Investigating the early role of oxidative stress in Alzheimer's disease: Insights from a transgenic model of the amyloid pathology and fluorescence imaging methods

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Dedicated to my loving family:

Christie, Robyn, Taylor, Avery, and Max

Abstract

With an aging population, the negative impact of age-related diseases such as Alzheimer's disease (AD) will only grow. Alzheimer's disease (AD) is the leading cause of dementia in the elderly and there are no cures nor preventative treatments. AD has an extended pre-symptomatic stage spanning decades which offers a promising therapeutic window. However, it is presently impossible to unquestionably diagnose AD during this early stage in the general population. Consequently, basic science research on pathological mechanisms that initiate and exacerbate disease progression during the earliest, pre-plaque stage would be insightful for biomarker development and disease-modifying therapies.

Studies from our laboratory using a transgenic rat model of the AD-like amyloid pathology and *post-mortem* human brain material, demonstrated that neurons burdened with Aβ exhibited increased gene and protein expression of inflammatory markers. This early neuroinflammation, which vastly differs from the classical inflammatory process during late, post-plaque stages, motivated our investigation of oxidative stress, which can be a cause and consequence of inflammation. Oxidative stress is elevated during post-plaque stages of AD, but its earliest role in AD remains uncharacterized. As such, Chapter 2 investigates neuron-specific gene and protein expression of oxidative stress-related targets in our rat model during a pre-plaque stage when neuroinflammation is incipient. We show that intraneuronal Aβ- (iAβ) burdened neurons exhibited evidence of DNA damage and had upregulated DNA repair and antioxidant genes and proteins, while oxidative damage trended to increase, suggesting this timepoint preceded a fully realized redox imbalance. Our findings reveal that inflamed iAβ-burdened neurons increase expression of oxidative stress-related genes, likely in response to elevated reactive oxygen species (ROS). Importantly, ROS production is upstream of oxidative stress responses including modulation of gene expression. Therefore, our next goal was to develop methodologies for reliably studying ROS.

Quantifying ROS is technically challenging since they are short-lived and include diverse chemical species. Therefore, detection methods must be specific to the ROS of interest. With this in mind, we utilized the fluorogenic probe, H_4BPMHC (developed by the McGill Cosa laboratory), that quantifies lipid peroxyl radicals, a form of lipid-associated ROS which neurons are vulnerable to. After optimizing culturing and imaging conditions in primary neurons, we validated *in vitro* sensitivity of this method by subjecting neurons to varying antioxidant loads over time then imaging them under stressed and non-stressed conditions. In sum, H_4BPMHC was sensitive enough to detect differences between our experimental

conditions. **Chapter 3** presents the proof-of-concept for using H_4BPMHC to study lipid peroxyl radicals in neurodegenerative disease models.

Finally, building on our expertise from live cell imaging, **Chapter 4** outlines the development of a methodology for studying ROS in *ex vivo* hippocampal slices using two-photon microscopy. Existing ROS detection methods have limited spatial and temporal resolution that real-time *in situ* imaging would overcome. Towards this goal, this chapter provides key considerations, limitations, and potential pitfalls when quantifying ROS in complicated but biologically relevant systems.

Overall, we show that a neuronal oxidative stress response occurs during the early, pre-plaque amyloid pathology and demonstrate the rigor necessary for developing methods of ROS quantification in disease-relevant models. This interdisciplinary work will provide a solid foundation and path forward for future studies investigating the earliest AD pathology as well as the role of oxidative stress in health and disease.

Résumé

Avec une population vieillissante, l'impact des maladies liées à l'âge comme la maladie d'Alzheimer (MA) ne fera que croître. La MA est la principale cause de démence chez les personnes âgées et il n'existe aucun remède ni traitement préventif. Cette maladie a un stade pré-symptomatique s'étendant sur des décennies, offrant une fenêtre thérapeutique prometteuse. Cependant, il est encore impossible de diagnostiquer la MA à ce stade dans la population générale. La recherche fondamentale sur les mécanismes pathologiques initiant et exacerbant la progression de la MA au cours des stades précoces serait donc bénéfique pour la découverte de biomarqueurs et le développement de thérapies.

Des études de notre laboratoire utilisant un modèle de rat transgénique comportant la pathologie amyloïde de la MA, ainsi que du matériel cérébral humain *post-mortem*, ont démontré que les cellules chargées d'Aβ intraneuronal (iAβ) présentaient une expression accrue de gènes et protéines inflammatoires. Cette neuroinflammation précoce, qui diffère de l'inflammation au cours des stades tardifs, a motivé notre étude sur le stress oxydatif, qui est élevé au cours des stades post-plaque. Nous avons donc étudié l'expression de gènes et protéines liés au stress oxydatif dans les neurones de notre modèle de rat au cours d'un stade pré-plaque lorsque l'inflammation débute (Chapitre 2). Nous démontrons que les neurones chargés d'iAβ présentaient des signes de dommages à l'ADN et que les gènes et protéines impliqués dans la réparation de l'ADN et l'action d'antioxydants étaient régulés à la hausse, tandis que les dommages oxydatifs avaient tendance à augmenter, suggérant que ce moment a précédé un déséquilibre redox complet. Nos résultats révèlent que les neurones inflammés et chargés d'iAβ ont une expression accrue de gènes liés au stress oxydatif, probablement en réponse à des espèces réactives de l'oxygène (ROS). Surtout, la production de ROS est en amont des réponses au stress oxydatif, y compris la modulation de l'expression des gènes. Ainsi, notre prochain objectif était de développer des méthodes pour étudier les ROS.

Quantifier les ROS est techniquement difficile dû à leur courte durée et les nombreux types existants. Ainsi, les méthodes de détection doivent être spécifiques aux ROS d'intérêt. Nous avons utilisé la sonde fluorogène, H_4BPMHC (développée par le laboratoire Cosa à McGill), qui quantifie les radicaux peroxyles lipidiques, une forme de ROS associée aux lipides auxquels les neurones sont vulnérables. Après avoir optimisé les conditions de culture et d'imagerie, nous avons validé la sensibilité *in vitro* de cette méthode en soumettant les neurones à des charges antioxydantes variables au fil du temps, puis en les imageant dans des conditions stressées et non stressées. Somme toute, la sonde H_4BPMHC était assez sensible

pour détecter des différences entre nos conditions. Le **Chapitre 3** présente la preuve de concept pour l'utilisation de H_4BPMHC pour étudier les radicaux peroxyles lipidiques dans des modèles de maladies neurodégénératives.

Enfin, le **Chapitre 4** décrit le développement d'une méthodologie pour étudier les ROS dans des coupes d'hippocampe *ex vivo* en utilisant la microscopie à deux photons. Les méthodes de détection de ROS existantes ont une résolution spatiale et temporelle limitée que l'imagerie *in situ* en temps réel permettrait de surmonter. Ce chapitre fournit des considérations, limitations et défis potentiels lors de la quantification des ROS dans des systèmes complexes et biologiquement pertinents.

Dans l'ensemble, nous montrons qu'une réponse au stress oxydatif neuronal se produit au cours de la pathologie amyloïde précoce pré-plaque. La rigueur est nécessaire pour développer des méthodes de quantification des ROS dans des modèles pertinents pour la maladie. Ce travail interdisciplinaire fournira une base solide pour de futures études portant sur les stades précoces de la MA et sur le rôle du stress oxydatif dans la maladie.

Translation by: Anne-Sophie Pépin

Author Contributions

"Science is a collaborative effort. The combined results of several people working together is often much more effective than could be that of an individual scientist working alone."

- John Bardeen

Morgan K Foret (MKF) was the lead investigator and first author of the manuscripts in this thesis, and of this dissertation. MKF designed experiments in collaboration with ACC, and GC, in addition to SDC, KJP, and RL for specific manuscripts. MKF troubleshooted and optimized protocols, collected data, and analyzed results. MKF wrote initial drafts of all manuscripts and generated figures.

Dr. A. Claudio Cuello (ACC) was the corresponding author for the manuscripts and contributed original ideas for the academic direction of this thesis, providing intellectual guidance. ACC revised and contributed to all manuscripts.

Dr. Gonzalo Cosa (GC) was the corresponding author for chapters 3 and 4. Although not officially, GC served as a co-supervisor, and contributed original ideas for experiments and projects, as well as intellectual guidance.

CHAPTER 2: Oxidative Stress-Related Genes are Dysregulated in Aβ-burdened Neurons of a Rat Model of Alzheimer's Disease.

Foret MK, Do Carmo S, Orciani C, Welikovitch L, Huang C, Cuello ACC. *In preparation for submission.*

SDC contributed to the project design and rationale. SDC performed the DCF assay and assisted in editing the manuscript.

CO assisted with perfusions and tissue collection and assisted in producing data for the glutathione reductase assay.

LW provided laser capture material.

CH assisted in breeding and genotyping rats for this study.

CHAPTER 3: Effect of Antioxidant Supplements on Lipid Peroxidation Levels in Primary Cortical Neuron Cultures

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SDC contributed intellectually to the project and to manuscript editing.

RL contributed to the project design, live cell imaging experiments, H_4BPMHC purification by HPLC, data analysis through designing the ImageJ macro, and illustrations.

LEG contributed by designing H_4BPMHC and helping optimize live cell imaging experiments with neuronal cultures.

WZ contributed by helping optimize live cell imaging experiments with neuronal cultures.

CHAPTER 4: Developing a Method for Real-Time Imaging of Lipid Peroxyl Radicals in *Ex Vivo* Hippocampal Slices

Foret MK,* Jodko-Piórecka K,* Audet N, Hooshmandi M, Do Carmo S, Khoutorsky A, Cosa G, Cuello AC. *In preparation for submission*.

*KJP contributed intellectually to the project and experimental design, performed all experiments with MKF, assisted in troubleshooting and optimizing slice preparation and imaging, assisted in preparing probes and contributed to the writing and editing of the manuscript.

NA assisted in optimizing imaging conditions with the two-photon microscope.

MH assisted in designing and setting up the system to generate hippocampal slices as well as the buffer composition.

SDC contributed intellectually to the project.

AK assisted by providing technical expertise for generating hippocampal slices.

* indicates equal contribution

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II. Wilson EN, Do Carmo S, Welikovitch LA, Hall H, Flores Aguilar L, **Foret MK**, Iulita MF, Jia DT, Marks AR, Allard S, Emmerson JT, Ducatenzeiler A, Cuello AC. (2020). NP03, a Microdose Lithium Formulation, Blunts Early Amyloid Post-Plaque Neuropathology in McGill-R-Thy1-APP Alzheimer-Like Transgenic Rats. *Journal of Alzheimer's Disease*. 73(2): 723-739. DOI.

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V. Malcolm JC, Emmerson JT, Welikovitch L, **Foret MK**, Cuello AC. Oxidative Stress and Alterations in Glutamate Transport Potentiate White Matter Changes in Tauopathy. *In preparation.*

VI. Orciani , Do Carmo S, **Foret MK**, Hall H, Bonomo Q, Lavagna A, Huang C, Cuello AC. ANAVEX 3-71, an M1/sigma-1 Receptor Agonist, Prevents Cognitive Deficits and Alzheimer's Disease-like Hallmarks in a Transgenic Rat Model. *In preparation.*

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"Live now; make now always the most precious time. Now will never come again."

- Jean-Luc Picard, "Inner Light".

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Table of Contents

List of Figures

Introduction

Chapter 2: Oxidative Stress-Related Genes are Dysregulated in Aβ-burdened Neurons of a Rat Model of Alzheimer's Disease

Chapter 3: Effect of Antioxidant Supplements on Lipid Peroxidation Levels in Primary Cortical Neuron Cultures

Chapter 4: Developing a Method for Real-Time Imaging of Lipid Peroxyl Radicals in *Ex Vivo* Hippocampal Slices

List of Tables

Chapter 2: Oxidative Stress-Related Genes are Dysregulated in Aβ-burdened Neurons of a Rat Model of Alzheimer's Disease

Chapter 3: Effect of Antioxidant Supplements on Lipid Peroxidation Levels in Primary Cortical Neuron Cultures

List of Abbreviations

4HNE	4-hydroxy-2-nonenal
αTTP	α-tocopherol binding transport protein
ABAD	Aβ-binding alcohol dehydrogenase
ABAP	2,2'-azobis(2-methyl-propionaminidine) dihydrochloride
aCSF	artificial cerebrospinal fluid
ADAM	a disintegrin and metalloprotease
AD	Alzheimer's disease
ADDL	Aβ-derived diffusible ligand
AICD	APP intracellular domain
AO	antioxidants
ApeI	apurinic/apyrimidinic endodeoxyribonuclease I
APP	amyloid precursor protein
AU	airy unit
BACEI	beta-site APP cleaving enzyme I
BBB	blood-brain-barrier
BER	base excision repair
BDE	bond dissociation energy
BODIPY	boron dipyrromethene
CAA	cerebral amyloid angiopathy
CAI	cornu ammonis I
CMARC	Canadian Council on Animal Care
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
CTF	carboxy-terminal fragment
CTCF	corrected total cell fluorescence
CypD	cyclophilin D
DAMP	damage-associated molecular patterns

DG	dentate gyrus
DIV	day *in vitro*
DMSO	dimethylsulfoxide
Drp1	dynamin-1-like protein
DS	Down Syndrome
DSB	double-strand break
EM	electron microscopy
Ercc2	excision repair cross-complementing 2
Ercc6	excision repair cross-complementing 6
EPR	electron paramagnetic resonance
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERO	ER oxidoreductin
ETC	electron transport chain
EthD-1	ethidium homodimer 1
fAD	familial Alzheimer's disease
Fancc	fanconi anaemia complementation group C
FBS	fetal bovine serum
FDA	Food and Drug Administration
FEN1	flap endonuclease 1
FRTA	free radical theory of aging
FTD	frontotemporal dementia
GCL	glutamate-cysteine ligase
Gclm	glutamate-cysteine ligase modifier subunit
GFAP	glial fibrillary acidic protein
GPx7	glutathione peroxidase 7
GR	glutathione reductase
GSH	glutathione
GST	glutathione-S-transferase
GWAS	genome-wide association studies

HOMO	highest occupied molecular orbital
HPLC	high pressure liquid chromatography
HR	homologous recombination
iAβ	intraneuronal amyloid beta
IDE	insulin-degrading enzyme
IdhI	isocitrate dehydrogenase I
Ift172	intraflagellar transport 172
IHC	immunohistochemistry
iNOS	inducible nitric oxide synthase
IRP	iron regulatory protein
IWG	International Working Group
LCM	laser capture microdissection
LDE	lipid-derived electrophile
LOAD	late onset Alzheimer's disease
LOX	lipoxygenase
LTP	long-term potentiation
LUMO	lowest unoccupied molecular orbital
mAb	monoclonal antibody
MAM	mitochondrial-associated membranes
MCI	mild cognitive impairment
MDA	malondialdehyde
MFRTA	mitochondrial free radical theory of aging
mPTP	mitochondrial permeability transition pore
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
NADPH	nicotinamide adenine dinucleotide phosphate
NCI	non-cognitively impaired
nDNA	nuclear DNA

NEP	neprilysin
NER	nucleotide excision repair
NFT	neurofibrillary tangles
NGS	normal goat serum
NHEJ	non-homologous end joining
NIA-AA	National Institute on Aging-Alzheimer's Association
NLRP3	NOD-like receptor protein 3
NMDA	N-methyl-D-aspartic acid
NOX	NADPH oxidase
NSAID	non-steroidal anti-inflammatory drugs
OCDL	oxidatively induced clustered DNA lesion
OGG1	8-oxoguanine glycosylase
ONE	4-oxo-2-nonenal
pAb	polyclonal antibody
PAMP	pathogen-associated molecular pattern
Parp1	poly(ADP-ribose) polymerase 1
PBS	phosphate-buffered saline
PCAD	pre-clinical Alzheimer's disease
Pcna	proliferating cell nuclear antigen
PDI	protein disulfide-isomerase
PET	positron emission tomography
PeT	photon-induced electron transfer
PHF	paired helical filaments
PM	plasma membrane
PND	post-natal day
Polβ	polymerase beta
PUFA	polyunsaturated fatty acid
PV	parvalbumin
RAGE	receptor for advanced glycation end products
RNS	reactive nitrogen species

ROI	region of interest
ROS	reactive oxygen species
RTA	radical trapping antioxidant
sAD	sporadic Alzheimer's disease
sAPPα	soluble APP alpha
SEM	standard error of the mean
SIRT3	sirtuin 3
sLOAD	sporadic late onset Alzheimer's disease
SOD	superoxide dismutase
Sqstm	sequestosome
SSB	single-strand break
SSBR	single-strand break repair
TBARS	thiobarbituric acid-reactive substances
TCA	tricarboxylic acid cycle
TGN	trans-Golgi network
Tg	transgenic
UPR	unfolded protein response
UPS	ubiquitin-proteasome system
Wt	wild type
XPD	xeroderma pigmentosum complementation group D

Chapter 1

I. Introduction

> "There are no small problems. Problems that appear small are large problems that are not understood"
>
> - Santiago Ramon y Cajal

Preamble

Dementia impacts approximately 50 million people worldwide, and this number is expected to increase to 150 million by 2050.[1] In Canada, there are currently 500,000 people living with dementia which is expected to double by the year 2030. Importantly, this estimation accounted for population growth, and therefore represents an increase in total population but also an increase in the aging population who are susceptible to dementia.[2] The cost of dementia not only impacts the individuals with the disease but also their families and caregivers. Providing care is psychologically, physically, and emotionally taxing, and becomes more difficult for the caregiver with time as the individual progresses to advanced stages of dementia.[3-7] Economically, the cost of dementia in Canada was around $10 billion in 2016 and climbing,[8] while in the USA it was $818 billion in 2015,[9-10] and globally in 2019 it was an estimated $1.3 trillion (USD).[11]

As Alzheimer's disease (AD) is the leading cause of dementia in the elderly, it is a primary research focus, especially now since currently available treatments offer limited therapeutic benefits—only delaying dementia for 6 to 12 months—and hundreds of clinical trials for AD have failed. The main criticism for failed clinical trials was that they were conducted too late in the disease progression when the brain was irreparably damaged. Since then, clinical trials have been designed to target earlier disease stages and this year (2021), after almost 20 years since the last approval, the FDA approved the therapeutic, aducanumab (Section 1.7.1), as a disease-modifying drug for AD. However, this approval has been met with a great deal of controversy, thus, the Phase IV data will be telling to determine whether aducanumab is as promising as some expected. The accelerated path to the approval of aducanumab has also

offered lessons regarding the use of AD biomarkers as part of inclusion criteria, and outcome measures.[12-13]

Despite the setbacks, progress has been made; research funding for AD has increased, with the US government providing $3.2 billion in 2021.[14] We have also learned that AD has an extended pre-symptomatic phase, spanning decades, which offers a promising therapeutic window to explore and take advantage of. Presently, it is not possible to undeniably determine if an individual will definitively progress to AD, but the race to determine reliable biomarkers and key pathological mechanisms for these more elusive early stages is advancing. Furthermore, as sporadic AD makes up a majority of cases compared to familial AD, research has identified genetic risk factors associated with sporadic forms. Indeed, the heterogeneity of sporadic AD adds another layer of complexity and emphasizes the importance of elucidating key early pathological mechanisms to better understand the spectrum of AD.

In sum, there is an urgent need for earlier diagnosis, and more effective treatments for Alzheimer's disease. This thesis focuses on a piece of the puzzle, namely, the amyloid pathology and how it relates to oxidative stress during early pathological stages, preceding amyloid plaque deposition. This work also provides methodological contributions towards studying oxidative stress in the context of disease, aiming to facilitate future studies in the understanding the earliest AD pathology.

"The history of the sciences is a great fugue, in which the voices of the nations come one by one into notice."

- Johann Wolfgang von Goethe

I.I Alzheimer's Disease Throughout History

Presently, Alzheimer's disease (AD) is the leading cause of dementia in the elderly. The brain pathology of this disease was first described just over a century ago. In 1907 Alois Alzheimer and Oskar Fischer reported seminal discoveries, that eventually culminated in defining Alzheimer's disease as we know it today. In addition to these contributions, other scientists at the time, namely Emil Kraepelin, Gaetano Perusini, Francesco Bonfiglio among others were also involved in these early studies.[15-17]

Alois Alzheimer (1864 - 1915) was born in southern Germany, later spending time in Frankfurt, Heidelberg, and Munich serving as a physician and neuropathologist. During his time in Frankfurt, he gained an appreciation for how clinical practice and laboratory research complemented each other, obtaining expertise in histopathological techniques from his colleague Franz Nissl[18] while serving as a medical resident then senior physician at the Hospital for the Mentally Ill and Epileptics. Alzheimer was invited to Heidelberg by Emil Kraepelin, one of the most prominent and influential psychiatrists in Germany, who held the chair of psychiatry there, it is noteworthy that Nissl had a role in making this offer for Alzheimer possible.[19] Shortly after Alzheimer's move to Heidelberg, he followed Kraepelin to Munich to become head of the neuroanatomy laboratory where some of his colleagues included the equally illustrious researchers, Alfons Jakob and Hans Gerhard Creutzfeldt who characterized Creutzfeldt-Jakob disease.[20] Other notable pupils of Alzheimer and contributors to the earliest AD research in Munich included Gaetano Perusini and Francesco Bonfiglio.[20-21]

Alzheimer's most influential findings resulted from his work between 1901 to 1906 when he followed the case of Auguste D, a 51-year-old woman from Frankfurt who, strikingly, exhibited clinical and neuropathological characteristics of senile dementia despite her young age.[20] He published his results in 1907 entitled "Über eine eigenartige Erkankung der Hirnrinde" (On an Unusual Illness of the Cerebral Cortex[22]).[23] In his publication, Alzheimer extensively documented her clinical symptoms including behavioural abnormalities and her progressive cognitive decline, noting that her case did not fit any of the existing clinical

classifications.[20] Following her passing in 1906, Alzheimer went on to study the neuropathological components of her ailment. Aside from reporting brain atrophy and arteriosclerotic changes in the vasculature, Alzheimer noted the characteristic neuropathology of AD today: amyloid plaques, described at the time as "minute miliary foci" composed of an unknown "substance" and tau neurofibrillary tangles, described as neurofibrils with characteristic thickness and distribution in the cortex affecting up to one third of cortical neurons.[20, 22] Based on these clinical and neuropathological observations, Alzheimer concluded that Auguste D's ailment was a novel condition involving a form of presenile dementia, occurring before age 65.

In years to follow other publications by Bonfiglio (1908)[16] and Sarteschi (1909)[24] reported other cases of presenile dementia, then a later a publication in 1909 by Perusini, who was guided by Alzheimer, "On histological and clinical findings of some psychiatric diseases of older people", revisited Auguste D's case along with three other cases of presenile dementia. This report further elaborated on the neuropathological characteristics of presenile dementia that exhibited a likeness to senile dementia.[21] Subsequently, the eponym *Alzheimer's disease* was assigned to Alois Alzheimer by Emil Kraepelin in his eighth edition of a "Psychiatrie: Ein Lehrbuch für Studierende und Ärzte" (1910).[15] Here, Kraepelin discussed the discrepancies between cases of senile dementia from the rarer form of presenile dementia as reported by Alzheimer in 1907. Although Alzheimer was unable to identify the composition of the "miliary foci" and the fibrillar changes, his observations were key in subsequent investigations of this ill-understood form of dementia.

Importantly, this observation of "miliary foci" (the present-day amyloid plaque) by Alzheimer was not the first. In 1892, Paul Blocq and Georges Marinesco at the Salpêtrière Hospital in Paris published findings from an elderly patient with epilepsy, describing structures in the cerebral cortex as 'amas ronds' or "round heaps" that they attributed to glial sclerosis.[25] Later, in 1898, Emil Redlich at the University of Vienna described two cases of senile dementia with miliary sclerosis which he also suggested were of glial origin and even referred to them as 'plaques'.[26] However, unlike Alzheimer, neither Blocq and Marinesco, nor Redlich linked these miliary foci as primary contributors to the clinical symptoms of their patients.

This introduction would be incomplete without mention of Oskar Fischer. The fate of Fischer's legacy and recounts of his equally meaningful contributions to AD research rested in the hands of history. Long-term acknowledgement of Fischer's contributions was impeded

by the political unrest affecting his institution (the German University in Prague) and lack of potential successors. Conversely, Alzheimer's legacy was fortunate in that his mentor, Kraepelin, was regarded as one of the most influential psychiatrists of the time. As well, Alzheimer's institute in Munich thrived for years following his time there, allowing his successors to continue his line of research, unlike the case of Oskar Fischer.[27] Regardless, as with any formidable historical figure, in any context, the threads of history remain tangled, to be unwound by those who succeed them.

Oskar Fischer (1876 – 1942) was born in a small town called Slaný, near to Prague, and similar to Alzheimer's training, Fischer obtained a medical degree and then worked in the Department of Psychiatry from 1900 – 1919 in Prague.[27] Furthermore, key to both Alzheimer's and Fischer's observations of neurofibrils was the use of the superior Bielschowsky's silver staining method.[28] In the same year of Alzheimer's publication where he described the presence of plaques and tangles, Fischer described neuritic plaques for the first time in a study that included 16 cases of senile dementia. Of significance, Fischer noted the presence of not only plaques but also mature plaques, containing a core surrounded by abnormal neurites. This description served as the first account of the neuritic plaque (drusige Nekrosen) and Fischer further linked the presence of these more advanced plaques to the clinical diagnosis of prebyophrenia (considered a subtype of dementia at the time). Fischer concluded that senile dementia associated with the presence of plaques, differed from prebyophrenia which required the presence of both plaques and neuritic plaques. Unlike other publications reporting plaques, or miliary foci, Fischer did not agree that they were of glial origin and proposed that the lesions may be the cause of the dementia observed clinically rather than a secondary event.[27] Fischer's subsequent publication in 1910 demonstrated the characterization of plaques in the brains of 275 patients and healthy controls, relating the stages of plaque development to disease progression, which was a novel concept at the time. This publication also included the first comprehensive illustration of present day cerebral amyloid angiopathy. Finally, in Fischer's 1912 paper, among other findings, he concluded that the plaque consisted of a proteinaceous metabolic brain product.[27]

Until the 1970s, pre-senile dementia or Alzheimer's disease (occurring before age 65) and senile dementia were considered separate ailments until Robert Katzman's work. Katzman observed that a majority of cases of senile dementia indeed had AD pathology and a comparable clinical progression, bringing to light that this one disease, Alzheimer's disease, was the 4th or 5th common cause of death in the USA at the time.[29]

I.2 Neuropathology of Alzheimer's Disease

The classical neuropathological hallmarks of AD include: 1) extracellular amyloid plaques, and 2) intracellular neurofibrillary tangles where both the amyloid plaques and tau tangles serve as definitive markers for post-mortem diagnosis of AD.

I.2.1 Extracellular Amyloid Plaques

The exact composition of the 'miliary foci' or plaques observed by Alzheimer, Fischer and others was unknown for some time following their initial discoveries. By the 1960s, electron microscopy data acquired by Michael Kidd and Robert Terry provided some insight into the structure of these plaques as containing a filamentous core material with radial filaments extending outwards, that were different from the paired helical filaments of neurofibrillary tangles. They further noted that the extracellular plaque material exhibited convincing similarities to amyloid, which had been suggested by Divry in 1927.[30-32] However, it was only in the mid-1980s that researchers began to ascertain the exact molecular identity of the plaques. Glenner and Wong isolated and sequenced a 4.2 kDa protein purified from the β-pleated fibrillary cerebrovascular deposits in post-mortem AD and Down syndrome (DS) brains.[33-34] Their results were confirmed by Masters *et al.* the following year, who then referred to the protein as A_4, however, today this protein is known as amyloid β (Aβ).[35]

The discovery of the Aβ peptide offered insight into the progressive nature of the AD pathology, where extracellular aggregation of Aβ peptides into plaques worsened with disease progression. Furthermore, it was discovered that Aβ peptides had differing lengths, with each peptide exhibiting varying aggregation capabilities that corresponded to toxicity, notably $A\beta_{40}$ and $A\beta_{42}$ are the most prominent species, with $A\beta_{42}$ being more prone to aggregation[36-38] (Section I.4).

As noted by Fischer, the plaques he observed assumed different morphologies that were associated with earlier or advanced disease stages. Presently, amyloid plaques are broadly characterized into either diffuse or dense-core (neuritic) plaques depending on the morphology, and the staining profiles with Thioflavin-S and Congo Red which detect the presence of β-pleated sheets, characteristic of amyloid fibrils where the hydrogen bonds are parallel to the fibril axis.[39] Diffuse plaques are both Thioflavin-S and Congo Red negative and as the name implies, they assume an amorphous shape when stained for Aβ and range from 50 to several hundred microns in diameter.[40] These plaques can be present in cognitively intact individuals and may serve as precursors to the mature dense-core plaques as they are prevalent during early stages of Aβ deposition.[41-45] However, the presence of diffuse plaques

does not necessarily mean AD will result. The ability of certain individuals to maintain cognitive performance despite amyloid load also supports the concept of neural reserve.[41] Neural reserve is the capacity, flexibility, and efficacy that allows an individual to be more capable of coping with disruptions caused by brain pathologies.[46]

Conversely, dense-core (neuritic) plaques are both Thioflavin-S and Congo Red positive, are smaller being as low as 10 microns in diameter and are associated with other pathological markers of AD including synapse loss, neuronal loss, glia activation, and dystrophic neurites. These mature plaques, coincide with some degree of cognitive impairment, thus representing later stages of AD and can have a semi-quantitative score for AD pathological diagnosis unlike diffuse plaques which do not have diagnostic value.[44] The cores of dense plaques are often in contact with activated microglia and unlike diffuse plaques, dense-core plaques contain higher levels of $A\beta_{42}$.[47] In addition to Aβ, plaques often contain many other proteins including ApoE, ApoJ (clusterin), ubiquitin, metal ions such as zinc, copper, and iron, as well as extracellular matrix components.[40] The corona of dense-core plaques contains dystrophic neurites, containing lipofuscin, degenerating mitochondria, and synaptic vesicles suggesting axonal origins, it also contains tau proteins and astrocytic processes.[40]

A large body of literature exists on studying Aβ plaques. However, it is important to note that the progression of amyloid deposits in the brain does not correlate with cognitive decline as well as tau pathology does, rather, amyloid plaque deposition is less predictable than neurofibrillary tangles (NFTs) (Section 1.2.2).[44] Furthermore, with the failure of amyloid-targeting clinical trials (see Section 1.7.1), it is now clear that the earliest, pre-plaque stages of AD—when amyloid exists as monomers and oligomers—are most relevant for developing biomarkers and therapeutics. Regardless, amyloid plaques are a key component of the AD pathology that have aided in out understanding of the evolving pathogenesis of AD.

1.2.1.1 Vasculature Pathology

Cerebral amyloid angiopathy (CAA), or the deposition of Aβ on the walls of leptomeningeal and cortical blood vessels,[48] occurs in up to ~80% of AD cases with varying severity.[49-51] However, it is important to note that CAA is associated with other clinical syndromes aside from AD and a diagnosis can only be confirmed with neuropathological examination.[48]

CAA is observed in cortical capillaries, small arterioles, mid-sized arteries and leptomeningeal arteries while venules, veins and white-matter arteries are not affected,[44] notably, when capillary Aβ deposition is observed this is classified as CAA type I which is often widespread

in the neocortex and hippocampus, conversely, in the absence of capillary Aβ deposition it is considered CAA type II. Compared to dense-core plaques, in CAA, the more soluble and less aggregation-prone $A\beta_{40}$ rather than $A\beta_{42}$ accumulates in the vessels, this could be explained by the increased solubility of $A\beta_{40}$, allowing for diffusion of $A\beta_{40}$ from the brain parenchyma to vessel walls.[44, 51] The morphology of vessels and degree of Aβ-positive staining indicates the severity of deposition and is classified into different stages. In sum, although beyond the scope of this introduction, vascular pathology in AD plays an important role in disease progression that requires further investigation, especially for earlier, pre-symptomatic disease stages to better define contributions of CAA versus parenchymal Aβ deposition.[52]

I.2.2 Tau Neurofibrillary Tangles

In humans, there are a total of six tau isoforms in that are generated by alternative splicing, including three isoforms with four microtubule binding repeats (termed 4R) and three isoforms that lack the second repeat (termed 3R). Tau is primarily present in neurons but has a low expression in glial cells. Under physiological conditions, tau proteins serve to bind and stabilize neuronal microtubules, helping direct axonal growth and regulate axonal transport among other functions.[53-54] Indeed, localization of tau to axons rather than dendrites also aids in neuron polarization.[55]

Neurofibrillary tangles (NFTs) were observed by Fischer and Alzheimer along with amyloid plaques. Then in the 1960s, electron microscopy studies revealed that NFTs were composed of paired helical filaments (PHFs),[56-57] which were later discovered to be aggregates of tau protein.[58-61] The tau that comprised these PHFs in AD included both 3R and 4R tau,[62] and was highly modified, with the most common post-translational modification being hyperphosphorylation, causing tau to lose the ability to bind microtubules.[63] Other modifications to tau such as ubiquitination, truncation, glycation, nitration, and oxidation have also been observed and can enhance aggregation of abnormal tau.[63-64] Aggregation of abnormal tau within neurons eventually leads to cell death, forming 'ghost' tangles.[65]

The *MAPT* gene encodes the protein tau, and although no mutations in *MAPT* lead to AD, mutations in this gene cause other forms of neurodegeneration including, frontotemporal dementia (FTD)[66-68] among others. Furthermore, tau NFTs are a key hallmark of the AD neuropathology, especially since, unlike the deposition of amyloid plaques, NFT brain deposition is spatially and temporally predictable, and correlates with cognitive decline in AD, ultimately serving as a more effective marker of neurodegeneration in the AD brain.[69-70] This predictable brain deposition of tau pathology was extensively documented by Braak and

Braak (1991) and classified into 6 Braak stages. During stages I-II, tau inclusions appear in the transentorhinal region followed by the limbic system in stages III-IV. Finally, in stages V-VI, advanced tau deposition appears in the neocortex and is associated with the latest stages of AD dementia.[71]

Although not covered extensively in this introduction, for a review on the status of tau in neurodegenerative diseases and AD see the following references.[72] [73-74]

I.2.3 Neurodegeneration

In addition to extracellular amyloid plaques and intracellular NFTs, the AD brain undergoes gross atrophy, resulting from extensive synapse loss (see Davies *et al.* (1987)[75] and work by Scheff *et al.*[76-79]) and neuronal death. With increasing disease severity in AD, brain volume decreases, ventricles expand, and cognitive decline accelerates.[80] Even during earlier stages of AD that precede cognitive decline, atrophy of vulnerable brain regions is observed,[81-83] and can aid in predicting which individuals will develop AD.[84] Cortical thinning has also proven to be a useful measure of mapping disease progression, while subtle thinning during asymptomatic stages in familial AD cases has been detected.[85]

I.2.4 Neuroinflammation

Although amyloid and tau pathology dominated the AD research field for much of the 20th century, neuroinflammation has recently emerged as a key pathological characteristic of the AD brain. Importantly, early contributions to the field included work by Nissl (1899)[86] suggesting that glial cells in the brain had similar functions to that of peripheral macrophages, as well as the early observation by Fischer that deposition of the now known amyloid plaques in AD brains resulted in a local inflammatory reaction.[86-87] Following these observations, minimal progress was made regarding neuroinflammation, in part due to the concept of the brain being immune-privileged.[88]

It was not until the 1980s that research on neuroinflammation gained momentum through seminal work by Eikelenboom and colleagues that showed the presence of immunoglobulins and complement proteins in amyloid plaques.[89] This was followed by a multitude of influential studies that provided convincing evidence for the role of neuroinflammation in the AD brain. This included work by the McGeers who demonstrated the presence of the terminal membrane attack complex in amyloid plaques,[90] the observation that microglia recruited towards plaques expressed IL-1β in AD and DS brains by Griffin,[91] and work by Rogers showing that Aβ could bind complement receptors.[92]

Since these and many other studies not mentioned here, an abundance of progress has been made towards understanding the role of neuroinflammation in the brain,[93] and in early and late AD beyond the scope of this introduction (reviewed by Cuello (2017)).[94] As example, epidemiological evidence demonstrated that individuals on chronic NSAIDs (non-steroidal anti-inflammatory drugs) exhibited a decreased prevalence of AD.[95-96] Additionally, genetic evidence through recent genome-wide association studies (GWAS) has identified immune-related genetic loci and variants that alter the risk of developing sporadic AD (Section 1.5.2).[97]

Of relevance to this thesis, this inflammatory hypothesis of AD that is linked to amyloid brain deposition (reviewed by McGeer and McGeer (2013))[98] is also closely tied to oxidative stress since inflammation can both be a cause and consequence of oxidative stress.

I.3 How is Alzheimer's disease diagnosed today?

Despite advancements in diagnosing AD over the last century, it is still necessary to perform a *post-mortem* autopsy to diagnose definite AD which then shifts the diagnosis from "probable" AD to "definite" AD.[99] More recently, with the advancement of biomarker research, in the AD field, there has been a conceptual shift towards understanding AD as a continuum, which has fueled debates regarding the role of biomarkers in defining and diagnosing AD.[100]

Criteria for diagnosing possible or probable AD was first developed in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association working group (known as the NINCDS-ADRDA criteria).[101-102] This criteria had a sensitivity of 81% and specificity of 70%[103] and utilized clinical and cognitive endpoints including memory loss over time, absence of other ailments, progressive atrophy identified by CT scans, and CSF testing to rule out other explanations for cognitive decline. In this criteria, probable AD was a more confident diagnosis than possible AD. Additionally, this criteria recommended recording age of onset, whether the case was familial or sporadic, and whether there were other coexisting conditions (e.g.: Down syndrome or Parkinson disease) were present.[101]

In 2011, the NINCDS-ADRDA criteria was revised by the National Institute on Aging-Alzheimer's Association (NIA-AA) to update the diagnostic guidelines for the symptomatic or 'clinical' stages of AD, while also providing researchers with a common terminology for describing their findings.[104] The main differences from the 1984 criteria were: (1) recognizing the stages of AD, including preclinical AD, prodromal or mild cognitive impairment (MCI), and AD, and (2) including AD biomarkers. Importantly, MCI diagnosis was intended for clinical and research use while preclinical AD characterization was only for research use.[102] Other revisions in 2011 included: adding the genetics of autosomal dominantly inherited AD (outlined in Section I.5.1), designating the *antemortem* biological changes as AD pathophysiological processes as being separate from the clinical disease, including magnetic resonance imaging (MRI), positron emission tomography (PET), and cerebrospinal fluid (CSF) assays in diagnosing MCI and AD, and defining "atypical" and "mixed" AD (clinical, neuroimaging or biochemical evidence of a non-AD contributing disorder, e.g.: Lewy body disease, cerebrovascular disease).[103]

The International Working Group (IWG) also developed and modified criteria which had some similarities to that from the NIA-AA but also contained key differences on staging,

nomenclature, and interpretations of biomarkers. As example, the 2007 research framework was the first to propose AD as being a clinical-biological entity which combined *in vivo* biomarkers with clinical phenotypes, it also extended the definition to prodromal AD stages. Then in 2010 the IWG made recommendations for pre-symptomatic classifications of AD including asymptomatic at-risk for individuals with biomarker evidence of AD, and pre-symptomatic for individuals who had monogenic AD mutations. This was followed by 2014 guidelines that required cognitive symptoms in addition to AD biomarker signatures for diagnosing AD. Also in 2014, a position paper was published outlining guidelines for AD diagnosis for research purposes and included specific recommendations for diagnostic criteria for atypical AD, mixed AD, and preclinical stages of AD.[105]

In early 2016, the IWG and NIA-AA came to a consensus on classifying preclinical AD which had gained attention in the field as being the most promising therapeutic window for preventative or disease-modulating strategies. This publication addressed issues including, 1) how to define preclinical AD (e.g.: sporadic vs. familial AD), 2) what biomarkers could identify preclinical AD most reliably, 3) what factors influence preclinical AD, and 4) how to detect the transition from preclinical to clinical AD.

Later, in 2016, for research purposes, the classification scheme A (amyloid β) / T (tau) / N (neurodegeneration) was introduced to establish an unbiased descriptive classification system that was separate from the clinically defined diagnostic schemes of the IWG and NIA-AA.[106] This scheme addressed four key issues in the use of biomarkers for AD by 1) incorporating tau PET, 2) having no preference towards one pathological mechanism over another (e.g.: no bias towards amyloid or tau as being causal), 3) including of all individuals regardless of biomarker findings, and 4) not specifying disease labels or diagnostic classification. The A/T/N scheme organized seven major biomarkers into these three groups, notably, the 2011 NIA-AA criteria only divided biomarkers into two groups, being amyloid and tau-associated neurodegeneration. The ATN system involved assigning the presence (+) or absence (-) of amyloid (cortical amyloid PET ligand binding, or low CSF $A\beta_{42}$), tau (elevated phosphorylated tau and cortical tau PET ligand binding), and neurodegeneration (CSF total-tau, FDG PET hypometabolism, and atrophy on MRI).

Using the ATN system, the NIA-AA updated their guidelines in 2018 as a research framework for observational and interventional research, emphasizing that the intended use was not for clinical care, or for diagnostic criteria. Through this framework, they advocated for defining Alzheimer's disease as a **biological construct** and no longer required cognitive

symptoms to denote AD.[104] Despite only being intended for research, these 2018 NIA-AA guidelines generated challenges in the use of biomarkers in clinical practice that were highlighted by Dubois *et al.* (2021) from the International Working Group.[107] They outlined key limitations to a solely biological definition of AD including the risk of confusing presence of Alzheimer's brain lesions and Alzheimer's disease, the low predictive accuracy of solely amyloid and tau, the potential presence of other neurodegenerative processes which will result in positive AD biomarkers, and the arbitrary thresholds of biomarkers depending on the site or study. In light of these and other limitations, the authors argued that AD diagnosis should be restricted to those who have positive biomarkers in addition to AD-specific cognitive phenotypes, while biomarker-positive but cognitively healthy individuals should only be considered at-risk for AD. Other authors have also echoed the importance of caution when interpreting and using biomarker assays since many assays still have analytical challenges where the weight of each biomarker remains unresolved, and longitudinal assessments are often needed for understanding their proper use for aiding diagnosis but not for being diagnostic themselves (Figure 1.1).[102] A summary of the progression of AD diagnosis can be found in Figure 1.2 adapted from Dubois *et al.* (2021).[107]

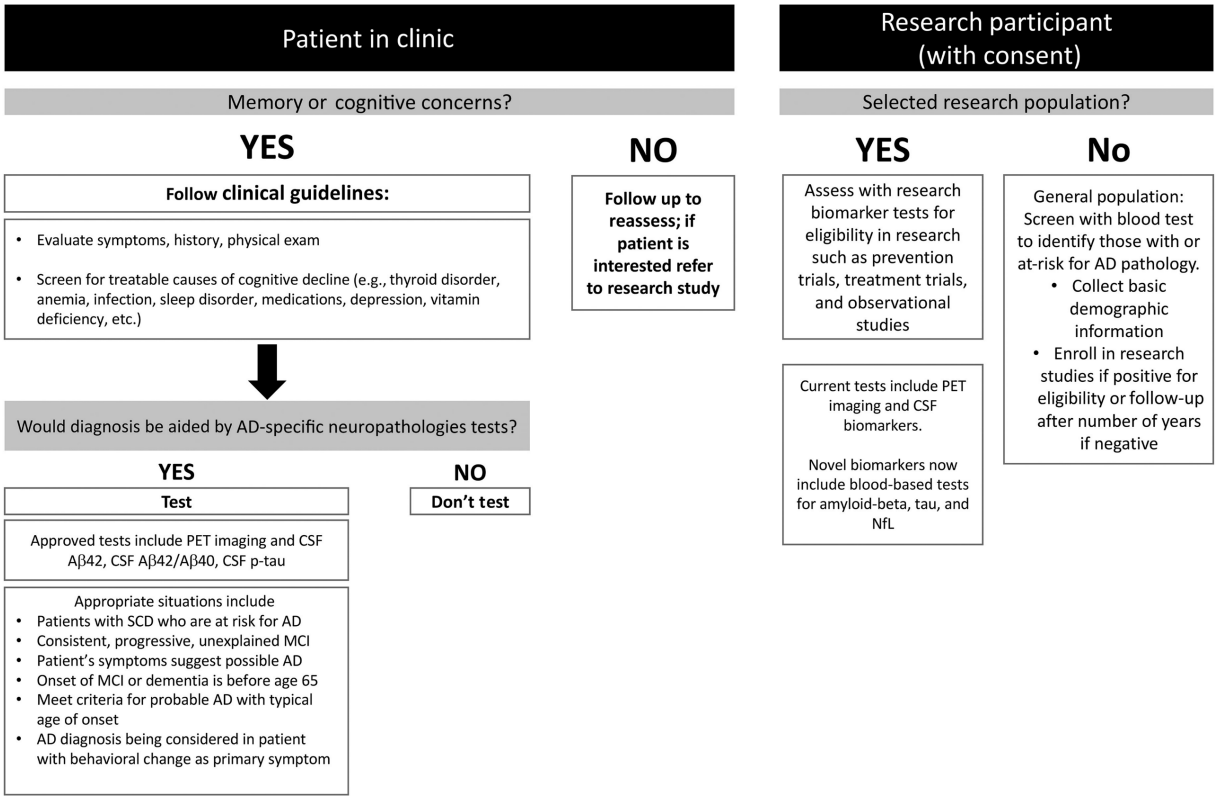


Figure 1.1: Using Alzheimer's disease biomarkers.

Adapted from Budelier and Bateman (2020).[102] Proposed clinical and research use of AD biomarkers. Reprinted from The Journal of Applied Laboratory Medicine, Vol. 5, Budelier M, Bateman RJ, Biomarkers of Alzheimer Disease, 194-208., Copyright (2020), with permission from Elsevier.

	NINCDS–ADRDA (1984)[2]	IWG (2007)[3]	IWG (2010)[4]	NIA–AA (2011)[5,6]	IWG (2014)[7]	IWG–AA (2016)[8]	NIA–AA (2018)[1]	IWG (2021)
Applicable settings	Research and clinical	Research	Research	Research and clinical	Research	Research	Research	Research and clinical
Clinical requirements	Dementia (memory changes and another cognitive impairment)	Amnestic syndrome of a hippocampal type	Amnestic syndrome of a hippocampal type, posterior cortical variant, logopenic variant, or behavioural–frontal variant	Mild cognitive impairment (amnestic or non-amnestic) or dementia	Amnestic syndrome of a hippocampal type, posterior cortical variant, logopenic variant, or behavioural–frontal variant	None	None	Amnestic variant, posterior cortical atrophy, logopenic variant primary progressive aphasia, behavioural or dysexecutive frontal variant, corticobasal syndrome, semantic and nonfluent variants of primary progressive aphasias*
Biological requirements	None	CSF biomarkers, MRI atrophy, ^{18}F-fluorodeoxyglucose PET hypometabolism, amyloid PET positive, or Alzheimer's disease autosomal dominant mutation	Pathophysiological markers: CSF changes (low CSF Aβ42, high phosphorylated tau, or high total tau) or amyloid PET positive	Amyloid β marker (CSF or PET) or marker of degeneration (CSF tau, phosphorylated tau, ^{18}F-fluorodeoxyglucose-PET, and T1-weighted MRI)	CSF amyloid β and tau or amyloid PET positive	Amyloid β marker (CSF or PET) and tau marker (CSF or PET)	Amyloid β marker (CSF or PET) and tau marker (CSF or PET)	Amyloid β marker (CSF or PET) and tau marker (CSF or PET)

ADRDA=Alzheimer's Disease and Related Disorders Association (now the Alzheimer's Association) Work Group. IWG=International Working Group criteria. IWG–AA=International Working Group and Alzheimer's Association joint criteria. NIA–AA=US National Institute on Aging and Alzheimer's Association joint criteria. NINCDS=US National Institute of Neurological and Communicative Disorders and Stroke criteria.
*Cognitively unimpaired individuals are considered at-risk for Alzheimer's Disease.

Table 1: **Details of successive proposed criteria for Alzheimer's disease diagnosis**

Figure 1.2: Criteria for Alzheimer's disease diagnosis.
Adapted from Dubois *et al.* 2021.[107] Timeline showing changes to the criteria used for diagnosing Alzheimer's disease. ADRDA (Alzheimer's Disease and Related Disorders Association (now the Alzheimer's Association)) Work Group, IWG (International Working Group criteria). IWG–AA (International Working Group and Alzheimer's Association joint criteria). NIA–AA (US National Institute on Aging and Alzheimer's Association joint criteria). NINCDS (US National Institute of Neurological and Communicative Disorders and Stroke criteria).[107] Reprinted from The Lancet, Vol. 20, Dubois B *et al.*, Clinical diagnosis of Alzheimer's disease: recommendations of the International Working Group, 484-496, Copyright (2021), with permission from Elsevier.

