

IMPERIAL COLLEGE LONDON

Unravelling the Progression of
Unfolded Protein Response
In a Model System of
Familial Alzheimer's disease

Stefani, Ioanna Chrysoula

October 2014

Department of Chemical Engineering and Chemical Technology

Imperial College London

South Kensington Campus

London SW7 2AZ

A thesis submitted to Imperial College London in partial fulfilment of the requirements of the degree of
Doctor of Philosophy

Abstract

Alzheimer's disease (AD) is the most common form of dementia disorders and, yet, there is no preventative or curative treatment. It is associated with the progressive loss of memory and cognition and clinically divided into sporadic and familial forms. Familial Alzheimer's disease (FAD) has predominantly a genetic predisposition with inherited mutations in the amyloid- β precursor protein (APP) and presenilin genes, which promote APP processing through the amyloidogenic pathway. This results in the release of the A β peptide, a major neurotoxic agent in AD progression.

Accumulation of unfolded and misfolded disease-specific proteins (including A β and tau proteins) in neuronal cells perturbs endoplasmic reticulum (ER) homeostasis, leading to the onset of a cellular stress cascade called unfolded protein response (UPR), markers of which are upregulated in AD brain specimens. This suggests a possible role for ER stress in activation and the pathogenesis of AD.

The research aimed to investigate the dynamic response of the UPR in an experimental model system of the disease combined with a computational model. For this purpose human neuroblastoma cell lines overexpressing the wild-type (APP_{WT}) and two mutant forms of APP (APP_{MUT}) associated with FAD were generated. Gene expression analysis of UPR markers revealed that overexpression of APP induces preconditioning of ER stress in all cell lines but with a stronger response in FAD-associated mutants. The progression sequence of UPR in APP_{WT} and APP_{MUT} was investigated in a time-course manner following the application of chemical stress. The results revealed that APP_{MUT} exhibited the highest global response to chemically induced stress with a similar pattern. A computational model of the mammalian UPR was then generated and used to understand the dynamics of UPR. The model was able to reproduce our experimental data, which included pre-existing genetic factors (mutations in APP-associated with FAD) and a mimic of environmental triggers (induction of stress) consequently triggering the stress response. It suggested a different protein load and magnitude of transcriptional activation upon stress among the three cell lines. This was followed by *in silico* case studies exploring the effect of drugs targeting different branches of the UPR.

This study proposes a novel multidisciplinary platform that could be further used for the development of therapeutics for AD. As the familial and sporadic form of the disease have similar neuropathological characteristics, drugs efficacious for FAD will also be beneficial for the most common form of AD.

Declaration of originality

I declare that this thesis is my own work, and certify that all material, which is not, my own work has been appropriately acknowledged.

Ioanna Chrysoula Stefani

London, U.K.

Copyright Declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.

This thesis is dedicated to my beloved parents

Vangelis & Aleka Stefani

Acknowledgments

I am grateful to several people for helping and motivating me in a way or other and making my Ph.D time an unforgettable experience.

First of all, I would like to express my deepest thanks to my two supervisors Dr. Cleo Kontoravdi and Dr. Karen Polizzi for their continuous support and guidance. I would like to thank Cleo for the detailed, fruitful and constructive discussions throughout my Ph.D. I am grateful that she challenged me to become better and achieve my maximum. I would like to thank Karen for her valuable advice and feedback as well as her encouragement and trust. Her guidance along with her meticulous attention to detail enabled me to consistently improve my skills including technical writing.

In addition, I would like to extend my gratitude to the members of the Dr. Kontoravdi's and Dr. Polizzi's lab: Sarantos Kyriakopoulos, François-Xavier Blaudin de Thé, Philip Jędrzejewski, Susie/Si Sou, Ioscani Jimenez del Val, Felix Jonas, Oleksiy Klymenko, Kealan Exley, Kate Royle and Cher Goey. A huge thanks goes to Christos Sagonas for his continuous support and never ending encouragement at all aspects. His impact was tremendous on the successful completion of my Ph.D.

I am also deeply thankful to my mum and dad for their love, continuous support and confidence in me and my brother who encouraged me at all times. A special thanks goes to my dad for inspiration throughout my life and for motivating me to stay determined, ambitious and focused in order to achieve my dreams and goals.

Contents

Abstract.....	2
Declaration of originality	3
Copyright Declaration	4
Acknowledgments.....	6
Contents.....	7
List of Figures	12
List of Tables	14
List of abbreviations.....	15
List of model nomenclature.....	20
Chapter 1.....	23
Introduction	23
1.1 General background.....	23
1.2 Alzheimer’s disease (AD).....	27
1.3 Aim and objectives.....	28
1.4 Structure of the thesis	30
Chapter 2.....	33
Literature Review	33
2.1 Epidemiology of neurodegenerative diseases	33
2.2 Clinical characteristics.....	34
2.2.1 Alzheimer’s disease.....	35
2.2.2 Parkinson’s disease	37
2.2.3 Huntington’s disease.....	39
2.3 The role of amyloid precursor protein in Alzheimer’s disease	40
2.3.1 APP structure	40
2.3.2 APP proteolytic processing	41

Contents

2.4	The role of A β in Familial Alzheimer's disease.....	42
2.4.1	Different FAD-associated APP mutations	42
2.4.2	Contributions of post-translational modifications in familial Alzheimer's disease	44
2.5	Characteristics of neuronal cells	47
2.5.1	Cellular physiology	47
2.5.2	ER structure and function	48
2.6	The unfolded protein response	49
2.6.1	IRE1 signalling	50
2.6.2	ATF6 signalling	52
2.6.3	PERK signalling	53
2.6.4	Overview of the UPR.....	54
2.7	ER stress link with apoptosis.....	56
2.7.1	ER stress signalling pathways inducing apoptosis	56
2.7.2	Role of Translational Attenuation.....	59
2.7.3	Influence of XBP1 unspliced protein in the UPR.....	59
2.8	ER stress link with Alzheimer's disease.....	62
2.9	ER related stressors in AD.....	64
2.9.1	Reactive Oxygen Species.....	65
2.9.2	Calcium Dyshomeostasis.....	66
2.10	Treatment of ER-stress induced diseases	68
2.10.1	Proposed treatments related to ER stress mechanisms	68
2.11	Potential targets for future drug development and clinical implications.....	71
2.12	Model organisms in AD.....	73
2.12.1	SK-N-SH cells	73
2.13	Computational modelling in biology.....	74
2.14	Previous UPR models	76
2.14.1	Raden model for IRE1 in yeast	76
2.14.2	Pincus model for IRE1 in yeast.....	77
2.14.3	Trusina model for PERK.....	79
2.14.4	Rutkowski model for BiP, CHOP and GADD34	80
2.14.5	Erguler model.....	82
Chapter 3		84

Contents

Materials and Methods.....	84
3.1 Cell lines and growth conditions.....	84
3.2 Constructs of mutant APP ₆₉₅	85
3.2.1 Site directed mutagenesis.....	85
3.2.2 Digestion of the PCR product.....	86
3.2.3 Plasmid transformation and isolation.....	86
3.2.4 Sequencing.....	87
3.3 Generation of a stable cell line	87
3.4 Freeze/Thaw	88
3.5 Optimisation of cellular differentiation	88
3.6 Stress protocol	89
3.7 Cell viability and metabolic activity	89
3.7.1 Trypan Blue exclusion method.....	89
3.7.2 MTS assay.....	90
3.8 Molecular Biology techniques.....	90
3.8.1 RNA isolation and cDNA construction.....	90
3.8.2 Optimisation of qRT-PCR conditions.....	91
3.8.3 Primer design	91
3.8.4 Quantitative reverse transcription polymerase chain reaction.....	92
3.8.5 Housekeeping genes	93
3.8.6 Agarose gel electrophoresis.....	93
3.9 Protein methods	93
3.9.1 SDS-PAGE and Western blot	93
Chapter 4.....	95
A cell model system of familial Alzheimer's disease.....	95
4.1 Introduction	95
4.2 Methodological diversity in the generation of stable cell lines.....	97
4.3 Results.....	98
4.3.1 Generation of the construct	98
4.3.2 DNA purification for sequencing.....	99
4.3.3 DNA purification for transfection	100
4.3.4 Optimising a transfection protocol for SK-N-SH cells	100

Contents

4.3.5	Optimisation of total RNA extraction protocol	102
4.3.6	Optimisation of primer annealing temperatures.....	103
4.3.7	Amplification efficiencies	105
4.3.8	Housekeeping genes	107
4.4	Discussion.....	121
4.5	Conclusion.....	124
Chapter 5.....		125
Progression of UPR in a cell model system of familial Alzheimer's disease		125
5.1	Introduction	125
5.2	Results.....	127
5.2.1	Cell's initial response to stress is an attempt at adaptation.....	127
5.2.2	UPR progression in response to ER stress has a different pattern in FAD cells.....	130
5.2.3	APP _{MUT} exhibit a similar UPR activation profile	137
5.2.4	Mutant cells exhibit stronger upregulation of UPR markers in response to ER stress.....	141
5.3	Discussion.....	145
5.3.1	Sequence of UPR activation revealed in a FAD cell model in response to ER stress	146
5.3.2	The key players in UPR activation induced by ER stress	147
5.4	Conclusion.....	150
Chapter 6.....		152
Computational model of the mammalian UPR network		152
6.1	Introduction	152
6.2	General characteristics of the computational model	154
6.2.1	Model assumptions.....	155
6.3	Model development.....	157
6.3.1	Protein folding dynamics	157
6.3.2	IRE1 pathway	160
6.3.3	PERK pathway	162
6.3.4	ATF6 pathway.....	165
6.4	Formation of the XBP1 protein complex	165
6.4.1	Degradation of XBP1 complex	167
6.4.2	Translation of XBP1 complex	168
6.5	Model calibration.....	169

Contents

6.5.1	Model calibration	171
6.6	Model simulation results	175
6.7	Simulation analyses reflecting the UPR mechanism.....	179
6.7.1	ATF6 sensitivity analysis.....	179
6.7.2	Quantitative analysis of unfolded protein load	181
6.7.3	Effect of XBP1 protein complex	182
6.8	The computational model used as a basis to explore treatments <i>in silico</i>	184
6.8.1	Case study 1: Effect of salubrinal	185
6.8.2	Case study 2: Effect of lithium and valproate (VPA)	187
6.9	Discussion.....	190
6.10	Conclusion.....	191
Chapter 7.....		193
Discussion.....		193
7.1	Summary of results	193
7.2	Main conclusions	197
7.3	Limitations.....	199
7.4	Future directions.....	201
7.5	Outlook	204
References		205
Appendix I: Plasmid and primers for site-directed mutagenesis.....		228
Appendix II: Primer amplification efficiencies		230
Appendix III: Mammalian UPR Code in gPROMS®		233

List of Figures

Figure 1: Protein folding and quality control in the endoplasmic reticulum.....	26
Figure 2: Overview of the mechanisms involved in the progression of Alzheimer's disease	37
Figure 3: Overview of mechanisms involved in the progression of Parkinson's disease.....	38
Figure 4: Overview of protein trafficking and stress activation in Huntington's disease	40
Figure 5: APP metabolic processing pathway	42
Figure 6: Familial Alzheimer's disease associated APP mutations.....	43
Figure 7: Schematic representation of the generation of A β peptides by APP proteolytic processing	46
Figure 8: Structure of a neuron with the respective functions of each part	48
Figure 9: Structural domains of the three ER-transmembrane stress sensors: IRE1, ATF6 and PERK	50
Figure 10: The UPR signalling cascades in response to ER stress	55
Figure 11: ER stress pathways mediating cell death.....	58
Figure 12: Functions of unspliced XBP1u protein	60
Figure 13: Structure of XBP1u and XBP1s mRNA.....	61
Figure 14: The implication of A β in Alzheimer's disease	65
Figure 15: Raden model for IRE1 activation regulated by BiP in yeasts.	77
Figure 16: Pincus schematic model for IRE1 activation	79
Figure 17: Trusina model of the UPR A) in yeasts and B) in mammalian cells.....	80
Figure 18: Rutkowski model of the mammalian UPR	81
Figure 19: Erguler model comprising the complete mammalian UPR.....	83
Figure 20: Schematic representation of the site-directed mutagenesis procedure.....	85
Figure 21: pCI-neo mammalian expression vector and sequence reference points.	98
Figure 22: DNA isolation from E. coli culture.....	99
Figure 23: DNA purification.....	100
Figure 24: Representative pictures of successfully or unsuccessfully transfected SK-N-SH cells.....	101
Figure 25: Evaluation of the quality of a total RNA	103
Figure 26: PCR amplification products.....	105
Figure 27: Quantification of APP expression	109
Figure 28: Microscopic inspection of differentiated APP _{WT}	111
Figure 29: Biochemical characteristics of differentiated APP _{WT}	112
Figure 30: Microscopic inspection of differentiated APP _S	113
Figure 31: Chaperones expression induced by APP overexpression	114
Figure 32: PERK activation induced by APP overexpression.....	115
Figure 33: IRE1 and ATF6 activation induced from APP overexpression.....	116
Figure 34: Molecular structure of tunicamycin (TM) isolated from <i>Streptomyces lysosuperficus</i>	118
Figure 35: Expression of UPR markers in non-transfected cells	119
Figure 36: Structure of the MTS tetrazolium compound and the conversion to formazan product.	119

List of Figures

Figure 37: Examining MTS conditions.....	120
Figure 38: Optimisation of MTS assay for TM treated cells.....	121
Figure 39: UPR signalling throughout time	126
Figure 40: Schematic representation of the UPR-genes induced by ER stress.....	127
Figure 41: Effect of TM on proliferation and cell viability in a FAD cell-model system.....	128
Figure 42: Expression profile of ER chaperones within each cell line during TM-treatment	131
Figure 43: Expression profile of PERK markers within each cell line during TM-treatment.....	134
Figure 44: Expression profile of IRE1 and ATF6 markers within each cell line during TM-treatment.....	135
Figure 45: Expression profile of the chaperones between the cell lines during TM-treatment	137
Figure 46: Expression profile of PERK markers between the cell lines during TM-treatment	139
Figure 47: Expression profile of IRE1 and ATF6 markers between the cell lines during TM-treatment ..	140
Figure 48: Expression profile of chaperones in APP _{MUT} compared to APP _{WT}	142
Figure 49: Expression profile of PERK markers in APP _{MUT} compared to APP _{WT}	143
Figure 50: Expression profile of IRE1 and ATF6 in APP _{MUT} compared to APP _{WT}	144
Figure 51: Complete map of the mammalian UPR model	154
Figure 52: Reaction map of XBP1 protein complex formation integrated in the model.....	166
Figure 53: Stability of pXBP1u and pXBP1s in Hella cells [337].....	167
Figure 54: Degradation rates of pXBP1u forms in HeLa cells	168
Figure 55: Formation of pXBP1u, pXBP1s and XBP1 mRNA following TM-treatment in HeLa cells.....	169
Figure 56: Comparison of model simulation results with experimental data for BiP and XBP1s mRNA..	176
Figure 57: Comparison of model simulation results with experimental data for ATF4 and CHOP mRNA	177
Figure 58: Comparison of model simulation results with experimental data for XBP1T mRNA.	178
Figure 59: Sensitivity analysis output for ATF6 pathway for each cell line.....	180
Figure 60: Quantitative analysis of the unfolded protein distribution	181
Figure 61: Effect of XBP1 protein complex formation rate on the XBP1s protein levels	183
Figure 62: Effect of salubrinal during TM-treatment.....	187
Figure 63: Effect of valproate during TM-treatment.	189

List of Tables

Table 1: Summary of clinical characteristics of three neurodegenerative disorders	34
Table 2: Computational UPR models	76
Table 3: Outline of the experimental setup.....	96
Table 4: Quality assessment of RNA isolated with different kits using spectroscopy	102
Table 6: Coefficient of variation expressed as a percentage (%CV) in the qRT-PCR replicates.....	107
Table 7: Evaluation of the variation.....	108
Table 8: Cell viability at zero and 24 h of TM-treatment	129
Table 9: ANOVA results (p-values) of the time course stress experiment within each cell line.....	136
Table 10: ANOVA results (p-values) of the time course stress experiment between the cell lines.	141
Table 11: ANOVA results (p values) of the time course stress experiment of APP _{MUT} relative to APP _{WT}	145
Table 12: Outline of the computational setup.....	153
Table 13: Parameter values taken from literature sources.	170
Table 14: Initial conditions used for model simulation	171
Table 15: Steps followed for parameter estimation.....	172
Table 16: Estimated parameter values.	173
Table 17: (a) The standard deviation values of the estimated parameters.....	174
Table 18: (b) The standard deviation values of the estimated parameters	174
Table 19: Drug targets for ER stress regulation	184

List of abbreviations

4μ8C: 4-methyl umbelliferone 8-carbaldehyde

Aβ: amyloid-β

AD: Alzheimer's disease

AICD: APP intracellular domain

ANOVA: Analysis of variance

APP: Amyloid-β precursor protein

APP_{WT}: SK-N-SH cells overexpressing wild-type APP₆₉₅

APP_S: SK-N-SH cells overexpressing Swedish APP₆₉₅

APP_{S-I}: SK-N-SH cells overexpressing Swedish-Indiana APP₆₉₅

Apaf-1: Apoptotic protease activating factor 1

APP: Amyloid precursor protein

ASK1: Apoptosis signal-regulating kinase

ATF4: Activating transcription factor 4

ATF6: Activating transcription factor 6

ATP: Adenosine triphosphate

AU: Arbitrary units

BAX: Bcl-2-associated X protein

BAK: Bcl-2 antagonist/killer

Bcl-2: B-cell lymphoma 2

BiP: Binding immunoglobulin protein

BIX: BiP Inducer X

bZIP: Basic region-leucine zipper motif

Ca²⁺: Calcium

CHOP: CCAAT/enhancer-binding protein (C/EBP) homologous protein

CMV: cytomegalovirus

CNS: Central nervous system

C_t: Cycle threshold with respect to qRT-PCR

CTFs: C-terminal membrane embedding fragments

CSP: Caspase

CTR: C-terminal region

CV: Coefficient of variation

CytC: Cytochrome C

DTT: Dithiothreitol

eIF2 α : Eukaryotic translation initiation factor-2 α

ER: Endoplasmic Reticulum

ERAD: ER associated degradation

ERp72: Endoplasmic reticulum protein 72

ERSE: ER stress response element

FAD: Familial Alzheimer's disease

FBS: Foetal bovine serum

G418: Geneticin selection antibiotic

GADD34: Growth arrest and DNA damage-inducible protein-34

GFP: Green Fluorescence protein

GRP94: glucose-regulated protein 94

HD: Huntington's disease

HSP90: heat shock protein 90

HTT: Huntingtin gene

Htt: Huntingtin protein

IRE1: Inositol-requiring protein-1

JIK: c-Jun NH₂-terminal inhibitory kinase

JNK: c-Jun N-terminal kinase

KO: Knock-out

MAO: Monoamine oxidase

MEFs: Murine primary mouse embryo fibroblasts

mtHtt: Mutant Huntingtin protein with larger polyglutamine tracts

mtHttN: N-terminal truncations of the Huntingtin protein with expanded repeats

NES: Nuclear export domain

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NFM: Neurofilament medium polypeptide

NFT: Neurofibrillary tangle

NLS: Nuclear localization signal

Nrf-2: Nuclear factor erythroid 2-related factor

OD: Optical density

PC12: pheochromocytoma cell line

PBA: Phenylbutyric acid

PCR: Polymerase chain reaction

PCSP: Procaspase

PD: Parkinson's disease

List of abbreviations

PDI: Protein disulfide isomerase

PERK: Protein kinase R-like ER kinase

PP1c: Protein phosphatase 1 catalytic subunit

pPERK: Phosphorylated PERK

p-eIF2 α : Phosphorylated eukaryotic initiation factor- α

pre-mRNA: Premessenger RNA

PS: Presenilin genes

qRT-PCR: Quantitative real time PCR

RA: Retinoic acid

RIDD: Regulated IRE1-dependent decay

ROS: Reactive oxygen species

S1P/S2P: Site 1 or site 2 protease, Golgi resident

sAPP: Soluble APP derivative

SDS: Sodium dodecyl sulphate

SORL1: Sortilin-related receptor gene

Tet: Tetracycline

Tg: Thapsigargin

TM: Tunicamycin

TF: Transcription factor

TRAF2: Tumor necrosis factor-receptor associated factor 2

uORFs : Upstream open reading frames

UPR: Unfolded protein response

UPS: Ubiquitin proteasome degradation system

UPRE: Unfolded protein response element

List of abbreviations

VPA: Valproate

XPB1: X-box binding protein 1

XPB1s: Spliced XPB1

XPB1T: Total XPB1

XPB1u: Unspliced XPB

List of model nomenclature

Latin symbols

$ATF4$	ATF4 protein (molecules)
$ATF4_m$	ATF4 mRNA (molecules)
$ATF6_A$	ATF6 protein (molecules)
$ATF6/B$	ATF6-BiP complex (molecules)
B	BiP protein (molecules)
B_m	BiP mRNA (molecules)
C_{AB}	Binding rate of BiP to ATF6 receptor ($\text{molecule}^{-1} \text{sec}^{-1}$)
C_{IB}	Binding rate of BiP to IRE1 receptor ($\text{molecule}^{-1} \text{sec}^{-1}$)
C_{PB}	Binding rate of BiP to PERK receptor ($\text{molecule}^{-1} \text{sec}^{-1}$)
C_{RB}	Binding rate of BiP to ATF6 or/and IRE1 or/and PERK ($\text{molecule}^{-1} \text{sec}^{-1}$)
C_{UB}	Binding rate of BiP to unfolded proteins ($\text{molecule}^{-1} \text{sec}^{-1}$)
$CHOP$	CHOP protein (molecule)
$CHOP_m$	CHOP mRNA (molecule)
$eIF2\alpha$	eIF2 α protein (molecule)
IRE	IRE1 protein (molecule)
IRE_A	Activated IRE1 protein (molecule)
IRE/B	IRE1-BiP complex (molecule)
K_U	Unfolded protein load (molecule sec^{-2})
n	Constant for decay rate of activated IRE1 (dimensionless)
N_{ATF4_m}	Constant for transcription rate of ATF4 mRNA under UPR (dimensionless)
N_{B_m}	Constant for transcription rate of BiP mRNA under UPR (dimensionless)
N_{CHOP_m}	Constant for transcription rate of CHOP mRNA under UPR (dimensionless)

List of model nomenclature

N_{ATF6_A}	Constant for magnitude of stress response following ATF6 activation (dimensionless)
$peIF2\alpha$	Posphorylated eIF2 α protein (molecule)
$PERK_A$	PERK protein (molecule)
$PERK/B$	PERK-BiP complex (molecule)
S_U	Rate of unfolded protein translation (molecule sec ⁻¹)
t	Time (s)
U	Unfolded protein (molecule)
U/B	Folding complex (molecule)
X_{Total}	Total XBP1 mRNA (molecule)
X_m^s	Spliced XBP1 mRNA (molecule)
X_m^u	Unspliced XBP1 mRNA (molecule)
X^s	Spliced XBP1 protein (molecule)
X^u	Unspliced XBP1 protein (molecule)

Greek symbols

α_0	Constant for transcription rate of BiP, ATF4 and CHOP mRNA (dimensionless)
α_1	Constant for transcription rate of BiP, ATF4 and CHOP mRNA (dimensionless)
β_{ATF4}	Translation rate of ATF4 mRNA (sec ⁻¹)
β_{ATF4_m}	Transcription rate of ATF4 (molecule sec ⁻¹)
β_{ATF6}	Formation rate of ATF6 (molecule sec ⁻¹)
β_B	Translation rate of BiP mRNA (sec ⁻¹)
β_{B_m}	Basal transcription rate BiP mRNA (molecule sec ⁻¹)
β_{CHOP}	Translation rate of CHOP protein (sec ⁻¹)
β_{CHOP_m}	Basal transcription rate of CHOP mRNA (molecule sec ⁻¹)
$\beta_{eIF2\alpha}$	Phosphorylation rate of eIF2 α (sec ⁻¹)
$\beta_{X_m^s}$	Splicing rate of XBP1 mRNA (sec ⁻¹)
β_{X^s}	Translation rate of XBP1s

List of model nomenclature

$\beta_{X_m^u}$	Basal transcription rate of unspliced XBP1 mRNA (molecule sec ⁻¹)
β_{X^u}	Translation rate of XBP1u
β_{X^c}	Protein complex formation rate of XBP1c
γ_{ATF4}	Decay rate of ATF4 protein (sec ⁻¹)
γ_{ATF4_m}	Decay rate of ATF4 mRNA (sec ⁻¹)
γ_B	Decay rate of BiP protein (sec ⁻¹)
γ_{B_m}	Decay rate of BiP mRNA (sec ⁻¹)
γ_{CHOP}	Decay rate of CHOP protein (sec ⁻¹)
γ_{CHOP_m}	Decay rate of CHOP mRNA (sec ⁻¹)
$\gamma_{eIF2\alpha}$	eIF2 α dephosphorylation rate (sec ⁻¹)
γ_{fold}	Protein folding rate (sec ⁻¹)
γ_{AB}	Dissociation rate of BiP from ATF6 (sec ⁻¹)
γ_{IB}	Dissociation rate of BiP from IRE1 (sec ⁻¹)
γ_{PB}	Dissociation rate of BiP from PERK (sec ⁻¹)
γ_{RB}	Dissociation rate of BiP from ATF6 or/and IRE1 α or/and PERK (sec ⁻¹)
γ_s	Decay rate of spliced XBP1 mRNA (sec ⁻¹)
γ_u	Decay rate of unspliced XBP1 mRNA (sec ⁻¹)
γ_{UB}	Dissociation rate of BiP from unfolded protein (sec ⁻¹)
γ_{X^u}	Decay rate of unspliced XBP1 protein (sec ⁻¹)
γ_{X^s}	Decay rate of spliced XBP1 protein (sec ⁻¹)
$\gamma_{X_m^s}$	Modified decay rate of spliced XBP1 mRNA (sec ⁻¹)

Chapter 1

Introduction

1.1 General background

Cells throughout their lifetime must have the ability to sense a range of environmental changes such as hypoxia, nutrient deprivation, oxidative stress, calcium (Ca^{2+}) dyshomeostasis, radiation and microbial pathogens and adapt to them by mounting a distinct and effective response [1, 2]. Endogenous sources of stress such as those arising from the endoplasmic reticulum (ER) of cells overloaded with misfolded proteins in the ER lumen have also been identified and are of great importance. Cells have evolved several mechanisms to counteract the noxious effects, restore homeostasis and promote survival. However when the stress is unresolved or chronic they trigger programmed cell death (apoptosis) [3, 4]. Each subcellular compartment senses stress conditions differently and thus elicits complex responses accordingly.

Programmed cell death is a highly orchestrated molecular process the regulation of which is vital for several physiological functions and homeostasis control in multicellular organisms [5]. Several lines of evidence have showed that dysregulation in this mechanism causes or contributes to a range of human diseases. For instance, cell-cycle arrest defects may induce uncontrolled proliferation as seen in cancer, whereas increased apoptosis may contribute to neurodegenerative disorders [3]. Apoptosis involves a series of biochemical events, which lead to morphological changes that include cell shrinkage, cytoplasmic and chromatin condensation, nuclear fragmentation, chromosomal DNA fragmentation, plasma membrane blebbing and cell

death. These changes result in the release of membrane-enclosed particles known as apoptotic bodies which are rapidly engulfed by phagocytes and cleared out without eliciting an inflammatory response [6].

Eukaryotic cells comprise a set of membrane-enclosed organelles, where around half of the total membrane area is enclosed by the ER, forming a continuous network with the nuclear envelope. The ER is the exclusive site of synthesis of secreted and membrane proteins in mammalian cells. The newly synthesised proteins, after entering the ER undergo post-translational modifications such as N-glycosylation and are then folded into their secondary and tertiary conformation through non-covalent interactions, in order to be able to carry out their tasks. The native three-dimensional structure of proteins, which is the state that is energetically more favourable, is in some cases stabilised with disulphide bonds, interactions that are mainly present in the extracellular proteins rather than the intracellular ones. The folding processes occur under both physiological and stress conditions. They involve a range of molecular chaperones and folding enzymes that aid the ER quality control process (Figure 1), but the complexity of this multisystem often makes it prone to errors [7]. Perturbation of the ER-associated functions that affect protein folding causes the accumulation of unfolded and misfolded proteins in the ER lumen, a cellular conditions known as ER stress [8].

The ER quality control system comprises several specialised molecular chaperones and folding enzymes, the five predominant ones named glucose-regulated protein 94 (GRP94), binding immunoglobulin protein (BiP/GRP78), protein disulphide isomerase (PDI/ERp59), endoplasmic reticulum protein 72 (ERp72) and calreticulin [9, 10]. GRP94 is a member of the 90 kDa heat shock protein (HSP90) family and is itself a chaperone. It is the most abundant protein in the ER lumen, transcriptionally co-regulated with BiP and induced by glucose deprivation or under conditions of significant accumulation of unfolded proteins. GRP94 can also interact with substrates after they have been released from BiP, showing its ability to identify elements with more complex folding intermediates [11]. BiP is a master regulator of ER stress due to its function as a major ER chaperone exhibiting anti-apoptotic properties as well as monitoring the activation of the UPR [12]. It regulates protein folding by recognising unfolded regions on

proteins containing exposed hydrophobic residues and suppressing their aggregation [13]. PDI catalyses the disulfide bond formation and it may act as a chaperone like BiP as demonstrated by *in vitro* and *in vivo* studies [14]. ERp72 shares sequence identity with PDI at discrete regions and its function involves facilitating disulfide bond formation and exchange. The last important chaperone is calreticulin, one of the major Ca^{2+} binding ER proteins, which also acts as a chaperone responsible for the folding of glycoproteins and nascent peptides [15, 16].

There are several diseases caused by genetic mutations, which affect the folding process leading to the accumulation of misfolded proteins. An overwhelming load of misfolded proteins in the ER lumen during protein synthesis increases the risk of aggregation and may further compromise ER functionality. Despite the cellular mechanisms assisting the protein folding process, a significant amount of newly synthesised polypeptides, fail to acquire correctly their native conformation and must be rapidly degraded. To date, there are no known ER-resident proteases involved in the elimination of misfolded proteins present in the ER lumen [17]. It is believed that these proteins re-translocate in the cytosol where they are targeted for ubiquitin-mediated protein degradation by the proteasome through a process of events collectively called ER associated degradation (ERAD) system (Figure 1) [18]. In some cases misfolded proteins have structural constraints that prevent their ability to translocate and enter the cylindrical proteolytic chamber. Such proteins are not optimal ERAD clients and entrance in the secretory pathway and the mediated autophagy may occur via the lysosomal degradation system [19]. Cells' ability to counteract the excessive deposits of misfolded proteins is vital and required for maintaining its functional and structural integrity [7].

Emerging evidence suggests that stress perturbing ER physiology, is involved in diverse pathological conditions ranging from brain disorders, cancer, inflammation, autoimmune disorders to diabetes and neoplasia affecting millions of people worldwide. Mammalian cells cope with this complex ER stress adaptive response by activating a transcriptional response transduced from the ER to nucleus and cytosol collectively called the unfolded protein response (UPR) [20, 21].

Introduction

The UPR is triggered by the activation of three major stress sensors named inositol-requiring protein-1 (IRE1), activating transcription factor 6 (ATF6) and protein kinase R-like ER kinase (PERK) and three main downstream responses (Figure 1) [22-24]. Firstly, the cellular machinery reduces the protein load entering the ER with transient adaptation, achieved by attenuating protein translation. Secondly, it increases the capacity of the ER to cope with the unfolded proteins with a long-term adaptation, which entails increasing transcription of UPR target genes such as chaperones. In cases where ER stress cannot be alleviated, a third mechanism is activated, apoptosis, to eliminate damaged cells and protect the organism [21]. Irreversible pro-apoptotic signalling events cause chronic cellular damage, a characteristic of neurodegenerative diseases [25]. The pathways involved in ER stress have therefore been proposed as potential targets for therapeutic intervention [26].

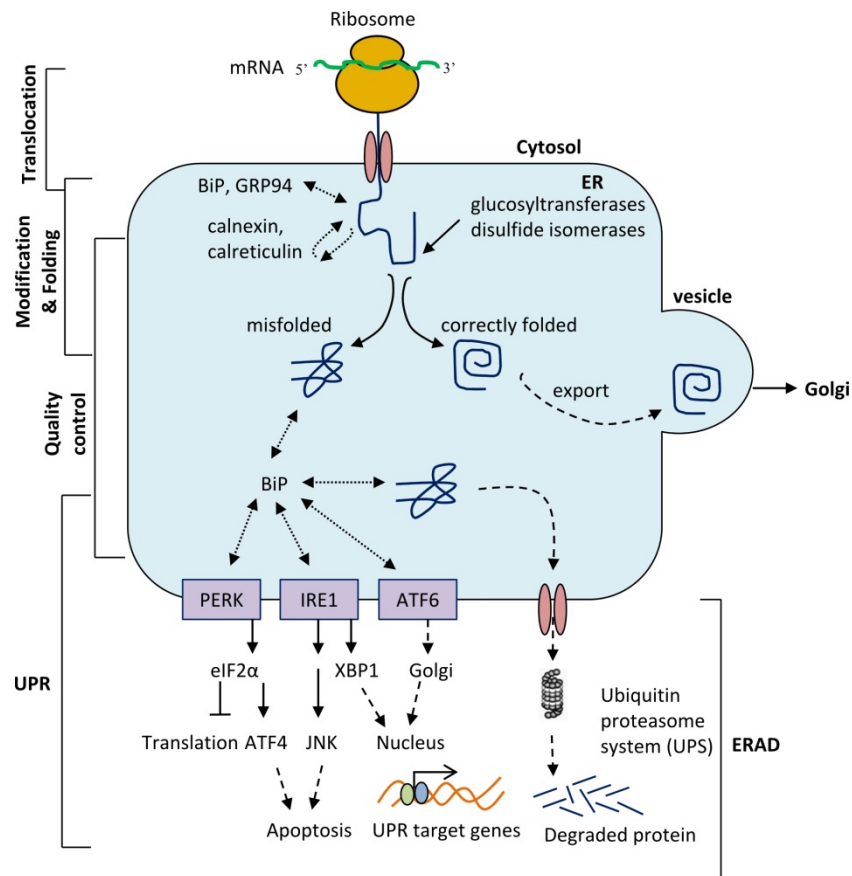


Figure 1: Protein folding and quality control in the endoplasmic reticulum. Solid arrows correspond to enzymatic catalysis, dotted arrows correspond to protein interaction and dashed arrows correspond to transport.

1.2 Alzheimer's disease (AD)

AD was first reported on the 3rd of November 1906 by a German psychiatrist and neuroanatomist, Dr. Alois Alzheimer. It is a devastating and progressive dementia disorder affecting mainly the elderly population and has a doubling incidence every five years beyond age 65. More specifically, the disease affects one in 10 people aged over 65 and around 50% of people over 85 [26].

Alzheimer's at the clinical level is classified into two main types, based on the age of the onset and the genetic predisposition. Sporadic AD is the most common form of the disease, has a late onset and is caused from a synergistic effect of both environmental and genetic factors. On the other hand, FAD accounts for around 5% of cases, has an early onset and manifests as a dominant inherited trait. Regardless of the mechanism of action, both forms exhibit phenotypic similarities and share a final pathophysiologic mechanism of action [27, 28]. Nevertheless, the mechanistic relationships between the two AD forms described remains to be elucidated [29, 30].

AD at the neuropathological level is characterised by the progressive and irreversible loss of neuronal cells and synapses in the cortex and hippocampal gray matter of the brain. The disease relies on the accumulation and aggregation of extracellular amyloid- β ($A\beta$) peptides, the main component of amyloid plaques (also known as senile plaques) and of intracellular deposits of hyperphosphorylated microtubules-associated tau protein, the principal component of neurofibrillary tangles (NFT).

The first hallmark of AD is the accumulation of $A\beta$ plaques, extracellular fibrous-containing aggregates composed of insoluble amyloid fibrils. Although plaques are found extracellularly, $A\beta_{42}$ peptides also accumulate intracellularly and are known to correlate better with the severity of the toxicity caused in the brain [31]. The $A\beta$ peptide ranges from 39 to 43 amino-acids residues in length and derive from the parental proteolytic processing of the amyloid precursor protein (APP). The APP is localised in the ER, the Golgi network, the plasma membrane and the early and late endosomes where the $A\beta$ is processed. Missense mutations

identified in the APP lead to progressive overproduction of A β deposits also causing dysfunction in the degradation mechanism, resulting in the pathogenesis of the disease [32].

The second hallmark of AD is the accumulation of neurofibrillary tangles, intracellular deposits composed of the hyperphosphorylated microtubule-associated tau protein. Tau is a microtubule stabilizing protein and phosphorylation disrupts its ability to regulate the axonal transport. This leads to the accumulation of NFT, causing dysfunction of neuronal cytoskeleton and of the neuronal transport system. A β protein may directly inhibit the degradation of hyperphosphorylated tau by the proteasome, implicating these two proteins in the progression of the disease [33].

Brain tissue analysis from AD patients has showed the upregulation of UPR markers, suggesting an involvement of ER stress activation in AD. However, the involvement of the pathway in disease progression is still speculative as the majority of research data obtained so far are either correlative or based on *in vitro* experiments [34]. This project focuses on unravelling the progression of the UPR in familial Alzheimer's disease (FAD), using a systematic approach of experimentation and mathematical modelling.

1.3 Aim and objectives

The progressive pro-apoptotic role of ER stress by activating the UPR signalling has highlighted this stress mechanism as a potential cause of neurodegeneration and its potential as a prominent therapeutic target. Given a possible role of ER stress in AD pathogenesis, the present study aimed to assess the progression of the UPR induced by ER stress in FAD, using an experimental approach coupled with a kinetic model. SK-N-SH neuroblastoma cells subjected to pre-existing genetic factors (mutations in genes associated with FAD), neuronal differentiation and a mimic of environmental triggers (induction of chemical stress) were assessed. Targets for the signalling pathway of the UPR were selected as such to provide specificity of the arm

activated and distributed according to the progression of the UPR. Emphasis was placed on the early timing of the response following the application of chemical stress.

The specific objectives to conduct this analysis were the followings:

1. Generate a cell model system of FAD with human SK-N-SH neuroblastoma cells overexpressing wild-type APP₆₉₅ or the FAD-associated mutants Swedish and Swedish-Indiana APP₆₉₅. Following confirmation of successful transfections, specific primers for UPR markers were designed to analyse and characterise the gene expression changes of stress markers of three parallel ER arms governed by IRE1, PERK and ATF6 receptors.
2. Investigate whether overexpression of APP₆₉₅ induces any pre-upregulation in the levels of ER stress markers and if so whether these levels differ between cells overexpressing wild-type and FAD-mutant APP₆₉₅ protein. The mRNA was isolated and subjected to quantitative real time PCR (qRT-PCR) for gene expression analysis. This was performed in a stress-free environment, and allowed us to evaluate whether the APP₆₉₅ overexpression and/or mutation itself was enough to cause preconditioning of ER stress.
3. Assess the progression sequence of UPR in the FAD cell model system under an environmental stress stimulus. In order to obtain a neuronal-like phenotype, optimisation of the differentiation (with retinoic acid exposure) and of artificial induction of stress by the addition of tunicamycin were evaluated. The response of the system was investigated by analysing the UPR mRNA levels following the application of chemical stress at different time points over the course of 24 h.
4. Understand the dynamics of the elaborate UPR system by constructing a model for the mammalian UPR. This model was based on mass-action kinetics, resulting in ordinary differential equations integrating the response of three signalling branches; PERK, IRE1 and ATF6. In order to find the parameters allowing the model fitting to the experimental data, the software gPROMS ModelBuilder[®] was used to run the estimations. The model was fitted to the relative mRNA experimental data of the first eight hours of the TM time-course stress experiment for each of the APP_{WT}, APP_S and APP_{SI}.

5. Examine whether our mathematical model can capture the biological effects of known therapeutics. *In silico* experimentation was performed in a series of case studies compounds and simulated for each of the cell lines individually.

1.4 Structure of the thesis

This project focused on identifying the progression and dynamics of the UPR with respect to FAD. Chapter 1 provides a general background of the molecular mechanism and the disease studied as well as the aim and objectives of the thesis. A thorough literature review was discussed in Chapter 2, initially describing the epidemiology and the clinical characteristics of the three most common neurodegenerative diseases but with a focus on AD (2.1 and 2.2). Subsequently, it discusses the main factors affecting AD, while focusing on the role of the amyloid precursor protein in this disease (2.3 and 2.4). As neurodegenerative disorders affect neuronal cell death, these cells were characterised to better understand their structure as well as the function of the ER (2.5). The aim of the project was to investigate the dynamic UPR-induced from ER stress in FAD thus an extended review on the UPR was made with a detailed description of each of the pathways activated along with the effect of their activation (2.6). To understand the link between FAD and UPR, the pathways inducing apoptosis were not only listed but also described (2.7). Eventually the link of ER stress with AD as well as the stressors related with AD were presented with the use of the respective evidence from previous studies (2.8 and 2.9). As there is no cure for AD, it is important to know the proposed treatments related to the UPR and the potential targets for future drug development (2.10 and 2.11). Furthermore, the literature analysis consisted on identifying some of the possible experimental (both in vitro and in vivo) and computational models already developed to study the UPR along with their limitations (2.12 and 2.13).

Following the literature review, we presented in Chapter 3 the materials and methods used to achieve the experimental objectives of our project. The next two chapters describe and discuss

the results obtained by the experimental model developed with the use of the aforementioned methodological approaches.

Chapter 4 presents the approach taken to develop a stable cell model system of FAD with SK-N-SH cells overexpressing either the wild-type of FAD-associated APP mutations. The optimising transfections and RNA extraction protocol were also presented. Then, we discussed the optimisation of the primer annealing temperatures, amplification efficiencies but also identified out of a range of housekeeping genes, the best one to use for our cell model system in the real-time quantification reactions. Although SK-N-SH cells can be differentiated with several agents, we present the possible molecules that can be used and the choice of the most adequate one in our system and the respective dosing and number of days requiring incubation. These were validated with neuronal and biochemical characterisation of our cells treated with different amount and/or days with RA. After establishing these protocols, cells were evaluated to test whether overexpression of wild-type of mutant FAD-associated APP pre-upregulated the UPR induced by ER stress. As the project aimed to evaluate the progression of UPR during stress, evaluation of the possible stress inducing drugs was performed and presented. In order to achieve our objectives related to cells' metabolic activity, the MTS assay conditions were also optimised with respect to the effect of different chemicals and the number of cells used (4.2). The results were then discussed (4.3) and summarised in the conclusion section (4.4).

Chapter 5 focuses on the progression of UPR in the experimental model of FAD. Differentiated cells were analysed for the stress response imposed by the intra and extra cellular stresses at six different time points with respect to their viability (with trypan blue exclusion method), their metabolic activity (with MTS assay) and the gene expression levels of several UPR markers (with qRT-PCR). The results presented demonstrate at first the cells' initial response to stress to be an attempt to adaptation and the UPR progression to have a different pattern in FAD cells. The qRT-PCR analysis illustrates the fold increase of the UPR markers downstream of PERK, IRE1 and ATF6 within each cell line during TM treatment (5.2.2). Then the same data are presented in a way to illustrate the fold increase of the UPR markers between the cell lines during TM treatment (5.2.3). Finally, the data are normalised to the wild-type cells at each time point of

TM treatment as we wanted to evaluate whether mutant cells exhibit a stronger upregulation of the UPR marker (5.2.4). The statistical analysis for each of the aforementioned sections was also presented accordingly. The results were discussed and included the sequence of UPR activation as well as the key players in UPR induced by ER stress (5.3). A conclusion in this section summarised the findings.

Chapter 6 describes the development of our computational model of the mammalian UPR network. Firstly, the importance of the generation of such a system is described (6.1) followed by the general characteristics of the kinetic model along with the assumptions made (6.2). Next, the development of the model was presented with the description of the protein folding dynamics, the IRE1, the PERK and ATF6 pathways (6.3). This was followed by a detailed description of the formation, the degradation and the translation of XBP1 complex (6.4). The calibration of the model was described while including the parameter values used, the initial conditions, and the estimated parameter values (6.5). The simulation results of the model were presented and compared with the experimental data for each of the APP_{WT}, APP_S and APP_{S-I} cell lines. Description of the simulation analysis reflecting the UPR mechanism was achieved with an ATF6 sensitivity analysis, a quantitative analysis of unfolded protein load and an understanding of the effect of XBP1 protein complex. The model was then used as a basis to explore treatments *in silico*. Three case studies are presented and include the effect of salubrinal and of lithium and valproate. A conclusion, which summarised all of the aforementioned on the computational model, was presented in the end (6.9).

Chapter 7 is the final discussion, which provides a summary the experimental and computational results demonstrating the novelty of the project followed by the main conclusions gained from these two systems, the limitations and the future directions.

Chapter 2

Literature Review

2.1 Epidemiology of neurodegenerative diseases

Neurodegenerative disorders describe a range of conditions characterised by cellular and molecular changes that occur in specific brain regions leading to loss or dysfunction of the nerve populations. Although they exhibit different clinical symptoms and neuronal loss at specific brain regions, they share common characteristics including excessive deposition of misfolded proteins, cytoskeletal and mitochondrial dysfunction, suggesting similar therapeutic intervention for their cure. Such conditions include Alzheimer's (AD), Parkinson's (PD) and Huntington's (HD) diseases [35]. The number of people affected is rapidly increasing and, as the world population ages, they are projected to reach epidemic proportions imposing a major public health issue. An average of 65.7 million people will be living with dementia by 2030 and the estimated worldwide cost for 2010 was already US\$604 billion as reported in the World Alzheimer Report 2010. Along with the high economic costs of professional dementia care come high social costs including decreased quality of life for patients and stress placed on family members who serve as informal caregivers [26].

2.2 Clinical characteristics

Numerous neurodegenerative disorders show accumulation of abnormal protein aggregates in the brain over time and this usually dictates the hallmark of the disease. The clinical manifestations reflect the distinct regions of the brain affected, and are apparent when more than 80% of the neuronal cells are lost. Thus, cerebral cortex atrophy (i.e. AD) results in memory loss and progressive aphasia, while defects in the basal ganglia (i.e. PD, HD), a region controlling the motor system, results in movement disorders. Even though most of these diseases have specific prime brain targets, they can often disrupt other parts of the brain as the disease progresses [26] (summarised in Table 1).

Table 1: Summary of clinical characteristics of three neurodegenerative disorders: 1) Alzheimer's, 2) Parkinson's and 3) Huntington's diseases.

	(a) Alzheimer's	(b) Parkinson's	(c) Huntington's
Protein	-A β -Tau	- α -synuclein	-Huntingtin
Clinical characteristics	-Senile plaques -Neurofibrillary tangles	-Lewy bodies -Lewy neuritis	-Glutamine repeats of HTT gene
Location	-Neurons of many regions of the brain	-Dopaminergic neurons	-Cortical neurons
Areas of the brain affected	-Entorhinal cortex -Basal forebrain -Hippocampal gray matter -Amygdala	-Substantia nigra	-Basal ganglia
Symptoms	-Memory loss -Progressive aphasia	-Tremor at rest -Bradykinesia -Muscular rigidity -Postural instability	-Chorea/dystonia -Dementia -Depression
Population affected	25-30 million	4 million	5-8 cases/100,000
General fact	1 in 3 seniors die with AD or another Dementia disorder		

2.2.1 Alzheimer's disease

AD is an incurable neurodegenerative and terminal disease characterised by impairment of cognitive abilities and changes in behaviour and personality [36]. It is the most common form of dementia disorder affecting 5-10% of over 65s and decreasing their life expectancy by half [37, 38]. It affects approximately 25 to 30 million people worldwide; it is the sixth leading cause of death in the USA where every 67 seconds an individual develops the disease. AD is anticipated to triple in prevalence by 2050 [29, 39].

Patients with AD exhibit brain shrinkage in regions involved in memory, learning and emotional behaviours resulting from neuronal degeneration [40]. The course of the disease is unique in each person, yet there are several common symptoms and patterns that emerge. It is histopathologically characterised by the accumulation of A β plaques, NFT, progressive neuronal and synaptic loss on the entorhinal cortex, basal forebrain, hippocampal gray matter and amygdala [41-43]. Central to AD pathophysiology are mutations in three different genes and variation in another gene all responsible for the susceptibility of several neuronal cellular changes including oxidative damage, production of reactive oxygen species (ROS) and inflammation (Figure 14) [44]. These genes include amyloid- β precursor protein (APP), presenilin1 (PS-1), presenilin 2 (PS-2) and apolipoprotein E4 (ApoE4) localised on chromosomes 21, 14, 1 and 19 respectively. These genes are associated with FAD, an inherited form of early-onset AD [45].

Recent studies have reported that single nucleotide polymorphism in the neuronal sortilin-related receptor gene (SORL1) located on chromosome 11 are implicated in the susceptibility of late onset AD. SORL1 expressed in the central and the peripheral nervous system is involved in the intracellular trafficking of APP as seen *in vitro* and *in vivo* experiments [46]. Underexpression of SORL1 has been demonstrated to cause overexpression of A β , increasing the risk of developing AD [47]. Additionally, this gene was found to be differentially expressed in people affected with AD compared with the controls providing evidence of being causally associated with the late onset AD [48].

Mutations in genes encoding transmembrane proteins that form part of the γ -secretase complex alter the extensive APP proteolytic processing and result in the extracellular production of short and long A β fragments named A β_{40} and A β_{42} , respectively. A β_{40} is the most abundant peptide, whereas A β_{42} is more hydrophobic and therefore prone to aggregation into fibrils. It has been shown that intraneuronal A β_{42} peptides form soluble oligomers that are the key toxic species precede mature fibril plaque formation and may be a significant step in the deposition of extracellular A β and formation of plaques in the brain [49, 50]. A β accumulation can also occur within cells. Not only can extracellular A β be re-internalised, but there is also evidence of A β_{40} and A β_{42} being produced in the endosome system, interacting with SORL1 that is linked to late onset AD [51], and along the secretory pathway of neuronal cells [52-55].

AD is also characterised as a tauopathy due to accumulation of abnormally folded and aggregated tau protein. Tau protein undergoes chemical changes; becomes hyperphosphorylated and results in a decrease of tau binding to microtubules. This causes dysfunction of neuronal cytoskeleton, disintegration of the neuronal transport system and thus degeneration [56]. There is also evidence that intracellular accumulation of tau protein may be promoted by the accumulation of A β , which has been shown to inhibit the ubiquitin–proteasome system [57]. An overview of the interconnectivity between A β oligomer formation, ER and oxidative stress, Ca²⁺ dyshomeostasis and tauopathy is provided in Figure 2 whereas the generation of A β peptides to various subcellular compartments and the cytosol is provided in Figure 7.

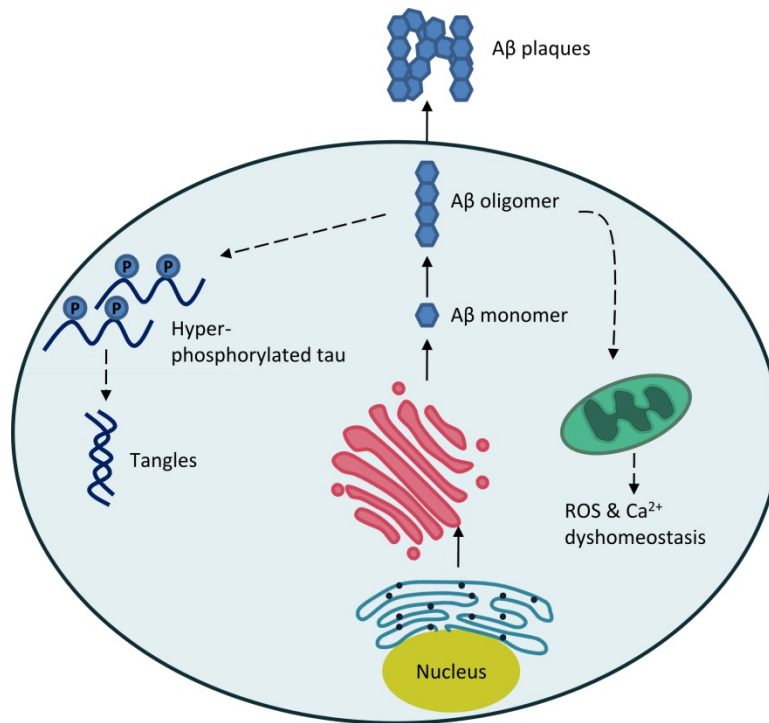


Figure 2: Overview of the mechanisms involved in the progression of Alzheimer's disease: Mutations in genes encoding transmembrane proteins that form part of the γ -secretase protease complex, result in the production of short and long amyloid- β fragments named $A\beta_{40}$ and $A\beta_{42}$, respectively. Intraneuronal $A\beta_{42}$ peptides are more hydrophobic and therefore prone to forming soluble oligomers that are the key toxic species. Oligomers precede mature fibril plaque formation and may be a significant step in the deposition of extracellular $A\beta$ and formation of plaques in the brain. Progressive accumulation of intracellular $A\beta$ in mitochondria is associated with altered mitochondrial membrane potential, a reduced rate of oxygen consumption and increased release of ROS. Compromised ER activity also leads to excessive Ca^{2+} in the cytosol, which is taken up by the mitochondria, dysregulating Ca^{2+} signalling. There is also evidence that intracellular accumulation of tau protein may be promoted by the accumulation of $A\beta$, which has been shown to inhibit the ubiquitin–proteasome system (UPS) [26]. Reproduced with permission.

2.2.2 Parkinson's disease

PD is the second most common form of neurodegenerative disorders after AD and affects 4 million people worldwide [58]. It is a late onset disease with multifactorial aetiology and a prevalence of 16–19 cases per 100,000 people in a male to female ratio of 1.5:1 [59]. It progressively degenerates the motors of the substantia nigra, an area in the basal ganglia where dopaminergic neurons are found. The clinical phenotype of PD is parkinsonism and is characterised by tremor at rest, bradykinesia, muscular rigidity and postural instability [59, 60].

Neuropathologically, PD is characterised by the death of these dopaminergic neurons, which results in neurotransmission failure, and the progressive formation and accumulation of fibrillar cytoplasmic spherical and rod-shaped protein aggregates called Lewy bodies and Lewy neurites, respectively, which are mainly composed of the presynaptic nerve terminal α -synuclein as well as ubiquitin proteins as seen in Figure 3 [61]. Even though familial PD is known to occur due to mutations in seven different genes including α -synuclein, parkin, leucine-rich repeat kinase 2 and PTEN-induced putative kinase 1 [59, 62, 63], the vast majority of PD cases are sporadic and believed to occur due to a combination of genetic and environmental factors. Due to the similarity of the pathological symptoms between familial and sporadic PD, it has been postulated that both types of patients undergo a similar cascade of events [64].

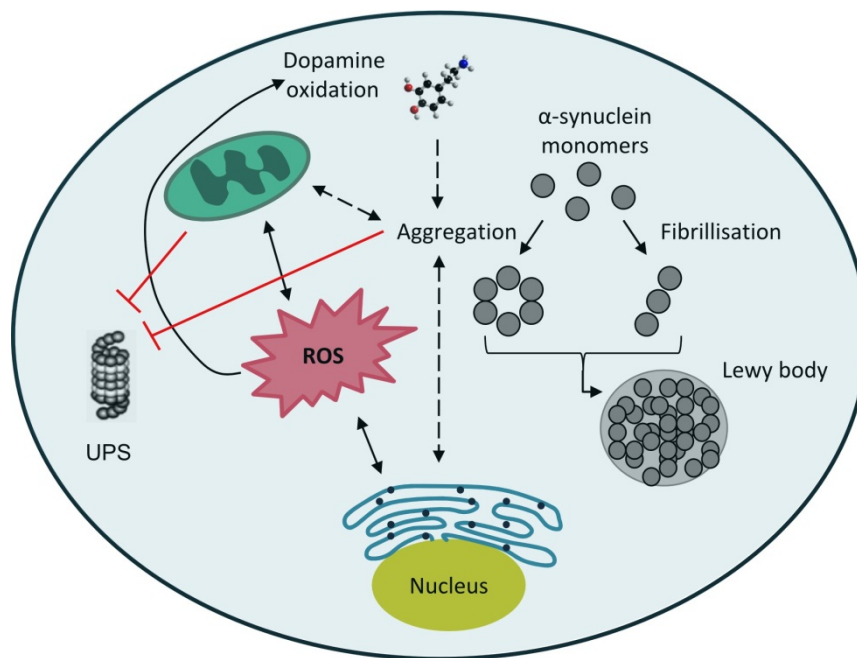


Figure 3: Overview of mechanisms involved in the progression of Parkinson's disease: Dysfunction of the mitochondria and the ER is the main manifestation of PD, even though the temporality of events is still poorly understood, partly because of the interdependence of the role of these two organelles. Overexpression of wild-type α -synuclein, a membrane protein that goes through the ER during normal processing, or expression of mutant proteins that are prone to misfolding (due to a variety of genetic and environmental factors) leads to activation of ER stress. Aggregation of α -synuclein results in the progressive formation of spherical and rod-shaped protein aggregates called Lewy bodies and Lewy neurites, respectively. Overexpression of α -synuclein is usually detected by dopamine neurons early enough to trigger a defence mechanism against its toxicity. However, the increased production of ROS due to ER stress and mitochondrial dysfunction, which is predominant in PD, impairs dopamine function, rendering the cells more susceptible to the effects of protein

aggregation. These symptoms are exacerbated by the fact that a fraction of inherited PD can be attributed to mutations in the gene for Parkin, a protein thought to be part of the ubiquitin proteasome degradation system (UPS), which therefore impede the degradation of malformed proteins [26]. Reproduced with permission.

2.2.3 Huntington's disease

HD is a late-onset autosomal dominant inherited disorder that affects approximately 5-8 per 100,000 people in their third to fifth decade and has a 10-20 year lifespan after the onset of the disease. Pathologically, HD is characterised by a gradual premature loss of the medium spiny neurons of the striatum and, to a lesser extent, cortical neurons mainly in the basal ganglia, which manifests in patients as involuntary motor movements (chorea), dementia, and in some cases psychiatric disturbances including depression [65-67]. It is the most well-known of the 'polyglutamine disorders' characterised by an expansion of the number of glutamine repeats (CAG) in the 5' coding region of Huntingtin gene (HTT) located on the short arm of chromosome 4 that encodes the Huntingtin protein (Htt) (Figure 4). The number of CAG repeats is highly polymorphic and varies between 11 to 34 repeats under normal conditions, but it is expanded beyond 40 to 180 copies in HD [68]. Mutation in this gene is completely penetrant and leads to aggregation of Htt in the cytoplasm and the nucleus (cell body), which is toxic to the cells and eventually results in cell death [69, 70]. Although genetic mutations are significantly responsible for the onset of the disease with an inverse correlation between the number of glutamine repeats and the age of onset [71], environmental factors have also been demonstrated to affect the age onset variation [72].

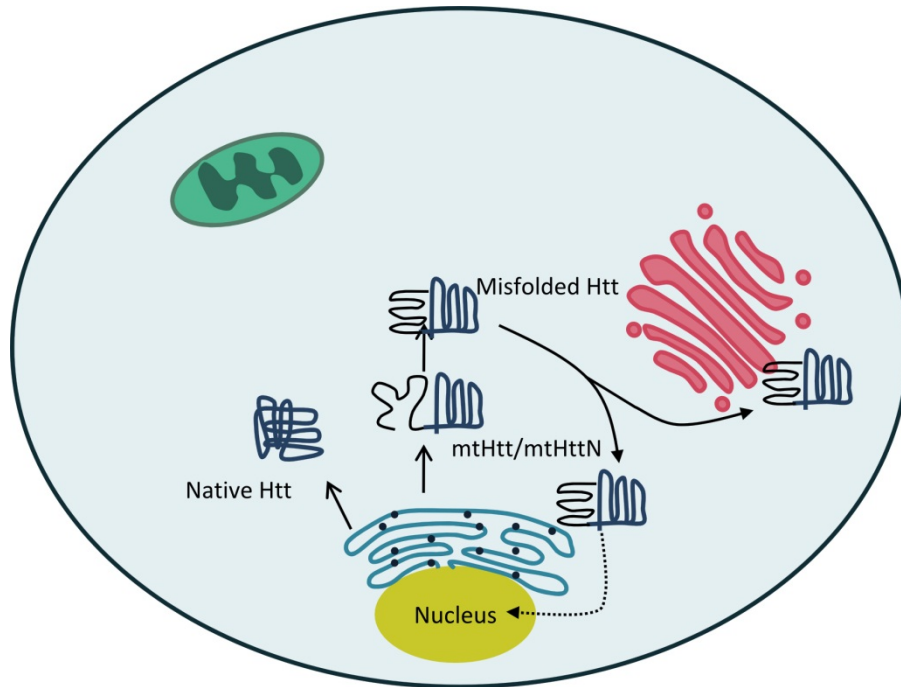


Figure 4: Overview of protein trafficking and stress activation in Huntington's disease: Expression of mutant Huntingtin (mtHtt) protein with larger polyglutamine tracts or N-terminal truncations of the Huntingtin protein with expanded repeats (mtHttN) triggers ER stress. Upon stress activation, Huntingtin appears to detach from the ER membrane and translocate to the nucleus. The first 17 or 18 amino acids form an amphipathic alpha helix that associates with membraneous organelles in the cytoplasm, including the ER, Golgi, and mitochondrial membranes. Studies have also demonstrated the colocalisation of Huntingtin aggregates and Ubiquitin-proteasome system (*UPS*) components, as well as impairment of *UPS* function in cells expressing proteins with expanded polyglutamine domains of pathological size [26]. Reproduced with permission.

2.3 The role of amyloid precursor protein in Alzheimer's disease

2.3.1 APP structure

APP is a highly conserved type-I single pass transmembrane glycoprotein composed of 695–770 amino acid residues and belongs to a superfamily of proteins that includes the APP-like proteins APLP1 and APLP2 [73, 74]. APP has a large hydrophobic extracellular N-terminal domain, a single transmembrane composed of 23 amino acids and a small intracellular C-terminal domain [75]. Alternative mRNA splicing of exon 7, 8 and 15 generates eight APP isoforms of which the most prevalent ones are APP₆₉₅, APP₇₅₀ and APP₇₅₁. APP₆₉₅ is predominantly expressed in the

central nervous system (CNS), whereas the other two isoforms are ubiquitously expressed in peripheral tissues [76]. Most APP forms contain a protease inhibitor domain (encoded by exon 7) responsible for inhibiting trypsin and other coagulation factors. APP₆₉₅ lacks this inhibitor indicating that this isoform is found almost exclusively in the brain [77]. Although the primary function of APP is unknown, it has been implicated in the regulation of synaptic formation and synaptic function [78].

2.3.2 APP proteolytic processing

Strong evidence from neuropathological and genetic studies implicates a product of APP metabolism, A β , as a major contributor in the pathogenesis of AD. APP can be proteolytically cleaved adjacent to the lipid bilayer through three different proteases named α -, β - and γ -secretase. Cleavage by α - or β - secretase at the luminal/extracellular domain yields large soluble APP derivatives (sAPP α or sAPP β) and sheds almost the entire ectodomain generating membrane-tethered α - or β - C-terminal membrane embedding fragments (CTFs) [79].

APP processing can occur via two separate pathways; the non-amyloidogenic or the amyloidogenic pathway as demonstrated in Figure 5. Under physiological conditions, APP undergoes non-amyloidogenic processing. Cleavage occurs by α -secretase at the amino acid residue 17 of the A β domain resulting in the release of a soluble NH₂ fragment (sAPP) and preventing the formation of the toxic A β molecule. Following the extracellular cleavage, the COOH domain produced is cleaved by γ - secretase within the transmembrane domain resulting in a short 3 kDa peptide termed p3 and the release of the APP intracellular domain (AICD). In the amyloidogenic pathway, APP first cleavage occurs by the β -site APP cleaving enzyme at the extracellular domain leading to the release of sAPP β . This is followed by the γ -secretase cleavage in the transmembrane domain leading to the generation of AD-associated A β peptides with various sizes, with A β ₄₀ and A β ₄₂ being the most common ones [40, 80, 81]. This is a key event in the processing of AD pathology.

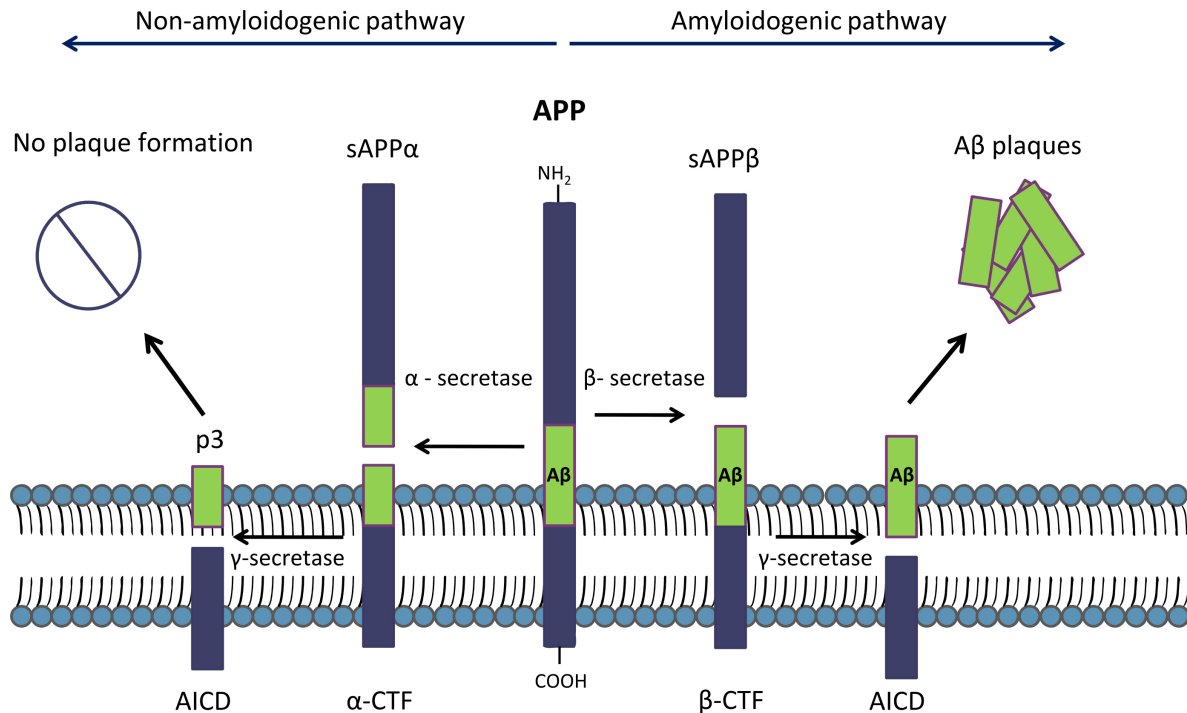


Figure 5: APP metabolic processing pathway. APP can undergo sequential proteolytic cleavage by α -, β -, and γ -secretases. Depending on whether A β peptides are generated from the APP, the pathway is named non-amyloidogenic or amyloidogenic. a) In the non-amyloidogenic pathway APP process occurs by α -secretase cleavage at amino acid number 17 of the A β domain and yields a soluble N-terminal (NH₂) APP α fragment (sAPP α) and a transmembrane C-terminal (COOH) fragment (α -CTF) which has neuroprotective as well as neurotrophic functions. The COOH domain produced from α -cleavage is subsequently cleaved by γ -secretase within the transmembrane domain and generates a non-amyloidogenic short peptide of three kDa termed p3 and an APP intracellular domain (AICD). b) In the amyloidogenic pathway APP is cleaved by β -secretase and yields a secreted N-terminal fragment APP ectodomain (sAPP β). The intracellular COOH domain is subsequently cleaved by γ -secretase and generates multiple A β peptides, the most frequent being A β ₄₀ and A β ₄₂.

2.4 The role of A β in Familial Alzheimer's disease

2.4.1 Different FAD-associated APP mutations

A subclass of FAD is due to dominant missense mutations in APP and presenilin genes, which alter the extensive APP proteolytic processing by γ -secretase complex resulting in an increased A β ₄₂/A β ₄₀ ratio [82-84]. A β ₄₂ has been previously demonstrated *in vivo* to aggregate into fibrils more quickly than A β ₄₀, which may explain why small changes in this ratio induce AD

pathogenesis [82, 83]. The most common FAD cases are due to missense mutations in APP in codons 670 (K670N) and 671 (M671L) (Swedish mutation) or in codon 717 (V717F) (Indiana mutation), affecting not only the cleavage by γ -secretase, which increases $A\beta_{42}$ secretion, but also the catabolic pathways responsible for the degradation of these fragments [84-86]. Previous studies have shown that cells carrying the Swedish APP mutation have a six- to eight-fold increase in the $A\beta_{40}$ and $A\beta_{42}$ production compared to cells expressing wild-type APP. This increase is caused from the abnormal APP cleavage at the β -secretase cleavage site [87]. On the other hand, the Indiana mutation affects the γ -secretase cleavage site and enhances the relative ratio of $A\beta_{42}/A\beta_{40}$ [86, 88]. Other FAD mutations have also been found including London (V717I) [89], Austrian (T714I) [90], Florida (I716V) [91], Italian (E693K) [92], Arctic (E22G) [93], Flemish (A692G) [92], and Iowa (D23N) [94]. These mutations were isolated from families living in different regions around the world that exhibited an early-onset AD (Figure 6) [86, 95].

