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Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brain of Diet-Induced Obese Mice

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Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brain of Diet-Induced Obese Mice

by

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Thesis

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Dedication

This work is dedicated to my family: to my parents, Vincenzo Saieva and Giovanna Stassi, and to my brother Vito. Thank you for your endless encouragement and unconditional love, even when thousands of miles kept us apart for a long time.

This work is also dedicated to all the people who are struggling in their PhD journey, have fallen in depression, feel lost and useless. Here is an example of someone who made it, even facing depression, anxiety, lack of motivation, disbelief in himself.

YOU CAN MAKE IT!

And a special dedication to the city in which I grew up, Piana degli Albanesi (Palermo), with the words of a poet from this city, Giuseppe Schirò, written in the Arbëresh language I speak in addition to the Italian.

"*Po të mbahij Arbëreshë e të ruani gluhën tënë me kujdes e me të dashur si një gjë të shejtëruamë, si më t'mirën nga të dhënat e t'yn Zoti, e ashtu edhe veset çë na lanë ata të parët, gojëdhënat edhe ndienjat*" *(*from *Te dheu I huaj*, 1891)

("But always Arbëresh preserve yourself and preserve our language with care and love, like a holy thing, like the best of God's gifts, and so also the customs that our ancestors left us, the traditions and the sentiments") (from "In foreign land", 1891)

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Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brain of Diet-Induced Obese Mice

Publication No._____

Salvatore Saieva, MS

The University of Texas Medical Branch, 2021

Supervisor: Giulio Taglialatela

Obesity is one of the most prominent risk factors for Alzheimer's Disease (AD); obesity and AD share several pathological features, in particular the neuroinflammation. Hippocampal neuroinflammation, in rodents, correlates with poor memory performance, while in humans, growing evidence shows that obesity increases three times the risk of developing AD. The overall objective of this work is to reduce the impact of obesity on neuroinflammation, that in turn may lead to dementia. In last years, near-infrared (NIR) light has been proposed as potential treatment, showing improvement of learning and memory in both humans and animal models. Previous work demonstrated that a transcranial delivery of NIR light reduced Aβ and Tau pathology and improved memory function in mouse models of AD. Here, it was tested whether NIR light may prevent obesity-induced neuroinflammation, in a diet-induced obese mouse model of obesity. After 13 weeks of high-fat diet (HFD), the mice received the NIR light treatments for 4 weeks in single daily sessions of 90 seconds each. The immunostaining investigations performed on brain slices to evaluate glial activation revealed that in both hippocampus and parietal/occipital cortex, HFD caused increased expression of CD68 (activated microglia) and GFAP (astrocytic marker), whereas NIR light reverses this increase. On the other hand,

the same investigations displayed no change in frontal cortex. Furthermore, I evaluated the effects of light on cytokines in hippocampus and frontal cortex, by using quantitative real-time PCR analyses: in the hippocampus, HFD caused the increase of pro-inflammatory IL-1β and TNF-α, as well as of the anti-inflammatory IL-10, while NIR light lowers their levels. Also, BDNF resulted upregulated in HFD mice treated with NIR light, compared to HFD not-treated mice, thus suggesting that the NIR light triggers neuroprotective effects resulting in reduced neuroinflammation. Interestingly, the levels of cytokines and BDNF were unchanged in the frontal cortex. Collectively, this data suggests that neuroinflammation is reduced by NIR light and it is a reversible process that may be targeted to prevent neurodegeneration. Moreover, NIR light poses as a potential preventive and non-invasive therapeutic approach against obesity-induced CNS deficits that are known to concur to AD neuropathological cascade.

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α-MSH	alpha-melanocyte stimulating hormone
AD	Alzheimer's Disease
AgRP	Agouti-Related Protein
AMPK	Adenosine Monophosphate Kinase
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
ApoE	Apolipoprotein E
APP	Amyloid (β) Precursor Protein
ARC	Arcuate Nucleus
ASC	Apoptosis-associated Speck-like containing a CARD domain
ATP	Adenosine triphosphate
AUC	Area Under the Curve
Aβ	Amyloid β
BBB	Blood-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
CCL	Chemokine (C-motif) ligand
CCO	Cytochrome C Oxidase
CD	Cluster of Differentiation
CGRP	Calcitonin-Gene Related Protein
cm^2	square centimeters
CN	Calcineurin
CNS	Central Nervous System
COX-2	Cyxlooxygenase-2
CR1	Complement Receptor 1

CREB	cAMP (cyclic Adenosine MonoPhosphate) response element - binding
Cu	Copper
CX3CL	Chemokine (C-X3-C motif) Ligand
CX3CR	Chemokine (C-X3-C motif) Receptor
DAM	Disease Associated Microglia
DAMP	Damage-Associated Molecular Patterns
DAP12	DNAX Activation Protein of 12 kDa
DAPI	4′,6-diamidino-2-phenylindole
DG	Dentate Gyrus
DIO	Diet-Induced Obese
DNA	Deoxyribonucleic Acid
EOAD	Early Onset AD
ERK	Extracellular signal-Related Kinase
fAD	familial (form of) AD
FAD(H_2)	Flavin Adenosine Dinucleotide (reduced)
FCTX	Frontal Cortex
FINGER	Finnish Geriatric (Inervention)
FOXO	Forkhead box protein O
GaAlAs	Gallium Aluminum Arsenide
GFAP	Glial Fibrillar Acidic Protein
GI (tract)	Gastrointestinal (tract)
GLP-1	Glucagon-Like Peptide-1
GSK3β	Glycogen Synthase Kinase 3 beta
He-Ne	Helium-Neon
HFD	High-Fat Diet
HIF-1	Hypoxia Inducible Factor-1
Iba-1	Ionized calcium-binding adapter-1
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-10	Interleukin-10

IL1R1	Interleukin-1 Receptor-1
IL-1β	Interleukin-1beta
iNOS	inducible Nitric Oxide Synthase
IPGTT	IntraPeritoneal Glucose Tolerance Test
IWL	Interfacial Water Layer
IκB	Inhibitor of κB
J	Joule
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
LANDO	LC-3 Associated Endocytosis
LED	Light Emitting Diode
LLLT	Low-Level Laser Therapy
LOAD	Late Onset AD
LPS	Lipopolysaccharide
LRP-1	LDL (low density lipoprotein)-related protein 1
LTP	Long-Term Potentiation
MAPK	Mitogen-Activated Protein Kinase
MCI	Mild Cognitive Impairment
MCP	Monocyte Chemoattractant Protein-1
MGnD	Microglial neurodegenerative (phenotype)
mRNA	messenger Ribonucleic Acid
MS	Multiple Sclerosis
mTOR	mammalian Target of Rapamycin
NAD(H)	Nicotinamide Dinucleotide (reduced)
NADP(H)	Nicotinamide Dinucleotide Phosphate (reduced)
NFTs	Neurofibrillary Tangles
NF-κB	Nuclear Factor kappa-light chain-enhancer of activated B cells
NGF	Nerve Growth Factor
NIH	National Institute of Health
NIR (light)	Near Infra-Red (light)

NLRP3	NLR [NOD (Nucleotide-binding Oligomerization Domain-like) Like Receptor] family Pyrin domain containing 3
nm	nanometers
NMDA	N-methyl-D-aspartate
NO	Nitric Oxide
NRF-2	Nuclear Factor Erythroid-2 (NFE-2)-related factor-2
NSAIDs	Non Steroid Anti-Inflammatory Drugs
P2RY12	Purinergic Receptor P2RY
p75NTR	p75 Neurotrophin Receptor
PAMP	Pathogen-Associated Molecular Patterns
PBM	Photobiomodulation
PBN	Parabrachial Nucleus
PBS	Phosphate Buffer Solution
PD	Parkinson's Disease
PI3K	PhosphoInositide 3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
POCTX	Parietal/Occipital Cortex
POMC	Pro-Opiomelanocortin
PPAR	Peroxisome Proliferator-Activated Receptor
PSEN	Presenilin
PUFA	Poly-Unsaturated Fatty Acids
q-RT-PCR	quantitative Real Time Polymerase Chain Reaction
RAGE	Receptor for Advanced Glycation End-products
RC	Regular Chow
Ref-1	Redox Protein
ROS	Reactive Oxygen Species
SAPK	Stress-Activated Protein Kinase
SCI	Spinal Cord Injury
SD	Standard Deviation
SDF-1	Stromal cell-Derived Factor-1

SNc	Substantia Nigra pars compacta
SOCS	Suppressor of Cytokine Signaling
SOD	Superoxide Dismutase
STAT	Signal Transducer and Activator of Transcription
T2DM	Type 2 Diabetes Mellitus
TBI	Traumatic Brain Injury
TBS	Tris Buffer Solution
TH	Tyrosine Hydroxilase
TLR	Toll-Like Receptor
Tmem119	Transmembrane protein 119
TNFR	Tumor Necrosis Factor Receptor
TNF-α	Tumor Necrosis Factor-alpha
TREM2	Triggering Receptor Expressed on Myeloid cells 2
TrkB	Tropomyosin receptor kinase B
TRP	Transient Receptor Potential
TYROBP	Tyrosine Kinase-binding Protein
W	Watts
WHO	World Health Organization

Chapter 1. Literature Review

ALZHEIMER'S DISEASE

Introduction

Alzheimer's Disease (AD) is the most common form of dementia, characterized by progressive loss of memory and cognitive decline (Scheltens *et al.*, 2016; Lane, Hardy and Schott, 2018). In general, two forms of AD have been described so far: late-onset AD (LOAD), that comprises the vast majority of cases and occurs past 65 years of age, and the early-onset AD (EOAD), that includes around 5% of AD cases and appears earlier than 65 years of age (Long and Holtzman, 2019). The EOAD encompasses rare forms of familial AD (fAD) due to genetic mutations on one of three specific genes, amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) (Lane et al., 2018). From a neuropathological point of view, two major hallmarks characterize AD: the extracellular deposition of amyloid-β (Aβ), with consequent formation of neuritic plaques, and the aggregation of intraneuronal neurofibrillary tangles (NFTs), composed of hyperphosphorylated Tau (Figure 1.1) (Selkoe, 2011; Long and Holtzman, 2019; Alzheimer's Association, 2020). The deposition of such aggregates occurs throughout several years before the manifestation of any clinical symptoms (Figure 1.3) (Huang and Mucke, 2012). In fact, the formation of these aggregates is preceded by the formation of soluble oligomers, that are considered the most toxic species driving the preclinical phase of the disease (Dineley *et al.*, 2010; Lasagna-Reeves *et al.*, 2012; Cline *et al.*, 2018; Soria Lopez, González and Léger, 2019).

Based on the progressive deposition of the amyloids that is accompanied by the gradual development of the symptoms, three phases of the disease have been identified: preclinical AD, mild cognitive impairment (MCI) due to AD, and dementia due to AD, with this last phase further divided in mild, moderate and severe (Figure 1.2) (Alzheimer's Association, 2021). The preclinical phase, the silent and asymptomatic phase, may last for over 20 years

before the appearance of the first symptoms and is characterized by the growth and development of the typical hallmarks of AD (Crous-Bou *et al.*, 2017), when neuritic plaques start forming and accumulating (Soria Lopez, González and Léger, 2019). MCI phase has gained growing interest because in this stage the individuals can still perform their daily activities independently, hence any therapeutic strategy could prevent any further development to full-blown dementia (Alzheimer's Association, 2021).

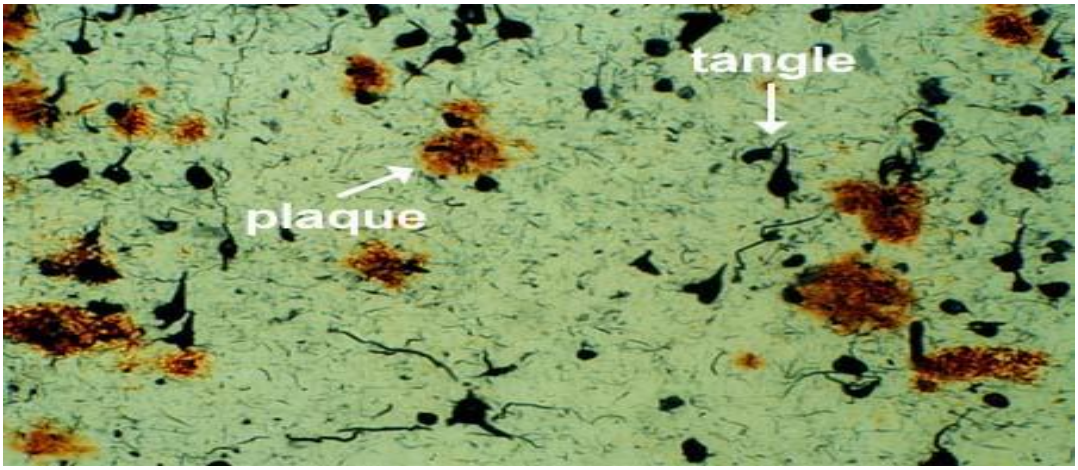

Figure 1.1: Deposits of amyloid plaques and neurofibrillary tangles
Amyloid-β plaques and hyperphosphorylated Tau are the two major hallmarks of AD (available at http://ladu.lab.uic.edu).

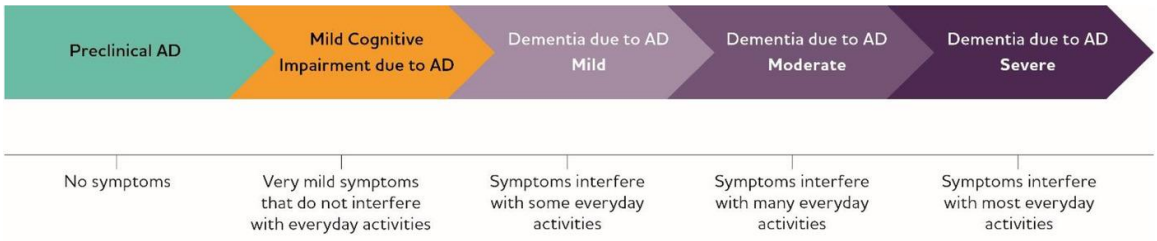

Figure 1.2: Phases of AD
It is possible to distinguish different phases in the development of AD, depending upon severity of symptoms (adapted with permission from 2021 Alzheimer's disease facts and figures, Alzheimer's & Dementia, 2021).

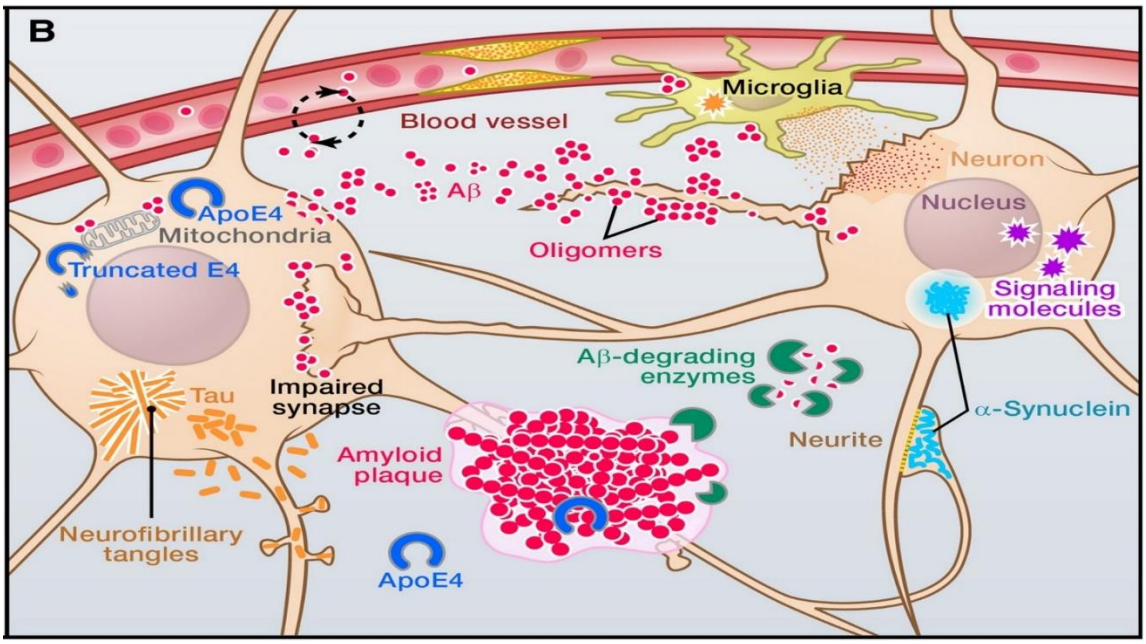

Figure 1.3: Representation of the events occurring in AD over time.
Several events occur long time before the deposition of plaques and tangles, including synapse loss, neuronal death and neuroinflammation.
Reproduced with permission from (Huang and Mucke, 2012).

However, several risk factors may accelerate the onset and exacerbate the progression of AD (Barnes and Yaffe, 2011; A. Armstrong, 2019; Alzheimer's Association, 2020, 2021), thus suggesting that multiple pathological mechanisms are involved in the AD development (Figure 1.3) (Musiek and Holtzman, 2015). AD comprises a plethora of events such as mitochondrial dysfunction, synapse failure and loss, oxidative stress and neuroinflammation that substantially contribute to the onset and progression of the disease (Querfurth & LaFerla, 2010). Moreover, while neuronal and synapse loss accompany tangle deposition and may be even considered a marker of AD severity (Querfurth and LaFerla, 2010), Aβ aggregates characterize pre-symptomatic phase (Lane, Hardy and Schott, 2018). Compelling evidence suggests that mitochondrial dysfunction is a major condition in neurodegenerative diseases (Querfurth and LaFerla, 2010): in aging, mitochondria dynamics that help maintaining structure function of mitochondria through fusion and fission are impaired, thus leading to less generation of energy and neurodegeneration. Moreover, mitochondrial turnover is impaired in aging and defects in

mitochondrial biogenesis cause accumulation of non-functional mitochondria that are a major source of reactive oxygen species (ROS). The consequent increase of oxidative stress and inflammation, as well as damage to lipids, proteins, DNA ultimately impairs ATP production and whole cell metabolism. Also, the antioxidant activity is impaired by the loss of functional mitochondria, where ROS are usually generated from the activity of coenzymes such as $FADH_2$, NADH and by the electron transport chain, and are immediately scavenged by antioxidant enzymes, like superoxide dismutase (SOD) (Bhatti *et al.*, 2020).

AD hypotheses

Several hypotheses have been proposed to explain the progression of AD pathology (Liu *et al.*, 2019). The most accepted theory is the amyloid hypothesis (Figure 1.4) (Selkoe, 2011), which suggests a central role for Aβ accumulation in driving the disease (Lane, Hardy and Schott, 2018), due to an imbalance between Aβ production and clearance (Querfurth and LaFerla, 2010), in light of the discovery that familial AD is characterized by mutations on genes that induce overproduction of Aβ (Soria Lopez, González and Léger, 2019). The formation of NFTs and neurodegeneration are considered downstream events that may involve other mechanisms, such as neuroinflammation (Lane, Hardy and Schott, 2018).

Nonetheless, revisions of the amyloid hypothesis have been proposed (Musiek and Holtzman, 2015; Selkoe and Hardy, 2016; Cline *et al.*, 2018). While evidence strongly supports the importance of Aβ aggregation in the AD pathogenesis, it seems that Aβ is necessary but not sufficient for the progression of the pathology, considering the minimal correlation between amyloid deposition and the extent of the cognitive decline (Musiek and Holtzman, 2015; Long and Holtzman, 2019). Moreover, the poor anatomical and temporal correlation between Aβ deposition, tangle formation, neuronal loss and clinical manifestations has contributed to cast doubts over the amyloid hypothesis (Musiek and

Holtzman, 2015). In fact, although it is unclear how Aβ and Tau pathology are correlated, it has been demonstrated that Tau aggregates remain confined in the limbic region in people who do not develop cognitive decline and do not show amyloid plaques, while Tau spreads in cortical regions in presence of Aβ pathology, with consequent dementia development. Likewise, the Tau-mediated neuronal degeneration occurs in the hippocampus only when Aβ plaques have established, thus suggesting the need of an Aβ trigger to have Tau-induced neurotoxicity, despite the absence of anatomical colocalization (Musiek and Holtzman, 2015).

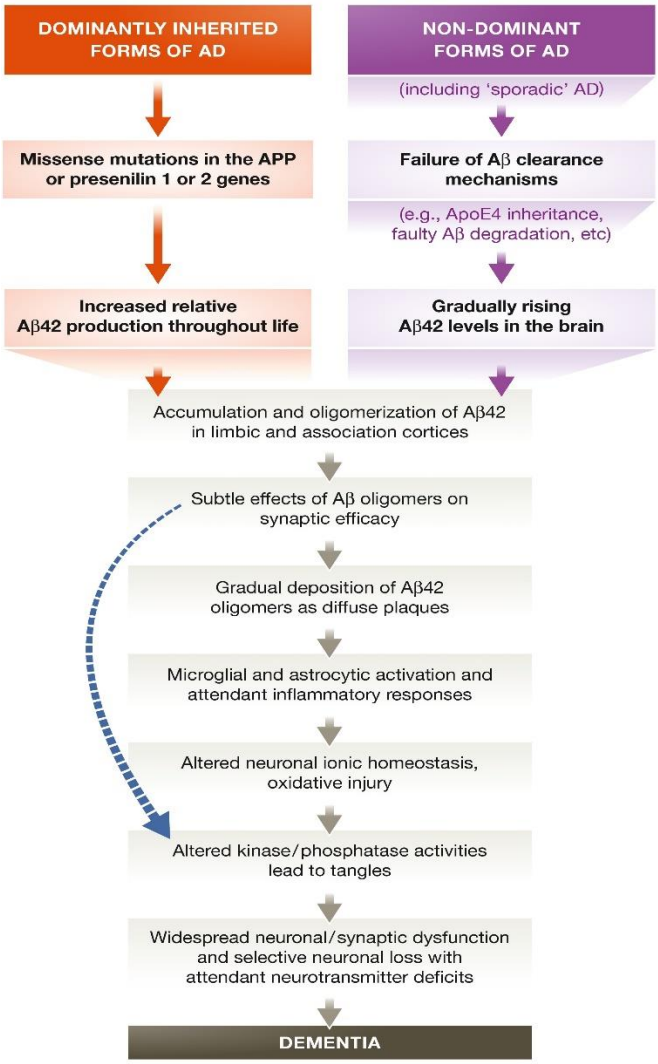

Figure 1.4: Sequence of major events occurring in AD according to the amyloid hypothesis Reproduced with permission from (Selkoe and Hardy, 2016).

To explain the link between Aβ deposition and the formation of NFTs four additional mechanisms have been proposed: 1) Aβ promotes activation of specific kinases, such as glycogen synthase kinase-3 beta (GSK3β), that hyperphosphorylates Tau leading to its aggregation; 2) Aβ aggregates trigger neuroinflammation, which in turn causes increased production of proinflammatory cytokines that stimulate Tau hyperphosphorylation; 3) Aβ accumulation impairs proteasomal activity, thus slowing Tau protein degradation and inducing its excessive phosphorylation; 4) Aβ causes defects in axonal transport that lead to mislocalization of Tau protein with consequent activation of its hyperphosphorylation (Silva *et al.*, 2019).

However, although compelling evidence suggests a crucial role of Aβ in initiating the pathology, the mechanism by which Aβ deposition promotes Tau aggregation is still not clear. A partial explanation of the mechanistic link between Aβ plaques and Tau aggregation comes from the observation whereby the toxic Aβ species driving the development of AD are the soluble Aβ oligomers (Selkoe, 2011), known to inhibit long-term potentiation (LTP), cause synaptic dysfunction and ultimately neuronal death (Lane, Hardy and Schott, 2018). Moreover, Aβ oligomers do not necessarily colocalize with Aβ plaques, correlate with cognitive decline more closely than plaques, and are correlated to the appearance of Tau pathology. However, the recognition of the important role of Aβ oligomers in promoting the disease is still not sufficient to explain the long prodromal, asymptomatic, preclinical phase of AD, where plaques can be detected despite no sign of neurodegeneration (Musiek and Holtzman, 2015). The detection of Aβ oligomers has led to the idea that they are the initiators of the disease, bringing to the proposal of the amyloid-β oligomer hypothesis (Cline *et al.*, 2018). Aβ oligomers precede the formation of plaques, but carry a toxic role that massively contributes to the progression of the pathology resulting in behavioral impairments and neuropathological outcomes (Cline *et al.*, 2018). Moreover, Aβ oligomers trigger a plethora of events such as Tau pathology, axonal

transport impairment, synapse loss, oxidative stress, neuroinflammation and insulin resistance (Cline *et al.*, 2018). The overwhelming evidence, from human and animal studies, showing a crucial role of Aβ oligomers in promoting AD development led to the hypothesis that Aβ oligomers are necessary and sufficient to cause AD and memory dysfunctions, thus suggesting that the therapeutic approaches should be directed towards the oligomers rather than targeting the plaques, in light of the weak correlation between plaques and cognitive decline (Cline *et al.*, 2018). Also, AD is not just a neuronal pathology but rather includes responses from astrocytes, microglia and vasculature, all events driven by Aβ oligomers (Figure 1.5); Aβ oligomers can trigger astrogliosis and astrocyte activation, impair astrocyte-mediated glutamate transport that leads to synaptic impairment; moreover, microglia might be attracted to plaques by surrounding the oligomers, thus switching to a pro-inflammatory phenotype that further contributes to oligomer formation, neuroinflammation, synapse loss and overall neurodegeneration (Cline *et al.*, 2018).

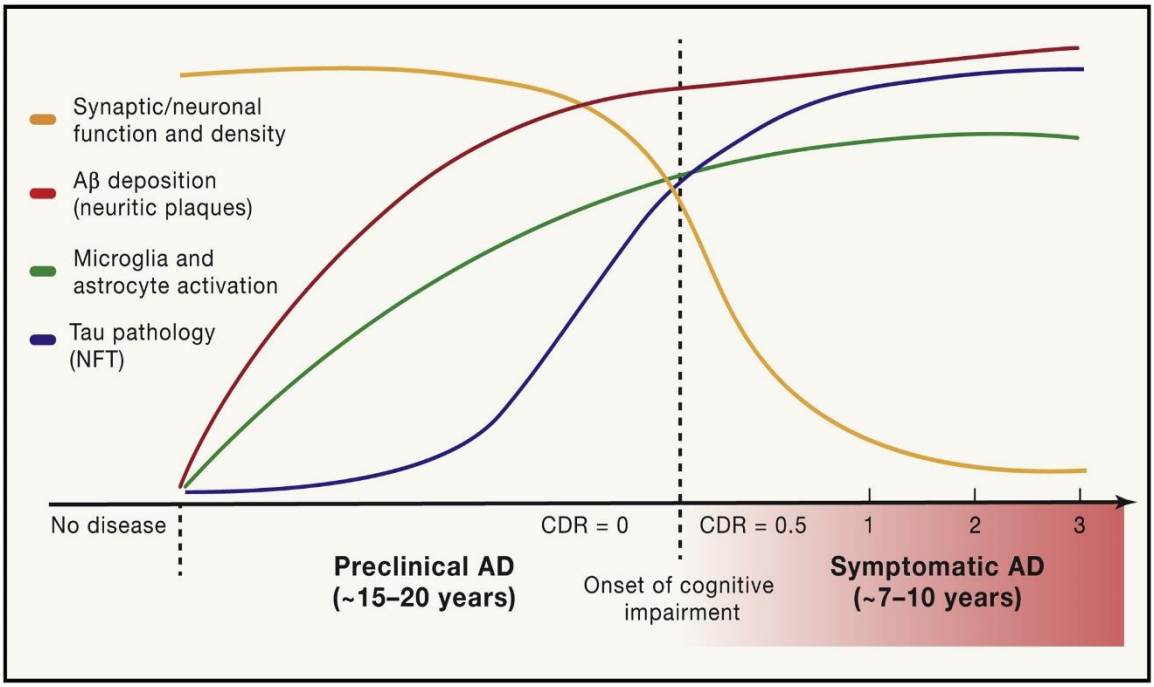

Figure 1.5: Major events in AD and their development over time
Reproduced with permission from (Long and Holtzman, 2019).

Despite this evidence, the correlation between Tau pathology and progression of cognitive impairment has led to the hypothesis that cognitive decline and neurodegeneration in AD are driven by Tau spreading (Long and Holtzman, 2019): this hypothesis is known as Tau propagation hypothesis (Liu *et al.*, 2019). Tau propagates throughout the AD brain with a precise patterns, although the cognitive symptoms become evident only when Tau starts propagating from the entorhinal cortex to the neocortex (Long and Holtzman, 2019; Silva *et al.*, 2019). Also, while Aβ deposition occurs first and in regions like frontal lobes, the neuronal death begins in the entorhinal cortex and in the hippocampus, where Aβ aggregates are relatively few (Musiek and Holtzman, 2015). It is also known that while Aβ starts accumulating in the frontal cortex and spreads in the most internal areas of the brain, Tau shows an opposite pattern of spreading. This apparent anatomical disconnect between Aβ aggregation, Tau deposition and neuronal loss is still not clear (Musiek and Holtzman, 2015). The propagation of Tau occurs in a prion-like fashion; therefore, it spreads out in a relatively quick period through mechanisms yet to be fully understood. Tau is hyperphosphorylated at many sites thus conferring it aggregation properties that cause microtubule disruption and impairment of signal transmission, which in turn seems to mediate Aβ-induced neurotoxicity, especially at synapses (Liu *et al.*, 2019).

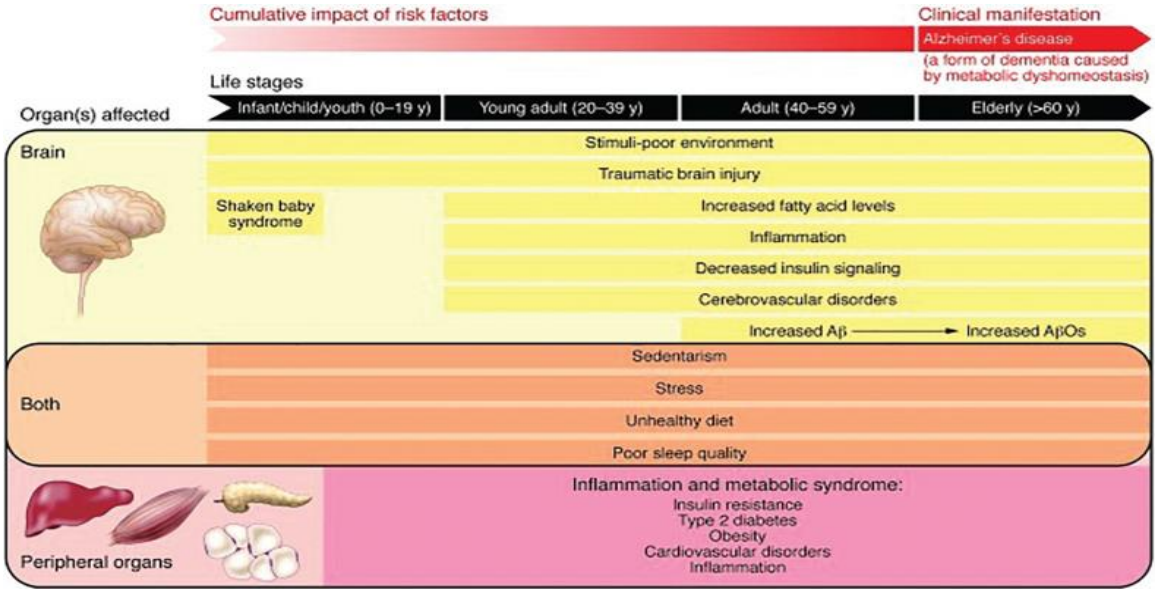

Figure 1.6: Impact of risk factors on AD development
From (Cline *et al.*, 2018).
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As previously mentioned, there are several factors that may accelerate and exacerbate AD progression, such as oxidative stress, neuroinflammation, mitochondrial dysfunction, which are normally considered downstream of Aβ aggregation. On the other hand, neuroinflammation, oxidative stress and mitochondrial dysfunction can induce Aβ accumulation, thus aggravating Aβ pathology (Musiek and Holtzman, 2015). Although it may seem obvious to consider these conditions as pathogenetic factors for AD, it is worthwhile to underline that neuroinflammation, oxidative stress and mitochondrial dysfunction characterize other neurodegenerative diseases, while Aβ pathology is exclusive of AD (Musiek and Holtzman, 2015). However, since the contribution of these prodromal factors in promoting the pathology is important, researchers have suggested other hypotheses that are centered on either mitochondrial dysfunction, oxidative stress or neuroinflammation: mitochondrial cascade hypothesis (that includes oxidative stress) and the inflammatory hypothesis (Liu *et al.*, 2019). The mitochondrial cascade hypothesis was suggested after the observation that in AD the number of mitochondria is decreased: oxidative stress due to mitochondrial damage occurs and seems preceding and even

promoting Aβ deposition, driven by the overproduction of ROS and insufficient antioxidant response. Moreover, the mitochondrial turnover, also known as mitophagy, is impaired in AD, thus leading to higher Aβ aggregation (Liu *et al.*, 2019). The inflammatory hypothesis arises from the observation that astrocyte and microglia activation, crucial steps of neuroinflammation, occur in AD brains: in particular, the amount of microglia around plaques and tangles in AD patients is much higher than control patients. Also, Aβ pathology is linked to neuroinflammation and Aβ aggregates causing increased release of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) through binding to specific microglial receptors (Liu *et al.*, 2019). A partial modification of the inflammatory hypothesis is the amyloid cascade-inflammation hypothesis: the sequential appearance of amyloid plaques and NFTs has suggested that these two events are connected by the microglial activation, which would act as a bridge connecting the Aβ aggregation to Tau hyperphosphorylation and subsequent accumulation (Leng and Edison, 2021). This hypothesis comes from the observation that different species of Aβ aggregates induce glial activation and the production of pro-inflammatory cytokines, that may lead to neuronal death; the toxic oligomers are the species that induce the strongest response by microglia (Leng and Edison, 2021). Neuroinflammation and its role in neurodegeneration will be further discussed in a later section of this thesis.

Risk factors of AD.