I.4 APP Processing

Accumulating evidence for the role of Aβ in AD provided impetus for studies on how this peptide was generated from APP. APP is a type-I membrane protein containing a long extracellular N-terminal region and a short intracellular C-terminal region. In the brain, APP_{695} is the prominent isoform (other APP splice variants are expressed in different tissues) where the full-length protein is predicted to play a role in development through axonal transport and signalling functions, possibly through the binding of different ligands which then dictate subsequent cleavage of the full-length protein. [108-109]

APP is proteolytically cleaved by the proteases, α-, β-, and γ-secretase resulting in two proteolytic pathways of APP, one of which leads to generation of the toxic Aβ peptide implicated in AD (the amyloidogenic pathway), whereas the other pathway does not result in Aβ generation (non-amyloidogenic pathway) (not reviewed here is the η-secretase pathway, see Willem *et al.* (2015)[110]). In the amyloidogenic pathway, APP is first cleaved by β-secretase, (Beta-site APP cleaving enzyme 1 (BACE1)) resulting in the extracellular release of soluble APP β (sAPPβ) comprising the amino-terminus, and leaving behind the membrane-bound carboxy-terminal fragment (CTF) CTF99. The CTF99 fragment is subsequently cleaved by γ-secretase to generate extracellular Aβ (that is released) and an APP intracellular domain (AICD).[111-112]

Conversely, non-amyloidogenic APP processing begins with cleavage by an α-secretase from the ADAM (a disintegrin and metalloprotease) family of proteins including ADAM 9, 10, 17 or 19. α-secretase cleavage occurs within the Aβ region of APP, thus preventing any formation of Aβ[111] and instead results in release of soluble APPα (sAPPα) which has physiological roles in cortical synaptogenesis [113] and synaptic transmission.[114-115] The remaining membrane bound portion of APP (CTF83) is then cleaved by γ-secretase resulting in release of a p3 peptide leaving behind an APP intracellular domain (AICD).[116] For a review of each proteolytic product, see Nhan *et al* 2014.[117]

γ-secretase is a complex of four subunits including presenilin 1 or presenilin 2, nicastrin, anterior pharynx defective (APH) 1a or 1b and the PS enhancer (PEN)-2 and displays a high degree of heterogeneity[118] whereby six functional γ-secretase complexes are known.[112] Cleavage by γ-secretase is also variable and can occur between amino acid residues 37-43 of the Aβ region, resulting in Aβ fragments of differing lengths although the most common and relevant forms for the AD pathology are either 40 or 42 long products ($Aβ_{40}$ and $Aβ_{42}$

respectively).[111] The preference favoring the more toxic Aβ42 is in part explained by the GxxxG motif in APP's transmembrane sequence.[119-120]

APP trafficking within the cell and the secretases discussed above govern the location of Aβ generation. APP follows the secretory pathway from the endoplasmic reticulum (ER), to the Golgi apparatus, trans-Golgi network (TGN), to axons and dendrites and to the plasma membrane (PM). Typically, α-secretase cleaves APP at the plasma membrane,[109, 111] where APP is short-lived and quickly internalized into endosomes. This endocytosed APP can be recycled to the PM and the TGN while some is degraded by lysosomes.[111] Importantly, proteins that interact with APP at the cell surface and either accelerate endocytosis or retain APP at the cell surface also influence the degree of amyloidogenic or non-amyloidogenic processing, such proteins include ApoE receptors.[121]

BACE1 trafficking follows a similar trajectory to APP where BACE1 undergoes post/co-translational modifications in the ER, then reaches the plasma membrane where it associates with lipid rafts that may aid amyloidogenic processing. BACE1 is then internalized, targeted to endosomes and recycled to the TGN. [116] The pH within endosomes provides an ample environment for the aspartyl protease activity of BACE1 (optimum around pH 4.5) resulting in increased preference for initiating the amyloidogenic pathway.[111, 122] Importantly, the fully assembled γ-secretase complex has also been shown to localize to the plasma membrane and in endosomes where it cleaves the CTF99 fragment to generate AICD and Aβ, with additional evidence of Aβ production at axonal synapses of neurons.[111]

Extracellular Aβ can be taken up via α7nAch receptors, low density lipoprotein receptor-related protein 1 (LRP), the receptor for advanced glycation end products (RAGE), and NMDA receptors (N-methyl-D-aspartate).[123] Conversely, extracellular degradation of Aβ occurs through the enzyme neprilysin (NEP) [124-125] which preferentially cleaves oligomeric $A\beta_{1-42}$ and $A\beta_{1-40}$[126] as well as through insulin-degrading enzyme (IDE),[127-130] among other enzymes (reviewed in Zuroff *et al.* 2017).[131]

I.4.1 Intraneuronal Oligomeric Amyloid Beta

The presence of Aβ intracellularly in neurons has previously been controversial due to technical reasons, namely the specificity of Aβ-antibodies used and whether they cross-reacted with full-length APP protein or other cleavage products (Section I.4.1).[123] Accumulation of **soluble** Aβ peptides intraneuronally is an early event in the AD pathology that precedes plaques (insoluble Aβ)[132-134] where secreted Aβ can be endocytosed or it can be produced

within neurons. This accumulation of intraneuronal Aβ is especially pathological when the primary form produced is $A\beta_{42}$ which is known to readily aggregate into even more toxic soluble oligomeric species.[112] Of note, Aβ peptides are present at low (picomolar) concentrations in the healthy brain and are thought to modulate various functions including synaptic activity, memory, neuronal survival, antioxidant responses, calcium transport, and BBB integrity. Conversely, higher concentrations at the nanomolar and micromolar level are associated with neurotoxicity.[135] This is especially relevant when evaluating the physiological and pathological relevance of studies that exogenously apply Aβ to their experimental models. In many cases, overtly high Aβ concentrations are used, thus limiting the relevance of the findings to the human condition.[136] Furthermore, in addition to concentration, the nature of the Aβ oligomers is important as they can be small (dimers and trimers), medium lengths (9 to 12mers, Aβ-derived diffusible ligands (ADDLs)) and higher mass oligomers termed protofibrils, among other forms.[137-138] Notably, these most toxic soluble amyloid species are not detectable in the brain by PET (positron electron tomography) which detects larger amyloid deposits such as plaques, and are instead detected in CSF or blood.[139] The complexity associated with Aβ conformations and their related toxicity is especially relevant when designing therapeutic strategies for AD.

I.5 The Etiology of Alzheimer's Disease

The lexicon used to classify AD has evolved over time and continues to undergo modifications as research advances.[99-100] AD is primarily divided between familial and sporadic origins (fAD and sAD) where the former involves genetic mutations as causative factors in AD, and the latter lacks these known mutations.[100] An additional factor in characterizing AD is the onset of clinical symptoms, divided into early onset, occurring before the age of 65 and late onset, occurring after the age of 65. The most common familial cases are early onset and termed early onset autosomal dominant AD (eADAD). However, in some cases there are familial mutations resulting in late-onset AD (fLOAD).[140-142] Similarly, sporadic AD is often late onset (sLOAD) but there are exceptional cases that are early onset (sEOAD).[142]

I.5.1 Familial Alzheimer's Disease

Familial Alzheimer's disease accounts for ~1% of all AD cases, is characterized by an autosomal dominant inheritance pattern and typically results in early disease onset, occurring before 65 years of age.[143-144] However, there are some familial cases of AD that have a later onset (fLOAD). Presently, there are over 300 mutations in three key genes that cause early-onset autosomal dominant Alzheimer's disease (eADAD) [145] including *PSEN1* mutations accounting for a majority (~81%) of eADAD cases, followed by *APP* mutations (~14%), and then *PSEN2* mutations accounting for only ~6% of cases.[143-144] Each of these mutations are implicated in Aβ production and played a large role in the formulation of the amyloid hypothesis of AD which has dominated the field (Section 1.6).

I.5.1.1 APP Mutations

In studies preceding Katzman's distinction between senile dementia and AD in 1976, there were reports of a potential autosomal dominant inheritance pattern of dementia that showed increased penetrance with age.[29, 146-148] Despite these studies of families affected by AD (or senile dementia), the gene(s) contributing to this supposed inheritance pattern remained elusive. Then, identification of the A_4 peptide (Aβ) in AD and DS-AD plaques by Glenner and Wong in 1984 provided a foundation for cloning the gene from which this peptide was derived. Importantly, the occurrence of AD in individuals with DS also suggested a leading role for chromosome 21, which is triplicated in DS. In 1987, two groups identified the *APP* gene on chromosome 21, that produced the full-length APP (amyloid precursor protein) from which the Aβ peptide was generated via proteolytic cleavage.[149-151]

Subsequent publications throughout the 1990s identified mutations in the *APP* gene that caused familial AD were found to increase Aβ peptide generation from APP through proteolytic processing and influenced the propensity of Aβ to aggregate in a pathologic manner. These mutations in the *APP* gene included the London mutation which was the first to be discovered (V171I, increased $A\beta_{42/40}$ ratio and $A\beta_{42}$)[152], Indiana mutation (V717F, increased $A\beta_{42/40}$ ratio)[153], Florida mutation (I716V, increased $A\beta_{42/40}$ ratio and $A\beta_{42}$)[154], and the Swedish mutation (actually a double mutation, KM670/671NL, increased total Aβ, increased $A\beta_{42}$ and $A\beta_{40}$)[155] among others. Numerous mutations localized to key cleavage sites of the full-length APP protein, as example, the Swedish mutation impacts cleavage of the APP protein to favor a more pathologic pathway initiated by BACE1 as discussed below.[111] Other mutations were located within the Aβ sequence where they enhanced self-aggregation of Aβ peptides as in the Dutch mutation (E693Q), which also exhibited extensive Aβ deposition in the vasculature leading to CAA.[156-158] These findings directly implicated APP processing in AD, for an extensive review of pathogenic *APP* mutations and their effects on Aβ production see Hunter and Brayne (2017),[115] and Cruts *et al.* (2012).[159]

I.5.1.2 PSEN Mutations

In addition to chromosome 21 linkage where *APP* mutations were found, other studies of EOFAD had linkage to chromosome 14[155, 160-162] and chromosome 1.[163] Shortly after these studies, mutations in *PSEN1*[164-166] and *PSEN2*[167-168] were discovered in EOFAD individuals. These genes encode the subunits of γ-secretase (PS1 and PS2 respectively) which are responsible for the catalytic activity of γ-secretase. As there are a multitude of *PSEN* mutations resulting in EOFAD, the exact mechanisms by which these mutations lead to the AD pathology is variable and depend on where in the sequence the mutation is located.[169] Arguments exist for a loss of function of PS1 as being the driver for the AD pathology [170-171] but also for a gain of function resulting in increased toxic Aβ species, particularly $A\beta_{42}$.[172-174] However, it should be noted that suggesting a full loss of function likely does not occur in EOFAD and rather one mutated copy of *PSEN* may serve to initiate Aβ seeding events that drive the pathology despite the presence of one wild type (Wt) copy of *PSEN*.[173]

Patient populations with fAD mutations offer an invaluable opportunity to study the extended pre-symptomatic stages of AD. Furthermore, these fAD mutations have been integral for generating cellular and transgenic animal models to study AD. The insight gathered from fAD studies has been leveraged towards understanding sporadic AD (sAD) which has a more elusive etiology.

I.5.2 Sporadic Alzheimer's Disease

The strongest risk factor for AD is age, where prevalence increases at an exponential rate after age 65 and then doubles every 5 years from age 65 to 90.[175-176] Other risk factors for AD relate to lifestyle including physical inactivity, poor diet, and smoking which impact vascular health, leading to hypertension, hypercholesterolaemia, diabetes, and obesity. Additionally, lack of cognitive training (less education), depression, and psychological factors (loneliness, stress, sleeping disorders) present an increased risk of AD.[177] Importantly, these are modifiable risk factors that can be addressed by lifestyle changes that promote overall physical and psychological health.[178] The recent FINGER study (Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability) was the first large multidomain lifestyle intervention study to show improved cognition in an at-risk group.[179] Subsequent studies of similar nature including MAPT (French Multidomain Alzheimer Preventive Trial) and PreDIVA (Dutch Prevention of Dementia by Intensive Vascular Care) had negative primary outcomes but following post-hoc analyses, some beneficial effects were detected.[175] Aside from multidomain interventions that target broader, lifestyle-related risk factors for AD, there also exist a number of genetic risk factors for sLOAD.

Before the exact cause of familial AD came to light, it was acknowledged that a number of AD cases did not exhibit an autosomal dominant inheritance pattern, suggesting potential environmental or otherwise unknown factors contributing to the disease.[180] As with familial AD, sporadic AD has variability between cases whereby a majority of cases have a late onset (after 65 years of age) known as sporadic late onset AD (sLOAD), while a only a handful of sporadic cases present at early onset (sEOAD).[142]

In the case of sLOAD, the *APOE* gene, located on chromosome 19, was one of the earliest genetic risk factors identified for this late-onset form of AD.[181-182] ApoE is a glycoprotein highly expressed in the liver and CNS glial cells, primarily astrocytes[183] and microglia,[184] but can also be produced by neurons.[185] ApoE serves as a lipid carrier, a regulator of lipid homeostasis, and is implicated in Aβ deposition and clearance.[97, 121] As the *APOE* gene has three alleles, ε2, ε3, and ε4 that have varying frequencies in the population, 8.4%, 77.9%, and 13.7% respectively,[143] early studies investigated which allele was most strongly associated with AD. It was discovered that two copies of the ε4 allele increased the risk of developing sLOAD by 10- to 15-fold, lowering the average age of onset to 68 years, and one copy of the ε4 allele increased the risk by 3- to 4-fold with an average age of onset of 76 years.[121, 186-188] The ε4 allele was also associated with heavier Aβ plaque load, CAA burden, increased

brain atrophy, and faster disease progression.[97, 189-190] This association of the ε4 allele with early AD pathology was also observed in individuals with Down syndrome[191-193] and recently reported to be associated with AD clinical and biomarker changes in DS.[194] Conversely, the Apoε2 variant was found to be protective against AD.[195] In the early 1990s ApoE was found to be a component of Aβ plaques and NFTs[196-197] and was observed to bind Aβ *in vitro*, where the ε4 variant bound more readily than the ε3 variant.[182, 198] However, certain *in vitro* studies on ApoE-Aβ interactions were contradictory and dependent on the methods of preparing Aβ and ApoE. As example, the lipidation state of ApoE—which is regulated by ATP-binding cassette A1, (ABCA1) —differs between isoforms and impacts interactions with Aβ. ApoE also has limited interactions with soluble Aβ but has increasing interactions as the Aβ aggregation state advances.[199] More recently, ApoE has been implicated in mediating Aβ clearance, through cellular uptake of Aβ by glia, extracellular degradation by insulin-degrading enzyme (IDE) or through transport across the blood-brain-barrier (BBB). In these contexts the ε4 variant was less efficient at clearing Aβ.[200-201]

Strategies to identify other risk loci in AD including genetic linkage studies, candidate gene studies, genome-wide association studies (GWAS),[202-203] and whole-genome sequencing have identified over 30 AD risk loci, while validating the risk associated with *APOE4* (summarized in Figure 1.3 and Figure 1.4). Through GWAS studies, the associations between millions of single nucleotide polymorphisms (SNPs) with either risk of AD or endophenotypes revealed that a number of these genes were related to the immune system (*CLU, CR1, ABCA7, CD33, EPHA1* and *MS4A*), synaptic function (*PICALM, CD33, CD2AP, EPHA1*, and *BIN1*), and lipid metabolism (*CLU* and *ABCA7*) while others were associated with APP metabolism (endosome recycling) (*SORL1* and *CASS4*) and tau (*CASS4* and *FERMT2*).[204-207] In 2013, a variant (R47H) in *TREM2* (triggering receptor expressed on myeloid cells 2) was found to increase the risk of AD by 2-3 fold.[208-209] However, TREM2 variants are not very common.[199] The protein product of this gene is a receptor of the immunoglobulin superfamily and is expressed on macrophages, including microglia in the CNS, further linking the immune system to AD.[97] Despite the identification of numerous risk loci, *APOE4* remains the primary genetic risk factor for sLOAD.[97, 199] A full understanding of the role ApoE plays in AD has yet to be elucidated. This was exemplified by a case study where a rare *APOE3* mutation, denoted as the Christchurch mutation (R154S or *APOE3ch*) was recently discovered to confer resistance to a *PSEN1* mutation (E280A). This *PSEN1* mutation typically results in MCI at 44 years of age and dementia onset at 49 years of age, but due to the presence of this specific *APOE3* mutation, this individual only developed MCI in her seventies. It was further

shown that the *APOE3ch* variant had altered Aβ binding properties, resulting in less $A\beta_{42}$ aggregation.[210-211]

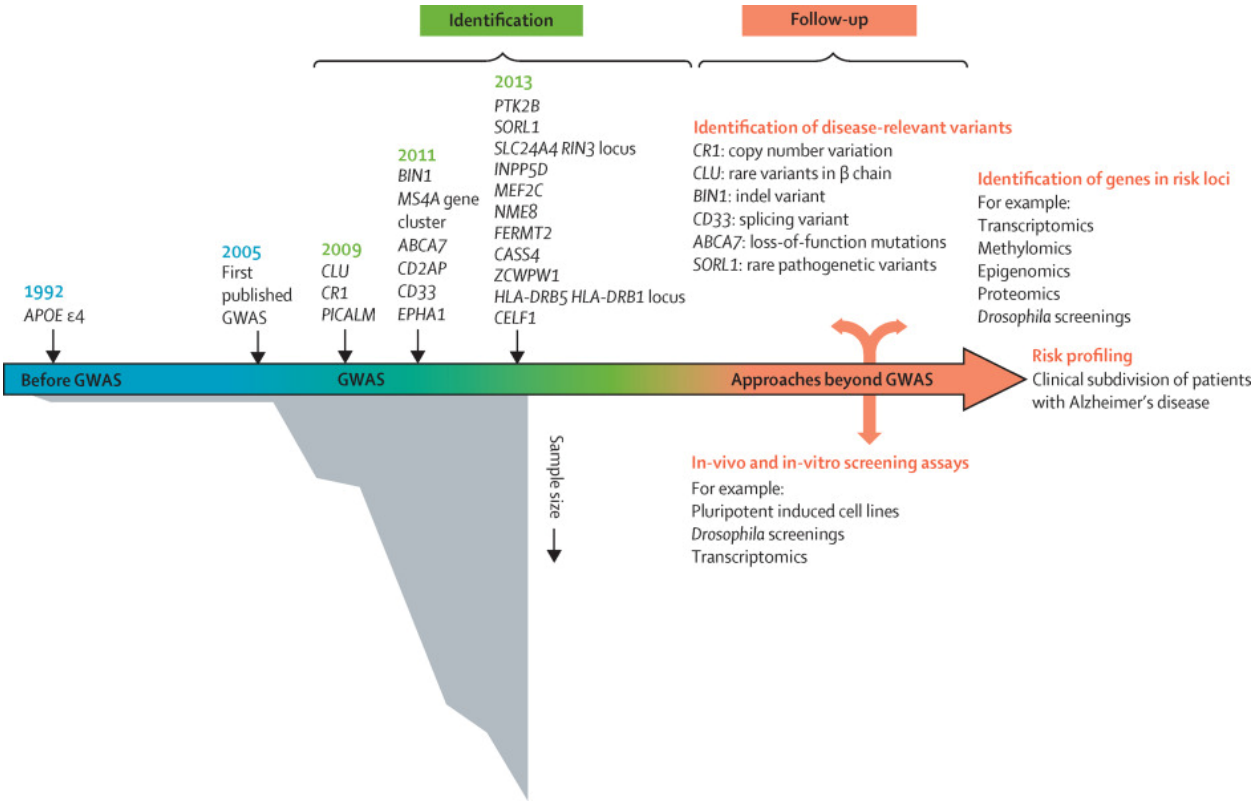


Figure 1.3: Discovery timeline of risk factors for Alzheimer's disease.
Adapted from Cuyvers *et al.* 2016,[205] showing risk gene identification in Alzheimer's disease research. Identification by GWAS is shown in green, while follow-up studies related to these GWAS genes are shown in red, and sample size in grey below the timeline. Reprinted from The Lancet Neurology, Vol. 15, Cuyvers E, Sleegers K., Genetic variations underlying Alzheimer's disease: evidence from genome-wide association studies and beyond, 857-868, Copyright (2016), with permission from Elsevier.

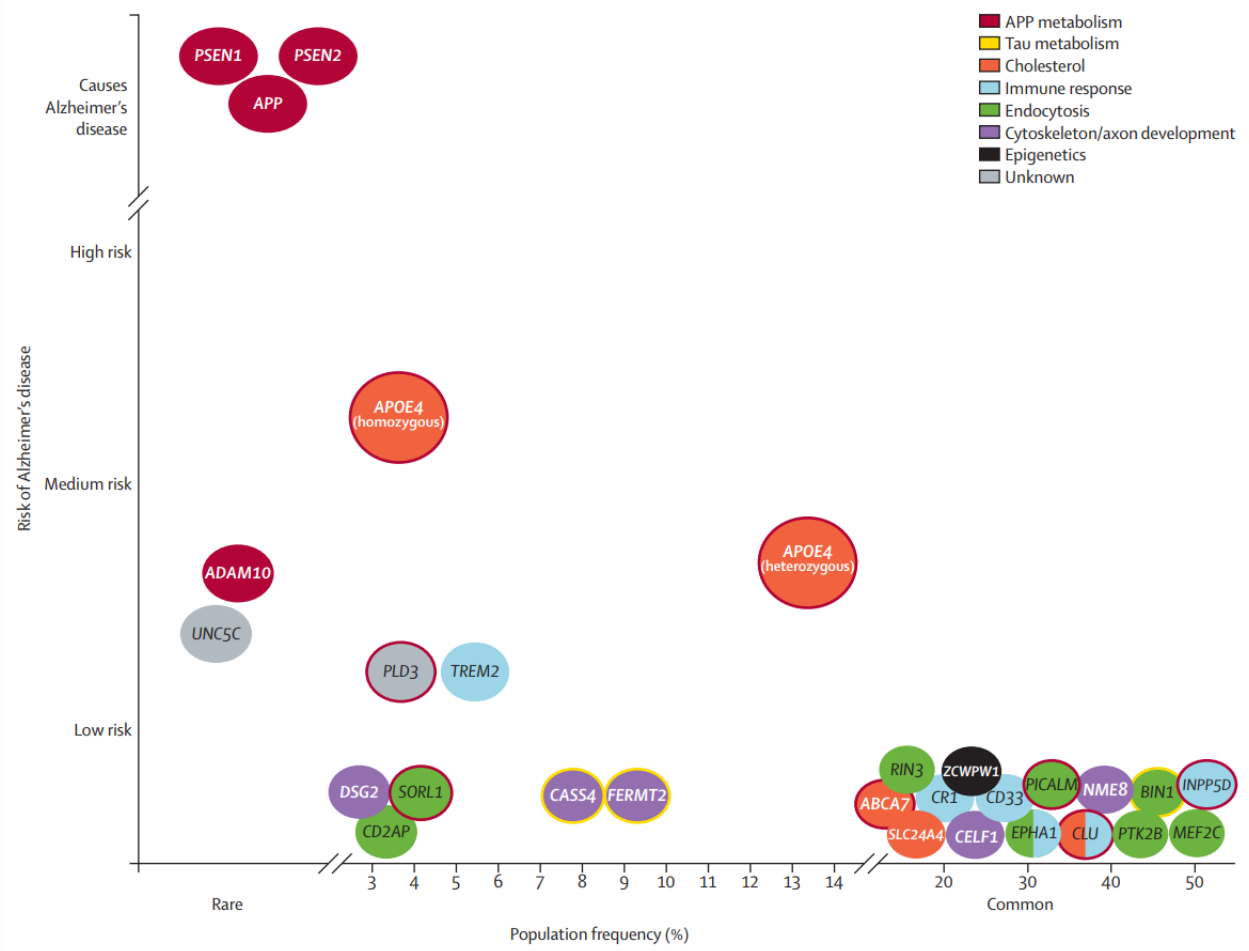


Figure 1.4: Frequency of genetic risk factors for Alzheimer's disease.
Adapted from Scheltens *et al.* 2016,[212] (which adapted the figure from Karch and Goate 2015)[213] showing the main pathways that genes causing increased risk for AD are implicated in. Dual coloring indicates genes implicated in multiple biological pathways. Reprinted from The Lancet, Vol. 388, Scheltens P, *et al.*, Alzheimer's disease, 505-517, Copyright (2016), with permission from Elsevier.

Numerous hypotheses have been formulated to explain the etiology of Alzheimer's disease and its primary pathogenesis. The prevailing hypothesis which has dominated the field for decades is the amyloid cascade hypothesis since both familial and sporadic AD implicate the Aβ peptide. However, with the recent failure of amyloid-targeting therapeutics other hypotheses are slowly coming to the forefront and will be discussed below.

I.6 The Amyloid Cascade Hypothesis

As outlined in previous sections, early AD research centered on the role of Aβ and mutations associated with altered APP metabolism. These observations led to the conception of the amyloid cascade hypothesis by Hardy, Allsop, and Selkoe in 1991[214-215] which was coined by Hardy and Higgins in 1992.[216] This hypothesis aimed to explain the etiology of both familial and sporadic AD, and as the name implies, it focused on Aβ as the key culprit in initiating and driving AD with neurofibrillary tangles, cell death, and dementia as being a consequence of Aβ accumulation. Several lines of evidence in support of a primary role for amyloid include:

I. **Autosomal dominant inheritance pattern in genes impacting Aβ production.** eADAD mutations in *APP, PSEN1* and *PSEN2* increase the production of Aβ through the amyloidogenic pathway or increase the aggregation propensity of Aβ peptides produced (i.e.: $A\beta_{42}$ is more prone to aggregation than $A\beta_{40}$) which cause AD.[204]

II. **Triplication of *APP* in Down syndrome increases AD risk.** Individuals with Down syndrome, who have a triplication of chromosome 21 and consequently triplication of the *APP* gene, develop AD and overexpress Aβ from an early age.[217]

III. Incomplete triplication of chromosome 21 in DS decreases the risk for developing the AD pathology.[218]

IV. **Protective mutation in *APP* gene.** The Icelandic mutation in the *APP* gene (A673T) first identified in 1993[219] was later confirmed to be a protective against AD and age-related cognitive decline.[220]

V. **ApoE contributes to Aβ load in sLOAD.** ApoE is implicated in Aβ deposition and clearance where the ε4 allele results in increased risk of developing sLOAD by decreasing clearance and increasing deposition of Aβ in the brain.[221]

Although the amyloid hypothesis has undergone some revisions over the years to place more focus on the neurotoxicity oligomeric Aβ,[138, 222-223] it remains controversial. It also has weaknesses in it's simplicity as it discounts the other alterations in the APP proteolytic system that result from mutations.[115] Furthermore, in the last two decades, there have been over 200 failed trials for AD therapeutics,[224] with most of these strategies targeting amyloid. These failures have fuelled the conception of alternative hypotheses to explain the etiology of AD.

I.7 Treating Alzheimer's Disease

Up until this year, there were only four approved drugs for symptomatic treatment of AD, namely donepezil (approved in 1997), rivastigmine (2000), galantamine (2001), and memantine (2003).[225] Of these, three are acetylcholine esterase inhibitors that increase brain levels of acetylcholine (in line with the cholinergic hypothesis of AD reviewed by Francis *et al.* (1999)[226]), while memantine is an NMDA (N-methyl-D-aspartate) receptor antagonist that targets glutamate excitotoxicity.[227] Although these approved treatments for AD improve cognitive symptoms for a certain period of time (6 to 12 months), they do not modify the course of the disease.[228]

This year (2021), the first anti-amyloid targeting drug (aducanumab, Biogen) was approved by the FDA. Despite a seemingly promising step forward, this approval has stirred controversy in the AD research community. This was because many experts did not agree that the clinical trial data conclusively showed that aducanumab could slow cognitive decline.[229-231] Aducanumab is a human IgG1 monoclonal antibody (mAb) directed against Aβ oligomers, it was derived from healthy aged doners who were cognitively normal with the rationale being that these individuals had evaded AD. Early on, aducanumab showed great promise, with dose-dependent and time-dependent decrease in amyloid plaques as well as a slowed rate of cognitive decline for certain measures in 2016.[232] However, in March of 2019, aducanumab was discontinued due to lack of efficacy.[233] Later in 2019, the dataset was revisited and Biogen claimed that a certain subset of patients receiving high doses of aducanumab showed slowed cognitive decline.[234] In light of these updated results, aducanumab went on to be approved by the FDA in 2021. While some prominent AD researchers are optimistic of this approval,[235] many others are concerned that this 'success' for aducanumab will distract from other pathological targets being explored and tested in clinical trials. Ultimately, only the Phase 4 trial data will determine whether aducanumab has any positive effects on delaying cognition.

I.7.1 The Controversy of Amyloid-Targeting Clinical Trials and Therapies

Early anti-amyloid immunotherapies focused on targeting plaques, and included both active and passive immunization strategies, where the former involved administration of an amyloid antigen and the latter involved administration of a monoclonal or polyclonal humanized anti-Aβ antibody.[138] Active immunization against Aβ was first tested in 2002 by Elan Pharmaceuticals and was known as AN-1792. Although this vaccine decreased Aβ deposits in AD patient brains, there were no cognitive or clinical benefits.[236] Furthermore, around 6% of trial participants developed meningoencephalitis due to a T cell-mediated response.[237] An

early critique of anti-amyloid therapies was that they were administered late in the AD continuum and thus, even though they effectively decreased brain amyloid load, neuronal damage was too advanced to achieve any cognitive benefits, while others argued that rather than targeting plaques, other more toxic amyloid species (such as oligomers) needed to be targeted.[138, 238-239] As a result, more recent amyloid-targeting trials involved targeting other amyloid species through passive immunization strategies such as solanezumab (soluble monomeric Aβ, but not fibrillar, tested in the DIAN-TU trial), gantenerumab[240] (Aβ oligomers and fibrils, tested in the DIAN-TU trial), and crenezumab (pentameric oligomeric and fibrillary Aβ).[138] The DIAN-TU trial with solanezumab and gantenerumab started as a Phase 2 biomarker study in December 2012 then progressed to a Phase 3 study assessing cognition. The trial included two populations, an asymptomatic mutation carrier group who were many years away from symptom onset, as well as a symptomatic mutation carrier group with early stage dementia. Importantly, partway through the trial, the AD field recognized the need for higher doses of anti-Aβ antibodies, and as such, doses were increased fivefold for gantenerumab and fourfold for solanezumab. Unfortunately, this was late in the disease progression for participants who originally enrolled in the symptomatic group, thus, their cognition saw no improvements. The initial results from the DIAN-TU trial were discouraging in early 2020,[241] but by April 2020,[242] with additional analysis of the asymptomatic group, gantenerumab showed promise for reducing brain amyloid plaque load, normalizing CSF $A\beta_{42}$, and normalizing CSF total tau and p-tau181, which is an AD-specific form. This led to an open-label extension of the trial for gantenerumab, while solanezumab was abandoned.[242]

Aside from directly reducing Aβ levels, other amyloid-targeting strategies aimed to reduce the total amount of Aβ produced by using γ-secretase inhibitors, such as semagacestat, which was the only γ-secretase inhibitor to reach Phase 3 clinical trials. In this case, cognition seemed to worsen in the trial participants, and these adverse effects were likely explained by the numerous other cleavage targets that γ-secretase has, leading to off-target effects. For this reason, more recently, instead of inhibition, researchers are trying to disrupt specifically the γ-secretase cleavage site on APP.[243] β-secretase inhibitors have also been tested (verubecestat) but did not have efficacy for either mild to moderate AD.[225] For a recent review of the different amyloid-targeting strategies see Panza *et al.* (2019) (Figure 1.5).[138]

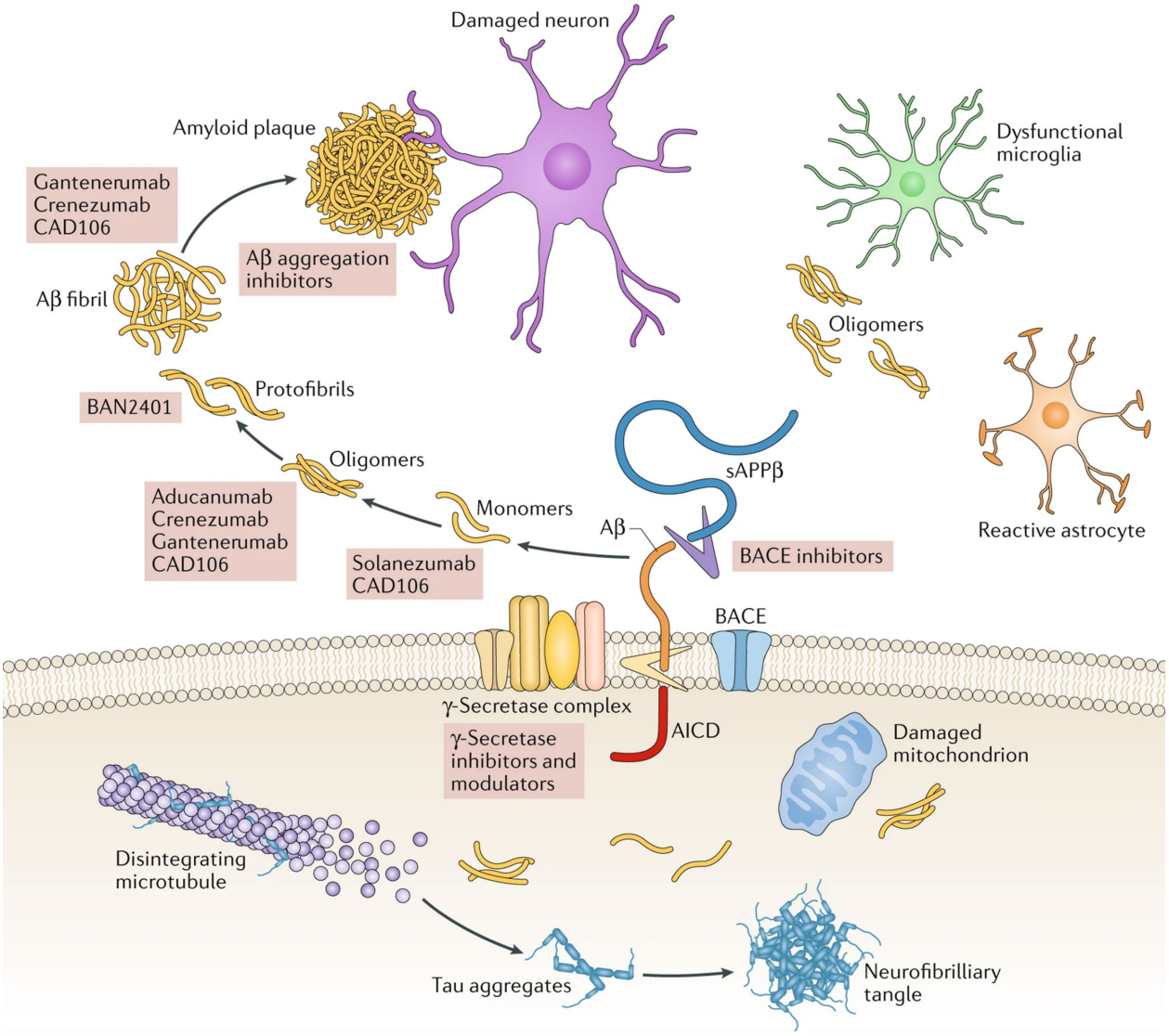


Figure 1.5: Amyloid-targeting therapeutic strategies.
Adapted from Panza et al. 2019.[138] Anti-amyloid-β drugs and their mechanisms of action for treating Alzheimer's diease. AICD: APP intracellular domain. Reprinted from Nature Reviews Neurology, Vol. 15, Panza F, Lozupone M, Logroscino G et al., A critical appraisal of amyloid-β-targeting therapies for Alzheimer disease, 73-88, Copyright (2019), with permission from Elsevier.

A major critique of these and other trials is that monotherapy approaches ignore other aspects of the AD pathology that contribute to disease initiation and progression. Furthermore, even if soluble Aβ is removed, levels can be replenished by plaques which are reserves for soluble Aβ species. Indeed, combined therapies addressing these pitfalls and considering other pathological targets will need to be considered moving forward.

I.8 Alternate Hypotheses for Alzheimer's Disease

Alzheimer's disease is a progressive neurodegenerative disease which has an extended pre-symptomatic period that lasts decades, (Figure 1.6), this promising therapeutic window remains under characterized at the cellular and molecular levels and despite the extensive body of research built over the last century towards understanding AD, there are still no preventative treatments nor cures.

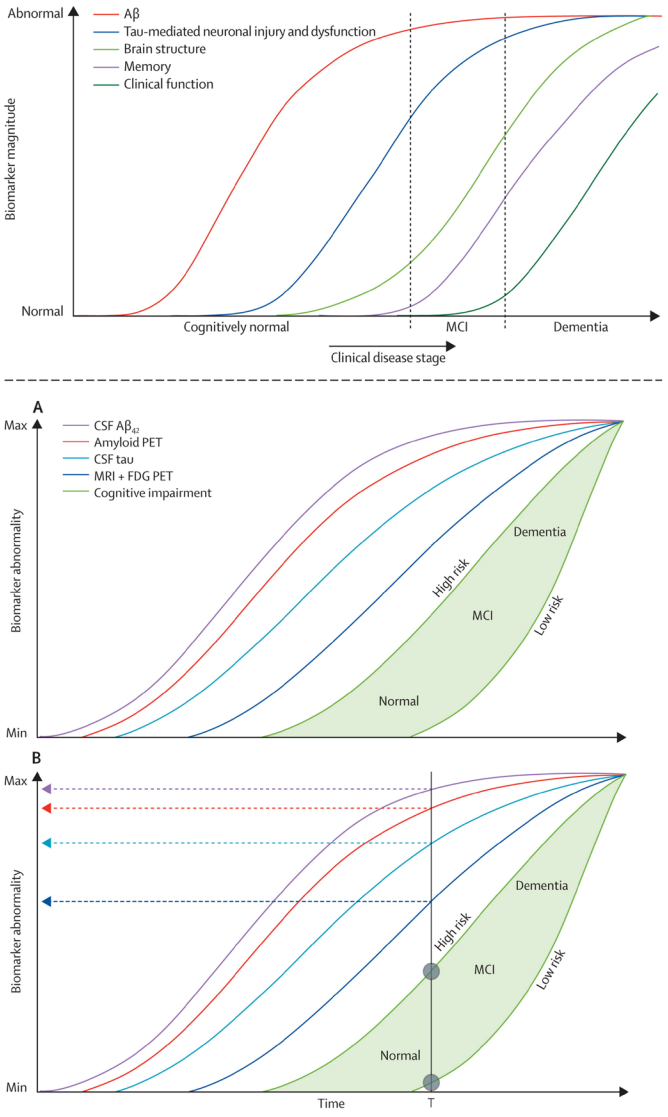


Figure 1.6: Alzheimer's disease continuum.
As modified from Jack Jr. *et al.* (2013) (Figures 1 and 5).[244] (Top) Showing the Jack Jr. *et al.* 2010 model of AD biomarkers. (A and B) Updated model from 2013. Reprinted from The Lancet Neurology, Vol. 12, Jack Jr CR *et al.*, Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers, 207-216, Copyright (2013), with permission from Elsevier.

With the failure of amyloid-targeting clinical trials (Section 1.7.1), attention has shifted towards alternative disease aggravating mechanisms that may contribute to disease initiation and progression, especially in the case of sporadic AD. As a result, a number of alternative hypotheses and clinical trial strategies for AD have emerged[245] including the cholinergic hypothesis (a thorough review of the development of this hypothesis is provided by Hampel *et al.* (2019))[246-249] the inflammation hypothesis,[98] the antimicrobial protection hypothesis,[145] the mitochondrial cascade hypothesis,[250] the calcium homeostasis hypothesis,[251] among many others. Indeed, there also exist sex differences in AD, with women representing 65% of total deaths due to dementia,[11] although this will not be elaborated on here, for a key review on this topic see Ferretti *et al.* (2018).[252]

Although beyond the scope of this thesis to discuss all other hypotheses, some are summarized in Figure 1.7 and Figure 1.8,[143] while those most relevant to this thesis are described in greater length.

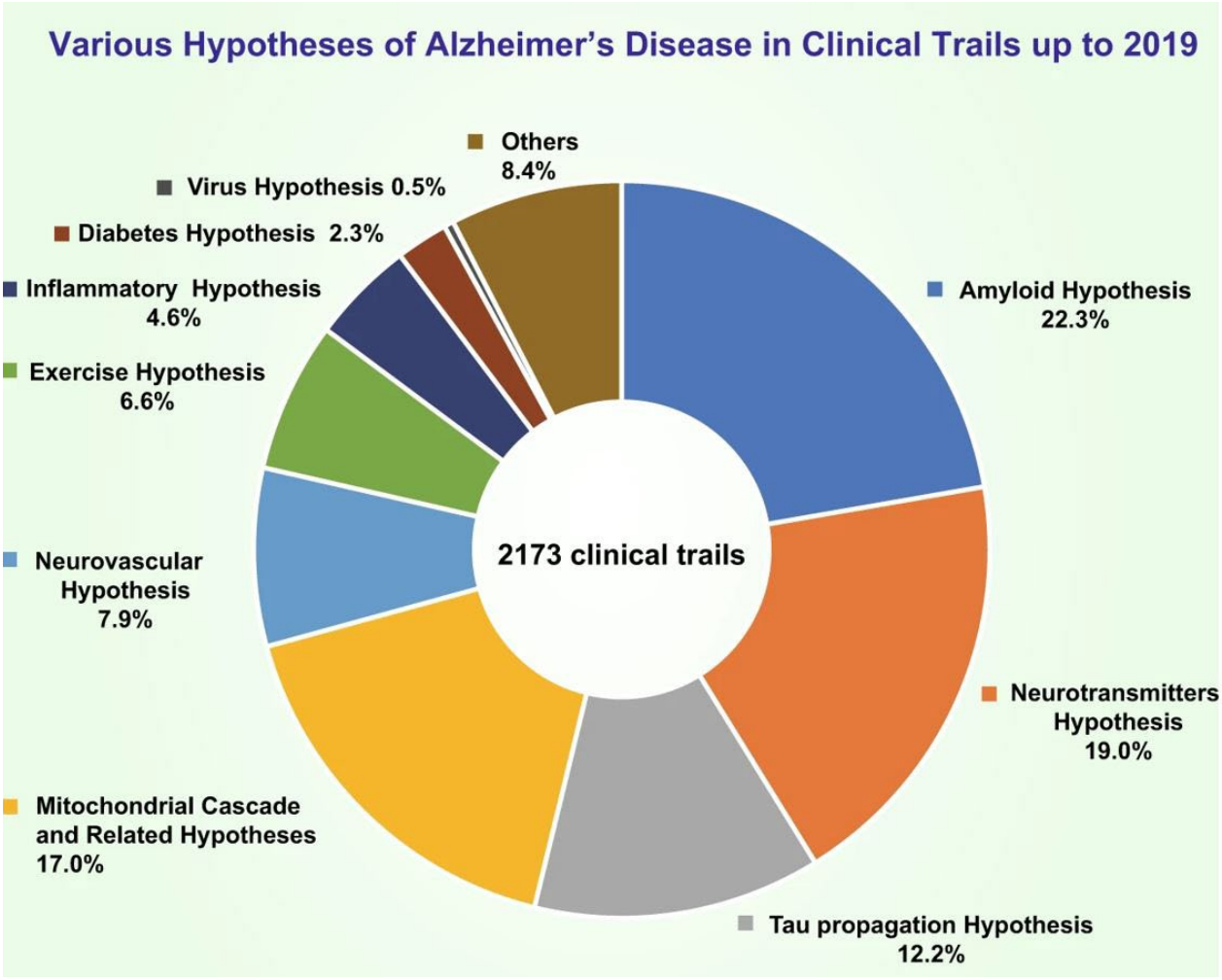


Figure 1.7: Clinical trials and their relation to alternate AD hypotheses 2019.
Adapted from Liu *et al.* 2019,[143] showing alternate hypotheses of Alzheimer's disease that are being tested in clinical trials as of 2019. Reprinted from Springer Nature, Nature Signal Transduction and Targeted Therapy, Vol. 4, Liu PP, Xie Y, Meng XY, *et al.*, History and progress of hypotheses and clinical trials for Alzheimer's disease, 1-22, Copyright (2019).

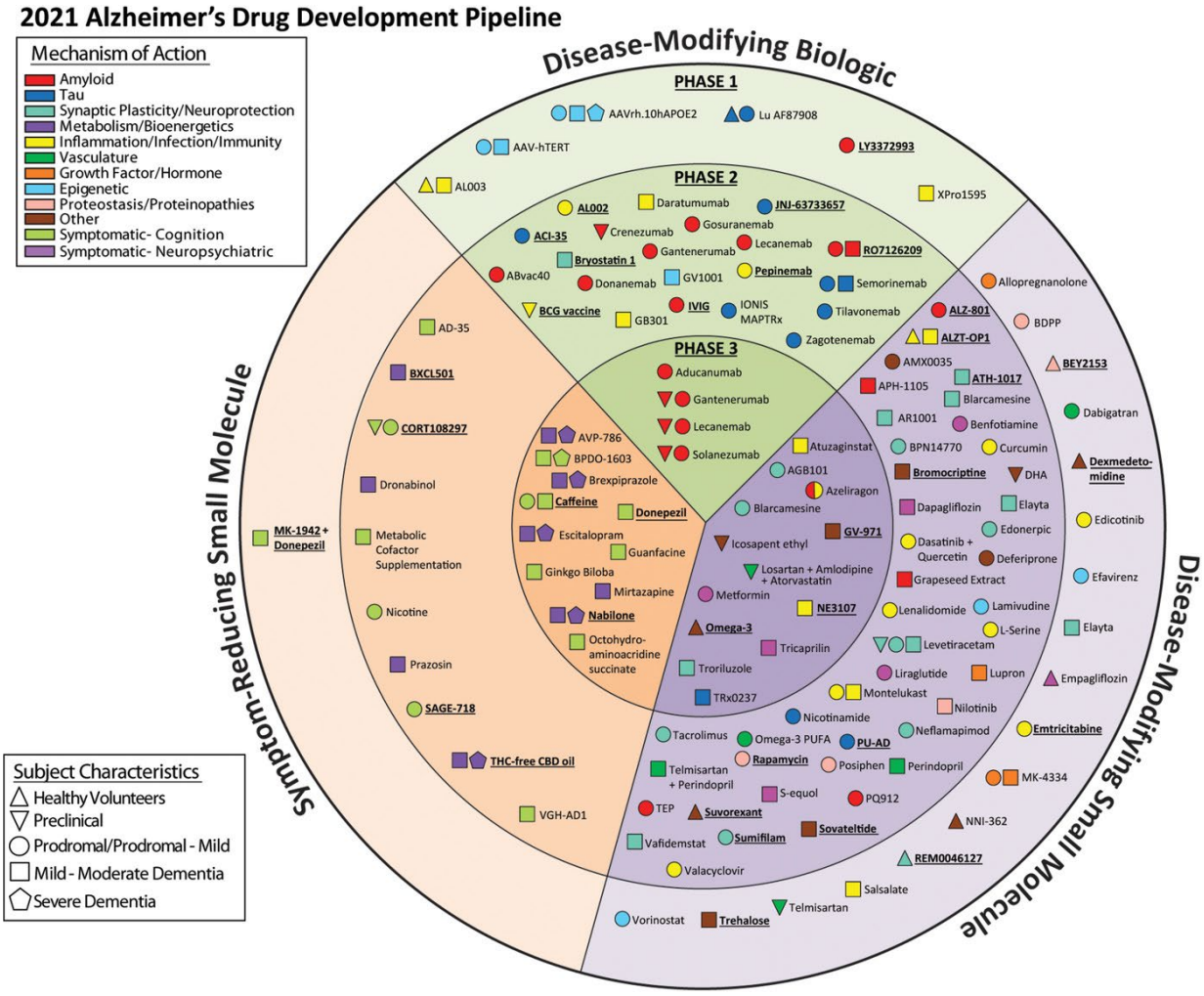


Figure 1.8: Clinical trials for Alzheimer's disease 2021.

Adapted from Cummings *et al.* 2020,[253] showing a detailed representation of clinical trials and stages of trials (Phases 1-3) for Alzheimer's disease therapeutics that test various alternative hypotheses of AD (identified by the 'mechanism of action' box). Reprinted with permission from Wiley Periodicals, Inc. on behalf of Alzheimer's Association, Alzheimer's & Dementia: Translational Research & Clinical Interventions, Vol. 6, Cummings J, *et al.*, Alzheimer's disease drug development pipeline: 2020, e12050, Copyright (2020).

I.8.1 Mitochondrial Cascade Hypothesis

The mitochondrial cascade hypothesis was first introduced by Swerdlow and Khan in 2004 and aimed to address the etiology of sLOAD.[250] It was based on experimental evidence showing that mitochondria from AD individuals differed both structurally and functionally from those of non-AD individuals.[254] The original paper emphasized that the efficiency of the electron transport chain (ETC) determined the basal levels of mitochondrial reactive oxygen species (ROS) in the cell and over the course of the human life span, this mitochondrial ROS (determined by both genetic and environmental factors) would lead to subsequent damage to DNA, RNA, lipids and proteins. When this damage surpassed a critical threshold, cellular responses culminated in the AD pathology observed in sLOAD.[250] The hypothesis claimed that each individual's genes encoding ETC proteins (both nuclear and mitochondrial DNA) determined their baseline mitochondrial function, ROS levels, and resilience, which in turn defined how their mitochondria changed with age – if mitochondria accumulated damage at a faster rate, the critical threshold would be surpassed sooner, initiating pathological mechanisms.[255] This hypothesis was revisited recently and acknowledged that current data supports both a primary and secondary (consequential) role for the mitochondrial cascade hypothesis in AD, further emphasizing that both the amyloid and mitochondrial hypotheses could co-exist. The authors also extended its scope to the influence of non-brain mitochondria and differences observed between AD and non-AD individuals.[254]

I.8.2 The Free Radical Theory of Aging and the Oxidative Stress Hypothesis

The oxidative stress hypothesis for AD was influenced in part by the free radical theory of aging (FRTA) which was coined by Harman in 1954 and based on work by Rebeca Gerschman and Daniel Gilbert.[256-257] This theory outlined that the aging process is driven by accumulation of oxidative damage from excessive ROS over time and has since undergone scrutiny in recent decades due to conflicting evidence regarding the benefit of antioxidants for longevity.[258] The FRTA overlooked complexities related to redox regulation that were not in existence at the time of its conception,[259] but despite the shortcomings, this theory encouraged key research on aging and oxidative stress and was updated to include the role of mitochondria in aging (mitochondrial MFRTA).[260-261] However, as reviewed by Stuart *et al.* (2014) the MFRTA also requires a more nuanced perspective on the role of ROS in redox signaling rather than solely on the potential for oxidative damage.[262] Harman additionally suggested a role for this theory in AD, especially when considering that age is the greatest risk factor of AD.[263]

The oxidative stress hypothesis was formulated based on evidence of elevated oxidative stress in the AD brain compared to the non-AD brain, namely, (1) increased iron and copper contributing to Fenton chemistry and generation of ROS (discussed further in Section 1.10.1), (2) increased protein oxidation (3) DNA oxidation (Section 1.10.3.2) and (4) lipid peroxidation and associated by-products (Section 1.10.3.1) (5) dysregulated energy metabolism contributing to excitotoxicity and loss of calcium homeostasis leading to increased oxidative stress and (6) evidence of Aβ-induced free radical generation.[264-267] Importantly, at the time, it was acknowledged that the role of ROS in AD could both be a primary and secondary event. However, it was not yet known at the time, and is still unresolved, what the initiating factor was in AD.[264] This hypothesis extends to other neurodegenerative processes and similarly to the mitochondrial cascade hypothesis, which suggested a cumulative effect of oxidative damage over time accounting for the progressive nature and late-onset of sporadic AD.[264] Furthermore, since oxidative stress is known to increase with aging, and as aging is the primary risk factor for LOAD it is likely that oxidative stress would play a leading role.

I.9 A Refined Hypothesis – Synergy of Amyloid Beta, Inflammation, and Oxidative Stress

Since the coining of the amyloid hypothesis, evidence has accumulated against the notion that insoluble amyloid plaques are the main drivers of AD, namely that: (1) amyloid plaque deposition does not correlate with neuronal cell death, synaptic loss,[268] tau pathology or cognitive impairment,[269] (2) cognitively healthy individuals can have amyloid plaque deposits without AD, (3) soluble $A\beta_{42}$ levels better correlate with synaptic changes and cognitive impairment[133, 270-272] while plaques may instead serve as a means to sequester more toxic soluble Aβ species indicated by the observation that as plaques increase soluble species decrease[273] and (4) as mentioned in the previous section, amyloid-targeting therapies have resulted in numerous failures.

Soluble Aβ oligomers (also known as amorphous aggregates, micelles, protofibrils, prefibrillar aggregates, ADDLs, Aβ*56, globulomers, amylospheroids, "tAβ" (toxic soluble Aβ), "paranuclei," and annular protofibrils as reviewed in Glabe (2008)),[137] began to garner research interest when an early study by Oda *et al.* (1995) emphasized that smaller, soluble Aβ complexes which accumulate to form insoluble plaques, may exert neurotoxicity independent of plaques and in fact enhance Aβ-mediated toxicity based on their ability to diffuse.[268] Since this study, numerous groups have shown that soluble oligomeric $A\beta_{42}$ exerts enhanced toxicity to neurons *in vitro* and *in vivo*.[274-277] These oligomeric species were also shown to be produced intraneuronally as discussed in Section 1.4.1.[278] Indeed, intraneuronal Aβ would favor aggregation due to the higher metal ion concentrations, lower pH in certain organelles, and the limited space within the cell thus, increasing likelihood of interactions.[133] However, this notion of intracellular Aβ required much convincing in the scientific community.

