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Guillaume WENDT

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Assessment of neuroprotective effects of gamma-hydroxybutyrate and neurosteroids on cellular models of Alzheimer's disease

THÈSE dirigée par :

M. MENSAH-NYAGAN Ayikoé, Guy	Professeur, Université de Strasbourg, France
M. SCHMITT Manfred J.	Professeur, Universität des Saarlandes, Allemagne

RAPPORTEURS :

M. MÜLLER Uli	Professeur, Universität des Saarlandes, Allemagne
M. KIRSCH Matthias	HDR, Universität Freiburg, Allemagne

AUTRES MEMBRES :

M. DUFOUR André	Professeur, Université de Strasbourg, France
M. DIEHL Björn	CR, Universität des Saarlandes, Allemagne
Mme KEMMEL Véronique	MCF, Université de Strasbourg, France

A mon père

A ma famille

A mon épouse et mes deux filles

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Abstract

This PhD work showed that GHB and neurosteroids efficiently protect neuroblastoma cells against nerve cell death caused by Alzheimer's disease etiological factors including amyloid precursor protein overexpression and oxidative stress. Interestingly, an additive action of GHB and allopregnanolone was identified that may result from the combination of partial stimulation of anti-apoptotic protein expression induced by both compounds. GHB protective effect was blocked by aromatase inhibitors, suggesting that GHB may also induce neuroprotection via the activation of neurosteroidogenesis. Finally, we have used a yeast-based MMP activity assay to check whether GHB and neurosteroids can regulate the activity of human MMP-2 and MMP-9, which both control Aβ peptide degradation. Although we cannot yet conclude from our preliminary results, further improvement of the experimental setup in combination with RT-qPCR and western analyzes in human neuroblastoma cells will help to determine the modulatory action of GHB and neurosteroids on MMP activity and/or expression. Together, our data suggest that GHB and neurosteroids may be used to develop combined neuroprotective strategies against neuronal loss in Alzheimer disease.

Zusammenfassung

In der vorliegenden Doktorarbeit konnte gezeigt werden, dass Gamma-Hydroxybutyrat (GHB) und Neurosteroide effektiv in der Lage sind, Neuroblastoma Zellen vor den ätiologischen Faktoren der Alzheimer-Krankheit, darunter insbesondere durch oxidativen Stress und Überexpression von Amyloid-Precursor-Proteinen verursachten Zelltod, zu schützen. Interessanterweise wurde eine additive neuroprotektive Wirkung von GHB und Allopregnanolon gegen den durch oxidativen Stress induzierten Zelltod beobachtet. Diese additive Wirkung ist vermutlich auf eine spezifische Aktivierung von anti-apoptotischen Signalwegen durch GHB und Allopregnanolon zurückzuführen. Die Schutzwirkung von GHB wurde durch Aromatase-Inhibitoren blockiert, was darauf schließen lässt, dass GHB möglicherweise die Neurosteroidogenese aktiviert. Abschließend wurde mit Hilfe eines Hefe-basierten MMP-Aktivitätstests überprüft, ob GHB und/oder Neurosteroide die Aktivität von humanem MMP-2 bzw. MMP-9, welche den Abbau von Aβ-Peptiden kontrollieren, direkt beeinflussen. Auch wenn mit dem verwendeten Testsystem noch kein signifikanter Effekt von GHB und Neurosteroiden beobachtet wurde, sollte eine weitere Optimierung des Testsystems kombiniert mit RT-qPCR und Western-Analysen an humanen Neuroblastoma Zellen dazu beitragen, mögliche regulatorische Effekte von GHB und Neurosteroiden auf die MMP-Aktivitat und -Expression zu bestimmen. Zusammenfassend deuten die vorliegenden Daten darauf hin, dass GHB und Neurosteroide möglicherweise als kombinierte Neuroprotektiva in der Alzheimer-Therapie Anwendung finden könnten.

Abreviations

AD	Alzheimer's disease
$[Ca^{2+}]_i$	Intracellular calcium
17β-HSD	17β-Hydroxysteroid dehydrogenase
3α,5α-THP	3α, 5α-Tetrahydroxyprogesterone or allopregnanolone
3α-DIOL	3α-androstanediol
3α-HSOR	3α-Hydroxysteroid oxidoreductase
3β-HSD	3β-Hydroxysteroid dehydrogenase
5α-R	5α-Reductase
ABAD	Aβ binding protein alcool dehydrogenase
ACE	Angiotensin converting enzyme
acetyl-CoA	Acetyl-Coenzyme A
ADAM	A-disintegrin and metalloprotease
AF1	Activation function 1 domain
AF2	Activation function 2 domain
AICD	APP intracellular domain
AMPAR	α-amino-3-hydroxy-5-methyl-4- isoxazoleproprionic acid receptor
ANOVA	Analysis of variance
AOX1	Alcohol oxidase 1
AOX2	Alcohol oxidase 2
AP	Allopregnanolone
Apaf-1	Apoptotic protease activating factor 1
APH1	Anterior pharynx defective
APP	Amyloid precursor protein
APP-CTF_β	APP carboxy-terminal fragment
APPwt	Wild-type APP
APS	Ammonium persulfate
AR	Androgens receptors
ARG	Arginine
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate

Aβ	Beta-amyloid peptides
BACE-1	β-site APP cleaving enzyme 1
Bax	Bcl-2 associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell Lymphoma 2
BiP	Binding immunoglobulin protein
BIS	N,N'-methylene-bis-acrylamide
BMG	Buffered Minimal Glycerol
BMM	Buffered Minimal Methanol
BSA	Bovine serum albumine
cAMP	Cyclic adenosine monophosphate
caspases	Cysteine-dependent, aspartate-specific proteases
CAT	Catalase
CDK5	cyclin-dependent kinase 5
cDNA	Complementary DNA
CHOP	C/EBP-homologous protein
CNS	Central nervous system
COX	Cytochrome c oxidase
CREB	cAMP response element binding protein
Cyt c	Cytochrome c
DBD	DNA-binding domain
DHEA	Dehydroepiandrosterone
DHP	Dihydroprogesterone
DHT	Dihydrotestosterone
DMEM	Dubelcco’s modified eagle medium
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double strand DNA
E2	Estradiol
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EIF2α	Eukaryotic translation initiation factor 2α

ER	Endoplasmic reticulum
E2R	Estrogens receptor
ERAD	ER associated degradation
ERE	Estrogen response elements
ERK2	Extracellular signal-related kinase 2
ETC	Electron transport chain
FACS	Fluorescence activated cell sorting
FAD	Familial Alzheimer's disease
$FADH_2$	Flavin adenine nucleotide
FITC	Fluorescein
FURA-2 AM	FURA-2 acetoxymethyl ester
GABA	γ-aminobutyric acid
$GABA_A$-R	γ-aminobutyric acid type A receptors
$GABA_B$-R	γ-aminobutyric acid type B receptors
GABA-T	GABA-transminase
GADD153	DNA damage inducible gene 153
GHB	Gamma-hydroxybutyrate
GHBh1	GHB human receptor type 1
GHB-R	GHB-receptor
GHB-T	GHB-transporter
GPCRs	G protein-coupled receptors
GPX	Glutathione peroxidase
GR	Glucocorticoids receptor
GRP78	Glucose-regulated protein 78kDa
GSK3β	Glycogen synthase kinase 3β
HIS	Histidine
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HMGR	HMG-CoA reductase
HRP	Horse raddish peroxidase
IDE	Insulin degrading enzyme
IMM	Inner membrane
IMS	Inter-membrane space
IP3R	Inositol triphosphate receptor

IRE1α	Inositol-requiring enzyme 1α
JNK	JUN amino-terminal kinase
LBDs	Ligand binding regions
LTD	Long term depression
LTP	Long term potentiation
MAMs	Mitochondria-ER associated membranes
MAPK	Mitogen activated protein kinase
MAPs	Microtubule-associated proteins
MCI	Mild cognitive impairment
MMP-2	Matrix Metalloproteinase 2
MMP-9	Matrix Metalloproteinase 9
MOMP	Mitochondrial outer membrane permeabilization
MR	Mineralocorticoids receptor
mRNA	Messenger ribonucleic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NADH	Nicotine adenine dinucleotide
NEP	Neprylysin
NFTs	Neurofibrillary tangles
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	Nuclear localization signal
NMDAR	N-methyl-D-aspartate receptor
NR2A	NMDAR subunit 2A
NR2B	NMDAR subunit 2B
NR	Nuclear receptors
OD	Optical density
OxPhos	Oxidative phosphorylation
P450c17	Cytochrome P450c17 or 17α-hydroxylase
P450scc	Cytochrome P450side chain-cleavage
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDH	Pyruvate dehydrogenase
PERK	Protein kinase RNA-like ER kinase
PE	Phycoerythrin

PI3K/Akt	Phosphatidylinositol 3-kinase and protein kinase B
PNS	Peripheral nervous system
PPP	Pentose phosphate pathway
PR	Progestins receptors
PSD95	Post-synaptic density protein 95
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PTPC	Permeability transition pore complex
PVDF	Polyvinylidene
PXR	Pregnane xenobiotic receptor
RFU	Relative fluorescence intensity
RIPA buffer	Radio Immunoprecipitation assay buffer
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-qPCR	Real time quantitative polymerase chain reaction
RyR	Ryanodine receptor
SAD	Sporadic Alzheimer's disease
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide Gel Electrophoresis
SEM	Standart error of the means
SERCA pumps	Sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPases
SOD	Superoxide dismutase
SRCs	Steroid receptor coactivators
SSA	Succinic semialdehyd
SSADH	Succinic semialdehyde dehydrogenase
SSR	Succinic semialdehyde reductase
StAR	Steroidogenic acute regulatory protein
STAT3	Signal transducer and activator of transcription 3
SUC	Succinate
TBS	Tris Buffered Saline
TC	Tunicamycin
TCA	Tricarboxilic acid

TEMED	-N,N,N',N'-tetramethylethylene diamine
THDOC	Tetrahydrodeoxy-corticosterone
THG	Thapsigargin
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumor necrosis factor α
TRIzol	Guanidinium thiocyanate-phenol-chloroform
Trxr-1	Thioredoxin reductase 1
TSPO	Translocator protein (18-kDa)
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPR	Unfolded protein response
UQ	Ubiquinone
VDAC	Voltage-dependent anion channel
VIAAT	Vesicular inhibitory amino acid transporter
XBP-1	X-box binding protein 1
YNB	Yeast nitrogen base
α7-nAChR	α7-nicotinic-acetylcholine receptor
αKGD	α-ketoglutarate dehydrogenase

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1. Introduction

1.1. Alzheimer's disease

1.1.1. Clinical definition and etiological factors

Alzheimer's disease (AD), which was first described by Alois Alzheimer in 1907 (Alzheimer, 1907), is the most frequent cause of dementia in Western societies affecting more than 35 million people worldwide, including 860,000 in France and 1.2 million in Germany (Sperling et al., 2011). Thus, AD represents a major health concern identified as a research priority in several countries. The clinical course of AD represents a continuum including three phases; the Preclinical phase, the mild cognitive impairment (MCI) phase and the dementia itself (see Fig. 1) (Sperling et al., 2011). MCI is an intermediate state in which persons have more memory problems than subtle mnesic disturbances considered as normal for their age, but their symptoms are not severe as in AD patients. As the disease progresses (usually a period of 7 to 10 years), AD patients become vegetative and totally dependent for all bodily functions (Petersen et al., 1999). Since there are no curative treatments available yet, the final issue of the pathology is always the death of patients due to secondary complications.

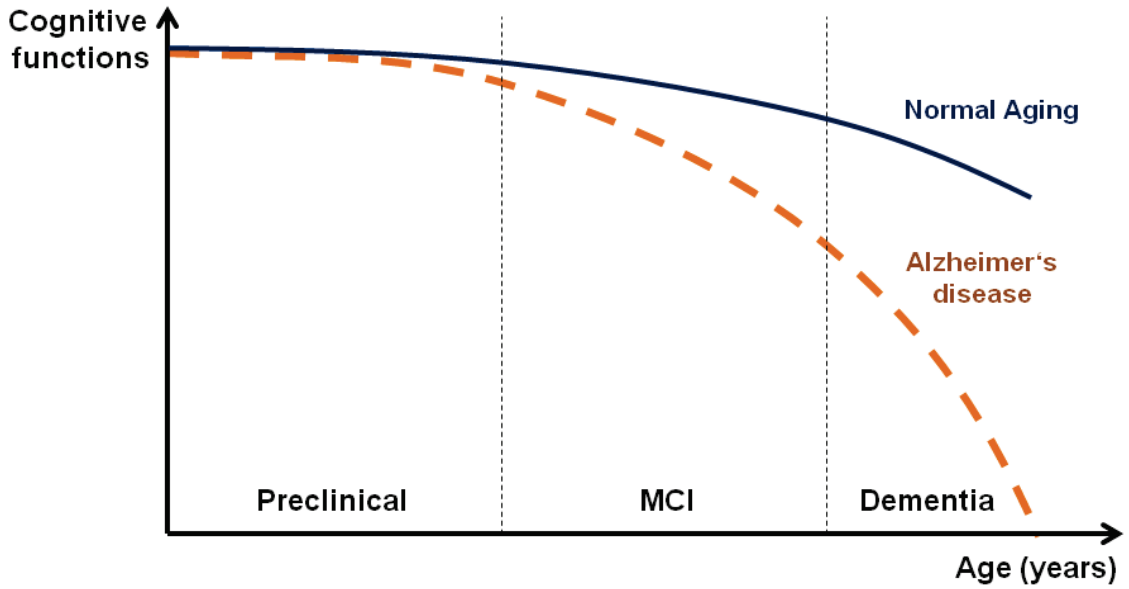

Figure 1 : Clinical course of Alzheimer's disease. The preclinical phase is asymptomatic. Thereafter, mild cognitive impairment is an intermediate state in which persons have more memory problems than normal cases, but the symptoms are not as severe as the symptoms of Alzheimer's dementia. In dementia, symptoms intensify and people become vegetative and totally dependent for all bodily functions.

1.1.2. Biological hallmarks and mechanisms

Many molecular lesions have been detected in AD, but two major hallmarks are the accumulation of missfolded proteins such as extra-cellular hydrophobic beta-amyloid peptides (Aβ) and intra-cellular neurofibrillary tangles (NFTs) (Fig 2). Amyloid plaques and NFTs are common features of rare familial AD (FAD) forms (less than 1% of all cases) as well as of sporadic AD (SAD) patients representing the majority of all cases (Querfurth and LaFerla, 2010, Ballard et al., 2011).

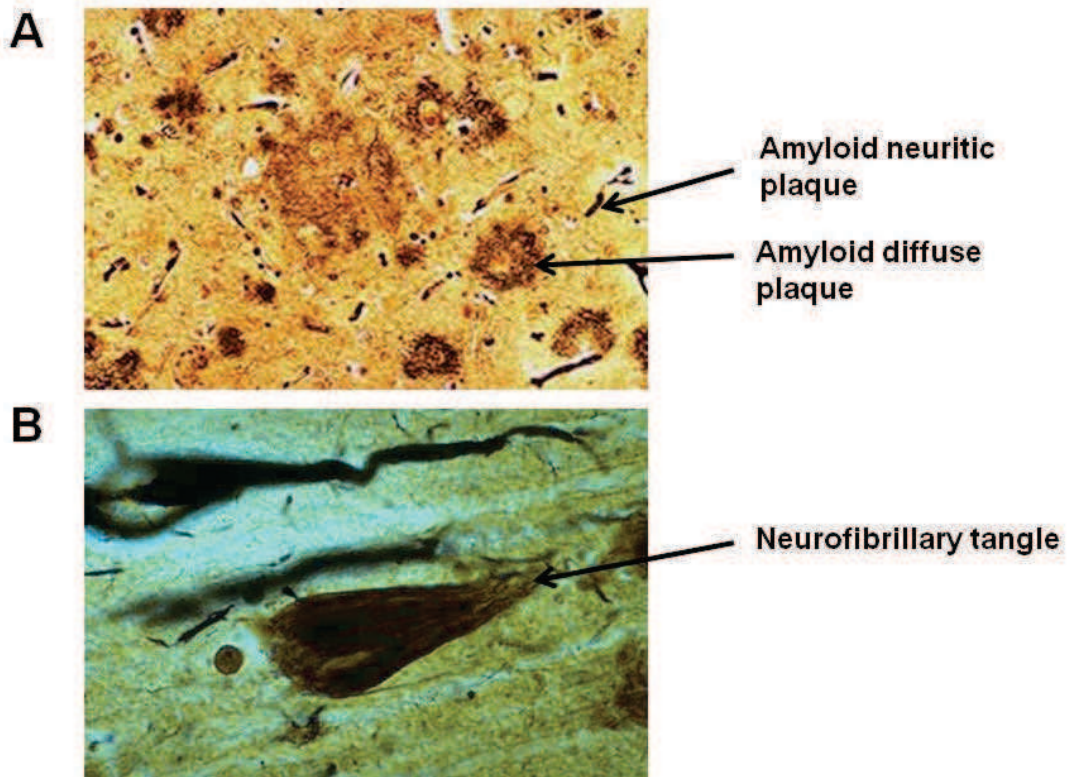

Figure 2 : Amyloid plaques and neurofibrillary tangles in human cerebral cortex of Alzheimer's disease patient. (A) Beta-amyloid peptides deposits either in diffuse plaques and in plaques containing elements of degenerating cells, termed neuritic plaques. (B) Neurofibrillary tangles results of intracellular deposition of hyperphosphorylated tau protein. (Adapted from http:// http://library.med.utah.edu/).

1.1.2.1. *Amyloid peptides*

Beta-amyloid peptides result from the multistep proteolytic cleavage by secretases of amyloid precursor protein (APP), a membrane-bound precursor. APP can be processed in two different cleavage-pathways : (i) the amyloidogenic pathway leads to the formation of hydrophobic Aβ peptides (De Strooper et al., 2010) and (ii) the non-amyloidogenic pathway, generating hydrophilic fragments. These two pathways are physiologically balanced in non pathological conditions, but in case of AD, the amyloidogenic pathway is over-activated. Although several hypotheses are suggested in the literature, the mechanisms leading to this over-activation remain

unclear (De Strooper, 2010, De Strooper et al., 2010, Querfurth and LaFerla, 2010, Ballard et al., 2011).

In the amyloidogenic pathway, APP is cleaved by *β*-secretase and *γ*-secretase (Fig. 3). *β*-secretase releases the ectodomain $APPs_{\beta,}$ and the remaining APP carboxy-terminal fragment ($APP\text{-}CTF_{\beta}$) is subsequently cleaved by *γ*-secretase which generates extracellular Aβ peptides and APP intracellular domain (AICD). The biological functions of $APP_{s\beta,}$ Aβ and AICD are poorly understood, although Aβ release is associated with decreased synaptic activity and abnormal neurotransmission (Kamenetz et al., 2003). AICD has been proposed to be a transcription factor (Cao and Sudhof, 2001) but this suggestion is controversial (Hebert et al., 2006, Waldron et al., 2008).

The non-amyloidogenic pathway involves *α*-secretase activity which releases $APPs_{\alpha}$ ectodomain and carboxy-terminal $APP\text{-}CFT_{\alpha}$ is thereafter processed by *γ*-secretase generating a small p3 fragment and AICD (Fig. 3). It has been shown that $APPs_{\alpha}$ ectodomain may exert neuroprotective effect and synapse-promoting action, but the mechanisms involved are most non elucidated (Bandyopadhyay et al., 2007). *α*-Secretase is a family of membrane-bound metalloproteases. Several members of the “A disintegrin and metalloprotease” or ADAM family have been implicated as *α*-secretases. ADAM-9, -10, and -17 appear to be the major members of the *α*-secretase family (De Strooper, 2010). In contrast to the *α*-Secretase family, *β*-Secretase activity is mainly due to one enzyme : the *β*-site APP cleaving enzyme 1 (BACE-1). BACE-1 is a membrane-bound aspartyl protease, and its genetic inactivation results in a dose dependent decrease of Aβ generation and deposition in various APP-overexpressing mouse models (Laird et al., 2005, McConlogue et al., 2007, Kobayashi et al., 2008). *γ*-Secretase is a multi-enzymatic complex composed

of four proteins that are present at equal stoechiometry : Presenilin 1 (PSEN1) or Presenilin 2 (PSEN2), nicastrin, anterior pharynx defective 1 (APH1) and presenilin enhancer 2. In total, there are four different γ-secretase complexes allowing therefore several cleavage possibilities of APP (De Strooper, 2010).

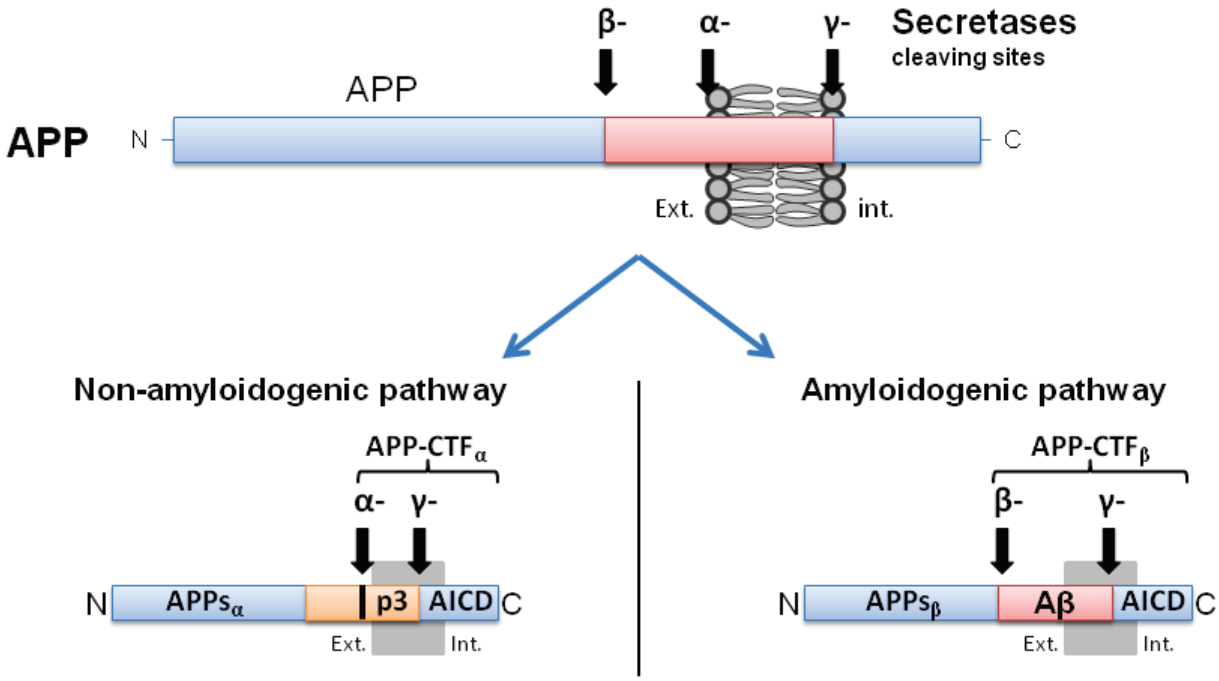

Figure 3 : Proteolysis of Amyloid Precursor Protein. Full length APP can be cleaved by α-, β- and γ-secretases. Cleaving sites are indicated. Cleavage by α-secretase yields a soluble ectodomain ($APPs_{\alpha}$) and a membrane-bound carboxyterminal fragment ($APP\text{-}CTF_{\alpha}$) which after cleavage by γ-secretase yields p3 and the APP intracellular domain (AICD). β-secretase (also known as β-site APP cleaving enzyme 1, BACE-1) yields $APPs_{\beta}$ and $APP\text{-}CTF_{\beta}$, which is then processed by γ-secretase into Aβ and AICD. Adapted from De Strooper, 2010.

It is important to realize that Aβ peptides consist of a heterogenous mixture of peptides having different solubility, stability and biological/toxic properties. C-terminal heterogeneity is generated by the γ-secretase complex itself. This protease cleaves

APP at different positions (Fig. 4), generating a variety of peptides consisting of 38 to 43 amino acids. Additional heterogeneity is due to further extracellular enzymes (Kumar et al., 2011) resulting in a mix of more than 20 Aβ peptides that all participate to different putative functions in the normal brain and also to oligomerization and fibrillization in the AD brain. Aβ40 is continuously produced in both healthy and AD brain, whereas other Aβ peptides such as Aβ42 are continuously produced at much higher levels in AD brains (Benilova et al., 2012).

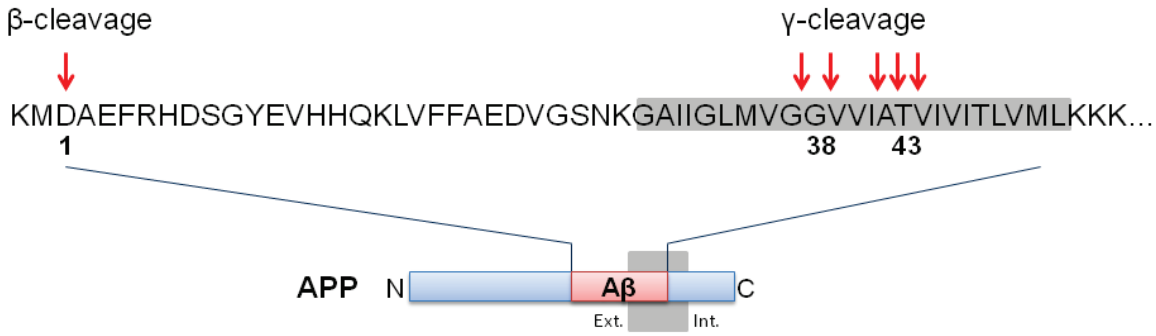

Figure 4 : Generation of Aβ peptides from APP. The sites of secretases-mediated cleavage are indicated with arrows, and the transmembrane domain of APP is highlighted in grey. γ-secretase-mediated cleavages produce a pool of Aβ peptides, varying in their lenght and hydrophobicity. Adapted from Benilova et al., 2012.

The well known peptide is Aβ42 which was firstly described in FAD. C-terminal fragments of Aβ42 provided the conditions initiating polymerization mechanisms leading to the formation of amyloid plaques (Jarrett et al., 1993a, b).

Aβ monomers (~4kDa) spontaneously self aggregates into multiple coexisting physical forms. One form consists of oligomers (2 to 6 peptides), which merge into intermediate assemblies (Kayed et al., 2003). Aβ can also grow into fibrils, which

assembly β-sheets to form the insoluble fibers of advanced amyloid plaques (Fig. 5). Soluble oligomers and intermediate amyloid are the most neurotoxic form of Aβ.

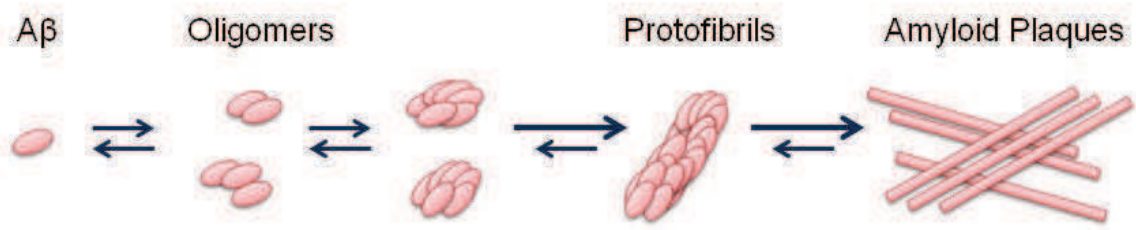

Figure 5 : Aggregation of Aβ into oligomers, fibrils and plaques. Due to its hydrophobicity, Aβ monomers self aggregate into various oligomers or larger conformation until Amyloid Plaques. Most of toxicity is assumed by the oligomeric forms. Adapted from De Strooper, 2010.

The fact that many research studies used solutions containing only a single type of Aβ in order to investigate aggregation and toxicity properties may not be appropriate to clarify AD pathogenesis. Indeed, Aβ accumulation and toxicity are likely to be strongly mediated by the concomitant presence of various Aβ species. In addition to Aβ42, Aβ43 is increased in some FAD, while shorter peptides are decreased (Portelius et al., 2010). Moreover, recent evidence show that Aβ polymerization represents a complex “melting pot“ of different Aβ species which occurs via metastable intermediaries in a process called “nucleated conformational conversion” (Benilova et al., 2012).

1.1.2.1.1. Biological clearance of amyloid peptides from the brain

The physiological Aβ fractional clearance rate is estimated to be about 8% per hour (Bateman et al., 2006). Physiological parameters, like blood and cerebrospinal fluid in the brain together with various “clearance receptors” are implicated in the removal of Aβ, which can also be taken up by endothelial cells of the blood-brain-barrier (De Strooper, 2010). Defects in these clearance processes appear to be relevant for the accumulation of Aβ on the blood vessels walls, causing vascular abnormalities and angiopathy observed in AD brains.

The proteolytic machinery in cerebral tissues also contribute to the clearance of Aβ peptides and fibrils. Knockout experiments of specific proteases demonstrated the increase of cerebral concentrations of Aβ peptides (Table 1).

Table 1 : Most proteases involved in Aβ turnover.

Protease	Type protease	Membrane bound (M) or secreted (S)	Degrading Aβ monomers (M), Oligomers (O) or fibrils (F)	References
Neprylysin (NEP)	Zn^{2+}	**M**	**M, O**	(Iwata et al., 2001)
Matrix Metalloproteinase 2 (MMP-2)	Zn^{2+}, Ca^{2+}	**S**	**M, O, F**	(Yin et al., 2006)
Matrix Metalloproteinase 9 (MMP-9)	Zn^{2+}, Ca^{2+}	**S**	**M, O, F**	(Yan et al., 2006)
Insulin degrading enzyme (IDE)	Zn^{2+}	**M and S**	**M**	(Farris et al., 2003)
Angiotensin converting enzyme (ACE)	Zn^{2+}	**M**	**M**	(Zou et al., 2007)
Plasmin	Serine protease	**S**	**M, O, F**	(Jacobsen et al., 2008)

Matrix-Metalloproteinases are secreted zinc- and calcium-dependent endopeptidases. MMP-2 and MMP-9, also known as gelatinases, are secreted and activated in the extra-cellular compartment. They modulate various physiological functions including neuritic outgrowth and matrix remodeling (Page-McCaw et al., 2007, Ould-yahoui et al., 2009) but they are also involved neuroinflammation or tumor invasiveness (Curran and Murray, 1999, Yong, 2005). MMP-2 and MMP-9 are expressed at low levels in the brain, but their astrocytal expression can be stimulated by Aβ peptides. Knockout of MMP-2 results in increased concentration of Aβ peptides in the soluble fraction of hippocampus and cortex, while infusion of broad-spectrum of MMPs inhibitor in the brain also induced an increase of Aβ levels (Yin et al., 2006).

In both FAD and SAD, there is a clear unbalance between the production and the clearance of Aβ peptides. Therefore, one therapeutic strategy may be the up-regulation of the brain expression/activity of amyloid degrading enzymes such as MMP-2 or MMP-9.

1.1.2.2. *Neurofibrillary tangles*

Neurofibrilary tangles (NFTs) are filamentous inclusions found in cell bodies and apical dendrites in AD and other neurodegenerative disorders generally termed tauopathies (Lee et al., 2001). Usually, the number of NFTs reflects the severity of the disease. NFTs are mainly composed of highly phosphorylated and aggregated forms of the tau protein which belongs to the family of microtubule-associated proteins (MAPs) abundant in axons (Grundke-Iqbal et al., 1986). Tau promotes the assembly and stability of microtubules and vesicle transport in axons. Hyperphosphorylation of Tau leads to an insoluble form which lacks affinity for

microtubules and self-associate into paired helical filaments and finally form the NFTs (Fig. 6). Both Aβ and Tau undergo nucleation-dependent fibril formation.

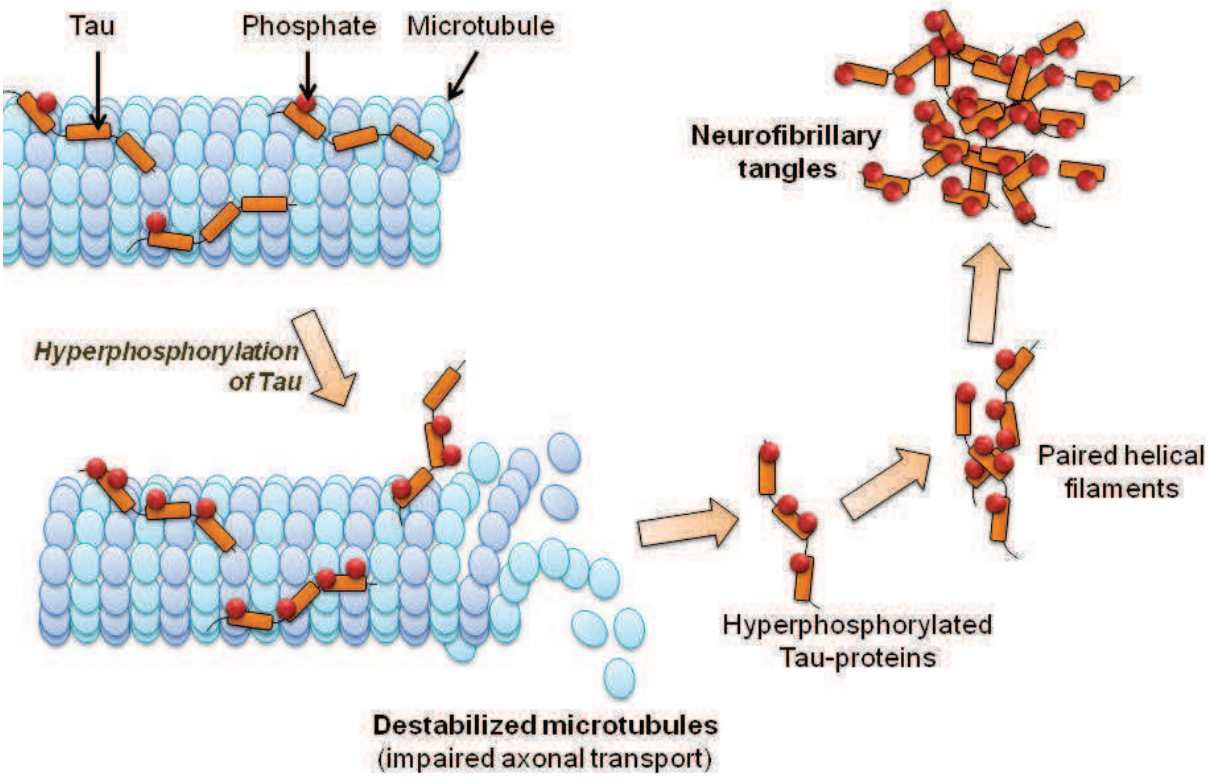

Figure 6 : Formation of paired helical filaments and neurofibrillary tangles. Tau binding promotes microtubules assembly and stability. Excessive kinases and reduced phosphatases activities lead to an hyperphosphorylation of Tau and induces its detachment and self-aggregate. Adapted from Querfurth and LaFerla, 2010.

Like Aβ oligomers, intermediate aggregates of hyperphosphorylated Tau are cytotoxic and impair cognition (Santacruz et al., 2005, Khlistunova et al., 2006). Tau mutations do not occur in AD and increased levels of phosphorylated Tau and total Tau in the cerebrospinal fluid correlate with the reduction of cognitive performance (Wallin et al., 2006). Evidence show that Aβ peptides accumulation precedes and drives Tau-aggregation (Gotz et al., 2001). Moreover, Aβ toxicity requires the presence of endogenous Tau (Roberson et al., 2007, Ittner et al., 2010, Ittner and Gotz, 2011). Tau contains 80 serine and threonine residues and 5 tyrosines that can

be phosphorylated. In physiological conditions, there are 2-3 Mol of phosphate per Mol of Tau. But in AD brain, there are 7-8 Mol of phosphate per Mol of Tau. Many phosphokinases, including glycogen synthase kinase 3β (GSK3β), cyclin-dependent kinase 5 (CDK5), and extracellular signal-related kinase 2 (ERK2) and mitogen activated protein kinase (MAPK) have been investigated as potential targets to reduce Tau phosphorylation (Takashima, 2009).

If Aβ peptides colonize synchronously predilection sites in the brain of AD patients (in hippocampus, amygdala and prefrontal cortex), NFTs develop an spread in a predictable manner. Braak and collaborators organized AD progression into six stages based on the distribution of NFTs (Braak and Braak, 1996). During the first stage, NFTs are observed in the transentorhinal and *Cornu Ammonis* (CA) regions of the hippocampus. The number of NFTs increases in Braak stage II. Stages I and II together are called the “transentorhinal stage”. Brains of normal non-demented aged subjects are often categorized as Braak stages I and II. In Braak stage III and IV, called the “limbic stage”, many NFTs appear in the entorhinal cortex, and tangles are found in the entire limbic system, including hippocampal regions CA1-4 and the amygdala. During the limbic stage, patients show various AD-specific symptoms, such as memory impairment, reduced spatial cognition and increased anhedonia, as a result of neuronal dysfunctions in the limbic system. In Braak stages V and VI, called the “isocortical stage”, NFTs are present in the cerebral cortex, where they impair neuronal functions, leading to dementia. The increasing spread of NFTs from the transentorhinal cortex to the limbic system, and finally to the cortex, correlates with the severity of cognitive impairment. Samuel et al. reported that the number of NFTs in the hippocampal formation correlates with the degree of dementia and that synapse loss is a key determinant of dementia in AD (Samuel et al., 1994).

1.1.3. Biological consequences of Amyloid peptides and neurofibrillary tangles

1.1.3.1. *Synaptic failure and axonal transport impairment*

Aging itself causes synaptic loss (Masliah et al., 2006), therefore, it is easy to understand that AD is primarily a disorder of synaptic transmission (Selkoe, 2002). As aforementioned, NFTs and synaptic loss are pivotally involed in AD physiopathology. In parallel to Braak spreading of the disease, hippocampal synapses begin to decline in MCI patients (Scheff et al., 2007). Some evidence reveal a 25% decrease of presynaptic vesicle synaptophysin in mild AD (Masliah, 2001, Masliah et al., 2001). In the last Braak stages, dramatic synaptic loss is positively correlated with dementia (Terry et al., 1991).

Aβ peptides are known to impair the “long term potentiation” (LTP), an experimental indicator of memory formation (Palop and Mucke, 2010). Subsequently, signaling molecules important to memory are also inhibited. Many studies investigated the effects of Aβ peptides on excitatory synaptic transmission, that is tightly regulated by the number of active N-methyl-D-aspartate receptor (NMDAR) and the α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptor (AMPAR). NMDAR activation has a central role in memory, by inducing LTP or long term depression (LTD), depending on the extent of the resultant intracellular calcium ($[Ca^{2+}]_i$) rise in the dendritic spines and the downstream activation of intracellular cascades (Kullmann and Lamsa, 2007). Activation of post-synaptic NMDARs and large increases in ($[Ca^{2+}]_i$) are necessary for LTP, whereas internalization of NMDARs, activation of perisynaptic NMDARs and lower increase in $[Ca^{2+}]_i$ are necessary for LTD. LTP induction promotes recruitment of AMPARs and growth of dendritic spines, whereas LTD induces spine shrinkage and synaptic loss (Kullmann and Lamsa, 2007).

Pathologically elevated Aβ may indirectly cause a partial block of NMDARs and induce LTD and synaptic loss. Although the mechanisms underlying Aβ-induced LTD remain poorly understood, they may involve the internalization or desensitization of receptors. Aβ effects on synaptic function may be mediated by the activation of presynaptic α7-nicotinic-acetylcholine receptor (α7-nAChR) and perisynaptic activation of NMDARs, inducing excessive Ca^{2+} inwards and downstream effects on many intracellular cascades including the activation of GSK3β and MAPK signaling pathways. Aβ-induced synaptic depression may result from NMDAR activation followed by NMDAR desensitization, internalization and by the stimulation of perisynaptic NMDARs or metabotropic glutamate receptors. These processes lead to a chronic increase in excitotoxic Ca^{2+} inwards, which can also lead to cell death.

Several data involve oligomeric Aβ in synapse failure, but until recently, only little was was known about the role of Tau in these dysfunctions. Ittner and colleagues described a dendritic function of Tau, mediating Aβ toxicity via NMDAR conformation (Ittner et al., 2010). Tau is able to target the src kynase Fyn to the dendrite, where it phosphorylates the NMDAR subunit NR2B, thereby facilitating complex formation with the post-synaptic density protein 95 (PSD95) and allowing excitotoxic over-activation of NMDAR by Aβ.

Moreover, neurons are elongated cells, and in order to maintain neuronal functions and excitability, they need efficient delivery of cellular organelles (such as mitochondria, endoplasmic reticulum, lysosome..) from the soma to the axon, dendrite and synapse. The delivery of organelles is purely based on microtubules, which serves as “rail tracks”, motor protein (such as kynesin or dynein) that represent engines, and organelles as cargoes which are directed to the cell-periphery or back again to the soma. Tau is known to facilitate the anterograde axonal transport of

organelles such as mitochondria (Gotz et al., 2006). In AD neurons, the detachment of hyperphosphorylated tau from the microtubules leads to impaired axonal transport of organelles (such as mitochondria targeted to the synapses).

1.1.3.2. *Neuroinflammation*

Biochemical markers of activated microglia and reactive astrocytes are increased in the brain of AD patients (Wyss-Coray and Mucke, 2002). In physiological conditions, phagocytic microglia engulf and degrade Aβ. However, the large and constant production of Aβ in the pathological case chronically activate microglia, leading to the release of a myriad of damaging chemokines and cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor α (TNF-α) (Akiyama et al., 2000). The binding of Aβ peptides to advanced glycosylation end products receptors (expressed by microglia) amplifies the generation of cytokines, glutamate and nitric oxide (Yan et al., 1996).

1.1.3.3. *Loss of calcium regulation*

Loss of calcium regulation is common to several neurodegenerative disorders. In AD, elevated concentrations of cytosolic calcium stimulate the amyloidogenic pathway (Isaacs et al., 2006). The chronic state of glutamatergic receptor activation is thought to aggravate neuronal damage in AD. NMDAR activation increases cytosolic calcium, which in turn stimulates calcium release channels in the endoplasmic reticulum. Evidence also show that Aβ could form voltage-independent cation channels in lipid membranes, resulting in calcium uptake and degeneration of neuritis (Lin et al., 2001). Deregulation of cytosolic calcium concentration can lead to a “calcium

overload", which is capable to induce mitochondrial dysfunctions, oxidative stress and also cell death.

1.1.4. Oxidative stress in Alzheimer's disease

The brain requires 20% of total blood flow and 25% of the body's glucose utilization. Due to their excitable properties and their important need in energy, neurons have a large number of mitochondria either in the soma or in synaptic ends. Since neurons have a limited glycolytic activity, mitochondria play an essential role in bioenergetics thanks to adenosine triphosphate (ATP) production. Also, mitochondria are key producers of reactive oxygen species (ROS). Unbalance between ROS formation and reduction may lead to the deleterious oxidation of lipids, proteins and DNA, also called "oxidative stress".

In neurons or glial cells, ATP is produced aerobically through the tricarboxilic acid (TCA) cycle and the oxidative phosphorylation (OxPhos), also called respiratory chain, in mitochondria. TCA cycle is composed of 8 enzymatic steps and converts carbohydrates and free fatty acids into ATP. It also yields electrons in form of reduced hydrogen carriers, nicotine adenine dinucleotide (NADH) and flavin adenine nucleotide ($FADH_2$). These compounds enter subsequently as coenzymes into the OxPhos, which is composed of electron transport chain or ETC (enzymatic complexes I-IV) and an ATP-synthase (complex V). These five enzyme complex are functionally interconnected via mobile electron carriers ; cytochrome c and ubiquinone/coenzyme Q. Enzymatic complexes and electron carriers are localized at the inner mitochondrial membrane (Fig. 7). In addition to flavin and nicotinamides, ETC involves cytochromes and metal ions clusters (iron and copper) to transfer electrons in a sequence of oxido/reductions steps, leading to a proton gradient

across the inner membrane which finally drives ATP synthesis via the complex V. In parallel, the complexes transfer electrons to oxygen and produce water. OxPhos is not totally efficient and up to 2% of electrons are incompletely reduced to yield O_2^-, instead of H_2O (Eckert et al., 2013, Sutherland et al., 2013).

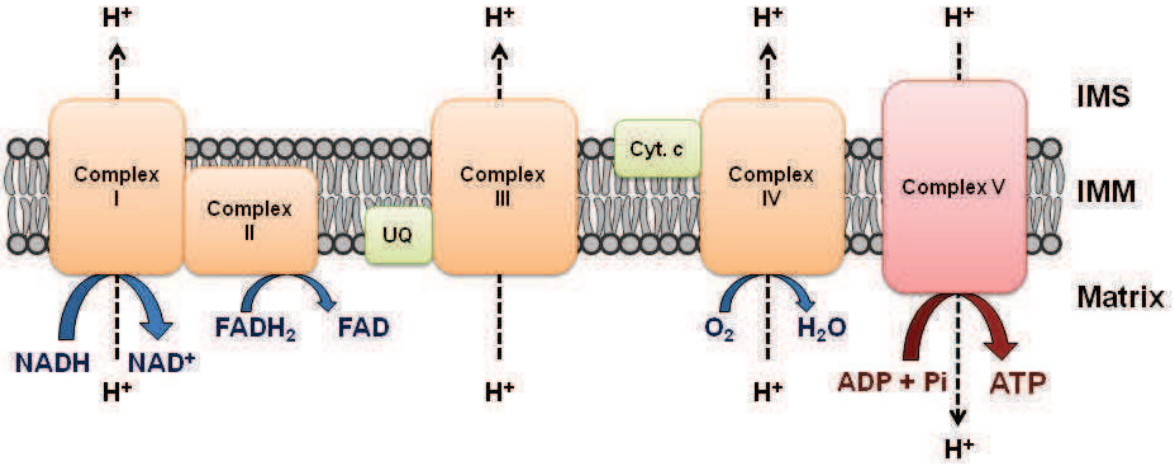

Figure 7 : Schematic representation of the oxidative phosphorylation system. Complex I (NADH-ubiquinone oxidoreductase) and II (succinate dehydrogenase) also belong to the tricarboxilic acid cycle (TCA) and receive electrons from NADH and $FADH_2$, respectively. Thereafter, electrons are driven from complexes by the mobile electron carrier ubiquinone (UQ) and cytochrome c (Cyt c) to the final acceptor, molecular oxygen. In parallel, a proton gradient is established across the inner membrane (IMM) in complexes I, III and IV. This gradient is utilized by complex V to generate ATP. IMS : inter-membrane space. Adapted from Eckert et al., 2013.