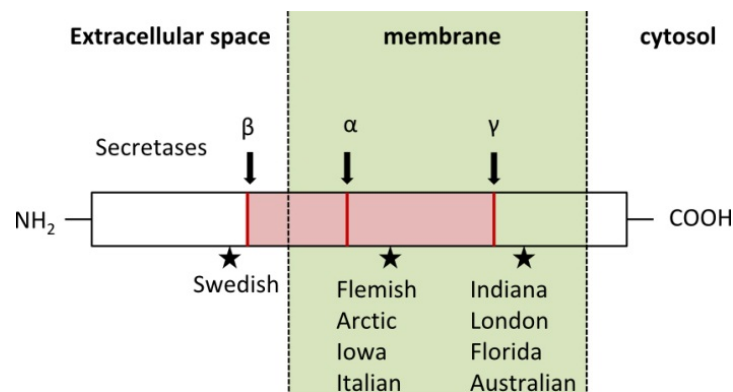


Figure 6: Familial Alzheimer's disease associated APP mutations. Early onset FAD mutations are marked with an asterisk. Most mutations are clustered around the secretases sites. The $A\beta$ sequence is the one highlighted in red.

Several transgenic mice expressing a single or numerous FAD-associated APP mutations have been previously developed [96]. One of the most well studied transgenic mouse-model is the Tg2576 developed by Hsiao *et al.* [97] overexpressing the Swedish APP mutation driven by the hamster prion protein promoter. These mice exhibited an age-dependent $A\beta_{40}$ and $A\beta_{42}$ increase, which caused the formation of senile plaques, as seen in AD-brain patients. $A\beta$

plaques were not formed until 11 months of age, which through time spread in cortical and limbic regions [97]. At the age of four years, they exhibited a decrease in spine density and impairment in memory probably caused by the synaptic dysfunction. At the early age of four to five months, they also showed an increase in the ratio of $A\beta_{42}/A\beta_{40}$ [98]. Intraneuronal accumulation of $A\beta$ was increased during aging, and was correlated with abnormal synaptic transmission before the formation of $A\beta$ plaques at the age of 18 months [99].

Other FAD transgenic mice were also generated, in which their APP expression was driven by strong APP promoters. Chishti *et al.* [100] developed TgCRND8 mice, overexpressing both the Swedish and the Indiana APP mutations, driven by a prion promoter. They exhibited an aggressive clinical phenotype and at the early age of three months developed parenchymal $A\beta$ deposits, following by formation of $A\beta$ plaques inducing neuritic dystrophy at the age of five months. The mice generated an increased load of $A\beta_{42}$ relative to $A\beta_{40}$, with the excess of $A\beta_{42}$ being associated with behavioural deficits at six month of age. These included impairment in learning and memory with respect to spatial recognition. NFT were not detected and neurodegeneration was absent [100].

2.4.2 Contributions of post-translational modifications in familial Alzheimer's disease

APP is synthesised in the ER, subjected to various post-translational modifications such as glycosylation, phosphorylation and sulfation during the intracellular transit through the secretory pathway that precedes its transport to the cell surface. The APP exists in two isoforms the immature of around 110 kDa and the mature of around 130 kDa. The immature APP is mainly N-glycosylated and is concentrated in the ER and the cis-Golgi compartment. On the other hand, the mature APP is N- and O-glycosylated as well as tyrosyl-sulfated, localised in the trans-Golgi and on the plasma membrane [101].

Tienari *et al.* [102] treated transfected hippocampal neurons with TM, a N-glycosylation inhibitor inducing misfolding of the wild-type APP protein, providing evidence for the axonal sorting of wild type and mutant APP. Another study by Yazaki *et al.* [103] used CHO cells and

demonstrated that deletion of the two N-glycosylation sites of the asparagine sites (Asn467 and Asn496) of APP decreases its secretion. These supported the hypothesis that correct glycosylation is essential for both axonal sorting and processing of the APP. Importantly, both the Swedish and the London APP mutations had alterations in the N-glycosylation of APP, with a high number of N-acetylglucosamine (GlcNac) residues.

In addition to N-glycosylation, O-glycosylation is also required for the correct APP function. As O-glycosylation is necessitated for cleavage by γ -secretase [104], its interference may lead to increased sAPP and decreased production of A β . APP cleavage mediated by α -, β - and γ -secretases in most cases occurs after the O-linked glycosylation. This takes place during the passage of APP through the Golgi where O-glycosylation takes place or in trans-Golgi compartments during the processing of the APP in the secretory pathway [105]. In agreement with this, brains from AD patients showed an increased mRNA expression of the GlcNac transferase III (enzyme responsible for the addition of N-acetylglucosamine residue in O-glycosidic linkage) compared to healthy individuals [106].

A fraction of nascent APP molecules in the ER may be proteolytically cleaved by γ -secretase to yield A β_{40} /A β_{42} products. From the newly synthesised APP, only a fraction of around 10% has been shown to reach the plasma membrane, as the majority transits to the Golgi apparatus and the trans-Golgi network [107]. This organelle is another site where A β processing can occur and crucial for AD pathogenesis (Figure 7). For instance, the APP Swedish and PS-1 mutations mainly generate A β molecules in the Golgi [108]. When at the plasma membrane, the majority of APP molecules are cleaved by α -secretase or by the β - and γ -secretases together, thus either releasing the s-APP or generating A β fragments, respectively. Uncleaved APP can get re-internalised within minutes due to the presence of the 'YENPTY' internalization motif located near the C-terminus site of APP (residues 682-687 of APP₆₉₅ isoform). The internalisation can lead to recycling of the APP to the cell surface or to sorting it for degradation via the ERAD system [109, 110]. This internalisation process may also result in the generation of A β fragments.

It is now clear that APP processing may occur in various subcellular compartments generating A β . These include organelles along the intracellular secretory pathway such as the ER, trans-Golgi, plasma membrane, early, late and recycling endo-lysosomes [111, 112]. Evidence from post-mortem brain tissue analysis has shown that aggregated A β fragments generated are produced extracellularly and/or are accumulated intracellularly. Intracellular A β accumulation acts as a key factor for the progression of AD [113]. An increased intracellular accumulation of such misfolded proteins may cause the proteasome to reach a saturation point and promote autophagy as a compensatory response. This in turn leads to dysfunction of the ERAD and contributes to the increase of misfolded proteins, which together with the autophagy decline promote chronic ER stress in several neurodegenerative diseases [114].

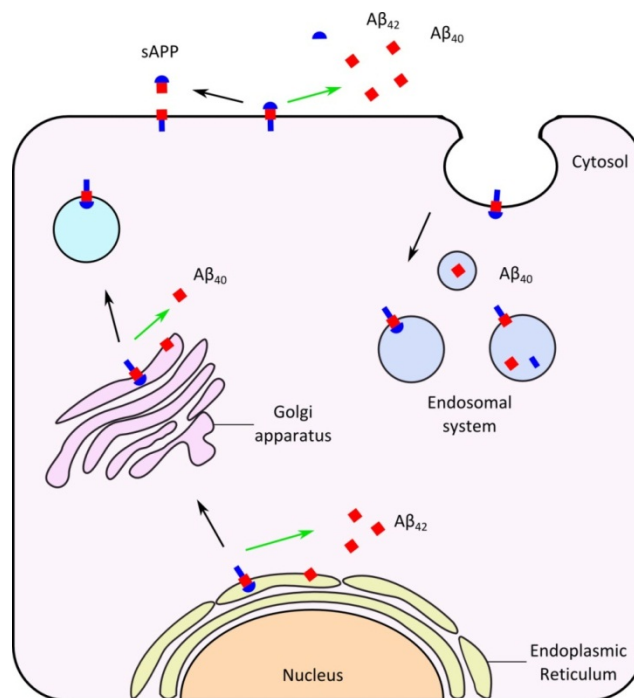


Figure 7: Schematic representation of the generation of A β peptides by APP proteolytic processing. APP is synthesised and secreted in the ER and can be processed through the amyloidogenic pathway (green arrows) leading to production of A β (red squares) and the non-amyloidogenic (black arrows) pathway leading to secreted APP. The APP can be proteolytically cleaved in the ER releasing A β molecules. When it transits to the Golgi, it can be cleaved to produce A β or it may continue to traffic in the plasma membrane. When APP is in the plasma membrane, its cleavage is mostly favoured by α -secretase and generating the sAPP (blue hemicycle with red tail) or it is processed by β - and γ -secretases generating A β peptides. The intact APP in the plasma membrane can get re-internalised into endosomes where it can either get recycled to the PM or degraded through a process that may also lead to A β production.

Intraneuronal A β ₄₂ toxic peptides form soluble oligomers that precede mature fibril plaque formation and could be a critical step in the deposition of extracellular A β and formation of plaques in the brain [45, 115]. The APP mutations cause A β toxicity, which can directly result in synaptic impairment but can also increase hyperphosphorylation of microtubules-associated tau protein and cause aggregation and formation of intracellular tangles. These A β peptides present in senile plaques and the intracellular aggregates of tau protein present in neurofibrillary tangles in the brains of AD patients are the hallmarks of AD and are responsible for induction of cellular toxicity [42, 116, 117].

2.5 Characteristics of neuronal cells

2.5.1 Cellular physiology

The mammalian CNS consists of two major cell populations; neurons and glial cells associated with the execution of the cognitive functions of the brain. Differentiated neurons are known to be post-mitotic as they are incapable of dividing even under chemical stimulation. As the CNS circuit remains fixed, neuronal growth (mitotic division) or repair cannot occur even after damage has occurred in chronic conditions such as neurodegenerative diseases. Therefore, the molecular mechanism responsible for the mitotic inactivity of the already differentiated neurons is a crucial feature faced in the brain-related diseases [118].

Neurons have a highly regionalised organisation where subcellular domains have distinct functional importance. These comprise: a) the cell body (soma) which is the metabolic centre responsible for the protein synthesis and the preservation of the whole cell mass, b) branching dendrites, which function in receiving and processing electrochemical inputs, and c) axons, acting as the information transmitters, spreading over distances bigger than the cell's diameter. Neurites (axons and dendrites) have a cytoplasmic volume greater compared to the cell body [119]. Preserving the complex morphology and the cellular subspecialisation of nerve cells is

essential for their numerous functions. The majority of the neuronal macromolecules are synthesised in the cell body whose function is dependent upon the correct function of three intracellular systems. These comprise: a) the rough ER, which utilises the attached ribosomes for the synthesis of the backbone polypeptides, b) the smooth ER and c) the Golgi apparatus a principal site for membrane differentiation, secretory vesicle formation and addition of prosthetic groups (i.e. sugars, lipid, sulphate) to a protein complex (Figure 8) [119, 120].

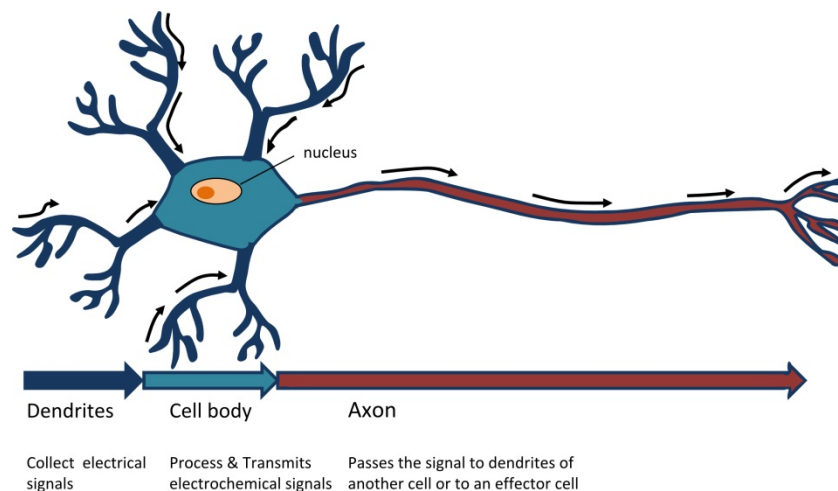


Figure 8: Structure of a neuron with the respective functions of each part.

2.5.2 ER structure and function

The ER is a specialised subcellular compartment responsible for the synthesis, post-translational modification and transport of secreted and transmembrane proteins to their target cellular sites. The majority of these nascent proteins translocate into the ER lumen as unfolded polypeptide segments. Immediately after entering the ER, many oligosaccharides but most commonly N-linked glycans are added to modify the protein chains. Once modified, proteins are folded into their secondary and tertiary structure and in many cases assemble into a multimeric assembly. The secondary protein conformation is stabilised by hydrogen bonds while the tertiary by hydrophobic interactions between amino-acid side chains and in some

cases through disulfide bonds, the latter mainly found in extracellular proteins for maintaining their native state [121].

The oxidising environment of the ER lumen (relative to the cytosol) promotes the formation of disulfide bonds and further facilitates protein folding. Additionally, ER-resident chaperones such as BiP function to inhibit the protein aggregation process by directly leading the proteins to folding pathways. The ER also serves as the major intracellular Ca^{2+} storage compartment, is responsible for the biosynthesis of lipids (i.e. steroids, cholesterol), and for executing the biogenesis and signalling of the organelle with the nucleus and the cytosol.

Execution of quality control ensures that only the properly folded proteins are transferred to their target cellular location, thus ensuring cellular homeostasis. Folding of the proteins into their proper tertiary structure is essential for their biological functions. A number of physiological and pathological conditions can affect protein folding, leading to activation of ER stress.

2.6 The unfolded protein response

ER stress caused by the accumulation of abnormally folded proteins in the ER activates the UPR to counteract the build-up of the un/misfolded proteins. This is a multi-branched pathway that is activated in the ER, acting via three distinct signalling mechanisms to attempt to restore homeostasis. The first involves the upregulation of protein folding chaperones and proteolytic enzymes, while the second causes the suspension of protein synthesis (translational attenuation). The former facilitates the process of protein folding, while the latter helps the ER cope with the stress by decreasing the amount of proteins that enter the folding process [122, 123]. The third mechanism triggers apoptosis if the stress has not yet been resolved. There are therefore several possible outcomes of ER stress, some protective and some detrimental.

In higher eukaryotes the UPR is regulated by at least three different classes of ER-transmembrane stress transducers. These include the IRE1 (IRE1 α and IRE1 β), ATF6 (ATF6 α and ATF6 β) and PERK (Figure 9). In the absence of ER stress, these three proteins are sequestered in an inactive state by binding BiP, an ER chaperone. Under conditions of ER stress, however, BiP interacts preferentially with misfolded proteins and dissociates from IRE1/ATF6/PERK, thereby priming UPR activation. The three UPR arms regulate the transcription of distinct transcription factor (TF) and the signalling cascades that control several responses downstream of the UPR.

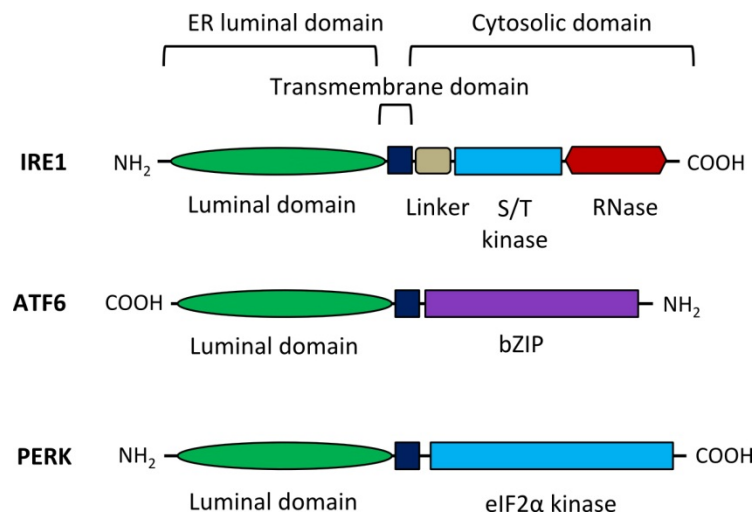


Figure 9: Structural domains of the three ER-transmembrane stress sensors: IRE1, ATF6 and PERK. S/T: serine/threonine kinase, bZIP: basic region-leucine zipper motif, eIF2 α : eukaryotic translation-initiation factor.

2.6.1 IRE1 signalling

IRE1 is a type-I transmembrane protein consisting of an ER luminal domain, a single transmembrane domain and a cytoplasmic domain subdivided into the autophosphorylating serine/threonine kinase and the carboxy-terminal endoribonuclease (RNase) domain (Figure 9). The mammalian genome encodes two isoforms of the IRE1; IRE1 α and IRE1 β . The IRE1 α is ubiquitously expressed in most cells and tissues with high expression levels in the pancreas and the placenta. In turn, IRE1 β is expressed in the intestinal epithelial cells. Deletion of either or both IRE1 isoforms from mouse genome in cell culture, did not affect the transcription of several UPR markers following ER stress. Deletion of IRE1 α (hereafter referred to IRE1) in mice

animal models resulted in embryonic lethality, while deletion of IRE1 β did not cause any developmental effect.

Upon activation, the luminal domains of the IRE1-monomers dimerise/oligomerize, bringing the cytoplasmic domains in close proximity allowing autophosphorylation to occur. This activates their endoribonucleolytic activity, which catalyzes the unconventional splicing of a 26-base intron from X-box binding protein 1 (XBP1) mRNA. This reaction causes a translational frameshift that changes a stop codon in the mRNA and generates the spliced XBP1 (XBP1s), a functionally active TF. XBP1 is capable of binding to different cis-acting elements and enhancing the transcription of UPR-related genes. Binding to the ER stress-response element (ERSE) promoters in the nucleus induces expression of ER chaperones such as BiP, GRP94 and calreticulin while binding to the unfolded protein response element (UPRE) –only dependent on XBP1- regulates the expression of genes that trigger ERAD components [124]. In contrast to XBP1s, unspliced XBP1 variant (XBP1u) is translated into an unstable protein that negatively regulates XBP1s and ATF6 signalling. In addition to mediating XBP1 mRNA splicing, IRE1 also employs the regulated IRE1-dependent decay (RIDD) process to degrade mRNAs encoding ER-targeted protein and reduce the load of protein influx and unfolded protein load in the ER during stress. Hence, activation of RIDD is also responsible for regulating cell fate [125]. In a transition phase between the adaptive and apoptotic responses, the mediated XBP1 mRNA splicing event is attenuated. If stress cannot be resolved, IRE1 activates the proapoptotic caspase 2 (CSP2) signalling causing induction of cell death [125]. IRE1 activation also regulates the c-Jun N-terminal kinase (JNK) pathway mediated by the stress responses. This subsequently leads to the activation of several cell death pathways (described in 2.7.1).

Although IRE1 acts as a positive regulator in cell survival by the splicing of XBP1 mRNA, it may also trigger programmed cell death. In contrast to mammalian cells, yeast studies have revealed that activation of IRE1 branch may also occur by the direct interaction of misfolded proteins with its luminal portion [25]. Kimata *et al.* [126] investigated the yeast IRE1 activation by using mutant IRE1 with a deleted BiP binding site. As this deletion did not affect the IRE1 activation,

they showed that BiP was not the only factor triggering IRE1 activation and thus that IRE1 could recognise a variety of sensors.

The phosphorylation sites are essential for the RNase splicing function of IRE1. Prischi *et al.* [127] elucidated the role of the phosphorylation status and the ligand binding upon the splicing activity of the IRE1. Three distinct phosphorylated regions of IRE1 were purified which were the linker, the activation loop and the RNase domain. They demonstrated that phosphorylation of the activation loop was essential to attain high levels of splicing *in vitro*, and mutation in this site resulted in loss of XBP1 splicing compared to the wild-type IRE1. On the other hand, the linker and the RNase domain showed little or no effect on the catalytic activity of the splicing reaction by IRE1.

2.6.2 ATF6 signalling

ATF6 is a type II ER transmembrane protein, and in the cytosolic region encodes a basic leucine zipper domain (bZIP), which under physiological (non-stressed conditions) is located in the ER (Figure 9). In mammalian cells, ATF6 is encoded by two isoforms ATF6 α and ATF6 β , which are ubiquitously expressed. ATF6 null mice do not experience any phenotypic abnormalities under normal conditions. Analysis of ATF6 α (hereafter ATF6) deficient cells that experienced ER stress revealed their inability to recover homeostasis, while ATF6 β knock-out (KO) did not have any phenotypic abnormalities, even under conditions of stress. Finally, mice with gene KO deficient in both of the two isoforms were lethal at the embryonic stage, suggesting an essential role for these genes in the embryonic development [128].

In response to ER stress, ATF6 protein translocates from the ER to the Golgi apparatus through vesicular transport in a coat protein complex II vesicle and sequentially undergoes cleavage by site 1-(S1P) and 2-(S2P) Golgi resident proteases. This cleavage causes the release of the transcriptionally active cytosolic N-terminal 50 kDa fragment (p50) that contains the DNA binding bZIP, dimerization and transactivation domains. p50 translocates to the nucleus where it binds to and activates the ERSE consensus sequence CCAAT-N9-CCACG (N9 represents a 9 bp

region rich in GC) found in the promoter of several UPR-target genes including BiP, CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) and XBP1 [129, 130].

2.6.3 PERK signalling

PERK is a type-I ER transmembrane protein similar in size to IRE1, but with a low degree of sequence similarity. As their structure is similar, it has been postulated that they share a similar mechanism in response to stress. Indeed, PERK like IRE1, undergoes oligomerisation following ER stress-induced dissociation of BiP, which leads to the trans-autophosphorylation and activation of the PERK kinase module. Activated PERK phosphorylates the eukaryotic translation initiation factor-2 α (eIF2 α) at serine 51 residue (Ser51) and decreases protein synthesis by inhibiting the binding of the initiator methionyl tRNA (met-tRNA) to the ribosomal initiation complex [127, 131]. Therefore by reducing the amount of active eIF2 α (and thus, the rate of translation initiation), PERK signalling prevents the further accumulation of proteins in the ER.

Coincident with repression of global translation, p-eIF2 α selectively enhances the translation of a number of mRNAs containing short upstream open reading frames (uORFs) in the 5' untranslated region. One of these proteins is the activating transcription factor-4 (ATF4) that plays a role in the adaptation to stress by regulating the transcription of several genes. These include genes counteracting oxidative stress such as nuclear factor erythroid 2-related factor (Nrf-2), chaperones such as BiP, the pro-apoptotic CHOP as well as amino-acid transporters [131]. In addition to translational regulation, ATF4 expression is also subjected by transcriptional control. ER stress induces transcription of ATF4 through a mechanism that is not well understood, leading to more mRNA available for preferential translation. Thus both transcriptional and translational control mechanisms enhance the expression of ATF4 and its downstream genes [132, 133].

The downstream gene CHOP can trigger apoptosis in stressed cells but can also upregulate the expression of growth arrest and DNA damage -inducible protein-34 (GADD34). The latter forms part of the protein phosphatase 1 that dephosphorylates eIF2 α following resolution of ER

stress, thus restoring general protein translation in the ER. Importantly both ATF4 and CHOP contain a bZIP protein domain and can bind to promoters to induce the expression of apoptotic target genes [134].

The importance of p-elf2 α has been illustrated in several studies. Gene knock-in mouse models heterozygous for the inserted Ser51 mutation of the elf2 α phosphorylated region, were lethal within 18 h of birth, due to hypoglycaemia. Furthermore, a new function of CHOP was introduced by a recent study by Han *et al.* [135], which was to interact with ATF4 and bind to promoter sequence of genes promoting increase of protein synthesis. Furthermore, they provided evidence for a cooperative ATF4 and CHOP mechanism of action, which was confirmed with qRT-PCR analysis. This illustrated that deletion of either ATF4 or CHOP in tunicamycin (TM) treated cells attenuated the expression of target genes responsible for the apoptotic program. As the expression of both ATF4 and CHOP is upregulated prior to cell death, they postulated that the increase in protein synthesis and oxidative stress are required signals to induce apoptosis. This suggests that reducing protein synthesis may be a step in therapeutic intervention [135].

2.6.4 Overview of the UPR

Misfolded proteins in the lumen of the ER are recognised by three ER transmembrane components: PERK, IRE1 and ATF6 (Figure 10). Activation of the PERK pathway results in the phosphorylation of eIF2 α , which represses protein synthesis [136, 137] but can also result in the transcription of ATF4, in turn triggering the expression of the pro-apoptotic factor CHOP. IRE1 signalling is the most conserved pathway and a key element responsible for the duration as well as the magnitude of the UPR [138, 139]. When activated it leads to the unconventional splicing of a small intron from the XBP1 forming the XBP1s mRNA [140]. The resulting protein is an active TF, which upregulates the genes associated with the ERAD pathway as well as chaperones and foldases. Once activated ATF6 is translocated to the Golgi apparatus, where it is cleaved by resident proteases releasing the p50 TF. This protein induces the expression of XBP1, along with ER chaperones [124].

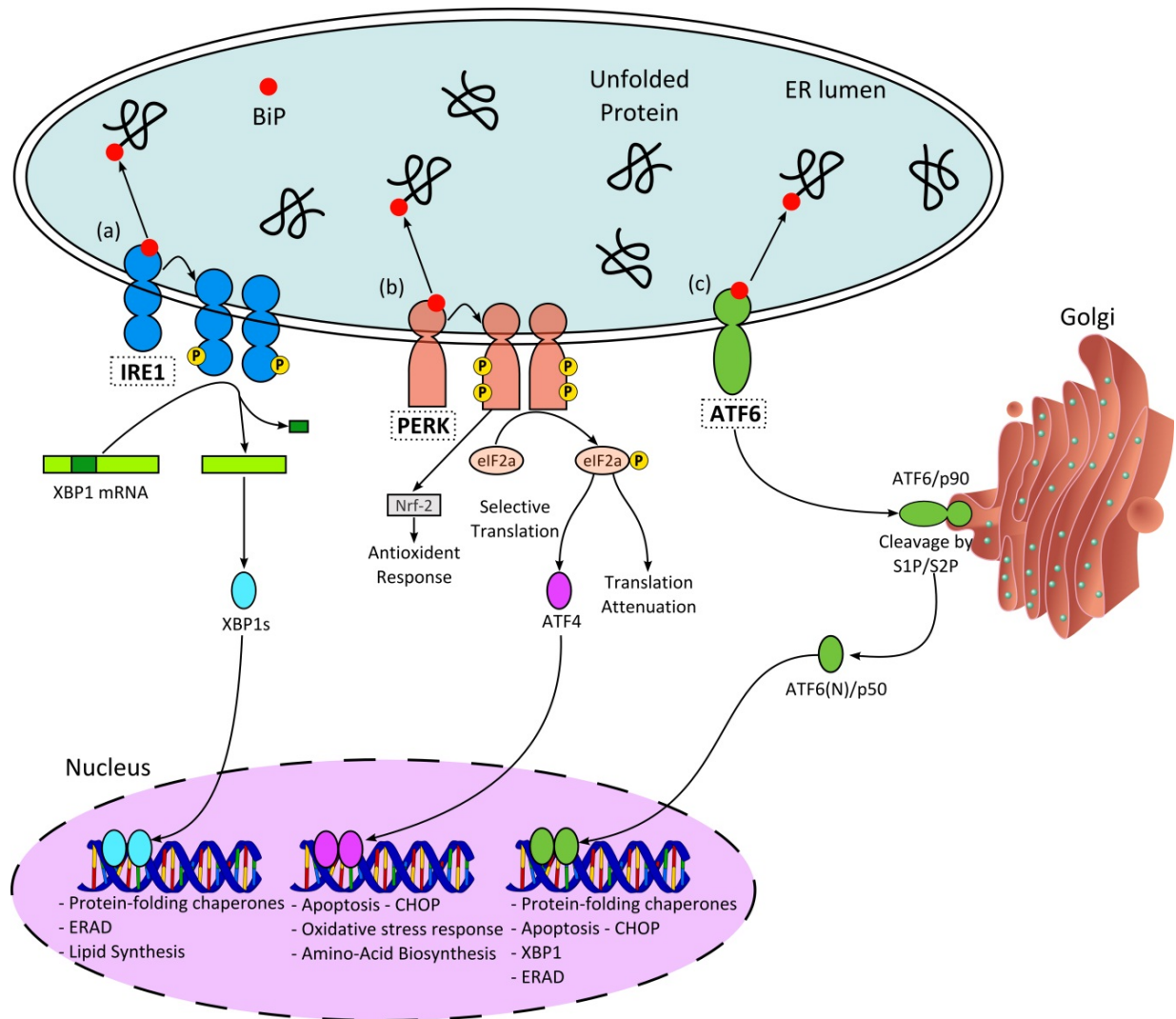


Figure 10: The UPR signalling cascades in response to ER stress. The ER stress-associated accumulation of misfolded proteins in the ER lumen is counteracted by the activation of stress transducer proteins (IRE1, PERK, and ATF6). Activation of the former proteins is triggered by the dissociation of BiP from them and its binding to unfolded/misfolded proteins in the ER lumen. BiP dissociation causes oligomerization and autophosphorylation of stress transducers, each triggering a distinct downstream response. 1) Activation of IRE1 induce the dimerization and trans phosphorylation of its cytosolic kinase domain which in turn activates the endonuclease domain of XBP1 transcription factor mRNA, generates spliced XBP1 (XBP1s) and upregulates the expression of ER chaperones. 2) PERK like IRE1 when activated undergoes oligomerization, which results in transphosphorylation of the alpha subunit of eIF2 kinase - a eukaryotic translation initiation factor - which upregulates the expression of ER chaperones and inhibits translation initiation preventing further protein accumulation in the ER. PERK can also induce apoptosis in terminally stressed cells. Lower levels of eIF2α promote expression of the activating transcription factor-4 (ATF4), which drives CHOP protein expression. 3) ATF6 under ER stress is transported from the ER to the Golgi apparatus where it undergoes cleavage by site 1 and site 2 Golgi resident proteases. This cleavage causes the release of N- terminal cytosolic portion p50. This

cytosolic fragment p50 contains the DNA binding, dimerization and transactivation domain and translocates to the nucleus where it activates molecular chaperone genes, which contain the ERSE domain.

2.7 ER stress link with apoptosis

2.7.1 ER stress signalling pathways inducing apoptosis

Analyses of post-mortem brain tissue samples, of *in vivo* and *in vitro* experimental models have demonstrated the involvement of apoptosis and signs of ER stress in the aforementioned disorders [141]. Saying this, it is challenging to convincingly show the effect of apoptosis in diseased brains, as experiments cannot be done directly in humans. For these reasons, the evidence confirming the role of ER stress induced apoptosis in neurodegeneration comes from studies on animal and cell culture models. However as neuronal death happens very quickly varying from many hours to a day it is difficult to identify cells with apoptotic characteristics [142].

Apoptosis is activated after a prolonged ER stress response when cells pro-survival mechanisms aiming to reduce the protein load and re-establish homeostasis fail. The point at which the cells trigger apoptosis from the prolonged ER stress response remains unknown. Cell death pathways can be either mitochondrion- dependent or independent. The ER serves as a site where pro-apoptotic signals are formed through multiple mechanisms. These include: 1) CHOP, activated from ATF6 and PERK pathways, 2) IRE1 mediated tumour necrosis factor receptor-associated factor 2 (TRAF2)- activating apoptosis signal regulating kinase (ASK1) cascade 3) the B-cell lymphoma 2 (Bcl-2)-associated X protein (BAX) / Bcl-2 antagonist/killer (BAK) dependent Ca^{2+} release and 4) activation of procaspase (PCSP12) which are further described below. Under excessive ER stress, CHOP is one of the most overexpressed genes. Although it is induced from ATF6 and PERK activation, the latter pathway is central for its activation [143]. Studies have shown that the induction of the TF CHOP does not directly promote apoptosis. It has been shown that a number of CHOP-induced genes including GADD34 and the ER oxidase 1a (ERO1 α) induce hyperoxidising ER stress conditions causing an increase in unfolded protein load in the

ER and thus promoting ER stress [144]. On the other hand, overexpression of CHOP itself does not trigger apoptosis following TM and thapsigargin (Tg) treatment [145]. These findings postulate that additional CHOP independent mechanisms are responsible for regulating the cell death response downstream of the PERK pathway.

It has been previously demonstrated that upon IRE1 activation, the adaptor molecule TRAF2 is recruited and regulated by c-Jun NH₂-terminal inhibitory kinase the JIK [146]. This subsequently triggers ASK1, a ubiquitously expressed mitogen-activated protein kinase kinase kinase, which activates the downstream c-Jun N-terminal kinase (JNK) pathway and subsequently leading to cell death. The role of ASK1 has been demonstrated in ASK1^{-/-} primary neurons and MEFs cells which exhibited resistance in response to stress stimuli and were defective in JNK activation and in the induction of cell death [147]. Association of the TRAF2 to IRE1 receptor also mediates activation of PCSP12 by its release from this caspase.

Up to date studies have proposed two pathways by which the cellular Bcl-2 associated proteins Bax/Bak may induce apoptosis. The first mechanism involves the ER membrane, where upon stress both Bax and Bak undergo a conformational change allowing the opening of the channel pore and the release the Ca²⁺ ion fluxes into the cytoplasm [148]. Nakagawa *et al.* in [149] used mixed glial cells from embryonic mice treated with TM or with Tg to provide more evidence on the above idea. Treatment of the cells with the stress agents caused an increase in the Ca²⁺ concentration in the cytoplasm from micromolar (μM) to milimolar (mM) levels, which subsequently activated the Ca²⁺ dependent cysteine protease enzyme (m-Calpain) responsible for activating the mammalian neuronal pheochromocytoma cell line PCS12. Moreover, MEFs cells lacking m-calpain showed reduced ability to cleave and activate PCSP12 cells as well as an increased resistance in the ER stress associated apoptosis [150] like the CSP12 deficient cells [151]. Even though CSP12 and CHOP deficient mice did not present any physiological defects, deletion of these genes provided protection against genetic modifications and environmental ER stress stimuli [151, 152].

The Ca²⁺ flux release from the ER also leads to Ca²⁺ entry into the mitochondria causing depolarization of the inner mitochondria membrane and subsequently release of cytochrome C

(CytC) from the mitochondria into the cytosol. Studies have shown that the expression of Bax or Bak is also required for CytC release [153] by promoting emptying the ER Ca^{2+} stores. This triggers the mitochondria Ca^{2+} uptake, which induced CytC release. CytC binds to apoptotic protease activating factor 1 (Apaf-1) and in the presence of deoxyribonucleotide adenosine triphosphate dATP/ATP promotes the assembly of an oligomeric complex named the apoptosome, which induces activation of PCSP9. CSP9 in turn activates effector caspases such as CSP3 causing DNA fragmentation and apoptosis (Figure 11) [154-157].

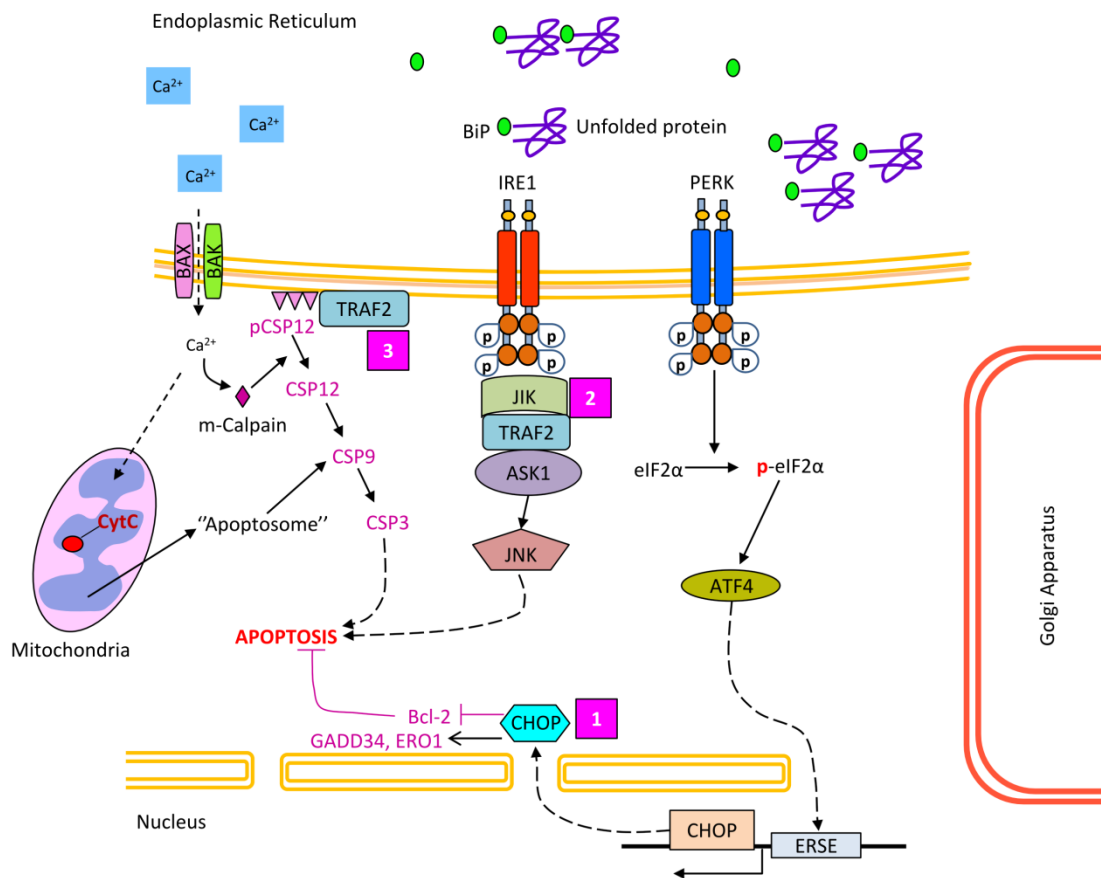


Figure 11: ER stress pathways mediating cell death. 1: Activation of the PERK and ATF6 pathways trigger to the expression of CHOP, which inhibits the expression of the proapoptotic Bcl-2 and promotes GADD34 and ERO1 α expression. The GADD34 and ERO1 α promote the formation of misfolded proteins and induction of stress. **2:** IRE1 activation in response to ER stress binds to TRAF2 with the aid of JIK adaptor molecule and activates ASK1 and JNK. JNK phosphorylates Bcl-2 causing its suppression (not shown) leading to mitochondria induced apoptosis. **3:** The recruitment of the adaptor TRAF2 from IRE1 also enables its dissociation from the ER-resident procaspase 12 (PCSP12) allowing its activation. In addition, upon ER BAX and BAK in the ER membrane undergo conformational changes promoting Ca^{2+} release from the ER lumen to the cytosol, which subsequently activates

m-Calpain and the PCSP12 eventually leading to CSP9 and CSP3 activation. Cytosolic Ca^{2+} fluxes can be also taken up by the mitochondria inducing the release of the CytC in the cytoplasm. This promotes the formation of the 'apoptosome', which activates caspases and leads to cell death.

2.7.2 Role of Translational Attenuation

Translational attenuation is mediated by PERK via phosphorylation of eIF2 α and destruction of its ability to form the pre-ribosomal initiation complex. The capacity for translational attenuation exists in all metazoan cells, but PERK is particularly abundant in cell types that synthesise and secrete large levels of proteins (e.g. immune cells, pancreatic β -cells). For these cells, translational attenuation plays a large role in their survival by preventing nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) induced apoptosis [158]. *In vitro* experiments with neuronal cells have shown that long-lasting downregulation of translation in stressed neurons exposed to an energy crisis may play a key role in the pathological process culminating in neuronal cell injury. This occurs by preventing them from responding to transient ischemia by synthesising neuroprotective proteins [159]. However, this inhibition of protein synthesis also affects the proteins with rapid turnover such as cyclins involved in cell cycle progression. This then leads to cell cycle arrest, which gives the cell time to cope with the adverse conditions. Although suppression of protein synthesis is considered to be a proactive mechanism, experimental findings show that prolonged suppression eventually leads to programmed cell-death [151, 160-163].

2.7.3 Influence of XBP1 unspliced protein in the UPR

XBP1 is a major regulator of the accumulation of unfolded proteins in the ER. Its expression occurs by unconventional mRNA splicing carried out by phosphorylated IRE1, a site-specific endonuclease. The XBP1 pre-messenger RNA (pre-mRNA) is cleaved at two sites by unconventional splicing (in the cytoplasm) mediated by IRE1 endonuclease. Subsequently, the two exons are ligated by a specific RNA ligase to form the mature RNA and excise the 26-nucleotide intron [164]. During translation the pre-mRNA encodes XBP1u protein (pXBP1u). The

hydrophobic region (HR2) of pXBP1u, attaches the protein to the ER membrane and may recruit the XBP1u mRNA to localise it in close proximity to the ER membrane (and to IRE1 receptor) as part of the mRNA-ribosome-nascent chain complex. This mechanism facilitates the IRE1 mediated XBP1 mRNA splicing (Figure 12). Moreover, the presence of the translational pausing motif in the C-terminal region (CTR) ensures termination of translation enabling the splicing process. It is evident that upon translation of XBP1 mRNA the pre-mRNA and mature mRNA encode different proteins named pXBP1s and pXBP1u the size of which are 54 and 33 kDa, respectively [165].

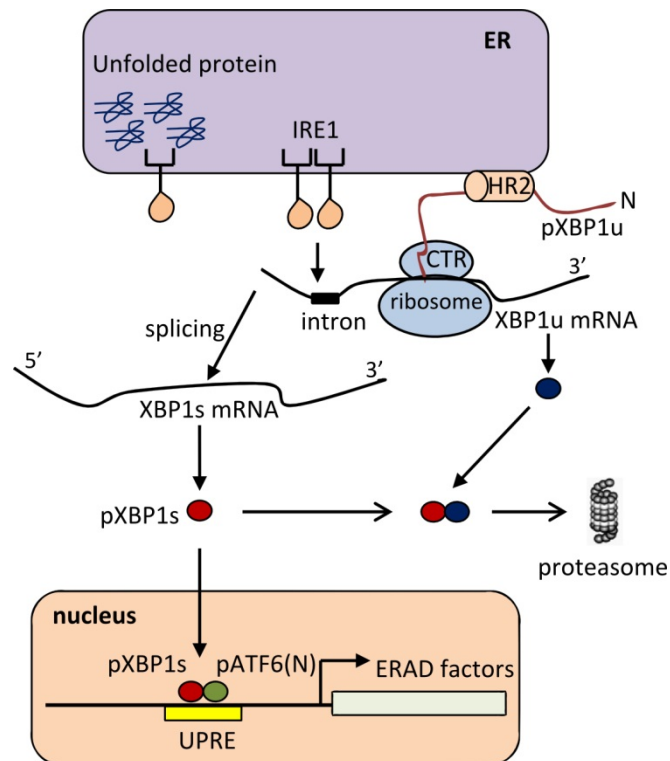


Figure 12: Functions of unspliced XBP1u protein. XBP1u mRNA induces translation of XBP1u protein (pXBP1u), which binds to spliced XBP1 protein (pXBP1s) and promotes its degradation. Under the translation process, a hydrophobic region (HR) of pXBP1u -which is a membrane targeting signal- places the protein into close proximity to the ER membrane and IRE1 receptor as part of the mRNA ribosome nascent chain complex. The pXBP1u interacts with the ribosome with the C-terminal region (CTR); a translational pausing motif that inhibits translation and facilitating IRE1 mediated XBP1 splicing.

Both pXBP1s and pXBP1u contain a nuclear localization signal (NLS) required for its translocation to the nucleus (Figure 13). The pXBP1s also has a transcriptional activation domain that mediates DNA binding to the ERSE and UPRE sites. Binding to these elements induces transcription of ER chaperones genes and of other ERAD components [166, 167]. As pXBP1s is responsible for the transcription of its own genes during the stress response, its stabilization and accumulation is part of a positive feedback loop [167].

On the other hand, the pXBP1u contains a nuclear export signal (NES) and a degradation motif (Figure 13). These enable the protein to shuttle between the nucleus and the cytoplasm and facilitate its proteasomal degradation in the cytoplasm. Besides that, it may also interact with and sequester pXBP1s from the nucleus to the cytoplasm, forming the pXBP1u-pXBP1s complex (pXBP1c) and enhancing its degradation. Thus, pXBP1u acts as a dominant negative regulator of the endogenous pXBP1s. This mechanism causes transcriptional repression of target genes, which are normally activated during the recovery phase of the stress response by the TF [164, 166].

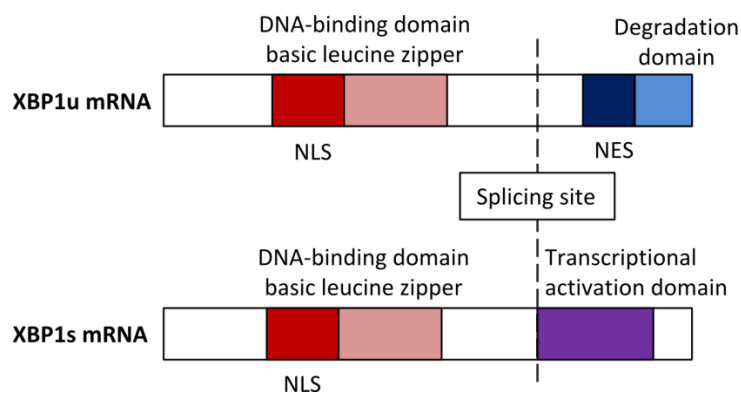


Figure 13: Structure of XBP1u and XBP1s mRNA, which translate the pXBP1u and pXBP1s respectively. The XBP1u mRNA contains a nuclear localization signal (NLS), a nuclear export signal (NES) and a degradation domain. The XBP1s mRNA contains a NLS and a transactivation domain.

2.8 ER stress link with Alzheimer's disease

The ER stress response is upregulated in neurons of patients with AD, with elevated levels of BiP and phosphorylated PERK (pPERK) detected in the brain tissue of patients compared with controls. For instance, Hoozemans *et al.* [42] demonstrated the activation of UPR in human brain specimens of AD patients. For this study 15 control cases (age range 41-91, mean age 76) and 18 clinically diagnosed with AD (age range 65-90, mean age 78), were analysed. Markers of ER stress activation including BiP and pPERK were used to investigate UPR activation in the temporal cortex and hippocampus. Their data demonstrated significant increase in the overall protein expression levels of BiP in AD neurons of the hippocampus and temporal cortex compared to control cases. pPERK was only observed in AD post-mortem material, which strengthens the hypothesis of ER stress activation in AD human brains [42]. Furthermore, the accumulation of unfolded protein in the ER lumen, phosphorylation of eIF2 α , inhibition of protein synthesis, and Ca²⁺ depletion from the ER lumen in tissues from mice subjected to transient focal cerebral ischemia suggested an active role for the ER in neuronal cell death after ischemia [168-172]. In addition, experiments in neuroblastoma cells demonstrated that high levels of APP expression pre-prime the cells for UPR induction and appeared to result in shunting toward apoptosis as detected by the upregulation of proapoptotic marker CHOP [173]. Colombo *et al.* [174] further showed that JNK is a proapoptotic output of the activated ATF6 and IRE1 signalling, as by mediating APP phosphorylation and increasing A β secretion contributes to ER stress mediated apoptosis.

A number of factors can contribute to ER stress activation in AD. First, accumulation of aggregation-prone mutant APP in the ER and/or Golgi apparatus has been demonstrated to occur in some cases of FAD. Additionally, Ferreiro *et al.* [175] showed that A β peptides trigger ER stress by stimulating the release of Ca²⁺ from ER stores. Oh *et al.* [176] have also shown that A β peptides inhibit the proteasome, which is essential for the ERAD of misfolded proteins. This may be one reason why AD-related mutations in PS-1 can lead to a different APP processing and greater A β production, and therefore potentiate ER stress-induced apoptosis. Mutations in APP are also known to promote apoptosis via ER stress specific caspases, namely CSP12 in mice

and CSP4 in humans, suggesting that ER stress may contribute to neurodegeneration in AD. Indeed, Nakagawa *et al.* [151] showed that murine neuronal cells lacking CSP12 are resistant to A β -induced cell death. Moreover, wild-type APP (as well as some PS-1 mutations) has also been shown to upregulate the expression of the apoptotic-triggering factor CHOP [177].

In addition to the intracellular effects of amyloid and its aggregates, recent work has implicated the binding of some forms of extracellular oligomeric A β to the plasma membrane as one of the mechanisms of amyloid-mediated toxicity [178]. A β binds to the phospholipid membrane itself and disrupts it [179] by causing the formation of pores called A β channels leading to increased ion transport and possibly Ca²⁺ dyshomeostasis [180, 181]. It has been proposed that the ion imbalances (including Ca²⁺) caused from the A β channels, may be an early event in the A β -induced neurotoxicity [182]. There is also strong evidence that extracellular A β oligomers can be re-internalised [112] via a variety of mechanisms, including interaction with lipid rafts and their associated receptors or by endocytosis [183, 184].

A link between extracellular A β oligomers and ER stress has been demonstrated in cell culture. Human embryonic kidney HEK293 cells cultured in the presence of extracellular oligomers (but not fibrils) allowed their internalisation and resulted in mild ER stress in some but not all members of the cell population as measured by increased XBP1 splicing. However, in cases where cells were treated with mild amounts of a stressing agent (like TM) in addition to the presence of oligomers, a marked decrease in viability was observed. This suggested that extracellular A β oligomers worsen the ER stress in the already pre-primed cells, by inducing higher levels of apoptosis than if treated under each condition separately [185]. In a pathological scenario, this could mean that the presence of A β oligomers could diminish the capacity of the cell to deal with normal insults effectively and cause the ER stress response to be overactive, leading to neuronal cell loss.

A second link between ER stress and the presence of extracellular A β oligomers comes through the lipid rafts. Lipid rafts contain a lot of cholesterol, depletion of which has been shown to slow A β production, by altering the rate of α -secretase processing. High levels of cholesterol, conversely, cause an increase in the amyloidogenic processing of APP, leading to a higher

accumulation of A β ₄₂ oligomers [184]. During the course of ER stress activation, lipid biosynthesis, including cholesterol synthesis, is upregulated as the ER attempts to expand in order to accommodate the overcrowding due to unfolded proteins [186]. This activation is independent of the actual lipid content of the cells. Therefore, it could easily lead to hypercholesterolemia, and thus, further production of toxic amyloid species.

As a whole, the aforementioned evidence of ER stress in AD suggests that a possible treatment avenue for neurodegeneration is to increase the ability of the UPR to deal with stress by preconditioning the response, upregulating chaperones to enhance protein folding, and/or blocking apoptosis by targeting the appropriate enzymes with small molecules [187]. However, it is also known that some genetic mutations associated with AD, such as in the PS genes, perturb the ER function and likely reduce its capacity to deal with stress by shifting the balance of cellular fate toward the apoptotic pathways rather than recovery [187]. Specifically, mutations in PS-1 are associated with increased susceptibility to ER stress and aberrant UPR signalling, while a PS-2 splicing variant associated with sporadic AD also interferes with UPR signalling via IRE1 [166, 188]. Indeed, PS-1, which localises to the ER membrane, is proposed to form complexes with BiP and UPR stress transducers, precluding BiP dissociation and preventing transducers from sensing the folding environment of the ER [189, 190].

2.9 ER related stressors in AD

The deposition of A β aggregates in AD has been demonstrated to induce aberrant Ca²⁺ signaling activating ER stress as well as oxidative stress (Figure 14). As A β aggregates can result in neuronal apoptosis, they have an important role in the underlying AD pathology.

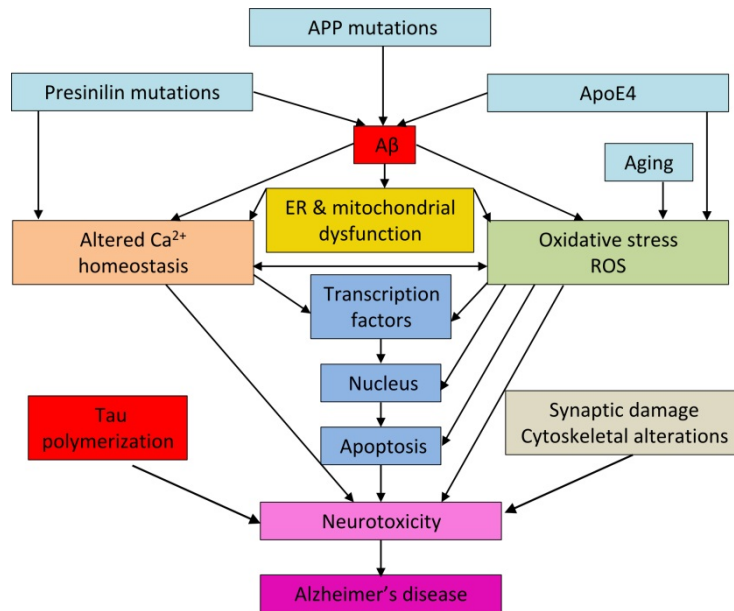


Figure 14: The implication of Aβ in Alzheimer's disease. Increased Aβ deposits are formed either by genetic mutations (i.e. APP, presenilin genes) or the presence of apolipoprotein-4 (ApoE4) gene or other signalling pathways such as oxidative stress or calcium (Ca²⁺) dyshomeostasis. It can affect the ER causing ER stress and alter the mitochondria flux balances. Alternative it may directly induce oxidative stress reflected by the ROS accumulation and Ca²⁺ dyshomeostasis (altering mitochondria fluxes and inducing oxidative stress). These show the pathways through which Aβ may implicate neuronal cell death and eventually Alzheimer's disease.

2.9.1 Reactive Oxygen Species

Growing evidence indicates oxidative stress to be linked with AD initiation and progression as increased concentration of ROS has been detected in post-mortem brain tissue samples of AD patients [191]. However, it is not known whether oxidative stress is a cause of neuronal cell death or a downstream manifestation of other pathological pathways.

ROS are cytotoxic products that can arise from two different organelles, the ER and the mitochondria. Under normal physiological conditions, ROS are released within the ER as part of the natural function for proper oxidative protein folding. Accumulation of these ROS could damage important ER-resident chaperones such as BiP, PDI, and calreticulin, resulting in impairment of function [192-194]. On the other hand, mitochondria release ROS as a natural by-product of aerobic metabolism. The mitochondria play a prominent role in the regulation of cellular metabolism, production of ATP and are intricately involved in buffering cellular levels of

Ca^{2+} and in cross- signalling with the ER. Deficiencies in mitochondrial function could thus significantly affect cell viability, Ca^{2+} homeostasis and ER function. The involvement of mitochondria in neurodegeneration centres on the inhibition of the electron transport chain [195, 196] and the concomitant increased release of the ROS. These species in turn damage mitochondrial proteins such as mitochondrial DNA, thus reducing the efficiency of oxidative phosphorylation [197].

It has been proposed that progressive accumulation of A β in mitochondria is associated with diminished enzymatic activity of respiratory chain complexes and therefore altered mitochondrial membrane potential, a reduced rate of oxygen consumption [198] and the associated increased release of ROS [197]. Accumulation of nitrotyrosine and protein carbonyls caused from oxidative protein damage along with markers of oxidised mitochondrial and nuclear DNA such as 8-hydroxy-2'-deoxyguanosine of cortical areas and the cerebellum were increased in AD brains [199]. These suggest oxidative stress to be an important factor contributing in the development and progression of AD.

2.9.2 Calcium Dyshomeostasis

Accumulating evidence suggests that many FAD associated PS mutations perturb intracellular Ca^{2+} mechanisms with the most significant signalling cascade disrupted involving the ER Ca^{2+} homeostasis. This impairment occurs in both FAD and sporadic cases prior to the manifestation of the disease hallmarks [200].

The concentration of Ca^{2+} in the cell is finely tuned by several mechanisms keeping it low in the cytosol ($\sim 10^{-7}$ M), whereas its concentration in the extracellular medium ($\sim 10^{-3}$ M) and in the ER lumen is high. These concentration gradients across the plasma and ER membrane allow Ca^{2+} to be used as a signal. There are three key transport mechanisms in maintaining Ca^{2+} homeostasis. Firstly, Ca^{2+} pumps in the plasma membrane help in keeping cytosolic Ca^{2+} concentration low in resting cells. Neuronal cells have an additional Ca^{2+} transport protein on their plasma membrane, the sodium-calcium pump mechanism that couples the efflux of Ca^{2+} to the influx of

sodium (Na^+). A second high affinity, ATP-dependent pump exists on the ER membrane, which enables the transport of Ca^{2+} into the ER against a steep concentration gradient and finally a low-affinity, high capacity pump on the inner mitochondrial membrane, responsible for restoring Ca^{2+} concentration after a signalling event. This third pump relies on the mitochondrial membrane potential generated by oxidative phosphorylation [8].

Even though recent evidence points to the ability of neurons to maintain Ca^{2+} homeostasis at early stages of neurodegeneration (AD in particular), possibly by altering its handling in the ER [201], prolonged dysregulated Ca^{2+} signalling has been shown to lead to neuronal cytoskeleton alteration, synaptic loss and neuritic atrophy. Compromised ER activity leads to excessive Ca^{2+} in the cytosol, which is taken up by the mitochondria. This build-up eventually leads to opening of mitochondrial permeability-transition pore and apoptosis. The mitochondrial pro-apoptotic pathway acts via the multidomain proapoptotic molecules Bax or Bak that form the main gateway of the Ca^{2+} -mediated apoptosis. Although the mechanism by which they are activated is incompletely understood [202, 203], it is known that activation of either Bax or Bak is sufficient to trigger apoptosis [204]. Their activation is manifested as intramembranous homooligomerization, resulting in the permeabilization of the outer mitochondrial membrane and, in turn, releasing the intermembrane space proteins including CytC. The latter binds with Apaf-1 and CSP9 forming a post-mitochondrial apoptosome complex that amplifies effector caspase activation.

It has been suggested that the role of the ER and mitochondria in Ca^{2+} homeostasis may contribute to premature death of neurons. Ca^{2+} buffering processes require energy and compromised mitochondrial activity may therefore impair the restoration of homeostasis. Even in cases where mitochondrial function is not initially affected, the high metabolic demand incurred by the cell's effort to restore Ca^{2+} homeostasis would inevitably increase oxidative phosphorylation and, thus, the production of ROS. In parallel, ROS could damage ER proteins, while the decrease in ATP levels due to reduced efficiency of oxidative phosphorylation would compromise ER and proteasomal function, causing the accumulation of misfolded proteins [205]. As Ca^{2+} dysregulation leads to failure of synaptic functions and neuronal loss,

characteristics of neurodegenerative disorders, it would be vital to elucidate its mechanism of action in the impairment of ER Ca^{2+} homeostasis for the development of therapeutics at early stages of the disease pathogenesis [206].

2.10 Treatment of ER-stress induced diseases

2.10.1 Proposed treatments related to ER stress mechanisms

Because ER stress is involved in so many different diseases including many highly prevalent ones such as neurodegeneration, diabetes, and cancer, the development of drugs that affect UPR signalling has attracted a lot of interest. In principle, it should be possible to manipulate any of the three branches of the ER stress response for the desired effect, but it has been difficult to find specific treatments in practice because of the high degree of cross signalling between the pathways. Some promising discoveries have been made in the past decade, however, which suggest that ER stress represents a valid drug target [207]. In the specific case of neurodegenerative diseases, it is likely that interfering with ER-stress induced apoptosis would be a means of slowing disease progression.

One logical strategy is to increase protein-folding capacity by stimulating the overexpression of protein folding chaperones without the associated activation of other, more detrimental, ER stress events. Related to this is the use of small molecule chaperones to directly affect the solubility and/or folding of the accumulated proteins. Both of these strategies appear promising. A body of evidence from the field of psychiatry suggests that certain mood-altering drugs used in the treatment of diseases such as bipolar disorder are able to selectively increase BiP levels without triggering ER stress. These include lithium [208, 209], valproate (VPA) [209, 210], and possibly very high (supratherapeutic) doses of carbamazepine [210], but not lamotrigine [209]. The effects of VPA appear to be particularly noticeable in the frontal cortex, an area affected by AD, and the CA1 region of the hippocampus [210]. A study of the mechanism of VPA showed that it also increases the expression of protein disulfide isomerase

and the HSP90 homologue GRP94, two folding chaperones, as well as calreticulin, which acts in protein quality control [211]. Interestingly, the effect of VPA seems mainly to act through lipid/ER membrane biosynthesis by inhibiting the enzyme glycogen synthase kinase 3 α [211]. How this is linked to differential upregulation of chaperones remains unclear.

Similarly, Kudo *et al.* [189] screened a library of 10,000 compounds for the potential to upregulate BiP expression using a reporter assay. They identified one compound, which they named BiP Inducer X (BIX, 1-(3, 4-dihydroxyphenyl)-2-thiocyanate-ethanone), which selectively increased BiP expression levels and protected cultured neuroblastoma cells from chemically induced ER-stress mediated death. Further testing of BIX showed that it was also neuroprotective in a mouse model of cerebral ischemia. BIX appeared to function via ATF6 binding to the ERSE response element and slightly increased overexpression of GRP94, calreticulin, and CHOP [189]. A similar high-throughput screen of antioxidant compounds to combat ER stress, identified one, norbergenin-11-caproate, which increased BiP expression about five-fold. The effect appeared to be at least partially independent of ROS production [212].

Small molecule chaperones, low-molecular weight chemical compounds that help promote protein folding; have also shown promise in their ability to alleviate ER stress by helping to fold accumulated proteins in the ER. These include phenylbutyric acid (PBA), taurine derivatised ursodeoxycholic acid, and glycerol [213-215]. Both PBA and glycerol prevent the formation of protein aggregates and plaques in cell culture models of AD, leading to a demonstrably lower level of oxidative stress [215]. PBA has the ability to alleviate ER-stress induced defects in APP trafficking and γ -secretase processing, without a significant increase in amyloid production [216]. PBA also protects against ER-stress induced cell death after ischemic shock both in neuroblastoma cell culture and mouse models. The protection was clearly associated with a reduction in PERK signalling, resulting in lower levels of CHOP and CSP12 activation. Interestingly, the effect of PBA in this study appeared to rely on the presence of functional mitochondria, as cell treated with mitochondrial inhibitors were not protected [213]. This suggested a possible hurdle in using PBA to treat diseases where mitochondrial function is

clearly impaired (e.g. Alzheimer's disease), although depending on the timing of the pathological events, it could be useful in treating early stage disease (i.e. before mitochondrial function is lost).

Another approach has focused on screening compound libraries, natural products or their derivatives for the ability to prevent ER-stress mediated apoptosis. Salubrinal, which acts selectively on the PERK-mediated signalling pathway, was discovered in a high-throughput screen of 19,000 small molecules for compounds that protected against ER-stress induced apoptosis in PC-12 cells. Salubrinal is a selective inhibitor of ER-stress mediated apoptosis. It enhances eIF2 α phosphorylation levels by inhibiting the eIF2 α phosphatases. It decreases translation rates without, however, triggering UPR target genes such as XBP1 and chaperones (BiP, GRP94) and finally enhances downstream ATF4 signalling. While data on salubrinal have been mixed [217] key *in vivo* as well as *in vitro* [218] studies suggested its potential in the treatment of neurodegenerative conditions including AD [219], PD [220] and HD [221] by preventing apoptosis. Salubrinal therefore inhibits protein synthesis without activating the transcription-mediated cascade of the UPR. In addition, another compound named guanabenz also acts on the eIF2 α -ATF4 arm [222, 223] by inactivating just the stress-induced eIF2 α phosphatase complex of the GADD34 associated with the protein phosphatase 1 catalytic subunit (GADD34-PP1c complex) and thus showing selectivity for ER stress activation only [222, 224]. These observations illustrate that guanabenz is a better pharmacological compound in the long term due to its specificity, as salubrinal may cause unwanted effects due to the unrestrained translational attenuation [223].

Other high-throughput screens have isolated several compounds with potential to decrease ER stress-mediated death in cell culture models with chemically induced ER stress [225-230]. Many of the compounds that have been identified are flavones [231-233]. Interestingly, the biochemical effects of the compounds appear to vary widely. Some appear to have very non-specific effects on ER stress activation, effectively suppressing the activation of the entire set of responses [233, 234]. Others have very clear specific effects, often outside of the main ER stress signalling pathways including targeting ATF4 activation and the Nrf-2 antioxidant response

without activating ER stress [231], inhibiting glycogen synthase 3 [232], or activating the anti-inflammatory response [235]. The latter observation, while somewhat surprising, is consistent with the observation that non-steroidal anti-inflammatory medications can decrease ER stress mediated cell death in culture [236]. While many of the results need to be confirmed in tests that are more representative of disease conditions, this suggests that ER stress can be alleviated by manipulating other biochemical pathways which link to ER homeostasis, rather than by targeting the three transmembrane signalling proteins directly.

Finally, a recent study tested the ability of existing medications to affect ER stress signalling. Sanz *et al.* [237] explored the ability of monoamine oxidase (MAO) inhibitors, which are currently used to alleviate PD symptoms, on ER stress activation in cell culture. Pre-treatment with a MAO inhibitor decreased XBP1 splicing and expression of CHOP and reduced cell death following subsequent chemical induction of ER stress [237]. This suggests that some current treatments of PD symptoms may work through ER stress signalling.

2.11 Potential targets for future drug development and clinical implications

In addition to the treatment strategies discussed above, another strategy for developing new therapeutics that target ER stress could be to try and define the cellular control points that determine the point of no return for cells and irreversibly initiate apoptosis. In one very important study, Lin *et al.* (2007), showed that prolonging IRE1 signalling improved cellular viability and delayed the onset of apoptosis, suggesting that developing drug targets that stimulate IRE1 signalling could be a viable strategy for preventing cell loss. The importance of IRE1 as a target was also recognised by Bouhcecareilh *et al.* [238], who developed a high-throughput screening assay to monitor the dimerization/oligomerization and phosphorylation properties of the cytosolic domain of IRE1. They showed *in vitro* that dimerization/oligomerization of the cytosolic domain of IRE1 correlates with the autophosphorylation ability of this domain and its endoribonuclease activity towards XBP1 mRNA [238]. The assay could be used to test compounds for their ability to modulate IRE1

activity. An inhibitor of the IRE1 activation was found to be the 4μ8C (4-methyl umbelliferone 8-carbaldehyde) compound, an aldehyde acting by binding covalently to a unique IRE1 endonuclease residue, lysine 907 (K⁹⁰⁷), via an imine forming a Schiff base. This binding causes the blockade of any substrate binding to the IRE1 active site and thus inhibits IRE1 activation and subsequent gene expression downstream of this receptor [239].

Recently, Wang *et al.* [240] identified small molecules that inhibit PERK mediated phosphorylation *in vitro* using a virtual library screen. Following this, the group investigated the structure activity relationships for the most potent inhibitors and, based on these, pinpointed the desired and necessary inhibitor characteristics, in the hope that this will accelerate selective inhibitor design. A potent and selective PERK inhibitor was found to be GSK2606414 with the chemical name 1-(5-(4-amino-7-methyl-7H-pyrrolo [2, 3-d] pyrimidin-5-yl) indolin-1-yl)-2-(3-(trifluoromethyl) phenyl) ethanone molecule. This drug was orally administered to mice, showing its ability to penetrate the blood-brain barrier and spread widely to all areas of the brain [241]. The pharmacological effect was inhibition of the PERK signalling and delay of the prion disease affected mice, thus exhibiting a neuroprotective effect. Toxicity of the drug was not investigated as the mice were sacrificed early due to weight loss and high levels of glucose in the blood [241].

ER stress is believed to be involved in a number of diseases apart from neurodegeneration, including cancer and diabetes. As such, its molecular mechanisms, functional implications and treatment methods are the topic of on-going studies. As mentioned above, various small molecules are being tested for their ability to halt the response progression. Interventions at the point of mutant/overexpressed protein interaction with glucose regulated chaperones and foldases may not be a sufficiently targeted approach and can affect general cellular homeostasis. Drug development for cancer applications focuses on promoting the pro-apoptotic features and therefore selecting molecules that inhibit the cell survival IRE1-XBP1 pathway. This pathway mediates cell survival under conditions of hypoxia that induce ER stress. However, drug development and clinical studies on neurodegenerative disorders need to focus on promoting the survival mechanisms and inhibiting the pro-apoptotic pathways downstream

of XBP1 splicing. Essentially, it would be useful to develop therapeutics that would modulate the same signalling pathway, but with opposite effects to treat different diseases.

2.12 Model organisms in AD

Scientists use a wide variety of model organisms for their experimental investigations, which range across the evolutionary tree of life. More specifically, they range from relative simple organisms such as yeast and bacteria up to mammalian cells and animal models [242]. Although animal models are essential in the pharmacological studies, they provide many challenges. They are relatively expensive as well as labour- and time-intensive as they require a lot of care and necessitate a high volume of screens with several generations of breeding [243].

Progress has been made in elucidating AD pathology and the genes implicated by developing genetically modified mouse models. These models have allowed the understanding of many molecular mechanisms and are essential in the assessment of novel therapeutics [244]. Although these studies bring us a step forward in elucidating AD, they are not suited for interactions of gene products and their analysis is restricted to a limited number of genes, as not every gene can be KO. Alternatives to the animal models are cell culture based models. They are highly used as they permit high throughput screening, which help the discovery of important compounds and drug targets. These systems act as unique tools in elucidating genes and proteins affecting the progression of the disease [245]. Several cell models have been established in order to study the role of ER stress in neurodegeneration, with the most common ones employed being those with genetic mutations [26, 246].