Despite the initial focus on extracellular amyloid species in the form of plaques, concurrently, other researchers observed intracellular Aβ deposits *in vitro*[279-281], and *in vivo*.[282]. Later studies, also observed intraneuronal Aβ accumulation in AD animal models,[283-286] individuals with MCI,[132] DS,[134, 287] and AD[123, 288-290] with the prominent species detected to be $A\beta_{42}$[291] within neurons.[292] It should be acknowledged that controversy over the presence of intracellular Aβ existed since the earliest studies employed antibodies that had cross-immunoreactivity with APP and APP-cleavage products that would also be intracellular.[123, 133] As example, the commonly used antibody 6E10 antibody that is directed against residues 4-10 of Aβ also recognizes full-length APP, and CTFs. Since then, other more specific antibodies have been

developed to recognize Aβ while avoiding cross-reactivity with full-length APP,[133] although some of these antibodies still can recognize other cleavage products such as p3 (from α- the γ-secretase cleavage) or CTFs from β-secretase cleavage, there are others that are specific to Aβ.[293] Furthermore, aggregation-specific antibodies, recognizing monomers, oligomers, and fibrils also offered insight into the presence of intraneuronal Aβ.[294-295] Other evidence for intracellular amyloid accumulation was obtained through immunoEM studies showing the presence of $A\beta_{42}$ in endosomes,[296] mitochondria,[297-298] and neuronal projections.[132] Intraneuronal Aβ accumulation, prior to plaque formation, has been linked to cognitive and LTP deficits in mouse and rat models of AD.[284-285, 293, 299-300]

Shifting away from the classical amyloid hypothesis that postulated a central role for insoluble, extracellular Aβ plaques in driving AD, a refined hypothesis focused on **soluble, intracellular** Aβ as being a driver of AD has been formulated.[299] Importantly, these soluble Aβ peptides progressively accumulate during the extended pre-plaque stage of AD, eventually aggregating to form the well-known insoluble, extracellular plaques. This asymptomatic period, that spans decades in humans, offers a valuable therapeutic window. However, our understanding of soluble Aβ accumulation and the toxic mechanisms (inflammation, oxidative stress etc.) exerted by this accumulation at the earliest, silent, stages of AD is incomplete and could provide guidance towards combined preventative or established therapies.

The next step is to elucidate the exact mechanisms exerted by iAβ and the alternate hypotheses described above provide some guidance, namely, early accumulation of intraneuronal Aβ has been shown to coincide with inflammation and oxidative stress in mouse and rat models of AD, including our laboratory's transgenic rat model.[286, 301-303,219] However, the exact role of oxidative stress during the earliest stages of pre-symptomatic AD remains to be determined. Importantly, oxidative stress is linked to numerous aspects of the early, pre-plaque AD pathology: 1) inflammatory events can both be a cause or consequence of oxidative stress, 2) mitochondrial and metabolic dysfunction observed in AD are linked to oxidative stress, and 3) Aβ has also been known to directly result in ROS production. Lastly, aging, the largest risk factor for sLOAD is associated with elevated oxidative stress in the brain. Teasing apart the primary and secondary contributions of oxidative stress to the AD pathology is a great significance. However, it is essential to assess these contributions on multiple levels from different perspectives. Since familial AD models provide a starting point and framework for studying the AD pathology in a controlled manner, Aβ-induced oxidative

stress can be studied using these models with the knowledge then extended and tested in other AD models before extrapolating to the human condition. Indeed, it is possible also that future preventative or therapeutic strategies will move beyond monotherapies and become more effective through combined treatments, targeting multiple aspect of the pathology. The next section will cover essential concepts related to oxidative stress and how to study it in the context of AD.

I.10 Oxidative Stress in Alzheimer's Disease

The term reactive oxygen species (ROS) refers to reactive molecules derived from oxygen that act as oxidizing agents such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) among others.[304-305] ROS can include oxygen radicals as well as nonradicals (two-electron oxidation) that can be converted into radicals. Different ROS are often derived from each other or exist in an equilibrium with one another, making it irrelevant to consider each of them in isolation.[306] However, equally so, each ROS molecule is unique and exhibits varying reactivities within the cell, as determined by availability of substrates and the rate constants of those reactions, which influences their diffusion radii (Figure I.9). This is an important detail since in a number of publications, the generic use of the term ROS and the grouping together of these unique molecules does not allow for a biologically significant understanding of their chemical mechanisms and often oversimplifies conclusions.[306] Other factors that determine how each ROS impacts the cell include, whether the ROS is membrane permeable, if there are concentration gradients across membranes, the concentrations of antioxidants, and where the ROS is produced, among other factors.[307-308] These characteristics make ROS elusive, as they are short-lived species implicated in both physiology through redox signalling (for extensive reviews on the physiology of redox signaling see Forman *et al.* (2014) [309] as well as Sies and Jones (2020))[310] and pathology through oxidative stress and damage.[311] As example, hydrogen peroxide (H_2O_2) has a lower reactivity compared to other ROS and thus, can diffuse great distances across cellular membranes, exerting widespread effects on biomolecules (Figure I.9). Importantly, reactive nitrogen species (RNS) also play a key role in health and disease. For a comprehensive review of RNS see.[312-313]

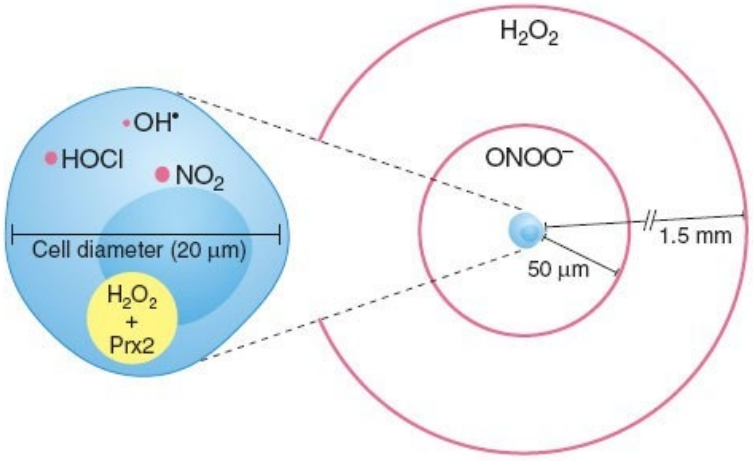


Figure 1.9: Diffusion distances for different ROS.
Adapted from Winterbourne 2008,[308] showing the diffusion distances of different cellular oxidants, assuming a cellular glutathione concentration of 2 mM. The yellow circle indicates the diffusion distance of hydrogen peroxide when in the presence of peroxiredoxin 2 (Prx2) at a concentration of 20 μM. For added details related to how these distances were located refer to Winterbourne 2008. Reprinted with permission from Springer Nature Customer Service Centre GmbH: Nature Chemical Biology, Vol. 4, Winterbourn C, Reconciling the chemistry and biology of reactive oxygen species, 278-286, Copyright (2008).

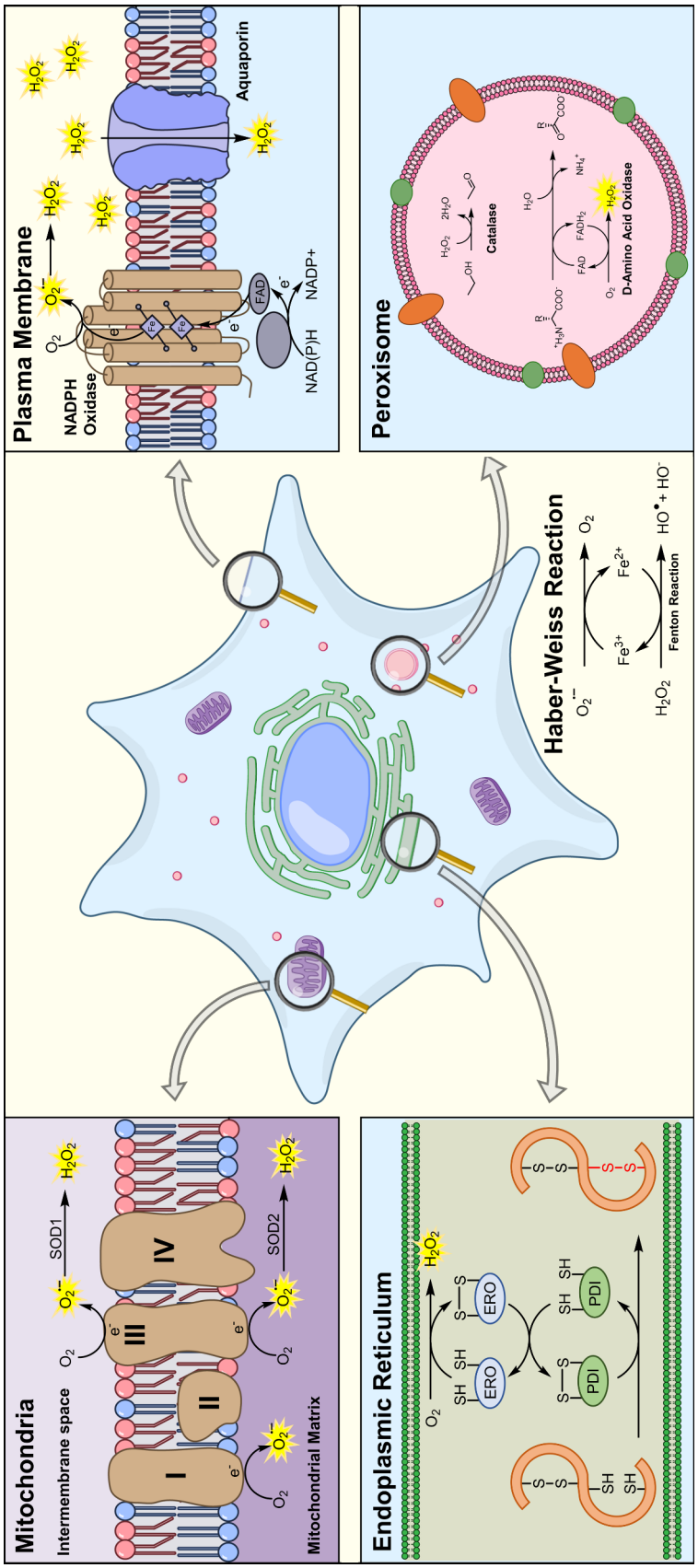
Oxidative stress was coined by Helmut Sies in 1985 and describes the imbalance between oxidant production and the antioxidant defences that control oxidant levels. This imbalance results in oxidative damage to proteins, lipids, DNA, RNA, and other biomolecules, ultimately disrupting cellular functions.[314] Notably, imbalances in ROS are upstream of subsequent oxidative damage (Section1.10.3), while antioxidants and cellular repair mechanisms (Section 1.11) serve to protect the cell from excessive ROS and oxidative damage. In the context of Alzheimer's disease (AD), oxidative stress and oxidative damage have been implicated at later, post-plaque stages, while alternative hypotheses related to the etiology of AD have also considered the importance of oxidative stress in the AD pathology. Therefore, questions remain regarding the earliest role of neuronal oxidative stress during pre-plaque stages. Notably, neurons are particularly vulnerable to oxidative stress for a number of reasons:

- High brain O_2 concentration[315]
- Large metabolic demand of neurons[316]
- Elevated levels of polyunsaturated fatty acids (PUFAs) in neuronal membranes which are specifically vulnerable to lipid peroxidation[315]
- Low ratio of antioxidant to pro-oxidant enzymes in the brain[315, 317-318]
- High brain iron content[315] (e.g.: oligodendrocytes contain the highest levels of iron compared to other cells in the brain)[319-320]
- Reliance on error-prone DNA repair pathways such as non-homologous end joining (NHEJ) as opposed to replication-associated DNA repair[316]

The subsequent sections will outline essential background information on ROS and oxidative stress and how they directly relate to AD.

I.10.1 Major Cellular Sources of ROS

Since ROS are short-lived due to their reactivity, the sites of ROS production in the cell determine what molecules and cellular functions are potentially disrupted or vulnerable to oxidative insult. The major sources of ROS in the cell include mitochondria, the plasma membrane, the endoplasmic reticulum, and peroxisomes (Figure 1.10).



Caption on next page.

Figure 1.10: Major cellular sources of ROS.
Adapted from Foret *et al.* 2020.[321] Reprinted with permission from Chemical Reviews, Vol. 120, Foret MK, Lincoln R, *et al.*, Connecting the "Dots": From Free Radical Lipid Autoxidation to Cell Pathology and Disease, 12757-12787, Copyright (2020) American Chemical Society.

Mitochondria generate ROS when electrons leak from the electron transport chain (ETC) during mitochondrial respiration. Most often they leak from complex I (NADH-ubiquinone-cytochrome reductase) or complex III (ubiquinone-cytochrome reductase) and subsequently react with molecular oxygen to form superoxide.[322] Once superoxide is generated, it is converted to hydrogen peroxide by superoxide dismutase (SOD) enzymes. SOD enzymes have three isoforms located in different cellular compartments, SOD1 is cytoplasmic and in the mitochondrial intermembrane space, while SOD2 is in the mitochondrial matrix and SOD3 is extracellular. Notably, quantitative estimates of superoxide produced by the body are often exaggerated due to confounding experimental conditions. Regardless, the brain accounts for 20% of bodily oxygen consumption as neurons have high energy requirements, resulting in considerable superoxide production and highlighting the vulnerability of neurons to oxidative stress.[306, 321, 323] Mitochondria also generate ROS through oxidative folding of certain proteins in the intermembrane space. This process of protein folding is termed the mitochondrial intermembrane space import and assembly (MIA) system.[324-327]

At the **plasma membrane** NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX) enzymes which are multiprotein complexes that extracellularly produce superoxide from molecular oxygen or in the case of NOX4 hydrogen peroxide is generated from superoxide.[306] In mammals, there are seven different NOX isoforms including NOX1-5, DUOX1 (dual oxidase), and DUOX2, each isoform is comprised of different subunits which have key phosphorylation sites, the isoforms have varied tissue expression, cellular localization, and differing activation mechanisms from transcriptional regulation to responsiveness to cellular calcium (Ca^{2+}) concentrations.[328-329] Originally, NOX2 was discovered in neutrophils as it played a key role in innate immunity and the phagocytosis of invading microbes, also termed 'respiratory burst'.[330] More recently, there is emerging evidence for a role of NOX enzymes in the CNS cell types including neurons, astrocytes and microglia as part of redox signalling functions.[306, 329, 331]

In the **endoplasmic reticulum** where oxidative protein folding occurs, hydrogen peroxide is produced when cysteine residues of proteins are oxidized to form disulfide bonds. This process involves both the protein disulfide-isomerase (PDI) enzyme and the flavoprotein oxidase ERO (ER oxidoreductin).[306]

Similarly, in **peroxisomes**, hydrogen peroxide is produced as a by-product of lipid catabolism such as β-oxidation of fatty acids.[332] Furthermore, these different sources of ROS are linked whereby peroxisomes, mitochondria and ER membranes form contact sites with ROS-driven intercompartmental signaling pathways.[333]

Many of these sources of ROS generate superoxide (radical oxidant) and hydrogen peroxide (non-radical, or two-electron oxidant). Superoxide is not as reactive as its name implies,[334] as example, the reaction rate constant of superoxide with superoxide dismutase is high ($>10^9$ M^{-1} s^{-1})[335] thus, few other reactions can out-compete this rate indicating a primary role for superoxide as a precursor to hydrogen peroxide for redox signalling rather than a cellular oxidant.[309] However, superoxide is unique and can act as a reductant, where it can outcompete reactions with superoxide dismutatses by reacting with nitric oxide at a reaction rate of $\sim 10^{10}$ M^{-1} s^{-1} to produce peroxynitrite, which is an especially harmful oxidant in the cell if produced at sufficient levels.[306] Furthermore, in the context of extracellularly released superoxide, in the absence of sufficient SOD, reactions with other substrates may prevail.[309] Other one-electron oxidation reactions by superoxide can also occur with iron/sulfur proteins including aconitase and dehydrastases which results in disrupted enzyme function but also the release of iron and hydrogen peroxide which can initiate Fenton reactions.[306] Although hydrogen peroxide is not particularly reactive when compared to other ROS, when in the presence of transition metals such as iron or copper,[336] reductive cleavage of hydrogen peroxide generates the most oxidizing radical in biological systems, the hydroxyl radical (HO•),[337] by the Haber-Weiss reaction. As a first step, in the presence of iron (III), superoxide is oxidized to molecular oxygen resulting in production of iron (II). Then next step, involving the Fenton reaction, proceeds when iron (II) is oxidized back to iron (III) by hydrogen peroxide, thus forming a hydroxyl radical and a hydroxide.[338-339] Overall, the net reaction is the reduction of hydrogen peroxide to a hydroxyl radical by superoxide with molecular oxygen (O_2) as a by-product. Importantly, there is an abundance of literature investigating the role of iron storage and metabolism and how dysregulation generates oxidative stress[336] during late, post-plaque stages of AD, as well as in transgenic rodent models of AD.[340-341] Notably, brain iron content increases with age and correlated with decreased cognitive function in AD,[342] while iron deposits were associated with the characteristic amyloid plaques (as high as ~1 mM of Fe(III))[343] and neurofibrillary tangles in the AD brain.[344] Additionally, iron regulatory protein 2 (IRP-2) but not IRP-1 is increased in the AD brain and associated with senile plaques and neurofibrillary tangles.[340] IRPs control intracellular iron homeostasis by regulating the iron

storage protein ferritin.[345] However, whether iron dyshomeostasis is important for driving the early pre-plaque AD pathology is unresolved.

I.10.2 Lipid Chain Autoxidation

Lipid chain autoxidation is a form of lipid peroxidation caused by oxidant attack (free-radical mediated) on unsaturated lipids (containing carbon-carbon double bonds) to form lipid hydroperoxides. Importantly, lipid peroxidation can also be mediated by enzymes, namely, cyclooxygenases (COX),[346] lipoxygenases (LOX),[347-348] and cytochrome P450 (CYP450)[349] enzymes which generate several lipid-derived compounds such as oxylipins that exert widespread effects on the cell. However, these enzymatic pathways will not be discussed here.

As outlined previously (Section I.10.1), sites of ROS production in the cell occur in proximity to lipid membranes. This is of relevance to AD because certain lipids are vulnerable to free radical attack, especially polyunsaturated fatty acids (PUFAs) which are prominent in neuronal membranes.[315] PUFAs are susceptible to free radical attack because, compared to saturated lipids that have H-C bonds of 100 kcal/mol, PUFA H-C bonds that are adjacent to carbon-carbon double bonds have a lower bond dissociation energy (BDE) of an estimated 80 kcal/mol.[350] This process is especially detrimental to the cell as it is self-propagating, therefore, once initiated lipid membranes are vulnerable to modification until the substrates are consumed or the process is terminated. There are three main steps of lipid chain autoxidation namely, 1) initiation, 2) propagation, and 3) termination or inhibition (Figure I.11).[321, 351] During initiation, a free radical, such as a hydroxyl radical, abstracts a hydrogen atom from the bis-allylic position (adjacent to carbon-carbon double bonds) on the lipid chain resulting in a carbon-centered radical. This is followed by propagation, when the carbon-centred radical (L•) reacts with molecular oxygen at near diffusion-controlled rates to form a lipid peroxyl radical (LOO•). The resulting lipid peroxyl radical can then propagate the chain reaction by abstracting a bis-allylic hydrogen atom from an adjacent PUFA, thus generating a lipid hydroperoxide (LOOH) and a newly formed carbon-centred radical. Propagation is the rate-limiting step of lipid chain autoxidation since abstraction of the hydrogen atom by lipid peroxyl radicals is relatively slow.[352] Lastly, termination can occur when two lipid peroxyl radicals react to yield nonradical products. More commonly, inhibition occurs through the action of radical trapping antioxidants (RTAs). As an example, the lipophilic RTA α-tocopherol (also known as vitamin E), has an O-H bond with a bond dissociation energy (BDE) of 78 kcal/mol, allowing for abstraction of this hydrogen atom which outcompetes abstracton of a hydrogen atom on another PUFA (BDE 80 kcal/mol).

This single hydrogen atom abstraction results in a phenoxyl radical (since the O-H on α-tocopherol is on a phenol) which then couples with another lipid peroxyl radical (LOO•). Overall, the net reaction allows for one α-tocopherol to scavenge two lipid peroxyl radicals, generating one lipid hydroperoxide and the two-electron oxidized, nonradical α-tocopherone (Figure 1.11). This process of lipid chain autoxidation ultimately damages lipid membranes, impairing membrane fluidity and function.

Notably, some of the lipid hydroperoxides generated are converted to more stable products including isoprostanes and neuroprostanes, of which there are many isoforms. These metabolites can serve as *in vivo* biomarkers of oxidative stress in neurodegenerative diseases and levels were found to be elevated in CSF and brain tissue of AD patients.[353-355] Other studies have also investigated isoprostane levels in plasma and urine of NCI, MCI, and AD patients although these peripheral quantifications are also sensitive to diet, exercise, and body mass index making them more susceptible to confounding factors[356-359] with technical challenges highlighted in a review by Halliwell and Lee (2009).[360] Indeed, isoprostanes are also shown to increase in other neurodegenerative diseases, therefore, rather than adding diagnostic value, they may offer prognostic value through longitudinal monitoring of patients.[355] Importantly, although isoprostanes are prostaglandin-like compounds, they are formed from free-radical initiated oxidation of arachidonic acid and not enzymatically, whereas prostaglandins are formed by COX-dependent oxidation.[360]

Alternatively, the lipid hydroperoxides are converted to reactive by-products such as lipid-derived electrophiles (LDEs) by a variety of mechanisms.[361-362] These reactive by-products can further react with and damage biomolecules in the cell as outlined in Section 1.10.3.1. The oxidative damage and adducts formed by these by-products have also been extensively studied in the AD brain.

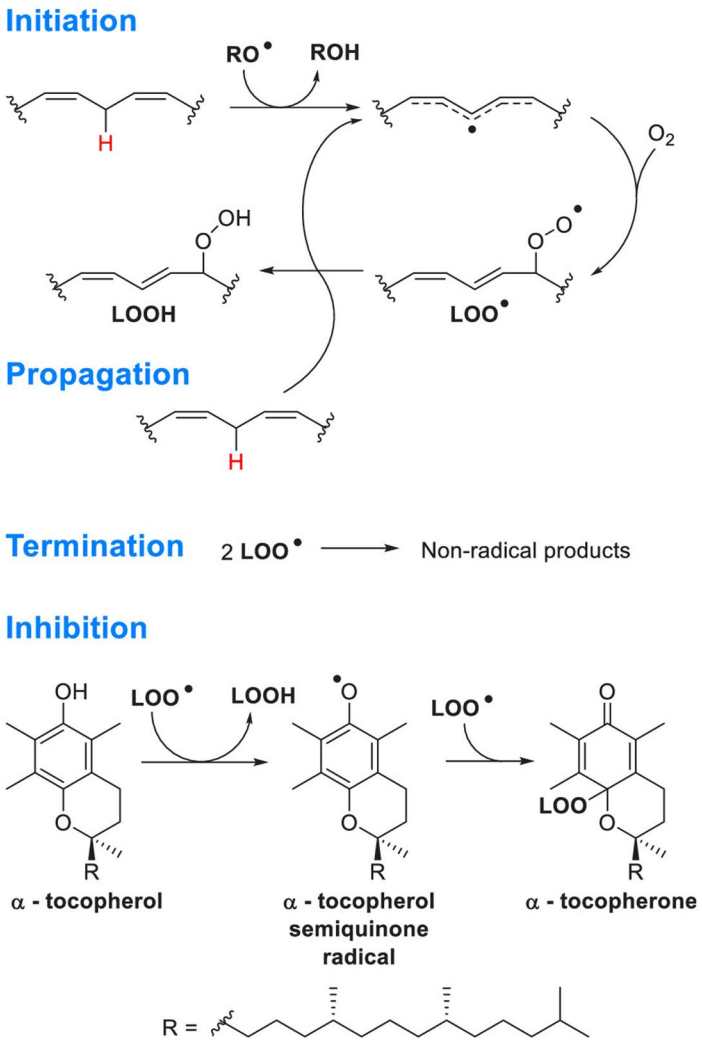


Figure 1.11: Lipid chain autoxidation.

From Foret *et al.* 2020.[321] Figure showing the four steps of free radical initiated lipid peroxidation (lipid chain autoxidation). Reprinted with permission from Chemical Reviews, Vol. 120, Foret MK, Lincoln R, *et al.*, Connecting the "Dots": From Free Radical Lipid Autoxidation to Cell Pathology and Disease, 12757-12787, Copyright (2020) American Chemical Society.

I.10.3 Oxidative Damage in Alzheimer's Disease

Aging is typically associated with increased oxidative damage including protein oxidation[363-365, 366] and DNA damage.[367] However, a number of studies have also reported the presence of oxidative damage to proteins, lipids, and nucleic acids (DNA and RNA), in both transgenic animal models of AD[368-378] and in the brains of individuals with AD.[379-392] More recently, oxidative damage has been investigated in the brains of individuals with during MCI,[393-395] and with DS,[396-397] especially since chromosome 21 has a number of genes implicated in redox homeostasis.[398-399] There are key markers of oxidative damage to various biomolecules that are outlined in the context of AD in the following sections,

I.10.3.1 Oxidative Damage to Lipids and Proteins – Reactive Electrophiles

As mentioned in Section I.10.2 non-enzymatic (lipid chain autoxidation) and enzymatic lipid peroxidation generate reactive by-products known as lipid derived electrophiles (LDEs). One of the most studied LDEs is 4-hydroxy-2-nonenal (4HNE), an α,β-unsaturated aldehyde, which was discovered in the 1960s[400] and is now known to be a toxic second messenger to lipid peroxidation but also serves as a signaling molecule for responding to oxidative stress.[401-403] Depending on the concentration and site of 4HNE production, different redox signaling pathways can become activated.[404] Lipid derived electrophiles (LDEs) such as 4HNE react with cellular nucleophiles such as cysteine, lysine, or histidine residues in proteins resulting in protein adducts such as Michael adducts, Schiff-bases, or protein crosslinking.[321, 402, 405] The hard-soft acid base model serves to explain the preference for either Michael adduct or Schiff-base formation. Simply put, hard species have a high charge density, making them more difficult to polarize (positive for electrophiles, negative for nucleophiles), while soft species have a diffuse charge density and are easier to polarize.[406-407] Notably, 4HNE is a soft electrophile with a diffuse charge density and reacts efficiently with cysteine which is also a soft nucleophile via Michael addition, this characteristic makes cysteine residues on proteins especially vulnerable to 4HNE attack. Conversely, the amine of lysine is a hard base and instead forms a Schiff-base product.[321] Numerous other LDEs are formed through lipid peroxidation including malondialdehyde (MDA), acrolein, and 4-oxo-2-nonenal (ONE).[408] As with 4HNE, these other electrophiles can form protein adducts that ultimately disrupt cellular function.[409] In the case of ONE, this reactive electrophile was observed to be more neurotoxic than 4HNE which was attributed to the different, more rapid characteristics of reactivity, indeed, ONE is a softer electrophile and thus will react more readily with cysteine.[410]

In Alzheimer's disease, oxidative damage caused by LDEs has been observed in transgenic animal models of AD,[368-378] in the brains of individuals with Down Syndrome (DS),[396-397] mild cognitive impairment (MCI),[393-394] and AD.[379-389] Aside from global increases in 4HNE, many studies have also investigated which proteins are modified in the AD brain, some of note include neprilysin (degrades Aβ),[411] and as reviewed by Butterfield and Halliwell (2019) glycolytic enzymes, tricarboxylic acid cycle (TCA) and ATP biosynthesis (thus contributing to decreased metabolism and ATP production).[311] As well, although this thesis primarily focuses on amyloid, tau can also undergo modifications by 4HNE which contributes to filament formation. A note of caution, that numerous studies have used the thiobarbituric acid-reactive substances (TBARS) assay for detecting lipid peroxidation products such as MDA.[412] However, as reviewed by multiple authors in the redox community, TBARS is non-specific when used with biological samples[413] since these samples contain other metabolites that can react with the reagent, resulting in overestimation of MDA due to lack of specificty.[355, 414]

Overall, the AD brain is burdened with oxidatively modified proteins resulting from lipid peroxidation and LDEs.[415-416] However, this form of oxidative damage is downstream of initiating ROS and propagating lipid peroxyl radicals, therefore, it is important to understand at what timepoint in the pathology these upstream ROS and lipid peroxyl radicals are elevated in the earliest amyloid pathology, as well as what cell types or cellular locations (eg: axons, dendrites etc) are most affected.

I.10.3.2 Oxidative DNA Damage

Every day, an estimated ~10^5 DNA lesions occur in the mammalian genome and ~10^4 of these are oxidized bases and single strand breaks.[417] When unrepaired, these lesions can be mutagenic, whereby C → 5-OHU lesions cause G-C → A-T transitions, G → 8-oxoG lesions cause G-C → T-A transversions. SSBs can also result in nonligatable termini such as 3' phosphoglycolate, 3' phosphoglycolaldegyde, 3' phosphate and/or 5'OH, 5' phosphosugar derivatives.[417]

Over 20 oxidized base adducts can be formed by ROS reactions with DNA, where the most common adduct is 8-hydroxyguanine (8-OHG).[418] This is because guanine has the lowest oxidation potential of the DNA bases and thus, it is readily oxidized[419]. Oxidation of C8 on guanine results in a C8-OH adduct radical that then follows different pathways: (i) ring opening then reduction to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua); (ii) reduction of the C8-OH to 7-hydro-8-hydroxyguanine that undergoes ring opening to form

FapyGua; (iii) oxidation of C8-OH to form 8-OHG.[418] For a comprehensive review on oxidative DNA damage and reaction mechanisms refer to Cooke *et al.* (2003)[420] and Cadet and Wagner (2013).[421]

In the context of AD, broadly, DNA (both nuclear (nDNA) and mitochondrial (mtDNA)) and RNA damage was observed to be increased in different brain regions and disease stages. Importantly, mtDNA is especially susceptible to oxidative insult due to lack of protection, as there are no histones like with nDNA,[422] mtDNA is also in close proximity to the ETC where ROS such as superoxide are produced and as a result mtDNA undergoes 5-10 fold more damage that nDNA,[423-424] as well as 20-100 fold higher mutation rates than nDNA.[425] Indeed, 8-oxo-dG was found to be increased at a faster rate in brain mtDNA compared to nDNA with normal aging in humans.[426] In AD patients, an early study found a 2-fold increase in DNA strand breaks in the cerebral cortex of AD patients compared to controls.[427] Later, another group reported elevated levels of 8-oxo-dG in mtDNA but not nDNA in the parietal cortex of AD brains.[428] However, later studies that assessed more brain regions and various nucleic acid oxidation adducts including 8-hydroxyadenine, 8-oso-dG, 5-hydroxycytosine, thymine glycol, Fapy-guanine, Fapy-adenine, 5-hydroxyuracil, and reported elevated levels these oxidative DNA adducts in both brain mtDNA[392] and nDNA[429] in AD patients.[390-392] RNA oxidation, specifically 8-oxo-G, was also found to be increased in the hippocampus of AD brains compared to controls,[430-431], and in the brains of individuals with DS as assessed by immunohistochemistry (IHC).[432] Notably, as with mtDNA, RNA is also without histones, is exposed as single strands, and ribosomal RNA is close to mitochondria and therefore ROS.[433] During MCI stages, both nDNA[395] and mtDNA[395] from different brain regions had elevated levels of oxidative damage, as did RNA.[434] Other studies also assessed oxidative DNA and RNA damage during pre-clinical AD (PCAD) stages, which were defined as being non-cognitively impaired but with AD pathology, and showed elevation in oxidative DNA damage[392, 418] but had conflicting results for RNA damage where one study reported elevated damage during PCAD[418] while another reported no elevation during PCAD.[423]

Lipid derived electrophiles such as 4HNE, ONE, and acrolein (Section 1.10.3.1) can also react with DNA forming adducts (Figure 1.12) which are also elevated in the AD brain.[435-436] The highly reactive, α,β-unsaturated aldehyde, 4-hydroxy-2-nonenal (4HNE), can react with deoxyguanosine via Michael addition on the exocyclic amino group, subsequently through ring closure of N-1 onto the aldehydic group to form the bulky exocyclic 1-N^2-propanodeoxyguanosine adduct.[434] Deoxyguanosine over the other three nucleosides has the most reactivity to 4-HNE, and second most reactive was dA.[436]

Importantly, when comparing results of the aforementioned studies, they differ in a number of components that makes comparison difficult including but not limited to: post-mortem interval, technique for oxidative adduct detection from mass spectrometry, HPLC (high pressure liquid chromatography), to IHC, number of samples per experimental group, brain regions studied, and oxidative adducts assessed. Regardless, it is clear that the MCI and AD brain is burdened with oxidative nucleic acid damage. Looking forward, new methods for quantifying and mapping DNA damage[437] are emerging and overcome technical challenges, thus, allowing for identification of vulnerable regions in the genome. Already, these techniques have revealed that DNA damage distribution is highly dependent on chromatin and transcription factor binding and are summarized in a review by Mingard *et al.* (2020).[419, 438]

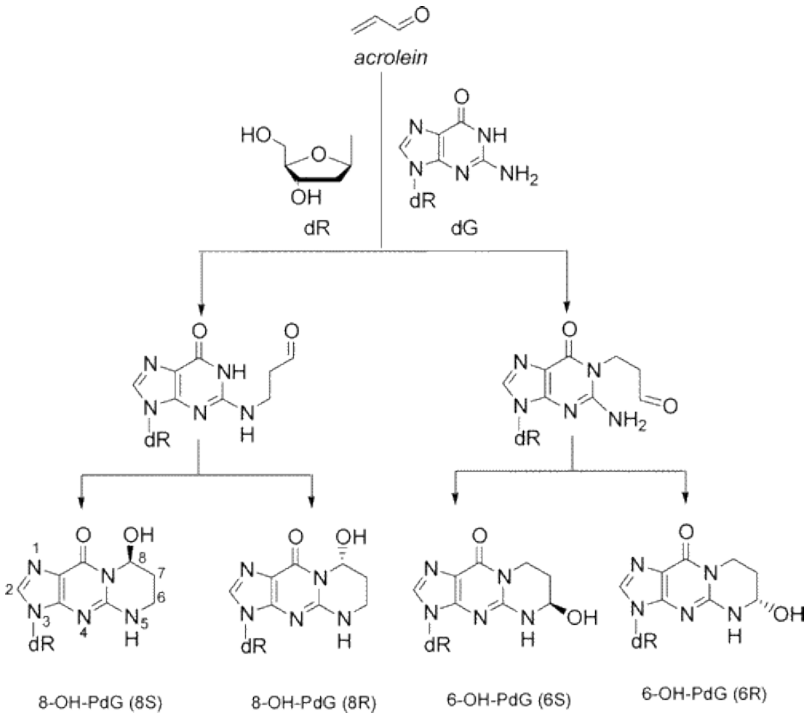


Figure 1.12: DNA adducts formed from the reactive electrophile acrolein.
Reprinted with permission from Analytical Chemistry, Vol. 77, Liu X, Lovell MA, Lynn BC, Development of a method for quantification of acrolein− deoxyguanosine adducts in DNA using isotope dilution-capillary LC/MS/MS and its application to human brain tissue, 5982-5989, Copyright (2005), American Chemical Society.

Oxidative damage by ROS or LDEs resulting in adducts can also lead to more severe types of DNA damage such as single-stranded DNA breaks (SSBs) and double-stranded DNA breaks (DSBs), especially when oxidative lesions are clustered.[439] Notably, formation of SSBs compared to DSBs occurs at a ratio of 2000:1.[439-440] DSBs can also occur during transcription, meiosis, and replication stress.[419, 441]

When DNA double strand breaks (DSBs) occur, the histone variant H2AX is rapidly phosphorylated at serine 139 to form γ-H2AX,[442] which then accumulates at DSBs as foci.[443-445] These γ-H2AX positive foci are one of the earliest markers of DSBs and can be visualized by immunofluorescence.[446-447] Studies conducted in AD mouse models showed that 1.5-month and 6-month old heterozygous APP (hAPP) J20 mice, the latter exhibiting cognitive deficits, had higher baseline levels of neuronal γ-H2AX foci in hippocampal and cortical neurons.[448] This study also showed that neuronal activity, stimulated by exploratory behavior, increased DSBs in the dentate gyrus (DG) of Wt and hAPP J20 mice as indicated by increased γ-H2AX foci. Foci numbers returned to baseline levels 24-hours after behavioral experiments in Wt mice but did not fully recover in hAPP J20 mice.[448] Similarly, in AD brains there were elevated numbers of γ-H2AX positive foci in both MCI and AD brains compared to cognitively healthy individuals.[446] Indeed, when assessing DSBs by γ-H2AX foci, one needs to consider any confounding factors such as cell cycle staging and replication induced DSBs among other important technical considerations as outline by Lobrich *et al.* (2010).[447] As neurons are post-mitotic this is less likely to create issues. However, there is evidence that at later disease stages of AD, neurons may re-enter the cell cycle.[449]

These studies clearly show that oxidative damage is elevated in the MCI and AD brain. However, the earliest contributions of oxidative damage and the upstream ROS during pre-symptomatic, pre-plaque stages remain to be known. This includes both what types of ROS and oxidative damage are elevated first in AD and in which cell types are affected first. Before tackling these pertinent questions, the next section briefly discusses key antioxidant defense and repair mechanisms that cells use to combat ROS and oxidative damage.

I.11 Antioxidants and Repair Mechanisms

Normal cellular function relies on a redox balance between ROS production, LDE/adduct formation, and subsequent elimination and detoxification by antioxidants or repair mechanisms. Antioxidants are substances that, at low concentrations compared to those of an oxidizable substrate,[413] prevent or remove oxidative modifications on substrates, and their capacity to do so is highly dependent on the molecular context.[306] As example, antioxidants may be lipophilic like vitamin E (α-tocopherol) or hydrophilic such as vitamin C (ascorbate) or glutathione (GSH) and therefore, they can protect against lipophilic or hydrophilic ROS to different degrees. Furthermore, antioxidants are only protective if their reaction products are less reactive than the initial substrate, this highlights the complexity of the concept of redox balance and the role of antioxidants in health and disease.[306] Forman *et al.* (2015) also highlighted that small molecules with claimed antioxidant activity, often cannot outcompete intracellular enzymatic antioxidants which have rate constants 5-fold higher than their nonenzymatic counterparts, with the exception of vitamin E (α-tocopherol).[414, 450] This highlights the importance of the context in which the antioxidant molecule is in and interpretations of data when assessing antioxidant activity. Finally, it should be noted that having more antioxidants is not always beneficial, physiological redox signaling is a balance between oxidants and antioxidants, thus too much of their entity can be disruptive to the cell.[451]

In terms of cellular enzymatic antioxidants in the cell,[452] these include: SODs (superoxide dismutases), catalase,[453-455] peroxiredoxins,[456-458] thioredoxins,[459-463] and heme oxygenase.[240, 464-467] While non-enzymatic antioxidants include glutathione (GSH), α-tocopherol, and ascorbate. GSH, also plays an important role in detoxifying LDEs, this occurs when glutathione-S-transferases (GST) catalyze the conjugation between GSH and the LDE. Studies have explored these antioxidants and detoxification mechanisms[468] as well as relevant redox signaling pathways which are additionally linked to inflammatory responses (e.g.: Nrf2[469] and NF-κB[470]) in the context of AD, with a number of these studies conducted at later disease timepoints.[471-472] Indeed, in our transgenic rat model of the AD-like amyloid pathology (Section 1.12) at post-plaque stages there was upregulation of NF-κB in hippocampal homogenates, while Nrf2 was increased at 3 months (pre-plaque) then decreased at 12 months (post-plaque).[303]

I.I.I.I DNA Repair Mechanisms

As discussed in Section I.10.3.2 there are various types of DNA damage and without effective repair, nDNA and mtDNA mutations can accumulate and result in neuronal death.[391] Neurons are particularly vulnerable to DNA damage as they are transcriptionally active as well as post-mitotic and utilize the more error-prone, non-homologous end joining (NHEJ) for double strand break (DSB) repair. Furthermore, as neurons are metabolically active, mtDNA is particularly susceptible to oxidative insult[473] and additionally does not have as versatile repair mechanisms as nDNA.[474] Indeed, synaptic mitochondria have exhibited exacerbated pathology and damage when compared to somatic mitochondria in Tg mouse models of AD.[298]

Some key DNA repair mechanisms include nucleotide excision repair (NER), base excision repair (BER), and Non-homologous end joining (NHEJ), homologous recombination (HR) and mismatch repair among others. As oxidative DNA damage has been reported to be elevated in AD as discussed in Section I.10.3.2, DNA repair gene expression, protein levels, and activities have also been shown to be decreased in aging[475] and in AD,[476-478] Importantly, as these pathways are complex, it is not always a simple matter of higher expression or protein levels, many of them are also phosphorylation dependent and can undergo oxidative modifications that would alter their efficacy at repairing oxidative DNA damage. Furthermore, many of these studies focused on late stages of the AD pathology (for a review of different DNA repair proteins studied in AD see Bucholtz and Demuth (2013)).[478]

I.12 Studying Aβ-Associated Oxidative Stress During the Earliest AD Pathology

Taking together the previous sections on Alzheimer's disease and oxidative stress, both fields are complex and rich with unanswered research questions. Of relevance to this thesis, our interest lies in studying the early, pre-plaque amyloid pathology of AD, when soluble Aβ oligomers accumulate intraneuronally and neuroinflammation is incipient.[301-302, 479] This stage of the disease is a promising therapeutic window, offering valuable insight into potential biomarkers and therapeutic targets for AD. Since the AD field has primarily focused on studying late disease stages when plaques are already present, and only recently began focusing on earlier disease stages, much of the literature related to oxidative stress in AD is also at late, post-plaque timepoints. Therefore, this thesis focuses on the role of oxidative stress during pre-plaque stages of the amyloid pathology. Furthermore, as neuronal membranes are vulnerable to lipid chain autoxidation (free radical initiated lipid peroxidation), and the AD brain has extensive burden of by-products associated to lipid peroxidation-induced damage, we have an interest in the upstream ROS, (lipid peroxyl radicals) responsible for this downstream damage.

Towards our goal of studying oxidative stress in the early amyloid pathology of AD, with a focus on lipid peroxyl radicals, we utilized two key research tools:

I. the McGill-R-Thy1-APP transgenic (Tg) rat model exhibiting an AD-like amyloid pathology and developed in the Cuello laboratory,[286] and

II. the fluorogenic probe H_4BPMHC that detects lipid peroxyl radicals developed in the Cosa laboratory, by Dr. Lana E. Greene.[480]

The McGill-R-Thy1-APP rat model exhibits an AD-like amyloid pathology, expresses the human *APP* gene with two familial AD mutations (Swedish double mutation and Indiana mutation) under the thymocyte antigen promoter (Thy1.2) which confines expression to the CNS.[286] As noted in Section (1.5.1), the Swedish mutation increases total Aβ production[481] while the Indiana mutation increases production of $A\beta_{42}$ over $A\beta_{40}$.[482] Rat models of human diseases offer additional advantages over mouse models since rats have a broader behavioural display, are less stressed with human handling, have less confounding factors influencing their behaviour, larger brains which makes surgery and handling easier,[483] and are physiologically, genetically, and morphologically closer to humans.[484-485]

An added advantage of this Tg model is that it exhibits an extended pre-plaque phase unlike other more aggressive rodent models of AD. In homozygous animals, intraneuronal human

Aβ is observed at 1 week of age in the cortex. This pool of soluble Aβ then accumulates and forms soluble oligomers detectable intraneuronally at 3 months of age.[293] Insoluble plaques then form only by approximately 9 months of age starting in the subiculum[293, 486] then spreading to the hippocampus and other cortical regions, finally reaching the olfactory bulb, amygdala, and thalamus by 18 months of age, a timepoint also where reduced neuronal counts in the subiculum were observed.[486]

During pre-plaque timepoints in this Tg rat model, there are LTP impairments and behavioral deficits in the morris water maze, novel object recognition and location, and fear conditions at 3 months of age.[479, 487-488] We also observed evidence of inflammatory processes driven by Aβ-burdened hippocampal neurons,[302, 479] increased 4HNE and 3-nitrotyrosine in cortical homogenates at 5 months of age,[489] increased TBARs reactivity at 3 months, and a trend to increased GSH also at 3 months.[303]

At post-plaque stages in this rat model, there are similar characteristics to AD including decreasing levels of CSF $A\beta_{42}$ levels, brain hypometabolism, decreased hippocampal volume and decreased neuron numbers.[490-491]

Indeed, studies on heterozygous McGill-R-Thy1-APP rats have also been performed, while this thesis utilized only homozygous animals, for a review of data from heterozygous rats see the following references.[492-495]

I.12.1 Studying ROS Using Fluorogenic Strategies

A recent publication by Forman *et al.* (2015) with the author list including prominent researchers in the redox community, outlined some essential guidelines for those entering this field from varied research backgrounds, including ensuring more rigorous use of fluorescent probes for ROS detection and more careful interpretations of the data.

A useful example the authors note is that of the most common commercial fluorogenic probe, $DCFH_2$-DA (dichlorodihydrofluorescein diacetate) which has generated a great deal of confusion around what this probe detects and what the fluorescence signal means for the redox state of the cell.[468] $DCFH_2$-DA enters the cell, is cleaved by esterases ($DCFH_2$) then following two-electron oxidation generates the fluorescent product, DCF. Importantly, many publications utilized this probe to reflect the levels of hydrogen peroxide in the cell. However, $DCFH_2$ does not react directly with hydrogen peroxide, superoxide or nitric oxide, and instead reacts with other free radicals, therefore, this probe cannot be used to directly quantify hydrogen peroxide. Another pitfall of this probe is that it can produce ROS itself, resulting

in artifactual amplification. This can happen following one-electron oxidation when the intermediate $DCF^{\bullet-}$ reacts with molecular oxygen (O_2) to form superoxide followed by hydrogen peroxide, which can propagate Fenton reactions generating radicals the probe reacts with. Adding to this issue, there are catalysts of $DCFH_2$ oxidation in the cell (e.g.: free iron, hemoglobin, catalase, and modified cytochrome c among others) that exhibit peroxidase-like activity with $DCFH_2$ resulting in one-electron oxidation, thus generating a "peroxidase" cycle.[496-497] Figure 1.13 from Ziolonka and Kalyanaraman (2012) shows the complexity of $DCFH_2$ reaction with other cellular entities.[497-498] As Forman *et al.* (2015) suggest, ultimately, the fluorescence signal generated by $DCFH_2$-DA likely reflects presence lysosomal iron released into the cytosol and peroxidase-catalyzed oxidation of the probe itself.[414, 499] Kalyanaraman *et al.* (2012) highlights that there is still useful information to be taken from data generated with this fluorogenic probe, namely, DCF can be a general redox indicator, to assess redox-active iron in the cell and cellular glutathione levels,[500-502] while another study used $DCFH_2$ to study oxidative burst in neutrophils, a context where the pitfalls of this probe are not as pronounced due to the large amount of superoxide and myeloperoxidase to ensure efficient catalysis of probe oxidation resulting in less artefactual fluorescence signal.[503-504] Kalyanaraman *et al.* (2012) also provide a useful table indicating proper use of various common fluorogenic probes and recommended interpretations. Wardman (2007) is also a useful resource for selecting fluorescent and luminescent probes in biology research, the below points are rephrased from this work as they are pertinent to appreciating the complexity of probe selection:[496]

- **Probe reactivity:** What does the probe react with, how fast, how specifically?
- **Catalyst:** Is a catalyst needed for the reaction and if so, will the concentration vary during the experiment?
- **Probe intermediates:** If the probe requires an initial reaction to produce a reactive intermediate what species are responsible for this reaction and what defines the probe response?
- **Reactions with probe intermediates:** If probe intermediates are formed, do they react with molecular oxygen to form superoxide or with antioxidants?
- **Probe distribution:** How is the probe distributed intracellularly and extracellularly but also amongst organelles? If distributed in organelles, how does differing pH affect the probe?

- **Antioxidants:** Does the probe react with cellular antioxidants and how do these rate constants compare to that of the reaction with the species of interest? Can these differences be quantified? Do reactive intermediates also react with antioxidants?

- **Photochemical reactions:** Is the product measured photostable or does it generate superoxide through quenching of excited states with cellular reductants?

- **Instrumental artefacts:** Technical considerations like inner filter effects, Rayleigh scattering and second-order diffraction artefacts.[496]

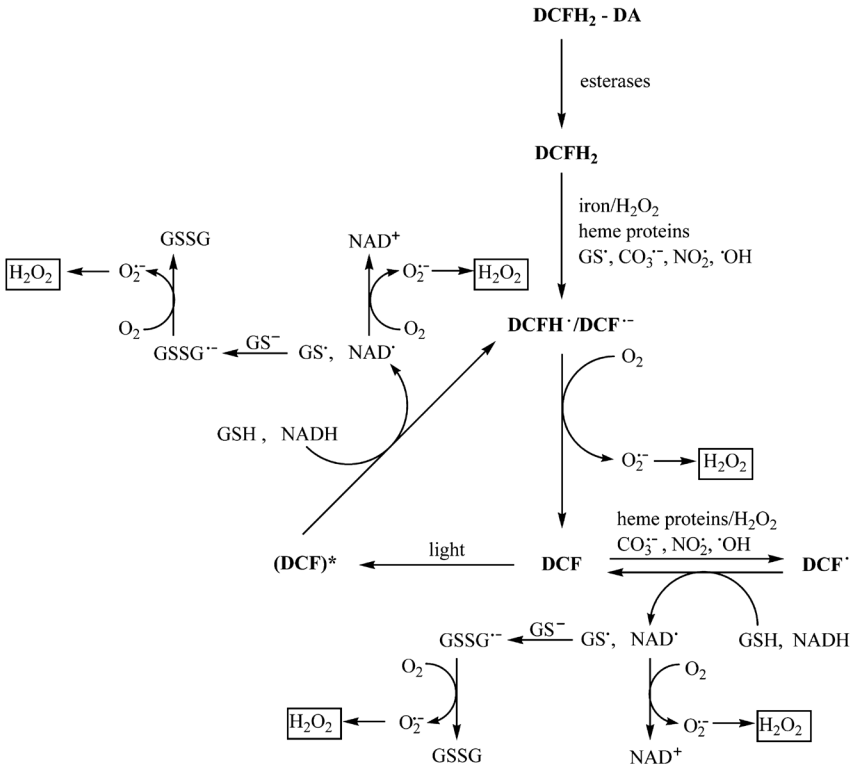


Figure 1.13: Redox chemistry of $DCFH_2$.
Reproduced from Zielonka and Kalyanarman 2008.[498] Reprinted from Free Radical Biology and Medicine, Vol. 45, Zielonka J and Kalyanaraman B, "ROS-generating mitochondrial DNA mutations can regulate tumor cell metastasis"—a critical commentary, 1217-1219, Copyright (2008), with permission from Elsevier.

These guidelines highlight the importance of specificity and results interpretation when studying ROS real-time using fluorescent strategies. As we were interested in studying lipid-derived ROS, specifically lipid peroxyl radicals that participate in lipid chain autoxidation which neurons are vulnerable to, we utilized the fluorogenic probe H_4BPMHC for studying this specific type of ROS. Although several methodologies exist for studying lipid chain autoxidation and its effects such as HPLC, mass spectrometry, and electron paramagnetic

resonance (EPR),[355] these methods are typically destructive and have limitations in the information they provide on the rate (temporal resolution) and location (spatial resolution) of ROS production. Conversely, fluorescence imaging enables higher temporal and spatial resolution of real-time ROS production when the appropriate fluorogenic probes and experimental conditions are employed. In the following sections, the basics of fluorescence and details regarding the fluorogenic probe H_4BPMHC will be discussed.