The greatest risk factor for AD is aging: the risk of developing AD increases dramatically with age reaching 33% in people 85 years of age and older (Alzheimer's Association, 2021). AD may be also aggravated by genetic, family history and environmental factors; although these factors seem to be linked, around 70% of AD risk can be ascribed to genetic factors. In fact, genome-wide association studies have revealed that around 20 genes regulating cholesterol and lipid metabolism, immune and inflammatory response, endosomal-vesicle recycling may increase the risk of AD development (Scheltens *et al.*,

2016). For example, the *APOE* gene, coding for the Apolipoprotein E (ApoE) is the single biggest genetic risk factor for sporadic AD (Lane, Hardy and Schott, 2018; Long and Holtzman, 2019): Apolipoprotein E is able to bind lipids and participate in transport and delivery of lipids to target sites. In the brain, ApoE protein is expressed primarily in astrocytes and to a lesser degree in microglia (Long and Holtzman, 2019) and exists in three isoforms, ε2, ε3, ε4 that differ for 1 or 2 amino acids (Masters *et al.*, 2015). Carrying just one copy of the ε4 allele increases the risk of AD by 2- to 4- fold, while having both ε4 copies increases the risk up to 12-fold, compared to ε2 or ε3 carriers (Soria Lopez, González and Léger, 2019; Alzheimer's Association, 2021). ApoE contributes to Aβ pathology by directly binding Aβ plaques or by inducing its aggregation; moreover, ApoE regulates Aβ clearance by competitively binding Low Density Lipoprotein (LDL) Receptor Related Protein 1 (LRP1), which is a typical Aβ receptor (Long and Holtzman, 2019). Additionally, ApoE increases the risk of AD development by modulating Tau-induced neurodegeneration in animal models of AD and by regulating immune and microglial response involved in Aβ clearance and degradation (Long and Holtzman, 2019). Another protein involved in lipid and cholesterol metabolism that has been identified as risk factor for AD when mutated is ATP Binding Cassette Subfamily A Member 7 (ABCA7) (Selkoe and Hardy, 2016). This is a lipid transporter expressed in neurons, microglia and peripheral macrophages that promotes lipid efflux to apolipoproteins and regulates phagocytosis (Selkoe and Hardy, 2016). ABCA7 mutation leads to a loss-of-function that seems to impair Aβ clearance: mice where ABCA7 was knocked out show dramatic increase of Aβ burden (Selkoe and Hardy, 2016).

In AD, the immune response against Aβ plaques is a well described feature that is considered a crucial step of AD pathogenesis (Selkoe and Hardy, 2016). Recently, some components of the immune system have been identified as prominent risk factors for AD (Selkoe and Hardy, 2016). Another important example is Triggering Receptor Expressed On Myeloid Cells 2 (TREM2), microglial receptor involved in Aβ clearance, whose

mutations greatly increase the risk of developing AD (Scheltens *et al.*, 2016; Long and Holtzman, 2019).

Interestingly, people whose first-degree relatives developed AD are at higher risk of AD, compared to families whose history does not contemplate AD cases; this observation is independent of the presence of ApoEε4 allele (Alzheimer's Association, 2021).

Besides the described risks associated with age, genetics and family history, there are several lifestyle related risk factors associated with AD, namely type 2 diabetes and insulin-resistance, obesity, hypertension, physical and mental inactivity, depression, smoking, low education attainment, and diet, which are considered modifiable risk factors (see Table 1.1) (Scheltens *et al.*, 2016; Crous-Bou *et al.*, 2017; Livingston *et al.*, 2017, 2020; A. Armstrong, 2019; Bhatti *et al.*, 2020). Noteworthy, the Lancet Commission on Dementia, Prevention, Intervention and Care has suggested that targeting such modifiable risk factors might prevent or delay up to 40% of AD cases (Figure 1.6) (Livingston *et al.*, 2017, 2020; Alzheimer's Association, 2021). Hypertension, diabetes and obesity (associated with high cholesterol and high body mass index) may be considered more specifically cardiovascular related risk factors (Imtiaz *et al.*, 2014), while cardiovascular disease, hypertension, obesity, diabetes and hyperlipidemia may be defined as acquired risk factors, as well (Silva *et al.*, 2019). Regardless of their classification, identifying such risk factors has allowed the medical and scientific community to consider AD a potentially preventable disease and no longer an inevitable consequence of aging (Imtiaz *et al.*, 2014).

Therefore, since an effective therapy is still missing, reducing the dementia risk is crucial to decrease the incidence of AD (Scheltens *et al.*, 2016; Livingston *et al.*, 2017, 2020). Also, non-pharmacological treatments aimed at limiting the progression of neurodegenerative diseases have been proposed, such as physical activity, caloric restriction, anti-oxidants diet supplements, nutraceuticals: these approaches may primarily target co-morbidities like diabetes, obesity, cardiovascular diseases, which are all risk

factors for AD. Therefore, any treatment that reduces the incidence of these pathologies may potentially reduce the risk of dementia, including AD (Bhatti *et al.*, 2020).

	Relative risk for dementia (95% CI)	**Risk factor prevalence**
Early life (<45 years)		
Less education	1.6 (1.3–2.0)	40.0%
Midlife (age 45–65 years)		
Hearing loss	1.9 (1.4–2.7)	31.7%
Traumatic brain injury	1.8 (1.5–2.2)	12.1%
Hypertension	1.6 (1.2–2.2)	8.9%
Alcohol (>21 units/week)	1.2 (1.1–1.3)	11.8%
Obesity (body-mass index ≥30)	1.6 (1.3–1.9)	3.4%
Later life (age >65 years)		
Smoking	1.6 (1.2–2.2)	27.4%
Depression	1.9 (1.6–2.3)	13.2%
Social isolation	1.6 (1.3–1.9)	11.0%
Physical inactivity	1.4 (1.2–1.7)	17.7%
Diabetes	1.5 (1.3–1.8)	6.4%
Air pollution	1.1 (1.1–1.1)	75.0%

Table 1.1: Relative risk of modifiable risk factors for AD
Adapted with permission from (Livingston *et al.*, 2020).

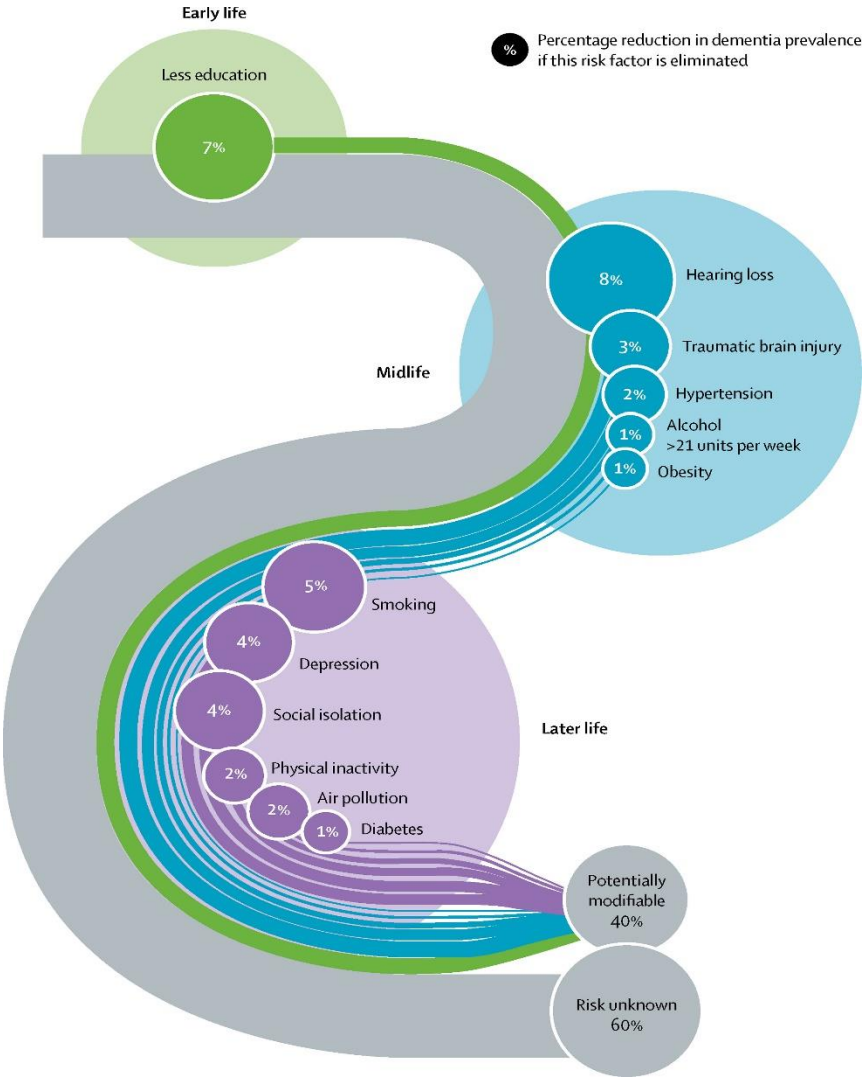


Figure 1.7: Percentage of population that could be saved from developing dementia. If the depicted risk factors were all tackled together, there is the potential possibility that up to 40% of the population could be prevented from developing dementia, including AD. Reproduced with permission from (Livingston *et al.*, 2020).

Current therapeutic strategies and risk factor reduction for AD

In 2013, the G8 has stated that AD should be considered a global priority and research efforts should be encouraged to obtain a therapy by 2025 (Scheltens *et al.*, 2016). Sadly, no disease-modifying treatment for AD is available yet, and the few approved drugs provide only limited symptomatic relief (Joe and Ringman, 2019). Acetylcholinesterase

inhibitors (donepezil, galantamine and rivastigmine) increase the availability of acetylcholine at synapses by inhibiting the enzyme (acetylcholinesterase) that cleaves it. They provide modest benefits especially in mild to moderate AD (Lane, Hardy and Schott, 2018), even though their beneficial effects decline after the first year of treatment (Scheltens *et al.*, 2016). Memantine is another pharmacologic treatment: it is an antagonist of N-methyl D-aspartate (NMDA) glutamate receptors, thus reducing excitotoxicity of glutamate, although the overall benefits (such as reduction of patient agitation) are modest (Lane, Hardy and Schott, 2018). Another drug, more specifically a monoclonal antibody that targets Aβ, aducanumab, has been recently approved by Food and Drug Administration (FDA) (Food and Drug Administration, 2021): this drug may potentially slow AD progression (Alzheimer's Association, 2021; Cummings *et al.*, 2021), although its clinical trials were initially discontinued for futility, uncertainties about the validity of the studies still stand and criticism against the FDA approval has been raised (Vaz and Silvestre, 2020; Nisticò and Borg, 2021). Other concurrent psychiatric symptoms, such as depression, anxiety, are often present, but difficult to treat, and there is limited evidence of possible beneficial pharmacological treatment to reduce them (Lane, Hardy and Schott, 2018), although selective serotonin reuptake inhibitors are currently used to counter depression and mood disorders (Soria Lopez, González and Léger, 2019). Moreover, in late-stages of dementia psychosis, agitation and aggression may develop, however their treatment results difficult and there are no drugs with clear indication of use in patients with dementia (Lane, Hardy and Schott, 2018).

Many therapies aimed at lowering either Aβ or Tau were developed with the goal to slow the disease progression, such as active and passive immunization against Aβ or Tau, secretase inhibitors to reduce Aβ production, Tau kinase inhibitors, agents that reduce Tau expression (Long and Holtzman, 2019); unfortunately, the proposed drugs targeting the amyloids or their biosynthetic pathways have so far proven unsuccessful (Graham, Bonito-Oliva and Sakmar, 2017; Long and Holtzman, 2019; Oxford, Stewart and Rohn, 2020; Vaz

and Silvestre, 2020). In fact, many of the proposed drugs that have failed phase 3 of testing trials have been evaluated in patients with mild to moderate AD, that most likely already represents an advanced stage of the disease, with significant and irreversible synaptic neuronal loss in place. Perhaps, treatments in cognitively normal people at high risk of developing AD, namely before any irreversible impairment arises, may result in better outcomes (Crous-Bou *et al.*, 2017; Long and Holtzman, 2019). Along these lines, a possible alternative approach might be targeting at Aβ oligomers, which drive the pathology in preclinical phase: proponents of the amyloid-β oligomers AD hypothesis suggest that an immunotherapeutic approach against Aβ oligomers would even be beneficial after AD onset (Cline *et al.*, 2018).

Target type	**Name**
Cholinergic	Donepezil
Cholinergic	Galantamine
Cholinergic	Rivastigmine
Glutaminergic	Memantine
Amyloid-β	Aducanumab

Table 1.2: Current approved drugs for AD

Considering that neuroinflammation is one of the earliest events characterizing AD, researchers have tested the use of anti-inflammatory drugs aimed at targeting the early stages of the pathology, especially in light of epidemiologic studies that found reduced risk of AD in people taking chronic ibuprofen for long period of times, compared to people who did not use anti-inflammatory drugs (in 't Veld *et al.*, 2001; Vlad *et al.*, 2008; Kempuraj *et al.*, 2016). There may be three approaches to counteract neuroinflammation: 1) suppressing the pro-inflammatory properties of microglia, 2) modulating microglia

phenotype to induce a shift towards an anti-inflammatory phenotype, 3) influencing microglial priming in the early phases of the disease (Leng and Edison, 2021). To suppress the pro-inflammatory activity of microglia, several clinical trials proposed the use of corticosteroids, non-steroid anti-inflammatory drugs (NSAIDs) and selective cyclooxygenase-2 (COX-2) inhibitors, however relevant clinical studies failed to show any improvement of cognitive symptoms in patients with mild to moderate AD (Heneka *et al.*, 2015; Graham, Bonito-Oliva and Sakmar, 2017; Long and Holtzman, 2019; Leng and Edison, 2021). Moreover, the use of the antibiotic minocycline was proposed, given some interesting results in animal models that displayed the inhibition of pro-inflammatory cytokines upregulation, but unfortunately the human clinical trials failed once again. Other ongoing studies are targeting different components of the inflammatory system, such as NLR (NOD (nucleotide-binding oligomerization domain) like-receptor) family pyrin domain containing 3 (NLRP3), caspase 1, or directly the pro-inflammatory cytokines. Drugs like VX-765 (caspase-1 inhibitor), etanercept (anti-TNF antibody), antibodies against IL-1, directed at different components of the neuroinflammation, are still under investigation (Leng and Edison, 2021). An alternative target might the receptor for advanced glycation endproducts (RAGE), expressed in microglia and whose activation leads to neuroinflammation and oxidative stress. RAGE is upregulated in AD, is able to bind Aβ and seems to mediate the toxic effects of Aβ oligomers. Hence, targeting this receptor might be beneficial for cognitive improvement (Graham, Bonito-Oliva and Sakmar, 2017). However, the only drug that was developed to target RAGE, azeliragon, was discontinued after that phase 3 clinical trials failed to provide meaningful benefits to patients (Burstein *et al.*, 2019). Another possibility might be to interfere with the phenotypic changes of microglia that confer pro-inflammatory properties to microglia with the evolution of the pathology: subcutaneous administrations of anti-inflammatory cytokines or molecules known to reduce inflammatory mediators (resveratrol) have been proposed; also, peroxisome proliferator-activated receptor-gamma (PPARγ)-agonists have

been studied, since PPARs are involved in microglia phenotypic changes. However, two PPARs agonists (rosiglitazone and pioglitazone) have not been further investigated due to safety concerns of the drugs (Leng and Edison, 2021). Several clinical trials have suggested that dietary integrations (with folic acid and omega 3 fatty acid supplementation) reduce the impact of neuroinflammation in AD by reducing inflammatory markers in CSF and plasma in AD patients. This reduction might reduce microglial priming, especially in people with high risk of developing AD. Also, patients with midlife obesity, insulin resistance and elevated LDL levels develop an inflammatory condition that may ultimately lead to AD, therefore such dietary intervention may prevent AD development (Imtiaz *et al.*, 2014). In the last decade, alternative approaches of targeting AD risk factors have been proposed, such as physical exercise, antioxidant supplements, healthy diets, and the use of nutraceuticals (Norton *et al.*, 2014; Livingston *et al.*, 2017, 2020; Bhatti *et al.*, 2020). For examples, studies conducted in patients at high risk of AD took into account several criteria (multidomain intervention) such as diet, cognitive training, exercise, throughout a long observational time (up to 10 years). These studies showed that a broad intervention on lifestyle habits and cognitive training led to better performances for treated patients compared to those not treated, thus suggesting that reducing the impact of risk factors on brain function could prevent the onset of cognitive dysfunction (Masters *et al.*, 2015; Crous-Bou *et al.*, 2017). Another interesting study, the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) study, in which were combined diet intervention, exercise and vascular risk management helped to maintain cognitive function in a population at risk of dementia. However, a clinical trial that combined omega 3 fatty acid supplementation physical activity, cognitive training and nutritional advice there were no positive outcomes (Crous-Bou *et al.*, 2017). Therefore, further studies are needed to understand how these approaches can be developed and to whom can be applied (Imtiaz *et al.*, 2014; Leng and Edison, 2021).

Dietary interventions are also known to reduce Aβ formation, Tau hyperphosphorylation, oxidative stress, thus potentially delaying or even preventing the occurrence of AD (Bhatti *et al.*, 2020). Molecules like poly-unsaturated fatty acids (PUFA), flavonoids, curcumin, resveratrol, vitamins are the most common dietary interventions utilized to attempt reducing the risk of developing AD. PUFA are important components of neuronal cell membranes: they influence membrane fluidity, which is in turn important for membrane-associated functions. Abnormalities of PUFA have been associated with AD and psychiatric disorders, studies employing omega-3 supplements have generated conflicting results, so that further investigations are needed (Bhatti *et al.*, 2020). There are medical foods (Axona and Souvenaid), rich in short and medium chain fatty acids, PUFA, phospholipids, vitamins, that showed cognitive benefits in AD and MCI patients, although these positive outcomes have been evaluated in a short time (45-90 days or 24 weeks after the beginning of the dietary intervention) (Joe and Ringman, 2019).

Curcumin is a molecule isolated from turmeric, an Asian spice that is considered neuroprotective and has shown anti-inflammatory, antioxidant and antibacterial activity. The frequent use of curcumin in Indian populations has been associated with lower incidence of AD in this population. Hence, many studies have focused on whether curcumin can protect the brain from degenerating, however returning conflicting outcomes needing further evaluation (Lo Cascio *et al.*, 2019; Bhatti *et al.*, 2020).

Flavonoids are natural compounds with polyphenolic structure commonly present in vegetables, fruits, wine, tea that have antioxidant, anti-inflammatory and anti-carcinogenic properties, thus having potential therapeutic use in AD. Quercetin, found in red wine, onions, green tea, apples, berries, that carry neuroprotective activity by modulating Nuclear factor erythroid (NFE)-2-related factor 2 (Nrf-2), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/AKT pathways (Bhatti *et al.*, 2020). The use of cocoa, plant rich in flavonoids has showed beneficial effects in AD prevention, as suggested by a clinical trial conducted in a

cohort of people over 65 years old where 48 months of cocoa consumption lowered the risk of developing AD by 41% (Lamport *et al.*, 2015). Anthocyanin, rich in soybean seeds, and caffeine reduce oxidative stress, neurodegeneration and memory impairments in animal models of AD, likely through modulation of PI3K/Akt and extracellular signal-regulated kinase/cAMP (cyclic adenosine monophosphate) response element-binding (Erk/CREB) pathways (Bhatti *et al.*, 2020). Resveratrol, rich in grapes and red wine, is another flavonoid whose antioxidant and anti-inflammatory activity has been tested in AD animal models and also in human clinical trials showing very promising neuroprotective results (Bhatti *et al.*, 2020).

Intake of healthy foods such as vegetables and fruits is correlated with better cognitive performance in studies conducted in developed countries, where usually the incidence of obesity is high (Leigh and Morris, 2020). The Mediterranean diet seems to be protective for the brain in terms of reduced risk of AD and reduced brain atrophy, compared to people who do not use this type of diet: the antioxidant and anti-inflammatory properties of this diet help preventing diseases like diabetes, obesity and cardiovascular diseases, prominent risk factors of AD, thus providing protection against cognitive decline (Nday, Eleftheriadou and Jackson, 2018; Bhatti *et al.*, 2020; Leigh and Morris, 2020). Conversely, diets rich in red meats, fried food, with lower intake of whole grains foods do amplify the risk of developing AD (Leigh and Morris, 2020). Caloric restriction has been proposed as non-pharmacologic intervention that seems to be effective in brain aging: in fact, it improves metabolic health by counteracting the harmful effects of ROS and oxidative damage (Bhatti *et al.*, 2020). Several studies showed that long-term caloric restriction can ameliorate AD pathology and cognitive decline, thus suggesting a preventive role: in a study conducted in female Tg2576, caloric restriction for 12.5 months significantly reduces Aβ load and γ-secretase in female Tg2576 mice, while did not have effect on α- and β-secretase and overall levels of APP (Schafer *et al.*, 2015); another study performed in male and female 3xTg mice demonstrated that 14 months of caloric restriction significantly

ameliorates learning and memory deficits, as well as reduces Aβ burden and Tau phosphorylation, although total Tau levels were unchanged (Halagappa *et al.*, 2007).

Physical activity might reduce the prevalence of AD by around 8% possibly due to its beneficial effects on other known risk factors of AD (Norton *et al.*, 2014). Other observations suggest that exercise in midlife reduces the risk of AD development, while investigations in animal models show that exercise increases neurogenesis and decreases the risk of AD, likely through upregulation of brain-derived neurotrophic factor (BDNF) and by decreasing levels of Aβ and Tau (Yau *et al.*, 2014; Hüttenrauch *et al.*, 2016; Choi *et al.*, 2018; Liu and Nusslock, 2018; Gerberding *et al.*, 2019; Meng, Lin and Tzeng, 2020). As more studies confirm the association between AD and diabetes or obesity, and the need of healthy diets to prevent AD, researchers have proposed to reposition (or repurpose) some drugs that are currently used to treat metabolic diseases, such as statins or intranasally injected insulin, however these studies have proven so far inconclusive (Graham, Bonito-Oliva and Sakmar, 2017; Nday, Eleftheriadou and Jackson, 2018).

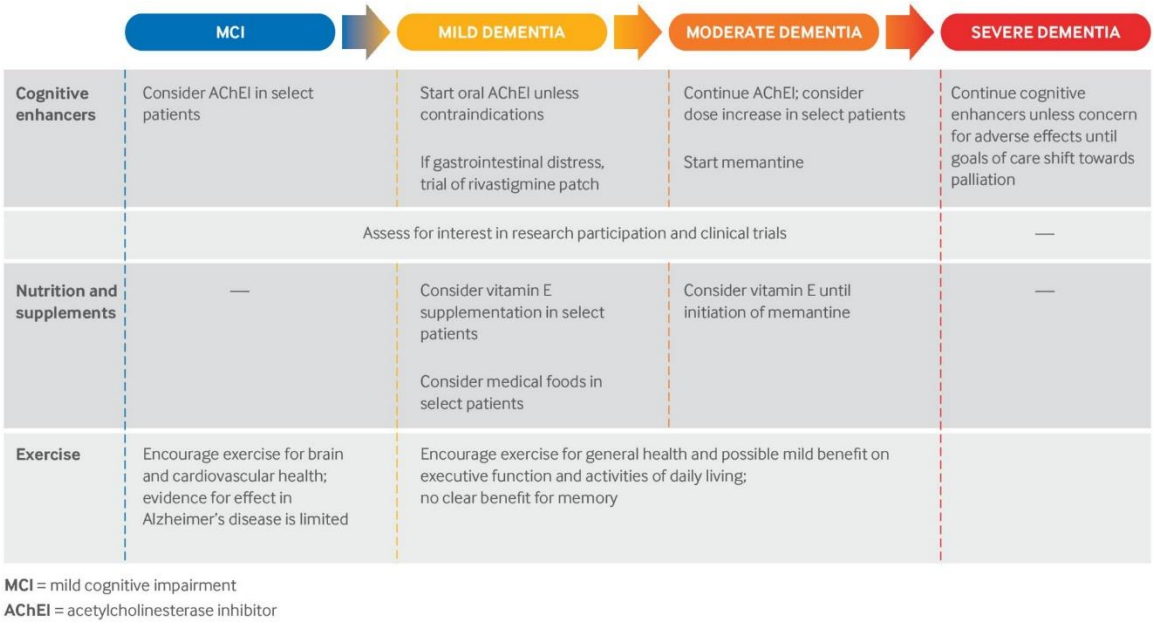


Figure 1.8: Possible strategies to prevent or manage AD
Reproduced with permission from (Joe and Ringman, 2019).

NEUROINFLAMMATION

Neuroinflammation in AD

Neuroinflammation is an immune response that occurs in the CNS following ischemia, trauma, injury or infection (Leng and Edison, 2021) as well as other events (metabolic, neoplastic, developmental) that interfere with CNS homeostasis (Morales *et al.*, 2016). Neuroinflammation affects all CNS cell types, including neurons (Shabab *et al.*, 2017), and is characterized by the production by competent cells of the innate immune system of pro-inflammatory cytokines, ROS and other molecules, known to induce synapse damage, inhibition of neurogenesis and neuronal death (Leng and Edison, 2021). Usually, neuroinflammation has beneficial effects in terms of activating mechanisms of tissue repair and removal of debris (Kwon and Koh, 2020), including upregulation of BDNF in astrocytes which is mediated by moderate levels of TNF-α, thus providing neurotropic and neuroprotective activity (Figure 1.8) (Kempuraj *et al.*, 2016); however, when this response is prolonged in time, a chronic inflammatory situation establishes thus leading to damage and degeneration and, ultimately, to increased vulnerability to pathology (Figure 1.8) (Morales *et al.*, 2016). Noteworthy, neuroinflammatory mechanisms are normally involved in complement cascade and microglial-mediated synapse pruning in healthy brain development (Morales *et al.*, 2016).

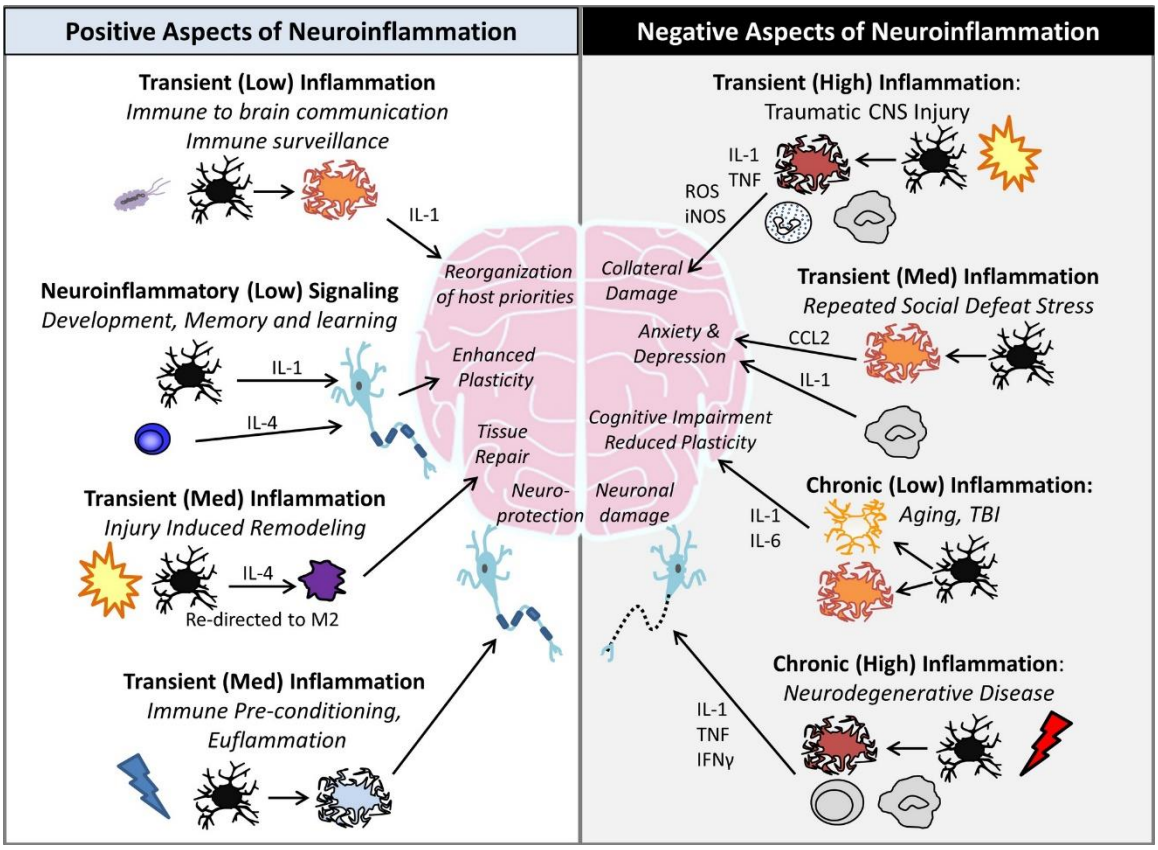


Figure 1.9: Positive and negative aspects of neuroinflammation
Reproduced with permission from (DiSabato, Quan and Godbout, 2016).

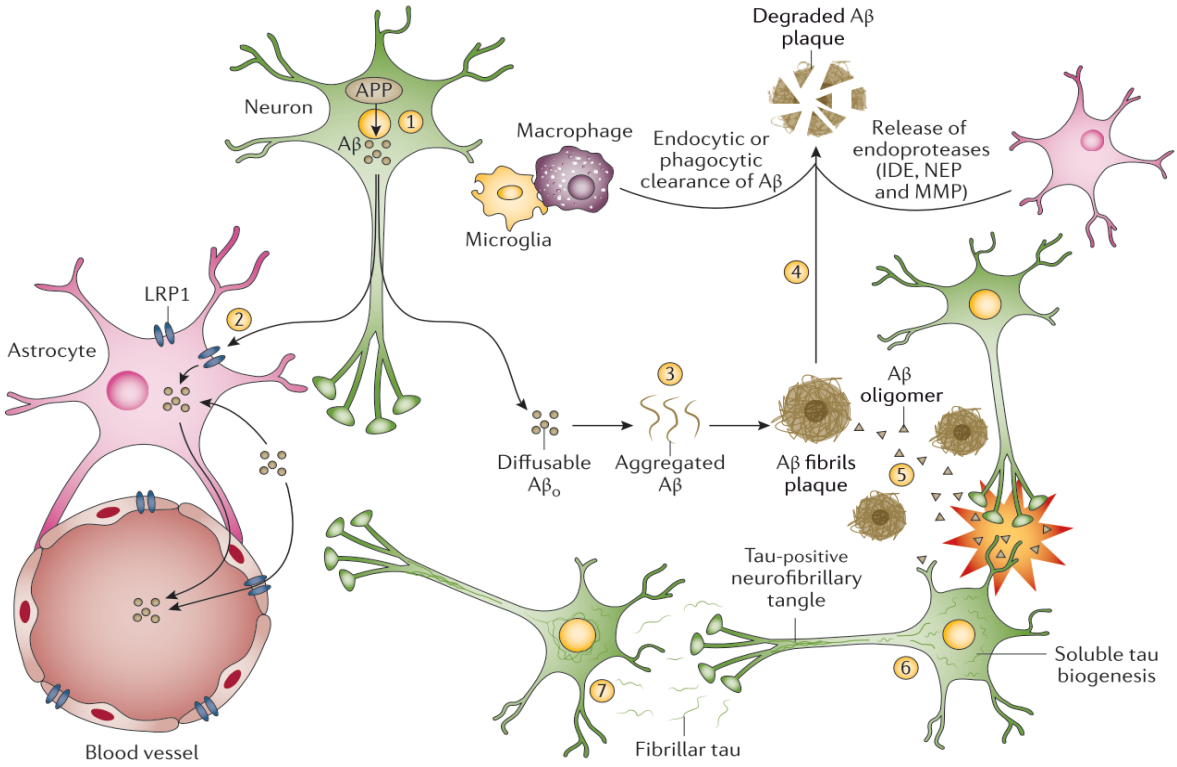


Figure 1.10: Progression of AD and involvement of microglia and astrocytes
Reproduced with permission from (Masters *et al.*, 2015).

Neuroinflammation and the role of the immune system have recently become an extensive area of research in the AD field and other neurodegenerative diseases. Neuroinflammation is considered the third neuropathological correlate of AD, after Aβ and Tau (Graham, Bonito-Oliva and Sakmar, 2017), believed to strongly contributing to the progression of the disease (Leng and Edison, 2021). Some systemic pathologies, such as obesity and diabetes can induce neuroinflammation through circumventricular organs or disruption of the blood-brain barrier (BBB) (Leng and Edison, 2021) and exacerbate the contribution of neuroinflammation to the development of AD (Heneka *et al.*, 2015). Another prominent contributor of neuroinflammation is aging. Aging leads to a low-grade systemic inflammation that causes in turn increased BBB permeability, impaired glial signaling and a low-grade chronic inflammation into the CNS. The chronic inflammation ultimately reduces glial efficacy in providing CNS protection, and paves the way to

neurodegeneration and cognitive decline (Kempuraj *et al.*, 2016). Astrogliosis and microgliosis, characteristics of neuroinflammation, are well described features of AD and several genetic mutations associated with microglial function have been identified as risk factors for AD (Long and Holtzman, 2019). According to the amyloid cascade-inflammatory hypothesis, neuroinflammation is considered a bridge connecting Aβ to Tau toxicity: microglia activation is present in preclinical condition and in MCI, thus suggesting that is an early event characterizing the pathology (Leng and Edison, 2021). There are studies demonstrating that the injection of Aβ into the brain of primate models did not induce any pathology, however the simultaneous injection of lipopolysaccharide and Aβ initiated the Aβ-driven pathology (Leng and Edison, 2021). Interestingly, data from human *post-mortem* studies revealed no relevant alteration of microglia in asymptomatic individual with high load of plaques, thus suggesting that neuroinflammation is required, in addition to Aβ, to initiate AD pathology (Leng and Edison, 2021). Moreover, an excessive phagocytic activity by microglia can lead to inappropriate synapse pruning, thus causing synaptic damage and neurotransmission impairments (Leng and Edison, 2021).