Low molecular weight anti-oxidant as glutathione and metal-containing enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), act to control the cellular redox environment. The first reducing barrier against ROS is SOD which converts O_2^- to H_2O_2. Then, CAT and GPX transfer H_2O_2 to oxygen and water. Iron and copper, which are also abundant in the brain, may facilitate H_2O_2 conversion into other ROS or may promote Aβ peptide formation (von Bernhardi and Eugenin, 2012, Greenough et al., 2013).

1.1.4.1. *Amyloid peptides and oxidative stress*

In the ageing brain, OxPhos not only becomes less efficient, but there is a substantial decrease in anti-oxidant enzymes expression/activity (Kowald, 2001). Also, Aβ peptides are potent mitochondrial poisons, which especially affect the synaptic pool (Mungarro-Menchaca et al., 2002). Aβ and APP itself were reported to accumulate in mitochondria in the brain of AD patients (Pavlov et al., 2009). Aβ peptides alter key mitochondrial enzymes (Hauptmann et al., 2006) such as cytochrome c oxidase (COX/Complex IV), pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (αKGD), leading to bioenergetic disturbances as evidenced in AD patients (Mosconi et al., 2008). Exposure of human neuroblastoma SH-SY5Y cells to Aβ peptides revealed a significant decrease of Complex IV activity (Lim et al., 2010). Moreover, decreased brain levels of antioxidant enzymes such as SOD or CAT are also observed in AD (Aksenov et al., 1998). The combination of ROS elevation to antioxidant enzyme decrease evidenced in brain tissues from AD patients suggests that oxidative stress may be a central element of AD physiopathology. Cu^{2+} ions have also been shown to render Aβ more toxic. Indeed, dimeric conformation of Aβ42 appear as potent inhibitor of COX, but only in the presence of Cu^{2+} (Crouch et al., 2005).

The mitochondrial Aβ binding protein alcool dehydrogenase (ABAD) can also bind to Aβ42 present in the cortical mitochondria of APP transgenic mice (Lustbader et al., 2004, Yan et al., 2007). In mice double transgenic for ABAD and APP, toxic effects of Aβ was increased compared to APP single transgenic mice. This interaction promotes the leakage of ROS, mitochondrial dysfunction, cell death, as well as spatial learning and memory deficits (Lim et al., 2010, Lim et al., 2011, Grimm et al.,

2012, Gotz et al., 2013a). It has also been shown that the specific inhibition of ABAD in human neuroblastoma SH-SY5Y cells was able to reduce Aβ-induced cell loss and to decrease ROS levels (Lim et al., 2011).

Another process to induce oxidative stress is the binding of Aβ to NMDAR which evokes calcium inwards. As aforementioned, Aβ cause an overactivation of NMDAR, which in turn lead to a downstream excessive production of nitric oxide and the generation of reactive nitrogen species (RNS), that are also involved in oxidative stress and damageable for the cells (Gotz et al., 2010).

1.1.4.2. *Tau and oxidative stress*

Hyperphosphorylated Tau has been shown to block mitochondrial transport which leads to energy deprivation and oxidative stress at the synapse end (Reddy, 2011). It was also reported that the overexpression of Tau inhibited kinesin-dependent transport of many organelles, including peroxisomes, which are important actors for the buffering of oxidative stress (Stamer et al., 2002). In a drosophila model overexpressing a tauopathy-related mutant form of human tau (tau R406W), reduction of gene expression of mitochondrial SOD2 and thioredoxin reductase 1 (Trxr-1) enhanced Tau-induced neurodegenerative histological abnormalities and neuronal apoptosis. In contrast, overexpression of antioxidant enzyme and treatments with vitamin E decreased Tau-induced neuronal cell death (Dias-Santagata et al., 2007). Moreover, in cortical neurons derived from transgenic rats model expressing a human truncated form of Tau, ROS were increased and antioxidants significantly attenuated this elevation of ROS (Cente et al., 2006, Cente et al., 2009). Altogether, these observations confirm that oxidative stress is, at least in part, a mediator of Tau-induced neurodegeneration.

In transgenic R5 mice over-expressing human P301L mutant Tau, biochemical consequences of tauopathy have been investigated using proteomics followed by functional validation (David et al., 2005). These mutant mice showed mainly a deregulation of mitochondrial respiratory chain complex components and antioxidant enzymes. The functional analysis revealed an age-dependent mitochondrial dysfunction and increased ROS levels.

To summarize, Aβ and hyperphosphorylated Tau mainly induce oxidative stress by disrupting the normal functioning of the electron transport chain. Aβ alter the complex IV while Tau preferentially modulates the complex I (Fig. 8).

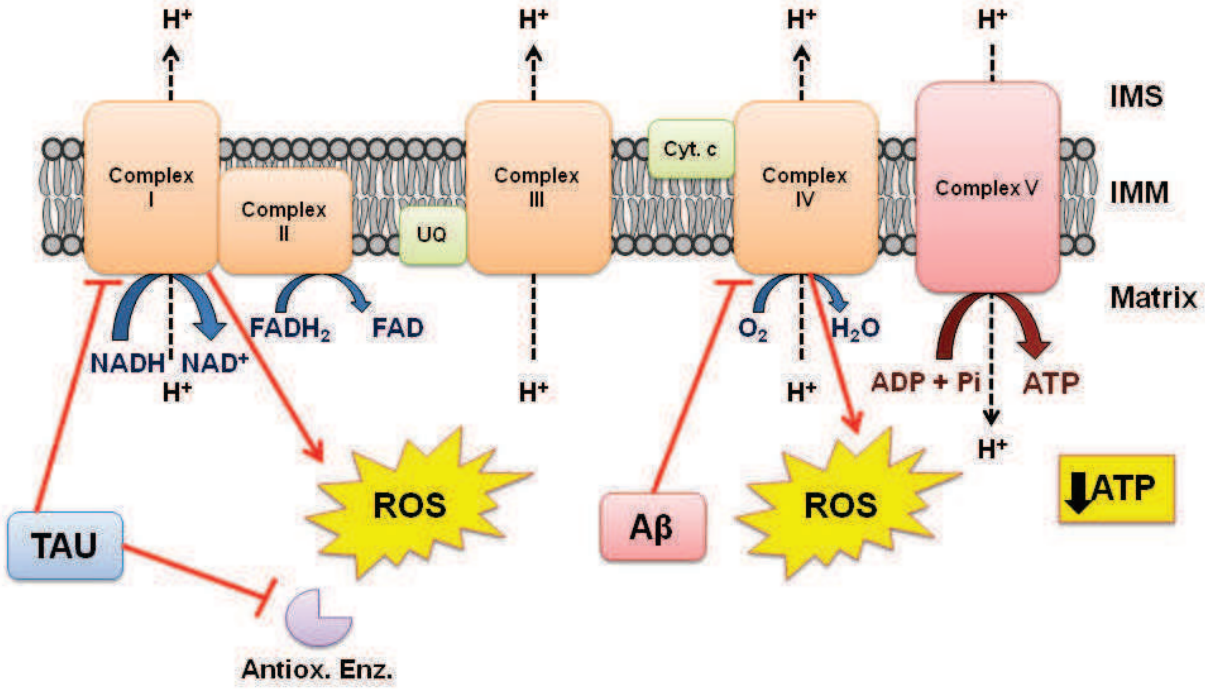

Figure 8 : Impairment of the electron transport chain in Alzheimer's disease
In AD, abnormalities in the functioning of electron transport have been observed, mostly in complex I and IV, leading to a decreased production of ATP and enhanced reactive oxygen species (ROS) levels. Tau impairs complex I and decreases the levels of antioxidant enzymes such as superoxide dismutase 2 (SOD2) or thioredoxin reductase 1 (Trxr-1). Aβ specifically alter complex IV. Both alterations of complex I and IV induce generation of ROS and, subsequently, oxidative stress. IMS, intermembrane space ; IMM inner mitochondrial membrane ; UQ, ubiquinone or coenzyme Q ; Cyt. c, cytochrome c ; Antiox. Enz., Antioxidant enzymes. Adapted from Eckert et al., 2011.

1.1.4.3. *Oxidative stress-induced apoptosis*

Cell death occurs by necrosis or apoptosis. These two mechanisms have distinct histological and biochemical signatures. In necrosis, the stimulus of death (Ischemia for example) is itself the direct cause of the demise of the cell. By contrast, in apoptosis, the stimulus of death (e.g., ROS) activates a cascade of molecular events that orchestrate the “clean” destruction of the cell (Hotchkiss et al., 2009). Apoptotic cell death, also known as programmed cell death, is a feature of various neurodegenerative disorders, such as AD (Mattson, 2000). Apoptosis results from two biochemical cascades, known as the extrinsic and the intrinsic or mitochondrial pathway. Intrinsic apoptosis is caused by various intracellular stressors, such as oxidative stress or calcium overload. Interplay between pro-apoptotic and anti-apoptotic members of the Bcl-2 (B-cell Lymphoma 2) proteins family control the mitochondrial apoptotic pathway. The major executioners in the apoptotic program are proteases known as caspases (cysteine-dependent, aspartate-specific proteases) (Hengartner, 2000). Caspase-9 regulates the beginning of this intrinsic pathway, which comes into play after intracellular sensors indicate overhelming cell damage (oxidative stress-induced damages and calcium overload in case of AD). ROS ultimately lead to increased mitochondrial permeability, thereby promoting the release of pro-apoptotic cytochrome c from the intermembrane space of mitochondria into the cytosol. Active caspase-9 in turn activates effectors caspases such as caspase-3, -6 and -7, that herald demolition of the cell by degrading numerous proteins and DNA.

A balance between pro-apoptotic and anti-apoptotic Bcl-2 family members determines cell survival. The major pro-survival members, Bcl-2 and Bcl-xl are

localized on the mitochondrial outer membrane and on the endoplasmic reticulum perinuclear membrane. Bcl-2 and Bcl-xl act by inhibiting the pro-apoptotic members through heterodimerization. Bax (Bcl-2 associated X protein) is a pro-apoptotic member of the Bcl-2 family that is widely expressed in the nervous system (Mattson, 2000). Bax has a pore-forming activity which triggers the permeability transition pore complex (PTPC) that activates the mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c in the cytosol (Galluzzi et al., 2012a, Galluzzi et al., 2012b, Kole et al., 2013). Cytochrome c and the apoptotic protease activating factor 1 (Apaf-1) are involved in the formation of the apoptosome which triggers caspase-9/caspase-3 proteolytic cascade and subsequent DNA fragmentation and cell shrinkage (Fig. 9).

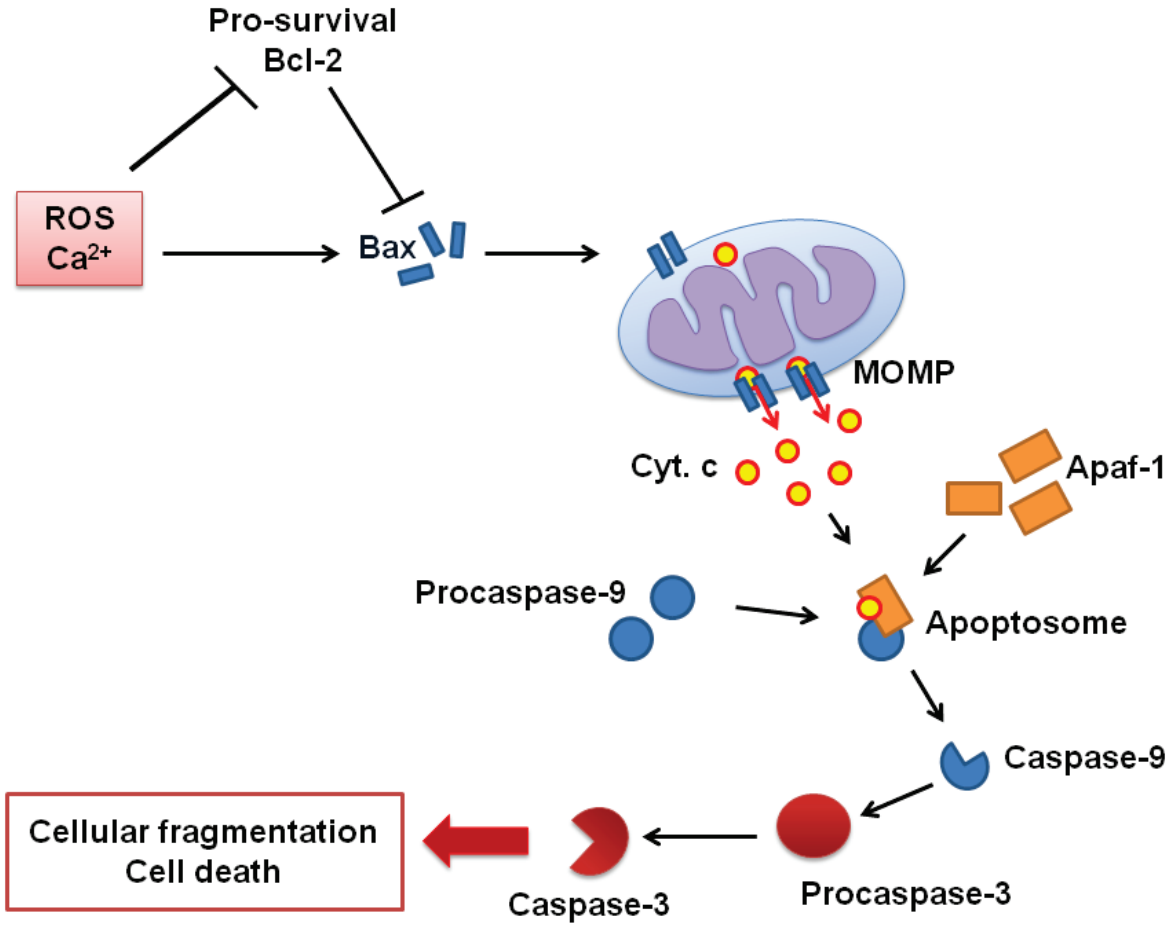

Figure 9 : Intrinsic mitochondrial pathway of apoptosis induced by oxidative stress or calcium overload. Mitochondrial pathway of apoptosis is initiated by intracellular stressors such as reactive oxygen species ROS or calcium overload. The inhibition of anti-apoptotic Bcl-2 leads to the pore-forming ability of Bax (pro-apoptotic factor) on mitochondrial outer membrane. Subsequently, mitochondrial outer membrane permeabilization (MOMP) is induced, that allows release of cytochrome c (Cyt. c). Together with the apoptotic protease activating factor 1 (Apaf-1) and Procaspase-9, Cyt. c drives the formation of the apoptosome and activate procaspase-9 into the initiatory caspase-9 which in turn elicits the activation of the effector caspase-3, leading to apoptotic features like cell shrinkage and DNA fragmentation. Adapted from Kole et al., 2013.

1.1.4.4. *Oxidative stress, amyloidogenesis and Tau : a vicious circle*

As aforementioned, it has been reported that Aβ-evoked toxicity generates cellular ROS such as H_2O_2 (Behl et al., 1994, Tabner et al., 2005) which may be suppressed by the activation of SOD or ROS scavengers (Miranda et al., 2000). Conversely, oxidative stress increases Aβ levels, a process which leads to the perpetuation of a vicious circle (Misonou et al., 2000, Oda et al., 2010). Indeed, Aβ partially target mitochondria to induce mitochondrial dysfunction and oxidative stress which in turn may lead proteins to adopt β-pleated sheet conformations.

Tau induces oxidative stress by affecting the respiratory chain complex I (see Part. 1.1.4.2., pages 32-33). But, growing evidence revealed that oxidative stress may have a role in the hyperphosphorylation and polymerization of Tau. In Tg2576 AD transgenic mice bearing the “Swedish mutation” of APP, deficiency in mitochondrial SOD2 or reduction of cytoplasmic SOD1 induced Tau hyperphosphorylation (Melov et al., 2007, Murakami et al., 2011), suggesting that ROS may play a critical role in Tau hyperphosphorylation.

Altogether, these data strongly suggest that oxidative stress may play a major role in AD pathogenesis. However, it remains unclear whether oxidative stress precedes Aβ and Tau hyperphosphorylation or whether it is a sub-product of these two aggregates. Whatever is the answer to this question, the reduction of oxidative stress may constitute a promising strategy for the development of effective therapies against AD (Fig. 10).

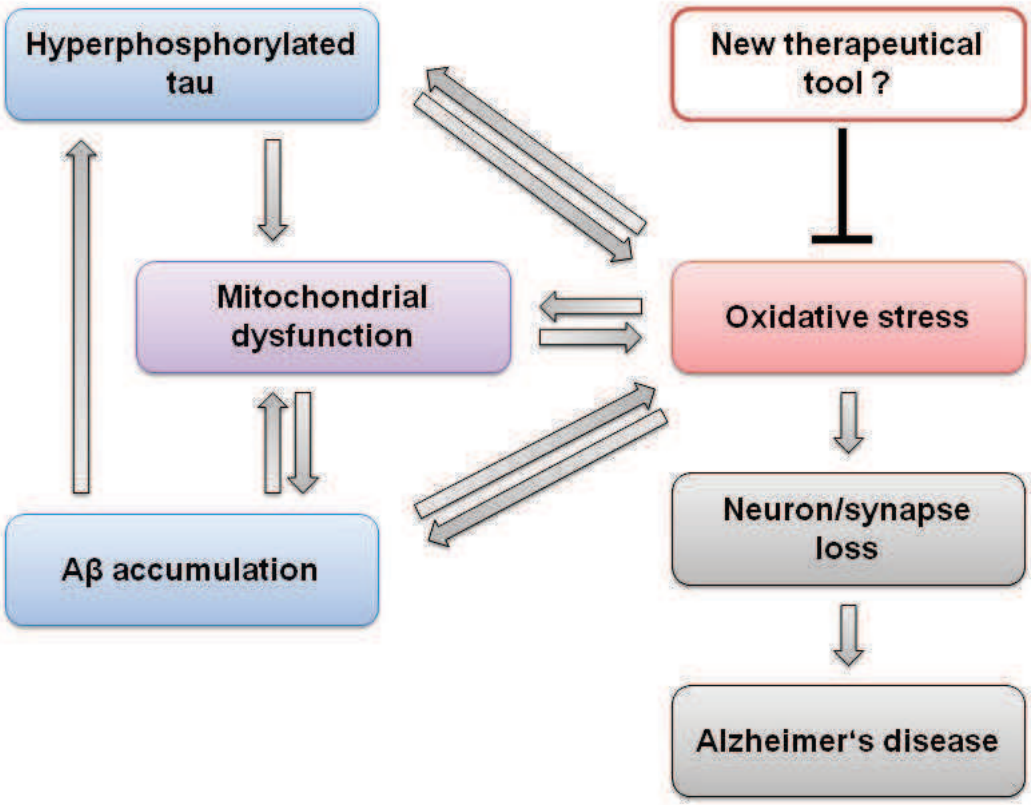

Figure 10 : Central role of oxidative stress in Alzheimer's disease. Both Aβ and hyperphosphorylated Tau are able to induce oxidative stress either directly or by disrupting mitochondrial respiratory chain. In turn oxidative stress can promote the formation/aggregation of Aβ and the hyperphosphorylation/accumulation of Tau, implicating the generation of a "vicious circle". Oxidative stress can activate the mitochondrial/endogenous pathway of apoptosis, leading to the neuronal cell loss and patho-physiological features of AD. Finally, based upon the essential role of oxidative stress in AD pathogenesis, antioxidant compounds reducing significantly oxidative stress may offer the possibility to develop efficient therapeutical strategies against AD.

1.1.5. Endoplasmic reticulum stress in Alzheimer's disease

1.1.5.1. *ER functions under normal and pathological conditions*

The endoplasmic reticulum (ER) is the main intracellular compartment which is a membrane–enclosed reticular network connecting the nuclear envelope to the golgi complex (Baumann and Walz, 2001). It has mainly three cellular functions : (i) protein folding, quality control, translational modification and transport to the golgi complex, (ii) storage and maintenance of cellular calcium homeostasis, and (iii) synthesis of lipids and cholesterol.

Several ER-chaperones proteins such as glucose-regulated protein 78kDa (GRP78, also known as Binding Immunoglobulin Protein, BiP), calnexin and calreticulin bind to the hydrophobic domain of newly synthesized proteins, allowing correct folding and inhibition of protein aggregation. Disulfide bond formation and N-linked glycosylation also play important role in protein folding and are favored by the oxidative environment of the ER and by calcium.

The ER is also the main intracellular Ca^{2+} storage site. As aforementioned, many ER chaperones are Ca^{2+} dependent. Under physiological conditions, the luminal Ca^{2+} concentration is as high as in the extracellular domain (~1-2 mM) while the concentrations in the cytosol are about ten thousand fold lower (~0.1 µM). depending on the cell type, maintenance of ER Ca^{2+} homeostasis may involve ryanodine receptor (RyR) or inositol triphosphate receptor (IP3R). Ca^{2+} influx are regulated by the sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPases (SERCA pumps). Also, Ca^{2+} homeostasis is a critical component of cellular signaling and survival, especially in neurons which are excitable cells.

Besides fatty acids, cholesterol biosynthesis is also a determinant of ER. Cholesterol is a crucial component of mammalian membranes, and also a precursor for steroid hormones and neurosteroids. The first steps of cholesterol synthesis are the conversion of three molecules of acetyl-Coenzyme A (acetyl-CoA) into one 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the cytoplasm. Then, HMG-CoA is converted to mevalonate by the HMG-CoA reductase (HMGR) exclusively in the ER. This is the rate limiting step which is subject to complex regulation. Thereafter, mevalonate is converted into cholesterol by a multistep processing in the ER (Canevari and Clark, 2007).

Because ER is a sensor of cellular homeostasis, different types of insults such as protein misfolding, redox unbalance, lipid/cholesterol dyshomeostasis can induce ER stress. In particular, failure of protein folding quality control is harmful to cell survival and therefore ER can initiate the unfolded protein response (UPR) to counteract this detrimental situation (Hetz and Mollereau, 2014). The UPR consists of two central components, a group of stress sensors localized at the ER membrane, and downstream transcription factors that influence gene expression to ensure adaptation to stress or the induction of cell death by apoptosis. In mammalian cells, there are three classes of ER stress sensors : inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Fig. 11). UPR induces a rapid translational inhibition that is controlled by PERK through the phosphorylation and inhibition of the eukaryotic translation initiation factor 2α (eIF2α). This allows the buffering of misfolded protein aggregation in the ER by decreasing the entrance of newly synthesized proteins in the ER. Moreover, eIF2α phosphorylation stimulates the activating transcription factor 4 (ATF4) which controls

the expression of numerous genes involved in apoptosis, autophagy and antioxidant responses (Hetz et al., 2013).

Activation of IRE1α implies its dimerization and autophosphorylation leading to the activation of its RNase domain. Then, it catalyses the splicing of mRNA encoding the transcription factor X-box binding protein 1 (XBP-1) which controls the expression of genes involved in protein folding, ER associated degradation (ERAD) or lipid synthesis (Hetz et al., 2011). Also, IRE1α activates the Jun amino-terminal kinase (JNK) and subsequently induces apoptosis.

Upon ER stress, ATF6 is translocated to the Golgi where it is processed to a transcription factor that also regulates expression of genes involved in ERAD pathway. ATF6 can also form heterodimers whith XBP-1 in order to control expression of various genes (Hetz and Mollereau, 2014).

Under conditions of chronic and irreversible ER stress, the UPR may also induce apoptosis through distinct overlapping cellular signaling mechanisms, including the upregulation of transcription factor C/EBP-homologous protein (CHOP, also known as DNA damage inducible gene 153, GADD153) which upregulates the pro-apoptotic members of the Bcl-2 family. PERK activation and the downstream induction of ATF4 can lead to the upregulation of CHOP which in turn inhibits the anti-apoptotic factor Bcl-2. Subsequently, the pro-apoptotic factor Bax engages the mitochondrial pathway of apoptosis (see Part 1.1.4.3., pages 34-35).

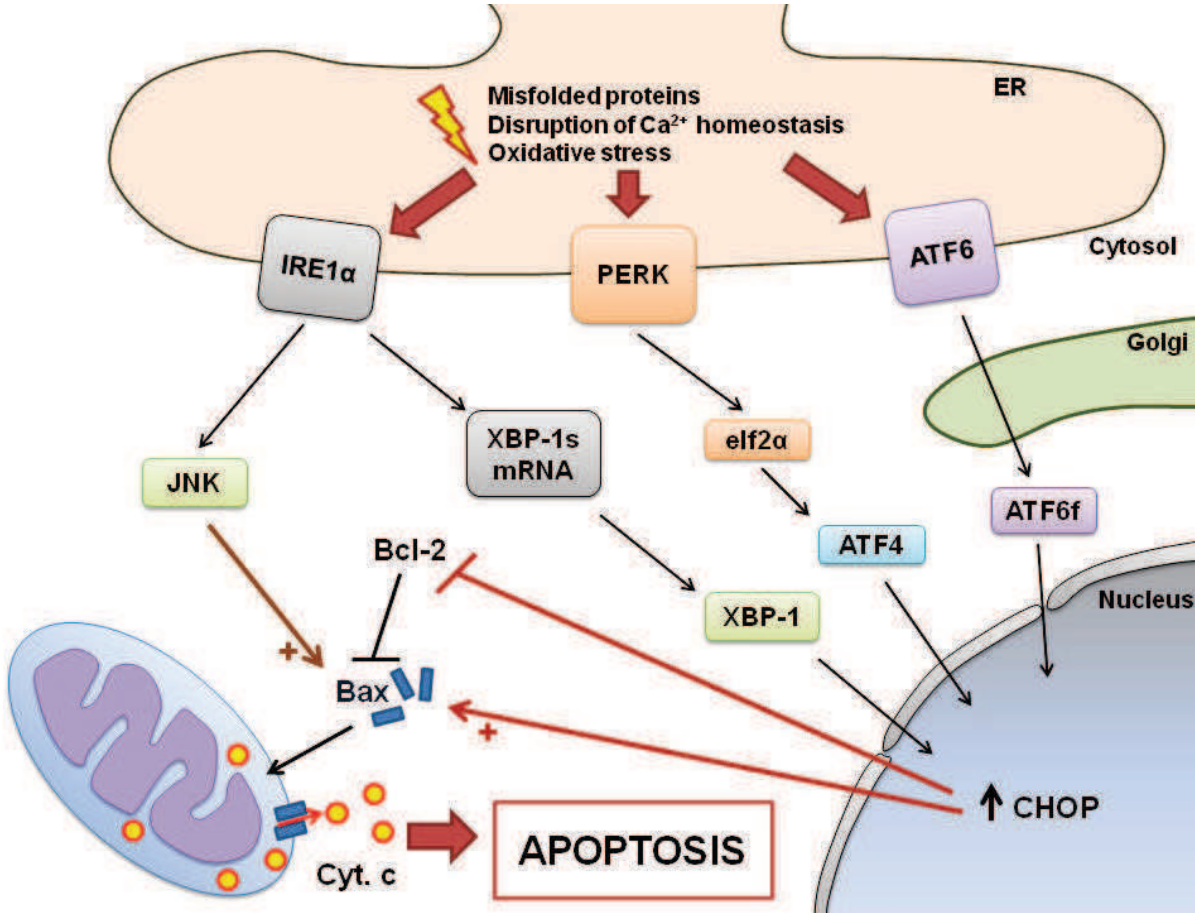

Figure 11 : ER stress and its induction of apoptosis. ER stress leads to activation of the UPR sensors, PERK, IRE1α and ATF6. Activation of PERK leads to inhibition of translation by eIf2α phophorylation and the activation of the transcription factor ATF4. Activation of IRE1α is associated with non conventional splicing of XBP-1 which translocates to the nucleus to modulate the expression of various genes involved in ER functioning. Upon its activation, ATF6 translocates to the Golgi apparatus where it is activated by proteolysis. Thereafter, in the nucleus, ATF6 activate the transcription of XBP-1 in order to control the expression of various genes. Under chronic and irreversible ER stress, the UPR sensors activate the JNK pathway and CHOP, which alter the balance between pro-apoptotic and anti-apoptotic Bcl-2 family members, leading to the activation of the intrinsic/mitochondrial apoptosis pathway. ER, Endoplasmic reticulum ; IRE1α, inositol-requiring enzyme 1α ; PERK, protein kinase RNA-like ER kinase ; ATF6, activating transcription factor 6 ; AFT6f, processed activating transcription factor 6 ; XBP-1, X-box binding protein 1 ; eIF2α, eukaryotic translation initiation factor 2α ; JNK, Jun amino-terminal kinase ; ATF4, activating transcription factor 4 ; CHOP, C/EBP-homologous protein. Adapted from Doyle et al., 2011.

1.1.5.2. *Endoplasmic reticulum stress in Alzheimer's disease*

Several studies have reported manifestations of ER stress in post mortem brain samples from AD patients. These observations demonstrate the splicing of XBP-1 mRNA in AD temporal cortex and hippocampal tissues (Lee et al., 2010). In addition, the same study showed enhanced expression of ER chaperones such as GRP78 or the pro-apoptotic transcription factor CHOP in AD postmortem brain tissues, suggesting an important role of ER stress in AD etiology.

Interconnections between ER stress, Aβ and NFTs have been shown in various experimental models. For example, reduction of Aβ is correlated with attenuated ER stress and vice versa (Prasanthi et al., 2011, Marwarha et al., 2013). Aged PSEN2 mutant mice revealed inhibited BACE-1 activity in brain tissues upon treadmill exercise. This was accompanied by a down-regulation of PERK, eIF2α, XBP-1 as well as CHOP and caspase-3 (Kang et al., 2013). GRP78 and XBP-1 levels increased in neurons treated with Aβ (Costa et al., 2013). Also, exposure of SK-S-SH cells to Aβ induced activation of the PERK pathway and upregulation of CHOP expression (Lee et al., 2010). Tunicamycin-evoked ER stress generated Aβ production in RGC-5 cells via the stimulation of BACE-1 and PSEN1 (Liu et al., 2014), suggesting that ER stress may be the cause or consequence of Aβ toxicity (Prasanthi et al., 2011, Cornejo and Hetz, 2013, Marwarha et al., 2013, Hetz and Mollereau, 2014, Liu et al., 2014). Moreover, AICD was shown to enhance CHOP expression in HEK 293 cells (Takahashi et al., 2009) and high levels of markers for UPR activation were detected in neurons with NFTs (Hoozemans and Scheper, 2012, Nijholt et al., 2012).

Interestingly, *In vitro* studies showed that the induction of UPR by exposure of primary cortical neuronal culture of rats to Aβ oligomers correlated with the generation of tau phosphorylation (Resende et al., 2008). Altogether, these data reveal the existence of a close link between ER stress and the etiological mechanisms of AD or tauopathies (Fig. 12).

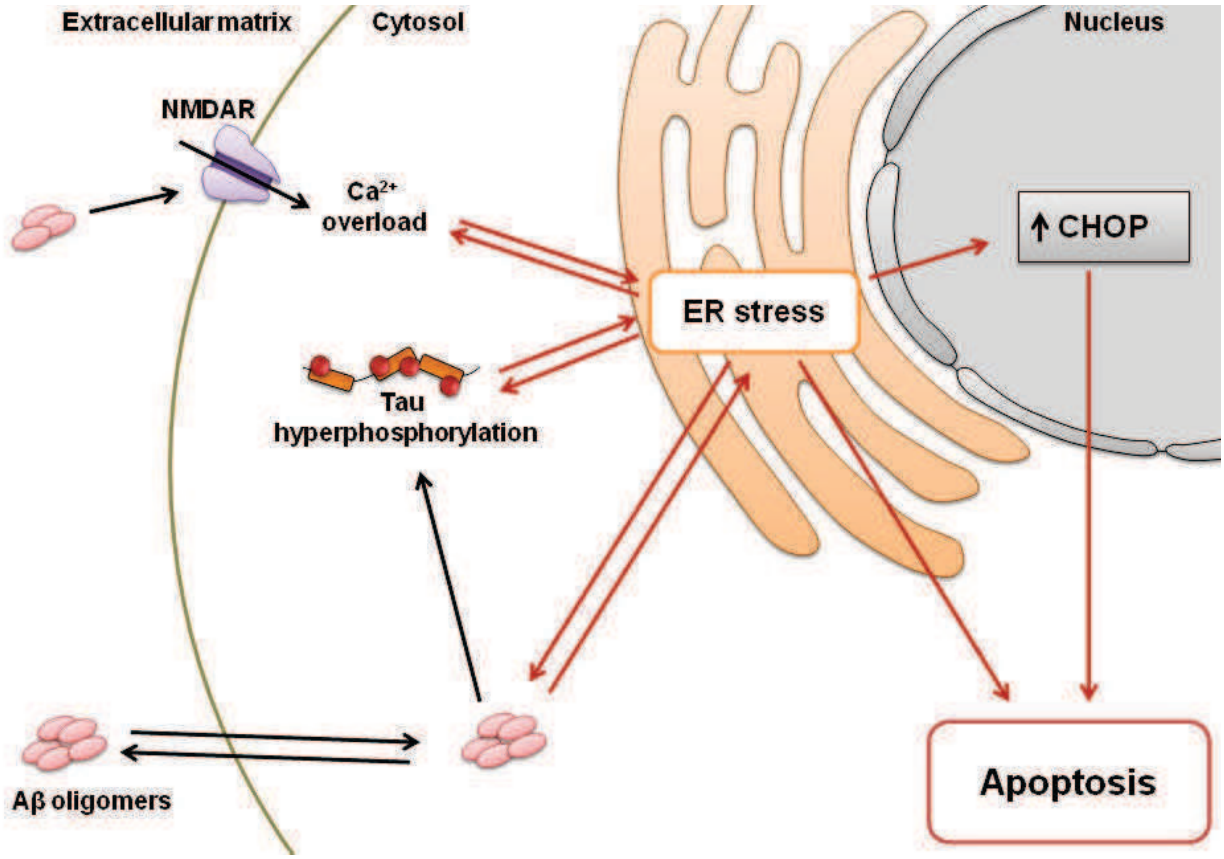

Figure 12 : Endoplasmic reticulum stress-mediated cell death in Alzheimer's disease. Aβ peptides aggregates and alter NMDAR functioning, inducing calcium overload into the cytoplasm. In addition, release of calcium from the ER can also trigger calcium overload, which initiates the mitochondrial pathway of apoptosis. Aβ may also induce chronic ER stress, which in turn promotes Aβ formation. The same vicious circle is observed with hyperphosphorylated Tau. The chronic induction of ER stress by Aβ or hyperphosphorylated tau leads to the activation of pro-apoptotic events controlled by the UPR-induced activation of CHOP. ER, Endoplasmic reticulum ; NMDAR, N-methyl-D-aspartate receptor ; UPR, unfolded protein response ; CHOP, C/EBP-homologous protein. Adapted from Cornejo and Hetz, 2013.

1.1.5.3. *Interplay between Endoplasmic Reticulum and Mitochondria in Alzheimer's disease*

The ER and mitochondria are closely connected. They join together at multiple contact sites to form specific domains, termed mitochondria-ER associated membranes or MAMs (Marchi et al., 2014). MAMs promote interorganelles lipids transfer but are also involved in Ca^{2+} ions exchange that regulate several biological processes such as protein folding in ER, production of ATP in mitochondria (Denton et al., 1988, Rowland and Voeltz, 2012, Vannuvel et al., 2013) and activation of Ca^{2+} dependent enzymes that activate cell death pathways. Interactions between ER and mitochondria may shape intracellular calcium signals and modulate synaptic and integrative neuronal activity (Mironov and Symonchuk, 2006). Various ER or mitochondria bound proteins have play a key role in the maintenance of close network between ER and mitochondria and in the formation of calcium channels involved in the specific transfer of calcium from ER to mitochondria. These proteins include ER resident Ca^{2+} channel inositol triphosphate receptor (IP3R) and the mitochondrial voltage-dependent anion channel (VDAC) (Rowland and Voeltz, 2012, Vannuvel et al., 2013). Recent findings reveal that MAM-associated proteins expressions are increased in young AD transgenic mice and in the brain of AD patients, suggesting that in early stages of AD, the elevation of ER-mitochondria interface proteins may reflect a neuronal stress response (Hedskog et al., 2013). Because ER and mitochondria are tightly bound, ER stress may affect and modify mitochondrial bioenergetics and *vice versa.* Thus, interplaying mechanisms between ER stress and mitochondrial functions are pivotal for neurodegenerative processes occurring in AD. Indeed, Aβ exposure of primary hippocampal neurons affected the

MAM region by disturbing the Ca^{2+} regulatory system and this process led to ER stress, mitochondrial dysfunctions and apoptosis (Hedskog et al., 2013).

It is noteworthy to recall that ER stress-evoked mitochondrial dysfunction is characterized by ROS formation increase and pro-apoptotic factors activation (Malhotra and Kaufman, 2007). Since hyperphosphorylated tau and Aβ induce ROS generation and ER stress, interconnection between ER and mitochondria make ER stress a partner of the vicious circle linking Aβ, NFTs and cellular stresses (Fig. 13). Consequently, development of efficient therapies should take into account this vicious circle by reducing oxidative stress, ER stress and/or Aβ accumulation.

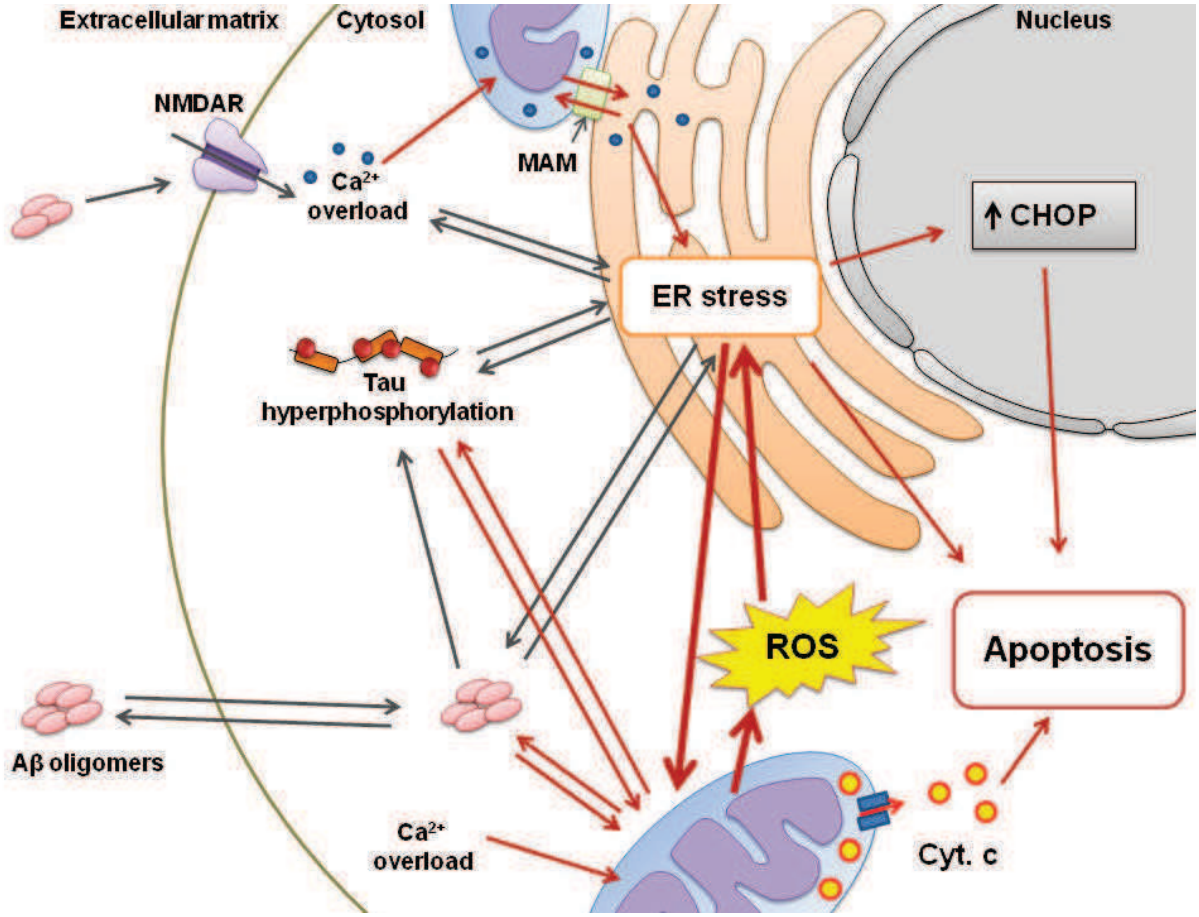

Figure 13 : Interplay between endoplasmic reticulum and mitochondria in Alzheimer's disease. Aβ peptides and hyperphosphorylated tau can either induce ER stress or mitochondrial impairment, leading to reactive oxygen species formation and oxidative stress. Both ER stress and oxidative stress are able to promote Aβ formation and tau hyperphosphorylation. Due to their close communication via mitochondria-ER-associated membranes (MAMs), ER stress and oxidative stress accentuate each other in a positive feed forward loop. Impaired calcium flux between ER and mitochondria also play a key role in this vicious circle leading to cell death by intrinsic apoptosis pathway. MAM, mitochondria-ER-associated membranes ; Cyt.c, cytochrome c ; ROS, reactive oxygen species ; ER, Endoplasmic reticulum ; NMDAR, N-methyl-D-aspartate receptor ; UPR, unfolded protein response ; CHOP, C/EBP-homologous protein. Adapted from Cornejo and Hetz, 2013.

1.2. Neurosteroids

1.2.1. Definition and mode of actions

The nervous system activity is modulated by steroids compounds which regulate the development, growth and maturation of nerve cells (McEwen, 1994). The term neuroactive steroid is generally used to designate any steroid exhibiting a neuroactive effect, whether this steroid is a synthetic molecule (exogenously synthesized) or an endogenous compound produced in the body by steroidogenic tissues. Therefore, the neuroactive steroid family includes synthetic exogenous steroids, hormonal steroids produced by peripheral or classical endocrine glands (adrenals, gonads) and steroids synthesized by neuronal or glial cells that are designated “neurosteroids” (Baulieu, 1998, Mensah-Nyagan et al., 1999). The biosynthesis of neurosteroids occurs directly in the CNS or peripheral nervous system, either *de novo* from cholesterol, or by *in situ* metabolism of circulating steroid precursors (Fig. 14) (McEwen, 1994, Baulieu, 1998, Mensah-Nyagan et al., 1999, Schumacher et al., 1999). The main criterion required to consider a steroid as a neurosteroid is its production in neurons and/or glial cells of the CNS or PNS independently from the activity of endocrine steroidogenic glands including the adrenals and the gonads. The process of neurosteroid biosynthesis (neurosteroidogenesis) is a well-conserved mechanism evidenced in non mammalian vertebrates and in all mammals including humans (Mensah-Nyagan et al., 1998, Mensah-Nyagan et al., 1999, Tsutsui et al., 1999, Mellon and Vaudry, 2001, Tsutsui et al., 2013). These data suggest that neurosteroids may control several key physiological functions.

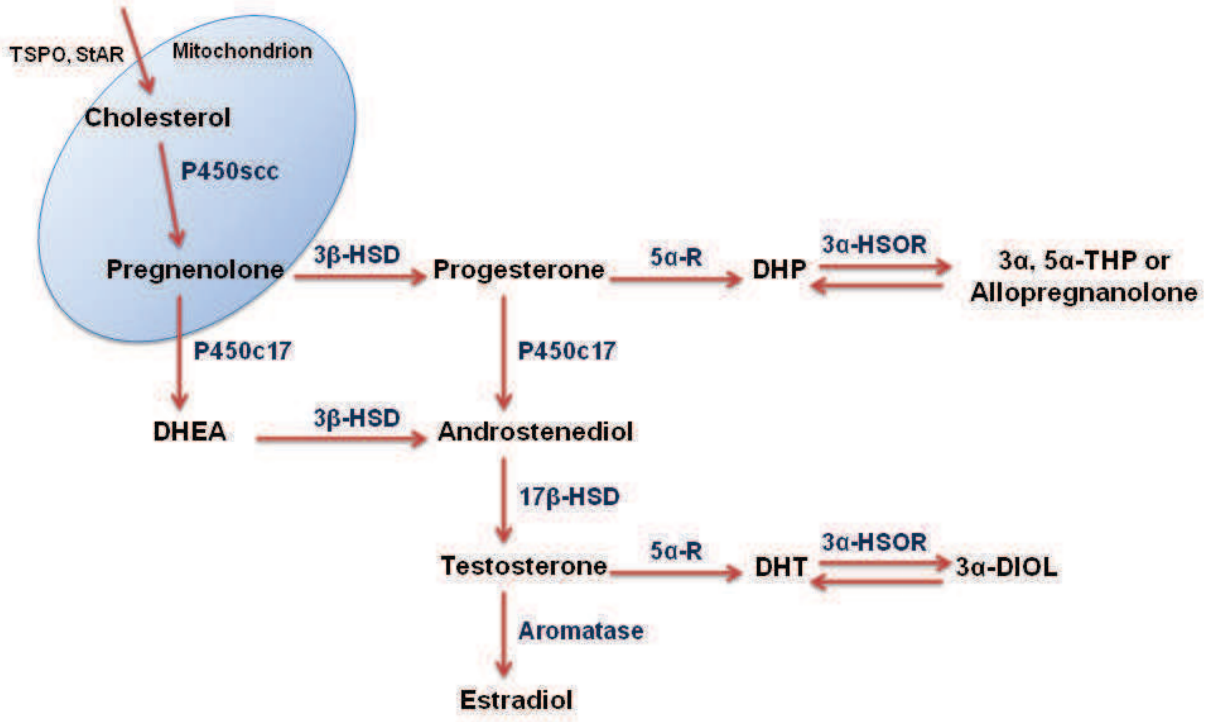

Figure 14 : Biochemical pathways leading to neurosteroids biosynthesis. The initial transport of cholesterol into the mitochondria is mediated by the complex StAR and TSPO. Steps of neurosteroidogenesis are shown. StAR, Steroidogenic acute regulatory protein ; TSPO, translocator protein (18-kDa) ; P450scc, Cytochrome P450side chain-cleavage ; P450c17, Cytochrome P450c17 or 17α-hydroxylase ; 3β-HSD, 3β-Hydroxysteroid dehydrogenase ; 5α-R, 5α-Reductase ; 17β-HSD, 17β-Hydroxysteroid dehydrogenase ; 3α-HSOR, 3α-Hydroxysteroid oxidoreductase ; DHEA, Dehydroepiandrosterone ; DHP, Dihydroprogesterone ; DHT, Dihydrotestosterone ; 3α-DIOL, 3α-androstanediol ; 3α,5α-THP, 3α,5α-Tetrahydroxyprogesterone also known as allopregnanolone. Adapted from Schaeffer et al., 2010.