I.12.1.1 Fluorescence Basics

The process of fluorescence occurs when electromagnetic radiation (light) of certain energy is absorbed by a molecule, resulting in excitation of an electron from the highest occupied molecular orbital (HOMO) (ground state) to the lowest unoccupied molecular orbital (LUMO) (excited state). After this excitation, energy dissipation occurs via multiple mechanisms, followed by relaxation back to the ground state, resulting in an emitted photon of longer wavelength (lower energy) than the initial excitation wavelength. This discrepancy in excitation and emission wavelength as shown in Figure 1.14, also known as the Jablonski diagram reproduced from Berezin and Achilefu (2010).[505-508]

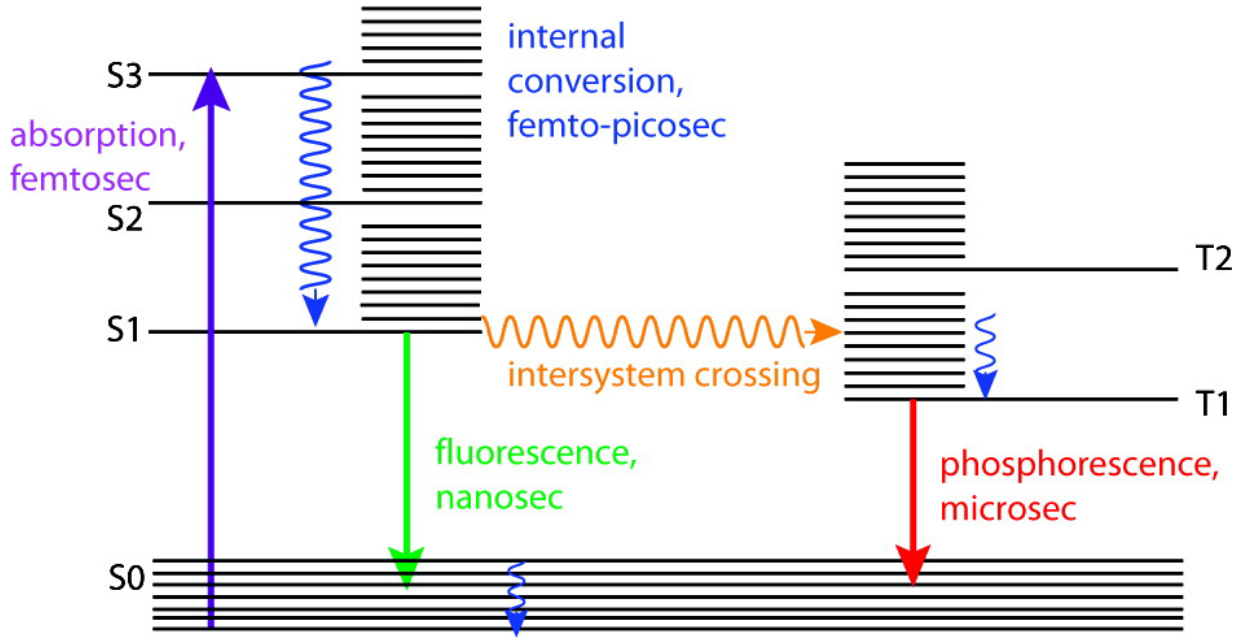


Figure 1.14: Jablonski diagram.
Adapted from Berezin and Achilefu (2010).[505] Jablonski diagram with time scales showing excitation of an electron from the ground state (S0) to a higher energy level (in this case S3). Radiative decay processes are shown in solid arrows while non-radiative decay processes are in curved arrow. Note 'S' indicates singlet state and 'T' indicates triplet state. Reprinted from Chemical Reviews, Vol. 110, Berezin MY and Achilefu A, Fluorescence lifetime measurements and biological imaging, 2641-2684, Copyright (2010) American Chemical Society.

I.12.1.2 Design of H4BPMHC – Inspiration from Vitamin E

Vitamin E (α-tocopherol) is the most active lipid-soluble antioxidant in the body,[509-510] and comprises two different classes of lipophilic antioxidants, tocopherols and tocotrienols which are classified based on their chromanol head group and phytyl tails. This chromanol head group enables antioxidant activity and depending on placement of methyl groups assumes and α, β, γ, or δ classification (Figure I.15).[511] The phytyl chain determines partitioning in lipid membranes and is classified as a tocopherol if there are no double bonds (saturated), or as a tocotrienol if there are double bonds present (unsaturated).[512] The most active form of vitamin E is α-tocopherol,[509, 513] and is also the most common form found in human tissue. Vitamin E is an effective radical trapping antioxidant (RTA) against lipid chain autoxidation (Section I.10.2) because the rate constant of this scavenging reaction is higher than the chain propagation rate, this quality makes it ideal for studying lipid peroxyl radicals, were some of the properties leveraged by Dr. Greene as described below.

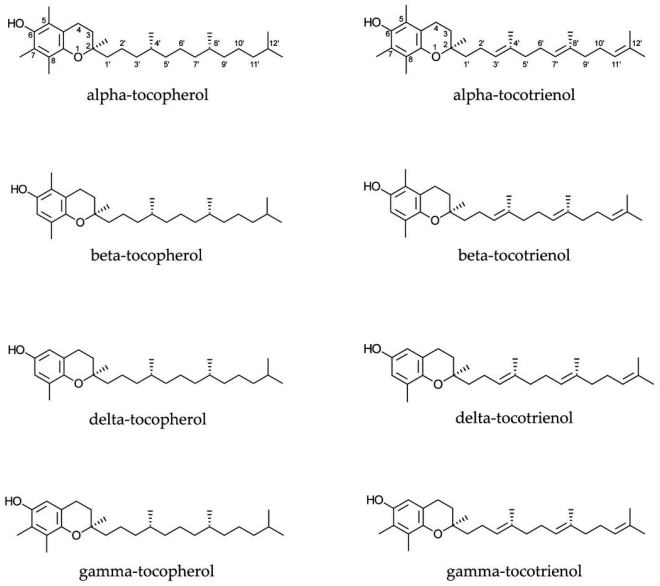


Figure I.15: Structure of vitamin E.
Reproduced from Shahidi and Costa De Camargo (2016).[511] Reprinted from International Journal of Molecular Sciences, Vol. 17, Shahidi F and Costa De Camargo, Tocopherols and Tocotrienols in Common and Emerging Dietary Sources: Occurrence, Applications, and Health Benefits, 1745, Copyright (2016), with permission from MDPI.

Fluorescence strategies to detect certain chemical entities requires that when the fluorophore reacts with the target of interest, it undergoes a detectable change such as: an increase in intensity from a quenched to fluorescent state (off/on), a decrease in intensity from fluorescent to quenched state (on/off), a shift in emission wavelength (ratiometric probes),

or a change in fluorescence lifetime where H_4BPMHC falls under the category of off/on probes. Certain photo-physical properties of fluorophores are integral to their applications in biological systems, in addition to brief descriptions of these characteristics, their relevance to the design of H_4BPMHC is listed below:

- Fluorescence quantum yield (ϕ_f) is the probability that the molecule will fluoresce, or the ratio of photons emitted to the photons absorbed, thus, as the quantum yield approaches one, this means almost all photons absorbed are then emitted and the process is highly efficient. However, there are various ways in which energy can be lost by non-radiative (non-fluorescent) means (Figure 1.14, curved arrows), thus diminishing the number of photons emitted. As seen in Equation 1.1, k_r is the radiative rate constant, while k_{dec} is the total decay processes, both radiative k_r and non-radiative k_{nr}. Non-radiative decay processes include internal conversion, intersystem crossing, or decay by various means of quenching. Notably, those non-radiative processes can be leveraged when designing fluorogenic probes that require an 'off' state.[480, 514]

Equation 1.1:

$$\phi = \frac{k_r}{k_r + k_{nr}}$$

- Fluorescence intensity which is determined by the extinction coefficient (ε) and the radiative rate constant (k_r).
    - (ε) is an intrinsic property of the fluorophore controlled by the electronic structure and can be determined by using Beer's Law (Equation 1.2) which measures the ability of the molecule to absorb a photon of a certain wavelength. *A* indicates absorbance of the molecule, *l* indicates the pathlength of the light, and *C* is the concentration of the molecule.[514]

Equation 1.2:

$$A = \varepsilon l C$$

- Fluorescence lifetime (τ) is the time that the fluorophore spends in the excited state before emitting a photon, and is the inversely proportional to the sum rate constants of radiative (k_r) and non-radiative (k_{nr}) processes (Equation 1.3).[505]

Equation 1.3:

$$\tau = \frac{1}{k_r + k_{nr}}$$

- Dynamic range is dictated by the minimal fluorescence intensity in the 'off' state (quantum yield approaching zero) and the maximal fluorescence intensity in the 'on' state (quantum yield approaching one). A larger dynamic range allows for more sensitive detection of differences which is essential for biological systems. In the case of H4BPMHC the quantum yields (ϕ_f) of the 'off' and 'on' states was 0.001 and 0.700-1.000 respectively,[480] allowing for a darker 'off' state than previous generations of fluorogenic probes from the Cosa laboratory.

H4BPMHC was designed with two segments, one being a reporter fluorophore, a boron dipyrromethene (BODIPY), and the second being a trap/receptor segment in this case a chromanol moiety, modelled after the antioxidant vitamin E (α-tocopherol) Figure 1.16. The BODIPY fluorophore allows for partitioning into lipid bilayers where the radicals of interest are located, narrow absorption and emission bands and high photostability.[515] Then, for the trap/receptor segment, the antioxidant activity of α-tocopherol, possible through its chromanol moiety, is highly efficient at trapping lipid peroxyl radicals and thus offers an ideal design for a fluorogenic probe aimed to detect this type of ROS.[480]

Being an off/on probe, H4BPMHC used the mechanism of photoinduced electron transfer (PeT) to enable an initial quenched (off) state preceding interaction with lipid peroxyl radicals. In general, PeT involves the exergonic, energetically favorable electron transfer from an electron donor to an electron acceptor, which are defined by their orbital energies. For H4BPMHC, the BODIPY fluorophore serves as an electron acceptor while the chromanol moiety is the electron donor. In the bottom panel of Figure 1.16 from Greene *et al.* (2017), the 'off' state is possible due to PeT quenching: first an electron in the HOMO of the BODIPY fluorophore is excited to the LUMO, then because the HOMO of the chromanol moiety is a higher energy than that of the BODIPY HOMO, an electron from the chromanol HOMO is donated. This reduction of the BODIPY prevents the initial excited electron from emitting a photon and thus quenches the fluorescence. However, when the chromanol moiety reacts with two lipid peroxyl radicals ($ROO^{\bullet}$), oxidizing it to a chromanone, the energy of the chromanol HOMO orbital decreases below the level of the BODIPY HOMO, preventing donation of an electron and enabling fluorescence.[480] Notably the highly exergonic electron transfer from the chromanol to the BODIPY enables a dark 'off' state (quantum yield approaching zero).[480, 516]

Another added advantage of H4BPMHC is that it does not require a high concentration for use, and has been previously used in HeLa cells at a concentration of 100 nM, around 50-fold less than other commercially available ROS-detecting probes.

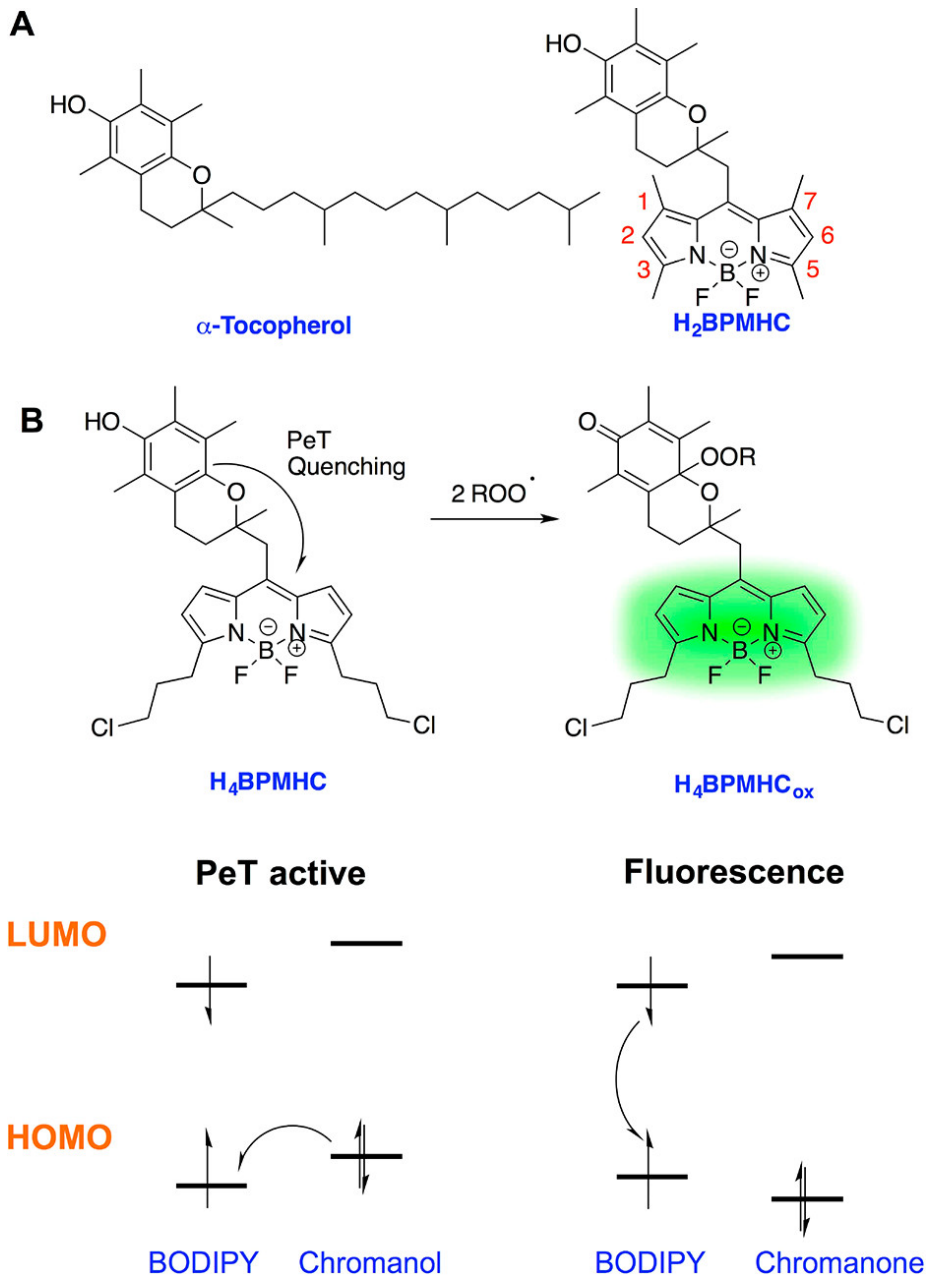


Figure 1.16: Photo-induced electron transfer in H_4BPMHC.

Adapted from Greene *et al.* (2017).[480] **A.** Structure of Vitamin E (α-tocopherol) and H_2BPMHC, the previous generation of fluorogenic tocopherol probe in which H_4BPMHC was based. **B.** Structure of the newer generation of probe, H_4BPMHC and a proposed mechanism of PeT by which it fluoresces upon reacting with two lipid peroxyl radicals ($ROO^{\bullet}$). Reprinted with permission from Journal of American Chemical Society, Vol. 139, Greene LE, Lincoln R, Cosa G, Rate of Lipid Peroxyl Radical Production during Cellular Homeostasis Unraveled via Fluorescence Imaging, 15801-15811, Copyright (2017) American Chemical Society.

I.13 Research Goals and Scope of Thesis

Through this introduction, it is apparent that Alzheimer's disease is a global health priority which has a complex pathology that is challenging and costly to study in humans especially considering the decades-long pre-symptomatic period. Furthermore, the physiology and pathology of ROS is also technically challenging to study and still has many unanswered questions in the field. While both these research fields are expansive, the role of oxidative stress-related processes in AD offers promising insight into pathological mechanisms during the earliest disease stages. This thesis sheds light on the role of oxidative stress during the earliest amyloid pathology of AD and provides the reader with tools for developing methods to study ROS real-time in physiologically relevant systems to AD. Towards these goals, we have taken into consideration the following points:

- Transgenic animal models reflect certain key aspects of the AD pathology and allow for studying early, pre-symptomatic stages that are difficult to assess in humans, offering substantive insight into health and disease. Furthermore, transgenic rat models offer advantages over transgenic mouse models.[483]

- Our laboratory has demonstrated the presence of a pre-plaque inflammatory process that is driven by Aβ-burdened neurons.[301-302, 479] As oxidative stress is closely tied to inflammation, we aim to build on this previous work.

- Fluorogenic probes offer valuable spatial and temporal resolution for studying ROS real-time in biologically relevant systems.

- Lipid peroxyl radicals represent an important ROS in the context of AD as neurons are especially vulnerable to lipid chain autoxidation.[315]

In light of the issues highlighted in the Introduction, I embarked on studies addressing the experimental aims listed below:

Aim 1: Investigate alterations in oxidative stress-related gene and protein expression in iAβ-burdened neurons that are known to exhibit an incipient inflammatory profile.

Aim 2: Validate the use of H_4BPMHC, a lipid peroxyl radical detecting fluorogenic probe, in primary neuronal cultures to assess its potential for studying neurodegenerative diseases.

Aim 3: Develop a methodology for using H_4BPMHC to study lipid peroxyl radical generation in *ex vivo* hippocampal slices.

II. Chapter 2

Oxidative Stress-Related Genes are Dysregulated in Aβ-burdened Neurons of a Rat Model of Alzheimer's Disease

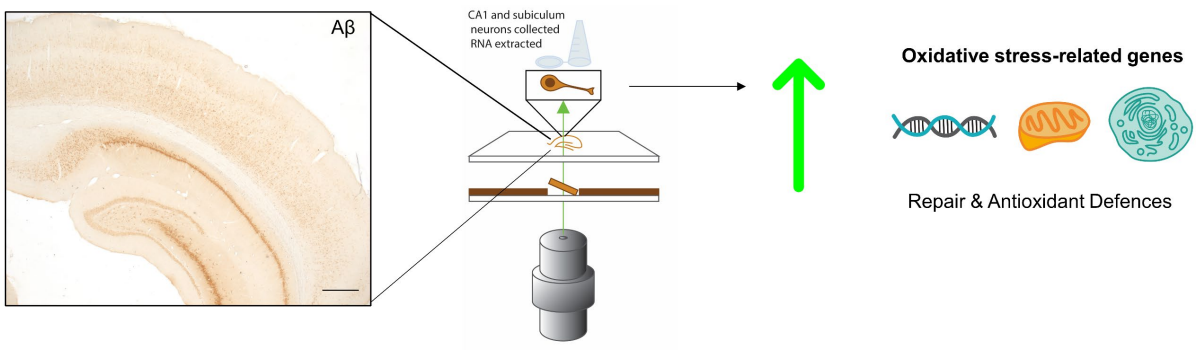


Figure credit: MK Foret, N Yousefpour

Manuscript in preparation for submission. "Oxidative Stress-Related Genes are Dysregulated in Aβ-burdened Neurons of a Rat Model of Alzheimer's Disease" Foret, M.K.; Do Carmo, S.; Orciani, C.; Welikovitch, L.; Huang, C.; Cuello, A.C. *In preparation.*

Preface

As discussed in the introduction (Chapter 1), our laboratory has previously shown in the McGill-R-Thy1-APP rat model of the AD-like amyloid pathology, that intraneuronal Aβ accumulates before plaque formation and cognitive decline, and coincides with a neuron-driven inflammatory process. In the next chapter, the aim was to further leverage this transgenic model to investigate oxidative stress-related changes in gene and protein expression specifically in these iAβ-burdened neurons. Our goal was to determine which genes/proteins and/or pathways are the earliest contributors to the CNS Aβ amyloid pathology, while also assessing relevant forms of oxidative damage in these neurons. A study which, ultimately provides new perspectives for understanding the earliest amyloid pathology in AD.

II.I Abstract

Alzheimer's disease (AD) has an extended pre-symptomatic period that can span decades. Understanding the pathological mechanisms that initiate and drive disease processes will assist in the discovery of early therapeutic targets. Neurons are especially vulnerable to the oxidative stress occurring in AD. Still, the earliest role of neuronal oxidative stress, long before extracellular amyloid plaque deposition, remains elusive. Here, we investigated oxidative stress-related gene and protein expression specifically in hippocampal neurons burdened with intraneuronal amyloid beta (iAβ) using the McGill-R-Thy1-APP transgenic rat model of the AD-like amyloid pathology. A time at which iAβ-burdened neurons exhibit increased expression of inflammatory cytokines, as previously shown by our lab. We found that iAβ-burdened neurons expressed higher levels of genes related to DNA repair and antioxidant defenses including *Ercc2*, *Fancc, Sod2, GR*, and *Idh1*. At the protein level, as assessed by immunofluorescence, we observed an elevation in neuronal levels of XPD (gene product of *Ercc2*) as well as elevated DNA double-strand breaks. We also observed a trend to increase in neuronal levels of 4HNE which indicates protein oxidation. The results of the present study indicate that a neuronal oxidative stress response occurs during the early pre-plaque amyloid pathology when a neuron-driven inflammatory process is incipient.

II.2 Introduction

The amyloid hypothesis has dominated Alzheimer's disease (AD) research and clinical trials for decades.[223] Additional pathological mechanisms underlying the early AD pathogenesis point towards a role for reactive oxygen species (ROS) and oxidative stress. Oxidative stress is implicated in numerous neurodegenerative diseases including AD,[368, 373, 378] and markers of oxidative damage have been observed in transgenic animal models of AD, as well as the brains of individuals with mild cognitive impairment (MCI),[393-394] Down Syndrome (DS),[396-397] and AD.[383, 387, 517] Recently, using a proteomics approach, our lab showed that cellular stress occurs during pre-plaque stages of the AD-like amyloid pathology,[303] and is accompanied by a neuron-driven inflammatory response.[302] However, how the earliest accumulation of neuronal amyloid β (Aβ) affects processes related to oxidative stress and vice versa remains unknown.

Although ROS have a physiological role, imbalances in ROS production, antioxidant levels or activity, and redox signaling can culminate in cellular damage and disease through modifications to biomolecules including proteins, lipids, and nucleic acids.[321, 518] Neurons are especially vulnerable to oxidative stress and accumulation of oxidative damage due to: 1) high brain O_2 concentration, 2) the large metabolic demand of neurons influencing mitochondrial ROS production[316, 433] 3) the elevated concentration of polyunsaturated fatty acids (PUFA) in neuronal membranes which are vulnerable to lipid peroxidation, 4) the low ratio of antioxidant to pro-oxidant enzymes in the brain,[315, 317] 5) high brain iron content[519] which can contribute to ROS production via the Fenton reaction,[315] and 6) the reliance on error-prone DNA repair pathways such as non-homologous end joining (NHEJ) in place of replication-associated DNA repair.[316, 446]

Preceding extracellular amyloid plaque formation, Aβ accumulates intraneuronally (iAβ) as monomeric and oligomeric forms.[132, 288-289, 520] This accumulation of iAβ results in deleterious effects including synaptic abnormalities,[296] long-term potentiation impairment,[284, 521-522] cognitive decline[286, 293, 300, 521, 523-525] and initiation of inflammatory processes.[301-302, 479, 526] However, the exact mechanisms by which iAβ exerts pathologic, disease-accelerating effects during early, pre-plaque stages of AD, and how neuron-derived oxidative imbalances contribute to this process remain unknown. As the asymptomatic, pre-plaque stages of AD span decades, studying the earliest pathologic mechanisms that coincide with iAβ accumulation will offer insight into potential preventative therapeutic strategies to halt or delay the AD pathology.

In the context of AD, there are a number of potential mechanisms where oxidative stress can be induced by Aβ. As example, intracellular Aβ has been shown to generate ROS by inserting into cellular membranes and initiating lipid peroxidation through its methionine 35 residue.[527-530] Aβ has also been observed to insert into mitochondrial membranes disrupting their function,[298, 531-533] and interactions between Aβ and copper can indirectly lead to hydroxyl radical production.[534-535] Furthermore, Aβ can bind RAGE (receptor for advanced glycation end products) which activates downstream pathways that indirectly lead to oxidative stress.[536-537] Lastly, Aβ-mediated disruption of NMDA receptor function can result in calcium dyshomeostasis which can lead to oxidative stress.[538-539]

During pre-plaque stages of AD, Aβ accumulates intraneuronally as monomers then oligomers, which are the most toxic form,[132] and this is recapitulated in the McGill-R-Thy1-APP rat.[286, 293, 302] In this study, using the McGill-R-Thy1-APP transgenic (Tg) rat, we explored alterations in oxidative stress-related genes in iAβ-burdened hippocampal neurons at pre-plaque timepoints. We used laser-capture microdissection (LCM) to extract iAβ-burdened hippocampal neurons to assess neuron-specific oxidative stress-related gene expression. Importantly, this same LCM material was also utilized in a recently published work that demonstrated upregulation of inflammatory gene expression in these Aβ-burdened neurons.[302]

II.3 Materials and Methods

II.3.1 Animals and tissue collection

Animal work was approved by the McGill Animal Care Committee and followed guidelines established by the Canadian Council on Animal Care (CMARC). McGill-R-Thy1-APP rats overexpressing the human *APP* gene with both the Swedish and Indiana mutations under the murine Thy1.2 promoter, and their wild-type (Wt) littermates were used for this study.[286] Rats (male and female) were housed in humidity-controlled and temperature-controlled rooms with 12 hour light/dark cycles and given *ad libidum* access to food and water. At 5 months of age, rats were deeply anesthetized with intraperitoneal injections containing a mix of chloral hydrate and sodium pentobarbital (6.5 mg chloral hydrate and 3 mg sodium pentobarbital per l00 g body weight), then transcardially perfused with ice-cold saline solution (pH 7.4) for two minutes.

Brains were extracted, and one hemisphere was either: 1) flash frozen in isopentane over dry ice and stored at –80 ºC for laser capture microdissection (LCM) experiments or 2) dissected and snap frozen (hippocampus, cortex, cerebellum) then stored at –80 ºC for biochemistry experiments. The other hemisphere was post-fixed at 4 ºC in 4% paraformaldehyde (PFA) (in 0.1 M phosphate buffer, PB) for 24 h, then saturated with 30% sucrose (dissolved in 0.1 M PB) and coronally sectioned at 40 μm using a freezing sledge microtome (Leica, SM 2000R, Germany). Sections were stored in cryoprotectant solution (37.5% v/v ethylene glycol, 37.5% w/w sucrose in phosphate-buffered saline (PBS)) at –20 ºC, pH 7.4 until used for IHC experiments.

II.3.2 Laser capture microdissection and RNA isolation

Brain tissue that was flash frozen in isopentane over dry ice was sectioned at 10 μm using a Leica CM3050S cryostat and thaw-mounted onto 1.0-mm PEN membrane-covered glass slides that were irradiated for 30 minutes with UV light (MembraneSlide 1.0 PEN; Carl Zeiss). Sections were dehydrated at -20 ºC for 30 minutes and stored at -80 ºC for later use. Mounted sections were immersed in 95% ethanol, rehydrated using decreasing ethanol concentrations, stained with Cresyl violet for 1 minute, and finally dehydrated with increasing ethanol concentrations followed by xylene. Laser capture microdissection (LCM) was performed after Cresyl violet staining where the pyramidal layer of CA1 and subiculum was collected from 40 tissue sections per animal using the PALM MicroBeam (Carl Zeiss). UV laser settings were: 75 cut energy, 70 cut focus, 12 auto-LPC dot-size. Neurons were identified by diffuse Cresyl violet staining and collected in PCR tubes with an opaque adhesive cap, collection tubes were changed every 2 hours (AdhesiveCap 200 Opaque; Carl

Zeiss). Microdissected neuronal samples were incubated with RLT lysis buffer (RNeasy Mini kit, Qiagen) for 30 minutes and RNA was extracted using a RNeasy Mini kit (Qiagen) then stored at -80 ºC for later use. To confirm the neuronal enrichment of the isolated mRNA, cDNA was used to measure the relative expression of neuron-specific MAP2 (microtubule associated protein 2) and TUBB3 (βIII-tubulin), microglia/macrophage-specific IbaI (ionized calcium binding adaptor molecule 1) and CD13, astrocyte-specific GFAP (glial fibrillary acidic protein), and oligodendrocyte-specific MBP (myelin basic protein) transcripts, using the ΔΔCT method. The housekeeping genes were as follows: ACTB (β-actin), CYC1 (cytochrome c 1) and RPL13 (60s ribosomal protein L13) as reported in[302].

II.3.3 RT² Rat Oxidative Stress Profiler PCR Array

The quality of RNA isolated from microdissected neuronal material was verified using an RNA 6000 Pico Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA), whereby all samples resulted in an RNA Integrity Number (RIN) higher than 7.0.[302] Isolated RNA was converted to cDNA using the RT² PreAMP cDNA Synthesis Kit and amplified using RT² Rat Oxidative Stress PreAMP Pathway Primer Mix (PBR-065Z, Qiagen). Expression of 84 oxidative stress-related genes was assessed by qRT-PCR (50 thermo cycles total) for each animal using the RT² Rat Oxidative Stress Profiler PCR Array (PARN-065ZD, Qiagen), a CFX Connect Real Time cycler (Bio-Rad) and cycle conditions recommended by Qiagen. Relative expression of each gene was calculated by the $\Delta\Delta C_T$ method, standardized with five housekeeping genes and using the recommended control values from the RT² PreAMP cDNA Synthesis Handbook, where C_T values above 35 were considered a negative call. As part of the RT² Profiler PCR Array, three internal controls were included: a genomic DNA contamination control, a reverse transcription control, and a positive PCR control. See Table S2.1 for the housekeeping genes used in the RT² Rat Oxidative Stress PCR Array.

II.3.4 Immunohistochemistry

II.3.4.1 Brightfield Immunohistochemistry

Fixed free-floating, 40 μm coronal brain sections were washed with PBS to remove cryoprotectant. Then, endogenous peroxidase activity was quenched using a solution of 3% H_2O_2, and 10% methanol in PBS for 30 minutes. Following washes with PBS and PBS containing 0.2% Triton-X-100 (PBS-T), sections were blocked for 1 hour at room temperature (RT) in 10% normal goat serum (NGS) in PBS-T. Sections were incubated with primary antibodies overnight at 4 ºC in 10% NGS: anti-Aβ (McSA1, 1:1000, Medimabs, Canada). Sections were then washed with PBS-T and incubated with rabbit-anti-

mouse (produced in-house, Table S2.5) (1:25) for 1 hour RT. After washing, sections were incubated for 1 hour with mouse anti-horseradish peroxidase (1:30) that was pre-incubated for 30 minutes with horseradish peroxidase (HRP) (5 μg/ml, 1:200) (MAP kit, Medimabs, Canada). Sections were then washed, and the staining was developed using 0.06% of 3,3′-diaminobenzidine (DAB) (Sigma-Aldrich, Germany) and 0.02% H_2O_2 to initiate the reaction. Sections were mounted on pre-cleaned Super Frost (Fisher) gelatin-coated slides, air-dried, dehydrated using increasing ethanol concentrations, cleared with xylene and coverslipped with #1.0 coverslips and Entellan (EM Science, USA).

II.3.4.2 Immunofluorescence

Fixed free-floating, 40 μm coronal brain sections were washed using PBS to remove cryoprotectant. For certain primary antibodies (namely: anti-Fancc, anti-γH2AX, anti-Idh1, anti-Sod2, and anti-XPD) sections underwent heat-mediated antigen retrieval and were incubated at 80 ºC in 10 mM citrate buffer (pH 6.0) for 30 minutes. After 20 minutes of cooling at RT, sections were washed using PBS and the standard protocol was resumed. Sections were permeabilized using 50% ethanol for 20 minutes, washed with PBS-T, and blocked for 1 hour at RT in 10% NGS. Sections were incubated with primary antibodies (Table S2.4) overnight at 4 ºC in 5% NGS. After primary antibody incubation, sections were washed with PBS-T and incubated with varying combinations of Alexa Fluor 488 (goat-anti-mouse), Alexa Fluor 568 (goat-anti-rabbit), and/or Alexa Fluor 647 (goat-anti-guinea pig) (all at 1:800, Thermo Fisher Scientific) for 2 hours RT. Following washes, to reduce autofluorescence, sections were incubated for 5 minutes with 0.3% Sudan black in 70% ethanol. Sections were then washed three times for 5 minutes each in PBS-T, then three times for 5 minutes each in PBS. In some experiments, sections were then incubated with DAPI (0.1 μg/ml) for 5 minutes and washed with PBS. Sections were then mounted on pre-cleaned Super Frost (Fisher) gelatin-coated slides and coverslipped with #1.5 coverslips and Aqua-Poly/Mount (Polysciences). Note that for γH2AX experiments TBS and TBS-T (0.5% triton-X-100) was used instead of PBS.

II.3.5 Microscopy and Image Analysis

II.3.5.1 Brightfield Imaging

For brightfield imaging, an Axio Imager M2 microscope with an AxioCam 506 color digital camera, and ZEN Imaging software (ZEN Blue; Zeiss, Germany).

II.3.5.2 Fluorescence Imaging

Confocal images were acquired using an LSM800 Confocal Microscope AxioObserver (Zeiss, Germany) and a 20X Plan Apochromat objective lens (NA = 0.80) with ZEN Imaging software (ZEN Black). To allow quantitative comparisons, images were acquired with the same microscope settings, adjusted specifically for each marker assessed. Z-stacks from 2-3 sections per animal were acquired for CA1 (three image regions) and the subiculum (one image region) with intervals of either 1 μm or 2 μm as determined by the marker of interest. Depending on the fluorophores in each experiment, diode lasers of 405, 488, 561, and/or 640 nm were imaged sequentially from longest to shortest wavelength, all with a pinhole size equivalent to 1 airy unit (AU) for each respective wavelength. 16-bit images (312.5 x 312.5 μm) were acquired with a pixel dwell of 0.76 μs and an averaging of four by line (1 pixel = 0.31 μm). Signal was detected using a Gallium arsenide phosphide (GaAsP) PMT with emission wavelengths of 450-495 nm (405 laser), 500-550 nm (488 laser), 575-650 nm (561 laser no 647 fluorophore), 571-620 nm (561 laser with 647 fluorophore), 650-700 nm (640 laser). To quantify γH2AX+ neurons, five images of CA1 and two images of the subiculum were acquired using the 20X Plan Apochromat (NA = 0.80) with 1 pixel = 0.21 μm. Qualitative images at higher magnifications were acquired using a 63X Plan Apochromat (NA = 1.40) oil immersion objective (pixel = 0.05 μm).

II.3.5.3 Image Analysis

Custom, automated ImageJ macros were created for each target investigated (Figure S2.2). Briefly, regions of interest (as example CA1 pyramidal neurons) were identified by the NeuN channel and a mask/region of interest (ROI) was generated using this channel. This was followed by quantification of signal intensity using a sum of the z-stack in the channel of interest, thus avoiding any bias in mask/ROI generation. However, for Idh1 quantification in astrocytes, the Idh1 immunoreactivity was used to generate a mask for ROI selection since GFAP and S100β (data not shown) did not capture all relevant Idh1 immunoreactive areas.

Background corrections were performed for XPD, Fancc, GR, 8-oxo-dG and Idh1 as follows: 1) two regions of interest (ROIs) were selected from the summed z-stack (50x50 pixels in dimension, usually above the CA1 pyramidal layer and not in areas lacking tissue), 2) these intensity values were divided by area (accounting for number of z-stacks) and then averaged generating a mean background value. This value was then subtracted from the intensity measurement generated from neuronal areas of interest. SOD2 and 4HNE were not background corrected since areas lacking immunoreactivity were not reliably found (e.g.: SOD2 localizes to mitochondria which are widespread in the hippocampus, and 4HNE is generated by lipid peroxidation which is associated with lipid membranes that are also widespread in the hippocampus).

II.3.6 Glutathione Reductase Assay

Following the Glutathione Reductase (GR) Assay Kit (ab83461, Abcam) with slight modifications, twenty micrograms of cortical tissue from Wt and Tg animals was homogenized in 200 µl of assay buffer on ice then sonicated twice in the span of five seconds and centrifuged at 10,000 g for 15 minutes (4 ^{o}C). Protein concentration of the supernatant was quantified using a Lowry assay and each sample was aliquoted and diluted to a total of 100 µl at 5 µg/µl. The samples were then pre-treated with 3% H_2O_2 for 5 minutes at RT followed by catalase to stop the reaction. Samples were then added in duplicates (60 µg/well determined through pilot experiments) to a 96-well plate along with the appropriate TNB standard and positive control as provided in the kit. Reduced glutathione (GSH) reacts with 5,5'-Dithiobis (2-nitrobenzoic acid) DTNB in the reaction mix to generate TNB which has an absorbance maximum at 405 nm. The reaction mix was then added to all sample wells and the absorbance (OD_{450}) was measured every minute for 60 minutes. Glutathione reductase activity was calculated using the linear range of the curve with T_1 at 1 minute and T_2 at 15 minutes. First, the baseline absorbance was subtracted (T_0 which preceded the addition of the reaction mix) and then these corrected absorbance values at T_1 and T_2 were used to calculate $\Delta A_{405nm} = A_2 - A_1$. This absorbance value (ΔA_{405nm}) was applied to the TNB standard curve to obtain ΔB (the change in TNB concentration in nmol). The below equation was then used to calculate the mU/ml of GR activity where V represents the amount of sample added per well:

$$GR\ Activity = \frac{\Delta B}{(T_1 - T_2) \times 0.9 \times V} \times\ Sample\ dilution\ factor = mU/ml$$

II.3.7 DCF Assay

The DCF ROS/RNS fluorogenic Assays (Abcam, ab238535) was used to determine the levels of ROS and RNS by measuring the fluorescence intensity in cortical extracts from Wt compared to Tg rats. The assay applies a fluorogenic probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ), which is based on similar chemistry to 2', 7'-dichlorodihydrofluorescein diacetate. 10-20 mg of cortical tissue were homogenized in 20 volumes of PBS by sonication on ice. Insoluble particles were removed by centrifugation at 10,000 g for 5 min. Supernatants were used to perform the assay following the manufacturer's instructions. The DCFH-DiOxyQ probe was added to the supernatants in the presence of the catalyst for 30 min and then fluorescence intensity was measured at ex/em 480/530 nm using a Synergy 2 (Bio Tek Instruments, USA). Concentration of H_2O_2 in the sample was calculated from the H_2O_2 standard curve in μM and normalized by the protein content.

II.3.8 Quantitative PCR of Hippocampal Homogenate

II.3.8.1 RNA Isolation and cDNA Synthesis

Fifteen to twenty mg of cortical tissue was cut for RNA extraction using the RNeasy Mini Kit (Qiagen, 74104) following the manufacturer's instructions. Quality of isolated RNA was confirmed by obtaining RNA Integrity Numbers (RIN) using a RNA 6000 Pico Kit and an Agilent 2100 Bioanalyzer (Agilent Technologies), all samples had RINs higher than 7.0. To synthesize cDNA, 500 ng of RNA was used for reverse transcription using iScript Reverse Transcription Supermix (Bio-Rad, 1708841) according to the manufacturer's instructions with the thermal cycle as follows: 5 minutes at 25 ºC, 20 minutes at 46 ºC and 1 minute at 95 ºC.

II.3.8.2 Quantitative Real-Time PCR

Quantitative real-time PCR was performed using a total reaction volume of 10 μl, containing 2 μl of diluted cDNA, SsoAdvanced Universal SYBR Green Supermix (1x) (Bio-Rad), and a final concentration of 0.25 μM or 0.5 μM of forward and reverse primers (designed using Primer-BLAST, Table S2.2), with a CFX Connect Real-Time Cycler and CFX manager (Bio-Rad). Cycling conditions were as follows: 30 seconds at 95 ºC, then 40 cycles of 10 seconds at 95 ºC, 30 seconds at 60ºC followed by a melt curve from 65 ºC to 95 ºC at 0.5 ºC intervals. Gene expression fold change was quantified using the $2^{(-\Delta\Delta CT)}$ method with HPRT and GAPDH as housekeeping (HK) genes and primer sequences as follows: HPRT forward 5'-CAGGCCAGACTTTGTTGGAT-3', reverse 5'-

TCCACTTCCGCTGATGACAC-3', GAPDH forward 5'-TGATGGGTGTGAACCACGAG-3', reverse 5'TCATGAGCCCTTCCACGATG-3'.

II.3.9 Statistical Analysis

The software GraphPad Prism version 9 (La Jolla, USA) was utilized for statistical analyses. The D'Agostino and Pearson omnibus normality test was used to assess normal distribution of the data. Graphed data is presented as mean values ±SEM and two-tailed t-tests were performed for the two-group comparisons. Significance was set to $p < 0.05$.

II.4 Results

Pre-plaque, Aβ-burdened hippocampal neurons had differentially expressed DNA repair and antioxidant response genes.

We investigated the impact of pre-plaque, intraneuronal Aβ (iAβ) accumulation on oxidative stress-related gene expression in hippocampal neurons using laser capture microdissection (LCM) and qRT-PCR (Figure 2.1A-C). Pyramidal neurons from CA1 and subiculum were isolated from Wt and Tg McGill-R-Thy1-APP rats, at 5 months of age, a time point in which Aβ accumulates within neurons (iAβ) but no plaques are present.[286] Intraneuronal Aβ load was confirmed with IHC using the antibody McSA1 which specifically recognizes N-terminal amino acids 1-12 of human Aβ without cross-reacting to APP or its cleavage products.[293] McSA1 immunoreactivity showed that hippocampal neurons in Tg rats were burdened with intraneuronal Aβ (Figure 2.1A, 1B) while Wt rats had no immunoreactivity.[293, 540] RNA from these laser-captured Aβ-burdened neurons was isolated for qRT-PCR and the expression of 84 genes related to oxidative stress was quantified and compared between Wt and Tg animals. All the samples had a RIN value above 7.0 to ensure quality of extracted mRNA (see [302] for raw data). Out of the 84 genes, five genes, namely, *Ercc2*, *Fancc*, *Sod2*, *GR*, and *Idh1* were significantly upregulated in Tg hippocampal neurons as compared to Wt neurons (Figure 2.1D), with six genes showing trends to increase in Tg neurons, including *GPx7*, *Ift172*, *Sqstm1*, *Ercc6*, *Gclm*, and *Prnp* (Figure S2.1).

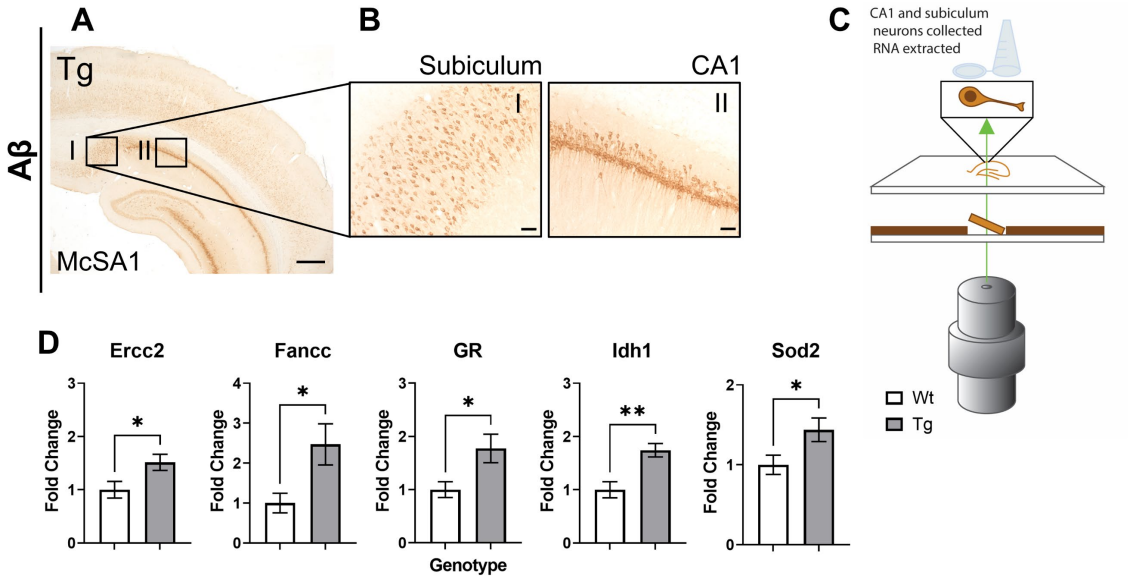


Figure 2.1. Laser captured Aβ-burdened neurons have increased expression in genes related to oxidative stress response.

A. Representative image of McSA1 immunoreactivity in Tg hippocampus. **B.** McSA1 immunoreactivity in Tg subiculum (I) and CA1 (II) from A. **C.** Schematic depicting laser capture microdissection of CA1 and subiculum neurons from Tg Aβ-burdened neurons and Wt neurons not burdened with Aβ. **D.** Differentially expressed genes including *Ercc2*, *Fancc*, *Sod2, GR*, and *Idh1* in Aβ-burdened Tg hippocampal neurons as compared to Wt neurons. Fold changes were normalized to Wt expression. Scale bars represent 500 μm in A, 50 μm in B. Error bars indicate SEM. two-tailed t-test, *$p < 0.05$, **$p < 0.01$.

DNA repair protein, XPD (*Ercc2*) was increased in transgenic subiculum neurons while Fancc levels remained unchanged.

To determine whether the changes observed in oxidative stress-related transcripts (Figure 2.1D) corresponded to changes at the protein level in these neurons, we performed immunofluorescence labelling using antibodies recognizing each protein of interest in combination with NeuN which specifically identifies mature neurons.[541] We then quantified immunoreactivity of each protein of interest in areas overlapping with NeuN immunoreactivity. We adapted our experiments to include DAPI labelling for nuclei when cellular localization of the protein needed to be considered (Figure S2.2). Consistent with our results at the transcript level, we found elevated levels of XPD (a product of the gene *Ercc2*) in Tg subiculum neurons, while levels in CA1 neurons remained unchanged from the Wt (Figure 2.2). Regarding Fancc, while RNA levels were upregulated in Tg hippocampal neurons (Figure 2.1D) at the protein level, no differences were observed. Furthermore, no difference in Fancc levels were detected at either cytoplasmic or nuclear localizations of hippocampal neurons between Wt and Tg samples (Figure 2.2D-F).

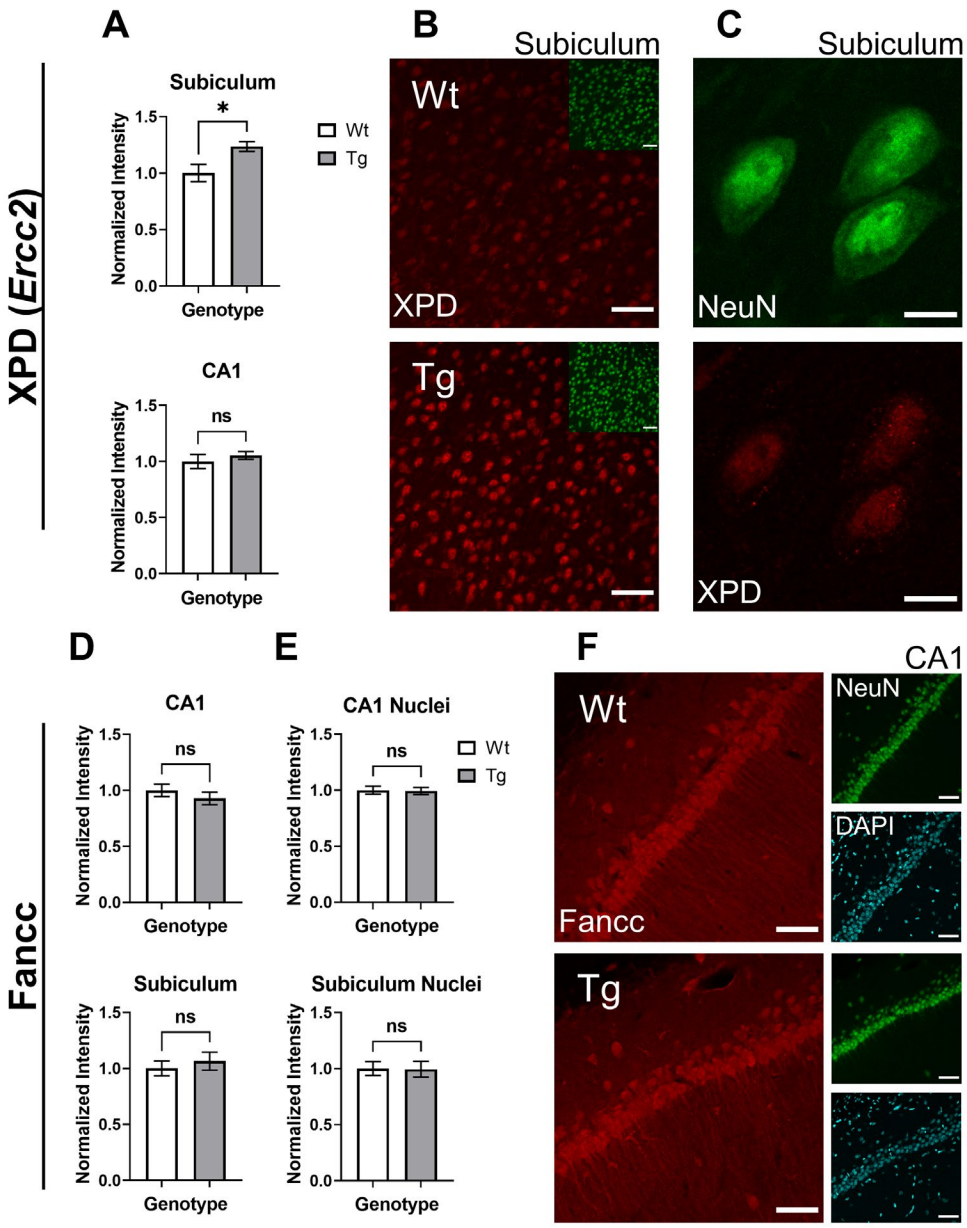


Figure 2.2. Protein levels of XPD (gene product of *Ercc2*) and Fancc in hippocampal neurons.
A. Quantification of XPD immunoreactivity in subiculum and CA1 neurons of Wt and Tg rats normalized to Wt fluorescence intensity. **B.** Representative images of XPD immunoreactivity (red) in Wt and Tg subiculum neurons with an inset showing NeuN in green. **C.** Higher magnification images of XPD immunoreactivity (red) in subiculum neurons with NeuN in green. **D.** Quantification of Fancc immunoreactivity in CA1 and subiculum neurons of Wt and Tg rats normalized to Wt fluorescence intensity. **E.** Quantification of Fancc immunoreactivity in the nuclei of CA1 and subiculum neurons normalized to Wt fluorescence intensity. **F.** Representative images of Fancc immunoreactivity (red) in Wt and Tg CA1 neurons with NeuN in green, and DAPI in cyan. Error bars represent SEM. Scale bars represent 50 μm in B and 10 μm in C. ns = non-significant, two-tailed t-test, *$p < 0.05$.

Hippocampal expression of other DNA repair genes showed alterations in *Fen1*.

In light of our findings in hippocampal neurons, where two of the five upregulated genes (*Ercc2* and *Fancc*) were implicated in DNA repair, we next investigated the status of other DNA repair genes in the Tg hippocampus as compared to Wt including *Ape1* (apurinic/apyrimidinic endodeoxyribonulease 1), *Cdk5* (cyclin dependent kinase 5), *Ercc3* (excision repair cross-complementing 3), *Fen1* (flap endonuclease 1), *Parp1* (poly(ADP-Ribose) polymerase 1), *Pcna* (proliferating cell nuclear antigen), *Polβ* (polymerase beta), and *Sirt3* (sirtuin 3) that play roles in base excision repair (BER), non-homologous end joining (NHEJ) and other DNA repair pathways. Towards this objective, qRT-PCR of cDNA isolated from hippocampal homogenates was performed. We found that there was an increase in expression of the gene *Fen1*, and a trend to increase in the gene *Ercc3* (gene product XPB).