2.12.1 SK-N-SH cells

Biedler, developed human neuroblastoma SK-N-SH cells in 1973 from the brain tissue of a 4-year old girl patient, who suffered from neuroblastoma. They were originally established from metastatic neuroblastoma tissue preserved *in vitro* and have now been extensively used in

assessing neurotoxicity and neuroprotection [247]. SK-N-SH cells exhibit biochemical characteristics of neuronal cells and have an average doubling time of 44 h [247, 248]. Their neurochemical characteristics are a) their high dopamine- β -hydroxylase activity (an enzyme present only in the nervous system), b) their neurochemical markers and c) induction of possible damage caused from A β treatment [247, 249]. The methods developed for culturing such cells during extended periods gave the luxury to discover many biological phenomena.

2.13 Computational modelling in biology

Modelling is the process of constructing and representing simplified versions of biological phenomena via mathematical simulations. Computational approaches combined with experimental ones are essential to get a thorough understanding of the complex biological systems and the molecular interactions involved in fatal diseases such as AD and cancer [250]. Such approaches are pivotal to understand better how such complex molecular mechanisms are connected and how their interruption may contribute to the development and progression of the disease [251].

Generation of such models is achieved by choosing specific mathematical strategies in conjunction with selective software tools. Execution of a model over time (simulation), allows not only assessing specific hypotheses with *in silico* experimentation but also forecasts outcomes that can be validated by *in vitro* and *in vivo* studies.

A high fidelity model is characterised by its ability to provide information on the behaviour and dynamics of a cellular process without the need to test it in real life. For such a model, it seems ideal to include all biological reactions mechanisms but in reality, representation of too many of them may be counterproductive. This may be due to the high underlying computational demand and to overparameterisation, as the number of parameters used typically exceeds the number of variables that can be measured. Realistically, most of the biological mechanisms are often too convoluted or simply unknown and so experimental data are not available. As

formulation of the model must be accomplished with the available literature data, it is often constrained [252].

Prior to selecting a modelling approach, it is essential to understand the nature of the underlying reactions of the biological system of interest. The system behaviour may be solved with different modelling approaches. In a deterministic approach, the output is uniquely defined by the initial conditions and the parameter values used. However, the behaviour of systems characterised by low concentrations of reactants or that of extremely small systems comprised of a small number of molecules is intrinsically stochastic. Hence, although deterministic models in this case may provide reasonable results for long-term observations they are not applicable in general. Instead, one has to resort to stochastic simulation approaches that incorporate the random nature of individual events into the computed output. As stochastic models add more computational complexity, the simpler approach (deterministic behaviour) is preferred.

Deterministic models describe processes at several levels of temporal variations. These are defined as i) dynamic models describing time-dependent measurements and ii) static (or steady state) ones describing time-independent measurements. Most of the biological reactions have dynamic processes as they describe time-dependent phenomena such as environmental changes or essential dynamic cellular functions. Modelling dynamics of cascades that capture the aforementioned changes depend on differential equations (ordinary or partial, time-dependent differential equations) [250, 253]. In cases where the timing and frequency of measurements is repetitive and occurs under the same rather than varying conditions, the model has poor dynamic. Thus, dynamic models may also have constraints even with the presence of a plethora of data.

Another consideration to be made is the scale, choosing either single-cell or cell-population model. A collective behaviour in the cell population can be further characterised as homogenous or heterogenous the latter incorporating a more realistic behaviour but adding computational complexity. Analysis of heterogeneous cell population system is permitted by flow cytometry, a powerful single-cell characterization technique [254].

In studies where the question to be answer depends on non-homogenous cell populations, the simplest computational approach is preferred. For instance, expansion of polyclonal cultures to study the UPR progression induced by ER stress, would generate time-course data incorporated in a heterogenous cell-population model.

2.14 Previous UPR models

To date, there are five mathematical models describing the ER stress and UPR signalling in yeasts and mammalian cells, as shown in Table 2.

Table 2: Computational UPR models.

UPR model	Pathway	System	Year	Reference
Raden	IRE1	Yeast cells	2005	[255]
Pincus	IRE1	Yeast cells	2010	[138]
Trusina	PERK	Yeast & mammalian cells	2008	[158]
Rutkowski	BiP, CHOP & GADD34	Mammalian cells	2006	[256]
Erguler	IRE1, PERK & ATF6	Mammalian cells	2013	[257]

2.14.1 Raden model for IRE1 in yeast

Activation of the IRE1 pathway was first described by a model for yeast by Raden *et al.* [255]. The model assumed that BiP dissociates from IRE1 causing IRE1 activation by dimerization and autophosphorylation (Figure 15). The authors acknowledged that this model was unable to reproduce the experimental data from a stress-induced experiment in *Saccharomyces cerevisiae* [255]. Thus, they concluded that the single BiP-IRE1 interaction by itself is not sufficient to describe the activation of the IRE1 pathway. The dual activation of IRE1 has been demonstrated in both biological and model systems [255, 258]. Gardner *et al.* [258] proposed

that in yeast, IRE1 may sense the unfolded or misfolded proteins in a BiP independent manner through direct binding with an N-terminal luminal domain.

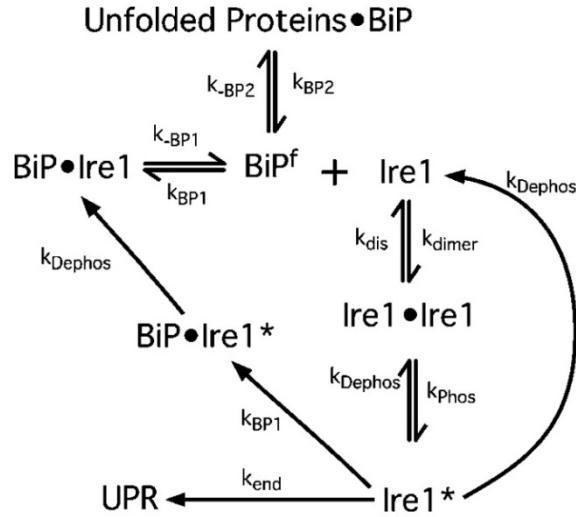


Figure 15: Raden model for IRE1 activation regulated by BiP in yeasts. BiP^f is the pool of free BiP, Ire1 is the kinase inactive Ire1 (nonphosphorylated IRE1), while Ire1^* is the activated Ire1 (phosphorylated IRE1) [255].

2.14.2 Pincus model for IRE1 in yeast

Pincus *et al.* [138] developed a mechanistic model based on in *Saccharomyces cerevisiae* to better understand the IRE1 pathway. This model justified the role of unfolded proteins in activating IRE1 in yeasts. This model was based on the experimental observations of Gardner *et al.* [138] and so corroborated the assumption of two-step activation mechanism for IRE1 in yeast. The first step involved BiP dissociation from IRE1 and its binding to unfolded proteins. Next, IRE1 directly sensed the unfolded proteins with its peptide-binding domain and activated the IRE1-dependent UPR pathway, as has been previously experimentally confirmed [258].

More specifically, the constructed model shown in Figure 16 considered three forms of the IRE1: a) free inactive IRE1_i , b) inactive IRE1 in a complex with BiP ($\text{IRE1}_i \bullet \text{BiP}$) and c) active IRE1 in a complex with an unfolded protein ($\text{IRE1}_a \bullet \text{UP}$). The unfolded proteins can activate the IRE1 either in their reduced (UP_r) or oxidised state (UP_o), but only the proteins in the oxidised state

get folded (FP). The number of unfolded proteins depends on the unfolded protein flux as well as the redox potential, determined by the ratio of Ero1 versus dithiothreitol (DTT). The Ero1 (transcribed by the yeast HAC1 TF, the XBP1 in mammals) is a molecule oxidising the proteins and inducing their proper folding while DTT does not make part of the natural system and was used to disrupt homeostasis. DTT impairs homeostasis by catalysing the reduction of disulphide bonds causing the production of unfolded proteins that trigger ER stress. IRE1 activation occurs by its direct interaction with the unfolded proteins inducing the splicing of HAC1 (a yeast TF similar to XBP1 in mammals) and upregulating the production of Ero1 and BiP. BiP, which allows the protein folding process, exists in three forms: a) monomer (BiP), b) in a complex with IRE1 ($\text{BiP} \bullet \text{Ire1}_i$) and c) bound to unfolded proteins ($\text{BiP} \bullet \text{UP}$) (Figure 16). Although BiP is able to bind both UP_r and UP_o proteins, it only facilitates the folding of the former ones. The interactions of the model were described as simple chemical equilibrium and the different variables were taken either from the literature or from experimental data.

Three mutant yeast cells lines created to validate the model, which were the followings: a) wild-type, b) IRE1 bipless (where the IRE1 lacked the BiP interaction motif) and c) HAC1 Δ (which lack the ability to increase BiP and ERO1 activity). The model was able to predict the role of BiP in IRE1 activation and to fit *a posteriori* the data from a DTT titration time-course experiment in the wild-type, IRE1 bipless and HAC1 Δ .

This model is explicative as it describes the IRE1 activation. It is also predictive as it was able to demonstrate the role of BiP in IRE1 activation and deactivation as well as to reproduce the data from the mutant cell lines *a posteriori*. Overall, this is a dynamic UPR model showing the dual role of BiP in activating/deactivating the IRE1. BiP acts as 1) a buffer lowering the sensitivity of the system by preventing UPR activation in the low concentration of unfolded proteins and 2) ends the UPR upon low unfolded protein concentration by binding the IRE1. However the authors recognise that the model is a simplification of reality and that other functions of IRE1 need to be included [138].

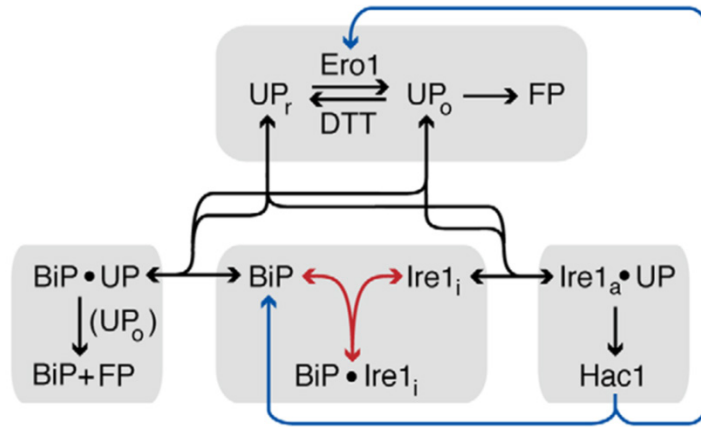


Figure 16: Pincus schematic model for IRE1 activation. Blue arrows show the feedback points and red arrows the IRE1/BiP interactions. UP: unfolded proteins, UP_r: unfolded proteins in their reduced state, UP_o: unfolded proteins in their oxidised state, FP: folded proteins [138].

2.14.3 Trusina model for PERK

A simplified model created by Trusina *et al.* showed the importance of PERK [158] (Figure 17). It described the UPR in yeasts and in mammalian cells by presenting all protein interactions with a single variable and several reaction rate constants. This model explained 1) the dynamics of PERK and IRE1 receptors in mammalian UPR, 2) how the absence of PERK in yeasts (also observed in extreme cases of mammalian non-secretory cells) affects the stress response and 3) the effects caused from both the synthesis of chaperones and translational attenuation on preventing stress.

The assumptions made were the following: a) translational attenuation was activated prior to eIF2 α phosphorylation which occurs five minutes later according to experimental observations and b) inactivation of IRE1 and PERK activity occur by the chaperones in a similar manner. Previous studies have shown that chaperones may act as inhibitors towards their receptors causing inhibition of their dimerization and their activation [259].

The changing rate of the free unfolded proteins was dependent on several factors. These consisted of: 1) the translation rate of the proteins entering the ER, 2) the induction of stress caused either by physiological changes or by artificial induction in the lab, 3) the influence of

the chaperones in folding/unfolding and degradation of unfolded proteins and 4) the dilution process of the unfolded proteins during division of the cells.

This model showed the importance of the PERK pathway through its ability to reduce the load of unfolded proteins caused by the inhibition of translation and thus of the proteins entering the ER. They demonstrated that upon low ER stress, translational attenuation is not required, as is the case in non-secretory cells like neurons. On the other hand, they postulated that for cells pre-conditioned to ER stress as occurs in AD, this mechanism is necessary in order to inhibit cell death. Finally, this model was not well detailed and was solely a test to understand the importance of translational attenuation in the monitoring of ER stress [158].

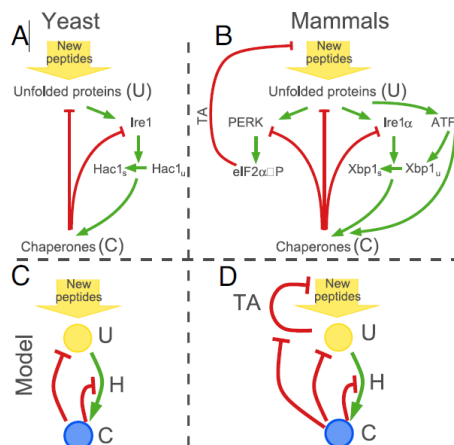


Figure 17: Trusina model of the UPR A) in yeasts and B) in mammalian cells. The model is presented C) in the absence of translation attenuation (TA) whereas in D) with the translation attenuation (TA) mechanism. Green arrows indicate positive regulation while red ones indicate negative regulation [158].

2.14.4 Rutkowski model for BiP, CHOP and GADD34

Rutkowski *et al.* [256] constructed a simple UPR model describing transient activation of BiP, CHOP and GADD34 under mild ER stress. They demonstrated that an adaptive response to ER stress is tuned by the discrepancy in stability of the prosurvival (i.e. chaperones) and proapoptotic (i.e. CHOP and GADD34) mRNAs and proteins. The experimental system used involved the treatment of MEFs cells with TM or Tg in order to induce stress. They

demonstrated that the mRNA and protein of ATF4 and GADD34 (upstream and downstream of CHOP) as well as of CHOP was unstable and degraded quickly. On the other hand the mRNA and protein of BiP, GRP94, p58^{IPK} (a cellular inhibitor of PERK) were quite stable showing little degradation. A model consisting of the ATF6-BiP and ATF4-CHOP-GADD34 axis was generated in which the mRNA and degradation rates were manipulated in order to determine the effects of their protein expression.

The model is based on experimental data and on the following assumptions. These included: 1) XBP1 splicing acts as a marker for the level of stress as has been previously been demonstrated [260], 2) upon ER stress ATF4 and the activated form of ATF6 are expressed and degraded at an equal rate (simplified assumption) and 3) all proteins in the model would be translated and degraded at a uniform rate constant (simplified assumption).

One of the model's limitations is that it does not consider some of the feedback regulatory mechanisms such as the GADD34 that mediates dephosphorylation of the eIF2 α or p58^{IPK} inhibiting PERK-mediated translational attenuation. Finally, from this model they observed an adaptive behaviour of the UPR and suggested that this response is the result of the differential stability of chaperones compared to the rest of the UPR players. However, some important regulatory events required for the adaptation and recovery state are missing.

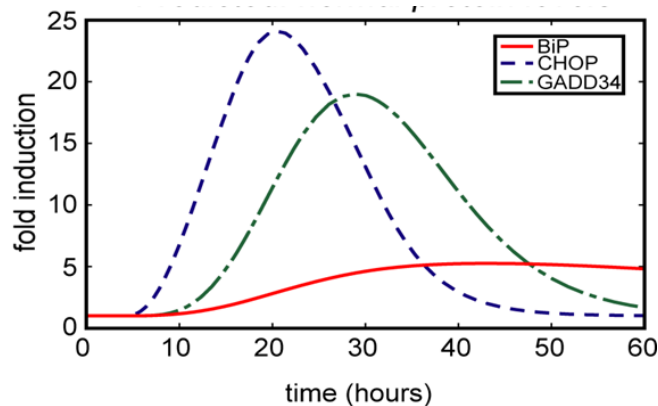


Figure 18: Rutkwoski model of the mammalian UPR. It predicts protein expression of BiP, CHOP, GADD34 using experimentally derived degradation rates [256].

2.14.5 Erguler model

Recently, Erguler *et al.* [257] created a mechanistic model with the aid of ordinary differential equations and reproduced a detailed version of the mammalian UPR combining all three signalling cascades (Figure 19). They linked the severity of the UPR state, translational attenuation and apoptosis.

The model is based on four elements that are linked to each other and are as follows: 1) the control of the activation of the receptors was assumed to occur from BiP by acting as a positive regulator based on competitive interaction to the receptor and the unfolded proteins. However, BiP was also able to act as a negative regulator by switching the active complex to its monomeric form, 2) the states that UPR can adopt were assigned from low to intermediate and high activities. During the intermediate state, the UPR oscillated between translational attenuation and induction of apoptotic stimuli. Under severe or prolonged stress, the system was able to cause continuous eIF2 α phosphorylation (p-eIF2 α). This in turn resulted in the quick ATF4 activation and the inhibition of translation attenuation mediated by PERK, 3) the IRE1 and ATF6 receptors, mediated the adaptive response by controlling the molecular dynamics of XBP1 and the expression of BiP and 4) evaluation of the timing of the apoptosis process, was achieved by using the apoptotic model [261] based on the mitochondrial BAX/BAK/BH3 while also assuming that CHOP inhibits Bcl-2 expression and activates the Bcl-2-interacting mediator. Emphasis was given on ER stress situations where the ERAD system was incapable of decreasing the unfolded protein load in the ER. Furthermore, by separating UPR activation from its response, the connection between the inputs and outputs was assessed. The majority of parameter values were not determined from experimental data but instead assigned as arbitrary units (AU). The model was justified by simulating the UPR in arbitrary stress scenarios.

Finally, this model suggested the ability of the UPR to switch from survival to death by generating oscillations and the timing of apoptosis. It also showed how mild ER stress may protect cells by preventing apoptosis and provided a novel approach for therapeutic intervention of UPR-associated disorders. However, this study lacked quantitative experimental data and thus the experimental validation was not possible.

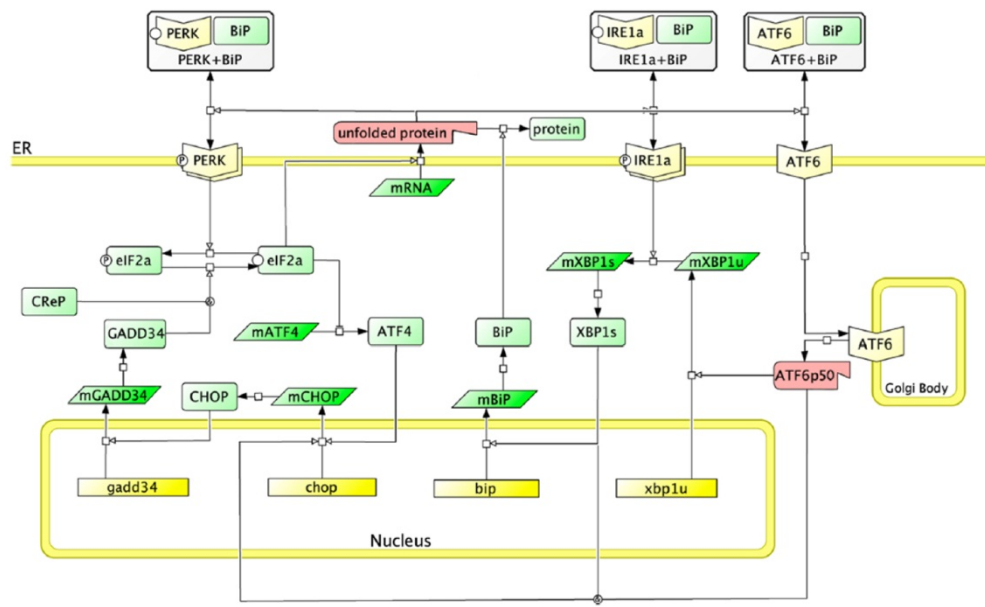


Figure 19: Erguler model comprising the complete mammalian UPR. It describes the UPR using 27 species, which are connected with 62 biochemical reactions and 82 parameters in total. The reactions take place in four cellular compartments including the nucleus, the cytoplasm, the endoplasmic reticulum and the Golgi apparatus [257].

Chapter 3

Materials and Methods

3.1 Cell lines and growth conditions

SK-N-SH human neuroblastoma cells (ATCC® HTB-11™, New York, USA) either non-transfected or overexpressing wild-type, Swedish or Swedish-Indiana APP₆₉₅ were maintained in 75-cm² tissue culture flasks in Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, Dorset, UK) supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich, Dorset, UK). Cells were grown in a monolayer in a humidified incubator at 37 °C with 5% CO₂ (NuAire) (Triple Red, Long Crendon, UK). Each culture was seeded in a new tissue culture flask at a density of 7.2x10⁵ cells/ml and passaged every three to four days. Cells were dissociated from culture flasks by incubating with 5 ml 0.25% trypsin (Invitrogen Ltd, Paisley, UK) for 5 min at 37 °C, followed by the addition of 5 ml fresh culture medium to neutralise trypsin. Cells were then centrifuged at 2,000 rpm for 5 min to separate the trypsin-containing solution. The supernatant was discarded; cell pellets were re-suspended in fresh medium containing FBS in a 1:4 dilution and cultures were seeded in new culture flasks. Viable cell concentration was determined by light microscopy using the trypan blue dye exclusion method.

3.2 Constructs of mutant APP₆₉₅

3.2.1 Site directed mutagenesis

Generation of Point Mutants –Swedish mutation: from lysine (AAG) to asparagine (AAC) and from methionine (ATG) to leucine (CTG) at amino acid residues 670 and 671, respectively, and of the Indiana mutation: from valine (GTC) to phenalanine (TTC) at amino acid residue 717 were generated. These were introduced to APP₆₉₅ cDNA in pCI-neo mammalian expression vector (Promega, Hampshire, UK) by site-directed mutagenesis using the QuikChange™ kit (Stratagene Ltd, Cambridge, UK) according to the manufacturer's instructions (Figure 20).

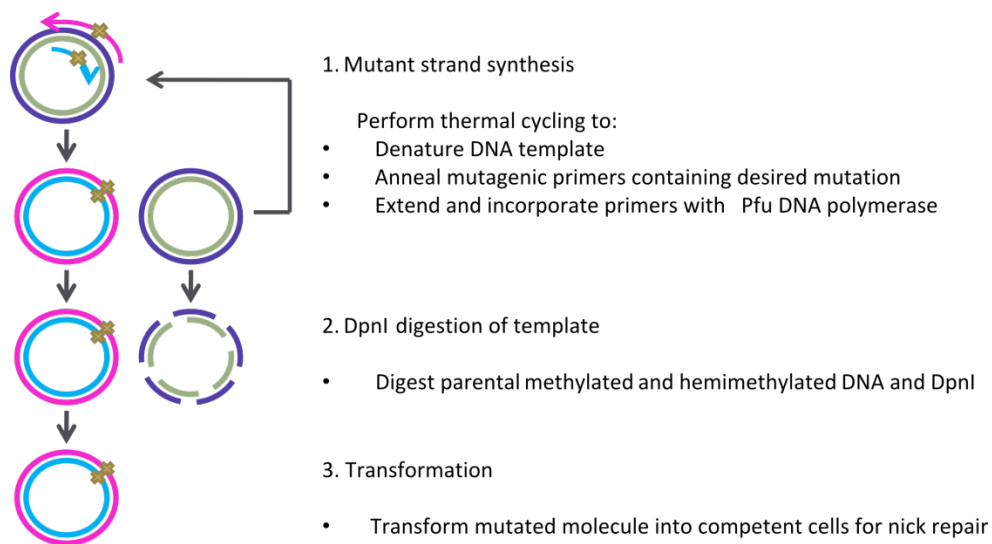


Figure 20: Schematic representation of the site-directed mutagenesis procedure.

Primers were designed according to human APP₆₉₅ sequence. Each mutation was inserted separately and the Swedish double mutant plasmid was used as a template for insertion of the Indiana mutation in a second round of polymerase chain reaction (PCR) to produce the Swedish-Indiana variant. In a final volume of 50 μ L, the following substances 1 μ L of DNA template (5 ng/ μ L), 1.25 μ L of each 100 ng/ μ L oligonucleotide primer, 1 μ L of 10 mM dNTP (pre-mixed), 40.5 μ L distilled water and 1 μ L of Pfu DNA polymerase (2.5 U/ μ L) were mixed. The

sample was incubated for 5 min at 98 °C in order to denature the DNA secondary structure and enable annealing of oligonucleotide primers. Then the amount of 1 µL of 2.5 U/µL Pfu DNA polymerase was added to the reaction mix in order to amplify each of the primers that were complementary to opposite strands of the vector, resulting in a final volume of 50 µL according to the manufacturer's recommendations. The sample was then immediately amplified with PCR. A control reaction containing no DNA polymerase was included to gauge the level of background reaction and to monitor template removal by *DpnI*. The reactions were run for 18 cycles 95 °C for 30 sec, 50 °C for 1 min, 68 °C for 10 min. Samples were left at 15 °C when PCR cycles finished.

3.2.2 Digestion of the PCR product

Following the amplification reaction, the plasmid was treated with 1 µL *DpnI* (10 U/µL) endonuclease, digesting the parental DNA template and selecting for the plasmid containing the mutated synthesised DNA. After mixing, the solution was incubated at 37 °C for 1 h to digest the parental double stranded DNA and thus to select for the plasmid containing the desired mutation.

3.2.3 Plasmid transformation and isolation

A volume of 1 µl of the DNA plasmid from the PCR reaction was added to 50 µl of chemically competent XL-1 Blue Supercompetent (Stratagene Ltd, Cambridge, UK) cell aliquots. The cells were incubated on ice for 20 min, followed by a heat pulse at 42 °C for 45 sec to induce DNA uptake. Following the heat pulse, the reaction was placed on ice for 2 min supplemented with 0.5 ml of LB at room temperature and was then incubated at 37 °C for 1 h and 30 min with shaking at 225 rpm. A volume of 250 µl of the reaction was plated on LB-ampicillin agar plates and the rest of the transformation reaction was stored at 4 °C.

For each transformation, three single colonies were selected at random from a selective plate and grown into a 10 ml LB medium containing 10 µl of ampicillin antibiotic. The culture was

incubated overnight at 37 °C with vigorous shaking. Several APP₆₉₅ plasmid candidates containing the mutation of interest were isolated using Qiagen Miniprep Purification kit (Qiagen Ltd, West Sussex, UK) according to manufacturer's instructions. Confirmation of the right plasmid was only possible after sequencing.

3.2.4 Sequencing

DNA sequencing with the APP₁₄₄₁ sequencing primer and two vector-specific primers at a concentration of 10 µmol/µl confirmed the fidelity of the mutated constructs. This allowed to ascertain that each DNA contained only the desired mutation without any other mutations (Eurofins MWG, Cambridge, UK). Oligonucleotide sequences used for mutagenesis and the sequencing are shown in the supplementary information (Appendix I).

3.3 Generation of a stable cell line

SK-N-SH cells stably expressing either the wild-type APP₆₉₅ (APP_{WT}), the Swedish mutation alone (APP_S) or APP with both the Swedish and the Indiana mutation (APP_{S-I}) were obtained by transfection using Lipofectamine 2000TM (Invitrogen Ltd, Paisley, UK) according to the manufacturer's protocol. More specifically, four days prior to transfection, cells were plated in serum-containing growth medium in six-well plates (1.5 ml per well) to obtain 95% confluence. For each well, 246 µl of OptiMEM was combined with 1 mg/µl DNA and allowed to sit at room temperature for 5 min. Then 238 µl of OptiMEM was combined with 12 µl of 1 mg/ml Lipofectamine 2000TM and allowed to sit at room temperature for 5 min. The two solutions were then mixed and incubated at room temperature for 20 min and then 500 µl of this solution was plated in each well. A control well was used for each plate, containing OptiMEM and Lipofectamine solution but not the DNA plasmid.

At 48 h post transfection, colonies were selected using 500 µg/ml of G418 (Sigma-Aldrich, Dorset, UK). When cells in the control well died and the transfected cells reached confluence,

the latter were progressed to 25 m² tissue culture flasks and subsequently into 75 m² tissue culture flasks. The stable SK-N-SH cells overexpressing APP_{WT}, APP_S and APP_{S-I} were maintained in the same medium as the non-transfected SK-N-SH cells, supplemented with 50 µg/mL of G418.

3.4 Freeze/Thaw

Cells for long-term storage were placed in cryovials suspended in a solution of 5% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) and 95% complete growth medium at a concentration of 10⁷ cells/ml. The vials were subsequently placed in a cryo-freezing “Mr Frosty” container with isopropyl alcohol and stored at -80 °C for 24 h prior to transferring the cells in liquid nitrogen (at -160 °C) for freezing them down. For revival, cells were thawed rapidly in a water bath of 37 °C, mixed with 4 ml fresh media (10% FBS/90% MEM) and centrifuged at 2000 rpm for 5 min. DMSO-containing supernatant was aspirated and the cell pellet was re-suspended in fresh FBS-containing growth medium.

3.5 Optimisation of cellular differentiation

Cells were grown in 6-well plates to reach 90% confluence and were then induced for differentiation with all-trans retinoic acid (RA) (Sigma-Aldrich, Dorset, UK). Treatment with a range of RA concentration including 3 µM, 5 µM and 10 µM was performed to evaluate the optimal amount needed for their differentiation. Phenotypic characteristics were assessed by the following tools 1) microscopic examination of the overall shape of the cells and 2) transcripts (mRNA levels) of differentiation markers with quantitative real time PCR (qRT-PCR) in order to determine the successful differentiation of the cultures. The mRNA levels were assessed with qRT-PCR as described below.

3.6 Stress protocol

For all experiments, SK-N-SH cells ~80% confluent were used in order to rule out the influence of overgrowth inducing stress. Cultures were differentiated for 5 days using 10 μ M RA supplemented to the culture medium. On the day of stimulation, the culture medium was renewed 1 h before the stress experiment to create uniform conditions. Cells were stimulated with 1 μ g/mL TM [130] (Sigma-Aldrich, Dorset, UK) in culture medium containing RA.

3.7 Cell viability and metabolic activity

3.7.1 Trypan Blue exclusion method

Cells were trypsinised and following their neutralisation were mixed with trypan blue. Cells were resuspended in trypan-blue solution and counted in a haemocytometer under a light microscope. Live and dead cells wells were counted by looking at their clear cytoplasm or their blue staining respectively. In order to avoid counting errors arising from incorrect dilutions, it was ensured that the cells counted in each 1 mm² chamber were in the range of 15-50. Identification of live and dead cells as well as enumeration was possible with the following calculations:

1. The total number of cells was defined by considering the following:

- a) The number of cells in each big corner square of the haemocytometer being equal to: *The number of cells* $\times 10^4$ cells/ml.

- b) Total cell count = $\frac{\text{Number of cells} \times \text{Dilution}}{\text{Number of squares counted}} \times 10^4$ cells/ml

2. Total percentage of viable cells was calculated with the formula:

$$\% \text{ Viability} = \frac{\text{Total number of live cells}}{\text{Total cell count}} \times 100$$

3.7.2 MTS assay

In vitro cytotoxicity of TM was determined with the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphenyl)-2H- tetrazolium, inner salt)) tetrazolium salt/phenazine methosulfate solution (Promega, Hampshire, UK) according the manufacturer's instructions. Differentiated cells in 6-well plates were exposed to TM (1 µg/mL) for 0 h to 24 h. MTS solution (0.25 mg/mL) was added, incubated for 2 additional hours at 37 °C. The optical density (OD) was measured directly at 490 nm using an automated ELx808™ absorbance microplate reader (BioTek, Bedfordshire, UK).

3.8 Molecular Biology techniques

3.8.1 RNA isolation and cDNA construction

Total RNA was isolated from actively growing cell cultures by harvesting them either in 1) 100 µL cell lysis buffer from the Cell Sure cDNA kit (Bioline Ltd, London, UK), 2) in 400 µL RNA lysis from the peqGOLD (Pepqlab, Southampton, UK) or 3) 400 µL lysis reagent from the High Pure RNA isolation kit (Roche, Applied Science), all followed by spin column purification according to the manufacturer's instructions. RNA integrity was verified electrophoretically with GelRed staining and by quantification of the OD₂₆₀/OD₂₈₀ nm absorption ratio ranged from 1.8-2.2 with NanoDrop 1000 (Fisher Scientific, Loughborough, UK). The cDNA was prepared by reverse transcription of 1 µg total RNA using the Sensiscript® reverse transcription kit (Qiagen Ltd, West Sussex, UK).

3.8.2 Optimisation of qRT-PCR conditions

The concentration of cDNA generated was determined by measuring the optical density at 260 nm using the NanoDrop 1000 and samples were then stored at -20 °C. Conditions for all primer pairs were optimised by gradient PCR at 8 different temperatures and verified by gel electrophoresis (using the appropriate primers and goTaq DNA polymerase) and melting curve analysis to assess whether the primers amplified only the target amplicon.

3.8.3 Primer design

For each of the target genes analysed with qRT-PCR, a number of primer pairs were designed using Primer-Blast (NCBI, Bethesda MD, USA) until a single PCR amplification product of interest was generated at 64.5 °C. The designed considerations were a) the amplicon size 60-500 bp; the smaller the better b) the melting temperature of the product and c) the GC content (the number of G's and C's in the primer as a percentage of the total bases) to be 40-60%. All primer stocks were diluted to a concentration of 10 µM.

Primer efficiencies were determined by performing serial dilutions of cDNA 2-fold up to 6 points followed by qRT-PCR reactions performed in duplicate using 4 different cDNA concentrations. Efficiency calculation was performed according to Pfaffl *et al.* [262] and primers were all optimised to have a similar annealing efficiency at 64.5 °C.

The average Cycle threshold (C_t) values with respect to qRT-PCR (an average calculated from the triplicate qPCR reactions) obtained during the amplification reactions and determined by Mastercycler ep realplex software (Eppendorf, Stevenage, UK) were plotted against the $\log_{10}$ of the cDNA dilutions. The equation from the linear regression line was used to test the efficiency and the coefficient of determination (R^2), which indicated how well the experimental data fitted the line (>0.99) were assessed (Appendix II).

1. Amplification efficiency was calculated by the following formula:

$$E = 10^{\frac{1}{-g}}$$

Where g is the slope of the linear regression

2. The percentage of the efficiency was calculated as:

$$\%E = (E - 1) \times (100\%)$$

3. Coefficient of determination: >0.98

The quantity of the PCR product would ideally double after each exponential cycle of the amplification, which is a 2-fold increase in the copy number after each cycle. In other words the values of E were considered to be within the range of 1.93 to 2.08. Primers whose reaction was reaching efficiency close to 100% (close to $E = 2$) and a great R^2 were used for gene expression analysis.

In order to verify that the amplified product is the desired one, melting curve analysis was performed immediately after the qRT-PCR reaction. Finally, the size of the amplicon was confirmed via agarose gel electrophoresis.

3.8.4 Quantitative reverse transcription polymerase chain reaction

Individual qRT-PCR reactions were performed using a mastermix containing SYBR® Green JumpStart™ Taq ReadyMix™ (Qiagen Ltd, West Sussex, UK), 100 ng cDNA and a final primer concentration of 1600 nM. Each reaction was performed in triplicate in 96-well plates (Fisher Scientific, Loughborough, UK) sealed with thermal film (Bioline, London, UK) and run in Mastercycler® ep Realplex 4S device (Eppendorf, UK). The conditions for the 2 step PCR were as follows: single denaturation cycle at 92 °C for 2 min followed by 40 cycles of 92 °C for 30 sec, 64 °C for 30 sec and a final extension cycle of 72 °C for 1 min. Amplified products were validated by melting curve analysis (62.7 °C to 92 °C with 0.025 °C per second increment). C_t values were determined by the Mastercycler ep Realplex software with a threshold of 100 (arbitrary fluorescence units). For each cell line, the mRNA levels of the ER stress markers (e.g. BiP, CHOP) mRNA levels were normalised for the expression of the endogenous housekeeping gene 18S rRNA.

3.8.5 Housekeeping genes

Three potential housekeeping genes were tested which were β -actin, GAPDH and 18S rRNA (Invitrogen Ltd, Paisley, UK).

3.8.6 Agarose gel electrophoresis

Electrophoretic mobility of DNA fragments was assessed on a 1-3% (w/v) gel electrophoresis. Agarose particles were added in 1 x TAE solution (40 mM Tris-acetate, 1 mM EDTA pH 8.0) and heated up until the particles were dissolved. The solution was cooled down to 60 °C and 0.05 μ l GelRed/ml (GelRed™ nucleic acid stain) was added to stain the DNA bands. For each gel, 5 μ l per lane of the DNA Hyperladder V (500 bp) (Bioline, London, UK) was loaded to reference the size of the observed fragments. Samples which were mixed with GoTaq® reaction buffer 5X (Promega, Madison, USA), loaded and electrophoresed at 120 V for 45 min. Gels were cast in a horizontal Gel Electrophoresis apparatus (BioRad, Hempstead, UK). They were then imaged by placing them in a UV light box, illuminated with a short UV and visualised using ImageJ software (Java image processing program).

3.9 Protein methods

3.9.1 SDS-PAGE and Western blot

Cells that were stored at -80 °C in a freeze mix containing 10% DMSO at a final concentration of 7.5×10^6 were thawed. They were then centrifuged at 2,000 rpm for 5 min and pellets were harvested using the Mem-PER® extraction kit (Fisher Scientific, Loughborough, UK). For analysis concentrated NuPage sample buffer (4X) and 1M DTT were added to the samples which were heated at 90 °C for 10 min. Equal amounts of protein were analysed on a gradient 4-20% precise protein gel (Fisher Scientific, Loughborough, UK) in Tris-Hepes running buffer at 120 V for 50 min. The proteins were transferred to a PVDF membrane (Merck Milipore, Hertfordshire, UK) at 25 V for 1 h 30 min using a semi-dry transfer system (BioRad, Hempstead,

UK). The WesternBreeze® Chemiluminescent Kit (Life Technologies, Paisley, UK) was used for western blotting. Membranes were incubated with 1:200 diluted rabbit monoclonal APP (Fisher Scientific, Loughborough, UK), and with 1:5000 diluted mouse monoclonal β -actin (Abcam, Cambridge, UK) the last one used as a loading control. Primary antibodies were detected using alkaline phosphatase-conjugated secondary antibodies and bands visualised using a LAS-3000 luminescent image analyser (Fuji Photo Film).

3.10 Statistical analysis

Statistical analysis was conducted using SPSS software (Statistical Package for Social Sciences SPSS Inc., Chicago, IL). To assess whether chemical treatment had a significant effect on the mRNA expression levels between the time points of a specific cell line (within a cell line) or the time points between the cell lines (between the cell lines) the one-way analysis of variance (ANOVA) was used. In order to compare the variances among treatments and determined whether they were equal or not (homogeneous or heterogeneous respectively), Levene's test was applied. When the variances were homogeneous ($p \geq 0.05$) ANOVA was employed, and when they presented heterogeneity ($p < 0.05$) Welch ANOVA procedure was applied. To find the relationships (equal or unequal means) of the different subgroups *post-hoc* analysis was used. In the case where ANOVA test set results indicated significant difference ($p < 0.05$) Tukey's *post-hoc* test was used. In case where Welch ANOVA was used and indicated significant effects ($p < 0.05$), Games-Howell *post-hoc* was used. Significance has been considered at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, as indicated in each case.

Chapter 4

A cell model system of familial Alzheimer's disease

4.1 Introduction

No treatment is available to cure AD, so a worldwide quest for innovative cures is under way in order to slow down, stop or even prevent this perplexing disease. Strong evidence implicates ER stress in AD, making it a possible treatment avenue. For instance, AD diminishes the ER's ability to deal with stress and this may possibly account for a shift in the balance of cellular fate towards cell death rather than survival. Increasing the chaperones to enhance protein folding and/or inhibit cell death with specific small-molecules [26] may provide a likely therapeutic avenue. In order to do this, it is necessary to develop convenient tools, which would detect the cellular responses and work as the framework for investigating such effects. Such tools include experimental (Table 3) and computational (Table 12) models allowing for the quick screening of small molecules for drug discovery.

While up to date biochemical and gene expression analyses have been successful in uncovering the UPR mechanisms, research on the UPR remains hampered by the lack of clear biological and computational models representing these features in mammalian cells [138]. Furthermore, Ron and Walter [25] proposed that the quantitative evaluation is essential in the understanding of the timing of the UPR.

Key pathological hallmarks of FAD are APP-linked mutations affecting the APP breakdown and subsequently the onset and the progression of AD. Therefore, an *in vitro* model of neuroblastoma cells stably expressing wild-type APP₆₉₅ isoform (APP_{WT}) and cells harbouring the APP₆₉₅ Swedish (APP_S) or Swedish-Indiana (APP_{S-I}) mutation was generated (Table 3). Swedish mutation located at the N-terminus of the A β domain favours the cleavage of APP by β -secretase as seen *in vitro* experiments [263], and is associated with elevated levels and deposition of A β ₄₂ in AD brains [264]. On the other hand Indiana mutation is, located in the transmembrane domain of APP and associated with abundant senile plaques, NFT and mild cerebral amyloid angiopathy in AD brains [265]. The latter mutation was used in conjunction with the Swedish in order to evaluate their synergistic effect in UPR, a complex network implicating three main downstream signalling pathways. The UPR markers along with their biological relevance were assessed as presented in Table 3.

Table 3: Outline of the experimental setup.

Type of model studied	UPR Signalling branches	UPR markers assessed	Biological relevance
Experimental • WT • S • S-I	IRE1, ATF6	BiP, GRP94	Chaperones (protein-folding)
	IRE1	Spliced XBP1	Encodes pXBP1s, a TF for ER chaperones & ERAD genes
	ATF6	Total XBP1	Encodes pXBP1u, a negative regulator of pXBP1s
		CHOP	Pro-apoptotic stimulus
	PERK	ATF4	Inhibits protein synthesis
		Nrf-2	Antioxidant response
		CHOP	Pro-apoptotic stimulus

4.2 Methodological diversity in the generation of stable cell lines

Several transfection methods have been developed to introduce foreign nucleic acids into the cells depending on the cell type and the purpose of the study. When short-term effects of the gene expression are studied transient transfection is preferred, while for long-term effects such as evaluation of wild-type or mutant APP overexpression in human neuroblastoma cells, stable transfection is preferred. There are multiple approaches that can be taken to generate stably transfected mammalian cells, broadly categorised into biological (i.e. virus-mediated), chemical (i.e. cationic polymer) and physical (i.e. microinjection). They all have their own advantages and disadvantages so the best approach is based on the experimental design choice.

The approach used to generate our FAD cell model system was based on the chemical – liposome mediated transfection – an easy to use, non-viral and highly efficient method, which is commonly used in SK-NSH cells. Hence, a polyclonal SK-N-SH cell model system constitutively overexpressing APP-associated mutations (further explained in 4.3.4) was generated. However, a major drawback of this method is the variability in transfection efficiency among different cells, which have distinct gene-expression patterns.

A possible approach to take in overcome this problem would be to clone the cells with one of the major strategies named cloning ring technique, dilution technique or the robotic cell transfer. The cloning procedure will thus enable us to obtain a pure culture of a particular cell type of interest. Alternatively, the transfection vector may contain a fluorescent protein such as GFP reporter gene activated from the APP promoter. The GFP-tagged cells may be analysed with fluorescence activated cell sorting (FACS) and provide information on the levels of the target gene. Another method could also be the use of an inducible type of overexpression of wild type or mutant APP. More specifically, APP expression would rely on an additive-controlled transcription activation of the APP gene. The most common inducible systems are the Tet-off and Tet-on, in which transcription remains either inactive or active respectively in the presence of tetracycline (Tet) or Doxocycline antibiotics.

4.3 Results

4.3.1 Generation of the construct

As many previous studies have demonstrated that mutations in APP protein are critical for the formation of plaques in FAD, site-directed mutagenesis was used to produce mutated APP₆₉₅ recombinant plasmids. The wild-type APP₆₉₅ cloned into the commercially available pCI-neo expression vector (Figure 21) was kindly provided by Dr. Jane Saffell. Site-directed mutagenesis was then performed. Expression of wild-type or mutant APP₆₉₅ in the host human neuroblastoma cells was controlled by the human cytomegalovirus (CMV) strong constitutive promoter of the pCI-neo vector [266]. The neomycin phosphotransferase gene allowed for the generation of stable transfectants by selecting the cells with the use of G418 antibiotic (Figure 21).

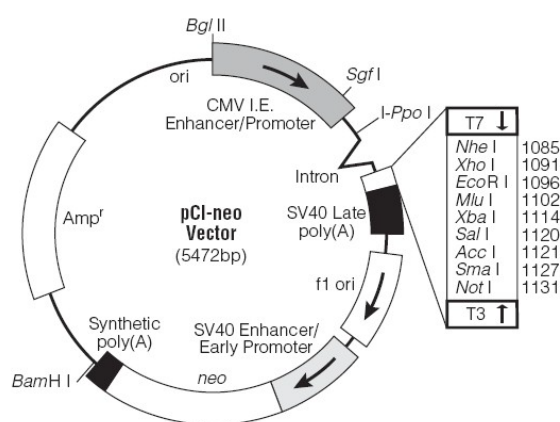


Figure 21: pCI-neo mammalian expression vector and sequence reference points. Neo=neomycin phosphotransferase, CMV I.E.=cytomegalovirus immediate-early (Promega, Hampshire, UK).

The first two mutations were performed by PCR using complementary primers with the desired mutation inserted into the wild-type APP₆₉₅ containing plasmid (Appendix I). Inserting the Indiana mutation form into the Swedish mutated plasmid generated the double mutation. Mutant DNA was in all cases amplified with the high fidelity proofreading enzyme 'Pfu polymerase', which generated a PCR product with a nicked circular DNA. Following the PCR, a

restriction enzyme *DpnI* specifically targeted for methylated and hemi-methylated DNA sites digested the template (non-parental) DNA while leaving the amplification product untouched. Both the wild-type and the mutant APP₆₉₅ containing plasmids were transformed into *E. coli*, where they were methylated and therefore susceptible to *DpnI* digestion. Finally, amplification of the APP₆₉₅ mutant DNA product of 8000 bp long was successful in all cases (Figure 22).

4.3.2 DNA purification for sequencing

The targeted APP₆₉₅ plasmids were transformed in *E. coli* and subsequently purified using three minipreps for each of the transformed DNAs. In order to examine whether the DNA from the miniprep products was sufficient to proceed with the analysis, samples were assessed on a 1% w/v agarose gel.

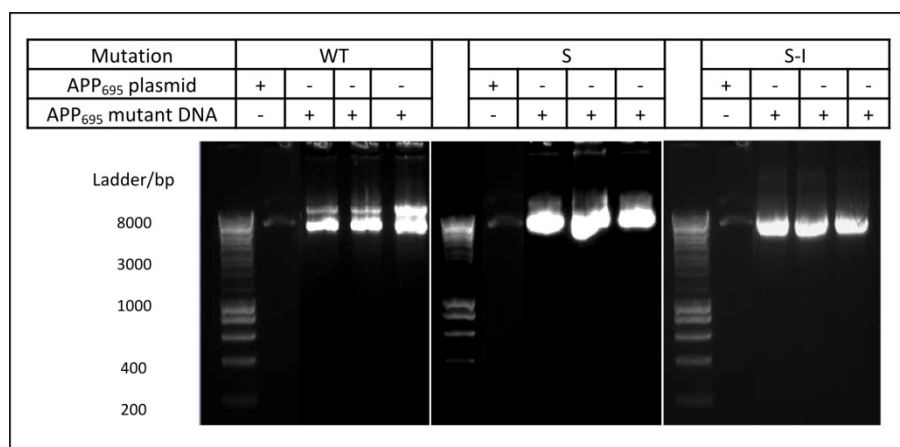


Figure 22: DNA isolation from *E. coli* culture with Qiagen miniprep. Bands of 8000 bp long were selected and sequenced in order to test if the plasmid has incorporated the desired mutation. All miniprep bands are very bright showing the high concentration of the plasmid. The bands in the first well correspond to the APP₆₉₅ plasmid while the other three bright bands correspond to APP₆₉₅ mutant DNA product.

Figure 22 clearly shows that all vectors (APP₆₉₅ plasmids) have the same size and are equally bright, thus have approximately the same concentration. In the wild-type vector there are two PCR amplified products in the three corresponding lanes; from which the larger band

corresponds to the non-supercoiled DNA. Mutant and wild-type APP₆₉₅ variants with the expected 8000 bp band size were validated by DNA sequencing.

4.3.3 DNA purification for transfection

Maxiprep Qiagen kit was used to purify the DNA prior to transfection. Assessment of the DNA purity was conducted by measuring the samples 260/280 absorbance yielding ratios of ~1.8. The purified samples were analysed on a 1% agarose gel. The PCR amplification bands had a single intact product and were very bright, which depicted that all APP₆₉₅ plasmids had a high DNA concentration as seen from Figure 23. These plasmids were then used for transfection.

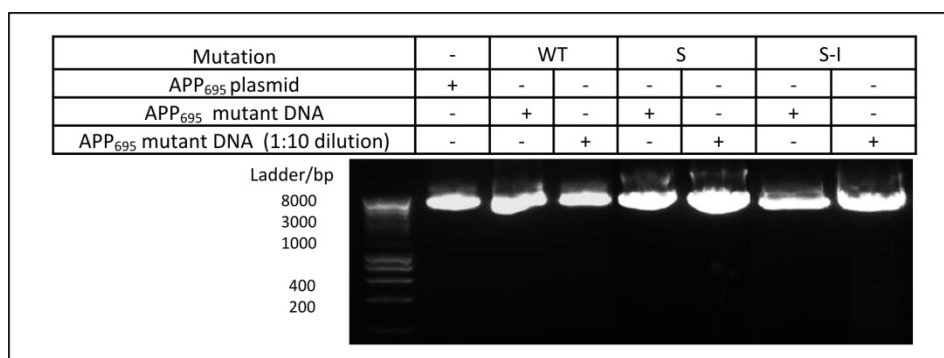


Figure 23: DNA purification with Qiagen maxiprep. All bands are 8000 bp and they are very bright corresponding to high DNA concentration.

4.3.4 Optimising a transfection protocol for SK-N-SH cells

Purified DNA was transfected into neuroblastoma cells with the Lipofectamine™ 2000 serum-compatible transfection reagent. A range of different DNA:Lipofectamine ratios were tested prior to the cell line development. Cells that contained the recombinant plasmid with the desired mutation were selected with G418. As the recombinant plasmid contained a G418 resistance gene, cells that did not incorporate the plasmid were not resistant to the antibiotic and thus did not grow. SK-N-SH cells overexpressing the APP_{WT} and the mutant forms including APP_S and APP_{S-I} were successfully created.

Surprisingly, following transfection some of the cells had an altered neuronal morphology. Studies by Granic *et al.* [267] on transfected human telomerase reverse transcriptase primary neurons showed that overexpression or mutation in PS-1 gene (which alters APP processing) induce defects in chromosomal segregation and aneuploidy. From this they hypothesised, that secretion of A β peptide alone could be responsible for the cell cycle defects [267]. The aforementioned observation led us to speculate that overexpression or mutations of APP₆₉₅ in SK-N-SH cells may be responsible for causing cellular morphological changes and thus mitotic defects (Figure 24). As the cells imaged in the microscope were under a blue fluorescent field, a blue background was present which however did not affect the cellular structures observed.

Confirmation of the aneuploidy hypothesis in the transfected cells could occur with further experiments such as a karyotype or fluorescence in situ hybridization analysis directly after harvesting of the cells. The first technique could allow the identification of chromosome abnormalities at the metaphase, while the second to assess chromosomes at all phases of the cell cycle. Such experiments could provide us conclusive proofs on these speculations. However, as these experiments were outside of the scope of this project, they were not conducted.

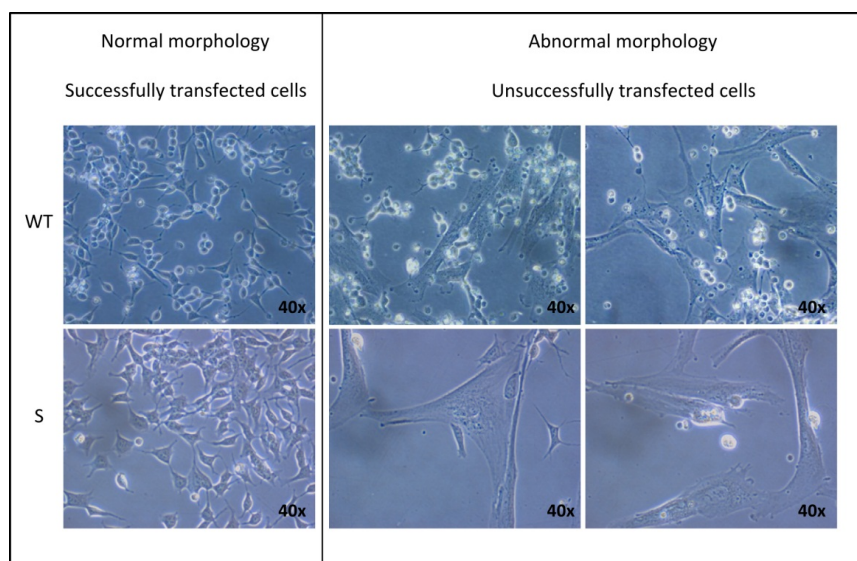


Figure 24: Representative pictures of successfully or unsuccessfully transfected SK-N-SH cells with APP₆₉₅. In the case of successful transfection, the 40X magnification microscopy pictures of cells overexpressing wild-type

(APP_{WT}) or Swedish (APP_S) APP demonstrated their neuronal shape and thus their normal morphology. However, in the case of unsuccessful transfected cells, the 40X magnification pictures of both APP_{WT} and APP_S depicted that cells bear morphological defects and thus an abnormal morphology.

4.3.5 Optimisation of total RNA extraction protocol

Total RNA isolation was achieved by lysing an actively grown SK-N-SH culture. A range of RNA extraction kits was evaluated for their ability to produce RNA with high yield and purity, an essential requirement for the cDNA synthesis. Spectrophotometric analysis of the RNA isolated from a) the CellSure b) peqGOLD and c) High Pure isolation determined each of the kits' efficiency reported in Table 4.

Table 4: Quality assessment of RNA isolated with different kits was conducted using spectroscopy, from cells overexpressing the wild-type (WT), Swedish (S) or Swedish-Indiana (S-I) APP₆₉₅ variant.

Cell type		WT	S	S-I
(a) Cell Sure kit				
RNA concentration (ng/μl)		108.5	88.9	109.1
Absorbance	260/280	1.27	1.32	1.28
	260/230	1.54	1.62	1.54
(b) peqGOLD kit				
RNA concentration (ng/μl)		98.9	95.9	152.1
Absorbance	260/280	1.31	1.34	1.35
	260/230	1.62	1.59	1.55
(c) High Pure isolation kit				
RNA concentration (ng/μl)		266.2	294.5	282.5
Absorbance	260/280	2.02	2.01	2.00
	260/230	2.11	2.09	2.29

The integrity of the total RNA isolated with the most successful protocol 'high pure isolation kit' was evaluated by gel electrophoresis (Figure 25). The RNA visualised on the denaturing gel had discrete and sharp 28S and 18S ribosomal RNA (rRNAs) bands and a wide band below the 18S rRNA representing the 5S tRNA. The mass ratio of 28S: 18S rRNA was of 2:1.

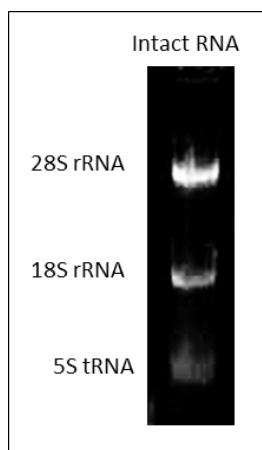


Figure 25: Evaluation of the quality of a total RNA sample isolated with the High Pure RNA kit by gel electrophoresis.

As the 'high pure isolation kit' provided the most successful RNA extraction, it was selected for all subsequent experiments. The isolated RNA was reverse transcribed into cDNA and was used to amplify specific genes including housekeeping genes (i.e. β -actin and 18S rRNA) as well as UPR markers (i.e. BiP and CHOP). Each of the amplification products was assessed by gel electrophoresis (Figure 26).

4.3.6 Optimisation of primer annealing temperatures

In order to investigate the mRNA levels of 18S rRNA, β -actin, APP, ATF4, BiP, CHOP, GAPDH, GRP94, Nrf-2, XBP1T, XBP1s via qRT-PCR, the specific conditions (annealing temperature and number of cycles) for each of the genes were tested via gradient PCR from 50-65 °C. The amplified temperatures tested were the followings: 49.5, 49.8, 51.7, 54.7, 59.7, 62.7, 64.4 and 64.5 °C.

Determination of the cellular mRNA levels of the housekeeping genes 18S rRNA (Figure 26a) β -actin (Figure 26c) and GAPDH (Figure 26g) was achieved by firstly designing specific primers to amplify a 171 bp, 383 bp, and 102 bp product respectively. The PCR at 64.5 °C amplified a single bright band of the correct size for each of the housekeeping genes. Next, evaluation of whether the transfections were successful was achieved by a comparison of the APP mRNA levels of the

transfected with the nontransfected cells. To do this, primers specific for APP (Figure 26c) were designed to amplify a 199 bp product and did so successfully at 64.5 °C as seen by the bright and intact PCR product on the gel electrophoresis.

Analysis of the PERK activation was achieved by investigating ATF4 (Figure 26d), CHOP (Figure 26f) and Nrf-2 (Figure 26i) expression levels. Specific primers for these genes were designed to amplify a 226 bp, 62 bp and 171 bp PCR product respectively. Gel electrophoresis provided evidence that all of the aforementioned primer pairs successfully amplified the correct product. The primers for CHOP although they did not amplify the template at 54.7 °C, an amplification product with the correct size was observed at 64.5 °C.

In order to assess the expression of the chaperones BiP (Figure 26e) and GRP94 (Figure 26h), specific primers were designed to amplify a 232 and 220 bp product respectively, which did so as determined by the yield of the PCR product.

Analysis of ATF6 activation was performed in part by examining the XBP1T and CHOP mRNA levels whereas of IRE1 activation by the XBP1s mRNA levels. Primers of the XBP1T were designed on one side of the intron so that the amplifying products derived from the XBP1s and XBP1u were of the same size (single band). The first primer pair designed to produce a 513 bp product (Figure 26j), however it amplified more than one band at 64.5 °C, which was the highest temperature used. For this reason, a second primer pair was designed to amplify the 441 bp long XBP1T (Figure 26k). At a low temperature (54.7 °C), a very faint band at the correct size was visible while at 64.5 °C the product was brighter. In a similar manner primers designed to amplify the XBP1s transcripts, produced a very faint band at a 54.7 °C while at 64.5 °C a brighter band was present (Figure 26l).

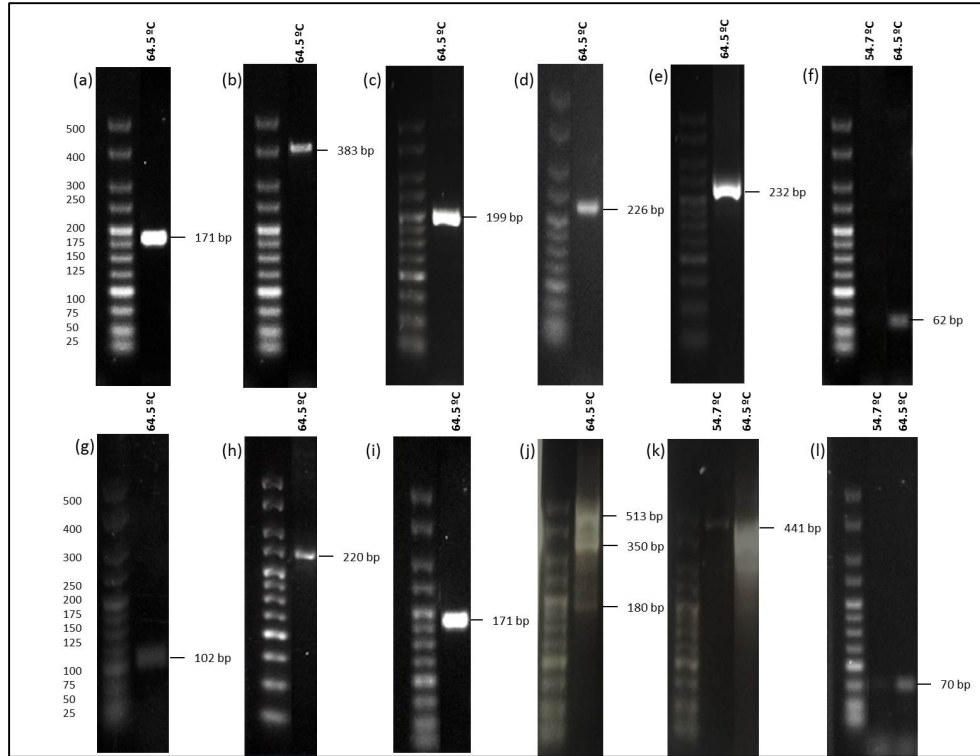


Figure 26: PCR amplification products of primers amplifying the house-keeping genes and the UPR markers with the following order: (a) 18S rRNA, (b) β -actin, (c) APP, (d) ATF4, (e) BiP, (f) CHOP, (g) GAPDH, (h) GRP94, (i) Nrf-2, (j-k) total XBP1, (l) spliced XBP1.

4.3.7 Amplification efficiencies

Amplification efficiencies for all primer pairs were determined by PCR. This was calculated by preparing serial dilutions of the initial cDNA template [262]. Following RNA extraction from SK-N-SH cells, the mRNA was reverse transcribed and the cDNA was diluted to the following concentrations: 0.05, 0.1, 0.5, 1, 1.5 and 2 $\mu\text{g}/\mu\text{l}$ which were then used for the generation of a standard curve and calculation of the corresponding efficiencies (Appendix II).

Table 5: Primer pair characteristics.

Gene	Primer Pair		Amplicon size (bp)	T _m (°C)	N° of cycles	Amplification efficiency	GenBank accession N°
18S rRNA	F	GTAACCCGTTGAACCCCAT	171	64.5	40	1.96	M10098
	R	CCATCCAATCGGTAGTAGCG					
β-actin	F	AAACTGGAACGGTGAAGGTG	383	64.5	40	1.95	NM001017992.2
	R	AGAGAAAGTGGGGTGGCTTTT					
APP	F	GGCCCTGGGAGAACTACATCA	199	64.5	40	1.93	NM201414
	R	AATCACACGGAGGTGTGTCA					
ATF4	F	TCAAACCTCATGGGTTCTCC	226	64.5	40	1.93	NM001675
	R	TGTCATCCAACGTGGTCAG					
BiP	F	TGGTGTTTGACCTGGGTGGCGGA	232	64.5	40	1.96	NM005347
	R	AGGGCCTTGGCCTTTTCTACCTCG					
CHOP	F	CTCTGGCTTGCTGACTGA	62	64.5	40	1.96	NM004083
	R	GCTCTGGGAGGTGCTTGT					
GAPDH	F	CCACTCCTCCACCTTTGAC	102	64.5	40	1.93	NM002046
	R	ACCCTGTTGCTGTAGCCA					
GRP94	F	TGGGAAGAGGTTCCAGAATG	220	64.5	40	1.95	NM003299
	R	GTTGCCAGACCATCCGTACT					
Nestin	F	AGCCCTGACCACTCCAGTTTAG	128	64.5	40	2.06	NM006617 [268]
	R	CCCTCTATGGCTGTTTCTTCTCT					
NFM	F	GTCAAGATGGCTCTGGATATAGAAATC	104	64.5	40	2.08	NM 001105541 [269]
	R	TACAGTGGCCAGTGATGCTT					
Nrf-2	F	GCGACGGAAGAGTATGAGC	161	64.5	40	1.96	NM 001145412
	R	GTTGGCAGATCCACTGGTTT					
XBP1T	F	AGGCCAGTTGTACCCCTCC	441	64.5	40	1.96	NM 001079539
	R	CCCAGCTCCGGAACGAGGTCA					
XBP1s	F	CCGCAGCAGGTGCAGG	70	64.5	40	1.97	NM005080
	R	GAGTCAATACCGCCAGAATCCA					

To confirm precision and evaluate reproducibility of the qRT-PCR, inter-assay variation was tested for all transcripts. The degree of variation was calculated for each gene by dividing the mean of the cycle threshold (C_t) values by the standard deviation (SD), giving the coefficient of variation (CV), which was presented as a percentage (%CV). All of the qPCR experiments displayed less than 3.9% dispersion of their C_t values indicating high expression stability between both the samples and the replicates.

Table 6: Coefficient of variation expressed as a percentage (%CV) in the qRT-PCR replicates (n=3).

Gene of interest/ %CV	Cell type		
	WT	S	S-I
18S rRNA	0.43	0.42	0.25
APP	0.24	0.47	0.97
ATF4	0.55	1.09	0.31
BiP	0.94	0.77	1.35
CHOP	0.18	0.52	0.20
GRP94	0.51	1.36	0.36
Nrf-2	0.45	0.54	0.49
Total XBP1	0.36	0.54	1.33
Spliced XBP1	0.90	0.82	0.70

4.3.8 Housekeeping genes

Quantification of the expression levels of a target gene requires the parallel transcript quantification of a control gene for the endogenous normalisation, which is critical for data evaluation. The most widely used endogenous control is β -actin as it represents a cornerstone of the internal architecture of the cell necessary for cellular function. However, studies have shown that its expression may not be stable in certain conditions such as under morphological cellular changes during neuronal differentiation [270].

Thus, three potential housekeeping genes were tested and compared in both differentiated and non-differentiated cells: β -actin, GAPDH and 18S rRNA. The stability of the expression of the housekeeping genes under differentiated experimental conditions was evaluated with the %CV (Table 7) and provided evidence that 18S rRNA had the least variability. The amplification efficiency obtained for 18S rRNA was 1.96 and the R^2 was 0.99. Out of these observations, 18S rRNA was judged the most suitable normalization factor for this work. Previous studies have also demonstrated 18S rRNA to be a superior normalizer by displaying the lower variation in response to a given stimulus in a wide range of cell types [271, 272].

Table 7: Evaluation of the variation (%CV) of (a) all the housekeeping genes compared between the different cell lines (APP_{WT}, APP_S and APP_{S-I}) and (b) 18S rRNA variation in APP_{WT} in response to a stimulus of cellular differentiation (RA= retinoic acid) or ER stressors (TM=tunicamycin).

(a) Gene of interest/Ct values	Cell type			%CV
	WT	S	S-I	
β -actin	18.15	16.16	14.32	11.82
GAPDH	17.94	15.25	16.20	8.29
18S rRNA	13.80	12.30	13.21	5.77

(b) Gene of interest/Ct values	Conditions used in WT			%CV
	Control	RA	TM	
18S rRNA	13.85	14.98	13.38	5.84

4.2.4 Quantification of APP expression in transfected cells

To establish a model system of FAD-associated genetic predisposition, three human SK-N-SH neuroblastoma cell lines were created, overexpressing wild-type (APP_{WT}) and mutant; Swedish (APP_S) and Swedish-Indiana (APP_{S-I}, collectively called APP_{MUT}) APP. Verification of the stable transfections was first examined with qRT-PCR using template from the different cell lines and APP primers. The APP mRNA levels were normalised to the housekeeping control 18S rRNA and the expression levels of the transfected cells were then compared to the non-transfected cells. The data confirmed that the APP expression in transfected cell lines was in all cases more than two-fold higher compared to the non-transfected cells (Figure 27a). Consistent with the qRT-PCR results, Western Blot showed overexpression of APP₆₉₅ in transfected cells thus defining the success of the transfection (Figure 27b). Thus, as was expected, the transfected cells demonstrated an increased expression of APP levels compared to non-transfected cells both at the mRNA and protein level.

