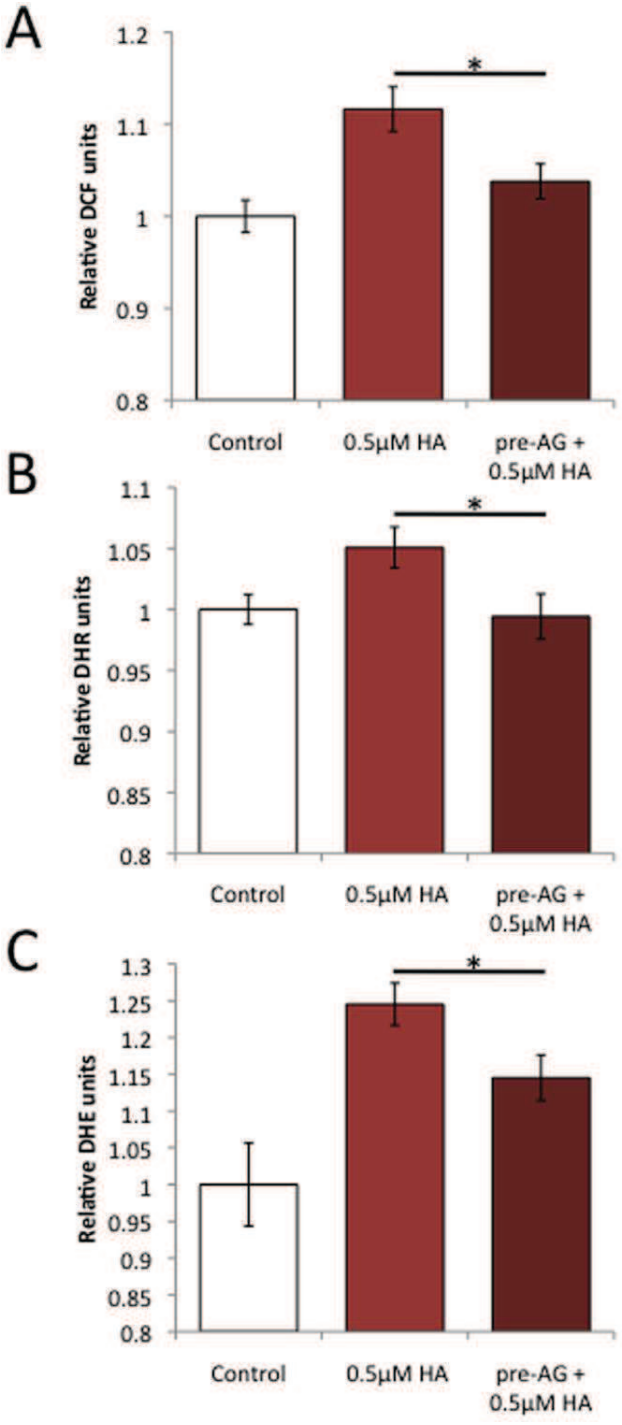

Figure 9. AG18051 pre-treatment prevents ROS generation also induced by HA. As for Aβ, HA causes reduced cellular (A) as well as mitochondrial ROS (B), e.g. reduced mitochondrial superoxide anion radicals (C). Levels are restored to vehicle upon pre-treatment with AG18051. *, $P<0.05$; ***, $P<0.001$.
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prevention of ROS formation caused by Aβ42, which in turn impairs mitochondrial function.