Microglia are important components of CNS constituting up to 10% of the total cell population (DiSabato, Quan and Godbout, 2016) and are the first line of defense to pathological insults in the brain (Shabab *et al.*, 2017; Kwon and Koh, 2020); microglia are involved in immune defense and maintenance of the CNS by modulating developmental synaptic pruning, neuronal apoptosis, synaptic plasticity and immune surveillance and are the main actor participating in the neuroinflammation cascade (Morales *et al.*, 2016; Leng and Edison, 2021). Ramified microglia constantly keep CNS under control by surveying the entire brain in search of any damage (Heneka *et al.*, 2015; Graham, Bonito-Oliva and Sakmar, 2017; Guzman-Martinez *et al.*, 2019), whereas the one associated with pathology is generally more amoeboid (Leng and Edison, 2021). However, their phenotype differs across brain regions and lifespan, with amoeboid phenotypes in regions lacking BBB, longitudinal ramification across the fibers, and extensively ramified microglia near the

neuropil. Noteworthy, these differences are dynamic since microglia can change their morphology depending upon endogenous and exogenous factors. These morphologic differences are also accompanied by differences in transcriptome and proteome (Nichols *et al.*, 2019). When an insult occurs, microglia respond by activating the danger-associated molecular patterns (DAMP) or pathogen-associated molecular patterns (PAMP) that ultimately recognize pathogens, debris or abnormal protein, like Aβ (Heneka *et al.*, 2015; Leng and Edison, 2021). Microglia remove these toxic elements through phagocytosis, pinocytosis or receptor-mediated mechanisms, and by activating the pro-inflammatory pathways to release pro-inflammatory molecules, hence starting a neuroinflammatory process (Shabab *et al.*, 2017; Leng and Edison, 2021). This mechanism is aimed at the maintenance of neuronal plasticity, protection and remodeling of synapses, through the release of neurotrophic factors, such as BDNF (Heneka *et al.*, 2015). The recognition of the harmful stimulus at the microglia cell surface leads to microglia activation and to the beginning of the neuroinflammatory cascade, which includes microglia morphologic changes, proliferation and migration to injury site, followed by phagocytosis and activation of pro- or anti-inflammatory cytokines (Shabab *et al.*, 2017). One of the most studied pathways, related to the microglia activation, is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) one. This pathway is activated following binding of a ligand to the cell membrane receptor belonging to the Toll-like receptors (TLRs) family, or alternatively, from the binding of pro-inflammatory cytokines such as IL-1β, TNF-α to their specific receptors. NF-κB is associated in the cytoplasm with an inhibitory molecule, inhibitor of κB (IκB), and it needs to translocate into the nucleus to exert its action: when a proper stimulus arrives, phosphorylation of IκB occurs with consequent cleavage of the complex IκB/NF-κB. At this point, NF-κB is able to get into the nucleus, bind to specific promoters and induce the transcription of specific genes (Shabab *et al.*, 2017). Interestingly, NF-κB may be activated by the binding of BDNF to the receptor tropomyosin receptor kinase B (TrkB), thus activating the pathway that liberates NF-κB from its binding

to the inhibitory subunit IκB. It is known that BDNF can also bind the receptor p75 neurotrophin receptor (p75NTR), whose activation is involved in pro-apoptotic and pro-inflammatory pathways, including JNK and NF-κB (Lima Giacobbo *et al.*, 2019); moreover, several studies in AD models noticed that the increase of pro-inflammatory cytokines is accompanied by a decrease of BDNF and, in particular, IL-1β upregulation turns down BDNF expression (Tong *et al.*, 2012; Lynch, 2015; Lima Giacobbo *et al.*, 2019). Once microglia remove the danger, the immune response is over, however, especially in aged brains or during pathology, including AD and neurodegenerative diseases, the microglial efficiency reduces and these processes may be impaired (Morales *et al.*, 2016; Graham, Bonito-Oliva and Sakmar, 2017; Leng and Edison, 2021). Also, in ageing brains, microglia are less ramified, hence reducing its capacity of surveying the CNS (Heneka *et al.*, 2015). An important role in maintaining chronic and detrimental neuroinflammation is held by cytokines, in particular TNF-α and IL-1β; their levels increase in the initial stages of an inflammatory condition and then drive the progression of the pathology by activating intracellular pathways after the binding to their specific receptors, interleukin-1 receptor-1 (IL-1R1) for IL-1β and tumor necrosis factor receptors (TNFRs) for TNF-α (Shabab *et al.*, 2017). Furthermore, IL-1β and TNF-α mediate BBB disruption, recruitment of cell-adhesion molecules, and are able to induce inducible nitric oxide synthase (iNOS), thus leading to increased production of NO. The excessive presence of iNOS is indicative of a pathological state as well as increased nitration of biomolecules, that further increases oxidative stress in the cell. Inflammation causes oxidative stress and ensuing oxidative damage which in turn cause the overproduction of ROS by microglia (Querfurth and LaFerla, 2010; Heneka *et al.*, 2015; Shabab *et al.*, 2017). ROS are diffusible species, capable to trigger neuroinflammation if not promptly removed from the cells, thus further contributing to neurodegeneration; moreover, mitochondria are a source of ROS which can activate NF-κB-mediated neuroinflammatory cascade. When mitochondria function is impaired, like in AD and other neurodegenerative diseases, ROS

production increases, with subsequent accumulation in the cytosol and activation of neuroinflammation. Other pathways involved in neuroinflammation are the ones leading to prostaglandin formation, catalyzed by COX-1 and COX-2 enzymes; the PI3K/Akt/mammalian target of rapamycin (mTOR) that is involved in NF-κB activation and translocation; and MAPK pathway which includes stress-activated protein kinases/Jun amino terminal kinases (SAPK/JNK) (Shabab *et al.*, 2017).

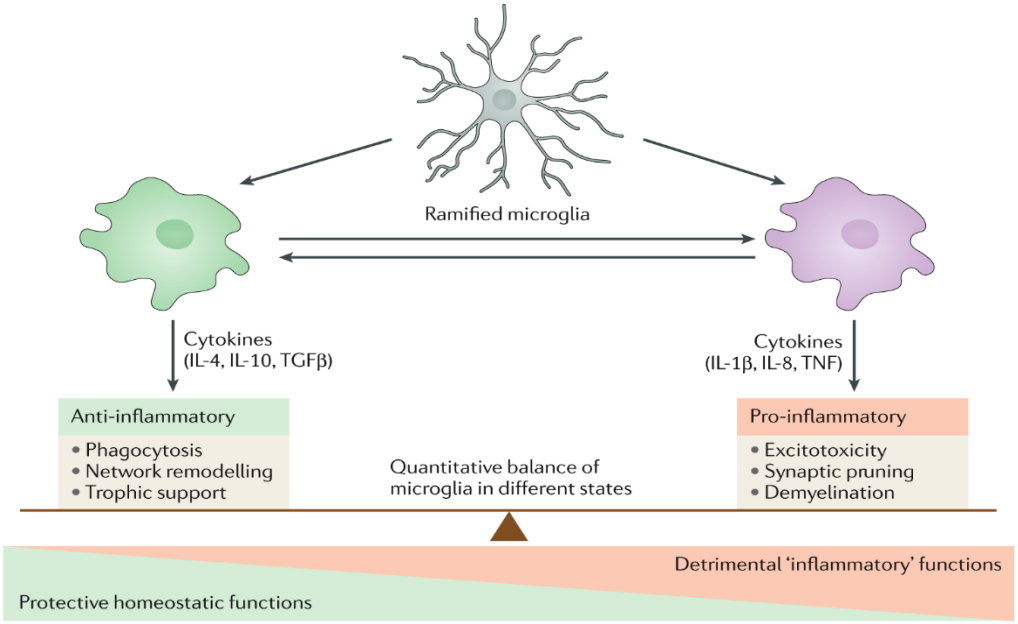


Figure 1.11: Different phenotypes of microglia.
In general, microglia are defined as M1 pro-inflammatory and M2 anti-inflammatory, although it is known that microglia acquire several other phenotypes between these two ends.
Adapted with permission from (Leng and Edison, 2021).

Microglia are defined by two major phenotypes (Figure 1.10): M1, that is associated with release of pro-inflammatory cytokines and may lead to impaired phagocytosis, due to the inhibition of phagocytic activity by pro-inflammatory cytokines (Kwon and Koh, 2020); and M2, characterized by the secretion of anti-inflammatory cytokines with neuroprotective activity (Heneka *et al.*, 2015). In reality, microglia adopt a broad range of

phenotypes, depending upon the pathology state and the regional differences within the brain (Heneka *et al.*, 2015; Kwon and Koh, 2020), as occurs in late stages of AD, where microglia undergo more dramatic morphologic changes, compared to the early stages of the pathology (Leng and Edison, 2021): this microglia has been generally defined a dystrophic microglia (Bisht *et al.*, 2016; Nichols *et al.*, 2019; Stratoulias *et al.*, 2019). In AD, the acquisition of a different phenotype depends also on the vicinity to plaques or tangles and on the response to either Aβ or Tau deposits. Interestingly, the appearance of Tau aggregates seems to shift microglia towards a pro-inflammatory change that sustains the progression of the pathology. Furthermore, proteomic analyses in AD mouse models have confirmed that the disease progression correlates with modifications in microglia phenotype, which progressively transforms from a homeostatic state to a disease-associated phenotype (Leng and Edison, 2021). In AD-transgenic animal models unique phenotypes of microglia, associated with neuritic plaques, have been identified: disease-associated microglia (DAM) and microglia neurodegenerative phenotype (MGnD), both showing upregulation of inflammatory genes and downregulation of homeostatic ones (Hansen, Hanson and Sheng, 2018; Long and Holtzman, 2019; Nichols *et al.*, 2019), such as TREM2 (Leng and Edison, 2021). One of the mechanisms underlying the appearance of DAM and MGnD is the downregulation of the homeostatic genes *Cx3cr1* (C-X3-C motif chemokine receptor 1), *P2ry12* (purinergic receptor P2RY12) and *Tmem119* (transmembrane protein 119), and the upregulation of genes implicated with phagocytosis, perhaps involving epigenomic phenomena that may be either TREM2 dependent or TREM2 independent (with the only difference consisting in the appearance of MGnD only when apoptosis takes place) (Guzman-Martinez *et al.*, 2019; Nichols *et al.*, 2019). It has also been suggested that microglia undergo a three-phase process that starts with the (1) homeostatic phenotype, evolves with an (2) intermediate stage, and eventually with the (3) DAM stage (Guzman-Martinez *et al.*, 2019). Furthermore, *post-mortem* histochemical analyses from human brains have revealed that microglia are strongly associated with Aβ

plaques (Long and Holtzman, 2019; Leng and Edison, 2021). Additionally, microglia may acquire diverse phenotypes spatially and temporally, suggesting that the regional and temporal differences among the different areas in terms of pathology response might be related to the presence of different microglial phenotypes (Figure 1.11). It is even conceivable that the heterogeneity of microglia might underlie the spatial and temporal progression of AD (Leng and Edison, 2021). Moreover, there is compelling evidence suggesting that in AD microglia undergo a process of priming, in which the presence of chronic low-level stimuli is responsible of an altered state of microglia providing altered, excessive or not efficient inflammatory responses to the chronic stimulation (Leng and Edison, 2021).

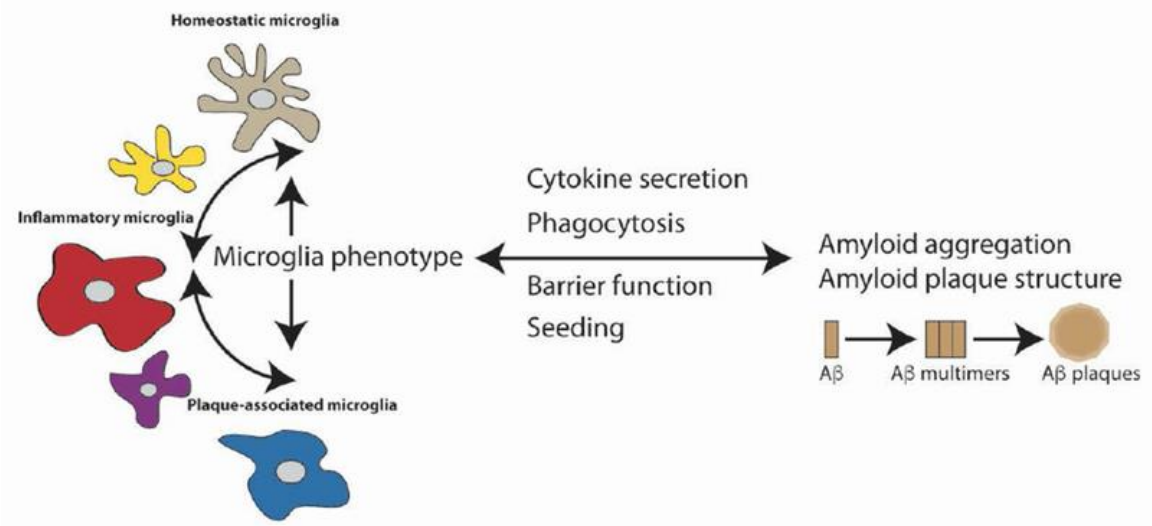


Figure 1.12: Diagram of the several microglia phenotypes identified in presence of plaques
Reproduced with permission from (Nichols *et al.*, 2019).

Noteworthy, one of the genes involved in microglial function whose mutation is considerably associated with increased risk of AD is TREM2 (Scheltens *et al.*, 2016; Long and Holtzman, 2019). Other genes regulating immune function that have been found associated with increased risk of AD are CD33 and Complement Receptor 1 (CR1) (Selkoe

and Hardy, 2016). The discovery of three mutations involving microglia function that increase the risk of developing AD is considered by some as a demonstration of the crucial role carried by neuroinflammation in inducing the disease (Leng and Edison, 2021). The overexpression of CD33 is correlated with increased risk of AD, thus causing reduced microglia activation and phagocytosis and increased Aβ pathology, while its knockout in 5xFAD mice improves pathology and cognitive performance (Griciuc *et al.*, 2013; Selkoe and Hardy, 2016; Long and Holtzman, 2019). TREM2 is expressed in microglia and promotes phagocytosis and modulation of inflammatory response, however a rare mutation, the R47H, causes the formation of a loss-of-function variant that dramatically increases the risk of AD (Long and Holtzman, 2019). The importance of TREM2 is confirmed in mouse models of AD where the depletion or the genetic knock-out of TREM2 leads to a reduced burden of Aβ in young mice and an increase, likely age-driven in older mice, accompanied by increased amounts of dystrophic neurites around plaques (Long and Holtzman, 2019), with microglia failing to circle Aβ fibrils and increased apoptosis (Hansen, Hanson and Sheng, 2018); also, TREM2 seems to have a role in suppressing amyloid toxicity and preventing Tau spreading, at least in the early stages of the pathology: in fact, it has been suggested that microglia surround amyloid aggregates, in a process mediated by TREM2 and ApoE, thus compacting Aβ fibrils into potentially packed and less toxic aggregates (Hansen, Hanson and Sheng, 2018; Long and Holtzman, 2019).

Microglia remove Aβ through a mechanism that is called LANDO (LC-3-associated endocytosis) that also recycles Aβ receptors (Figure 1.12); if this mechanism is genetically knocked down, there is increased Aβ deposition, microgliosis and increased release of pro-inflammatory cytokines, synapse dysfunction and Tau hyperphosphorylation (Long and Holtzman, 2019). Moreover, phagocytosis of Aβ by microglia leads to further activation of microglial cells: when Aβ binds to specific receptors (either TLRs or CD14 or CD36) (Nichols *et al.*, 2019; Leng and Edison, 2021), activation of the inflammasome complex NLRP3 occurs, which in turn leads to IL-1β release and caspase-1 activation (Long and

Holtzman, 2019) as well as morphologic changes of microglia (Leng and Edison, 2021). NLRP3 activation is followed by assembling of monomers of apoptosis-associated speck-like protein containing a CARD domain (ASC), which form fibrils that in turn lead to caspase-1 recruitment and its consequent activation. ASC further aggregates and can be leaked extracellularly, where can be taken up by other microglial cells or can bind other Aβ aggregates, thus contributing to the cross-seeding of Aβ oligomers and to their spreading. Therefore, microglia role may have opposite effects: restricting the spread of amyloid pathology or exacerbating its spreading (Long and Holtzman, 2019; Leng and Edison, 2021). In fact, the phagocytic elimination of Aβ is considered too slow to be effective, and is mostly directed at plaques with no effect on oligomers that, rather, induce production of pro-inflammatory cytokines that inhibit microglia phagocytosis (Leng and Edison, 2021). Importantly, microglia lacking TREM2 and CD68 has been identified as well, thus confirming that an inefficient microglia with compromised phagocytic capacity establishes in AD, therefore the pathology further worsens (Heneka *et al.*, 2015; Leng and Edison, 2021). The internalization of Aβ triggers other mechanisms, such as the scavenger receptors, thus activating NF-κB, JNK and MAPK pathways that lead to IL-1β upregulation; alternatively, the binding of Aβ to TLR2 receptor causes TNF overproduction (Leng and Edison, 2021).

Tau oligomers can also trigger microglia activation: animal models of AD and tauopathies are characterized by microglia associated with Tau tangles which appear dystrophic and therefore not effective in removing Tau aggregates. These dystrophic changes seem to be triggered by chronic Aβ accumulation and precede the onset of Tau pathology. Moreover, microglia can contribute to the spreading of Tau aggregates through phagocytosis and exosomes secretion, similarly to their role in Aβ spreading (Leng and Edison, 2021). In the amyloid cascade-inflammatory hypothesis, glia activation is considered a bridge linking Aβ and Tau pathology: one link might be NLRP3 activation, while another one seems to be the pathway TREM2-DAP12 (DNAX activation protein of 12kDa). Interestingly, some

studies suggest that while the binding of Aβ to TREM2 induces protective responses by microglia, the binding of Tau to TREM2 triggers Tau-mediated neurodegeneration through the TREM2-DAP12 pathway: this observation suggests that microglia activation is protective from neurodegeneration in MCI, while accelerates the pathology in late stages of AD when the role of Tau becomes predominant (Perry and Holmes, 2014; Morales *et al.*, 2016; Nichols *et al.*, 2019; Leng and Edison, 2021).

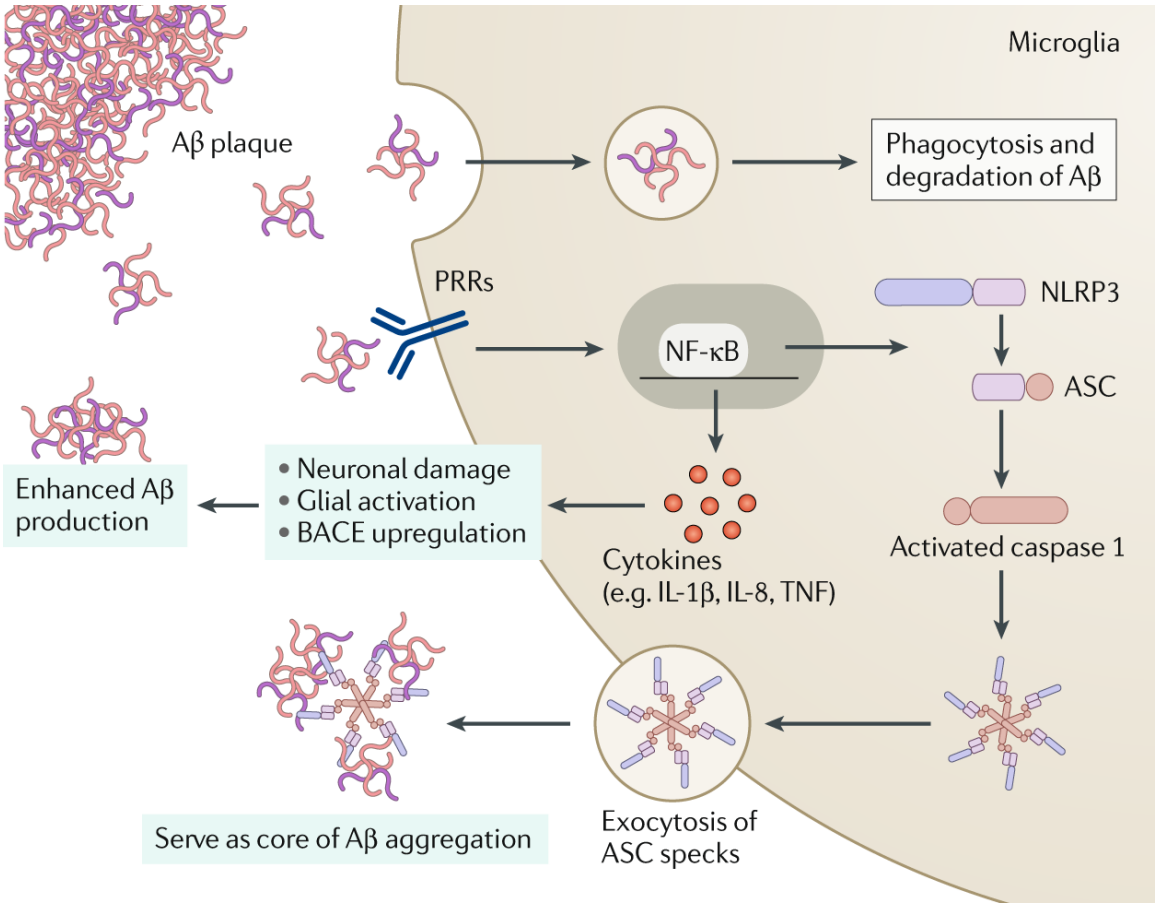


Figure 1.13: LANDO mechanism of microglia-mediated Aβ phagocytosis
Reproduced with permission from (Leng and Edison, 2021).

Microglia activation has negative correlations with brain integrity and functional activity, evaluated as hippocampal volume and neuronal glucose metabolism, in AD patients; also, there is a negative correlation between microglia activation and cognitive function but not

with the amyloid load in AD patients. Moreover, Aβ induced microglia activation leads to the increased release of pro-inflammatory (and cytotoxic) cytokines, such as the cytokines TNF-α, IL-1β and interleukin-6 (IL-6), the chemokine (C-C motif) ligand-2,3,4 (CCL2, CCL3, CCL4), activation of caspase-1 with the inflammasome NLRP3, upregulation of inducible nitric oxide synthase (iNOS) and nicotinamide dinucleotide phosphate (NADPH) oxidase. This leads to the consequent overproduction of reactive nitrogen species and ROS, respectively, which provoke further damage to proteins, lipids and nucleic acids by oxidization or addition of nitrogen moieties (nitrosylation, S-nitrosylation, tyrosine formation) (Heneka *et al.*, 2015; Kempuraj *et al.*, 2016; Leng and Edison, 2021).

Cline and colleagues indicate a decisive role for astrocytes in the clearance of excessive levels of Aβ (Cline *et al.*, 2018). Astrocytes are the most common glial cells in CNS (Kwon and Koh, 2020) that preside at CNS homeostasis by contributing to the regulation of the cerebral blood flow, providing metabolic and neurotrophic support, participating at synapse formation, plasticity and transmission (Morales *et al.*, 2016; Leng and Edison, 2021). The pathological response of astrocytes involves morphology changes, like microglia, aimed at protecting neurons and CNS homeostasis from injury, that is usually known as astrogliosis (Figure 1.13) (Heneka *et al.*, 2015). Astrocytes can acquire two different phenotypes, known as A1 and A2: the former activates pro-inflammatory pathways that lead to cell apoptosis, while the latter is the "protective" phenotype (Leng and Edison, 2021). Similarly to microglia, astrocytes may display a broad spectrum of phenotypes, depending on stage of the disease and localization within the brain (Kwon and Koh, 2020), as confirmed by studies using RNA-sequencing and morphologic analyses, that revealed heterogeneity even within the same brain region (Giovannoni and Quintana, 2020). Aβ exposure may trigger pro-inflammatory cytokine release, thus activating neuroinflammation and astrocyte activation (Heneka *et al.*, 2015). In fact, the A1-astrocytes are abundant in AD, as revealed by *post-mortem* analyses that display their presence in co-localization with amyloid plaques. In this scenario, astrocytes show the

diminished clearance efficiency that may exacerbate the amyloid pathology and the disease development (Leng and Edison, 2021). Also, pro-inflammatory astrocytes have been linked with synaptic degeneration and glutamate dysregulation that are responsible in turn for neuronal excitotoxicity (Kwon and Koh, 2020). Astrocyte activation is not entirely clear: it is believed that surface receptors, such as TLRs, are involved (Morales *et al.*, 2016) although the overall process seems highly heterogenous (Giovannoni and Quintana, 2020). Astrocyte activation leads to a morphology change, similarly to activated microglia, with consequent increased expression of glial fibrillar acidic protein (GFAP), usually identified as sign of astrocyte activation (Morales *et al.*, 2016), although the extent of such upregulation depends on type of injury and proximity to the injury (Giovannoni and Quintana, 2020). The pathways involved in astrocyte activation are Janus kinase/signal transducer and activator of transcription (JAK/STAT3), calcineurin (CN), NF-κB and MAPK, although only JAK/STAT seems holding a crucial role in astrocyte activation, while the other modulate astrocyte activity (Giovannoni and Quintana, 2020).

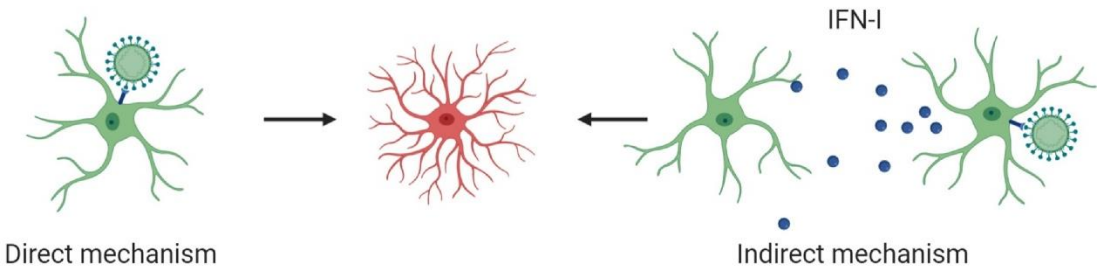


Figure 1.14: Schematic mechanisms of astrocyte activation
Adapted with permission from (Giovannoni and Quintana, 2020).

Some studies have suggested that astrocytes, microglia and neurons are synchronized in promoting neurodegeneration: the activation of NF-κB in astrocytes leads to the release of the C3 component of the complement, which in turn binds on neuronal and microglial surface receptors to induce neuronal dysfunction and microglial activation (Morales *et al.*, 2016; Leng and Edison, 2021). At the same time, activated microglia can induce astrocytes

to shift towards the A1 phenotype by releasing pro-inflammatory cytokines: astrocytes and microglia can even create a positive feedback loop in the pro-inflammatory conditions of AD that ultimately exacerbates the neuroinflammation (Guzman-Martinez *et al.*, 2019; Leng and Edison, 2021). On the other hand, microglia and astrocyte create a positive feedback that leads to the acquisition of their neuroprotective phenotypes (Kwon and Koh, 2020). Notably, the crosstalk neuron-microglia is impaired during AD: normally, the pathways involving chemokines and their respective receptors maintain microglia in an homeostatic state, however both chemokines and chemokine receptors are decreased in AD, thus suggesting a loss of regulation for microglia homeostasis (Leng and Edison, 2021).

A recent meta-analysis study explained the spatial and temporal distribution of neuroinflammation in AD: neuroinflammation is more pronounced in AD rather than MCI, following an increased microglia activation as the disease progresses. The study demonstrates that microglia activation begins in the neocortex in MCI stage, then spreads towards ventral areas of the brain, even reaching cerebellum, affecting especially the temporal areas where the Tau pathology starts, and becomes dominant in the late stages of the pathology (Figure 1.14) (Bradburn, Murgatroyd and Ray, 2019; Leng and Edison, 2021). Given this evidence, Leng and Edison suggest in their review that the chronic inflammatory background that occurs in aging provides mild stimuli that provoke microglia priming, followed by a period of potential protection provided by activated microglia in order to cope with Aβ accumulation (Leng and Edison, 2021). Priming is an exaggerated response to a second inflammatory stimulus much higher than the first one; in other words, an event that creates conditions whereby microglia chronically develop a more pro-inflammatory phenotype, can cause heightened responses by microglia itself when a second inflammatory event occurs (Perry and Holmes, 2014). For example, the systemic inflammation caused by obesity primes the microglia to shift towards a pro-inflammatory phenotype, therefore the presence of amyloids may cause an excessive neuroinflammatory

response that ultimately makes microglial process ineffective and further accelerates the progression of the disease (Perry and Holmes, 2014). Notably, an ineffective Aβ clearance, in combination with increasing Tau deposition as the disease evolves, impairs microglial function to a point that microglia activation becomes detrimental, especially in late stages of AD (Leng and Edison, 2021). Aging and traumatic injuries seem to contribute to a reduction in microglia efficacy, thus inducing chronic neuroinflammation that ultimately leads to neurodegeneration (DiSabato, Quan and Godbout, 2016; Suescun, Chandra and Schiess, 2018).

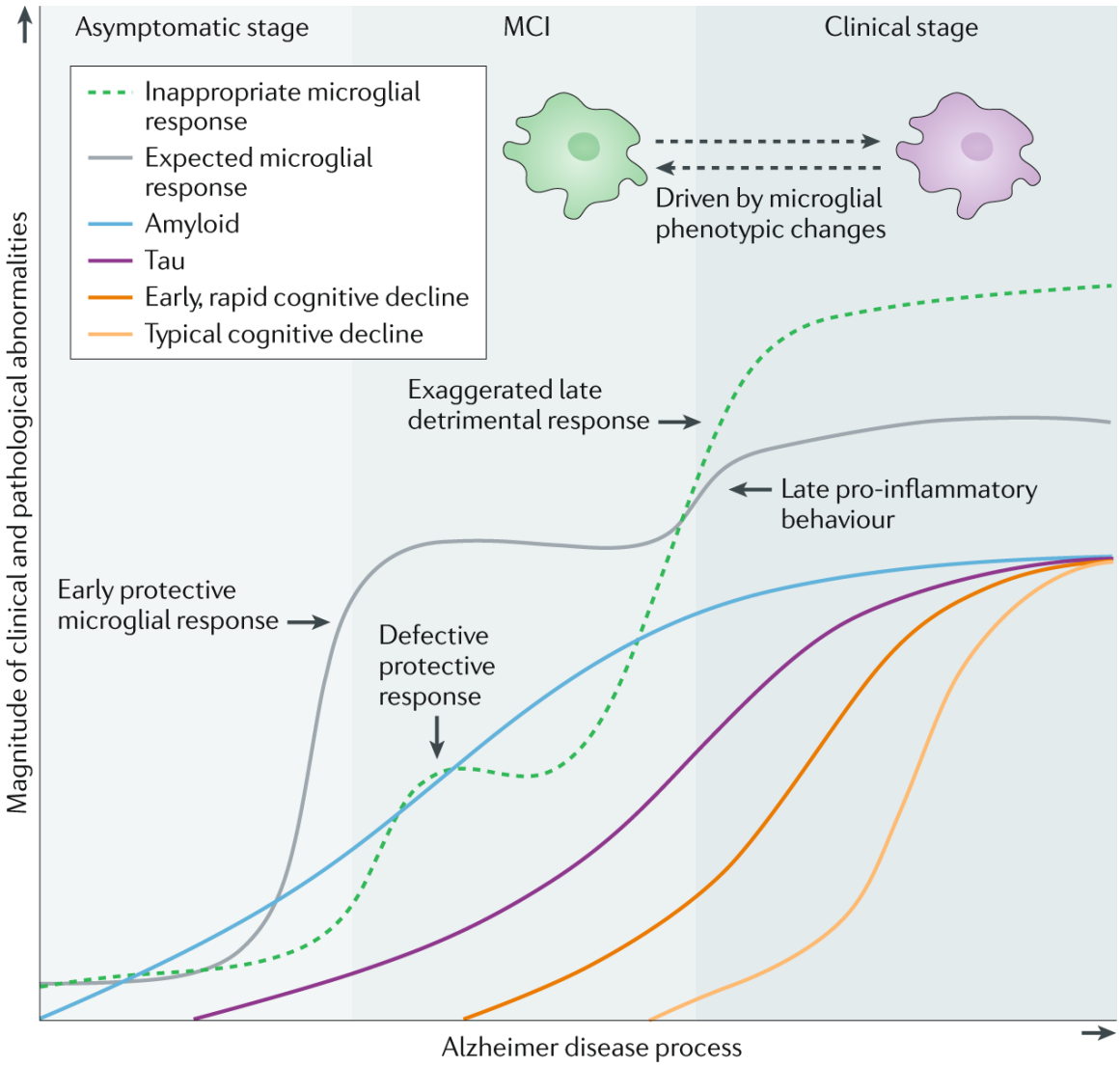


Figure 1.15: Morphologic changes in microglial activation affect Alzheimer disease progression.
Reproduced with permission from (Leng and Edison, 2021).

Obesity: an "inflammatory" condition

Obesity is a medical condition defined as an excessive accumulation of adipose tissue that may pose serious threats for life, due to an imbalance between energy intake and energy expenditure (World Health Organization (WHO), 2016). It has emerged as a serious health concern in 20th century due to the increased food supply and availability that characterizes the most developed countries, but also due to reduced physical activity and increased use of drugs that may cause weight gain (Heymsfield and Wadden, 2017). Moreover, obesity is associated with a decreased life expectancy: it is believed that between 5 and 20 years of life may be lost, depending on the pathologies that accompany obesity and to the severity of the obese condition itself (Blüher, 2019). Obesity has a multifactorial pathogenesis, due to genetic, lifestyle, environmental and familiar factors (Figure 1.15) (Bray *et al.*, 2016): there are at least eleven rare monogenic forms of obesity, including deficiency in leptin and melanocortin-4 receptors, which are expressed in the hypothalamus and regulate energy homeostasis. Over 300 loci have been identified with increased risk of obesity (Heymsfield and Wadden, 2017), although they account for 2-5% of all obesity cases (Blüher, 2019). Moreover, obesity might be inherited, as shown by several studies that demonstrate a rate of inheritance for high body mass index (BMI) oscillating from 40% to 70% (Blüher, 2019). The obesity diagnosis is usually assessed analyzing the BMI, an indicator that has good correlation with the body fat, although it is not considered highly reliable (Nuttall, 2015; Bray *et al.*, 2016), because it does not consider individual variances in skeletal muscle, lean-fat mass composition, ethnic differences and medical conditions (Schwartz *et al.*, 2017). Despite the limited reliability of this parameter (some have suggested to consider BMI as a screening measurement rather than a diagnostic method (Bray *et al.*, 2016)), the WHO establishes that a person is considered overweight when the BMI is greater or equal than 25 Kg/m^2, while is considered obese when BMI > 30 Kg/m^2 (Bray *et al.*, 2016; WHO, 2016). Obesity is strongly associated with other co-morbidities,

such as type 2 diabetes mellitus (T2DM), cardiovascular diseases and neurodegenerative disorders (Haslam and James, 2005; Blüher, 2019).