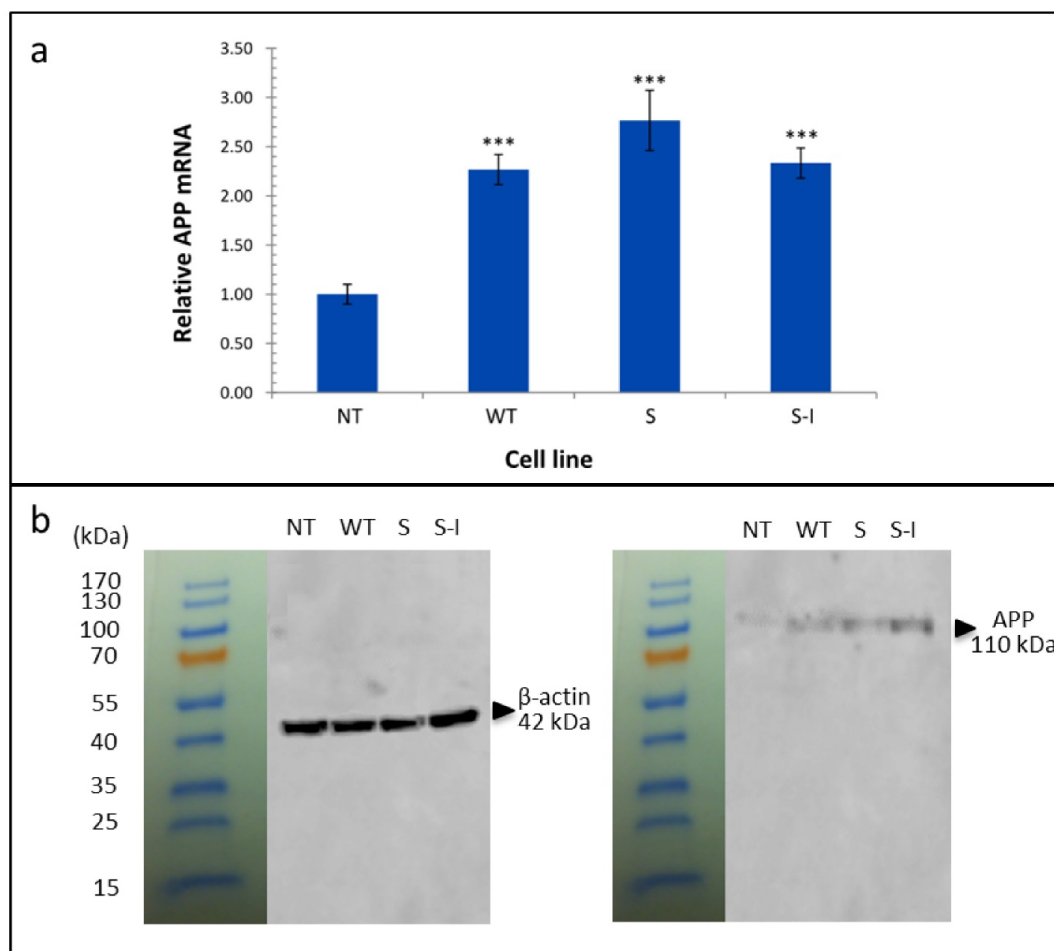


Figure 27: Quantification of APP expression. (a) Expression levels of APP mRNA in APP_{WT}, APP_S and APP_{S-I} measured with qRT-PCR and compared to the non-transfected (NT) cells (mean=3; $\pm$ SD) and (b) Western blot indicating APP overexpression in stably transfected SK-N-SH cells. β -actin was used as a loading control. The protein ladder was visualised using chromogenic detection (with white light) while the APP and β -actin proteins with chemiluminescence (with UV) in the same image analyser. Statistical significance of $p < 0.001$ was denoted with three stars.

4.2.5 Cell differentiation

SK-N-SH cells can be differentiated with the treatment of various agents. All-trans-retinoic acid (RA), a vitamin A-derived small lipophilic molecule is the most common differentiation agent used that influences both non-transfected and transfected cells. It co-ordinately promotes cellular differentiation and regulates gene expression mechanisms [273-275]. Several studies have reported the use of RA for cell differentiation, each of them using different concentrations and incubation times. For instance, Irving *et al.* [276] induced neuronal differentiation with a 1

μM RA concentration for five and nine days, while Wainwright *et al.* [277] used 10 μM RA with a treatment of three to 14 days, demonstrating differentiation to occur after day three.

To optimise the differentiation protocol cells were tested with 3 μM , 5 μM and 10 μM RA in order to assess the amount needed. Cells were considered as differentiated when they had at least one process (neurite) that was bigger in length than the cell body (Figure 28). Previous studies demonstrated that differentiated post-mitotic neurons derived either from wild-type or V642-APP (London type mutation) knock-in embryonic stem cells exhibit similar characteristics [278], suggesting that the use of either APP_{WT} or APP_{MUT} for identifying the ideal RA treatment conditions would provide us with the same outcome. Therefore, APP_{WT} were used in order to optimise the differentiation conditions, which were kept constant for all the cell lines. Each experiment involved seven observations of APP_{WT} from three biological replicates while always comparing them back to the control (untreated) cells using light microscopy. Control cells as seen in Figure 28 were highly dense after nine days of culture and exhibited a spherical shape. A clearer picture of their spherical shape was observed when the control cells were cultured for fewer days (five days) as seen in APP_S cells in Figure 30, where cell confluence was lower compared that after nine days of culture.

Treatment of APP_{WT} with RA for nine days induced their differentiation process to some extent even under the conditions where the lowest RA amount (3 μM) was used. It also resulted in a decreased confluence caused possibly either by growth inhibition or by higher rates of cell death rates. However, a higher RA concentration seemed necessary to achieve a higher number of cells with neuronal characteristics. Indeed as seen in Figure 28, a 10 μM of RA treatment was required to induce the phenotypical processes in most cells including neuronal differentiation and neurite outgrowth.

A cell model system of familial Alzheimer's disease

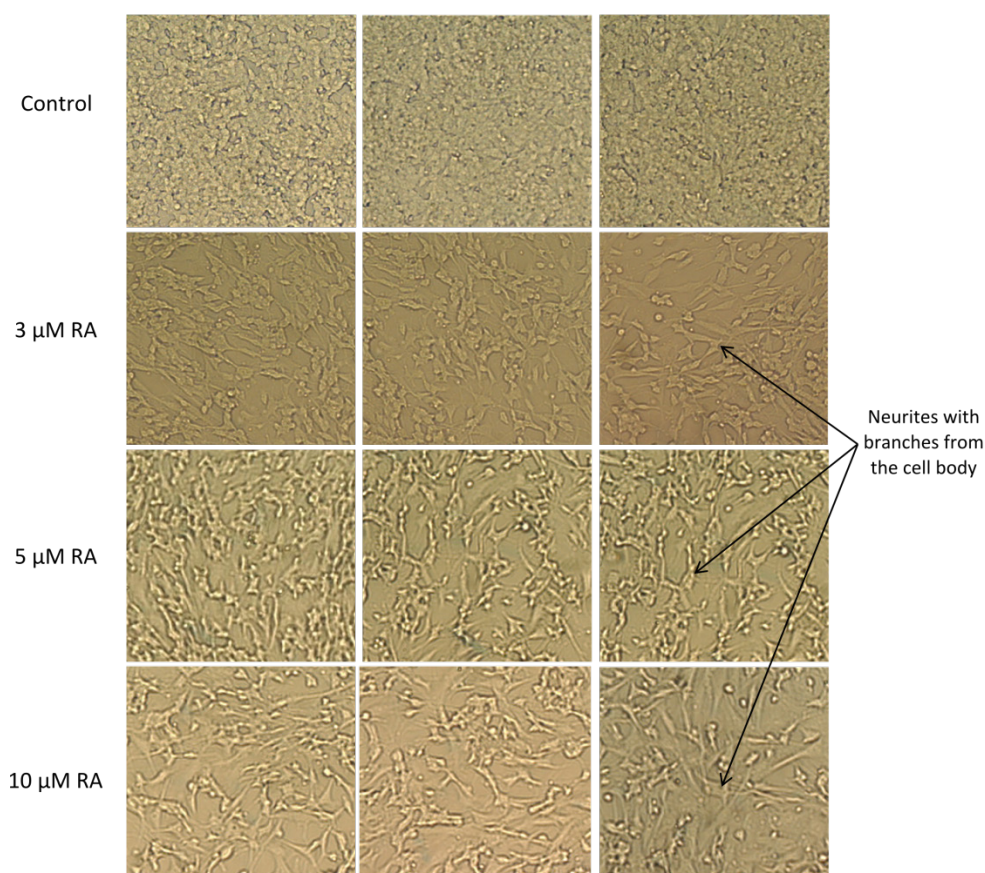


Figure 28: Microscopic inspection of differentiated APP_{WT}. Technical replicates of cells cultured with different RA concentrations for nine days.

Neuronal characterisation is often evaluated primarily by the morphological features and then by the biochemical properties (gene expression). Many reports have described a wide range of neuronal markers to aid the cellular characterisation process [279]. Some of the most common markers used for neuronal differentiation include the nestin and the neurofilament medium polypeptide (NFM) genes.

Nestin is an intermediate filament protein of the cytoskeleton expressed early in the development of the CNS and induces survival and cell proliferation [280]. During the cellular differentiation, nestin is replaced by other specific intermediate filaments and its mRNA expression gets quickly downregulated [281]. On the other hand, NFM is encoded by the intermediate filament protein and is expressed in a wide range of neuronal cells providing both axonal and structural support while also modulating the axonal diameter [282]. Studies of

synovium-derived mesenchymal stem cells differentiated with RA treatment into neuronal cells showed upregulation of their NFM transcript levels [283]. Therefore, qRT-PCR analysis of nestin and NFM was used to confirm their differentiation. For this, cells were treated with RA for nine days with 3, 5 and 10 mM RA (Figure 29).

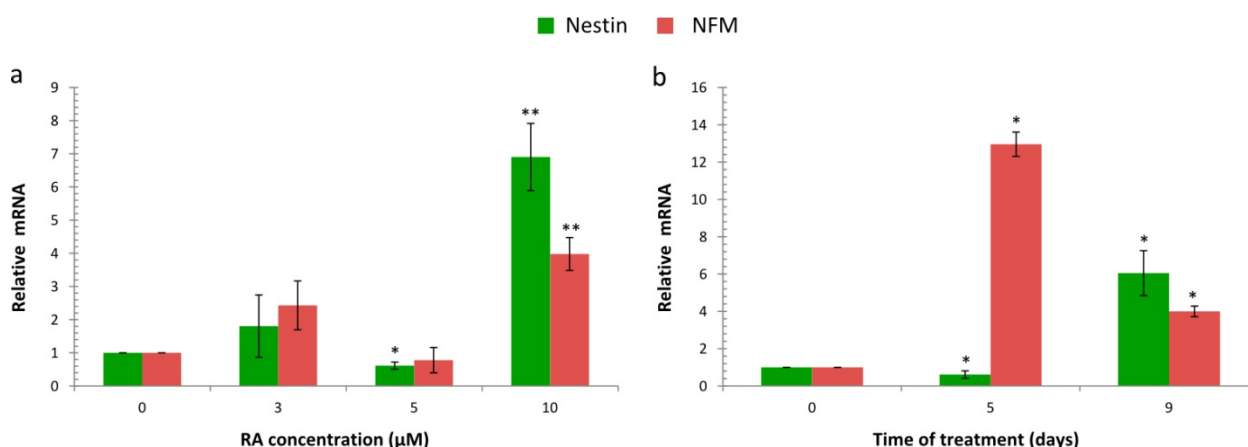


Figure 29: Biochemical characteristics of differentiated APP_{WT}. Relative expression levels of Nestin and NFM in APP_{WT} under a) nine days of RA-treatment altering the RA concentration, b) 10 μM RA-treatment at different days of treatment. Data are presented in relation to the mRNA levels of a) the zero RA concentration or b) zero hours of RA treatment (n=3; mean ±SD). Statistical significant differences of the different treatments compared with the zero concentration or time point *p<0.05, **p<0.01, ***p<0.001.

Figure 29a illustrated that the amount of 5 μM RA was enough to significantly downregulate the nestin mRNA expression levels after nine days of treatment. However, the NFM marker, which was supposed to be upregulated upon cellular differentiation, remained low. Treatment of cells with an increased RA concentration of 10 μM induced significant upregulation of NFM transcripts, as expected, but nestin mRNA levels were also high. As it has been previously demonstrated that nestin expression can be re-induced following injury of the CNS [284], we speculated that the increase in nestin mRNA levels might have been caused by a stress factor such as the overexpression of APP₆₉₅.

Following the optimisation protocol, the amount of 10 μM RA was used and cells were treated for a shortened period of time (five days) as well as for nine days used as a reference to the

previous experiment. Figure 29b illustrated that cells treated with 10 μ M RA for five days; exhibited significant downregulation of nestin transcripts as well as significant upregulation of NFM transcripts, an expected biochemical profile corresponded to neuronal cells.

Subsequently of the biochemical analysis, morphological evaluation of the cells treated with 10 mM RA for five days was verified. Figure 30 shows that untreated cells (control) exhibited a clear rounded body while RA-treated cells possess distinguished axons and have generated neuronal networks.

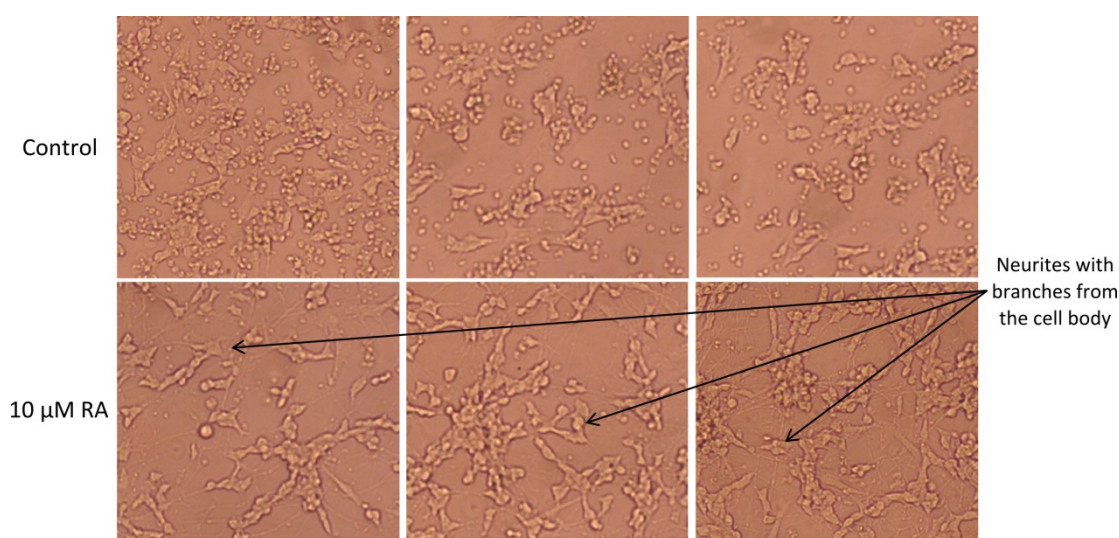


Figure 30: Microscopic inspection of differentiated APPs. Cells were treated with 10 μ M RA for five days.

The biochemical analysis in combination with the physiological study confirmed that 10 μ M of RA for five days were the optimal conditions required to induce markedly morphological and gene expression changes and thus to successfully generate SK-N-SH cells with a neuronal identity. These conditions were also the ones used by Chafekar *et al.* [173] to differentiate SK-N-SH cells overexpressing either the WT or Indiana APP₆₉₅ isoform.

4.2.6 Pre-upregulation of ER stress in APP transfected cells

Based on microscopic evaluation, cell confluence monitoring did not demonstrate any major differences with regards to the rate of growth and the viability of the transfected APP₆₉₅ cells and the non-transfected cells. Despite that, whether APP₆₉₅ overexpression and/or APP₆₉₅ point-mutations pre-upregulate ER stress in our FAD-cell lines, as has been previously suggested [74, 173] was not yet known. In order to assess whether transfected cells pre-upregulated ER stress, a range of seven markers of the UPR signalling were assessed with qRT-PCR.

Initially, the expression of two chaperones (BiP and GRP94) was investigated the levels of which were compared between the non-transfected and the transfected with APP₆₉₅ cells. The data (Figure 31) indicated that only APP_S exhibited a significant 4.5-fold increase in BiP expression levels when compared to the non-transfected cells.

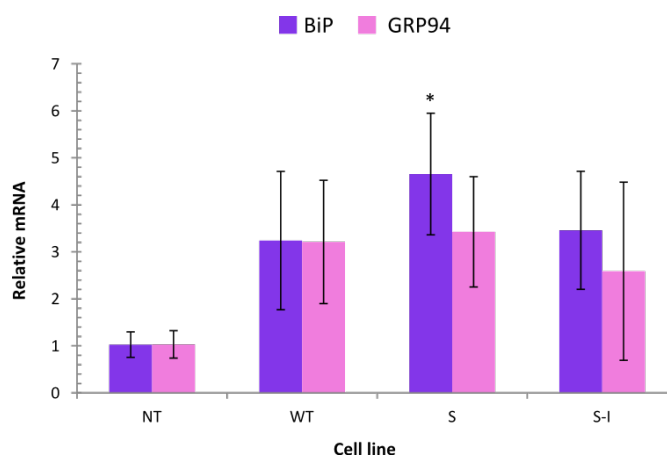


Figure 31: Chaperones expression induced by APP overexpression. The results are presented in relation to the mRNA levels of the non-transfected cells (n=3; mean $\pm$ SD). Statistical significant differences of APP_{WT} APP_S and APP_{S-I} compared with the non-transfected cells are indicated *p<0.05, **p<0.01, ***p<0.001.

Following several experimental approaches, Laing *et al.* [285] indicated that ER-stress induced apoptosis mediated by the PERK signalling selectively promoted ATF4 and CHOP expression while other studies also demonstrated PERK activation to promote survival through increased

Nrf-2 expression [286]. We thus evaluated PERK signalling through expression of ATF4, Nrf-2 and CHOP mRNA (Figure 32). Our results indicated APP_{WT} and APP_S to overexpress ATF4 relative to the non-transfected cells (p-value of 0.037 and 0.001, respectively) while the ATF4 mRNA levels of APP_S were also significant upregulated when compared to the transfected APP_{WT} and APP_{S-I} (p=0.04 and 0.01, respectively). The 6.5-fold upregulation of ATF4 transcripts in APP_S probably further led to a pre-upregulation of CHOP mRNA of ~8-fold (p=0.032) compared to non-transfected cells. Therefore, preliminary data provided an indication of the PERK activation signalling in APP_{WT} and APP_S, a pathway mainly responsible for translation attenuation. However, as ATF4 is regulated at a translational level, analysis of its protein levels would be a better indication of PERK activation induced by APP overexpression.

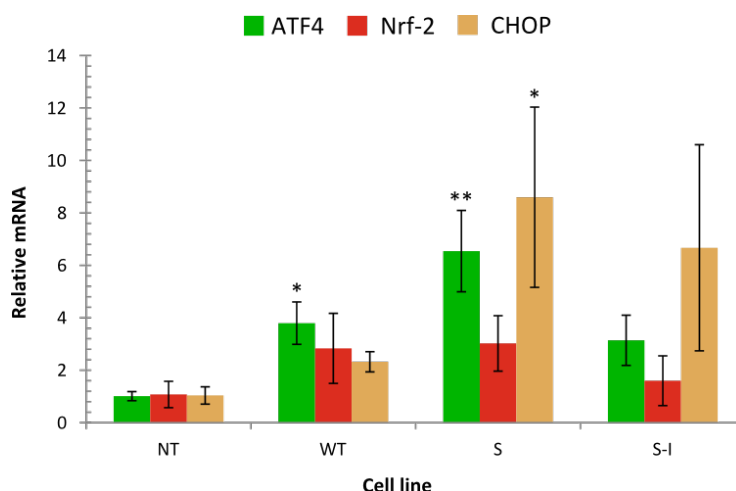


Figure 32: PERK activation induced by APP overexpression. The results are presented in relation to mRNA levels of the non-transfected cells (n=3; mean $\pm$ SD). Statistical significant differences of APP_{WT}, APP_S and APP_{S-I} compared with the non-transfected cells are indicated *p<0.05, **p<0.01, ***p<0.001.

The IRE1 ER-stress sensor is an important UPR regulator as it induces splicing of XBP1 mRNA affecting cellular fate. On the other hand, XBP1T is regulated by the ATF6 activation, making analysis of both XBP1s and XBP1T of high importance. The results showed XBP1s transcripts to be significantly overexpressed by a ~7-fold in both APP_S and APP_{S-I} compared to non-transfected cells (p=0.004 and 0.003, respectively) (Figure 33). The XBP1s transcripts in APP_{S-I} were also significantly higher than in APP_{WT} (p=0.043). This led us to hypothesise that the UPR

in mutant cells may be activated through the IRE1 pathway, which is responsible for the upregulation and expression of ER chaperones. On the other hand, total XBP1 mRNA levels were upregulated in all transfected cells when compared to the control (non-transfected cells) ($p=0.002$ for APP_{WT} and $p=0.001$ for APP_{MUT}), suggesting possible ATF6 activation in all cell lines.

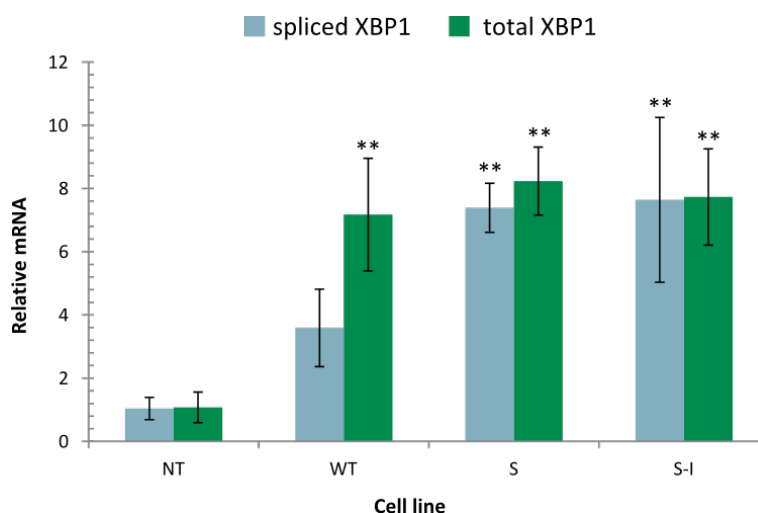


Figure 33: IRE1 and ATF6 activation induced from APP overexpression. The results are presented in relation to mRNA levels of the non-transfected cells ($n=3$; mean $\pm$ SD). Statistical significant differences of APP_{WT} APP_S and APP_{S-I} compared with the non transfected cells are indicated * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

These preliminary data indicated the differences in the UPR genes expression levels between the non-transfected and the transfected cells under a non-induced environment. They suggested a pre-upregulation of the UPR markers caused by the overexpression of APP_{WT} or APP_{MUT}. The upregulated ATF4, XBP1s and XBP1T genes seen are known to also be responsible for restoration of cellular homeostasis [287]. On the other hand the pro-apoptotic CHOP marker exhibited an 8-fold increase only in the APP_S ($p=0.032$).

A possible explanation of the ER stress pre-upregulation observed in APP_S and APP_{S-I} may have resulted from the formation and accumulation of misfolded proteins. Their formation could have resulted from the APP mutant mediating protein folding destabilization [7] while their accumulation from the re-internalisation of protein aggregates by endocytosis [288].

Nishitsuji *et al.* [74] have previously reported E693Δ APP mutation to increase ER stress without the additional treatment of any agents. More specifically, analysis of p-eIF2α protein levels in mock-transfected cells (positive control) and cells overexpressing wild-type and E693Δ APP mutant demonstrated increased phosphorylation of eIF2α in the mutant cells. Wild-type cells also had elevated p-eIF2α levels compared to the positive control. In parallel, BiP protein levels were more abundant in mutant than wild-type cells and demonstrated that mutant cells have an increased ER stress response [74]. These show that APP_{WT} alone or APP_{MUT} can pre-upregulate ER stress, which is in accordance with our observations.

4.2.7 Artificial induction of ER stress

Several drugs are available to use in order to model specific stress responses and to perturb the protein folding process in the ER. Examples include DTT, which affects disulphide bond formation, TM, which inhibit protein glycosylation, brefeldin A, which blocks the transport from the ER to the Golgi apparatus, and Tg, which inhibits the sarco/ER Ca²⁺ ATPase pump causing disruption of disulphide bond formation and thus mediating depletion of Ca²⁺ in the ER stores [289, 290]. TM and Tg mechanisms of action do not rely on altering the ER redox state of the cells. This is in contrast to the DTT mechanism, which perturbs the ER by reducing the oxidising species and altering the redox state, thus making DTT a non-specific ER stress-inducing agent [291, 292].

As our aim was to investigate the UPR activation caused directly by the accumulation of unfolded proteins in the ER and not promoted by reductive stress, DTT was ruled out from our choices. A reductive environment (induced by DTT) could have not only lead to accumulation of misfolded proteins but also could have affected several protein functions and influence numerous cellular processes (i.e. mitochondrial dysfunction) [293]. For these reasons, TM (Figure 34), known for its ability to activate all three UPR branches and induce translation attenuation as well as cell death was chosen [294, 295].

Induction of stress in our cell-model system was achieved by treatment with the TM ER stress-inducing agent. This agent is composed of a mixture of ten or more individual nucleotide sugar

analogues fatty-acylated with N-linked acyl chains varying in length and degree of unsaturation. It is naturally produced by several *Streptomyces* bacteria species such as *Streptomyces clavuligerus* and *Streptomyces lysosuperficus*. TM-treatment is used to block the N-linked glycosylation, which inhibits protein folding at a slow rate, and activates the UPR [256, 296].

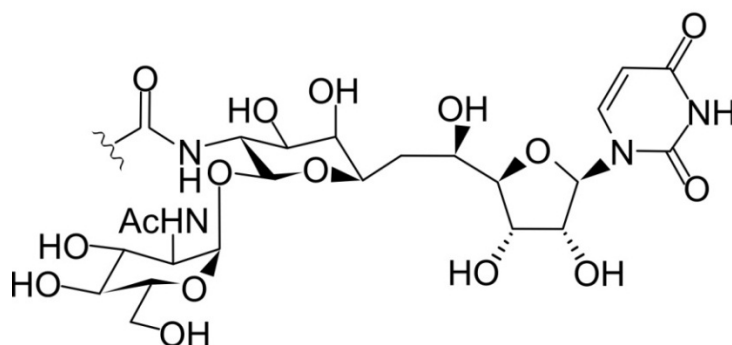


Figure 34: Molecular structure of tunicamycin (TM) isolated from *Streptomyces lysosuperficus*.

4.2.8 Stress activation using TM-ER stress inducing agent

An 'artificial' induction of ER stress in our SK-N-SH cells was carried out with TM-treatment. The concentration of TM used was 1 $\mu\text{g}/\text{ml}$ as it has been previously demonstrated to activate ER stress in differentiated SK-N-SH cells [290]. Oda *et al.* [290] showed that this concentration was the minimum amount needed to upregulate chaperones in a time-dependent manner and to cause a significant decrease of the cell viability at 24 h. Additionally, Samali *et al.* [130] used the same TM concentration to induce ER stress while Waechter *et al.* [297] showed that 1 $\mu\text{g}/\text{ml}$ of TM inhibited protein glycosylation of neuroblastoma cells by 80-94%. In agreement with these findings, our data demonstrated that at 24 h of TM-treatment, the mRNA expression levels of the chaperones BiP and GRP94 were indeed significantly upregulated compared to untreated cells (Figure 35).

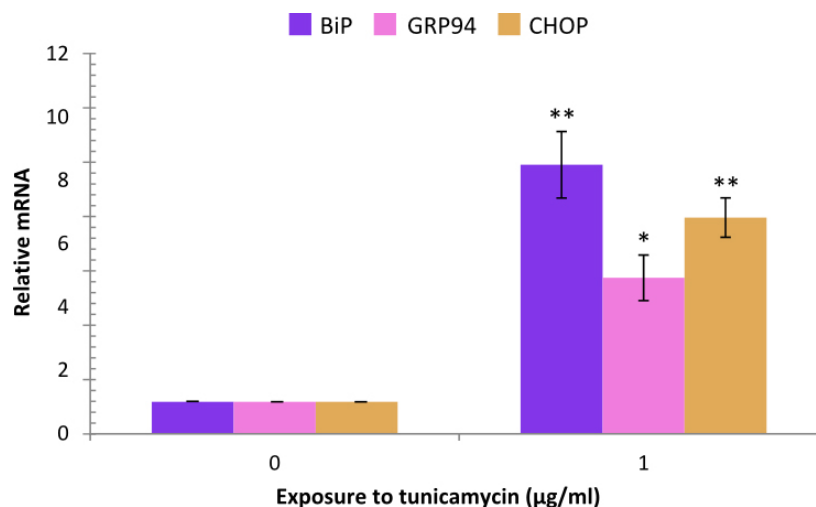


Figure 35: Expression of UPR markers in non-transfected cells. Cells were either non-stressed (0 $\mu\text{g/ml}$ TM), or treated with 1 $\mu\text{g/ml}$ of TM for 24 h. The results are presented in relation to mRNA levels of non-stressed cells for each respective gene (n=3; mean $\pm$ SD). Statistical significant differences of the UPR associated mRNA levels of cells treated with TM compared with the non-treated cells are indicated *p<0.05, **p<0.01, ***p<0.001.

4.2.9 Optimisation for the MTS assay

In order to assess the metabolic activity of cells, the MTS colorimetric assay was used. This assay is based on the bioreduction of MTS tetrazolium compounds into coloured formazan by mitochondrial dehydrogenases (Figure 36). During this assay, only the viable and metabolically active cells reduce/cleave the MTS reagent into a water-soluble formazan product (from yellow to dark blue) indicating the number of cells whose metabolism is still active [298]. It further allows quantification of the number of cells whose metabolism has been disrupted, by a deduction of the signal from the controls (non-TM treated cells).

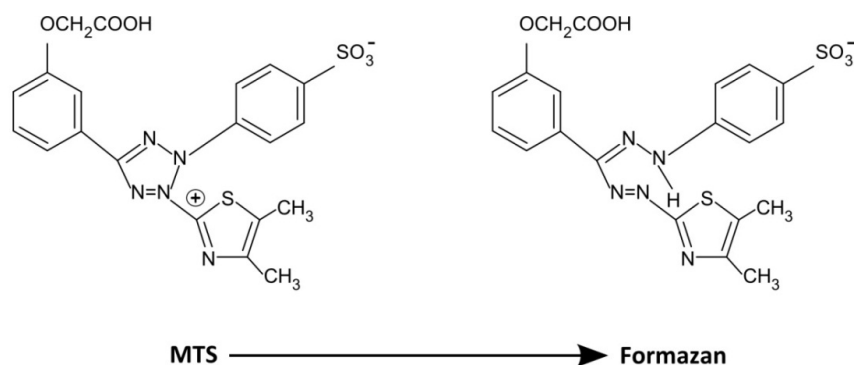


Figure 36: Structure of the MTS tetrazolium compound and the conversion to formazan product.

As different cell lines have different metabolic activities, a serial cell dilution was performed in 96 well microplates to test specifically whether our cell lines exhibited a linear relationship between the cell number and the signal produced. Recordings of the absorbance (Figure 37a) illustrated a good linearity ($R^2=0.99$) with the SK-N-SH cells cultured.

In order to eliminate the possibility of chemicals affecting the absorbance measurements, the recordings of non-transfected cells were evaluated with the MEM alone or in a combination of MEM and specific chemicals used including TM, RA, or the antibiotic G418. These drugs exhibited a very low background absorbance therefore they did not have a significant effect on the signal produced (Figure 37b).

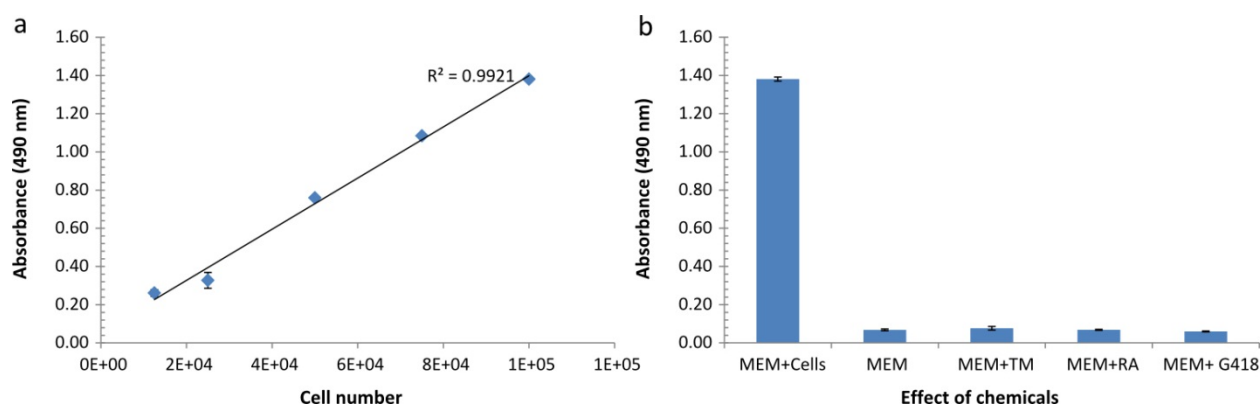


Figure 37: Examining MTS conditions. a) Linear relationship between cell number and absorbance values, b) effect of different chemicals in the background signal. (n=3; mean±SD)

Cell numbers must be sufficient in order to provide information on possible differences in their metabolic activity under specific conditions, but overgrowth must be avoided as it leads to activation of various stresses. A total number of 5×10^4 cells/well in a 96 well microplate was used to determine and compare the absorbance at 490 nm of differentiated versus non differentiated non-transfected cells. There was no variability observed in the readings between the non-neuronal and neuronal cells (cells with RA). The non-transfected cells (without RA) were treated with TM for 24 h. This treatment induced a significantly lower absorbance,

indicating a decrease in the metabolic activity caused by either cell cycle arrest or apoptosis (Figure 38a).

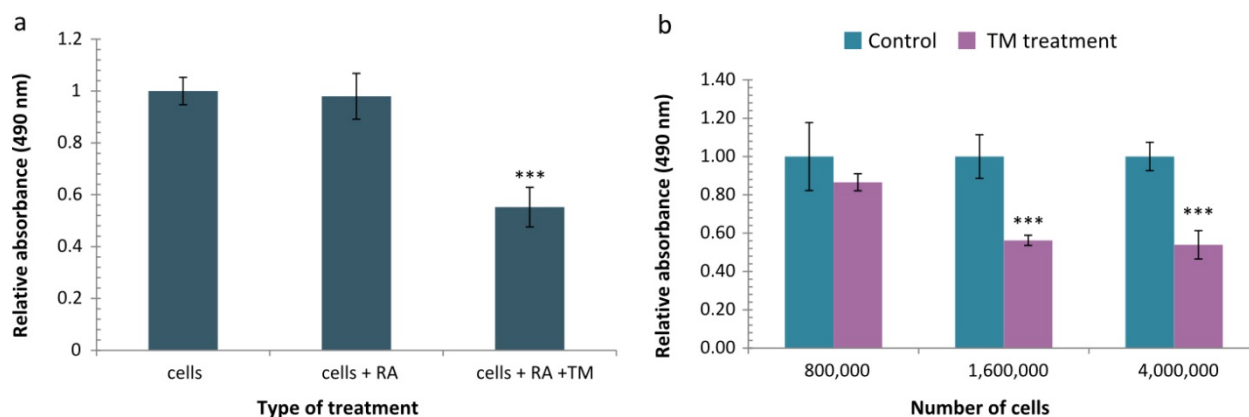


Figure 38: Optimisation of MTS assay for TM treated cells. a) Analysis of the effect of TM-treatment in differentiated SK-N-SH cells. The results are presented in relation to the relative absorbance of the non-chemically treated cells ($n=3$; mean $\pm$ SD). b) Optimisation of the lower cell number used to capture the TM-induced effect in the MTS assay. The results are presented in relation to the relative absorbance of control cells ($n=3$; mean $\pm$ SD). Statistical significant differences of cells treated with TM and/or RA compared with the non-treated cells are indicated * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

The manufacturer of the MTS kit provides a protocol for performing the assay with cells in 96-well plates. However, as the experiments involved neuronal cells and consistency between seeding densities among the different experiments was preferred, cells were plated in 6-well plates. A range of cell numbers was assessed in different wells prior to the addition of the dye. The total number of 1.6×10^6 cells was demonstrated to be the lowest needed in the 6-well plates in order to be able to detect a significant effect from the TM-induction when compared to the untreated (control) non transfected cells (Figure 38b).

4.4 Discussion

To investigate the UPR induction mechanism in response to ER stress in AD pathology, a cell model system was generated with human neuroblastoma SK-N-SH cells overexpressing either

wild-type, Swedish or Swedish-Indiana APP₆₉₅. Verification of successful transfection was performed with qRT-PCR and Western blot analysis. Expression of APP was significantly higher in APP_{WT}, APP_S and APP_{S-I} relative to non-transfected cells, which confirmed the successful generation of a FAD-model system. The qRT-PCR data illustrated APP_S to exhibit a slight but significant higher increase of APP levels relative to APP_{WT} and APP_{S-I}. The non-transfected cells were expressing APP, which was expected as the respective endogenous protein is ubiquitously expressed [299].

The role of APP in the induction of ER stress-induced apoptosis is a matter of current debate. Previous studies have indicated that in the absence of chemical ER stress inducing agents (i.e. TM, DTT), overexpression of APP alone can overwhelm or disrupt the protein folding machinery triggering ER stress [300]. More specifically, Nishitsuji *et al.* [300] showed that in the absence of a pharmacological agent, the UPR markers (BiP, p-eIF2 α) and cell death markers (CSP4) were upregulated in HEK293 cells that were overexpressing wild-type or Swedish APP [300]. Furthermore, Takahashi *et al.* [301] found that overexpression of APP leads to induction of CHOP mRNA and reduction of cellular viability.

We thus initially examined whether SK-N-SH cells overexpressing wild-type or FAD-mutant APP exhibited ER-stress signalling downstream UPR activation and whether its expression was differential relative to the APP mutations. Our findings showed that APP overexpression alone could trigger the pre-upregulation of ER stress. Analysis showed that not all genes were significantly upregulated compared to the parental cell line (non-transfected). Overexpression of ATF4 transcript was observed in both APP_{WT} and APP_S, increase of XBP1s transcript in APP_S and APP_{S-I} while increase of BiP and CHOP mRNA only in APP_S. The data also suggested that the highest pre-upregulation of UPR markers occurred in the APP_S cells. The stronger response was exhibited by APP_S as seen by the CHOP transcripts that had a seven-fold increase. It is worth noting that the magnitude of the stress response was lower compared to the magnitude of stress from TM-induction (seen in Chapter 5), illustrating that cells were mildly stressed. The above data provide evidence that even in the absence of chemical ER stressors (i.e. TM)

overexpression of the wild-type and FAD-associated APP₆₉₅ activates stress signalling and it does so with a low magnitude.

Findings from other studies suggested that APP overexpression does not induce ER stress activation. Kogel *et al.* [302] demonstrated with the use of rat pheochromocytoma cell line PC12 that expression of wild-type or mutant APP has no effect on BiP and CHOP expression and thus on UPR activation. In agreement with the aforementioned study were the findings of Chafekar *et al.* [173]. SK-N-SH human neuroblastoma cells overexpressing wild-type or Swedish APP did not exhibit any differential basal levels of the UPR markers (BiP, CHOP and XBP1s) when compared to the parental cell line [173].

The fact that various studies have reported different effects caused from the APP overexpression may be due to several reasons. Firstly, the expression levels of the APP probably differ in between the different studies. A higher APP expression level could probably induce the highest intracellular A β concentration. High concentration of the toxic A β may result to its aggregation as well as to the inhibition of the APP neuroprotective effect, which could eventually triggering stress. Secondly, the densities of cells studied vary among the studies. Cells cultured in high confluence may exhaust the nutrients in the media and cause stress, while a lower cell number may be insufficient to observe the stress effect.

As the next goal of the project (Chapter 5) involved the evaluation of the 'artificial' induction of ER-stress, human neuroblastoma cells were differentiated to obtain morphological and biochemical characteristics of mature neurons. This was achieved by optimisation of the RA treatment protocol. Schule *et al.* [303] also reported that RA-differentiated cells consist of a better cellular model system compared to undifferentiated cells. This was stated as non-differentiated cells were demonstrated to be more susceptible to toxicity compared to the differentiated ones and thus less suitable for the toxic studies in the nervous system [303].

Next, a specific pharmacological agent was chosen to artificially induce ER stress. For this effect, TM, which inhibits the early steps in the biosynthesis of N-linked glycans in the ER, was used. Based on previous studies in SK-N-SH cells, the amount of TM was confirmed along with the duration of treatment in our system. Metabolic activity of the cells during various time points of

stress was assessed with the MTS assay. This allowed us to optimise the conditions for conducting the MTS assay for our cell system. Overall, the above experiments enabled us to establish the conditions required for evaluating the stress response triggered by chemical induction, which is presented in the following chapter.

4.5 Conclusion

The findings from the characterization of UPR markers suggest that overexpression of APP₆₉₅ alone can induce mild preconditioning of ER stress and that overexpression of APP_{MUT} induced a stronger stress response than APP_{WT}. Further work is necessary to confirm these observations. The next chapter discusses the chemical activation of ER stress, which was carried out to examine whether overexpression of APP_{WT} and APP_{MUT} exacerbates ER stress and to elucidate the timing of pro-survival versus pro-apoptotic signals triggered by UPR.

Chapter 5

Progression of UPR in a cell model system of familial Alzheimer's disease

5.1 Introduction

Despite the increased number of studies focused on the role of individual steps initiated by distinct UPR branches, the timing and progression of the ER stress response in mammalian cells is not well understood. For instance, it is not well known whether all three ER stress transmembrane receptors (IRE1, PERK, and ATF6) are activated at the onset of ER stress or whether their induction follows a sequential order. Previous data have suggested that in response to ER stress, activation of the ATF6 signalling is rapid and precedes XBP1 splicing mediated by IRE1. Subsequently, the activated ATF6 pathway triggers transcriptional induction of ER chaperone genes prior to the IRE1-XBP1 pathway [304]. Lin *et al.* [305] have confirmed ER stress to activate all three UPR branches and to promote survival. The way that the pro-apoptotic and anti-apoptotic responses integrate to choose between a survival and death decision remains unknown. Under conditions of prolonged stress, IRE1 and ATF6 signalling were attenuated while PERK pathway remained activated (Figure 39) [305]. It is not well understood whether dissociation of BiP is simultaneous from the three transmembrane receptors or whether IRE1 activation takes longer because XBP1 processing has multiple steps. It also remains unclear whether all IRE1, ATF6 and PERK pathways are fully activated or whether there is a synergistic effect to promote the response if the stress has not been completely resolved.

The timing of the stress response is important to identify specific steps that allow fine-tuning of the ultimate fate of the cell (survival or apoptosis). Identification of such key steps could help to pave the way for developing novel small target molecules for therapeutics. This chapter describes the investigation of the timing of stress induction by analysing the system's response at early time points following the application of chemical stress.

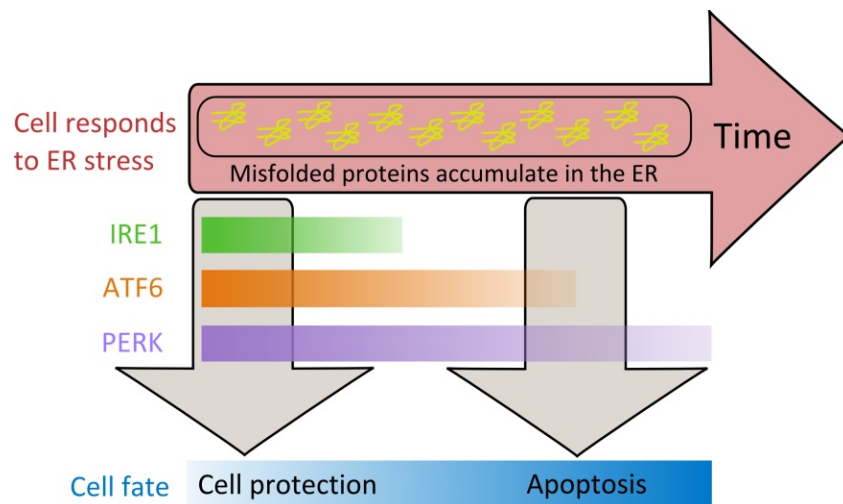


Figure 39: UPR signalling throughout time. Accumulation of misfolded proteins in the ER induces stress, which induces the activation of the UPR. This response induces the activation of three ER membrane sensors (IRE1, ATF6 and PERK) at different times in order to first restore homeostasis or trigger cell death if the response is prolonged. Figure adapted from [305].

The SK-N-SH human cell lines overexpressing either the wild-type or mutant APP protein were differentiated (with RA) and chemically treated with TM (a well-characterised ER stress inducer), the latter preventing protein glycosylation and causing a build-up of unfolded proteins in the ER [84, 306]. Differentiated cells validated by their morphology and expression of neuronal markers, were analysed for the stress response imposed by the intra-and extra-cellular stresses (mutations and TM-treatment, respectively) at six different time points (0 h, 3 h, 4 h, 6 h, 8 h, 24 h). Seven UPR genes were examined including chaperones and markers in the UPR pathway downstream of all three ER-sensors receptors (IRE1, PERK, and ATF6) and apoptotic factors. This allowed us to assess the specific signalling pathways activated from cells over-expressing different APP isoforms. By inducing ER stress and capturing the response at

early time points in a quantitative manner, we sought to better understand the progression of UPR in FAD.

Initially, cell viability at each time point was determined by the trypan blue exclusion method and metabolic activity was assessed with the MTS assay. Subsequently, qRT-PCR was used to analyse the gene expression levels of genes BiP, GRP94, ATF4, Nrf-2 spliced XBP1 (XBP1s), total XBP1 (XBP1T), and CHOP from a population of n=5 from two independent experiments.

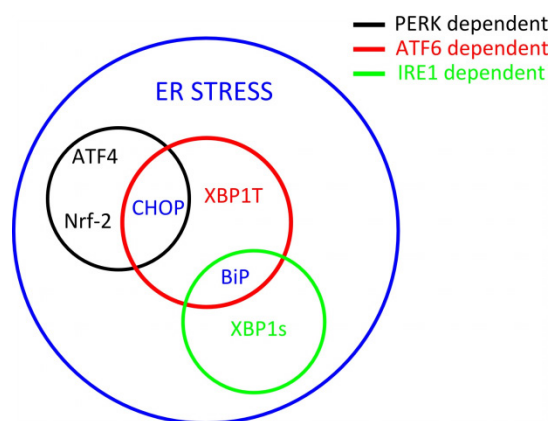


Figure 40: Schematic representation of the UPR-genes induced by ER stress (ATF4, Nrf-2, CHOP, XBP1T, XBP1s and BiP) and the interconnectivity between the different ER-stress receptors (IRE1, PERK and ATF6)

5.2 Results

5.2.1 Cell's initial response to stress is an attempt at adaptation

Evaluation of the effect of TM-treatment on the metabolic activity and the cellular viability was achieved with the MTS and trypan-blue exclusion methods, respectively. MTS assay data indicated a reduction in metabolic activity at six hours of TM-treatment in all APP_{WT}, APP_S, and APP_{S-I} (p=0.029, p=0.002 and p=0.001, respectively) with respect to zero hours (Figure 41a). This decrease was ~20-25% in all cell lines, with the lowest activity being measured in APP_S. This drop in absorbance was unusual, as the data of up to four hours of TM treatment didn't indicate any gradual decrease in the cells' metabolic activity. Further investigation in this drop

would include the evaluation of the cells' activity at five as well as seven hours of TM-treatment. However, as the scope of the project was not focused on the cells metabolic activity from a stress stimulus, these time points were not investigated. At eight hours of TM-treatment, APP_S and APP_{S-I} appeared to recover, as cells exhibited a significant up-regulation of their metabolic activity when compared to the six hours samples ($p=0.027$ and $p=0.011$, respectively). Although the APP_{WT} also seemed to recuperate at eight hours, the increase of the metabolic activity was not statistically significant. This may possibly be explained by the fact that at six hours of TM-treatment, APP_{WT} had the lowest average reduction in activity (with respect to zero hours) of all the cell lines. However, 24 h of TM-treatment showed a significant ~40% drop of the absorbance in all cell lines when compared to zero hours, which could be resulted either from cell cycle arrest or cell death.

Further analysis with the trypan-blue dye exclusion method showed that, with one exception (APP_S at six hours of TM-treatment), cell viability was not significantly decreased up to eight hours (Figure 41b). After 24 h of treatment only the APP_S had a significantly reduced viability compared to the starting population.

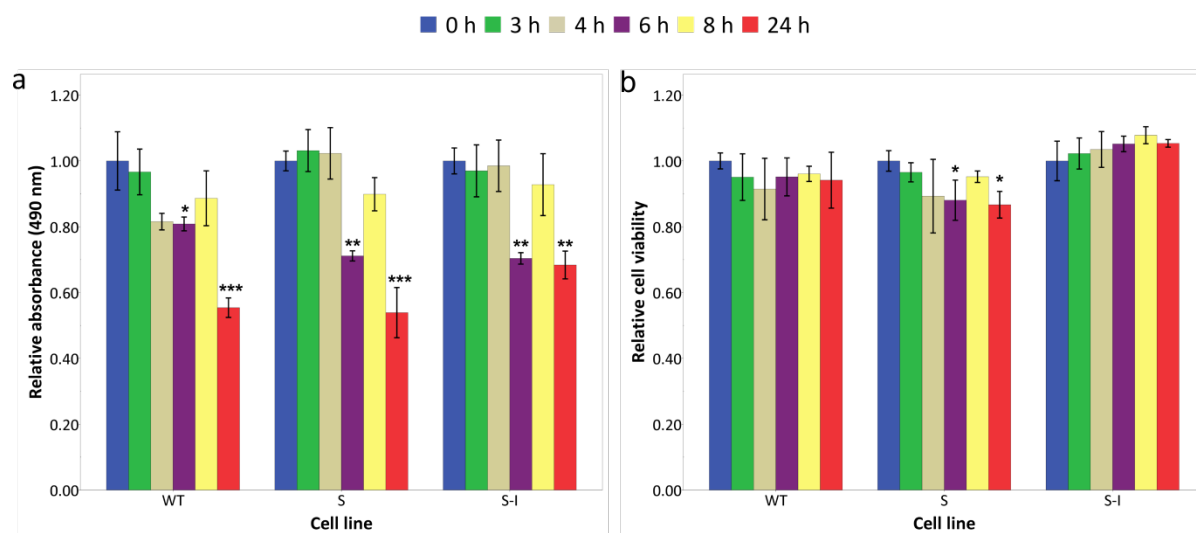


Figure 41: Effect of TM on proliferation and cell viability in a FAD cell-model system. Cell proliferation and viability of differentiated stressed cells were analysed by (a) MTS assay and (b) trypan blue dye exclusion method following the incubation of cells with TM for the indicated time points. Values represent the means for (a) $n=3$; $\pm$ SD and independent of (b) representing two independent experiments where the technical replicates for each experiment were $n=3$ and $n=2$ respectively $\pm$ SD. For 24 h the means are from the $n=3$ only. The results

are presented in relation to the zero hour TM-treatment. Statistical significant differences demonstrated on the graph are from cells treated with TM for zero hours compared with the cells treated with TM for 3 h, 4 h, 6 h, 8 h or 24 h respectively * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

However, as the blue coloration of cells with the trypan blue dye indicated cell death but not cell lysis, the live cells count was also evaluated (Table 8). All three cell lines revealed a significant decrease in the living cell count at 24 h of TM-treatment compared to the starting population, which for APP_{WT} and APP_S was $p<0.001$ while for APP_{S-I} was $p<0.05$. These data suggested that a number of cells died and some of them lysed during the 24 h of stress induction mediated by TM. Hence, prolonged stress could also lead to cell lysis induced from swelling and plasma membrane rupture of the cells. Taken together the data provide evidence that sustained toxicity as caused from 24 h TM-treatment induces cell death.

Table 8: Cell viability at zero and 24 h of TM-treatment for n=3. Student t-test analysis revealed a significant difference between the 0h and 24 h stress induction in all of the three cell lines. $p<0.05$ for APP_{WT} and APP_S (*) and $p<0.01$ for APP_{S-I} ().**

TM-treatment		Cell type & Live cell count		
		WT	S	S-I
0 h	Mean	1.81×10^6	2.95×10^6	3.68×10^6
	SD	2.23×10^5	5.32×10^5	3.02×10^5
24 h	Mean	1.18×10^6	1.72×10^6	1.95×10^6
	SD	1.25×10^5	3.12×10^5	4.20×10^5

At first, the data suggested that by eight hours of TM-treatment the viability and metabolic activity of all cell lines was temporally restored to basal levels. However, 24 h of TM-treatment induces death as seen by the drop in the metabolic activity, cell viability and the viable cell count. Taken together the data provide evidence that at eight hours of ER stress mediated by TM, an adaptation of the stress mechanism was induced in all cell lines but that sustained toxicity (24 h of TM-treatment), induces apoptosis.

5.2.2 UPR progression in response to ER stress has a different pattern in FAD cells

Analysis of the gene expression levels was conducted with qRT-PCR. For a given time point, the marker gene was initially normalised against the 18S rRNA housekeeping gene to address the variability between all the samples. This was followed by comparison with the results at zero hours to identify the fold change against the control sample. Thus all values presented below are expressed as the relative values with reference to the zero hour time point for each cell line, hereafter $APP_{WT,0h}$, $APP_{S,0h}$, $APP_{S-L,0h}$. Melting curve analysis was performed in all qRT-PCR samples to ascertain that only a specific single product was amplified from the reaction. A sharply defined single peak was produced in all apart from the 24 h time point samples. At 24 h most of the melting curves showed a peak at a temperature other than the expected one or no peak at all, probably as a result of cell death and degradation of cellular mRNA [307]. For this reason, samples of this time point were not analysed further to avoid the inaccurate representation of transcript levels.

The UPR responsive genes BiP, GRP94, ATF4, Nrf-2, XBP1s, XBP1T and CHOP were upregulated following TM-treatment indicating that the stress response was activated (Figures 42-44). In depth analysis of individual pathways both within (comparison of the mRNA levels following TM-treatment within a specific cell line) and among (comparison of the mRNA levels following TM-treatment between the cell lines) cell lines at each time point revealed differences in the stress-induced molecular mechanism triggered by FAD mutations.

The expression profile of UPR markers within each of the cell lines at each time point of TM-treatment was investigated first. This was initiated by examining the expression of BiP and GRP94. BiP is a major component of ER stress signalling; it acts prior to GRP94 and can be a negative UPR regulator by binding to ER stress receptors and keeping them in an inactive state while its depletion [308] leads to the activation of the three UPR arms. BiP was upregulated in all cell lines confirming the activation of the UPR from either the IRE1 or ATF6 pathways [309].

Although BiP was upregulated in all cell lines, it exhibited a different activation profile in each of them (Figure 42a). APP_{WT} exhibited a 2-fold increase of BiP transcripts ($p < 0.05$) at six hours post TM-induction with respect to $APP_{WT,0h}$ and while the average fold-change in BiP levels was still

elevated at eight hours, this was not statistically significant. APP_S induced a 3-fold increase of BiP expression at three hours of TM-treatment with respect to APP_{S,0h}. This response was sustained throughout the eight hours TM time-course induction and peaked at six hours post-induction with a 16-fold increase (three-, four-, and eight-hours of TM-treatment: $p < 0.05$, six-hours: $p < 0.01$) with respect to APP_{S,0h}. APP_{S-I} upregulated BiP at four hours of TM-treatment and its expression peaked at eight hours with a ~16-fold increase when compared to APP_{S-I,0h}.

Next, GRP94 mRNA, which often works in concert with BiP [310] was examined. GRP94 is generally expressed upon high demand of chaperones and under conditions where misfolded protein intermediates may have a deleterious effect [308]. In our time-course experiment, GRP94 transcript levels were increased at six hours in APP_S and at eight hours of TM-treatment in APP_{S-I} with respect to APP_{S,0h} and APP_{S-I,0h} respectively (Figure 42b).

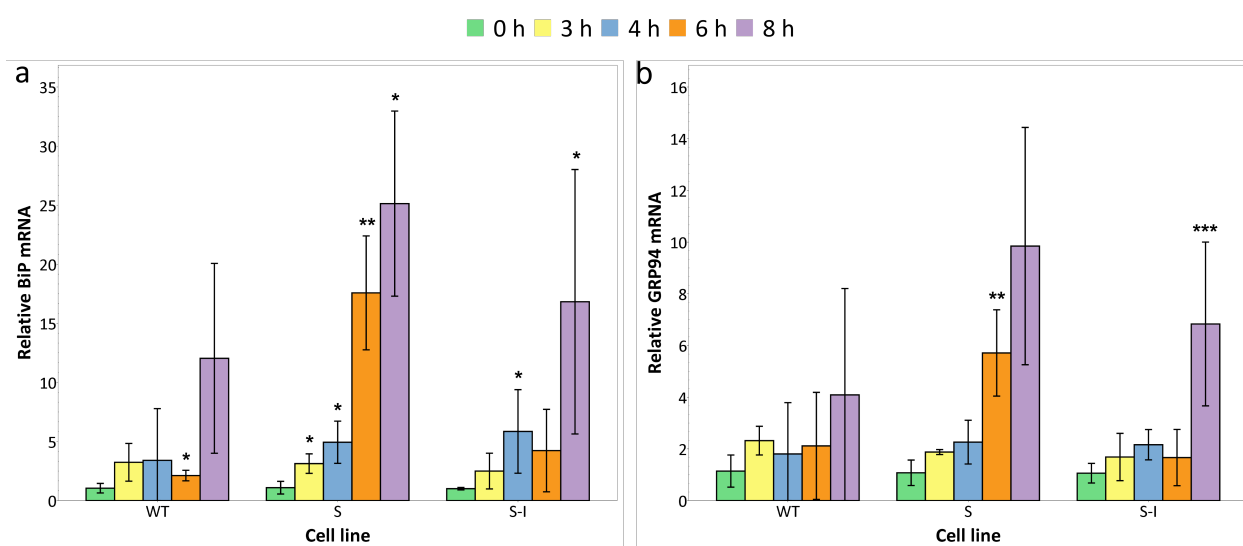


Figure 42: Expression profile of ER chaperones within each cell line during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were $n=3$ and $n=2$ respectively and the error bars correspond to $\pm$ SD. The stars represent in APP_{WT}, APP_S and APP_{S-I} represent the statistical significance compared to APP_{WT,0h}, APP_{S,0h} and APP_{S-I,0h} respectively. Significant p-values are denoted as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PERK activation was followed by assessing the contribution of the ATF4-CHOP and Nrf-2 signalling axes. ATF4 TF is responsible for triggering transcription of UPR markers for adaptation

such as GADD34, which induces translational recovery, while it may also promote CHOP expression leading to apoptosis [311]. At this point it is important to highlight that CHOP may also be stimulated by ATF6 activation, as a pro-survival mechanism (unlike PERK-mediated CHOP expression) [312]. In addition to ATF4-CHOP, PERK may also activate Nrf-2 acting as an antioxidant TF by inhibiting CHOP expression and promoting survival [313]. Some findings have suggested that the response from the two PERK-dependent UPR target genes namely ATF4 and Nrf-2 converge and magnify the PERK response [313]. Although the p-eIF2 α promotes translation of ATF4 TF, this TF is also subjected to translational control as discussed in section 2.6.3. Following PERK activation, this study focuses on investigating the ATF4 mRNA changes and the downstream target genes.

APP_{WT} revealed a 6-fold increase in CHOP expression at three hours of TM-treatment ($p < 0.01$) with respect to APP_{WT, 0h}, which was sustained throughout the time-course experiment with the peak expression being 11-fold at eight hours of TM-treatment (Figure 43). As ATF4 mRNA was not upregulated in this cell line, we hypothesised that CHOP expression was induced throughout the ATF6 pathway. However, as ATF4 is induced from p-eIF2 α at a translational level and CHOP induced by ATF6 is not known to be pro-apoptotic [312] while APP_{WT} data indicated at that at 24 h they underwent apoptosis, we suggest that the CHOP expression at the late time points (i.e. six and eight-hours) may be partially attributed to PERK activation. In addition, PERK is known to facilitate the synthesis of ATF6 and its trafficking to the Golgi [314], strengthening our hypothesis of PERK activation. The fact that ATF4 expression at the later time points (six and eight hours of TM-treatment) was not significantly upregulated was probably either due to the high variation observed and/or the transcriptional levels not reflecting the protein increase.

APP_S exhibited activation of both ATF4-CHOP and Nrf-2 signalling arms during the stress response. ATF4 mRNA showed a 7.5-fold statistically significant increase at six and eight hours of TM-treatment ($p < 0.001$ at both six and eight hours) compared to APP_{S, 0h}. CHOP transcripts were significantly upregulated from three to eight hours of TM-induced stress ($p < 0.001$ at all-time points) with an increase going from around 6- to ~62-fold respectively relative to APP_{S, 0h}.

We speculated again that CHOP activation at early time points (three hours) where ATF4 was not upregulated was the result of ATF6 pro-survival mechanism. It may also be plausible; ATF4 to be upregulated at the protein level but not reflected with the mRNA data. On the other hand CHOP activation at the later time points (six- and eight-hours) where ATF4 was upregulated was due to signalling of the PERK pro-apoptotic mechanism.

APP_{S-I} displayed a 6-fold increase in ATF4 mRNA expression at eight hours of TM-treatment ($p < 0.05$) with respect to APP_{S-I,0h}. On the other hand CHOP transcripts increased from 7.5-fold at three-hours to 50-fold at eight hours. Similarly with APP_S, we suggested that expression of CHOP at early time points was due to ATF6 activation while the latter time points from PERK effect. Nrf-2 transcripts were not increased throughout the response implying that the Nrf-2 pathway was not activated in this cell line.

Progression of UPR in a cell model system of familial Alzheimer's disease

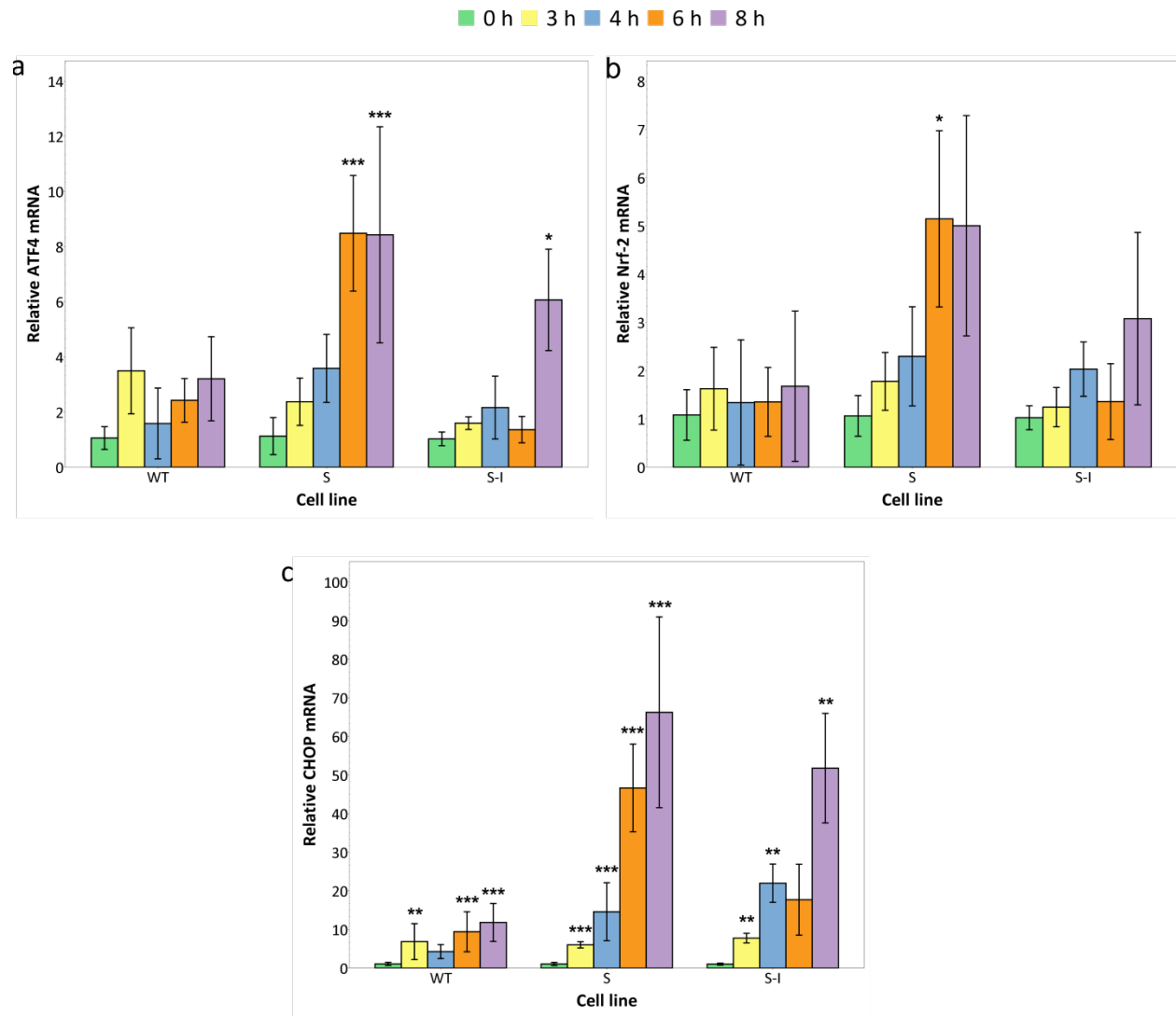


Figure 43: Expression profile of PERK markers within each cell line during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were $n=3$ and $n=2$ respectively and the error bars correspond to $\pm$ SD. The stars in APP_{WT} , APP_S and APP_{S-I} represent the statistical significance compared to $APP_{WT,0h}$, $APP_{S,0h}$ and $APP_{S-I,0h}$ respectively. Significant p-values are denoted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

IRE1 activation induces XBP1 splicing, thus XBP1s mRNA levels were measured with qRT-PCR to detect the activity of this pathway (Figure 44a). APP_{WT} exhibited a significant XBP1s expression at eight hours of TM-treatment compared to $APP_{WT,0h}$. Analysis of APP_S showed upregulation of XBP1s from a 2.5-fold increase at three hours to ~13-fold at eight hours of TM-treatment compared to $APP_{S,0h}$. Similarly, APP_{S-I} exhibited increased levels of XBP1s relative to $APP_{S-I,0h}$ at three hours of TM-treatment followed by its attenuation at the later time points.

Progression of UPR in a cell model system of familial Alzheimer's disease

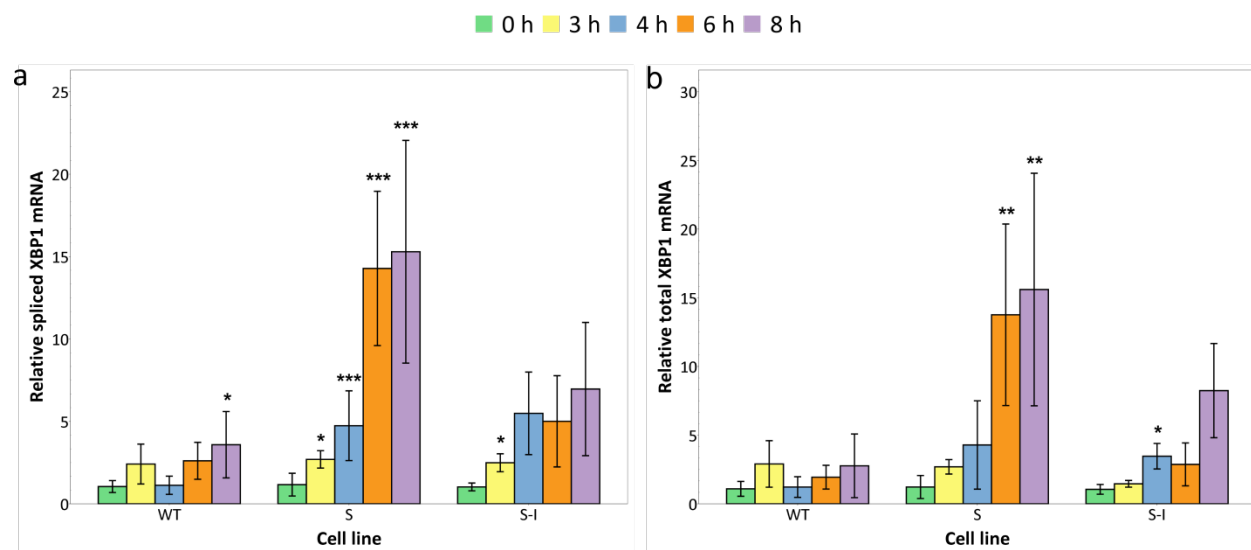


Figure 44: Expression profile of IRE1 and ATF6 markers within each cell line during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were n=3 and n=2 respectively the error bars correspond to $\pm$ SD. The stars in APP_{WT}, APP_S and APP_{S-I} represent the statistical significance compared to APP_{WT,0h}, APP_{S,0h} and APP_{S-I,0h} respectively. Significant p-values are denoted as *p<0.05, **p<0.01, *p<0.001.**

Activation of the ATF6 pathway was measured by monitoring the total XBP1 transcripts, while also considering the CHOP mRNA levels induced by PERK and/or ATF6. BiP mRNA levels were also considered for this analysis as their expression is induced by IRE1 and ATF6 signalling [315]. APP_{WT} did not exhibit XBP1T overexpression, but induced CHOP mRNA in the absence of ATF4 transcripts from three to eight hours; as well as of BiP at six hours post TM-induction. This suggests that APP_{WT} activates the ATF6 pathway in response to TM-induced ER. APP_S induced increased expression of XBP1T transcript from six to eight hours of TM-treatment when compared to APP_{S,0h} (p<0.01 in both cases), which peaked at eight hours with a ~13-fold increase (Figure 44b). As activation of CHOP at three hours was not a result of PERK signalling because ATF4 was only expressed from six hours of TM-treatment, we suggested that ATF6 activation was induced from three hours of TM-treatment and continued throughout the time-course experiment (up to eight hours), where BiP mRNA was also expressed. In APP_{S-I} XBP1T activation was detected at four hours with an approximately 8-fold increase compared to APP_{S-I,0h}. The CHOP mRNA levels induced at three and four hours of TM-treatment were not a result of the ATF4-CHOP axis (PERK), which was activated at eight hours. Instead, the expression

of CHOP in addition with BiP transcripts elevated at four hours, suggested ATF6 signalling to occur at three and four hours of TM-treatment. Our data provide evidence of ATF6 activation prior to PERK while also coincide at further time points (in APP_{MUT}), possibly caused by PERK enhancement of ATF6 signalling.