1.2.1.1. *Genomic actions of neurosteroids*

Neuroactive steroids including neurosteroids exert classically their physiological effects through nuclear receptors (NR)-mediated gene transcription. These NR include the glucocorticoids receptors (GR), mineralocorticoids receptors (MR), estrogens receptors (E2R), androgens receptors (AR) and progestins receptors (PR) (Beato and Klug, 2000). Recently, a focus has been on characterizing the role of the pregnane xenobiotic receptor (PXR), a NR involved in allopregnanolone signaling

(Frye et al., 2014b, a, c), which acts as a transcriptions factor for neurosteroidogenic enzymes. Steroid hormones receptors are functionally composed of three critical modular domains : a hormone-independent activation function 1 (AF1) domain, a DNA-binding domain (DBD) and a hormone dependent activation function 2 (AF2) domain that is activated allosterically upon ligand binding (Stanisic et al., 2010). The general outline of functional and structural domains of steroids nuclear receptors are described in Fig. 15.

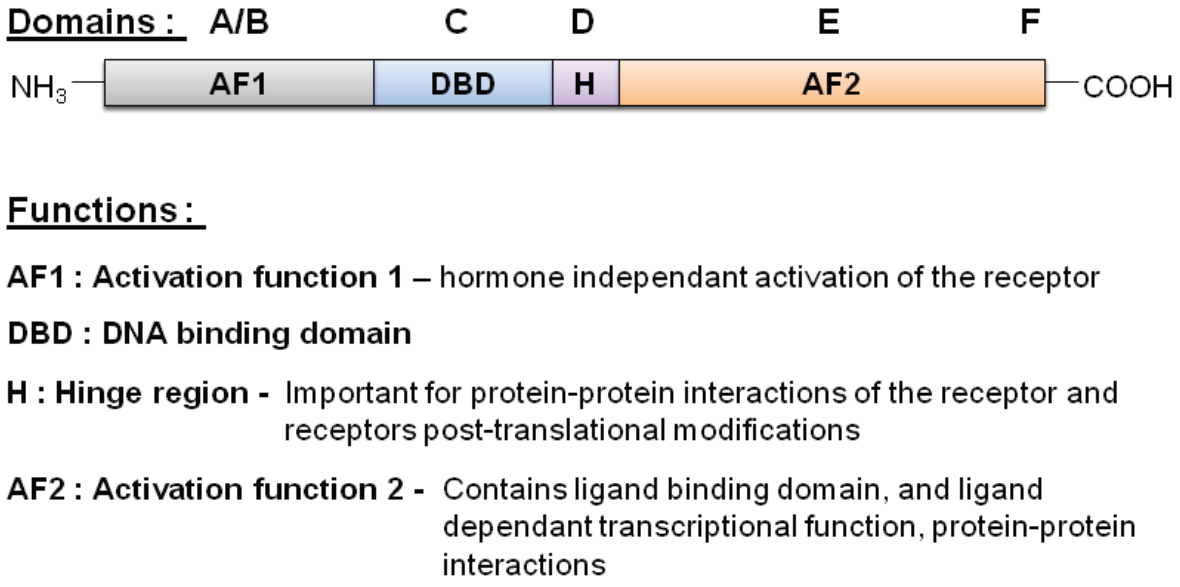

Figure 15 : General outline of functional and structural domains of steroid hormones nuclear receptors. Structural domains (A-F) and corresponding functional modalities are depicted. From Stanisic et al., 2010.

The A and B domains are contained within the receptor's AF1 and are implicated in the hormone-independent transcriptional activation of the receptor. The C domain represents the DBD and is composed of two zinc-finger motifs that are responsible for DNA sequence-specific binding to hormone response elements (e.g. Estrogen response elements, ERE). The D domain or hinge region is a linker between the DNA and ligand binding regions (LBDs) of steroid hormones NR. Functionally, it contains a nuclear localization signal (NLS) and is implicated in interaction with coregulator

molecules. The E domain is responsible for ligand binding and doubles as the ligand-activated AF2 domain. In case of E2R, the LBD is composed of 12 alpha helixes, five of these form a hydrophobic ligand-binding cleft in Estrogens receptors (Bourguet et al., 2000, Srinivasan et al., 2013). In the example of E2R, upon binding to Estradiol, this region undergoes a conformational change involving helix 12 displacement over the opening of the ligand binding pocket. This change allows specific interactions with LXXLL helical motifs available in coactivator structures such as steroid receptor coactivators which remodel chromatine and facilitate the access of the transcriptional machinery to DNA (Han et al., 2009).

1.2.1.2. *Non genomic actions of neurosteroids*

Genomic actions of steroids develop relatively slowly (over minutes to hours) and may persist after the metabolism of steroidal ligands in the brain. However, it is well-known that various steroids may also induce immediate changes (within seconds) on neuronal excitability within a timescale that excludes genomic mechanisms of action. In the 1940s, Seyle showed that certain pregnane steroids can induce rapid sedation and anesthesia. Since this pioneer study, numerous investigations showed that neuroactive steroids and neurosteroids are able to control neuronal excitability and activity via membrane bound receptors such as γ-aminobutyric acid type A ($GABA_A$) receptors, NMDA receptors and sigma receptors (Losel and Wehling, 2003, Belelli and Lambert, 2005, Maurice et al., 2006, Mellon, 2007). Because neurosteroids are directly synthesized in the nervous system, they could act in a paracrine, or autocrine manner on these receptors to modulate nerve cell activities (Patte-Mensah and Mensah-Nyagan, 2008).

1.2.2. Evidence for neuroprotective effects of neurosteroids in neurodegenerative disorders

Several evidence revealed that neurosteroids exert neurotrophic and neuroprotective effects in various experimental models of neurodegenerative disorders (Belelli and Lambert, 2005, Patte-Mensah et al., 2005, Borowicz et al., 2011, Gravanis et al., 2012, Panzica et al., 2012, Brinton, 2013). Previous publications from our laboratory have highlighted the neuroprotective effects of neurosteroids such as allopregnanolone, 3α-androstenediol or DHEA against neuropathic pain in animal models (Kibaly et al., 2008, Mensah-Nyagan et al., 2008, Meyer et al., 2008, Mensah-Nyagan et al., 2009, Patte-Mensah et al., 2010, Meyer et al., 2011, 2013, Patte-Mensah et al., 2014). Progesterone and its metabolites were also shown to attenuate neuronal loss after motor neuron axotomy and to promote remylenisation in the CNS and the PNS (Garay et al., 2007, Garay et al., 2009, Kipp and Beyer, 2009). Furthermore, in rat middle cerebral artery occlusion model, both progesterone and allopregnanolone significantly improved behavioral and cognitive performances (Sayeed et al., 2007). The administration of allopregnanolone or progesterone after traumatic brain injury decreased apoptotic DNA fragmentation, caspase-3 and Bax proapoprotic protein expression and ameliorated cognitive performances (Djebaili et al., 2004, Djebaili et al., 2005). Growing evidence also suggested the potential of neurosteroids including allopregnanolone as regenerative agents in the brain (Brinton, 2013).

Several investigations were particularly focused on the assessment of neurosteroid involvement in AD pathophysiological mechanisms in order to develop neurosteroid-based neuroprotective strategies against AD-related symptoms. Reduced blood levels of steroids hormones have been associated with aging and increased risk of

AD. In particular, cholesterol concentration decreased and dehydroepiandrosterone (DHEA) level increased in AD patients brain were correlated with Braak neuropathological stages (Naylor et al., 2008). Evidence showing that DHEA mediates neuroprotection against Aβ-evoked toxicity (Cardounel et al., 1999) suggests that the increased level of DHEA in AD patient brain may represent an adaptative process to cope with undergoing neurodegenerative process as AD.

A consistent body of evidence from animal studies indicate that estrogens may also exert a neuroprotective action in AD. Estradiol synthesis from testosterone was induced in astrocytes by aromatase enzymatic activity in response to injury and estradiol exerted a preventive role on AD-like neuropathology (Garcia-Ovejero et al., 2005, Carroll et al., 2007, Garcia-Segura, 2008). Immunocytochemistry also showed that aromatase expression was upregulated only in brain areas which are strongly affected in AD (Ishunina et al., 2005). This selective upregulation in brain regions undergoing neurodegeneration suggests a possible compensatory mechanism to increase neuroprotection. In agreement with this hypothesis, *in vitro* analysis performed in our laboratory revealed that the overexpression of wild-type APP (APPwt) or mutant P301L tau in SH-SY5Y cells induced an up-regulation of estradiol formation (Schaeffer et al., 2006a). Also, estradiol protects rat cerebellar granule cells against Aβ-induced toxicity by decreasing pro-apoptotic Bax protein expression and by reducing cytochrome c release and caspase-3 activation via the inhibition of JNK pathway (Napolitano et al., 2014). Moreover, estradiol was shown to promote Aβ clearance through Insulin-degrading enzyme activation in rats neurons or via MMP-2 and MMP-9 up-regulation in human SH-SY5Y neuroblastoma cells (Jayaraman et al., 2012, Merlo and Sortino, 2012). These data are perfectly consistent with the results

showing that brain estrogen deficiency accelerates Aβ plaque formation in AD animal models (Yue et al., 2005).

Accumulating evidence in humans points towards a high potential of allopregnanolone to counteract AD-related symptoms. Indeed, reduced plasma and brain levels of allopregnanolone were found AD patients in several studies (Marx et al., 2006, Naylor et al., 2010). Treatments of triple transgenic AD mice with allopregnanolone was able to reverse neurogenic and cognitive deficits by promoting neurogenesis in the subventricular zone of hippocampus (Wang et al., 2010). Also, allopregnanolone reduced Aβ-oligomers accumulation in triple transgenic AD mice hippocampus by decreasing ABAD expression and restoring cholesterol homeostasis through the increase of the key enzyme HMG-CoA-Reductase (Chen et al., 2011a). Furthermore, allopregnanolone improved memory both in triple transgenic AD mice and wild-type mice (Singh et al., 2012). Altogether, these data strongly support the idea that neurosteroids, particularly allopregnanolone may offer interesting perspectives for the development of effective therapies against AD and other neurodegenerative disorders.

1.3. Gamma-hydroxybutyrate (GHB)

1.3.1. GHB : an endogenous neuromodulator

γ-hydroxybutyric acid (GHB) is a molecule which was first synthesized in 1960 by the French anesthesiologist H. Laborit, who attempted to generate an easily brain-permeable form of the principal inhibitory neurotransmitter GABA (Laborit et al., 1960). Three years later, GHB was reported to be an endogenous constituent of the mammalian brain and a GABA metabolite (Bessman and Fishbein, 1963). GABA is translocated into the mitochondria and transformed to succinic semialdehyde (SSA)

by a GABA-transminase (GABA-T). Thus, GABA can also be metabolized through the citric acid cycle via conversion into succinate by the enzyme succinic semialdehyde dehydrogenase (SSADH) (Maitre, 1997). Deficiency in SSADH is associated with elevated levels of GHB and GABA (Gibson et al., 1998). SSA is then transported from the mitochondria to the cytoplasm and is transformed into GHB by succinic semialdehyde reductase (SSR) which uses NADPH as co-substrate. Inhibition of SSR also diminishes the levels of GHB (Rumigny et al., 1981). Via a Ca^{2+}-dependent exocytose, GHB is released into the synaptic cleft where it may act on multiple targets. The elimination of GHB after intraventricular administration occurs in less than five minutes (Maitre, 1997). GHB is reported to be loaded into synaptic vesicles via the vesicular inhibitory amino acid transporter (VIAAT), which also transports GABA and glycine (Bay et al., 2014). Besides this classical removal, GHB is also transported out of the synaptic cleft by a Na^{+}-dependent plasma membrane transporter and is then reconverted into SSA by a GHB dehydrogenase (Benavides et al., 1982, Maitre, 1997, Maitre et al., 2000) (Fig. 16).

The majority of reported pharmacological and behavioral effects of exogenous GHB are mediated by $GABA_B$ receptors. This receptor is a member of the G protein-coupled receptors (GPCRs) which leads to the hyperpolarization of neurons by opening of post-synaptic G protein-coupled inwardly rectifying potassium channels and decreases in adenylate cyclase activity and calcium conductance (Bay et al., 2014). Indirect effects may also occur if GHB is converted to GABA, which in turn activates GABA transmission. Nevertheless, binding to $GABA_B$ receptors in synaptosomes persisted in the presence of GHB dehydrogenase inhibitors (Maitre, 1997). In addition to $GABA_B$ receptors, recent data suggest that GHB may bind on specific σ4βδ $GABA_A$ receptors subtypes (Absalom et al., 2012), but this observation

remain controversial (Connelly et al., 2013). Nevertheless, because of the structural similarity between GHB and GABA and the sedative properties of GHB, it seems reasonable to suggest GHB interactions with $GABA_A$ receptors (Fig. 16).

Besides the GABA receptors, GHB was reported to have its own receptors. In 2003, our laboratory reported the cloning of a GHB receptor in rat brain (Andriamampandry et al., 2003b). Using the analogue NCS-400, a protein from solubilized rat brain membranes was isolated by affinity purification. The presence of a consensus G-protein binding in the amino acid sequence suggest that the expressed GHB receptor is a member of GPCRs. Later, in 2007, a human GHB receptor was also isolated from a human frontal cortex by our institute (Andriamampandry et al., 2007). This receptor (GHBh1) may also be a GPCR, more precisely a G_i/G_o coupled GPRC. More recently, GHB was reported to induce calcium and cAMP signaling in differencied NCB-20 neurohybrydoma cells confirming the existence of specific GPCRs for GHB (Coune et al., 2010) (Fig. 16).

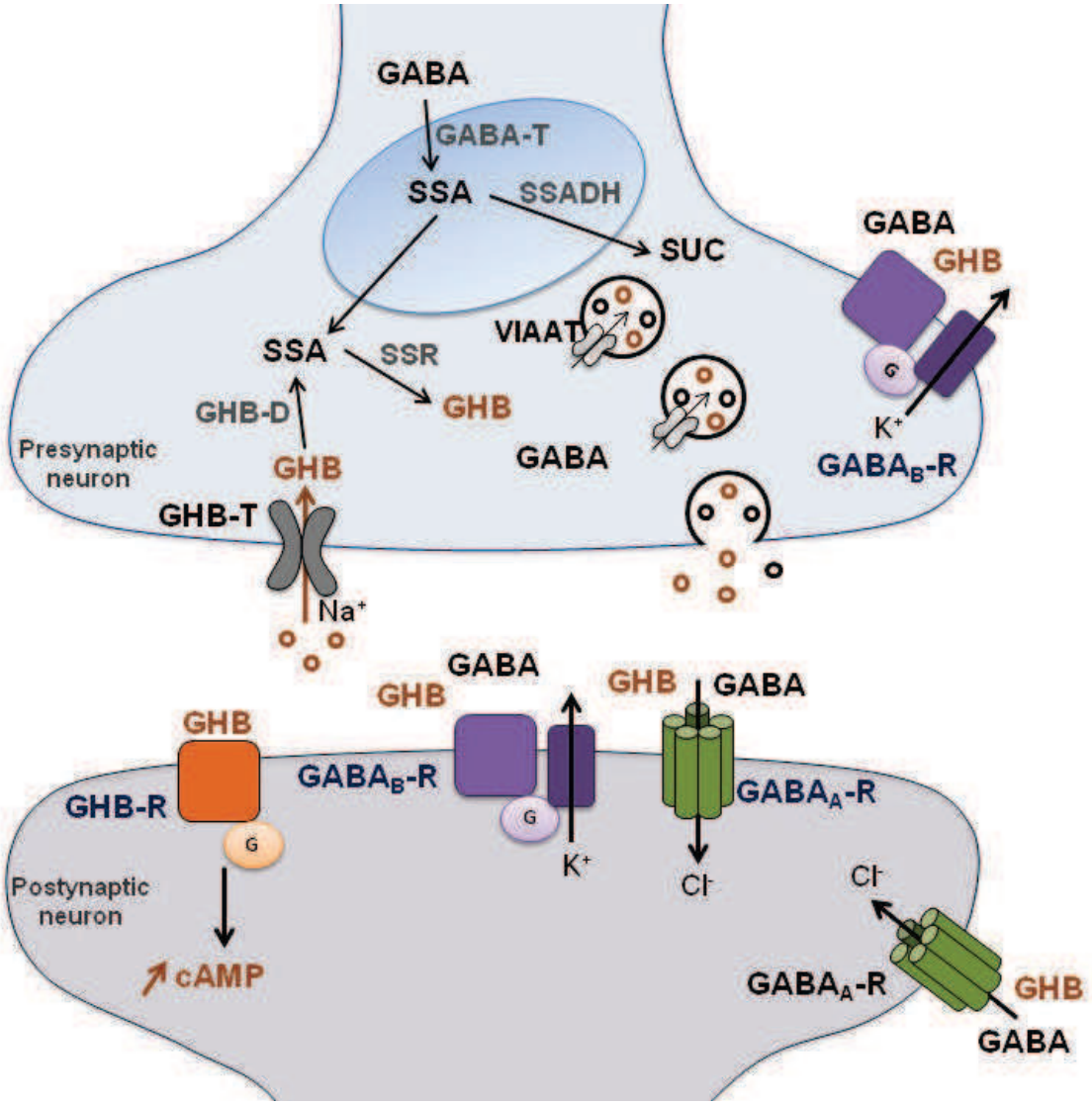

Figure 16 : Overview of a GABA/GHB synapse. GHB is a GABA metabolite which is converted into succinic semialdehyde (SSA) by a GABA-transaminase in the mitochondria. SSA is then converted into GHB in the cytosol by a succinic semialdehyde reductase (SSR). After loading into synaptic vesicles by the vesicular inhibitory amino acid transporter (VIAAT) and exocytosis, GHB can act on metabotropic $GABA_B$-receptors ($GABA_B$-R) or on the ion channel GABAA-receptor ($GABA_A$-R). Besides the GABAergic system, GHB binds on its own G-protein-coupled receptor (GHB-R) which positively modulates cAMP. GHB is reuptaken by a GHB transporter and is then reconverted into SSA by a GHB-T, GHB-transporter ; GHB-D, GHB-dehydrogenase ; SSADH, succinic semialdehyde dehydrogenase ; SUC, succinate ; cAMP, cyclic adenosine monophosphate. Adapted from Bay et al., 2014.

1.3.2. Gamma-hydroxybutyrate as potential neuroprotective agent

GHB was first used as an anesthetic agent because of its ability to easily cross the blood brain barrier. Moreover, this molecule, which induces slow waves sleep (Lapierre et al., 1990, Van Cauter et al., 1997) appears crucial for neuronal recuperation from wakefulness (Tononi and Cirelli, 2003). Therefore, GHB was clinically used to treat sleep disorders such as catalepsy/narcolepsy and demonstrated efficiency in this context (Mamelak et al., 2004, Thorpy, 2005). Because of the reparative aspects of sleep, GHB was also observed to attenuate pain, fatigue and sleep disturbance in fibromyalgia patients (Russell, 1999, Russell et al., 2009, Russell et al., 2011, Spaeth et al., 2013).

A protective effect of GHB has been shown in animal models of brain ischemia/hypoxia as well as in head injury-induced coma in humans (Escuret et al., 1977, Wolfson et al., 1977a, Lavyne et al., 1983, Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004). More precisely, GHB was observed to limit histological and functional consequences of a focal ischemic or excitotoxic insult of the brain confirming the potential of GHB as a neuroprotective agent (Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004). However, no mechanism underlying this protection was demonstrated.

In vivo studies in rodent revealed that GHB is able to diminish cerebral metabolism, particularly to shift glucose metabolism towards the pentose phosphate pathway (PPP) via the activation of glucose-6-phosphate dehydrogenase which induces the formation of NADPH, an essential cofactor of numerous antioxidant enzymes including gluthatione reductase/catalase (Taberner et al., 1972, Taberner, 1973, Russell et al., 1999, Mamelak, 2007). Consequently, GHB also appears as an

interesting agent for the reduction of oxidative stress. Furthermore, ischemic reperfusion injury has been attributed in large part to ROS formation, lipid peroxidation and the increase of intracellular calcium (Alvarez et al., 2014). Therefore, the protection exerted by GHB in brain ischemia models could be attributed to the reduction of oxidative stress and calcium overload, which are also major characteristics of AD physiopathology, but *in vitro* data supporting this hypothesis are completely missing. However, recent data of our laboratory showed that a single pharmacological dose of GHB modulates the brain expression of multiple genes including various genes encoding for amyloid degrading enzymes (Neprilysin) or for programmed cell death factors (Kemmel et al., 2010). These results strongly suggest GHB involvement in neuroprotection.

1.4. GHB and neurosteroids : a potential link

GHB and neurosteroids share similarities in their molecular targets. As aforementioned, GHB and neurosteroids may modulate $GABA_A$ receptors. Therefore, these two classes of molecules may act together in GABAergic synapses. $GABA_A$-receptor activation was repeatedly shown to induce neuroprotective effects in various brain injuries models (Farber et al., 2003, Elsersy et al., 2006, Mensah-Nyagan et al., 2009). Also, the enhancement of GABAergic transmission is known to be neuroprotective against neuroinflammation- or oxidative stress-evoked damages (Farber et al., 2003, Elsersy et al., 2006, Querfurth and LaFerla, 2010, Dias et al., 2014).

Interestingly, it has clearly been demonstrated that GHB increased allopregnanolone and tetrahydrodeoxy-corticosterone (THDOC) production in the rat brain (Barbaccia et al., 2002). The selective $GABA_B$ receptor antagonist SCH50911 prevented the

stimulatory action of GHB on neurosteroids formation, while the $GABA_B$ receptor agonist baclofen mimicked it. These results suggest that GHB, via $GABA_B$ receptor-mediated mechanisms, increases the brain concentrations of neurosteroids such as allopregnanolone and THDOC that positively modulate the $GABA_A$ receptor (Barbaccia et al., 2005). Because Ca^{2+} and cAMP signaling which are activated by GHB proper receptor (GHB-R) are also well known to stimulate various neurosteroid-synthesizing enzymes, it is reasonable to hypothesize that GHB may upregulate neurosteroidogenesis via the activation of GHB-R (Beaudoin et al., 1997, Maitre et al., 2000, Kimoto et al., 2001, Morita et al., 2004, Trbovich et al., 2004, Coune et al., 2010, Udhane et al., 2013).

Altogether, these data suggest that GHB and neurosteroids may act synergistically or additively to protect nerve cells against degenerative mechanisms or neurotoxicity-induced neuronal cell loss.

1.5. PhD project

1.5.1. Objectives

As aforementioned, direct interactions occur between GHB and neurosteroidogenesis but also between the mechanisms of actions of GHB and neurosteroids in the nervous system. Therefore, we hypothesized that GHB may exert neuroprotective effects through the modulation of neurosteroidogenesis. It is also possible that GHB and neurosteroids act additively or synergistically to protect nerve cells against deleterious mechanisms leading to neurodegenerative disorders, including AD.

The purpose of the thesis was therefore to :

(1) Investigate the ability of separated or concomitant GHB and neurosteroid treatments to protect human neuroblastoma cells against death mechanisms evoked by AD etiological factors such as (i) oxidative stress-induced apoptosis, (ii) abnormal expression of wild-type APP (APPwt) or (iii) ER stress.

(2) Verify if GHB-evoked neuroprotection is mediated by the modulation of neurosteroidogenesis.

(3) Check whether GHB and/or neurosteroids may be able to stimulate the activity and/or expression of two amyloid peptide regulatory enzymes : MMP-2 and MMP-9.

1.5.2. Experimental models

To reach our objectives, we have used the human neuroblastoma SH-SY5Y cells which possess all of the neuronal features and have been well demonstrated as a relevant cellular model for the investigation of molecular and biochemical mechanisms involved in AD (Biedler et al., 1978, Tanaka et al., 1995, Li et al., 1996b, Misonou et al., 2000, Rhein et al., 2009). In particular, the transfection of SH-SY5Y cells with AD brain hallmarks reproduced accurately cellular abnormalities involved in AD physiopathology such as toxic Aβ accumulation, mitochondrial dysfunctions, ROS generation, oxidative stress, apoptosis and decreased cell viability (Tanaka et al., 1995, Li et al., 1996a, Misonou et al., 2000, Hoerndli et al., 2007, Qin and Jia, 2008, Rhein et al., 2009, Zampese et al., 2011, Wan et al., 2012, Gotz et al., 2013b, Monroy-Ramirez et al., 2013). Most importantly, SH-SY5Y cells have also been characterized as neurosteroid-producing cells expressing several key steroidogenic enzymes (Melcangi et al., 1993, Schaeffer et al., 2006b, Schaeffer et al., 2008a,

Schaeffer et al., 2008c). Interestingly, previous results from our laboratory have shown that the overexpression of APPwt or the transfection of mutant tau P301L or normal hTau40 proteins significantly affected the process of neurosteroid production in SH-SY5Y cells (Schaeffer et al., 2006a, Patte-Mensah et al., 2012).

For the induction of oxidative stress-evoked cell death mechanisms, we have treated SH-SY5Y cells with hydrogen peroxide (H_2O_2) which is well established in the literature as the best pharmacological substance to generate relevant experimental models to investigate the cellular effects of oxidative stress (Finkel and Holbrook, 2000, Chen et al., 2011b, Mouton-Liger et al., 2012).

Regarding the assessment of ER stress-induced cell damages, SH-SY5Y were treated either with tunicamycin, an inhibitor of protein N-glycosylation, or thapsigargin, a calcium SERCA-pump specific blocker (King and Tabiowo, 1981, Thastrup et al., 1990, Li et al., 1993, Yoshida et al., 2006, Ghosh et al., 2012, Hetz et al., 2013).

In order to determine the effects of GHB and neurosteroids on the activity of MMP-2 and MMP-9, we optimized a yeast based high throughput MMP activity assay based on *Pichia pastoris* cell surface expression of human MMP-2 and MMP-9 (Diehl et al., 2011).

2. Materials and methods

2.1. Neuroblastoma cell line SH-SY5Y

SH-SY5Y is a thrice cloned subline of the neuroblastoma cell line SK-N-SH which has been established in 1970. A neuroblast like subclone of SK-N-SH, named SH-SY, was subcloned as SH-SY5, which was subcloned a third time to produce the SH-SY5Y line, first described in 1978 (Biedler et al., 1978). The cloning process involved expansion of individual cells or a small group of cells that expressed neuron-like characteristics. SH-SY5Y cells possess numerous neuronal characteristics and are well recognized as a relevant cell model for the study of molecular and biochemical mechanisms involved in AD (Tanaka et al., 1995, Li et al., 1996a, Misonou et al., 2000, Rhein et al., 2009).

In order to develop a cell model which can mimick Aβ accumulation evoked by the overexpression of APP, the wild-type (wt) or non mutated APP was stably transfected into SH-SY5Y cells (Scheuermann et al., 2001). Briefly, APPwt was cloned into the pCEP4 vector (Invitrogen/ITC Biotechnology, Heidelberg, Germany). Stably expression of APPwt was obtained by transfection of SH-SY5Y cells using Lipofect AMINE plus (ITC Biotechnology, Heidelberg, Germany) and selection with hygromycin. Empty pCEP4 vector was also transfected in SH-SY5Y cells that constitute the control cells. Native SH-SY5Y were purchased from Sigma-Aldrich (Saint-Quentin, France). The morphological aspects of these three cell lines are represented in Fig. 17.

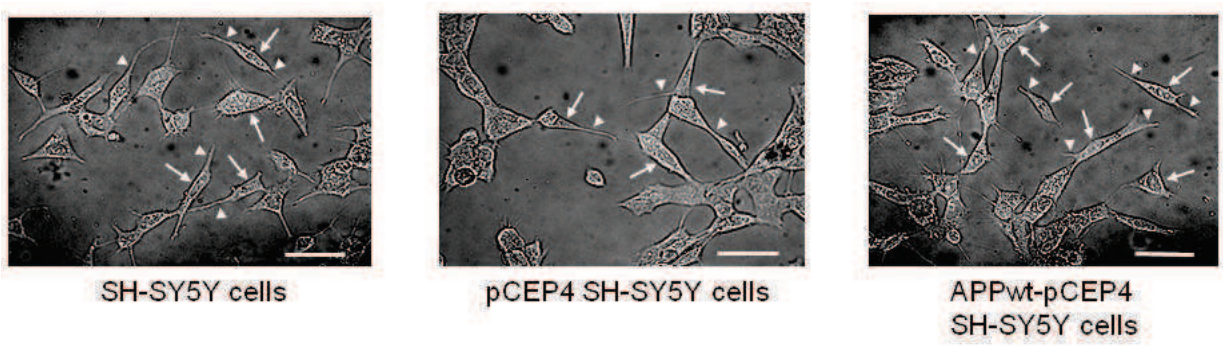

Figure 17 : Morphology of native and transfected SH-SY5Y cells. Native SH-SY5Y cells exhibit a neuroblast-like morphology with differentiated perykaria and occasional short neurites (arrowheads). Cells transfected with pCEP4 vector alone or harboring APPwt cDNA exhibit a similar morphology to that of native cells. Scale bars = 50 μm. Adapted from Schaeffer et al., 2006.

2.2. Yeast : *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast which is widely used in biotechnology for recombinant protein production because of its high growth rate and its ability to grow on simple and inexpensive culture media. As unicellular eukaryote, *P. pastoris* combines the advantage of being a eukaryotic expression host bearing the capability to perform complex processing and posttranslational modifications of higher eukaryotic proteins. In many cases, foreign protein expression is driven from promoters derived from the *P. pastoris* alcohol oxidase genes *AOX1* or *AOX2* which both allow efficient methanol induced protein expression (Daly and Hearn, 2005).

For the analysis of human MMP-2/-9 activity in a yeast-based bioassay, a recombinant *P. pastoris* strain (KM71) was used having the genotype *arg4 his4 aox1::ARG4*. Transformation of this histidine auxotrophic yeast strain was performed with an expression plasmid carrying the *HIS4* gene such that transformants could be selected by growth on media lacking histidine.

2.3. Material

2.3.1. Antibodies

The antibodies used in this PhD thesis are listed in table 2.

Table 2 : Antibodies

Primary antibody	Developed in	Provider	Reference
Anti β-actin	Mouse	Abcam, Cambridge, UK	ab8226
Anti Bax	Rabbit	Abcam, Cambridge, UK	ab7977
Anti Bcl-2	Mouse	Santa-Cruz, Dallas, USA	sc-509
Anti CHOP	Rabbit	Santa Cruz, Dallas, USA	Sc-793
Anti GRP78	Rabbit	Abcam, Cambridge, UK	ab32618
Anti XBP-1	Rabbit	Santa Cruz, Dallas, USA	sc-7160
Anti α-Tubulin	Mouse	Sigma-Aldrich, Saint-Quentin, France	T8203
Secondary antibody	**Developed in**	**Provider**	**Reference**
Anti Mouse IgG-HRP	Goat	PARIS anticorps (Compiègne, France)	BI2413C
Anti Rabbit IgG-HRP	Goat	PARIS anticorps (Compiègne, France)	BI2407

2.3.2. RNA oligonucleotides

The oligonucleotides used for RT-qPCR were purchased from Eurogentec (Angers, France). The related sequences are represented in table 3.

Table 3 : Oligonucleotide sequences

Gene	Sens	5'-3' sequence	References
3α-HSOR	forward	GGTGAGACGCCACTACCAAA	(Stoffel-Wagner et al., 2000)
	reverse	TCTAGCTAGCTGAAGTTGCCA	
5α-Reductase	forward	ATACCAAGGGGAGGCTTATTTGAA	(Luchetti et al., 2006)
	reverse	CTCCATTTCAGCGTATTTAGGTAC	
TSPO	forward	AGGCTTCACAGAGAAGGTTGTGGT	(Luchetti et al., 2006)
	reverse	AGTTGAGTGTGGTCGTGAAGGCCA	
MMP-2	forward	AGATCTTCTTCTTCAAGGACCGGTT	(Munaut et al., 2003)
	reverse	GGCTGGTCAGTGGCTTGGGGTA	
MMP-9	forward	GCGGAGATTGGGAACCAGCTGTA	(Munaut et al., 2003)
	reverse	GACGCGCCTGTGTACACCCACA	
XBP-1 unspliced	forward	ACTCAGACTACGTGCACCTCTGCA	(Jiang et al., 2012)
	reverse	GAGAAAGGGAGGCTGGTAAGGAAC	
XBP-1 spliced	forward	GGCTCGAATGAGTGAGCTGGAACA	(Jiang et al., 2012)
	reverse	CAACTGGGCCTGCACCTGCT	
GRP78 - BiP	forward	CATCACGCCGTCCTATGTCG	(Jiang et al., 2012)
	reverse	CGTCAAAGACCGTGTTCTCG	
CHOP	forward	TTCTCTGGCTTGGCTGACTGA	(Jiang et al., 2012)
	reverse	TGGTCTTCCTCCTCTTCCTCCT	
β-Actin	forward	TGGCACCCAGCACAATGAA	(Jiang et al., 2012)
	reverse	CTAAGTCATAGTCCGCCTAGAAGCA	
Aromatase	forward	TGCAGGAAAGTACATCGCCAT	(Schaeffer et al., 2008c)
	reverse	TCCTTGCAATGTCTTCACGTG	
Vimentine	forward	TGGCACGTCTTGACCTTGAA	(Sharma et al., 2009)
	reverse	GGTCATCGTGATGCTGAGAA	
Bax	forward	TTTGCTTCAGGGTTTCATCC	(Qin et al., 2009)
	reverse	GCCACTCGGAAAAAGACCTC	
Bcl-2	forward	ACGACTTCTCCCGCCGCTAC	(An et al., 2010)
	reverse	CCCAGCCTCCGTTATCCTG	

2.3.3. Chemicals, reagents and material

The most important chemicals, reagents and devices are represented in table 4.

Table 4 : Reagents, kits and devices

Reagents	Provider
Dubelcco's modified eagle medium (DMEM)	Sigma-Aldrich (Saint-Quentin, France)
Penicillin/streptomycin	Sigma-Aldrich (Saint-Quentin, France)
Hygromycin	Sigma-Aldrich (Saint-Quentin, France)
Hydrogen peroxyde	Sigma-Aldrich (Saint-Quentin, France)
Thapsigargin	Sigma-Aldrich (Saint-Quentin, France)
Tunicamycin	Sigma-Aldrich (Saint-Quentin, France)
TRIzol reagent	Sigma-Aldrich (Saint-Quentin, France)
Trypan blue	Sigma-Aldrich (Saint-Quentin, France)
MTT (Thiazolyl Blue Tetrazolium Blue)	Sigma-Aldrich (Saint-Quentin, France)
GHB	Sigma-Aldrich (Saint-Quentin, France)
Allopregnanolone	Steraloids (Newport, USA)
Estradiol	Sigma-Aldrich (Saint-Quentin, France)
FURA-2 / AM	Invitrogen (Heidelberg, Germany)
Kits	**Provider**
iQ SYBR Green super mix	Biorad (Hercules, USA)
iScript cDNA synthesis kit	Biorad (Hercules, USA)
In Situ cell death detection kit, fluorescein (TUNEL reagent)	Roche Diagnostics (Mannheim, Germany)
Phycoerythrin (PE) conjugated rabbit anti-active caspase-3 Antibody / detection kit	BD Pharmingen (Heidelberg, Germany)
BCA kit	Sigma-Aldrich (Saint-Quentin, France)
Clarity Electro Chemoluminescence substrate Kit	Biorad (Hercules, USA)
cOmplete® Protease inhibitor cocktail tablets	Roche Diagnostics (Mannheim, Germany)
Enzcheck Gelatinase/collagenase assay Kit	Invitrogen (Heidelberg, Germany)
Device	**Provider**
Spectrophotometer Multiscan GO 1510	Thermo Scientific (Villebon s. Yvette, France)
Microplate fluorescence reader-Fluoroskan ascent CF	Thermo Scientific (Villebon s. Yvette, France)
Automatic cell counter TC20	Biorad (Hercules, USA)
Flow cytometer Navios	Beckman Coulter (Indianapolis, USA)
Confocal LEICA TCS-SP microscop	Leica (Nanterre, France)
Olympus IX70 microscope (Calcium imaging)	Olympus (Tokyo, Japan)
Thermocycler - iCycler	Biorad (Hercules, USA)
Chemidoc MP - Imager	Biorad (Hercules, USA)

2.4. Cell culture

2.4.1. SH-SY5Y cells

2.4.1.1. *Routine culture*

Human neuroblastoma SH-SY5Y cells were grown at 37°C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mm Glutamax and 1% (v/v) Penicillin/Streptomycin. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP_{695} (APPwt) or the expression vector pCEP4 (Invitrogen**,** Europe) alone (control vector) using lipofect AMINEplus (Invitrogen, Europe). Transfected APPwt cells were grown in DMEM standard medium supplemented with 300 µg/ml hygromycin.

For the split, cells were washed in Phosphate Buffered Saline (PBS) (see recipe in Part. 2.7.1), resuspended in DMEM medium and scraped. Then cells were diluted in new dishes preloaded with DMEM medium (generally a dilution of 1:3 / 1:4).

2.4.1.2. *Freezing and unfreezing of SH-SY5Y cells*

Cell lines in continuous culture are prone to genetic drift. Therefore, it is crucial that they are frozen down as a seed stock and preserved for long term storage. Cryopreserving of cultured cells is accomplished by storing them in liquid nitrogen or at -80°C in the presence of a cryoprotective agent such as dimethylsulfoxide (DMSO). DMSO decreases the freezing point of the medium and also allows a slower cooling rate, reducing the risk of ice crystal formation which may cause cell damages or death. The freezing medium recipe is described as follows.

Freezing Medium :

- 30 % Fetal calf serum
- 10 % DMSO
- 60 % DMEM (supplemented with 0.5 % Penicillin/streptomycin)

Before the freezing, a confluent dish is selected and washed twice with PBS. Then, the cells are resuspended in normal DMEM, scraped and harvested in a 50 ml falcon. Cells are counted with the TC20 automatic counter. Centrifugation was performed at room temperature at 300g for 10 min. The supernatant was removed, and the cells resuspended in the freezing medium. For each cryovial, 2 millions cells are deposed in 1.8 ml. Then, the cryovials are stored in a “freezing container” which is placed in liquid nitrogen or at -80°C freezer.

To unfreeze a seed stock, the medium described below was used.

Unfreezing medium :

- 80 % DMEM (supplemented with 0.5 % Penicillin/streptomycin)
- 20 % fetal calf serum

A seed stock cryovial is removed from liquid nitrogen or -80°C and warmed in a double boiler at 37°C. In parallel, unfreezing medium was also warmed in a double boiler at 37°C. When the cells are solubilized, they are resuspended in 10 ml of unfreezing medium. Then, the cells are centrifugated at 300g for 10 min at room temperature. The supernatant was removed, the cells were successively resuspended in 6 ml of unfreezing medium, seeded in a 25 cm^2 flask and placed at 37°C under 5% CO_2 atmosphere.

2.4.2. Pichia Pastoris

2.4.2.1. *Culture media*

The following media were used for the culture of *Pichia pastoris*.

Histidine drop out (d/o) medium (20 mM, pH 7.0) :

Glucose	2 %
Sodium-*Di*hydrogen phosphate dihydrate	3.12 g/l
*Di*Sodium-hydrogen phosphate dihydrate	3.56 g/l
Ammonium sulfate	0.5 %
Histidine Drop out mix	0.85 g/l
YNB (yeast nitrogen base)	1.7 g/l
(Agar	1.2 %)

Glucose and YNB were separately autoclaved and combined after sterilization.

Yeast Nitrogen Base (YNB) :

YNB w/o amino acids and ammonium sulfate	34 g/l

YNB was diluted in dH_2O. The solution was sterile filtered before use.

Histidine drop out mix :

Adenine	0.4 g	Phenylalanine	1.0 g
Arginine	0.4 g	Threonine	4.0 g
Histidine	***0 g***	Tryptophane	0.4 g
Isoleucine	0.6 g	Tyrosine	0.6 g
Leucine	2.0 g	Uracile	0.4 g
Lysine	0.6 g	Valine	3.0 g
Methionine	0.4 g		

The powder mix was stored at 4°C.

BMG (Buffered Minimal Glycerol) medium :

Glycerol	2 %
Ammonium sulfate	1 %
YNB	3.4 %
Biotin	4×10^{-5} %
1 M Potassium phosphate buffer pH 6.0	10 %

Glycerol and ammonium sulfate were dissolved in dH_2O and autoclaved. Thereafter, the biotin solution and YNB were added. Addition of potassium phosphate buffer was done immediately before use. The medium was stored at room temperature (RT).

BMM (Buffered Minimal Methanol) medium :

Methanol	1 %
Ammonium sulfate	1 %
YNB	3.4 %
Biotin	4×10^{-5} %
1 M Potassium phosphate buffer pH 6.0	10 %

Ammonium sulfate was dissolved in dH_2O and autoclaved. Thereafter, biotin and YNB were added. Addition of methanol and potassium phosphate buffer was done immediately before use. The medium was stored at 4°C.

1 M potassium phosphate buffer, pH 6.0 :

Potassium *Di*hydrogen phosphate	106.1 g/l
*Di*Potassium hydrogen phosphate	212.3 g/l

500 x Biotin solution :

Biotin	20 %

Biotin was dissolved in dH2O, sterile filtered and stored at 4°C.

2.4.2.2. *Pichia pastoris culture*

Pre-precultures of *P. pastoris* were seeded on Histidine d/o agar plates and incubated at 30°C. After growth of selected colonies, a single colony of each strain was inoculated into liquid His d/o medium and cultivated in shaking flasks over night at 220 revolutions per minute (RPM) and 30°C (the conditions of incubation of the yeast strains are similar after this step). Then, precultures were grown in BMG until they reached an optical density at 600 nm (OD_{600}) of at least 50. Induction of MMP-2/-9 expression was achieved by shifting the cells into BMM at a feeding rate of 1%

(vol/vol) methanol twice a day. After 96 h of methanol induction, cells were harvested by centrifugation, washed with sterile H_2O and lyophilized before being used in the MMP bioassay (see Part 2.8., pages 88-89). Each vial contained 2 ml of yeast diluted in H_2O with an OD_{600} of 5 before lyophilization.

2.4.3. Cell counting

For all experiments, SH-SY5Y cells were counted before seeding, so that the same number of cells was seeded in each conditions. The cells were counted thrice in the automatic TC20 cell counter (BioRad, Hercules, USA) and the average of these counts were used for the next step of seeding. Briefly, after scraping and resuspension of cells in DMEM, the cells were homogenized by gentle vortex. Then, 3 x 10 µl were deposed into the three counting slides which were inserted into the TC20 cell counter. The cell diameter range selected for the counting was from 5 to 25 µm. Since the optimal cell concentration range of the counter is between 1 x 10^5 and 5 x 10^6 cells, the cell suspension was either diluted or concentrated (by centrifugation at 300g for 8 min, and resuspension in smaller volume) after two counts (if the result was out of this range). Real cell number was also deduced with the dilution/concentration factor used before the counting.

For crude estimations of yeast cell numbers, optical density was measured at 600 nm (OD_{600}) in a Ultraspec 2100 pro, UV/visible spectrophotometer (Amersham Bioscience). Cultures were diluted such as the observed OD_{600} was <1.0. In this range, an OD_{600}=1.0 is approximately equal to 3 x 10^7 cells/ml. The resulting OD_{600} was corrected by the dilution factor used for the measurement.

2.5. Cell viability assays

2.5.1. Trypan blue exclusion method

The trypan blue exclusion method is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes including trypan blue, whereas dead cells do not. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye.

In our study, SH-SY5Y cells were seeded into a 24-well plate (10^5 cells per well) and incubated for 48 h at 37°C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum. Native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells were treated with various concentrations of H_2O_2 ranging from 0 to 1 mM. After 24 h, cells were detached from the plate using a 0.05% trypsin-EDTA solution and immediately after DMEM supplemented with serum was added. Equal volumes of the cell suspension and 0.4% (v/v) trypan blue in PBS were mixed. Ten microliters of each mixture was transferred to a non-gridded disposable Countess® chamber Slide and the cells were scored using a Countess® automated cell counter (Invitrogen, Heidelberg, Germany). Each probe was counted twice. The percent of cell survival was calculated by the Countess® software (Invitrogen, Heidelberg, Germany) as the number of living cells divided by total cell number (including dead and living cells).

2.5.2. MTT viability assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a colorimetric assay to assess cell viability. Mitochondrial NADPH-dependent oxidoreductase reflects the number of viable cells by reducing MTT into a purple

insoluble formazan crystal (Fig. 18). After solubilization of this crystal with DMSO, the absorbance at 550 nm is spectrophotometrically quantified.