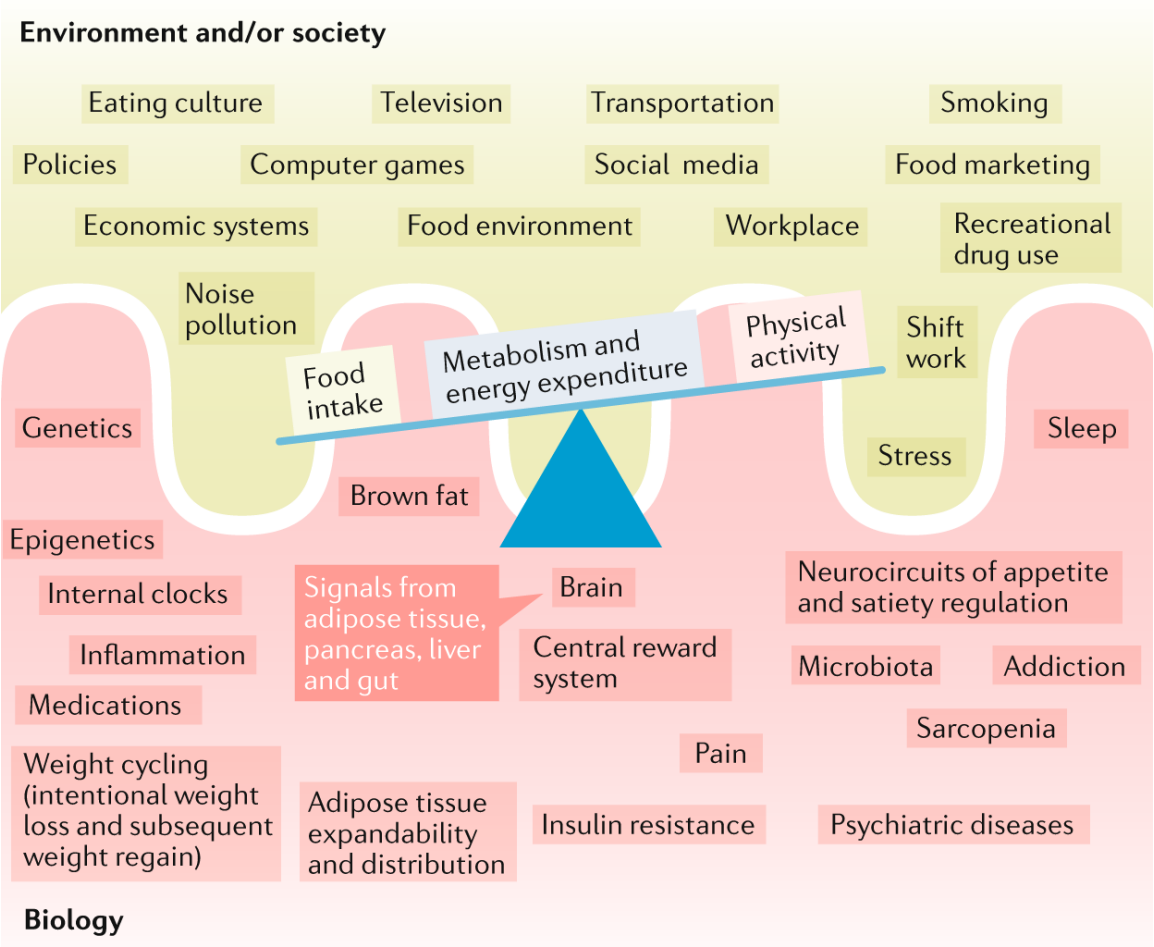


Figure 1.16: Complex of factors that contribute to the development of the obesity
Reproduced with permission from (Blüher, 2019).

From a pathophysiological point of view, obese people have an increased accumulation of lipids, mainly triglycerides, in the adipose tissue, as well as increased volumes of liver, skeletal muscle and other organs (Heymsfield and Wadden, 2017). The adipocytes normally control energy homeostasis and regulate storage and utilization of nutrients; also, adipocytes synthesize and secrete hormones like leptin and adiponectin, whose release rates strictly depend on the amount of fat that is distributed. Based on this correlation, the

more fat is accumulated, the more leptin is released, while adiponectin decreases as fat increases (Heymsfield and Wadden, 2017; Crispino *et al.*, 2020). An important feature of adipose tissue in obesity states is the increased presence of macrophages and other immune cells that secrete pro-inflammatory cytokines and contribute to the typical insulin resistance that characterizes obesity (Heymsfield and Wadden, 2017). In fact, chronic overfeeding cause adipocyte expansion with consequent hypertrophy; when adipose tissue expandability becomes low, adipocytes result hypertrophic, so are less able to store and take up fat, due to inhibition of lipoprotein lipase (Leigh and Morris, 2020) and prone to metabolic stress, thus activating pro-inflammatory pathways that in turn cause insulin resistance and higher release of fatty acids (Crispino *et al.*, 2020). Additionally, expanded adipocytes lack oxygen and receive decreased blood supply, thus causing cell death and recruitment of macrophages in the adipose tissue. The infiltrated macrophages remove cell debris and release additional pro-inflammatory cytokines, that aggravates insulin resistance and inflammation (Kacířová *et al.*, 2020; Leigh and Morris, 2020). The massive release of fatty acids, due to adipose tissue impairment, allows them to bind TLRs receptors that further activate pro-inflammatory mechanisms (such as NF-κB translocation to the nucleus with consequent production of TNF-α and IL-6) thus exacerbating insulin resistance, lipolysis and altering secretion of leptin and adiponectin (Figure 1.16) (Crispino *et al.*, 2020). The excess of lipids is distributed in many compartments with the subcutaneous adipose tissue storing most of them. Moreover, higher amounts of visceral adipose tissue surround many organs, thus potentially contributing to the metabolic impairments typical of obesity and other simultaneous conditions, like hypertension. Additionally, obese people show higher resting energy expenditure, increased cardiac output, increased blood pressure and higher volume of pancreatic β-cell compared to non-obese people (Heymsfield and Wadden, 2017). Moreover, in obesity mitochondrial function is heavily affected, thus contributing to the inflammatory cascade: the impairment of the mitochondrial activity leads to increased production of ROS that is not properly counteracted by efficient

antioxidant response, with ensuing oxidative damage, metabolic derangements and activation of pro-inflammatory mechanisms. Specifically, liver and skeletal muscle show considerable mitochondrial dysfunction that reduces oxidation of fatty acids, impairs glucose metabolism, increases fatty acids spillover and reduces insulin sensitivity. Moreover, the overload of fatty acids coming from high-caloric/high-fat diet results overwhelming for mitochondria, whereby an excessive β-oxidation activity occurs, which in turn results in a massive flow of electrons that in turn increases ROS production, activates NF-κB and other pro-inflammatory molecules.

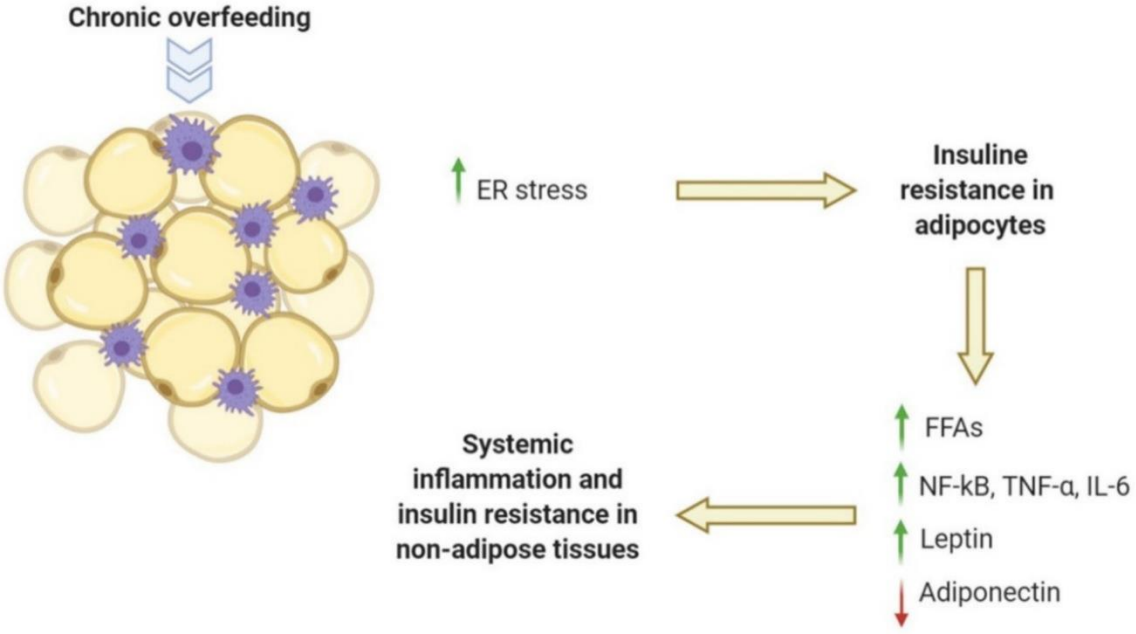


Figure 1.17: Overfeeding and Adipose tissue limited expandability leads to a systemic low-grade inflammation
From (Crispino *et al.*, 2020). Open access license for reprinting.

Also, a promoting role for oxidative stress and metabolic alterations is attributed to 5' adenosine monophosphate-activated protein kinase (AMPK), normally inhibited in the hypothalamus by the anorexigenic hormones leptin and insulin. In obesity states, when hypothalamic leptin and insulin sensitivity dramatically decreases, activation of AMPK causes hyperphagia, thus promoting obesity (Crispino *et al.*, 2020).

Central regulation of food intake and energy expenditure involves hypothalamus that receives information coming from adipose tissue, liver, pancreas and other organs, thus constantly monitoring the energetic needs of the organism (Heymsfield and Wadden, 2017). The hypothalamus is in fact the area of the CNS that governs and modulates energy expenditure, food intake, and glucose metabolism; the hypothalamic neurons project to the brain areas that modulate sympathetic and parasympathetic nervous system, which in turn control peripheral organs involved in metabolism (Cai, 2013). The arcuate nucleus (ARC) of the mediobasal hypothalamus is the region that monitors the metabolic signals coming from periphery (hormones like leptin and insulin, or the ones secreted by gastrointestinal (GI) tract) (Cai, 2013). In ARC two sets of neurons preside at energy expenditure, regulate food intake and in general control energy balance, AgRP neurons and POMC neurons (Schwartz *et al.*, 2017). The AgRP neurons exclusively express the agouti-related protein, from which they derive their name. These neurons are expressed in conditions of negative energy balance and weight loss and are normally inhibited by insulin and leptin; in condition of fasting the levels of insulin and leptin are low, so AgRP neurons are no longer inhibited and drive the need to eat, thus providing a strong stimulus to feeding and perhaps activating reward mechanisms. Adjacent to AgRP neurons, there are the pro-opiomelanocortin (POMC) expressing neurons, that release the anorexic neuropeptide α-melanocyte–stimulating hormone (α-MSH): when this hormone binds melanocortin-4 receptors on neurons of the paraventricular hypothalamic nucleus, and at the same time leptin stimulates POMC neurons, food intake is reduced. Therefore, when AGRP neurons are activated in fasting conditions, POMC are inhibited, and vice versa during feeding (Schwartz *et al.*, 2017). Furthermore, the central regulation of feeding behavior is supported by signals coming from the GI tract: in fact, feeding causes the release of gut-derived hormones such as glucagon-like peptide 1 (GLP-1) and cholecystokinin that promote satiety. These hormones activate an ascending circuit that recruits vagal afferent neurons projecting signals from GI tract to hindbrain areas, for example the nucleus of the

solitary tract. In turn, some neurons in this tract convey signals to the parabrachial nucleus (PBN), in which the calcitonin-gene related ($CGRP^{PBN}$) protein expressing neurons play a crucial role: these neurons receive several information related to food consumption that influences also secretion of GLP-1, cholecystokinin and gastric distension. Importantly, these neurons are implicated not only in satiety, but also in anorexia, since AgRP neurons inhibit $CGRP^{PBN}$ ones when feeding is stimulated. The contribution of POMC and $CGRP^{PBN}$ in limiting food intake is different but converging: while the former have a physiological role in limiting food intake over a long time, the latter have a crucial role in individual meal termination (Schwartz *et al.,* 2017). Hypothalamus regulation of appetite behavior is impaired in obesity: consumption of high-fat diet (HFD) can cause structural changes to hypothalamic neurons as early as 3 days after beginning of HFD, with consequent impairment of cytoskeleton and synaptic plasticity: in particular the anorexigenic POMC neurons show strong loss of synapses, together with a massive increase of microglial activity in the ARC, that is prodromal to obesity-induced neuroinflammation (Crispino *et al.,* 2020).

Obesity as risk factor for AD

Obesity is one of the most prominent risk factors for AD. Compelling evidence has established that mid-life obesity correlates with lower cognitive performance and increased risk for AD (Whitmer *et al.*, 2005; Beydoun, Beydoun and Wang, 2008; Anstey *et al.*, 2011; Xu *et al.*, 2011; Smith *et al.*, 2011; Letra, Santana and Seiça, 2014; Chuang *et al.*, 2016; Kivimäki *et al.*, 2018; Nday, Eleftheriadou and Jackson, 2018; Singh-Manoux *et al.*, 2018; Lloret *et al.*, 2019). This increased risk is independent of other co-morbidities, such as cardiovascular diseases (Miller and Spencer, 2014). Moreover, the increased risk of AD posed by obesity has a positive correlation with cognitive decline when obesity occurs in mid-life; however, obesity in late life (age >65 years) does not show a clear correlation with cognitive impairment (Miller and Spencer, 2014). Interestingly, Miller and Spencer

reviewed several studies in which mid-life obesity is associated with brain atrophy and decreased hippocampal volume, strong predictors of cognitive decline and dementia: the atrophy of the brain and the reduced hippocampal volume due to mid-life obesity amplify the risk of AD (Miller and Spencer, 2014). In another study, the measurement of the sagittal abdominal diameter determined that people with a larger diameter have 3-fold increased risk of developing dementia (Pugazhenthi, Qin and Reddy, 2017). Interestingly, also overweight people have increased risk of developing AD, even if it is about half of the risk shown by obese people (Pugazhenthi, Qin and Reddy, 2017). Different aspects of brain function have been analyzed in correlation with obesity and several studies have identified that executive function, episodic memory, attention, intelligence, cognitive flexibility and processing speed are negatively associated with BMI and adiposity (Leigh and Morris, 2020). Noteworthy, also the type of diet is correlated to cognitive function: in fact, HFDs increase the odds of developing AD by 2-fold in non-demented adults past 70 years of age, while intake of unhealthy foods for four years causes smaller hippocampal volumes in people between 60 and 64 years of age (Leigh and Morris, 2020). These observations have also been investigated in animal models of obesity: several studies have demonstrated in rodents decreased learning and memory function upon high-fat feeding, as well as a reduction of synaptic plasticity in hippocampus and cerebral cortex, increased apoptosis and impairment of BBB (Molteni *et al.*, 2002; Stranahan *et al.*, 2008; Miller and Spencer, 2014). There is a U-shaped correlation between body weight and cognitive function, with both low and high weight posing higher risk for AD (Crous-Bou *et al.*, 2017; Silva *et al.*, 2019). Moreover, AD leads to body weight loss in the preclinical AD, thus suggesting that the correlation between body weight and AD is a consequence of mid-life obesity (Crous-Bou *et al.*, 2017), while obesity in late age does not correlate with AD (Silva *et al.*, 2019). It is worthwhile to mention that concurrent pathologies, such as T2DM, may contribute to strengthen the obesity-AD association (Crous-Bou *et al.*, 2017). Several pathological mechanisms linking AD and obesity have been proposed from investigations performed in

animal models: among the plethora of mechanisms that have been identified, neuroinflammation has gained growing interest in recent years. (Cai, 2013; Erion *et al.*, 2014; Aguilar-Valles *et al.*, 2015; Hao *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Spencer *et al.*, 2017; Busquets *et al.*, 2017; Guillemot-Legris and Muccioli, 2017; Pugazhenthi, Qin and Reddy, 2017; Kacířová *et al.*, 2020; Leigh and Morris, 2020).

Obesity-induced neuroinflammation

Obesity initiates a peripheric inflammation, due to the increased presence of macrophages in the adipose tissue, with consequent higher release of pro-inflammatory cytokines and free fatty acids, which in turn lead to liver and muscle insulin resistance. Moreover, the amount of adipose tissue-secreted pro-inflammatory cytokines is proportional to the amount of fat tissue. This increased release of pro-inflammatory cytokines brings to a state of systemic and chronic low-grade inflammation, also known as metainflammation (or "metabolic inflammation") (Lumeng and Saltiel, 2011; Mraz and Haluzik, 2014; Chen, Zhang and Huang, 2016; Heymsfield and Wadden, 2017), which is both cause and consequence of the obesity (Miller and Spencer, 2014). Adipose tissue normally secretes a broad group of hormones, such as leptin and adiponectin, in addition to cytokines; in obesity, the secretion of these hormones is significantly affected. Leptin and adiponectin are key-mediators of the cross-talk between adipose tissue and brain (Nday, Eleftheriadou and Jackson, 2018), although having opposite roles: when fat accumulates leptin increases, while adiponectin decreases; likewise, when fat mass reduces adiponectin levels are higher, while leptin ones drop (Heymsfield and Wadden, 2017). Also, leptin is involved in hippocampal development and function, promotes neuronal plasticity, modulates LTP by acting on NMDA receptors, provides neuroprotection through the Jak-2/STAT pathway with consequent reduction of mitochondrial oxidative stress; adiponectin displays similar protective actions by inhibiting pro-apoptotic pathways and by promoting suppression of ROS-induced toxicity (Nday, Eleftheriadou and Jackson, 2018).

HFD-induced obesity leads to neuroinflammation (Cai, 2013). In fact, HFD causes activation of inflammatory pathways in the neurons of the hypothalamic ARC, due to increased circulating free fatty acids that are able to cross BBB and cause leptin and insulin-resistance, likely due to fatty acids-mediated activation of JNK and NF-κB pro-inflammatory pathways (Lumeng and Saltiel, 2011; Rahman, Bhusal, *et al.*, 2018). In fact, the ARC and the median eminence of the hypothalamus, the area postrema and the subfornical organ have a fenestrated BBB, thus exposing the hypothalamus to circulating nutrients and peripheral signals (such as pro-inflammatory cytokines derived from peripheral circulation) which allow the hypothalamus itself to regulate food intake and feeding behavior (Miller and Spencer, 2014; Klein *et al.*, 2019). The excessive presence of free fatty acids, especially after prolonged periods of HFD feeding, on one hand disrupts BBB integrity. Disrupted BBB allows the penetration of massive amounts of fat to the brain (Leigh and Morris, 2020), in particular the saturated fatty acids (such as palmitic acid and stearic acid). The excess of free fatty acids leads to microglia activation (via TLR4 signaling) in the hypothalamic ARC and at the mediobasal hypothalamus, with consequent increased expression of inflammatory cytokines that further exacerbates neuroinflammation, which ultimately provokes neurodegeneration (Figure 1.17) (Cai, 2013; Miller and Spencer, 2014; Schwartz *et al.*, 2017; Rahman, Bhusal, *et al.*, 2018; Crispino *et al.*, 2020). Consequently, the role of the hypothalamus in regulating food intake and control body weight is impaired, thus further contributing to the peripheral insulin resistance, peripheral tissue damage and weight gain (Lumeng and Saltiel, 2011; Cai, 2013; Schwartz *et al.*, 2017). From a mechanistic point of view the IKKβ/NF-κB pathway is mainly involved in neuroinflammation via TLR receptors and/or TNF receptors in microglial cells: binding of fatty acids or cytokines to TLRs or TNFRs triggers an intracellular pathway that activates IKKβ kinases to phosphorylate IκB proteins, thus promoting NF-κB translocation into the nucleus, normally inhibited by non-phosphorylated IκB. Also, the IKKβ/NF-κB pathway upregulates the suppressor of

cytokine signaling-3 (SOCS3), that in turn suppresses leptin and insulin signaling (Cai, 2013). Alternatively, JNKs, part of MAPKs family, are involved in hypothalamic neuroinflammation: MAPKs phosphorylate JNKs and this activation ultimately brings to production of pro-inflammatory cytokines through JNK-modulated action of nuclear transcription factors, such as c-Jun, forkhead box protein O4 (FOXO4), nuclear hormone receptors (Rahman, Bhusal, *et al.*, 2018).

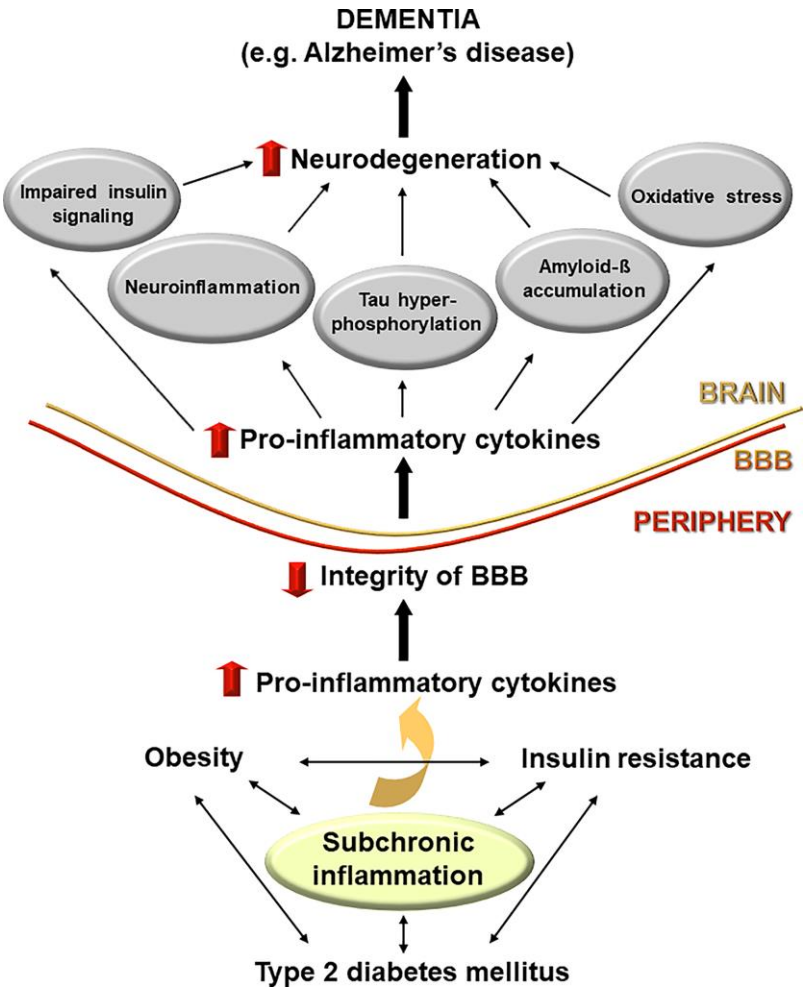


Figure 1.18: Consequences of obesity-induced inflammation on brain function
Reproduced with permission from (Kacířová *et al.*, 2020).

Remarkably, activated astrocytes contribute to the progression of the neuroinflammation by altering hypothalamic neuronal activity: it has been proposed that in obesity astrocytes

remodel synapses by modifying their interaction with synaptic terminals, thus causing altered sensitivity to nutrients and hormones, which is further impaired by the increased release of pro-inflammatory cytokines (Rahman, Bhusal, *et al.*, 2018). Another aspect to consider in obesity-induced neuroinflammation is the crosstalk between microglia and astrocytes: in fact, in the hypothalamus of rodent models of obesity these cells have been shown in close proximity, thus suggesting intercellular communication that may mediate the neuroinflammatory cascade. Several chemokines, such as CX3CR1 (fractalkine), monocyte chemoattractant protein-1 (MCP-1/CCL2), stromal cell-derived factor (SDF-1/CXCL12) have been identified as mediators of the interglial communication. Moreover, it has been proposed a mechanism whereby lipid-laden astrocytes start releasing pro-inflammatory cytokines such as IL-1β, TNF-α, MCP-1, thus starting neuroinflammation; by inducing microglia activation and additional release of pro-inflammatory factors (Rahman, Kim, *et al.*, 2018). It is even conceivable that peripheral and central inflammation eventually converge in a common pathway (Leigh and Morris, 2020), considering also that peripheric inflammation, independently of and associated with obesity, is correlated with cognitive decline and dementia, including AD, as well as higher plasmatic levels of pro-inflammatory cytokines are correlated with decreased cognitive function (Miller and Spencer, 2014). An indirect confirmation of a peripheral trigger for neuroinflammation comes from a study conducted by Erion *et al*, where transplantation of adipose tissue from obese mice in non-obese ones causes neuroinflammation, while its removal from obese mice reduces the expression of inflammatory markers, particularly in the hippocampus (Erion *et al.*, 2014). In fact, the CNS impairment due to HFD is not limited to the hypothalamus or the hypothalamic nuclei regulating appetite, but rather encompasses other brain areas that are involved in cognitive function (Solas *et al.*, 2017; Leigh and Morris, 2020): while short-time HFDs in rodent models of obesity show only disruption of hypothalamus and neuroinflammation of this area after just 3 days (Baufeld *et al.*, 2016; Rahman, Kim, *et al.*, 2018), longer HFD regimens (>2 weeks) lead to

neuroinflammation spreading in other areas, such as hippocampus, amygdala and frontal cortex (Puig *et al.*, 2012; Erion *et al.*, 2014; Miller and Spencer, 2014; Spencer *et al.*, 2017; Cavaliere *et al.*, 2019). In particular, the effects of HFD-induced neuroinflammation on the hippocampus manifest with increased levels of TNF-α and higher expression of microglial markers, as well as involve impaired synaptic plasticity, which is prodromal to cognitive deficits (Miller and Spencer, 2014; Crispino *et al.*, 2020). Furthermore, it has been proposed that in the hippocampus of diet-induced obese (DIO) wild-type mice, long-term HFDs (16 weeks) and very long-term HFDs (around 6 months) trigger Aβ deposition, accompanied by increased glia activation, increased release of pro-inflammatory cytokines, cognitive deficits, anxiety-like and depressive-like behaviors (Puig *et al.*, 2012; Almeida-Suhett *et al.*, 2017; Busquets *et al.*, 2017); likewise, HFD induces Tau hyperphosphorylation in the hippocampus, in association with neuroinflammation and an increased release of pro-inflammatory cytokines, after just 3 days of HFD (Nakandakari *et al.*, 2019). Interestingly, microglia activation induced by HFD leads to synaptic stripping in the hippocampus of DIO mice, suggesting that HFD-induced microglia activation ultimately provokes hippocampal dysfunction due to the synapse removal exerted by microglia; however, this synaptic loss is reversible if the HFD is stopped and mice are fed with a regular chow (Hao *et al.*, 2016). In hippocampi of severely obese individuals, high levels of APP have been identified, while obese individuals in midlife have shown increased plasmatic levels of Aβ, thus confirming the increased risk of AD for people with obesity (Nday, Eleftheriadou and Jackson, 2018).

Another interesting aspect that has been extensively investigated in correlation with obesity and neuroinflammation is the role of BDNF (Figure 1.18): in rodent models of obesity it has been demonstrated a downregulation of BDNF, with consequent reduction of cognitive activities, especially in the hippocampus where BDNF contribution in synaptic plasticity and memory process is prominent (Molteni *et al.*, 2002; Stranahan *et al.*, 2008; Karimi, Motamedi and Ranjbar, 2018; Sona *et al.*, 2018; Cavaliere *et al.*, 2019; Crispino *et al.*,

2020). Moreover, BDNF seems to have an important role in energy metabolism by reducing food intake, therefore HDF-induced BDNF reduction leads to a dysregulation of food intake and ultimately to weight gain and possibly obesity (Abidin *et al.*, 2018; Ramalho *et al.*, 2018).

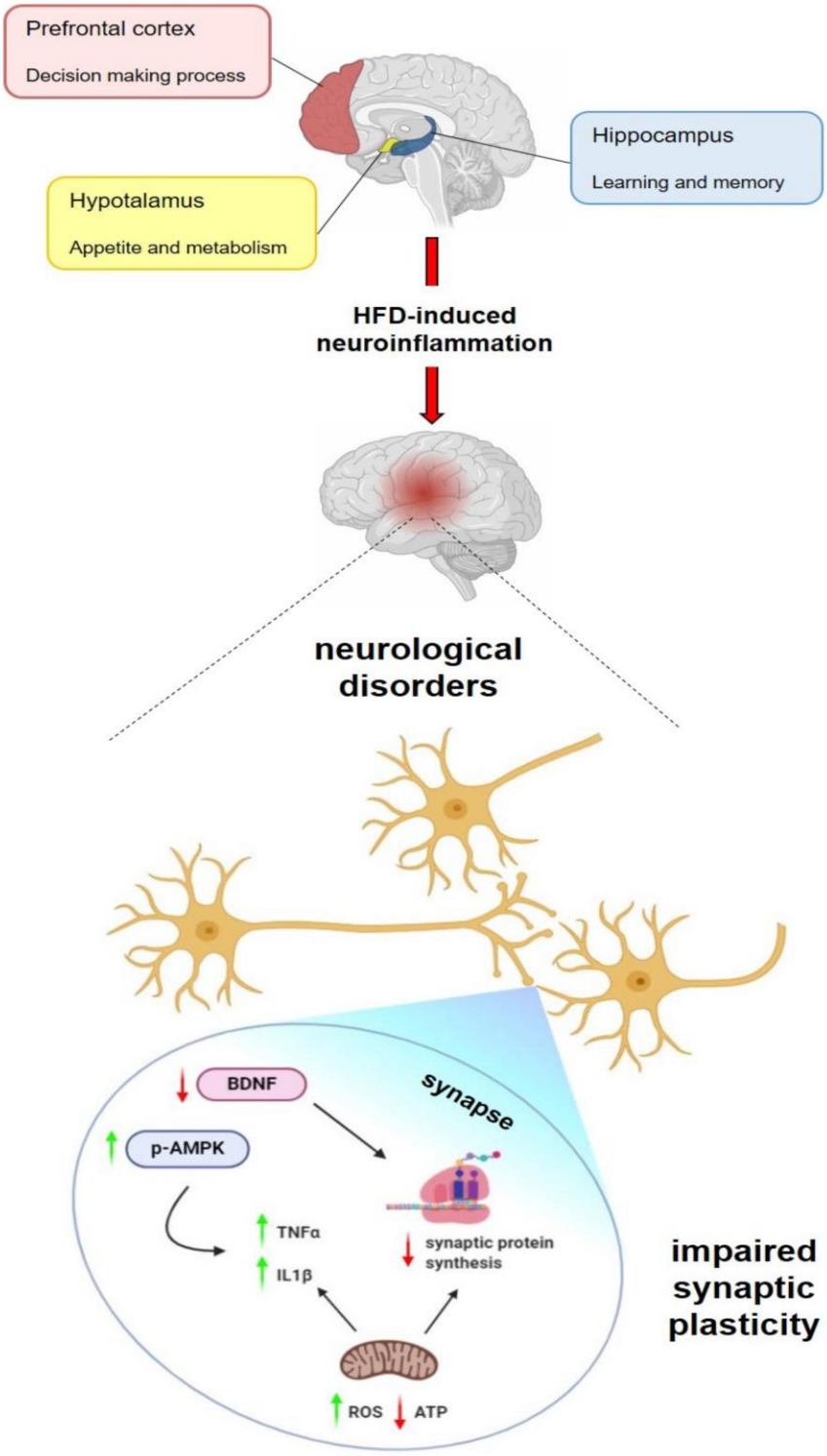


Figure 1.19: Molecular mechanisms involved in obesity-induced neuroinflammation From (Crispino *et al.*, 2020). Open access license for reprinting.

NEAR-INFRARED (NIR) LIGHT: PRINCIPLES AND APPLICATIONS

In the last decade, many different strategies aimed at alleviating obesity-induced neuroinflammation – and, possibly, preventing neurodegeneration and dementia - have been proposed, i.e.: intrahippocampal infusions of insulin (Gladding *et al.*, 2018), NSAIDs (Solas *et al.*, 2017; Rahman, Bhusal, *et al.*, 2018; Rahman, Kim, *et al.*, 2018), modulators of feeding and reward circuits and hormones involved in food intake (Pipatpiboon *et al.*, 2012, 2013; Nuzzo *et al.*, 2019), probiotics that regulate gut microbiota (Solas *et al.*, 2017), (recently recognized as possible mediators of obesity-induced neuroinflammation (Kaur *et al.*, 2020)), physical activity (Kang *et al.*, 2016; Han, Leem and Kim, 2019), dietary intervention (either through diet reversal (Hao *et al.*, 2016) or through use of antioxidant food) (Nuzzo *et al.*, 2018, 2020; de Mello *et al.*, 2019; Zhuang *et al.*, 2019; Di Bonaventura *et al.*, 2020), resveratrol (B.-L. Xu *et al.*, 2018), ursolic acid (Guillemot-Legris and Muccioli, 2017), simvastatin (Wu *et al.*, 2019).

In this study, I propose a novel strategy that has recently been developed and proposed as potential treatment for several CNS pathologies: the near-infrared (NIR) light, used in photobiomodulation (PBM) or low-level laser therapy (LLLT). One definition of PBM is "the use of monochromatic or quasi-monochromatic light from a low power laser or light emitting diode (LED) source to modify or modulate biological functions" (Enengl, Hamblin and Dungel, 2020). The light sources can be coherent, such as the lasers, non-coherent (non-filtered lamps and LEDs) or a combination of both (Avci *et al.*, 2013). The light can modulate cells and tissues activity thanks to the presence of chromophores, namely molecules able to absorb light whose excitation activates pathways that may potentially have a therapeutic effect (Hashmi *et al.*, 2010; Enengl, Hamblin and Dungel, 2020).

The first lasers were discovered in 60's of last century and soon after the first "therapeutic" benefits of these light sources were noticed: hair growth, wound healing and resolution of

skin ulcers; since then, NIR lights and in general LLLT have been extensively exploited to prevent cell death, inflammation and pain (Hashmi *et al.*, 2010). The LLLT/PBM has been used for decades in the medical field and comprises the electromagnetic radiations that fall in the range between 600 and 1100 nm, with power density below 500 mW/cm^2 and energy between 1 and 60 J/cm^2 (Salehpour, Mahmoudi, *et al.*, 2018; Ramezani *et al.*, 2021). The value of 500 mW/cm^2 is the limit above which heating becomes an issue, although heating produced in the tissue depends also on the wavelength, and any excess of heating is removed by the automatic increase of blood flow due to light absorption (Ramezani *et al.*, 2021). Power density (irradiance) and energy density (fluence) are two important parameters to take into account as well as the light wavelength: the optical power of any light is measured in Watts, W, however is reported as power density considering the point of application and the working distance (cm^2); the energy is measured in Joule (J) and is often reported as dose or energy density or fluence, measured in J/cm^2, to take into account the treated area; as far as regards the wavelength of the light, it must be considered that the optical tissue window spans from 650 nm to 1200 nm to allow the light to travel through the skull, namely through a transcranial administration (Enengl, Hamblin and Dungel, 2020). The limits of the optical tissue window are defined by the light absorption of hemoglobin and water: according to several observations the most suitable wavelength are around 810 nm and 660 nm (Enengl, Hamblin and Dungel, 2020), although studies conducted in human subjects have led to beneficial effects using also 1064 nm lights (Gonzalez-Lima and Barrett, 2014; Blanco, Maddox and Gonzalez-Lima, 2017). The wavelength of 1064 nm, according to simulations, has an absorption similar to those at 700-900 nm and penetration depth similar to those at 600-900 nm. As mentioned before, the heating produced by the light in the 600-900 nm range is negligible, in fact these lasers are referred to as cold lasers and, in general, NIR light lasers have good penetration through skin and soft/hard tissue, thus making them suitable for pain relief, wound healing, tissue regeneration, and as anti-inflammatory treatments (Enengl, Hamblin and Dungel, 2020;

Ramezani *et al.*, 2021). In addition, this approach is non-invasive and does not induce major side effects (Saltmarche *et al.*, 2017; Hamblin, 2019; Enengl, Hamblin and Dungel, 2020).