Table 9: ANOVA results (p-values) of the stress experiment comparing the mRNA levels between the TM-treatment time points of a specific cell line. The highlighted colours illustrate the (p) significance: a) Green corresponds to $p < 0.05$ (*), b) Yellow corresponds to $p < 0.01$ () and c) red corresponds to $p < 0.001$ (***).**

Gene	Time (h)	3			4			6			8		
		WT	S	S-I	WT	S	S-I	WT	S	S-I	WT	S	S-I
BiP	0	0.151	0.015	0.097	0.755	0.033	0.010	0.049	0.007	0.005	0.153	0.011	0.005
	3				1.000	0.350	0.168	0.606	0.011	0.831	0.269	0.015	0.014
	4							0.957	0.014	0.819	0.322	0.018	0.203
	6										0.200	0.427	0.083
GRP94	0	0.175	0.091	0.537	0.175	0.157	0.090	0.175	0.012	0.660	0.175	0.056	0.000
	3				0.175	0.841	0.781	0.175	0.031	1.000	0.175	0.077	0.001
	4							0.175	0.034	0.665	0.175	0.087	0.010
	6										0.175	0.422	0.001
ATF4	0	0.102	0.882	0.032	0.895	0.383	0.331	0.071	0.000	0.653	0.139	0.000	0.016
	3				0.303	0.892	0.807	0.665	0.002	0.848	0.998	0.002	0.024
	4							0.729	0.012	0.626	0.427	0.013	0.031
	6										0.839	1.000	0.017
CHOP	0	0.002	0.000	0.001	0.011	0.000	0.003	0.000	0.000	0.067	0.000	0.000	0.006
	3				0.916	0.015	0.011	0.860	0.000	0.274	0.326	0.000	0.010
	4							0.391	0.000	0.882	0.076	0.000	0.034
	6										0.867	0.617	0.017
Nrf-2	0	0.849	0.284	0.052	0.849	0.225	0.052	0.849	0.030	0.052	0.849	0.076	0.052
	3				0.849	0.858	0.052	0.849	0.057	0.052	0.849	0.138	0.052
	4							0.849	0.107	0.052	0.849	0.237	0.052
	6										0.849	0.076	0.052
XBP1T	0	0.168	0.990	0.320	0.168	0.869	0.014	0.168	0.006	0.233	0.168	0.002	0.091
	3				0.168	0.986	0.033	0.168	0.018	0.395	0.168	0.005	0.106
	4							0.168	0.052	0.941	0.168	0.015	0.234
	6										0.168	0.977	0.177
XBP1s	0	0.402	0.013	0.011	1.000	0.000	0.070	0.277	0.000	0.133	0.024	0.000	0.126
	3				0.460	0.339	0.217	0.999	0.000	0.395	0.547	0.000	0.261
	4							0.323	0.004	0.998	0.030	0.003	0.952
	6										0.703	1.000	0.891

5.2.3 APP_{MUT} exhibit a similar UPR activation profile

Following the observation that all cell lines activated IRE1 and ATF6 but only APP_{MUT} triggered PERK activation, an analysis of the differences in the magnitude of the response between the FAD cell lines was performed. As explained in the 5.2.2 section, each of the genes was normalised against the 18S rRNA and compared with the respective transcript levels at zero hour of TM-treatment. Thus, the aim in this section was to compare the fold changes of APP_{MUT}, with APP_{WT}. This let us evaluate how APP₆₉₅ mutations affect the magnitude of the stress response.

In APP_S the expression of the two chaperone genes (BiP and GRP94) demonstrated a significant fold increase at six hours of TM-treatment relative to both the APP_{WT} and APP_{S-I} with an eight- (p<0.001) and 4-fold (p<0.001) increase, respectively (Figure 45a).

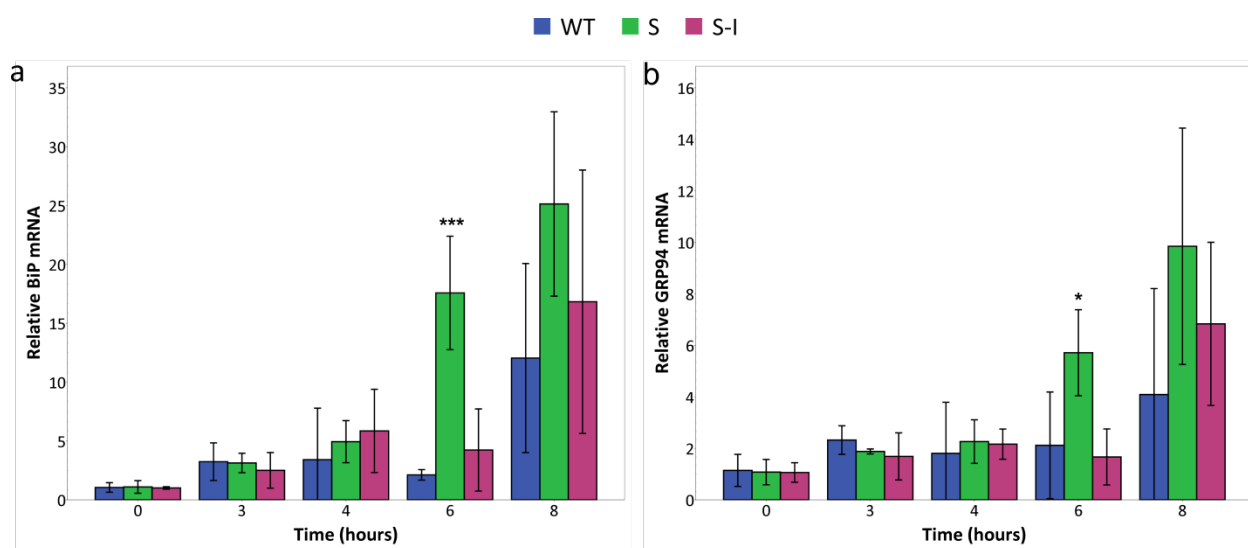


Figure 45: Expression profile of the chaperones between the cell lines during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were n=3 and n=2 respectively and the bars correspond to ±SD. The stars represent the fold change compared to the APP_{WT} at zero hours. Significant p-values are denoted as *p<0.05, **p<0.01, ***p<0.001.

The magnitude of the PERK response was evaluated by the fold changes of the ATF4 and CHOP. As CHOP is also simulated by ATF6 signalling, specific PERK-signalling fold changes were

investigated only at times when both ATF4 and CHOP were activated. In APP_S activation of ATF4-CHOP arm was stimulated at six and eight hours while in APP_{S-I} at eight hours of TM-treatment. APP_S at six hours exhibited a significant fold change of ATF4 relative to both APP_{WT} and APP_{S-I} ($p<0.01$), and at eight hours displayed ATF4 increase only with respect to APP_{WT} ($p<0.05$) (Figure 46a).

The fold increase of CHOP was significant higher in APP_S at six- and eight- hours of TM-treatment with a fold increase of 5- ($p<0.05$) and of 5.5- ($p<0.001$), respectively compared to APP_{WT}. Increase of the CHOP transcript in APP_{S-I} was also significant higher at eight hours (the time point at which both ATF4 and CHOP were seen to be upregulated in section 5.2.2) and was 4.5 times higher ($p<0.001$) with respect to the APP_{WT} (Figure 46c). These results indicate that APP_{MUT} activated PERK with a higher magnitude (Figure 43c) compared to APP_{WT} (Figure 46c).

Progression of UPR in a cell model system of familial Alzheimer's disease

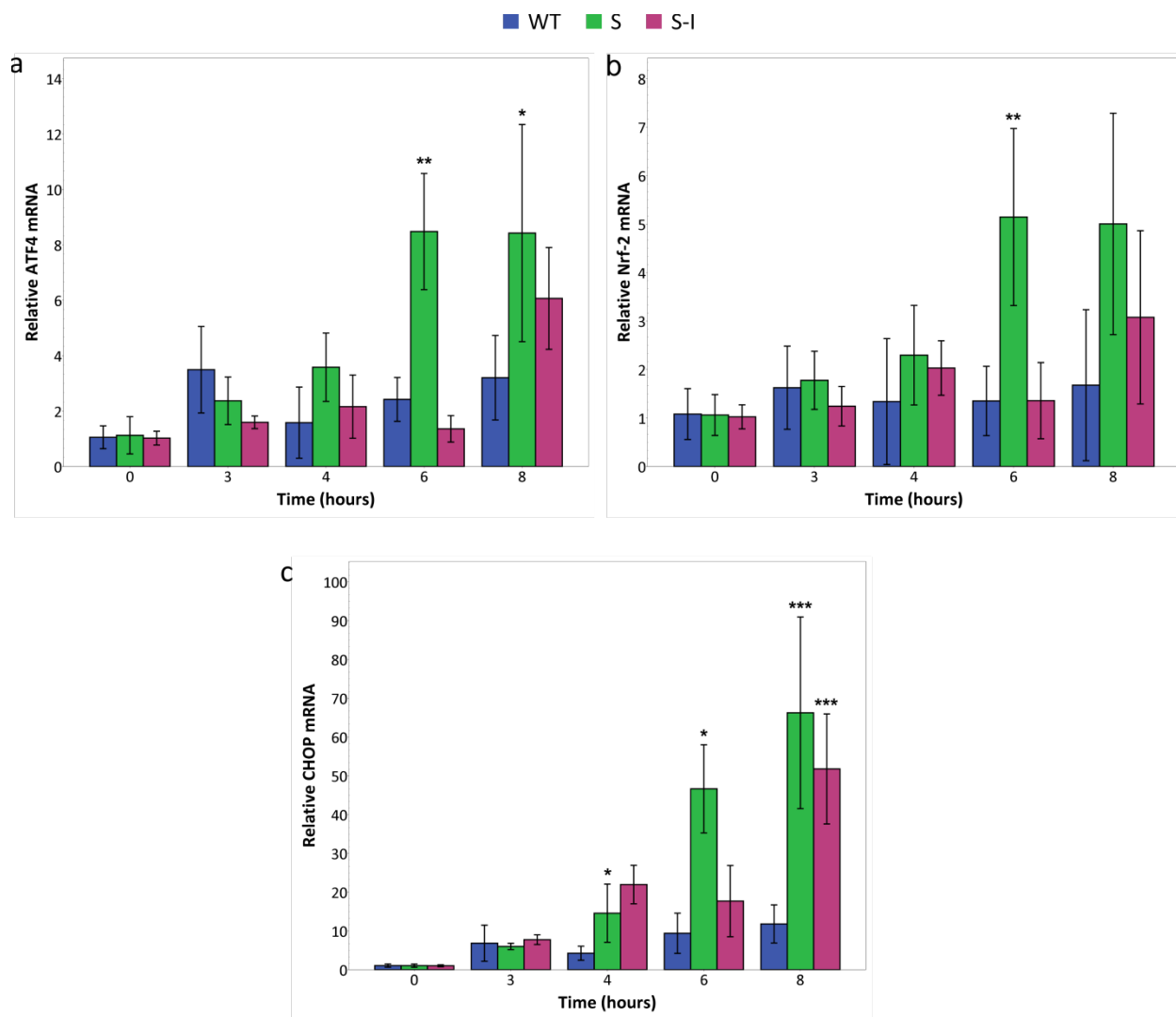


Figure 46: Expression profile of PERK markers between the cell lines during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were $n=3$ and $n=2$ respectively and the error bars correspond to $\pm$ SD. The stars represent the fold change compared to the APP_{WT} that has been normalised h APP_{WT,0h}. Significant p-values are denoted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Next the differences in IRE1 expression markers between cell lines at each time point of TM-treatment were assessed by measuring the fold changes of the XBP1s. APP_S was previously shown to activate IRE1 from three to eight hours of TM-treatment. The XBP1s fold change of APP_S when compared with the respective APP_{WT} levels exhibited a significant increase from four to eight hours of TM-treatment ($p<0.001$) while when compared with APP_{S-I} demonstrated a significant increase at six- and eight hours ($p<0.01$). The pattern observed was expected as IRE1 pathway at these time points was not activated in APP_{S-I} (Figure 47a). APP_{S-I} analysis revealed

an increase of the XBP1 mRNA fold change at four hours only with respect to APP_{WT} ($p < 0.01$). These data also demonstrated that APP_S activated the IRE1 branch with the highest stress magnitude.

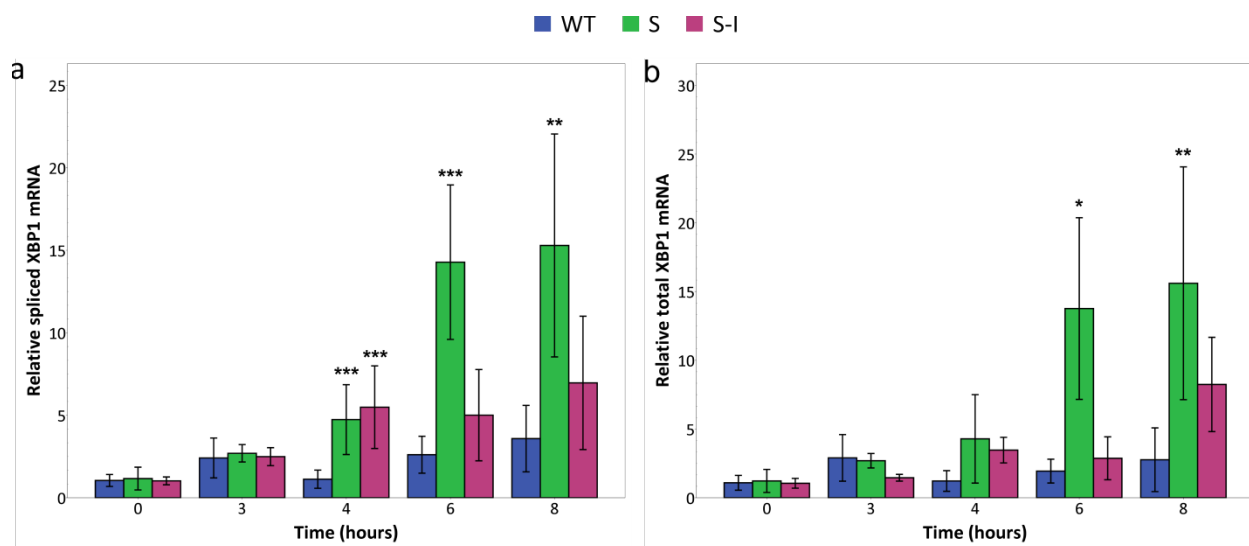


Figure 47: Expression profile of IRE1 and ATF6 markers between the cell lines during TM-treatment. The means are from two independent experiments where the technical replicates for each experiment were $n=3$ and $n=2$ respectively and the bars correspond to $\pm$ SD. The stars represent the fold change compared to the APP_{WT}. Significant p-values are denoted as * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$.**

The magnitude of the ATF6 response between the cell lines was evaluated by monitoring the XBP1T transcripts, but also by considering the fold changes of CHOP at early time points and of BiP levels at all time-points. APP_S demonstrated ATF6 activation from three to eight-hour post TM-treatment. The activation at three- and four-hours was mainly observed by CHOP (in the absence of ATF4) and by BiP expression while at six and eight hours by XBP1T transcripts. When compared to APP_{WT}, the fold increase of CHOP at four hours of TM-treatment was 5-fold higher in APP_S ($p < 0.05$), while the fold increase of XBP1T at six and eight hours was of a magnitude of 7- ($p < 0.05$) and 5.5-fold ($p < 0.01$) higher, respectively in APP_S. Finally, comparison of APP_S with APP_{S-I} revealed that at eight hours APP_S had a 5-fold increase in XBP1T ($p < 0.05$), (Figure 47b). This was expected, as at eight hours, APP_{S-I} did not have activated the ATF6 pathway as

suggested in section 5.2.2. With respect to ATF6 pathway, APP_S revealed a stronger activation of this pathway.

Table 10: ANOVA results (p-values) of the time course stress experiment between the cell lines. The highlighted colours illustrate the (p) significance: a) Green corresponds to $p < 0.05$ (*), b) Yellow corresponds to $p < 0.01$ (), and c) red corresponds to $p < 0.001$ (***)**

Time (h)	Cell line	0		3		4		6		8	
		WT	S	WT	S	WT	S	WT	S	WT	S
BiP	S	0.942		0.658		0.536		0.000		0.114	
	S-I	0.942	0.942	0.658	0.658	0.536	0.536	0.666	0.000	0.114	0.114
GRP94	S	0.964		0.279		0.469		0.011		0.127	
	S-I	0.964	0.964	0.279	0.279	0.469	0.469	0.949	0.007	0.127	0.127
ATF4	S	0.943		0.059		0.061		0.004		0.023	
	S-I	0.943	0.943	0.059	0.059	0.061	0.061	0.087	0.003	0.242	0.368
CHOP	S	0.981		0.107		0.024		0.012		0.000	
	S-I	0.981	0.981	0.107	0.107	0.001	0.110	0.243	0.012	0.000	0.575
Nrf-2	S	0.975		0.432		0.336		0.001		0.055	
	S-I	0.975	0.975	0.432	0.432	0.336	0.336	0.997	0.001	0.055	0.055
XBP1T	S	0.899		0.963		0.077		0.034		0.008	
	S-I	0.899	0.899	0.963	0.963	0.077	0.077	0.512	0.042	0.064	0.585
XBP1s	S	0.921		0.856		0.000		0.000		0.005	
	S-I	0.921	0.921	0.856	0.856	0.000	0.879	0.485	0.002	0.510	0.039

5.2.4 Mutant cells exhibit stronger upregulation of UPR markers in response to ER stress

Each of the UPR-associated genes normalised to 18S rRNA, was now compared against APP_{WT} at each of the time points of TM-treatment. Thus, all values presented below are expressed as the relative values with reference to the APP_{WT} at each time-point individually. This analysis was performed in order to strengthen our hypothesis that APP_{MUT} cells exhibited a stronger ER stress response after TM administration- than the APP_{WT}.

Analysis of the expression levels of chaperones illustrated their upregulation in APP_{MUT} compared to APP_{WT}. More specifically, APP_S exhibited an increase in both BiP and GRP94 at six hours which was of around 4-($p < 0.01$) and 2.2-fold ($p < 0.01$), respectively. At the same time point (six hours) the BiP mRNA was also around 3-fold ($p < 0.01$) higher compared to APP_{S-I}. Consistent with these data are the ones from the APP_{WT} comparison (Figure 45) as they also

showed APP_S to have increased levels of BiP and GRP94 at six hours of TM-treatment. Analysis of APP_{S-I} showed that it displayed increased BiP transcripts levels at four hours and of GRP94 at eight hours of TM-treatment with a magnitude of 4.5-($p<0.001$) and of around 4-fold ($p<0.05$), respectively (Figure 48) compared to APP_{WT}. These results indicated that APP_{MUT} induced the expression of chaperones with at higher levels compared to APP_{WT}.

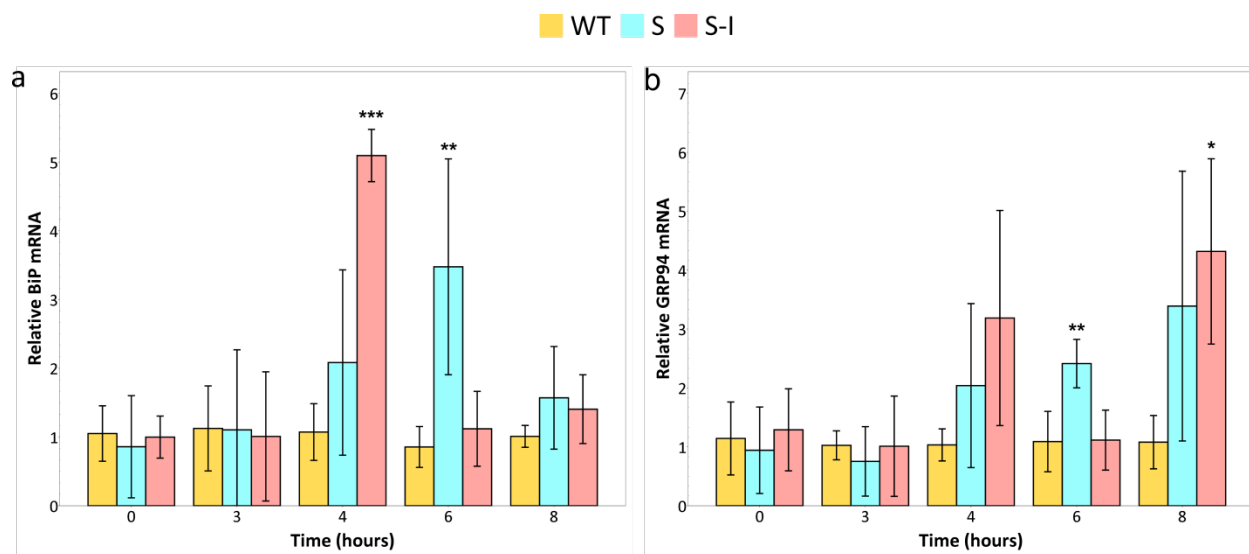


Figure 48: Expression profile of chaperones in APP_{MUT} compared to APP_{WT}. The means are from two independent experiments where the technical replicates for each experiment were n=3 and n=2 respectively and the error bars correspond to $\pm$ SD. The stars represent the fold change compared to the APP_{WT}. Significant p values are denoted as * $p<0.05$, ** $p<0.01$, *** $p<0.001$

PERK activation in the APP_{MUT} was demonstrated in Figure 43. Comparison of the magnitude of the PERK signalling was investigated by analysis of ATF4, Nrf-2 and CHOP mRNA levels. APP_S exhibited significant $\sim$ 2.5-fold upregulation of the ATF4 and Nrf-2 transcripts at six hours and $\sim$ 2.5-fold of CHOP mRNA at eight hours of TM-treatment with respect to APP_{WT} ($p<0.001$, $p<0.01$ and $p<0.01$, respectively) (Figure 49). In addition, APP_S also had significantly increased Nrf-2 transcript levels of around 2-fold ($p<0.05$) compared to APP_{S-I} at six hours of TM-treatment. Similar with these conclusions were the ones deduced from the comparison of APP_S with APP_{WT,0h} (Figure 46) demonstrating elevated levels of ATF4, Nrf-2 and CHOP at six hours of TM-treatment. APP_{S-I} displayed a 3.5-and 2.5-fold increase ($p<0.05$) in the Nrf-2 and CHOP

levels, respectively, at eight hours of TM-treatment with respect to APP_{WT,0h}. Interestingly, APP_{S-I} also displayed increased mRNA expression of CHOP at eight hours with respect to APP_{WT,0h} (Figure 46c).

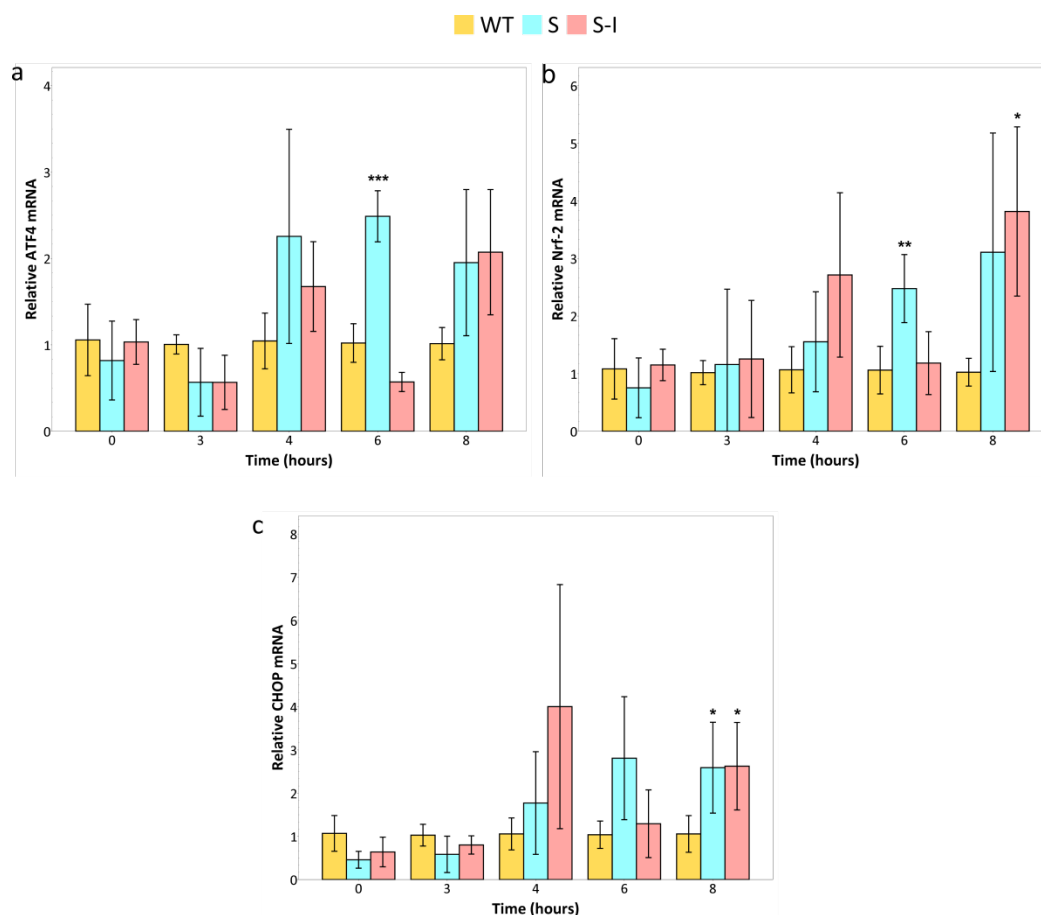


Figure 49: Expression profile of PERK markers in APP_{MUT} compared to APP_{WT}. The means are from two independent experiments where the technical replicates for each experiment were n=3 and n=2 respectively and the error bars correspond to $\pm$ SD. The stars represent the fold change compared to the APP_{WT}. Significant p values are denoted as *p<0.05, **p<0.01, ***p<0.001.

Comparison of the differential magnitude of IRE1 activation among cell lines was achieved by assessing the XBP1s mRNA levels. APP_S attained a ~2.5-fold increase (p<0.01) of XBP1s transcripts at six hours of TM-treatment with respect to APP_{WT} (Figure 50) and of around 2-fold (p<0.05) with respect to APP_{S-I}. Similarly, APP_S exhibited an upregulated XBP1s mRNA fold increase relative to APP_{WT,0h} as well as to APP_{S-I} (Figure 47a). These observations strengthen the

hypothesis that APP_{MUT} exhibited a stronger activation than APP_{WT} of the IRE1 branch, but also suggested APP_S to have the strongest magnitude of this response.

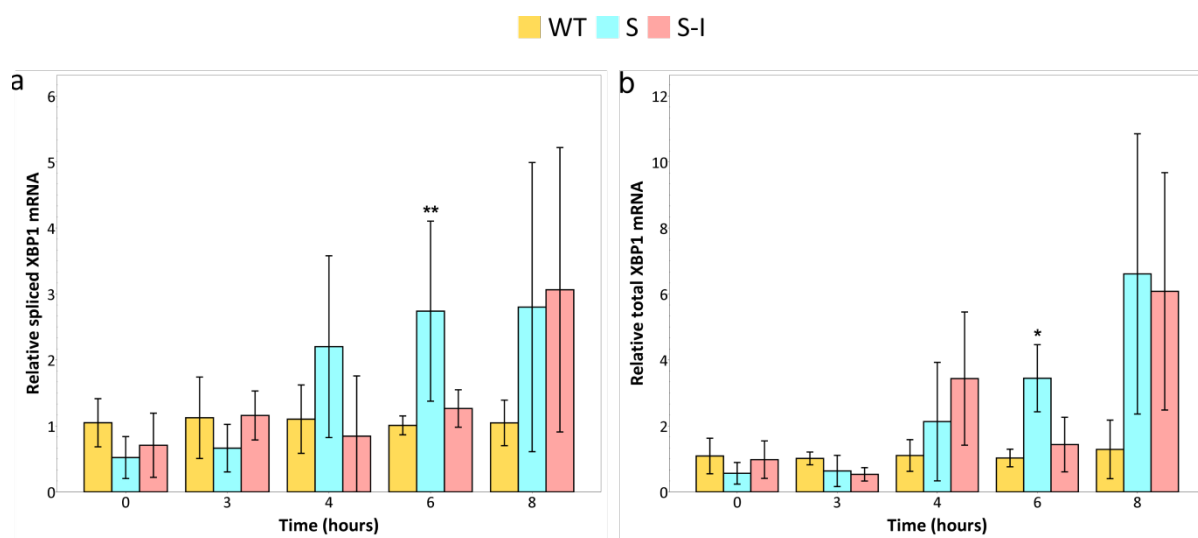


Figure 50: Expression profile of IRE1 and ATF6 in APP_{MUT} compared to APP_{WT}. The means are from two independent experiments where the technical replicates for each experiment were n=3 and n=2 respectively and the error bars correspond to ±SD. The stars represent the fold change compared to the APP_{WT}. Significant p values are denoted as *p<0.05, **p<0.01, ***p<0.001.

Evaluation of the ATF6 response among the cell lines was performed by analysis of the XBP1T mRNA levels as well as of CHOP (at early time points) and BiP mRNA. APP_S which activated the ATF6 from three to eight hours, exhibited a significant 3.5-fold (p<0.05) increase of XBP1T at six hours with respect to APP_{WT} and of ~2.5-fold (p<0.05) with respect to APP_{S-I} (Figure 50). Similarly, when XBP1T transcripts of APP_S were compared with the fold changes of APP_{WT,0h}, they demonstrated an increase, which was also evident when compared to APP_{WT} at six hours (Figure 47). APP_S also had significant higher mRNA levels of BiP at six hours compared to APP_{WT}. On the other hand, APP_{S-I} that activated ATF6 from three to four hours post TM-induction, demonstrated an increase of BiP mRNA at four hours, which was significant higher compared to APP_{WT}.

Table 11: ANOVA results (p values) of the time course stress experiment of APP_{MUT} relative to APP_{WT}. The highlighted colours illustrate the (p) significance: a) Green corresponds to p<0.05 (*), b) Yellow corresponds to p<0.01 () and c) red corresponds to p<0.001 (***).**

Time (h)	Cell line	0		3		4		6		8	
		WT	S	WT	S	WT	S	WT	S	WT	S
BiP	S	0.835		0.684		0.000		0.007		0.263	
	S-I	0.835	0.835	0.684	0.684	0.182	0.000	0.921	0.009	0.263	0.263
GRP94	S	0.730		0.397		0.098		0.002		0.175	
	S-I	0.730	0.730	0.397	0.397	0.098	0.098	0.996	0.003	0.018	0.745
ATF4	S	0.575		0.051		0.072		0.000		0.056	
	S-I	0.575	0.575	0.051	0.051	0.072	0.072	0.052	0.000	0.056	0.056
CHOP	S	0.053		0.115		0.124		0.098		0.018	
	S-I	0.053	0.053	0.115	0.115	0.124	0.124	0.098	0.098	0.013	0.996
Nrf-2	S	0.366		0.812		0.111		0.003		0.176	
	S-I	0.366	0.366	0.812	0.812	0.111	0.111	0.928	0.005	0.027	0.812
XBP1T	S	0.331		0.229		0.105		0.011		0.063	
	S-I	0.331	0.331	0.025	1.000	0.105	0.105	0.584	0.023	0.063	0.063
XBP1s	S	0.144		0.212		0.116		0.001		0.285	
	S-I	0.144	0.144	0.212	0.212	0.116	0.116	0.511	0.006	0.010	0.120

ER stress in our cell model system caused by the inhibition of N-linked glycosylation revealed that different FAD mutations activate the UPR in a specific manner. Comparison of transcript levels of UPR markers from distinct pathways at each time point of TM-treatment was performed both within and among cell lines.

Our results demonstrated that downstream activation of the IRE and ATF6 pathways were seen in each of the three cell lines with the progression being different among them, but induction of all three branches (IRE1, ATF6 and PERK) was only demonstrated in APP_{MUT}. It was also confirmed that the magnitude of the response was significantly higher in APP_{MUT} than APP_{WT} cells.

5.3 Discussion

In this chapter, we investigated the transcript levels of UPR targets downstream of the IRE1, PERK, and ATF6 pathways, namely BiP, GRP94, XBPT, XBP1s, Nrf-2, ATF4 and CHOP. These markers were investigated in a time-course manner by TM-treatment of differentiated

neuroblastoma cells overexpressing either the wild-type or mutant APP₆₉₅ isoforms; including APP695_{K670N/M671L} (APP_S) and APP695_{V717F} (APP_{S-I}) experimentally. Using gene expression (qRT-PCR), metabolic and cell viability analysis (MTS assay in combination with trypan blue exclusion method), the data suggested differences in the UPR mechanism and the time-scale of the response. Cells overexpressing APP₆₉₅ bearing FAD genetic mutations activated ER stress more strongly compared to APP_{WT}.

5.3.1 Sequence of UPR activation revealed in a FAD cell model in response to ER stress

Analysis of the APP_{WT} revealed that the ATF6 branch was activated from three to eight hours of TM-treatment as indicated by the upregulation of CHOP marker in the absence of ATF4 expression (with a pro-survival function), (Figure 43). At this point, we also speculate that the PERK arm may also be activated but not reflected in the ATF4 transcriptional levels. The IRE1 arm was activated at eight hours of TM-treatment as seen by the XBP1s activation (Figure 44).

We then examined the stress response in APP_S, which appeared to be different and with a higher magnitude compared to APP_{WT}. Transcriptional analysis provided evidence of IRE1 activity starting at three hours and remaining active up to eight hours of TM-treatment (Figure 44). At the same time-points (three to eight hours) of stress induction, the cells also activated the ATF6 branch. This was seen by the transcriptional upregulation of CHOP mRNA (in the absence of ATF4), of BiP mRNA as well as by the transcriptional upregulation of XBP1T (Figure 44). Activation of PERK was suggested to occur at the later time points; six and eight hours of TM-treatment as seen by the pro-apoptotic CHOP mRNA expression levels. All three pathways remained active at eight hours of TM-treatment.

We next checked the order of UPR activation in APP_{S-I}. Early activation of the IRE1 pathway was observed by monitoring the active XBP1s mRNA at three hours of TM-treatment, which was followed by a drop in its transcript level (Figure 44). ATF6 was also activated early in the time-course experiment as seen by CHOP, BiP and/or XBP1T transcripts (Figure 44). Finally, APP_{S-I} stimulated PERK activation at eight hour of TM-induced ER stress (Figure 43).

Further comparisons between the magnitudes of the response between the cell lines, revealed APP_{MUT} to stimulate a stronger response compared to APP_{WT}. In order to determine the magnitude of the pro-apoptotic stimuli, the levels of the pro-apoptotic CHOP TF mRNA were evaluated in all cell lines. Although CHOP was transcriptionally upregulated with statistical significance in all cell lines at eight hours of TM-treatment, we detected the highest increase in APP_S, which exhibited a 62-fold increase compared to APP_{S,0h} hour time point. The APP_{S-I} were shown to have a 50-fold increase compared to APP_{S-I,0h} and lastly the APP_{WT} had the lowest increase which was of 11-fold with respect to APP_{WT,0h}. The higher CHOP transcripts in APP_{MUT} suggested a possibly higher cell loss in the cells carrying the mutant APP₆₉₅ variant. However, at 24 of TM-treatment there was not any indication of significant cell loss between APP_{WT} and APP_{MUT}. A higher difference in the cell loss between APP_{WT} and APP_{MUT} could probably be captured at a time point after 8 h and before 24 h of TM-treatment when cells have been directed to apoptosis.

The data supported the following sequence of UPR activation:

1. APP_{WT} induced ATF6 activation at three hours continuing throughout the duration of the experiment, and IRE1 activation at eight hours of TM-treatment.
2. APP_S initially exhibited IRE1 and ATF6 activation at three hours, which was sustained throughout the time-course, and PERK activation at six and eight hours of TM-treatment.
3. APP_{S-I} displayed IRE1 and ATF6 activation at three hours, with the ATF6 pathway being activated at the four hours too, followed by PERK activation at eight hours of TM-treatment.

5.3.2 The key players in UPR activation induced by ER stress

Several genes are responsible for protecting the ER from any physiological input. Molecular chaperones have a vital role in controlling protein quality and assisting with the folding process

of misfolded proteins. Quantification of BiP transcript levels during the time course induction showed that all cell lines exhibited upregulation of BiP after TM-treatment but with a different activation profile, showing that overexpression of the wild-type APP₆₉₅ is sufficient to trigger its expression (Figure 42a). The earliest upregulation of BiP as well as the highest transcript increase was detected in APP_S cells. More specifically, at six hours of TM-treatment the fold increase of BiP mRNA was significantly higher in APP_S compared to both APP_{WT} and APP_{S-I} ($p < 0.001$).

In addition to BiP, other chaperones are also needed for the protein folding control under stress conditions. As such, GRP94 another major UPR target was upregulated only in FAD-associated cell lines and observed to work in conjunction with BiP. More specifically, GRP94 was found to be expressed at the same time as BiP as its transcripts were elevated at six hours in APP_S and at eight hours in APP_{S-I} of TM-treatment (Figure 42). As GRP94 has a key role under conditions necessitating an increased demand of protein folding machinery, this suggested that the unfolded protein load in APP_S and APP_{S-I} is higher compared to APP_{WT} during the time course induction.

Transcription of several genes during the UPR is controlled by XBP1s, ATF4 and the truncated-ATF6 transcription factor [316], where each of them define a specific signalling pathway. IRE1 activation in both APP_S and APP_{S-I} was rapid as monitored by the increased XBP1s mRNA at three hours of TM-treatment. The early IRE1 signalling observed is supported by the Lin *et al.* [305] findings, suggesting this pathway to be set in motion in the beginning of the UPR response. Furthermore, the fact that XBP1 splicing was reported to occur during cellular adaptation strengthens our speculation of cellular adaptation at eight hours of TM-treatment. Previous reports have revealed that unmitigated cellular stress promotes the activation of IRE1, but persistent stress attenuates its activity. IRE1 is a key step in the cell fate decision of survival or cell death, with its prolonged activation being related to enhanced survival [185]. The fact that IRE1 which was previously reported to occur during cellular adaptation [79] remained activated at eight hours in APP_S, may be an indicator of the enhanced survival strengthening our speculation of cellular adaptation at eight hours of TM-treatment.

Strong and sustained CHOP activation was revealed in all genetically modified cell lines from the beginning of the TM-treatment. Our data showed that CHOP was activated at a very early time points (three hours) but also at later time points (eight hours) of TM-treatment in cells carrying both the mutant and wild-type APP₆₉₅ variant. These reinforced the key role of CHOP signalling in both its pro- and anti-apoptotic properties by ATF6 or PERK signalling respectively in the UPR.

APP_{MUT} cells provided strong evidence of PERK activation with a high magnitude at eight hours of TM-treatment. These data were strengthened by the observation of Hoozemans and co-workers in post-mortem brain material that demonstrated the presence of higher levels of phosphorylated (activated) PERK in diseased compared to healthy individuals [42]. Exposure of APP_S to TM also induced Nrf-2 overexpression, leading to transcription of genes regulating redox homeostasis. This upregulation occurred at six hours of TM-treatment and thus in parallel with the ATF4 activation. These observations are supported by previous findings showing ATF4 and Nrf-2 to act together following the eIF2 α phosphorylation step [313]. The same group postulated that ATF4 and Nrf-2 pathways converge and act together to amplify the PERK response. Our data showed that pro-apoptotic CHOP had the highest activation in APP_S; suggesting a possible cross talk between ATF4 and Nrf-2 markers leading to an increased expression of the downstream target genes. Importantly, Nrf-2 mRNA was not upregulated in APP_{WT} or in APP_{S-L}, strengthening the hypothesis that the overall amplified PERK response in APP_S resulted from the synergistic effect of ATF4 and Nrf-2. As this activation was indicated only in APP_S, it reinforced the suggestion that these cells accumulated more unfolded proteins leading to higher stress and thus activating the PERK arm with a higher amplification signal. Saying this though, the significance of Nrf-2 in the progression of neurodegenerative diseases is still under investigation. Its activation plays presumably a protective role in the detoxification of ROS that promote apoptosis [317].

Metabolic activity analysis demonstrated a drop at six hours of TM-treatment, which was however not accompanied by a further decrease during stress-induction (i.e. eight hours) suggesting a possible adaptation of cells to the stress response, through the upregulation of

specific UPR markers. However, at 24 h both metabolic activity and cell viability data provided evidence of cell death. Therefore, although cells appeared to achieve a new steady state of homeostasis at eight-hours, the prolonged stress-induction (24 h) resulted in apoptosis.

As distinct changes in transcription of several genes may affect the progression of the disease [318], it was shown *in vitro* that different FAD mutations have transcriptional differences in the key regulators. The different transcriptional profiles suggest the presence of a customised response in the different cell types. Although the ER tries to restore homeostasis through transcriptional changes, it also requires short-term responses to ease the effects of stress by translational pause. However, re-activation of global translation along with the contribution of transcriptional mechanisms is essential for cells to avert death and for homeostasis to be re-established. In addition, faithful translation of the mRNA of the UPR target genes upregulated under induction of stress is essential as part of the transcriptional UPR response [316, 319].

5.4 Conclusion

A substantial amount of evidence suggests that stress induction in cells overexpressing wild-type and mutant APP₆₉₅, leads to a differential sequence of the UPR activation. The data indicated, the pattern of upregulation in APP_S and APP_{S-I} to be similar, whereas in APP_{WT} to differ. APP_{MUT} activated all three arms of the stress response (IRE1, ATF6 and PERK) with APP_S keeping them all active at eight hours while APP_{S-I} induced their activation at different time points of the TM time-course experiment. Furthermore, the APP_{MUT} were generally more stressed than APP_{WT} and demonstrated a different UPR mechanism. However, we postulated that these differences might be the result of an increased variability of the UPR expression markers, masking a similar type of response exhibited with a lower magnitude in the APP_{WT} cells. It was also demonstrated that the cells' activity appeared to adapt but following a prolonged stress induction (24 h) cells died showing their over-commitment to apoptosis. The data suggest that the tripartite response is structured as such to control stress via distinct branches, providing a customised approach to cells. A prolonged stress response appears to

have detrimental effects and make the cells unable to fully restore homeostasis and to activate pro-apoptotic pathways. However, it remains unclear what controls the balance of pro-apoptotic and anti-apoptotic stimuli or whether and how this response is translated at a protein level.

Chapter 6

Computational model of the mammalian UPR network

6.1 Introduction

The UPR forms a highly interconnected network regulating a number of genes in response to ER stress which encode proteins essential for the decrease of the cellular unfolded protein load [206]. It is difficult to address the role of UPR activation and its involvement in AD with experimental approaches for several reasons. Firstly, clinical symptoms of AD usually occur when the disease has already progressed. For instance, diagnostic biomarkers may already be upregulated at the time of the diagnosis, since the beginning of the pathologic process may take many years before the clinical onset [320]. Second, as AD affects the brain tissue, it is difficult to disentangle the onset and disease progression over time. When techniques for monitoring these activities become available, they would require longitudinal studies starting prior of the disease clinical manifestations and continuing to the most advance stages or even until death has occurred. Although *in vivo* and *in vitro* models have been established to interrogate the UPR mechanism, it is difficult to deduce their relevance in humans. A first step towards that could be adopting a ‘systems’ approach, with both biological experimentation and mathematical modelling. This would allow isolating the UPR signalling pathways and analysing the complexity of the system at hand by focusing on how component interactions control the UPR-system behaviour in different diseases. This information would help identify the effects of

specific mutations, specific points for intervention and promising therapeutic avenues. Several initiatives towards the application of systems biology principles to medicine have been made recently [321, 322].

This chapter describes the development of a mammalian UPR computational model using semi-quantitative data of an eight-hour TM-time course stress experiment, for each of the APP_{WT}, APP_S and APP_{S-I} cell lines. The fold change of the five UPR markers (BiP, XBP1s, XBP1T, ATF4 and CHOP) was multiplied by the corresponding initial values of the model -which were their steady state concentrations obtained by simulating the model starting from an initial condition of zero- in order to be adjusted with the same scale. Following the scaling process, the 14 parameters associated with the three UPR pathways were estimated such that the model output tracked the values of five aforementioned UPR markers. The simulations and parameter estimation were performed using the gPROMS[®] software version 3.5.3 (Process Systems Enterprise, Ltd, London, UK) using the DAEs (differential-algebraic equations) solver “DASOLV” and parameters values exported from the literature. Subsequently, the model was used to perform case studies on the effect of drugs known to target UPR progression, which demonstrate the potential of complimenting experimental analyses with modelling to probe the system (Table 12).

Table 12: Outline of the computational setup.

Type of model studied	Signalling branches	Experimental data used	UPR dynamics & Case studies
Computational • WT • S • S-I	IRE1	BiP	Effect of lithium & valproate in FAD
		Spliced XBP1	Effect of XBP1 complex formation
	ATF6	Total XBP1	
	PERK	ATF4	Effect of salubrinal
		CHOP	

6.2 General characteristics of the computational model

The model was developed using a top-down approach. It was formulated based on single-cell deterministic mass-action kinetics, resulting in a set of ordinary differential equations that describe known features of the IRE1, PERK and ATF6 pathways. In this model we took into account: 1) the key UPR markers downstream of these receptors which were described as variables and defined as number of molecules and 2) their link in regulating one another's functions described with the rate equations. The complete map of reactions and signals considered in the model is illustrated in Figure 51.

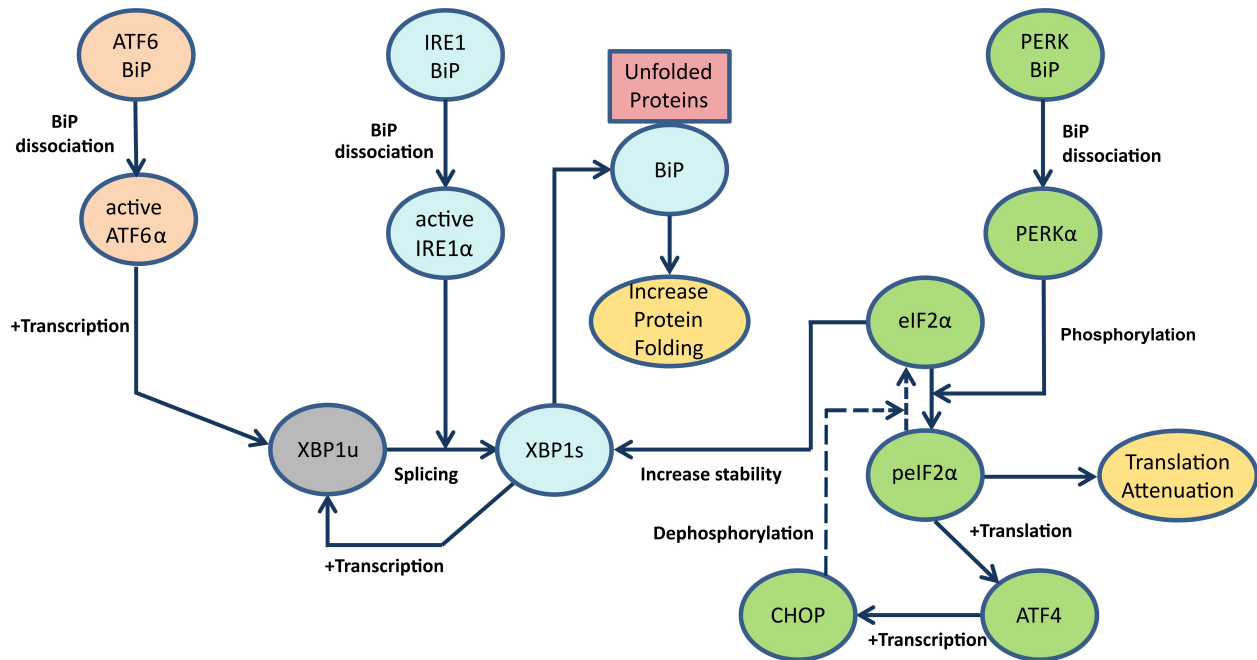


Figure 51: Complete map of the mammalian UPR model. Each arrow represents the reaction rate of an algebraic equation. The input of the system is the rate of translation of unfolded proteins (red square) and the output is represented by i) an increase of the protein folding or ii) translation attenuation (yellow circles) responsible for restoring ER homeostasis or pro-apoptotic signals (transcription of CHOP).

Several biochemical features were incorporated in the model and are as follows: a) association and dissociation rates of BiP from the UPR receptors triggering the activation or inhibition of the stress cascade, b) transcription of the following components: BiP, ATF4, CHOP, unspliced

XBP1, and spliced XBP1, c) translation of the following components: BiP, unspliced XBP1 and spliced XBP1, ATF4 and CHOP mRNA, d) phosphorylation/dephosphorylation of eIF2 α protein and, finally, e) degradation rates of BiP, ATF4, CHOP, unspliced XBP1 and spliced XBP1.

More specifically, BiP dissociation from the receptors induces their activation while its binding induces the deactivation of that particular branch of the stress response. Activation of the IRE1 pathway was examined by studying the XBP1 components, activation of the PERK pathway by studying the phosphorylated eIF2 α and the downstream ATF4 and CHOP genes while activation of the ATF6 pathway by studying the XBP1u.

6.2.1 Model assumptions

The development of the computational model was based on the following assumptions:

- a) All three ER transmembrane sensors (IRE1, PERK, and ATF6) were bound to BiP initially. This assumption was made in order to have our system in starting from a point without stress.
- b) The activation of IRE1, PERK and ATF6 stress receptors occurs upon the dissociation of BiP from them. BiP therefore acts as a modulator for the activation and deactivation of the ER stress receptors.
- c) The binding and dissociation constants between BiP and the three receptors take the same values for all pathways. The values for IRE1 were extracted from the literature by the model of UPR in yeast by Pincus *et al.* [138] while PERK and ATF6 constants were assumed to follow the same kinetics. As the binding constant of BiP with IRE1 used by Pincus *et al.* was taken from an experimental study based on mammalian cells [323], we believe that the values used were valid for our mammalian UPR model. Although we assumed that all three receptors had the same rate constants, our experimental data showed a different activation profile between the receptors. To model this activation

we introduced a time-delay in IRE1 and PERK pathways, which was in accordance with the time profile of our experimental data (described in 6.3.1a).

- d) The IRE1 double activation mechanism was neglected. The mammalian IRE1 isoforms (IRE1 α and IRE1 β) have been demonstrated to activate the UPR differently [324]. IRE1 α , which is ubiquitously expressed, is activated/deactivated by the BiP dissociation/association. IRE1 β found in the epithelium of the gastrointestinal tract is activated with a dual mechanism as follows: i) the direct interaction of unfolded proteins and ii) the dissociation/association of BiP. As we were examining the UPR in AD, we investigated and subsequently modelled the IRE1 α (that has been referred as IRE1 from Chapter 2), which is ubiquitously expressed. For this reason, the single activation mechanism was chosen.
- e) Changes in mRNA levels were assumed to have a linear correlation with the equivalent protein levels. Although in biological reality this linear relationship is not always the case, this assumption was made because of the lack of protein level data [325].
- f) PERK phosphorylation of eIF2 α promotes the translation of ATF4 mRNA [314]. As ATF4 is also subjected to transcriptional changes, we investigated ATF4 mRNA data, even though the exact mechanism of these changes remains unknown.
- g) The number of IRE1 molecules in the ER used (2.56×10^2) was reported by Pincus *et al.* [138] taken from a study on yeast by Ghaemmaghami *et al.* [326]. The number of PERK and ATF6 molecules in the ER was chosen to be the same as the number of IRE1 molecules. Evaluation of the numbers of molecules of the three receptors could be experimentally achieved by blotting. Furthermore, when this information becomes available, kinetic constants of BiP to the receptors (association and dissociation rates) could be assessed providing information on the natural activation procedure (which receptor is activated first).

- h) The translation rate of BiP mRNA (β_B) was selected to be the same as in yeast, which is 0.25 sec^{-1} [138].
- i) The decay rate of BiP protein (γ_B) is $1.39 \times 10^{-4} \text{ sec}^{-1}$ [327].
- j) The initial values for ATF4, CHOP, eIF2 α , U, U|B were chosen arbitrarily such that the initial state of the single-cell model reflects steady-state conditions.
- k) CHOP expression was induced from PERK signalling only. In reality CHOP expression, is transactivated by both PERK and ATF6 [328]. More specifically, PERK mediates expression of pro-apoptotic CHOP while ATF6-associated signals induced facilitate the pro-survival mechanism [312].

6.3 Model development

6.3.1 Protein folding dynamics

The first event we wanted to capture in our model was translational attenuation, which is activated by the PERK signalling pathway. With regards to the protein folding dynamics, the following key features were taken into account to capture this event.

- a) Following our experimental data analysis from the TM time course experiment, it was observed that the cells were stressed at around three hours of TM-treatment. This was specifically seen by the upregulation of the transcript levels of UPR markers. For this reason, in order to capture this behaviour in the computational model, all cell lines exhibited a time delay in the onset of the stress response. This was introduced in the model by inserting the time lag (τ) in the beginning of the ER stress to fit the experiments, a technique widely used in modelling and proven to be valid [329]. We also model a time-delay in the transcription of ATF4 and CHOP mRNA in order to fit the experimental data. This delay was

added to these genes, as we had no data to follow and modify the eIF2 α phosphorylation as opposed to the data for XBP1 splicing.

- b) Previous studies have showed that several UPR regulatory genes exhibit their upregulation both prior to and post- induction of ER stress. In a similar manner, the proteins that are upregulated due to UPR activation, i.e. chaperones, ATF4 and CHOP, are not affected by translational attenuation in our model [330].
- c) Activated PERK (pPERK) acts as a kinase that induces phosphorylation of the ' α ' subunit of eIF2 α . In our model, translational attenuation is dependent on phosphorylation of eIF2 α [331].

A progressive onset of stress was created, mimicking the accumulation of unfolded proteins in the ER. Therefore, the rate of unfolded proteins synthesised in the ER, S_u , is given by the following equation:

$$\frac{d[S_u]}{dt} = K_u t \quad (1)$$

Where K_u represents the cumulative load of unfolded proteins and t is time. Translational attenuation was modelled by following the approach proposed by Trusina *et al.* [158]. S_u (quantity of unfolded proteins arriving in the ER per seconds) was scaled according to the extent of eIF2 α phosphorylation:

$$\text{translation attenuation: } \frac{[peIF2\alpha]}{[eIF2\alpha]_{(0)}} S_u \quad (2)$$

The $[peIF2\alpha]$ stands for the quantity concentration of phosphorylated eIF2 α , whereas $[eIF2\alpha]_{(0)}$ for the initial quantity of the total eIF2 α protein.

The equation related to the unfolded protein is as follows:

$$\frac{d[U]}{dt} = \frac{[peIF2\alpha]}{[eIF2\alpha]_{(0)}} S_U - C_{UB}[U][B] + \gamma_{UB}[U|B] + \gamma_B[U|B] \quad (3)$$

Where $[U]$ represents the concentration of unfolded proteins, C_{UB} is the binding rate constant of unfolded protein binding to BiP, $[B]$ is the concentration of free BiP, γ_{UB} is dissociation rate of BiP from unfolded protein, γ_B is the decay rate of BiP protein, and $[U|B]$ is the concentration of unfolded proteins bound to BiP. Therefore, the material balance for the unfolded protein-BiP complex is:

$$\frac{d[U|B]}{dt} = C_{UB}[U][B] - \gamma_{UB}[U|B] - \gamma_B[U|B] - \gamma_{fold}[U|B] \quad (4)$$

Where γ_{fold} is the rate of protein folding. BiP mRNA is given by:

$$\frac{d[B_m]}{dt} = \beta_{Bm} + N_{Bm}f(X_m^s) - \gamma_{Bm}[B_m] \quad (5)$$

The binding of the spliced XBP1 TF to the UPRE promoter was modelled as a Hill function. This was a convenient mathematical way to describe XBP1s transcription in the regulation of the other UPR genes.

$$f(X_m^s) = \frac{[X_m^s]^2}{\alpha_0 + \alpha_1[X_m^s] + [X_m^s]^2} \quad (6)$$

Where $[B_m]$ is the concentration of BiP mRNA, β_{Bm} is its basal transcription rate, N_{Bm} is a constant for the upregulation of BiP transcription under the UPR, and γ_{Bm} is the decay rate of BiP mRNA. $[X_m^s]$ is the concentration of spliced XBP1 mRNA and α_0, α_1 are constants. Similarly, the material balance for BiP is:

$$\frac{d[B]}{dt} = \beta_B[B_m] - \gamma_B[B] - C_{RB}[U][B] + \gamma_{RB}[U|B] + \gamma_{fold}[U|B] \quad (7)$$

Where β_B is the translation rate of BiP mRNA, C_{RB} is the rate of BiP binding to the free receptor, and γ_{RB} is the rate of dissociation of BiP from the inactive receptor.

6.3.2 IRE1 pathway

The description of the IRE1 pathway follows the model proposed by Pincus *et al.* [138] for UPR activation in yeast, which consists of a single-stranded response mediated by IRE1. It has been proposed that IRE1 in yeast [332] and IRE1 β in the mammalian cells [324] may be activated by dissociation from BiP or by subsequent interaction of its peptide-binding domain on its luminal ER side with unfolded proteins [324, 332]. These scenarios represent the single and double activation mechanism, respectively. As in here the IRE1 α arm was modelled, the single activation mechanism was employed.

Under non-stressed conditions, IRE1 is present in a complex with BiP ($IRE|B$) the material balance for which is:

$$\frac{d[IRE|B]}{dt} = C_{IB}[B][IRE_A] - \gamma_{IB}[IRE|B] \quad (8)$$

Where $[IRE_A]$ the concentration of activated IRE1, C_{IB} is the binding rate of BiP to activated IRE1, and γ_{IB} is the dissociation rate of BiP from IRE1. The concentration profile of activated IRE1 over time is described by:

$$\frac{d[IRE_A]}{dt} = -C_{IB}[B][IRE_A] + \gamma_{IB}[IRE|B] \quad (9)$$

While studying the behavior of IRE1 activation, the model clearly demonstrated that there is a link between the different UPR modules and thus that they are not exclusive of each other. More specifically, the BiP component is present in the protein folding module, the IRE1 activation and is itself affected by transcription of UPR signalling branches.

The transcription of the unspliced XBP1 mRNA (X_m^u) is up-regulated by the activation of the ATF6 branch, prior to its splicing by the IRE1 pathway [333]. Thus, the material balance for XBP1 mRNA is a function of ATF6 activation and is described with the following equation:

$$\frac{d[X_m^u]}{dt} = \beta_{X_m^u} + N_{ATF6\alpha} f(ATF6_\alpha) \quad (10)$$

Where $\beta_{X_m^u}$ the basal transcription rate of unspliced XBP1 mRNA, $N_{ATF6\alpha}$ is the constant representing the magnitude of stress response following ATF6 activation, and $f(ATF6_A)$ is the following activation Hill function:

$$f(ATF6_A) = \frac{[ATF6_A]^2}{\alpha_0 + \alpha_1[ATF6_A] + [ATF6_A]^2} \quad (11)$$

Where $[ATF6_A]$ is the concentration of activated ATF6 and α_0, α_1 are constants. The material balance for XBP1s mRNA is a function of IRE1 activation and is represented as follows:

$$\frac{d[X_m^s]}{dt} = -\gamma_{X_m^s}[X_m^s] + \beta_{X_m^s} \min([IRE_A], [X_m^u]) \quad (12)$$

Spliced XBP1 mRNA stability is dependent on the level of phosphorylation of $eIF2\alpha$. To capture this feature, we introduced a modified decay rate of XBP1s mRNA ($\gamma_{X_m^s}$) that is linearly dependent on the concentration of phosphorylated $eIF2\alpha$. The approach used was that at 0% phosphorylation, the mRNA decay rate would be the same as the one of the unspliced form taken from Majumder *et al.* [167]. In contrast, at full phosphorylation, the decay rate would be

the one of the spliced form as given in the same study. The decay rate of XBP1s mRNA would therefore become:

$$\gamma_{X_m^s} = \gamma_u + (\gamma_s - \gamma_u) \frac{[peIF2\alpha]}{[eIF2\alpha]_{(0)}} \quad (13)$$

Where $\begin{cases} \gamma_u = 5.7 \times 10^{-4} \text{ sec}^{-1} \\ \gamma_s = 8.7 \times 10^{-5} \text{ sec}^{-1} \end{cases}$

This new feature also allowed us to create a link (other than BiP and unfolded proteins) between the two pathways (PERK and IRE1); such links are known to be multiple. Finally, the addition of the downstream pathway of PERK with mRNAs and protein such as ATF4 and eIF2 α helped us create a negative regulation loop in the PERK pathway, through the CHOP-mediated dephosphorylation of phosphorylated eIF2 α (equation 16) [334].

6.3.3 PERK pathway

While the PERK activation mechanism requires the dissociation of BiP [335], there is some debate on whether the unfolded proteins are also engaged with its activation of the luminal domain through direct binding [336]. Although some studies have shown direct activation of unfolded proteins with PERK [332], there is not enough evidence to prove that this mechanism takes place. In our model, PERK is bound to BiP under non-stressed conditions. In the presence of increased levels of unfolded proteins in the ER, BiP dissociates from this complex and PERK becomes activated. The material balances for the complex ($PERK|B$) and the activated PERK ($PERK_A$) are:

$$\frac{d[PERK|B]}{dt} = C_{PB}[B][PERK_A] - \gamma_{PB}[PERK|B] \quad (14)$$

$$\frac{d[PERK_A]}{dt} = -C_{PB}[B][PERK_A] + \gamma_{PB}[PERK|B] \quad (15)$$

Where C_{PB} is the binding rate of BiP to activated PERK and γ_{PB} is its dissociation rate from the complex. Following on from PERK activation, $eIF2\alpha$ is phosphorylated at the Ser51 residue [258] as part of the translation attenuation mechanism. The material balances for the phosphorylated ($peIF2\alpha$) and non-phosphorylated $eIF2\alpha$ species are as follows:

$$\frac{d[peIF2\alpha]}{dt} = \beta_{eIF2\alpha} \min([PERK_A], [eIF2\alpha]) - \frac{[CHOP]}{[CHOP]_{max}} \gamma_{eIF2\alpha} [peIF2\alpha] \quad (16)$$

$$\frac{d[eIF2\alpha]}{dt} = -\beta_{eIF2\alpha} \min([PERK_A], [eIF2\alpha]) + \frac{[CHOP]}{[CHOP]_{max}} \gamma_{eIF2\alpha} [peIF2\alpha] \quad (17)$$

Where $\beta_{eIF2\alpha}$ is the rate of phosphorylation and $\gamma_{eIF2\alpha}$ the rate of dephosphorylation. Dephosphorylation of p-eIF2 α is mediated by GADD34 [334], which is directly activated by CHOP [260]. The ratio of $\frac{[CHOP]}{[CHOP]_{max}}$ was therefore used as a modulator of the CHOP-mediated dephosphorylation rate.

As part of the UPR, ATF4 is transcribed and translated. The former event is subject to regulation by the level of phosphorylated eIF2 α , which is described as a Hill function in the material balance below:

$$\frac{d[ATF4_m]}{dt} = \beta_{ATF4_m} + N_{ATF4_m} f(peIF2\alpha) - \gamma_{ATF4_m} [ATF4_m] \quad (18)$$

$$\text{With } f(peIF2\alpha) = \frac{[peIF2\alpha]^2}{\alpha_0 + \alpha_1 [peIF2\alpha] + [peIF2\alpha]^2}$$

Where β_{ATF4_m} is the basal transcription rate of ATF4, N_{ATF4_m} is a constant for the upregulation of ATF4 transcription under the UPR, and γ_{ATF4_m} is the decay rate of ATF4 mRNA. The material balance for ATF4 protein is:

$$\frac{d[ATF4]}{dt} = \beta_{ATF4}[ATF4_m] - \gamma_{ATF4}[ATF4] \quad (19)$$

Where β_{ATF4} is the translation rate of ATF4 mRNA and γ_{ATF4} is the decay rate of ATF4 protein.

The next effect of the PERK pathway is the expression of CHOP. The CHOP mRNA ($CHOP_m$) mass balance is described as a function of ATF4 and is given by:

$$\frac{d[CHOP_m]}{dt} = \beta_{CHOP_m} + N_{CHOP_m}f(ATF4) - \gamma_{CHOP_m}[CHOP_m] \quad (20)$$

where

$$f(ATF4) = \frac{[ATF4]^2}{\alpha_0 + \alpha_1[ATF4] + [ATF4]^2} \quad (21)$$

β_{CHOP_m} and γ_{CHOP_m} are the transcription and decay rates of CHOP mRNA, respectively. N_{CHOP_m} is a constant for the upregulation of CHOP transcription under the UPR. Finally, the levels of CHOP are:

$$\frac{d[CHOP]}{dt} = \beta_{CHOP}[CHOP_m] - \gamma_{CHOP}[CHOP] \quad (22)$$

Where β_{CHOP} is the translation rate of CHOP mRNA and γ_{CHOP} is the decay rate of CHOP protein.

6.3.4 ATF6 pathway

Under homeostatic conditions, ATF6 is bound to BiP. In the presence of increased levels of unfolded protein in the ER, BiP dissociates from this complex and ATF6 translocates to the Golgi where it gets cleaved by caspases [333]. This results in the release of its activated truncated form. The material balances for the complex ($ATF6|B$) and the activated ($ATF6_A$) are:

$$\frac{d[ATF6|B]}{dt} = C_{AB}[B][ATF6_A] - \gamma_{AB}[ATF6|B] \quad (23)$$

$$\frac{d[ATF6_A]}{dt} = -C_{AB}[B][ATF6_A] + \gamma_{AB}[ATF6|B] \quad (24)$$

Where C_{AB} is the binding rate of BiP to activated ATF6 and γ_{AB} is the dissociation constant of BiP from ATF6.

6.4 Formation of the XBP1 protein complex

As described in section 2.7.3, XBP1 is a major regulator in the UPR. Its function is vital as upon formation of the XBP1u-XBP1s complex (XBP1c), it acts as a negative regulator for the XBP1s TF, which promotes the synthesis of ER chaperones (Figure 13). The dynamic interplay of the complex formation in our model is illustrated in Figure 52.

Computational model of the mammalian UPR network

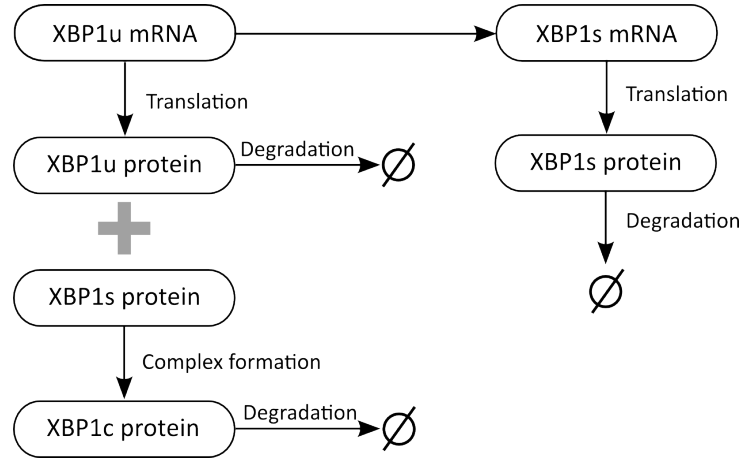


Figure 52: Reaction map of XBP1 protein complex formation integrated in the model. The following letters stand for the different forms of XBP1 where u: unspliced, s: spliced and c: complex.

In order to replicate this mechanism *in silico*, the equations 25 and 26 were introduced in the model to describe the translation of spliced and unspliced XBP1 mRNA and the formation of the protein complex.

The material balance for XBP1 unspliced is:

$$\frac{d[X^u]}{dt} = \beta_{X^u} [X_m^u] - \beta_{X^c} [X^u][X^s] - \gamma_{X^u} [X^u] \quad (25)$$

Where $[X^u]$ is the concentration of XBP1u protein (pXBP1u), $[X^s]$ is the concentration of XBP1s protein (pXBP1s), β_{X^u} is the translation rate of XBP1u mRNA, β_{X^c} is the rate of XBP1 complex formation, γ_{X^u} is the decay rate of pXBP1u.

Similarly, the material balance for XBP1 spliced is:

$$\frac{d[X^s]}{dt} = \beta_{X^s} [X_m^s] - \beta_{X^c} [X^u][X^s] - \gamma_{X^s} [X^s] \quad (26)$$

Where γ_{X^s} is the decay rate of pXBP1s.

6.4.1 Degradation of XBP1 complex

Yoshida *et al.* [337] used transfected HeLa cells overexpressing either the spliced or unspliced XBP1 mRNA form and examined their half-lives with immunoprecipitation of the cell lysates (Figure 53). The experimental degradation study showed that although these proteins have different degradation rates, these rates do not vary significantly to each other as both forms disappear within four hours.

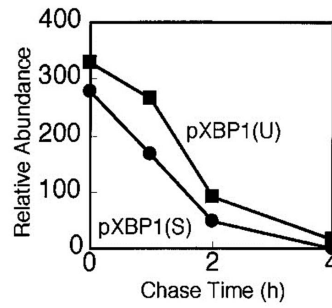


Figure 53: Stability of pXBP1u and pXBP1s in Hella cells [337].