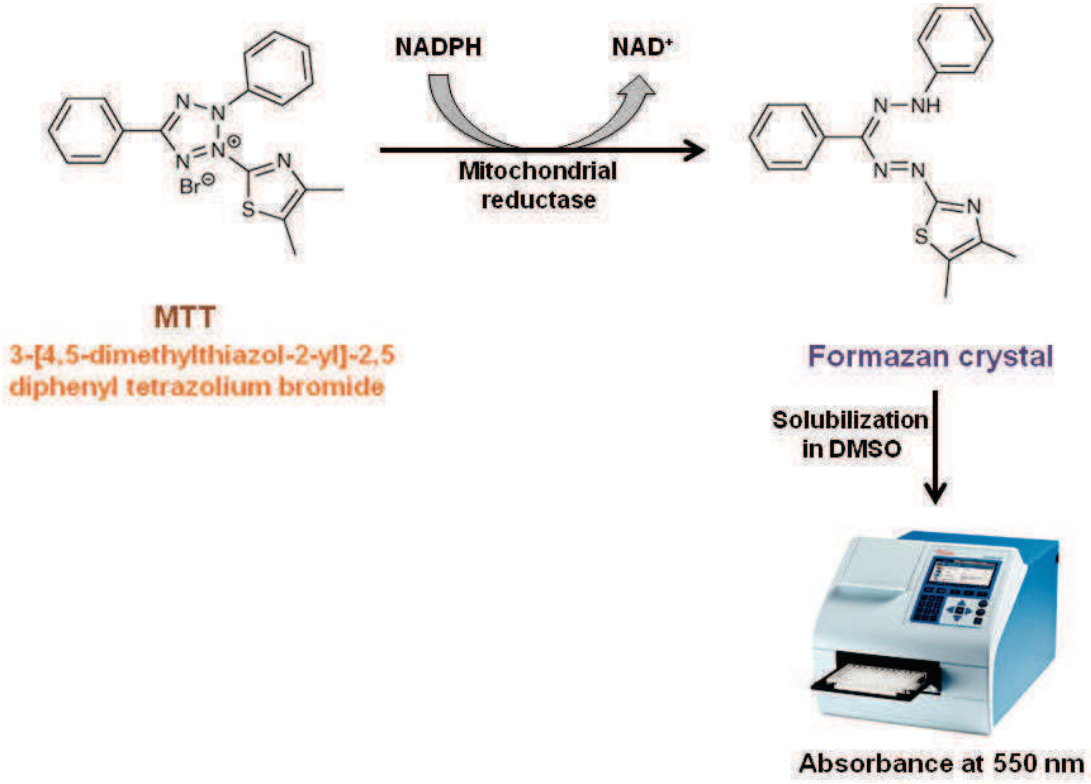

Figure 18 : MTT assay principle. MTT, a yellow tetrazole, is reduced to insoluble purple formazan in living cell mitochondria. After solubilization in DMSO, absorbance at 550 nm is measured in order to determine the percentage of viable cells.

Native and genetically modified SH-SY5Y cells were seeded at $5x10^4$ cells per well into 96-well plates and allowed to attach in DMEM medium w/o phenol red. After 48 h, neuroblastoma cells were incubated under the following conditions :

- Effects of GHB and neurosteroids on cell survival under normal/physiological conditions :

Normal culture medium completely devoid of stressor but containing graded doses of GHB (0, 50, 200, 500 and 1000 µM), allopregnanolone (0, 250, 500 and 1000 nM) or estradiol (0, 250, 500 and 1000 nM) was administrated for 24 h or 48 h.

- Effects of GHB and neurosteroids on cell survival under oxidative stress conditions

In a first step, the cells were exposed to culture medium containing H_2O_2 at various concentrations (0, 0.05, 0.1, 0.2, 0.5, 0.7, 1, 1.5, 2, 4 and 10 mM) for 24 h or 48 h in order to determine effective H_2O_2 doses and adequate incubation time inducing a significant percentage of cell loss. This step was also usefull to establish the sensitivity profile of each category of cells (native, APPwt-transfected and control vector-pCEP4-transfected SH-SY5Y cells) to oxidative stress-evoked toxicity.

In a second step, the cells were incubated with a dose of H_2O_2 capable of killing about 60-90% of native or genetically modified SH-SY5Y cells in the absence or presence of increasing concentrations of GHB, allopregnanolone or estradiol administered alone for 24 h or 48 h in order to determine eventual neuroprotective effect.

Additional experiments were performed by exposing the cells for 24 h or 48 h to H_2O_2 dose capable of killing 60-90% SH-SY5Y cells in the concomitant presence of GHB and allopregnanolone or GHB and estradiol in order to determine eventual additive/synergistic neuroprotective effects of these compounds.

- Effects of GHB and neurosteroids against ER stress-induced cell loss

The cells were first exposed for 24 h to the culture medium containing tunicamycin (0, 1, 5, 10 or 20 µg/ml) or thapsigargin (0, 0,25, 0,5, or 1 µg/ml) in order to determine effective tunicamycin, or thapsigargin doses inducing a significant percentage of cell loss as well as the sensitivity profile of each category of cells (native, APPwt-transfected and control vector-pCEP4-transfected SH-SY5Y cells) to ER stress-evoked toxicity.

Afterwards, the cells were incubated for 24 h with tunicamycin or thapsigargin (concentration killing about 50% of SH-SY5Y cells) in the presence or absence of GHB, allopregnanolone or estradiol administered alone or concomitantly (GHB+ Allopregnanolone or GHB+Estradiol).

In all of the conditions aforementioned, MTT (diluted in DMEM w/o phenol red) was added to each well at the end of the incubation period (24 h or 48 h). The final concentration of MTT in each well was of 0.4 g/l. Thus, the cells were incubated for 3 h at 37°C under 5% CO_2 atmosphere. Thereafter, the medium was removed from the 96-well plates and formazan crystals were diluted in 200 µl DMSO per well. Finally, the absorbance at 550 nm was measured and the background noise (absorbance at 650 nm) subtracted for each probe. Moreover, the absorbance of the medium alone was also subtracted. MTT signal detected for each cell type in basal condition (in absence of stressor) was arbitrary set at 100 %. This basal signal reflecting the total number of living cells in each cell type was the reference that served for the accurate determination of dose-dependent effects of H_2O_2 at 24 h or 48 h. The cell survival percentage is obtained by using the following calculation.

$$Cell\ survival\ (\%) = \left(\frac{ABSORBANCE\ sample - ABSORBANCE\ medium}{ABSORBANCE\ untreated\ cells - ABSORBANCE\ medium}\right) \text{x } 100$$

2.6. RT-qPCR

2.6.1. RNA extraction

RNA extraction with TRIzol, or Guanidinium thiocyanate-phenol-chloroform allows the maintenance of RNA integrity during cell lysis. Addition of chloroform and centrifugation separates the solution into organic and aqueous phases. RNA which

remains only in the aqueous phase can be recovered after precipitation with isopropyl alcohol (Fig. 19).

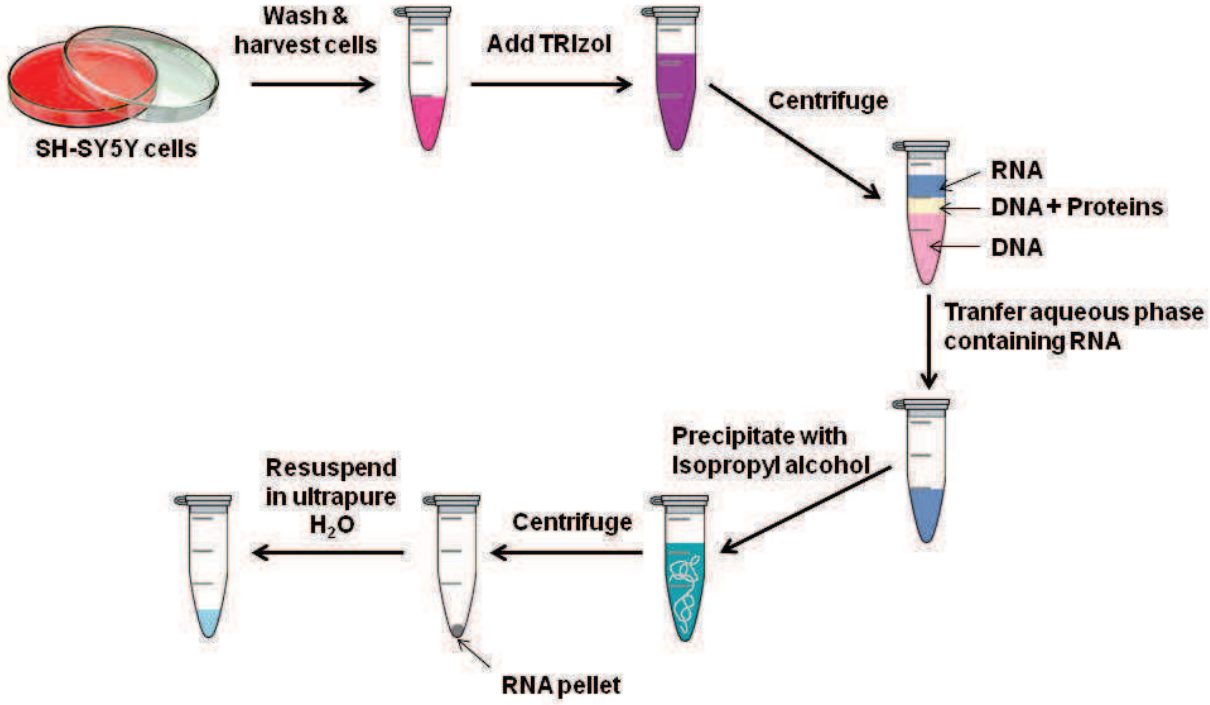

Figure 19 : Principle of TRIzol RNA extraction.

SH-SY5Y cells were seeded at 50 x 10^4 cells/ml in 10 cm dishes and allowed to attach in DMEM medium for 48 h. After 24 h of treatments, the cells were washed twice with cold PBS and centrifuged at 300 g for 8 min. The supernatant was removed and 400 µl TRIzol reagent was added in each vial which was gently homogenized and incubated at RT for 10 minutes. Then, 200 µl chloroform was added per ml of TRIzol used (80 µl chloroform for 400 µl of Trizol in this case). The samples were gently homogenized without vortexing and were incubated at RT for 10 min. Thereafter, the samples were centrifuged at 12.000g for 15 min at 4°C. Aqueous phase was transferred into RNAse free eppendorfs and 500 µl isopropyl alcohol was added per ml of TRIzol used (200 µl in our experiments) and samples were homogenized and incubated for 15 min at RT. The samples were centrifuged at

12,000 g for 15 min at 4°C and the supernatant was removed. RNA pellets were washed with a 80% ethanol solution and were centrifuged again at 12,000 g for 15 min at 4°C. Finally, each RNA pellets were resuspended in 50 µl ultrapure water and were stored at -80°C before use.

2.6.2. RNA concentration and quality determination

Nucleic acids were quantified using UV absorption detected by a spectrophotometer (Thermo Scientific, Villebon sur Yvette, France). The absorbance was measured at 260 and 280 nm. The concentration of nucleic acid was determined using the Beer-Lambert law. An OD_{260} of 1.0 is equivalent to about 40 µg/ml of RNA. RNA has its absorption maximum at 260 nm whereas aromatic amino acids have an absorption maximum at 280 nm. Consequently, the quality of RNA was determined by optical density absorption ratio OD_{260nm} / OD_{280nm} >1.7. In this work, a 1:50 dilution of RNA in ultrapure water was used for measurements of the optical densities. Each sample was measured thrice.

2.6.3. Reverse transcription

In this step, RNA is reverse transcribed into complementary DNA (cDNA) in a reaction using a reverse transcriptase. This cDNA is used as the template for the next quantitative polymerase chain reaction (qPCR). In order to achieve this, we used the iScript cDNA synthesis kit (BioRad, Hercules, USA), which contains a modified Moloney Murine Leukemia Virus (MMLV)-reverse transcriptase characterized by its lower RNase H activity.

Reverse transcription was performed with 2 µg RNA. For each sample, the reaction mix was the following :

5x iScript reaction mix	8 µl
iScript reverse transcriptase	2 µl
Nuclease-free water	x µl
RNA template (2 µg)	x µl
Total volume	**40 µl**

The reaction mix were incubated in a thermal iCycler (BioRad, Hercules, USA) with the following reaction protocol :

5 minutes at 25 °C
30 minutes at 42 °C
5 minutes at 85 °C
Hold at 4°C

If not directly used, cDNA samples were stored at -80°C.

2.6.4. Real Time quantitative PCR (RT-qPCR)

In conventional PCR, the amplified product, or amplicon, is detected with an end-point analysis, by running DNA on an agarose gel at the end of the reaction. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in “real time”.

Real time PCR is made possible by including in the reaction a fluorescent molecule that reports an increase in the amount of DNA with a proportional increase of fluorescent signal. The fluorescent chemistries employed for this purpose include DNA-binding dyes and fluorescently labeled primers or probes. In this thesis, we used the SYBR green label (BioRad, Hercules, USA) which is a DNA-binding dye.

SYBR green binds nonspecifically to double strand DNA (dsDNA) and exhibits fluorescence only when bound to dsDNA. Therefore, the overall fluorescent signal from the reaction is proportional to the amount of dsDNA present. Specific forward and reverse primers (described in Part. 2.2.2) were designed to amplify a 75-200 bp product. Thus, only specific genes expression are quantified.

For each sample, the reaction mix was the following :

Step	**Volume per reaction**	**Final concentration**
Forward primer	Variable	320 nM
Reverse primer	Variable	320 nM
iQ SYBR Green supermix 2x	12.5 µl	1x
cDNA template	5 µl of 1:10 solution	1/50 of 2µg RNA used in RT
Nuclease free water	Variable	-
Total reaction volume	**25 µl**	

To validate the RT-qPCR data, a standard curve was constructed with successive dilutions of cDNA so that the range of template concentrations used for the standard curve encompassed the entire range of template concentrations in the samples analyzed. Moreover, the specificity of the amplification was controlled by a melting curve ranging from 55 to 95°C allowing the identification of a single peak corresponding to each amplicon.

The thermal cycling protocol was performed with an iCycler (BioRad, Hercules, USA) and is described as followed :

Cycling step	Temperature	Hold time (min:sec)	Number of cycles
Initial denaturation and polymerase activation	95°C	3:00	1
Denaturing	95°C	0:15	40
Annealing (primer-designed)	55-64°C	0:30	
Extension	72°C	0:30	
Melt curve	55-95°C (in 0.5°C increments)	0:30	1

Starting quantities of interest genes were analyzed by using the iCycler iQ optical system software (BioRad 3.1 Version). All samples were analyzed in triplicate and reported to the vimentine or actine genes (housekeeping genes) in the same plate in order to ensure an accurate calculation. Gene expression levels were obtained by relative quantification normalized to the housekeeping genes.

2.7. Protein based analysis

Besides RNA levels, protein levels were also analyzed. In order to achieve this, SDS-Page and western analysis were performed.

2.7.1. Samples preparation – Protein Extraction

After 24 h or 48 h treatments in 10 cm dishes, SH-SY5Y cells were rinsed twice in cold PBS, harvested and transferred into 50 ml falcon. Then, the cells were centrifuged at 300 g for 8 minutes, the supernatant was removed, and each sample was resuspended in 200 µl lysis buffer (Radio Immunoprecipitation assay buffer, RIPA buffer). Samples were kept on ice for 1 h and vortexed every 10 minutes and

were then clarified by centrifugation at 4°C for 30 min at 12,000 g. The supernatant was saved for protein analysis and western blotting. Total protein concentration was determined by the bicinchoninic acid (BCA) assay (see Part 2.7.2., page 83). Then, protein samples were diluted in Laemmli buffer and denatured by warming for 5 minutes at 95°C (see Part 2.7.3., page 83).

1x PBS :

Sodium chloride	137 mM
Potassium Chloride	2.7 mM
*Di*Sodium-hydrogen phosphate dihydrate	10 mM
Potassium *Di*hydrogen phosphate	2 mM

These reagents were dissolved in ultrapure water, pH was adjusted to 7.4 with hydrogen chloride. Then, the PBS was autoclaved and stored at 4°C.

RIPA buffer :

Tris-HCl	50 mM
NaCl	150 mM
EDTA	1 mM
IGEPAL (NP-40)	1 %
Na-Deoxycholate	1 %
SDS	0.1 %

This solution was steril filtered and stored at 4°C. Before use, 1 tablet of cOmplete® Protease inhibitor (Roche, Mannheim, Germany) was added per 10 ml of RIPA buffer.

2.7.2. Assessment of protein level with BCA assay

The BCA assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total proteins which is a modified form of the Lowry method (Hartree, 1972). This method combines the reduction of Cu^{2+} to Cu^{+} by protein in alkaline medium (the biuret reaction) with the sensitive and selective detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid (Smith et al., 1985, Redinbaugh and Turley, 1986). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one of cuprous ion. This water-soluble complex exhibit a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a broad working range (20-2,000 µg/ml). Protein concentration is calculated with a reference curve obtained for a standard protein (Bovine Serum Albumin in this thesis) ranging from 0 to 2,000 µg/ml.

Each standard probe and protein sample was measured thrice. Before the measurement, samples were prepared in two distinct dilutions (1:10 and 1:20) with ultrapure water. In parallel, the BCA working reagents (Sigma-Aldrich, Saint-Quentin, France) was prepared by adding 1 part of reagent B to 50 parts of reagents A. 25 µl of standard or sample were deposed in 96-well plates and 200 µl of BCA working reagent was added per sample. Then, the 96-well plate was incubated for 30 minutes at 37°C and the absorbance at 562 nm was measured with the multiscan Go 1510 spectrophotometer (Thermo Scientific, Villebon s. Yvette, France).

2.7.3. SDS-PAGE

Sodium dodecyl sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was first described in the end of the sixties (Shapiro et al., 1967) and is a method used to separate components of a protein mixture based on their size. The technique is

based upon the principle that a charged molecule will migrate in an electric field toward an electrode with opposite sign. In PAGE, proteins charged negatively by the binding of the anionic detergent SDS separate within a matrix of polyacrylamide gel in an electric field according to their molecular weight. Polyacrylamide is formed by the polymerization of the monomer molecule-acrylamide crosslinked by N,N'-methylene-bis-acrylamide (BIS). Free radicals generated by ammonium persulfate (APS) and a catalyst acting as an oxygen scavenger (-N,N,N',N'-tetramethylethylene diamine [TEMED]) are required to start the polymerization since acrylamide and BIS are nonreactive by themselves or when mixed together. The distinct advantage of acrylamide gel systems is that the initial concentrations of acrylamide and BIS control the hardness and degree of cool, crosslinking of the gel. The hardness of a gel in turn controls the friction that macromolecules experience as they move through the gel in an electric field, and therefore affects the resolution of the components to be separated. Hard gels (12-20% acrylamide) retard the migration of large molecules more than they do with small ones. In certain cases, high concentration acrylamide gels are so tight that they exclude large molecules from entering the gel but allow the migration and resolution of low molecular weight components of a complex mixture. Alternatively, in a loose gel (4-8% acrylamide), high molecular weight molecules migrate much farther down the gel and, in some instances, can move right out of the matrix.

In this thesis, 45 µg of proteins per sample were fractionated by 4-20% TGX SDS pre-casted polyacrylamide gels (BioRad, Hercules, USA). Proteins were diluted in reducing Laemmli buffer (added with β-mercaptoethanol) and heated for 5 min at 95°C. Precision Plus Protein Dual color standards (BioRad, Hercules, USA) was

used as protein ladder. The electrophoresis was performed with an electric field of 180 V (constant voltage) in Tris/glycine/SDS running buffer.

Laemmli buffer 1x :

Tris-HCl	62.5 mM
Glycerol	25 %
SDS	2 %
Bromophenol Blue	0.01 %
β-mercaptoethanol	5 %

pH was adjusted to 6.8 with hydrogen chloride. β-mercaptoethanol was added directly before the use. This buffer was stored at room temperature.

Tris/glycine/SDS running buffer 1 x :

Tris, pH 8.3	25 mM
Glycine	192 mM
SDS	0.1 %

pH was adjusted to 8.3 with hydrogen chloride. This buffer was stored at 4°C.

2.7.4. Western analysis

Western blotting identifies, thanks to specific antibodies proteins that have been separated from another according to their size by SDS-PAGE (see Part 2.7.3., pages 83-85). The blot is a membrane of polyvinylidene (PVDF). The gel from SDS-PAGE is placed next to the membrane and application of an electrical current allows the protein in the gel to transfer towards the membrane where they adhere. The membrane is then a replica of the gel's protein pattern, and is subsequently stained with a primary antibody which are recognized by a secondary antibody. In this thesis, secondary antibody were conjugated with horse radish peroxidase and detected by

chemoluminescence. Before the transfer (blotting), PVDF membranes (BioRad, Hercules, USA) were activated with 100 % ethanol.

2.7.4.1. *"Semi dry" blotting*

In a semi dry transfer, the gel and the membrane are sandwiched between two stacks of filter paper that are in direct contact with plate electrodes. The membrane is closed to the positive electrode and the gel closest to the negative electrode. The term "semi dry" refers to the limited amount of transfer buffer, which is confined to the two stacks of filter paper. In semi dry system, the distance between the electrodes is limited only by the thickness of the gel and membrane sandwich. As a result, high electric field strengths and high-intensity blotting conditions are achieved. In this thesis, semi dry transfers were performed with a Transblot Turbo transfer system (BioRad, Hercules, USA) at 1 A constant and up to 25 V for 30 minutes.

Transfer buffer 1x :

Tris-HCl	25 mM
Glycine	192 mM
SDS	0.1 %
Ethanol	20 %

Ethanol was added directly before the use. The buffer was stored at 4°C.

2.7.4.2. *Immunodetection*

After the protein transfer to the membrane, this membrane is blocked thanks its incubation with a blocking buffer for 1 h at room temperature. Then, the membrane is incubated overnight at 4°C with primary antibodies (listed in part 2.2.1) diluted in blocking buffer (see Table 5).

Table 5 : Antibodies and dilutions used in western analyses

Primary antibody	Developed in	Provider/Product n°	Dilution
Anti β-actin	Mouse	Abcam, Cambridge, UK/ ab8226	1 : 1000
Anti Bax	Rabbit	Abcam, Cambridge, UK/ ab7977	1 : 800
Anti Bcl-2	Mouse	Santa-Cruz, Dallas, USA/ sc-509	1 : 200
Anti CHOP	Rabbit	Santa Cruz, Dallas, USA/ Sc-793	1 : 1000
Anti GRP78	Rabbit	Abcam, Cambridge, UK/ ab32618	1 : 500
Anti XBP-1	Rabbit	Santa Cruz, Dallas, USA/ sc-7160	1 : 500
Anti α-Tubulin	Mouse	Sigma-Aldrich, Saint-Quentin, France/ T8203	1 : 2000
Secondary antibody	**Developed in**	**Provider/Product n°**	**Dilution**
Anti mouse IgG-HRP	Goat	PARIS anticorps (compiègne, France)/ BI2413C	1 : 2000
Anti Rabbit IgG-HRP	Goat	PARIS anticorps (compiègne, France)/ BI2407	1 : 2000

At the end of the incubation with primary antibodies, the membranes were washed 3 x 10 minutes (washing buffer) and were incubated with secondary antibodies (diluted in blocking buffer) for 1 h at room temperature. Then, the membranes were washed again 3 x 10 minutes. Finally, the membranes were incubated for 5 minutes with Clarity Western electrochemoluminescence substrate (BioRad, Hercules, USA). Specific chemoluminescence signals were detected with a ChemiDoc MP imaging System (BioRad, Hercules, USA). The relative intensity of bands was densitometrically determined by using the Image Lab software (BioRad, Hercules,

USA). All data from three independent experiments were expressed as the ratio to densitometric values of the corresponding β-actin or α-tubulin control.

Washing buffer 1x:

Tween 20	0.05 %
10 x TBS	10 %

This buffer was stored at 4°C

10 x TBS (Tris Buffered Saline) :

Tris-HCl	1 M
NaCl	1 M

pH was adjusted to 7.4 with hydrogen chloride. This buffer was stored at 4°C.

Blocking Buffer :

Non fat dry milk	5 %

The dry milk was diluted in washing buffer. This buffer was stored at 4°C.

2.8. MMP-2/-9 activity assay with recombinant yeast

Gelatinase activity of human MMP-2 and -9 recombinantly expressed on the outer cell wall of *P. pastoris* was measured as previously described (Diehl et al., 2011) by using a modified and specifically adapted version of a commercial EnzChek Gelatinase/collagenase assay (Invitrogen, Heidelberg, Germany). A schematic outline of this yeast bioassay is shown in Fig. 20.

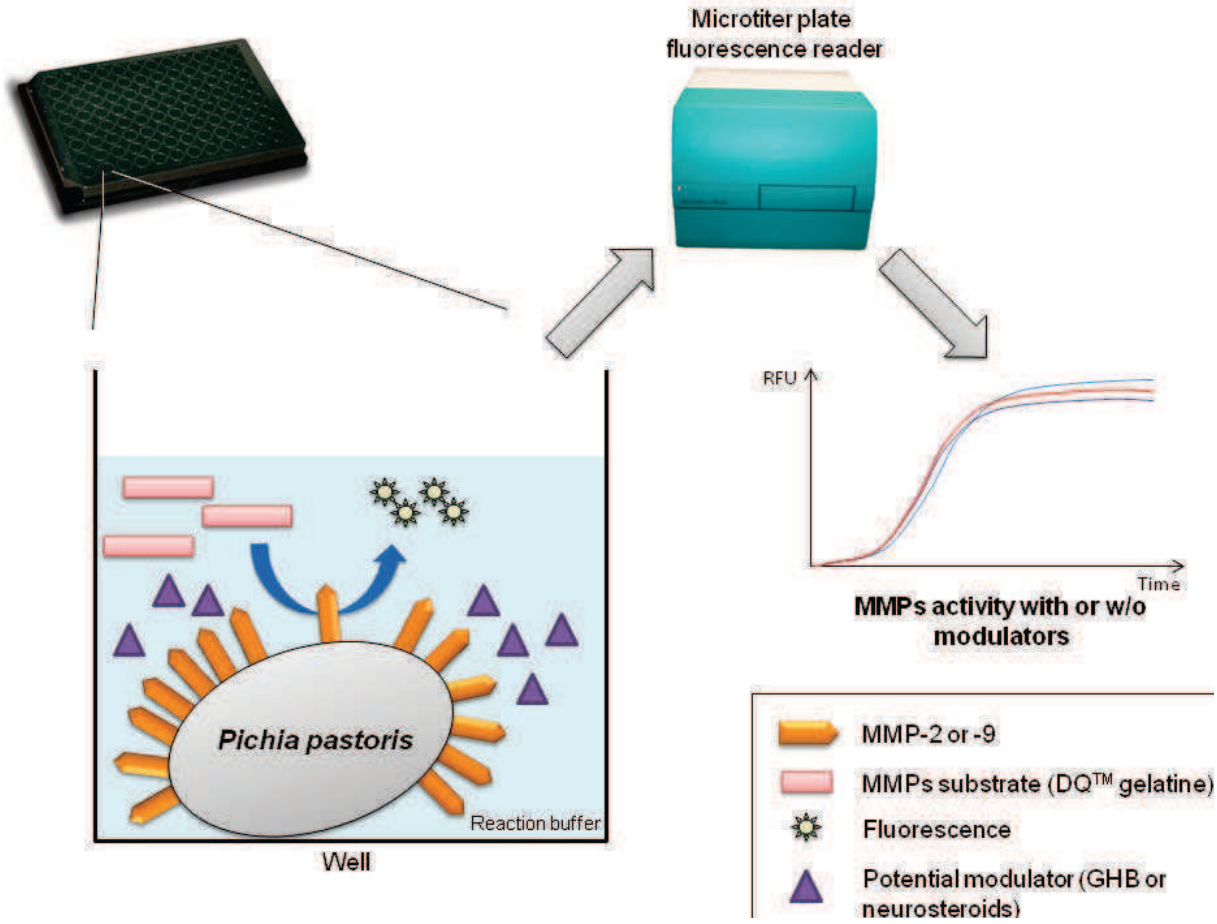

Figure 20 : Schematic outline of the MMP-2/-9 bioassay used in this thesis on the basis of recombinant *P. pastoris* cells. Yeast cells expressing human MMP-2 or MMP-9 on the outer cell surface are resuspended in reaction buffer at the same optical density in a 96-well plate. Cells are exposed to potential modulator (GHB and/or neurosteroids) and incubated with a DQ™ gelatine substrate which becomes fluorescent when cleaved by MMPs. Green fluorescence is continuously recorded over 20 hours in an automated microtiter plate reader (RFU, relative fluorescence intensity; MMP, matrix metalloproteinase; adapted from Diehl et al, 2011).

To perform the assay, lyophilized cells were resuspended in reaction buffer at an optical density (OD_{600}) of 5. GHB and neurosteroids were diluted in reaction buffer at 4-fold the concentration that was supposed to be tested. 100 µg/ml of fluorescein-conjugated DQ® (MMP substrate) was also diluted in reaction buffer. 100 µl of cells, 50 µl of modulator and 50 µl of MMP substrate were deposed in each well of a black 96-well plate. Fluorescence detection was recorded at 37°C for 20 hours with 20 minutes intervals between each measurement and with 19 min 30 s of shaking in

between (700 rpm, 1 mm diameter) by a Labsystems Fluororoskan Ascent CF microtiter plate reader (excitation at 485 nm and emission measured at 527 nm). The effect of each tested modulator was measured in 4 wells per MMP and all experiments were repeated three times. Relative intensity of fluorescence was collected and analyzed with Microsoft Excel.

Reaction buffer :

Tris	50 mM
NaCl	150 mM
$CaCl_2$	5 mM

This solution was stored at 4°C.

2.9. Flow cytometry- and microscopy-based methods

2.9.1. Flow cytometry (FACS) assessment of activated Caspase-3 and TUNEL labeling

The occurrence of apoptosis in SH-SY5Y cells was investigated using (i) the TUNEL technique coupled with fluorescein (FITC) and (ii) the activated caspase-3 approach combined with phycoerythrin (PE). The apoptotic fluorescent signal was therefore assessed by flow cytometry (FACS) using the Roche Diagnostics TUNEL kit or the BD Pharmingen™ PE Active Caspase-3 Apoptosis kit.

Briefly, SH-SY5Y cells were seeded in 100 mm² Petri dishes and treated for 24 h as previously described. Following the treatment, the cells were detached, centrifuged for 10 min at 1,000 g (room temperature) and re-suspended with PBS supplemented with 0.5% BSA before being gently fixed in buffer (4% paraformaldehyde in PBS) for 1h. Thereafter, the cells were submitted to 2 centrifugation steps (10 min at 1,000 g) separated by a washing step using PBS supplemented with 0.5% BSA. The cells

were then incubated in permeabilization buffer (0.1% Triton X-100 in 0.1 % sodium citrate) for 2 min on ice. An additional round of washing and centrifugation was performed before incubating the cells with PE-rabbit anti-activated caspase-3 antibody (BD Pharmingen cat: 550821) for 1h at 37°C. At the end of the incubation, the cells were washed and centrifuged again for 10 min at 1,000 g. Supernatants were removed and the TUNEL-FITC reaction mixture (50 µl) was added to each pellet. After incubation in darkness for 1 h at 37°C, the cells were washed and suspended in PBS before being analyzed by a flow cytometer (Navios, Beckman Coulter, Indianapolis IN USA). A concentration of 10^4 cells of each sample were assessed and the fluorescence was quantified using CXP® software (Beckman-Coulter, Indianapolis IN, USA). The data were analyze with Kaluza® software (Beckman-Coulter, Indianapolis IN, USA).

Negative controls were performed by omitting Phycoerythrin rabbit anti active Caspase-3 antibody and/or dUTP coupled to fluorescein. Untreated cells obtained after suspension in standard growth medium were used to verify the absence of autofluorescence. TUNEL-FITC and activated caspase-3-PE positive signals were selectively and specifically assessed by flow cytometry. However, additional control experiments were performed by merging the two types of fluorescent signals (TUNEL-FITC and caspase-3-PE) in order to rule out eventual overlapping and use only either the specific TUNEL-FITC or the specific activated caspase-3-PE positive signal for data analysis.

2.9.2. Confocal microscope analysis of apoptotic signals

Oxidative stress- or APPwt-overexpression-evoked apoptotic signal in SH-SY5Y cells was also visualized with a confocal laser scanning microspcope. Briefly,

neuroblastoma cells were seeded onto slides at a density of $1x10^5$ cells and then treated with GHB (500 µM) alone, H_2O_2 alone (0.1 mM for APPwt-transfected cells or 0.7 mM for control vector-pCEP4-transfected and native cells) or with GHB (500 µM) and H_2O_2 (0.1 mM or 0.7 mM) for 1 h, 6 h, 24 h or 48 h. After treatments, the cells were fixed in 4% paraformaldehyde for 1h, washed with PBS and permeabilized with 0.1% sodium citrate and 0.1% Triton X-100. The slides were then incubated with the TUNEL-FITC reaction mixture for 1 h at 37°C. Negative controls were performed by omitting TdT. Also, positive controls were made by incubating the cells with DNAse (3 UI/ml in Tris-HCl, pH 7.5 in 1 mg/ml BSA solution) during 10 min. Images were captured using a LEICA TCS-SP confocal inverted microscope. All analyses were carried out in comparable areas under the same optical and light conditions. Black-and-white images were digitized and viewed on a computer with LAS software (Leica).

2.9.3. Calcium $[Ca^{2+}]_i$ imaging

Intracellular calcium was measured by using FURA-2 acetoxymethyl ester (FURA-2 AM), a membrane permeable derivative of the ratiometric calcium FURA-2 which is rapidly metabolized by cytoplasmic esterases, leading to the active dye FURA-2. The Ca^{2+} unbound form of FURA-2 gets excited at 380 nm and the Ca^{2+} bound form of FURA-2 at 340 nm. The emitted light is measured at 510 nm. In the presence of augments concentrations of free calcium, the fluorescence intensity at 340 nm increases, wheras the fluorescence intensity at 380 nm decreases. Therefore, the 340 nm/380 nm ratio increases. To assess this ratio in SH-SY5Y cells, we incubated them with 1 µM FURA-2 AM (Invitrogen, Karlsruhe, Germany) at 37°C under 5% CO_2 atmosphere for 30 min. Afterwards, the cells were washed with PBS and placed at

RT for 10 min before use. The coverslip was assembled into a sandwich chamber allowing a complete solution exchange in less than 1 s. Stock solutions of thapsigargin (THG) were prepared in DMSO. Two external solutions were prepared :

External solution A (with calcium) :

NaCl	155 mM
$CaCl_2$	0.5 mM
$MgCl_2$	2 mM
Glucose	10 mM
HEPES	5 mM

The pH was adjusted to 7.4 with NaOH and the solution was steril-filetered before storage at 4°C.

External Solution B (without Calcium) :

NaCl	155 mM
$MgCl_2$	2 mM
Glucose	10 mM
HEPES	5 mM

The pH was adjusted to 7,4 with NaOH and the solution was steril-filtered before storage at 4°C.

Thapsigargin, and GHB were diluted either in solution A and B (with or without Ca^{2+} respectively). Before incubation with thapsigargin, the cells were incubated first with solution with solution A then with solution B. Then, the cells were successively exposed to solution B containing 1 µg/ml thapsigargin and then followed with solution A containing 1 µg/ml thapsigargin. 500 µM GHB was either added simultaneously with thapsigargin or pre-incubated 2 h before the measurement. The cells were also incubated for 2 h with 500 nM Allopregnalolone prior to measurement. Fluorescent signals were measured with an Olympus IX 70 microscope (Olympus, Tokyo, Japan) equipped with a 20x objective and were alternatively excited at 340 and 380 nm.

Fluorescence emission (510 nm) were captured each 5 s and the data were analyzed with the TILL® Vision software.

2.10. Statistical analysis

Statistical significance of differences between means was determined using one way ANOVA followed by Student's *t*-test for repeated measurements. Dose-effect parameters (EC_{50} values) were determined by non-linear regression of the experimental data using the GraphPad-Prism program (GraphPad-Prism, San Diego, CA, USA). The goodness of fits was estimated by the R squared value (>0.7).

3. Results

3.1. Effects of GHB and/or neurosteroids against oxidative stress- and APPwt-overexpression-induced cell death

3.1.1. Effect of H_2O_2-induced oxidative stress on native and genetically modified SH-SY5Y cell viability

A time and dose-dependent study was performed to investigate H_2O_2 effect on native, APPwt- and control vector-pCEP4-transfected SH-SY5Y cell viability. The doses of H_2O_2 ranged from 0 to 10 mM and the incubation times were 24 h and 48 h (Fig. 21). MTT signal detected for each cell type in basal condition (absence H_2O_2) was arbitrary set at 100 %. H_2O_2 significantly decreased the viability of each category of neuroblastoma cells in a dose and time dependent manner but it appeared that native, APPwt- and control vector-pCEP4-transfected SH-SY5Y cells are differently sensitive to oxidative stress. Indeed, different concentrations of H_2O_2 killing 50% of cells (EC_{50} values) were observed for the 3 categories of cells after 24 h or 48 h exposure to H_2O_2. At 24 h, EC_{50} values were 0.55 ± 0.09 mM, 0.50 ± 0.08 mM and 0.044 ± 0.002 mM for native, control vector-pCEP4-transfected and APPwt-transfected SH-SY5Y cells, respectively. At 48h, the EC_{50} was 0.51 ± 0.21 mM (native), 0.55 ± 0.11 mM (control vector-pCEP4-transfected) or 0.053 ± 0.01 mM (APPwt-transfected). More importantly, it appeared that 0.7 mM of H_2O_2 was capable of killing about 70% of native or control vector-pCEP4-transfected SH-SY5Y cells while only 0.1 mM of H_2O_2 was sufficient to induce the same percentage (70%) of death in APPwt-transfected SH-SY5Y cells, indicating that APP overexpression significantly enhances cellular susceptibility to oxidative stress.

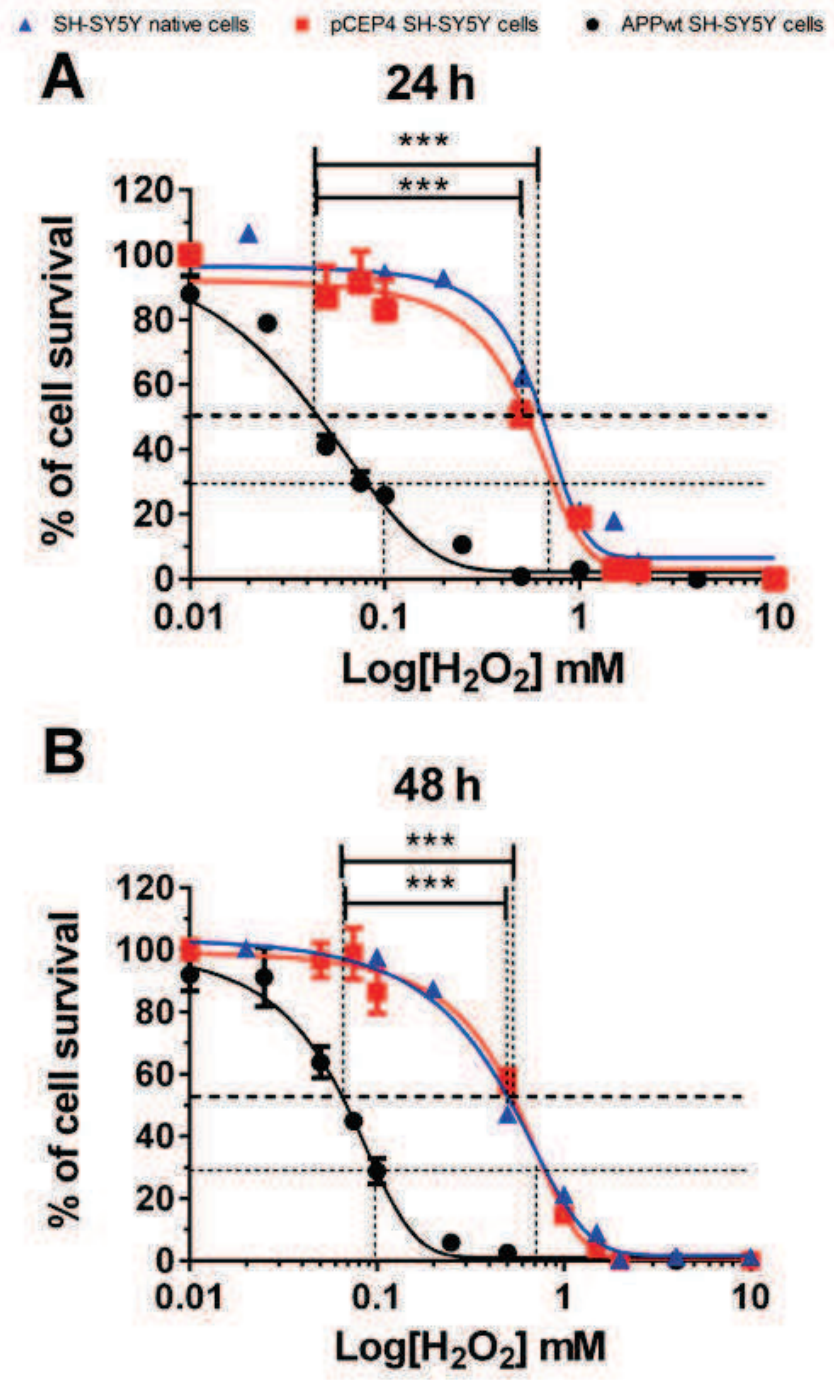

Figure 21 : Dose-response and time-course studies of the effect of H_2O_2 on native (▲), control vector-CEP4-transfected (■) and APPwt-transfected (●) SH-SY5Y cells viability. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. H_2O_2 concentration evoking 50 % of cell survival corresponds to the EC_{50} (mM) for each cell type (line with thick and bold dotted). The dose of H_2O_2 (mM) causing 70% of cell death is indicated for each cell type by the slight dotted line. Each value on the graph represents the mean ± S.E.M. of cell survival of eight independent experiments. ***p<0.001.

3.1.2. Trypan blue exclusion and MTT assessments of control and APPwt-overexpressing SH-SY5Y cell viability and survival

MTT reduction assay is known as a sensitive assay for the measurement of cell viability and proliferation based upon the mitochondrial reduction of tetrazolium salt into insoluble formazan product. However, MTT method only measures living cell percentage while trypan blue exclusion assays allow the determination of both living and dead cell percentages. Therefore, we compared the profiles of the dose-dependent effects exerted by graded concentrations of H_2O_2 on cell survival percentages assessed by both MTT and trypan blue methods. We observed that the profiles obtained with MTT and trypan blue assays fit very well as the R square was higher than 0.98 for each cell type investigated. These observations were also confirmed by Bland-Altman statistical analysis which revealed no significant difference between the data obtained with the two methods. Because the trypan blue exclusion method also allowed the determination of cell death percentages, we observed that in basal condition (absence of H_2O_2), overexpression of APP induced 30 % of death in APPwt-transfected SH-SY5Y cells while the dead cell percentage was non-significant in native (4.4 ± 6.4 %) and control vector-pCEP4-transfected (3.4 ± 4.5 %) cells (Fig. 22).

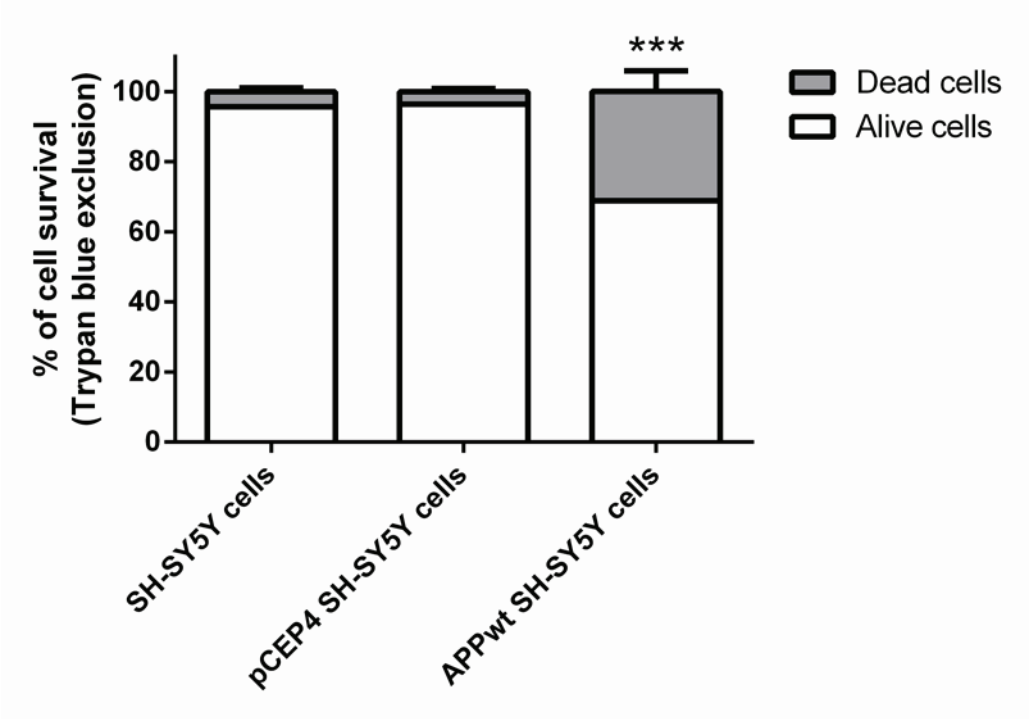

Figure 22 : Basal percentages of dead in control and APPwt-overexpressing-cells. Basal cell death percentages were determined by trypan blue exclusion assays. Each value on the graph represents the mean ± S.E.M. of cell survival of eight independent experiments. ***p<0.001.

3.1.3. Assessment of basal level of apoptotic signal in native, control vector-pCEP4- and APPwt-transfected cells

Because our trypan blue exclusion and MTT investigations revealed that native and genetically modified SH-SY5Y cells, which did not have the same viability in normal conditions, are also differently sensitive to oxidative stress, we decided to determine the basal level of apoptotic signal in each cell type. Flow cytometry assessment showed that the basal levels of both TUNEL-FITC- (Fig. 23A) and activated caspase-3-PE- (Fig. 23B) positive signals were significantly elevated in APPwt-transfected cells compared to native and control-vector-transfected cells. Furthermore, reverse transcription, quantitative real-time PCR and western blot experiments demonstrated that mRNA and protein basal ratios of key apoptotic modulators Bax (pro-apoptotic) / Bcl-2 (anti-apoptotic) were also higher in APPwt-transfected cells than in the controls

(Fig. 23C, D). Altogether, these results show that, in the absence of H_2O_2, overexpression of APPwt induced a *per se* substantial amount of apoptosis leading to a reduced viability of APPwt-transfected cells compared to native and control-vector transfected SH-SY5Y cells.