The most typical chromophores in biological tissues are water, hemoglobin, myoglobin, cytochromes, flavins, melanin and other pigments, lipids (Figure 1.19) (Salehpour, Cassano, *et al.*, 2019). Water is relatively transparent to visible and NIR irradiations, however it increases its absorption logarithmically, for example the absorption at 970-980 nm is 200 times higher than the one at 600 nm. In fact, at 900-1100 nm water becomes the most important chromophore. Though, LLLT and NIR light do not produce significant amount of heat at 900-1100 nm: one explanation may come from the concepts of "nanostructured water" or "interfacial water" whereby the water located in this "exclusion zone" absorbs light, thus causing changes in viscosity and pH. Since these zones are usually located within the membranes, these biophysical changes may have consequences on the ion channels embedded in this water, hence activating different mechanisms. On the other hand, the "bulk" water does not absorb light like the one in the exclusion zone, therefore the changes induced by light absorption are not spread in the whole tissue and heating of the tissue does not occur, as it would be expected if all water molecules absorbed the light in the same fashion (Hamblin, 2019; Salehpour, Cassano, *et al.*, 2019). Among the other mentioned chromophores, oxygenated hemoglobin has the highest peaks of absorption on the visible range, then at 924 nm in the NIR interval, while non-oxygenated hemoglobin has two distinct peaks in the NIR region (758 and 914 nm); myoglobin has similar patterns of absorption as hemoglobin, while lipids have absorption peaks in the range 430-1100 nm, flavins have a maximum peak at 450 nm and absorb light up to 520 nm. Cytochromes, such as cytochrome c oxidase (CCO), have absorption peaks that depend on the oxidation states of their heme and copper centers (Figure 1.20). CCO is a complex protein composed of thirteen polypeptides with two heme centers (a and a_3) and two copper centers (Cu_A and Cu_B), that can be either oxidized or reduced, thus displaying up to sixteen different

oxidation states, that imply slight changes in light absorption spectrum (Hamblin, 2019): CCO absorbs in the red (heme a, 605 nm; Cu_A reduced, 620 nm; heme a_3/Cu_B, 655 nm), far-red/NIR (Cu_B oxidized, 680 nm), and NIR spectrum (Cu_B reduced, 760 nm; Cu_A oxidized, 825 nm) and despite having a high molar excitation coefficient, it does not have significant effects on tissue absorption because its relative abundance is low, compared to hemoglobin (Salehpour, Cassano, *et al.*, 2019). CCO is located at the end of the electron transport chain that takes place at the mitochondrial membrane and mediates the transfer of electrons from cytochrome c to molecular oxygen through a series of redox reactions: the result of the electron transfer is the generation of a proton gradient, crucial for the production of adenosine triphosphate (ATP) by ATP synthase (Salehpour, Cassano, *et al.*, 2019).

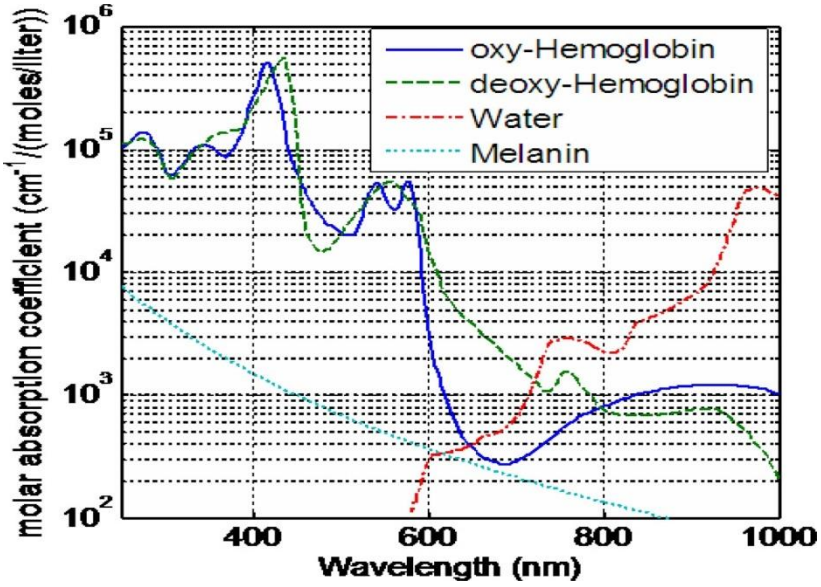


Figure 1.20: Molar absorption coefficient of typical chromophores in biological tissues Reproduced with permission from Sandell & Zhu, 2011.

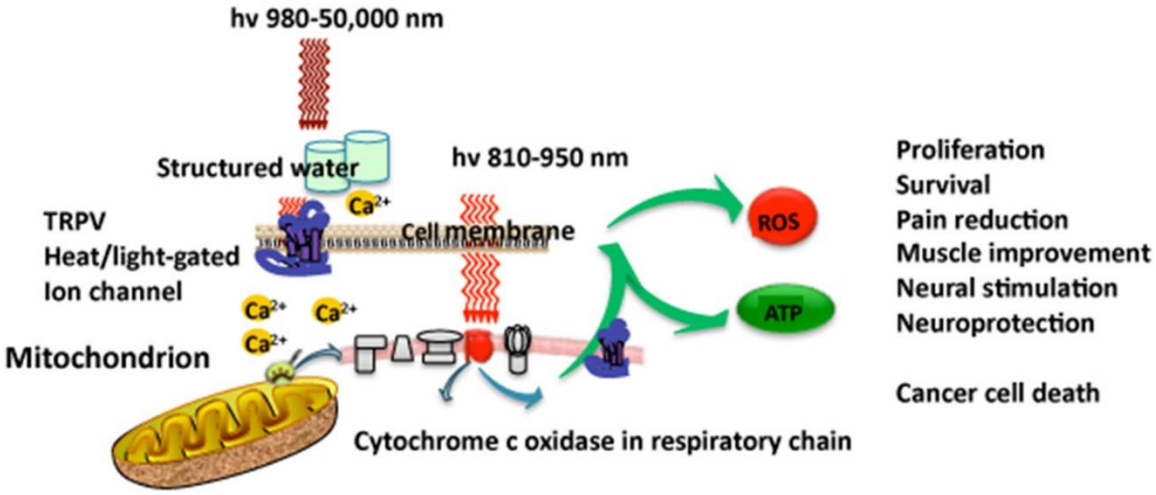


Figure 1.21 Schematic representation of the effects induced by NIR light in a cell Reproduced with permission from (Tsai and Hamblin, 2017).

To determine the most effective wavelength for LLLT/PBM, it must be considered that laser lights have three different interactions with the tissues, absorption, reflection, and scattering (or dispersion), that depend on type of tissue, presence of fat, bone and muscle, abundance of molecules able to absorb different wavelengths (Ramezani *et al.*, 2021). Moreover, scattering, transmittance, reflection of lasers, and in general light penetration inside the body depend on wavelength, irradiance, exposure time, exposed area, coherence of light source (Salehpour, Mahmoudi, *et al.*, 2018; Ramezani *et al.*, 2021); for example, a light in the range 600-900 nm significantly increases the transmittance, with hard tissue, like the bone, compared to soft tissues. In addition, NIR light seems to penetrate in deeper areas of the body, compared to other types of light, reaching distance of 1-5 cm from the application site (Ramezani *et al.*, 2021). Interesting studies have established the amount of energy that is transmitted through a human or an animal skull, the distance travelled by the light and the penetration through the biological barriers. It must be noted that the toughest challenge in this field of research is the considerably higher penetration of the light in animal models compared to humans, therefore the following reports from literature do not allow to establish conclusive criteria over the choice of the better parameters for the

treatment of a specific condition: further investigations are needed to determine and standardize this approach. In this regard, Avci *et al.* described which aspects make this approach still controversial: the elucidation of the exact mechanisms by which the light transduces the signals to induce a biological effect and the great variation in the dosimetry parameters: wavelength, irradiance or power density, pulse structure, coherence, polarization, energy, fluence, irradiation time, contact vs non-contact application, and repetition regimen (Avci *et al.*, 2013). The choice of wavelength is surely paramount when a light therapy wants to be explored; however, the beneficial effects found with a large set of wavelengths does not help in finding the most efficient one, although it seems that irradiations in the NIR range (600-850 nm) are preferred because of their overall most effective outcomes (Salehpour, Mahmoudi, *et al.*, 2018).

One of the most important parameters is surely the energy and the energy density, referred to as fluence, which is calculated by multiplying the irradiance (W/cm^2) by time (seconds) and is defined as the amount of energy per unit of area (J/cm^2) (Salehpour, Mahmoudi, *et al.*, 2018). Studies performed in human subjects have shown wide ranges of fluence, from 10 to 60 J/cm^2, with the interval 10-30 J/cm^2 used in neurological disorders, 13-84 J/cm^2 for psychological disorders and a wide 15-60 J/cm^2 interval for healthy subjects. Notably, different application sites were studied and the forebrain in human remains the preferred one because it lacks hair, which constitute a barrier for the light passage (Salehpour, Mahmoudi, *et al.*, 2018). The criterium of the irradiance depends on other factors, such as delivered dose, number of sessions, duration of treatments and interval between treatment sessions; nonetheless, it was mentioned earlier that values above 500 W/cm^2 cause heating of the tissue, that is undesirable (Salehpour, Mahmoudi, *et al.*, 2018; Ramezani *et al.*, 2021). The type of light used in PBM is important and it is referred to as coherence: in fact, while lasers generate coherent light (ranging from many meters to few mm), LED devices emit non-coherent light; also, the lasers are monochromatic, while LEDs have wider bandwidths; finally it must be noted that lasers have deeper penetration, compared to

LEDS, however LEDs are less expensive and can illuminate wider surfaces (Salehpour, Mahmoudi, *et al.*, 2018). Treatment duration, intervals during session and repetition regimens are other crucial criteria that must be considered, although no studies have been specifically tailored to address this aspect: however, it is reasonable to believe that just one treatment is not enough to have beneficial effects, as well as prolonged treatments may not generate improvements (Salehpour, Mahmoudi, *et al.*, 2018) Another important aspect, that has been extensively investigated in order to establish the most effective, is the energy absorption: Lapchak *et al*. found that approximately 40% of the energy derived from a light with wavelength of 808 nm (and power density of 700 mW/cm^2) is transmitted through the mouse skull, while human skulls seem to receive approximately 4.2-4.7% of the energy from the same light source (Lapchak *et al.*, 2015). Another crucial criterium is the penetration: Salehpour *et al.* established an average penetration of 60-70% in a C57Bl6/J mouse skull for a NIR light (630-810 nm) and 0.2-10% penetration in a human skull with wavelengths around 808-810 nm (Salehpour, Cassano, *et al.*, 2019). Interestingly, simulations studies reported by Salehpour *et al.* have found that an irradiation from a 670 nm light showed best results because it was able to reach deeper structures, although the presence of hair, follicles that may affect the scattering was not taken into account during these studies. Moreover, the authors report that the majority of studies (including studies on human subjects) employ a 800-810 nm wavelength, because it allows the achievement of deepest penetration, although the most important parameter is the amount of energy that is delivered with the light treatment: when the energy at the cortical region is in the interval 1-20 J/cm^2 the biological mechanisms that lead to neuroprotection are activated (Salehpour, Cassano, *et al.*, 2019). Notably, Purushothuman *et al.* claim that their 670 nm treatment reaches 2.5% of the transmitted light reaches the cortex of a C57Bl6/J mouse (Purushothuman *et al.*, 2014). Investigations by Tedford *et al.* conducted in eight unfixed sectioned *post-mortem* human brains established that a 808 nm light applied in 20 different application sites is able to penetrate 40-50 mm of the head (Tedford *et al.*, 2015). A

previous study reported that a light with wavelength of 830 nm applied in 3 different sites (frontal, temporal and occipital area) of a cadaveric human skull shows better transmission, thus delivering much more energy, compared to a 633 nm light (Jagdeo *et al.*, 2012), while another investigations found that lights with longer wavelength (780 and 835 nm), penetrate deeper in several human tissues, including *post-mortem* brain, compared to 633 and 675 nm lights (Stolik *et al.*, 2000). Noteworthy, the effect of NIR light is biphasic, following the principle of hormesis: low doses are stimulatory, while high doses are less effective and even harmful (Enengl, Hamblin and Dungel, 2020). A better explanation of this effect comes from the Arndt-Schulz law: according to this law, light at very low doses does not determine any effect, whereas small but larger irradiations provides beneficial effects because it is able to overcome a threshold above which the delivery of light produces biological effects; however, much higher doses do not cause any effect or rather damage the tissue (Salehpour, Mahmoudi, *et al.*, 2018). There are in fact studies that have tried to establish the maximum tolerated dose as well as the minimum effective dose: one interesting study was conducted by Gonzalez-Lima and Barrett, where rats were illuminated with a 660 nm light (power density 9 mW/cm^2) at different times (20-40-60 minutes); in this study, the authors found that the higher upregulation of CCO is obtained with the shorter treatment (therefore providing the least amount of energy) and no effect with the longest, thus confirming that NIR light shows the principles of hormesis when delivered to the brain (Gonzalez-Lima and Barrett, 2014). The biphasic effect is an important feature of LLLT: in fact, either increasing the power of the irradiation or the time of application may provoke damages to the tissue, while low energy, low powers or short times of illumination have beneficial effects (Hashmi *et al.*, 2010). Enengl *et al.* reviewed several studies to establish which are the most effective application time, fluence and/or irradiance to obtain the most effective outcome when applied to the brain: they concluded that to have a minimum effective energy of 5-10 J/cm^2 at the cortex, fluences around 25-60 J/cm^2 should be applied; therefore, the application times are different, depending on the

amount of fluence and irradiance that is requested to be delivered to observe a successful effect. For example to have irradiance of 250 mW/cm^2 4 minutes of treatment should be enough, while to have 100 mW/cm^2 the treatment should last 10 minutes (Enengl, Hamblin and Dungel, 2020).

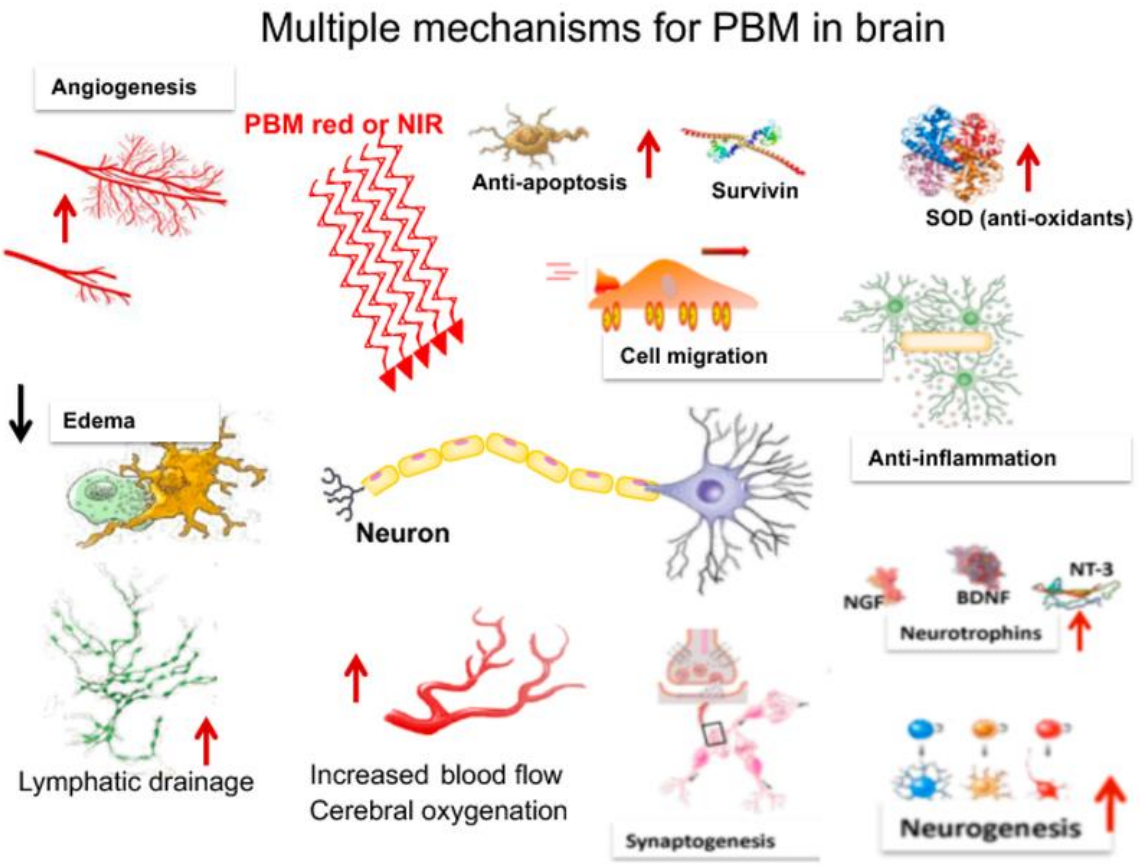


Figure 1.22: Mechanisms modulated by NIR light
From (Hamblin, 2019). Open access license for reprinting.

Several mechanisms have been suggested to explain the beneficial effects of LLLT and NIR light transcranial deliveries on brain function (Figure 1.21): in fact, it increases cerebral blood flow and the cellular energy metabolism as well as the antioxidant defenses, thus providing neuroprotection through modulation of pro- and anti-apoptotic molecules, anti-inflammatory signals and neurotrophic factors. Hence, the improvement of these

functions leads to the amelioration of cognitive function and sleep patterns as well as provides anti-depressant effect (Salehpour, Mahmoudi, *et al.*, 2018). The beneficial effect of LLLT and NIR light transcranial deliveries on brain function can be explained with the bioenergetics, since many molecules able to absorb light in the wavelength of NIR belong to the mitochondrial respiratory chain, (Gonzalez-Lima and Barrett, 2014). As mentioned earlier, NIR light can be absorbed by specific photoacceptor molecules, known as chromophores (Ramezani *et al.*, 2021). In this regard, one of the most important chromophores is cytochrome C oxidase (CCO), also known as mitochondrial Complex IV. In fact, light absorbed by CCO in the NIR region increases its enzymatic activity and likely its expression, thus improving mitochondrial activity in terms of energy metabolism thanks to increased oxygen consumption, that in turn leads to increased ATP production. In addition, there is increased mitochondrial membrane potential, accelerated electron transport, increased synthesis of NADH and NADPH oxidase, potential amelioration of cell survival mechanisms and overall prolonged homeostasis (Gonzalez-Lima and Barrett, 2014; Hamblin, 2019; Enengl, Hamblin and Dungel, 2020; Ramezani *et al.*, 2021). Moreover, it is possible that light absorption by CCO causes dissociation of nitric oxide (NO) from CCO itself: in fact, when a cell is stressed, NO may replace oxygen in binding CCO and slow down mitochondrial respiration activity, therefore the dissociation of NO from CCO may restore normal production of ATP, even improving it. The displacement of NO from its binding to CCO may lead to an increased blood flow (Figure 1.22): NO is diffusible gaseous molecule with known vasodilator properties, therefore its release may cause vasodilation that translates into increased blood flow in the area where the light is administered; consequently, the oxygenation of the tissue improves, as well (Thunshelle and Hamblin, 2016). Besides, it is known that LLLT increases angiogenesis, thus adding another element to the overall amelioration of the blood flow (Hamblin, 2019). The increased production of ATP may also lead to increased levels of cAMP, mediator of many cellular pathways.

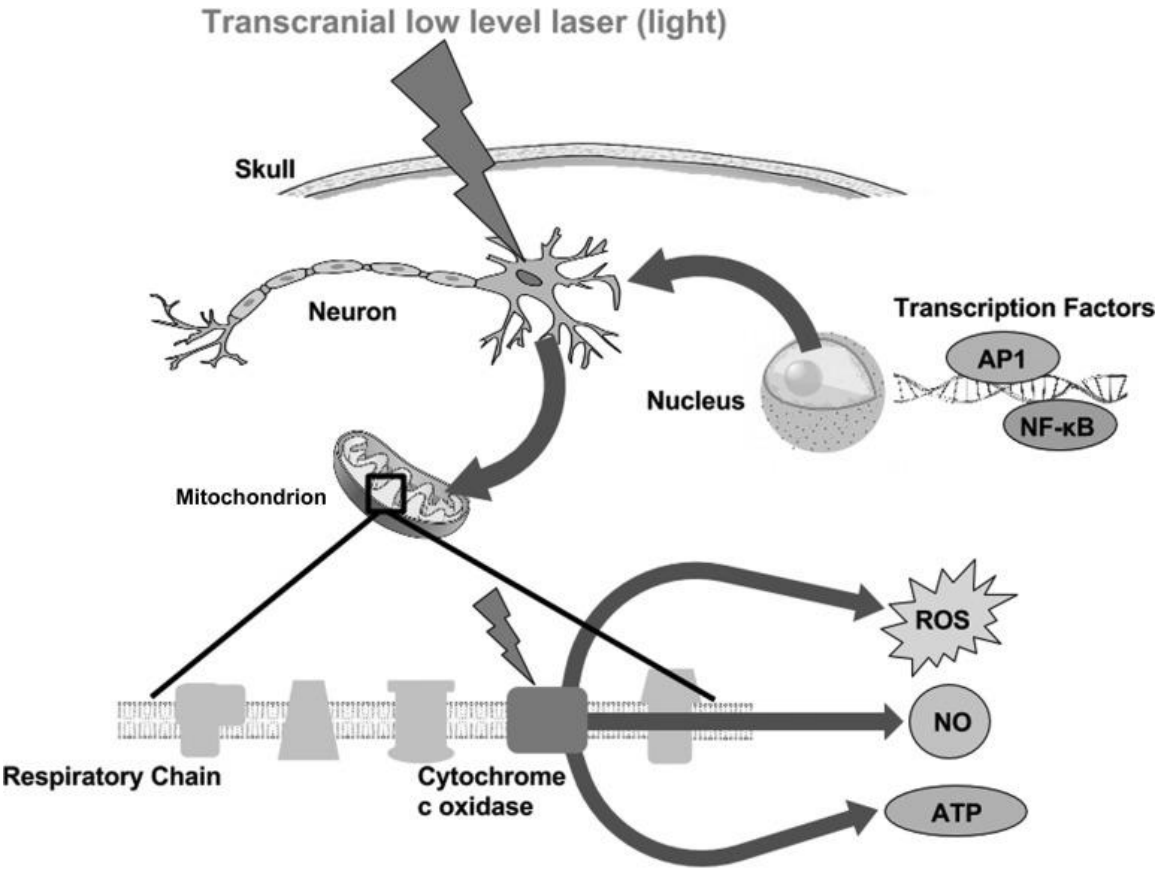


Figure 1.23: Mitochondria and CCO are the main targets of NIR light
Reproduced with permission from (Thunshelle and Hamblin, 2016).

Since LLLT and NIR light delivery promote higher oxygen metabolism, an increase of ROS occurs, that in turn activates NF-κB pathway with consequent upregulation of several molecules (Hashmi *et al.*, 2010; Hamblin, 2017): the increase of ROS changes the redox state of the cell and consequently redox-sensitive genes and transcription factors, such as redox factor-1 (Ref-1), cyclic adenosine monophosphate (cAMP)-response element (CREB), activator protein 1 (AP-1), p53, NF-κB, hypoxia-inducible factor (HIF-1), and HIF-like factor (Thunshelle and Hamblin, 2016). Apparently, this increase of ROS sounds paradoxical, however the modest rise in ROS levels is just enough to induce the antioxidant defense and the aforementioned activation of survival mechanism. (Ramezani *et al.*, 2021).

Moreover, LLLT induces release of calcium from mitochondria with consequent changes in cellular calcium metabolism. (Ramezani *et al.*, 2021). It is conceivable that the effects of the light involve also biophysics phenomena, such as interfacial water layers (IWL, may be similar to the "exclusion zone water" mentioned earlier): it is believed that when the light interacts with IWL into the mitochondria, the viscosity could be lower due to the irradiation, thus leading to higher speed of the "rotor", namely the ATP synthase, that would generate more ATP (Figure 1.20). Conversely, the presence of IWL on the cell membrane should lead to higher intake of nutrients, hence increased metabolic activity of the cell and consequently of the mitochondria occur (Hamblin, 2019). Moreover, other chromophores might be involved in the beneficial effects of the light, such as opsins, flavins and cryptochromes: opsins contain a cis-retinaldehyde that is converted to a trans-molecule by the light, thus triggering several biochemical pathways; flavins, such as riboflavin, flavin mononucleotide or flavin adenosine dinucleotide contain chromophores that can absorb light; cryptochromes are a particular class of flavoproteins abundant also in plants and animals, able to absorb blue-lights and involved in the regulation of the circadian clock (Hamblin, 2017). Potentially, also transient receptor potential (TRP) channels may mediate the biological effects of the light, however their ability to absorb NIR light is poor, as opposite to their ability to absorb green light, which does not have clinical utility (Hamblin, 2019). Interestingly, NIR light upregulates BDNF in rodent injury-models, thus suggesting that this effect may provide neuronal and synaptic protection, as well as promoting neurogenesis (Meng, He and Xing, 2013; Ghanbari *et al.*, 2017; Yan *et al.*, 2017; Heo *et al.*, 2019). The upregulation of BDNF involves the protein calcitonin gene-related peptide (CGRP), whose expression rises during injury, inflammation, acute and chronic pain. Higher levels of CGRP induce higher neuronal expression of BDNF and the phosphorylated form of the CREB; NIR light upregulates BDNF thanks to both promotion of CREB and ERK phosphorylation and activation of calcium-mediated pathways (Ramezani *et al.*, 2021). BDNF is an important factor that is

involved in maintenance of synaptic plasticity and neuronal structure: in fact, it is a mediator of synapsin-1, protein involved in synaptogenesis by accelerating the development of neuronal fibers and maintaining the contacts within the synapse. Consistently, Meng *et al.* have demonstrated the presence of denser branches and enhanced synaptic connectivity following the delivery of a 780 nm light (Meng, He and Xing, 2013; Hamblin, 2019). Furthermore, a study conducted in a rat model of traumatic brain injury (TBI) revealed that the delivery of a 810 nm light ameliorates the neurological function, and induces higher expression of BDNF in the subventricular zone and in the sub-granular layer of the hippocampal dentate gyrus (the two brain areas defined as the neurogenic niches), 7 days after injury, and returned to normal 28 days following the injury. On the other hand, the levels of synapsin-1 were significantly higher 28 days after injury, thus indicating that LLLT induces synaptogenesis and likely neurogenesis through a BDNF-mediated mechanism that involves synapsin-1 (Xuan *et al.*, 2015; Hamblin, 2019). Also, LLLT seems to inhibit GSK3β, enzyme that is involved in Tau hyperphosphorylation as well as in the promotion of apoptotic pathways; in fact, it has been proposed that light delivery increases the levels of Akt, which in turn increases the phosphorylation of GSK3β, thus causing its inhibition. On the other hand, the inhibition of GSK3β leads to activation and nuclear accumulation of β-catenin, that activates cell survival mechanisms; additionally, the inhibition of GSK3β impedes Bax to be phosphorylated and to translocate in the nucleus in response to pro-apoptotic signals (Hamblin, 2019). The antioxidant effects of the LLLT lead to the reduction of iNOS expression in animal models that display oxidative stress: iNOS expression may be induced by cytokines with consequent production of NO, which in turn may lead to the production of nitrogen radical species that together with ROS cause oxidative damage. Specifically, PBM reduces the levels of peroxynitrite and does not interfere with the other isoforms of NOS, such as the endothelial isoform, responsible for vasodilation (Salehpour, Farajdokht, *et al.*, 2018; Hamblin, 2019; Salehpour, Farajdokht, Mahmoudi, *et al.*, 2019).

NIR light has shown beneficial effects against neuroinflammation, and in particular has an effect on cytokines: NIR light decreases the levels of anti-inflammatory cytokines in mice frontal cortex and hippocampus, such as IL-1β, IL-6 and TNF-α (Zhang *et al.*, 2014; Salehpour, Farajdokht, Cassano, *et al.*, 2019; Salehpour, Farajdokht, Mahmoudi, *et al.*, 2019), as well as decreases glia activation, in different models of TBI and AD (De Taboada *et al.*, 2011; Lee, S. W. Lee, *et al.*, 2017; Salehpour, Mahmoudi, *et al.*, 2018). It was mentioned that the light may induce a little increase of ROS, that in turn activates NF-κB and consequent activation of redox-sensitive molecules. However, depending on the activation state of the immune cell, the delivery of light may either lead to NF-κB increase or decrease: if the cell has shifted towards an M1-like phenotype, the light administration reduces pro-inflammatory cytokines and NF-κB levels. It has also been reported a reduction of cyclooxygenase-2 following light delivery. Importantly, microglial shift towards M1 phenotype causes itself a metabolic shift: in fact, M1 microglia rely on glycolysis for energy purposes, while M2 microglia supply energy with oxidative phosphorylation. The difference in energy demands may reflect different activities, therefore may be indication of the different role of these two types of microglia in the development of a pathology; the effect of light on mitochondrial function could explain the microglia shifting from a pro-inflammatory state towards an anti-inflammatory upon treatment (Hamblin, 2019). This latter hypothesis is supported by the observations made by von Leden *et al*., after treatment of microglia cell cultures with a 808 nm wavelength: energy densities between 0.2 and 10 J/cm^2 induced a shift towards the anti-inflammatory phenotype, while irradiations with higher energy (4 to 30 J/cm^2) led to the acquisition of a more pro-inflammatory phenotype, thus confirming that NIR light has a biphasic effect and lower doses should be preferred; also, co-culture of either NIR-treated microglia or control-treated microglia with neurons showed that NIR-treated microglia induced a dose-dependent neuritic growth (Von Leden *et al.*, 2013). Interestingly, an *in vitro* investigation performed by Song *et al*., showed that microglia cell cultures challenged with LPS and

treated with a He-Ne light (632.8 nm) show reduction of the pro-inflammatory cytokine TNF-α, decreased levels of NO and downregulation of iNOS, as well as an improved phagocytic capacity: this improvement occurs thanks to the upregulation of proteins involved in actin polymerization and cytoskeleton remodeling, via a mechanism dependent on Src proteins and involving the pathway PI3K/Akt. Since in chronic neuroinflammation, microglia activity becomes detrimental and even toxic for neurons, LLLT is a potential tool to restore microglia physiological function in order to curb neuroinflammation-induced neurodegeneration (Song, Zhou and Chen, 2012).

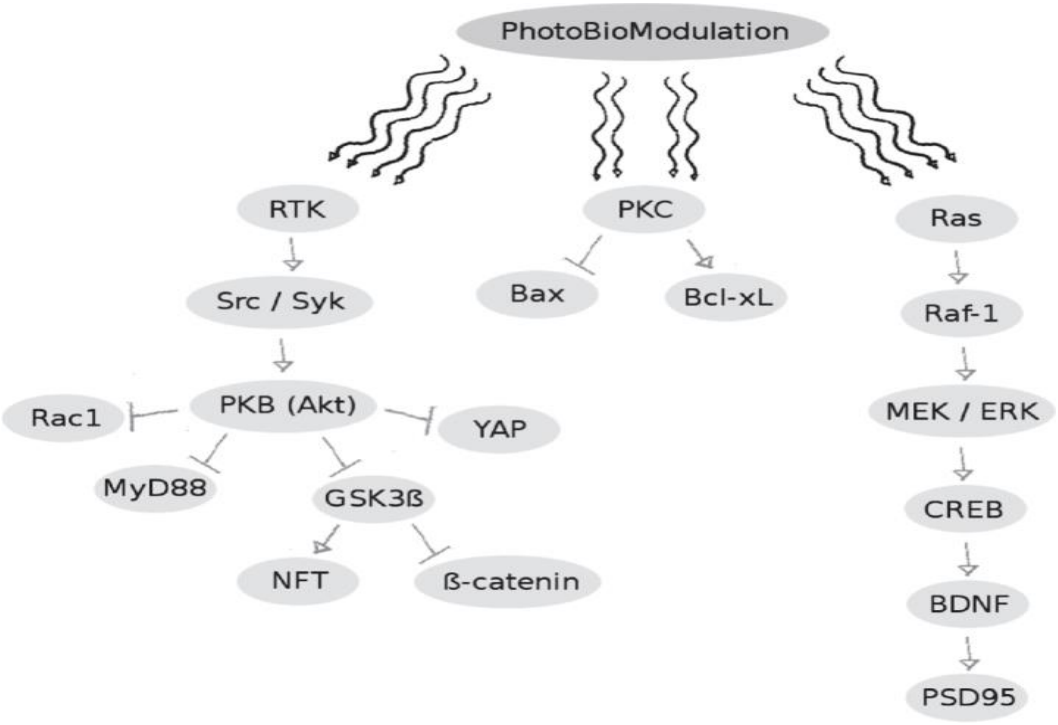


Figure 1.24: Biochemical pathways regulated by PBM
From (Enengl, Hamblin and Dungel, 2020). Open access license for reprinting.

Interestingly, LLLT and NIR light deliveries have also been explored in animal models of obesity in few studies, even though the light was not delivered into the brain, but rather in the abdominal area, in order to target adipose tissue. NIR light ameliorated insulin resistance (Silva *et al.*, 2020), improved glucose (Silva *et al.*, 2018) and lipid metabolism

in the adipose tissue (Gong *et al.*, 2020), reduced the release of free fatty acids and other lipids (Aquino *et al.*, 2013), although none of these studies investigated either the effects on the systemic inflammation that accompanies obesity or the obesity-induced neuroinflammation.