Table 2.1. Quantitative PCR of hippocampal homogenates.

Gene	DNA Repair Pathway	Fold-Change Tg	P value
Ape1	BER	1.14	0.3854
Cdk5	BER/Others	1.12	0.4985
Ercc3	NER	1.21	0.1129+
Fen1	**BER, NHEJ**	**1.31**	**0.0136***
Parp1	Multiple	1.00	0.9973
Pcna	Multiple	0.92	0.7682
Polβ	BER	1.10	0.3517
Sirt3	mtDNA repair	1.13	0.4735

* $p < 0.05$, + trend

Glutathione reductase protein levels trended to decrease in transgenic CA1 neurons while SOD2 levels remained unchanged.

Immunoreactivity for other proteins whose genes were upregulated in Tg hippocampal neurons, namely GR and SOD2, was assessed and we found that these proteins were not upregulated as their respective genes were. Glutathione reductase (GR) was unchanged in the subiculum while there was a trend to decrease in Tg CA1 neurons (Figure 2.3A, B), contrary to the increase in transcript levels (Figure 2.1D). The activity of GR in cortical homogenates was also assessed in 3-month and 5-month old Wt and Tg rats and was unchanged (Figure 2.3C). For SOD2, RNA levels were upregulated in Tg hippocampal neurons (Figure 2.1D) while at the protein level we did not observe differences as assessed by immunofluorescence (Figure 2.3G-I). On the other hand, parvalbumin positive (PV+) neurons displayed higher SOD2 immunoreactivity compared to parvalbumin negative (PV-) neurons in both the CA1 and the subiculum (Figure S2.3) therefore, PV- and PV+ neurons were analyzed separately.

However, no significant differences in SOD2 immunoreactivity between Wt and Tg neurons were observed.

Interestingly, hippocampal Idh1 immunoreactivity was more prominently expressed at the protein level in astrocytes rather than neurons (Figure S2.4A). When quantified, immunoreactivity of Idh1 in astrocytes did not differ between Wt and Tg (Figure S2.4B). To ensure the increase of *Idh1* in Tg neurons at the transcript level was not due to contaminating astrocytic material, total GFAP (glial fibrillary acidic protein) immunoreactivity in CA1 and subiculum ROIs (Figure S2.4C) as well as GFAP immunoreactivity overlapping with neuronal layers in CA1 and subiculum (Figure S2.4D) was quantified. No differences between Wt and Tg GFAP immunoreactivity were observed confirming that increased *Idh1* gene expression in captured neuronal material from Tg hippocampi was not due to increased gliosis. Indeed, as reported in Welikovitch *et al.* (2020) which utilized the same laser captured neuronal material, there was minimal astrocytic content in the LCM samples.[302]

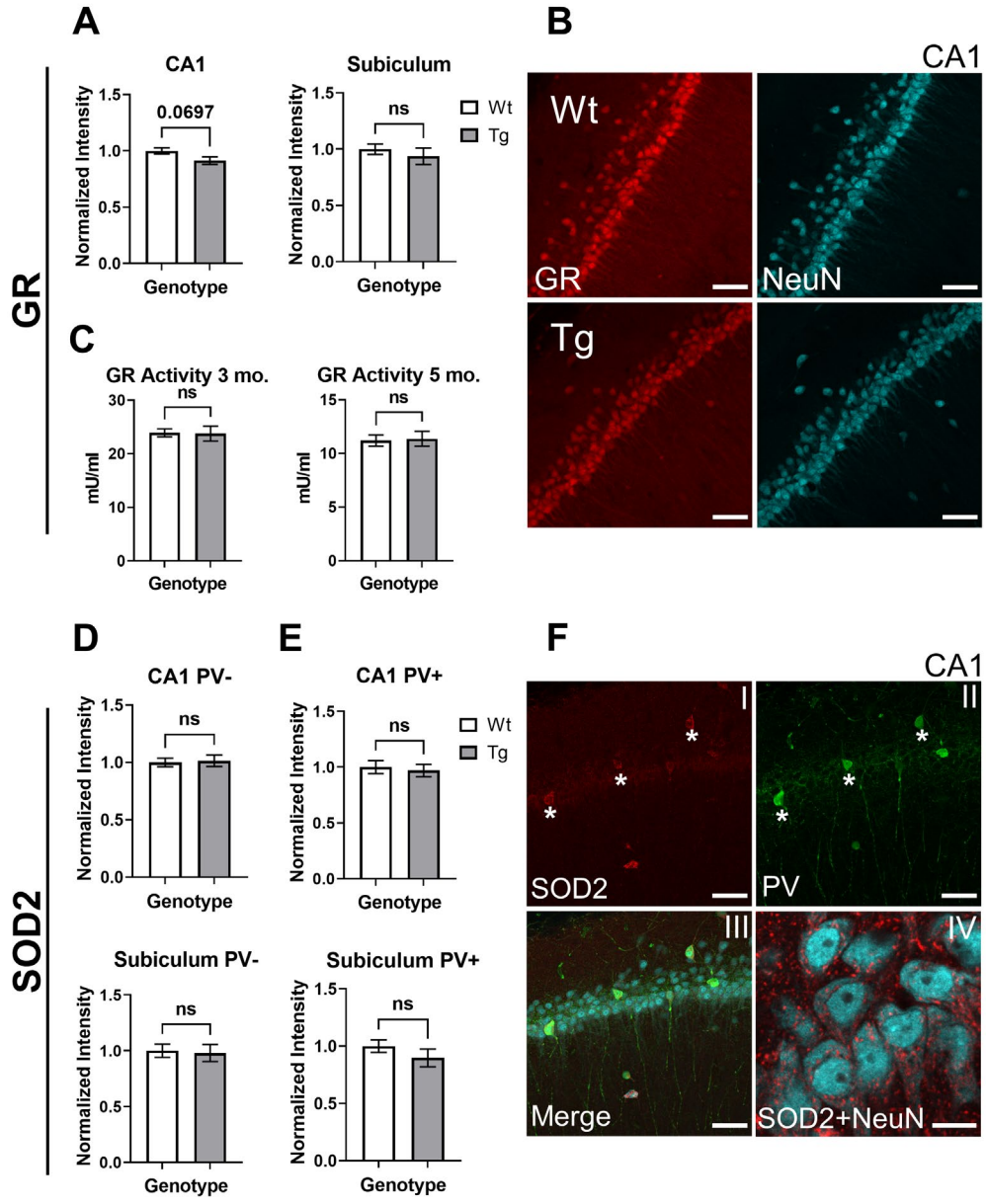


Figure 2.3. Protein levels of GR and SOD2 in hippocampal neurons.

A. Quantification of GR immunoreactivity in CA1 and subiculum neurons of Wt and Tg rats normalized to Wt fluorescence intensity. **B.** Representative images of GR immunoreactivity (red) in Wt and Tg CA1 neurons with NeuN in cyan. **C.** Enzyme activity of GR in 3-month and 5-month old Wt and Tg cortical homogenates. **D.** Quantification of SOD2 immunoreactivity in CA1 and subiculum neurons of Wt and Tg rats that had no parvalbumin (PV-) immunoreactivity. Values were normalized to Wt fluorescence intensity. **E.** Quantification of SOD2 immunoreactivity in CA1 and subiculum of Wt and Tg PV+ neurons normalized to Wt fluorescence intensity. **F.** Representative CA1 images of SOD2 immunoreactivity in red (I), PV+ neurons in green indicated by asterisks (*) (II), merged with NeuN in cyan (III). A higher magnification image of SOD2 (red) and NeuN (cyan) in CA1 (IV). Error bars represent SEM. Scale bars represents 50 μm in B, F (I-III) and 10 μm in F (IV). ns = non-significant, two-tailed t-tests.

At pre-plaque stages, transgenic hippocampal neurons showed evidence of incipient oxidative damage

To examine the extent of downstream oxidative damage in CA1 and subiculum neurons, γH2AX, 4HNE, and 8-oxo-dG immunoreactivity were quantified. Subiculum neurons burdened with Aβ had significantly higher numbers of γH2AX positive neurons (Figure 2.4A), while CA1 neurons showed a trend to increased numbers. γH2AX foci indicate the presence of double-strand DNA (dsDNA) breaks which can be caused by oxidative DNA damage.[542] Quantification of 4HNE immunoreactivity—which reflects levels of lipid peroxidation, a downstream form of oxidative damage—showed a trend to increase in CA1 Tg neurons. Next, oxidized DNA was assessed by probing for 8-oxo-dG. Notably, the antibody used is capable of recognizing both 8-oxo-dG and 8-oxo-G, thus, pre-treatment with either DNase or RNase can help elucidate RNA- or DNA- specific oxidation respectively. Indeed, pre-treatment of sections with RNase resulted in a decreased immunoreactivity (Figure S2.5) and recognition of DNA (nuclear and mitochondrial) rather than RNA oxidation. Nuclear, and cytoplasmic immunoreactivity of 8-oxo-dG in Tg and Wt hippocampal neurons was quantified using immunofluorescence (Figure 2.4C, D), and no differences between Tg and Wt neurons were found. However, interestingly, there was a trend to decrease in 8-oxo-dG immunoreactivity in CA1 neurons of Tg animals (Figure 2.4E). Indeed, oxidative damage such as 8-oxo-dG is a downstream consequence of many upstream processes that may, at this early pre-plaque timepoint still be managed by cellular antioxidant and repair mechanisms. The general redox status of 5 month cortical tissue was assessed using an assay employing the fluorescent probe DCF (dichlorodihydrofluorescin) (Figure S2.6) and no changes between Wt and Tg cortical homogenates were detected. Of note, interpretations of results from this particular probe must be exercised with caution, since it can react with various free radicals in the cell, is dependent on peroxidase activity and the availability of free iron among other factors.[543]

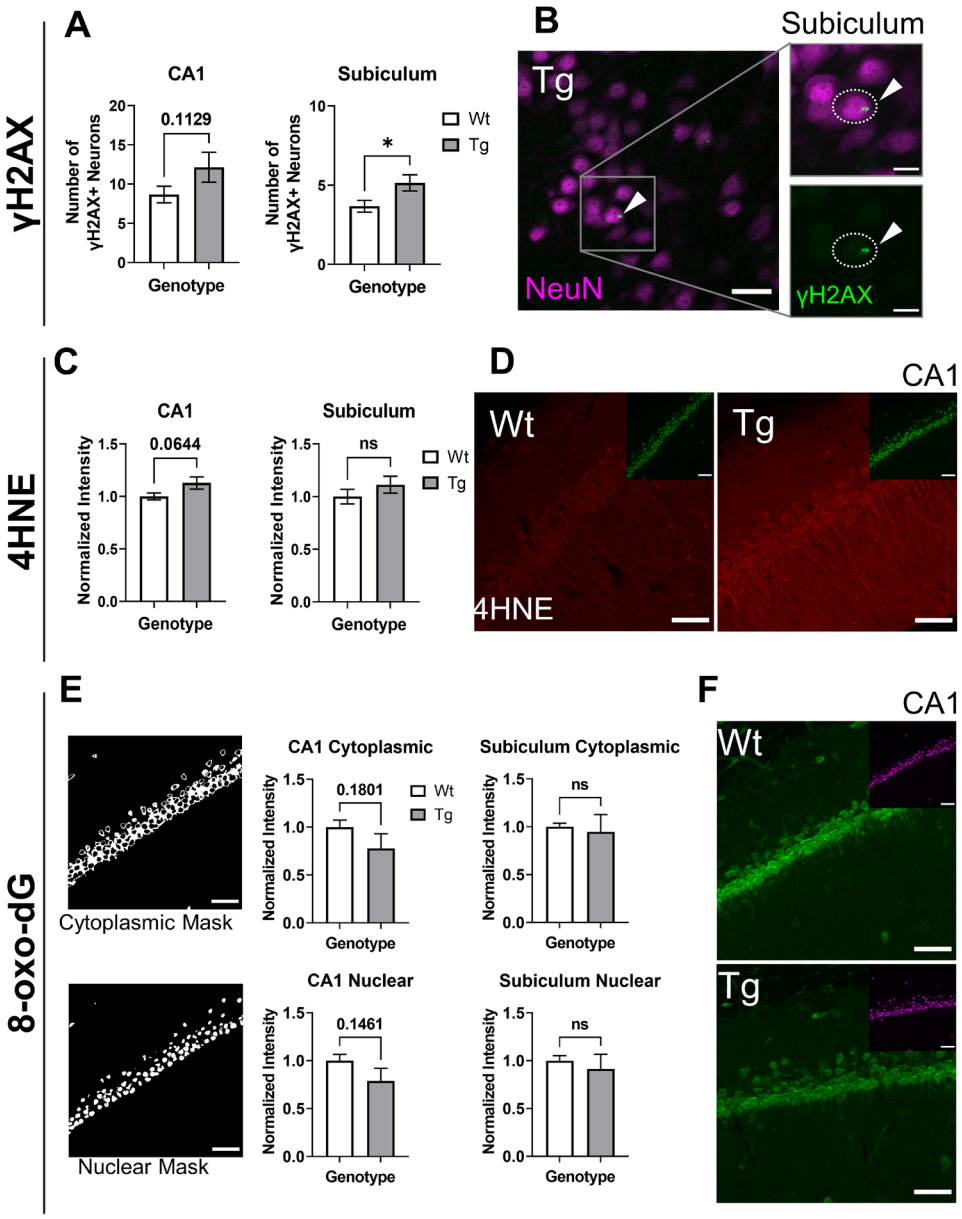


Figure 2.4. Damage in Aβ-burdened neurons.

A. Quantification of neurons with γH2AX-positive foci in CA1 and subiculum. **B.** Representative image of γH2AX-positive foci in green (arrowhead) in the Tg subiculum (NeuN in magenta). **C.** Quantification of 4HNE immunoreactivity in CA1 and subiculum of Wt and Tg neurons normalized to Wt fluorescence intensity. **D.** Representative images of 4HNE immunoreactivity (red) in Wt and Tg CA1 neurons with an inset showing NeuN in green. **E.** *Quantification of 8-oxo-dG immunoreactivity in CA1 and subiculum of Wt and Tg neurons normalized to Wt fluorescence intensity. The left-most panels show the cytoplasmic or nuclear masks used to distinguish cytoplasmic versus nuclear immunoreactivity for quantification. **F.** Representative image of 8-oxo-dG immunoreactivity in Wt and Tg CA1 neurons (green) with inset showing NeuN (magenta). Error bars represent SEM. Scale bars represent 50 μm. ns = non-significant, two-tailed t-tests, *$p < 0.05$.

II.5 Discussion

The present study showed an upregulation of genes responsible for DNA repair, and antioxidant response (both cytoplasmic and mitochondrial) in Tg neurons compared to Wt. There was also evidence of oxidative and DNA damage in these Tg neurons by IHC. At the protein level, XPD (*Ercc2*), which is involved in nucleotide excision repair (NER), was increased in Tg neurons compared to Wt.

Oxidative damage in the brain, caused through redox imbalance and oxidative stress increases with aging,[544] and has been implicated in neurodegenerative diseases including AD.[545] Adding to this, neuroinflammation has emerged as an early pathological mechanism in AD which is closely tied to oxidative stress.[302, 479] In AD, the decades preceding extracellular plaque formation and clinical symptoms remain uncharacterized, thus, understanding the role of neuronal oxidative stress at the earliest stages of AD would offer insight into disease progression. Indeed, antioxidant clinical trials for AD have not succeeded.[546-547]

During advanced, late stages of AD, the pathology is irreversible and therefore the opportunity to prevent or delay AD is during the earliest preclinical stages when the initial, disease aggravating oxidative stress occurs. However, without a complete understanding of the key players maintaining redox balance and managing oxidative stress at early disease stages, the appropriate dosage, targeting, combinations, and timing of antioxidant treatments will not be possible.[546]

Our laboratory recently discovered that during early, pre-plaque stages in the McGill-R-Thy1-APP rat model of the AD-like amyloid pathology, iAβ-burdened neurons increased gene and protein expression of inflammatory markers.[302] Using this same neuronal material, in this study we investigated alterations in oxidative stress-related genes coinciding with this upregulation of inflammatory markers and found that DNA repair and antioxidant genes were upregulated in these same neurons. Of note, the McGill-R-Thy1-APP rat model exhibits an AD-like amyloid pathology with a prolonged pre-plaque stage, which allows for studying the effects of gradual iAβ accumulation well before extracellular plaque formation.[286] Rats are also physiologically, genetically and morphologically closer to humans, with six tau isoforms, increased homology to ApoE and a wider behavioral display, compared to mice.[484]

Alterations in expression and activity of DNA repair genes and proteins, has been observed at late stages in the AD pathology in the brains of individuals with MCI, and AD,[542, 548-550] (reviewed in Bucholtz and Demuth (2013)),[551] and in transgenic rodent models of AD.[542, 552-

[553] In the present study, during pre-plaques stages, iAβ-burdened hippocampal neurons had increased expression of *Ercc2* and *Fancc* which are both implicated in DNA repair processes, namely nucleotide excision repair (NER) and interstrand crosslink repair, respectively.[554] However, Fancc may also play a role in other repair pathways,[555] response to oxidative DNA damage,[556] and the redox state of the cell.[557] While Fancc was not significantly elevated at the protein level (Figure 2.3D-F), one publication has reported rare variants in *Fancc* that were associated with entorhinal cortex thickness in participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort.[558] Conversely, in the present study, the gene product of *Ercc2* (XPD, Xeroderma pigmentosum complementation group D protein) was elevated in subiculum neurons (Figure 2.2). XPD is an ATP-dependent 5'-3' helicase, that plays a role in RNA polymerase II initiated transcription and in NER.[559] NER repairs a variety of bulky DNA lesions including those resulting from oxidative damage.[560]

Notably, previous studies have shown that *Ercc2* gene expression was increased in the brains of individuals with Down Syndrome (DS),[561] while XPD protein expression was increased in the brains of individuals with DS and AD.[562] Importantly, individuals with DS develop AD due to triplication of chromosome 21 which contains the *APP* gene, and causes excess Aβ production. As a result, individuals with DS exhibit progressive brain Aβ accumulation from as early as birth.[134, 563-565]

In the context of sporadic AD, a more recent study showed that polymorphisms in XPD were not associated with sporadic late-onset AD.[566] Interestingly, another gene that trended to increase in Tg neurons was *Ercc6* (Figure S2.1), which encodes the protein CSB. Like XPD, CSB plays a role in NER but also contributes to base excision repair (BER) which is responsible for repairing a wide variety of oxidative DNA damage.[567-568]

As two of the five differentially expressed genes in Tg iAβ-burdened hippocampal neurons involved DNA repair pathways, other genes implicated in various DNA repair pathways were investigated (Table 2.1). For these experiments hippocampal homogenates were used, thus, the main limitation to interpreting these results is that they are not neuron specific. Although we did not find differences between Wt and Tg expression of *Ape1*,[569-570] *Cdk5*,[569, 571-575] *Parp1*,[550] *Pcna*,[576-577] *Polβ*,[578-579] or *Sirt3*,[580-582] these targets have been implicated in AD at late stages. It would in future be of interest to assess protein and activity levels of these targets since gene expression is only part of the picture. There was a significant increase in the hippocampal expression of *Fen1* (flap endonuclease 1) (Table 2.1), which plays a role in BER[583] and non-homologous end joining (NHEJ).[584] Since neurons are post-mitotic, they

tend to repair double-strand breaks (DSB) using NHEJ over homologous recombination (HR), even though the former is more error prone.[585] Importantly, there was evidence of increased DSBs in the subiculum (discussed later and shown in Figure 2.4A, B). There was also a trend to increase in *Ercc3* gene expression (Table 2.1) which produces the protein XPB. This finding aligns with studies showing an increase in gene and protein expression of XPB in the brains of individuals with DS[561] and AD.[562]

Regarding other neuron-specific genes that were upregulated in the Tg hippocampus, *Sod2* expression increased in Tg neurons (Figure 2.1D) but showed no changes at the protein level (Figure 2.3G-I). However, since SOD2 is a mitochondrial antioxidant enzyme, our analysis at the protein level by IF was limited in that we could not quantify CA1 neuron-specific synapse levels of SOD2. Indeed, it could be possible that at the level of the synapse, there is SOD2 deficiency in the Tg hippocampus. Other publications have demonstrated that synaptic mitochondrial deficits precede non-synaptic mitochondrial deficits in Tg AD models including heterozygous McGill-R-Thy1-APP rats.[298, 493] Interestingly parvalbumin positive (PV+) neurons expressed significantly higher levels of SOD2 (Figure S2.3), likely due to the increased need for protection against oxidative stress related to increased activity.[586]

Transgenic hippocampal neurons exhibited an increase in *GR* (glutathione reductase), gene expression that trended to decrease at the protein level in CA1 while remaining unchanged in the subiculum (Figure 2.3A, B). GR activity levels in cortical homogenates at 3 and 5 months were also measured and no differences between Wt and Tg were observed, which aligns with results from another study that utilized a Tg mouse model of AD.[378] However, the limitation here is that we did not assess hippocampal, nor neuron-specific GR activity which may be important. One study assessing non-cognitively impaired (NCI), MCI and AD brain tissue including synaptosomal and mitochondrial fractions, showed that GR activity was only decreased in MCI and AD synaptosomal fractions.[587]

GR aids in replenishing glutathione (GSH) levels in the cell by catalyzing the conversion of oxidized glutathione (GSSG) back to reduced glutathione (GSH). Thus, the ratio of reduced to oxidized glutathione (GSH:GSSG) is indicative of the cellular redox state and found to be altered in aging,[588] in Tg rodent models of AD[378, 589] and peripherally in MCI and AD.[590] It is important for GSH to be replenished by GR since GSH is a highly abundant, non-protein antioxidant with intracellular concentrations ranging from 1-10 μM,[337, 450] (brain levels are from 1-3 μM).[591] GSH protects the cell by reacting with free radicals, but also by aiding glutathione peroxidases in breaking down hydrogen peroxide (H_2O_2).[592] Notably, the gene

GPx7 (glutathione peroxidase 7), which has functions in oxidative protein folding in the endoplasmic reticulum,[593] trended to increase in Tg hippocampal neurons (Figure S2.2). As well, the gene *Gclm* (an essential subunit for glutathione synthesis) trended to increase in Tg neurons (Figure S2.1). However, it should be acknowledged that there are key differences between the mechanisms by which *Gclm* transcription is activated in the rat and human.[594-595]

Another important function of GSH is to detoxify reactive electrophiles such as 4HNE.[596] In this case, GSH is enzymatically conjugated to 4HNE by glutathione-S-transferases, of which there are many isoforms. In the RT^2 qRT-PCR array, two glutathione-S-transferases were assessed (*Gstk1* and *Gstp1*) but not found to be significantly changed in Tg neurons (Table S2.3). When we assessed 4HNE immunoreactivity in the hippocampus, we found a trend to increase in CA1 (Figure 2.4C, D), CA1 was also the region in which GR had trended to decrease (Figure 2.3A, B), while there was no change in the subiculum.

Oxidative damage to DNA accumulates with aging[597] and can result in double stand breaks (DSBs) when there are multiple lesions in proximity to one another (within 20 bp), also known as oxidatively induced clustered DNA lesions (OCDLs).[439, 598] Additionally, independently of oxidative stress, non-dividing cells such as neurons can accumulate DSBs from transcription but also through abnormal activity.[448, 599-600] Of note, in AD, stimulation of glutamate receptors by Aβ binding can lead to oxidative stress[538] and hyperexcitability.[601-602] When DNA DSBs occur, the histone variant H2AX is rapidly phosphorylated at serine 139 to form γH2AX,[603] which then accumulates at DSBs as foci to help recruit repair proteins.[445, 604-605] These γH2AX positive foci are one of the earliest markers of DSBs and can be visualized using immunofluorescence.[447, 602] By immunofluorescence, there was a significantly increased number of neurons with γH2AX positive foci in the Tg subiculum, while Tg CA1 neurons showed a trend to increase compared to Wt neurons (Figure 2.4A). These results align with another study using hAPP-J20 mice that showed elevated levels of hippocampal and cortical neurons with γH2AX positive foci at 6-months of age when cognitive deficits were observed and few amyloid deposits were present. Even at 1.5-2 months, before cognitive impairment, the authors observed more neurons with γH2AX positive foci in the entorhinal cortex and dentate gyrus of Tg hAPP-J20 mice.[448] A more recent study also showed increased numbers of both neurons and astrocytes with γH2AX positive foci in the hippocampus and frontal cortex of MCI and AD brains.[602] Of importance, as highlighted by Shanbhag *et al.* (2019), earlier studies of γH2AX in AD brains quantified

pan-nuclear immunoreactivity rather than foci, where the former is indicative of neuronal activity and the latter is indicative of DSBs.[602]

Oxidatively modified nuclear and mitochondria DNA (nDNA, mtDNA) is elevated in the brains of individuals with DS,[606] pre-clinical AD (PCAD),[607-608] MCI,[609] and AD.[430-431, 610-613] In these post-mortem studies, the oxidative lesions reported included 8-OHdG, 8-OHdA, 5-OHC, FapyGua, FapyAde, and 5-OHdU in various brain regions using HPLC/ECD, GC/MS-SIM, and/or IHC to verify neuronal oxidative DNA damage since one limitation of numerous studies was the use of tissue homogenates that were not cell specific. Elevations in oxidative DNA damage varied between studies, but in most, mtDNA lesions were higher than nDNA[613] and often exhibited more marked differences between healthy controls and AD. However, it remains unknown whether this oxidative damage to nucleic acids plays an early role in the AD pathology and when oxidative stress affecting DNA overwhelms compensatory mechanisms. Here, 8-oxo-dG levels remained unchanged between Wt and Tg hippocampal neurons, with a possible trend to decrease in CA1 Tg neurons was observed (Figure 2.4E, F). This was contrasting to what we hypothesized. However, it is important to note that oxidative damage occurs downstream to ROS production, and it is possible that at this early time point, Aβ-burdened neurons are still able to manage and repair oxidatively modified nucleotides.

II.6 Conclusions

The present study revealed that early accumulation of iAβ, coinciding with a neuron-derived inflammatory response,[302] alters expression of oxidative stress-related genes including key DNA repair and antioxidant genes. These changes are likely in response to reactive oxygen species (ROS) production and an incipient redox imbalance. Furthermore, the lack of overt oxidative damage in these iAβ-burdened hippocampal neurons suggests that this pre-plaque timepoint precedes the fully-realized oxidative stress observed at late, post-plaque stages of AD and implicates DNA repair and antioxidant response during early pre-plaque stages. Our lab has previously shown, at the same pre-plaque timepoint, excessive hypomethylation in hippocampal neurons linked to iAβ accumulation which may also contribute to the observations made in this study.[614]

II.7 Supporting Information

The Supporting Information includes the following Figures and Tables:

- **Figure S2.1.** Genes trending to increase in hippocampal neurons.
- **Figure S2.2.** Image analysis methods.
- **Figure S2.3.** SOD2 immunoreactivity in hippocampal neurons.
- **Figure S2.4.** Area of GFAP immunoreactivity in the hippocampus.
- **Figure S2.5.** RNase pre-treatment for 8-oxo-dG assessment.
- **Figure S2.6.** DCF Assay.
- **Table S2.1.** Housekeeping genes for RT2 Rat Oxidative Stress Profiler PCR Array.
- **Table S2.2.** Primer sequences for quantitative real-time PCR (hippocampal homogenates).
- **Table S2.3.** Expression of oxidative stress-related genes in hippocampal neurons.
- **Table S2.4.** List of primary antibodies and dilutions for IHC and IF experiments.
- **Table S2.5.** List of secondary antibodies and dilutions for IHC and IF experiments.

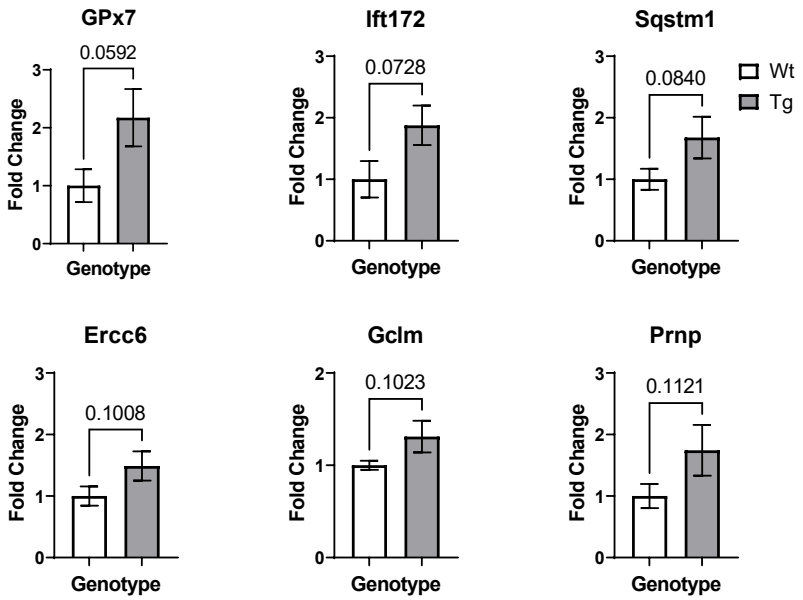


Figure S2.1. Genes trending to increase in hippocampal neurons.
Expression of genes in Aβ-burdened Tg hippocampal neurons as compared to Wt neurons including *GPx7, Ift172, Sqstm1, Ercc6, Gclm,* and *Prnp*. Fold changes were normalized to Wt expression. Error bars indicate SEM.

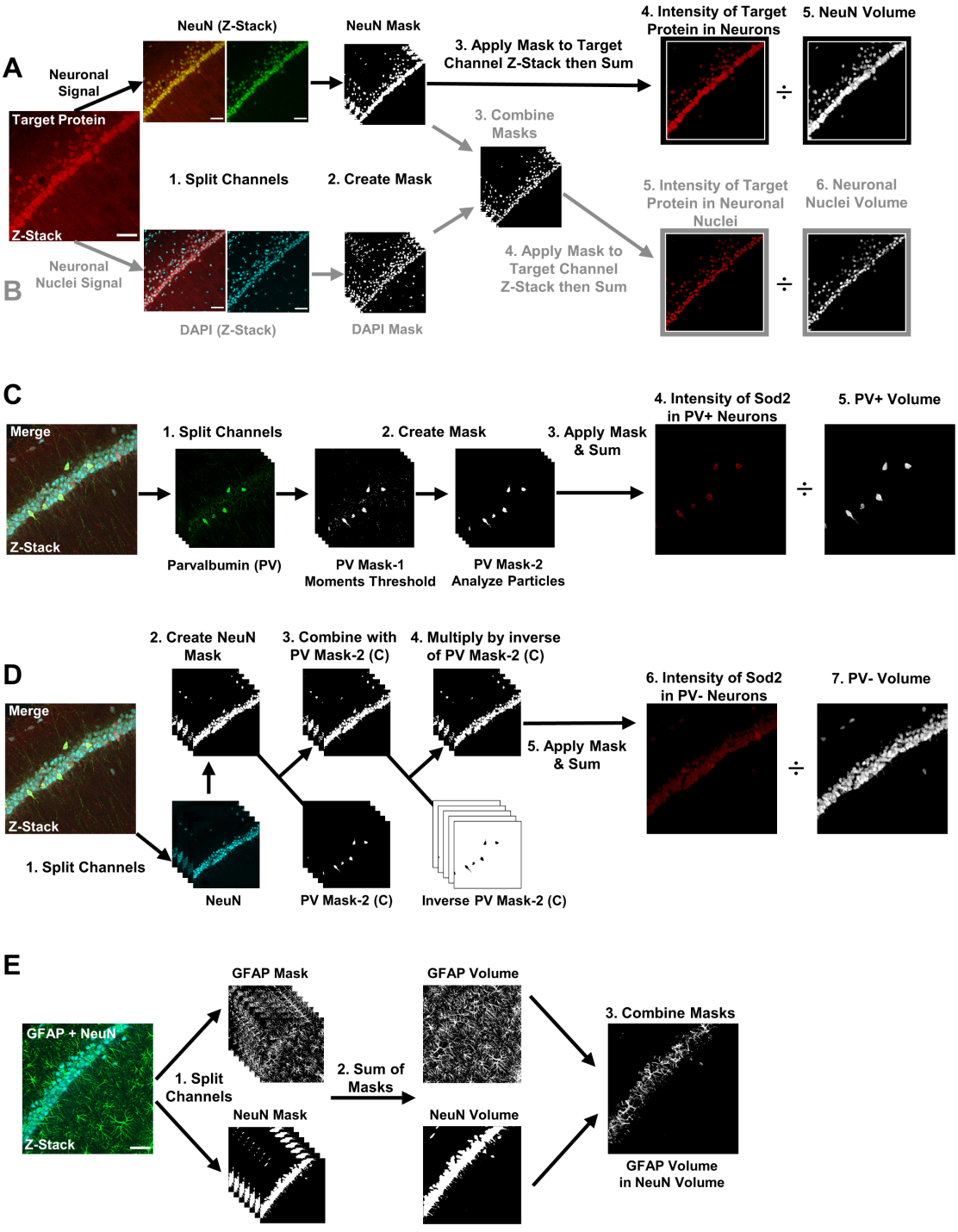

A
Neuronal Signal
Target Protein
Z-Stack
NeuN (Z-Stack)
NeuN Mask
3. Apply Mask to Target Channel Z-Stack then Sum
4. Intensity of Target Protein in Neurons
5. NeuN Volume
3. Combine Masks
1. Split Channels
2. Create Mask
5. Intensity of Target Protein in Neuronal Nuclei
6. Neuronal Nuclei Volume
4. Apply Mask to Target Channel Z-Stack then Sum
B
Neuronal Nuclei Signal
DAPI (Z-Stack)
DAPI Mask
C
Merge
Z-Stack
1. Split Channels
2. Create Mask
3. Apply Mask & Sum
4. Intensity of Sod2 in PV+ Neurons
5. PV+ Volume
Parvalbumin (PV)
PV Mask-1 Moments Threshold
PV Mask-2 Analyze Particles
D
Merge
Z-Stack
2. Create NeuN Mask
3. Combine with PV Mask-2 (C)
4. Multiply by inverse of PV Mask-2 (C)
5. Apply Mask & Sum
6. Intensity of Sod2 in PV- Neurons
7. PV- Volume
1. Split Channels
NeuN
PV Mask-2 (C)
Inverse PV Mask-2 (C)
E
GFAP + NeuN
Z-Stack
1. Split Channels
GFAP Mask
NeuN Mask
2. Sum of Masks
GFAP Volume
NeuN Volume
3. Combine Masks
GFAP Volume in NeuN Volume

Figure S2.2: Image analysis methods.

A. Quantifying target protein fluorescence intensity in CA1 and subiculum neurons using NeuN. ImageJ macro work-flow using z-stack image files. (1) Channels were split and a NeuN mask (2) was generated as follows: the NeuN z-stack was processed using despeckle and a z-projection of the average intensity (Fancc, GR) or maximum intensity (SOD2, XPD) was obtained. The threshold method Default (Fancc), Yen (GR), or Moments (XPD) was applied to obtain lower and upper threshold values. The raw NeuN z-stack was then despeckled twice and these threshold values were applied to the entire stack to generate a binary NeuN z-stack which was divided by 255 to obtain pixel values of 0 and 1. (3) This NeuN binary z-stack mask was multiplied by the z-stack of the target protein channel and summed to solely obtain (4) the fluorescence intensity within the neuronal volume. The NeuN volume (5) was obtained by taking the summed projection of the binary NeuN z-stack mask in step 2. Finally, fluorescence intensity was divided by the volume to obtain a mean fluorescence intensity.

B. Quantifying target protein fluorescence intensity in CA1 and subiculum neuronal nuclei using DAPI and NeuN similar to the process in A. The process for generating a NeuN binary mask were as described in A steps (1) and (2). (1) Channels were split and a DAPI mask (2) was generated as follows: the DAPI z-stack was processed using despeckle and a z-projection of the average intensity was obtained. The threshold method Default was applied to obtain lower and upper threshold values. The raw DAPI z-stack was then despeckled twice and these threshold values were applied to the entire stack to generate a binary DAPI z-stack which was divided by 255 to adjust the pixel values to 0 and 1. (3) This DAPI binary z-stack mask was then multiplied by the NeuN binary z-stack to obtain a binary z-stack mask of neuronal nuclei. (4) This neuronal nuclei binary z-stack mask was then multiplied by the z-stack of the target protein channel and summed to solely obtain (5) the fluorescence intensity within the neuronal volume. The neuronal nuclei volume (6) was obtained by taking the summed projection of the binary z-stack mask in step 3. Finally, fluorescence intensity was divided by the volume to obtain a mean fluorescence intensity.

C. Quantifying target protein fluorescence (specifically SOD2) in PV+ neurons of CA1 and subiculum. (1) Channels were split and a PV mask (2) was generated as follows: the PV z-stack was processed using despeckle and a z-projection of the maximum intensity was obtained. The threshold method Li was applied to obtain lower and upper threshold values. The raw PV z-stack was then despeckled twice and these threshold values were applied to the entire stack to generate a binary PV z-stack. Analyze particles (250 pixels^2) was then applied and the mask was divided by 255 to obtain pixel values of 0 and 1. (3) This PV binary z-stack mask was then multiplied by the z-stack of the target protein channel (SOD2) and summed to solely obtain (4) the fluorescence intensity within the PV neuronal volume. The PV volume (5) was obtained by taking the summed projection of the binary PV z-stack mask in step 2. Finally, fluorescence intensity was divided by the volume to obtain a mean fluorescence intensity.

D. Quantifying target protein fluorescence (specifically Sod2) in PV- neurons of CA1 and subiculum. (1) Channels were split and a NeuN mask (2) was generated as described in A. (3) This NeuN binary z-stack mask was then combined with the PV binary mask generated in C. This combined mask including NeuN and PV was then multiplied by the inverse of the PV mask to generate a mask that excluded PV+ neurons (4). (5) This modified NeuN binary z-stack designed to exclude PV+ neurons was then multiplied by the z-stack of the target protein channel (SOD2) and summed to solely obtain (6) the fluorescence intensity within the NeuN neuronal volume excluding PV+ neurons. The volume of this modified NeuN mask (7) was obtained by taking the summed projection of the binary z-stack mask in step 4. Finally, fluorescence intensity was divided by the volume to obtain a mean fluorescence intensity.

E. Assessing GFAP-positive processes in the vicinity of CA1 and subiculum neurons, also shown in Figure S4. (1) Channels were split and masks were generated for NeuN (z-projection maximum intensity, despeckle, triangle threshold, binary, fill holes, erode twice, divide by 255 to generate pixel values of 0 and 1 – for the total z-stack area multiply by the number of z-stacks) and for GFAP (gaussian blur with a sigma of 1, threshold applied to each image in stack (obtain threshold value using a mean threshold applied to the average z-stack), binary, divide by 255 to generate pixel values of 0 and 1). (2) Take the sum the masks, which represents the total neuronal (NeuN+) and astrocytic (GFAP+) areas. (3) To obtain the area of GFAP immunoreactivity in neuronal regions, combine the summed NeuN and GFAP masks by multiplying them (therefore only areas containing both NeuN and GFAP with pixel values of 1 will remain).

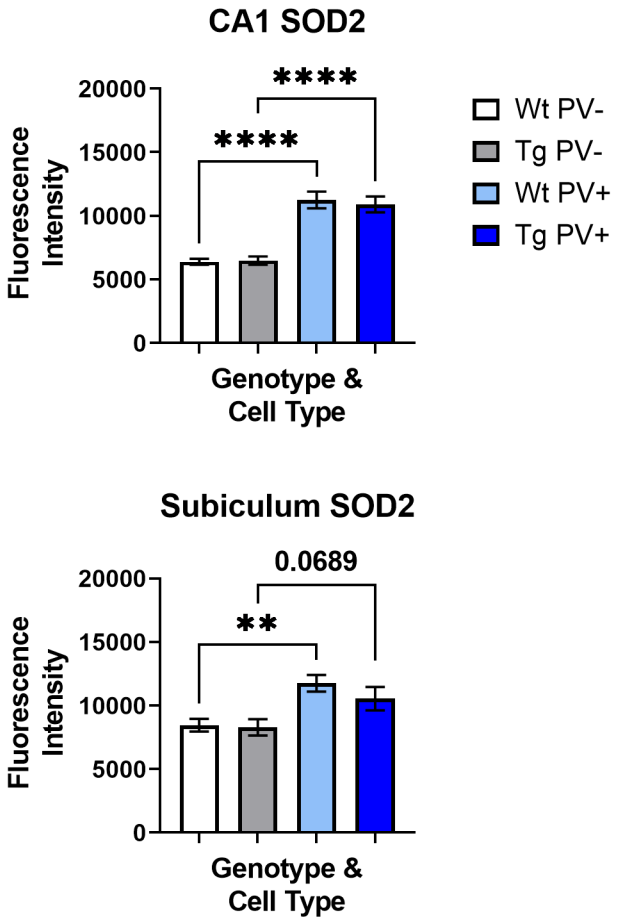


Figure S2.3: SOD2 immunoreactivity in hippocampal neurons.
SOD2 immunoreactivity in parvalbumin positive (PV+) and negative PV- hippocampal neurons. Error bars represent SEM. One-way ANOVA, Bonferroni correction, ****p<0.0001, **p<0.01.

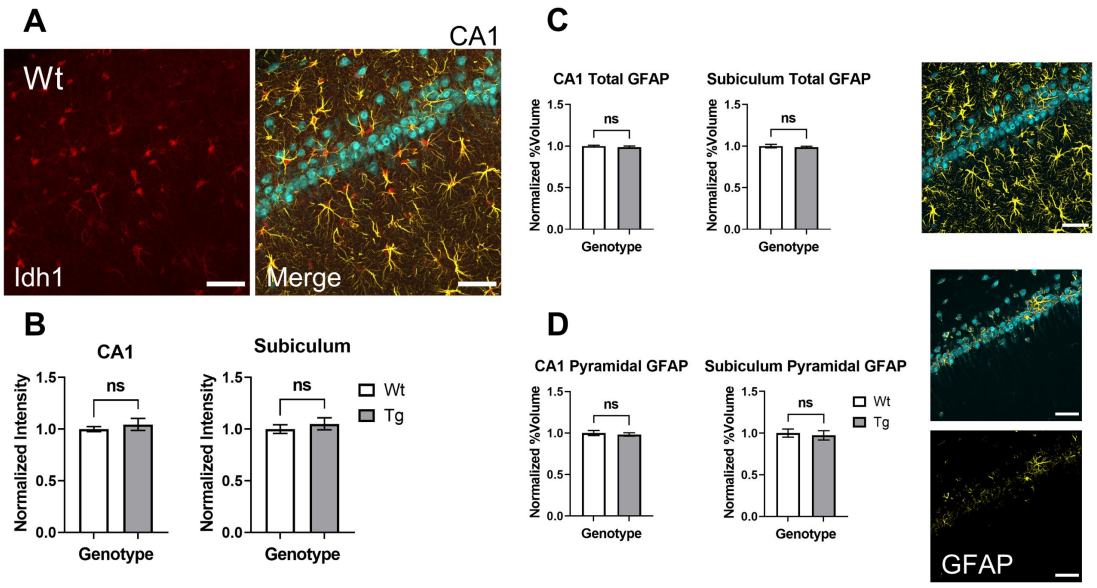


Figure S2.4: Area of GFAP immunoreactivity in the hippocampus.
A. Representative image of Idh1 immunoreactivity (red) in CA1 merged with NeuN (cyan) and GFAP (yellow). **B.** Quantification of Idh1 immunoreactivity in CA1 and subiculum of Wt and Tg astrocytes normalized to Wt fluorescence intensity. **C.** Total GFAP coverage in CA1 and subiculum image regions. Percent volume is normalized to the entire z-stack volume. Right panel shows representative image of NeuN (cyan) and GFAP (yellow) **D.** GFAP coverage in the CA1 pyramidal layer and subiculum neuron volumes. Only GFAP immunoreactivity (volume) that co-localized with NeuN immunoreactivity was quantified and then divided by the total NeuN volume. Right panels show representative images of GFAP (yellow) and NeuN (cyan). Scale bars represent 50 μm. Error bars represent SEM. Two-tailed t-tests. ns = non-significant.

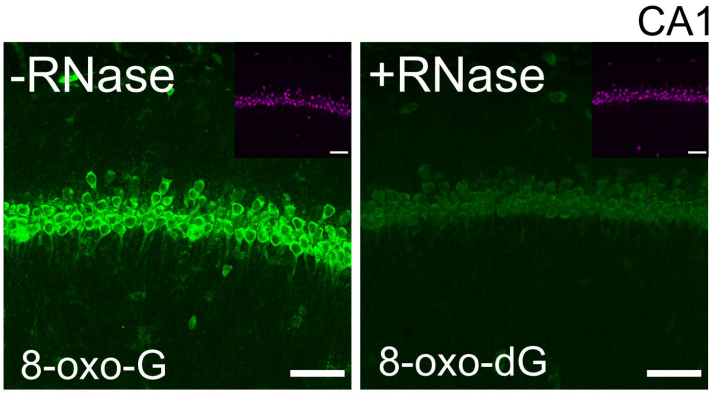


Figure S2.5. RNase pre-treatment for 8-oxo-dG assessment.
RNase pre-treatment diminishes 8-oxo-G immunoreactivity and reveals 8oxodG immunoreactivity. Comparison of pre-treatment without or with RNase, showing 8oxoG in green and NeuN as an inset (magenta) using the same imaging and analysis settings. Scale bar represents 50 μm.

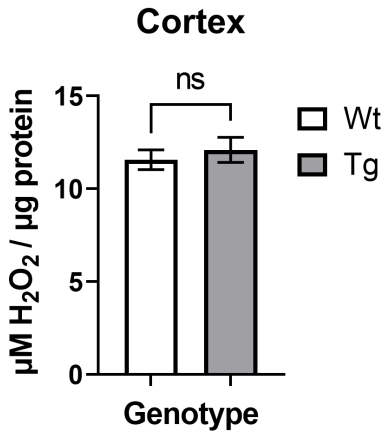


Figure S2.6. DCF Assay.
Quantification of fluorescence signal from cortical homogenates. Signal indicates the general redox status of the samples. Error bars represent SEM, two-tailed t-test, ns = non-significant.

Table S2.1. Housekeeping genes for RT² Rat Oxidative Stress Profiler PCR Array.

Abbreviation	Full name
Actb	β-Actin
B2m	β2 microglobulin
Hprt1	Hypoxanthine phosphoribosyltransferase 1
Ldha	Lactate dehydrogenase A
Rplp1	Ribosomal protein, large, P1

Table S2.2. Primer sequences for quantitative real-time PCR (hippocampal homogenates).

Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	Amplicon Length (bp)
Ape1	CGTTGGGAGGCAGCGTAGTA	CTTCTTGGTCTCTGGCTCGG	138
Cdk5	GTATCCCAGTCCGCTGCTAC	CTGTTCCTCAGTCGGTGTCC	224
Ercc3	CTGCCAGAAGCAAATGTCCTC	CTGCGACCATCCCTTTCTTG	109
Fen1	CGCTGGTAGGAAGAAGCCATT	ACCCTGACGAACAGCAATCA	182
Parp1	ACCACGCACAATGCCTATGA	AGTCTCCGGTTGTGAAGCTG	107
Pcna	TGCAGATGTACCCCTTGTTGT	CATCTTCGATCTTGGGAGCCA	83
Polβ	AATGAGTACACCATCCGCCC	GCGTCATTCACTCCTGTCCT	132
Sirt3	GGGCTTGAGAGAGCATCTGG	ACAACGCCAGTACAGACAGG	167

Table S2.3. Expression of oxidative stress-related genes in hippocampal neurons

Gene	Fold-change	P value	Gene	Fold-change	P value
Alb	0.80	0.5892	*Mb*	n/a	n/a
Als2	1.11	0.4903	*Mpo*	n/a	n/a
Aox1	n/a	n/a	*Ncf1*	n/a	n/a
Apc	0.98	0.8856	*Ncf2*	0.86	0.6859
Apoe	1.60	0.3346	*Ngb*	1.07	0.8676
Cat	1.10	0.7371	*Nos2*	1.63	0.4668
Ccl5	n/a	n/a	*Nox4*	n/a	n/a
Ccs	1.24	0.6024	*Noxa1*	n/a	n/a
Ctsb	1.38	0.1324	*Noxo1*	n/a	n/a
Cyba	n/a	n/a	*Nqo1*	0.98	0.9557
Cygb	1.03	0.9061	*Nudt1*	1.04	0.8607
Dhcr24	1.72	0.2212	*Park7*	1.33	0.3312
Dnm2	1.65	0.2114	*Prdx1*	0.99	0.8902
Duox1	2.02	0.2950	*Prdx2*	1.02	0.7977
Duox2	1.14	0.6058[+]	*Prdx3*	1.36	0.1910
Ehd2	0.82	0.6095[+]	*Prdx4*	1.07	0.7930
Epx	0.51	0.4634[+]	*Prdx5*	1.43	0.2359[+]
Ercc2	1.52	0.0365	*Prdx6*	1.57	0.1529
Ercc6	1.49	0.1008	*Prnp*	1.74	0.1121
Fancc	2.47	0.0392	*Psmb5*	1.19	0.3275
Fmo2	1.26	0.6620[+]	*Ptgs1*	3.03	0.0976
Fth1	1.11	0.3367	*Ptgs2*	1.07	0.9626[+]
Gclc	0.94	0.7249	*Rag2*	n/a	n/a
Gclm	1.31	0.1023	*Scd1*	1.90	0.1404
Gpx1	1.20	0.6994	*Sels*	1.12	0.3704[+]
Gpx2	n/a	n/a	*Sepp1*	0.74	0.4266
Gpx3	1.36	0.3864	*Serpinb1b*	0.83	0.5602
Gpx4	1.62	0.3008	*Slc38a1*	1.13	0.6425
Gpx5	n/a	n/a	*Slc38a5*	n/a	n/a
Gpx6	1.88	0.1893[+]	*Sod1*	0.98	0.9213
Gpx7	2.17	0.0592	*Sod2*	1.44	0.0351
GR	1.77	0.0200	*Sod3*	0.97	0.9324
Gstk1	1.09	0.7669	*Sqstm1*	1.68	0.0840
Gstp1	1.28	0.1552	*Srxn1*	1.24	0.2768
Hba-a2	1.59	0.5494	*Tpo*	n/a	n/a
Hmox1	0.93	0.3823[+]	*Txn1*	0.89	0.7486
Hspala	n/a	n/a	*Txnip*	2.04	0.5218
Idh1	1.74	0.0021	*Txnrd1*	1.49	0.1793
Ift172	1.88	0.0728	*Txnrd2*	1.39	0.1299
Krt1	n/a	n/a	*Ucp2*	1.51	0.4439
LOC367198	n/a	n/a	*Ucp3*	n/a	n/a
Lpo	0.50	0.4668	*Vim*	n/a	n/a

n/a indicates genes where qRT-PCR amplification was not efficient in enough samples from both groups.
[+]Mann Whitney test, otherwise t-tailed t-test.

Table S2.4: List of primary antibodies and dilutions for IHC and IF experiments.