Following the abundance of XBP1 protein motifs (measured in AU) using pulse chase analysis by Yoshida *et al.* (Figure 53), the calculation of the XBP1 degradation rates for pXBP1s (γ_{X^s}) and pXBP1u (γ_{X^u}) was as follows:

$$\gamma_{X^s} = \frac{290-40}{2h} = 2.08 \text{ min}^{-1} = 0.035 \text{ sec}^{-1}$$

$$\gamma_{X^u} = \frac{320-100}{2h} = 1.83 \text{ min}^{-1} = 0.030 \text{ sec}^{-1}$$

The values introduced in the model were 0.030 sec^{-1} for pXBP1u and 0.035 sec^{-1} for pXBP1s. Next, the complex degradation rate was determined. Studies have shown that degradation of the pXBP1s-pXBP1u complex by the proteasome occurs following the complex translocation to the cytoplasm. These motifs have different C-terminal regions while their only common domain is the leucine zipper in the N-terminus allowing DNA binding and dimerization [338]. However, the proteasomal degradation process requires the 209-261 region of pXBP1u, showing that its

presence is indispensable for the quick destruction of the complex (Figure 54) [166, 338]. In another study, Lee and co-workers [339] generated mutant pXBP1u forms where two or three lysine residues (necessary for ubiquitin dependent degradation) in the C-terminal domain were replaced with arginine residues. These mutations generated more stable forms of the pXBP1u expressed at higher levels, which is in accordance with the function of ubiquitin in regulating protein degradation. The stable pXBP1u inhibited the activity of pXBP1s TF, suggesting a potential role for pXBP1u in controlling the function of pXBP1s.

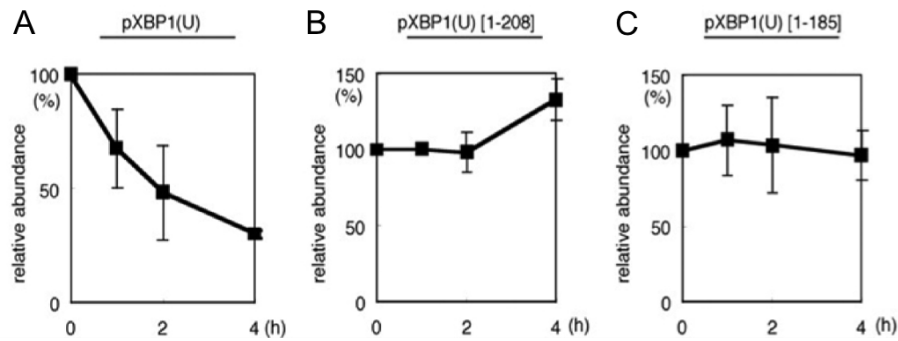


Figure 54: Degradation rates of pXBP1u forms in HeLa cells. Cells transfected with different forms of pXBP1 and treated with cycloheximide as indicated for four hours. The figure illustrates the cells expressing A: pXBP1u, B: pXBP1u (1-208) and C: pXBP1u (1-185) [338].

In agreement with the experimental evidence from the aforementioned studies, degradation of XBP1c, was assumed to occur at the same rate as pXBP1u i.e. faster than pXBP1s. In order to examine the role of pXBP1u in regulating the pXBP1s, the degradation and the translation rate constants for both molecules were determined.

6.4.2 Translation of XBP1 complex

The translation rates for pXBP1s (β_{Xs}) and pXBP1u (β_{Xu}) were calculated from data of a study of Yoshida and co-workers [166]. In that study, the accumulation of the XBP1 proteins was examined in the lysates of HeLa cells treated with TM in a 12 h time-course assay (Figure 55).

$$\beta_{X^s} = \frac{100-20}{7h} = 0.19 \text{ min}^{-1} = 3.2 \times 10^{-3} \text{ sec}^{-1}$$

$$\beta_{X^u} = \frac{100-10}{12h} = 0.125 \text{ min}^{-1} = 2.0 \times 10^{-3} \text{ sec}^{-1}$$

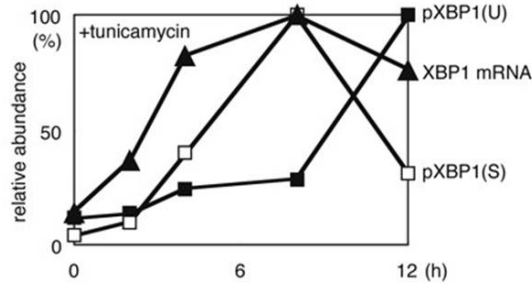


Figure 55: Formation of pXBP1u, pXBP1s and XBP1 mRNA following TM-treatment in HeLa cells [166].

As it was observed from the degradation rates (γ_{X^u} and γ_{X^s}), pXBP1u and pXBP1s exhibit different stability. Further analysis, this time on the translation rate constant, illustrated pXBP1u rate to be lower compared to the pXBP1s. Once the two translation rate constants were found, it only remained to determine the complex degradation rate. A sensitivity analysis was performed in section 6.7.1 to verify that the XBP1 complex behaviour is truthful and the magnitude of the response is in a roughly correct range.

6.5 Model calibration

In order to enable model simulation, most parameter values were determined based on literature values taken from Pincus *et al.* [138] and maintained constant for all three cell lines studied experimentally. Some of these parameters such as the binding rate of BiP to IRE1 receptor (taken by Pincus *et al.* [138]) were from studies on mammalian cells by Bertolotti *et al.* [323]. All values are shown in Table 13. Certain parameters were estimated using the experimental data presented in Chapter 5 using the maximum likelihood formulation of the parameter estimated entity in gPROMS (PSE Ltd., U.K.) as described in section 6.5.1. The initial

conditions used for model simulation are shown in Table 14. Finally, the values of parameter estimation for each of the three cell lines are presented in Table 16.

The parameters related to the kinetic rates of UPR progression were fixed for all the three cell lines. However the parameters representing the magnitude of the stress response, the folding rate and the unfolded protein load were re-estimated separately for each cell line. This was decided as 1) the key UPR markers (ATF4, BiP, CHOP and XBP1) provided a different upregulation profile upon UPR activation, 2) the folding rate of a protein is known to be affected by the mutation and 3) the APP_{MUT} appeared to be more stress than APP_{WT} and thus had probably a higher load of unfolded proteins.

Table 13: Parameter values taken from literature sources.

Model parameters	Value	Units	Reference
C_{AB}	1/0.35	$\text{mol}^{-1} \text{sec}^{-1}$	[138]
C_{PB}	1/0.35	$\text{mol}^{-1} \text{sec}^{-1}$	
C_{IB}	1/0.35	$\text{mol}^{-1} \text{sec}^{-1}$	
C_{RB}	1/0.35	$\text{mol}^{-1} \text{sec}^{-1}$	
C_{UB}	1/0.35	$\text{mol}^{-1} \text{sec}^{-1}$	
n	4.50	AU	
S_U	0	mol sec^{-1}	
β_{ATF4}	0.25	sec^{-1}	
β_B	0.25	sec^{-1}	
β_{CHOP}	0.25	sec^{-1}	
$\beta_{eIF2\alpha}$	0.16	sec^{-1}	
β_{X^s}	3.20×10^{-3}	sec^{-1}	[166]
β_{X^u}	2.0×10^{-3}	sec^{-1}	
γ_{ATF4}	7.70×10^{-3}	sec^{-1}	Value re-used from fitted γ_{CHOP}
γ_B	1.39×10^{-4}	sec^{-1}	[327]
$\gamma_{eIF2\alpha}$	8.33×10^{-4}	sec^{-1}	[138]
γ_{AB}	1.96×10^2	sec^{-1}	
γ_{IB}	1.96×10^2	sec^{-1}	
γ_{PB}	1.96×10^2	sec^{-1}	
γ_{RB}	1.96×10^2	sec^{-1}	
γ_{UB}	1.96×10^2	sec^{-1}	
γ_{X^s}	3.50×10^{-2}	sec^{-1}	[337]
γ_{X^u}	3.00×10^{-2}	sec^{-1}	

6.5.1 Model calibration

The experimental qRT-PCR data represented in relative values were first adjusted to the model data by a scaling process. As in our experimental results the actual mRNA quantities of the specific markers were not obtained, the relative values (AU) of the UPR markers were fitted to describe their fold increase. Scaling was achieved by multiplying the fold change experimental values with the values of the corresponding model variables when it reached its steady state, obtained by simulating the model starting from an initial condition of zero, which was assumed to be that at time zero. The standard deviation (σ) of the model was also transformed by applying the following formula:

$$\sigma(t) = \frac{\sigma(t=0)}{\text{Relative exp value}(t=0)} + \frac{\sigma(t)}{\text{Relative exp value}(t)} \times \text{model initial value} \quad (27)$$

Where $\sigma(t=0)$ is the initial value and $\sigma(t)$ is the value at time point (t) for each variable. Following the transformation of our data, they were incorporated in the parameter estimation entity in gPROMS. The complete raw code in gPROMS language is provided in Appendix III. The initial conditions used for model simulation are described in Table 14.

Table 14: Initial conditions used for model simulation.

Variable	Initial condition (molecules)	Reference
<i>ATF4</i>	0	Reflect steady state conditions
<i>ATF4_m</i>	3.16×10^3	Our experimental data (time 0 h)
<i>ATF6_A</i>	0	Reflect steady state conditions
<i>B</i>	4.30×10^5	[138]
<i>B_m</i>	2.00×10^2	Our experimental data (time 0 h)
<i>CHOP</i>	0	Reflect steady state conditions
<i>CHOP_m</i>	1.10×10^5	Our experimental data (time 0 h)
<i>eIF2α</i>	2.0×10^2	[138]
<i>IRE_A</i>	0	Reflect steady state conditions
<i>IRE/B</i>	2.56×10^2	[138]
<i>peIF2α</i>	0	Reflect steady state conditions
<i>PERK_A</i>	0	
<i>PERK/B</i>	2.56×10^2	[138]
<i>U</i>	0	Reflect steady state conditions
<i>U/B</i>	0	
<i>X_{Total}</i>	1.52×10^2	Our experimental data (time 0 h)
<i>X_s_m</i>	27.54	Our experimental data (time 0 h)

Parameters pertaining to the kinetic rates of UPR progression were estimated from the experimental data of the APP_S cells. Estimation was performed in order to find the parameters allowing the model to fit our experimental data. These were fixed for the other two cell lines, with the exception of six parameters that were estimated separately from the data set for each cell line. These comprised the parameters representing the magnitude of the stress response for each cell namely N_{Bm} , N_{ATF4_m} , N_{CHOP_m} , N_{ATF6_A} , the folding rate γ_{fold} and the unfolded protein load K_u . The difference in transcription rates under the UPR was evident from the different upregulation profiles of the key UPR indicators, namely BiP, ATF4 and CHOP. The change in the folding rate was appropriate as protein folding studies have shown that some of the site-mutations in the APP protein may affect its tertiary structure while others do not cause any effect in the folding shape [340]. As the rate at which proteins fold is determined by their native state [341], estimation of γ_{fold} was performed for each cell line separately. It was further hypothesised that the mutant APP proteins might require longer time to fold compared to the wild-type.

The parameters were fitted in the order shown in Table 15 to obtain the relevant initial estimates but not the final values. In this approach the values that were firstly estimated corresponded to the IRE1 pathway, which were the transcription and degradation rates of BiP and then of XBP1 (Step (i) and (ii) in Table 15). These were used to continue the estimation of parameters related to the PERK pathway. The parameters related to the PERK pathway were the ATF4 and CHOP transcription and decay rates and their estimation was done simultaneously (Step (iii) in Table 15).

Table 15: Steps followed for parameter estimation

Order of estimated parameters									
(i)	Parameters	(ii)	Parameters	(iii)	Parameters	(iv)	Parameters	(v)	Parameters
	β_{Bm}		$\beta_{X_m^s}$		β_{ATF4_m}		N_{Bm}		γ_{fold}
			$\gamma_{X_m^s}$		γ_{ATF4_m}		N_{ATF4_m}		
	γ_{Bm}		$\beta_{X_m^u}$		β_{CHOP_m}		N_{CHOP_m}		K_u
			$\gamma_{X_m^u}$		γ_{CHOP_m}		N_{ATF6_A}		

At step (iv) of Table 15 the parameters representing the magnitude of the stress response namely N_{B_m} , N_{ATF4_m} , N_{CHOP_m} , N_{ATF6_α} were fitted for each cell line separately, followed by the folding rate γ_{fold} and the unfolded protein load K_u presented in Table 16. A step-by-step approach was used to estimate the parameters. The optimal estimates were obtained as presented in Table 16, with the respective SDs as presented in Tables 17 and 18.

Table 16: Estimated parameter values.

Model Parameters	WT	S	S-I	Units
α_0	18.19×10 ⁴			AU
α_1	23.73			AU
β_{ATF4_m}	0.24			mol sec ⁻¹
β_{B_m}	5.00×10 ⁻²			mol sec ⁻¹
β_{CHOP_m}	41.43			mol sec ⁻¹
$\beta_{X_m^s}$	1.42×10 ⁻⁴			sec ⁻¹
$\beta_{X_m^u}$	9.19×10 ⁻³			mol sec ⁻¹
γ_{ATF4_m}	4.67×10 ⁻⁷			sec ⁻¹
γ_{B_m}	1.03			sec ⁻¹
γ_{CHOP}	7.70×10 ⁻³			sec ⁻¹
γ_{CHOP_m}	7.73×10 ⁻⁶			sec ⁻¹
$\gamma_{X_m^s}$	3.69×10 ⁻⁸			sec ⁻¹
$\gamma_{X_m^u}$	6.39×10 ⁻⁴			sec ⁻¹
N_{ATF4_m}	4.89	18	11	AU
N_{B_m}	4.00	1.84	1.48	AU
N_{CHOP_m}	71.88	378.30	138.76	AU
N_{ATF6_A}	1.00×10 ⁻²	1.09	0.57	AU
K_u	35.10×10 ³	76.19×10 ³	50.04×10 ³	mol sec ⁻¹
γ_{fold}	2.58×10 ⁻⁴	2.57×10 ⁻⁴	2.39×10 ⁻⁴	mol sec ⁻¹

Table 17: (a) The standard deviation values of the estimated parameters, the same for all three cell lines.

Fitted parameters	$\pm$ SD
WT / S / S-I	
β_{ATF4_m}	2.00×10^{-2}
β_{B_m}	4.00×10^{-3}
β_{CHOP_m}	1.94
$\beta_{X_m^s}$	1.22×10^{-5}
$\beta_{X_m^u}$	4.90×10^{-3}
γ_{ATF4_m}	9.98×10^{-5}
γ_{B_m}	9.95×10^{-6}
γ_{CHOP}	1.02×10^{-6}
$\gamma_{X_m^u}$	9.59×10^{-6}
$\gamma_{X_m^s}$	4.04×10^{-6}

Table 18: (b) The standard deviation values of the estimated parameters, different for each cell line.

Fitted parameters	$\pm$ SD
(a) WT	
N_{B_m}	2.29
N_{ATF4_m}	75
N_{CHOP_m}	109
N_{ATF6_A}	0.50
K_u	1.17×10^3
γ_{fold}	1.28×10^{-5}
(b) S	
N_{B_m}	9.00×10^{-2}
N_{ATF4_m}	3.80
N_{CHOP_m}	3.97
N_{ATF6_A}	7.00×10^{-3}
K_u	2.30×10^3
γ_{fold}	1.49×10^{-5}
(c) S-I	
N_{B_m}	9.62×10^{-2}
N_{ATF4_m}	1.89
N_{CHOP_m}	2.94
N_{ATF6_A}	0.11
K_u	1.41×10^3
γ_{fold}	1.19×10^{-5}

The UPR-related genes ATF4, BiP and CHOP, which exhibited changes in their transcription rates, also demonstrated different estimated values of the related parameters Table 16. More specifically, APP_S exhibited an increase of 3.68- and 5.25-fold in their N_{ATF4_m} and N_{CHOP_m} parameter values, respectively, when compared to APP_{WT}, while APP_{S-I} rates were in the same order of magnitude as APP_{WT}. Changes in N_{ATF6_A} were also observed and they were 76 and 57 times higher in APP_S and APP_{S-I}, respectively, compared to the values for the wild type cell line. The next parameter estimated was γ_{fold} , the values of which were found to be similar in all cell lines. Along with the protein folding rate, the rate of unfolded protein synthesis was also estimated separately in each of the cell lines. The program was run for each cell line individually to allow for the possibility that different cell lines have different unfolded protein load. Indeed, the changes in K_u showed a 3.83- and 1.43-fold increase in APP_S and APP_{S-I} when compared to APP_{WT}. All of the estimated parameter values used are presented in Table 16.

6.6 Model simulation results

The model was simulated with the initial conditions and parameter values presented in Table 14 and the results were compared to the experimental data. The agreement for the UPR target genes, namely BiP (Figure 56 a-c), XBP1s (Figure 56 d-f), ATF4 (Figure 57 a-c), CHOP (Figure 57 d-f) and XBP1T (Figure 58 a-c) for the APP_{WT} (a, b), APP_S (c, d) and APP_{S-I} (e, f) cell lines is satisfactory. Looking at the data obtained from the IRE1 markers in **Figure 56**, they demonstrate the model capability to reproduce the experimental data accurately.

Computational model of the mammalian UPR network

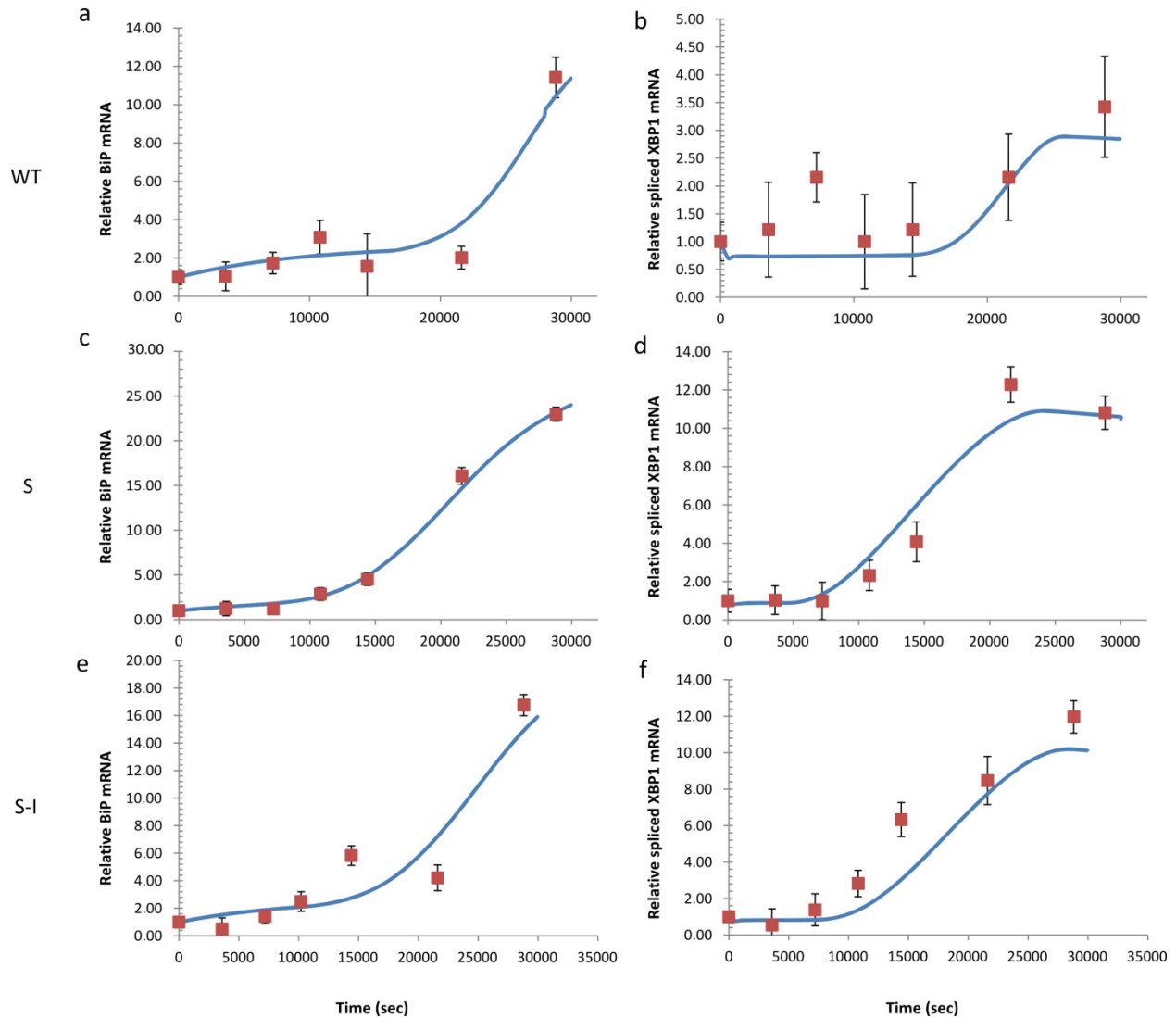


Figure 56: Comparison of model simulation results with experimental data for BiP and XBP1s mRNA. The dots correspond to the experimental data while the solid lines show the model simulation results.

Moving on to the data from the PERK pathway, Figure 57 suggested the need to improve the response of ATF4, which was very sharp, and the signal of the downstream target CHOP. This sharp increase might be associated with the induction of CHOP expression by both ATF6 and PERK pathways. More specifically, the experimental data suggested that CHOP mRNA at early time points of TM-treatment was induced from ATF6 and not from PERK signalling and demonstrated that PERK activation occurred at later time points of TM-treatment. This suggests the bend in ATF4 response (Figure 57 a, c and e) to be a reflection of the onset of PERK activation while the bend in CHOP (downstream of ATF4) (Figure 57 b, d and d) to be a sum of

both ATF6 and PERK signalling. By allowing the regulation of CHOP by ATF6 and PERK activation in the model, a smoother response may be obtained. Another explanation for the ATF4 response may be its upregulation at a protein level but not at the mRNA level.

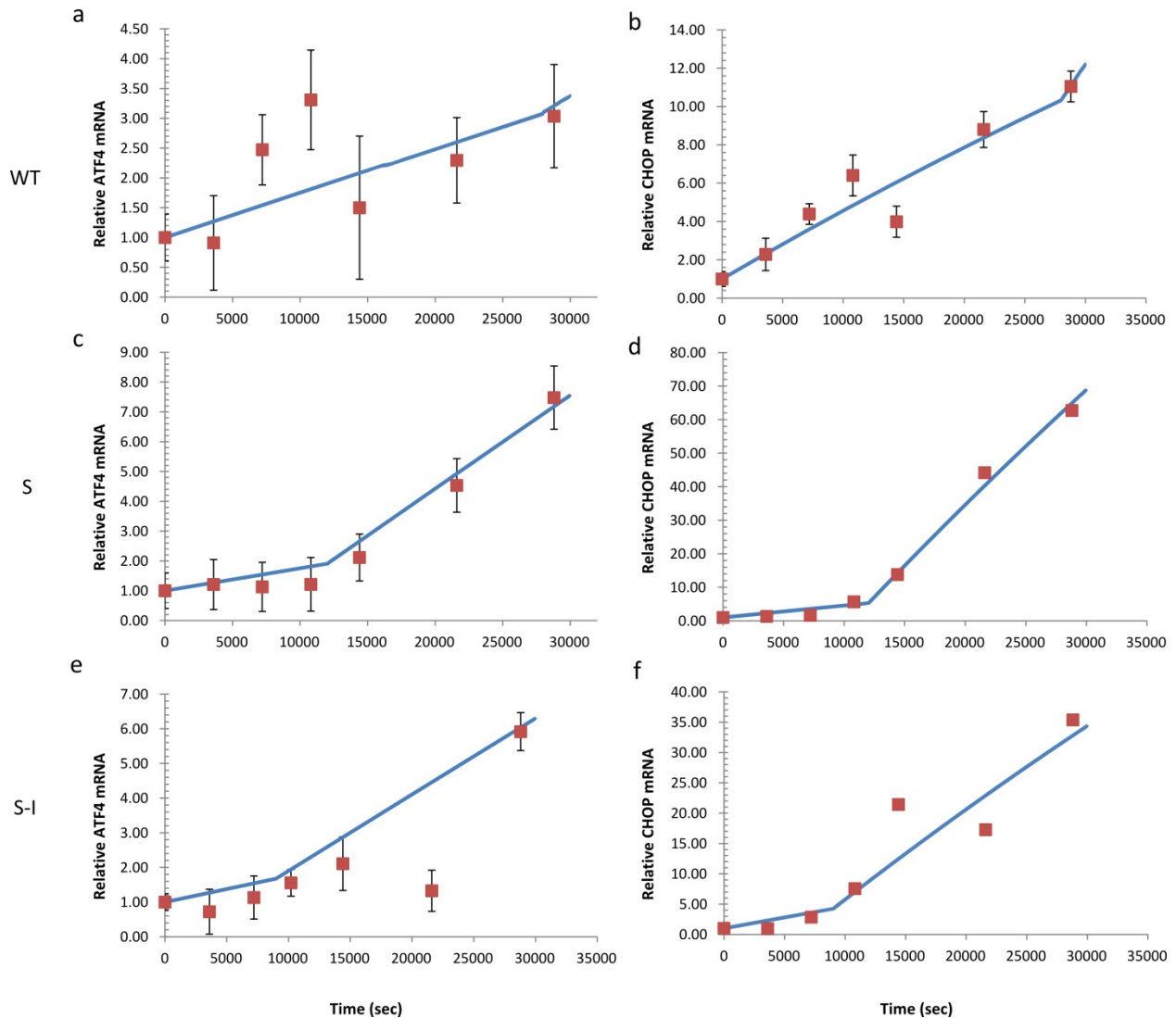


Figure 57: Comparison of model simulation results with experimental data for ATF4 and CHOP mRNA. The dots correspond to the experimental data while the solid lines show the model simulation results.

Looking at the data for the ATF6 pathway (Figure 58), a good fit of the XBP1T mRNA was achieved in the APP_S and APP_{S-I} cell lines and a less satisfactory fitting for APP_{WT} caused by the high variability of the experimental data. A better model response could be obtained by including protein data of the activated ATF6 molecule p50 that directly correlates its activation.

Computational model of the mammalian UPR network

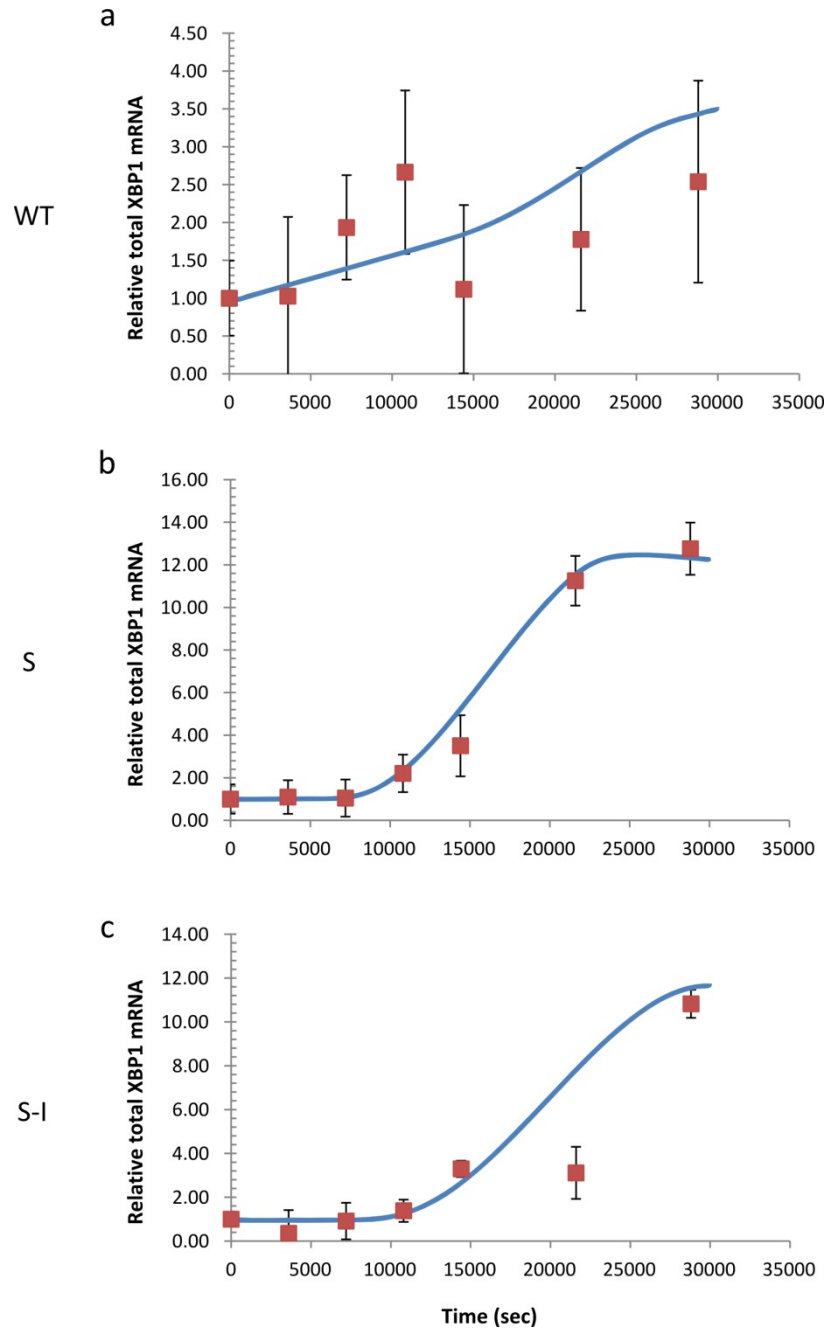


Figure 58: Comparison of model simulation results with experimental data for XBP1T mRNA. The dots correspond to the experimental data while the solid lines show the model simulation results.

The simulation results obtained show the model's ability to reproduce the desired shape of the UPR response for each of the three cell lines studied. It stands to reason that the computational

model can reflect biological reality and with further validation, could provide a better understanding of the UPR mechanism.

Along with the estimated parameter values, the respective SDs were also reported (Tables 17 and 18. In some cases such as for γ_{ATF4_m} the standard deviation (SD) was high reflecting the increased variation of the experimental data. In other parameters such as $\beta_{X_m^u}$ the high order of magnitude of SD showed that, either our experimental data were not sufficient to make the estimation or that the system was not sensitive enough to identify this parameter. As the model represents a highly complex biological system, it is evident that some of the parameters could not be accurately determined. Identification and assessment of the absolute rates of specific substrate molecules is a process that would require their isolation from the cell for accurate measurements.

Most biological systems are very complex and have many interconnected components, making them difficult to define. For this reason, the attempt to model the structure of models such as the mammalian UPR may not precisely reflect the biological relevance for all parameters estimated. Future model improvement would include a parameter sensitivity analysis prior to the estimation in order to find the key kinetic parameters of our model and fix them to their normal values.

6.7 Simulation analyses reflecting the UPR mechanism

6.7.1 ATF6 sensitivity analysis

The model simulations results of XBP1T reflect the experimental data with reasonable accuracy as shown in Figure 58. APP_S showed a pronounced increase in the magnitude of the stress response of ATF4 (N_{ATF4_m}), the pro-apoptotic marker CHOP (N_{CHOP_m}), as well as following ATF6 activation (N_{ATF6_A}). Due to the lack of experimental data for the ATF6 pathway, a sensitivity analysis was conducted in each of the cell lines by increasing the N_{ATF6_A} and

following the relative amount of XBP1T mRNA (Figure 59). This successfully showed that during stress, a higher N_{ATF6A} causes an increased expression of the XBP1T transcripts levels.

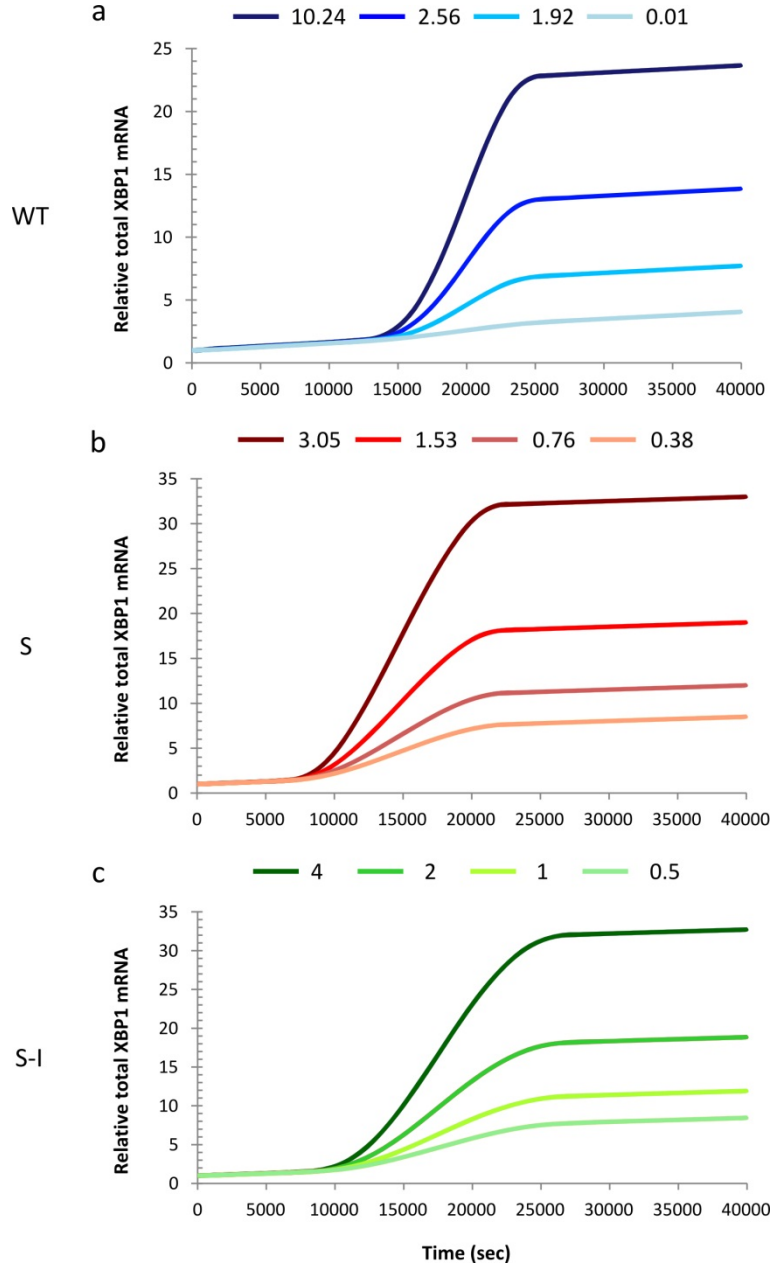


Figure 59: Sensitivity analysis output for ATF6 pathway for each cell line. Figures correspond to a) APP_{WT}, b) APP_S and c) APP_{S-I}. The plot illustrates the effect of the magnitude of stress response following ATF6 activation (N_{ATF6A}) on the relative amount of XBP1 total mRNA levels. The gradient colour scale reading (darker to lighter) reflects the higher to lower N_{ATF6A} values.

These simulation results were in agreement with recent studies by Yoshida *et al.* [337] which have illustrated that XBP1 mRNA regulation is induced by ATF6 activation under stress conditions, and is spliced upon IRE1 activation. Figure 59 also provided information on the magnitude of the ATF6 signalling in the different cell lines. APP_{WT} required a very high transcription rate of the ATF6 under UPR ($N_{ATF6A}=10.24$) to achieve a similar trend of the response with the other cell lines. This rate was three-fold higher relative to the APP_{MUT} ($N_{ATF6A}=3.05$ and 4 for APP_S and APP_{S-I}, respectively). However, the XBP1T mRNA levels in APP_{WT} were observed to be the lowest compared to the other cell lines. This provided further evidence that the magnitude of the stress response regarding ATF6 signalling was stronger in the APP_{MUT} cell lines.

6.7.2 Quantitative analysis of unfolded protein load

Subsequently, the simulation results for BiP (N_{Bm}) also suggested that APP_{WT} were more resistant to stress as they had a sharper transcriptional response of this gene and the lowest unfolded protein flux K_u compared to the FAD mutant cells. The latter parameter, the value of which is indicative of the cumulative load of unfolded proteins in the ER, demonstrated a fold increase of 2.18 and 1.43 in APP_S and APP_{S-I} respectively, when compared to APP_{WT} (Figure 60).

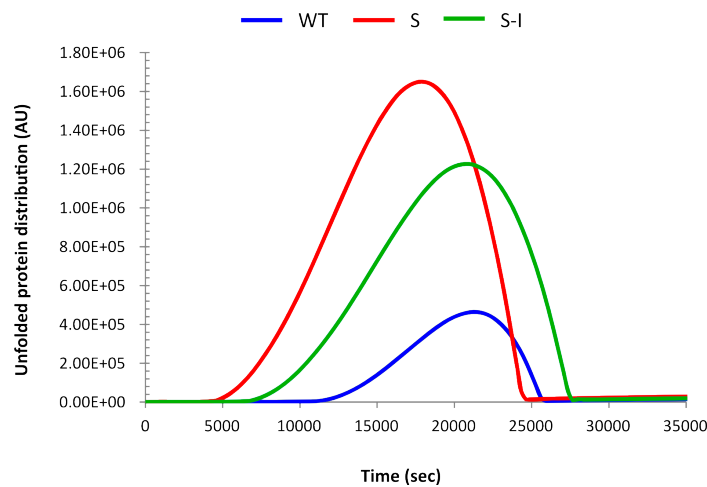


Figure 60: Quantitative analysis of the unfolded protein distribution. Protein load of APP_{WT}, APP_S, and APP_{S-I} following eight hours of TM-treatment. The plot shows the absolute levels of unfolded proteins during the stress time course.

6.7.3 Effect of XBP1 protein complex

Unspliced XBP1 mRNA can be translated and the resulting protein (pXBP1u) has been reported to form a complex with the XBP1s TF (pXBP1s). Specifically, pXBP1u, which shuttles between the nucleus and the cytoplasm, can heterodimerise with pXBP1s in the nucleus to form a complex (pXBP1u-pXBP1s) that is trafficked to the cytoplasm where it is degraded. The complex is therefore a negative regulator of pXBP1s [342]. Degradation of pXBP1s consequently causes reduced transcriptional activation of its target genes. Understanding this interplay may facilitate the modulation of the UPR in terms of intensity and duration [166, 337, 342].

In order to replicate this mechanism *in silico*, the equations 25-26 were introduced in the model to describe the translation of spliced and unspliced XBP1 mRNA and the formation of the protein complex. Different degradation rates were also introduced for each of the two proteins, 0.030 sec^{-1} for pXBP1u and 0.035 sec^{-1} for pXBP1s, the values of which were determined from an experimental degradation study described in section 6.4.1 [337]. Finally, the complex was assumed to degrade at the same rate as pXBP1u, i.e. faster than pXBP1s, in agreement with the experimental evidence in the aforementioned study.

A sensitivity analysis was then performed for the value of β_{X^c} , which represents the rate at which the complex is formed. We specifically assessed the impact of this value on the number of pXBP1s molecules (Figure 61). The simulation results showed that at the highest rate of XBP1 complex formation ($\beta_{X^c} = 5.5 \times 10^{-3} \text{ sec}^{-1}$), pXBP1s levels were significantly lower due to pXBP1s degradation within the complex. Furthermore, in (Figure 61) a quicker increase of the pXBP1s was observed from three to eight hours (12,000 to 18,000 sec), which reflected the duration of the stress response induced experimentally with TM. This observation is in accordance with the fact that the splicing process and thus accumulation of pXBP1s is a result of the ER stress response [166]. Finally, the aforementioned results clearly fit the theory, which suggests that pXBP1u functions as a negative regulator for pXBP1s by modulating its function [166]. This feature can improve the dynamic behaviour of the model allowing a better representation of the biological processes after appropriate experimental confirmation.

Computational model of the mammalian UPR network

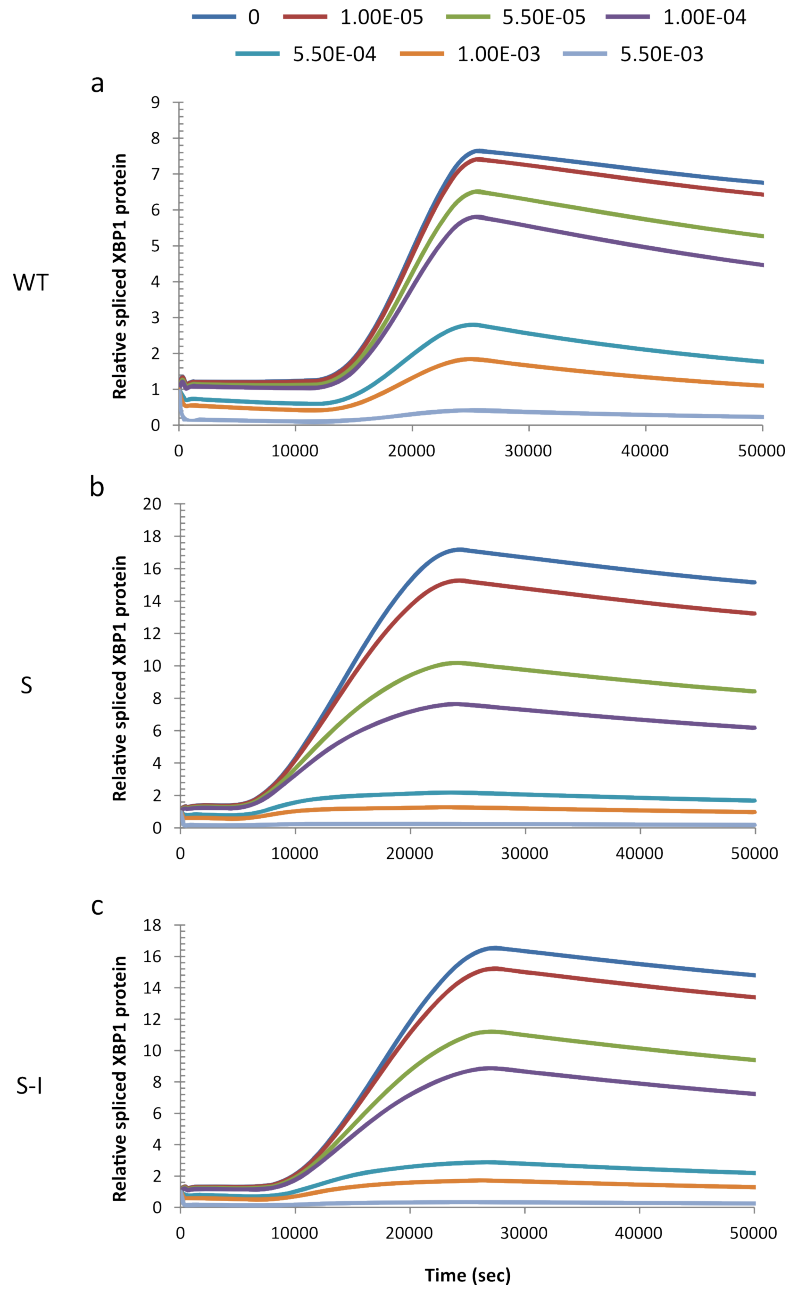


Figure 61: Effect of XBP1 protein complex formation rate on the XBP1s protein levels. Spliced XBP1 protein levels are upregulated during ER stress (12,000-18,000 sec) in a) APP_{WT}, b) APP_S and c) APP_{S-I}. The pXBP1s levels are directly affected by the XBP1 protein complex formation (β_{X^c}).

6.8 The computational model used as a basis to explore treatments *in silico*

Therapeutic strategies that modulate ER function represent a promising approach for prevention or treatment of neurodegeneration. Manipulation of the UPR can induce cellular adaptation and improvement of the disease phenotype [257, 343]. Specific targets for ER regulation identified through screening of several compounds could act as potential inhibitors of ER stress. These vary from inhibitors of the IRE1 pathway, PERK phosphorylation or protein synthesis through the ATF4 pathway.

An IRE1 inhibitor was found to be the 4 μ 8C [239], which blocks the splicing of XBP1 mRNA causing inhibition of protein synthesis of the downstream genes. Investigation of the PERK arm signalling demonstrated several inhibitors of PERK such as GSK2606414, [344] a compound inhibiting selectively the PERK phosphorylation and exhibiting neuroprotective functions. Pharmacological compounds that selectively increase the protective effects of the UPR acting through the eIF2 α -ATF4 arm are salubrinal [345] and guanabenz molecules [222, 223]. Salubrinal enhances eIF2 α phosphorylation levels by inhibiting the eIF2 α phosphatases while guanabenz selectively deactivates only the stress-induced eIF2 α phosphatase [222, 224]. Another strategy involves increasing protein-folding capacity without triggering other stress mechanisms. Selective mood altering drugs such as lithium [208, 346], VPA and supratherapeutic doses of carbamazepine are known to upregulate BiP expression without activating other stress markers [210] (Table 19).

Table 19: Drug targets for ER stress regulation

Drug	Targeted pathway	Effect	Ref.	Date
4 μ 8C	IRE1	Blocks XBP1s mRNA splicing	[239]	2012
GSK2606414	PERK	Inhibits pPERK/p-eIF2 α & translation attenuation	[344]	2013
Salubrinal Guanabenz	PERK	Enhances p-eIF2 α & decreases translation rates	[345] [222]	2005
Valproate Lithium	IRE1 ATF6	Upregulates BiP but not the other UPR markers	[208] [210]	2002

We subjected the mathematical model to a series of case studies representing the biological effect of known therapeutics in order to assess whether their effects can be captured *in silico*. The computational model for each of the cell lines was simulated individually for each case study.

6.8.1 Case study 1: Effect of salubrinal

Salubrinal inhibits eIF2 α dephosphorylation and protect cells from stress [345]. The computational model was modified to simulate this by removing the reaction of eIF2 α dephosphorylation (equation 17). In other words, once an eIF2 α molecule was phosphorylated, it would remain in that state. This had implications for equation 16, in which the influx of new proteins to the ER was reduced with a decreasing number of non-phosphorylated eIF2 α molecules. According to that equation, inhibition of eIF2 α dephosphorylation was expected to shut down protein translation. Indeed, the model simulation results in Figure 62 a, c and d demonstrated that salubrinal significantly reduced the load of unfolded proteins in cells. Consequently, the stress response was predicted to be less sharp compared to the response of the untreated cells.

As the accumulation of unfolded proteins in each cell line occurred at different time points from the stress induction, the effect of salubrinal was not simulated from the same time point. More specifically, dephosphorylation was halted at 14400 sec in APP_S, at 17000 sec in APP_{S-I} and lastly at 18500 sec of stress in APP_{WT}. The accumulation of unfolded proteins in the non-salubrinal treated cells was in accordance with Figure 60, validating the repeat of the above simulations of stress. In all cell lines, addition of salubrinal caused a sharp fall in the simulated unfolded protein distribution, demonstrating that the ultimate effect of this drug was the same independently of whether cells were carrying the wild-type or mutant APP₆₉₅ variant.

Simulation results also showed that BiP mRNA levels were upregulated upon the addition of salubrinal compared to the levels at the beginning of the response of the salubrinal treated cells (Figure 62b, d and f). These results are in agreement with the experimental observations of

Lee and co-workers [347] in SK-N-SH human neuroblastoma cells treated with A β , where salubrinal activated the PERK–eIF2 α pathway and significantly increased BiP levels. Therefore, they suggested that this compound could be used as a potential treatment in neurodegenerative conditions. Even though BiP mRNA levels were higher than prior to salubrinal addition, they were lower than in the control case, in which BiP mRNA was expected to increase further (Figure 62 b, d). This is in agreement with recent studies on a rat model of ischaemic preconditioning by Gao *et al.* [348], which showed that salubrinal inhibited the ER stress response and reduced the levels of BiP compared to the non-treated group. This is evident from Figure 62 b, d and f as salubrinal treated cells exhibited reduced BiP mRNA compared to the non-treated cells that were undergoing stress.

Finally, the model predicted that the overall trend and, thus, effect on the unfolded protein load and the BiP mRNA levels following addition of salubrinal was the same in all cell lines. Its experimental validation could demonstrate the consistency in the drug effect.

Computational model of the mammalian UPR network

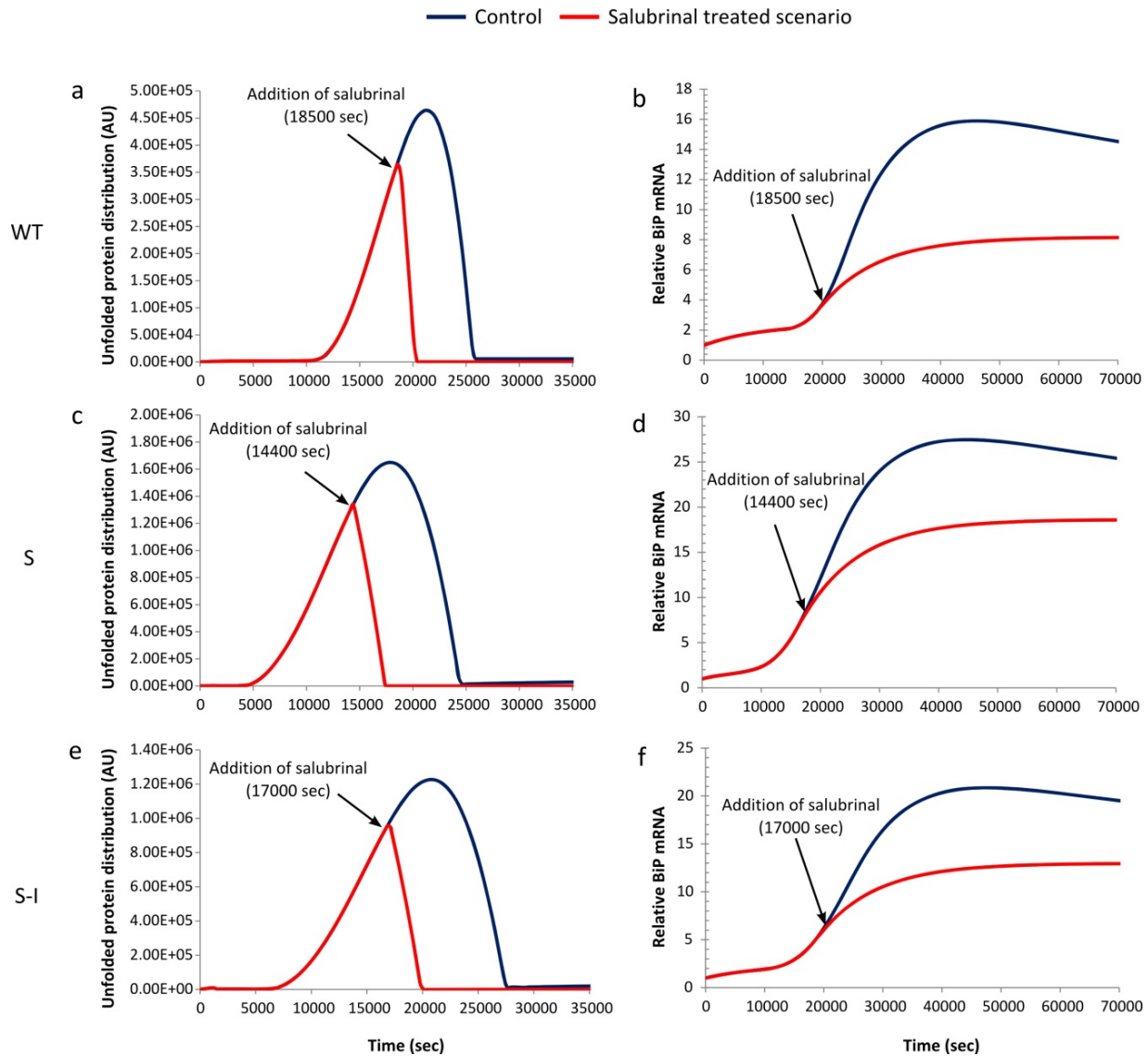


Figure 62: Effect of salubrinal during TM-treatment. Figures (a) and (b) correspond to APP_{WT}, (c) and (d) to APP_S, (e) and (f) to APP_{S-I} cells treated with or without (control) TM. The graphs show the simulation results for the distribution of (a) unfolded protein and (b) BiP mRNA levels in control (untreated) and in salubrinal-treated cells at 14000 sec.

6.8.2 Case study 2: Effect of lithium and valproate (VPA)

Pioneering studies have demonstrated that some mood stabiliser drugs such as lithium and VPA have a neuroprotective role against cell death. For instance, the addition of the aforementioned drugs in Tg-treated human neuroblastoma cells offered neuroprotection through a variety of cellular mechanisms [349]. Both of the aforementioned drugs have been

found to alter the expression of BiP mRNA and protein levels in neurons, bringing up the possibility of their therapeutic use [350].

We have performed a model-based case study to determine the level of BiP upregulation necessary in order to process all the proteins in the ER without activating the UPR. In practice, the value of parameter β_{Bm} , representing the transcription rate of BiP, was increased in the model for cells producing APP_{WT}, APP_S and APP_{S-I} until no further XBP1 mRNA splicing occurred.

The simulation results presented in Figure 61 illustrated that all three cell lines have the same pattern of response, which was that the higher the transcription rate of BiP the lower the spliced XBP1 mRNA. It was also clear that when the transcription rate of BiP was at the lowest level, the relative spliced XBP1 mRNA levels in APP_S and APP_{S-I} were demonstrated to be similar, while being significantly higher than levels exhibited by APP_{WT}. This observation reflected the reality of the experimental system with the mutant cells displaying a stronger response to UPR markers with respect to stress relative to APP_{WT}.

Results in Figure 61a showed that a seven-fold increase in BiP transcription would be sufficient for folding the APP_{WT} protein without inducing stress. This increase was within the capabilities of the system, which experimentally exhibited a 12-fold increase in BiP mRNA levels under conditions of stress. Similarly, a nine-fold increase in transcription of BiP would be enough for the folding of APP_S and APP_{S-I}, which experimentally displayed a 23- and 17-fold increase, respectively, in BiP mRNA during stress induction.

Computational model of the mammalian UPR network

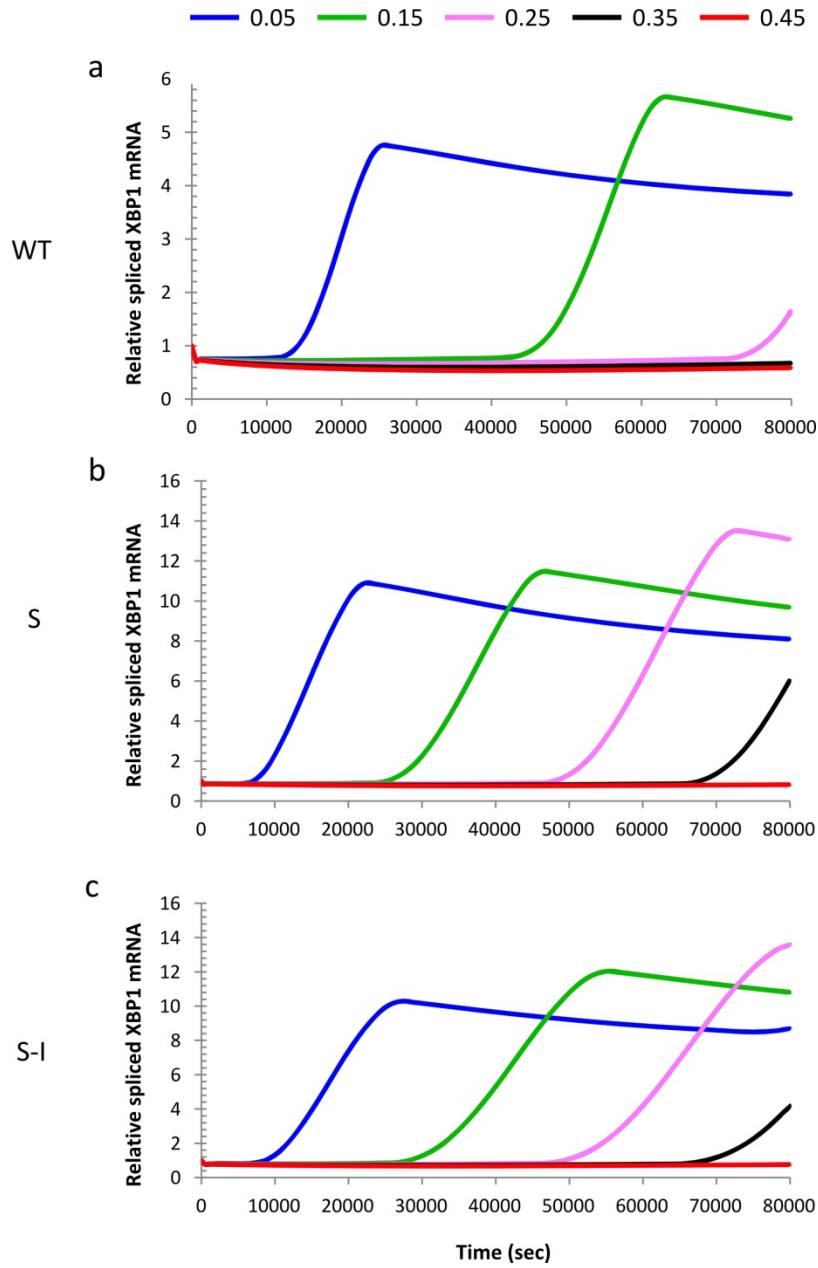


Figure 63: Effect of valproate during TM-treatment. The simulation results in (a) APP_{WT}, (b) APP_S and (c) APP_{S-I} show the effect of VPA on spliced XBP1 mRNA levels by means of varying BiP transcriptional rates (β_{B_m}).

6.9 Discussion

To better understand the dynamics of the elaborate mammalian UPR system, we constructed a computational model integrating the response of three signalling branches: PERK, IRE1 and ATF6. The model was fitted to the mRNA experimental data of the first eight hours of the TM-time course stress experiment for each of the APP_{WT}, APP_S and APP_{S-I} cell lines. As from the qRT-PCR experiments no absolute values were obtained, the data used for the computational model were semi-quantitative.

The developed kinetic model gave us not only the desired shape of the response, but was also able to fit the relative increase for the five mRNAs whose upregulation was measured experimentally (BiP, ATF4, total XBP1, spliced XBP1 and CHOP). Following the parameter estimation, our model was able to reproduce our experimental data. These included pre-existing genetic factors (mutations in APP-associated with FAD) and a mimic of environmental triggers (chemical induction of stress) consequently triggering the stress response. Although the comparison of the model simulation results with the experimental data was satisfactory, it may be valuable obtaining more experimental data from additional time points to produce more reliable fits especially for the PERK pathway. For instance, by introducing gene expression data from additional TM-treatment time points could be beneficial. These measurements could be performed from around three to eight hours (11,000 sec to 30,000 sec) of TM-treatment, which was the time where the response got sharper. An additional experimental method could involve investigating the dynamics of the PERK-BiP from its deactivation. These data would verify whether the Hill function was the right equation to use.

Simulation results of the model reflected the UPR mechanism and revealed its dynamics. Firstly, the sensitivity analysis performed for ATF6 demonstrated that an increase in the transcription under stress causes an increased expression of the XBP1T mRNA levels. Secondly, a quantitative analysis of the unfolded proteins in an eight hours TM-time course revealed a higher unfolded protein load in APP_{MUT} compared to APP_{WT} with highest load in APP_S. This is an interesting finding as the unfolded protein load was estimated from the data on UPR markers (rather than

APP expression data), but is still able to reflect the increased expression in APP_s measured by qRT-PCR. Thirdly, evaluation of the function of XBP1 protein complex *in silico* confirmed its function as a negative regulator of pXBP1s.

In silico analysis of salubrinal- and lithium/valproate-treatment case studies, successfully validated their therapeutic effects demonstrating the model's ability to reproduce the effect of known drugs. Simulation of the inhibition of eIF2a dephosphorylation caused by salubrinal, demonstrated an inhibition of the unfolded protein load, which is in accordance with the global translation attenuation effect of this drug. The data although they showed upregulation of BiP mRNA than prior to the addition of the drug, these levels remained lowered compared to the control cells, which are in agreement with previous experimental studies. Next, simulation of the lithium effect known to upregulate the levels of chaperones without activating the stress cascade revealed the fold increase of BiP mRNA required for the correct folding of APP_{WT} and APP_{MUT} upon induction of UPR.

6.10 Conclusion

The kinetic model that we developed reflects the biological reality of the UPR in mammalian cells and shows the dynamics effects of this molecular pathway which would have been difficult to obtain with solely an experimental approach. Following the fitting simulation results, the model was subjected to a series of case studies that were able to capture the therapeutic effect of several drugs. Therefore, this computational model could be used to evaluate the response to drugs with a known effect with respect to the UPR.

Although the simulations results we obtained are promising, the model is a simplification of the reality with one of the limitations being the use of mRNA data, which in reality do not linearly correlate with the protein levels. More specifically, BiP mRNA results did not capture the effect of ATF6 activation neither the increase of the unfolded protein load. Despite that, certain events including the XBP1 splicing occur at the mRNA level and thus changes in these events

were directly observed. To conclude, generation of a closer to reality UPR model would include validation at protein concentration level obtained possibly with the aid of protein detection tools such as ELISA (enzyme-linked immunosorbent assay), western blot or powerful techniques on detecting phosphorylation events such as immunohistochemistry and flow cytometry.

Chapter 7

Discussion

The aim of this project was to assess the progression of UPR in response to ER stress in FAD, using an experimental approach combined with a computational model. In particular, emphasis was placed on the early response following the application of chemical stress, an area that has been underexplored in the literature.

7.1 Summary of results

In the current study the first goal was to generate a cell model system of human neuroblastoma cells overexpressing either wild-type or FAD-associated APP₆₉₅ mutants, the latter constructs containing either a single mutation (APP_S) or a combination of two (APP_{S-I}). Successful gene expression and protein levels analysis confirmed the significant upregulation of APP₆₉₅ in the transfected cells compared to non-transfected, with qRT-PCR data suggesting a slight, but significant, APP increase in APP_S compared to APP_{S-I} and APP_{WT} (Chapter 4). These cell lines were used as the cell model system to provide quantitative data on i) whether FAD-mutations induce a preconditioning of ER-stress, ii) the timing of the UPR response, by treating the cells with an ER-stress inducer pharmacological agent and iii) the UPR dynamics by generating a mathematical model for mammalian UPR.

The expression of eight UPR-responsive genes was analysed. These included: a) the ER chaperones BiP and GRRP4, b) the gene downstream of IRE1 pathway XBP1s, in addition to BiP, which is regulated by XBP1s TF, c) genes downstream of PERK signalling: Nrf-2, ATF4 and the

apoptotic CHOP TF and d) the downstream target genes of the activated ATF6 TF, XBP1T, BiP and CHOP were monitored in the three cell lines. As activation of ER stress can be triggered both by overproduction of APP alone but also by pharmacological intervention (TM-treatment), both of these methods were employed for the purpose of this project (Chapters 4 and 5, respectively).

APP overexpression induced the upregulation of certain UPR markers even in the absence of additional stress. More specifically, APP_{WT} exhibited increased expression levels of ATF4 mRNA, APP_S of BiP, ATF4, XBP1s and CHOP mRNA and APP_{S-I} of XBP1s transcripts, all with respect to the non-transfected cells (Chapter 4). It is evident that all cell lines were preconditioned for ER stress. The APP_S that had higher APP levels compared to the other cell lines induced UPR with a higher magnitude. The increase of UPR markers observed in APP_S was still mild in comparison to the respective one following pharmacological treatment (Chapter 5). The data indicate that overexpression of APP induces ER stress preconditioning and that a higher APP load (as seen in APP_S) affects the level of the response. APP_{WT} and APP_S in a stress free environment also affect the mitochondria morphology, which among other dysfunctions trigger an increase in ROS in the cell [351]. As preconditioning of stress may provide a protective effect against the harmful effects stimulated by oxidative stress (as opposed to ER stress) [352], we postulated that activation of this mechanism might be beneficial for cells to counteract the increased of ROS production.

Evaluation of the early events in the progression of UPR triggered by ER stress was achieved by following the UPR expression markers of a FAD-cell model in a TM-time course assay. At eight hours of TM-treatment, the cells metabolic activity along with their viability was restored to basal levels confirming cellular adaptation to ER stress. However 24 h of TM-treatment caused cell death confirming that prolonged stress induces cellular toxicity and apoptosis [3].

Upregulation of the eight ER stress-induced UPR-responsive markers indicated activation of the UPR. An initial comparison of the fold increase within each of the cell lines, confirmed APP_{MUT} to activate all three ER-stress sensors while APP_{WT} to activate primarily the IRE1 and ATF6. The pattern of the response was similar in APP_{MUT} with an early IRE1 and ATF6 activation at three

hours of TM-treatment followed by PERK activation at eight hours while APP_{WT} demonstrated an early ATF6 activation followed by IRE1 activation at eight hours of TM-treatment. We postulated that all three pathways may be also activated in APP_{WT} but masked by the increased variability of the UPR expression markers and specifically of ATF4 transcripts. Therefore, as the effect is translational, it could lead to higher protein levels even if there is no effect on the mRNA levels.

Comparison of the fold increase in UPR markers among the cell lines provided further evidence that APP_{MUT} activate ER stress in a similar manner to each other and with a stronger magnitude compared to APP_{WT}. More specifically, at four hours of TM-treatment both APP_S and APP_{S-I} exhibited a significant fold increase of XBP1s, while at eight hours an upregulation of the fold levels of XBP1T and of the pro-apoptotic CHOP (ATF4-CHOP axis) respectively, compared to APP_{WT}. Relative analysis of the UPR-markers of APP_{MUT} to the APP_{WT} demonstrated the levels of chaperones to be upregulated in APP_{MUT}. More specifically BiP (at six and four hours of TM-treatment for APP_S and APP_{S-I}, respectively) and GRP94 (at six and eight hours of TM-treatment for APP_S and APP_{S-I}, respectively) transcripts were significantly higher. At eight hours of TM-treatment they both exhibited relative upregulation of CHOP as well as Nrf-2 mRNA relative to APP_{WT} transcripts at the same time point. On the other hand, relative overexpression of XBP1s and XBP1T transcripts compared to APP_{WT} at the same time point was only reported in APP_S. Therefore, the data confirmed APP_{MUT} to exhibit stress with higher magnitude and APP_S to display the highest stress response. The higher APP levels expressed by APP_S provide evidence that transcriptional variation may be the result of the higher amount of APP protein produced rather than caused by the specific mutation.

In order to obtain a more complete and accurate understanding of the elaborate mechanism of UPR, a computational model integrating the three known ER-stress signalling branches was generated (Chapter 6). The experimental data of the gene expression levels of five UPR markers (BiP, XBP1s, XBP1T, ATF4 and CHOP) from eight hours of TM-treatment (Chapter 5) were fitted for each of the APP_{WT}, APP_S and APP_{S-I}. Construction of the model was as such to allow evaluation of each of the UPR-signalling arms.

Several general considerations were made for this system and were successfully represented. First, the model demonstrated the high cross signalling of the three UPR signalling pathways. Second, the system was activated upon ER stress and was shut down when stress was resolved. Third, in case of prolonged stress the system restored homeostasis. Fourth, the model included the dynamic interplay of the translation of XBP1s and XBP1u mRNA and the formation of the XBP1 protein complex (XBP1c). Other important features were the incorporation of chaperones, which aid in the protein folding as well in the activation/deactivation process of the UPR. In addition, attenuation of protein synthesis was also included, lowering the amount of protein synthesised in the ER.

Additionally of the general features, some more specific UPR considerations were made prior to fitting the experimental data with the model. As our cells exhibited stress at around three hours of TM-treatment, a time lag was introduced to follow this effect. The key indicators of UPR named BiP, ATF4, CHOP and XBP1T demonstrated different transcription upregulation profiles during the TM-induction and thus different transcription rates were considered to represent the stress response. As mutations in proteins affect the rate at which they are folded, these rates were estimated separately for each of the cell lines. Taken these assumptions into consideration, the model was expected and indeed demonstrated to provide the correct shape of the response as well as to fit with satisfaction the experimental fold increase of the UPR-transcripts. Both the general as well as the more specific considerations provided details for the effective functioning of the UPR.

Model simulation also reflected the UPR mechanism and dynamics, which included a sensitivity analysis of ATF6, a quantitative analysis of the unfolded protein load and evaluation of the function of XBP1 protein complex. The model was further used to perform case studies on the effect of known drugs targeting the UPR progression, which included the effect of salubrinal, lithium, and valproate. It also replicated *in silico* the effect of the XBP1 complex formation. These studies successfully demonstrated the model's ability to replicate the effect caused by specific drugs while also predicting their outcome. By testing other molecules with known effect on the UPR, it could provide a better understanding of how different mutations affect the

activation of the response. This information gained could possibly assist in identifying point of interventions for therapeutics.

7.2 Main conclusions

Our analysis suggested that neuroblastoma cells overexpressing APP₆₉₅ bearing FAD associated-genetic mutations activated ER stress signalling differently, than those overexpressing wild-type APP₆₉₅. The genetic predisposition of these mutants might therefore be responsible for to their different effect on the UPR action.

As distinct changes in transcription of several genes can have an effect on the progression of the disease [353], here we proved *in vitro* that different FAD mutations have transcriptional differences in the key regulators BiP, GRP94, ATF4, XBP1, Nrf-2 and CHOP. This might be also the case for FAD patients. Our data also confirmed that ER stress can be triggered only within a few hours since upregulation of UPR markers occurred at around three hours of TM-treatment, which was in accordance with the findings of previous studies [292]. Although the ER tries to restore homeostasis through transcriptional changes, it also requires short-term responses to ease the effects of stress by translational pause. However, the re-activation of global translation along with the contribution of transcriptional events is required to prevent cell death and allow homeostasis to be re-established. Additionally, faithful transcription of the mRNA of UPR target genes upregulated under the induction of stress is also essential as part of the transcriptional UPR response [319, 354].