LLLT has shown beneficial effects in several rodent models of neurodegenerative disease, such as stroke (Hashmi *et al.*, 2010; Vogel *et al.*, 2021), sleep deprivation (Salehpour, Farajdokht, Mahmoudi, *et al.*, 2019), major depressive disorder (Salehpour and Rasta, 2017; Salehpour, Farajdokht, Cassano, *et al.*, 2019), TBI (Xuan *et al.*, 2015), multiple sclerosis (MS) (Gonçalves *et al.*, 2016), spinal cord injury (SCI) (Ramezani *et al.*, 2021) and Parkinson's Disease (PD) (Moro *et al.*, 2014; Oueslati *et al.*, 2015; El Massri *et al.*, 2017). Moreover, my laboratory and other have demonstrated that NIR light preserves the synapses from binding of toxic amyloid oligomers and improves the cognitive function of different AD-like mouse models (Comerota, Krishnan and Taglialatela, 2017; Lu *et al.*, 2017; Blivet *et al.*, 2018; Comerota *et al.*, 2018). In particular, LLLT has been proposed as a treatment for AD and several *in vitro* and *in vivo* studies have been performed showing interesting outcomes. Application of light in cell cultures of neuroblastoma reduced levels of Aβ aggregates (Enengl, Hamblin and Dungel, 2020); also, light treatment reduced Aβ-induced oxidative stress and neuroinflammation in rat cortical astrocytes (Enengl, Hamblin and Dungel, 2020). Likewise, NIR light (808 nm) reduced the toxicity in the rat hippocampus after intrahippocampal injection of Aβ peptide by improving hippocampal-dependent memory in behavioral tests, reducing oxidative stress, diminishing pro-apoptotic proteins, turning down neuroinflammation, restoring mitochondrial activity (in terms of fusion and metabolic activity) and by decreasing Tau pathology (Lu *et al.*, 2017). Similarly, mice who received intracerebroventricular injections of Aβ peptide and treated with a device that combines 3 different light sources showed amelioration of memory tasks, reduction of pro-inflammatory cytokines and reduced glia activation, as well as decrease of pro-apoptotic factors and phosphorylated Tau (Blivet *et al.*, 2018). PC-12 cells treated

with Aβ show reduction of apoptosis after light delivery (using a He-Ne light) at an energy of 2 J/cm^2 via activation of anti-apoptotic pathways mediated by protein kinase B (PKB) and protein kinase C (PKC): interestingly an irradiation for 5 minutes or 20 minutes decreases the levels of the pro-apoptotic protein Bax compared to cells treated with only Aβ, while a prolonged treatment with NIR light for 40 minutes does not lead to beneficial effects, thus showing a biphasic effect. Human neuronal cells challenged with Aβ and treated with He-Ne light with doses ranging from 0.5 J/cm^2 to 4 J/cm^2 showed upregulation of BDNF and Erk/CREB pathway with consequent prevention of dendritic atrophy (Meng, He and Xing, 2013). Two AD-like transgenic model, K3 and APP/PS1 mice, who show respectively deposition of either NFTs or Aβ plaques were treated in the same fashion as the mice treated in this project (a total of 20 times with a NIR light at 670 nm, energy 4 J/cm^2, for four weeks): the deposition of such aggregates is dramatically reduced as well as markers of oxidative stress are reduced after light treatment (Purushothuman *et al.*, 2014). An APP transgenic mouse model bearing the Swedish and the London mutation, that overexpresses Aβ peptides, was treated with a GaAlAs (Gallium-Aluminum-Arsenide) diode laser (wavelength ~808 nm) either continuously or with a pulsed delivery, thus receiving light doses with increasing irradiance and fluence (1.2 J/cm^2 to 12 J/cm^2 at cortical surface): after the treatment the mice display decrease amyloid load and improved memory function in behavioral tests, as well as reduced levels of pro-inflammatory cytokines and ameliorated mitochondrial function (De Taboada *et al.*, 2011). Previous works from my laboratory demonstrated that NIR light provides many beneficial effects in different AD-mouse models, treated with 90-seconds doses of 670 nm NIR light, transcranially applied, 5 days per week for 4 weeks. Synaptosomes isolated from hippocampi and frontal cortices of Tg2576, 3xTgAD and hTau mice have reduced levels of Aβ and/or Tau oligomers, and improved mitochondrial function; moreover, synaptosomes of wild-type mice treated with NIR light isolated from the same brain areas show reduced binding of Aβ oligomers compared to synaptosomes derived from untreated

wild-type mice; likewise, electrophysiology analyses of brain slices incubated with Aβ oligomers and obtained from brains of wild-type animals either treated or untreated with light, display improved LTP (Comerota, Krishnan and Taglialatela, 2017; Comerota *et al.*, 2018). Other studies regarding the effects of light delivery on AD *in vitro* and *in vivo* models are summarized in Tables 1.3, while studies using the same device used in this project are reported in Table 1.4.

Study/year	**Animal model/cell line**	**Light source and wavelength**	**Irradiation parameters and duration**	**Main findings**
***In vitro* models**				
(Duan *et al.*, 2003)	PC12 cell ($A\beta_{25–35}$-induced neurotoxicity)	LEDs, self-made GaAlAs 640 nm	0.05–1 mW/cm^2, 30–60 min, single irradiation	At 0.09 mW/cm^2 and 60 min diminished apoptosis and attenuated DNA fragmentation
(Yang *et al.*, 2010)	Primary astrocytes ($A\beta_{1–42}$-induced neurotoxicity)	Laser, He-Ne 632.8 nm	1.5 mW/cm^2, 16.2 J/cm^2, 3 h, single irradiation	Decreased oxidative stress; inhibited pro-inflammatory markers including IL-1β and iNOS
(Sommer *et al.*, 2012)	SH-EP and PC12 cells ($A\beta_{42}$-induced neurotoxicity)	Laser 670 nm	17.36 mW/cm^2, 1 J/cm^2, 1 min, single irradiation	SH-EP cells: reduced intracellular $A\beta_{42}$ aggregates. Increased ATP levels in $A\beta_{42}$-free SH-EP cells PC12 cells: small decrease in ATP levels in $A\beta_{42}$-challenged
(Liang, Liu and Xing, 2012)	SH-SY5Y, PC12, and HEK293T cells ($A\beta_{25–35}$-induced neurotoxicity)	Laser 632.8 nm	12.74 mW/cm^2, 2 J/cm^2, single irradiation	In all cell types: decreased apoptosis via Akt/GSK3b/b-catenin pathway
(Meng, He and Xing, 2013)	SH-SY5Y cell and mice	Laser 632.8 nm	12.74 mW/cm^2, 0.5, 1, 2, or 4 J/cm^2; with	At 2 J/cm^2: promoted cell survival and improved

	hippocampal primary neuron ($A\beta_{25–35}$ and $A\beta_{1–42}$-induced neurotoxicity)		corresponding duration of 0.7, 1.25, 2.5, and 5 min in the dark, respectively; single irradiation.	dendrite growth atrophy through up-regulation of BDNF mediated by activation of ERK/CREB signaling pathway
***In vivo* models**				
(De Taboada *et al.*, 2011)	AD (mouse APP transgenic model)	Laser, 808 nm	1.2, 6, or 12 J/cm^2 at cortex; fiber diameter of 3 mm, 2 min. Transcranially; At a point in sagittal suture, 4 mm caudal to coronal suture 3×/week for 6 months	Improved learning and memory in behavioral test; decreased CSF plasma and brain Aβ level; decreased brain inflammatory markers such as IL-1β, TNF-α, and TGF-β; increased ATP level and O_2 consumption
(Purushothuman *et al.*, 2014)	**AD (mouse K3 and APP/PS1 transgenic models)**	**LEDs, 670 nm**	**4 J/cm^2, 90 s, 5×/week for 4 weeks, Transcranially; Holding probe 1 to 2 cm above the head**	**K3 mouse: decreased phosphorylated tau and tangles in neocortex and hippocampus; decreased oxidative stress markers in neocortex; enhanced CCO expression patterns in neocortex and hippocampus APP/PS1 mouse: decreased number, size, and burden of Aβ plaques in neocortex and hippocampus**
(Purushothuman *et al.*, 2015)	**AD (mouse K3 and APP/PS1 transgenic models)**	**LEDs, 670 nm**	**4 J/cm^2, 90 s, 5×/week for 4 weeks, Transcranially; Holding probe 1 to 2 cm above the head**	**APP/PS1 mouse: reduced number, size and deposition of Aβ plaques in cerebellar cortex K3 mouse: decresed formation of NFTs, phosphorylated tau, oxidative stress, and**

				increased CCO expression in cerebellar cortex
(Lu *et al.*, 2017)	AD (rat $A\beta_{1-42}$ model)	Laser, 808 nm	25 mW/cm^2, 3 J/cm^2 at cerebral cortex, spot area of 1 cm^2, 2 min/day for 5 consecutive days, Transcranially; At the 3 mm posterior to eye and 2 mm anterior to ear	Suppressed neuronal degeneration; improved mitochondrial function through reduction of Bax/Bcl-2 ratio and increase of MMP, CCO activity and ATP levels; enhanced total antioxidant capacity; inhibited glial activation, proinflammatory cytokines production and tau hyperphosphorylation; suppressed pro-apoptotic signals; improved spatial learning and memory in behavioral tests
(Grillo *et al.*, 2013)	AD (mouse TASTPM model)	LEDs, 1072 nm	5 mW/cm^2, 1.8 J/cm^2, 6 min/day for 2 days, biweekly for 5 months, Full-body irradiation	Decreased APP, $A\beta_{1-40}$, $A\beta_{1-42}$, and phosphorylated tau proteins expression; decreased plaque deposition in dentate gyrus and cerebral cortex
(Farfara *et al.*, 2015)	AD (mouse 5XFAD transgenic model)	NR	400 mW, 1 J/cm^2, spot diameter of 0.3 cm, 6× (at 10-day intervals, for 2 months), Remote tissue irradiation; At the middle portion of the medial part of the tibia	Improved memory in object recognition and fear-conditioning tests; decreased Aβ burden in hippocampus

(da Luz Eltchechem *et al.*, 2017)	AD (rat $A\beta_{25-35}$ model)	LEDs, 627 nm	70 mW, 7 J/cm^2, 100 s/day for 21 days Transcranially; Holding probe at 1 cm from the frontal region of scalp	Improved motor skills at days 14 and 21; improved spatial memory in behavioral test at day 14; reduced amount of Aβ
(Comerota, Krishnan and Taglialatela, 2017)	**C57Bl6/J WT and Tg2576**	**LEDs, 670 nm**	**4 J/cm^2, 90 s, 5×/week for 4 weeks, Transcranially; Holding probe 1 to 2 cm above the head**	**WT mice: Reduced binding of Aβ oligomers to POCTX, FCTX and hippocampus synaptosomes. Preservation of LTP in brain slices from NIR light-treated and ex-vivo challenged with Aβ oligomers Tg2576: Reduced levels of $A\beta_{1-42}$ in synaptosomes from POCTX, FCTX, hippocampus, cerebellum. Improved mitochondrial activity and abundance**
(Comerota *et al.*, 2018)	**C57Bl6/J WT, hTau (P301L) and 3xTg**	**LEDs, 670 nm**	**4 J/cm^2, 90 s, 5×/week for 4 weeks, Transcranially; Holding probe 1 to 2 cm above the head**	**hTau: Reduced levels of Tau oligomers at hippocampal and cortical synaptosomes and total homogenates. Improved memory in behavioral tests Tg2576: Reduced levels of Tau oligomers at hippocampal and cortical synaptosomes and total homogenates.**

Table 1.3: Published *in vitro* and *in vivo* studies on effect of NIR/LLLT on AD models (Adapted with permission from (Salehpour, Mahmoudi, *et al.*, 2018)). **In bold studies where either the same wavelength used in this study or the same device here utilized are reported.**

Study/year	**Animal model/cell line**	**Light source and wavelength**	**Irradiation parameters and duration**	**Main findings**
***In vitro* models**				
(Wong-Riley *et al.*, 2001)	Cultured rat cortical neurons (TTX-induced neurotoxicity)	LEDs, GaAlAs 670 nm	50 mW/cm^2, 4 J/cm^2, 80 s	Increased CCO activity in all three metabolic categories of neurons (daily irradiation for 5 days); increased CCO activity in darkly reactive cell type (a single irradiation)
(Wong-Riley *et al.*, 2005)	Cultured rat visual cortical neurons (KCN-induced neurotoxicity)	LEDs, 670, 728, 770, 830, or 880 nm	50 mW/cm^2, 4 to 30 J/cm^2, 80 to 600 s	670 and 830 nm significantly increased CCO activity and ATP content back to control levels compared to 728, 880, and 770 nm (each at 4 J/cm^2) 670 nm: pre-irradiation at 30 J/cm^2

				reduced cell death
(Liang *et al.*, 2006)	Cultured rat visual cortical neurons (KCN-induced neurotoxicity)	LEDs, 670 nm	50 mW/cm^2, 30 J/cm^2, single irradiation	Pre-irradiation reduced cell death; reduced number of ssDNA-positive neurons; reduced caspase-3 and Bax levels, and increased Bcl-2 levels; reduced ROS production
(Liang *et al.*, 2008)	Cultured rat occipital cortical and striatal neurons (KCN- or MMP+- or rotenone-induced neurotoxicity)	LEDs, 670 nm	50 mW/cm^2, 4 J/cm^2, 80 s, 1 to 4x/day	Reduced apoptosis, NO production, nitrotyrosine expression, increased ATP and CCO activity
(Ying *et al.*, 2008)	Cultured rat visual cortical and striatal neurons (Rotenone- or MPP+-induced neurotoxicity)	LEDs, 670 nm	50 mW/cm^2, 4 J/cm^2, 80 s	decreased apoptosis in both types of neurons; increased ATP content in striatal neurons
(Giuliani *et al.*, 2009)	PC12 cell (H2O2-induced neurotoxicity)	Laser, 670 nm	0.005 or 0.011 mW/cm^2; 0.11, 0.22, 5.06 or 10.12 J/cm^2; 20 or 900 s, single irradiation	Enhanced axonal protection via stimulation of

				NGF-induced neurite outgrowth; rescued MMP (at all fluencies); increased cell viability (at 0.11 and 0.22 J/cm^2)
***In vivo* models**				
(Shaw *et al.*, 2010)	**PD (mouse MPTP model)**	**LEDs, 670 nm**	**40 mW/cm^2 at scalp, 5.3 mW/cm^2 inside skull, 0.47 J/cm^2 per irradiation (total of 4 irradiations over 30 h), 90 s, irradiation area of 10 cm^2; Transcranially; Holding probe at 1 cm from the head**	**Increased TH$^+$ cell numbers in SNc**
(Peoples *et al.*, 2012)	**PD (mouse MPTP model)**	**LEDs, 670 nm**	**5 J/cm^2 over 10 sessions, 90 s/session, Transcranially; Holding probe at 1–2 cm from the head**	**Increased TH$^+$ cell numbers in SNc**
(Shaw *et al.*, 2012)	**PD (mouse MPTP model)**	**LEDs, 670 nm**	**0.5 J/cm^2, 90 s, Transcranially;**	**Decreased Fos$^+$ cell numbers in STN and ZI after acute (~ 1 day) and chronic (5 weeks) MPTP insult**

(Moro *et al.*, 2013)	**PD (MPTP Balb/c and C57BL/6 mouse models)**	**LEDs, 670 nm**	**0.47 J/cm^2 per session (total of 4 sessions over 30 h), 90 s, Transcranially; Holding probe at 1–2 cm from the head**	**increased TH$^+$ cell numbers in SNc; improved locomotor activities**
(Johnstone *et al.*, 2014)	**PD (mouse MPTP models)**	**LEDs, 670 nm**	**40 mW/cm^2, 4 J/cm^2, 90 s, Head or body irradiation**	**increased TH$^+$ cell numbers in SNc; increased glial cell numbers in SNc**
(Moro *et al.*, 2014)	PD (mouse MPTP models)	LEDs, 670 nm coupled with an optical fiber	1.5 mW/cm^2 or 14.5 mW/cm^2, fiber diameter of 300 μm, continuous irradiation for 6 days Intracranially; Implant site: lateral ventricle	Increased TH$^+$ cell numbers in SNc
(El Massri *et al.*, 2016)	PD (monkey MPTP model)	Laser, coupled with an optical fiber 670 nm	10 mW; 25 or 35 J over 7 days; with 5 s ON/60 s OFF Intracranially; Implant site: near the SNc of both sides	Decreased number and cell size of astrocytes in both the SNc and striatum; decreased cell size of microglia in both the SNc and striatum
(Moro *et al.*, 2016)	PD (monkey MPTP model)	Laser, coupled with an optical fiber	670 nm 10 mW, 125 J for 25 days continuous irradiation, with 5 s ON/60 s OFF Intracranially; Implant site: a region close to	Improved PD signs; increased number of nigral Nissl-stained cells and density

			midline in the midbrain, encompassing the ventral tegmental area	of striatal TH^+ terminals
(Reinhart, Massri, *et al.*, 2016)	PD (rat 6-OHDA model)	LEDs, coupled with an optical fiber, 670 nm	333 nW or 0.16 mW, 634 mJ or 304 J, fiber diameter of 300 μm, for 23 consecutive days, (2×/day for 90 s). Intracranially; Implant site: a region near the SNc (including red nucleus and ventral tegmental area)	Decreased rotational behavior at days 14 and 21; increased TH^+ cell numbers in SNc
(Reinhart, El Massri, *et al.*, 2016)	PD (mouse MPTP model)	LEDs, 670 nm	5.3 mW/cm^2, ~0.5 J/cm^2 at midbrain, 90 s, 2×/day for 2, 4, or 6 days Transcranially; Holding probe at 1–2 cm from the head	Improved locomotor activity; increased TH^+ cell numbers in SNc (at all regimens)
(Reinhart *et al.*, 2017)	PD (mouse MPTP model)	LEDs, 670 and/or 810 nm	15 or 30 mW, total dosage of ~11 or 22 J, 45 or 90 s, 2×/day for 2 days, Transcranially; Full head irradiation	Improved locomotor activity and increased TH^+ cell numbers in SNc
(El Massri *et al.*, 2017)	PD (mouse and monkey MPTP model, and rat 6-OHDA model)	Laser, coupled with an optical fiber 670 nm	0.16 mW for mouse and rat, and 10 mW for monkey, fiber diameter of 300 μm, continuous	Increased number of TH^+ cells, TH^+ terminal density, and GDNF

			irradiation for 2 (mouse), 23 (rat) and 5 (monkey) days. Intracranially; Implant sites: lateral ventricle in mouse, a midline region of midbrain in rat and monkey	expression patterns in striatum of monkey
(El Massri *et al.*, 2018)	Young and aged C57Bl6/J mice	LEDs, 670 nm	NR	Reduced number of Iba-1^+ and $GFAP^+$ cells in aged mice upon light treatment
(Quirk *et al.*, 2012)	TBI (rat)	LEDs 670 nm	50 mW/cm^2, 15 J/cm^2, 5 min, 2 sessions/day for 72 h or 10 days, Transcranially; Holding probe at 0.5 cm above the head	Improved locomotor activity (at 10 days post-irradiation); increased Bcl-2 and GSH, and decreased Bax expression levels

Table 1.4: Published *in vitro* and *in vivo* studies with 660-670 nm wavelength (Adapted with permission from (Salehpour, Mahmoudi, *et al.*, 2018)). **In bold studies where the same device here utilized is reported.**

NIR light treatments have also been tested in humans, where they showed improvement of executive function, anxiety disorders, depressive behaviors: in two studies aimed at understanding whether the application of light improves memory and/or executive function, cognitively intact volunteers received light in one (Gonzalez-Lima and Barrett, 2014) or two locations (Blanco, Maddox and Gonzalez-Lima, 2017) of the right portion of

their foreheads at a wavelength of 1064 nm (high wavelength should maximize tissue penetration) and irradiance of 250 mW/cm^2 for 4 minutes per site, for a total fluence of 60 J/cm^2 per site. The treatment improved executive function (Blanco, Maddox and Gonzalez-Lima, 2017), increased psychomotor vigilance and enhancement in memory tasks (Gonzalez-Lima and Barrett, 2014). Noteworthy, Gonzalez-Lima and Barrett report that the 1064 nm laser produced negligible heat and did not cause any damage; moreover, the authors measured the absorption of the 1064 nm light applied in the forehead of a *post-mortem* brain, concluding that about 2% of the light was able to pass through the bone, hence the amount of energy that reaches the frontal cortex is about 1.2 J/cm^2 (compared to the initial 60 J/cm^2 applied) (Gonzalez-Lima and Barrett, 2014). Another study demonstrates that a delivery of a 810 nm light, with irradiance of 250 mW/cm^2 and fluence of 60 J/cm^2, resulted in an effective energy of 2.1 J/cm^2 reached by the dura of the brain and reduction of depression and anxiety (Enengl, Hamblin and Dungel, 2020). Ramezani *et al*. report that lights with wavelengths falling in the range 600-890 nm cross skin an muscle to reach injury site but wavelengths around 670 nm should be preferred over the others to obtain pain relief, because light absorption by CCO is much higher at this wavelength (Ramezani *et al.*, 2021). On the other hand, other studies report that wavelengths around 700 nm are not much effective, while a combination of 665 and 810 nm seems to provide the best outcomes, considering that CCO has two distinct absorption intervals in this range of wavelengths (Enengl, Hamblin and Dungel, 2020). In this regard, another study performed in healthy older adults showed that a delivery of a mixed light with LEDs at 633 nm and others at 870 nm, (energy density 20 J/cm^2, power density 44.4 mW/cm^2) for 7.5 minutes improves reaction time, mental flexibility and word fluency (Chan *et al.*, 2019). Interestingly, in a small study 5 AD patients received a combined transcranial and intranasal treatment for 12 weeks with a 810 nm LED light delivered at the nodes of the default mode network (bilateral mesial prefrontal cortex, precuneus/posterior cingulate cortex, angular gyrus, and hippocampus). Patients showed

remarkable improvement in brain function, sleep patterns and anxiety, and reduced anger episodes, with no side effect. Nonetheless, the study lacked a placebo-group, so that further studies are needed to understand whether the reported improvements are significant (Saltmarche *et al.*, 2017).

Chapter 2. Aim of the project

Given the literature evidence reviewed in the previous chapter and considering the staggering number of failed attempts in obtaining an efficient therapy against dementia, including AD (Graham, Bonito-Oliva and Sakmar, 2017; Joe and Ringman, 2019; Long and Holtzman, 2019; Oxford, Stewart and Rohn, 2020; Vaz and Silvestre, 2020), alternative strategies aimed at preventing the occurrence of AD should be pursued (Imtiaz *et al.*, 2014; Scheltens *et al.*, 2016; Crous-Bou *et al.*, 2017; Livingston *et al.*, 2017, 2020; Joe and Ringman, 2019). For example, targeting those early events that may lead to neurodegeneration, i.e. neuroinflammation, might prevent the onset of cognitive decline and ultimately dementia. In more general terms, reducing the incidence of the risk factors that may cause AD may be an efficient approach: for example, reducing obesity, T2DM, and cardiovascular diseases by promoting healthy lifestyle, balanced diets and physical exercise not only could reduce these diseases but likely reduce the risks that they pose on AD development.

Obesity and AD share several pathological mechanisms and neuroinflammation is one of the most common pathological links between them (Cai, 2013; Miller and Spencer, 2014; Guillemot-Legris and Muccioli, 2017; Crispino *et al.*, 2020; Kacířová *et al.*, 2020; Leigh and Morris, 2020). Therefore, curbing obesity-driven neuroinflammation could be an effective strategy to prevent its impact on brain function. The overall aim of this project is to find a strategy to mitigate the contribution of neuroinflammation to the development of dementia, including AD. In particular, I focused the attention on two main features of neuroinflammation, namely the activation of microglia and astrocytes and the increased release of inflammatory cytokines. Obesity-induced neuroinflammation can be viewed as a consequence of the systemic low-grade inflammation that establishes in peripheral organs (Heymsfield and Wadden, 2017; Pugazhenthi, Qin and Reddy, 2017; Crispino *et al.*, 2020; Kacířová *et al.*, 2020). This inflammatory state (also known as metainflammation) is due

to the metabolic impairment of adipose tissue, that causes large release of free fatty acids, responsible of inducing systemic insulin resistance, and to the massive release of pro-inflammatory cytokines by malfunctioning adipocytes that ultimately trigger neuroinflammation (Lumeng and Saltiel, 2011; Mraz and Haluzik, 2014; Heymsfield and Wadden, 2017). As described in the previous chapter, the hypothalamus is the area of the brain responsible of sensing the levels of nutrients and hormones coming from the circulation, thus activating those mechanisms regulating food intake and energy expenditure (Schwartz *et al.*, 2000; Cai, 2013; Heymsfield and Wadden, 2017). If the hypothalamus receives large amounts of pro-inflammatory cytokines and free fatty acids, microglia and astrocytes in this area respond to the presence of these molecules by activating a neuroinflammatory cascade that ultimately impairs the hypothalamic control of energy homeostasis (Cai, 2013; Baufeld *et al.*, 2016; Rahman, Bhusal, *et al.*, 2018; Rahman, Kim, *et al.*, 2018). However, longer periods of high-caloric food intake sustain the neuroinflammation, which ultimately spreads out to other brain areas, especially the hippocampus, with serious consequences on learning and memory processes, and an overall increased risk of AD (Puig *et al.*, 2012; Erion *et al.*, 2014; Hao *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Busquets *et al.*, 2017; Cavaliere *et al.*, 2019).

In AD, neuroinflammation is a typical feature of the pathology and has two opposite roles: in the early phases, glia cells try to protect the CNS by phagocytizing Aβ, while their response results detrimental in late stages of AD when the role of Tau becomes predominant, thus exacerbating and accelerating the neurodegeneration (Nichols *et al.*, 2019; Leng and Edison, 2021). It must be noted that usually neuroinflammation is a short event that resolves soon after the stress that caused its activation; however, if the triggering cause is not removed the neuroinflammatory cascade is continuously activated, thus leading to chronic neuroinflammation. Hence, any treatment aimed at reducing the impact of neuroinflammation on brain function may prevent the formation of detrimental events prodromal to neurodegeneration (DiSabato, Quan and Godbout, 2016; Kempuraj *et al.*,

2016; Morales *et al.*, 2016; Graham, Bonito-Oliva and Sakmar, 2017; Kwon and Koh, 2020; Leng and Edison, 2021).

As far as regards the link between obesity and CNS-related deficits, it is established that an impaired adipose tissue, due to excessive fat accumulation, starts releasing massive amounts of pro-inflammatory cytokines that cause a low-grade peripheral inflammation (Heymsfield and Wadden, 2017; Schwartz *et al.*, 2017; Crispino *et al.*, 2020). This inflammatory state ultimately causes neuroinflammation, triggered by penetration of pro-inflammatory cytokines and free fatty acids, which in turn exacerbate neuroinflammation. When neuroinflammation becomes chronic, synaptic and neuronal derangements occur, thus paving the way to neurodegeneration (Cai, 2013; Erion *et al.*, 2014; Miller and Spencer, 2014; Aguilar-Valles *et al.*, 2015; Guillemot-Legris *et al.*, 2016; André *et al.*, 2017; Solas *et al.*, 2017; Guillemot-Legris and Muccioli, 2017; Denver, Gault and McClean, 2018; Crispino *et al.*, 2020; Kacířová *et al.*, 2020). Therefore, I tried to find a strategy to limit the effects of obesity-induced neuroinflammation on brain function, that possibly may prevent neurodegeneration and cognitive decline

To pursue the aim of targeting obesity-induced neuroinflammation, I tested the hypothesis that a transcranial delivery of NIR light reduces glia activation and inflammatory cytokines in hippocampus and cortex of DIO mice, thus alleviating obesity-induced neuroinflammation, and likely its impact as risk factor for AD. Transcranial delivery of NIR light has been recently developed as a novel, non-invasive strategy to improve brain function, as pioneer studies in humans and extensive investigations in animal models suggest (Gonzalez-Lima and Barrett, 2014; Farfara *et al.*, 2015; Blanco, Maddox and Gonzalez-Lima, 2017; Comerota, Krishnan and Taglialatela, 2017; Lu *et al.*, 2017; Saltmarche *et al.*, 2017; Comerota *et al.*, 2018; Salehpour, Cassano, *et al.*, 2019). Moreover, previous work from my laboratory has shown the efficiency of a transcranial delivery of NIR light in reducing the concentration of toxic oligomers in synaptosomes of several AD animal models, reducing the binding of the oligomers to the synapses and

improving mitochondrial function (Comerota, Krishnan and Taglialatela, 2017; Comerota *et al.*, 2018).

Therefore, I wanted to test whether this approach can be proposed against those early events, such as neuroinflammation due to obesity, that may lead to cognitive decline, thus preventing the onset of dementia, including AD.

In the following chapters, the results of the proposed strategy against two main aspects of neuroinflammations are presented, namely glia activation and expression levels of inflammatory cytokines.

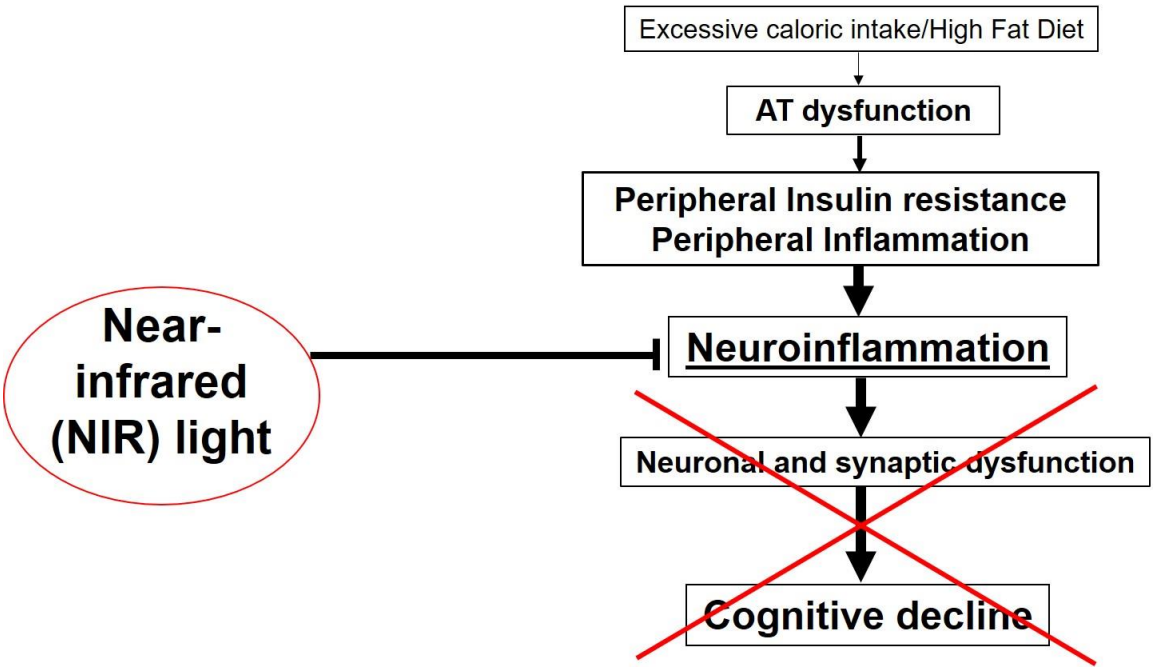


Figure 2.1: Experimental approach and Aim of the project

Chapter 3. Near Infrared-Light Reduces Glia Activation

INTRODUCTION

One of the most studied mechanisms that characterizes both obesity and neurodegenerative diseases is neuroinflammation (Cai, 2013; Martin-Jiménez *et al.*, 2017; Crispino *et al.*, 2020). Rodent models of obesity have shown that an early hypothalamic neuroinflammation arises following 2-3 days of HFD (André *et al.*, 2018; Carraro *et al.*, 2018; N. Xu *et al.*, 2018), then it spreads out in other brain areas, such as hippocampus (Hao *et al.*, 2016; Kim *et al.*, 2018; Tsai *et al.*, 2018; Vinuesa *et al.*, 2018; Wu *et al.*, 2018) and cortex (Pistell *et al.*, 2010; Jayaraman, Lent-Schochet and Pike, 2014; Cavaliere *et al.*, 2019), when the animals are kept under HFD for a long time (up to 6 months). Interestingly, mixed animal models of obesity and AD show that HFD and obesity worsen behavioral and cognitive deficits, synapse dysfunction and overall neurodegeneration, in addition to the aggravation of neuroinflammation (Takalo *et al.*, 2014; Nam *et al.*, 2017; Sah *et al.*, 2017).

To study the HFD/obesity-induced neuroinflammation it was chosen a DIO mouse model, namely a wild-type mouse fed chronically a HFD (Wang and Liao, 2012; King and Bowe, 2016). As described earlier, targeting the modifiable risk factors of dementia is a likely strategy to prevent the onset of this dramatic pathology (Livingston *et al.*, 2017, 2020); in this study, it was employed an animal model that resembles a condition – the obesity – that poses serious threats for CNS integrity and occurs earlier than any evident sign of cognitive dysfunction, regardless of any genetic manipulation. Notably, the DIO mouse model develops obesity, hyperglycemia and insulin resistance, but also decreased neurogenesis, impaired synaptic transmission (Sallam *et al.*, 2015; Krishnan *et al.*, 2020), and neuroinflammation, events that are all present in dementia, including AD, therefore this model gives the unique opportunity to study obesity-induced CNS deficits (Fadel and Reagan, 2016). In this study, the mice were fed a HFD for total 17 weeks, including during

the 4-weeks NIR light treatment which started after 13 weeks of HFD. In fact, to establish obesity-induced neuroinflammation in hippocampus and cortex, HFD has to be administered for at least 12 weeks in order to induce obesity as well as persistent neuroinflammation, as demonstrated by several groups (Puig *et al.*, 2012; Hao *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Busquets *et al.*, 2017; de Aquino *et al.*, 2019; Suárez *et al.*, 2019; Zhu, Zhu and Wang, 2019).

To reduce the deleterious effects of HFD on brain, I explored the possibility that NIR light may reduce the neuroinflammation determined by obesity. My laboratory previously demonstrated that a transcranial delivery of NIR light for 4 weeks decreases synaptic vulnerability to toxic oligomers in the hippocampus and the cortex of different AD-like mouse models (Comerota, Krishnan and Taglialatela, 2017; Comerota *et al.*, 2018), therefore I investigated whether NIR light treatments could also be effective as a preventative strategy for neurodegeneration by targeting specific risk factors. Moreover, it is known that NIR light reduces neuroinflammation in animal models of TBI or genetic models of Aβ-pathology (De Taboada *et al.*, 2011; Zhang *et al.*, 2014; Lee, S.-W. W. Lee, *et al.*, 2017), therefore the overall purpose of this study consisted on investigating whether the proposed treatment is effective in the DIO model and eventually considered as a protective strategy against cognitive deficits, given the pathological link between obesity and dementia.

METHODS

Animals and diet

Twenty male mice from the strain C57Bl6/J were utilized in this study. The mice were purchased from Jackson Laboratories (cat# 000664, Bar Harbor, ME, USA) at four weeks of age and five mouse/cage were housed together. After one week of acclimatation with free access to water and food, the mice were randomly divided in two groups of ten mice

each (Figure 3.1), one fed with regular chow (RC) (cat #7012, Teklad, Madison, WI, USA), the other fed a HFD (cat# D12492, Research Diet, New Brunswick, NJ, USA) for a total of seventeen weeks, with water and food *ad libitum*. The composition of each diet is described in Table 2.1. At the 12th week the mice were subjected to the intraperitoneal glucose tolerance test (IPGTT, details in section IPGTT). At the 13th week of diet treatment, the mice were further subdivided in four groups (n=5/experimental group): RC-fed not treated (RC SHAM), RC-fed treated (RC NIR), HFD-fed not treated (HFD SHAM), HFD-fed treated (HFD NIR). The mice underwent NIR light treatments for four weeks (details in section "NIR light treatments"), after which the mice were euthanized by exposure to isoflurane, followed by transcardial perfusion with 1X phosphate buffer solution (PBS). After the perfusion, the mice brains were quickly removed and separated in two hemispheres, one destined for quantitative real-time PCR, the other intended for histological investigations; the former hemisphere was dissected in major regions (frontal cortex, hippocampus, midbrain, parietal/occipital cortex, cerebellum) and each region was stored at -80 °C until future use; the latter hemisphere was post-fixed in 10% buffered formalin (cat# 245-684, Fisher, Waltham, MA, USA) for 48 hours at 4 °C, then the brain samples were transferred in 1X PBS + 0.01% sodium azide at 4 °C to avoid degradation until the next step. The brain samples were shipped in this buffer to Neuroscience Associates (Knoxville, TN, USA) for tissue processing. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas Medical Branch. All methods were performed in accordance with the guidelines and regulations of the Committee. Animals were housed under USDA standards (12:12 hour light dark cycle, food and water ad libitum) at the UTMB vivarium.