Target	Source	Category Number	Species and Clonality	Dilution
4HNE	Abcam	Ab46545	Rabbit polyclonal	1:250
8-oxo-dG	Trevigen/R&D	4354-MC-050	Mouse monoclonal	1:500
Fancc	LS-Bio	LS-C331704	Rabbit polyclonal	1:100
γH2AX	Abcam	Ab26350	Mouse monoclonal	1:1000
GFAP	Novus	SPM507	Mouse monoclonal	1:2000
GR	ThermoFisher	PA5-29945	Rabbit polyclonal	1:250
Idh1	Abcam	ab172964	Rabbit monoclonal	1:100
NeuN	EMD Merck Millipore	MAB377X	Mouse monoclonal (AF 488)	1:500
NeuN	EMD Merck Millipore	ABN90P	Guinea pig polyclonal	1:1000
NeuN (with γH2AX)	Abcam	ab177487	Rabbit monoclonal	1:2500
Aβ	Medimabs	McSA1	Mouse monoclonal	1:1000
Parvalbumin	EMD Merck Millipore	MAB1572	Mouse monoclonal	1:5000
SOD2	Abcam	ab68155	Rabbit monoclonal	1:100
XPD	Abcam	ab111596	Rabbit polyclonal	1:500

Table S2.5: List of secondary antibodies and dilutions for IHC and IF experiments.

Fluorophore Conjugate	Source	Category Number	Species and Clonality	Dilution
n/a	In-house	n/a	Rabbit-anti-Mouse	1:25
Alexa Fluor 488	ThermoFisher	A-11029	Goat-anti-Mouse Polyclonal Highly Cross-Adsorbed	1:800
Alexa Fluor 568	ThermoFisher	A-11036	Goat-anti-Rabbit Polyclonal Highly Cross-Adsorbed	1:800
Alexa Fluor 647	ThermoFisher	A-21450	Goat-anti-Guinea pig Polyclonal Highly Cross-Adsorbed	1:800

III. Chapter 3

Effect of Antioxidant Supplements on Lipid Peroxidation Levels in Primary Cortical Neuron Cultures

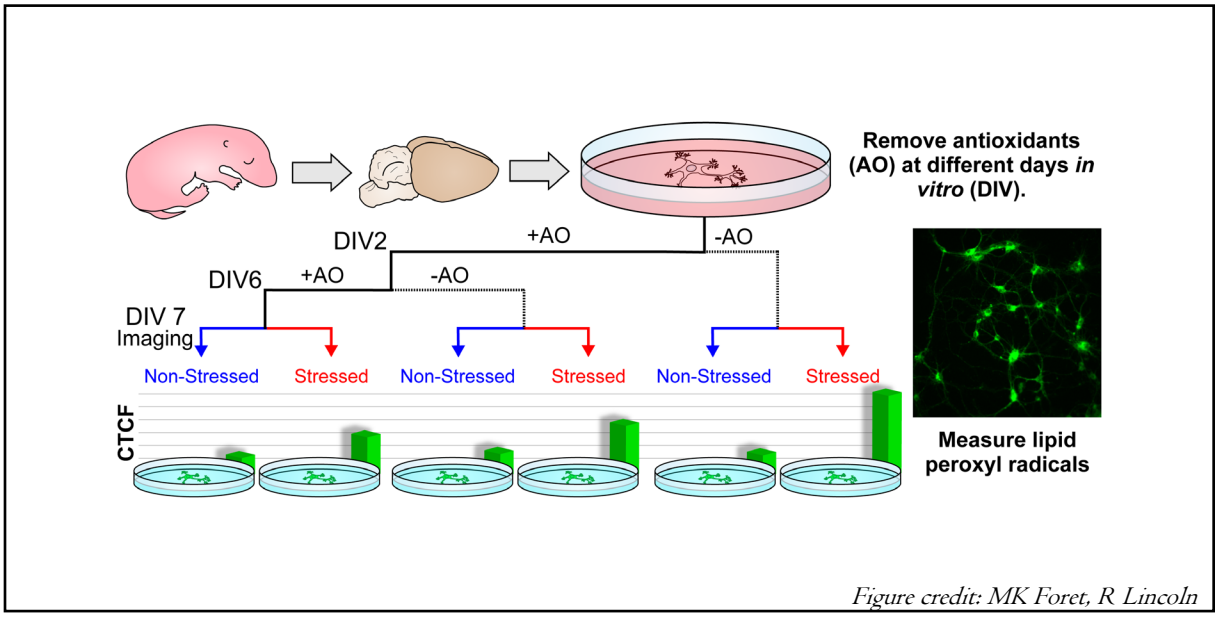


Figure credit: MK Foret, R Lincoln

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Preface

In Chapter 2 we assessed downstream alterations in neuron-specific oxidative stress-related gene and protein expression in response to early intraneuronal Aβ accumulation. Through this next chapter, we direct our focus upstream, towards the specific ROS that likely contribute to downstream oxidative damage and response in neurons, namely, lipid peroxyl radicals. As fluorescence imaging of ROS in real-time offers valuable spatial and temporal resolution in biologically relevant systems, we collaborated with Dr. Gonzalo Cosa, whose laboratory developed fluorogenic probe H_4BPMHC for studying lipid peroxyl radical production. In this Chapter, we optimize and validate the use of this probe, modeled after the antioxidant Vitamin E (α-tocopherol), in cerebral cortex neurons isolated from post-natal day 1 rats.

III.1 Abstract

Oxidative stress, specifically lipid peroxidation, is a major driving force in neurodegenerative processes. However, the exact role of lipid peroxidation remains elusive as reliable real-time detection and quantification of lipid peroxyl radicals proves to be challenging in vitro and in vivo. Motivated by this methodological limitation, we have optimized conditions for real-time imaging and quantification of lipid peroxyl radical generation in primary neuron cultures using the lipophilic , α-tocopherol analog probe, 8-((6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)-methyl)-1,5-di(3-chloropropyl)-pyrromethene fluoroborate (H_4BPMHC). By subjecting neurons to different antioxidant conditions in the presence and absence of lipid peroxidation inducing stressors (Haber-Weiss reagents), we maximized H_4BPMHC sensitivity and confirmed its potential to temporally resolve subtle and marked differences in lipid peroxidation levels in real-time. Herein we report imaging and quantification of homeostatic and induced lipid peroxidation in primary neuron cultures, supporting the use of this probe for investigating healthy and diseased states. Overall these results provide the necessary foundation and impetus towards using H_4BPMHC for elucidating and mapping lipid peroxyl radical contributions to ROS-associated pathological processes in neurons.

III.2 Introduction

Reactive oxygen species (ROS) are key contributors to states of both health and disease [615]. They maintain cell signaling pathways but can also induce or exacerbate pathological processes through oxidative damage. Numerous studies have ascribed oxidative stress as being a major driving force for neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) among others [264, 616-618]. Specifically, lipid peroxidation by-products have been observed in the brains of individuals with these ailments [379, 619] and may also serve as supplemental biomarkers for AD [620]. However, this only provides a view of downstream effects caused by lipid peroxidation, where direct detection of initial events, the true initiator of pathological cascades, in real time is lacking.

Lipid membranes in neurons are rich in poly-unsaturated fatty acids (PUFAs) containing allylic hydrogen atoms that are particularly vulnerable to attack by ROS, specifically free radicals. PUFA reaction with free radical initiators triggers a lipid autooxidation chain reaction where lipid peroxyl radicals are chain carriers and lipid peroxidation results (Figure 3.1A). Lipid peroxidation modifies lipid bilayers [402] and its side effects, associated to by-products, may also involve damage to proteins and DNA.

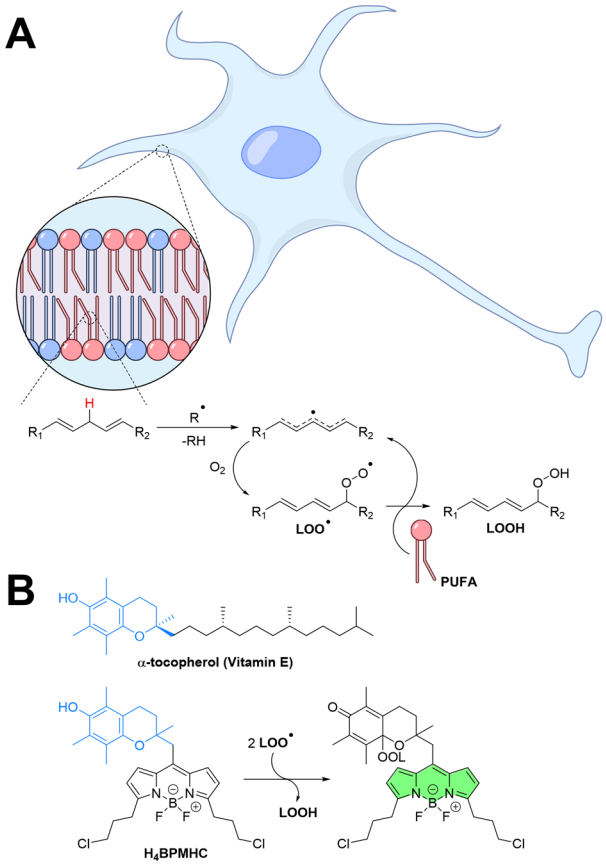

Figure 3.1. Lipid chain autoxidation.
A. Autocatalytic oxidation of polyunsaturated fatty acid (PUFA). **B.** Structure of α-tocopherol (Vitamin E) and the fluorogenic antioxidant H4BPMHC. The radical trapping moiety is shown in blue.

Our understanding of lipid peroxidation resulting from lipid peroxyl radical formation at the cellular level is currently limited as reliable, real-time quantification of ROS levels and subsequent oxidative damage *in vitro* and *in vivo* is an arduous task [621]. Further, suboptimal experimental conditions easily introduce confounding factors that can increase ROS and oxidative damage or create artifacts that skew results, leading to misguided conclusions [622]. Cell culture is inherently oxygen rich and experimental conditions can lead to ROS generation. Equally important are the effects of antioxidants added to culture media in attempt to minimize effects of the oxygen rich environment.

The recently published fluorogenic probe 8-((6-hydroxy-2,5,7,8-tetramethylchroman-2-yl)-methyl)-1,5-di(3-chloropropyl)-pyrromethene fluoroborate (**H4BPMHC**) is a promising experimental tool for studying lipid peroxyl radical levels that overcomes the limitations associated with indirect detection of lipid peroxyl radical formation (Figure 3.1B) [480]. This α-tocopherol analogue, a two-segment trap-reporter probe bearing a BODIPY reporter

chromophore, directly reports through fluorescence emission enhancement on lipid peroxyl radicals, the chain-carrying species of lipid autoxidation, allowing for the spatio-temporal monitoring of lipid peroxidation events in real-time [515, 623]. Characterized in HeLa cells, the high sensitivity of **H4BPMHC** supports detection of subtle changes in lipid peroxidation levels over time under cellular homeostasis using a modest probe concentration of 100 nM, well below concentrations found in neuron culture media and physiological levels in the rat and human brain [624-627].

Herein, we report on the application of **H4BPMHC** in primary rat cortical neuron cultures to monitor lipid peroxyl radical formation and the protective role that antioxidant supplements have over time, further characterizing the ideal parameters for maximizing **H4BPMHC** accuracy and sensitivity in this primary neuron system. To assess the probe sensitivity to newly generated lipid peroxyl radicals under a range of antioxidant loads we specifically induced lipid peroxidation using the Haber-Weiss reagents cumene hydroperoxide (100 μM) with copper(II) sulfate (10 μM). To establish subtle changes in lipid peroxyl radical levels associated with the antioxidant load under cellular homeostasis (no inducer of peroxidation), we deprived neurons of antioxidants in the media at different time points during the culturing period prior to imaging. A picture emerges illustrating that neurons consume the antioxidant in the media, where deprivation of antioxidants for one day has a minor impact in the rate of lipid peroxidation yet culturing for five days with no antioxidant results in increased membrane peroxidation.

Our results demonstrate the successful application of **H4BPMHC** in primary neuronal cultures through reliable quantification of lipid peroxyl radicals under varying levels of stress, creating a platform for assessing lipid peroxidation events in real time *in vitro*. These results lay a foundation for utilizing **H4BPMHC** to detect subtle changes in healthy and disease states in more physiologically relevant cell culture systems ultimately elucidating the role of lipid peroxyl radicals in neurodegeneration.

III.3 Methods

III.3.1 Purification of H4BPMHC

The compound **H4BPMHC** was synthesized according to previously reported procedures [480] and purified by HPLC as follows: HPLC measurements were carried out on an Agilent Technologies Infinity II 1260 equipped with absorption (G7115A) and fluorescence (G7121B) detectors. A 3 mM solution of the compound was prepared in 45/55 water:acetonitrile. To the **H4BPMHC** sample, the faster-eluting antioxidant 2,2,5,7,8-pentamethyl-6-chromanol (30 mM) was added to prevent oxidation of the stock solution, as well as serve as a scavenger of oxidizing species on the HPLC column. 20 µl injections were purified by HPLC on a ZORBAX RRHD Eclipse Plus Phenyl-hexyl 2.1 × 50 mm (1.8 µm) column (Agilent) by isocratic elution of 45/55 water:acetonitrile at a flow rate of 1.2 mL min^{-1}. The absorbance of the eluate was monitored at 254 and 509 nm, and the fluorescence of the eluate was monitored at 520 nm following excitation at 485 nm. **H4BPMHC** devoid of contaminations was collected, and the concentration of the eluate was determined by UV-Vis absorption spectroscopy recorded on a Hitachi U-2800 UV-Vis-NIR spectrophotometer and a 1 cm x 1 cm quartz cuvette. The samples were aliquoted, and the solvent removed under reduced pressure. The samples were stored at -20°C until used.

A word of caution to those willing to adopt this probe. **H4BPMHC** should be handled with care to avoid pre-oxidation of the dye, as we have highlighted in a recent review [623].

III.3.2 Primary neuronal cultures

Isolation of Cells

All procedures were carried out in accordance with the guidelines set out by the Canadian Council of Animal Care and were approved by the Animal Care Committee of McGill University.

Solutions for primary neuron cultures supplied by ThermoFisher Scientific included Neurobasal-A (10888-22), B-27 Supplement with antioxidants (17504044), and B-27 Supplement minus AO (10889038). Hibernate A (HA) and Hibernate A minus calcium (HACA) were supplied by Brainbits, and papain (LS003119) from Worthington Biochemical Corporation. OptiPrep (D1556), poly-D-lysine (PDL) (P6407), and cytosine arabinoside (C1768) were obtained from Sigma-Aldrich.

Two separate litters of Wistar rats were used as biological replicates for the live cell imaging experiments. Before isolation, µ-Slide 8 well glass bottom dishes (ibidi, #80827) were coated

with PDL for 2 hours at room temperature (RT), washed three times with autoclaved water and stored at 4° until used. Cerebral cortex neurons were isolated from post-natal day (PND) 1 Wistar rats using a protocol modified from Brewer and Torricelli (2007) [628]. Briefly, dissected cerebral cortices were collected on ice in solution containing Hibernate A (with calcium), supplemented with B-27 Supplement (with antioxidants), and L-glutamine (0.5 mM final), termed HABG solution. The tissue was cut into small ~1 mm^3 pieces and placed in tubes on ice containing HABG. Tissue was digested at 30°C for 25 minutes using papain that was prepared with HA minus calcium incubated at 37°C for 20-30 minutes, filter sterilized then supplemented with L-glutamine (0.5 mM). After digestion, tissue was triturated with fire polished pipettes, the tissue was allowed to settle, and the supernatant was collected. HABG was added to the tissue, it was triturated and collected again. The combined supernatants were layered onto OptiPrep density gradients prepared that day. The layered gradients were centrifuged (800 g, 15 minutes, 22°C) and the fraction enriched for neurons was collected in HABG and centrifuged once more (200 g, 5 minutes). The neuronal pellet was resuspended in Neurobasal A media with B-27 Supplement (with antioxidants), 1% penicillin/streptomycin, and L-glutamine (5 mM), and seeded at 32,000 cells cm^{-2}.

Maintaining Primary Neurons and Antioxidant Conditions

All cells were cultured in Neurobasal A media with B-27 Supplement (with antioxidants), 1% penicillin/streptomycin, and L-glutamine (5 mM) up until day *in vitro* (DIV) 2. At this timepoint, B-27 Supplement minus antioxidant (defined as −AO, lacking vitamin E, vitamin E acetate, superoxide dismutase, catalase, and glutathione) was used for a fraction of the wells until the imaging day on DIV 7 (these cells are referred to as −AO DIV 2). At DIV 6 other wells were then cultured in antioxidant free B-27 (−AO DIV 6) until the imaging day on DIV 7. A third batch of cells remained in Neurobasal A media supplemented with B-27 Supplement until imaging on DIV 7. For reliable comparison between treatments, every dish had cells cultured with antioxidant (+AO) and cells that lacked antioxidant from either DIV 2 or DIV 6 (−AO) (Figure 3.2). To minimize astrocyte proliferation in the cultures they were treated with cytosine arabinoside (AraC) from DIV 2 – 3 (5 μM) which was reduced to (2.5 μM) from DIV 3 – 6.

III.3.3 Microscopy

All fluorescence and differential interference contrast (DIC) imaging was performed using a wide-field microscopy setup consisting of an inverted microscope (Nikon Eclipse Ti) equipped with a Perfect Focus System (PFS) and an air objective. A stage-top incubator

(Tokai Hit) was used to maintain the cells at 37°C (5% CO_2) in a humidified atmosphere. Switching between DIC and fluorescence channels was done using a motorized filter block turret. The switching time was approximately 2 seconds. For fluorescence imaging, the diode laser output (405, 488, or 561 nm) of a laser combiner (Agilent Technologies, MLC-400B) was passed through a multiband clean-up filter and coupled into the microscope objective using a multiband beam splitter. Fluorescence was spectrally filtered with an emission filter. The optical configurations for the specific fluorophores are given in Table 3.1.

Table 3.1. Optical configurations for microscopy experiments.

	Objective	Laser[a]	Excitation Filter	Beam Splitter	Emission Filters
H_4BPMHC	Nikon CFI Plan Apo VC 20x, NA = 0.75	488 nm, 0.1 mW		ZT488/640rpc	ZET488/640m
EtHD-1		561 nm, 0.06 mW	ZET405/488/561/647x	ZT405/488r/561/640rpc	ZET405/488/561/647M
DAPI	Nikon CFI Plan Apochromat Lambda 10x, NA = 0.45	405 nm, 0.5 mW	ZET405/488/561/647x	ZT405/488r/561/640rpc	ZET405/488/561/647M, ET460/50m
β3Tubulin (Alexa Fluor 488)		488 nm, 0.3 mW	ZET405/488/561/647x	ZT405/488r/561/640rpc	ZET405/488/561/647M
GFAP (Alexa Fluor 594)		561 nm, 0.5 mW	ZET405/488/561/647x	ZT405/488r/561/640rpc	ZET405/488/561/647M

[a]Power measured out of the objective.

III.3.4 Imaging lipid peroxidation with H_4BPMHC

Neurons regardless of their treatment were imaged on DIV 7. Each ibidi dish was imaged as follows: The culture media was removed, and the cells were washed with Live Cell Imaging Solution (LCIS) (ThermoFisher Scientific, A14291DJ). LCIS does not contain phenol red or added metal ions, avoiding potential interferences. This choice of media further ensures a stable pH even outside of a CO_2 atmosphere. 200 µL of LCIS was added to each well and an image was acquired for each imaged region (488 nm excitation, 300 ms exposure, 0.1 mW power), to establish the autofluorescence of the neurons in that region. 100 µL of **H_4BPMHC** (300 nM stock in 1% DMSO in LCIS) was then added to each well and incubated for 10 minutes for the probe to partition into the neurons (the final concentration of **H_4BPMHC** was 100 nM in 0.33% DMSO). Following this initial incubation, the solution containing the probe was then removed, and the neurons were washed with LCIS before fresh LCIS (200 µL) was added. **H_4BPMHC** was allowed to equilibrate (partition out of the neurons) for an additional 10 minutes before the start of image acquisition.

The **H_4BPMHC**-stained neurons were then spiked with a 100 µL volume of either LCIS (non-stressed condition) or a chemical cocktail (stressed condition) consisting of cumene

hydroperoxide (300 μM) with copper(II) sulfate (30 μM)) in LCIS to induce lipid peroxidation through Haber-Weiss chemistry. The final concentration of cumene hydroperoxide and copper(II) sulfate was 100 μM and 10 μM, respectively. Two regions per well were imaged every 3 minutes over the course of 60 minutes (488 nm excitation, 300 ms exposure, 0.1 mW power). Following completion of the **H4BPMHC** experiment, a subset of wells from each condition were labelled with 1 μM ethidium homodimer-1 (ThermoFisher Scientific, E1169) for 20 minutes to ensure viability and imaged (300 ms exposure, 0.1 mW power). Remaining wells were then fixed as described below.

III.3.5 Immunocytochemistry

Astrocyte Content: On DIV 7, following imaging, neurons were washed with phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde for 12 minutes. After more PBS wash steps, to detect contaminating astrocytes, neurons were incubated with primary antibody against GFAP (glial fibrillary acidic protein) (abcam, ab33922; 1:10,000) overnight at 4°C, then for 2 hours RT with goat-anti-rabbit Alexa Fluor 594 (1:1000) secondary antibody (ThermoFisher Scientific, A11037) followed by DAPI for 5 minutes for nuclei identification.

Neuron and Astrocyte Content: To avoid **H4BPMHC** interference with green fluorescent secondary antibody, a separate ibidi dish containing neurons that underwent the same antioxidant pre-treatments and that were not imaged with **H4BPMHC** was immunostained on DIV 7 with both β-III-Tubulin primary antibody (1:2,000) for neurons (Promega, G7129) and GFAP (1:10,000) overnight at 4°C. Secondary antibodies were added for 2 hours RT including goat-anti-rabbit Alexa Fluor 594 at 1:1000 and goat-anti-mouse Alexa Fluor 488 at 1:800 (ThermoFisher Scientific, A11029) followed by DAPI for 5 minutes. Two image regions per well were taken. Control experiments were performed separately to ensure reliable detection of desired antigens. These included labelling in the absence of primary antibodies or with one primary antibody and both secondary antibodies.

III.3.6 Data analysis

All microscopy images were processed using FIJI image processing package [629].

To quantify the fluorescence of **H4BPMHC**, a FIJI macro was developed to calculate single-neuron corrected total cell fluorescence (CTCF) vs. time trajectories [480]. For each frame, the CTCF was calculated as follows:

$$CTCF = \text{RawIntDen}_{\text{neuron}} - (\text{Area}_{\text{neuron}} \times \text{BkgFluorescence}) \qquad \text{(Eq.3. 1)}$$

where $RawIntDen_{neuron}$ is the raw integrated density of the pixels within a region of interest (ROI) containing the neuron, $Area_{neuron}$ in pixels of the ROI, and BkgFluorescence is the averaged mean gray values of three nearby 8-pixel by 8-pixel regions containing no cells. The CTCF-time trajectories were corrected by subtracting the CTCF value of autofluorescence acquired prior to H_4BPMHC staining.

To determine the distribution of the data, initial fluorescence CTCF values were compiled across antioxidant conditions and treatments and the D'Agostino and Pearson omnibus normality test was performed (GraphPad Prism Software), accounting for skewness and kurtosis. Data was then transformed to logarithms (Figure S3.5) and tested again for normality. The extreme studentized deviate (ESD) outlier test was performed and indicated no presence of outliers. Geometric averages of CTCF values in each group (+AO non-stressed, +AO stressed, −AO DIV 6 non-stressed, −AO DIV 6 stressed, −AO DIV 2 non-stressed, and −AO DIV 2 stressed) were calculated and presented.

The CTCF vs. time trajectories for cells of each condition (antioxidant and Haber-Weiss reagent exposure) across biological and technical replicates were averaged geometrically to plot changes in fluorescence over time (Figure 3.4A).

III.4 Results and Discussion

Towards our goal of measuring neuronal lipid peroxyl radical levels using the probe **H4BPMHC** we first established enriched neuron cultures from PND 1 rat cerebral cortices using a modified protocol from Brewer and Torricelli (2007) [628]. Immunocytochemistry experiments were used to verify neuronal content at the DIV 7 time point when the cells were imaged. Immunostaining with GFAP (astrocytes) and β-III-tubulin (neurons) showed minimal astrocyte contamination (<1%, Supplementary Figure S3.1) allowing for assessment of solely neuronal lipid peroxyl radical levels.

To visualize the rates of lipid peroxyl radical production in the neuron cultures, on DIV 7 the cultures were incubated with 100 nM of the fluorogenic antioxidant **H4BPMHC** in Live Cell Imaging Solution (LCIS) without serum or supplements to ensure maximal probe partitioning [480]. Serums and supplement components commonly used in cell culture media, such as serum albumins can bind BODIPY dyes [630]. In our initial report, we have shown that serum supplements compete with cellular membranes for **H4BPMHC**, thus hindering probe partitioning into cells [480]. By excluding these components from the imaging solution, a short partition-in time of 10 minutes was possible, further reducing any masking reactions that may take place during incubation periods.

To modulate the antioxidant load, neurons were cultured under three different conditions where antioxidants were either kept (+AO) or removed at DIV 2 (−AO DIV 2) or DIV 6 (−AO DIV 6) (Figure 3.2). This was done by utilizing either B-27 Supplement containing vitamin E, vitamin E acetate, superoxide dismutase, catalase, and glutathione or B-27 minus AO which lacks these antioxidants. While the precise concentrations of these antioxidants in B-27 Supplement are proprietary, they have been estimated [625] to be similar to that reported for the B-18 Supplement also developed by Brewer et al. (1989) which contains: 2.3 μM vitamin E, 2.1 μM vitamin E acetate, 0.077 μM superoxide dismutase, 0.010 μM catalase, and 3.2 μM glutathione [624]. This method of early antioxidant removal in primary cortical neuron cultures was validated by Perry *et al.* (2004) who showed that antioxidants are only required for the first 24 hours of culturing [631].

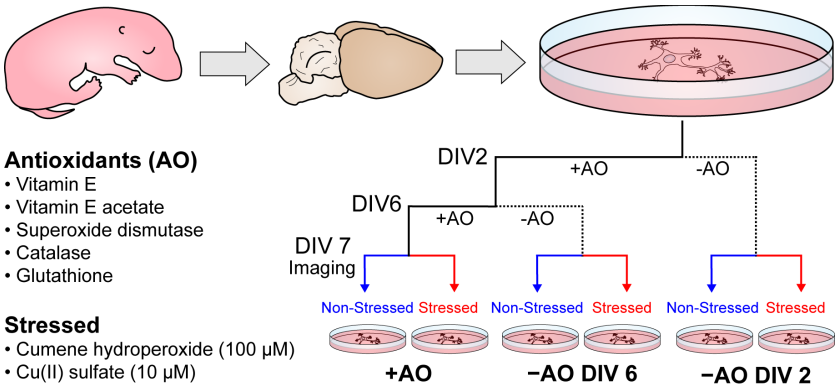

Figure 3.2. Experimental design.
Primary cortical neurons (cerebral cortex mantle highlighted in brown) were isolated from PND 1 rat pups from two separate litters and cultured in three different antioxidant conditions up until the imaging day DIV 7. The antioxidant conditions were altered by using B-27 Supplement containing the five antioxidants listed on the left portion of the scheme, or by using B-27 Supplement minus AO, lacking these components. The conditions were as follows: (1) +AO or with antioxidants (2) −AO DIV 6, deprived of antioxidants since DIV 6 or 1 day before imaging and (3) −AO DIV 2, deprived of antioxidants since DIV 2. On DIV 7 neurons were imaged in the presence (stressed) or absence (non-stressed) of Haber-Weiss reagents (100 μM cumene hydroperoxide + 10 μM copper(II) sulfate).

To image neurons under stressed and non-stressed conditions, they were either treated with Haber-Weiss reagents to induce high levels of oxidative stress via lipid peroxyl radical generation, or without this chemical insult, mimicking conditions of cellular homeostasis. Lipid peroxidation was induced in half of the wells (stressed) by the addition of cumene hydroperoxide (100 μM) with copper(II) sulfate (10 μM). Results were compared to those from wells not treated with Haber-Weiss reagents (non-stressed). The cells were imaged for 60-minutes in both cases. The resulting fluorescence images are shown in Figure 3.3 and Supplementary Video S1.

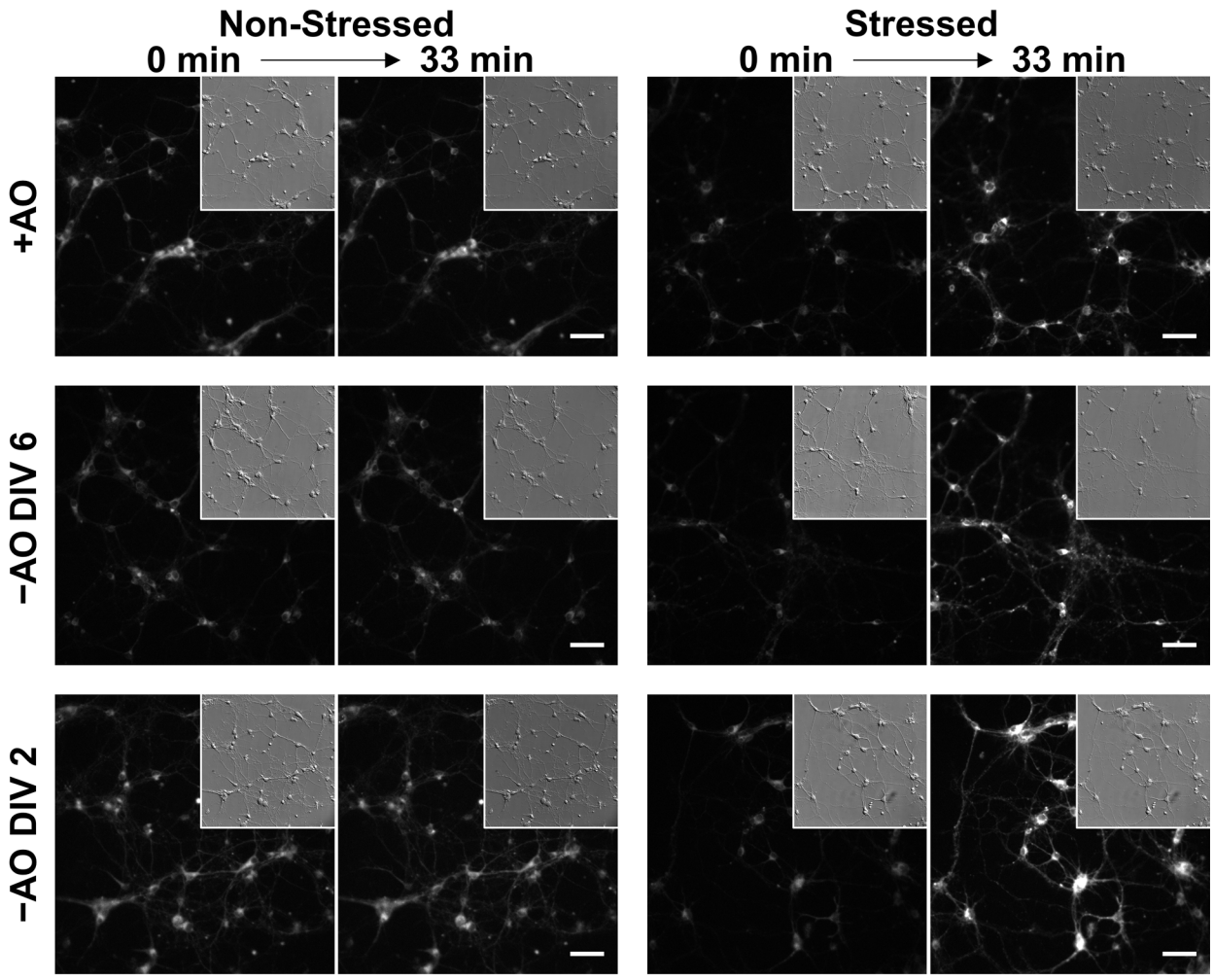

Figure 3.3 Live cell imaging.
Representative fluorescent images of cortical neurons across the three antioxidant conditions. Images of DIV 7 neurons in the presence of 100 nM H_4BPMHC acquired at 0 and 33 minutes following treatment with (Stressed) and without (Non-Stressed) cumene hydroperoxide (100 μM) and copper(II) sulfate (10 μM) to induce lipid peroxidation. Images were acquired via widefield microscopy with 20× magnification. The excitation wavelength was 488 nm (0.1 mW) and the fluorescence emission was spectrally filtered with a ZET480/640m emission filter. Inset shows compressed DIC images for the same region. Scale bars are 50 μm.

Qualitatively, we observed a small change in H_4BPMHC fluorescence intensity over time in the non-stressed neurons. A slight increase in fluorescence was thus observed in neurons that lacked the B-27 antioxidants since DIV 2 (−AO DIV 2), when compared to those with antioxidants for the entire culturing period (+AO) and those that lacked antioxidants since DIV 6 (−AO DIV 6) (Figure 3.3). These changes were more prominent to the naked eye in processes than in neuronal cell bodies.

For neurons treated with Haber-Weiss reagents, changes in emission intensity over time were prominent. These neurons underwent a significantly greater increase in fluorescence intensity over time compared to non-stressed samples, with marked differences in H_4BPMHC fluorescence between all three antioxidant conditions. Neurons that lacked the B-27 antioxidants since DIV 6 were noticeably brighter than those with antioxidants the entire culturing period. Neurons cultured without antioxidants since DIV 2 exhibited the largest fluorescence enhancement indicating a greater sensitivity to the chemically induced lipid peroxyl radicals. Both neuronal cell bodies and processes exhibited enhancements in fluorescence intensity. In all three antioxidant conditions, the maximum intensity was achieved at 33-minutes, which corresponded to the onset of membrane blebbing in the DIC images (Figure S3.2). We posit that blebbing is a marker of cell death, and following this point, no significant lipid peroxyl radical formation was observed. This blebbing was not seen in the non-stressed neurons even after the 60 minutes.

To verify that the non-stressed neurons maintained viability for the entire 60-minute imaging session, we stained with ethidium homodimer-1 (EthD-1) after the imaging with H_4BPMHC was complete (Figure S3.3). EthD-1 is a marker for non-viable cells and exhibits fluorescence only in dead or dying cells. For all antioxidant conditions, the non-stressed neurons in the absence of Haber-Weiss reagents remained viable as judged from the outcome of EthD-1 imaging experiments. While some labelling was visible in cells, or cell fragments, these are likely from astrocytes impacted by culturing in the presence of cytosine arabinoside (AraC), where no labelling was recorded in the neurons selected for analysis.

Following our qualitative analysis, to quantify the fluorescence signal overt time within individual neurons, and in turn the load of lipid peroxyl radicals, single neuron corrected total cell fluorescence (CTCF) (Equation 3.1) vs time trajectories were calculated from the fluorescence movies (Figure S3.4). Here we measured CTCF from neuronal cell bodies, where each neuron intensity-time trajectory was corrected for local changes in the background. Additionally, the CTCF value for each neuron was corrected for autofluorescence. This type of cell-by-cell analysis allowed us to avoid debris etc., and further allowed for screening of any subpopulations or outlier neurons within each experimental condition (Figure S3.5).

To assess the statistical distribution of the single-neuron fluorescence trajectories we recorded, a histogram was constructed using the CTCF value at 0-minutes from every neuron trajectory, for neurons across all conditions (Figure S3.6A). This histogram of initial CTCF values exhibited a non-gaussian distribution ($p < 0.0001$), the data instead was consistent

with a Log-normal distribution ($p = 0.8239$) (Figure S3.6B) [632]. Given this result, the geometric averages were calculated from the neuron CTCF-time trajectories, for each culture condition (antioxidant and Haber-Weiss reagents exposure).

Consistent with the qualitative observations, drastic quantitative differences in fluorescence (CTCF) values, indicative of lipid peroxyl radical levels, were observed between antioxidant conditions when neurons were in the presence of Haber-Weiss reagents (Figure 3.4A). Neurons that lacked the B-27 antioxidants since DIV 6 had elevated CTCF values compared to those with antioxidants the entire culturing period, whereas those without antioxidants since DIV 2 exhibited the sharpest increase and highest CTCF values overall (Figure 3.4A). The CTCFs of all cells in the presence of Haber-Weiss reagents eventually plateaued at the 33-minute time point (Figure 3.4A).

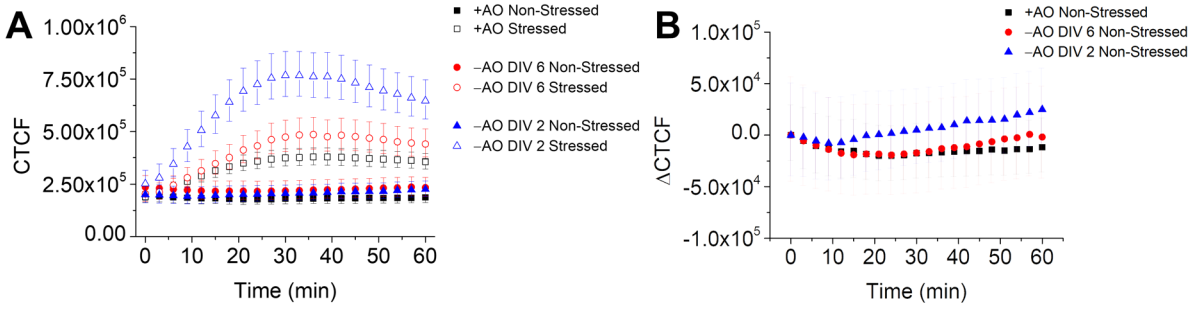

Figure 3.4. Lipid peroxidation levels in primary neurons cultured with (+AO) and without (−AO) antioxidants.

A. CTCF values over 60 minutes. Data points correspond to geometric averages from neurons across two different litters of rats and 2-3 technical replicates (+AO Non-Stressed n = 140, +AO Stressed n = 139, −AO DIV 6 Non-Stressed n = 57, −AO DIV 6 Stressed n = 57, −AO DIV 2 Non-Stressed n = 67, −AO DIV2 Stressed n = 59). Error bars correspond to 95% confidence intervals. (B) Change in CTCF (lipid peroxidation) levels in healthy neurons.

To assess antioxidant consumption during the culturing period leading up to DIV 7, the maximal CTCF values at 33-minutes were plotted against DIV without supplement antioxidants (i.e. DIV 2 corresponds to five days without supplement), (Figure 3.5). The maximal signal of **H4BPMHC** reflects the competition of the probe with the cellular antioxidant reserves for the generated lipid peroxyl radicals. The resulting graph showed a correlation where longer periods without antioxidants resulted in higher cell fluorescence intensities ($r^2 = 0.991$). The linear trend reflected the gradual consumption of supplement antioxidants over time in culture. This led to diminished antioxidant reserve in the cells and higher sensitivity of **H4BPMHC** to the chemically induced lipid peroxyl radicals.

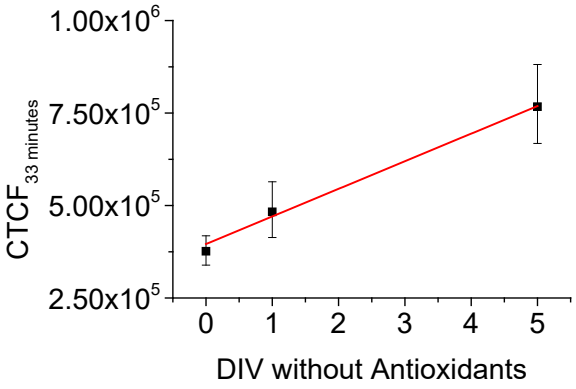

Figure 3.5. Antioxidant deprivation in culture over time.
Correlation between maximal CTCF values and days deprived of antioxidants obtained from stressed neurons at 33-minutes with a linear fit ($r^2 = 0.991$). Data points show geometric averages with 95% confidence intervals. Five days and one day without antioxidants corresponds respectively to −AO DIV 2 and −AO DIV 6 samples.

Importantly, while H4BPMHC could successfully detect large changes in lipid peroxyl radical levels in stressed neurons, subtle changes in CTCF were observed between non-stressed neurons across the antioxidant conditions. To tease apart these differences, we calculated the change in CTCF values in the non-stressed neurons (Fig. 4B). An initial decrease in CTCF fluorescence was observed at early time points, arising from some probe partitioning out of the neurons due to the change of buffer volume at the beginning of the experiment (while no Haber-Weiss reagents were added, a volume of LCIS was added to maintain a consistent concentration of probe compared to the Haber-Weiss experiments). Importantly, while the neurons with antioxidants over the entire culturing period showed no intensity enhancement at later time points, neurons that lacked B-27 antioxidants showed a steady increase in intensity that, while within the 95% confidence intervals, points to an increased level of lipid peroxyl radical generated under cellular homeostasis. We observed that the inflection point where intensity goes from decreasing to increasing for −AO DIV 2 neurons occurred much earlier than for −AO DIV 6 neurons, where intensity remained flat for +AO. The early removal of antioxidants for DIV 2 samples allowed for detecting subtle levels of lipid peroxyl radical generation that appeared to be masked in the +AO condition. The −AO DIV 6 neurons did exhibit a slight increase above the +AO condition towards the end of the imaging session, although this occurred much later than the −AO DIV 2 neurons.

Reflecting both elevated levels of lipid peroxyl radicals (stressed conditions) and decreased antioxidant load, these results highlighted the importance of testing antioxidant removal before oxidative stress measurements. Our results under non-stressed conditions also illustrate

that subtle levels of lipid peroxyl radical production under homeostasis may be detected in neurons. These latter set of results highlight the potential for resolving small changes in the rates of lipid peroxyl radical generation, that may prove essential in studying differences between healthy and diseased neurons, circumstances where we believe these small differences will be exacerbated and thus able to be detected with **H4BPMHC**.

III.5 Conclusions

Lipid peroxidation is known to play an important role in neurodegenerative diseases. Successfully imaging and quantifying the formation of lipid peroxyl radicals *in vitro* and *in vivo* will enable a better understanding of pathogenic mechanisms induced or exacerbated by lipid peroxidation. Towards this goal, we have created a platform for assessing lipid peroxidation events in enriched primary neuron cultures with the lipophilic probe **H4BPMHC**, to monitor the temporal evolution of lipid peroxyl radicals and the protective role that antioxidant supplements have on the cultures. Through careful control of cell culture, imaging, and analysis methodologies, we have described ideal parameters for maximizing **H4BPMHC** accuracy and sensitivity in this primary neuron system. Our results show that **H4BPMHC** successfully detected differences in lipid peroxyl radical levels between antioxidant conditions in the presence of induced lipid peroxidation (Haber-Weiss reagents), or under cellular homeostasis. The latter supports potential for studying healthy and diseased neurons where differences may be small but exaggerated relative to homeostatic processes. This work ultimately lays the foundation for utilizing **H4BPMHC** for the real-time detection of elusive lipid peroxyl radicals in neurodegenerative diseases.

III.6 Supporting Information

The Supporting Information includes the immunohistochemistry of the primary neurons (Figure S3.1), membrane blebbing of neurons under stressed condition at 33-minute time point (Figure S3.2), ethidium homodimer-1 labelling post-imaging (Figure S3.3), an example of single neuron CTCF calculation (Figure S3.4), the single neuron CTCF-time trajectories (Figure S3.5), CTCF data distribution for 60-minute living imaging experiment with **H4BPMHC** (Figure S3.6).

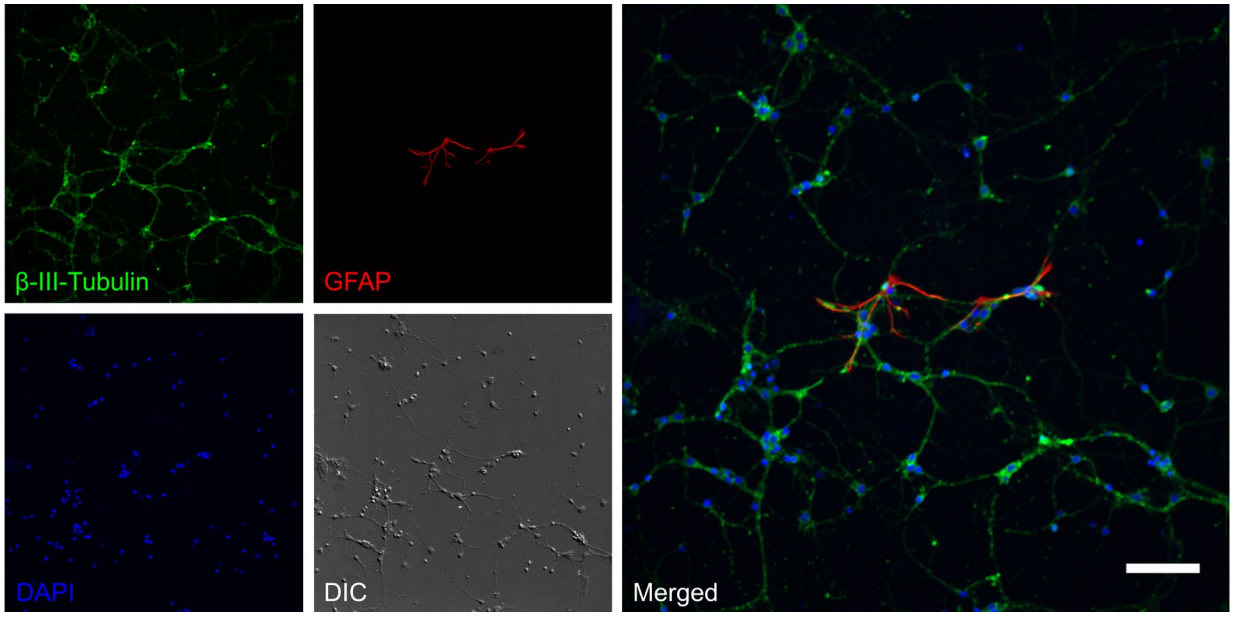

Figure S3.1. Immunocytochemistry of primary neurons.
Widefield fluorescence and DIC images of DIV 7 primary neurons never exposed to **H4BPMHC**, immunostained with antibodies against β-III-Tubulin (green, neurons) and GFAP (red, astrocytes), and labelled with DAPI (blue, nuclei). Scale bar is 100 μm.

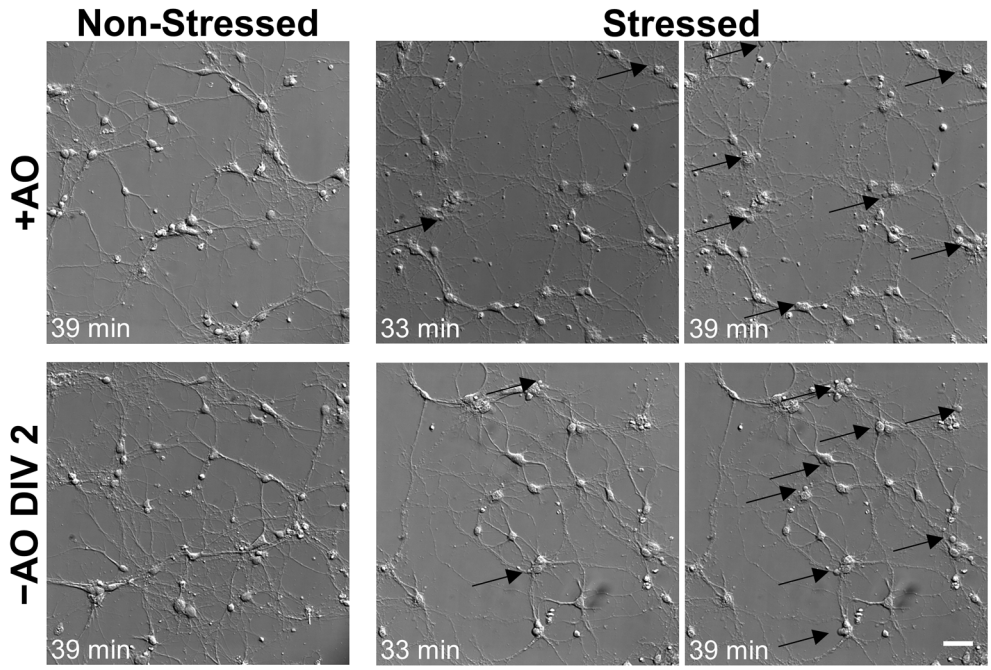

Figure S3.2. Membrane blebbing of neurons under stressed condition after 33-minute time point.
Neurons stressed with cumene hydroperoxide (100 μM) and copper(II) sulfate (10 μM) first exhibited membrane blebbing at 33 minutes (Middle panels) which also corresponded to maximum fluorescence signal (Figure 3.4A). This did not occur in untreated non-stressed neurons (Left panels). Black arrows indicate areas of membrane blebbing. Scale bar is 50 μm.

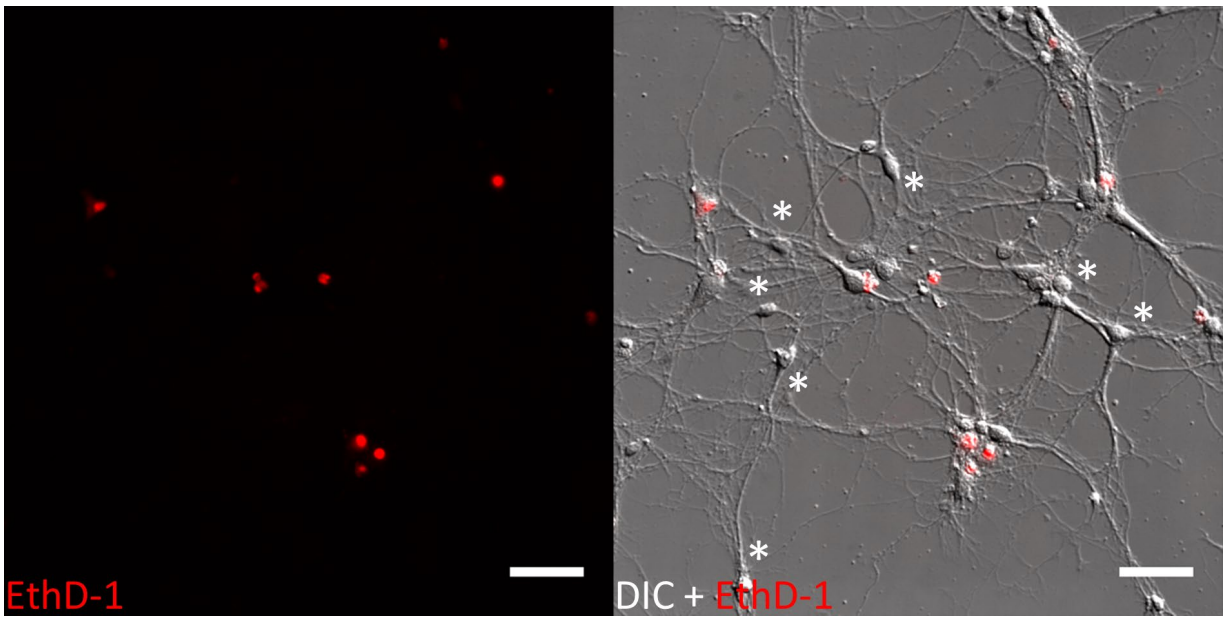

Figure S3.3. Ethidium homodimer-1 labelling post imaging +AO Non-Stressed neurons.
Left panel: EthD-1 fluorescence. The excitation wavelength was 561 nm (0.1 mW) and the fluorescence emission was spectrally filtered with a ZET405/488/561/647M emission filter. Right panel: DIC image of neurons (without Haber-Weiss reagents) in +AO condition with overlay of ethidium homodimer-1 (EthD-1) fluorescence. Examples of neurons used for analysis are indicated with white asterisks (*). Scale bars are 50 μm.