That a defective or insufficient mitochondrial function might play a potentially pathogenic role in another epidemic disease, Type 2 diabetes mellitus (T2DM), has emerged in recent years [59,60]. The association of diabetes with obesity and inactivity indicates an important, and potentially pathogenic, link between fuel and energy homeostasis and the emergence of metabolic disease. Given the central role for mitochondria in fuel utilization and energy production, mitochondrial dysfunction at the cellular level can impact whole-body metabolic homeostasis [61]. T2DM is characterized by HA deposition in the pancreas, and AD by Aβ deposition in the brain [59]. Aβ and HA share a common β-sheet secondary structure, a strong determinant in toxicity [62,63]. Supporting this notion we have shown previously that Aβ and HA share a mitochondrial toxicity profile [52]. In the present study we found that HA caused toxicity and impaired metabolic functions. When we co-incubated HA with AG18051 using the same conditions as for Aβ42, AG18051 significantly decreased levels of LDH compared to just HA alone, suggesting that HA toxicity is in part mediated by ABAD (Fig. 8A). Interestingly, co-incubation of AG18051 with HA did not significantly change levels of MTT absorbance, an established assay of mitochondrial function (Fig. 8B) [64]. We have previously shown that to mitochondria, at equimolar concentration, HA is more toxic than Aβ42 [52]. This suggests that a higher concentration of AG18051 may be required to restore the HA-induced mitochondrial toxicity. Interestingly, while the MTT assay is a reliable test for metabolic impairment, it does not always precisely reflect neuroprotective effects, suggesting that the LDH assay is more accurate in determining neurotoxicity [65]. It is therefore possible that the neuroprotective effects of AG18051 against HA treatment differs from that of Aβ42. By extrapolation, this also means that HA and Aβ42 may exert a differential toxicity on ABAD. This is a possibility, as exposure to HA did not alter estradiol levels, different from Aβ42 (Fig. 1A).

Pre-treatment of SH-SY5Y cells with AG18051 prevented both the cellular and mitochondrial ROS formation induced by Aβ42 and HA suggesting that ABAD is involved in their mechanism of toxicity. We have shown previously that increased ROS generation and reduced mitochondrial complex IV activity was the common mechanism of toxicity of HA and Aβ42 [52]. This is in agreement, in the case of Aβ, with other reports showing that Aβ results in ROS generation and reduction of complex IV in AD mouse models and that there is a direct involvement of ABAD in these processes [17,66,67]. Now it appears that HA ROS generation is also mediated by ABAD. However, while AG18051 may be protecting against most of the toxic effects of Aβ42, it does so only partially for HA. This indicates that HA may have additional toxicity pathways unrelated to ROS generation or ABAD.

The mechanism of protection by AG18051 is probably through its inactivation of ABAD's catalytic activity. Inactive mutants of ABAD do not enhance the toxicity of Aβ that is observed when wildtype ABAD is over-expressed, despite the fact that Aβ still binds to the mutant ABAD with the same affinity as wild-type [68]. It has also been reported that siRNA-ABAD and the subsequent reduction of ABAD expression prevents the toxic effects of Aβ in SH-SY5Y cells induced to over-express ABAD by corticosterone and Aβ [67]. But why would the catalytic activity of ABAD be harmful when Aβ is bound to it? In view of the participation of ABAD in mitochondrial RNAse P, which is responsible for the centrally important processing of the mitochondrial ETC mRNA [37], one possibility is that ABAD's primary function is as an RNAse P and that Aβ and HA induce a toxic gain of function related to its catalytic activity compromising its RNAse P function. This would result in aberrant processing of the ETC mRNA, a dysfunctional ETC and ROS generation. Aβ may do this directly by binding to ABAD and, while there is no

evidence that HA directly interacts with ABAD, our study shows that ABAD is nevertheless key to the toxicity of HA.

In conclusion, we extend previous findings on the role of mitochondria, and in particular the mitochondrial enzyme ABAD, in mediating Aβ42 toxicity. We established a neuroprotective effect of AG18051, a novel ABAD-specific inhibitor, and showed that it promotes cell survival in part by preventing the generation of ROS and stabilizing estradiol levels. Our findings extend previous studies suggesting ABAD activity as a suitable biomarker for impaired brain functions. We further present AG18051 and related compounds for consideration in therapeutic strategies targeting AD.

Author Contributions

Conceived and designed the experiments: Y-AL AE JG. Performed the experiments: Y-AL AG MG. Analyzed the data: Y-AL AE AGM-N JEV LMI JG. Contributed reagents/materials/analysis tools: JEV. Wrote the paper: Y-AL JG.

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