Following the experimental analysis, we established a computational model that explores the role of UPR in mammalian cells expressing wild type APP and FAD-associated APP mutations. Our model was able to reproduce our experimental data, which included pre-existing genetic factors (mutations in APP-associated with FAD) and a mimic of environmental triggers (chemical induction of stress) consequently triggering the stress response. The model gave us not only the desired shape of the response, but was also able to fit the relative increase for the five mRNAs

whose upregulation was measured experimentally (BiP, ATF4, XBP1T, XBP1s and CHOP). Another utility for this model would be to use it in order to identify novel specific proteins in the stress response as targets for therapeutic intervention. For instance, salubrinal drug acts selectively on the PERK-mediated signalling pathway by specifically inhibiting the phosphatases responsible for its dephosphorylation. This compound maintains elevated eIF2 α phosphorylation levels providing protection to cells against stress-induced apoptosis [345]. Thus if the mechanism of action is known, the present model can be used to simulate the corresponding effect.

The switch to apoptosis in ER stress remains unclear; however, evidence shows that it is linked with the timing of the UPR [305]. Lin *et al.* [305] showed that a tailored combination of individual UPR branches determine susceptibility to stress which is in accordance with our data. More specifically, our experimental data depicted a stronger stress response in the cells carrying the mutant APP₆₉₅ providing evidence for FAD mutations affecting the cellular susceptibility to stress. Our data also validated APP_{MUT} to exhibit a strong induction of CHOP transcripts, which subsequent to a sustained UPR activation (24 h of TM-treatment) triggered apoptotic cell death. Several lines of evidence suggest APP_{WT} to be more resistant to apoptosis compared to FAD-associated APP_{MUT} [355] [355]. Previous studies revealed APP_{MUT} to generate increased A β ₄₂ levels inhibiting the protective effects of the wild-type APP [355]. Hence, we hypothesise that the significant upregulation of UPR markers in APP_{MUT} may have occurred as a result of the increased accumulation of the toxic intraneuronal A β ₄₂ peptides in the ER. These toxic molecules by forming intracellular soluble oligomers increased the APP_{MUT} susceptibility to stress.

Our data demonstrated that the cells carrying the Swedish APP₆₉₅ mutation exhibited the strongest response to stress. This molecular process was probably the result of the slight, but significant, higher expression of APP in APP_S compared to APP_{S-I}. In addition, the model also depicted that APP_{MUT} had a higher unfolded protein flux compared to APP_{WT} with APP_S having the highest amount of unfolded proteins. APP_{MUT} exhibited elevated levels of GRP94, usually expressed upon high demand of chaperones and thus under conditions of increased unfolded

protein load. This strengthens the model simulation results showing higher accumulation of unfolded proteins in cells carrying the FAD APP₆₉₅ mutations. In the case of APP_{WT}, the computational model also demonstrated a sharper transcriptional response of BiP under conditions of stress, confirming to have the lowest unfolded protein load compared to APP_{MUT}. Thus, the effect captured with the UPR transcript data (strongest UPR activation) was in accordance with the model results, validating further our system.

Nevertheless, *in silico* analysis illustrated that even in APP_{MUT}, the levels of unfolded proteins returned to their initial values upon UPR activation, showing that UPR successfully resolves stress. This adaptive mechanism was also evident from the increase in transcription of the UPR markers during the stress response. Each of the cell lines customised this upregulation showing their attempt to gain adaptation. However, in an extensive or chronic stress situation (where stress is not alleviated), neuronal cells could get irreversibly damaged resulting in cell death [34, 356] as was observed at 24 h of TM-treatment.

The metabolic recovery observation was confirmed experimentally and computationally in the case study of cells treated with an inhibitor of eIF2 α de-phosphorylation inducing down-regulation of unfolded proteins. Hence, *in silico* analysis of drug-treatment scenarios validated that the concentration of unfolded protein was decreased to the initial levels. This study thus proposes a multidisciplinary platform that could be used in future development strategies for therapeutics with respect to UPR, to inhibit the onset or/and progression of AD.

7.3 Limitations

Even though the cell model system we studied provided information on the progression of UPR in FAD, it had several limitations. One of the main drawbacks was the use of polyclonal cell lines. Each of the stably transfected cell lines were composed of a heterogeneous mixture of cells, which probably had different susceptibility to the TM stress stimulus. More specifically, during the TM time course treatment, some cells within the same culture might have

experienced stress differently altering the background and increasing the variability of the expression changes of UPR-related genes. These possible effects caused by the non-homogenous cell populations make it harder to provide solid conclusions on the UPR profile as well as our ability to observe trends. The development of a monoclonal FAD-cell line could reduce this variability, as all cells would be derived from a single colony and would have the same levels of expression throughout the population. In that case, TM addition should affect all cells in a culture in the same way causing the same level of accumulation of the UPR markers.

Evidence on the progression and the dynamics of UPR was provided based on mRNA experimental data from *in vitro* and *in silico* studies. Despite the fact that the transcription of a gene provides information on the gene expression patterns, the level of transcription gives only a rough estimate of the protein expression levels. This is because the mRNA levels of a target gene that may be produced in abundance either can be rapidly degraded or be inefficiently translated. Therefore, increased mRNA levels do not necessarily demonstrate high protein levels. Importantly, some UPR events occur at a protein level. For instance, the stress induced p-eIF2 α upregulates ATF4 at a translation level. Our analysis on the PERK arm during UPR evaluated the ATF4 mRNA levels which are known to be upregulated upon stress as well as evaluated the transcripts of its downstream target genes (i.e. CHOP). Future investigation of the ATF4 protein levels would ascertain the activation of the PERK pathway.

Another limitation in this project is the activation of ATF6, which was evaluated by quantitative analysis of the expression levels of its direct targets and not of the p50 TF. Although direct analysis of ATF6 protein cleavage was attempted with WB, the outcome was unsuccessful probably due to the very low concentration of the ATF6 protein in this cell line (data not shown). In previous studies, evaluation of ATF6 activation under the induction of stress was accomplished either with a flag-tagged form of the full length ATF6 [185, 357] or with overexpression of the reported gene [358]. As our model cells were already genetically modified (with the APP mutations), it was preferred not to induce any further stress on the secretory pathway by including the overexpression of ATF6. To ascertain ATF6 activation, a flag-

tagged form of the full length ATF6 could be generated in our cell model system and through quantification of the fluorescence verify its progression in the UPR.

7.4 Future directions

The experimental data showing the increase of the UPR markers during stress demonstrated APP_S to exhibit the highest magnitude of the stress response. Subsequently the computational model provided evidence of APP_S having the highest unfolded protein load compared to APP_{WT} and APP_{S-L}. This could be validated by experimental studies such as with the use of BLItz™ system, which uses fluorescence to measure the thermal stability of a protein in real-time kinetics. Thus, by measuring the unfolded transition temperature of a protein, it could provide real-time quantification of the relative percentage of folded versus unfolded proteins in a time-course assay. This assay could be performed at each of the time points of TM-treatment time-course experiment in all cell lines and the obtained signal could be subtracted from time zero hour. A control pre-heated sample could be used as a reference for the 100% unfolded protein load. This experiment could provide us information on the relative changes of the amount of unfolded proteins in the system throughout the progression of ER stress.

Following analysis of the UPR adaptation phase, a subsequent investigation of the late stages of ER stress could be performed. This would require analysis of the activated apoptotic CPS such as CSP12 which has been previously suggested to be essential in the ER stress induced apoptosis [151] eventually activating the CSP3.

Additionally with the effect of unfolded proteins in inducing ER stress, an altered ER redox environment or Ca²⁺ concentration could also trigger stress and induce the generation of its stress-mediated ROS [359]. As these intracellular oxidants may be induced in upstream events of the stress response and have deleterious consequences by creating vicious cycles in the onset and the progression of AD, their detection is of high importance. A semi-quantitative method for detecting oxidised proteins in a western blot format is with the use of the

commercial OxyBlot™ kit (Millipore, Massachusetts, USA). Oxidative stress can cause changes in the levels of glutathione (GSH) - an important antioxidant - indicating the potential of cells undergoing cell death, making the measurement of their levels also critical. GSH changes in our *in vitro* model can be detected at different time points of TM-treatment by using the GSH/GSSG-Glo™ assay kit (Promega, Hampshire, UK). This assay would measure the total glutathione in the reduced (GSH) and oxidised state (GSSG) and subsequently the ratio of GSH/GSSG would be estimated. This technique would allow us to evaluate the link between ER stress and oxidative stress.

As the oxidative species which play an important role in the disease have short half-lives and alter quickly the cellular redox state, it would be ideal to monitor the redox changes in real-time. The development of biosensors could be used as a tool to measure redox alterations in our FAD model system. In addition, of the redox changes, such a tool will be able to directly detect changes in gene expression levels from IRE1, PERK, and ATF6 and assess which of them is/are active after a specific treatment. Generation of these biosensors would be achieved by transfecting the cells with a construct vector containing a fluorescent protein (e.g GFP) reporter gene and promoter element for the target genes involved in the UPR (e.g chaperones). They would also contain inserted reporter elements such as UPRE or ERSE that have binding motifs specific for XBP1 and ATF6 transcription factors. The set of biosensors should contain different natural promoter sequences such as for chaperones, anti-oxidant and ERAD transcription factors influenced by ER stress. The data generated (level of fluorescence) would then be tested against the already obtained qRT-PCR data in order to correlate the fluorescent sensor with the gene expression data. This approach would allow us to follow the ER stress activation of the biosensors by the fluorescence levels. If the results are comparable, the biosensor system could be further used to test causes of and treatments for FAD.

Changes in the environmental conditions causing ER stress can be measured by the increase in fluorescence. More specifically we would be able to test different conditions which occur in cells of FAD patients, including hypoxia [360] and glucose deprivation [361, 362]. Therefore, we will be able to examine the time points and the levels of glucose deprivation that cause stress

and results in cell death. Monitoring with high-throughput screening assays cellular events that can change over time or under different conditions could give us spatiotemporal information of neurodegenerative diseases. This could eventually help us determine the key steps needed to be targeted by small molecule therapies.

Even though the ER organelle may act as a site generating several apoptotic signals, the mitochondria are also implicated at different stages of the ER stress response ranging from the adaptation process to cell death. It would thus be of great importance to investigate this interplay between ER dyshomeostasis and mitochondrial dysfunction at the beginning of ER stress. This could be achieved by measuring the UPR markers such as BiP (by western blot) and CytC release concentration with the Cytochrome C ELISA kit (MBL, Watertown, MA, USA) for ER and mitochondria, respectively.

Although our computational model gives a good representation of the biological system studied, we are conscious that it is oversimplified. A high fidelity model of the mammalian UPR could be achieved by estimating protein-level parameters when they become available, as the increases in mRNA levels are not always indicative of the changes at the protein levels. Protein data will also allow a better fit. More specifically, they will enable the fitting of more constants including of the $\text{eIF2}\alpha$ of the PERK pathway, whose fitting was not possible in our model due to the lack of data. Furthermore, quantification of several mRNA decay kinetic profiles would increase accuracy for some of the estimated degradation parameters (i.e. for ATF4 mRNA). This would involve blocking the new transcriptional events and measuring the rate at which the mRNA is degraded. This would let us establish the individual decay profiles of the mRNA levels and include these experimental data to our system.

To reflect the complexity of the UPR system better, negative regulators should also be included in the model. For instance the GADD34 protein stimulated by CHOP and dephosphorylating $\text{eIF2}\alpha$ in a negative feedback loop that causes translational repression in response to ER stress [363]. Another of great-importance feature to consider in the model would be the influence of ER stress in a dose dependent manner. This experimental approach would involve treating the cells with a variety of stressors (such A β or TM) in a range of different concentrations and

incorporating the data in the model. These data could provide information on the changes in the dynamics of the accumulation of unfolded proteins in the ER and deliver additional quantitative data on the impact of ER stress in FAD.

Medical intervention of AD patients sharing similar symptoms but different mutations may be more efficient in certain subpopulations, emerging the need of a new era of personalised care [364]. The mammalian UPR model could be coupled with an individualised experimental system taking into consideration specific mutations. This approach may be a good basis to provide a better and safer drug discovery tailored to specific individuals. An individualised system of familial (carrying the E693Δ APP mutation) and sporadic AD was recently established [365] using induced pluripotent stem cells derived from AD patients. This study demonstrated that treatment of AD patients with the ER-stress specific docosahexaenoic acid, that failed clinical studies, could be effective in a subpopulation of AD patients [365]. This clearly shows that determination of patient-specific parameters would allow a more accurate identification of treatments based on personalised pharmacotherapeutics.

7.5 Outlook

In summary, we showed that FAD APP mutations trigger UPR more quickly than the cells overexpressing wild-type APP. The cells overexpressing APP_{MUT} were the most sensitive to stress, activating all three ER membrane receptors with a similar profile. Experimental and computational studies suggested that all cells exhibited an adaptation to stress by eight hours of TM-treatment but when the stress persisted, UPR signalling induced cell death. The computational model reflects the biological reality of ER stress activation in mammalian cells and can be used to test the behaviour and efficiency of various drugs with a known effect. In conclusion, the UPR is an attractive target for neurodegenerative diseases and future investigations may provide insight into the major challenge of finding a prominent target for drug discovery.

References

1. Spriggs, K.A., M. Bushell, and A.E. Willis, *Translational Regulation of Gene Expression during Conditions of Cell Stress*. Molecular Cell, 2010. **40**(2): p. 228-237.
2. Pagani, L., E. Dell'Amico, R. Migliavacca, M.M. D'Andrea, E. Giacobone, G. Amicosante, E. Romero, and G.M. Rossolini, *Evaluation of Brilliance CRE Agar for the detection of carbapenem-resistant Gram-negative bacteria (vol 41, pg 4264, 2013)*. New Microbiologica, 2013. **36**(4): p. 435-435.
3. Fulda, S., A.M. Gorman, O. Hori, and A. Samali, *Cellular stress responses: cell survival and cell death*. Int J Cell Biol, 2010. **2010**: p. 214074.
4. Todd, D.J., A.H. Lee, and L.H. Glimcher, *The endoplasmic reticulum stress response in immunity and autoimmunity*. Nature Reviews Immunology, 2008. **8**(9): p. 663-674.
5. Rodriguez, D., D. Rojas-Rivera, and C. Hetz, *Integrating stress signals at the endoplasmic reticulum: The BCL-2 protein family rheostat*. Biochimica Et Biophysica Acta-Molecular Cell Research, 2011. **1813**(4): p. 564-574.
6. Taylor, R.C., S.P. Cullen, and S.J. Martin, *Apoptosis: controlled demolition at the cellular level*. Nature Reviews Molecular Cell Biology, 2008. **9**(3): p. 231-241.
7. Valastyan, J.S. and S. Lindquist, *Mechanisms of protein-folding diseases at a glance*. Disease Models & Mechanisms, 2014. **7**(1): p. 9-14.
8. Lamond, A.I., *Molecular biology of the cell, 4th edition*. Nature, 2002. **417**(6887): p. 383-383.
9. Wearsch, P.A. and C.V. Nicchitta, *Endoplasmic reticulum chaperone GRP94 subunit assembly is regulated through a defined oligomerization domain*. Biochemistry, 1996. **35**(51): p. 16760-9.
10. Gidalevitz, T., F. Stevens, and Y. Argon, *Orchestration of secretory protein folding by ER chaperones*. Biochim Biophys Acta, 2013. **1833**(11): p. 2410-24.
11. Hebert, D.N. and M. Molinari, *In and out of the ER: protein folding, quality control, degradation, and related human diseases*. Physiol Rev, 2007. **87**(4): p. 1377-408.
12. Wang, M., S.A. Wey, Y. Zhang, R.S. Ye, and A.S. Lee, *Role of the Unfolded Protein Response Regulator GRP78/BiP in Development, Cancer, and Neurological Disorders*. Antioxidants & Redox Signaling, 2009. **11**(9): p. 2307-2316.
13. Zhang, Y., R. Liu, M. Ni, P. Gill, and A.S. Lee, *Cell Surface Relocalization of the Endoplasmic Reticulum Chaperone and Unfolded Protein Response Regulator GRP78/BiP*. Journal of Biological Chemistry, 2010. **285**(20): p. 15065-15075.
14. Kozlov, G., P. Maattanen, D.Y. Thomas, and K. Gehring, *A structural overview of the PDI family of proteins*. Febs Journal, 2010. **277**(19): p. 3924-3936.
15. Imaoka, S., *Chemical Stress on Protein Disulfide Isomerases and Inhibition of Their Functions*. International Review of Cell and Molecular Biology, Vol 290, 2011. **290**: p. 121-166.

References

16. Braakman, I. and N.J. Bulleid, *Protein Folding and Modification in the Mammalian Endoplasmic Reticulum*. Annual Review of Biochemistry, Vol 80, 2011. **80**: p. 71-99.
17. Claessen, J.H.L., L. Kundrat, and H.L. Ploegh, *Protein quality control in the ER: balancing the ubiquitin checkbook*. Trends in Cell Biology, 2012. **22**(1): p. 22-32.
18. Friedlander, R., E. Jarosch, J. Urban, C. Volkwein, and T. Sommer, *A regulatory link between ER-associated protein degradation and the unfolded-protein response*. Nature Cell Biology, 2000. **2**(7): p. 379-384.
19. Houck, S.A., H.Y. Ren, V.J. Madden, J.N. Bonner, M.P. Conlin, J.A. Janovick, P.M. Conn, and D.M. Cyr, *Quality Control Autophagy Degrades Soluble ERAD-Resistant Conformers of the Misfolded Membrane Protein GnRHR*. Molecular Cell, 2014. **54**(1): p. 166-179.
20. Scheper, W., D.A.T. Nijholt, and J.J.M. Hoozemans, *The unfolded protein response and proteostasis in Alzheimer disease Preferential activation of autophagy by endoplasmic reticulum stress*. Autophagy, 2011. **7**(8): p. 910-911.
21. Kaufman, R.J., *Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls (vol 13, pg 1211, 1999)*. Genes & Development, 1999. **13**(14): p. 1898-1898.
22. Tirasophon, W., A.A. Welihinda, and R.J. Kaufman, *A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells*. Genes & Development, 1998. **12**(12): p. 1812-1824.
23. Yoshida, H., K. Haze, H. Yanagi, T. Yura, and K. Mori, *Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors (vol 273, pg 33741, 1998)*. Journal of Biological Chemistry, 1999. **274**(4): p. 2592-2592.
24. Harding, H.P., Y.H. Zhang, and D. Ron, *Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase*. Nature, 1999. **397**(6716): p. 271-274.
25. Ron, D. and P. Walter, *Signal integration in the endoplasmic reticulum unfolded protein response*. Nature Reviews Molecular Cell Biology, 2007. **8**(7): p. 519-529.
26. Stefani, I.C., D. Wright, K.M. Polizzi, and C. Kontoravdi, *The Role of ER Stress-Induced Apoptosis in Neurodegeneration*. Current Alzheimer Research, 2012. **9**(3): p. 373-387.
27. Lippa, C.F., A.M. Saunders, T.W. Smith, J.M. Swearer, D.A. Drachman, B. Ghetti, L. Nee, D. PulaskiSalo, D. Dickson, Y. Robitaille, C. Bergeron, B. Crain, M.D. Benson, M. Farlow, B.T. Hyman, P. StGeorgeHyslop, A.D. Roses, and D.A. Pollen, *Familial and sporadic Alzheimer's disease: Neuropathology cannot exclude a final common pathway*. Neurology, 1996. **46**(2): p. 406-412.
28. Ray, W.J., F. Ashall, and A.M. Goate, *Molecular pathogenesis of sporadic and familial forms of Alzheimer's disease*. Molecular Medicine Today, 1998. **4**(4): p. 151-157.
29. Cruz, J.C. and L.H. Tsai, *Cdk5 deregulation in the pathogenesis of Alzheimer's disease*. Trends in Molecular Medicine, 2004. **10**(9): p. 452-458.
30. Dahm, R., *Alzheimer's discovery*. Current Biology, 2006. **16**(21): p. R906-R910.
31. Takahashi, R.H., E. Capetillo-Zarate, M.T. Lin, T.A. Milner, and G.K. Gouras, *Accumulation of Intraneuronal beta-Amyloid 42 Peptides Is Associated with Early Changes in Microtubule-Associated Protein 2 in Neurites and Synapses*. Plos One, 2013. **8**(1).
32. Dewachter, I., J. van Dorpe, K. Spittaels, I. Tesseur, C. Van Den Haute, D. Moechars, and F. Van Leuven, *Modeling Alzheimer's disease in transgenic mice: effect of age and of Presenilin1 on amyloid biochemistry and pathology in APP/London mice*. Experimental Gerontology, 2000. **35**(6-7): p. 831-841.
33. Iqbal, K., C.X. Gong, and F. Liu, *Microtubule-associated protein tau as a therapeutic target in Alzheimer's disease*. Expert Opinion on Therapeutic Targets, 2014. **18**(3): p. 307-318.

References

34. Matus, S., F. Lisbona, M. Torres, C. Leon, P. Thielen, and C. Hetz, *The stress rheostat: An interplay between the unfolded protein response (UPR) and autophagy in neurodegeneration*. Current Molecular Medicine, 2008. **8**(3): p. 157-172.
35. Durrenberger, P.F., F.S. Fernando, S.N. Kashefi, T.P. Bonnert, D. Seilhean, B. Nait-Oumesmar, A. Schmitt, P.J. Gebicke-Haerter, P. Falkai, E. Grunblatt, M. Palkovits, T. Arzberger, H. Kretzschmar, D.T. Dexter, and R. Reynolds, *Common mechanisms in neurodegeneration and neuroinflammation: a BrainNet Europe gene expression microarray study*. J Neural Transm, 2014.
36. Reddy, P.H., *Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease*. J Neurochem, 2006. **96**(1): p. 1-13.
37. Desai, A.K. and G.T. Grossberg, *Diagnosis and treatment of Alzheimer's disease*. Neurology, 2005. **64**(12): p. S34-S39.
38. Desai, A.K. and G.T. Grossberg, *Diagnosis and treatment of Alzheimer's disease*. Neurology, 2005. **64**(12 Suppl 3): p. S34-9.
39. Zhang, X.J. and W.H. Song, *The role of APP and BACE1 trafficking in APP processing and amyloid-beta generation*. Alzheimers Research & Therapy, 2013. **5**(5).
40. Mattson, M.P., *Pathways towards and away from Alzheimer's disease*. Nature, 2004. **430**(7000): p. 631-639.
41. Zhu, X.W., A.K. Raina, G. Perry, and M.A. Smith, *Alzheimer's disease: the two-hit hypothesis*. Lancet Neurology, 2004. **3**(4): p. 219-226.
42. Hoozemans, J.J.M., R. Veerhuis, E.S. Van Haastert, J.M. Rozemuller, F. Baas, P. Eikelenboom, and W. Scheper, *The unfolded protein response is activated in Alzheimer's disease*. Acta Neuropathologica, 2005. **110**(2): p. 165-172.
43. Wang, X.L., B. Su, G. Perry, M.A. Smith, and X.W. Zhu, *Insights into amyloid-beta-induced mitochondrial dysfunction in Alzheimer disease*. Free Radical Biology and Medicine, 2007. **43**(12): p. 1569-1573.
44. Reddy, P.H., *Amyloid precursor protein-mediated free radicals and oxidative damage: Implications for the development and progression of Alzheimer's disease*. Journal of Neurochemistry, 2006. **96**(1): p. 1-13.
45. Wagner, S.L. and B. Munoz, *Modulation of amyloid beta protein precursor processing as a means of retarding progression of Alzheimer's disease*. Journal of Clinical Investigation, 1999. **104**(10): p. 1329-1332.
46. De Strooper, B. and W. Annaert, *Proteolytic processing and cell biological functions of the amyloid precursor protein*. Journal of Cell Science, 2000. **113**(11): p. 1857-1870.
47. Andersen, O.M., J. Reiche, V. Schmidt, M. Gotthardt, R. Spoelgen, J. Behlke, C.A.F. von Arnim, T. Breiderhoff, P. Jansen, X. Wu, K.R. Bales, R. Cappai, C.L. Masters, J. Gliemann, E.J. Mufson, B.T. Hyman, S.M. Paul, A. Nykjaer, and T.E. Willnow, *Neuronal sorting protein-related receptor sorLA/LR11 regulates processing of the amyloid precursor protein*. Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(38): p. 13461-13466.
48. Lee, J.H., S. Barral, and C. Reitz, *The neuronal sortilin-related receptor gene SORL1 and late-onset Alzheimer's disease*. Current Neurology and Neuroscience Reports, 2008. **8**(5): p. 384-391.
49. Wirths, K.J., *A short proof of a theorem of Bertilsson by direct use of Lowner's method*. Arkiv for Matematik, 2001. **39**(2): p. 395-398.
50. Bayer, T.A., O. Wirths, K. Majtenyi, T. Hartmann, G. Multhaup, K. Beyreuther, and C. Czech, *Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport*. Brain Pathol, 2001. **11**(1): p. 1-11.

References

51. Rogaeva, E., Y. Meng, J.H. Lee, Y. Gu, T. Kawarai, F. Zou, T. Katayama, C.T. Baldwin, R. Cheng, H. Hasegawa, F. Chen, N. Shibata, K.L. Lunetta, R. Pardossi-Piquard, C. Bohm, Y. Wakutani, L.A. Cupples, K.T. Cuenco, R.C. Green, L. Pinessi, I. Rainero, S. Sorbi, A. Bruni, R. Duara, R.P. Friedland, R. Inzelberg, W. Hampe, H. Bujo, Y.Q. Song, O.M. Andersen, T.E. Willnow, N. Graff-Radford, R.C. Petersen, D. Dickson, S.D. Der, P.E. Fraser, G. Schmitt-Ulms, S. Younkin, R. Mayeux, L.A. Farrer, and P. St George-Hyslop, *The neuronal sortilin-related receptor SORL1 is genetically associated with Alzheimer disease*. Nat Genet, 2007. **39**(2): p. 168-77.
52. Cook, D.G., M.S. Forman, J.C. Sung, S. Leight, D.L. Kolson, T. Iwatsubo, V.M. Lee, and R.W. Doms, *Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells*. Nat Med, 1997. **3**(9): p. 1021-3.
53. Skovronsky, D.M., R.W. Doms, and V.M. Lee, *Detection of a novel intraneuronal pool of insoluble amyloid beta protein that accumulates with time in culture*. J Cell Biol, 1998. **141**(4): p. 1031-9.
54. Wild-Bode, C., T. Yamazaki, A. Capell, U. Leimer, H. Steiner, Y. Ihara, and C. Haass, *Intracellular generation and accumulation of amyloid beta-peptide terminating at amino acid 42*. J Biol Chem, 1997. **272**(26): p. 16085-8.
55. Hartmann, T., S.C. Bieger, B. Bruhl, P.J. Tienari, N. Ida, D. Allsop, G.W. Roberts, C.L. Masters, C.G. Dotti, K. Unsicker, and K. Beyreuther, *Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides*. Nat Med, 1997. **3**(9): p. 1016-20.
56. Iqbal, K., F. Liu, C.X. Gong, and I. Grundke-Iqbal, *Tau in Alzheimer disease and related tauopathies*. Curr Alzheimer Res, 2010. **7**(8): p. 656-64.
57. Almeida, C.G., R.H. Takahashi, and G.K. Gouras, *Beta-amyloid accumulation impairs multivesicular body sorting by inhibiting the ubiquitin-proteasome system*. J Neurosci, 2006. **26**(16): p. 4277-88.
58. Imaizumi, Y. and H. Okano, *Modeling human neurological disorders with induced pluripotent stem cells*. Journal of Neurochemistry, 2014. **129**(3): p. 388-399.
59. Farrer, M.J., *Genetics of Parkinson disease: paradigm shifts and future prospects*. Nature Reviews Genetics, 2006. **7**(4): p. 306-318.
60. de Lau, L.M., D. Verbaan, J. Marinus, and J.J. van Hilten, *Survival in Parkinson's disease. Relation with motor and non-motor features*. Parkinsonism Relat Disord, 2014. **20**(6): p. 613-6.
61. Betarbet, R., T.B. Sherer, G. MacKenzie, M. Garcia-Osuna, A.V. Panov, and J.T. Greenamyre, *Chronic systemic pesticide exposure reproduces features of Parkinson's disease*. Nature Neuroscience, 2000. **3**(12): p. 1301-1306.
62. Irizarry, M.C., W. Growdon, T. Gomez-Isla, K. Newell, J.M. George, D.F. Clayton, and B.T. Hyman, *Nigral and cortical Lewy bodies and dystrophic nigral neurites in Parkinson's disease and cortical Lewy body disease contain alpha-synuclein immunoreactivity*. Journal of Neuropathology and Experimental Neurology, 1998. **57**(4): p. 334-337.
63. Wakabayashi, K., S. Hayashi, A. Kakita, M. Yamada, Y. Toyoshima, M. Yoshimoto, and H. Takahashi, *Accumulation of alpha-synuclein/NACP is a cytopathological feature common to Lewy body disease and multiple system atrophy*. Acta Neuropathologica, 1998. **96**(5): p. 445-452.
64. Maguire-Zeiss, K.A. and H.J. Federoff, *Convergent pathobiologic model of Parkinson's disease*. Parkinson's Disease: The Life Cycle of the Dopamine Neuron, 2003. **991**: p. 152-166.
65. Kumar, P., H. Kalonia, and A. Kumar, *Huntington's disease: pathogenesis to animal models*. Pharmacological Reports, 2010. **62**(1): p. 1-14.
66. Kim, H.A., L. Jiang, H. Madsen, C.L. Parish, J. Massalas, A. Smardencas, C. O'Leary, I. Gantois, C. O'Tuathaigh, J.L. Waddington, M.E. Ehrlich, A.J. Lawrence, and J. Drago, *Resolving pathobiological mechanisms relating to Huntington disease: gait, balance, and involuntary movements in mice with targeted ablation of striatal D1 dopamine receptor cells*. Neurobiol Dis, 2014. **62**: p. 323-37.

References

67. Ross, C.A., E.H. Aylward, E.J. Wild, D.R. Langbehn, J.D. Long, J.H. Warner, R.I. Scahill, B.R. Leavitt, J.C. Stout, J.S. Paulsen, R. Reilmann, P.G. Unschild, A. Wexler, R.L. Margolis, and S.J. Tabrizi, *Huntington disease: natural history, biomarkers and prospects for therapeutics*. Nature Reviews Neurology, 2014. **10**(4): p. 204-216.
68. Giacomello, M., J.C. Oliveros, J.R. Naranjo, and E. Carafoli, *Neuronal Ca²⁺ dyshomeostasis in Huntington disease*. Prion, 2013. **7**(1): p. 76-84.
69. Kehoe, P., M. Krawczak, P.S. Harper, M.J. Owen, and A.L. Jones, *Age of onset in Huntington disease: sex specific influence of apolipoprotein E genotype and normal CAG repeat length*. Journal of Medical Genetics, 1999. **36**(2): p. 108-111.
70. Trottier, Y., D. Devys, G. Imbert, F. Saudou, I. An, Y. Lutz, C. Weber, Y. Agid, E.C. Hirsch, and J.L. Mandel, *Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form*. Nat Genet, 1995. **10**(1): p. 104-10.
71. Kremer, B., P. Goldberg, S.E. Andrew, J. Theilmann, H. Telenius, J. Zeisler, F. Squitieri, B. Lin, A. Bassett, E. Almqvist, and et al., *A worldwide study of the Huntington's disease mutation. The sensitivity and specificity of measuring CAG repeats*. N Engl J Med, 1994. **330**(20): p. 1401-6.
72. Andrew, S.E., Y.P. Goldberg, B. Kremer, H. Telenius, J. Theilmann, S. Adam, E. Starr, F. Squitieri, B.Y. Lin, M.A. Kalchman, R.K. Graham, and M.R. Hayden, *The Relationship between Trinucleotide (Cag) Repeat Length and Clinical-Features of Huntingtons-Disease*. Nature Genetics, 1993. **4**(4): p. 398-403.
73. Lee, M.S., S.C. Kao, C.A. Lemere, W.M. Xia, H.C. Tseng, Y. Zhou, R. Neve, M.K. Ahljianian, and L.H. Tsai, *APP processing is regulated by cytoplasmic phosphorylation*. Journal of Cell Biology, 2003. **163**(1): p. 83-95.
74. Nishitsuji, K., T. Tomiyama, K. Ishibashi, K. Ito, R. Teraoka, M.P. Lambert, W.L. Klein, and H. Mori, *The E693 Delta Mutation in Amyloid Precursor Protein Increases Intracellular Accumulation of Amyloid beta Oligomers and Causes Endoplasmic Reticulum Stress-Induced Apoptosis in Cultured Cells*. American Journal of Pathology, 2009. **174**(3): p. 957-969.
75. Saito, R., M. Kaneko, Y. Kitamura, K. Takata, K. Kawada, Y. Okuma, and Y. Nomura, *Effects of Oxidative Stress on the Solubility of HRD1, a Ubiquitin Ligase Implicated in Alzheimer's Disease*. Plos One, 2014. **9**(5).
76. Pagani, L. and A. Eckert, *Amyloid-Beta interaction with mitochondria*. Int J Alzheimers Dis, 2011. **2011**: p. 925050.
77. Ninomiya, H., J.M. Roch, M.P. Sundsmo, D.A. Otero, and T. Saitoh, *Amino acid sequence RERMS represents the active domain of amyloid beta/A4 protein precursor that promotes fibroblast growth*. J Cell Biol, 1993. **121**(4): p. 879-86.
78. Soldano, A. and B.A. Hassan, *Beyond pathology: APP, brain development and Alzheimer's disease*. Curr Opin Neurobiol, 2014. **27C**: p. 61-67.
79. Walter, J. and G. van Echten-Deckert, *Cross-talk of membrane lipids and Alzheimer-related proteins*. Mol Neurodegener, 2013. **8**: p. 34.
80. Rangachari, V., B.D. Moore, D.K. Reed, L.K. Sonoda, A.W. Bridges, E. Conboy, D. Hartigan, and T.L. Rosenberry, *Amyloid-beta(1-42) rapidly forms protofibrils and oligomers by distinct pathways in low concentrations of sodium dodecylsulfate*. Biochemistry, 2007. **46**(43): p. 12451-12462.
81. Parthasarathy, S., F. Long, Y. Miller, Y.L. Xiao, D. McElheny, K. Thurber, B.Y. Ma, R. Nussinov, and Y. Ishii, *Molecular-Level Examination of Cu²⁺ Binding Structure for Amyloid Fibrils of 40-Residue Alzheimer's beta by Solid-State NMR Spectroscopy*. Journal of the American Chemical Society, 2011. **133**(10): p. 3390-3400.
82. Kuperstein, I., K. Broersen, I. Benilova, J. Rozenski, W. Jonekheere, M. Debulpaep, A. Vandersteen, I. Segers-Nolten, K. Van der Werf, V. Subramaniam, D. Braeken, G. Callewaert, C.

References

- Bartic, R. D'Hooge, I.C. Martins, F. Rousseau, J. Schymkowitz, and B. De Strooper, *Neurotoxicity of Alzheimer's disease A beta peptides is induced by small changes in the A beta(42) to A beta(40) ratio*. Embo Journal, 2010. **29**(19): p. 3408-3420.
83. Kudo, T., T. Katayama, K. Imaizumi, Y. Yasuda, M. Yatera, M. Okochi, M. Tohyama, and M. Takeda, *The unfolded protein response is involved in the pathology of Alzheimer's disease*. Alzheimer's Disease: Vascular Etiology and Pathology, 2002. **977**: p. 349-355.
84. Katayama, T., K. Imaizumi, A. Honda, T. Yoneda, T. Kudo, M. Takeda, K. Mori, R. Rozmahel, P. Fraser, P. St George-Hyslop, and M. Tohyama, *Disturbed activation of endoplasmic reticulum stress transducers by familial Alzheimer's disease-linked presenilin-1 mutations*. Journal of Biological Chemistry, 2001. **276**(46): p. 43446-43454.
85. Park, S.J., A. Schertel, and S.S. Han, *Altered structure of synapse in a brain of the Swedish mutation which causes early-onset Alzheimer's disease: A FIB-SEM Microscopy study*. Journal of Neurogenetics, 2010. **24**: p. 38-39.
86. De Jonghe, C., C. Esselens, S. Kumar-Singh, K. Craessaerts, S. Serneels, F. Checler, W. Annaert, C. Van Broeckhoven, and B. De Strooper, *Pathogenic APP mutations near the gamma-secretase cleavage site differentially affect Abeta secretion and APP C-terminal fragment stability*. Hum Mol Genet, 2001. **10**(16): p. 1665-71.
87. Sung, H.Y., E.N. Choi, D. Lyu, I. Mook-Jung, and J.H. Ahn, *Amyloid Beta-mediated epigenetic alteration of insulin-like growth factor binding protein 3 controls cell survival in Alzheimer's disease*. PLoS One, 2014. **9**(6): p. e99047.
88. Murrell, J.R., A.M. Hake, K.A. Quaid, M.R. Farlow, and B. Ghetti, *Early-onset Alzheimer disease caused by a new mutation (V717L) in the amyloid precursor protein gene*. Arch Neurol, 2000. **57**(6): p. 885-7.
89. Goate, A., M.C. Chartierharlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, L. James, R. Mant, P. Newton, K. Rooke, P. Roques, C. Talbot, M. Pericakvance, A. Roses, R. Williamson, M. Rossor, M. Owen, and J. Hardy, *Segregation of a Missense Mutation in the Amyloid Precursor Protein Gene with Familial Alzheimers-Disease*. Nature, 1991. **349**(6311): p. 704-706.
90. Kumar-Singh, S., C. De Jonghe, M. Cruts, R. Kleinert, R. Wang, M. Mercken, B. De Strooper, H. Vanderstichele, A. Lofgren, I. Vanderhoeven, H. Backhovens, E. Vanmechelen, P.M. Kroisel, and C. Van Broeckhoven, *Nonfibrillar diffuse amyloid deposition due to a gamma(42)-secretase site mutation points to an essential role for N-truncated A beta(42) in Alzheimer's disease*. Human Molecular Genetics, 2000. **9**(18): p. 2589-2598.
91. Eckman, C.B., N.D. Mehta, R. Crook, J. Perez-tur, G. Prihar, E. Pfeiffer, N. Graff-Radford, P. Hinder, D. Yager, B. Zenk, L.M. Refolo, C.M. Prada, S.G. Younkin, M. Hutton, and J. Hardy, *A new pathogenic mutation in the APP gene (I716V) increases the relative proportion of A beta 42(43)*. Hum Mol Genet, 1997. **6**(12): p. 2087-9.
92. Nilsberth, C., A. Westlind-Danielsson, C.B. Eckman, M.M. Condron, K. Axelman, C. Forsell, C. Stenh, J. Luthman, D.B. Teplow, S.G. Younkin, J. Naslund, and L. Lannfelt, *The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A beta protofibril formation*. Nature Neuroscience, 2001. **4**(9): p. 887-893.
93. Nilsberth, C., A. Westlind-Danielsson, C.B. Eckman, M.M. Condron, K. Axelman, C. Forsell, C. Stenh, J. Luthman, D.B. Teplow, S.G. Younkin, J. Naslund, and L. Lannfelt, *The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation*. Nat Neurosci, 2001. **4**(9): p. 887-93.
94. Grabowski, T.J., H.S. Cho, J.P. Vonsattel, G.W. Rebeck, and S.M. Greenberg, *Novel amyloid precursor protein mutation in an Iowa family with dementia and severe cerebral amyloid angiopathy*. Ann Neurol, 2001. **49**(6): p. 697-705.

References

95. Krone, M.G., A. Baumketner, S.L. Bernstein, T. Wytttenbach, N.D. Lazo, D.B. Teplow, M.T. Bowers, and J.E. Shea, *Effects of familial Alzheimer's disease mutations on the folding nucleation of the amyloid beta-protein*. J Mol Biol, 2008. **381**(1): p. 221-8.
96. Schaeffer, E.L., M. Figueiro, and W.F. Gattaz, *Insights into Alzheimer disease pathogenesis from studies in transgenic animal models*. Clinics, 2011. **66**: p. 45-54.
97. Hsiao, K., P. Chapman, S. Nilsen, C. Eckman, Y. Harigaya, S. Younkin, F. Yang, and G. Cole, *Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice*. Science, 1996. **274**(5284): p. 99-102.
98. Jacobsen, J.S., C.C. Wu, J.M. Redwine, T.A. Comery, R. Arias, M. Bowlby, R. Martone, J.H. Morrison, M.N. Pangalos, P.H. Reinhart, and F.E. Bloom, *Early-onset behavioral and synaptic deficits in a mouse model of Alzheimer's disease*. Proc Natl Acad Sci U S A, 2006. **103**(13): p. 5161-6.
99. Takahashi, R.H., T.A. Milner, F. Li, E.E. Nam, M.A. Edgar, H. Yamaguchi, M.F. Beal, H. Xu, P. Greengard, and G.K. Gouras, *Intraneuronal Alzheimer abeta42 accumulates in multivesicular bodies and is associated with synaptic pathology*. Am J Pathol, 2002. **161**(5): p. 1869-79.
100. Chishti, M.A., D.S. Yang, C. Janus, A.L. Phinney, P. Horne, J. Pearson, R. Strome, N. Zuker, J. Loukides, J. French, S. Turner, G. Lozza, M. Grilli, S. Kunicki, C. Morissette, J. Paquette, F. Gervais, C. Bergeron, P.E. Fraser, G.A. Carlson, P. St George-Hyslop, and D. Westaway, *Early-onset amyloid deposition and cognitive deficits in transgenic mice expressing a double mutant form of amyloid precursor protein 695*. Journal of Biological Chemistry, 2001. **276**(24): p. 21562-21570.
101. Park, H.J., D. Shabashvili, M.D. Nekorchuk, E. Shyqyriu, J.I. Jung, T.B. Ladd, B.D. Moore, K.M. Felsenstein, T.E. Golde, and S.H. Kim, *Retention in Endoplasmic Reticulum 1 (RER1) Modulates Amyloid-beta (A beta) Production by Altering Trafficking of gamma-Secretase and Amyloid Precursor Protein (APP)*. Journal of Biological Chemistry, 2012. **287**(48): p. 40629-40640.
102. Tienari, P.J., B. DeStrooper, E. Ikonen, M. Simons, A. Weidemann, C. Czech, T. Hartmann, N. Ida, G. Multhaup, C.L. Masters, F. VanLeuven, K. Beyreuther, and C.G. Dotti, *The beta-amyloid domain is essential for axonal sorting of amyloid precursor protein*. Embo Journal, 1996. **15**(19): p. 5218-5229.
103. Yazaki, M., K. Tagawa, K. Maruyama, H. Sorimachi, T. Tsuchiya, S. Ishiura, and K. Suzuki, *Mutation of potential N-linked glycosylation sites in the Alzheimer's disease amyloid precursor protein (APP)*. Neuroscience Letters, 1996. **221**(1): p. 57-60.
104. Tomita, S., Y. Kirino, and T. Suzuki, *Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway - Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism*. Journal of Biological Chemistry, 1998. **273**(11): p. 6277-6284.
105. El Ayadi, A., E.S. Stieren, J.M. Barral, and D. Boehning, *Ubiquitin-1 regulates amyloid precursor protein maturation and degradation by stimulating K63-linked polyubiquitination of lysine 688*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(33): p. 13416-13421.
106. Akasaka-Manyá, K., H. Manyá, Y. Sakurai, B.S. Wojczyk, Y. Kozutsumi, Y. Saito, N. Taniguchi, S. Murayama, S.L. Spitalnik, and T. Endo, *Protective effect of N-glycan bisecting GlcNAc residues on beta-amyloid production in Alzheimer's disease*. Glycobiology, 2010. **20**(1): p. 99-106.
107. Haass, C., C. Kaether, G. Thinakaran, and S. Sisodia, *Trafficking and Proteolytic Processing of APP*. Cold Spring Harbor Perspectives in Medicine, 2012. **2**(5).
108. Joshi, G., Y.J. Chi, Z.P. Huang, and Y.Z. Wang, *A beta-induced Golgi fragmentation in Alzheimer's disease enhances A beta production*. Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(13): p. E1230-E1239.

References

109. Vassar, R., P.H. Kuhn, C. Haass, M.E. Kennedy, L. Rajendran, P.C. Wong, and S.F. Lichtenthaler, *Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects*. J Neurochem, 2014. **130**(1): p. 4-28.
110. Haass, C., C. Kaether, G. Thinakaran, and S. Sisodia, *Trafficking and proteolytic processing of APP*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006270.
111. Choy, R.W.Y., Z.L. Cheng, and R. Schekman, *Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid beta (A beta) production in the trans-Golgi network*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(30): p. E2077-E2082.
112. Chafekar, S.M., F. Baas, and W. Scheper, *Oligomer-specific A beta toxicity in cell models is mediated by selective uptake*. Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2008. **1782**(9): p. 523-531.
113. Wirths, O., G. Multhaup, C. Czech, V. Blanchard, S. Moussaoui, G. Tremp, L. Pradier, K. Beyreuther, and T.A. Bayer, *Intraneuronal A beta accumulation precedes plaque formation in beta-amyloid precursor protein and presenilin-1 double-transgenic mice*. Neuroscience Letters, 2001. **306**(1-2): p. 116-120.
114. Hetz, C. and B. Mollereau, *Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases*. Nat Rev Neurosci, 2014. **15**(4): p. 233-49.
115. Bayer, T.A., O. Wirths, K. Majtenyi, T. Hartmann, G. Multhaup, K. Beyreuther, and C. Czech, *Key factors in Alzheimer's disease: beta-amyloid precursor protein processing, metabolism and intraneuronal transport*. Brain Pathology, 2001. **11**(1): p. 1-11.
116. Hoozemans, J.J.M., E.S. van Haastert, D.A.T. Nijholt, A.J.M. Rozemuller, P. Eikelenboom, and W. Scheper, *The Unfolded Protein Response Is Activated in Pretangle Neurons in Alzheimer's Disease Hippocampus*. American Journal of Pathology, 2009. **174**(4): p. 1241-1251.
117. Imaizumi, K., K. Miyoshi, T. Katayama, T. Yoneda, M. Taniguchi, T. Kudo, and M. Tohyama, *The unfolded protein response and Alzheimer's disease*. Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2001. **1536**(2-3): p. 85-96.
118. Yoshikawa, K., *Cell cycle regulators in neural stem cells and postmitotic neurons*. Neuroscience Research, 2000. **37**(1): p. 1-14.
119. Grafstein, B. and D.S. Forman, *Intracellular transport in neurons*. Physiol Rev, 1980. **60**(4): p. 1167-283.
120. Broadwell, R.D. and A.M. Cataldo, *The Neuronal Endoplasmic-Reticulum - Its Cyto-Chemistry and Contribution to the Endomembrane System .1. Cell-Bodies and Dendrites*. Journal of Histochemistry & Cytochemistry, 1983. **31**(9): p. 1077-1088.
121. Ma, Y.J. and L.M. Hendershot, *ER chaperone functions during normal and stress conditions*. Journal of Chemical Neuroanatomy, 2004. **28**(1-2): p. 51-65.
122. Kozutsumi, Y., M. Segal, K. Normington, M.J. Gething, and J. Sambrook, *The Presence of Malfolded Proteins in the Endoplasmic-Reticulum Signals the Induction of Glucose-Regulated Proteins*. Nature, 1988. **332**(6163): p. 462-464.
123. Welch, W.J., *Mammalian Stress Response - Cell Physiology, Structure-Function of Stress Proteins, and Implications for Medicine and Disease*. Physiological Reviews, 1992. **72**(4): p. 1063-1081.
124. Yamamoto, K., H. Yoshida, K. Kokame, R.J. Kaufman, and K. Mori, *Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II*. Journal of Biochemistry, 2004. **136**(3): p. 343-350.
125. Chen, Y.N. and F. Brandizzi, *IRE1: ER stress sensor and cell fate executor*. Trends in Cell Biology, 2013. **23**(11): p. 547-555.

References

126. Kimata, Y., Y. Shimizu, D. Oikawa, Y. Kimata, and K. Kohno, *A role of BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1*. Cell Structure and Function, 2004. **29**: p. 94-94.
127. Prisci, F., P.R. Nowak, M. Carrara, and M.M.U. Ali, *Phosphoregulation of Ire1 RNase splicing activity*. Nature Communications, 2014. **5**.
128. Cao, S.S. and R.J. Kaufman, *Unfolded protein response*. Current Biology, 2012. **22**(16): p. R622-R626.
129. Kakiuchi, C., M. Ishiwata, A. Hayashi, and T. Kato, *XBP1 induces WFS1 through an endoplasmic reticulum stress response element-like motif in SH-SY5Y cells*. Journal of Neurochemistry, 2006. **97**(2): p. 545-555.
130. Samali, A., U. Fitzgerald, S. Deegan, and S. Gupta, *Methods for monitoring endoplasmic reticulum stress and the unfolded protein response*. Int J Cell Biol, 2010. **2010**: p. 830307.
131. B'chir, W., A.C. Maurin, V. Carraro, J. Averous, C. Jousse, Y. Muranishi, L. Parry, G. Stepien, P. Fafournoux, and A. Bruhat, *The eIF2 alpha/ATF4 pathway is essential for stress-induced autophagy gene expression*. Nucleic Acids Research, 2013. **41**(16): p. 7683-7699.
132. Dey, S., S. Savant, B.F. Teske, M. Hatzoglou, C.F. Calkhoven, and R.C. Wek, *Transcriptional Repression of ATF4 Gene by CCAAT/Enhancer-binding Protein beta (C/EBP beta) Differentially Regulates Integrated Stress Response*. Journal of Biological Chemistry, 2012. **287**(26): p. 21936-21949.
133. Dey, S., T.D. Baird, D.H. Zhou, L.R. Palam, D.F. Spandau, and R.C. Wek, *Both Transcriptional Regulation and Translational Control of ATF4 Are Central to the Integrated Stress Response*. Journal of Biological Chemistry, 2010. **285**(43): p. 33165-33174.
134. B'Chir, W., A.C. Maurin, V. Carraro, J. Averous, C. Jousse, Y. Muranishi, L. Parry, G. Stepien, P. Fafournoux, and A. Bruhat, *The eIF2alpha/ATF4 pathway is essential for stress-induced autophagy gene expression*. Nucleic Acids Res, 2013. **41**(16): p. 7683-99.
135. Han, J., S.H. Backa, J. Hur, Y.H. Lin, R. Gildersleeve, J.X. Shan, C.L. Yuan, D. Krokowski, S.Y. Wang, M. Hatzoglou, M.S. Kilberg, M.A. Sartor, and R.J. Kaufman, *ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death*. Nature Cell Biology, 2013. **15**(5): p. 481-+.
136. Ma, T., M.A. Trinh, A.J. Wexler, C. Bourbon, E. Gatti, P. Pierre, D.R. Cavener, and E. Klann, *Suppression of eIF2 alpha kinases alleviates Alzheimer's disease-related plasticity and memory deficits*. Nature Neuroscience, 2013. **16**(9): p. 1299-U185.
137. Scheper, W. and J.J. Hoozemans, *A New PERKspective on Neurodegeneration*. Sci Transl Med, 2013. **5**(206): p. 206fs37.
138. Pincus, D., M.W. Chevalier, T. Aragon, E. van Anken, S.E. Vidal, H. El-Samad, and P. Walter, *BiP Binding to the ER-Stress Sensor Ire1 Tunes the Homeostatic Behavior of the Unfolded Protein Response*. Plos Biology, 2010. **8**(7).
139. Stahl, S., J.M. Burkhart, F. Hinte, B. Tirosh, H. Mohr, R.P. Zahedi, A. Sickmann, Z. Ruzsics, M. Budt, and W. Brune, *Cytomegalovirus Downregulates IRE1 to Repress the Unfolded Protein Response*. Plos Pathogens, 2013. **9**(8).
140. Calton, M., H.Q. Zeng, F. Urano, J.H. Till, S.R. Hubbard, H.P. Harding, S.G. Clark, and D. Ron, *IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA (vol 415, pg 92, 2002)*. Nature, 2002. **420**(6912): p. 202-202.
141. Forman, M.S., J.Q. Trojanowski, and V.M. Lee, *Neurodegenerative diseases: a decade of discoveries paves the way for therapeutic breakthroughs*. Nat Med, 2004. **10**(10): p. 1055-63.
142. Mattson, M.P., *Apoptosis in neurodegenerative disorders*. Nature Reviews Molecular Cell Biology, 2000. **1**(2): p. 120-129.

References

143. Harding, H.P., I. Novoa, Y.H. Zhang, H.Q. Zeng, R. Wek, M. Schapira, and D. Ron, *Regulated translation initiation controls stress-induced gene expression in mammalian cells*. Molecular Cell, 2000. **6**(5): p. 1099-1108.
144. Harding, H.P., Y.H. Zhang, H.Q. Zeng, I. Novoa, P.D. Lu, M. Calton, N. Sadri, C. Yun, B. Popko, R. Paules, D.F. Stojdl, J.C. Bell, T. Hettmann, J.M. Leiden, and D. Ron, *An integrated stress response regulates amino acid metabolism and resistance to oxidative stress*. Molecular Cell, 2003. **11**(3): p. 619-633.
145. McCullough, K.D., J.L. Martindale, L.O. Klotz, T.Y. Aw, and N.J. Holbrook, *Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state*. Molecular and Cellular Biology, 2001. **21**(4): p. 1249-1259.
146. Lee, J. and U. Ozcan, *Unfolded Protein Response Signaling and Metabolic Diseases*. Journal of Biological Chemistry, 2014. **289**(3): p. 1203-1211.
147. Nishitoh, H., A. Matsuzawa, K. Tobiume, K. Saegusa, K. Takeda, K. Inoue, S. Hori, A. Kakizuka, and H. Ichijo, *ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats*. Genes & Development, 2002. **16**(11): p. 1345-1355.
148. Shamas-Din, A., J. Kale, B. Leber, and D.W. Andrews, *Mechanisms of Action of Bcl-2 Family Proteins*. Cold Spring Harbor Perspectives in Biology, 2013. **5**(4).
149. Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, and J. Yuan, *Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta*. Nature, 2000. **403**(6765): p. 98-103.
150. Tan, Y.F., N. Dourdin, C. Wu, T. De Veyra, J.S. Elce, and P.A. Greer, *Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis*. Journal of Biological Chemistry, 2006. **281**(23): p. 16016-16024.
151. Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, and J.Y. Yuan, *Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta*. Nature, 2000. **403**(6765): p. 98-103.
152. Zinszner, H., M. Kuroda, X.Z. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, and D. Ron, *CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum*. Genes & Development, 1998. **12**(7): p. 982-995.
153. Nutt, L.K., A. Pataer, J. Pahler, B.L. Fang, J. Roth, D.J. McConkey, and S.G. Swisher, *Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca²⁺ stores*. Journal of Biological Chemistry, 2002. **277**(11): p. 9219-9225.
154. Kadowaki, H., H. Nishitoh, and H. Ichijo, *Survival and apoptosis signals in ER stress: the role of protein kinases*. Journal of Chemical Neuroanatomy, 2004. **28**(1-2): p. 93-100.
155. Breckenridge, D.G., M. Germain, J.P. Mathai, M. Nguyen, and G.C. Shore, *Regulation of apoptosis by endoplasmic reticulum pathways*. Oncogene, 2003. **22**(53): p. 8608-8618.
156. Zhang, K.Z. and R.J. Kaufman, *The unfolded protein response - A stress signaling pathway critical for health and disease*. Neurology, 2006. **66**: p. S102-S109.
157. Brentnall, M., L. Rodriguez-Menocal, R.L. De Guevara, E. Cepero, and L.H. Boise, *Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis*. BMC Cell Biology, 2013. **14**.
158. Trusina, A., F.R. Papa, and C. Tang, *Rationalizing translation attenuation in the network architecture of the unfolded protein response*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(51): p. 20280-20285.
159. Mengesdorf, T., P.H. Jensen, G. Mies, C. Aufenberg, and W. Paschen, *Down-regulation of parkin protein in transient focal cerebral ischemia: A link between stroke and degenerative disease?* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(23): p. 15042-15047.

References

160. van Anken, E. and I. Braakman, *Endoplasmic reticulum stress and the making of a professional secretory cell*. Critical Reviews in Biochemistry and Molecular Biology, 2005. **40**(5): p. 269-283.
161. Urano, F., X.Z. Wang, A. Bertolotti, Y.H. Zhang, P. Chung, H.P. Harding, and D. Ron, *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1*. Science, 2000. **287**(5453): p. 664-666.
162. Srivastava, S.P., K.U. Kumar, and R.J. Kaufman, *Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase*. Journal of Biological Chemistry, 1998. **273**(4): p. 2416-2423.
163. Barone, M.V., A. Crozat, A. Tabaee, L. Philipson, and D. Ron, *Chop (Gadd153) and Its Oncogenic Variant, Tls-Chop, Have Opposing Effects on the Induction of G(1)/S Arrest*. Genes & Development, 1994. **8**(4): p. 453-464.
164. Uemura, A., M. Oku, K. Mori, and H. Yoshida, *Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response*. Journal of Cell Science, 2009. **122**(16): p. 2877-2886.
165. Vidal, R.L. and C. Hetz, *Unspliced XBP1 controls autophagy through FoxO1*. Cell Research, 2013. **23**(4): p. 463-464.
166. Yoshida, H., M. Oku, M. Suzuki, and K. Mori, *pXBP1(U) encoded in XBP1 pre-mRNA negatively regulates unfolded protein response activator pXBP1(S) in mammalian ER stress response*. Journal of Cell Biology, 2006. **172**(4): p. 565-575.
167. Majumder, M., C. Huang, M.D. Snider, A.A. Komar, J. Tanaka, R.J. Kaufman, D. Krokowski, and M. Hatzoglou, *A Novel Feedback Loop Regulates the Response to Endoplasmic Reticulum Stress via the Cooperation of Cytoplasmic Splicing and mRNA Translation*. Molecular and Cellular Biology, 2012. **32**(5): p. 992-1003.
168. Althausen, S., T. Mengesdorf, G. Mies, L. Olah, A.C. Nairn, C.G. Proud, and W. Paschen, *Changes in the phosphorylation of initiation factor eIF-2 alpha, elongation factor eEF-2 and p70 S6 kinase after transient focal cerebral ischaemia in mice*. Journal of Neurochemistry, 2001. **78**(4): p. 779-787.
169. DeGracia, D.J., R. Kumar, C.R. Owen, G.S. Krause, and B.C. White, *Molecular pathways of protein synthesis inhibition during brain reperfusion: Implications for neuronal survival or death*. Journal of Cerebral Blood Flow and Metabolism, 2002. **22**(2): p. 127-141.
170. Hu, B.R., M.E. Martone, Y.Z. Jones, and C.L. Liu, *Protein aggregation after transient cerebral ischemia*. Journal of Neuroscience, 2000. **20**(9): p. 3191-3199.
171. Nowak, T.S., R.L. Fried, W.D. Lust, and J.V. Passonneau, *Changes in Brain Energy-Metabolism and Protein-Synthesis Following Transient Bilateral Ischemia in the Gerbil*. Journal of Neurochemistry, 1985. **44**(2): p. 487-494.
172. Paschen, W. and A. Frandsen, *Endoplasmic reticulum dysfunction - a common denominator for cell injury in acute and degenerative diseases of the brain?* Journal of Neurochemistry, 2001. **79**(4): p. 719-725.
173. Chafekar, S.M., R. Zwart, R. Veerhuis, H. Vanderstichele, F. Baas, and W. Scheper, *Increased A beta(1-42) Production Sensitizes Neuroblastoma Cells for ER Stress Toxicity*. Current Alzheimer Research, 2008. **5**(5): p. 469-474.
174. Colombo, A., A. Bastone, C. Ploia, A. Scip, M. Salmona, G. Forloni, and T. Borsello, *JNK regulates APP cleavage and degradation in a model of Alzheimer's disease*. Neurobiology of Disease, 2009. **33**(3): p. 518-525.
175. Ferreira, E., R. Resende, R. Costa, C.R. Oliveira, and C.M.F. Pereira, *An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity*. Neurobiology of Disease, 2006. **23**(3): p. 669-678.

References

176. Oh, S., H.S. Hong, E. Hwang, H.J. Sim, W. Lee, S.J. Shin, and I. Mook-Jung, *Amyloid peptide attenuates the proteasome activity in neuronal cells*. Mechanisms of Ageing and Development, 2005. **126**(12): p. 1292-1299.
177. Copanaki, E., T. Schurmann, A. Eckert, K. Leuner, W.E. Muller, J.H.M. Prehn, and D. Kogel, *The amyloid precursor protein potentiates CHOP induction and cell death in response to ER Ca²⁺ depletion*. Biochimica Et Biophysica Acta-Molecular Cell Research, 2007. **1773**(2): p. 157-165.
178. Sakono, M. and T. Zako, *Amyloid oligomers: formation and toxicity of A beta oligomers*. Febs Journal, 2010. **277**(6): p. 1348-1358.
179. Valincius, G., F. Heinrich, R. Budvytyte, D.J. Vanderah, D.J. McGillivray, Y. Sokolov, J.E. Hall, and M. Losche, *Soluble Amyloid beta-Oligomers Affect Dielectric Membrane Properties by Bilayer Insertion and Domain Formation: Implications for Cell Toxicity*. Biophysical Journal, 2008. **95**(10): p. 4845-4861.
180. Lin, H., R. Bhatia, and R. Lal, *Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology*. Faseb Journal, 2001. **15**(13): p. 2433-2444.
181. Glabe, C.G. and R. Kaye, *Common structure and toxic function of amyloid oligomers implies a common mechanism of pathogenesis*. Neurology, 2006. **66**: p. S74-S78.
182. Kawahara, M., I. Ohtsuka, S. Yokoyama, M. Kato-Negishi, and Y. Sadakane, *Membrane Incorporation, Channel Formation, and Disruption of Calcium Homeostasis by Alzheimer's beta-Amyloid Protein*. Int J Alzheimers Dis, 2011. **2011**: p. 304583.
183. Lai, A.Y. and J. McLaurin, *Mechanisms of amyloid-Beta Peptide uptake by neurons: the role of lipid rafts and lipid raft-associated proteins*. Int J Alzheimers Dis, 2010. **2011**: p. 548380.
184. Rushworth, J.V. and N.M. Hooper, *Lipid Rafts: Linking Alzheimer's Amyloid-beta Production, Aggregation, and Toxicity at Neuronal Membranes*. Int J Alzheimers Dis, 2010. **2011**: p. 603052.
185. Chafekar, S.M., J.J. Hoozemans, R. Zwart, F. Baas, and W. Scheper, *A beta 1-42 induces mild endoplasmic reticulum stress in an aggregation state-dependent manner*. Antioxid Redox Signal, 2007. **9**(12): p. 2245-54.
186. Lee, J.N. and J. Ye, *Proteolytic activation of sterol regulatory element-binding protein induced by cellular stress through depletion of Insig-1*. Journal of Biological Chemistry, 2004. **279**(43): p. 45257-45265.
187. Paschen, W. and T. Mengesdorf, *Cellular abnormalities linked to endoplasmic reticulum dysfunction in cerebrovascular disease - therapeutic potential*. Pharmacology & Therapeutics, 2005. **108**(3): p. 362-375.
188. Katayama, T., K. Imaizumi, T. Manabe, J. Hitomi, T. Kudo, and M. Tohyama, *Induction of neuronal death by ER stress in Alzheimer's disease*. Journal of Chemical Neuroanatomy, 2004. **28**(1-2): p. 67-78.
189. Kudo, T., S. Kanemoto, H. Hara, N. Morimoto, T. Morihara, R. Kimura, T. Tabira, K. Imaizumi, and M. Takeda, *A molecular chaperone inducer protects neurons from ER stress*. Cell Death and Differentiation, 2008. **15**(2): p. 364-375.
190. Lindholm, D., H. Wootz, and L. Korhonen, *ER stress and neurodegenerative diseases*. Cell Death and Differentiation, 2006. **13**(3): p. 385-392.
191. Ferreiro, E., I. Baldeiras, I.L. Ferreira, R.O. Costa, A.C. Rego, C.F. Pereira, and C.R. Oliveira, *Mitochondrial- and endoplasmic reticulum-associated oxidative stress in Alzheimer's disease: from pathogenesis to biomarkers*. Int J Cell Biol, 2012. **2012**: p. 735206.
192. Haynes, C.M., E.A. Titus, and A.A. Cooper, *Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death*. Molecular Cell, 2004. **15**(5): p. 767-776.
193. Naidoo, N., *ER and aging-Protein folding and the ER stress response*. Ageing Research Reviews, 2009. **8**(3): p. 150-159.

References

194. Alfadda, A.A. and R.M. Sallam, *Reactive Oxygen Species in Health and Disease*. Journal of Biomedicine and Biotechnology, 2012.
195. Bueler, H., *Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease*. Experimental Neurology, 2009. **218**(2): p. 235-246.
196. Banerjee, R., A.A. Starkov, M.F. Beal, and B. Thomas, *Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis*. Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2009. **1792**(7): p. 651-663.
197. Fiskum, G., A. Starkov, B.M. Polster, and C. Chinopoulos, *Mitochondrial mechanisms of neural cell death and neuroprotective interventions in Parkinson's disease*. Parkinson's Disease: The Life Cycle of the Dopamine Neuron, 2003. **991**: p. 111-119.
198. LaFerla, F.M., K.N. Green, and S. Oddo, *Intracellular amyloid-beta in Alzheimer's disease*. Nature Reviews Neuroscience, 2007. **8**(7): p. 499-509.
199. Zhao, Y. and B.L. Zhao, *Oxidative Stress and the Pathogenesis of Alzheimer's Disease*. Oxidative Medicine and Cellular Longevity, 2013.
200. Honarnejad, K., C.K. Jung, S. Lammich, T. Arzberger, H. Kretschmar, and J. Herms, *Involvement of presenilin holoprotein upregulation in calcium dyshomeostasis of Alzheimer's disease*. J Cell Mol Med, 2013. **17**(2): p. 293-302.
201. Chakroborty, S., I. Goussakov, M.B. Miller, and G.E. Stutzmann, *Deviant Ryanodine Receptor-Mediated Calcium Release Resets Synaptic Homeostasis in Presymptomatic 3xTg-AD Mice*. Journal of Neuroscience, 2009. **29**(30): p. 9458-9470.
202. Scorrano, L., S.A. Oakes, J.T. Opferman, E.H. Cheng, M.D. Sorcinelli, T. Pozzan, and S.J. Korsmeyer, *BAX and BAK regulation of endoplasmic reticulum Ca^{2+} : A control point for apoptosis*. Science, 2003. **300**(5616): p. 135-139.
203. Cheng, E.H.Y., T.V. Sheiko, J.K. Fisher, W.J. Craigen, and S.J. Korsmeyer, *VDAC2 inhibits BAK activation and mitochondrial apoptosis*. Science, 2003. **301**(5632): p. 513-517.
204. Wei, M.C., W.X. Zong, E.H.Y. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K.A. Roth, G.R. MacGregor, C.B. Thompson, and S.J. Korsmeyer, *Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death*. Science, 2001. **292**(5517): p. 727-730.
205. Rockabrand, E., N. Slepko, A. Pantalone, V.N. Nukala, A. Kazantsev, J.L. Marsh, P.G. Sullivan, J.S. Steffan, S.L. Sensi, and L.M. Thompson, *The first 17 amino acids of Huntingtin modulate its sub-cellular localization, aggregation and effects on calcium homeostasis*. Human Molecular Genetics, 2007. **16**(1): p. 61-77.
206. Camandola, S. and M.P. Mattson, *Aberrant subcellular neuronal calcium regulation in aging and Alzheimer's disease*. Biochim Biophys Acta, 2011. **1813**(5): p. 965-73.
207. Kim, I., W.J. Xu, and J.C. Reed, *Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities*. Nature Reviews Drug Discovery, 2008. **7**(12): p. 1013-1030.
208. Wang, J.F. and L.T. Young, *Regulation of molecular chaperone GRP78 by mood stabilizing drugs*. Clinical Neuroscience Research, 2004. **4**(3-4): p. 281-288.
209. Shao, L., X.J. Sun, L. Xu, L.T. Young, and J.F. Wang, *Mood stabilizing drug lithium increases expression of endoplasmic reticulum stress proteins in primary cultured rat cerebral cortical cells*. Life Sciences, 2006. **78**(12): p. 1317-1323.
210. Bown, C.D., J.F. Wang, B. Chen, and L.T. Young, *Regulation of ER stress proteins by valproate: therapeutic implications*. Bipolar Disorders, 2002. **4**(2): p. 145-151.
211. Kim, A.J., Y.Y. Shi, R.C. Austin, and G.H. Werstuck, *Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3*. Journal of Cell Science, 2005. **118**(1): p. 89-99.