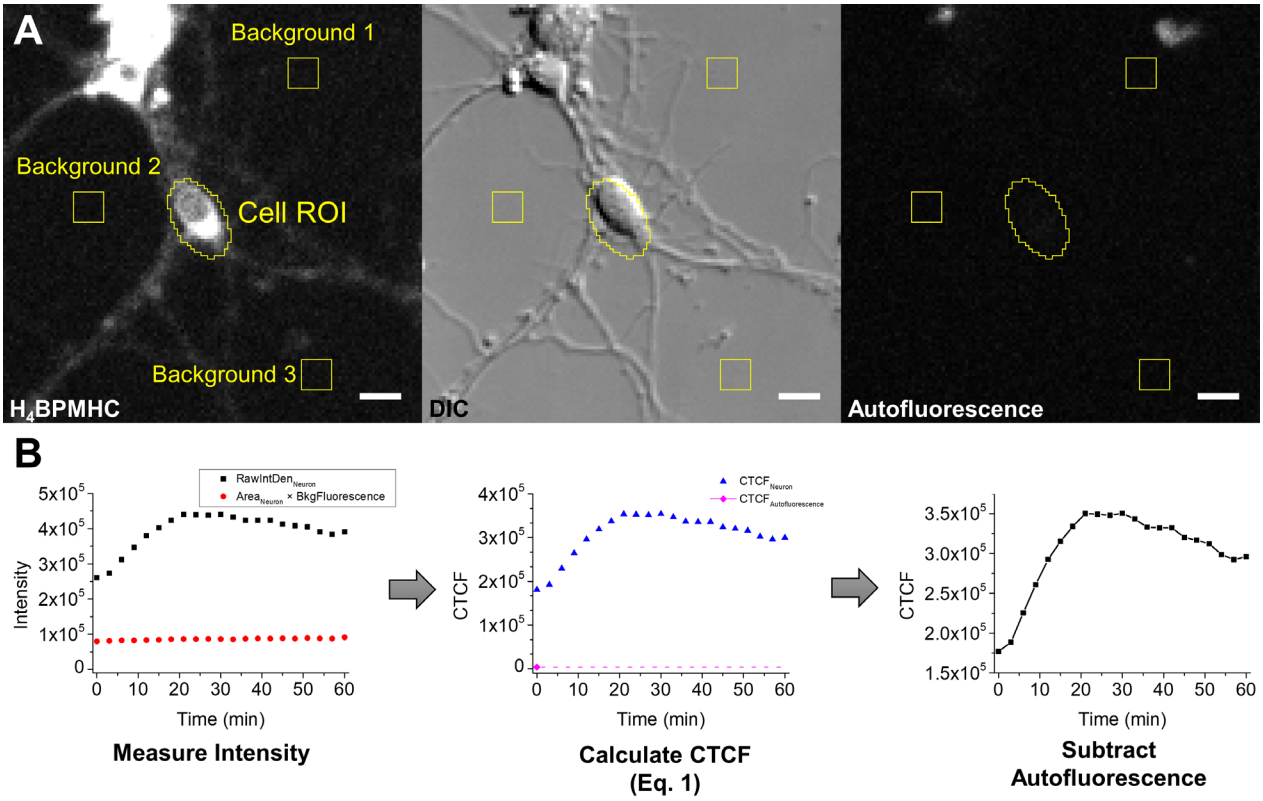

Figure S3.4. Example of single neuron CTCF calculation.

A. Left: Fluorescence and ROI selection of neuron and three background areas for calculating CTCF. Middle: DIC image with overlay of ROI selection. Right: Autofluorescence of the same ROI and background areas. Scale bars are 10 μm **B.** Left: Graph of raw fluorescence intensity of the individual neuron and background areas over 60 minutes used for CTCF calculation. Middle: CTCF of the single neuron calculated using Equation 3.1 plotted with the CTCF of the autofluorescence image. Right: Corrected single neuron CTCF after subtraction of autofluorescence CTCF.

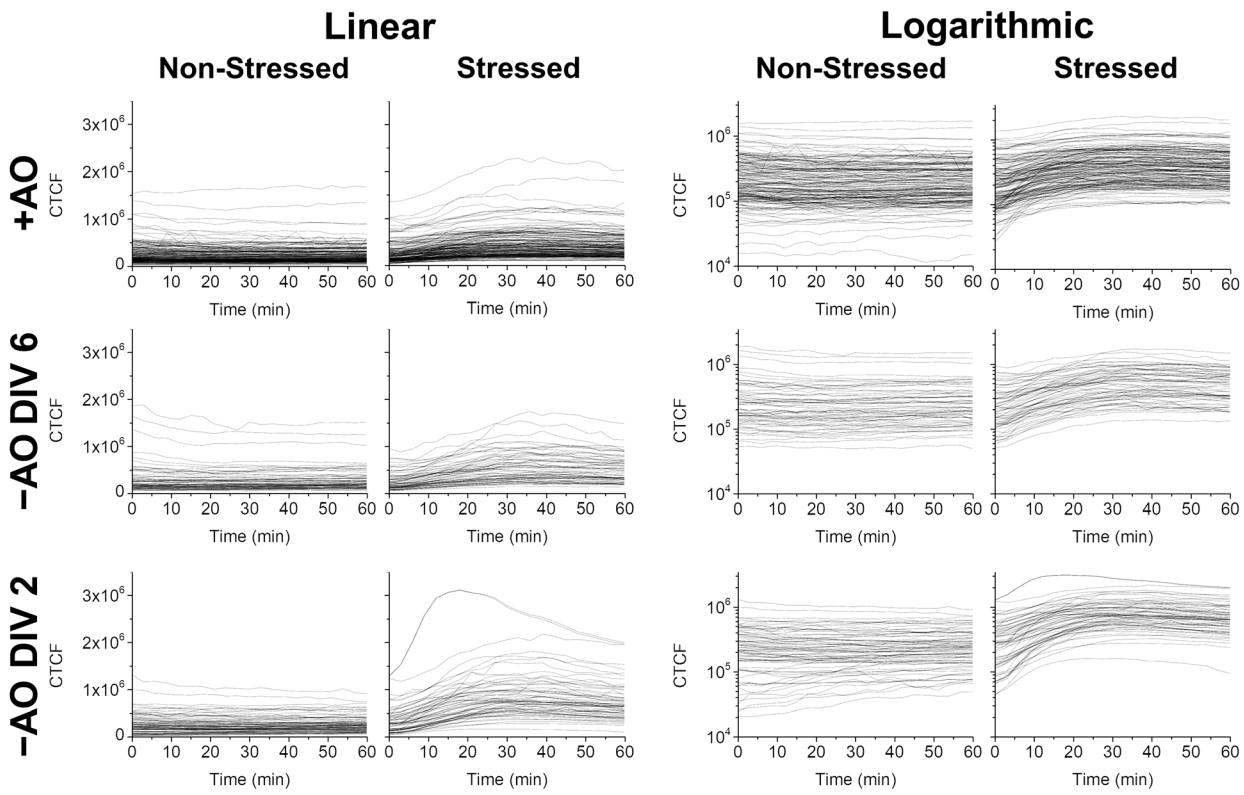

Figure S3.5. Single neuron CTCF-time trajectories.

Data corresponds to neurons across two different litters of rats and 2-3 technical replicates (+AO Non-Stressed n = 140, +AO Stressed n = 139, −AO DIV 6 Non-Stressed n = 57, −AO DIV 6 Stressed n = 57, −AO DIV 2 Non-Stressed n = 67, −AO DIV2 Stressed n = 59).

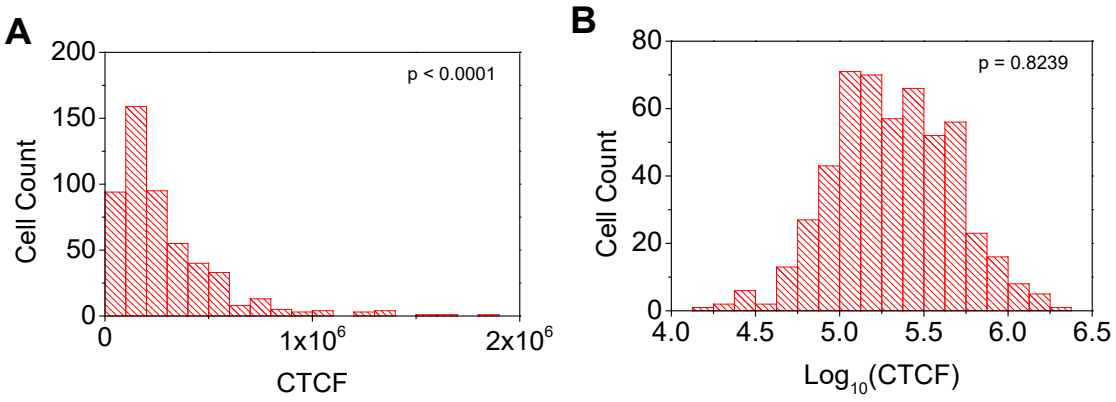

Figure S3.6. CTCF data distribution for 60-minute live imaging experiment with H4BPMHC. Histograms of cell counts corresponding to initial CTCF or $\log_{10}$(CTCF) values across the entire data set (N = 519 cells) divided into 20 bins. (A) Number of cells per range of CTCF values tended towards a lognormal rather than Gaussian distribution ($p < 0.0001$, D'Agostino and Pearson omnibus normality test). (B) CTCF values in (A) transformed to logarithmic values $p = 0.8239$.

Video S1. Fluorescence of cortical neurons across the three antioxidant conditions. DIV 7 neurons in the presence of 100 nM **H4BPMHC** across antioxidant treatments (+AO, −AO DIV 6, −AO DIV 2) following treatment with (Stressed) and without (Non-Stressed) cumene hydroperoxide (100 μM) and copper(II) sulfate (10 μM) to induce lipid peroxidation. Images were acquired via widefield microscopy with 20× magnification every 3 minutes for 60 minutes. The excitation wavelength was 488 nm (0.1 mW) and the fluorescence emission was spectrally filtered with a ZET480/640m emission filter. The video is sped up 180× (20s duration). Scale bar is 50 μm.

IV. Chapter 4

Developing a Method for Real-Time Imaging of Lipid Peroxyl Radicals in *Ex Vivo* Hippocampal Slices

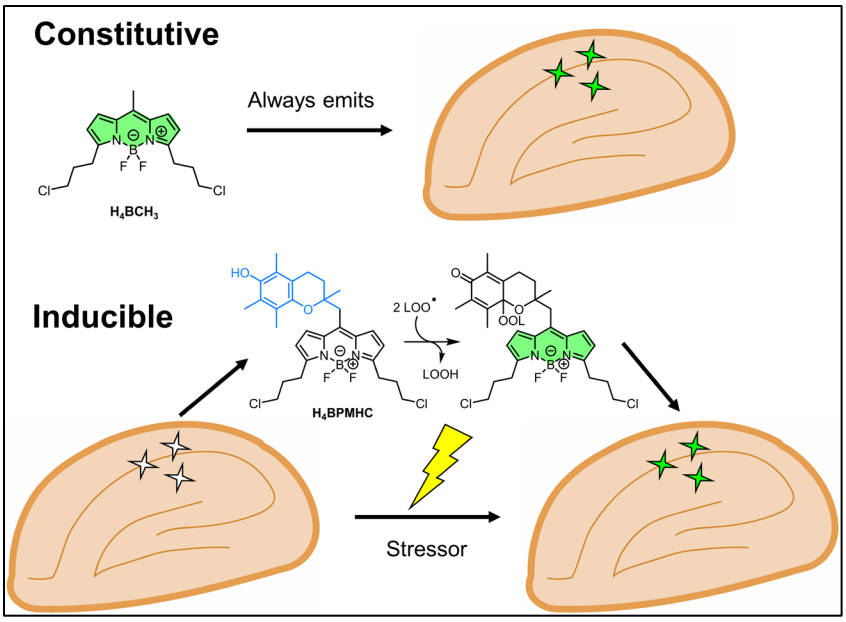

Figure credit: MK Foret

Manuscript in preparation for submission. "Developing a Method for Real-Time Imaging of Lipid Peroxyl Radicals in *Ex Vivo* Hippocampal Slices" Foret, M.K.*; Jodko-Piórecka, K.*; Audet, N.; Hooshmandi, M.; Do Carmo, S.; Khoutorsky, A.; Cosa, G.; Cuello, A.C. *In preparation for submission.*

Preface

In Chapter 3, we optimized the use of the fluorogenic probe H_4BPMHC in postnatal day primary neuronal cultures for detecting lipid peroxyl radicals real-time and assessing varying antioxidant loads in culture. This methodology offers advantages for quantitatively studying lipid peroxyl radicals real-time in vitro in neurodegenerative disease models such as primary neuronal cultures. However, an even more physiologically and disease-relevant system would involve a complex environment, such as *ex vivo* tissue preparations. In Chapter 4, we present the steps towards developing a method for utilizing H_4BPMHC and its constitutively fluorescing control probe H_4BCH_3 in *ex vivo* hippocampal slices. This chapter is presented in a format required for a journal specializing in methods development and thus assumes a layout different from the preceding manuscript chapters since the focus is placed on elaborate technical details.

IV.1 Abstract

This protocol describes the steps we used to troubleshoot the application of lipid peroxyl radical-detecting fluorogenic probes to *ex vivo* hippocampal slices. Detection of reactive oxygen species (ROS) in live tissue would offer valuable insight into physiological and pathological processes in a variety of contexts and research areas. Here, our aim is to provide readers with information that will assist in similar methods development, even with other fluorogenic probes, while providing key considerations and relevant references from the chemistry, biology, and microscopy perspectives to aid the process.

IV.2 Background

Reactive oxygen species (ROS) are radical and non-radical species derived from oxygen that act as oxidizing agents, and are implicated in both physiology through redox signaling as well as in disease through oxidative stress.[518] When ROS are excessively produced without sufficient antioxidant defenses and repair, they culminate in oxidative stress[314, 633] as indicated by downstream oxidative damage.[545] Although writing about ROS in a general sense is cautioned[414] they are considered elusive entities that readily react with biomolecules (lipids, proteins, nucleic acids, sugars) at varying rates based on their chemistry as well as their cellular location among other factors.[634] This transient quality makes reliable quantification of ROS challenging.[634] However, since ROS are upstream of oxidative damage, they offer insight into physiology and disease, including temporal and spatial information regarding the redox status of the cell or tissue of interest before overt oxidative damage occurs. This is valuable for understanding disease mechanisms in which oxidative stress is implicated and for administering relevant treatment before the excessive (and irreversible) damage occurs and therefore warrants efforts towards studying ROS real-time in physiologically relevant systems. Fluorescence imaging of ROS [635]overcomes temporal and spatial limitations of other ROS and/or oxidative damage detecting methods such as HPLC, mass spectrometry, and electron paramagnetic resonance (EPR)[636-637] and provides a way forward for studying ROS real-time.

Among different ROS present in the cellular milieu, peroxyl radicals are an especially interesting target for detection, because they act as chain carriers in the peroxidation of lipids – biomolecules especially sensitive towards oxidation. Thus, real-time monitoring of peroxyl radicals enables visualization of the initial steps of the oxidative stress cascade. Towards this goal, we have previously visualized and quantified lipid peroxyl radicals using the novel fluorogenic probe H_4BPMHC[635, 638]in HeLa cell cultures[635] as well as post-natal day primary neuronal cultures.[639] Lipid peroxyl radicals are of exceptional relevance to neuroscience since

neuronal membranes, that are enriched in polyunsaturated fatty acids (PUFAs), are particularly vulnerable to lipid peroxidation propagated by this type of ROS.

H_4BPMHC is comprised of a BODIPY conjugated to a chromanol moiety, modelled after α-tocopherol, an efficient radical trapping antioxidant. Initially, H_4BPMHC is quenched by photon-induced electron transfer (PeT), producing a dark off state, then, upon reaction with two lipid peroxyl radicals, PeT is inhibited and thus fluorescence is possible.[635]

In addition to the inducible, off/on probe H_4BPMHC, we have also utilized the constitutively fluorescent H_4BCH_3 probe (reported in Greene et al. 2017)[635] to serve as a benchmark for the maximum fluorescence signal, expected after oxidation of all available molecules of inducible probe (applied at the same concentration). This constitutive probe allowed for controlling probe diffusion in the tissue to optimize probe concentration, incubation time, imaging parameters, and aids quantification.

Currently, there are few publications employing non-commercial ROS detecting probes (e.g.: hydrogen peroxide) with ex vivo hippocampal slices[640-643] as well as studies utilizing cranial windows with ROS probes[644] (for a review of two-photon probes for tissue imaging see Kim and Cho (2015)).[645] However, as reliable quantification of ROS in biological systems and interpretation of results can be challenging, the details associated with such protocols are important to report (see Halliwell and Whiteman (2004),[637] Halliwell (2014),[646] Winterbourn (2014),[543] Forman et al. (2015)[414]).

In this communication, we report a new protocol developed to monitor lipid peroxyl radicals in ex vivo CNS slices using two-photon microscopy in addition to key considerations while troubleshooting this protocol. This work combines expertise from chemistry, biology, and microscopy to offer new perspective and references to apply the several concepts and procedures applicable to future investigations on the real-time formation of ROS in *ex vivo* slices.

IV.3 Materials and Reagents

IV.3.1 Probes

1. H_4BPMHC^{635}
2. $H_4BCH_3^{635}$
3. Hoechst (10 mg/ml stock, final use 2 μg/ml) (ThermoFisher Scientific, catalog number 33342)

IV.3.2 Dissection and Cutting of CNS Tissue Slices

1. Isoflurane (Baxter International Inc., catalog number CA2L9108)
2. Double-edge Prep Blades (Personna, catalog number 74-002)
3. Ice cold cutting solution (see Recipes)
4. Disposable razor blade (Personna, 94-120-2)
5. Large weigh boats
6. Bubbling stones (AutoMate Scientific, catalog number 01-40)
7. Rongeurs (Fine Science Tools, catalog number 16152-15)
8. Spatula (Fisherbrand, catalog number 2857510)
9. Petri dish (Falcon, catalog number 351029)
10. Scalpel (Feather #4)
11. Feather Surgical Blade #21 (Fisher Scientific, 08-918-5B)
12. Super glue (Lepage, catalog number 16625232)
13. Tubing clamps (Keck, KT 6 mm)
14. Pasteur pipette dropper
    a. Break the thin end of the glass Pasteur pipette then affix a dropper bulb onto this end.
    b. Alternatively, cut a 5 ml pipette tip at the 2 ml mark and use with a 5 ml pipettor.

IV.3.3 Incubations and Imaging of Tissue Slices

1. 12-well plate (Sarstedt, catalog number 83.3921)
2. Well carriers for 12-well plate (Corning, Netwell, 3520)
3. Tubing at varying diameters for bubbling setup
    a. (Saint Gobain Tygon S3, E3603 NSF 51)
    b. (Tygon, R-3608)
    c. (Becton Dickinson, 427406)
4. Aluminium foil
5. Beaker with nylon holder (as described in Papouin and Haydon (2018))[647] – for transferring slices to the microscope:
    a. Nylon tights
    b. Superglue (Lepage, catalog number 16625232)
    c. Soft plastic bottle, 6 cm in diameter
    d. Paper clips
6. 500 ml tall form beakers (PYREX, catalog number 1060-500)
7. Forceps (RWD, catalog number F12001-10)
8. Slice Hold-Down harp (Warner Instruments, catalog number 64-0252)

IV.3.4 Reagents

1. NaCl (Sigma Aldrich, catalog number: S7653)
2. KCl (Sigma Aldrich, BioUltra, catalog number: 60128)
3. $NaH_2PO_4 \cdot H_2O$ (Sigma Aldrich, catalog number: 71507)
4. $NaHCO_3$ (Sigma Aldrich, catalog number: S6297)
5. $MgSO_4 \cdot 7H_2O$ (Sigma Aldrich, catalog number: 63138)
6. $CaCl_2 \cdot 2H_2O$ (Sigma Aldrich, catalog number: 21097)

7. D-(+)-Glucose (Sigma Aldrich, catalog number: G7528)
8. Sucrose (Sigma Aldrich, catalog number: S7903)
9. $MgCl_2 \cdot 6H_2O$ (Sigma Aldrich, catalog number: M2670)
10. KH_2PO_4 (Sigma Aldrich, catalog number: 60218)
11. DMSO (Sigma Aldrich, catalog number D8418)
12. Ice-cold cutting solution (see Recipes)
13. Artificial cerebrospinal fluid (aCSF) (see Recipes)
14. ABAP (Sigma, catalog: 440914)
15. NMDA (Sigma)
16. Copper (II) sulfate (Sigma, catalog number: 203165)
17. Cumene hydroperoxide (Sigma, catalog number: 247502)

IV.3.5 Required Equipment

1. 95% O_2 / 5% CO_2 tank (MEGS)
2. pH meter (Hanna Instruments, B417)
3. Digital balance (Satorius, catalog number QUINTIX224-15)
4. Fume hood (Bedcolab)
5. Rat guillotine
6. Vibratome (Leica)
7. Water bath heater (Fisher Scientific)
8. Two-photon microscope (Leica SP8)
    a. 5x air objective, 25x HCX IRAPO (0.95 NA dipping objective, #506323)
    b. Two-photon laser (Coherent Cameleon Vision 2)
    c. Filters (BP 525/50, BP 460/50)

d. Detector (Leica HyD6 detector)

9. Imaging flow chamber (Pecon POC-R perfusion adaptor (open cultivation), catalog number 001021 (0727.121))
10. Peristaltic Pump (Isamtec peristaltic pump)
11. Tubing:
    a. Ismatec Pump Tubing 3-stop 0.76 mm ID (catalog number RK-96450-24)
    b. Masterflex L/S Precision pump tubing Tygon E-Lab E-3603 (catalog number RK-06509-13).
12. HPLC (Agilent Technologies Infinity II 1260, absorption (G7115A) and fluorescence (G7121B) detectors.
13. Spectrophotometer (Hitachi U-2800 UV–Vis–NIR)

IV.3.6 Software

1. Leica LAS X Version 3.5.2.18963

IV.4 Procedure

IV.4.1 Probe Preparation

1. H_4BPMHC and H_4BCH_3 can be synthesized according to previously reported procedures to make aliquots of 3 mM per 15 µl of DMSO.[635, 648]

IV.4.2 Setup

1. Assemble slice holder with nylon base similar to described in.[647]

    a. Cut the plastic bottle to generate a ring 5 cm in height.

    b. Stretch the nylon around one open end of the plastic ring to form a firm base, secure it with elastic bands and use super glue to affix the nylon. Do not use excessive glue and allow to dry for at least 24 hours.

    c. Cut off excess nylon with scissors.

    d. Rinse well with water before use to remove any excess glue.

    e. Cut 3-4 small holes at the top of the plastic ring to insert paper clips acting as hooks to ensure the holder does not sink to the bottom of the beaker.

    f. Assemble the elements in a 500 ml beaker with a tube and bubble rock air diffuser to bubble 95% O_2 / 5% CO_2.

2. Prepare aCSF and cutting solution fresh (see Recipes)

    a. Begin oxygenating the aCSF recovery solution in a 500 ml beaker containing the nylon nest, and in the 12-well plate containing well nets (3 ml per well) for at least 30 minutes before use and maintain at 32 ^{o}C by placing in a heated bath.

        i. Note: Two wells in the same row as the wells with nets should contain oxygenated aCSF (but lacking nets) for subsequent probe incubation. As opposed to transferring slices with a transfer pipette which also adds volume to the wells, we transferred them using the well nets to avoid variations in probe concentrations due to excess liquid being transferred. Furthermore, as these wells are used later in the protocol, they can begin to be oxygenated closer to the time of use.

b. Place cutting solution at -20 ºC (**not** -80 ºC)[647] for 20-30 minutes to create a slushy.

3. Prepare tools for dissection and cutting (vibratome blade and assembly)

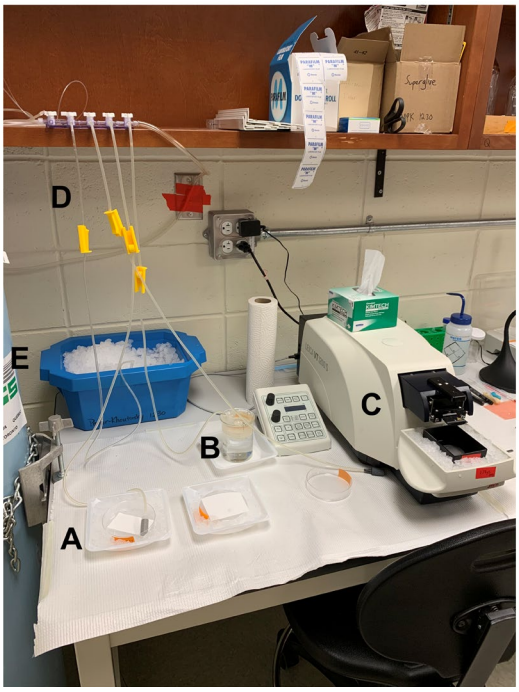

Figure 4.1. Dissection and cutting setup.
Example setup for dissection and cutting steps (heated water bath not shown, 500 ml beaker to be placed in the heated bath). **A.** Weigh boats with petri dish, and bubbling rock to be used for extraction and dissection of brain (ice and aCSF not shown). **B.** Bubbling aCSF for slice recovery. **C.** Vibratome equipped with blade for cutting brain slices. **D.** Multi-channel tubing for supplying oxygenated aCSF to dissection and cutting setups. **E.** Air tank (95% O_2, 5% CO_2).

IV.4.3 Dissection and Slice Generation

Coronal hippocampal slices (300 μm thickness) need to be generated and a brief protocol is outlined below with reference to Papouin (2018)[647] as a basic resource (Figure 4.1).

1. Prepare a small beaker with 150-200 ml of ice cold, bubbling cutting solution to place the brain in immediately after extraction
2. Anesthetize the rat using isoflurane and ensure no reflexes are present.
3. Rapidly decapitate the rat with a guillotine and dissect out the brain as indicated in other established protocols.[647]
4. Expose the skull using a razor blade.
5. Break and peel away the skull with rongeurs and/or curved forceps, taking care not to damage the brain.
6. Using a spatula dislodge the brain from the rest of the skull (taking care to cut the optic nerve or other nerves still remaining intact).
7. Immerse the isolated brain in ice-cold cutting solution.
    a. Note: Ensure the brain is removed and dissected rapidly and use chilled materials and solutions to handle it following isolation and during cutting.
8. Obtain the region of interest by using a razor blade to cut rostral and caudal sections (first removing the cerebellum then using the optic chiasma to remove the portion rostral to the hippocampus).
    a. Note: As in Figure 4.1, we used large weigh boats for ice in which we placed petri dishes with bubbling cutting solution for dissecting steps.
9. Glue the brain onto the cutting stage and immerse in ice cold, oxygenated cutting solution (do not pour directly on the brain but around it).
10. Cut 300 μm thick coronal sections and cut the bottom half of the coronal slice off with a scalpel such that the upper half contains the hippocampus and cortex.
11. Transfer slices using the modified transfer pipette to the 12-well plate containing well nets (one slice per well) (additional slices should be stored in 500 ml beaker) with bubbling aCSF at 32-33 ºC and let rest for 30 minutes (Figure 4.2).

a. Note: Avoid using a paintbrush to transfer slices as even soft paintbrushes can damage them.[647]

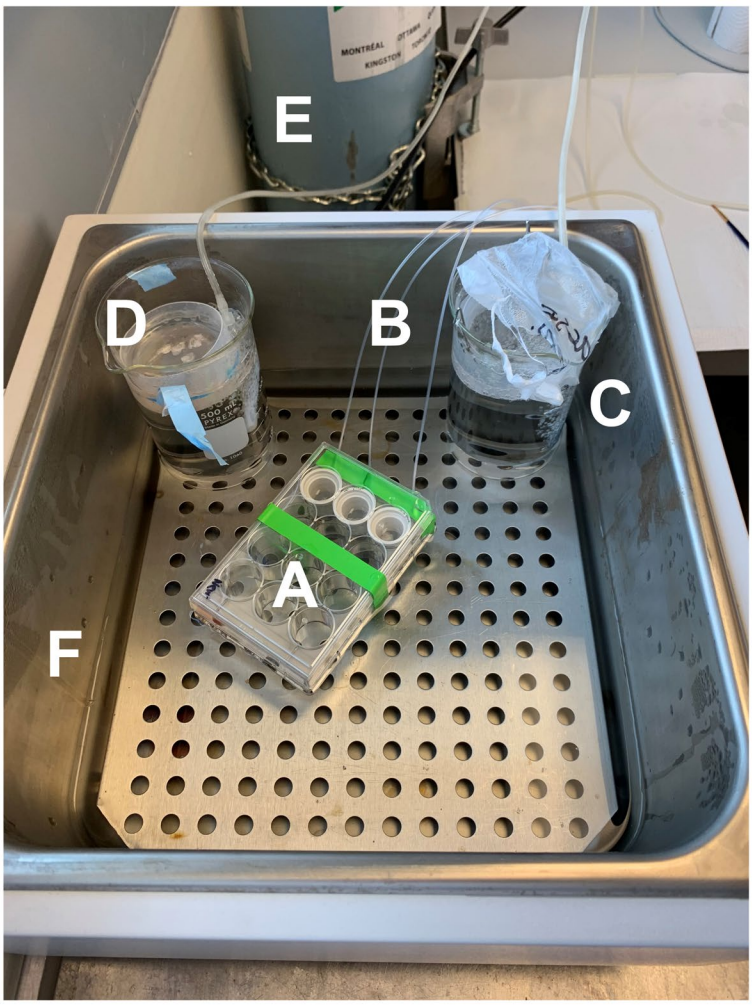

Figure 4.2. Incubation Setup.
Setup for incubating slices post-cutting in oxygenated aCSF at 32 ºC. **A.** 12-well plate equipped with well nets and bubbling lines (**B**). **C** and **D** show 500 ml beakers with the nylon slice holder used for transporting slices to the two-photon microscope. **E.** 95% O2 / 5% CO2 tank. **F.** Water bath.

IV.4.4 Probe Incubation

1. While slices are recovering for 30 minutes, prepare probe (H_4BPMHC and H_4BCH_3) dilutions:

    a. Dissolve probe aliquots in 15 µl of DMSO and vortex well to bring it to a concentration of 3 mM.

    b. Dilute probe stocks to 40 µM by adding 13.2 µl of 3 mM stock to 986.8 µl of aCSF for each 1 ml of probe needed.

    c. Pre-warm and protect from light.

2. Spike each well without a net with 1 ml of the 40 µM probe concentration to give a final probe concentration of 10 µM (each well should have 3 ml of oxygenated aCSF to which 1 ml of probe is added, giving a total of 4 ml per well)

3. Transfer the well nets containing slices over to the wells just spiked with probe. Cover the plate with aluminum foil to protect from light and incubate slices with the probe for 1-2 hours at 32 ºC.

    a. Note: Concentration and timing needs to be optimized depending on the properties of the probe (the probe lipophilicity will affect diffusion rates). For H_4BPMHC and H_4BCH_3 we used 10 µM probe, while in cell culture we utilized 0.1 µM of these probes. Concentration of 10 µM was chosen after performing initial studies with 1 µM, 5 µM, and 10 µM H_4BCH_3. This concentration also aligns with concentrations used in another publications using non-commercially available probes in *ex vivo* slices.[640]

4. While the probe is incubating, prepare the Hoechst dilution from a 10 mg/ml stock by diluting 1:10 (100 µl) with aCSF (900 µl) to make a 1 mg/ml solution. Then dilute 1:5000 with aCSF. As you need 2 ml of diluted Hoechst per well this is equivalent to 0.4 µl of the 1:10 dilution in every 2 ml.

5. Following probe incubation, transfer nets with slices to the wells containing the stressor (next section) or if not using a stressor then to the Hoechst wells for 15 minutes (final concentration of 0.02 µg/ml).

a. Note: Hoescht can aid in determining relevant brain regions, in our case, CA1 of the hippocampus. However, if animals used express fluorescent proteins in cells of interest, this would be an asset and Hoescht staining can be omitted.

IV.4.5 Stressor Incubation

1. After incubation with the inducible probe (H_4BPMHC) transfer the well nets with the slices to the adjacent wells containing the desired stressor diluted in aCSF (oxygenated) for 1 hour.

    a. 10 mM ABAP

    b. 10 μM NMDA

    c. 100 μM copper(II) sulfate with 1 mM cumene hydroperoxide

2. Following stressor incubation, transfer net with slices to the well containing Hoechst for 15 minutes (final concentration of 0.02 μg/ml).

IV.4.6 Imaging

1. The microscope, including the temperature control (32 ºC, and peristaltic pump with oxygenated aCSF should already be running by the time slices are brought to the microscope.

2. To transfer slices to the microscope, utilize the 500 ml beaker with the nylon net and bubbling aCSF. Transfer the slice to be imaged using the pasteur pipette dropper to the beaker with the net and bring to the microscope.

3. Use the pasteur pipette dropper to transport the slices to the imaging chamber (briefly shutting off the pump so that the slice does not float away) and use forceps to carefully place the slice holder harp on top of the slice in a manner compatible with imaging (e.g.: not covering the region of interest) (Figure 4.3).

4. To take z-stack images, use an excitation wavelength of 940 nm and emission range of 501-569 nm to image H_4BPMHC or H_4BCH_3 signal. For Hoechst, use an excitation wavelength of 720 nm and emission range of 406-497 nm. Speed 400, EOM gain 100-500.

a. Note: Alter imaging parameters as needed for different objective lenses, always image long wavelengths (lower energy) first then shorter wavelengths subsequently.

b. Note: It is important to maintain slice health when imaging, ensuring proper flow rate, temperature, and pH. For a review on considerations for live tissue imaging see.[649-652]

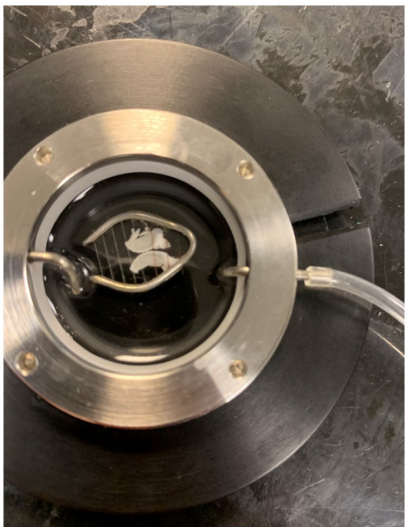

Figure 4.3. Flow chamber setup.
Flow chamber with two hippocampal slices held by a slice harp, in-flow of oxygenated aCSF is on the left side while out-flow is on the right.

IV.5 Data Analysis

1. Using the fluorescence intensity of the constitutive probe (H_4BCH_3) as a reference point for 100% of the signal (Figure 4.4), and the inducible probe (H_4BPMHC) under non-stressed basal conditions as the lowest possible signal you can gauge the dynamic range of the system.

2. Further correction to fluorescence intensities can be performed by subtracting any autofluorescence signal detected. Of note, in Figure 4.5, using the same imaging settings for the constitutive probe (H_4BCH_3) results in oversaturation of signal and was thus not shown. Importantly, this demonstrates that the dynamic range of the probe is large and can accommodate even greater stress levels than presented here.

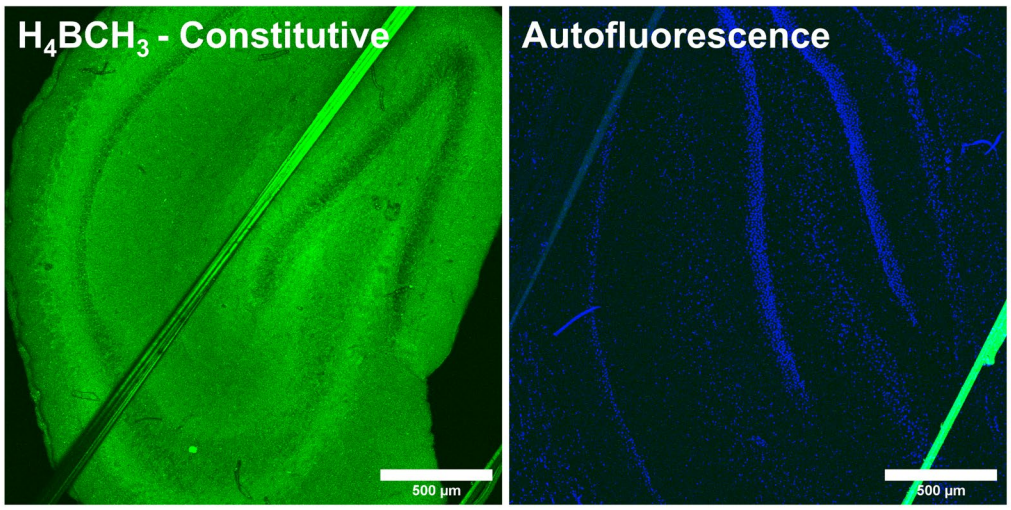

Figure 4.4 Constitutive and autofluorescence signal.
Example of maximal possible signal obtained with the constitutive probe (H_4BCH_3, left) compared to the autofluorescence signal (right) in a hippocampal *ex vivo* slices from rat. The right panel also shows Hoechst in blue as the autofluorescence signal was negligible. Strands over slices are from the harp holding down the slices. Scale bars represents 500 µm.

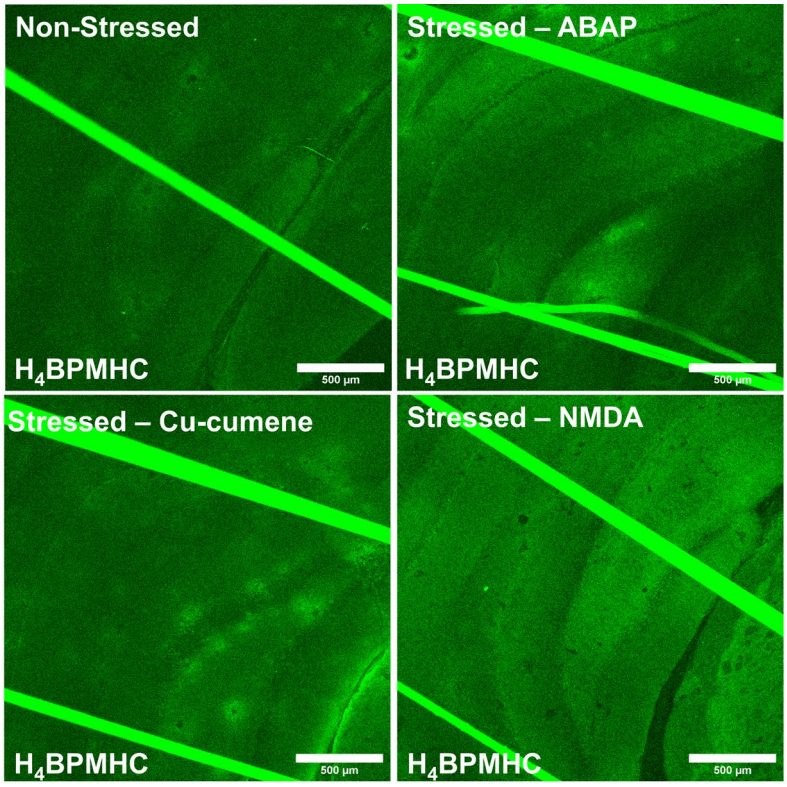

Figure 4.5. Stressor imaging.
Example of signal obtained following incubation with stressors (probe H_4BPMHC) in hippocampal *ex vivo* slices from rat. Of note, the constitutive probe (H_4BCH_3) signal was oversaturated when imaged with these settings and thus not shown. Strands over slices are from the harp holding down the slices. Scale bars represents 500 µm.

IV.6 Solution Recipes

Note: Use freshly prepared solutions.

Artificial Cerebrospinal Fluid (aCSF)

In 1 L of MilliQ water or ddH_2O dissolve:

- 7.25 g NaCl
- 0.37 g KCl
- 0.17 g $NaH_2PO_4 \cdot H_2O$
- 0.49 g $MgSO_4 \cdot 7H_2O$
- 2.18 g $NaHCO_3$
- 1.80 g D-(+)-Glucose
- 0.29 g $CaCl_2 \cdot 2H_2O$

Adjust pH to 7.4 and store at 4°C.

Cutting Solution

In 1 L of MilliQ water or ddH_2O dissolve:

- 0.186 g KCl
- 0.170 g KH_2PO_4
- 2.184 g $NaHCO_3$
- 1.802 g D-Glucose
- 43.13 g Sucrose
- 4 ml of 1 M stock $MgCl_2 \cdot 6H_2O$
- 0.1 ml of 1 M stock $CaCl_2 \cdot 2H_2O$

Bubble with 95% O_2 and 5% CO_2

IV.7 Notes

- Since the imaging setup requires flow of fresh oxygenated aCSF over the slice, it is also important to consider whether the endogenous stressor of interest or possibly the probe itself may be washed away. In our hands, our probes did not exhibit decreased fluorescence over time and only were 'pulled' out of the sample in the presence of serum (FBS) when tested in HeLa cells.[480]

- Depending on the probe used, the type of tissue and the type of ROS (or RNS) that is of interest to your study, the stressor will differ. As example, in the case of H_4BPMHC, which detects lipid-associated ROS, lipophilic free radical initiators such as AMVN (2,2'-azobis (2,4-dimethylvaleronitrile)) would be a promising positive control. Conversely, if the ROS of interest is hydrophilic, then the aqueous free radical initiator AAPH (2,2'-azobis(amidinopropane) dihydrochloride) may be a better alternative. In both cases, you need to consider diffusion rates of the stressor and how they relate to that of your probe, adjusting concentration and incubation time as needed. Hydrogen peroxide (H_2O_2) may also serve as an effective positive control as it has lower reactivities with biomolecules compared to other ROS, therefore, allowing H_2O_2 to diffuse greater distances. Additionally, NMDA (N-methyl-D-aspartic acid), inflammatory cytokines, or other biological stressors may also be employed.

- If the setup allows, you can also include the stressor in your imaging setup, such that it can be flowed over the slice as you are imaging. This would ensure you do not miss peak enhancements that the stressor causes.

IV.8 Acknowledgements

We thank Dr. Masha Prager-Khoutorsky for providing us with the vibratome setup and space for generating the hippocampal slices.

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V. Summary of Thesis and Contributions to Original Knowledge

Using a transgenic rat model of the AD-like amyloid pathology, the present work provides evidence for an early, pre-plaque oxidative stress response in Aβ-burdened neurons when a neuron-driven inflammatory response is incipient. The work then focuses on methodological developments towards studying and better understanding upstream ROS, namely, lipid peroxyl radicals in real-time. Overall, the contributions to original knowledge that this thesis provides are as follows:

1. I showed that a pre-plaque oxidative stress response occurs in Aβ-burdened neurons, which **coincided** with a neuron-driven inflammatory process but **preceded** overt oxidative damage. The strength of these findings is that they are specific to hippocampal CA1 and subiculum neurons and not glial cells as the material studied was obtained using laser capture microdissection (LCM).

    a. This was the first study to specifically implicate DNA repair genes and proteins in amyloid burdened neurons from the McGill-R-Thy1-APP rat during early pre-plaque stages.

    b. I identified that DNA repair genes (*Ercc2*, and *Fancc*) were upregulated in Aβ-burdened neurons.

    c. I showed that the protein XPD (*Ercc2*), that plays a role in nucleotide excision repair (NER), was upregulated in Aβ-burdened subiculum neurons, which also showed an increase in the number of neurons with double-strand DNA breaks (DSBs).

    d. I identified other potentially influential DNA repair and antioxidant response genes that trended to increase in Tg CA1 and subiculum neurons, including *GPx7, Ift172, Sqstm1, Ercc6, Gclm* and *Prnp*.

    e. I identified that expression of *Fen1* which plays a role in base excision repair (BER) increased in hippocampal homogenates from Tg rats, while *Ercc3* (NER) trended to increase.

    f. I discovered an upregulation of antioxidant-related genes *Sod2*, *GR*, and *Idh1* in Aβ-burdened hippocampal neurons.

g. I established that despite an increase in gene expression, the protein glutathione reductase trended to decrease in Aβ-burdened CA1 neurons, which also had a trend to increase in 4HNE immunoreactivity.

h. I developed multiple automated ImageJ macros for specifically and accurately quantifying immunoreactivity volumes in hippocampal neurons.

i. I developed ImageJ macros for assessing structural aspects of astrocytic immunoreactivity including the presence of GFAP-positive processes in the vicinity of CA1 and subiculum neurons.

2. I optimized and validated the fluorogenic probe H_4BPMHC for visualizing and quantifying lipid peroxyl radicals in neuronal cultures. This was the first time the probe was used in primary post-natal day rat neurons, thus extending its application beyond HeLa cell cultures.

    a. I demonstrated that H_4BPMHC had the sensitivity to detect variations in antioxidant load of neuronal cultures, serving as a proof-of-concept for using this probe towards studying neurodegenerative disease models.

    b. I contributed to developing a methodology for studying lipid peroxyl radical production real-time in a biologically relevant system that ultimately provides a foundation for future studies.

3. I laid a foundation and framework for developing a method to study lipid peroxyl radicals in *ex vivo* hippocampal slices using H_4BPMHC and H_4BCH_3.

    a. I optimized the use of these probes with two-photon microscopy, which has not been done previously.

    b. I troubleshooted the use of these probes in *ex vivo* hippocampal slices, which is the first time they were applied to tissue systems.

4. Given the intense efforts towards understanding the challenging subject of the earliest probable oxidative stress-related events during the earliest stages of the Alzheimer's-like Aβ pathology, my extensive discussions and readings allowed me to co-lead a comprehensive, interdisciplinary review on free radical initiated lipid autoxidation and its involvement in health and disease in a high impact journal (as published in *Chemical Reviews*. 2020. 120:12757-12787.)[321]

VI. General Discussion

"Above all, don't fear difficult moments. The best comes from them."

- Rita Levi-Montalcini

VI.I Advantages and Limitations of Rodent Amyloid Models

In general, animal models of disease provide useful insight into pathological mechanisms that are not possible in other research contexts. As example, cell culture is limited since it is not physiologically comprehensive. Of course, 3D cell culture methods,[653] primary cultures, co-cultures and the advancement of iPSC cultures[654-655] have improved the translational value of cell culture data. However, cell cultures miss the complex interactions of mature and fully differentiated cells integrated in a tissue as intricate as the CNS. In addition, for AD research, cell culture models are produced over a short amount of time, while in the human brain, AD progresses over the course of decades; thus, recapitulating the disease remains a challenge.[656] Conversely, studies in humans are also limited for AD since post-mortem data only represents one point in time, while longitudinal data collection is costly and provides limited information regarding molecular and cellular pathological mechanisms. Therefore, transgenic rodent models using mice and rats are useful research tools for studying aspects of the AD pathology. Indeed, these models require genetic (or other) manipulation since rodents do not develop AD. Some animals do develop amyloid plaques and cerebral amyloid angiopathy with age including squirrels, non-human primates, and dogs but they do not develop neurofibrillary tangles (NFTs).[657]

Since the discovery of the causative genes for AD, multiple rodent models (primarily mouse) have been generated. Typically, these models overexpress human mutated *APP* containing familial mutations in neurons. Regarding tau pathology, there are not any mutations in the tau gene (*MAPT*) that cause AD. Instead, *MAPT* mutations are linked to a familial form of frontotemporal dementia (FTD).[483] These human mutations (including those in *MAPT*) inspired the development of numerous rodent models for studying AD. Although not discussed in depth here, there are also knock-in models, as well as non-genetic rodent AD models whereby pathological proteins (amyloid and tau), brain lysates from AD patients or transgenic mice, are injected intracranially stereotaxically, for a review of these models see Götz *et al.* (2018).[483]

The strengths of these **overexpressing** mutated amyloid models are as follows:[483, 658]

- Transgenic animals develop a similar amyloid pathology as in the human brain, with progressive accumulation of intracellular Aβ, amyloid peptide and the formation of extracellular amyloid plaques in an age-dependent manner.
- Amyloid plaques are similar in structure to those in the human brain, progressing from diffuse to dense core plaques that accumulate various other proteins at later stages.
- Animals experience cognitive deficits which correlate with soluble Aβ rather than plaque load, similar to in humans. However, in humans, cognitive decline only occurs after extensive plaque deposition, explained by the considerable neural reserve of the human species.
- Rodent models recapitulate synapse loss in a neurotransmitter-specific manner, as reviewed by Bell and Cuello (2006).[659]
- The 3xTg-AD model developed by Oddo *et al.* (2003) containing *PSENI* (M146V), *APP* (Swedish), and *MAPT* (P301L) exhibited both plaque and initial tangle pathology.[284]

Major limitations of familial AD rodent models include:[483, 658, 660]

- Most AD cases are sporadic and not caused by familial AD mutations.
- Pathology develops relatively fast, and therefore, the contribution of aging (the largest risk factor for AD) is removed.
- Unlike humans, some models overexpress *APP* to varying degrees, the 5xFAD mouse model containing two mutations in *PSENI* (M146L, L286V) and three mutations in *APP* (Swedish, London, and Florida)[656] is especially aggressive and does not represent well the earliest human AD pathology.
- Human transgenes used do not contain all non-coding sequences.
- Insertion site and copy number of the transgene cannot be controlled.
- Rodents are heavily inbred and do not faithfully represent the heterogeneity of the human population.
- There has been a lack of successful translation of rodent data to therapeutic outcomes for humans.

To overcome some of these limitations, recently, an initiative called MODEL-AD (Model organism development and evaluation for late-onset Alzheimer's disease) was established to develop better mouse models for understanding LOAD and for preclinical data collection in order to test novel therapeutics.[660] However, this effort is conducted primarily in mice. When comparing mouse versus rat models, as outlined in the Introduction, rat models offer multiple benefits over mouse models including: 1) larger brain size and thus easier manipulation, 2) easier to handle, 3) have a broader behavioral display allowing for cognitive assessments, 4) exhibit more diverse social behavior 5) are physiologically, genetically and morphologically more similar to humans than mice (e.g.: 6 tau isoforms as humans, although not exactly the same ratios this is still more similar than mice, and more homology between rat and human ApoE). [484]

It is important to be cognizant of the limitations of these and other models when extrapolating findings. However, overall, transgenic rodent models of AD, including the McGill-R-Thy1-APP rat model, have been highly valuable tools for furthering our understanding of pathological mechanisms in AD as well as the contributions of oxidative stress to the AD continuum.

With these aspects in consideration, the work presented in this thesis focused on utilizing a transgenic well-established model of the AD-like amyloid pathology, rendering a closer approximation to that of the human pathology, with an extended pre-plaque stage that recapitulates key aspects of the silent pre-symptomatic stages of AD.

VI.2 How does Amyloid Beta Generate Oxidative Stress in Neurons?

VI.2.1 Aβ-Induced Reactive Oxygen Species

In the AD field, numerous studies have investigated potential mechanisms by which Aβ can lead to ROS production and oxidative stress, and there is no shortage of literature. The following sections will outline the variety of mechanisms in which Aβ can directly or indirectly lead to ROS production and place these mechanisms into the context of the present thesis.