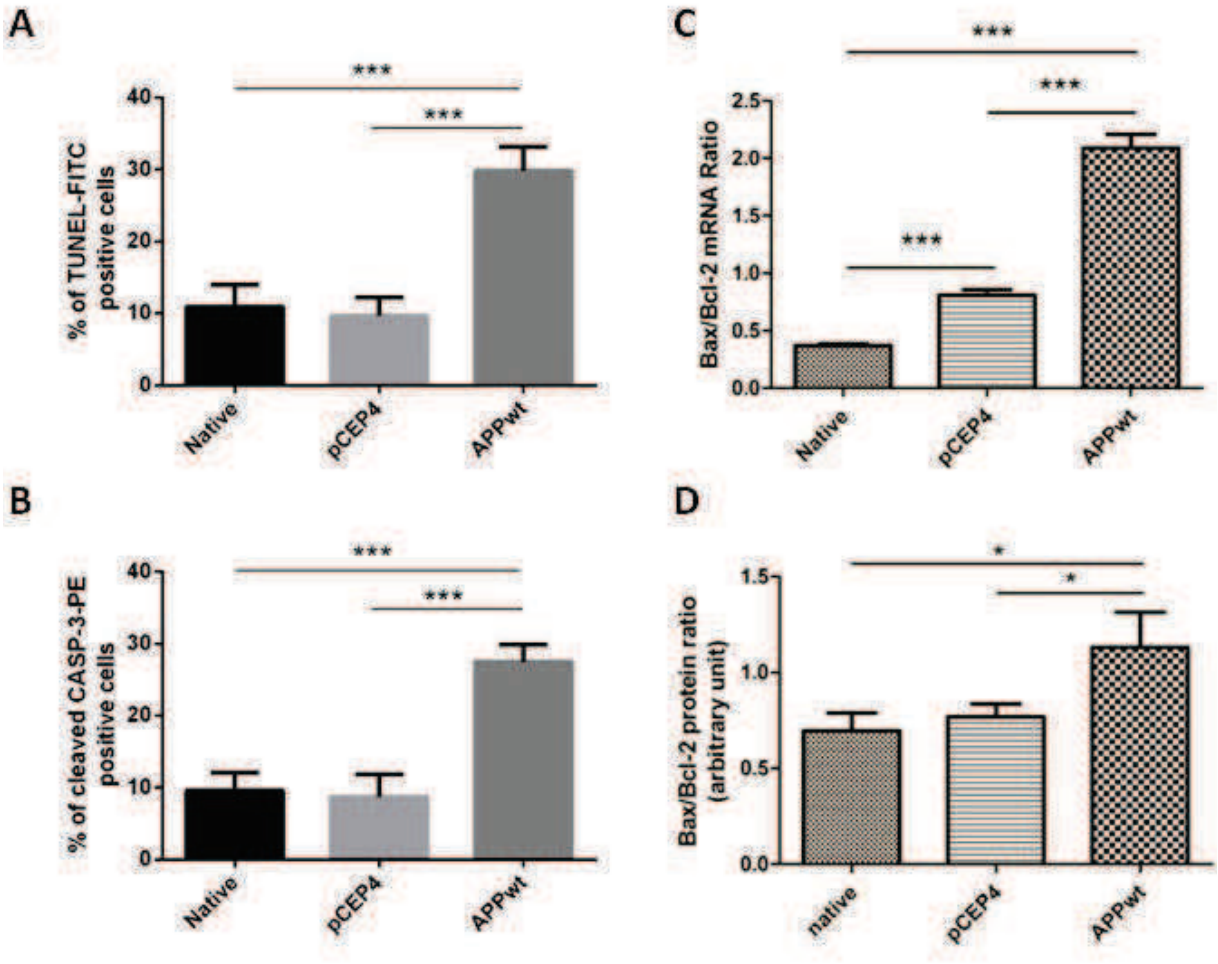

Figure 23 : Characterization of basal levels of apoptotic signal in native, control vector-pCEP4- and APPwt-transfected cells. (A, B) Flow cytometry quantitative assessment of TUNEL-FITC (A) and activated caspase-3-PE positive signals (B) in native and genetically modified SH-SY5Y cells. Both TUNEL-FITC and activated caspase-3-PE methods showed a basally elevated apoptotic signal in APPwt-transfected but not in control-vector-transfected and native SH-SY5Y cells. (C) Reverse transcription and qPCR assessment of Bax/Bcl-2 mRNA ratio in native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells. (D) Western blot semi-quantification of Bax/Bcl-2 protein ratio in native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells; β-actin was used as loading control. Each value represents the mean ± S.E.M. of three independent experiments (*p<0.05 ; ***p<0.001).

3.1.4. Protective effect of GHB against APPwt-overexpression-induced decreased cell viability

In basal conditions (absence of H_2O_2), while the percentage of cell death was non-significant in native and control-vector-transfected cells, about 30 % of cell loss was measured in the population of APPwt-transfected SH-SY5Y cells. Application of graded concentrations of GHB (0 to 1,000 µM) revealed that GHB at 500 µM induced about 30 % increase of APPwt-transfected SH-SY5Y cell viability without affecting the viability of control cells (Fig. 24). Therefore, it appears that, in normal conditions (absence of H_2O_2), GHB which did not promote the proliferation of native and control-vector transfected cells, prevented APPwt-transfected cell loss through a mechanism different from a simple activation of cell proliferation to compensate the loss.

3.1.5. Protective effect of GHB against H_2O_2-induced cell death

In H_2O_2-evoked oxidative stress conditions, we observed that H_2O_2 significantly decreased the viability of native and genetically modified cells in a dose dependent manner but it appeared that APPwt-transfected SH-SY5Y cells are the most sensitive (Fig. 21). Therefore, we decided to test the ability of GHB to protect against H_2O_2-evoked cell death in the drastic condition where more than 70% of cells are killed by H_2O_2-induced toxicity (Fig. 21 and 25). Consequently, we have treated native and control vector-pCEP4-transfected SH-SY5Y cells with 0.7 mM H_2O_2 and APPwt-transfected cells with 0.1 mM H_2O_2 in the absence or presence of graded doses of GHB ranging from 0 to 1,000 µM. The results showed that GHB protected efficiently all of the 3 categories of cells against H_2O_2-evoked death (Fig. 25). In particular, GHB at 500 µM appeared as the optimal concentration that reduced about fifty percent the number of native or genetically modified neuroblastoma cells killed by H_2O_2-toxicity (Fig. 25).

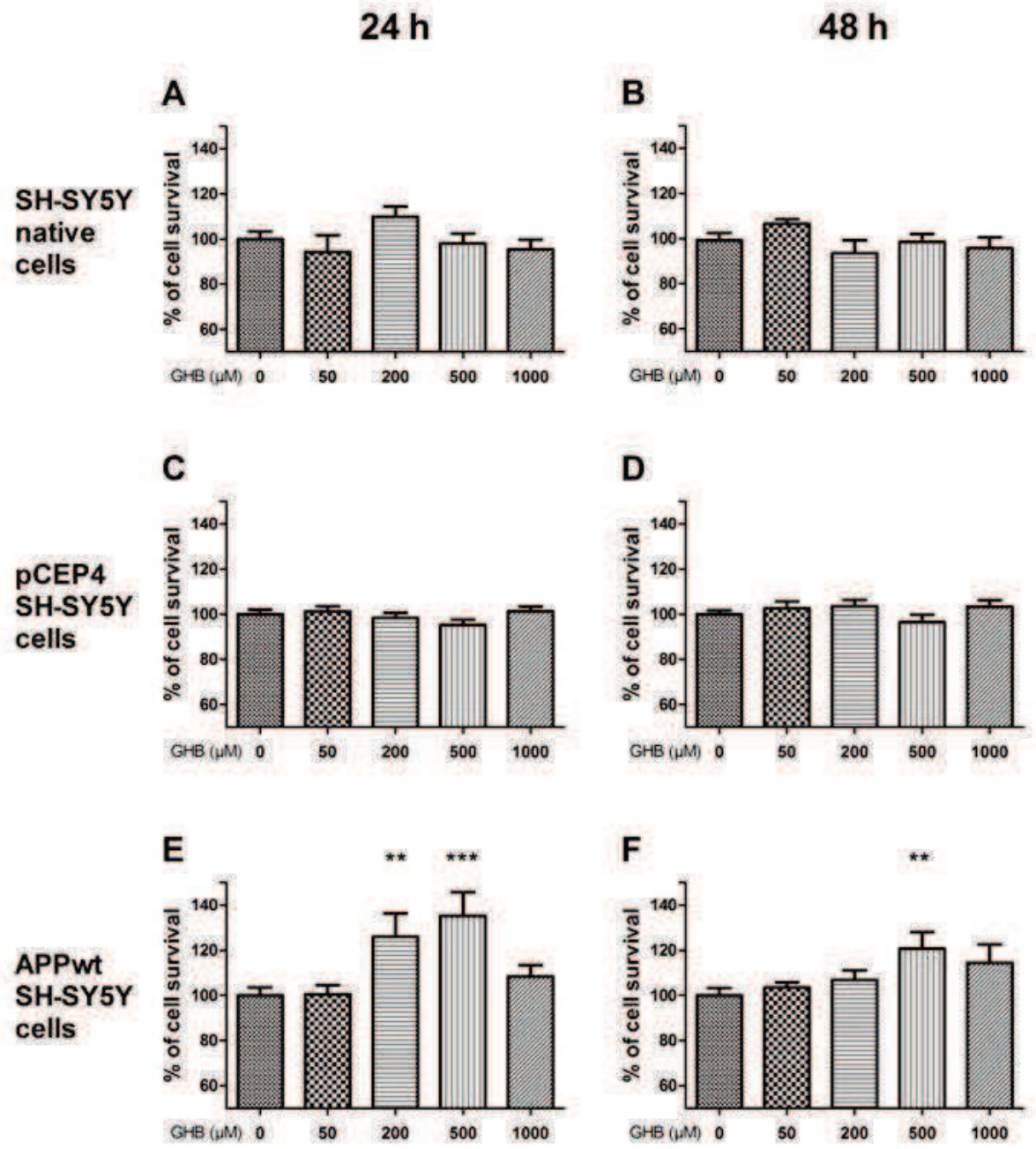

Figure 24 : Dose-response and time-course studies of the effect of GHB on native (A, B), control vector-pCEP4-transfected (C, D) and APPwt-transfected (E, F) SH-SY5Y cell viability. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A stimulatory effect of GHB (200 and 500µM) is detected only on APPwt-transfected SH-SY5Y cell viability and no proliferative effect is identified on the native or control vector-pCEP4-transfected SH-SY5Y cells viability. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value represents the mean ± S.E.M. of five independent experiments. $^{**}p<0.01$ and $^{***}p<0.001$.

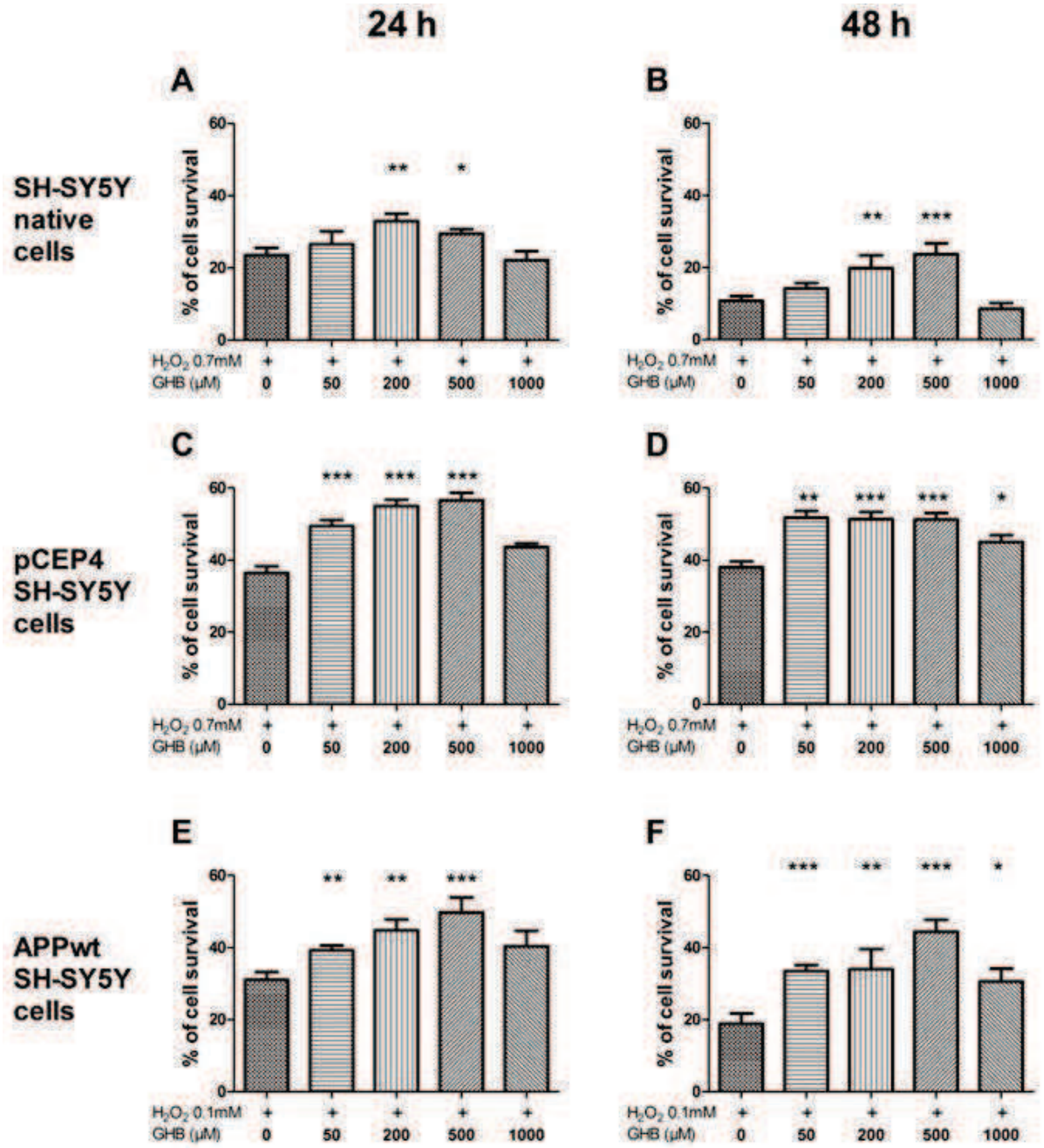

Figure 25 : Protective effect of GHB against H_2O_2-induced native (A, B), control vector-pCEP4-transfected (C, D) and APPwt-transfected (E, F) SH-SY5Y cell death. Native and genetically modified SH-SY5Y cells were exposed to H_2O_2 dose inducing more than 70% of cell loss and treated with graded concentrations of GHB for 24 h (A, C, E) or 48 h (B, D, F). MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A maximum of stimulatory effect of GHB is detected at 500 µM for all type of SH-SY5Y cells. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of five independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

3.1.6. Protective effect of GHB against APPwt-overexpression and H_2O_2-evoked apoptosis

It is well documented that H_2O_2 induces cell death via the activation of apoptosis in several cell types including neurons. Moreover, our aforementioned flow cytometry and molecular anlayzes showed that, in the absence of H_2O_2, overexpression of APPwt caused apoptosis leading to decreased viability of APPwt-transfected cells compared to control cells (Fig. 24). Therefore, to provide valuable insights into the elucidation of cellular mechanisms activated by GHB to counteract SH-SY5Y cells death, we investigated the effects of GHB on the levels of TUNEL-FITC-positive and activated caspase-3-PE-positive signals evoked by APPwt-overexpression or H_2O_2 in native and genetically modified cells (Fig. 26 and 28).

Quantitative flow cytometry (FACS) analyzes showed that GHB (500 µM) significantly reduced the levels of TUNEL-FITC-positive signal evoked by APPwt-overexpression in basal condition (-7 %) or by H_2O_2 treatment in native (-12 %), control vector-pCEP4 (-10 %)- and APPwt-transfected (-9%) SH-SY5Y cells (Fig. 26B). This effect was also qualitatively characterized by the left shift of the fluorescent signal caused by GHB (Fig. 26A).

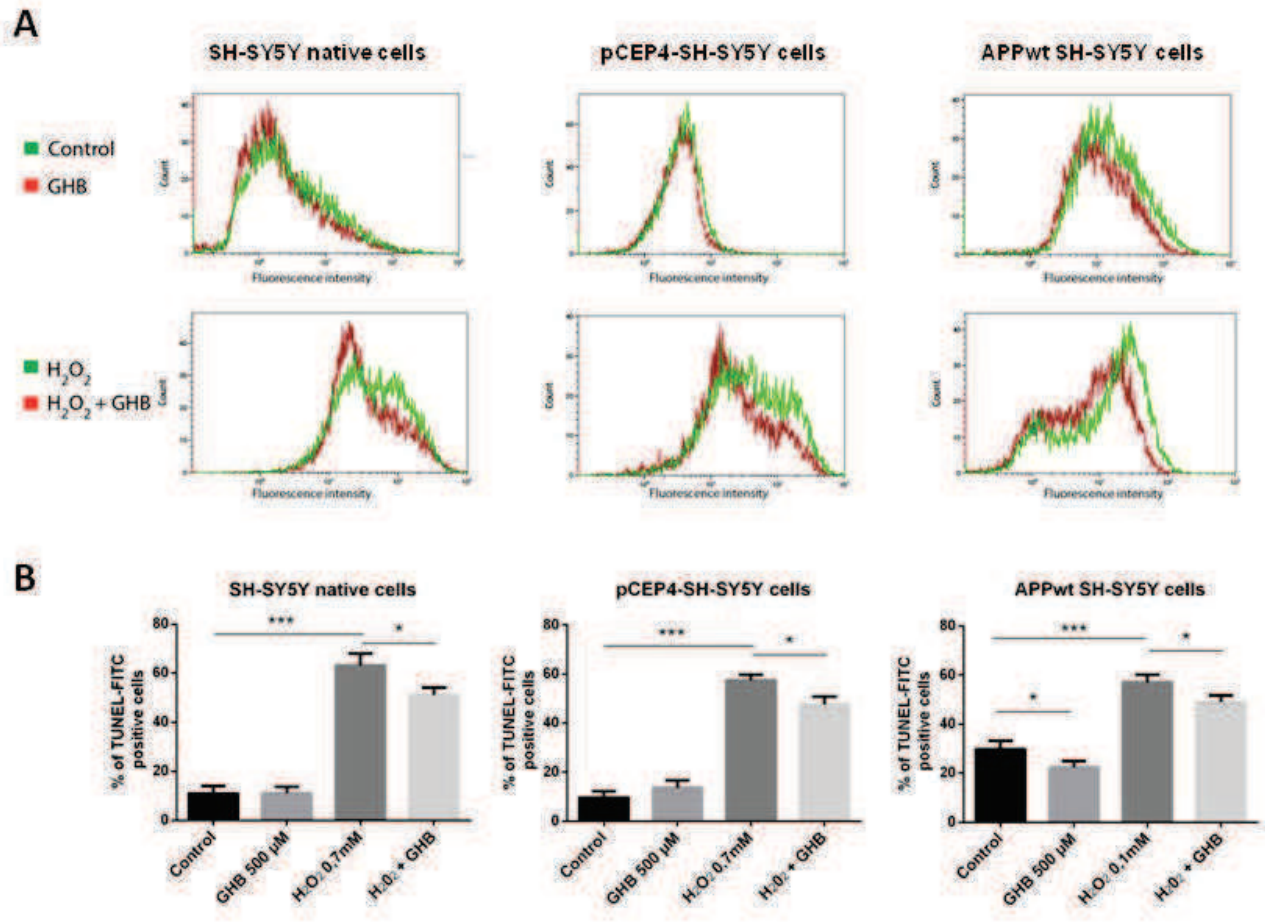

Figure 26 : Flow cytometry qualitative (A) and quantitative (B) assessment of GHB (500 µM for 24 h) effects on the levels of TUNEL-FITC staining in native, control-vector- and APPwt-transfected SH-SY5Ycells in the absence (basal) or presence of H_2O_2. Each value represents the mean ± S.E.M. of four independent experiments. **p*<0.05, ***p*<0.01 and ****p*<0.001.

The effect of GHB on the level of TUNEL-FITC-positive apoptotic signal was also investigated using a confocal scanning microscope (Fig. 27). Prior to the treatment of neuroblastoma cells with H_2O_2, we performed a series of control experiments to evaluate the kinetic and the basal apoptotic signal in each cell type. As shown in the Fig. 27, H_2O_2 treatment rapidly induced an apoptotic signal that was efficiently revealed by the TUNEL-FITC labeling. Indeed, after 1 h treatment, H_2O_2–evoked TUNEL-FITC staining was diffusely distributed in SH-SY5Y cell nucleus in a similar

manner as DNAse-induced apoptotic signal (positive control). From 6 to 24 h after H_2O_2 treatment, the TUNEL-FITC signal became heterogeneously distributed in the nucleus giving to the labeling an appearance of dispersed fluorescent dots (Fig. 27A). Interestingly, we observed that, in normal (absence of H_2O_2), the intensity of TUNEL-FITC-immunoreactivity was basally high in APPwt-transfected cells compared to the control cells which were devoid of TUNEL-FITC staining (Fig 27B). When the 3 cell types were exposed to H_2O_2, it became possible to detect TUNEL-FITC-positive labeling in these cells with an ascending intensity ranging from native SH-SY5Y cells (low), vector control-transfected (low) to APPwt-transfected (high) neuroblastoma cells (Fig. 27B). Treatment with GHB (500 µM) significantly decreased/suppressed TUNEL-FITC-positive signal evoked by APPwt-overexpression or H_2O_2 in neuroblastoma cells (Fig. 27B). Quantitative image analyses (Fig. 27C) were therefore combined with confocal microscope scanning to study the effect of GHB on H_2O_2-induced TUNEL-positive signal or apoptosis in native and genetically modified neuroblastoma cells. The results showed that GHB (500 µM) strongly decreased or suppressed H_2O_2-induced apoptotic signal in native, control vector-transfected and APPwt-transfected SH-SY5Y cells.

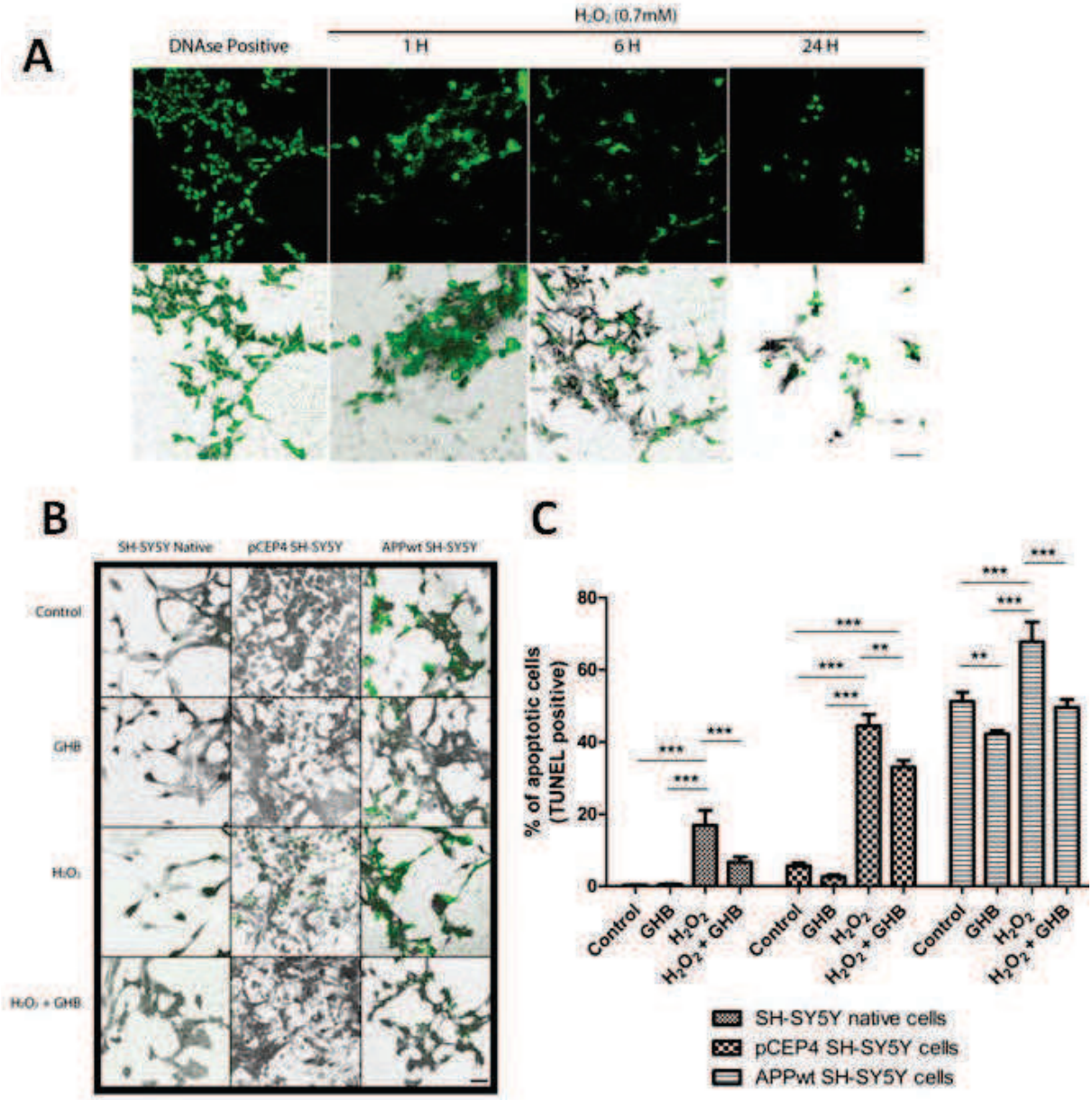

Figure 27 : Confocal laser scanning microscope analysis of GHB effects on the level of TUNEL-FITC-immunoreactive signal in native and genetically modified cells in the absence or presence of H_2O_2. (A) Qualitative time-course studies of H_2O_2-evoked TUNEL labeling in control vector-pCEP4-transfected SH-SY5Y cells. The photomicrographs show DNAse-induced apoptotic signal and the time-dependent distribution (1, 6 and 24 h) of H_2O_2-evoked TUNEL-positive labeling (green, first line of images) in SH-SY5Y cell cultures (gray, second line of images). (B) Effects of GHB on TUNEL labeling of control and APP overexpressing cells in presence or absence of H_2O_2. Scale bar=25 µm. (C) Quantitative analysis of TUNEL labelling in control and APPwt overexpressing cells. Each value represents the mean ± S.E.M. of four independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

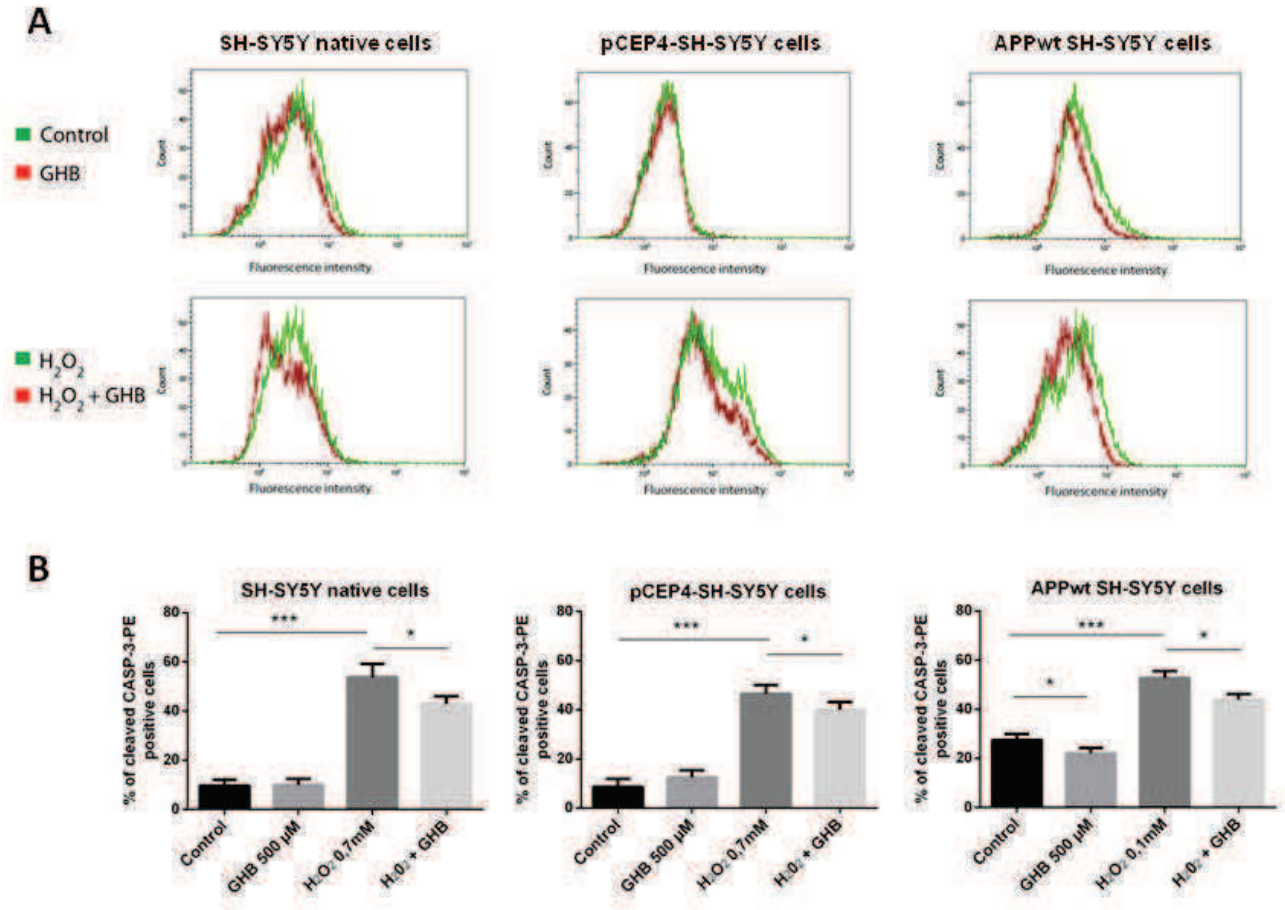

Figure 28 : Flow cytometry qualitative (A) and quantitative (B) assessment of GHB (500 µM for 24 h) effects on the levels of activated caspase-3-PE staining in native, control-vector- and APPwt-transfected SH-SY5Ycells in the absence (basal) or presence of H_2O_2. Each value represents the mean ± S.E.M. of four independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

Qualitative FACS analysis (Fig. 28A) and quantitative assessment (Fig. 28B) of activated caspase-3-PE staining by flow cytometry (FACS) demonstrated that GHB (500 µM) decreased also the levels of caspase-3-apoptotic signal induced by APPwt-overexpression in basal condition (-5.5 %) or by H_2O_2 in native (-10 %), control vector-pCEP4- (-7%) and APPwt-transfected (-9%) SH-SY5Y cells (Fig. 28).

In accordance with our flow cytometry results, reverse transcription combined with qPCR and western blot analysis also showed that, GHB (500 µM) treatment efficiently decreased the basally elevated Bax/Bcl-2 mRNA and protein ratios

detected in APPwt-transfected cells in the absence of H_2O_2 (Fig. 29). More importantly, we observed that GHB (500 µM) also reduced remarkably H_2O_2-evoked increase of Bax/Bcl-2 mRNA (Fig. 29A) and protein (Fig. 29B) ratios in native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells.

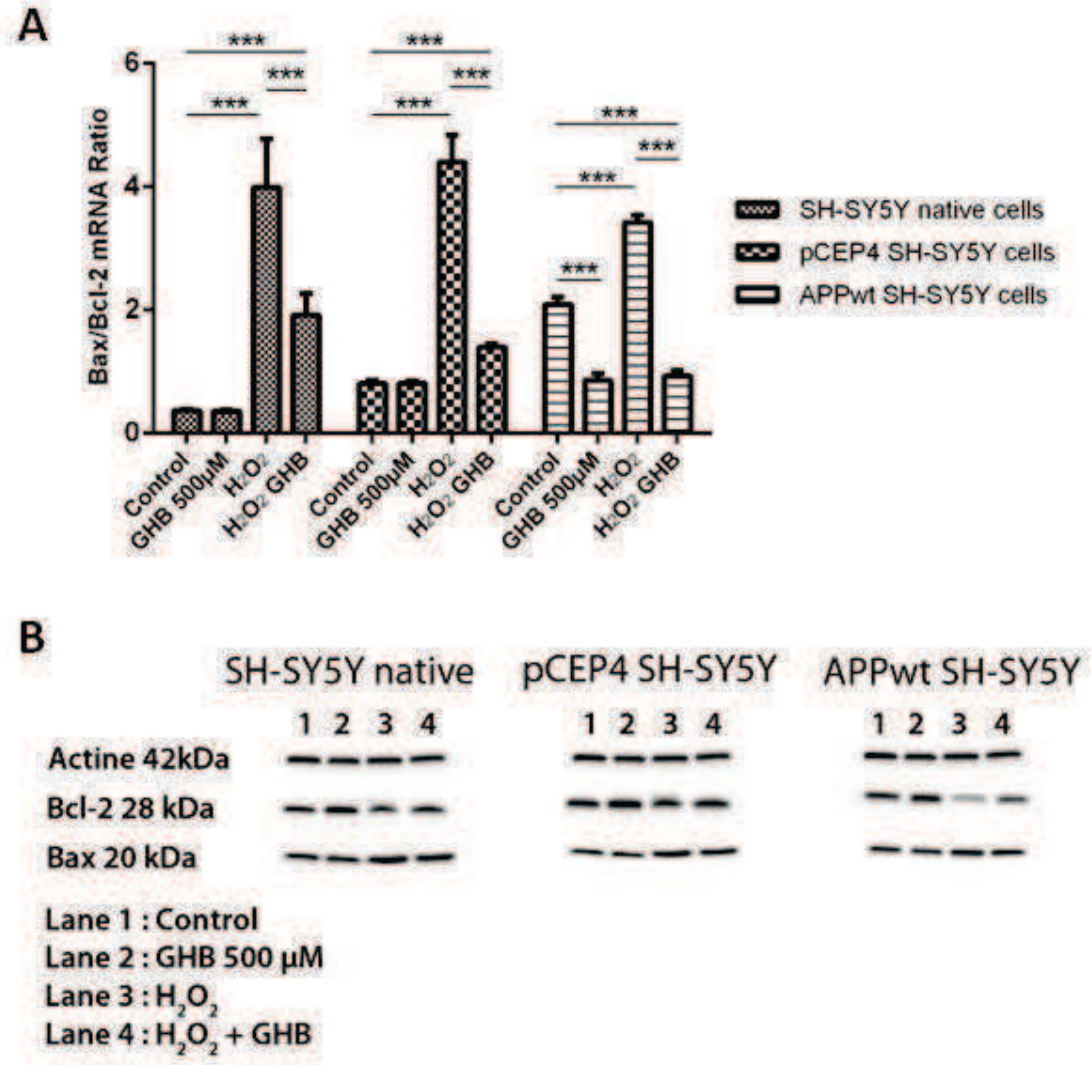

Figure 29 : Effects of GHB on the Bax/Bcl-2 ratio in absence or presence of H_2O_2 on control and APPwt overexpressing SH-SY5Y cells. (A) Effect of GHB (500 µM for 24h) on Bax/Bcl-2 mRNA ratio in native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells in the absence (basal) or presence of H_2O_2. (B) Western blot analysis of Bax, Bcl-2 and β-actin protein levels in native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells exposed or not to H_2O_2 (dose evoking 70 % of cell death) in the absence or presence of GHB at 500 µM for 24h. Each value represents the mean ± S.E.M. of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

3.1.7. Protective effects of neurosteroids against H_2O_2-induced cell loss

3.1.7.1. *Effects of Allopregnanolone*

Based upon the bibliographic knowledge of the potential of neurosteroids to exert neuroprotective effects (see Part 1.2.2., pages 52-53), we decided to test the ability of neurosteroids such as allopregnanolone to protect against H_2O_2-evoked cell death in the drastic condition where more than 70% of cells are killed by H_2O_2-induced toxicity (Fig. 30). Consequently, we have treated native, control vector-pCEP4- and APPwt-overexpressing SH-SY5Y cells with the same concentrations of H_2O_2 as aforementioned (0.7 mM H_2O_2 for native and pCEP4 cells and 0.1 mM H_2O_2 for APPwt-overexpressing cells) in the absence or presence of graded doses of allopregnanolone ranging from 0 to 1,000 nM. We observed that H_2O_2 treatments significantly reduced cell viability in all three cell lines (-70.2 ± 1.1 %, -80.1 ± 2.5 % and -85.7 ± 2 %, respectively in native, control vector and APPwt-overexpressing cells) (Fig. 30). The results also showed that allopregnanolone (250, 500 or 750 nM) efficiently protected all of the 3 categories of cells against H_2O_2-evoked death (Fig. 30). Indeed, allopregnanolone significantly decreased oxidative stress-induced cell loss. Particularly, the most effective concentration of allopregnanolone was 500 nM which respectively prevented about 14, 16 or 15 % of native, pCEP4- and APPwt-overexpressing cells against H_2O_2-evoked death (Fig. 29, $p<0.001$).

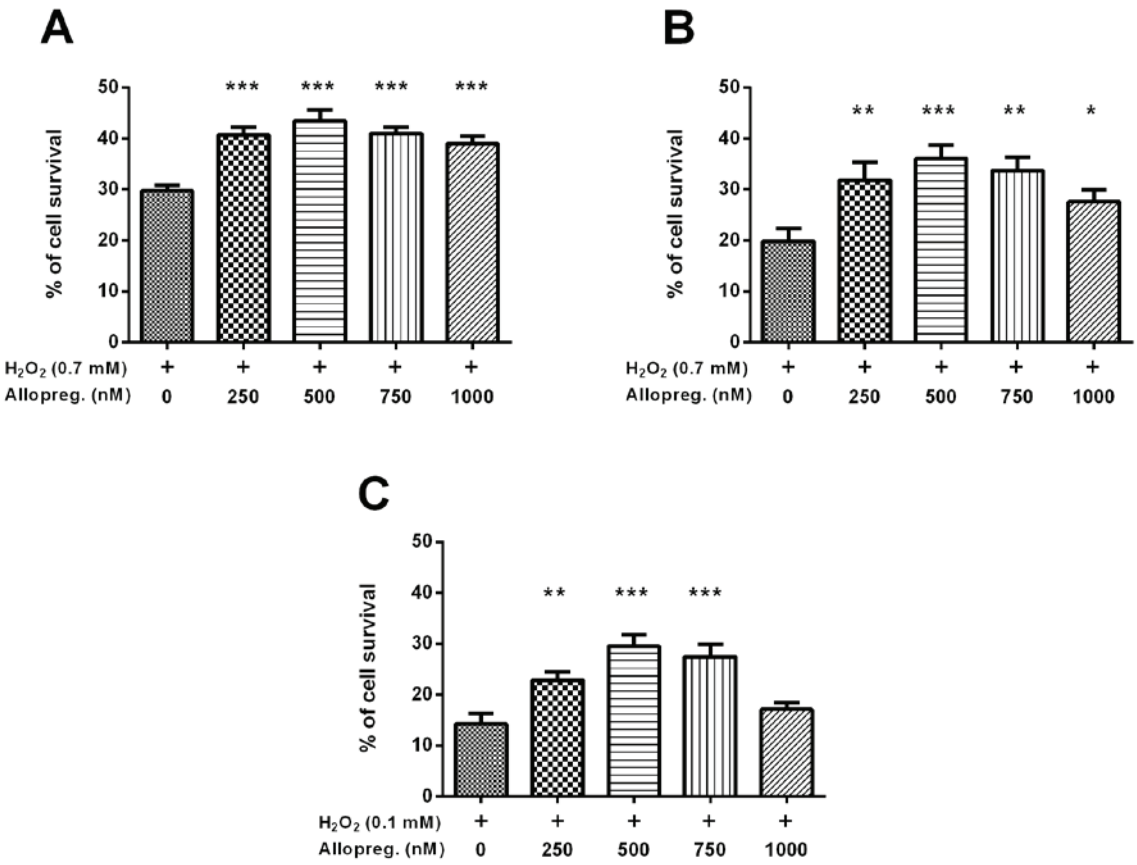

Figure 30 : Protective effect of allopregnanolone against H_2O_2-induced native (A), control vector-pCEP4-transfected (B) or APPwt-transfected (C) SH-SY5Y cell death. Native and genetically modified SH-SY5Y cells were exposed to H_2O_2 dose inducing more than 70% of cell loss and treated with graded concentrations of allopregnanolone for 24 h. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A maximum of protective effect of allopregnanolone is observed at 500 nM for all SH-SY5Y cell types. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of five independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

3.1.7.2. *Effects of Estradiol*

In addition to allopregnanolone, we have also tested the ability of another neurosteroid, estradiol, to prevent H_2O_2-evoked cell death. The three types of SH-SY5Y cells were also treated with the same concentrations of H_2O_2 (0.7 mM for native and control vector cells, and 0.1 mM for APPwt-overexpressing cells) in the presence or absence of graded concentrations of estradiol ranging from 0 to 1000

nM. Here again, H_2O_2 drastically reduced the cell viability in SH-SY5Y cells (-65.5 ± 1.4 %, -69.9 ± 2.7 % or -72.38 ± 2.7 %, respectively in native, control vector or APPwt-overexpressing cells) (Fig. 31). The neurosteroid estradiol was also able to reduce the H_2O_2-induced cell loss. The most efficient concentration of estradiol was also 500 nM and that prevented respectively 13%, 19% or 21 % respectively in native, control vector or APPwt-overexpressing cells against H_2O_2-induced cell death (p<0.001).

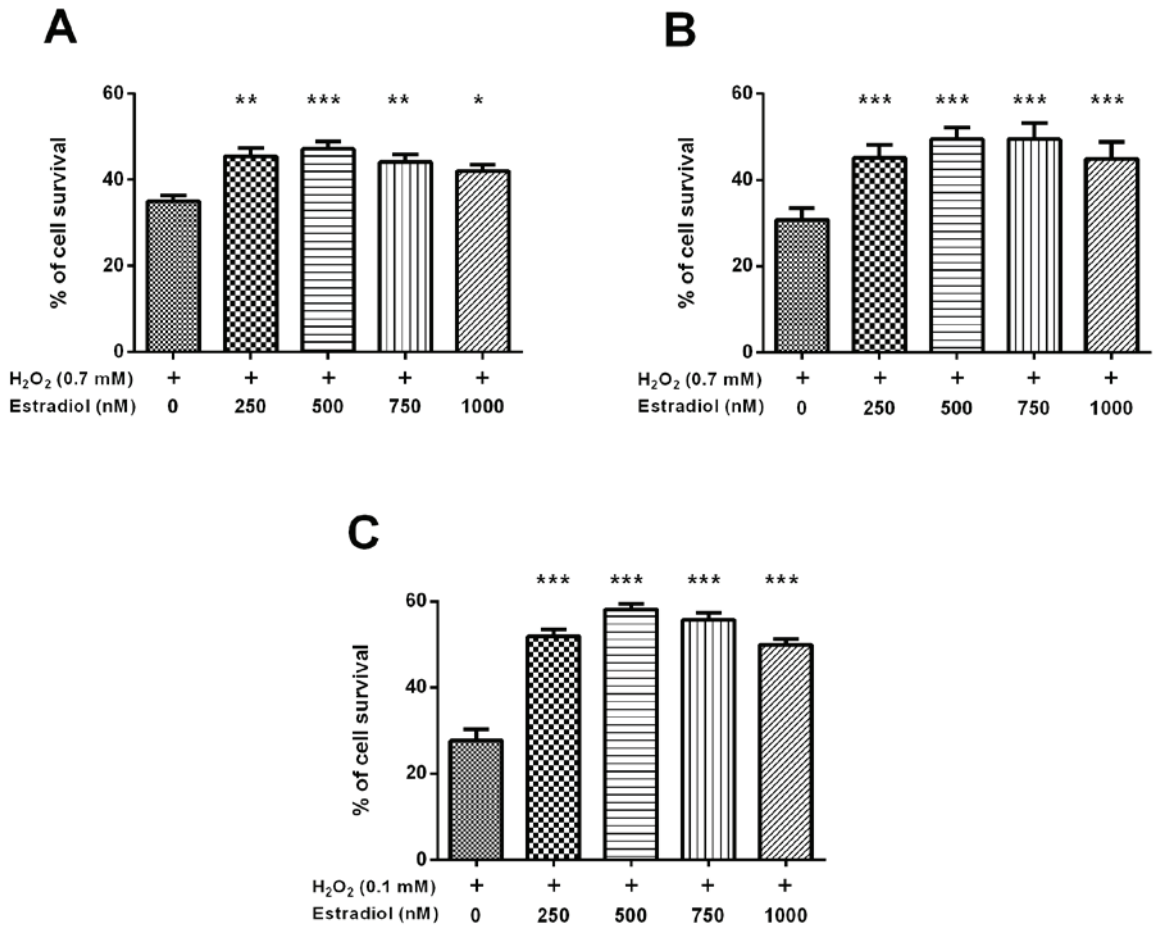

Figure 31 : Protective effect of Estradiol against H_2O_2-induced native (A), control vector-pCEP4-transfected (B) or APPwt-transfected (C) SH-SY5Y cell death. Native and genetically modified SH-SY5Y cells were exposed to H_2O_2 dose inducing more than 70% of cell loss and treated with graded concentrations of estradiol for 24 h. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A maximum of protective effect of estradiol is observed at 500 nM for all SH-SY5Y cell types. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of five independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

3.1.7.3. *Basal effects of neurosteroids on cell viability*

To determine whether the process of steroid-induced cell proliferation may contribute to the protective actions of allopregnanolone and estradiol against oxidative stress-evoked SH-SY5Y cell death, we investigated the effects of these two neurosteroids on SH-SY5Y cell viability in basal conditions. Therefore, in the absence of H_2O_2, the three SH-SY5Y cell types were treated for 24 h with allopregnanolone or estradiol (ranging from 0 to 1,000nM).

Allopregnanolone significantly stimulated the viability of all cell types (Fig. 32). The most stimulatory concentration 500 nM which induced respectively, +12.5%, +12.2% and +12.7% respectively in native, control vector- or APPwt transfected cell viability ($p<0.01$).