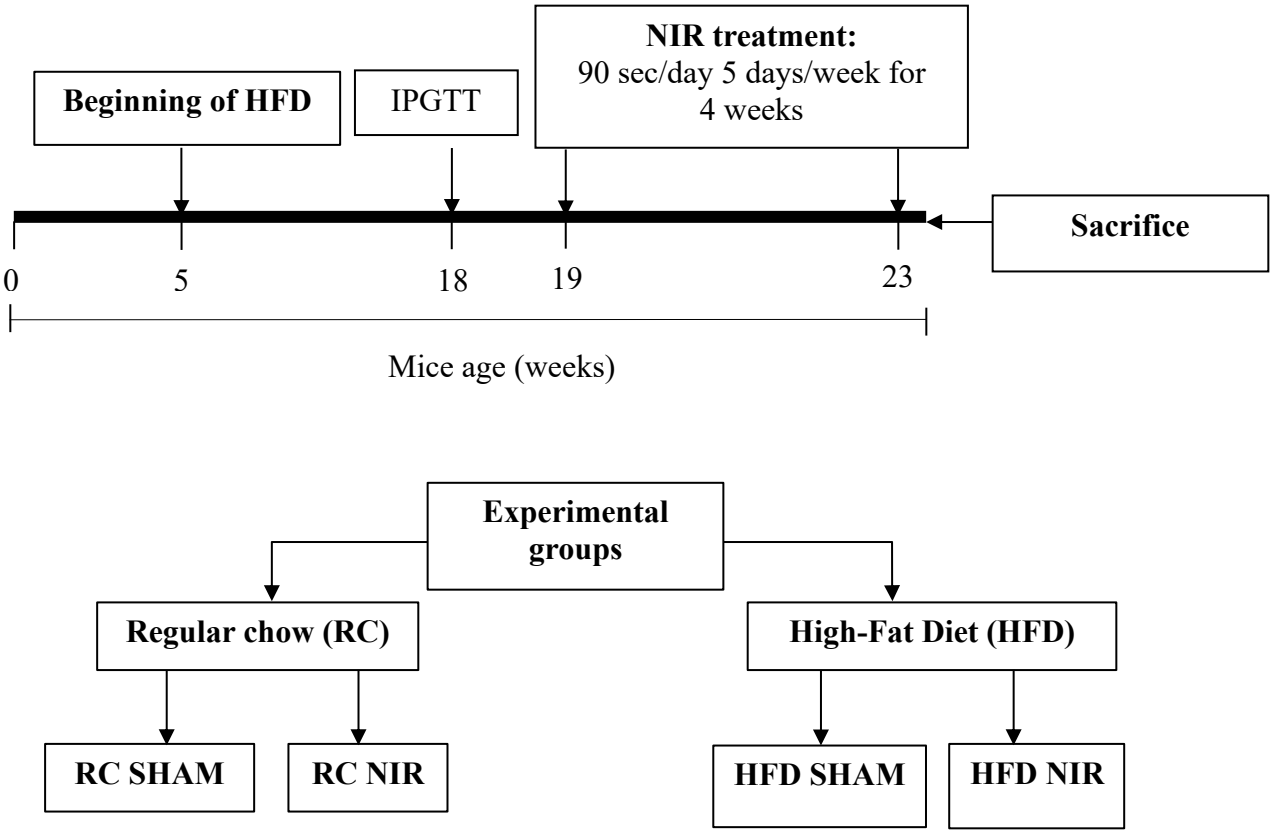


Figure 3.1: Experimental design.
In this scheme, the timeline of the manipulations and treatment administered to the mice and the experimental groups used in the study.

NUTRIENT COMPOSITION	RC (Teklad, 7012)	HFD (Research Diet, D12492)
Protein	19.1% Kcal	20% Kcal
Fat	5.8% Kcal	60% Kcal
Carbohydrate	44.3% Kcal	20% Kcal
Energy density	3.1 Kcal/g	5.21 Kcal/g
FAT COMPOSITION	**RC**	**HFD**
C16:0 Palmitic	0.6%	49.9%
C18:0 Stearic	0.2%	26.9%
C18:1ω9 Oleic	1.3%	86.3%
C18:2ω6 Linoleic	2.6%	72.7%
C18:3ω3 Linolenic	0.3%	5.1%
Total Saturated	0.8%	32.2%
Total Monounsaturated	1.3%	35.9%
Total Polyunsaturated	2.9%	31.9%

Table 3.1: Composition of diets administered to mice

Intraperitoneal glucose tolerance test (IPGTT)

This test was carried out twelve weeks after the beginning of HFD. Each mouse was transferred in a new cage, one per cage, and fasted for five hours. Subsequently, each mouse was briefly anesthetized, and the very tip of the tail was snipped in order to collect blood samples. A drop of blood was obtained from the tail vein to assess blood glucose levels at the baseline (time 0 minutes) using a Contour next glucometer (Ascensia Diabetes Care, Parsippany, NJ, USA). Maintaining the mouse under anesthesia, glucose (cat#

G8644, Sigma-Aldrich, St. Louis, MO, USA) was administered by intraperitoneal injection at a dose of 1 g/kg body weight, then the mouse was put back in its own cage, still with no access to food and water, until the end of the test. Blood glucose levels were further measured at 15, 30, and up to 120 minutes at 30 minutes intervals by milking the tail to obtain blood, while the awake mouse was restrained in order to offer its tail for the blood withdrawal. After the withdrawal each mouse was returned in its own cage, until the end of the test. Continuous pressure is applied over the tail until hemostasis was achieved every time blood was withdrawn and at the end of the test. Animals were followed for the duration of the study and for 30 minutes afterwards for any signs of bleeding. After this time, the mice were returned to their original cages and brought back to the vivarium.

NIR light treatments

Thirteen weeks after the beginning of HFD and one week following IPGTT, the mice were subjected to the NIR light treatments. The mice received one dose per day, five days per week, for four weeks. One dose of NIR light consisted of a 90 second treatment from a LED device (WARP 10; Quantum Devices, Barneveld, WI, USA); the light had a wavelength of 670 nm, irradiance 50 mW/cm^2 and fluence equal to 4 J/cm^2 per treatment (around 80 J/cm^2 of total energy was delivered over 4 weeks of treatments). The device was held on a flat support, hence slightly elevated from the surface to ensure sufficient space to allocate the animals. Each mouse was hand-restrained, with the head placed at approximately 1 cm from the light source. The body of each mouse was covered with aluminum foil to prevent light exposure to the periphery. The control groups (RC SHAM and HFD SHAM) were restrained in the same way and for the same amount of time, however the device was kept off (Figure 3.2). At the end of the treatment, the mice coming from the same cage were placed temporarily in a separate cage, in order to separate those who received the treatment from the ones who had not been treated yet, until all the mice from the same cage completed the treatments, then they were returned in the original cage.

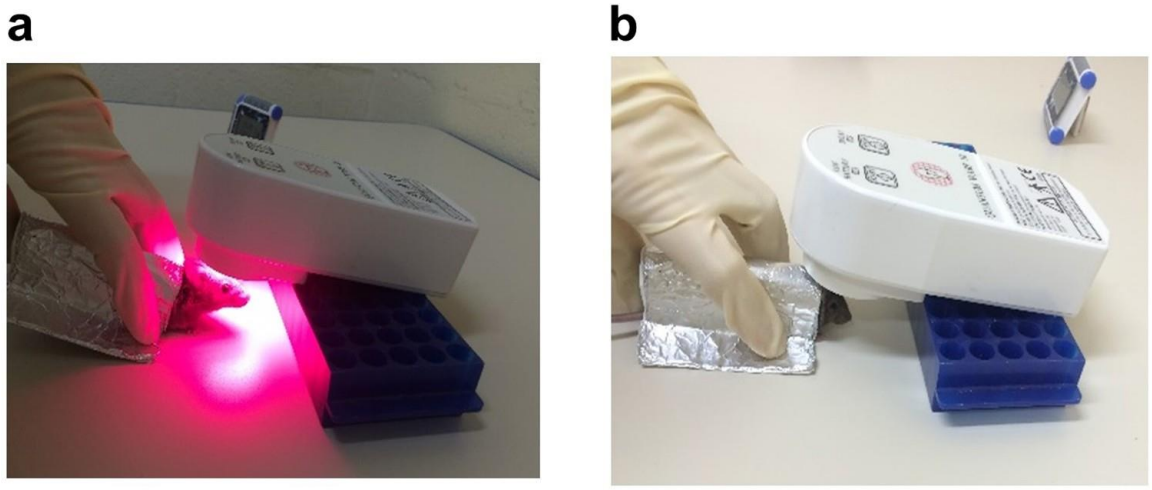


Figure 3.2: NIR light treatment procedure.
Each mouse was hand-restrained, with the head placed at approximately 1 cm from the light source. The body of each mouse was covered with aluminum foil to prevent light exposure to the periphery. From (Comerota, Krishnan and Taglialatela, 2017). Open access license for reprinting.

Tissue processing

One day after the end of the light treatments, the mice were euthanized as described in the section "Animals and diet". The brain samples intended for histological investigations were shipped to the company Neuroscience Associates. The following protocol was communicated by the company. Once the samples were received, they were examined, then treated overnight with 20% glycerol and 2% dimethylsulfoxide to prevent freeze-artifacts. The twenty specimens were then all together embedded in a gelatin matrix using MultiBrain® Technology (Neuroscience Associates, Knoxville, TN, USA). The block was rapidly frozen, after curing by immersion in 2-methylbutane chilled with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The MultiBrain block was sectioned at 35µ in the coronal plane through the cerebrum section of the mouse brain hemisphere (~9 mm in length). All cut sections were collected into a series of 24 cups containing antigen preserve solution (50% PBS pH 7.0, cat# 79383, Sigma-Aldrich, St Louis, MO, USA/50% Ethylene Glycol, cat# 107-21-1, Alfa Aesar, Ward Hill, MA,

USA/1% Polyvinyl Pyrrolidone, cat# 9003-39-8, Fisher Scientific, Fair Lawn, NJ, USA). One section was placed into each cup, beginning with cup #1 and processing numerically, until the last cup was used, then the process cycled back to cup #1. Each cup therefore contains 1 of every 24 cut sections; the adjacent cup contains every 24th section adjacent to those in cup #1 and so on. The cups containing the cut sections were shipped back to our lab for free-floating immunofluorescence. Upon delivery, the cups were stored at -20 °C until future use (Figure 3.3).

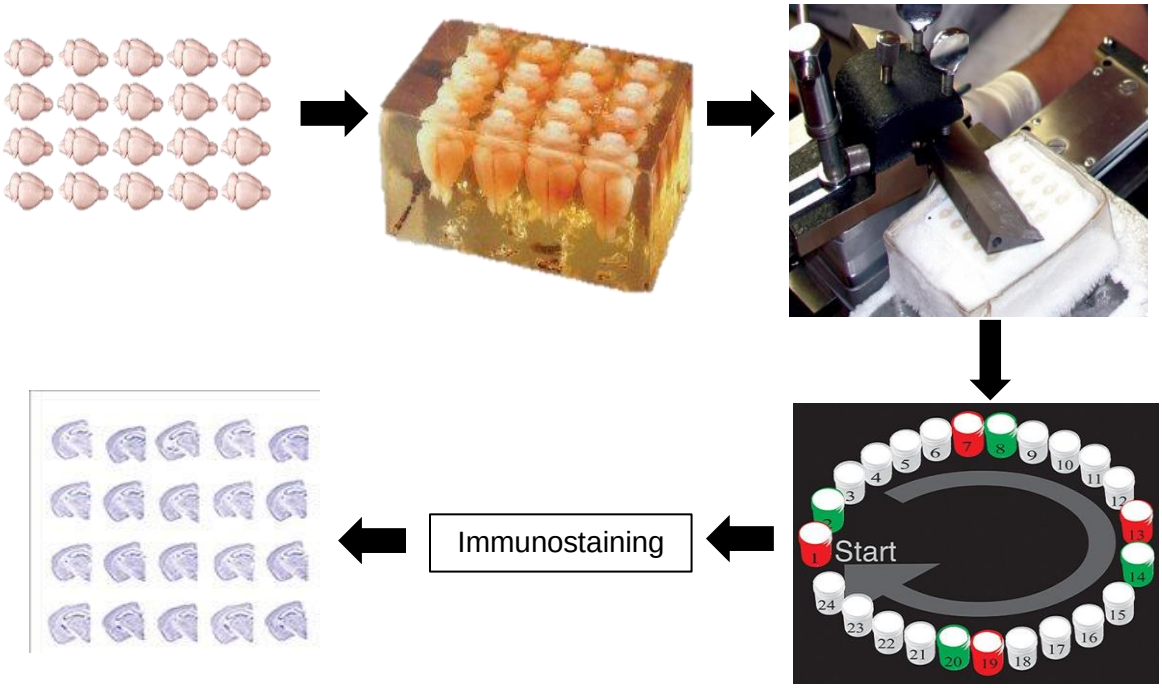


Figure 3.3: Steps of tissue processing.
The tissue followed the following steps: collection, post-fixation, examination, chilling, embedding, slicing, immunostaining, microscope acquisition

Free-floating immunofluorescence

The slices were harvested from adjacent cups and identified with a stereomicroscope with inverted light (Leica MZ12, Leica Microsystems, Buffalo Grove, IL, USA) to choose those slices that included the desired brain areas of investigation. Six sections per animal were

stained. After three brief washings – 5 minutes each – with 1X Tris Buffer Solution (TBS) (diluted from a 10X stock with composition: 200 mM TRIS, 1.5 M NaCl, pH 7.6) to remove excess of antigen-preserve solution, the non-specific binding sites were blocked with 5% bovine serum albumin (BSA, cat# A4503-100G, Sigma-Aldrich, Saint Louis, MO, USA)/10% normal goat serum (NGS, cat# S-26, Millipore, Temecula, CA, USA) in 1X TBS, containing also 0.5% Triton X-100 (cat# 802388. ICN Radiochemicals) and 0.05% Tween-20 (cat# AC233360010, Fisher Scientific, Fair Lawn, NJ, USA) to allow permeabilization, for 1h at room temperature. The slices were then incubated with the following primary antibodies containing 1.5% NGS in 1X TBS, overnight at 4 °C: rabbit anti-Ionized calcium-binding adapter-1 (Iba-1, dilution 1:200, cat# 019-19741 Wako, Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan), chicken anti-glial fibrillar acidic protein (GFAP, dilution 1:500, cat #GFAP Aves Labs, Davis, CA, USA), rat anti-cluster of differentiation 68 (CD68, dilution 1:500, cat# ab53444, Abcam, Cambridge, MA, USA). After three washings – 10 minutes each – with 1X TBS to remove residuals of the primary antibodies, the slices were incubated with the proper fluorescent chromophore Alexa-conjugated secondary antibodies (goat anti-rabbit Alexa Fluor 488, 1:400, cat# A-11008; goat anti-chicken Alexa Fluor 594, 1:400, cat# A-11042; goat anti-rat Alexa Fluor 594, 1:400, cat# A-11007, Invitrogen, Eugene, OR, USA) in 1X TBS containing 1.5% NGS to amplify the immune reaction between the antigen (on the sample) and the primary antibodies. Following three washings – 10 minutes each – with 1X TBS to remove residuals of secondary antibody, the slices were adhered onto glass slides (50x76x1.2 mm) by immerging the slices in a gelatinous solution (95% ethanol/1M ammonium acetate buffer pH 6.0/0.5% gelatin in distilled water). The slices were air dried for 15-30 minutes. Finally, the slices were treated with 0.3% Sudan Black in 70% ethanol for 10 minutes to remove lipofuscin autofluorescence, washed for three times – 3 minutes each – in deionized water to remove excess of the dye, covered using Fluoromount-G containing 4′,6-diamidino-2-phenylindole (DAPI, cat# 0100-20, SouthernBiotech, Birmingham, AL, USA) and sealed.

The slides were air-dried for 30 minutes and stored at 4 °C until the observation at the microscope.

Image acquisition

All the images were acquired with a Keyence BZ-X800 (Keyence Corporation, Osaka, Japan) microscope, by using 40X and immersion oil 60X objectives. From each of the two analyzed sections, 9 images from hippocampus (3 from cornu ammonis 3 (CA3), 3 from cornu ammonis (CA1) and 3 from dentate gyrus (DG), 4 images from parieto/occipital cortex (POCTX) and 5 images from frontal cortex (FCTX) for each mouse were analyzed. Each image was the result of 18-22 Z-stacks with a 2.0 µm pitch by using the Keyence Software.

Image analyses

The quantitative analyses of the images were performed using ImageJ software (https://imagej.nih.gov/ij, NIH, Bethesda, MD, USA), analyzing the intensity of fluorescence for each marker (Integrated Density, IntDen), when the overall distribution was studied. Representative images were composed in an Adobe Photoshop CC2021 format.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.4.3 software (GraphPad Software, San Diego, CA, USA). T-test with Mann-Whitney post-hoc test, one-way ANOVA with Tukey's post hoc test or two-way ANOVA with Sidak's multiple comparison test were used to detect significant differences between groups. Data were then expressed as means ± SD and for all statistical analyses $p < 0.05$ was considered as statistically significant.

RESULTS

Effect of HFD on body weight and glucose tolerance

DIO mice show increased body weight upon high-fat feeding, compared to regular chow-fed mice, which is gradual and becomes evident over time. Also, the blood glucose levels, and the glucose tolerance result altered, showing increased levels in HFD-fed mice compared to mice fed with a regular chow (RC) (Bagnol *et al.*, 2012; Wang and Liao, 2012). Ten mice were fed with a 60% kcal HFD and other 10 with a RC, as control ad libitum for 13 weeks before starting the NIR light deliveries, and for additional 4 weeks during the time of the NIR light treatment (see Figure 3.1 for experimental design). In order to establish whether the high-fat regimen caused the mentioned alterations during the 13-weeks period, the changes in body weight were evaluated (Figure 3.4A) and blood glucose levels (Figures 3.4B and 3.4C). The progressive difference in body weight was observed from week 1. The mice were weighted once a week and, at the time of the assignment to either group, there was no significant change in body weight between the groups; though, as of week 1 the mice fed a HFD had higher body weight compared to RC-fed mice (Figure 3.4A). Moreover, the difference in body weight became more evident throughout the course of our observations, reaching a plateau by week 11. In addition, blood glucose levels were assessed (Figure 3.4B): 12 weeks after the beginning of HFD, the mice were subjected to the intraperitoneal tolerance glucose test (IPGTT), which is a physiological test widely used to determine the effect of the diet on the metabolism, in particular on glucose utilization (Andrikopoulos *et al.*, 2008; Dinger *et al.*, 2018). The test showed no difference in blood glucose levels between RC-fed mice and HFD-fed mice at time 0 and 15, whereas from time 30 through 120 minutes, the test revealed higher levels of glucose in the blood of HFD-fed mice (Figure 3.4B), compared to mice in RC (confirmed also by the calculation of the area under the curve, AUC, for each group of mice, in Figure 3.4C), as expected.

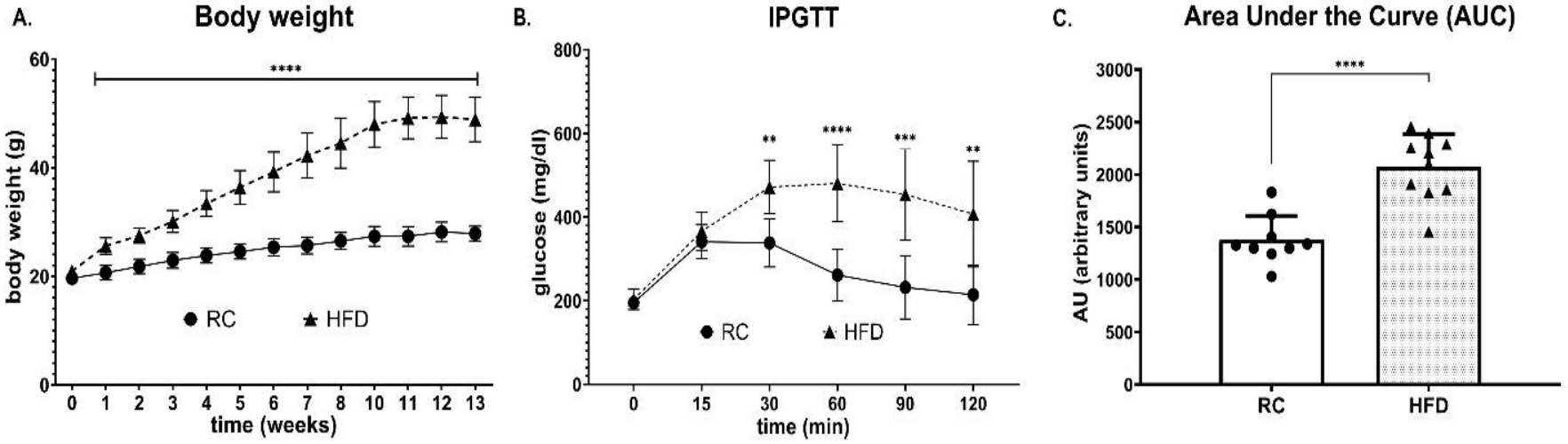


Figure 3.4: Assessment of body weight and blood glucose levels after 13 weeks of HFD.

A. Weekly evaluations of body weight show weight gain for mice under HFD, compared to RC-fed mice. Graph presented as mean ± SD. Each dot represents the average of 10 animals/group. Statistical analyses: two-way ANOVA with Sidak's post-hoc test. **** $p<0.0001$. B. Following 12 weeks of HFD, the blood glucose levels of the mice were evaluated through intraperitoneal glucose tolerance test (IPGTT). After 5h of fasting and following an intraperitoneal injection of glucose in each mouse, we observed that HFD induced higher blood glucose levels during the 2 hours after glucose administration, compared to controls. Graph presented as mean ± SD. Each dot represents the average of 9-10 animals/group per each time point. Statistical analyses: two-way ANOVA with Sidak's post-hoc test. ** $p<0.01$, *** $p<0.001$; **** $p<0.0001$. C. Area under the curve (AUC) of glucose vs. time graph. Graph presented as mean ± SD. Statistical analyses: one-tailed t-test with Mann-Whitney post-hoc test. Each dot represents a single animal. n= 9/10 animals/group.

Effect of NIR light on HFD-induced glia activation in the different brain areas of DIO mice

In order to evaluate whether obesity-induced glial activation is lowered by NIR light treatment, I analyzed the immunoreactive levels of Iba-1, typical marker to identify microglia (Yun *et al.*, 2018; Jurga, Paleczna and Kuter, 2020), CD68, which detects activated microglia (Jurga, Paleczna and Kuter, 2020) and GFAP, marker of astrocytes (Preston, Cervasio and Laughlin, 2019). I focused my attention on hippocampus and cortex, two areas highly impacted by HFD/obesity-induced neuroinflammation in both human and rodents (Cavaliere *et al.*, 2019; Zhu, Zhu and Wang, 2019; Crispino *et al.*, 2020). Specifically, I looked at different areas within hippocampus and cortex. For the hippocampus, different areas were separately analyzed: cornu ammonis 3 (CA3), cornu ammonis (CA1) and dentate gyrus (DG), while for the cortex were independently analyzed frontal cortex (FCTX) and parietal/occipital cortex (POCTX).

Hippocampus. In the hippocampal CA3, the quantification of the immunoreactive levels of Iba-1 (Figure 3.5A), CD68 (Figure 3.5A) or GFAP (Figure 3.5B) did not show any significant differences among the four groups of mice, with or without NIR treatment. Therefore, glia activation was neither induced by HFD nor modulated by NIR in CA3.

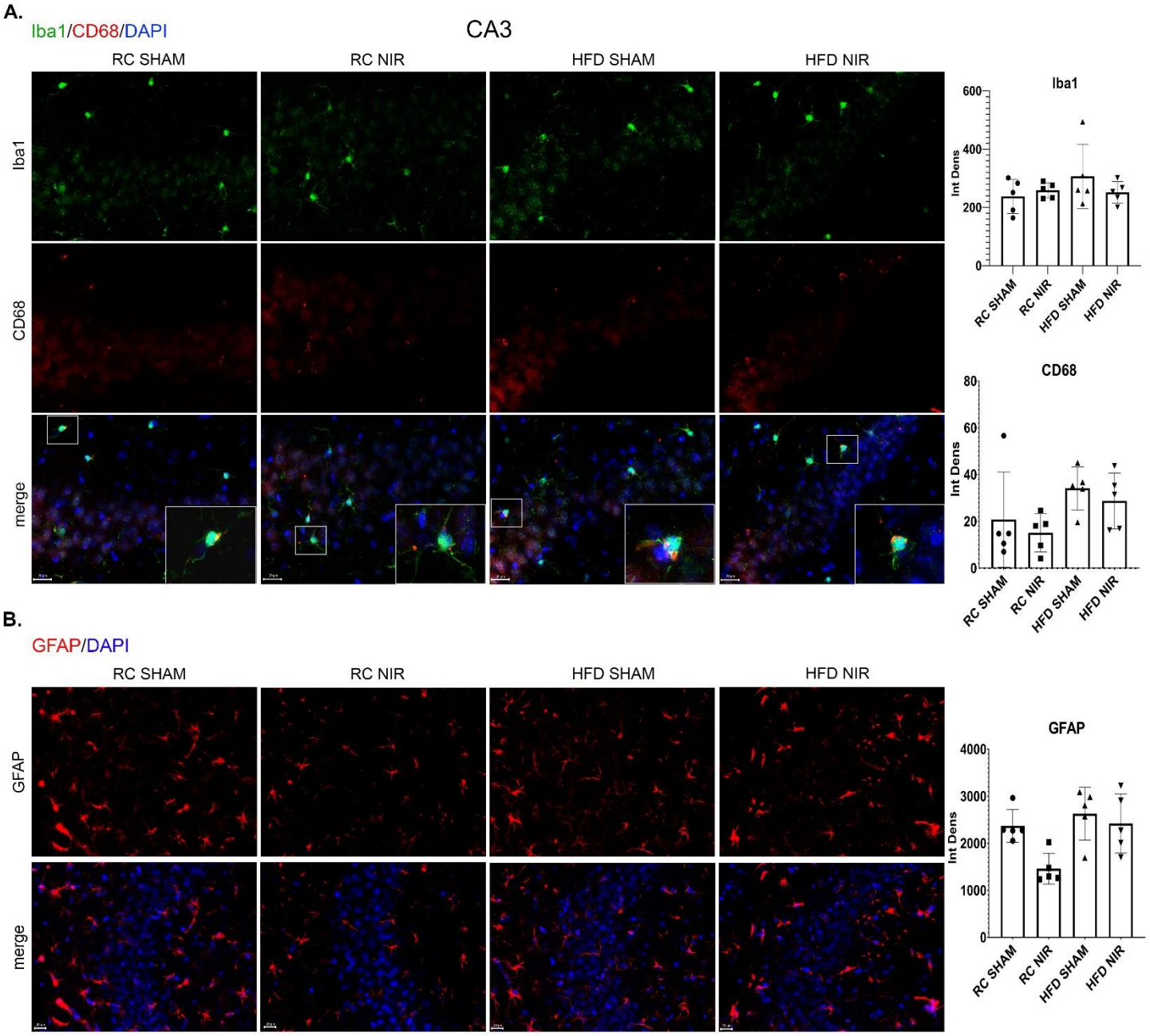
In the CA1 (Figure 3.6A-B), while Iba-1 levels were not changed among the groups, CD68 levels showed significant increase following HFD challenge in comparison to control animals (Figure 3.6A). Moreover, the immunoreactive levels of CD68 were reduced ($p=0.0541$) in HFD mice treated with NIR light treated compared with their untreated counterpart, thus indicating that the HFD-induced microglia activation in the CA1 is lowered by NIR light. In the same area, GFAP showed a similar trend (Figure 3.6B): an increase upon HFD feeding, compared to controls, that is then reduced after NIR light treatment. Therefore, this piece of evidence suggests that the glia activation induced by HFD can be alleviated by NIR light treatment in CA1.

In the DG similar results were observed (Figure 3.7A-B); in fact, while Iba-1 did not show significant changes, CD68 did show a dramatic increase in HFD-fed mice, compared to control mice, and a return to normal levels after NIR light treatment (Figure 3.7A). Similarly, GFAP immunoreactivity was elevated in HFD mice under high-fat dietary regimen, compared to control mice, and was lowered to normal levels in mice treated with the light, compared to HFD SHAM (Figure 3.7B). This evidence displays that NIR light can mitigate HFD-induced glia activation also in DG.

At the end of the analyses conducted for each hippocampal area, I collected all the data from CA3, CA1 and DG, and performed a statistical test to establish the overall outcome (Figure 3.8). Only CD68 and GFAP showed significant levels of reduction between the HFD SHAM and HFD NIR group. Therefore, I report that NIR light mitigates two of the three markers for hippocampal glia activation by HFD.

Overall, the conclusion is that the increased glia activation due to high-fat feeding that took place in DG and CA1 is reduced by NIR light treatment, while in CA3 there is no change induced by either HFD or NIR light delivery.

Cortex. In POCTX, I observed trends very similar to the hippocampus (Figure 3.9A-B). HFD induced higher microglia (Figure 3.9A) and astrocytes (Figure 3.9B) activation in HFD SHAM that was attenuated in the HFD NIR group. However, in the FCTX (Figure 3.10A-B) I did not detect any change caused by HFD or NIR, thus suggesting that this area of the brain reacted differently to the challenge from HFD.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Iba1/CD68/DAPI
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HFD SHAM
HFD NIR

Figure 3.5: Glia markers expression in CA3 of RC- and HFD-fed mice with or without light treatment.

A. Quantitative analyses of Iba-1 and CD68 show not significant changes of the microglia markers among the groups at the hippocampal CA3. Magnification: 60X. B. Quantitative analyses show no change for GFAP in hippocampal CA3. Magnification: 40X.
Images result of Z-stacks with pitch 2.0 μm. n= 5 animals/group. Graphs presented as mean ± SD. Each point is the average of 6 images/animal from 2 technical replicates. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$.

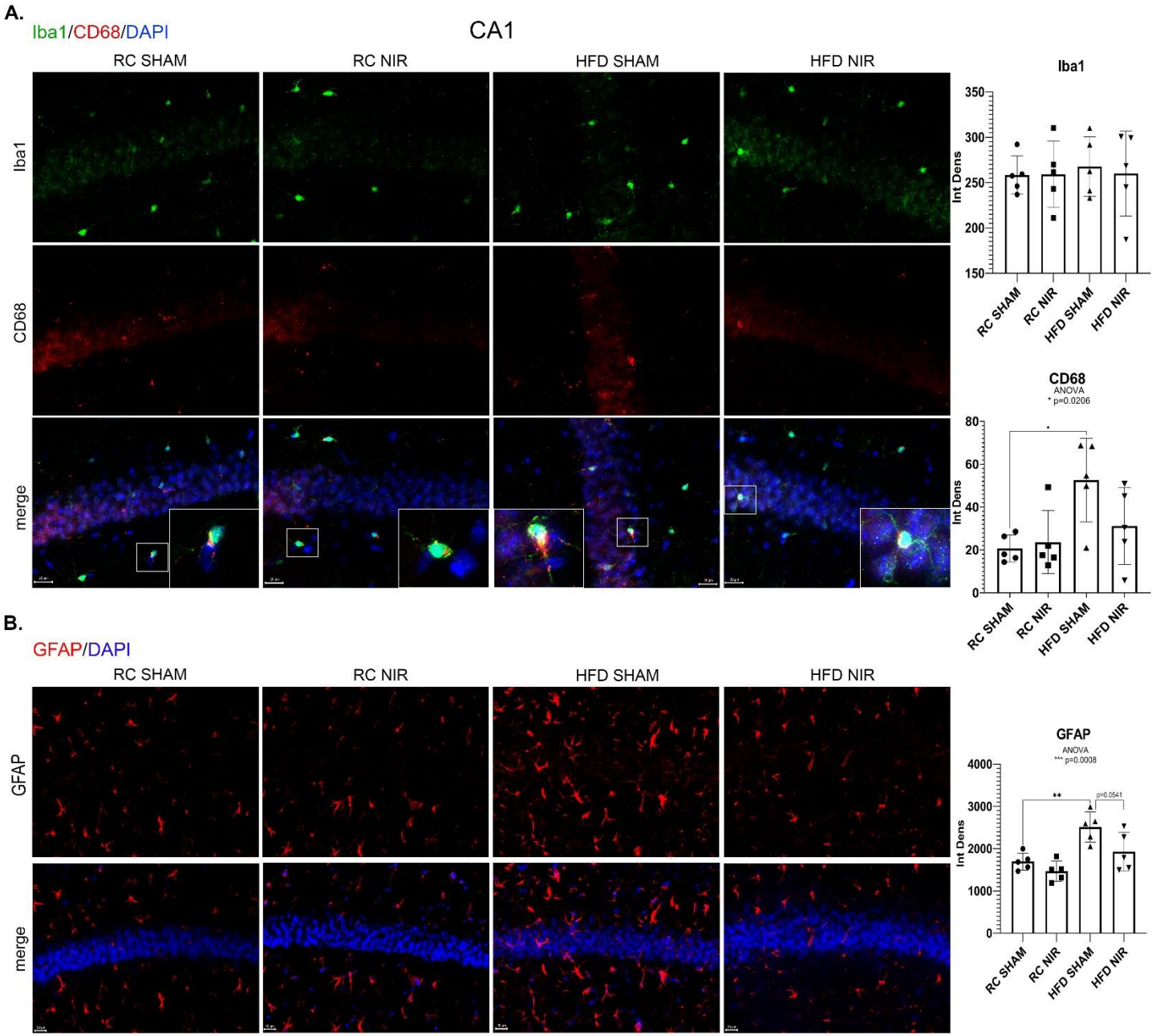

A.
Iba1/CD68/DAPI
CA1
RC SHAM
RC NIR
HFD SHAM
HFD NIR
Iba1
CD68
merge
Iba1
Int Dens
350
300
250
200
150
RC SHAM
RC NIR
HFD SHAM
HFD NIR
CD68
ANOVA
* p=0.0206
Int Dens
80
60
40
20
0
B.
GFAP/DAPI
GFAP
merge
GFAP
ANOVA
*** p=0.0008
** p=0.0541
4000
3000
2000
1000
0

Figure 3.6: Glia markers expression in CA1 of RC- and HFD-fed mice with or without light treatment.