VI.2.1.1 Direct ROS Generation

Early *in vitro* studies by Butterfield and colleagues demonstrated that exogenous Aβ induced oxidative stress in neurons and glia that was ameliorated with free radical scavenging antioxidants, and suggested a causal role for AD (summarized in Butterfield 1997).[267, 661] Many of these earlier studies utilized primary neuronal cultures incubated with varying Aβ fragments such as Aβ25-35, and Aβ40 at high concentrations (20-50 μM) over the course of days and measured oxidative stress using fluorogenic probes for ROS detection as well as electron paramagnetic resonance (EPR).[662-664]

Later studies by Butterfield and colleagues discovered that Aβ42 generated oxidative stress by inserting into cellular membranes and initiating lipid peroxidation (Introduction Section 1.10.2). They went on to investigate the mechanism by which Aβ42 could generate lipid-associated ROS and predicted with theoretical calculations that the glycine 33 residue could be attacked by a sulfur-based free radical generated by the methionine 35 residue on an adjacent Aβ42 peptide, thus generating a carbon-centred free radical which could then initiate lipid peroxidation.[665] They tested this predication by measuring neurotoxicity, oxidized proteins, and ROS in primary hippocampal neurons or isolated synaptosomes exposed to exogenous Aβ42 or Aβ42G33V. The latter showed no neuronal toxicity, significantly lower oxidized proteins and lower ROS as measured by the fluorogenic probe DCF indicating a potential role for the glycine 33 residue in Aβ-induced lipid peroxidation.[665] In a follow-up study, the group then tested the neurotoxicity of exogenous native $A\beta_{42}$ or the variant $A\beta_{42G37D}$ by exposing primary hippocampal neurons for 24-hours at varying concentrations of the peptides (1-10 μM). In this case, the G37D variant decreased the hydrophobicity of $A\beta_{42}$ where both neuronal toxicity and aggregation were decreased in neurons, highlighting the importance of membrane-associated oxidative stress.[527] Notably, methionine is easily oxidized

to both R- and S-stereoisomers of methionine sulfoxide, which can be reduced back to methionine, by methionine sulfoxide reductases. This characteristic of methionine has been suggested play a role in the cellular antioxidant defense as well as regulation of protein function since this oxidation is reversible.[666] Interestingly, methionine sulfoxide levels increase with aging[667] while the activity of methionine sulfoxide reductases decreases in AD.[668]

Other groups proposed an alternative mechanism for Aβ-induced ROS,[669] namely, production of hydrogen peroxide when Aβ42 traps molecular oxygen (O_2) and reduces Cu(II) to Cu(I) *in vitro*. The Cu(I) can react with hydrogen peroxide to produce hydroxyl radicals thus initiating lipid peroxidation.[534] Importantly, this study compared human and rat Aβ and only observed a reduction of copper by human Aβ. Follow-up studies by this group using primary hippocampal neurons showed that the presence of Cu(II) to exogenous $A\beta_{42}$ exposure potentiated neurotoxicity by facilitating Aβ-induced oxidative damage.[343]

Although these *in vitro* studies provided evidence for Aβ-mediated ROS production, specifically lipid peroxidation, there are limitations to them, namely the use of exogenous Aβ species, which does not recapitulate endogenous Aβ production and aggregation, as well as the use of Aβ concentrations that exceeded physiologically and pathologically relevant levels. As noted in the Introduction, concentrations in the nM rather than μM range are now considered more relevant to studying the pathology of Aβ. Butterfield and colleagues have since performed *in vivo* studies to further investigate the role of methionine 35 in Aβ-mediated oxidative stress. They used the PDAPP Tg mouse model of AD (Swedish and Indiana APP mutations) at 8 months of age (post-plaque) with and without the additional mutation, M631L (631 indicates the location in the full-length APP protein that corresponds to methionine 35 in the Aβ peptide). Leucine was selected as a substitution since it has a similar side chain length and hydrophobicity as methionine. In mice with the M631L mutation, markers of oxidative damage in the brain were returned to the level of Wt animals, while the PDAPP Tg mice lacking the M631L mutation had elevated levels of oxidative damage (4HNE and 3-NT). The M631L mutation resulted in decreased plaque deposition compared to regular Tg mice but did not improve cognition as measured by the Morris water maze (MWM) test.[530] Despite the lack of improvement on cognition, other studies have confirmed the importance of the methionine 35 residue of Aβ for generating oxidative stress in vitro.[670-674]

Another *in vivo* study by Matsumara et al. (2015) used electron paramagnetic resonance (EPR) to visualize and quantify free radicals in a transgenic (Tg) mouse model of AD at 3, 6, 9, 12 and 18 months, of note, in this model, plaques first appeared at 6 months of age. The EPR technique is useful as it directly detects ROS, allows in vivo brain imaging (currently not approved for use in humans), and it provides temporal resolution. However, as noted by other publications, even though EPR has these benefits, it is not sensitive enough to detect minute changes in ROS that are biologically relevant,[355] and this is apparent in the results of Matsumara et al. (2015) since they only detected changes in EPR data at 9 months of age well past plaque formation in the brain occurring at 6 months.[675]

A major gap that remains in fully understanding Aβ-mediated ROS production is the real-time temporal and spatial tracking of ROS production during pre-plaque stages of AD. In the present thesis, methodologies for assessing lipid peroxyl radical production in primary neuronal cultures and *ex vivo* hippocampal slices in real-time, offer a foundation for future investigations. Chapters 3 and 4 provide a proof-of-concept for future use of H_4BPMHC and related fluorogenic ROS-sensitive probes in relevant AD models. [676]

VI.2.1.2 Receptor of Advanced Glycation End Products

Indirect generation of ROS by Aβ can also occur through binding to the receptor for advanced glycation end products (RAGE). AGEs are a family of post-translational modifications generated through a Maillard reaction involving condensation between reducing sugars and protein amino groups resulting in oxidative damage to proteins and were found to be increased in the AD brain at sites of plaque and tangle formation.[311, 536] RAGE expression is known to be increased in AD and Aβ has been shown to bind AGE receptors on endothelial cells and neurons, initiating oxidative stress and NF-κB activation, as well as receptors on microglia resulting in cytokine production.[123, 537] Additionally, glycation of Aβ can slow conversion to fibrils, therefore lengthening the lifetime of toxic oligomeric species.[677]

VI.2.1.3 Mitochondrial Dysfunction

Many neurodegenerative diseases, including AD, exhibit mitochondrial dysfunction, highlighting the vulnerability of neurons to mitochondrial-associated insults.[678] In the context of AD, Aβ-induced mitochondrial dysfunction has been extensively studied[679] in rodent models (including the McGill-R-Thy1-APP rat)[493, 495] as well as in MCI and AD brains, including alterations in bioenergetics,[680-681] mitochondrial dynamics[533, 682-685] (namely increased

mitochondrial fission in AD), and mitophagy. As mitochondria are key sources of ROS, any disruption of their function can cause oxidative stress and studies have already investigated the benefits of mitochondrial-targeting antioxidant strategies for AD.[686] Indeed, neurons also have high energy demands, therefore, synaptic mitochondria are vulnerable to disruptions in their mitochondrial function and in mitochondrial transport to synapses where energy demands are highest. Of course, tau also plays a large role in the disruption of mitochondrial transport in AD as well.[678]

Importantly, in both rodent models of AD and in MCI and AD brains, APP and/or Aβ have been shown to be produced in mitochondrial membranes,[123, 532, 687-689] insert into mitochondrial protein channels,[690] and to bind mitochondrial proteins including ABAD (Aβ-binding alcohol dehydrogenase),[531] DrpI (dynamin-1-like protein, responsible for fission)[691] and CypD (cyclophilin D, part of the mitochondrial permeability transition pore mPTP) among others, thus contributing to mitochondrial dysfunction (often through calcium dyshomeostasis) and ROS generation. As example, Takuma et al. (2005) showed *in vitro* and *in vivo* using a Tg mouse model of AD, that Aβ binding to ABAD resulted in elevated ROS production,[692] decreased ATP and cell viability, and altered activity measured by electrophysiology.[693] Similarly, Du et al. (2008)[694] showed that Aβ interactions with CypD resulted in ROS generation and opening of the mPTP, which can result in cell death via calcium-induced mitochondrial swelling, more ROS generation, and loss of membrane potential.[695-696] Indeed, synaptic mitochondria have higher levels of CypD, making them especially vulnerable to pore opening and calcium dyshomeostasis.[697] Notably, ablating CypD-Aβ interactions improved mitochondrial function and learning and memory in a Tg mouse model of AD.[698]

Among other mitochondrial quality control mechanisms, mitophagy is an important process where mitochondria are degraded through autophagy and is also implicated in AD.[699] In relation to oxidative stress, when mitophagy is compromised, oxidative damage can accumulate in the cell.[678] Mitophagy can be initiated by different pathways including ubiquitin-mediated, receptor-mediated, lipid-mediated, stress-mediated (as example, through AGEs (advanced glycation end products) and ROS))[700-701] or even Aβ- and tau-induced mitophagy and involves the formation of an autophagosome which engulfs mitochondria, delivers them to the lysosome where they are degraded.[702-703]

Lastly, in AD patients, glucose metabolism, as measured by positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose,[704] has been shown to be decreased at pre-symptomatic

stages, while proteins implicated in mitochondrial bioenergetics are oxidatively modified in the AD brain (as reviewed by Butterfield and Halliwell (2019)).[311]

Overall, it is clear mitochondrial dysfunction is implicated in AD and likely contributes to the earliest pathology, being closely tied to Aβ accumulation. In Chapter 2, we found elevated expression of the mitochondrial antioxidant Sod2 in Tg Aβ-burdened hippocampal neurons. Although we did not observe this increase at the protein level, our analysis was limited to only assessing somatic SOD2 levels and not synaptic levels where a difference is more likely to be. Indeed, SOD2 likely plays an important protective role during the earliest stages of the amyloid pathology as transgenic APP mice heterozygous for SOD2 exhibit an exacerbated AD-like pathology.[705-706] Furthermore, SIRT3, which is responsible for deacetylating SOD2, enabling its function, was found to be decreased in AD,[699] in the present thesis we found no changes in *Sirt3* expression. However, it would be of interest to investigate protein levels and activity of SIRT3 since it plays an important role in the proper function of SOD2.

In addition to studying genes, proteins and pathways affiliated with mitochondrial dysfunction in AD, the next step towards understanding oxidative stress in AD is real-time imaging and quantification of these elusive reactive oxygen species. Towards this goal, in the introduction, it was outlined that fluorogenic probes can be chemically tailored to each biological question of interest, as example, previous generations of the α-tocopherol probes employed in this thesis have utilized mitochondrial-targeting moieties to specifically assess mitochondrial lipid peroxyl radicals.[676] It would be of interest to utilize this mitochondrial specific fluorogenic probe to assess how mitochondrial lipid peroxyl radical levels compare to those of the rest of the cell. Indeed, there is strong evidence that Aβ-burdened neurons are especially vulnerable to mitochondrial insults.

VI.2.1.4 ER Stress and Proteostasis

The endoplasmic reticulum is an important reservoir for calcium in the cell,[707] notably, calcium homeostasis also plays an important role in mitochondrial function and can also be disrupted due to Aβ-induced excitotoxicity and mPTP opening.[708-711] There is also a great deal of literature on calcium dyshomeostasis in AD.[712] Furthermore, the ER forms connections with mitochondria that allow for transport of lipids between these organelles,[713] termed mitochondrial-associated membranes (MAMs) where Aβ is also found.[714] Disruptions in calcium homeostasis and in MAMs have been observed in AD and are associated with ER stress.

The ER also maintains proteostasis in the cell through synthesis and quality control of misfolded proteins in order to prevent aggregation, including ER-associated degradation (ERAD where misfolded or unfolded proteins are transported to the cytoplasm where they are degraded by the ubiquitin-proteasome system (UPS).[715] When the load of misfolded or unfolded proteins exceeds the capacity of ERAD, another strategy termed the unfolded protein response (UPR) is used by the cell to respond to this overload.[707] When activated, as example, by Aβ, the UPR triggers multiple signaling pathways associated with inflammatory responses including NF-κB which can lead to downstream oxidative stress.

In AD, proteostasis is disrupted not only by Aβ accumulation and aggregation but also by oxidative modifications.[311] As example, ubiquitin carboxyl-terminal hydrolase L1, which is integral to the UPS was observed to be oxidatively modified in MCI and AD brains, ablating its function and resulting in the accumulation of damaged proteins.[716-717] Although this is at a later timepoint in the AD pathological continuum, it implicates oxidative stress and damage in disruption of proteostasis, which is studied in the context of AD and other neurodegenerative diseases.

One note of caution, however, in a recent review by Hasimoto and Saido (2018) they noted that not all transgenic rodent models of AD are ideal for studying ER stress since they have confounding factors such as overexpression which can lead to poor extrapolation of results to the human condition. As example, they noted that *APP*-knock-in models lacked the ER stress response observed in overexpression models.[718] This highlights an important point from Gotz *et al.* (2018) in Section 5.1, namely that transgenic AD models that are repurposed for studying different mechanisms than originally intended which can result in misleading interpretations and extrapolations.[483]

VI.2.1.5 Excitotoxicity

Many studies have observed that Aβ increases neuronal vulnerability to excitotoxicity.[719] Aβ interactions with NMDA receptors can cause chronic activation, leading to increased intracellular calcium levels. As a result, incoming physiological signals may not be distinguished, thus impairing synaptic plasticity, long-term potentiation, learning and. Furthermore, the calcium dyshomeostasis could cause cell stress and death of the postsynaptic neuron through the aforementioned mechanisms, including oxidative stress.[538, 720] Interestingly, other studies have implicated hippocampal neuron hyperexcitability to an increase in DNA double strand breaks (DSB). Notably, transgenic AD mice, during pre-

plaque stages exhibited elevated levels of DSBs above those associated with learning and memory.[448, 599-601] The link to DSBs and DNA repair in AD will be discussed further in a later section. However, these connections between hyperexcitability (which can be caused directly by Aβ), calcium dyshomeostasis and an increase in DSBs are of relevance to unraveling the earliest AD pathology. In Chapter 2 we reported our findings of an increase in DSBs in the subiculum of Tg rats, while there was a trend to increase in CA1.

VI.2.1.6 Inflammation

As discussed in the Introduction (Section I.2.4), although only gaining momentum in the AD research field in the 1980s, neuroinflammation is now considered a key pathological aspect of AD.[94] Inflammation is closely linked to oxidative stress where it can be a cause and consequence of oxidative stress. ROS are key signaling molecules for generating immune responses, while oxidatively modified biomolecules (termed oxidation-specific epitopes (OSEs)) are recognized by receptors for PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular patterns).[721] Additionally, there is evidence that the NLRP3 (NOD-like receptor protein 3) inflammasome can be activated by mitochondrial ROS as well as oxidized lipids which act as DAMPs.[722-723] Indeed, the NLRP3 inflammasome has been implicated in AD.[724-725]

It is important to note that glial cells contributing to neuroinflammatory processes have recently been recognized to have a spectrum of activation states. As example, previous definitions oversimplified microglial activation states into M1 and M2 phenotypes, while today it is recognized that activation is defined by specific cellular contexts and transcriptomic analyses,[726] with similar advancements being made for understanding activation states of astrocytes.[727] In the context of AD, there are numerous studies related to amyloid plaque-associated inflammation that is also associated with elevated oxidative stress and how microglia play a resolving role during this timepoint. Conversely, the early, pre-plaque role of microglia and the neuroinflammatory response likely play an exacerbating role.[94]

Recently, our lab demonstrated an inflammatory role for Aβ-burdened neurons during the pre-plaque amyloid pathology which was a foundation for the rationale behind this thesis. Notably, neuronal Aβ oligomer accumulation was associated with an increase in inflammatory gene and protein expression in neurons including: major histocompatibility complex II (MHCII), inducible nitric oxide synthase (iNOS), cluster of differentiation 40 (CD40), COX-2 (PTGS2), IL-1β, tumor necrosis factor alpha (TNFα), chemokine (C-X-

C Motif) ligand 3 (CXCL3),[301, 479] and more recently, chemokine (C–C) ligand 2 (CCL2), CCL3, and interleukin-6 (IL-6).[302] The present thesis I demonstrate that this pre-plaque inflammatory process coinciding with amyloid accumulation is also linked to upregulation of DNA repair and antioxidant genes. An important next step would be to assess the targets investigated in Chapter 2 at even earlier pre-plaque timepoints.

In this section a variety of mechanisms in which Aβ can generate or result in oxidative stress were presented. It remains to be known to what degree each pathway exacerbates the earliest AD pathology. However, based on the findings in this thesis, iAβ-burdened neurons exhibited increases in antioxidant and DNA repair genes, with incipient increases in oxidative damage. Importantly, other studies from our lab observed a neuron-driven inflammatory process also at the timepoint investigated in this work,[302] pointing towards an important role for these mechanisms.

VI.3 DNA Damage and Repair in Alzheimer's Disease

As discussed in Section I.10.3 of the Introduction, there are various types of DNA damage and without effective repair, nDNA and mtDNA mutations can accumulate and result in neuronal death.[391] Neurons are particularly vulnerable to DNA damage as they are transcriptionally active as well as post-mitotic and utilize the more error-prone, non-homologous end joining (NHEJ) for double strand break (DSB) repair. Furthermore, as neurons are metabolically active, mtDNA is particularly susceptible to oxidative insult[473] and does not have as versatile repair mechanisms as nDNA.[474] Indeed, synaptic mitochondria have exhibited exacerbated pathology and damage when compared to somatic mitochondria in Tg mouse models of AD.[298]

In this section, key DNA repair pathways will be outlined, followed by their relevance to AD including nucleotide excision repair (NER), base excision repair (BER), and Non-homologous end joining (NHEJ), since they relate to genes that were observed to be dysregulated in Aβ-burdened neurons from the McGill-R-Thy1-APP rat and were also related to the damage we observed, namely elevated DSBs and trend to decrease in 8-oxo-dG immunoreactivity (Chapter 2). Repair pathways not discussed include homologous recombination (HR) and mismatch repair among others although DNA repair proteins in these pathways have recently been implicated in AD and AD rodent models.[446, 728] Importantly, as oxidative DNA damage has been reported to be elevated in AD (Introduction, Section I.10.3), DNA repair proteins have also been shown to be decreased or less active in aging[475] and at late stages during MCI and AD.[478]

Nucleotide Excision Repair

In Chapter 2, genes implicated in NER including the gene *Ercc2* and its gene product XPD, were upregulated in Aβ-burdened hippocampal neurons. *Ercc6* (CSB) also trended to increase in these neurons, while *Ercc3* (XPB) trended to increase in hippocampal homogenates. Below an overview of NER is outlined followed by the significance of this work in the context of AD.

Nucleotide excision repair (NER) recognizes and repairs a wide spectrum of DNA lesions and thus has one of the most complex DNA damage recognition and verification processes.[729] NER can be divided into two different pathways that recognize damage through different methods, and then merge together as one pathway: 1) global genome repair (GG-NER), which repairs lesions throughout the genome, including heterochromatic, transcriptionally inactive regions[730] and 2) transcription-couped repair (TC-NER) which repairs lesions in euchromatic regions on the transcribed strand of DNA that would otherwise block progression of RNA polymerase II. Defects in GG-NER and TC-NER result in different diseases, where the former predisposes individuals to cancer and the latter results in UV sensitivity and premature aging. NER typically repairs DNA lesions that distort the DNA double helix where some substrates of NER include cyclobutene pyrimidine dimers (CPDs), pyrimidine-(6,3)-pyrimidone photoproducts (6-4PPs) generated from UV radiation, base adducts created by exogenous chemical agents (cisplatin, nezopyrene), base lesions caused by reactions with lipid peroxidation products (as example reactions with MDA produce malendialdehyde-related pyrimidopurinone adduct (M1G)), and ROS-induced lesions including cyclopurines.[731] These bulky lesions often hinder replication or transcription leading to replication fork collapse or a half of transcription, or they can be bypassed, resulting in errors.[731] Broadly, NER can be divided into the steps: 1) damage recognition, 2) incision and removal of the damage-containing oligonucleotide fragment, 3) gap filling, and 4) ligation. In GG-NER, more distorting lesions are recognized directly by the protein XPC (Xeroderma pigmentosum C) which is the main damage sensor in GG-NER and is stabilized by RAD23B and CETN2 (centrin 2). XPC indirectly detects DNA damage by binding to the undamaged strand opposite to the lesion and has increased affinity for unpaired DNA.[732] Numerous DNA lesions disrupt the DNA helix and result in unpaired regions that are detectable by XPC, which explains the broad range of lesions repaired by GG-NER.

While XPC can recognize UV-induced 6-4 pyrimidine-pyrimidone (6-4PP) lesions, it cannot recognize cyclobutene pyrimidine dimers (CPD), CPD is a poor substrate for XPC

since it does not destabilize the DNA helix sufficiently. This is an issue since CPDs are approximately twice as abundant as 6-4PPs. Therefore, recognition of CPDs occurs via the UV-DDB (ultra-violet radiation-DNA damage-binding protein) complex which directly binds to the lesions.[732] This complex is comprised of proteins DDB1 and DDB2 (XPE) as well as CUL4A (cullin 4A) and RBX (ring-box protein 1), where DDB2 contributes to the high affinity for UV-induced lesions and DDB1-CUL4A-RBX1 serves as an E3 ubiquitin ligase to remodel chromatin around the lesion while ubiquitination of XPC enhances its DNA binding activity.[729, 733-734]

Conversely, in TC-NER recognition occurs indirectly when transcription elongation by RNA Pol II is disrupted at a lesion. Stalling of transcription elongation is likely due to a broader range of lesions than those recognized by GG-NER such as oxidative DNA damage, which typically does not induce GG-NER and is repaired by BER. However, recent studies have brought to light the interplay between NER and BER pathways to work cooperatively in removing oxidative lesions.[730]

During TC-NER, the DNA-dependent ATPase CSB (gene *Ercc6*) transiently binds to RNA Pol II to monitor for regular pausing or transcription stalling.[735] If transcription stalling occurs, CSB remains bound to RNA Pol II and recruits CSA (*Ercc8*). CSA associates with DDB1, RBX1, and CUL4A forming the cullin 4-RING ubiquitin E3 ligase (CRL4) complex ($CRL4^{CSA}$) which becomes activated upon. Next, UVSSA (UV-stimulated scaffold protein A) and USP7 (ubiquitin-specific-processing protease 7), are recruited to the lesion and associate with CSA. CSB is ubiquitylated by $CRL4^{CSA}$ but USP7 de-ubiquitinylates to prevent CSB degradation. Next, TFIIH is recruited, USP7 dissociates and CSB is ubiquitinylated and degraded to enable RNA Pol II backtracking.[735] It is noteworthy that mutations in CSB that impact the ubiquitin-binding domain prolong binding to stalled RNA Pol II, ultimately hindering resumption of transcription. Therefore, the controlled removal of CSB is essential for TC-NER.

In post-mitotic cells such as neurons, TC-NER predominates over GG-NER.[736] DNA damage repair is primarily done on active genes rather than inactive genes in terminally differentiated cells, thus, GG-NER in these cells occurs on the non-transcribed strand of active genes.[735]

Following damage recognition in GG-NER and TC-NER, these pathways funnel into one, involving recruitment of the transcription factor II H (TFIIH) complex containing 10 subunits including a core (XPB, XPD, p62, p52, p44, p34 and p8) and the CAK complex

(Cdk7, cyclin H and MAT1). ATPase activity of XPB enables anchoring of the TFIIH complex to DNA, then recruitment of XPA releases the CAK (Cdk-Activating Kinase) complex from TFIIH. CAK usually suppresses the 5' to 3' helicase activity of XPD, but when CAK is released, XPD unwinds DNA and verifies the presence of a bulky DNA lesion (TFIIH scans the DNA for helicase blocking lesions). The Arch and Fe-S cluster domains of XPD form a channel that undamaged ssDNA can pass through but damaged ssDNA cannot.[732] XPA also aids in verifying the presence of DNA damage by binding to the single-stranded damaged nucleotides, while RPA (replication protein A) binds to the undamaged ssDNA, protecting it from endonucleases and directing the endonucleases to incise the damaged strand.[732] XPA then recruits the 5' endonuclease, ERCC1-XPF (*Ercc4*) which makes an incision on the damaged strand since RPA binding to the undamaged strand prevents any interaction with the undamaged strand. Next the 3' endonuclease XPG (*Ercc5*) excises the lesion as a 22-30 nucleotide-long strand.[729, 732] Once the damaged oligonucleotide is excised, PCNA (proliferating cellular nuclear antigen) recruits DNA polymerases (ε, δ, κ) to fill the gap in cooperation with RFC (replicating factor C), the process is completed with sealing of the nick by DNA ligase, either XRCC1-LIG3, FEN-LIG1 (flap endonuclease) complex or XRCC1-LIG3α in non-dividing cells.[729, 731, 734] Other DNA damage responses (DDRs) can be initiated by NER lesions when NER intermediates build up and NER is not able to keep up with all the DNA lesions.[732]

In Alzheimer's disease, deficits in NER proteins were observed, as example, Hermon *et al.* (1998) showed that in the brains of both AD and DS individuals, XPD (gene *Ercc2*) was elevated compared to control cases,[562] while another study showed increased mRNA levels of *Ercc2* in DS brains.[737] However, in a study of 97 AD and 101 non-AD age-matched controls, polymorphic variants of XPD did not increase risk for developing sporadic AD.[738] Indeed, these studies implicate *Ercc2* (XPD) at later timepoints, thus, our observation that this particular gene and protein are changed during pre-plaque stages is novel.

Interestingly, the gene *Ercc6* which encodes the protein CSB trended to increase in Tg hippocampal neurons. CSB has recently been found to play important role in base excision repair for repair of oxidative damage.[730] and was implicated in chromatin remodeling in response to oxidative stress.[739] Although the current work did not investigate epigenetic contributions to the pre-plaque pathology, our laboratory has previously shown that epigenetic modifications are present during pre-plaque stages in the McGill-R-Thy1-APP rat. The added evidence of DNA repair response in Aβ-burdened neurons demonstrated in Chapter 2 reinforces the significance of these findings and warrants future studies.[739]

VI.3.1 Base Excision Repair

In Chapter 2, the RT^2 qRT-PCR array did not specifically assay BER genes. However, in hippocampal homogenates, we observed an increase in *Fen1* expression which is implicated in BER. Although we did not see changes at the level of gene expression of *Ape1, Pnkp, Pnkp and Polβ* it is possible that if modest differences in neuronal expression of these genes existed, they may be diluted out by the other cell types in the hippocampal homogenates. Furthermore, as with APE1, this protein is highly dependent on phosphorylation and thus even though it is not changed at the level of the gene, it may be regulated in this manner, therefore it would be of interest to assess phosphorylation as well as oxidative modification of these proteins in future work. The next section will provide a brief overview of the BER pathway and implications in AD and in the context of this thesis.

BER has been extensively studied in the context of AD in humans (MCI, AD)[478, 579, 740] and in rodent models. Importantly, BER plays a large role in mtDNA repair and mitochondrial lysates from AD brains were observed to have decreased repair capacity for certain BER proteins.[741] Interestingly, Weissman *et al.* (2009) reported that although their group observed deficiencies in BER proteins in the MCI and AD brain, when they assessed BER protein activities in 3xTg male mice, they observed no differences at any timepoint assessed.[742]

As background, BER is essential for correcting smaller lesions that only have minor perturbations in the DNA helix, including oxidative damage. Numerous oxidized bases are recognized by only a handful of DNA glycosylases, and thus, each DNA glycosylase exhibits a broad and overlapping substrate range.[417] BER can be divided into short- and long-patch repair that vary based on the DNA polymerase used for gap filling and the ligases for ligation. In general, BER begins with recognition and excision by a lesion-specific DNA glycosylase (either mono- or bifunctional), which results in an apurinic/apyrimidinic (AP) site. The AP site can then be processed by bifunctional DNA glycosylases such as OGG1, MUTYH, NTH1, NEIL1-3 that have 3' AP lyase activity, allowing them to cleave this AP site while monofunctional DNA glycosylases rely on APE1 (apurinic/apyrimidinic endonuclease 1) to do so.[417] After this step, the SSB needs to be processed to obtain 3'OH and 5'P termini for subsequent DNA repair synthesis and ligation. To attain these 3'OH and 5'P termini there are multiple 3' and 5' processing enzymes part of different BER/SSBR pathways. In the case of monofunctional DNA glycosylases, APE1 (AP endonuclease 1) cleaves the DNA backbone, 5' to the abasic lesion resulting in a 5'dRP and 3'OH strand break product. These termini can be processed by Polβ to remove the 5'dRP, leaving a 3'OH and 5'P enabling gap

filling by Polβ then ligation. Conversely, after processing by bifunctional DNA glycosylases, the resulting 3' and 5' termini vary and are processed by APE1 or PNKP (polynucleotide kinase 3'-phosphatase). While other 3' processing enzymes for SSBR include TDP1 (tyrosyl-DNA phosphodiesterase 1) and APTX. Notably, mutations in these proteins TRP1 and APTX, lead to neurological disorders, SCAN1 (spinocerebellar ataxia with axonal neuropathy1) and AOA1 (ataxia-oculomotor apraxia 1), respectively, the impact on the nervous system may be due to reliance of neurons on TDP1 and APTX, elevated oxidative stress neurons are subjected to, high transcriptional demand, and/or the limited regenerative property of neurons.[743]

Long-patch BER (LP-BER) occurs when the 5'-terminal moiety is not a substrate for Polβ and other DNA polymerases (δ/ε) are recruited for gap filling. Since a number of replication-associated proteins are involved in LP-BER, these proteins are typically downregulated in non-dividing cells (such as neurons), thus, the SP-BER pathway is likely more prominent in non-dividing cells.[417, 731] SP- and LP-BER also both occur in the mitochondria.[744] However, one study reported that brain mitochondria (isolated from mice) had the lowest mitochondrial BER capacity,[745] while another study showed a decrease in mouse brain mitochondrial DNA glycosylase activities with age.[746]

In the broader context of AD, studies have shown that expression and activity of the DNA glycosylase OGG1 (8-oxoguanine glycosylase) was reduced in MCI and AD brains compared to controls[549, 747] while immunoreactivity for OGG1 was associated with NFTs, dystrophic neurites and reactive astrocytes.[748] Similarly, other DNA glycosylases were decreased in levels and activities in AD brains across various brain regions.[579]

In Chapter 2, a trend to decrease was observed in 8-oxo-dG immunoreactivity in Tg hippocampal neurons, which is primarily repaired by the BER pathway. Indeed, although the present work only investigated 8-oxo-dG—the most prominent oxidative insult—it is quite possible that other oxidative DNA adducts are also present during pre-plaque timepoints. Furthermore, as presented in Chapter 2, the slight trend to decrease in 8-oxo-dG in Tg hippocampal neurons while DNA repair genes and proteins increased may suggest that the at the pre-plaque timepoint investigated, the neurons were still able to manage the oxidative insults occurring in association with iAβ accumulation.

Studying these earliest pre-plaque timepoints is challenging in that modest changes in gene, protein and activity levels may indicate the tipping point of pathology acceleration, and offers

a snapshot of what is going on in the brain. This highlights the importance of the present work and the value of Chapters 3 and 4 since real-time live cell and tissue imaging offers further insight into the oxidative events occurring. To link modest but biologically significant changes in cellular DNA repair mechanisms and antioxidants with real-time ROS quantification and localization would be valuable. It is also worth noting that advances in studying DNA damage now allow for increased spatial resolution of oxidative insults.[419] Combinations of these cutting edge techniques would bring the field closer to a more comprehensive understanding of the earliest AD amyloid pathology and offer an exciting future ahead for the field.

VI.3.2 Non-homologous End Joining

In Chapter 2, an elevation in DSBs in subiculum Aβ-burdened neurons was observed. The presence of DSBs can indicate defects in upstream oxidative DNA damage repair but also deficits in repair of DSBs. To repair DSBs non-homologous end joining (NHEJ) or homologous recombination (HR) can be used. NHEJ plays a role in V(D)J (variable, diversity, joining) recombination in T and B cell maturation to generate multiple unique antigen receptors.[749-750] In the context of the CNS, since neurons are post-mitotic, they are more likely to utilize NHEJ for DSB repair since it can occur throughout all phases of the cell cycle, unlike HR which is restricted to S and G2 phases[731] even though NHEJ is known to be more error-prone[751] since it does not use the sister chromatid as a template as HR does. For reviews of HR as well as how HR or NHEJ are selected for DSB repair see.[752-754]

When DSBs occur, the first step in repair is to bring the broken ends back in proximity with one another since it is problematic if they diffuse away from each other this is done through binding of the Ku70-Ku80 heterodimer to the DSB, which then recruit other NHEJ proteins. End processing is then performed through the kinase activity of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), the nuclease activity of Artemis, nucleotide addition by polymerases (μ and λ), and modification of nucleotides by TDP1 and PNKP. Final ligation occurs with the XRCC4-DNA ligase 4 complex which is assisted by XLF.

NHEJ activity has been shown to decline with age, as assessed in cortical brain extract from rat,[755-757] while mice with defective NHEJ exhibited faster aging.[758] In the context of AD, *in vitro* data showed that $A\beta_{25-35}$ inhibited DNA-PK in PC12 cells, the authors predicted this was mediated by oxidative stress.[759] In humans, NHEJ activity, specifically that of DNA-PKcs, Ku, and XRCC4/DNA ligase 4 was decreased in cortical extracts from AD cases when compared to controls.[760]

As noted earlier in this general discussion, in addition to oxidative insults, DSBs can also form from transcription of certain early response genes in neurons,[599] as well as hyperexcitability which has also been linked to Aβ and oxidative stress.[446, 448, 728] It is possible that the increase in DSBs observed in Chapter 2 are a result of a combination of these factors.

Other Considerations

In Chapter 2, we also observed elevated levels of *Fancc* gene expression but not protein expression in Tg neurons. Fancc is implicated in DNA repair specifically for interstrand crosslinks (ICLs), where crosslinked bases are on opposite strands of the DNA duplex.[761] Mutations in Fancc among other Fanconi anemia (FA) genes leads to FA disease. Additionally, FA pathway genes including BRCA2 (FANCD1) and BRCA1 (FANCS) are implicated in breast and ovarian cancer. ICLs can be caused by exogenous sources such as chemotherapeutic agents but also endogenously by metabolites from alcohol, cigarette smoke, and dietary fat including acetaldehyde and MDA.[762] Unrepaired ICLs can result in DNA breakage and chromosomal rearrangements[761] and several proteins in the FA pathway are also involved in homologous recombination (HR). Aside from roles in DNA repair, FANCC modulates inflammatory responses in cells, including inflammation-associated ROS,[763] and in the context of AD, this topic is unexplored.[764-765]

Indeed, data assessing DNA damage and repair in the AD brain has largely been conducted at later time points of the AD pathology and thus could be consequential rather than causal. However, since aging is the primary risk factor for AD and in general, DNA repair decreases while oxidative stress increases with aging, this link reinforces the importance of investigating these pathways in neurons at earlier disease timepoints and in the context of sporadic AD.

The mechanisms by which Aβ directly induces mitochondrial DNA (mtDNA) damage are seemingly more clear than nDNA damage caused by Aβ, since the mitochondria are known sites of Aβ accumulation and ROS production. Indirectly, hyperexcitability induced by Aβ binding to NMDA receptors can lead to DNA damage mediated by calcium dyshomeostasis. Other indirect mechanisms of Aβ-mediated nDNA damage may stem from the multitude of oxidative stress-promoting pathways that Aβ is implicated in (Section 5.2) that can generate ROS or LDEs which subsequently react with nucleic acids and chromatin. However, more directly, there is evidence of Aβ translocation to the nucleus via pores[766] where it serves to regulate gene expression.[767-768] Nuclear transport of Aβ is a developing research field, with primarily *in vitro* evidence that remains to be explored. Our lab has also implicated epigenetic alterations in the early, pre-plaque pathology using the McGill-R-Thy1-APP rat model thus highlighting the changing chromatin landscape which is also susceptible to oxidative modifications.[487] Overall, early (pre-plaque) versus late (post-plaque) neuronal oxidative stress responses in AD are likely different as with the early and late neuroinflammatory responses. [94, 303]

VI.4 Cellular Antioxidants in Alzheimer's Disease

The earliest neuronal oxidative stress response, as outlined by the findings of Chapter 2, pointed towards a role for neuronal antioxidants during this timepoint. Therefore, in the subsequent section, key cellular antioxidant mechanisms will be outlined and placed in the context of AD and the present work.

VI.4.1 Non-enzymatic Cellular Antioxidants

In Chapter 2, we reported an elevation in the expression of *GR* (glutathione reductase) in Aβ-burdened neurons, as well as a trend to decrease in GR protein levels in CA1 neurons. Additionally, *Gclm* and *GPx7* trended to increase in expression. These genes are related to glutathione regeneration, synthesis, and activity in the cell. Glutathione brain content has been studied in the context of AD since it serves to protect the brain from oxidative insult. Specifically, GPx7 is implicated in oxidative protein folding in the ER, [593] tying it back to one mechanism by which Aβ can induce oxidative stress while, GCLM is an essential subunit needed for glutathione synthesis. [595, 769]

Glutathione (GSH) is the most abundant non-protein thiol in cells, with a high intracellular concentration of ~1-10 mM,[337, 450] (although only 1-3 mM in the brain)[318] and serves as an antioxidant, among other functions (e.g.: xenobiotic and eicosanoid metabolism, and gene expression).[45] GSH is comprised of glutamine, cysteine, and glycine, and is synthesized in two consecutive steps by the enzymes glutamate-cysteine ligase (GCL) which is the rate-limiting step, GCL is also allosterically inhibited by GSH, serving as a negative feedback loop. The second reaction in GSH synthesis is catalyzed by glutathione synthase (GS).[770] Importantly, cysteine levels are limited as they depend on cellular uptake of extracellular cystine by a cystine/glutamate antiporter x_c^-.[771] In the CNS, astrocytes supply GSH substrates to neurons by exporting glutathione to the extracellular space where the glutamyl group is enzymatically transferred onto an amino acid acceptor, leaving cysteinylglycine that is subsequently broken down into cysteine and glycine to then be taken up by surrounding neurons.[318, 772] GSH protects the cell from excessive ROS by reacts directly with radicals in an non-enzymatic manner,[773] but also serves as an electron donor for the reduction of peroxides as catalyzed by glutathione peroxidases (GPxs)[774] of which there are many isozymes, (some of which contain selenocysteine and thus, rely on dietary selenium intake, namely, GPx 1-4 and 6). For a review of GPx enzymes see Brigelius-Flohé and Maiorino (2013).[775] Notably, although catalases can also remove hydrogen peroxide, the levels in the brain are not as high as other tissues, thus GPxs likely play a more prominent role.[776] The product of GSH oxidation is glutathione

disulfide (GSSG) that is then converted back to GSH by glutathione reductase (GR) which transfers electrons from NADPH to GSSG, the ratio of reduced to oxidized GSH:GSSG is often used to assess the redox state of the cell or tissue.

With aging, GSH decreases in certain tissues, including the brain.[776] In the context of AD,[777] *in vitro* evidence using primary neurons from the 3xTg mouse model showed a decrease in GSH levels[778] that when compensated for, increased Tg neuron survival.[779] Similarly, in the 3xTg APP mouse model of AD at 3-5 months of age (preceding plaques) cortical levels of GSH levels decreased, while GPx activity and 4HNE increased.[378] In individuals with MCI and DS decreased plasma levels of GSH were observed,[590, 780] as well as decreased GSH:GSSG, glutathione-S-transferase (GST), increased GR expression but no change in activity in the brains of individuals with MCI.[781] GSH was also decreased in the AD brain, [782-784] and also decreased along with decreases in GCL and GS in blood from male AD patients but not female AD patients.[785] *In vitro* data[786] from astrocyte and microglia cultures suggest that decreased glial GSH levels may also contribute to neuroinflammatory processes.[787]

In addition to the other roles of glutathione (GSH) discussed above, it is also essential for detoxification of LDEs. The glutathione-S-transferase (GST) family of enzymes[596] catalyze conjugation of GSH to electrophilic substrates, which consumes cellular GSH[788-789] unlike the recycling of GSH when it reacts with radicals or through GPx-catalyzed reactions.[318] The conjugation product of GSH and 4HNE can additionally serve as a biomarker of lipid peroxidation.[790] Indeed, the levels and activity of GSTs were observed to be decreased in the brain and ventricular fluid of AD brains.[468]

Enzymatic Cellular Antioxidants

In terms of cellular enzymatic antioxidants in the cell,[452] these include: SODs (superoxide dismutases), catalase,[453-455] peroxiredoxins,[456-458] thioredoxins,[459-463] and heme oxygenase.[240, 464-467] Although not all with be discussed here, relevant literature has been referenced for these other key enzymatic antioxidants. In Chapter 2, we found elevated gene expression of *Sod2* in Tg neurons while protein levels remained unchanged. Indeed, a limitation of our assessment was that we did not assess synapse-specific levels nor enzymatic activity.

SOD enzymes were some of the first antioxidants characterized,[791] and are known to catalyze the dismutation of two superoxide radicals to hydrogen peroxide and molecular oxygen. There are three isoforms of SODs where SOD1 (copper-zinc or CuZnSOD) localizes to the cytoplasm and intermembrane space of the mitochondria, SOD2 (manganese or MnSOD) localizes to the mitochondria matrix and SOD3 which is extracellular. As mitochondrial dysfunction has been observed in AD, especially in synaptic over somatic mitochondria,[298, 493, 495] SOD2 is a target of interest. Furthermore, since Aβ is known to insert into mitochondrial membranes,[297, 532, 690-691, 792-793] it is possible that SOD2 plays a role in protecting neurons from Aβ-induced oxidative stress. There are multiple publications studying the effects of SOD2 in transgenic mouse models of AD, showing that overexpression of SOD2 prevents cognitive decline,[794] while a decrease in SOD2 (using $SOD2^{+/-}$ mice crossed with *APP* mice) accelerated onset of behavioural changes.[706] One study using brain tissue from AD patients found an increase in expression of antioxidant enzymes, including SOD2 but decreased activity, this highlights the importance of assessing not only gene or protein expression but also activity levels of enzymes.[795] Furthermore, as this study focused on later stages of AD when plaques are present, it could be possible that these enzymes were oxidatively modified and thus dysfunctional. When assessing the literature in relation to AD, it must be kept in mind that data acquired during later disease stages is more difficult to discern as being a cause or consequence of disease processes.

VI.4.2 Signaling Pathways

A number of the aforementioned antioxidant genes are regulated by transcription factors related to oxidative stress and inflammation such as Nrf2 and NF-κB which have been studied in the context of AD.[796-797]

Briefly, nuclear factor E2-related factor (Nrf2) is a transcription factor that mediates expression of antioxidant and detoxification enzymes through activating the antioxidant

response element (ARE). Under basal conditions, Nrf2 is sequestered in the cytoplasm through binding to Keap1 (Kelch-like ECH-associated protein 1) which facilitates Nrf2 degradation through the ubiquitin-proteasome system.[798] Keap1 binding to Nrf2 is via a cysteine-rich, and thus redox sensitive, region and when Keap1 undergoes oxidative modification by a reactive electrophile[799] or ROS, it releases Nrf2 allowing it to translocate to the nucleus where it serves as a transcription factor.[800] Of relevance to this work, Nrf2 regulates transcription of multiple antioxidant genes including two subunits of GCL, SODs 1-3, some members of the Prdx, GPx and GST enzyme families, as well as thioredoxin reductase, sulfiredoxin, and glucose-6-phosphate dehydrogenase.[801] At post-plaque stages in our transgenic rat model of the AD-like amyloid pathology there was upregulation of NF-κB in hippocampal homogenates. While Nrf2 was increased at 3 months (pre-plaque) then decreased at 12 months (post-plaque).[303]

In the context of this thesis, interestingly, there is increasing evidence that 8-oxo-dG lesions may also serve a purpose in facilitating oxidative stress gene activation including NF-κB among others.[419] Furthermore, NF- κB activation can lead to elevated expression of RAGE as well as increased expression of inflammatory cytokines which contribute negatively to the early, pre-plaque AD pathology (reviewed by Cuello 2017).[94] thus exacerbating effects of Aβ binding to RAGE. Indeed, the interconnected nature of oxidative stress to cellular transcription factors complicates the overall picture, and reinforces the importance of studying ROS in real-time and in a cell specific manner.

Potential Therapeutic Avenues for Alzheimer's Disease

In light of the aforementioned mechanisms by which amyloid can exert toxicity and lead to oxidative stress, one wonders about the potential of antioxidant therapies for preventing or delaying AD. This section will outline the landscape of AD clinical trials in general followed by a concise summary of the status of antioxidant trials for AD.

VI.4.3 Clinical Trials for Alzheimer's Disease

Generally, hundreds of clinical trials for AD have failed in the last decades, with a 99.6% failure rate from 2002-2012 and little improvement since then.[225, 802] As outlined in Cummings (2018)[802] numerous factors have contributed to these failures including:

- lack of reliable preclinical AD models (e.g.: numerous models rely on overexpression of mutated genes),
- failure of drugs reaching the brain at effective levels,
- small sample sizes,
- lack of target engagement (e.g.: if soluble Aβ is the primary target, endpoints assessing changes in levels of soluble Aβ species must be reliable and consistent across different studies),
- lack of biomarker data collected during trials (more biomarkers allow for better disease staging and broader assessment of outcomes; PET scans only detect larger Aβ plaques while CSF and blood biomarkers provide insight into soluble Aβ),
- variability in trial participants (e.g.: trials including mild to moderate AD patients have a great variability in age of disease onset as well as rate of disease progression which can mask potentially clinically relevant improvements, furthermore, biomarker changes in a heterogeneous patient population complicates the results further), and
- biases associated with post-hoc analyses (e.g.: the patient groups selected for post-hoc analyses were not randomly selected but selected based on a certain criteria).[802]

The AD research community has shifted focus from post-plaque stages when cognitive symptoms are present, pre-symptomatic stages of AD when soluble Aβ accumulates. These stages span decades and offer the most promising therapeutic window. However, as they are pre-symptomatic, this makes it challenging to know which individuals would eventually develop AD unless they are genetically predisposed. Current trials such as the Dominantly Inherited Alzheimer's Network trials unit (DIAN-TU)[803] therefore use a more homogenous

patient population who are predisposed to familial AD (fAD) driven by *APP* or *PSEN* mutations. In these trials, interventions were initiated at ages corresponding to pre-symptomatic and symptomatic stages. It is worth noting that as our understanding of pre-symptomatic pathological mechanisms in AD (especially sLOAD) improves, and our biomarkers become more reliable, clinical trial recruitment and endpoints will better assess disease modifying therapies.

Regarding antioxidant trials for AD, overall, they have been unsuccessful. One of the major antioxidants tested has been the dietary, lipophilic antioxidant vitamin E,[804] which was the inspiration for H4BPMHC.[480, 623, 676] As noted in the Introduction, vitamin E comprises both tocopherols and tocotrienols, whereby RRR-α-tocopherol is the most common form found in the brain and most efficient at trapping lipid peroxyl radicals.[311] The other forms of vitamin E (β, γ and ξ either tocopherol or tocotrienol) are less studied in vivo but γ-tocopherol has been shown to be more effective at scavenging RNS. [805] In addition to variations in the type of stressors trapped by these antioxidants, effective vitamin E uptake and transport to the brain must also be considered since there are multiple mechanisms for its transport including the protein α-tocopherol binding transport protein (αTTP) that has affinity only for the RRR-α-tocopherol.[806] Notably, the brain does have homeostatic mechanisms to maintain vitamin E levels.[807]

Aside from generalized issues with AD trials as listed above, those testing vitamin E had additional weaknesses including:[808] 1) incorrect dose, whereby at high levels vitamin E can exert pro-oxidant effects through generation of α-tocopherol radicals, and support the supplementation of additional types of antioxidants (such as aqueous vitamin C (ascorbic acid) or GSH), in order to reduce α-tocopherol radicals,[809] 2) wrong timing, as with other AD trials, administration was after cognitive symptoms appeared, 3) monotherapy, as mentioned since vitamin E can be pro-oxidant, it may be wise to supplement with other antioxidants, although trials that have done this for other diseases without finding efficacy and 4) wrong target, without a complete understanding of the key players maintaining redox balance and managing oxidative stress at early disease stages, as well as potentially synergistic combinational therapies (as example, anti-inflammatories or anti-Aβ therapies) the use of antioxidants or redox modifying molecules may not be successful.

VI.5 Other Considerations

VI.5.1 Tau and Oxidative Stress

Although the amyloid pathology has been the highlight of this thesis, links to tau and oxidative stress should also be acknowledged, even if briefly, since oxidative stress is an early marker of tauopathies.[810] As example, the phosphorylation states of tau have been shown to become altered due to mitochondrial ROS[811] which can activate certain kinases such as GSK-3β[812] while 4HNE modification to tau can promote aggregation.[415-416, 813-814] As well, of relevance to our findings related to DNA damage and repair, the role of nuclear tau in protecting DNA integrity in response to stress warrants further study.[815-816] As the interplay between the amyloid and tau pathologies in AD becomes more clear, it will also be of importance to understand the role of oxidative stress in this context.

VI.6 Concluding Remarks

Based on the present set of studies, the work presented highlights the benefit of interdisciplinary studies spanning the biology and chemistry of disease. Furthermore, this work offers valuable insight into the earliest oxidative stress response during the pre-plaque amyloid pathology of AD while highlighting the importance of fluorescence real-time imaging and quantification of ROS towards this goal.

In this thesis, we investigated the early, pre-plaque amyloid pathology of Alzheimer's disease using a transgenic rat model (McGill-R-Thy1-APP). Building on our previous investigations on amyloid-driven inflammation, this thesis assessed contributions of oxidative stress-related pathways at the same pre-plaque timepoint. We found that DNA repair and antioxidant-related gene expression were altered in hippocampal neurons at this timepoint when inflammation was incipient, and oxidative damage was emerging. We also presented two methodologies for studying upstream lipid peroxyl radicals in real-time in primary neuronal cultures and *ex vivo* hippocampal slices that can leverage the spatial and temporal resolution of fluorescence microscopy and will prove useful for elucidating the early AD pathology.

Regarding future work, this thesis lays a path forward for numerous influential studies some of which are outlined below:

- As a great deal of the literature related to oxidative stress in AD focused on post-plaque disease stages which have limited therapeutic potential. This work offers opportunity to further explore the pre-plaque stages which offer the most promising therapeutic window for biomarker detection and disease-modifying therapies.

- A comprehensive analysis of gene expression alterations in Aβ-burdened neurons would be of great interest. Current methodologies involving cell sorting combined with fluorescence quantification of specific ROS and subsequent RNAseq could offer answers towards the interplay between oxidative stress, DNA repair, inflammation, and other key mechanisms of the early AD pathology. Indeed, some groups have leveraged this workflow, albeit, using DCFDA.[817] If applied using H_4BPMHC the results would prove interesting to assess lipid peroxyl radical loads and how they relate to gene expression changes in health and disease.

- Notably, in addition to ROS-detecting probes, the Cosa laboratory recently reported a fluorogenic probe modelled after reactive electrophiles.[818] This experimental tool in combination with those quantifying specific forms of ROS would also offer insights into the dynamics of the earliest oxidative stress.

- Additionally, fluorogenic probes targeting different cellular compartments would also be of great interest to explore.[676]

- Here, we have primarily focused on neuron-associated oxidative stress responses. However, it must be acknowledged that all cells in the brain have the capacity to generate and ameliorate ROS and future studies investigating differential oxidative stress responses in varying cell types would be of great interest considering the complexity of redox signaling in health and disease. Leveraging transgenic rodent models expressing cell-specific fluorogenic markers would be an asset for future studies assessing differential effects of oxidative stress on various cells in the brain.

- Lastly, the contributions of reactive nitrogen species (RNS) have not been covered but play an equally important role in the CNS and in health and disease.

In sum, moving forward, the future of AD research must place focus and energy towards the earliest pathological stages whether amyloid, tau, oxidative stress, inflammation etc, moving away from the late, post-plaque, symptomatic stages in which it is nearly impossible to tease apart causal versus consequential factors. At such late timepoints, not only is there irreversible damage to the brain, but multiple comorbidities complicate further the advanced pathological scenario. Additionally, although not taken into consideration in this thesis, the study of sex differences in AD is essential, as is the study of sporadic AD. Although sporadic AD is more difficult to study, it accounts for the majority of AD cases. As a prediction, the future of therapies to prevent, delay or treat AD likely will be heavily influenced by individualized medicine and combination therapy that account for the heterogeneity and complexity of this neurodegenerative disorder.

"Any living cell carries with it the experiences of a billion years of experimentation by its ancestors. You cannot expect to explain so wise an old bird in a few simple words."

- Max Delbrück

VII. References

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