References

212. Suzuki, S., Y. Okuse, M. Kawase, M. Takiguchi, Y. Fukuyama, H. Takahashi, and M. Sato, *A norbergenin derivative inhibits neuronal cell damage induced by tunicamycin*. Biological & Pharmaceutical Bulletin, 2006. **29**(7): p. 1335-1338.
213. Qi, X., T. Hosoi, Y. Okuma, M. Kaneko, and Y. Nomura, *Sodium 4-phenylbutyrate protects against cerebral ischemic injury*. Molecular Pharmacology, 2004. **66**(4): p. 899-908.
214. Ozcan, U., E. Yilmaz, L. Ozcan, M. Furuhashi, E. Vaillancourt, R.O. Smith, C.Z. Gorgun, and G.S. Hotamisligil, *Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes*. Science, 2006. **313**(5790): p. 1137-1140.
215. Woltjer, R.L., W. McMahan, D. Milatovic, J.D. Kjerulf, F.S. Shie, L.G. Rung, K.S. Montine, and T.J. Montine, *Effects of chemical chaperones on oxidative stress and detergent-insoluble species formation following conditional expression of amyloid precursor protein carboxy-terminal fragment*. Neurobiology of Disease, 2007. **25**(2): p. 427-437.
216. Wiley, J.C., J.S. Meabon, H. Frankowski, E.A. Smith, L.C. Schecterson, M. Bothwell, and W.C. Ladiges, *Phenylbutyric Acid Rescues Endoplasmic Reticulum Stress-Induced Suppression of APP Proteolysis and Prevents Apoptosis in Neuronal Cells*. Plos One, 2010. **5**(2).
217. Hosoi, T. and K. Ozawa, *Endoplasmic reticulum stress in disease: mechanisms and therapeutic opportunities*. Clin Sci (Lond), 2010. **118**(1): p. 19-29.
218. Sokka, A.L., N. Putkonen, G. Mudo, E. Pryazhnikov, S. Reijonen, L. Khiroug, N. Belluardo, D. Lindholm, and L. Korhonen, *Endoplasmic reticulum stress inhibition protects against excitotoxic neuronal injury in the rat brain*. Journal of Neuroscience, 2007. **27**(4): p. 901-908.
219. Lee do, Y., K.S. Lee, H.J. Lee, H. Kim do, Y.H. Noh, K. Yu, H.Y. Jung, S.H. Lee, J.Y. Lee, Y.C. Youn, Y. Jeong, D.K. Kim, W.B. Lee, and S.S. Kim, *Activation of PERK signaling attenuates Abeta-mediated ER stress*. PLoS One, 2010. **5**(5): p. e10489.
220. Colla, E., P. Coune, Y. Liu, O. Pletnikova, J.C. Troncoso, T. Iwatsubo, B.L. Schneider, and M.K. Lee, *Endoplasmic Reticulum Stress Is Important for the Manifestations of alpha-Synucleinopathy In Vivo*. Journal of Neuroscience, 2012. **32**(10): p. 3306-3320.
221. Reijonen, S., N. Putkonen, A. Norremolle, D. Lindholm, and L. Korhonen, *Inhibition of endoplasmic reticulum stress counteracts neuronal cell death and protein aggregation caused by N-terminal mutant huntingtin proteins*. Exp Cell Res, 2008. **314**(5): p. 950-60.
222. Tsaytler, P., H.P. Harding, D. Ron, and A. Bertolotti, *Selective Inhibition of a Regulatory Subunit of Protein Phosphatase 1 Restores Proteostasis*. Science, 2011. **332**(6025): p. 91-94.
223. Hetz, C., E. Chevet, and H.P. Harding, *Targeting the unfolded protein response in disease*. Nature Reviews Drug Discovery, 2013. **12**(9): p. 703-719.
224. Hetz, C., E. Chevet, and H.P. Harding, *Targeting the unfolded protein response in disease*. Nat Rev Drug Discov, 2013. **12**(9): p. 703-19.
225. Suzuki, S., Y. Okuse, M. Kawase, M. Takiguchi, Y. Fukuyama, H. Takahashi, and M. Sato, *A norbergenin derivative inhibits neuronal cell damage induced by tunicamycin*. Biol Pharm Bull, 2006. **29**(7): p. 1335-8.
226. Tabata, Y., K. Takano, T. Ito, M. Iinuma, T. Yoshimoto, H. Miura, Y. Kitao, S. Ogawa, and O. Hori, *Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation*. Am J Physiol Cell Physiol, 2007. **293**(1): p. C411-8.
227. Takano, K., Y. Tabata, Y. Kitao, R. Murakami, H. Suzuki, M. Yamada, M. Iinuma, Y. Yoneda, S. Ogawa, and O. Hori, *Methoxyflavones protect cells against endoplasmic reticulum stress and neurotoxin*. Am J Physiol Cell Physiol, 2007. **292**(1): p. C353-61.
228. Takano, K., Y. Kitao, Y. Tabata, H. Miura, K. Sato, K. Takuma, K. Yamada, S. Hibino, T. Choshi, M. Iinuma, H. Suzuki, R. Murakami, M. Yamada, S. Ogawa, and O. Hori, *A dibenzoylmethane derivative protects dopaminergic neurons against both oxidative stress and endoplasmic reticulum stress*. Am J Physiol Cell Physiol, 2007. **293**(6): p. C1884-94.

References

229. Park, Y.J., Y.M. Jang, and Y.H. Kwon, *Isoflavones prevent endoplasmic reticulum stress-mediated neuronal degeneration by inhibiting tau hyperphosphorylation in SH-SY5Y cells*. J Med Food, 2009. **12**(3): p. 528-35.
230. Choi, A.Y., J.H. Choi, J.Y. Lee, K.S. Yoon, W. Choe, J. Ha, E.J. Yeo, and I. Kang, *Apigenin protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis*. Neurochem Int, 2010. **57**(2): p. 143-52.
231. Takano, K., Y. Tabata, Y. Kitao, R. Murakami, H. Suzuki, M. Yamada, M. Iinuma, Y. Yoneda, S. Ogawa, and O. Hori, *Methoxyflavones protect cells against endoplasmic reticulum stress and neurotoxin*. American Journal of Physiology-Cell Physiology, 2007. **292**(1): p. C353-C361.
232. Park, Y.J., Y.M. Jang, and Y.H. Kwon, *Isoflavones Prevent Endoplasmic Reticulum Stress-Mediated Neuronal Degeneration by Inhibiting Tau Hyperphosphorylation in SH-SY5Y Cells*. Journal of Medicinal Food, 2009. **12**(3): p. 528-535.
233. Choi, A.Y., J.H. Choi, J.Y. Lee, K.S. Yoon, W. Choe, J. Ha, E.J. Yeo, and I. Kang, *Apigenin protects HT22 murine hippocampal neuronal cells against endoplasmic reticulum stress-induced apoptosis*. Neurochemistry International, 2010. **57**(2): p. 143-152.
234. Takano, K., Y. Kitao, Y. Tabata, H. Miura, K. Sato, K. Takuma, K. Yamada, S. Hibino, T. Choshi, M. Iinuma, H. Suzuki, R. Murakami, M. Yamada, S. Ogawa, and O. Hori, *A dibenzoylmethane derivative protects dopaminergic neurons against both oxidative stress and endoplasmic reticulum stress*. American Journal of Physiology-Cell Physiology, 2007. **293**(6): p. C1884-C1894.
235. Tabata, Y., K. Takano, T. Ito, M. Iinuma, T. Yoshimoto, H. Miura, Y. Kitao, S. Ogawa, and O. Hori, *Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation*. American Journal of Physiology-Cell Physiology, 2007. **293**(1): p. C411-C418.
236. Hosoi, T., M. Sasaki, S. Baba, and K. Ozawa, *Effect of pranoprofen on endoplasmic reticulum stress in the primary cultured glial cells*. Neurochemistry International, 2009. **54**(1): p. 1-6.
237. Sanz, E., A. Quintana, J. Hidalgo, J.L. Marco, and M. Unzeta, *PF9601N [N-(2-propynyl)-2-(5-benzyloxy-indolyl) methylamine] confers MAO-B independent neuroprotection in ER stress-induced cell death*. Molecular and Cellular Neuroscience, 2009. **41**(1): p. 19-31.
238. Bouchecareilh, M., M.E. Caruso, P. Roby, S. Parent, N. Rouleau, S. Taouji, O. Pluquet, R. Bosse, M. Moenner, and E. Chevet, *AlphaScreen-based characterization of the bifunctional kinase/RNase IRE1alpha: a novel and atypical drug target*. J Biomol Screen, 2010. **15**(4): p. 406-17.
239. Cross, B.C.S., P.J. Bond, P.G. Sadowski, B.K. Jha, J. Zak, J.M. Goodman, R.H. Silverman, T.A. Neubert, I.R. Baxendale, D. Ron, and H.P. Harding, *The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule*. Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(15): p. E869-E878.
240. Wang, H., J. Blais, D. Ron, and T. Cardozo, *Structural Determinants of PERK Inhibitor Potency and Selectivity*. Chemical Biology & Drug Design, 2010. **76**(6): p. 480-495.
241. Moreno, J.A., M. Halliday, C. Molloy, H. Radford, N. Verity, J.M. Axten, C.A. Ortori, A.E. Willis, P.M. Fischer, D.A. Barrett, and G.R. Mallucci, *Oral Treatment Targeting the Unfolded Protein Response Prevents Neurodegeneration and Clinical Disease in Prion-Infected Mice*. Science Translational Medicine, 2013. **5**(206).
242. Gouw, J.W., J. Krijgsveld, and A.J.R. Heck, *Quantitative Proteomics by Metabolic Labeling of Model Organisms*. Molecular & Cellular Proteomics, 2010. **9**(1): p. 11-24.
243. Woodruff-Pak, D.S., *Animal models of Alzheimer's disease: therapeutic implications*. J Alzheimers Dis, 2008. **15**(4): p. 507-21.
244. LaFerla, F.M. and K.N. Green, *Animal Models of Alzheimer Disease*. Cold Spring Harbor Perspectives in Medicine, 2012. **2**(11).

References

245. Trinchese, F., S.M. Liu, P. Ninan, D. Puzzo, J.P. Jacob, and O. Arancio, *Cell cultures from animal models of Alzheimer's disease as a tool for faster screening and testing of drug efficacy*. Journal of Molecular Neuroscience, 2004. **24**(1): p. 15-21.
246. Schlachetzki, J.C.M., S.W. Saliba, and A.C.P. de Oliyeira, *Studying neurodegenerative diseases in culture models*. Revista Brasileira De Psiquiatria, 2013. **35**: p. S92-S100.
247. Biedler, J.L., L. Helson, and B.A. Spengler, *Morphology and Growth, Tumorigenicity, and Cytogenetics of Human Neuroblastoma Cells in Continuous Culture*. Cancer Research, 1973. **33**(11): p. 2643-2652.
248. Ba, F., P.K.T. Pang, and C.G. Benishin, *The establishment of a reliable cytotoxic system with SK-N-SH neuroblastoma cell culture*. Journal of Neuroscience Methods, 2003. **123**(1): p. 11-22.
249. Nam, D.T., M. Arseneault, N. Zarkovic, G. Waeg, and C. Ramassamy, *Molecular Regulations Induced by Acrolein in Neuroblastoma SK-N-SH Cells: Relevance to Alzheimer's Disease*. Journal of Alzheimers Disease, 2010. **21**(4): p. 1197-1216.
250. Fischer, H.P.P.D., *Mathematical Modeling of Complex Biological Systems: From Parts Lists to Understanding Systems Behavior*. Alcohol Res Health, 2008. **31**(1): p. 49-59.
251. Kitano, H., *Computational systems biology*. Nature, 2002. **420**(6912): p. 206-10.
252. Crampin, E.J., S. Schnell, and P.E. McSharry, *Mathematical and computational techniques to deduce complex biochemical reaction mechanisms*. Prog Biophys Mol Biol, 2004. **86**(1): p. 77-112.
253. Swameye, I., T.G. Muller, J. Timmer, O. Sandra, and U. Klingmuller, *Identification of nucleocytoplasmic cycling as a remote sensor in cellular signaling by databased modeling*. Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(3): p. 1028-1033.
254. Stamatakis, M., *Cell population balance and hybrid modeling of population dynamics for a single gene with feedback*. Computers & Chemical Engineering, 2013. **53**: p. 25-34.
255. Raden, D., S. Hildebrandt, P. Xu, E. Bell, F.J. Doyle, and A.S. Robinson, *Analysis of cellular response to protein overexpression*. Iee Proceedings Systems Biology, 2005. **152**(4): p. 285-289.
256. Rutkowski, D.T., S.M. Arnold, C.N. Miller, J. Wu, J. Li, K.M. Gunnison, K. Mori, A.A.S. Akha, D. Raden, and R.J. Kaufman, *Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins*. Plos Biology, 2006. **4**(11): p. 2024-2041.
257. Erguler, K., M. Pieri, and C. Deltas, *A mathematical model of the unfolded protein stress response reveals the decision mechanism for recovery, adaptation and apoptosis*. BMC Systems Biology, 2013. **7**.
258. Gardner, B.M. and P. Walter, *Unfolded Proteins Are Ire1-Activating Ligands That Directly Induce the Unfolded Protein Response*. Science, 2011. **333**(6051): p. 1891-1894.
259. Bernales, S., F.R. Papa, and P. Walter, *Intracellular signaling by the unfolded protein response*. Annual Review of Cell and Developmental Biology, 2006. **22**: p. 487-508.
260. Marciniak, S.J., C.Y. Yun, S. Oyadomari, I. Novoa, Y.H. Zhang, R. Jungreis, K. Nagata, H.P. Harding, and D. Ron, *CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum*. Genes & Development, 2004. **18**(24): p. 3066-3077.
261. Zhang, T., P. Brazhnik, and J.J. Tyson, *Computational analysis of dynamical responses to the intrinsic pathway of programmed cell death*. Biophys J, 2009. **97**(2): p. 415-34.
262. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR*. Nucleic Acids Research, 2001. **29**(9).
263. Perez, R.G., S.L. Squazzo, and E.H. Koo, *Enhanced release of amyloid beta-protein from codon 670/671 "Swedish" mutant beta-amyloid precursor protein occurs in both secretory and endocytic pathways*. Journal of Biological Chemistry, 1996. **271**(15): p. 9100-9107.

References

264. Mann, D.M.A., T. Iwatsubo, Y. Ihara, N.J. Cairns, P.L. Lantos, N. Bogdanovic, L. Lannfelt, B. Winblad, M.L.C. MaatSchieman, and M.N. Rossor, *Predominant deposition of amyloid-beta(42/43) in plaques in cases of Alzheimer's disease and hereditary cerebral hemorrhage associated with mutations in the amyloid precursor protein gene*. American Journal of Pathology, 1996. **148**(4): p. 1257-1266.
265. Ghetti, B., J. Murrell, M.D. Benson, and M.R. Farlow, *Spectrum of Amyloid Beta-Protein Immunoreactivity in Hereditary Alzheimer-Disease with a Guanine to Thymine Missense Change at Position 1924 of the App Gene*. Brain Research, 1992. **571**(1): p. 133-139.
266. Loser, P., G.S. Jennings, M. Strauss, and V. Sandig, *Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: Involvement of NF kappa B*. Journal of Virology, 1998. **72**(1): p. 180-190.
267. Granic, A., J. Padmanabhan, M. Norden, and H. Potter, *Alzheimer Abeta peptide induces chromosome mis-segregation and aneuploidy, including trisomy 21: requirement for tau and APP*. Mol Biol Cell, 2010. **21**(4): p. 511-20.
268. Zulewski, H., E.J. Abraham, M.J. Gerlach, P.B. Daniel, W. Moritz, B. Muller, M. Vallejo, M.K. Thomas, and J.F. Habener, *Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes*. Diabetes, 2001. **50**(3): p. 521-533.
269. Mareschi, K., M. Novara, D. Rustichelli, I. Ferrero, D. Guido, E. Carbone, E. Medico, E. Madon, A. Vercelli, and F. Fagioli, *Neural differentiation of human mesenchymal stem cells: evidence for expression of neural markers and eag K+ channel types*. Experimental Hematology, 2006. **34**(11): p. 1563-1572.
270. Rhinn, H., C. Marchand-Leroux, N. Croci, M. Plotkine, D. Scherman, and V. Escriou, *Housekeeping while brain's storming validation of normalizing factors for gene expression studies in a murine model of traumatic brain injury*. BMC Molecular Biology, 2008. **9**.
271. Aerts, J.L., M.I. Gonzales, and S.L. Topalian, *Selection of appropriate control genes to assess expression of tumor antigens using real-time RT-PCR*. Biotechniques, 2004. **36**(1): p. 84-6, 88, 90-1.
272. Bas, A., G. Forsberg, S. Hammarstrom, and M.L. Hammarstrom, *Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes*. Scand J Immunol, 2004. **59**(6): p. 566-73.
273. Sidell, N., T. Sarafian, M. Kelly, T. Tsuchida, and M. Haussler, *Retinoic acid-induced differentiation of human neuroblastoma: a cell variant system showing two distinct responses*. Exp Cell Biol, 1986. **54**(5-6): p. 287-300.
274. Pahlman, S., A.I. Ruusala, L. Abrahamsson, M.E. Mattsson, and T. Esscher, *Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbol ester-induced differentiation*. Cell Differ, 1984. **14**(2): p. 135-44.
275. Apostolatos, H., A. Apostolatos, T. Vickers, J.E. Watson, S. Song, F. Vale, D.R. Cooper, J. Sanchez-Ramos, and N.A. Patel, *Vitamin A metabolite, all-trans-retinoic acid, mediates alternative splicing of protein kinase C deltaVIII (PKCdeltaVIII) isoform via splicing factor SC35*. J Biol Chem, 2010. **285**(34): p. 25987-95.
276. Irving, H., P.E. Lovat, Q.C. Hewson, A.J. Malcolm, A.D.J. Pearson, and C.P.F. Redfern, *Retinoid-induced differentiation of neuroblastoma: Comparison between LG69, an RXR-selective analogue and 9-cis retinoic acid*. European Journal of Cancer, 1998. **34**(1): p. 111-117.
277. Wainwright, L.J., A. Lasorella, and A. Iavarone, *Distinct mechanisms of cell cycle arrest control the decision between differentiation and senescence in human neuroblastoma cells*. Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9396-400.

References

278. Abe, Y., K. Kouyama, T. Tomita, Y. Tomita, N. Ban, M. Nawa, M. Matsuoka, T. Niikura, S. Aiso, Y. Kita, T. Iwatsubo, and I. Nishimoto, *Analysis of neurons created from wild-type and Alzheimer's mutation knock-in embryonic stem cells by a highly efficient differentiation protocol*. J Neurosci, 2003. **23**(24): p. 8513-25.
279. Masland, R.H., *Neuronal cell types*. Current Biology, 2004. **14**(13): p. R497-R500.
280. Park, D., A.P. Xiang, F.F. Mao, L. Zhang, C.G. Di, X.M. Liu, Y.A. Shao, B.F. Ma, J.H. Lee, K.S. Ha, N. Walton, and B.T. Lahn, *Nestin Is Required for the Proper Self-Renewal of Neural Stem Cells*. Stem Cells, 2010. **28**(12): p. 2162-2171.
281. Dahlstrand, J., M. Lardelli, and U. Lendahl, *Nestin Messenger-Rna Expression Correlates with the Central-Nervous-System Progenitor-Cell State in Many, but Not All, Regions of Developing Central-Nervous-System*. Developmental Brain Research, 1995. **84**(1): p. 109-129.
282. Iwanaga, T., Y. Takahashi, and T. Fujita, *Immunohistochemistry of Neuron-Specific and Glia-Specific Proteins*. Archives of Histology and Cytology, 1989. **52**: p. 13-24.
283. Kashafi, E., H. Karimi Jashni, S. Erfaniyan, K. Solhjoui, A. Sepidkar, and H. Fakhryniya, *Transdifferentiation of human synovium-derived mesenchymal stem cell into neuronal-like cells in vitro*. Journal of Jahrom University of Medical Sciences, 2013. **11**(2): p. 31-38.
284. Hyder, C.L., K.O. Isoniemi, E.S. Torvaldson, and J.E. Eriksson, *Insights into intermediate filament regulation from development to ageing*. Journal of Cell Science, 2011. **124**(9): p. 1363-1372.
285. Laing, S., G. Wang, T. Briazova, C. Zhang, A. Wang, Z. Zheng, A. Gow, A.F. Chen, S. Rajagopalan, L.C. Chen, Q. Sun, and K. Zhang, *Airborne particulate matter selectively activates endoplasmic reticulum stress response in the lung and liver tissues*. Am J Physiol Cell Physiol, 2010. **299**(4): p. C736-49.
286. Cullinan, S.B., D. Zhang, M. Hannink, E. Arvisais, R.J. Kaufman, and J.A. Diehl, *Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival*. Molecular and Cellular Biology, 2003. **23**(20): p. 7198-7209.
287. Scortegagna, M., H. Kim, J.L. Li, H. Yao, L.M. Brill, J. Han, E. Lau, D. Bowtell, G. Haddad, R.J. Kaufman, and Z.A. Ronai, *Fine tuning of the UPR by the ubiquitin ligases Siah1/2*. PLoS Genet, 2014. **10**(5): p. e1004348.
288. Nagele, R.G., M.R. D'Andrea, W.J. Anderson, and H.Y. Wang, *Intracellular accumulation of beta-amyloid(1-42) in neurons is facilitated by the alpha 7 nicotinic acetylcholine receptor in Alzheimer's disease*. Neuroscience, 2002. **110**(2): p. 199-211.
289. Treiman, M., C. Caspersen, and S.B. Christensen, *A tool coming of age: thapsigargin as an inhibitor of sarcoendoplasmic reticulum Ca2+-ATPases*. Trends in Pharmacological Sciences, 1998. **19**(4): p. 131-135.
290. Oda, T., Y. Kosuge, M. Arakawa, K. Ishige, and Y. Ito, *Distinct mechanism of cell death is responsible for tunicamycin-induced ER stress in SK-N-SH and SH-SY5Y cells*. Neuroscience Research, 2008. **60**(1): p. 29-39.
291. Schuiki, I., L.L. Zhang, and A. Volchuk, *Endoplasmic Reticulum Redox State Is Not Perturbed by Pharmacological or Pathological Endoplasmic Reticulum Stress in Live Pancreatic beta-Cells*. Plos One, 2012. **7**(11).
292. Osowski, C.M. and F. Urano, *Measuring Er Stress and the Unfolded Protein Response Using Mammalian Tissue Culture System*. Methods in Enzymology: Unfolded Protein Response and Cellular Stress, Vol 490, Pt B, 2011. **490**: p. 71-92.
293. Rand, J.D. and C.M. Grant, *The thioredoxin system protects ribosomes against stress-induced aggregation*. Molecular Biology of the Cell, 2006. **17**(1): p. 387-401.
294. Lehrman, M.A., *Oligosaccharide-based information in endoplasmic reticulum quality control and other biological systems*. Journal of Biological Chemistry, 2001. **276**(12): p. 8623-8626.

References

295. Davenport, E.L., H.E. Moore, A.S. Dunlop, S.Y. Sharp, P. Workman, G.J. Morgan, and F.E. Davies, *Heat shock protein inhibition is associated with activation of the unfolded protein response pathway in myeloma plasma cells*. *Blood*, 2007. **110**(7): p. 2641-2649.
296. Price, N.P.J. and B. Tsvetanova, *Biosynthesis of the tunicamycins: A review*. *Journal of Antibiotics*, 2007. **60**(8): p. 485-491.
297. Waechter, C.J., J.W. Schmidt, and W.A. Catterall, *Glycosylation is required for maintenance of functional sodium channels in neuroblastoma cells*. *J Biol Chem*, 1983. **258**(8): p. 5117-23.
298. Quent, V.M.C., D. Loessner, T. Friis, J.C. Reichert, and D.W. Hutmacher, *Discrepancies between metabolic activity and DNA content as tool to assess cell proliferation in cancer research*. *Journal of Cellular and Molecular Medicine*, 2010. **14**(4): p. 1003-1013.
299. Ho, A. and T.C. Sudhof, *Binding of F-spondin to amyloid-beta precursor protein: a candidate amyloid-beta precursor protein ligand that modulates amyloid-beta precursor protein cleavage*. *Proc Natl Acad Sci U S A*, 2004. **101**(8): p. 2548-53.
300. Nishitsuji, K., T. Tomiyama, K. Ishibashi, K. Ito, R. Teraoka, M.P. Lambert, W.L. Klein, and H. Mori, *The E693Delta mutation in amyloid precursor protein increases intracellular accumulation of amyloid beta oligomers and causes endoplasmic reticulum stress-induced apoptosis in cultured cells*. *Am J Pathol*, 2009. **174**(3): p. 957-69.
301. Takahashi, K., T. Niidome, A. Akaike, T. Kihara, and H. Sugimoto, *Amyloid precursor protein promotes endoplasmic reticulum stress-induced cell death via C/EBP homologous protein-mediated pathway*. *Journal of Neurochemistry*, 2009. **109**(5): p. 1324-1337.
302. Kogel, D., R. Schomburg, T. Schurmann, C. Reimertz, H.G. Konig, M. Poppe, A. Eckert, W.E. Muller, and J.H.M. Prehn, *The amyloid precursor protein protects PC12 cells against endoplasmic reticulum stress-induced apoptosis*. *Journal of Neurochemistry*, 2003. **87**(1): p. 248-256.
303. Schule, B., R.A.R. Pera, and J.W. Langston, *Can cellular models revolutionize drug discovery in Parkinson's disease?* *Biochimica Et Biophysica Acta-Molecular Basis of Disease*, 2009. **1792**(11): p. 1043-1051.
304. Yoshida, H., T. Matsui, N. Hosokawa, R.J. Kaufman, K. Nagata, and K. Mori, *A time-dependent phase shift in the mammalian unfolded protein response*. *Developmental Cell*, 2003. **4**(2): p. 265-271.
305. Lin, J.H., H. Li, D. Yasumura, H.R. Cohen, C. Zhang, B. Panning, K.M. Shokat, M.M. LaVail, and P. Walter, *IRE1 signaling affects cell fate during the unfolded protein response*. *Science*, 2007. **318**(5852): p. 944-949.
306. Hitomi, J., T. Katayama, Y. Eguchi, T. Kudo, M. Taniguchi, Y. Koyama, T. Manabe, S. Yamagishi, Y. Bando, K. Imaizumi, Y. Tsujimoto, and M. Tohyama, *Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and A beta-induced cell death*. *Journal of Cell Biology*, 2004. **165**(3): p. 347-356.
307. Owens, G.P., W.E. Hahn, and J.J. Cohen, *Identification of mRNAs associated with programmed cell death in immature thymocytes*. *Mol Cell Biol*, 1991. **11**(8): p. 4177-88.
308. Eletto, D., A. Maganty, D. Eletto, D. Dersh, C. Makarewich, C. Biswas, J.C. Paton, A.W. Paton, S. Doroudgar, C.C. Glembotski, and Y. Argon, *Limitation of individual folding resources in the ER leads to outcomes distinct from the unfolded protein response*. *Journal of Cell Science*, 2012. **125**(20): p. 4865-4875.
309. Lee, A.S., *The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress*. *Methods*, 2005. **35**(4): p. 373-381.
310. Melnick, J., S. Aviel, and Y. Argon, *The Endoplasmic-Reticulum Stress Protein-Grp94, in Addition to Bip, Associates with Unassembled Immunoglobulin-Chains*. *Journal of Biological Chemistry*, 1992. **267**(30): p. 21303-21306.

References

311. Lai, E.D., T. Teodoro, and A. Volchuk, *Endoplasmic reticulum stress: Signaling the unfolded protein response*. Physiology, 2007. **22**: p. 193-201.
312. Szegezdi, E., S.E. Logue, A.M. Gorman, and A. Samali, *Mediators of endoplasmic reticulum stress-induced apoptosis*. Embo Reports, 2006. **7**(9): p. 880-885.
313. Cullinan, S.B. and J.A. Diehl, *Coordination of ER and oxidative stress signaling: The PERK/Nrf2 signaling pathway*. International Journal of Biochemistry & Cell Biology, 2006. **38**(3): p. 317-332.
314. Teske, B.F., S.A. Wek, P. Bunpo, J.K. Cundiff, J.N. McClintick, T.G. Anthony, and R.C. Wek, *The eIF2 kinase PERK and the integrated stress response facilitate activation of ATF6 during endoplasmic reticulum stress*. Molecular Biology of the Cell, 2011. **22**(22): p. 4390-4405.
315. Schroder, M. and R.J. Kaufman, *The mammalian unfolded protein response*. Annual Review of Biochemistry, 2005. **74**: p. 739-789.
316. Glover-Cutter, K.M., S. Lin, and T.K. Blackwell, *Integration of the Unfolded Protein and Oxidative Stress Responses through SKN-1/Nrf*. PLoS Genet, 2013. **9**(9): p. e1003701.
317. Milani, P., G. Ambrosi, O. Gammoh, F. Blandini, and C. Cereda, *SOD1 and DJ-1 Converge at Nrf2 Pathway: A Clue for Antioxidant Therapeutic Potential in Neurodegeneration*. Oxidative Medicine and Cellular Longevity, 2013.
318. Theuns, J. and C. Van Broeckhoven, *Transcriptional regulation of Alzheimer's disease genes: implications for susceptibility*. Hum Mol Genet, 2000. **9**(16): p. 2383-94.
319. Walter, P. and D. Ron, *The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation*. Science, 2011. **334**(6059): p. 1081-1086.
320. Perneczky, R., P. Alexopoulos, and A. Kurz, *Soluble amyloid precursor proteins and secretases as Alzheimer's disease biomarkers*. Trends in Molecular Medicine, 2014. **20**(1): p. 8-15.
321. Ashall, L., C.A. Horton, D.E. Nelson, P. Paszek, C.V. Harper, K. Sillitoe, S. Ryan, D.G. Spiller, J.F. Unitt, D.S. Broomhead, D.B. Kell, D.A. Rand, V. See, and M.R. White, *Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription*. Science, 2009. **324**(5924): p. 242-6.
322. Toettcher, J.E., A. Loewer, G.J. Ostheimer, M.B. Yaffe, B. Tidor, and G. Lahav, *Distinct mechanisms act in concert to mediate cell cycle arrest*. Proc Natl Acad Sci U S A, 2009. **106**(3): p. 785-90.
323. Bertolotti, A., Y.H. Zhang, L.M. Hendershot, H.P. Harding, and D. Ron, *Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response*. Nature Cell Biology, 2000. **2**(6): p. 326-332.
324. Oikawa, D., A. Kitamura, M. Kinjo, and T. Iwawaki, *Direct Association of Unfolded Proteins with Mammalian ER Stress Sensor, IRE1 beta*. Plos One, 2012. **7**(12).
325. Maier, T., M. Guell, and L. Serrano, *Correlation of mRNA and protein in complex biological samples*. Febs Letters, 2009. **583**(24): p. 3966-3973.
326. Ghaemmaghami, S., W. Huh, K. Bower, R.W. Howson, A. Belle, N. Dephoure, E.K. O'Shea, and J.S. Weissman, *Global analysis of protein expression in yeast*. Nature, 2003. **425**(6959): p. 737-741.
327. Axelsen, J.B. and K. Sneppen, *Quantifying the benefits of translation regulation in the unfolded protein response*. Physical Biology, 2004. **1**(3): p. 159-165.
328. Ma, Y.J., J.W. Brewer, J.A. Diehl, and L.M. Hendershot, *Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response*. Journal of Molecular Biology, 2002. **318**(5): p. 1351-1365.
329. Srividhya, J. and M.S. Gopinathan, *A simple time delay model for eukaryotic cell cycle*. Journal of Theoretical Biology, 2006. **241**(3): p. 617-627.
330. Ventoso, I., A. Kochetov, D. Montaner, J. Dopazo, and J. Santoyo, *Extensive Translatome Remodeling during ER Stress Response in Mammalian Cells*. Plos One, 2012. **7**(5).

References

331. Hetz, C., *The unfolded protein response: controlling cell fate decisions under ER stress and beyond*. Nature Reviews Molecular Cell Biology, 2012. **13**(2): p. 89-102.
332. Kimata, Y. and K. Kohno, *Endoplasmic reticulum stress-sensing mechanisms in yeast and mammalian cells*. Current Opinion in Cell Biology, 2011. **23**(2): p. 135-142.
333. Matus, S., L.H. Glimcher, and C. Hetz, *Protein folding stress in neurodegenerative diseases: a glimpse into the ER*. Current Opinion in Cell Biology, 2011. **23**(2): p. 239-252.
334. Novoa, I., H.Q. Zeng, H.P. Harding, and D. Ron, *Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2 alpha*. Journal of Cell Biology, 2001. **153**(5): p. 1011-1021.
335. Chakrabarti, A., A.W. Chen, and J.D. Varner, *A Review of the Mammalian Unfolded Protein Response*. Biotechnology and Bioengineering, 2011. **108**(12): p. 2777-2793.
336. Volmer, R., K. van der Ploeg, and D. Ron, *Membrane lipid saturation activates endoplasmic reticulum unfolded protein response transducers through their transmembrane domains*. Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(12): p. 4628-4633.
337. Yoshida, H., T. Matsui, A. Yamamoto, T. Okada, and K. Mori, *XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor*. Cell, 2001. **107**(7): p. 881-891.
338. Uemura, A., M. Taniguchi, Y. Matsuo, M. Oku, S. Wakabayashi, and H. Yoshida, *UBC9 Regulates the Stability of XBP1, a Key Transcription Factor Controlling the ER Stress Response*. Cell Structure and Function, 2013. **38**(1): p. 67-79.
339. Lee, A.H., N.N. Iwakoshi, K.C. Anderson, and L.H. Glimcher, *Proteasome inhibitors disrupt the unfolded protein response in myeloma cells*. Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(17): p. 9946-9951.
340. Lemmin, T., M. Dimitrov, P.C. Fraering, and M. Dal Peraro, *Perturbations of the Straight Transmembrane alpha-Helical Structure of the Amyloid Precursor Protein Affect Its Processing by gamma-Secretase*. J Biol Chem, 2014. **289**(10): p. 6763-74.
341. Baker, D., *A surprising simplicity to protein folding*. Nature, 2000. **405**(6782): p. 39-42.
342. Navon, A., A. Gatushkin, L. Zelcbuch, S. Shteingart, M. Farago, R. Hadar, and B. Tirosh, *Direct proteasome binding and subsequent degradation of unspliced XBP-1 prevent its intracellular aggregation*. Febs Letters, 2010. **584**(1): p. 67-73.
343. Inagi, R., *Endoplasmic reticulum stress as a progression factor for kidney injury*. Current Opinion in Pharmacology, 2010. **10**(2): p. 156-165.
344. Axten, J.M., J.R. Medina, Y.H. Feng, A. Shu, S.P. Romeril, S.W. Grant, W.H.H. Li, D.A. Heerding, E. Minthorn, T. Mencken, C. Atkins, Q. Liu, S. Rabindran, R. Kumar, X. Hong, A. Goetz, T. Stanley, J.D. Taylor, S.D. Sigethy, G.H. Tomberlin, A.M. Hassell, K.M. Kahler, L.M. Shewchuk, and R.T. Gampe, *Discovery of 7-Methyl-5-(1-([3-(trifluoromethyl)phenyl]acetyl)-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a Potent and Selective First-in-Class Inhibitor of Protein Kinase R (PKR)-like Endoplasmic Reticulum Kinase (PERK)*. Journal of Medicinal Chemistry, 2012. **55**(16): p. 7193-7207.
345. Boyce, M., K.F. Bryant, C. Jousse, K. Long, H.P. Harding, D. Scheuner, R.J. Kaufman, D.W. Ma, D.M. Coen, D. Ron, and J.Y. Yuan, *A selective inhibitor-of eIF2 alpha dephosphorylation protects cells from ER stress*. Science, 2005. **307**(5711): p. 935-939.
346. Shao, L., X. Sun, L. Xu, L.T. Young, and J.F. Wang, *Mood stabilizing drug lithium increases expression of endoplasmic reticulum stress proteins in primary cultured rat cerebral cortical cells*. Life Sci, 2006. **78**(12): p. 1317-23.

References

347. Lee, D.Y., K.S. Lee, H.J. Lee, D.H. Kim, Y.H. Noh, K. Yu, H.Y. Jung, S.H. Lee, J.Y. Lee, Y.C. Youn, Y. Jeong, D.K. Kim, W.B. Lee, and S.S. Kim, *Activation of PERK Signaling Attenuates A beta-Mediated ER Stress*. Plos One, 2010. **5**(5).
348. Gao, B., X.Y. Zhang, R. Han, T.T. Zhang, C. Chen, Z.H. Qin, and R. Sheng, *The endoplasmic reticulum stress inhibitor salubrinal inhibits the activation of autophagy and neuroprotection induced by brain ischemic preconditioning*. Acta Pharmacologica Sinica, 2013. **34**(5): p. 657-666.
349. Hiroi, T., H. Wei, C. Hough, P. Leeds, and D.M. Chuang, *Protracted lithium treatment protects against the ER stress elicited by thapsigargin in rat PC12 cells: roles of intracellular calcium, GRP78 and Bcl-2*. Pharmacogenomics Journal, 2005. **5**(2): p. 102-111.
350. Kakiuchi, C., S. Ishigaki, C.M. Osowski, S.G. Fonseca, T. Kato, and F. Urano, *Valproate, a Mood Stabilizer, Induces WFS1 Expression and Modulates Its Interaction with ER Stress Protein GRP94*. Plos One, 2009. **4**(1).
351. Wang, X.L., B. Su, S.L. Siedlak, P.I. Moreira, H. Fujioka, Y. Wang, G. Casadesus, and X.W. Zhu, *Amyloid-beta overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins*. Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(49): p. 19318-19323.
352. Li, J.M., J.J. Wang, and S.X. Zhang, *Preconditioning with Endoplasmic Reticulum Stress Mitigates Retinal Endothelial Inflammation via Activation of X-box Binding Protein 1*. Journal of Biological Chemistry, 2011. **286**(6): p. 4912-4921.
353. Theuns, J. and C. Van Broeckhoven, *Transcriptional regulation of Alzheimer's disease genes: implications for susceptibility*. Human Molecular Genetics, 2000. **9**(16): p. 2383-2394.
354. Glover-Cutter, K.M., S. Lin, and K. Blackwell, *Integration of the Unfolded Protein and Oxidative Stress Responses through SKN-1/Nrf*. Plos Genetics, 2013. **9**(9).
355. Esposito, L., L. Gan, G.Q. Yu, C. Essrich, and L. Mucke, *Intracellularly generated amyloid-beta peptide counteracts the antiapoptotic function of its precursor protein and primes proapoptotic pathways for activation by other insults in neuroblastoma cells*. Journal of Neurochemistry, 2004. **91**(6): p. 1260-1274.
356. Cardoso, S.M., E. Ferreira, C.R. Oliveira, and C. Pereira, *The crosstalk between the endoplasmic reticulum and mitochondria in Alzheimer's disease*. Febs Journal, 2005. **272**: p. 55-55.
357. Maiuolo, J., S. Bulotta, C. Verderio, R. Benfante, and N. Borge, *Selective activation of the transcription factor ATF6 mediates endoplasmic reticulum proliferation triggered by a membrane protein*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(19): p. 7832-7837.
358. Wang, Y., J.S. Shen, N. Arenzana, W. Tirasophon, R.J. Kaufman, and R. Prywes, *Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response*. Journal of Biological Chemistry, 2000. **275**(35): p. 27013-27020.
359. Bhandary, B., A. Marahatta, H.R. Kim, and H.J. Chae, *An Involvement of Oxidative Stress in Endoplasmic Reticulum Stress and Its Associated Diseases*. International Journal of Molecular Sciences, 2013. **14**(1): p. 434-456.
360. Wang, C.Y., J.W. Xie, T. Wang, Y. Xu, J.H. Cai, X. Wang, B.L. Zhao, L. An, and Z.Y. Wang, *Hypoxia-Triggered m-Calpain Activation Evokes Endoplasmic Reticulum Stress and Neuropathogenesis in a Transgenic Mouse Model of Alzheimer's Disease*. Cns Neuroscience & Therapeutics, 2013. **19**(10): p. 820-833.
361. Yao, J., S.H. Chen, Z.S. Mao, E. Cadenas, and R.D. Brinton, *2-Deoxy-D-Glucose Treatment Induces Ketogenesis, Sustains Mitochondrial Function, and Reduces Pathology in Female Mouse Model of Alzheimer's Disease*. Plos One, 2011. **6**(7).
362. Badiola, N., C. Penas, A. Minano-Molina, B. Barneda-Zahonero, R. Fado, G. Sanchez-Opazo, J.X. Comella, J. Sabria, C. Zhu, K. Blomgren, C. Casas, and J. Rodriguez-Alvarez, *Induction of ER stress*

References

- in response to oxygen-glucose deprivation of cortical cultures involves the activation of the PERK and IRE-1 pathways and of caspase-12.* Cell Death & Disease, 2011. **2**.
363. Kojima, E., A. Takeuchi, M. Haneda, F. Yagi, T. Hasegawa, K. Yamaki, K. Takeda, S. Akira, K. Shimokata, and K. Isobe, *The function of GADD34 is a recovery from a shutoff of protein synthesis induced by ER stress - elucidation by GADD34-deficient mice.* Faseb Journal, 2003. **17**(9): p. 1573-+.
 364. Jang, S.F., W.H. Liu, W.S. Song, K.L. Chiang, H.I. Ma, C.L. Kao, and M.T. Chen, *Nanomedicine-based neuroprotective strategies in patient specific-iPSC and personalized medicine.* Int J Mol Sci, 2014. **15**(3): p. 3904-25.
 365. Kondo, T., M. Asai, K. Tsukita, Y. Kutoku, Y. Ohsawa, Y. Sunada, K. Imamura, N. Egawa, N. Yahata, K. Okita, K. Takahashi, I. Asaka, T. Aoi, A. Watanabe, K. Watanabe, C. Kadoya, R. Nakano, D. Watanabe, K. Maruyama, O. Hori, S. Hibino, T. Choshi, T. Nakahata, H. Hioki, T. Kaneko, M. Naitoh, K. Yoshikawa, S. Yamawaki, S. Suzuki, R. Hata, S. Ueno, T. Seki, K. Kobayashi, T. Toda, K. Murakami, K. Irie, W.L. Klein, H. Mori, T. Asada, R. Takahashi, N. Iwata, S. Yamanaka, and H. Inoue, *Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness.* Cell Stem Cell, 2013. **12**(4): p. 487-96.

Appendix I: Plasmid and primers for site-directed mutagenesis

Sequences

1. APP₆₉₅ A33292.1

ATGCTGCCCCGGTTTGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGGAGGTACCCACT
GATGGTAATGCTGGCCTGCTGGCTGAACCCAGATTGCCATGTTCTGTGGCAGACTGAACATGCAC
ATGAATGTCCAGAATGGGAAGTGGGATTGAGATCCATCAGGGACCAAAACCTGCATTGATACCAAG
GAAGGCATCCTGCAGTATTGCCAAGAAGTCTACCCTGAACTGCAGATCACCAATGTGGTAGAAGCC
AACCAACCAGTGACCATCCAGAACTGGTGCAAGCGGGGCCGCAAGCAGTGCAAGACCCATCCCCAC
TTTGTGATTCCCTACCGCTGCTTAGTTGGTGAGTTTGTAAAGTGATGCCCTTCTCGTTCTTGACAAGTG
CAAATTCTTACACCAGGAGAGGATGGATGTTTGCGAACTCATCTTCACTGGCACACCGTCGCCAAA
GAGACATGCAGTGAGAAGAGTACCAACTTGCATGACTACGGCATGTTGCTGCCCTGCGGAATTGAC
AAGTTCCGAGGGGTAGAGTTTGTGTGTTGCCCACTGGCTGAAGAAAGTGACAATGTGGATTCTGCT
GATGCGGAGGAGGATGACTCGGATGTCTGGTGGGGCGGAGCAGACACAGACTATGCAGATGGGA
GTGAAGACAAAGTAGTAGAAGTAGCAGAGGAGGAAGAAGTGGCTGAGGTGGAAGAAGAAGAAG
CCGATGATGACGAGGACGATGAGGATGGTGTGAGGTAGAGGAAGAGGCTGAGGAACCCTACGA
AGAAGCCACAGAGAGAACCACCAGCATTGCCACCACCACCACCACCACAGAGTCTGTGGAAGA
GGTGGTTCGAGTTCCTACAACAGCAGCCAGTACCCCTGATGCCGTTGACAAGTATCTCGAGACACCT
GGGGATGAGAATGAACATGCCCATTTCCAGAAAGCCAAAGAGAGGCTTGAGGCCAAGCACCGAGA
GAGAATGTCCCAGGTCATGAGAGAATGGGAAGAGGCAGAACGTCAAGCAAAGAAGTGCCTAAAG
CTGATAAGAAGGCAGTTATCCAGCATTTCCAGGAGAAAGTGAATCTTTGGAACAGGAAGCAGCCA
ACGAGAGACAGCAGCTGGTGGAGACACACATGGCCAGAGTGGAAGCCATGCTCAATGACCGCCGC
CGCCTGGCCCTGGAGAACTACATACCGCTCTGCAGGCTGTTCTCCTCGGCCTCGTCACGTGTTCA
ATATGCTAAAGAAGTATGTCCGCGCAGAACAGAAGGACAGACAGCACACCCTAAAGCATTTCGAGC
ATGTGCGCATGGTGGATCCCAAGAAAGCCGCTCAGATCCGGTCCCAGGTTATGACACACCTCCGTG
TGATTTATGAGCGCATGAATCAGTCTCTCTCCCTGCTCTACAACGTGCCTGCAGTGGCCGAGGAGAT
TCAGGATGAAGTTGATGAGCTGCTTCAGAAAGAGCAAACTATTGAGATGACGTCTTGCCCAACAT
GATTAGTGAACCAAGGATCAGTTACGGAACGATGCTCTCATGCCATCTTTGACCGAAACGAAAACC

ACCGTGGAGCTCCTTCCCGTGAATGGAGAGTTCAGCCTGGACGATCTCCAGCCGTGGCATTCTTTTG
GGGCTGACTCTGTGCCAGCCAACACAGAAAACGAAGTTGAGCCTGTTGATGCCCCGCCCTGCTGCCG
ACCGAGGACTGACCACTCGACCAGGTTCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAG
TGAAGATGGATGCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTCTT
TGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGC
GACAGTGATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTG
GTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTA
CGAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAG

2. Indiana APP₆₉₅ mutation:

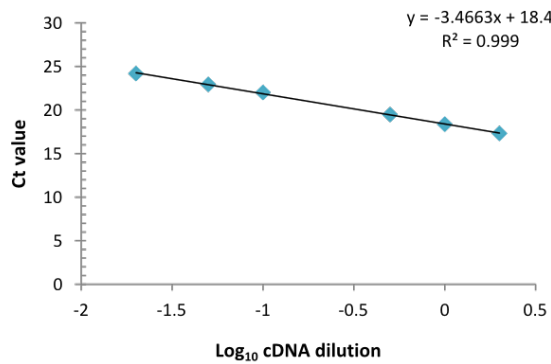
- a) V717F s GCGACAGTGATCTTCATCACCTTGG
- b) V717F as CCAAGGTGATATAGATCACTGTCGC

3. Swedish APP₆₉₅ mutation:

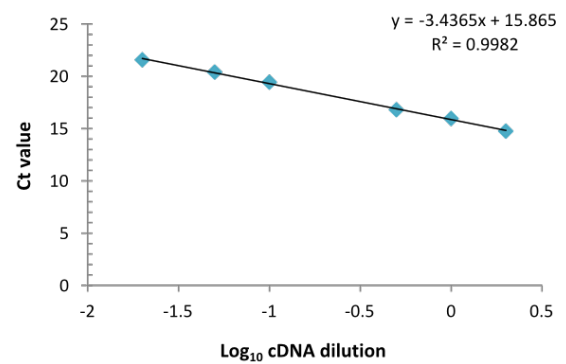
- a) K670N,M671L s GATCTCTGAAGTGAACCTGGATGCAGAATTC
- b) K670N,M671Las GAATTCTGCATCCAGGTTCACTTCAGAGATIC

Appendix II: Primer amplification efficiencies

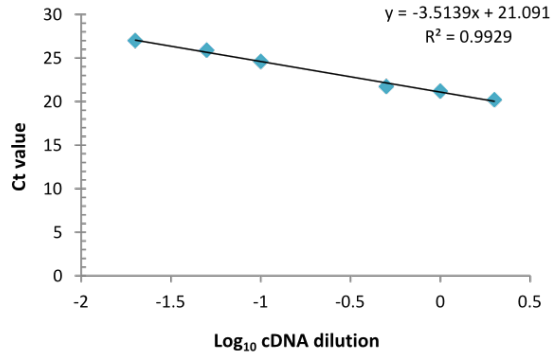
a) 18s rRNA



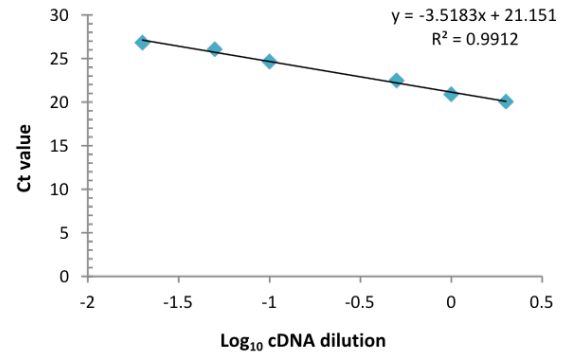
b) β -actin



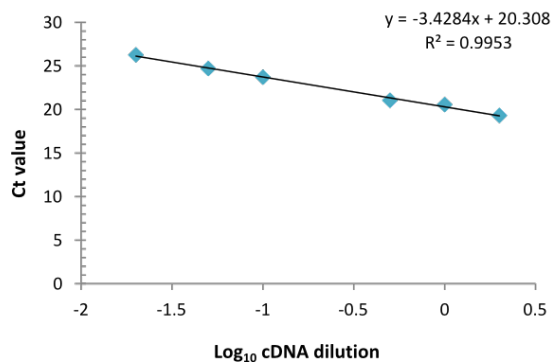
c) APP



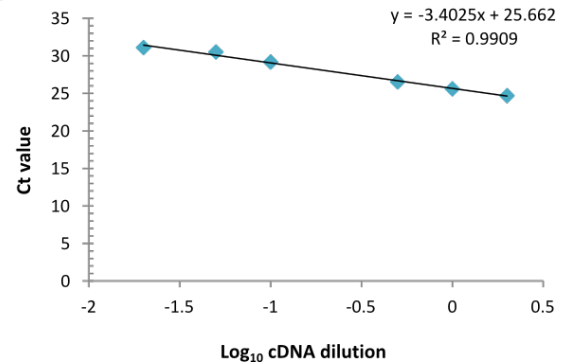
d) ATF4



e) BiP

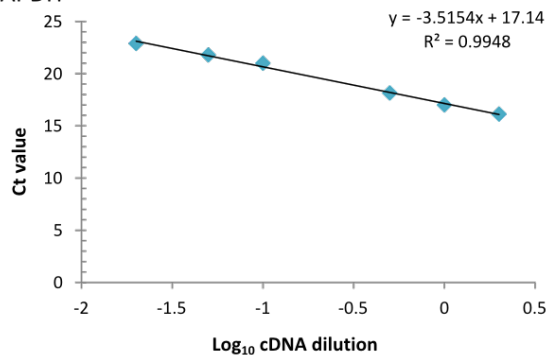


f) CHOP

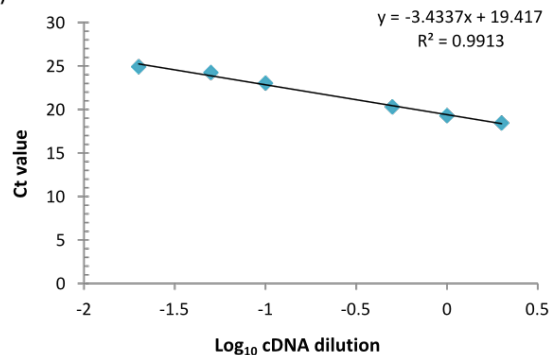


Appendix II: Primer amplification efficiencies

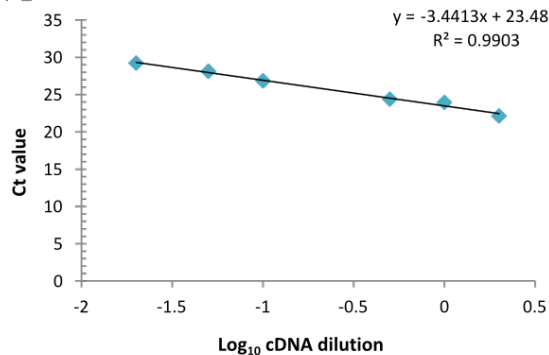
g) GAPDH



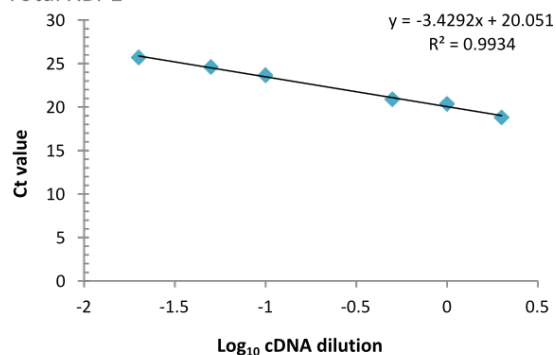
h) GRP94



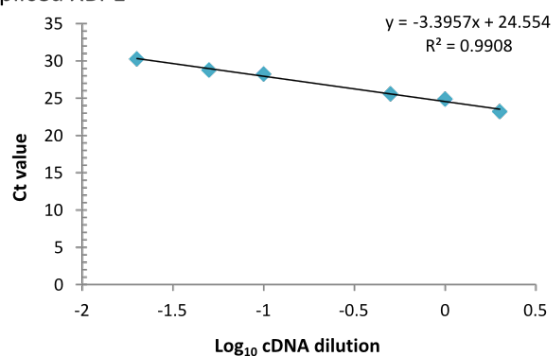
i) Nrf-2



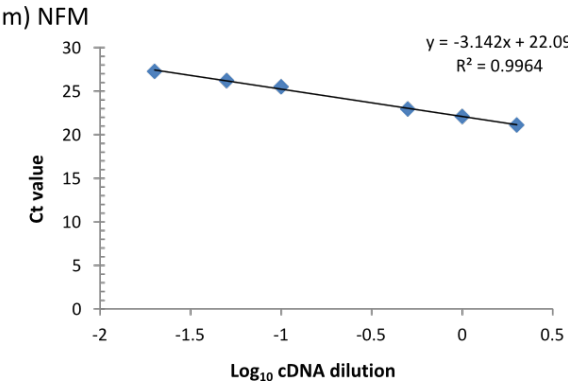
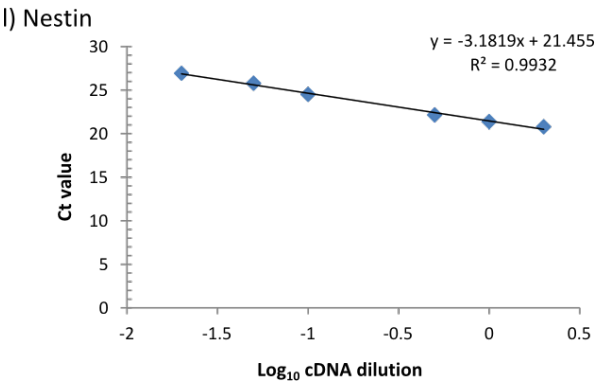
j) Total XBP1



k) Spliced XBP1



Appendix II: Primer amplification efficiencies



Appendix III: Mammalian UPR Code in gPROMS®

1. Computational model: Mammalian UPR for cells overexpressing Swedish APP695

PARAMETER

#Folding module

Cb as REAL

Cub AS REAL

Gub AS REAL

#Cd0 AS REAL

#Cpdi AS REAL

Gb AS REAL

#IRE1 activation, XBP1 splicing, splicing reporter module

Ci1b AS REAL

Gi1b AS REAL

Grsm as real

Brs as real

Grs as real

#ATF6 pathway

GAFB AS REAL

#BiP transcription module

Bb as real

#PERK activation/pathway

n AS real
 IO as real
 Cpkb AS REAL
 Gpkb AS REAL
 Cup AS REAL
 Be2a as real
 Ge2a as real
 GpE2a as real
 BpE2a as real

#ATf4 pathway

Bcp as real
 Gcp as real
 Ba4 as real
 Ga4 as real
 a0 as real
 a1 as real
 tau as real

#fit?

DISTRIBUTION_DOMAIN

DomainName AS [LowerBound : UpperBound]

DomainName AS ARRAY (Size < , ... >) OF [LowerBound : UpperBound]

UNIT

UnitName AS UnitModelName

UnitName AS ARRAY (Size || OrderedSet < , ... >) OF UnitModelName

PORT

```
# PortName AS ConnectionType
# PortName AS ARRAY ( Size || OrderedSet < , ... > ) OF ConnectionType
```

VARIABLE

```
t as concentration #time
Bm as mRNA #BiP mRNA
B as concentration #BiP
I1AB as concentration #Ire1 linked to BiP
I1A as concentration #Ire1 active
Xu1m as mRNA
pXu1 as concentration
pXs1 as concentration
pXc1 as concentration
Xs1m as mRNA

AFB as concentration #ATF6 linked to BiP
AFa as concentration #activated ATF6
#ATF6 as concentration #ATF6 active

#Ge2a as mRNA # for salubrinal
Xtotal as mRNA #total XBP1
U as concentration # unfolded protein
UB as concentration # folding complex
Rsm as mRNA #Reporter mRNA
Rs as concentration #reporter GFP
Pka as concentration #activated PERK
Pkb as concentration #PERK-BiP
E2a as concentration #eIF2a
pE2a as concentration #phospho eIF2a
Cp as concentration # CHOP
A4 as concentration #ATF$
Cpm as mRNA # CHOP mRNA
A4m as mRNA # ATF4 mRNA
Su as concentration # variable ER stress
#fitting
```

Na4m as fit

Ba4m as fit

Ga4m as fit

Bbm as fit

Nbm as fit

Gbm as fit

Bcpm as fit

Gcpm as fit

Ncpm as fit

GXu1m as fit

GXs1m as fit

GpXu1 as fit

GpXs1 as fit

GpXc1 as fit

BXs1m as fit

BXu1m as fit

BpXs1 as fit

BpXu1 as fit

BpXc1 as fit

ku as fit

Gfold as fit

NAFa as fit

rates

Rtransl, Rdecay, Rbind, Rdissoc AS rate

SELECTOR

SelectorName AS (Flag < , ... >) < DEFAULT Flag >

SelectorName AS ARRAY (Size || OrderedSet < , ... >) OF (Flag < , ... >) < DEFAULT Flag >

SelectorName AS DISTRIBUTION (DomainName < , ... >) OF (Flag < , ... >) < DEFAULT Flag >

```
# SET
# ParameterPath := Expression ;
# ParameterPath := [ Expression < , ... > ];
# ParameterPath := "<ForeignObjectClass::>ForeignObjectValue";
# ParameterPath := [ "Name" < , ... > ];
# DomainPath := [ BFDm || CFDM || FFDM || OCFEM , Order , NumOfElements ];
```

```
# BOUNDARY
# Boundary condition equations
```

```
# TOPOLOGY
# Unit connection equations
Selector
UpRegul as (NoRegul, Regul) DEFAULT NoRegul
Transcription as (NoTransc, Transc) default NoTransc
```

```
EQUATION
#time reporter
$t=1;
```

```
#PERK activation/pathway
```

```
$PkB=Cb*B*Pka-Gpkb*PkB;
$Pka=Gpkb*PkB-Cb*B*Pka;
```

```
$E2a=-Be2a*min(Pka,E2a)+Ge2a*pE2a ;
$pE2a=+Be2a*min(Pka,E2a)-Ge2a*pE2a;
```

```
#Salubrinal case study
```

```
#Case UpRegul OF
#When NoRegul :
```

```
#E2a=-Be2a*min(Pka,E2a)+Ge2a*pE2a ;
#$pE2a=+Be2a*min(Pka,E2a)-Ge2a*pE2a;
#Switch to Regul if t=15000;
#when Regul:
#$E2a=-Be2a*min(Pka,E2a);
#$pE2a=+Be2a*min(Pka,E2a)-Ge2a*pE2a;
#End
```

#ATF4 pathway

Case transcription of

When NoTransc :

\$A4m=Ba4m-Ga4m*A4m;

\$Cpm=Bcpm-Gcpm*Cpm;

Switch to Transc if t>tau;

When Transc :

(a0+a1*pE2a+(pE2a)^2)*\$A4m=Ba4m*(a0+a1*pE2a+(pE2a)^2+Na4m*(pE2a)^2)-

(a0+a1*pE2a+(pE2a)^2)*Ga4m*A4m;

\$Cpm=Bcpm+Ncpm/(a0+a1*A4+(A4)^2)*(A4)^2-Gcpm*Cpm;

End

\$A4=Ba4*A4m-GA4*A4;

\$Cp=Bcp*Cpm-Gcp*cp;

#protein folding dynamics

\$Su=ku/(3*3600);

#\$U=E2a/200*Su-Cb*U*B+Gub*UB-Cd0*D0*U+Cpdi*PDI*Ud+Gb*UB;

\$U=E2a/200*Su-Cb*U*B+Gub*UB+Gb*UB;

#\$UB=Cb*U*B-Gub*UB-Cd0*D0*UB+Cpdi*PDI*Ud+Gb*UB-Gfold*UB;

\$UB=Cb*U*B-Gub*UB+Gb*UB-Gfold*UB;

#Ire1, XBP1 mRNA and splicing reporter

\$I1AB=Cb*B*I1A-Gi1b*I1AB;

\$I1A=-Cb*B*I1A+Gi1b*I1AB;

\$Xs1m=-(GXu1m-(GXu1m-GXs1m)*pE2a/200)*Xs1m+BXs1m*min(I1A,Xu1m);

\$Rsm=-Grsm*Rsm+BXs1m*min(I1A,Xu1m);

\$Rs=-Grs*Rs+Brs*Rsm;

#new equations:

\$pXu1=BXu1m*Xu1m-BpXc1*(pXu1*pXs1)-GpXu1*pXu1;

\$pXs1=BXs1m*Xs1m-BpXc1*(pXu1*pXs1)-GpXs1*pXs1;

\$pXc1=BpXc1*(pXu1*pXs1)-GpXc1*pXc1;

#\$GpXs1=GpXs1*pXs1;

#\$GpXu1=GpXu1*pXu1;

#\$GpXc1=GpXc1*pXc1;

#BiP dynamics

Case UpRegul OF

When NoRegul :

\$Bm=Bbm-Gbm*Bm;

Switch to Regul if Xs1m>30;

when Regul:

(a0+a1*Xs1m+(Xs1m)^2)*\$Bm=Bbm*(a0+a1*Xs1m+(Xs1m)^2)+Nbm*(Xs1m)^2-

(a0+a1*Xs1m+(Xs1m)^2)*Gbm*Bm;

end

\$B=Bb*Bm-Gb*B-Cb*U*B+Gub*UB+Gfold*UB;

Rtransl = Bb*Bm ;

Rdecay = Gb*B ;

Rbind = Cb*U*B ;

Rdissoc = Gub*UB + Gfold*UB;

\$(a0+a1*Xs1m+(Xs1m)^2)*\$PDI=Bpdim*((a0+a1*Xs1m+(Xs1m)^2)+Npdim*(Xs1m)^2)-

(a0+a1*Xs1m+(Xs1m)^2)*Gpdim*PDI;

#\$PDI=Bpdi*PDI-G*PDI;

#ATF6 activation/pathway

\$AFB=Cb*B*AFa-GAFB*AFB;

\$AFa=-Cb*B*AFa+GAFB*AFB;

\$Xtotal=\$Xu1m + \$Xs1m;

```
$Xu1m* (a0+a1*AFa+(AFa)^2)=BXu1m* (a0+a1*AFa+(AFa)^2)+NAFa*(AFa^2); # -
GXu1m*Xu1m;
```

```
# $Xu1m* (a0+a1*AFa+(AFa)^2)= BXu1m*Xs1m/200-GXu1m*Xu1m-
BXs1m*min(I1A,Xu1m)+NAFa*(AFa^2);
```

```
# ASSIGN
```

```
# VariablePath := Expression ;
```

```
# VariablePath := [ Expression < , ... > ];
```

```
# PRESET
```

```
# < RESTORE " >
```

```
# VariablePath := InitialValue ;
```

```
# VariablePath := InitialValue : LowerBound : UpperBound ;
```

```
# INITIALSELECTOR
```

```
# SelectorPath := FlagPath ;
```

```
# SelectorPath := [ FlagPath < , ... > ];
```

```
# INITIAL
```

```
# Equations
```

2. Process: Mammalian UPR for cells overexpressing Swedish APP695

```
# ParameterName
```

```
AS INTEGER || REAL || LOGICAL < DEFAULT Value >
```

```
# ParameterName AS ARRAY ( Size < , ... > ) OF INTEGER || REAL || LOGICAL < DEFAULT Value >
```

```
# ParameterName AS FOREIGN_OBJECT < "ForeignObjectClass" > < DEFAULT "ForeignObjectValue" >
```

```
UNIT
```

```
ER as S_UPR_2
```

```
# UnitName AS ModelName
```

```
# UnitName AS ARRAY ( Size < , ... > ) OF ModelName
```

```
#MONITOR
```

```
# SYNTAX: VariablePathPattern ;
```

```
SET
```

```
within ER do
```

```
#Folding module
```

```
Cub :=.0350; #mol-2.s-1
```

```
Cb :=1/0.350; #mol2s1
```

```
Gub:= 196; #mol-1.s-1
```

```
Gb :=1.39e-4; #mol-2.s-1
```

```
#IRE1 activation, XBP1 splicing, splicing reporter module
```

```
Ci1b :=0.350; #mol-2.s-1
```

```
Gi1b :=196; #mol-1.s-1
```

Grsm :=8.33e-4; #mol-1s-1

Brs := 8.33e-3; #mol-1s-1

Grs :=3.47e-5;

#BiP trascription module

Bb :=0.25; #s-1

Gb :=1.39e-4; #s-1

#PERK activation/pathway

n :=4.5;

l0 :=45;

Cpkb :=0.350; #mol-2.s-1

Gpkb :=196; #mol-1.s-1

Cup :=2.33e-4; #mol-2.s-1

Be2a :=0.167; #s-1

GpE2a :=8.33e-4; #s-1

Ge2a :=8.33e-4; #s-1

Bpe2a :=1.5e-3; #s-1

#ATF6 pathway

GAFB:= 196; #mol-1.s-1

#ATf4 pathway

Bcp :=0.25; #s-1

Gcp :=7.7e-3; #s-1

Ba4 :=0.25; #s-1

Ga4 :=7.7e-3; #s-1

a0 :=181935 ;

a1 := 23.7363 ;

tau:=12000;

end

```
# ParameterPath := Expression ;
# ParameterPath := [ Expression < , ... > ];
# ParameterPath := "<ForeignObjectClass::>ForeignObjectValue";
# DomainPath := [ BFDM || CFDM || FFDM || OCFEM , Order , NumOfElements ];
```

```
# ASSIGN
```

```
# VariablePath := Expression ;
# VariablePath := [ Expression < , ... > ];
```

```
# PRESET
```

```
# < RESTORE " >
# VariablePath := InitialValue ;
# VariablePath := InitialValue : LowerBound : UpperBound ;
```

```
# INITIALSELECTOR
```

```
# SelectorPath := FlagPath ;
# SelectorPath := [ FlagPath < , ... > ];
```

```
ASSIGN
```

```
within ER do
```

```
Na4m :=18.0;
Ba4m :=0.24; #mol.s-1
Ga4m :=4.67112E-7; #s-1
Nbm :=1.8473; #
```

```
Bbm :=0.05;
Gbm :=1.03873E-4;
Ncpm :=378.305;
Gcpm :=7.73116E-6; #s-1
Bcpm :=41.435 #mol.s-1
Gfold :=2.5704E-4; #mol-1.s-1
ku:=76.123E3;
GXs1m :=3.69151E-8;
```

```

GXu1m :=6.37986E-4; #s-1
BXs1m :=1.4E-4; #0.0024; #0.000242457;
BXu1m :=0.00919444; #s-1
BpXs1 :=0.0032;
BpXu1 :=0.002;
GpXu1 := 0.03; #s-1
GpXc1 :=0.03;
Gpxs1 :=0.035;

BpXc1 :=1.33E-07;

NAFa := 1.09 ;

#Ge2a :=8.33e-4; #salubrinal

end

INITIAL

Within ER do
t=0;
B=430000;
#PDI=0;
l1AB=256; #256
l1A=0;
U=0;
UB=0;
Rs=0;
Rsm=0;
Pka=0;
PkB=256;
E2a=200;
pE2a=0;
Cp=0;
A4=0;
Su=0;
Xu1m=151.88618-27.547108;
pXu1=0.05; #

```

```
pXs1=0.07; #
pXc1=0.05;
```

```
AFB=256;
AFa=0;
```

```
#Fitted
Bm= 200;
Xs1m=27.547108;
Xtotal=151.88618;
A4m=3160.3965;
Cpm=109562.414;
end
```

```
# Equations
# STEADY_STATE
```

```
SOLUTIONPARAMETERS
REPORTINGINTERVAL := 300; #300 ;
OUTPUTLEVEL := 4 ;
gRMS := ON ;
BLOCKDECOMPOSITION := ON ;
```

```
SCHEDULE
# salubrinal case study
#Sequence
#Continue for 14400
#Reassign
#ER.Ge2a:=0;
#End
Continue for 40000
#End
```