The neurosteroid estradiol also significantly increased SH-SY5Y cell viability (Fig. 33). The most effective concentration was 750 nM what evoked, respectively, +16.7%, +12.2% or +6% increase of native, pCEP4- ($p<0.001$) or APPwt-transfected cells ($p<0.005$).

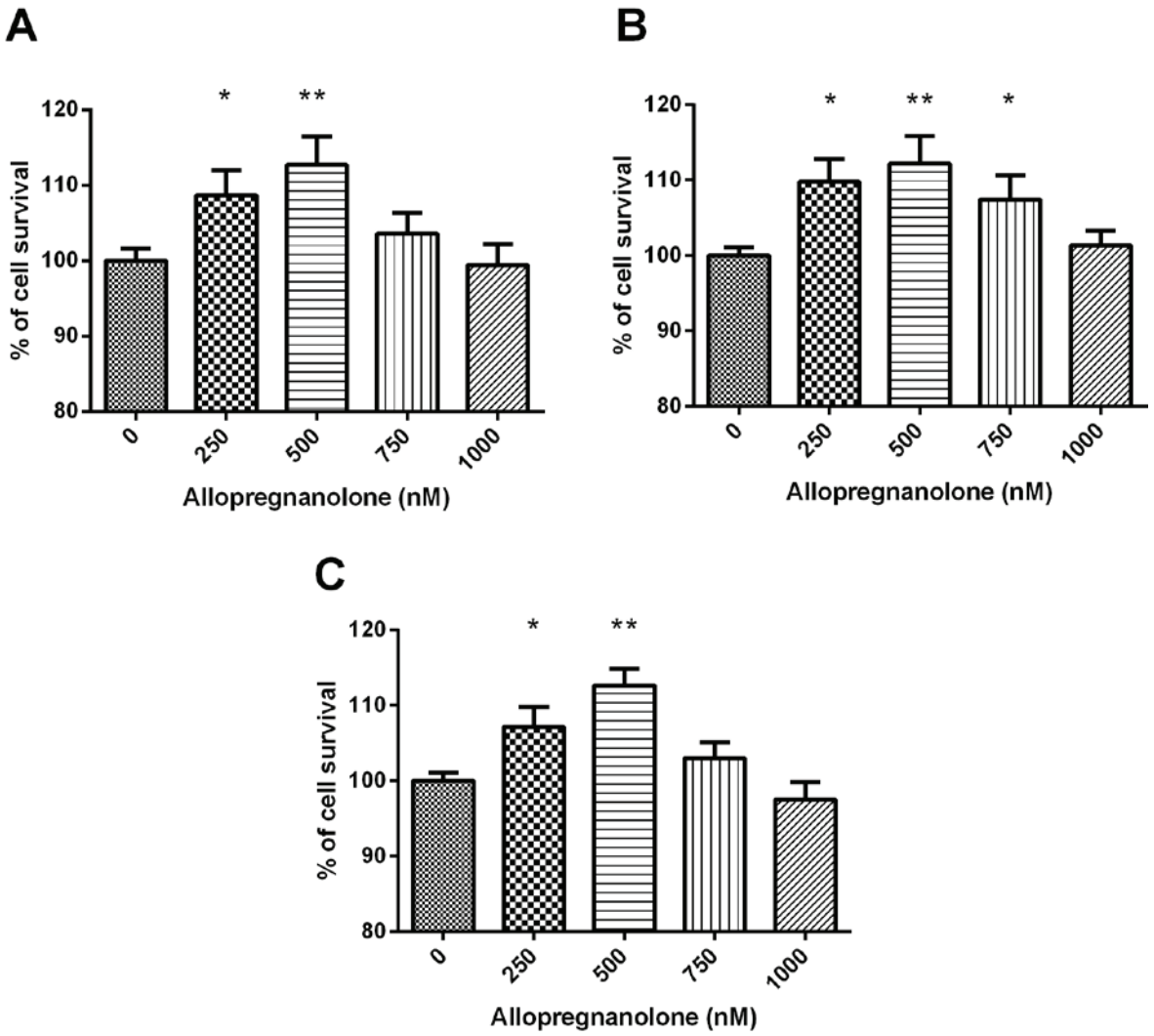

Figure 32: Dose-response study of the effect of allopregnanolone on native (A), control vector-pCEP4-transfected (B) or APPwt-transfected (C) SH-SY5Y cell viability. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A stimulatory effect of allopregnanolone (250 and 500 nM) is detected on all SH-SY5Y cell lines. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value represents the mean ± S.E.M. of five independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

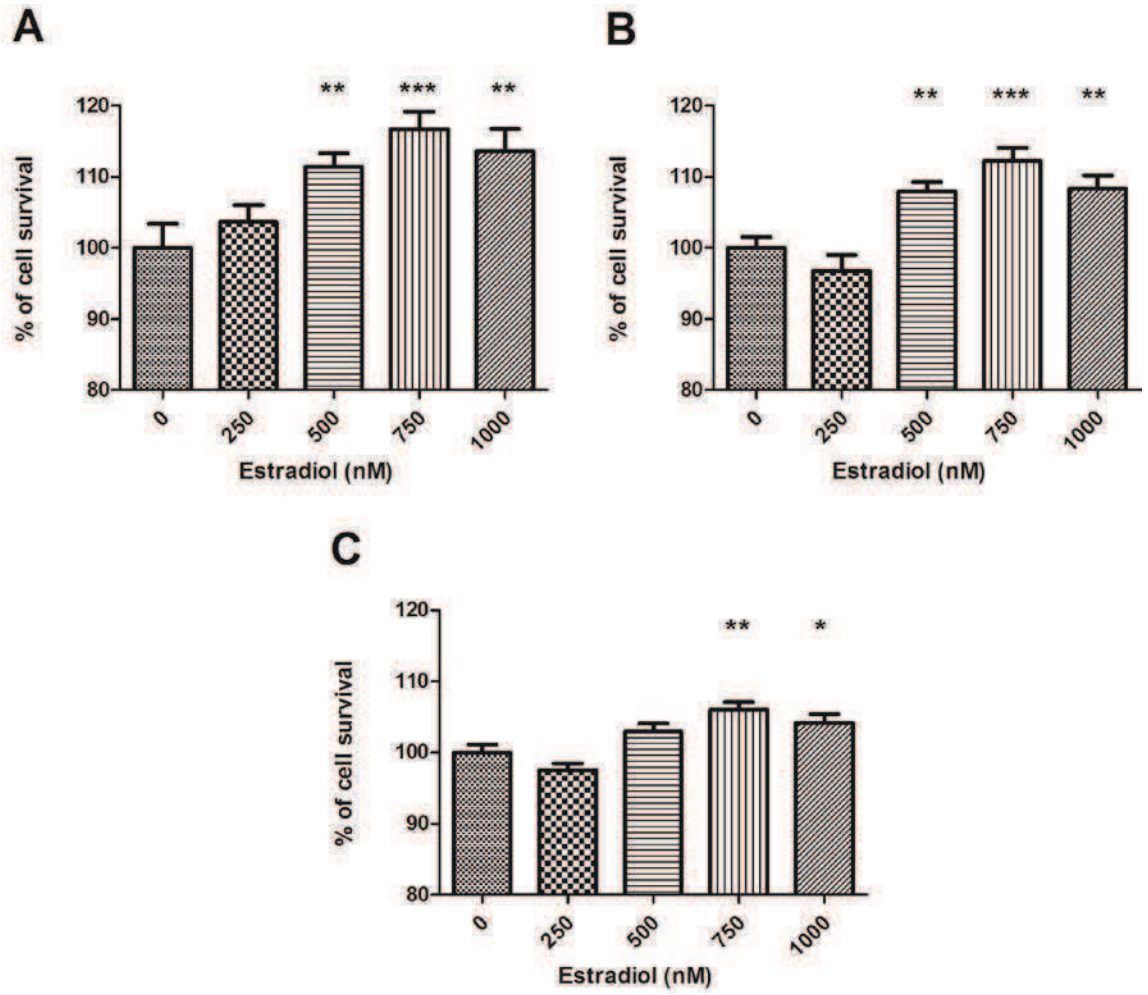

Figure 33 : Dose-response study of the effect of estradiol on native (A), control vector-pCEP4-transfected (B) or APPwt-transfected (C) SH-SY5Y cell viability. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). A stimulatory effect of Allopregnanolone (750 and 1,000 nM) is detected on all SH-SY5Y cell lines. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value represents the mean ± S.E.M. of five independent experiments. *p<0.05, **p<0.01 and ***p<0.001.

3.2. Effects of GHB and/or neurosteroids against ER stress-induced cell death

3.2.1. Effects of GHB or neurosteroids against tunicamycin-induced cell death

3.2.1.1. *Effects of tunicamycin on native SH-SY5Y cell viability*

Native SH-SY5Y cells were treated with graded concentrations of tunicamycin ranging from 0 to 20 µg/ml (Fig. 34). Tunicamycin decreased in a dose-dependent manner SH-SY5Y cell viability. A maximum of toxicity is reached with 10 µg/ml (-48%, $p<0.001$). This concentration was therefore used to assess the ability of GHB and neurosteroid allopregnanolone to protect against tunicamycin-evoked ER-stress.

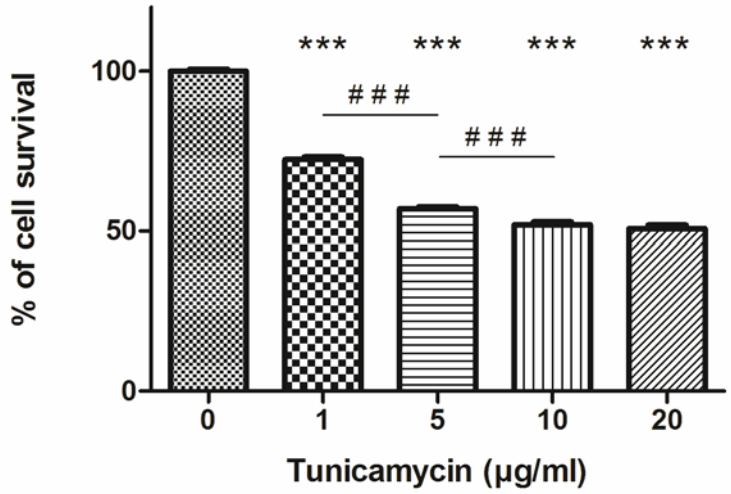

Figure 34 : Dose-response effect of tunicamycin on native SH-SY5Y cell viability. Cells were treated for 24h with graded concentrations of tunicamycin. MTT reduction assays was used to determine the cell viability which was expressed as percent of control (untreated cells). MTT signal assessed for each cell type in basal condition (absence of tunicamycin) is arbitrary set at 100 %. Tuncicamycin concentration evoking 50 % of cell survival corresponds to 10 µg/ml. Each value on the graph represents the mean ± S.E.M. of cell survival of five independent experiments. $^{###}p<0.001$, $^{***}p<0.001$.

3.2.1.2. *Effects of GHB or allopregnanolone against tunicamycin-induced cell death*

The ability of GHB and allopregnanolone to protect against tunicamycin-evoked cell death was investigated in the drastic condition where about 50% of cells are killed by tunicamycin (10 µg/ml). In a first strategy, SH-SY5Y cells were treated simultaneously with tunicamycin (10 µg/ml) and graded doses of GHB (0 to 1,000 µM) or Allopregnanolone (0 to 1,000 nM) and were incubated for 24 h at 37°C in the incubator before the MTT viability assay (Fig. 35A, B). A second strategy consisted of pre-treating the cells for 2 h with either GHB or allopregnanolone before adding tunicamycin and incubating the cells for 24 h at 37°C before the viability assay (Fig. 35C, D).

In both conditions of treatment, neither GHB nor allopregnanolone prevented or decreased tunicamycin-induced SH-SY5Y cell death.

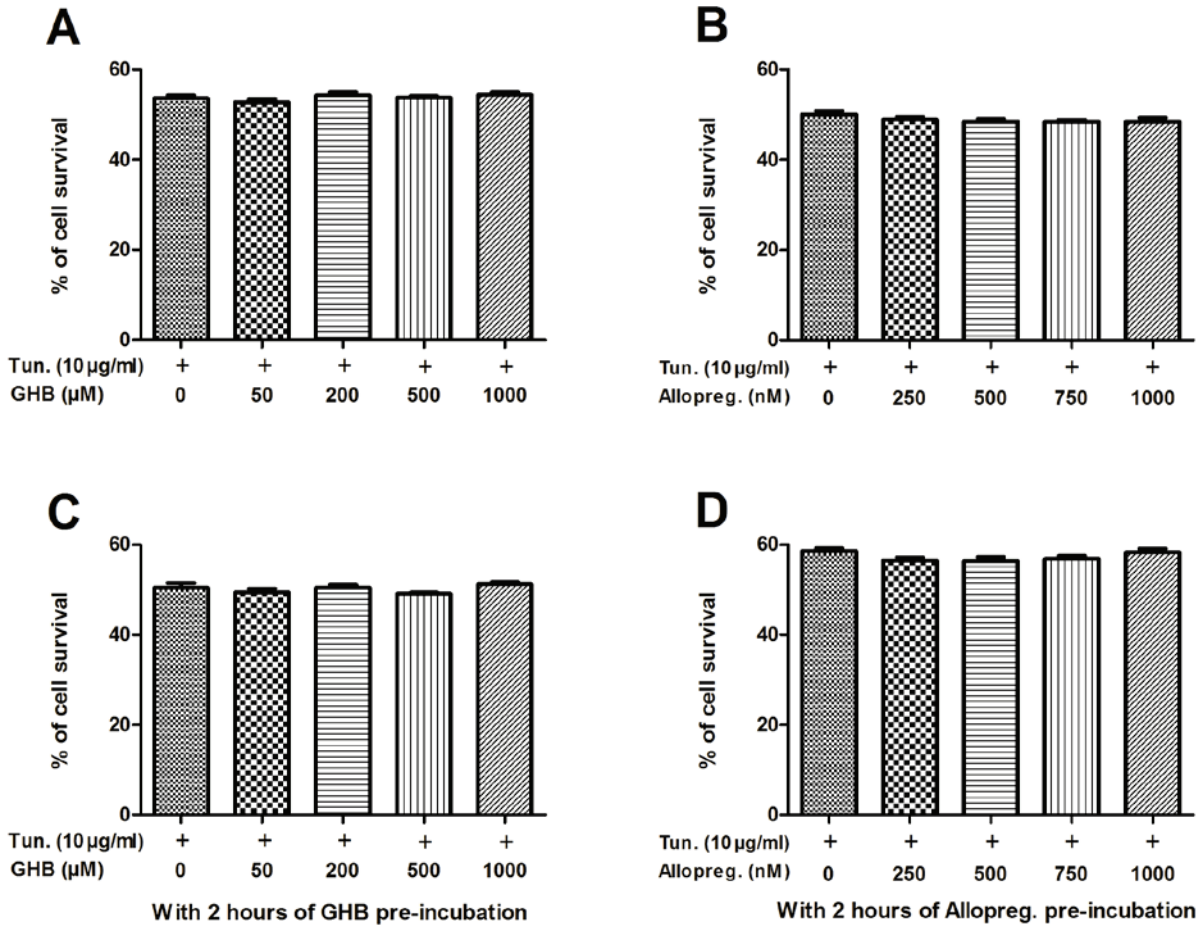

Figure 35 : Effects of GHB (A, C) and allopregnanolone (B, D) against tunicamycin-induced native SH-SY5Y cell death. (A, B) Native SH-SY5Y cells were exposed to tunicamycin dose inducing about 50% of cell loss with graded concentrations of GHB (A) or allopregnanolone (B). (C, D)The cells were pre-treated with GHB (C) or allopregnanolone (D) for 2 h before their exposure to tunicamycin. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). MTT signal assessed for each cell type in basal condition (absence of tunicamycin) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of five independent experiments.

3.2.2. Effects of GHB or neurosteroids against thapsigargin-induced cell death

3.2.2.1. *Effects of thapsigargin on native and genetically modified SH-SY5Y cell viability*

The cells were treated with graded concentrations of the calcium SERCA-pumps inhibitor thapsigargin (0 to 1 µg/ml) and incubated for 24 h at 37°C before MTT

viability assays. As shown in Fig. 36, thapsigargin significantly decreased the cell viability of native and genetically modified SH-SY5Y cell viability and no difference of sensitivity to thapsigargin was detected between the three cell lines. The most toxic concentration which killed more than 60% of cells was 1µg/ml (p<0.001). This dose was therefore used to assess the effects of GHB and neurosteroids against thapsigargin-induced cell death.

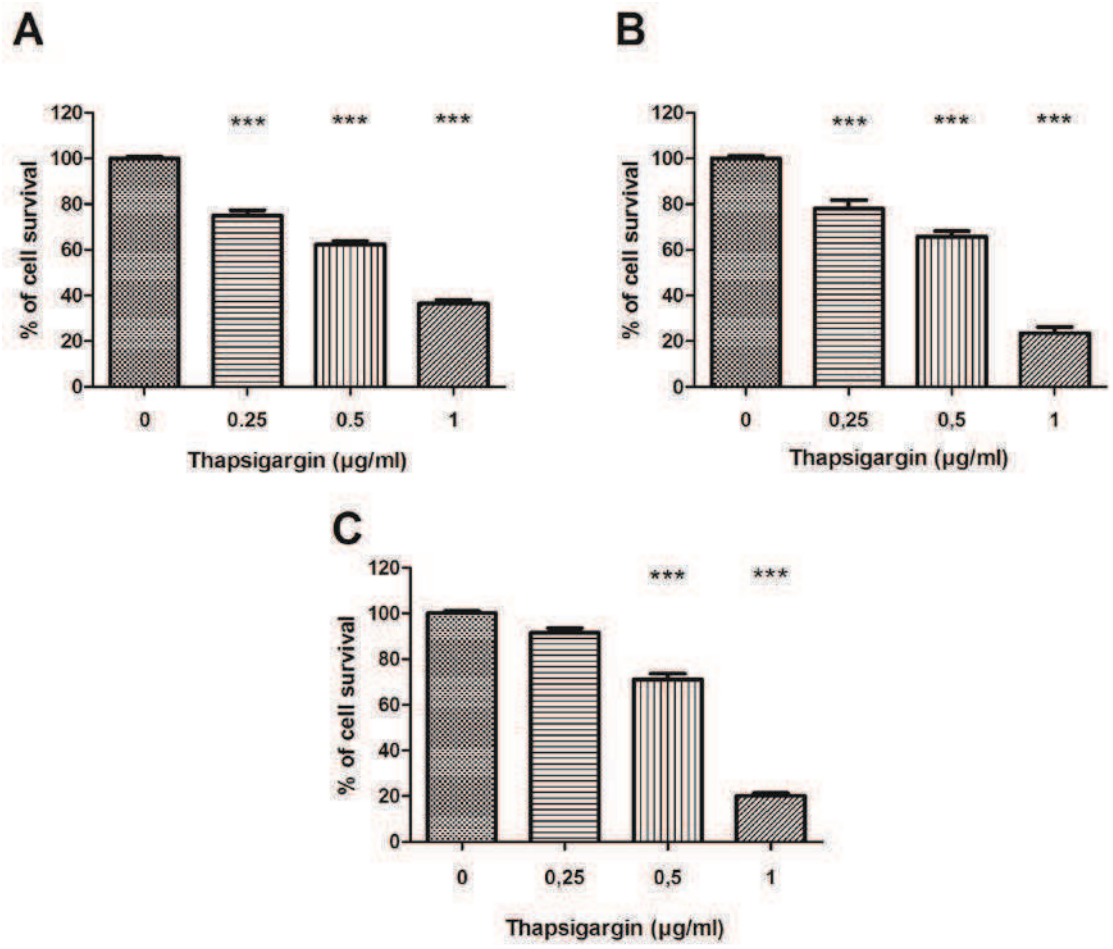

Figure 36 : Dose-response effect of thapsigargin on native (A), control vector (B) or APPwt transfected SH-SY5Y cells viability. The cells were treated for 24 h with graded concentrations of thapsigargin. Each value on the graph represents the mean ± S.E.M. of cell survival of five independent experiments. ***p<0.001.

3.2.2.2. *Effects of GHB against thapsigargin-induced cell death*

Native, pCEP4- and APPwt overexpressing cells were treated for 24 h with graded concentrations of GHB (0 to 1,000 µM) in the presence of 1µg/ml thapsigargin before MTT viability assay. Thapsigargin significantly decreased the viability of all SH-SY5Y cell types (-70%, - 69% or -73% in native, pCPE4 or APPwt-overexpressing cells, respectively, $p<0.001$) (Fig. 37). Any of the tested doses of GHB was able to prevent or reduce thapsigargin-evoked cell death (Fig. 36).

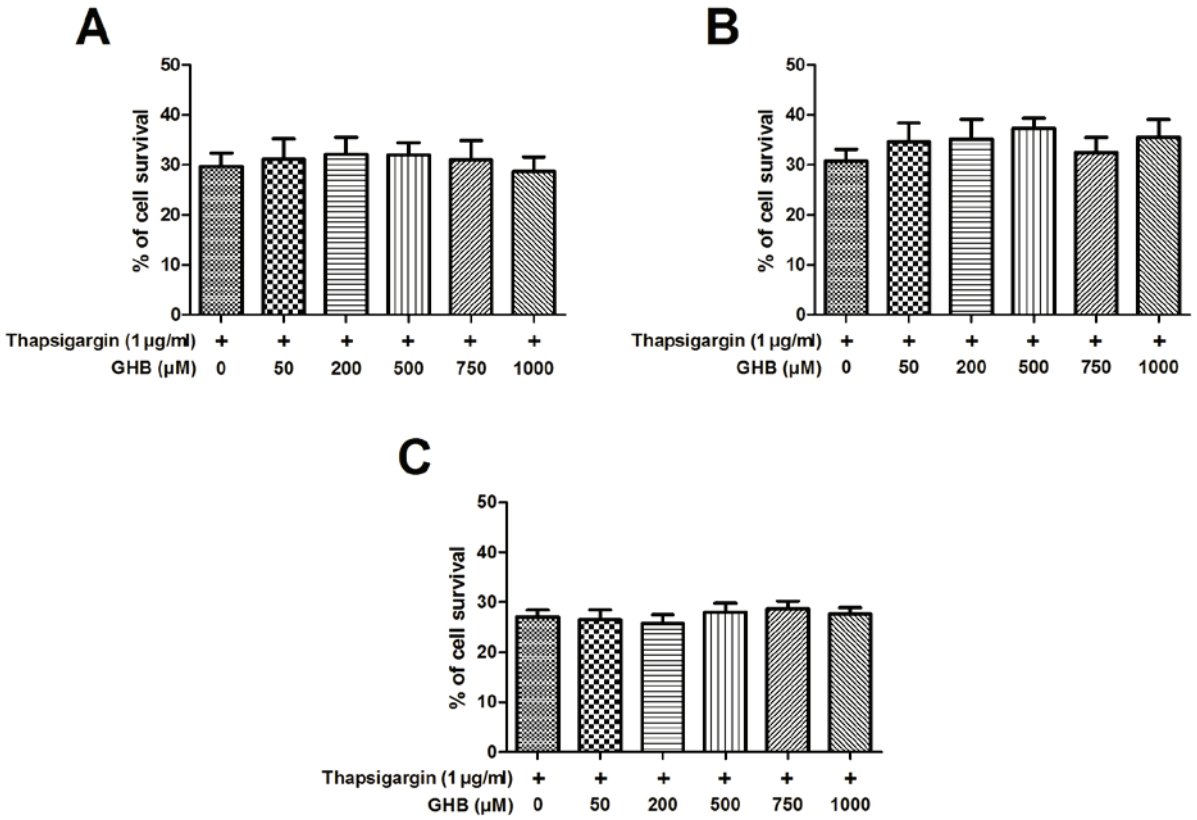

Figure 37 : Effects of GHB against thapsigargin-induced native (A), pCEP4- (B) or APPwt-transfected (C) SH-SY5Y cell death. SH-SY5Y cells were exposed to thapsigargin dose inducing more than 60% of cell loss and treated with graded concentrations of GHB. MTT signal assessed for each cell type in basal condition (absence of thapsigargin) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of three independent experiments.

3.2.2.3. *Effects of allopregnanolone against thapsigargin-induced cell death*

Native, pCEP4- and APPwt overexpressing cells were also for 24 h treated with graded concentrations of allopregnanolone (0 to 1,000nM) in the presence of 1 µg/ml thapsigargin before MTT viability assay. Thapsigargin significantly decreased the viability of all SH-SY5Y cell types (-64%, -69% or -68% in native, pCPE4 and APPwt-overexpressing cells, respectively, $p<0.001$) (Fig. 38). The tested doses of allopregnanolone also failed to prevent or reduce thapsigargin-induced cell death.

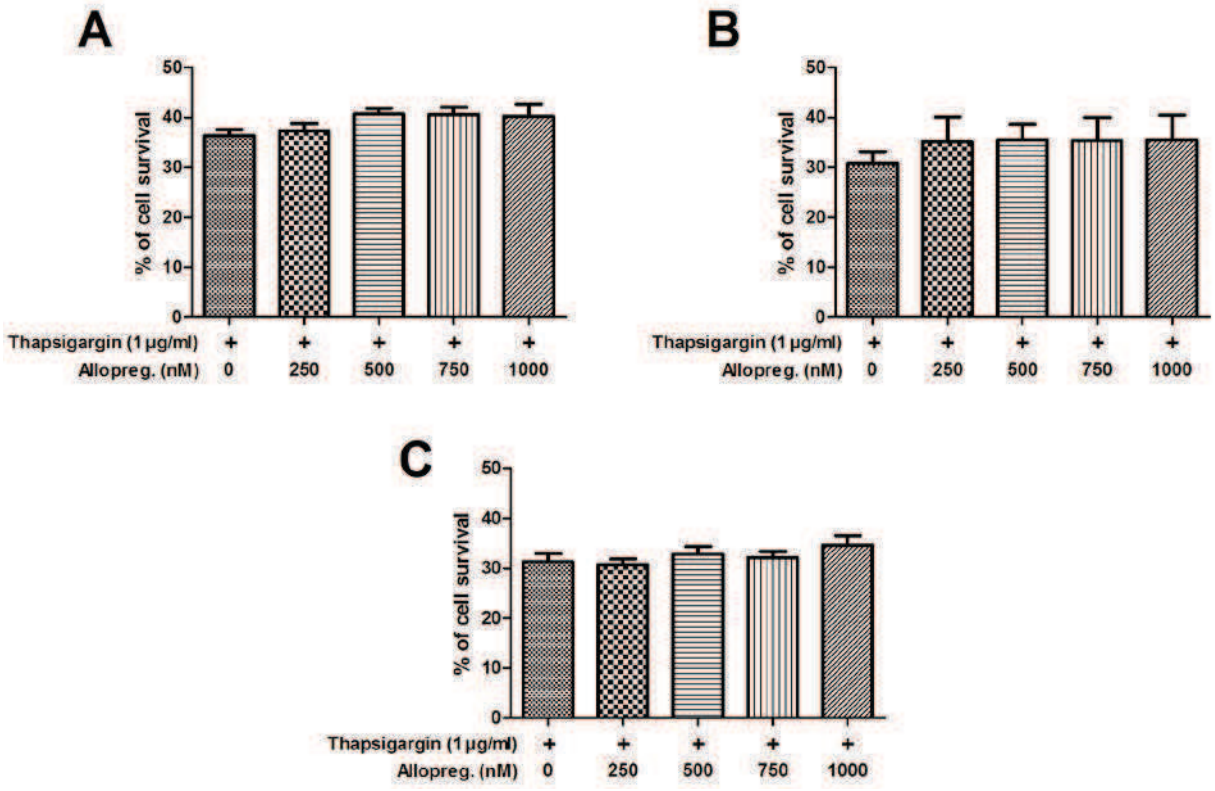

Figure 38 : Effects of alloregnanolone against thapsigargin-induced native (A), pCEP4- (B) or APPwt-transfected (C) SH-SY5Y cell death. SH-SY5Y cells were exposed to thapsigargin dose inducing more than 60% of cell loss and treated with graded concentrations of allopregnanolone. MTT signal assessed for each cell type in basal condition (absence of thapsigargin) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of three independent experiments.

3.2.2.4. *Assessment of concomitant actions of GHB and allopregnanolone against thapsigargin-induced cell death*

Because separated treatments of GHB or allopregnanolone administrated alone did not prevent thapsigargin-evoked cell death, we investigated whether the co-application of these two drugs may be effective to counteract the cell loss caused by thapsigargin. Therefore, native and genetically modified SH-SY5Y cells were concomitantly treated for 24 h with GHB (500 µM) and allopregnanolone (500 nM) in the presence of thapsigargin (1 µg/ml) before MTT viability assays. The results showed that the co-application of GHB and allopregnanolone was also unable to prevent or decrease thapsigargin-induced cell death like the separated treatments (Fig. 39).

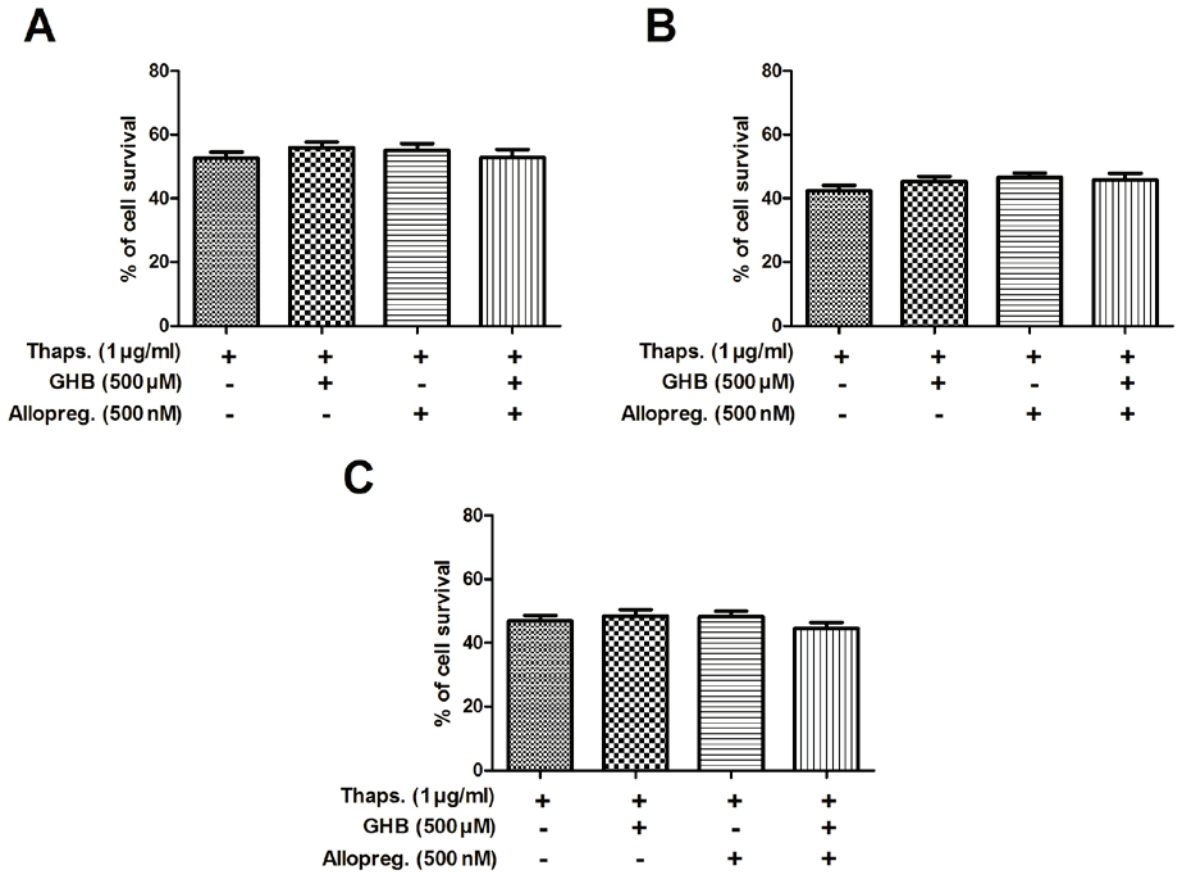

Figure 39 : Effects of separate and concomitant treatments of GHB and allopregnanolone against thapsigargin-induced native (A), control vector (B) and APPwt-transfected (C) SH-SY5Y cell death. SH-SY5Y cells were exposed to thapsigargin (1 µg/ml) and treated either separately or concomitantly with GHB (500 µM) and allopregnanolone (500 nM) for 24 h. MTT signal assessed for each cell type in basal condition (absence of thapsigargin) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of three independent experiments.

3.2.2.5. *Effects of estradiol against thapsigargin-induced cell death*

Preliminary experiments were performed in order to assess the effects of estradiol against thapsigargin-induced cell loss. In this case, only native SH-SY5Y cells were treated for 24 h with graded doses of estradiol in the presence of thapsigargin (1 µg/ml) before MTT viability assays. The preliminary results showed that estradiol, at the doses of 250 nM and 500 nM, significantly reduced thapsigargin-evoked cell death (Fig. 40).

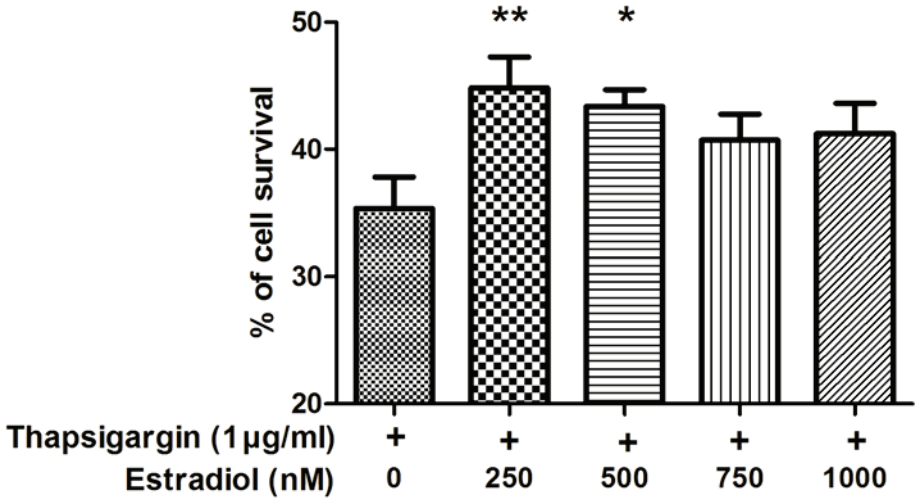

Figure 40 : Effects of estradiol against thapsigargin-induced SH-SY5Y cell death. Native SH-SY5Y cells were exposed to thapsigargin dose inducing more than 60% of cell loss and treated with graded concentrations of estradiol for 24 h. MTT reduction assays were used to determine the cell viability which was expressed as percent of control (untreated cells). MTT signal assessed for each cell type in basal condition (absence of thapsigargin) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of three independent experiments. * $p<0.05$, ** $p<0.01$.

3.2.3. Effects of GHB and neurosteroids on thapsigargin-induced cytosolic calcium changes

3.2.3.1. *Effects of allopregnanolone*

Native SH-SY5Y cells were pre-treated for two hours with allopregnanolone (500 nM) and incubated with external solutions containing 0.5 mM Ca^{2+} for 100 s. Then, the cells were transferred into a medium without calcium which contained ethylene glycol tetraacetic acid (EGTA), a calcium chelator in external solution in order to observe the basal cytosolic calcium level. At t=400s, thapsigargin (1 µg/µl) was added to in the same external solution in order to assess allopregnanolone action on cytosolic Ca^{2+} elevation evoked by the blockade of SERCA-pumps and progressive emptying of ER Ca^{2+} stores. Finally, at t=1,400s, the cells were again incubated with thapsigargin in the presence of extracellular calcium (0.5 mM). This experiment

aimed at investigating allopregnanolone eventual action on store operated calcium entry (SOCE) via the plasma membrane.

As shown in Fig. 41, thapsigargin induced a large cytosolic Ca^{2+} increase in absence and presence of external calcium as expected. Allopregnanolone at 500 nM did not modify calcium stores depletion or the SOCE. These data are consistent with the absence of allopregnanolone effect on thapsigargin-evoked cell viability decrease (see Part. 3.2.3.1., page 123).

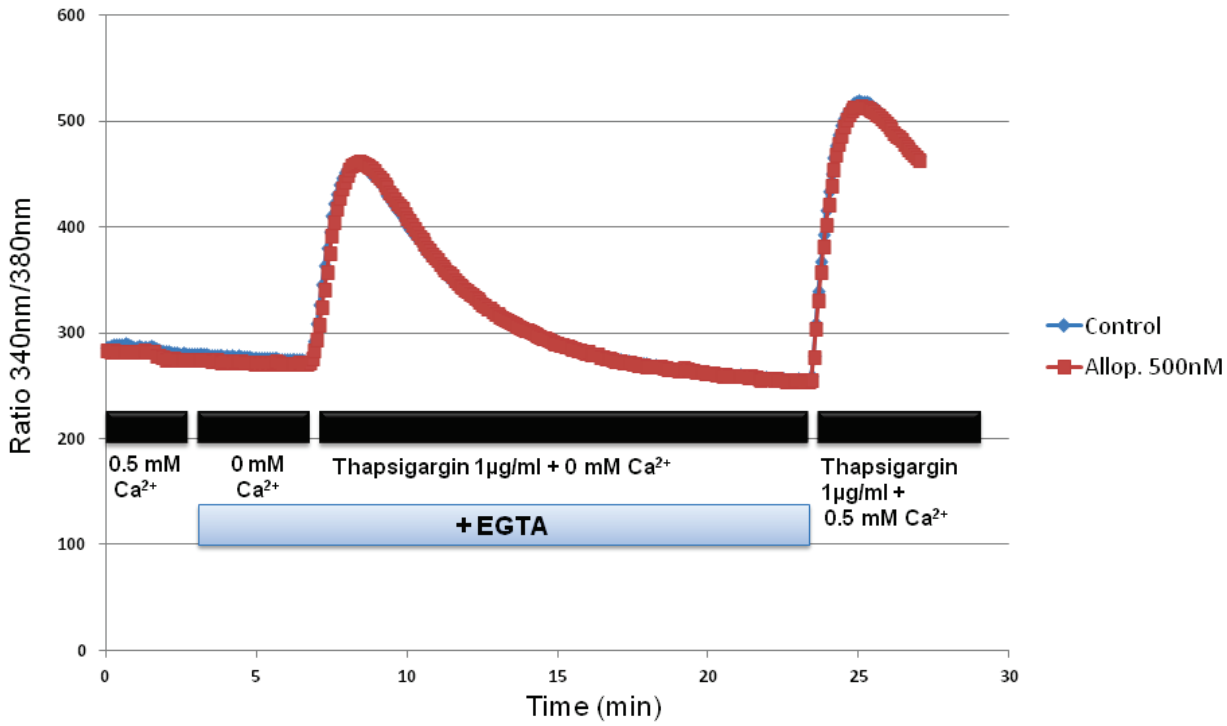

Figure 41 : Effects of allopregnanolone on thapsigargin-induced cytosolic calcium elevations. $[Ca^{2+}]_i$ responses before and after calcium store depletion by thapsirgargin (1 µg/ml, without external calcium) and after addition of thapsigargin in the presence of extracellular calcium (store operated calcium entry, SOCE). Cells were pre-incubated for 2 h with 500 nM allopregnanolone before the measurement. Each value is the mean of three independent experiments (50 cells/experiment were measured). EGTA, Ethylene glycol tetraacetic acid.

3.2.3.2. *Effects of GHB*

The same approach aforementioned was used to assess GHB action on thapsigargin-induced cytosolic calcium elevations. The results showed that GHB at 500 µM (administrated as pre-treatment or maintained permanently in the incubation medium) did not affect Ca^{2+} store depletion or SOCE (Fig. 42). These data are also consistent with the absence of GHB effect on thapsigargin-evoked cell loss (see Part. 3.2.3.2., page 125).

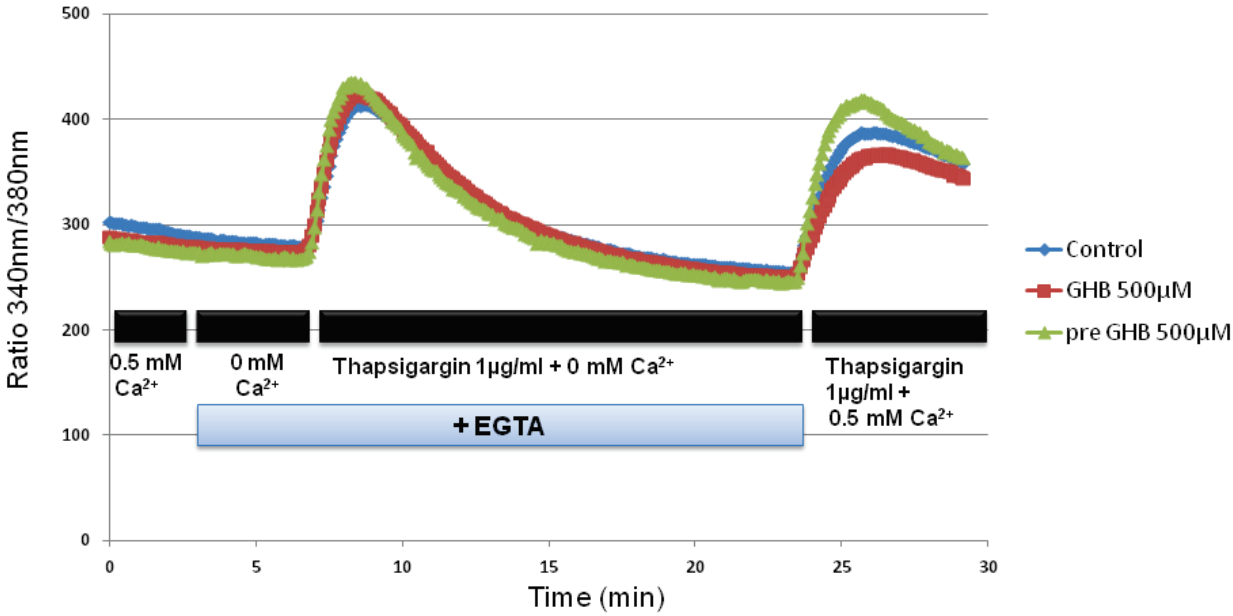

Figure 42 : Effects of GHB on thapsigargin-induced cytosolic calcium elevations. $[Ca^{2+}]_i$ responses before and following calcium store depletion by thapsirgargin (1 µg/ml, without external calcium) and after addition of thapsigargin in the presence of extracellular calcium ($[Ca^{2+}]_i$ response in case of store operated calcium entry, SOCE). Cells were either pre-incubated (for 2 h) with 500 µM GHB or permanently exposed to GHB (500 µM) during the entire measurement. Each value is the mean of three independent experiments (50 cells/experiment were measured). EGTA, Ethylene glycol tetraacetic acid.

3.2.4. Assessment of ER stress signaling proteins evoked by thapsigargin

Preliminary experiments were performed to assess the ability of thapsigargin to induce ER stress signaling proteins such as CHOP or XBP-1 (see Part 1.5.1., pages 39-42). Control vector and APPwt-transfected cells were treated for 24 h with thapsigargin (1 µg/ml) or the vehicle the total fraction of proteins was extracted. Western analysis reveal no difference of CHOP and XBP-1 expression levels in control vector and APPwt-transfected cells treated with the vehicle (Fig. 43 and 44). In contrast, thapsigargin treatment increased CHOP protein levels in both cell lines (Fig. 43) but did not affect XBP-1 cellular concentrations (Fig. 44).

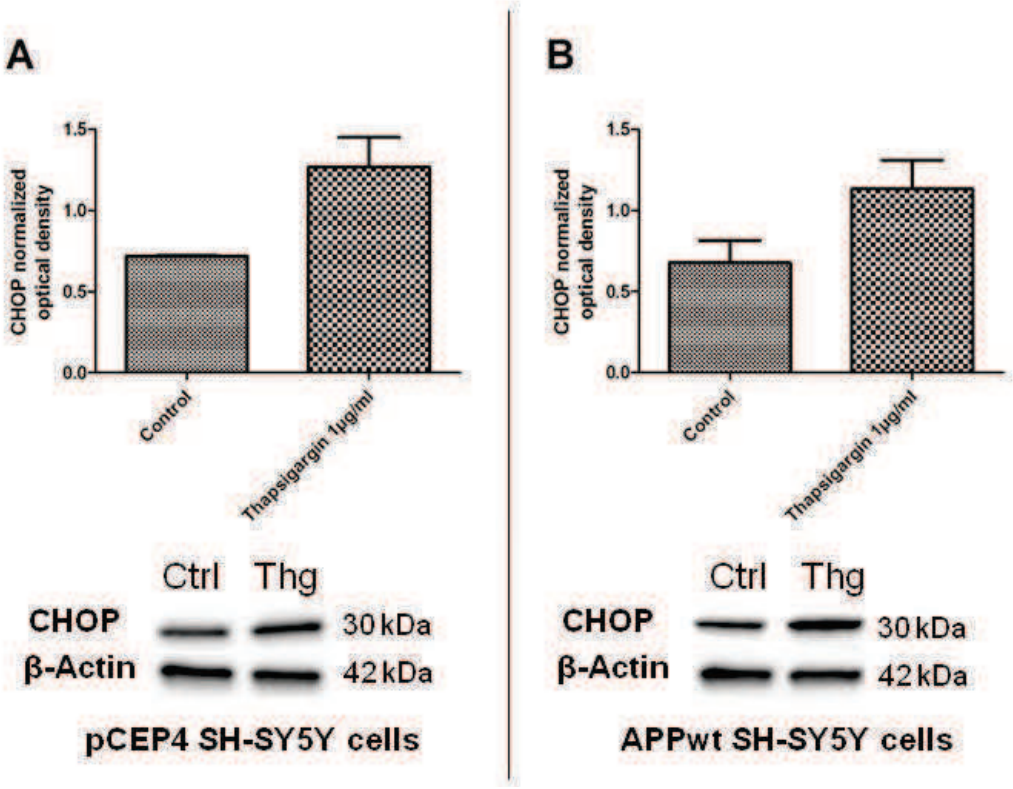

Figure 43 : Western blot analysis of CHOP protein levels in control vector- (A) and APPwt-transfected SH-SY5Y cells (B). Cells were exposed or not (vehicle) to Thapsigargin (dose evoking 60% of cell death) for 24 h. CHOP optical density was normalized to β-actin expression. Each value represents the mean ± S.E.M. of two independent experiments. Ctrl, control ; Thg, Thapsigargin. CHOP, C/EBP-homologous protein.

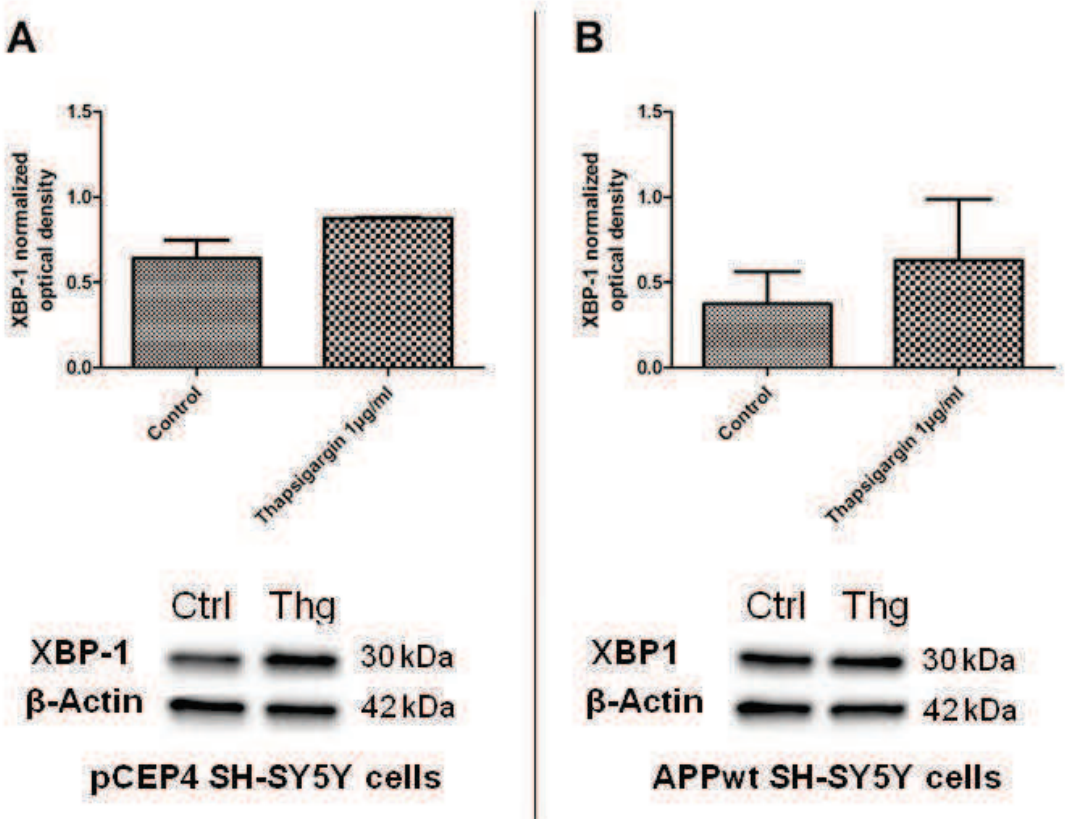

Figure 44 : Western blot analysis of XBP-1 protein levels in control vector- (A) and APPwt-transfected SH-SY5Y cells (B). Cells were exposed or not (vehicle) to Thapsigargin (dose evoking 60% of cell death) for 24 h. XBP-1 optical density was normalized to β-actin expression. Each value represents the mean ± S.E.M. of two independent experiments. Ctrl, control ; Thg, Thapsigargin ; XBP-1, X-box binding protein 1.

3.3. Neuroprotection by GHB and neurosteroids : additive or synergistic action ?

During this PhD program, the neuroprotective effects of GHB and neurosteroids were assessed in 3 different conditions that induce cell death : overexpression of APPwt, H_2O_2-induced stress and ER stress. It was interesting to observe that GHB and neurosteroids were mainly effective against APPwt-overexpression and H_2O_2-evoked cell death and did not exhibit a potent protective action against ER stress-induced cell loss except the promising preliminary effect observed for estradiol. Therefore, for the moment, to gain insight into the possible cooperation that may exist between GHB and neurosteroids for a potent neuroprotective action, we decided to use H_2O_2

experimental model to investigate the effects of concomitant administration of GHB and allopregnanolone or estradiol against oxidative stress-induced cell death.

3.3.1. Assessment of the neuroprotective action of GHB and neurosteroid co-treatments

Native SH-SY5Y cells were treated with different combinations of GHB + Estradiol or GHB + allopregnanolone. In the presence of 0.7 mM H_2O_2 (Fig. 45). Many combinations were tested but the Fig. 45 only shows the best representative neuroprotective concentrations of GHB and estradiol or allopregnanolone. The data revealed that, while the co-application of GHB (500 µM) and estradiol (500 nM) did not improve the level of neuroprotection exerted by GHB (500 µM) or estradiol (500 nM) administrated separately (Fig. 45A). The concomitant treatment of GHB (500 µM) and allopregnanolone (500 nM) exhibited a significant additive neuroprotective effect compared to GHB or allopregnanolone alone (Fig. 45B).

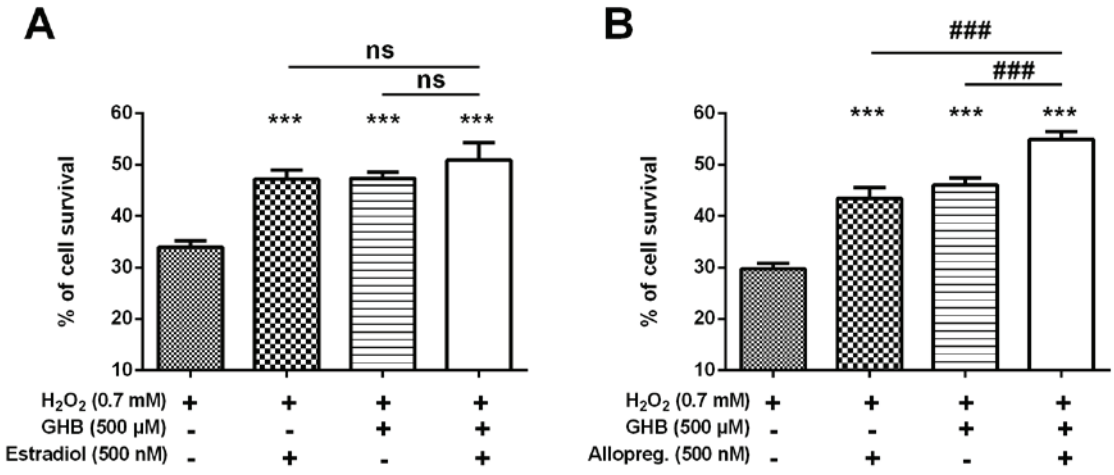

Figure 45 : Effects of separate and concomitant treatments of GHB and estradiol (A) or GHB and allopregnanolone (B) against H_2O_2-induced SH-SY5Y cell death. Native SH-SY5Y cells were exposed to H_2O_2 (0.7 mM) and treated either separately or concomitantly with GHB (500 µM) and allopregnanolone/estradiol (500 nM) for 24 h. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of four independent experiments. $^{***}p<0.001$, $^{###}p<0.001$.

3.3.2. Evaluation of GHB capacity to induce neuroprotection via the modulation of neurosteroid production

To test the hypothesis that GHB may exert neuroprotective action through the control of neurosteroidogenesis, we performed a series of preliminary experiments to assess the effect of GHB (500 µM) against H_2O_2-evoked cell death in the presence of fadrozole (10 µM), a specific inhibitor of aromatase, the key estradiol synthesizing enzyme (Falkson et al., 1992, Raats et al., 1992). The results show that fadrozole (10 µM) significantly decreased the effectiveness of neuroprotective effect exerted by GHB (500 µM) against H_2O_2-induced cell death (Fig 46).

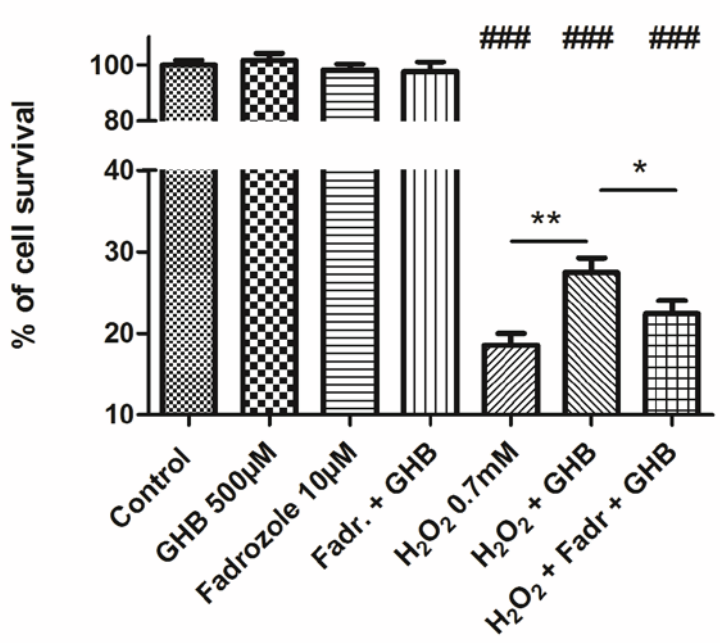

Figure 46 : Effects of GHB against H_2O_2-evoked cell death in the presence of fadrozole, a specific inhibitor of aromatase. SH-SY5Y cells were treated with GHB (500 µM) and H_2O_2 (0.7 mM) in presence or absence of Fadrozole (10 µM) for 24 h. MTT signal assessed for each cell type in basal condition (absence of H_2O_2) is arbitrary set at 100 %. Each value is the mean ± S.E.M. of two independent experiments. *p<0.05, **p<0.01, $^{###}p$<0.001 compared to control.

3.3.3. Effect of GHB on aromatase expression in SH-SY5Y cells

Our preliminary results revealing that fadrozole decreases GHB neuroprotective efficacy suggest that GHB may exert neuroprotection through the stimulation of aromatase expression or activity. To test this idea, we investigated the effects of GHB on aromatase mRNA levels in SH-SY5Y cells in the absence or presence of H_2O_2 (Fig. 46). The levels of transcripts encoding aromatase were examined by RT-qPCR in native SH-SY5Y cells treated with GHB (500 µM) in the absence or presence of H_2O_2 (0.7 mM). the preliminary results showed that, after normalization of RT-qPCR aromatase products to β-actin, GHB (500 µM), which increased aromatase expression in normal condition (+70%) also counteracted the decreased aromatase mRNA levels (-49%) evoked by H_2O_2 (Fig. 47).

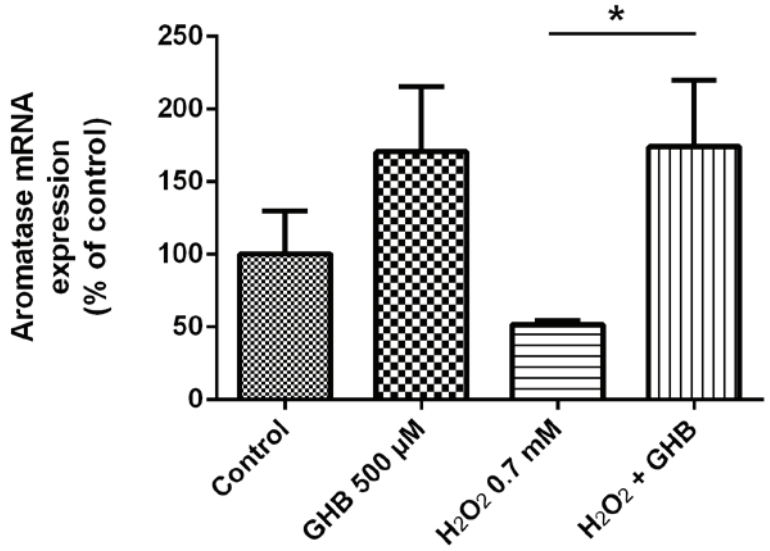

Figure 47 : Preliminary assessment of GHB effects on aromatase mRNA levels in native SH-SY5Y cells in the absence or presence of H_2O_2. Cells were treated for 24 h with GHB (500 µM) in the absence or presence of H_2O_2 (0.7 mM). Aromatase mRNA levels are expressed as a ratio of β-actin in SH-SY5Y cells. mRNA levels in control condition (absence of H_2O_2) is arbitrarily set at 100%. Each value is the mean ± S.E.M. of two independent experiments. *p<0.05.

3.4. Effects of GHB and/or neurosteroids on the activity and expression of beta amyloid degrading enzymes (MMP-2 and MMP-9)

3.4.1. Effects of GHB and/or neurosteroids on human MMP-2 and MMP-9 activity in yeast

3.4.1.1. *Validation of the yeast-based assay*

Since MMPs are members of the metalloproteinase family, we first intended to evaluate the suitability and accuracy of the yeast-based MMP-2/-9 activity assay by testing serial dilutions of phenanthroline which is a broad spectrum metalloproteinase inhibitor (Feder et al., 1971). Thus we incubated yeast expressing biologically active MMP-2 and MMP-9 with increasing concentrations of phenanthroline ranging from 0 to 500 µM in the presence of fluorescein-labeled gelatine as MMP substrate. Fluorescence detection was recorded at 37°C for 20 hours with 20 minutes intervals between each measurement. As shown in Fig. 48, fluorescence increased with time and phenanthroline efficiently inhibited MMP-2 and MMP-9 activities. Interestingly, MMP-2 and MMP-9 were differentially inhibited by phenanthroline: 62.5 µM phenanthroline was sufficient to abolish MMP-2 activity whereas 125 µM was necessary to obtain the same inhibition of MMP-9. Therefore, we chose inhibitor concentrations of 70 µM and 150 µM phenanthroline for MMP-2 and MMP-9 respectively as negative control in all experiments in which the potential effect of GHB and/or neurosteroids on MMP activity was analyzed.

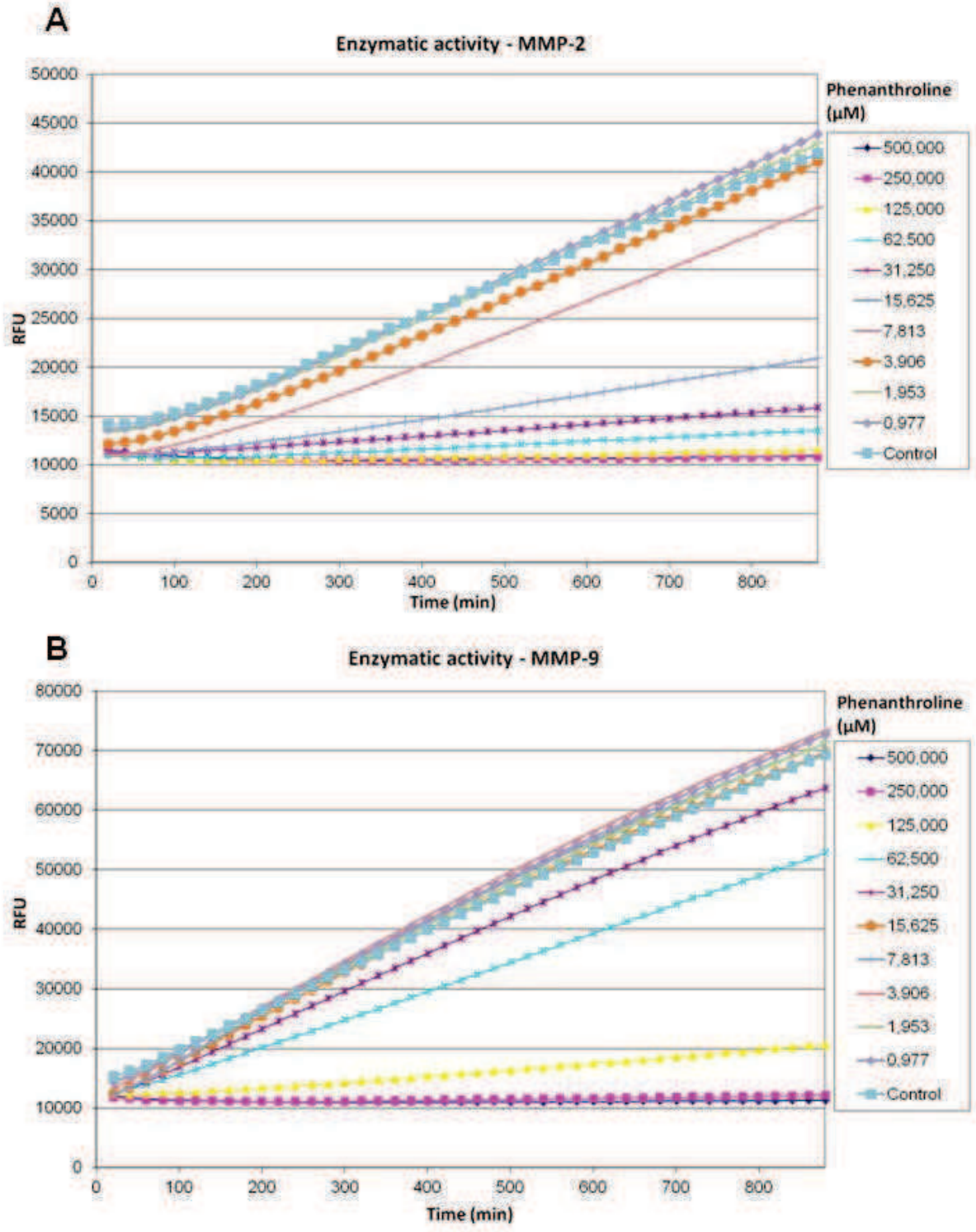

Figure 48 : Validation of the yeast-based MMP-2 (A) and MMP-9 (B) activity assay in the presence of increasing concentrations of the metalloproteinase inhibitor phenanthroline. Yeast expressing human MMP-2 or MMP-9 at the cell surface were deposed at the same optical density in a 96-well plate in reaction buffer. Cells were exposed to the indicated concentrations of phenanthroline in the presence of fluorescein-labeled gelatine as MMP substrate. Green fluorescence is recorded each 20 min for 20 h. Each value represents the mean of three independent experiments (RFU, relative fluorescence intensity. MMP, matrix metalloproteinase).

3.4.1.2. *Effects of GHB and/or neurosteroids on MMP-2 and MMP-9 activity*

In order to evaluate the effects of GHB, allopregnanolone and estradiol on MMP-2 and MMP-9 activity, we tested serial dilutions of both compounds using the parameters mentioned previously. Concentrations of phenanthroline determined in Part 3.4.1.1 were used as negative control of MMP-2 and MMP-9 activity in all experiments described in this Part.

We first assessed the effect of increasing concentrations of GHB ranging from 0 to 2,000 µM on MMP-2 and MMP-9 activity. However, no significant modulatory effect of GHB on MMP-2/-9 activity could be detected under the experimental conditions used in this bioassay (Fig. 49A, B). We likewise investigated the effect of increasing concentrations of allopregnanolone ranging from 0 to 1,000 nM on MMP-2 and MMP-9 activity, but again no significant effect was observed (Fig. 50A, B).

The same experiment was performed in the presence of increasing concentrations of estradiol which likewise did not show any modulatory effect on MMP activity in the range of up to 1,000 nM (Fig. 51A, B).

Finally, any possible effect of the simultaneous application of GHB and neurosteroids (500 µM GHB + 500 nM allopregnanolone or 500 µM GHB + 500 nM estradiol) on MMP-2 and MMP-9 activity could also be ruled out (Fig. 52A, B).

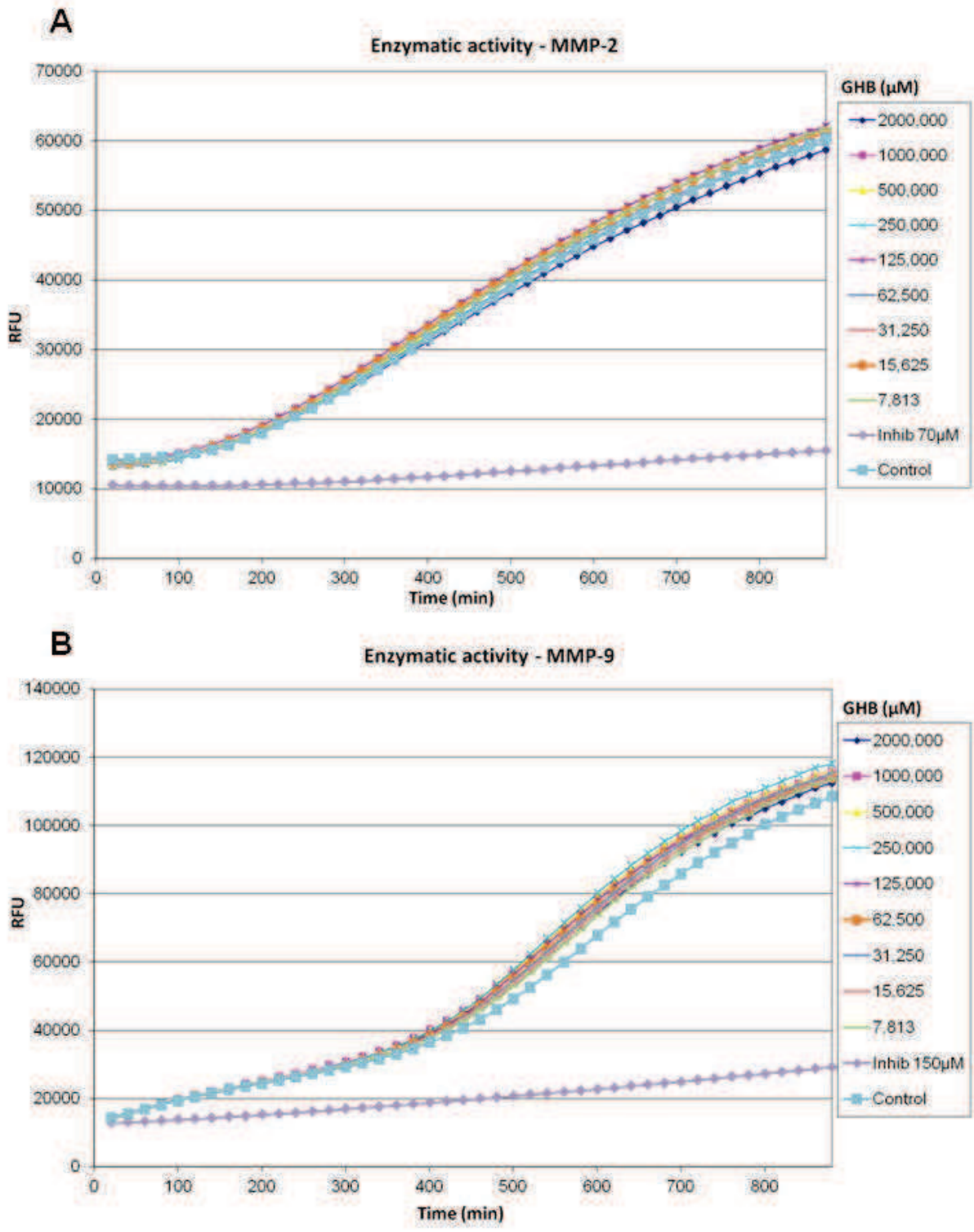

Figure 49 : Effects of GHB on MMP-2 (A) and MMP-9 (B) activity in yeast. Yeast expressing human MMP-2 or MMP-9 at the cell surface were deposed at the same optical density in a 96-well plate in reaction buffer. Cells were exposed to the indicated concentrations of GHB in the presence of fluorescein-labeled gelatin as MMP substrate. Phenanthroline was used as negative control and MMP inhibitor (Inhib.). Green fluorescence is recorded each 20 min for 20 h. Each value represents the mean of three independent experiments (RFU, relative fluorescence intensity. MMP, matrix metalloproteinase).

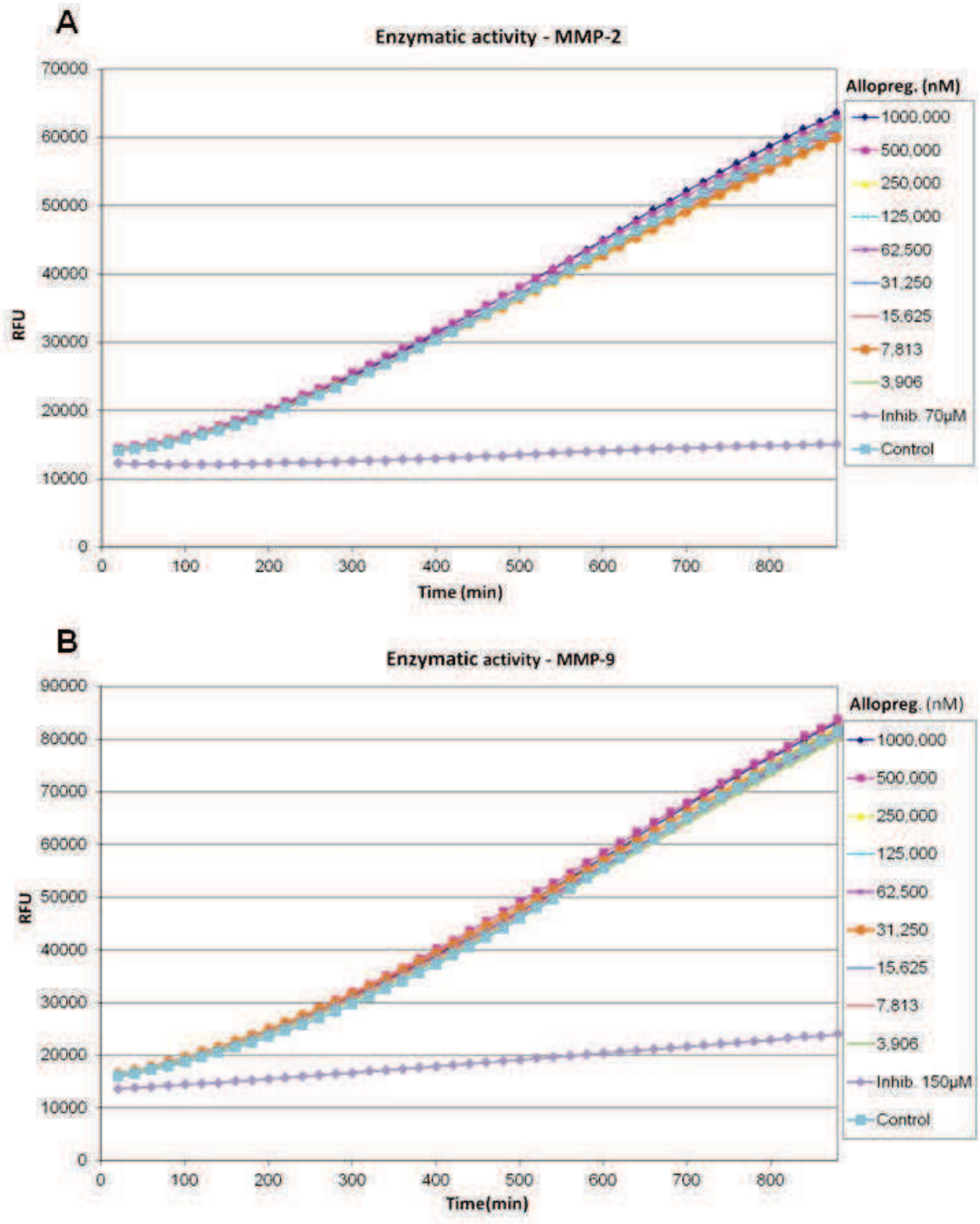

Figure 50 : Effects of allopregnanolone on MMP-2 (A) and MMP-9 (B) activity in yeast. Yeast expressing human MMP-2 or MMP-9 at the cell surface were deposed at the same optical density in a 96-well plate in reaction buffer. Cells were exposed to increasing concentrations of allopregnanolone in the presence of fluorescein-labeled gelatin as MMP substrate. Phenanthroline was used as negative control and MMP inhibitor (Inhib.). Green fluorescence is recorded each 20 min for 20 h. Each value represents the mean of three independent experiments (RFU, relative fluorescence intensity. MMP, matrix metalloproteinase).

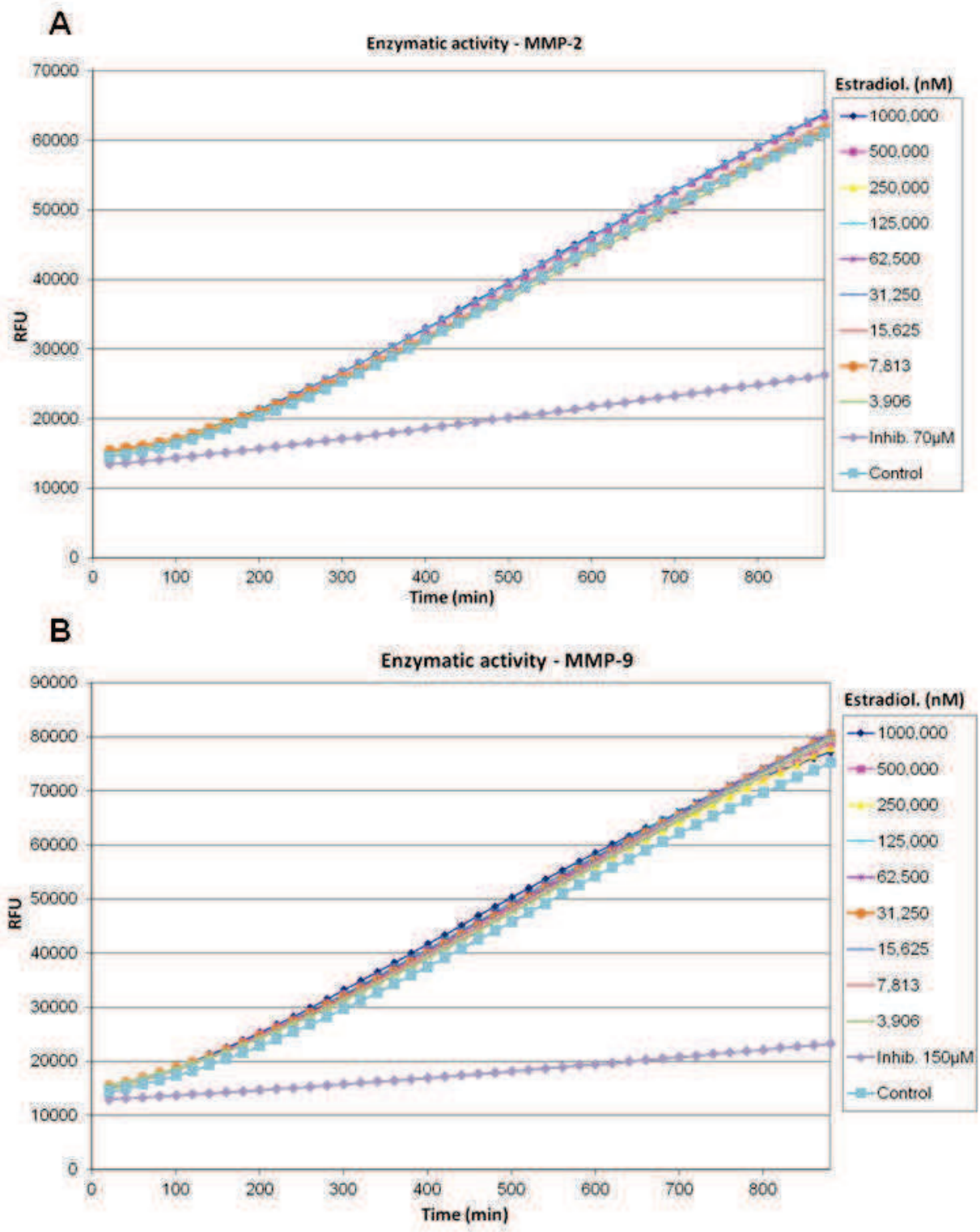

Figure 51 : Effects of estradiol on MMP-2 (A) and MMP-9 (B) activity in yeast. Yeast expressing human MMP-2 or MMP-9 at the cell surface were deposed at the same optical density in a 96-well plate in reaction buffer. Cells were exposed to increasing concentrations of estradiol in the presence of fluorescein-labeled gelatin as MMP substrate. Phenanthroline was used as negative control and MMP inhibitor (Inhib.). Green fluorescence is recorded each 20 min for 20 h. Each value represents the mean of three independent experiments (RFU, relative fluorescence intensity. MMP, matrix metalloproteinase).

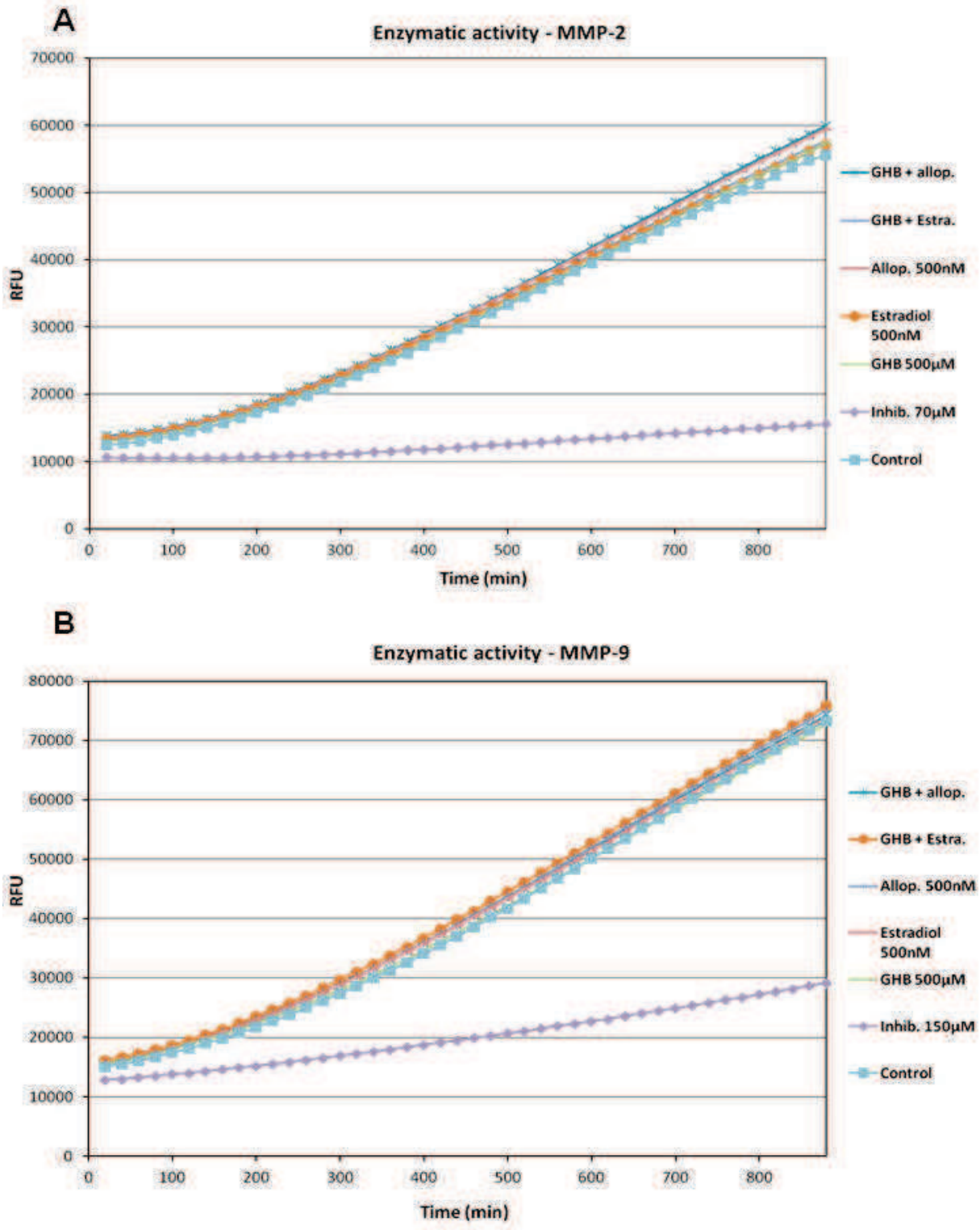

Figure 52 : Effects of GHB and neurosteroids on MMP-2 (A) and MMP-9 (B) activity in yeast. Cells were exposed to the indicated concentration of allopregnanolone, GHB or estradiol in the presence of fluorescein-labeled gelatin as MMP substrate. Phenanthroline was used as negative control and MMP inhibitor (Inhib.). Green fluorescence is recorded each 20 min for 20 h. Each value represents the mean of three independent experiments (RFU, relative fluorescence intensity. MMP, matrix metalloproteinase).

3.4.2. Effects of GHB on human MMP-2 and MMP-9 mRNA expression in SH-SY5Y cells

Preliminary assessments of the effects of GHB on MMP-2 and MMP-9 mRNA expression were performed in SH-SY5Y cells. The levels of transcripts encoding MMP-2 and MMP-9 were examined by RT-qPCR in native SH-SY5Y cells treated with GHB (500 µM) for 24 h. After normalization of the obtained MMP mRNA ratio to β-actin, it appeared that GHB (500 µM) did not change MMP-2 mRNA levels in SH-SY5Y cells (Fig. 53A). However, GHB (500 µM) significantly decreased the level of MMP-9 in SH-SY5Y cells (-72,7%, $p<0.05$), suggesting a differential effect of GHB on MMPs expression.

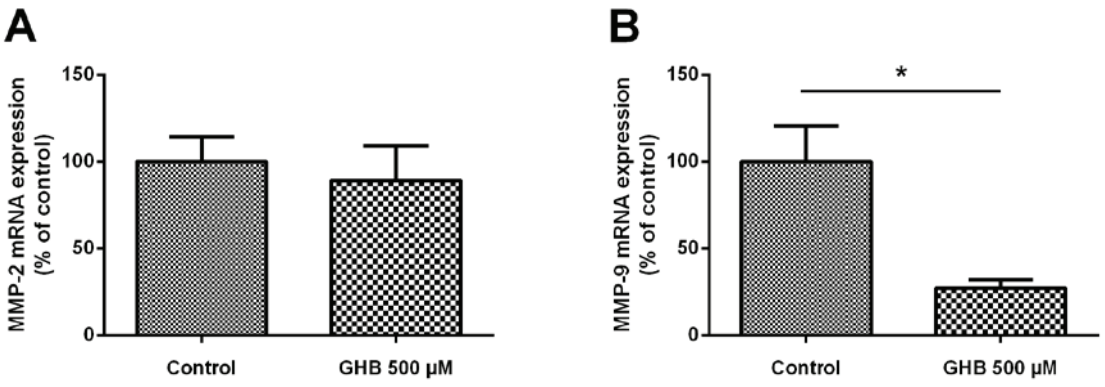

Figure 53 : Preliminary assessment of GHB effects on MMP-2 (A) and MMP-9 (B) mRNA expression in native SH-SY5Y cells. Cells were treated with 500 µM GHB for 24 h before RT-qPCR. MMP-2/-9 mRNA levels are expressed as ratio to β-actin in SH-SY5Y cells. mRNA levels under control condition (untreated) is arbitrarily set at 100%. Each value represents the mean ± S.E.M. of two independent experiments (MMP, matrix metalloproteinase; *$p<0.05$).

4. Discussion

In agreement with the objectives of this PhD thesis (See Part. 1.5.1., page 60), we investigated the ability of separated or combined treatments of GHB and neurosteroids such as allopregnanolone and estradiol to protect against cell death induced by key factors involved in AD physiopathology. Our studies, which provided some data about GHB action on neurosteroidogenesis, also determined wether GHB and/or neurosteroids modulate the activity and/or expression of MMP-2 and MMP-9 that regulate amyloid beta peptides in the brain.

4.1. Protective effects of GHB against oxidative stress-induced cell death

Numerous studies suggested the existence of neuroprotective effects of GHB but *in vitro* experimental proofs showing a direct protective action of GHB against cell death were until now unavailable (Wolfson et al., 1977b, Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004, Yosunkaya et al., 2004). As mentioned above (Part. 1.1.4), oxidative stress is a key element involved in neurodegenerative diseases (Beal, 1995, Giasson et al., 2002, Andersen, 2004). Thus, in a first step, we performed a dose and time-dependent study which revealed that H_2O_2, a potent oxidative stressor involved in pathogenic mechanisms of several neurodegenerative disorders, severely evoked death of native and genetically modified human neuroblastoma SH-SY5Y cells. This study also showed different EC50 values for H_2O_2 toxicity in each category of cells. More importantly, we observed that 70% of native and control vector-pCEP4-transfected cells were killed after exposure to 0.7 mM H_2O_2 while a dose seven times less (0.1 mM) was enough to kill the same percentage of APPwt-transfected cells. These results indicate that the over-expression of APPwt in SH-SY5Y cells enhanced

their vulnerability to oxidative stress. Our results, which were obtained thanks to the combination of two complementary methods such as trypan blue exclusion and MTT reduction assays, strongly consolidate previous observations suggesting that APP expression may be related to neuroblastoma cell susceptibility to oxidative stress (Matsumoto et al., 2006). This "hypersensitivity" to H_2O_2 could be, in part, explained by an abnormal production of Aβ peptides due to the overexpression of APP itself. Moreover, Aβ peptide cytotoxicity is known to be mediated by the endogenous production of H_2O_2 (Behl et al., 1994). Therefore, the exogenous exposition of H_2O_2 may exacerbate the endogenous oxidative stress existing occurring in APPwt-overexpressing cells.

In the drastic condition of survival when neuroblastoma cells were exposed to H_2O_2 dose killing more than 70% of cells, GHB was effective to rescue a significant number of SH-SY5Y cells in a dose-dependent manner. In particular, GHB at 500 µM protected about 50% of SH-SY5Y cells against H_2O_2-induced death. Clearly, these results demonstrate that GHB exerts a protective action *in vitro* which is consistent with *in vivo* data suggesting that GHB may control neuroprotection (Wolfson et al., 1977a, Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004, Yosunkaya et al., 2004, Kemmel et al., 2010). Interestingly, we observed that, 24 h after GHB single application, the doses of 200 and 500 µM efficiently rescued APPwt-transfected SH-SY5Y cells that were extremely vulnerable under oxidative stress condition. 48 h after GHB single administration, the protective efficacy of GHB at 200 µM disappeared while the dose of 500 µM remained effective in protecting against APPwt-overexpression-induced cell death. These results suggest the occurrence of a time-dependent catabolism of GHB in the incubation medium. Thus, GHB concentration of 200 µM, which was effective 24 h after administration, may completely be degraded

at 48 h justifying the disappearance of the protective effect. At the dose of 500 µM, which is 2.5 times higher than 200 µM, a substantial amount of GHB may remain present in the medium after 48 h (in spite of the time-dependent catabolism of GHB) and therefore continue to protect APPwt-transfected cells against APP-overexpression- and/or oxidative stress-evoked death. Several studies demonstrated that APPwt-transfected SH-SY5Y cells represent a relevant cellular model for the investigation of biochemical mechanisms involved in AD pathogenesis (Li et al., 1996b, Misonou et al., 2000, Scheuermann et al., 2001, Olivieri et al., 2002, Schaeffer et al., 2006a, Schaeffer et al., 2008b, Schaeffer et al., 2008c, Rhein et al., 2009, Rhein et al., 2010). Indeed, it is well documented that the production of Aβ from APP is a major event in AD pathogenic mechanisms. In normal conditions, APP is processed by a non-amyloidogenic pathway but under pathological situations, APP generates toxic Aβ accumulation which induces neuronal death (see Part. 1.1.2.1., pages 17-21). It has been suggested that oxidative stress evokes a shift of APP processing from the non-amyloidogenic to the amyloidogenic pathway, a mechanism which may explain the high vulnerability of APPwt-overexpressing SH-SY5Y cells to H_2O_2-induced death (Misonou et al., 2000, Oda et al., 2010, Ballard et al., 2011). Therefore, the strong protective effect exerted by GHB against H_2O_2-evoked APPwt-transfected cell death suggests that GHB may significantly inhibit the ability of H_2O_2 to shift APP normal processing towards the amyloidogenic pathway.

Oxidative stress, particularly H_2O_2, is well known for its capacity to trigger mitochondrial apoptosis pathway in several cell types including neurons (Dare et al., 2001, Chen et al., 2011b, Hayashi et al., 2012, Perez-Pinzon et al., 2012). In agreement with these data, our flow cytometry (FACS) analyzes of TUNEL-FITC and activated caspase-3-PE staining revealed apoptosis occurrence in native and

genetically modified human neuroblastoma cells after exposure to H_2O_2. Interestingly, a substantial basal amount (~30%) of activated caspase-3-PE- and TUNEL-FITC-positive labeling was detected in APPwt-transfected SH-SY5Y cells in the absence of H_2O_2, indicating that the over-expression of APP may facilitate Aβ accumulation and apoptosis in neuroblastoma cells. Consistently, it has been reported that APPwt-overexpression induced apoptosis in HEK 293 cells (Takahashi et al., 2009). Furthermore, we observed that H_2O_2 strongly enhanced the basal apoptotic signal in APPwt-transfected cells, a result which may also confirm the idea that H_2O_2-induced death may up-regulate the amyloidogenic pathway leading to increased apoptosis in neuroblastoma cells. In all categories of SH-SY5Y cells used (native or genetically modified), GHB was capable of reducing efficiently H_2O_2-induced apoptotic signal. In addition, the basally elevated activated caspase-3-PE- and TUNEL-FITC-positive labeling detected in APPwt-transfected cells in the absence of H_2O_2 was also significantly decreased by GHB treatment. Altogether, these results indicate that GHB protective action against cell death involves anti-apoptotic mechanisms.

Apoptosis is a complex cellular process characterized by multi-factorial pathways (Kerr et al., 1972, Chang et al., 1998, Hengartner, 2000, Kroemer and Reed, 2000, Mattson, 2000, Wu et al., 2001, Acehan et al., 2002, Adams and Shapiro, 2003). However, it is clearly established that proteins of Bcl-2 family crucially control apoptotic mechanisms (see Part 1.1.4.3., pages 34-35) (Gross et al., 1999, Mattson, 2000, Kuwana and Newmeyer, 2003, Petros et al., 2004, Garcia-Saez, 2012). Indeed, as aforementioned, some members of the Bcl-2 family such as Bcl-2 and Bcl-xl are anti-apoptotic factors while other members as Bax, Bid and Bcl-xs are pro-apoptotic proteins (Reed, 1998, Reed et al., 1998, Mattson, 2000, Newmeyer and

Ferguson-Miller, 2003). Consequently, the Bax/Bcl-2 ratio is conventionally used as a key index to evaluate the apoptotic status of cells (Jung et al., 2009, Zhang et al., 2009, Zarate et al., 2010). Interestingly, previous investigations revealed that the level of Bcl-2 protein significantly decreased in APPwt-transfected cells, suggesting a higher susceptibility to apoptosis (Matsumoto et al., 2006). Therefore, to get valuable insights into the mechanisms of action of GHB against neuroblastoma cell death, we combined reverse transcription, quantitative real-time PCR experiments and western blot analysis to determine the effects of GHB on Bax/Bcl-2 mRNA and protein ratios in native and genetically modified SH-SY5Y cells in the absence or presence of H_2O_2. Our investigations revealed that, in the absence of oxidative stress, Bax/Bcl-2 mRNA and protein ratios were elevated only in APPwt-transfected cells and this observation is perfectly consistent with our results obtained with flow cytometry analyzes of TUNEL-FITC- and activated caspase-3-PE labeling. In the absence of H_2O_2 (normal condition), application of GHB at 500 µM efficiently decreased the elevated basal Bax/Bcl-2 (mRNA and protein) ratios in APPwt-transfected SH-SY5Y cells, indicating that a possible mechanism activated by GHB to protect against cell death may be the promotion and/or the repression of Bcl-2 and Bax gene and protein expression, respectively. In support of this hypothesis, our results also show that, under oxidative stress condition, GHB was also capable of reducing significantly the increased Bax/Bcl-2 ratios evoked by H_2O_2 in native or genetically modified SH-SY5Y cells.

Since our MTT reduction assays revealed that GHB is devoid of proliferative action on neuroblastoma cells, anti-apoptotic effect through the down-regulation of Bax/Bcl-2 (mRNA and protein) ratios and/or caspase-3 activity appears as a pivotal mechanism activated by GHB to protect cell against death. Various data from our

laboratory and other groups demonstrated that GHB modulates G-protein coupled receptors and trigger intracellular signaling that may interact with the expression of Bcl-2 family proteins (Maitre, 1997, Maitre et al., 2000, Andriamampandry et al., 2003a, Crunelli et al., 2006, Andriamampandry et al., 2007, Coune et al., 2010, Kemmel et al., 2010). Further investigations will certainly help to specify or detail the interactions between GHB-evoked intracellular signaling and Bax/Bcl-2 expression and activities. It will also be interesting to check whether GHB-evoked neuroprotection of SH-SY5Y cells involves $GABA_A$-receptors. Indeed, it has recently been shown that GHB binds to $GABA_A$-receptors (Absalom et al., 2012) those activation reduces amyloid cytotoxicity and endogenous ROS (Lee et al., 2005, Brar et al., 2014).

Although our results strongly indicate that GHB protects SH-SY5Y cells via the activation of an anti-apoptotic mechanism, we cannot rule out the possibility that GHB may also be neuroprotective by exerting a direct anti-oxidative action against H_2O_2. Indeed, the acidic character of GHB molecular structure allows it to decrease H_2O_2 toxicity via an oxido-reductive reaction. Furthermore, it has been shown that GHB, which activated glucose-6-deshydrogenase and the pentose phosphate pathway, also increased the formation rate of NADPH which is the key cofactor of several anti-oxidative or reductive enzymes (Lopatin et al., 1984, Martins et al., 1986, Russell et al., 1999, Mamelak, 2007). Therefore, GHB may inhibit H_2O_2 toxicity thanks to its ability to facilitate the activity of various anti-oxidative enzymes. However, the fact that, in the absence of H_2O_2, GHB induced a protective effect and significantly reduced the basally elevated apoptotic signal in APPwt-transfected SH-SY5Y cells, strongly supports the idea that GHB may also protect nerve cells against death via an anti-apoptotic mechanism. In support of this hypothesis, we have recently observed

that a single acute pharmacological dose of GHB modulates the brain expression of multiple genes including various genes encoding for programmed cell death factors (Kemmel et al., 2010). In support of our data, another microarray study has also revealed that GHB efficiently down-regulated the expression of the apoptotic protease activating factor 1 (Apaf-1) which is pivotally involved in apoptosome formation (Schnackenberg et al., 2010).

4.2. Protective effects of allopregnanolone and estradiol against oxidative stress-induced cell death

Based upon the high neuroprotective potential of neurosteroids evidenced in various experimental models (Belelli and Lambert, 2005, Patte-Mensah et al., 2005, Borowicz et al., 2011, Gravanis et al., 2012, Panzica et al., 2012, Brinton, 2013), we assessed the ability of allopregnanolone (AP) and estradiol (E2) to prevent or counteract H_2O_2-induced cell death. Although various nerve cell models have been used to test the neuroprotective action of neurosteroids, the present PhD works provide the first assessment of AP and E2 protective effect against control vector and APPwt-transfected SH-SY5Y cells in oxidative stress conditions. A previous study of our laboratory has shown that E2 protected against H_2O_2-induced SH-SY5Y cell death but the genetically modified pCEP4- and APPwt-transfected cells were not used in that study (Schaeffer et al., 2008c). Therefore, it is interesting to observe that, beyond the confirmation of the capacity of E2 to protect against H_2O_2-induced native SH-SY5Y cell death, the present PhD study also reveals that E2 and AP effectively prevent pCEP4- and APPwt-transfected cells against oxidative stress-evoked death. More importantly, our results showed that, while micromolar doses of GHB (500 µM) were necessary to prevent H_2O_2-induced cell death, nanomolar concentrations (250 – 750 nM) of neurosteroids AP and E2 were enough to exert efficient neuroprotective

effects. Furthermore, our studies revealed that, in the absence of H_2O_2, GHB (500 µM) restored normal percentage of cell viability in APPwt-transfected cells but did not stimulate or increase the viability of control cells. In contrast, we observed that in basal conditions, E2 and AP increased native, pCEP4- and APPwt-transfected cell viability. Taken together, these data suggest that GHB and neurosteroids may not activate the same mechanisms to exert neuroprotective actions against H_2O_2-induced cell death even through common intracellular factors such as the proteins of the Bcl-2 family may be modulated via the signaling pathways evoked by GHB or neurosteroids. Indeed, it has been demonstrated that E2 may reduce oxidative stress-induced apoptosis through the up-regulation of Bcl-2 and Bcl-Xl proteins, a process that may also be activated by GHB (see Part 3.1.6., pages 103-108). However, E2 may induce anti-apoptotic effect through other mechanisms (probably not shared with GHB) such as the down-regulation of the JNK signaling pathway and the stimulation of the expression of mitochondrial respiratory complex (I and IV) or ATP synthase (Garcia-Segura et al., 1998, Nilsen and Diaz Brinton, 2003, Irwin et al., 2008, Nilsen, 2008, Simpkins and Dykens, 2008, Simpkins et al., 2010, Grimm et al., 2012, Napolitano et al., 2014). Moreover, various studies demonstrated that, acting through E2Rα or E2Rβ, E2 may induce neuroprotection via the activation of MAPK (mitogen activated protein kinase), PI3K/Akt (phosphatidylinositol 3-kinase and protein kinase B), STAT3 (signal transducer and activator of transcription 3) and CREB (cAMP response element binding protein) (Cimarosti et al., 2005, Jover-Mengual et al., 2007, Zhao and Brinton, 2007).

Regarding the neurosteroid AP, which also exhibited a potent neuroprotective effect against H_2O_2-induced cell death, several mechanisms may be discussed including those that can partially be common to AP and GHB or E2 and other processes that

can be specific to AP. Indeed, recent investigations showing that AP efficiently decreased glucose-induced oxidative stress and apoptosis, suggested that AP down-regulated Bax/Bcl-2 ratio and caspase-3 activation (Afrazi and Esmaeili-Mahani, 2014, Afrazi et al., 2014). It has also been shown that AP protected against H_2O_2-evoked oxidative stress in a cellular model of Niemann-Pick disease by decreasing ROS levels, lipid peroxidation and NFκB pathway-related apoptosis (Zampieri et al., 2009). Moreover, AP is a potent activator of $GABA_A$-receptor which the stimulation of, by specific agonists, has been shown to decrease ROS levels, caspase-3 activation and apoptosis (Lee et al., 2005).

To summarize, our results revealed that neurosteroids and GHB may activate common and/or specific factors to protect SH-SY5Y cells against H_2O_2-induced death. Also, our data suggest that a cooperation may exist between GHB- and neurosteroid-evoked signaling pathways for an optimal neuroprotective action not only under oxidative stress conditions but also in other experimental models such as ER stress-induced cell death which is also involved in AD physiopathology (Prasanthi et al., 2011, Cornejo and Hetz, 2013, Marwarha et al., 2013, Hetz and Mollereau, 2014, Liu et al., 2014).

4.3. Effects of GHB and neurosteroids against ER stress-induced cell loss

Our results showed that tunicamycin- (TC) or thapsigargin- (THG) induced ER stress caused a significant decrease of SH-SY5Y cell survival. These observations confirmed that TC and THG are effective ER stressors in our cellular experimental model. We did not have additional time to explore whether TC or THG induce also apoptosis in our model. However, other studies demonstrated that specific caspases

including caspase-4, -7 and -12 are activated during ER stress conditions and these data suggest that TC- or THG-evoked ER stress may cause apoptosis in SH-SY5Y cells (Nakagawa and Yuan, 2000, Nakagawa et al., 2000, Momoi, 2004, Liu et al., 2013, Hetz and Mollereau, 2014). Further experiments will however be necessary to confirm this hypothesis. Although they still preliminary, our data indicate that the tested doses of GHB (0-1,000 µM) and AP (0-1,000 nM) were unable to counteract TC- or THG-evoked SH-SY5Y cell loss. Because we did not have enough time, E2 was only tested against THG-evoked cell death and the results revealed that E2 (250 or 500 nM) efficiently counteracted THG-induced ER stress and cell loss. Additional experiments scheduled for the future will help to clarify whether or not TC- and THG-induced ER stress may differentially be sensitive to the action of E2, AP and GHB used separately or concomitantly. In particular, it will be useful to complete our current results on cytosolic calcium changes and ER stress signaling proteins (See Part. 3.2.3. and 3.2.4., pages 123-126) with additional experiments testing other doses of AP, E2 and GHB in the presence of lower concentration of TC and THG.

4.4. Interactions between GHB and neurosteroids for neuroprotective strategy

We investigated the effects of co-applications of GHB and AP or E2 against H_2O_2-induced cell death, an experimental model in which separated treatments of neurosteroids or GHB administrated alone were all effective (see Part 3.1., pages 95-114). We found that the co-application of GHB and E2 did not increase the efficacy of each drug used separately. In contrast, the co-treatment with GHB and AP significantly improved the level of neuroprotection via an additive action. These

results suggest the existence of positive interactions between GHB and AP. These interactions seem to not be synergistic since GHB and AP doses that were ineffective separately did not become neuroprotective when applied concomitantly. The positive interactions were reflected by the fact that the doses of GHB (500 µM) and AP (500 nM) that were neuroprotective separately exhibited a more potent protective action when they were co-administrated to SH-SY5Y cells under oxidative stress conditions. The mechanisms of actions underlying this additive effect of GHB and AP require further investigation before being elucidated. However, as aforementioned, various studies demonstrated that GHB and AP can activate similar intracellular signaling pathways (see Part. 1.4., page 59). Indeed, it has been shown that GHB acts via the $GABA_A$-receptor which is also a major receptor allosterically stimulated by AP (Lovick et al., 2005, Absalom et al., 2012, Bay et al., 2014). The additive effect may also result from the combination or complementary actions of specific pathways evoked by GHB and AP. For instance, while GHB activates its own G-protein coupled receptor to increase cAMP signaling that may trigger neuroprotective mechanisms (Andriamampandry et al., 2003a, Andriamampandry et al., 2007, Coune et al., 2010), this process may be added to the neuroprotective effect induced by AP via the activation of pregnane-xenobiotic-receptor (Frye et al., 2011, Jayaraman et al., 2012, Irwin and Brinton, 2014) or through the modulation of calcium channels (Brinton, 2013, Irwin and Brinton, 2014). Finally, another possibility may also be an addition of the stimulatory effect exerted by GHB and AP on anti-apoptotic proteins of the Bcl-2 family leading to a more potent decrease of Bax/Bcl-2 ratio when GHB and AP are administrated concomitantly. This hypothesis is supported by various results including our data which demonstrated the regulatory effects of GHB and AP on

Bax/Bcl-2 ratio (Afrazi and Esmaeili-Mahani, 2014, Afrazi et al., 2014, Wendt et al., 2014).

Interactions between GHB and neurosteroids cannot be restricted only to the conditions of co-administration of these compounds. Indeed, previous investigations, which demonstrated that GHB stimulated AP and THDOC production in the rat brain (Barbaccia et al., 2002, Barbaccia et al., 2005), strongly suggested that GHB may exert neuroprotective effects through the modulation of neurosteroid biosynthesis. In line with these results, our laboratory has shown that the inhibition of AP production in rat spinal cord *in vivo*, thanks to intrathecal injections of provera (pharmacological blocker of 3α-HSOR, the key AP synthesizing enzyme), exacerbated neuropathic symptoms (Meyer et al., 2008). Thus, it appears that the compounds such as GHB which increase AP production may exert beneficial or protective actions against neuropathological symptoms while the drugs, such as provera which decrease AP synthesis may facilitate neurodegenerative or deleterious action on nerve cells. Further investigations to assess the protective effect of GHB against H_2O_2-induced SH-SY5Y cell death in the presence of provera will help us to confirm this idea.

Another important study previously published by our laboratory revealed that the inhibition of endogenous E2 synthesis by letrozole (inhibitor of aromatase, the key E2-producing enzyme) significantly decreased SH-SY5Y cell viability and the addition of exogenous E2 counteracted the cell loss (Schaeffer et al., 2008c). This paper suggested that the compounds modulating endogenous E2 production in SH-SY5Y cells may control their survival in normal or stress conditions. Therefore, we checked whether GHB-evoked neuroprotective effect against H_2O_2-induced cell death may be mediated via the modulation of endogenous E2 biosynthesis or aromatase expression and/or activity in SH-SY5Y cells. We found that GHB-evoked

neuroprotective action was significantly reduced by fadrozole, a specific inhibitor of aromatase activity (Falkson et al., 1992, Raats et al., 1992). In addition, our preliminary RT-qPCR experiments revealed that GHB completely reverse the decreased aromatase mRNA level induced in SH-SY5Y cells by H_2O_2 treatment. These results, which need additional conformation, strongly suggest that GHB may protect SH-SY5Y cells against H_2O_2-evoked death through the increase of endogenous E2 production. Assessment of the effect of GHB on the level of endogenous E2 produced in SH-SY5Y cells in the presence or absence of H_2O_2 will also help to consolidate our data.

4.5. Effects of GHB and neurosteroids on MMP-2 and MMP-9 activity/expression

Besides the strategy to characterize compounds that may efficiently counteract oxidative or ER stress-induced neuronal death that are crucially involved in AD physiopathological mechanisms (Mattson, 2000, Lee et al., 2010, Querfurth and LaFerla, 2010, Reddy, 2011, Grimm et al., 2012, Cornejo and Hetz, 2013, Hetz and Mollereau, 2014), we also sought for molecules that may degrade or eliminate toxic Aβ peptides which pivotally induce neurodegeneration (Gotz et al., 2010, Querfurth and LaFerla, 2010, Ballard et al., 2011). Since various studies demonstrated that MMP-2 and MMP-9 are key regulators of Aβ peptide degradation, we have used the yeast based MMP activity assay to determine whether GHB, AP and E2 may directly modulate MMP-2 and MMP-9 activities. Probably because the basal level of MMP-2 and MMP-9 activities in this yeast model seems to be already elevated, it was not possible to evidence additional stimulation of these activities induced by separated or

concomitant administration of GHB, AP or E2. The yeast biological system expresses active human MMP-2 and MMP-9 on the surface of *Pichia pastoris* (Diehl et al., 2011).

Since MMPs physiologically have the potential to massively degrade various components of the extracellular matrix and surrounding tissue, MMPs are *in vivo* tightly regulated and controlled at three main levels : Firstly, many MMPs are only transcribed after cellular stimulation. Secondly, MMPs are initially expressed as inactive proenzymes, requiring additional processing to become biologically active. Thirdly, physiological inhibitors expressed and secreted by various cell types in tissue permit a fine tuning of MMP activity. These inhibitors include the tissue inhibitors of metalloproteinases (TIMPs) (Yong, 2005, Clark et al., 2008). Thus, it might be possible that GHB, allopregnanolone and/or estradiol modulate MMPs by acting at one or more of these three levels. MMP-2 and MMP-9 are zinc- and calcium-dependent proteases belonging to the gelatinase subfamily (Nagase and Woessner, 1999). Therefore, their activity can be detected by gelatine zymography. This technique resembles an electrophoretic method, using gelatine containing gels which permit the direct measurement of MMP activity in gels. Therefore, it would be interesting to study the basal expression of active MMP-2 and MMP-9 in native or APPwt-overexpressing SH-SY5Y cells. Moreover, further experiments investigating the effect of separate or concomitant treatment with GHB and neurosteroids such as allopregnanolone and estradiol on the levels of MMP mRNA, protein level and activity would open the possibility to eventually identify novel and specific inducers of these amyloid degrading enzymes. Estradiol was recently described to increase the activation and activity of both, MMP-2 and MMP-9 in SH-SY5Y cells, leading to the efficient degradation of Aβ peptides (Merlo and Sortino, 2012). In this study,

treatment with 10 nM estradiol for 15 hours caused an increase in MMP-2 and -9 mRNA and protein levels and also resulted in the conversion of pro-MMP-2 and -9 into their active forms. The authors also treated SH-SY5Y cells carrying the Swedish familial mutation of APP (which results in an overproduction of Aβ peptides) with estradiol and observed a significant reduction in the endogenous concentration of Aβ parallel to an increase in MMP-2 and MMP-9 activity, suggesting a prominent role of estradiol in MMP activation. Moreover, exogenous Aβ42-induced cell death was efficiently counteracted by estradiol, suggesting the role of MMP-2 and MMP-9 in neuroprotection against Aβ peptides.

MMP expression status in human AD brain is still controversially discussed. Reports show either unchanged, decreased or increased levels of both MMP-2 and MMP-9 in human brain tissue and plasma (Asahina et al., 2001, Baig et al., 2008, Horstmann et al., 2010). A most recent study demonstrated that MMP-9 concentration in the cerebrospinal fluid of AD and MCI patients is significantly decreased compared to control groups, while levels of MMP-2 and TIMPs were unchanged (Mroczko et al., 2014).

In our preliminary RT-qPCR experiments, we observed that 500 µM GHB did not change the mRNA expression level of MMP-2 in SH-SY5Y cells, while it significantly decreased MMP-9 mRNA expression. Although this observation is *per se* interesting, the obtained data needs to be further confirmed at mRNA and protein levels. In fact enhanced MMP activity in the CNS could have both, beneficial and detrimental effects. For example, MMP-9 is detrimental in acute insults to the nervous system such as stroke and spinal cord injury (Yong, 2005). Elevated expression of MMP-9 and MMP-2 following stroke-induced ischemia yields to an increased infarct size and disruption of the blood brain barrier (Cunningham et al., 2005, Machado et al., 2006,

Kook et al., 2013). However, MMPs including MMP-9 display beneficial effects on axonal regeneration, synaptic plasticity, remyelination and Aβ-induced toxicity (Yong, 2005, Agrawal et al., 2008, Fragkouli et al., 2014).

In case of stroke-induced ischemia, inhibition of MMP-9 is associated with attenuation of infarct size, better tissue recovery and a reduced risk of cerebral hemorrhages (Sumii and Lo, 2002, Agrawal et al., 2008). Therefore, if the results concerning the effects of GHB on MMP-9 are confirmed at mRNA, protein level and activity in SH-SY5Y cells, it would explain (at least in part) the protective effects of GHB observed in animal models of focal ischemic insults (Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004). A recent study implying allopregnanolone and progesterone also emphasizes this hypothesis. Indeed, the authors investigated the effects of these neurosteroids on blood brain barrier integrity and MMP-2 and -9 expression after middle cerebral artery occlusion in rats. They observed that both, progesterone and allopregnanolone attenuated blood brain barrier disruption and infarct size by down-regulating the expression of MMP-2 and -9 (Ishrat et al., 2010).

Thus, additional experiments investigating the effects of GHB, estradiol and allopregnanolone on MMP-2 and -9 expression, activation and activities in native and genetically modified SH-SY5Y cells are necessary to better understand their neuroprotective effects observed in AD or other CNS injury models.

5. Conclusions

Thanks to the combination of various pharmacological, cellular, molecular and biochemical methods, the present PhD report provides different series of results showing that GHB and neurosteroids, including AP and E2, efficiently protect against nerve cell death induced by key factors involved in AD physiopathological mechanisms. Indeed, the data described in this PhD manuscript, constitute the first experimental proofs demonstrating *in vitro*, a direct neuroprotective effect of GHB against neural cell death caused by major etiological determinants of AD such as the overexpression of APPwt (that generates toxic Aβ peptides accumulation) and oxidative stress which evokes apoptosis, neuronal loss and subsequent cognitive/memory deficits. Also, even though the neuroprotective potential of neurosteroids was previously in various experimental models, the present PhD study provides an original contribution to the literature by showing, for the first time that, neurosteroids AP and E2 are able to exert effective neuroprotective effects against pCEP4- and APPwt-transfected SH-SY5Y cells under oxidative stress conditions. Moreover, we obtained also a series of preliminary results related to the effects of GHB and neurosteroids AP and E2 against TC- and THG-induced ER stress that is also involved in cellular mechanisms of AD physiopathology. Except an effective action of E2 against THG-evoked ER stress and decreased viability of SH-SY5Y cells, the tested doses of GHB (micromolar) and AP (nanomolar) were unable to prevent ER stress-induced cell loss. Future experiments will help us to clarify whether GHB and neurosteroids are differentially effective against APPwt overexpression-, H_2O_2- and ER stress-induced cell death according to the dose regimens or whether GHB, AP and E2 may only counteract specifically some of the key physiopathological components of AD without having the ability to protect against the others.

Another important finding provided by the present work is the identification of an additive action of GHB and AP that significantly improves the neuroprotective effectiveness of these compounds when they are administrated concomitantly. Intracellular mechanisms underlying this additive effect will further be characterized. However, our current results strongly suggest that the potent neuroprotective action exerted by the co-administration of GHB and AP against H_2O_2-induced cell death may result (at least in part) from the addition in the intracellular compartment of the partial stimulatory effects induced by each one of these compounds on the expression of anti-apoptotic proteins of Bcl-2 family. Also, other interesting possibilities to be explored are : (1) the concomitant activation of GABAergic system by GHB (which can interact with $GABA_A$ or $GABA_B$ receptors depending on the concentration used) and AP (a positive allosteric modulator of $GABA_A$ receptor) that may enhance the neuroprotective efficacy of GHB and AP or (2) the combination of specific cellular pathways evoked by GHB and AP via GHB-GPCR and pregnane-X-receptor, respectively.

In perfect line of previous investigations of our laboratory suggesting that the substances modulating neurosteroid production in neural cells may control their viability (Schaeffer et al., 2006b, 2008c), the present PhD study provides additional results demonstrating that GHB-evoked neuroprotection is partially mediated through aromatase (the key E2-synthezising enzyme) activity. Therefore, it appears that, over the additive effects exerted by the co-application of GHB and AP to improve their neuroprotective efficacy, GHB can also induce neuroprotection through the stimulation of neurosteroidogenesis in the nerve cells.

Finally, we have also used a yeast-based MMP activity assay to check whether GHB and neurosteroids (AP and E2) may regulate MMP-2 and MMP-9 activities which

crucially control Aβ peptide degradation. At this stage, it is difficult to conclude from our preliminary data about the effects of GHB, AP and E2 on MMP-2 and MMP-9 activities in the yeast model since the basally elevated MMP activity level in this model did not facilitate the characterization of the specific actions of GHB and neurosteroids. Investigations scheduled for the future using RT-qPCR method to assess MMPs expressions in SH-SY5Y cells will certainly help to elucidate the situation.

Taken together, our results, which demonstrate the existence of positive interactions between GHB and neurosteroids AP or E2 in the protection of SH-SY5Y cells against APPwt-overexpression- or H_2O_2-evoked cell death, suggest that GHB and neurosteroids may be used to develop combined neuroprotective strategies to prevent or to counteract physiopathological processes leading to neuronal loss in AD.

6. Perspectives

As indicated in our discussion and conclusions, some of our results are preliminary and require additional works to be consolidated. In particular, one of the first perspectives is to clarify whether GHB and neurosteroids act differentially on APPwt-overexpression-, H_2O_2- and ER stress-induced cell death. Therefore, we will continue the experiments initiated with TC- and THG-evoked ER stress and assess in these conditions different dose ranges of GHB, AP and E2 but various other neurosteroids will also be tested separately or concomitantly with GHB. Furthermore, the effects of GHB and neurosteroids on the expression of proteins (XBP-1, CHOP…) involved in the signaling pathways evoked by TC and THG will be completely characterized thanks to the combination of molecular and pharmacological methods. In addition, interaction existing between these proteins and some of the factors determining apoptosis will be identified in order to establish whether the anti-apoptotic pathways activated by GHB and/or neurosteroids are similar, different or complementary to protect nerve cells against death.

One of the major finding of our study was the additive action of GHB and AP. Thus, a subsequent perspective will be the characterization of the mechanisms underlying this additive actions of GHB and AP. Pharmacological tools will be combined to biochemical investigations to determine the contribution of $GABA_A$-R, $GABA_B$-R and GHB-GPCR to the additive effect of GHB and AP. Furthermore, molecular methods will be developed to characterize intracellular signaling proteins as well as the interactions between these proteins in the development of GHB and AP additive effects.

Regarding the mediation of GHB-evoked neuroprotective effect via neurosteroidogenesis, we shall perform additional experiments (ELISA, GC-MS or LC-MS measurements) to assess the effect of GHB on endogenous E2 level newly-synthesized in SH-SY5Y cells in the absence or the presence of H_2O_2. These experiments will be correlated to MTT and flow cytometry assessments of cell viability and apoptotic signals, respectively. Also, as suggested in our discussion, it is possible that GHB controls neural cell viability via the modulation of AP biosynthesis. Therefore, one major perspective will also be to combine pharmacological and biochemical approaches to investigate the neuroprotective action of GHB in the presence of finasteride and provera, which respectively inhibit 5α-reductase and 3α-HSOR two key enzymes involved in AP production.

To further characterize and elucidate the effects of GHB and neurosteroids on MMP-2 and MMP-9 activity and/or expression, we will combine RT-qPCR, western blotting, gelatin zymography electrophoresis and enzymatic activity assays to determine the ability of these compounds to modulate MMPs activities/expression in SH-SY5Y cells.

Finally, after the characterization and confirmation of all of the effective compounds to be used separately or concomitantly, it will be interesting to test these compounds *in vivo* in animals models of AD such as the triple transgenic mice (carrying the $PSEN1_{KI}$-, TAU_{P301L} and APP_{swe}- mutations) or the tg2576 mice to confirming the ability of these molecules to decrease neuronal loss and to improve cognitive or memory functions.

7. References

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Descriptif synthétique en français des travaux de la these

La maladie d'Alzheimer (MA) est la cause la plus fréquente de démence dans les sociétés occidentales, affectant plus de 35 millions de personnes dans le monde, dont 860 000 en France et 1.2 millions en Allemagne (Querfurth and LaFerla, 2010, Sperling et al., 2011). Plusieurs types de lésions ont été décrits chez les patients Alzheimer mais l'hypothèse la mieux établie est l'accumulation excessive de protéines telles que les plaques amyloïdes extracellulaires ou la dégénérescence neurofibrillaire intracellulaire (DNF) observées dans les cerveaux de patients autopsiés (De Strooper, 2010, Querfurth and LaFerla, 2010, Ballard et al., 2011, Sperling et al., 2011). Les plaques amyloïdes sont dues à une accumulation de peptides beta-amyloïdes (Aβ), générés par protéolyse de l'amyloid precursor protein (APP) effectuée par des secrétases (Strooper and Annaert, 2001, De Strooper, 2010, De Strooper et al., 2010). La DNF est le résultat de l'agrégation de formes hyperphosphorylées de la protéine Tau (protéine associée aux microtubules). Les oligomères Aβ sont cytotoxiques et induisent l'hyperphosphorylation de Tau (Gotz et al., 2001, Lee et al., 2001, Santacruz et al., 2005, Reddy, 2011, Gotz et al., 2013). Les peptides Aβ et la DNF induisent un stress oxydant en perturbant la chaine respiratoire mitochondriale et l'homéostasie du calcium. Cependant, le stress oxydant est aussi décrit comme un élément précurseur de la formation de DNF et de peptides Aβ, établissant ainsi un cercle vicieux reliant Aβ, DNF et stress oxydant (Behl et al., 1994, Miranda et al., 2000, Misonou et al., 2000, Melov et al., 2007, Oda et al., 2010). Du fait de l'accumulation excessive de protéines dans la maladie, le stress du réticulum endoplasmique (RE) suscite également un intérêt scientifique (Ottani et al., 2004, Lee et al., 2010, Hetz et al., 2011, Cornejo and Hetz, 2013, Hetz

et al., 2013, Kang et al., 2013, Hetz and Mollereau, 2014). Les mécanismes associant RE et MA sont divers, complexes et impliquent des altérations de la quasi-totalité des acteurs de la voie de sécrétion de protéines et de la régulation du calcium intracellulaire (Prasanthi et al., 2011, Costa et al., 2012, Marwarha et al., 2013). Le RE et les mitochondries étant étroitement connectées, le stress du RE peut induire un stress oxydant et vice versa, faisant du stress du RE un membre à part entière du cercle vicieux reliant Aβ, DNF et stress cellulaires (Malhotra and Kaufman, 2007, Hedskog et al., 2013, Marchi et al., 2014). Un autre aspect physiopathologique est le déséquilibre entre la production et la clairance des peptides Aß. Les concentrations des protéases impliquées dans la dégradation des peptides Aß, comme la Neprilysine (NEP), les matrix métalloprotéinases - 2 et 9 (MMP-2/-9), sont diminuées dans les cerveaux de patients MA, conduisant à une accumulation accrue des peptides Aß et à la cytotoxicité (Yong, 2005, Yin et al., 2006, De Strooper, 2010, Merlo and Sortino, 2012). A ce jour, aucune thérapie efficace n'est disponible contre la MA. Il est donc crucial de trouver des molécules capables de réduire la perte cellulaire provoquée par le stress oxydant, le stress du RE ou par l'accumulation des peptides Aß résultant d'une baisse de l'expression et/ou de l'activité des protéases NEP, MMP- 2 et MMP- 9 .

Diverses données de la littérature suggèrent que les neurostéroïdes, qui exercent des effets neurotrophiques et neuroprotecteurs, pourraient constituer une piste intéressante à explorer pour le traitement des maladies neurodégénératives (Belelli and Lambert, 2005, Patte-Mensah et al., 2005, Borowicz et al., 2011, Gravanis et al., 2012, Panzica et al., 2012, Brinton, 2013). Par ailleurs, d'autres éléments bibliographiques indiquent que le gamma-hydroxybutyrate (GHB), un anesthésique utilisé en clinique humaine contre la narcolepsie et la cataplexie, pourrait avoir des

effets neuroprotecteurs (Escuret et al., 1977, Lavyne et al., 1983, Russell, 1999, Vergoni et al., 2000, Ottani et al., 2003, Ottani et al., 2004, Russell et al., 2009, Russell et al., 2011, Spaeth et al., 2013). Dans une récente étude, notre laboratoire a démontré qu'une dose thérapeutique du GHB modifie l'expression d'une série de gènes situés dans des régions cérébrales contrôlant les processus cognitifs et mnésiques (Kemmel et al., 2010). Parmi ces gènes, certains codent des protéases participant à l'élimination des peptides Aβ et d'autres sont impliqués dans les mécanismes de vie et de mort cellulaire. Ces données révèlent également que le GHB, par l'intermédiaire de récepteurs couplés aux protéines G, induit une signalisation calcique impliquant l'AMPc, second messager crucial pour le fonctionnement des enzymes de synthèse des neurostéroïdes (Andriamampandry et al., 2003, Andriamampandry et al., 2007, Coune et al., 2010). Par conséquent, nous avons émis l'hypothèse que le GHB pourrait exercer des effets neuroprotecteurs via la modulation de la neurostéroïdogénèse. Il est également possible que GHB et neurostéroïdes agissent de manière synergique ou additive pour protéger les cellules nerveuses contre les mécanismes cellulaires délétères conduisant aux maladies neurodégénératives comme la MA.

Nous nous sommes fixés trois objectifs dans le cadre de ce travail de thèse:

- (1) Etudier la capacité du GHB et des neurostéroïdes (administrés séparément ou de façon concomittante) à protéger les neuroblastomes humains SH-SY5Y contre la mort cellulaire induite par les facteurs étiologiques de la MA comme (i) le stress oxydant provoqué par le peroxyde d'hydrogène (H_2O_2), (ii) la sur-expression de la protéine APP non mutée (APPwt) ou (iii) le stress du RE.

- (2) Vérifier si les effets neuroprotecteurs du GHB sont médiés par la modulation de la neurostéroïdogénèse.

- (3) Evaluer la capacité du GHB et des neurostéroïdes à stimuler l'activité et/ou l'expression des protéases MMP-2 et MMP-9.

Pour modéliser expérimentalement le stress oxydant, les cellules SH-SY5Y ont été traitées avec de l'H_2O_2 pendant 24 et 48 h. Les tests de survie cellulaire MTT ont d'abord révélé que les cellules sur-exprimant l'APPwt sont plus vulnérables au stress oxydant que les cellules témoins (natives, ou vecteur vide). Nos résultats montrent aussi que le GHB exerce un effet neuroprotecteur contre la mort cellulaire induite par le stress oxydant ou par la sur-expression de l'APPwt. Pour déterminer les mécanismes de protection du GHB contre la mort cellulaire, il était important de savoir si le GHB exerçait simplement une action proliférative compensant la perte cellulaire liée à l'H_2O_2, ou si le GHB avait une action anti-apoptotique. Nous avons donc utilisé la technique de double marquage TUNEL et Caspase-3 activée en microscopie confocale et cytométrie de flux pour répondre à cette question. Les résultats ont d'abord révélé qu'en absence d'un traitement à l'H_2O_2, il existe un niveau basal d'apoptose significatif dans les cellules sur-exprimant l'APPwt contrairement aux cellules témoins (natives ou transfectées avec le vecteur vide) qui présentent un pourcentage d'apoptose négligeable. Ensuite, nous avons constaté que le traitement à l'H_2O_2 provoque l'apparition d'une apoptose significative dans les cellules témoins et augmente fortement le niveau d'apoptose existant déjà dans les cellules sur-exprimant l'APPwt. L'administration de GHB diminue significativement l'apoptose induite par la sur-expression de l'APPwt et par l'H_2O_2. En combinant les techniques de RT-qPCR et western blot, nous avons démontré que cette protection passe par la modulation du ratio Bax/Bcl-2, élevé en conditions de stress et réduit

par les traitements au GHB. Parallèlement, des tests MTT ont également prouvé que les neurostéroïdes comme l'oestradiol et l'allopregnanolone exercent aussi des effets neuroprotecteurs contre la cytotoxicité induite par l'H_2O_2. De plus, des applications concomitantes de GHB et d'allopregnanolone ont révélé l'existence d'un effet neuroprotecteur additif de ces deux composés contre la mort cellulaire induite par le stress oxydant. Bien que cela nécessitera des expériences complémentaires pour être confirmé, nos travaux suggèrent que l'effet additif neuroprotecteur du GHB et de l'alloprégnanolone pourrait résulter de la combinaison des stimulations partielles de l'expression des protéines anti-apoptotiques de la famille Bcl-2.

Un autre aspect important de la thèse était de vérifier si l'action neuroprotectrice du GHB et des neurostéroïdes est spécifique aux situations de stress évoquées par l'H_2O_2 ou si cet effet neuroprotecteur peut également s'exercer dans le cas de la mort cellulaire induite par le stress du RE. Nous avons alors provoqué le stress du RE en traitant les cellules SH-SY5Y avec la tunicamycine et la thapsigargine. Nos résultats ont d'abord permis de confirmer le traitement des cellules SH-SY5Y avec la tunicamycine ou la thapsigargine induit effectivement une nette diminution de la viabilité cellulaire. Nous avons ensuite constaté que les gammes de concentrations de GHB (de l'ordre de la micromolaire) et de neurostéroïdes (allopregnanolone, en concentrations nanomolaires) utilisées n'exercent pas d'effet neuroprotecteur contre la perte cellulaire évoquée par la tunicamycine. En revanche, nous avons observé que le neurostéroïde oestradiol (à 250 ou 500 nM) réduit significativement l'effet délétère exercé par la thapsigargine sur la survie cellulaire. Faute de temps, nous n'avons pas encore évalué la capacité de l'oestradiol à protéger également contre la mort cellulaire induite par la tunicamicyne. Des expériences complémentaires

permettront de s'assurer si l'expression de l'activité neuroprotectrice de l'oestradiol dépend de la nature du composé utilisé (tunicamycine ou thapsigargine) pour induire expérimentalement le stress du RE. Nous réaliserons également des études de biologie moléculaire axées sur les facteurs de transcription C/EBP-homologous protein (CHOP) et X-box-protein type 1 (XBP-1) impliqués dans l'apoptose liée au stress du RE (Cornejo and Hetz, 2013, Hetz et al., 2013, Hetz and Mollereau, 2014), pour déterminer si l'oestradiol et d'autres neurostéroïdes sont capables de moduler l'expression de ces facteurs CHOP et XBP-1. L'ensemble de ces données permettra de vérifier si le GHB et les neurostéroïdes exercent préférentiellement ou sélectivement leurs effets neuroprotecteurs contre l'apoptose ou la mort cellulaire induite par le stress oxydant, la sur-expression de l'APPwt ou par le stress du RE.

Les interactions entre le GHB et les neurostéroïdes ne se limitent pas uniquement aux actions pharmacologiques concomitantes telles que l'effet additif observé avec la co-administration du GHB et de l'alloprégnanolone. Compte tenu des résultats antérieurs de notre laboratoire qui suggèrent que les composés modulateurs de la neurostéroïdogenèse contrôlent les processus neurodégénératifs et/ou les symptômes neuropathiques (Patte-Mensah et al., 2003, Patte-Mensah et al., 2006, Schaeffer et al., 2006, Meyer et al., 2008, Patte-Mensah and Mensah-Nyagan, 2008, Schaeffer et al., 2008, Schaeffer et al., 2010), nous avons vérifié si les effets neuroprotecteurs du GHB contre le stress oxydant pourraient être relayés par une modulation de la synthèse locale de neurostéroïdes. La co-application de GHB et de Fadrozole (inhibiteur spécifique de l'aromatase, enzyme clé de synthèse d'oestradiol) diminue significativement la neuroprotection induite par le GHB. Des analyses de RT-qPCR ont démontré que le GHB stimule l'expression des ARNm codant l'aromatase dans les cellules SH-SY5Y. Ces résultats démontrent donc que le GHB induit la

neuroprotection via la stimulation de la néosynthèse d'oestradiol dans les cellules nerveuses.

Afin d'étudier *in vitro* les effets du GHB et des neurostéroïdes sur l'activité des MMP-2 et MMP-9, nous avons optimisé un test d'activité enzymatique sur la base de l'expression membranaire de ces protéases par la levure *Pichia pastoris* (Diehl et al., 2011). Les niveaux d'activité enzymatique de type protéase détectés dans notre modèle de levure sont fortement élevés. Ainsi, nos résultats préliminaires n'ont pas permis de révéler facilement d'éventuels effets stimulateurs supplémentaires induits par le GHB et les neurostéroïdes. En conséquence, nous avons prévu des travaux complémentaires avec la technique de zymographie couplée à la détection de l'activité des gélatinases pour approfondir nos connaissances sur les effets exercés par le GHB et les neurostéroïdes sur les activités des MMPs dans les cellules SH-SY5Y. Les résultats attendus seront importants pour compléter nos données issues des analyses RT-qPCR en cours de réalisation pour évaluer les effets du GHB et des neurostéroïdes sur l'expression des ARNm codant la MMP-2 et la MMP-9. En effet, nos travaux préliminaires de RT-qPCR suggèrent fortement que le GHB modulerait l'expression des ARNm codant la MMP-9 dans les cellules SH-SY5Y.

En conclusion, notre travail de thèse démontre l'efficacité neuroprotectrice du GHB et des neurostéroïdes contre l'apoptose et la mort cellulaire induites par le stress oxydant ou par la sur-expression de l'APPwt dans les neuroblastomes humains. Nos travaux révèlent aussi que l'effet neuroprotecteur du GHB et des neurostéroïdes s'exerce préférentiellement contre certains types de stress cellulaires, ce qui suggère une sélectivité d'action de ces composés dans la neuroprotection. Enfin, nous avons mis en évidence un effet neuroprotecteur additif du GHB et des neurostéroïdes ainsi

qu'une régulation positive par le GHB des voies de la neurostéroïdogenèse permettant la biosynthèse d'oestradiol.

L'ensemble de ces données ouvre des perspectives intéressantes pour le développement de stratégies neuroprotectrices innovantes basées sur l'utilisation concomitante ou alternée du GHB et des neurostéroïdes dans le traitement des maladies neurodégénératives comme la MA.

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Curriculum vitae

Guillaume Wendt : 29 Years old – Married

Education :

Since 11.2010	**Joint PhD in Neuroscience** Université de Strasbourg (France) – Universität des Saarlandes (Deutschland)
June 2013	**German certificate of academic teaching methodologies** ***(Hochschuldidaktik-Zertifikat)*** Universität des Saarlandes (Deutschland)
2008-2010	**Master of cell-integrated neuroscience** Université de Strasbourg (France)
2005 - 2008	**Bachelor of cell biology and physiology** Université de Strasbourg *2007-2008 : ERASMUS year in Germany (Universität des Saarlandes)*
2003-2005	**Faculty of Medicine** Université de Strasbourg (France)
2003	**Baccalauréat scientifique** Lycée Saint-Antoine (Phalsbourg, France)

Professional experience :

Since 11.2010	**Joint PhD in Neuroscience** ; *"Assessment of the neuroprotective effects of gamma-hydroxybutyrate and neurosteroids in cellular models of Alzheimer's disease".* **Universität des Saarlandes** – Molekular- und Zellbiologie Prof. Dr. M. J. Schmitt **Université de Strasbourg** – INSERM UMR_S U1119 Biopathologies de la myéline, neuroprotections et Stratégies thérapeutiques. Prof. Dr. A. G. Mensah-Nyagan
From 01.2010 to 06.2010	**Master Thesis** : *"Study of the neuroprotective effects of GHB in a cellular model of Alzheimer's disease".* **Université de Strasbourg** – EA4438 Equipe Stéroïdes, Neuromodulateurs et Neuropathologies. Prof. Dr. A. G. Mensah-Nyagan
From 03.2009 to 07.2009	**Practical course in neurobiology** **Université de Strasbourg** – EA4438 Equipe Stéroïdes, Neuromodulateurs et Neuropathologies. Prof. Dr. A. G. Mensah-Nyagan
From 06.2008 to 07.2008	**Practical course in neurobiology** **Universität des Saarlandes** – FR8.3 Zoologie/Neurobiologie Prof. Dr. U. Müller
From 01.2008 to 02.2008	**Practical course in molecular- and cell biology** **Universität des Saarlandes** – Molekular- und Zellbiologie Prof. Dr. M. J. Schmitt

Guillaume Wendt

Evaluation des effets neuroprotecteurs du gamma-hydroxybutyrate et des neurostéroïdes dans des modèles cellulaires de la maladie d'Alzheimer.

RESUME :

Cette thèse montre que le GHB et les neurostéroïdes protègent efficacement contre la mort neuronale induite par les facteurs étiologiques de la maladie d'Alzheimer, notamment la sur-expression de l'*amyloid precursor protein* et le stress oxydant. Nous avons identifié un effet additif du GHB et de l'alloprégnanolone qui pourrait résulter de la combinaison des stimulations partielles évoquées par ces molécules sur l'expression des protéines anti-apoptotiques. L'effet du GHB est bloqué par un inhibiteur de l'aromatase, suggérant que le GHB induirait la neuroprotection via la stimulation de la neurostéroïdogenèse. Pour étudier les effets du GHB et des neurostéroïdes sur l'activité des MMP-2 et MMP-9, qui dégradent les peptides amyloïdes, nous avons optimisé un test enzymatique basé sur l'expression de ces protéases dans la levure. Nos résultats préliminaires ne permettent pas encore de conclure mais leur amélioration et combinaison avec des données de RT-qPCR contribueront à déterminer l'action du GHB et des neurostéroïdes sur l'activité et/ou l'expression des MMP. Notre travail suggère que le GHB et les neurostéroïdes pourraient être associés pour élaborer des stratégies neuroprotectrices contre les pertes neuronales provoquées par la maladie d'Alzheimer.

Mots clés : Anti-apoptotique, Anti-oxydant, Neuroprotection, Maladie d'Alzheimer, GHB, Neurostéroïdes.

ABSTRACT :

This PhD work showed that GHB and neurosteroids efficiently protect against nerve cell death caused by Alzheimer's disease etiological factors including amyloid precursor protein overexpression and oxidative stress. Interestingly, we identified an additive action of GHB and allopregnanolone that may result from the combination of partial stimulations of anti-apoptotic protein expression induced by both compounds. GHB protective effect was blocked by aromatase inhibitor, suggesting that GHB may also induce neuroprotection via the activation of neurosteroidogenesis. Finally, we have used a yeast-based MMP activity assay to check whether GHB and neurosteroids regulate MMP-2 and MMP-9 activities which control Aβ peptide degradation. We cannot yet conclude from our preliminary results but their improvement and combination with RT-qPCR analyzes will help to determine the modulatory action of GHB and neurosteroids on MMP activity and/or expression. Together, our data suggest that GHB and neurosteroids may be used to develop combined neuroprotective strategies against neuronal loss in AD.

Keywords : Anti-apoptotic, Anti-oxydant drug, Neuroprotection, Alzheimer's disease, GHB, neurosteroids.