A. While Iba-1 remains unchanged in any condition, immunoreactive levels of CD68 show that microglia activation results elevated in HFD SHAM vs control groups, while it is reduced after NIR light treatment. Magnification: 60X. B. The analyses show that the increase of GFAP upon HFD regimen with respect to RC-fed mice is normalized by NIR light in the CA1 ($p=0.0541$). Magnification: 40X. Images result of Z-stacks with pitch 2.0 μm. n= 5 animals/group. Graphs presented as mean ± SD. Each point is the average of 6 images/animal from 2 technical replicates. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

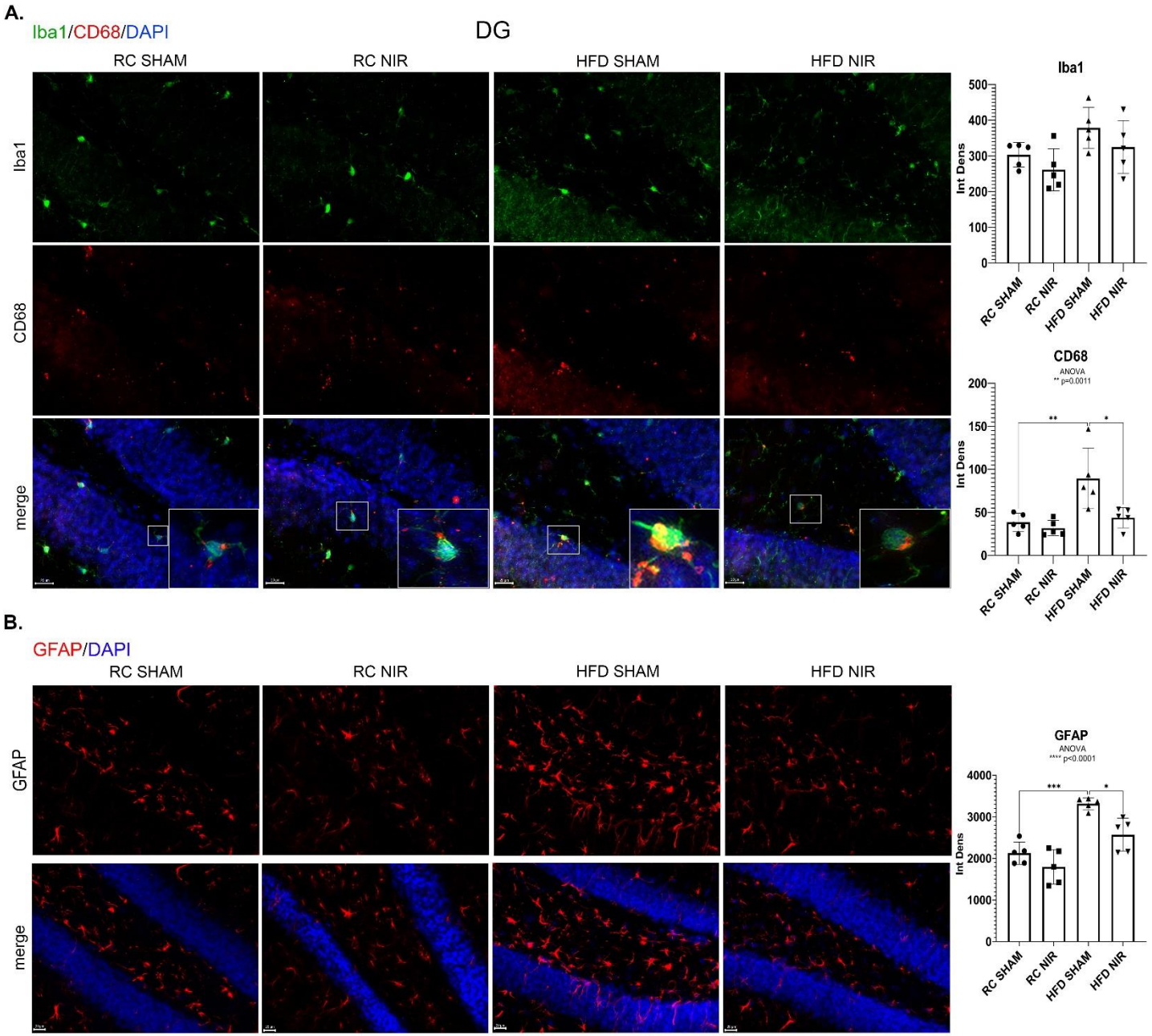
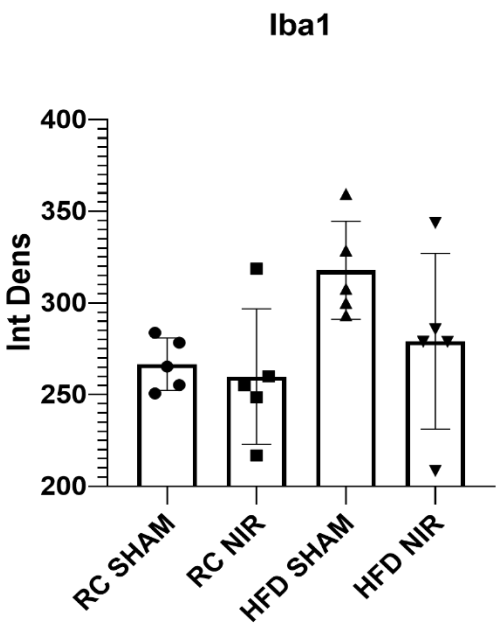
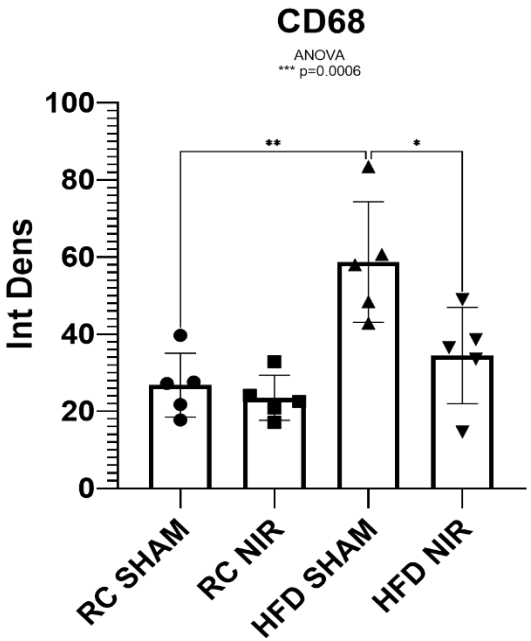

A.
Iba1/CD68/DAPI
DG
RC SHAM
RC NIR
HFD SHAM
HFD NIR
Iba1
CD68
merge
Iba1
Int Dens
500
400
300
200
100
0
RC SHAM
RC NIR
HFD SHAM
HFD NIR
CD68
ANOVA
** p=0.0011
200
150
100
50
0
**
*
B.
GFAP/DAPI
GFAP
GFAP
ANOVA
**** p<0.0001
4000
3000
2000
1000
***

Figure 3.7: Glia markers expression in DG of RC- and HFD-fed mice with or without light treatment

A. Quantification of immunofluorescent images taken in the DG shows that NIR light decreases HFD-induced microglia activation the DG. Iba-1 levels are not significantly changed among the groups. Magnification: 60X. B. In the DG, NIR light reduces also astrocyte activation induced by HFD, as the quantifications show. Magnification: 40X.
Images result of Z-stacks with pitch 2.0 μm. n= 5 animals/group. Graphs presented as mean ± SD. Each point is the average of 6 images/animal from 2 technical replicates. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.


Iba1
Int Dens
400
350
300
250
200
RC SHAM
RC NIR
HFD SHAM
HFD NIR


CD68
ANOVA
*** p=0.0006
Int Dens
100
80
60
40
20
0
**
*
RC SHAM
RC NIR
HFD SHAM
HFD NIR

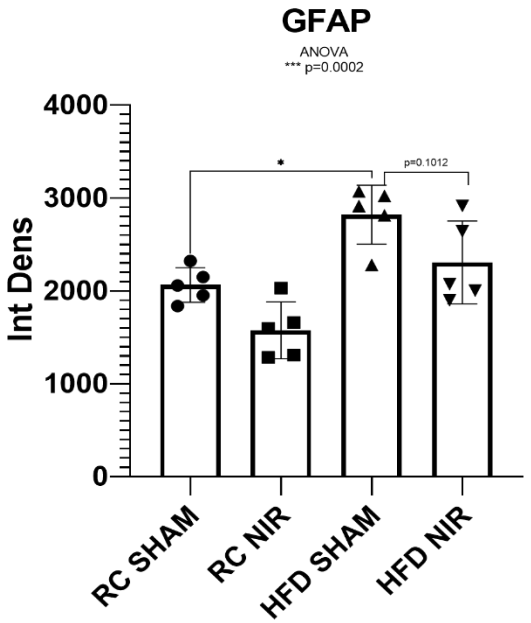

GFAP
ANOVA
*** p=0.0002
Int Dens
4000
3000
2000
1000
0
*
p=0.1012
RC SHAM
RC NIR
HFD SHAM
HFD NIR

Figure 3.8: Glia markers expression in the whole hippocampus of RC- and HFD-fed mice ± light treatment.

A. The overall analyses of all the immunofluorescence images taken in the hippocampal area establish that there is no significant change in Iba-1 immunoreactivity levels. (B.) CD68 results increased in HFD SHAM, compared to control mice, and normalized to levels close to RC-fed mice after NIR light treatment. (C.) The trend is very similar for GFAP, although there is no significant difference between HFD NIR and HFD SHAM.
Graphs presented as mean ± SD. Each point is the average of 18 images/animal from 2 technical replicates Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ****$p<0.0001$.

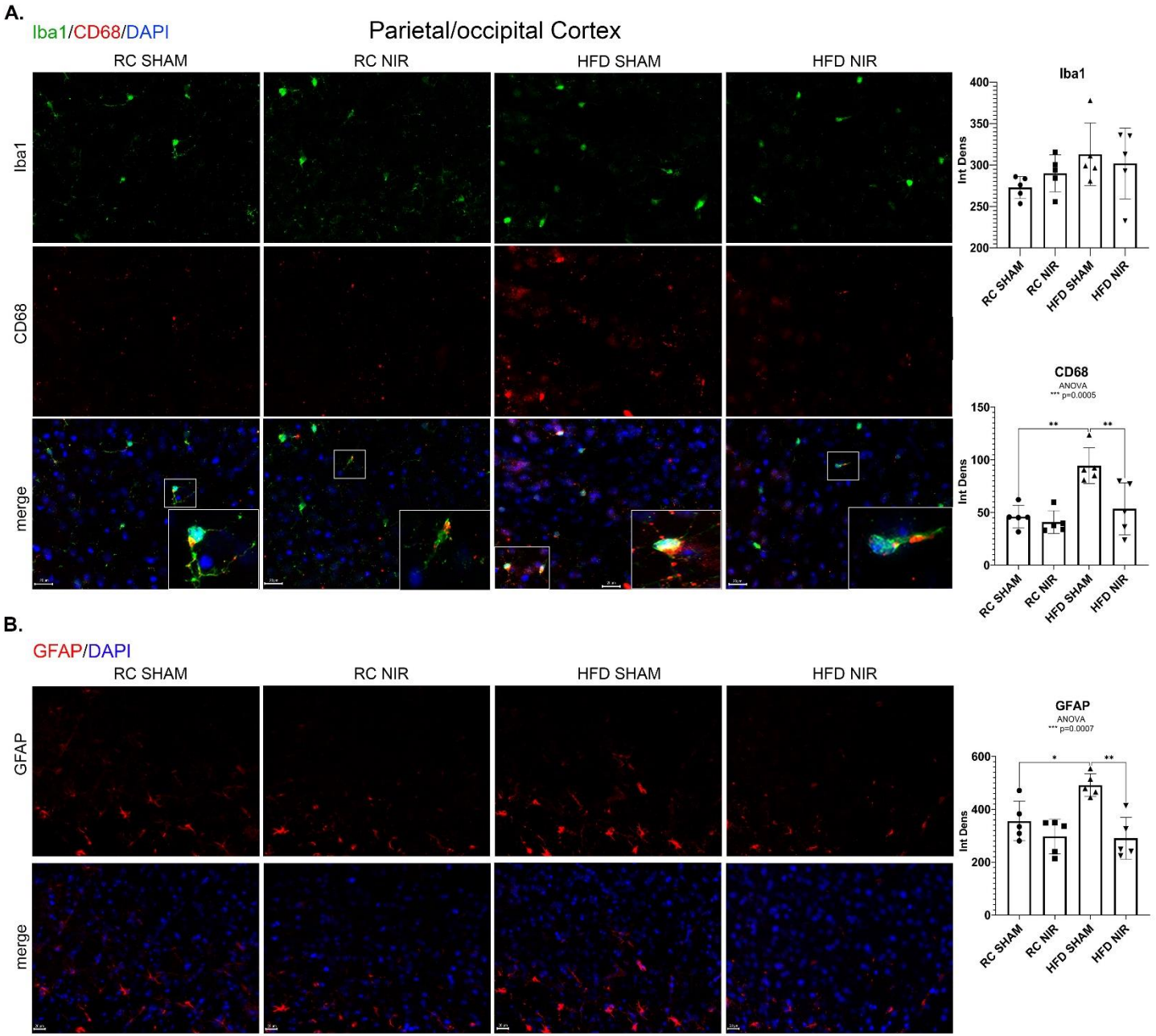

A.
Iba1/CD68/DAPI
Parietal/occipital Cortex
RC SHAM
RC NIR
HFD SHAM
HFD NIR
Iba1
CD68
merge
Iba1
Int Dens
400
350
300
250
200
RC SHAM
RC NIR
HFD SHAM
HFD NIR
CD68
ANOVA
*** p=0.0005
Int Dens
150
100
50
0
**
**
B.
GFAP/DAPI
RC SHAM
RC NIR
HFD SHAM
HFD NIR
GFAP
merge
GFAP
ANOVA
*** p=0.0007
Int Dens
600
400
200
0
*
**

Figure 3.9: Glia markers expression in POCTX of RC- and HFD-fed mice with or without light treatment.

A. In this area we observed not significant changes in Iba-1 expression among the groups, while there is a dramatic increase of microglia activation in HFD SHAM, in comparison to control groups. Conversely, NIR light reduces CD68 in HF-fed mice. Magnification: 60X. B. HFD-induced increase of GFAP is lowered by the NIR light to levels close to control animals. Magnification: 40X.
Images result of Z-stacks with pitch 2.0 μm. n= 5 animals/group. Graphs presented as mean ± SD. Each point is the average of 6 images/animal from 2 technical replicates. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$; *** $p<0.01$; *** $p<0.001$.

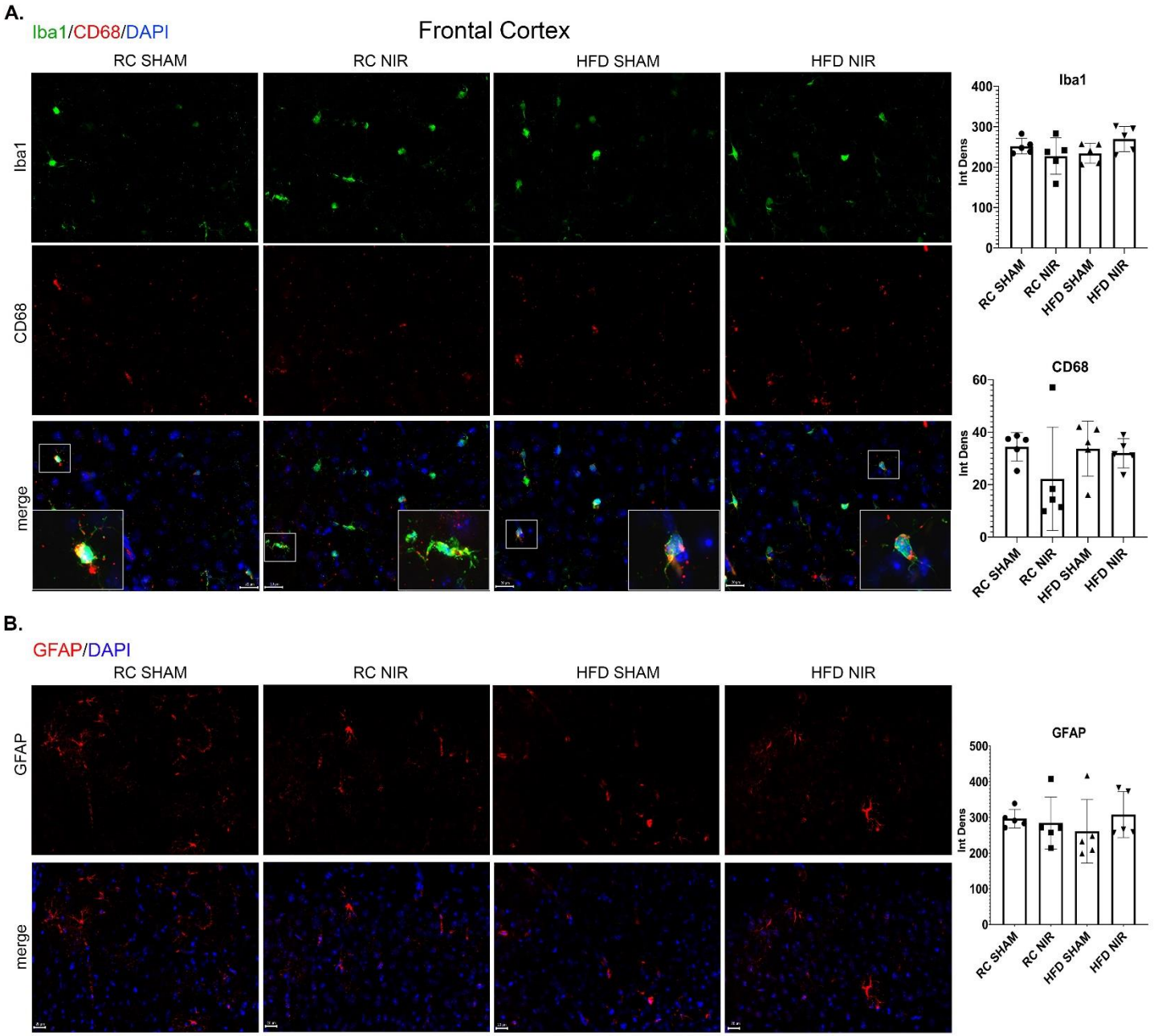

A.
Iba1/CD68/DAPI
Frontal Cortex
RC SHAM
RC NIR
HFD SHAM
HFD NIR
Iba1
CD68
merge
Iba1
Int Dens
400
300
200
100
0
RC SHAM
RC NIR
HFD SHAM
HFD NIR
CD68
Int Dens
60
40
20
0
RC SHAM
RC NIR
HFD SHAM
HFD NIR
B.
GFAP/DAPI
RC SHAM
RC NIR
HFD SHAM
HFD NIR
GFAP
merge
GFAP
Int Dens
500
400
300
200
100
0
RC SHAM
RC NIR
HFD SHAM
HFD NIR

Figure 3.10: Glia markers expression in FCTX of RC- and HFD-fed mice with or without light treatment

A. Quantitative analyses of microglia markers in FCTX did not show any significant difference for both the microglia markers among the groups. Magnification: 60X. B. GFAP did not show any change, as well. Magnification: 40X.
Images result of Z-stacks with pitch 2.0 μm. n= 5 animals/group. Graphs presented as mean ± SD. Each point is the average of 6 images/animal from 2 technical replicates. Statistical analyses: one-way ANOVA with Tukey's post-hoc test.

DISCUSSION

In this study I hypothesized that targeting those early events that may lead to neurodegeneration, such as obesity-induced neuroinflammation, might prevent the occurrence of cognitive decline and ultimately dementia. In particular, there is the need to understand whether is possible to target the neuroinflammation in specific areas that are highly engaged with memory and learning processes, such as hippocampus and cortex. Specifically, I tested the hypothesis that a transcranial delivery of NIR light reduces glia activation and inflammatory cytokines in hippocampus and cortex of DIO mice, thus alleviating obesity-induced neuroinflammation.

Obesity is a prominent risk factor for neurodegenerative diseases (Mazon *et al.*, 2017), including AD (Lloret *et al.*, 2019) and may exacerbate the onset and the progression of AD through several pathological mechanisms that are common to these two pathologies (Kiliaan, Arnoldussen and Gustafson, 2014; Aguilar-Valles *et al.*, 2015; Pugazhenthi, Qin and Reddy, 2017; Kacířová *et al.*, 2020). This suggests that curbing obesity-induced CNS deficits is a likely strategy to prevent neurodegeneration and ultimately dementia, including AD. One of the most studied mechanisms that characterizes both obesity and neurodegenerative diseases is neuroinflammation (Cai, 2013; Martin-Jiménez *et al.*, 2017; Crispino *et al.*, 2020). Rodent models of obesity have shown that an early hypothalamic neuroinflammation arises following 2-3 days of HFD (André *et al.*, 2018; Carraro *et al.*, 2018; N. Xu *et al.*, 2018), that spreads out to other brain areas, such as hippocampus (Hao *et al.*, 2016; Kim *et al.*, 2018; Tsai *et al.*, 2018; Vinuesa *et al.*, 2018; Wu *et al.*, 2018) and cortex (Pistell *et al.*, 2010; Jayaraman, Lent-Schochet and Pike, 2014; Cavaliere *et al.*, 2019), when the animals are kept in HFD feeding regimens for longer times (up to 6 months). Also, mixed animal models of obesity and AD show that HFD and obesity worsen behavioral and cognitive deficits, synapse dysfunction and overall neurodegeneration, in

addition to the aggravation of the neuroinflammation (Takalo *et al.*, 2014; Nam *et al.*, 2017; Sah *et al.*, 2017).

To study the HFD/obesity-induced neuroinflammation the DIO mouse model was employed, namely a wild-type mouse fed chronically a HFD (Wang and Liao, 2012; King and Bowe, 2016). This animal model resembles a condition – the obesity – that endangers CNS integrity and occurs earlier than any evident sign of cognitive dysfunction, regardless of any genetic manipulation. Notably, the DIO mouse model develops obesity, hyperglycemia and insulin resistance, but also decreased neurogenesis, impaired synaptic transmission (Sallam *et al.*, 2015; Krishnan *et al.*, 2020), and neuroinflammation, events that are all present in dementia, including AD, thus offering a unique opportunity to study obesity-induced CNS deficits (Fadel and Reagan, 2016).

Following the preliminary assessments necessary to assure that the HFD regimen had caused the typical impairments that are documented in literature (Reed *et al.*, 2000; Rees and Alcolado, 2005; Wang and Liao, 2012; King and Bowe, 2016), namely weight gain and hyperglycemia, I tested whether our treatment mitigates neuroinflammation. Glia activation was investigated through free-floating immunofluorescence; the results of my investigations show that, in the hippocampus and in the cortex, HFD induces microglia activation, as suggested by elevated levels of CD68, as well as increased astrocyte activation, indicated by increased levels of GFAP, as compared to RC-fed mice. I report regional differences within the hippocampus and the cortex, with the hippocampal CA3 and FCTX not impacted by the HFD.

To reduce the deleterious effects of HFD on brain, I explored the possibility that NIR light may reduce the neuroinflammation determined by obesity. My laboratory previously demonstrated that a transcranial delivery of NIR light for 4 weeks decreases synaptic vulnerability to toxic oligomers in the hippocampus and the cortex of different AD-like mouse models (Comerota, Krishnan and Taglialatela, 2017; Comerota *et al.*, 2018), while other groups demonstrated its effectiveness in reducing neuroinflammation in animal

models of TBI or genetic models of Aβ-pathology (De Taboada *et al.*, 2011; Zhang *et al.*, 2014; Lee, S. W. Lee, *et al.*, 2017). Based on this evidence, I investigated whether NIR light treatments could also be effective as a preventative strategy for cognitive decline and neurodegeneration by targeting specific risk factors for dementia. In this study, I focused on alleviating the impact of obesity on CNS.

The results that were obtained are consistent with data from the literature, where HFD leads to differential glial activation within different areas of the hypothalamus (Lainez *et al.*, 2018) and of the hippocampus (Tsai *et al.*, 2018), as well as from one brain area to another (including areas not included in the analyses here performed) (Baufeld *et al.*, 2016; Guillemot-Legris *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Denver, Gault and McClean, 2018). More specifically, the results here presented showing an increase of GFAP, marker of astrocytes, in CA1, DG and POCTX, following HFD, are consistent with data from the literature (Puig *et al.*, 2012; Baufeld *et al.*, 2016; Guillemot-Legris *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Busquets *et al.*, 2017; Denver, Gault and McClean, 2018; Tsai *et al.*, 2018; Zhu, Zhu and Wang, 2019; Di Bonaventura *et al.*, 2020; Hahm *et al.*, 2020), while studies for microglia are in some measure contradictory. In fact, while some study report that the levels of the microglial marker Iba-1 in the hippocampus were increased upon HFD (Almeida-Suhett *et al.*, 2017; Vinuesa *et al.*, 2018; Suárez *et al.*, 2019; Zhu, Zhu and Wang, 2019; Di Bonaventura *et al.*, 2020; Hahm *et al.*, 2020), other ones show that Iba-1 was not changed following a HFD regimen (Erion *et al.*, 2014; Guillemot-Legris *et al.*, 2016; Denver, Gault and McClean, 2018), like the analyses here conducted suggest; similarly, the studies conducted on the cortex regarding Iba-1 show either similarities (Baufeld *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Denver, Gault and McClean, 2018; Lainez *et al.*, 2018; Taga *et al.*, 2018) with the observations of this study, namely no change following high-caloric diet, or differences, specifically an increased expression of Iba-1 in the cortex (Suárez *et al.*, 2019; Di Bonaventura *et al.*, 2020; Hahm *et al.*, 2020).

Consequently, since significant differences with the Iba-1 marker in both hippocampus and cortex were not appreciated, I investigated a well-recognized marker of activated microglia (Cherry *et al.*, 2016; Korzhevskii and Kirik, 2016; Hendrickx *et al.*, 2017; Neri *et al.*, 2018; Allen *et al.*, 2019; Lananna *et al.*, 2020; van Vliet *et al.*, 2020), namely CD68, in order to assess the microglia activation state. The results here reported show that HFD induced higher microglia activation in CA1, DG and POCTX, while did not cause any evident change in CA3 and FCTX, coherently with the literature observations (Puig *et al.*, 2012).

The different reaction to the HFD that were noticed in this study may be explained in different ways. It is conceivable that such different outcomes from microglia investigations may be due to the different diet regimens (in terms of HFD type and time under HFD) that were administered to the animals, as suggested by Baufeld *et al.* (Baufeld *et al.*, 2016). Furthermore, Guillemot-Legris *et al.* suggest that the differential development of the neuroinflammation in the CNS can be attributed to both time-dependent impairments and distinct involvement of the blood-brain barrier permeability (Guillemot-Legris *et al.*, 2016). In fact, it is known that obesity causes increased permeability of BBB, due to reduced expression of proteins involved in maintenance of tight junctions (Kacířová *et al.*, 2020; Leigh and Morris, 2020), although these alterations seem to mainly involve the hypothalamus (for anatomic reasons) (Cai, 2013), the cerebellum (Guillemot-Legris *et al.*, 2016) and the hippocampus (de Aquino *et al.*, 2019), but not the cortex (Guillemot-Legris *et al.*, 2016). Therefore, I hypothesize that the HFD initially affects those regions where the BBB is altered, due to the increased levels of pro-inflammatory cytokines coming from the periphery and to the increased presence of fatty acids, which can cross BBB and bind receptors on microglial cells, thus leading to their activation that ultimately triggers the neuroinflammation (Cai, 2013; Miller and Spencer, 2014).

The findings shown in this study demonstrate that NIR light alleviates glia activation induced by HFD in CA1, DG and POCTX, by reducing the levels of microglial marker CD68 and astrocytic marker GFAP, while did not have any significant effect in CA3 and

FCTX. The differences between the distinct areas taken into account in this study suggest that the areas showing higher glial activation following HFD feeding have different "sensitivity" to the fat overload. On one hand, it is conceivable that FCTX and CA3 are less stimulated by HFD because the BBB in these areas is less disrupted or is less permeable, compared to CA1, DG and POCTX, thus receiving less stimulation by free fatty acids and pro-inflammatory signals that eventually result in no glia activation. Another plausible explanation suggests that a different population of microglia and astrocyte resides in these areas, implying that longer dietary regimens could lead to a shift of the FCTX-resident (or CA3-resident) glia towards a pro-inflammatory profile.

Taken together, these findings suggest that this strategy may be developed to target obesity-induced neuroinflammation and possibly prevent the neurodegeneration that ultimately leads to dementia. This mitigating effect on glia activation provided by NIR light is consistent with previous studies where a NIR light treatment is administered few hours or few days following injury (Gonçalves *et al.*, 2016; Lee, S.-W. W. Lee, *et al.*, 2017; Blivet *et al.*, 2018; Salehpour, Farajdokht, Cassano, *et al.*, 2019; Vogel *et al.*, 2021), thus suggesting the effectiveness of NIR light treatments following acute neuroinflammation. It is possible that the limited time (4 weeks) of NIR treatment is insufficient to completely address the effects of the chronic inflammation associated with 13 weeks of HFD, however, the effects observed are encouraging and warrant further investigation in the future. Though, it must be emphasized that in this study the light treatments initiated 13 weeks following HFD, therefore when the neuroinflammation has likely become chronic; moreover, during the 4-weeks light treatment, the mice were kept under hypercaloric feeding, hence indicating that the treatment is effective in lowering glia activation despite the continuous presence of stressors, consistently with previous investigations performed in aging mice and AD rodent models where NIR light proved effective in alleviating the neuropathology (De Taboada *et al.*, 2011; Lu *et al.*, 2017; Blivet *et al.*, 2018; El Massri *et al.*, 2018).

Chapter 4. Near Infrared-Light Modulates Inflammatory Cytokines

INTRODUCTION

The increased expression of inflammatory cytokines is an important feature of neuroinflammation. These molecules (chemokines, interleukins, TNFα, and many others) are produced by microglia and astrocytes in consequence of the presence of pathogens or following injuries that may threaten the homeostasis of the CNS (DiSabato, Quan and Godbout, 2016; Morales *et al.*, 2016; Shabab *et al.*, 2017; Rahman, Bhusal, *et al.*, 2018; Giovannoni and Quintana, 2020). While the production of these molecules has positive effects when it lasts for short periods and lead to repair or immune surveillance, an excessive production of inflammatory cytokines causes persistent inflammation and may ultimately lead to neurodegeneration (DiSabato, Quan and Godbout, 2016). Also, in HFD-induced obesity there is an increased expression of inflammatory cytokines in CNS that keeps fueling the neuroinflammation, initially in the hypothalamus, later on in the hippocampus and other brain areas (Miller and Spencer, 2014; Guillemot-Legris and Muccioli, 2017; Kacířová *et al.*, 2020), as well as to exacerbate the neurodegeneration (Erion *et al.*, 2014; Hao *et al.*, 2016). Another aim of this study consisted in understanding whether NIR light can influence inflammatory cytokines, hence alleviating another major feature of the neuroinflammation.

Also, HFD and obesity also induce downregulation of BDNF in the hippocampus and cortex of rodent models of obesity (Molteni *et al.*, 2002; Stranahan *et al.*, 2008; Park *et al.*, 2010; Pistell *et al.*, 2010; Karimi, Motamedi and Ranjbar, 2018; Ramalho *et al.*, 2018; Sona *et al.*, 2018; Cavaliere *et al.*, 2019; de Souza *et al.*, 2019; Zhuang *et al.*, 2019). In fact, BDNF, in addition to its known role in synaptic plasticity and maintenance of synaptic transmission and structure, plays an important role as regulator of energy metabolism by modulating food intake and weight gain, and increasing locomotor activity (Abidin *et al.*,

2018; Sona *et al.*, 2018; Crispino *et al.*, 2020). Moreover, in animal models that are known to develop neuroinflammation (Luo *et al.*, 2019; Kotagale *et al.*, 2020; Lim *et al.*, 2020; Sharma, Saini and Nehru, 2021; Tiwari *et al.*, 2021), BDNF levels are decreased, thus suggesting that neuroinflammatory conditions on their own may contribute in reducing the levels of the neurotrophic factor. Interestingly, NIR light can restore BDNF levels in *in vitro* (Meng, He and Xing, 2013; Yan *et al.*, 2017), *ex vivo* (Heo *et al.*, 2019) and *in vivo* models (Xuan *et al.*, 2015; Ghanbari *et al.*, 2017) in which the neuroinflammation had induced BDNF downregulation. Given these previous results, I tested whether also in our DIO model the light treatment could restore BDNF levels.

METHODS

Animals and diet

See Chapter 2, Section "Methods"

Intraperitoneal glucose tolerance test (IPGTT)

See Chapter 2, Section "Methods"

NIR light treatments

See Chapter 2, Section "Methods"

Quantitative Real Time Polymerase Chain Reaction (q-RT-PCR)

Total RNA was isolated using RNeasy® Mini kit (cat #74104, Qiagen, Germantown, MD, USA) from mice hippocampus and frontal cortex. Approximately 30-40 mg of tissue were homogenized with 600 µl of Buffer RLT, provided in the kit (proprietary composition) + 1% β-mercaptoethanol (cat# 1610710, Biorad, Hercules, CA, USA); each lysate was transferred in a 1.5 ml tube and centrifuged for 3 minutes at full speed. The resulting supernatant was transferred in another 1.5 ml tube and mixed with 1 volume of 70%

ethanol, immediately mixed by pipetting and transferred to the spin columns provided in the kit, and placed in a 2 ml collection tube. The mixture was subjected to centrifugation for 15 seconds at the speed of 10,000 rpm and the flow-through was discarded. To prevent DNA contamination an on-column DNase digestion was performed: 350 µl of the Buffer RW1 (proprietary composition) provided in the kit were added to the column spin followed by a centrifugation for 15 seconds at the speed of 10,000 rpm, after which the flow-through was discarded. At this point, in each column components of the kit RNAse free DNAse set were added (cat# 79254, Qiagen, Germantown, MD, USA): 80 µl of DNase I incubation mix (10 µl DNase I stock solution + 70 µl Buffer RDD (proprietary composition)) was added to each spin column for 15 minutes at room temperature. To remove the excess of DNase, 350 µl of Buffer RW1 were added to each spin column, followed by centrifugation for 15 seconds at 10,000 rpm of speed. After having discarded the flow-through, 500 µl of the Buffer RPE (proprietary composition) were dispensed in the spin columns, followed by centrifugation for 15 seconds at 10,000 rpm. After having discarded the flow-through other 500 µl of Buffer RPE were dispensed in the columns, followed by a centrifugation at 10,000 rpm for 2 minutes; then, the column was detached from the collection tube and placed in a new clean collection tube. To completely dry the column, another centrifugation for 1 minute at full speed was performed, after which the column was placed in a 1.5 ml collection tube. 50 µl of nuclease-free water (cat# W086-050, GenDepot) were added into the columns, with subsequent centrifugation for 1 minute at 10,000 rpm to elute the RNA. The RNA concentration was measured using NanoDrop 2000c (ThermoFisher Scientific, Waltham, MA, USA). Real-time PCR was performed to quantitate mRNA in RC SHAM, RC NIR, HFD SHAM and HFD NIR hippocampi and frontal cortices. The RNA samples were mixed with components of the QuantiFast® SYBR® Green RT-PCR Kit (cat #204154, Qiagen, Germantown, MD, USA) and the QuantiTect® Primer Assay (product #249900, Qiagen, Germantown, MD, USA – TNF-α, cat# QT00104006 – IL-1β, cat# QT01048355 – IL-10, cat# QT00106169 – BDNF cat # QT00097118 – β-actin, cat#

00095242, proprietary composition). In each well of a 96-well plate was dispensed a 25 µl mixture containing: 12.5 µl of the 2X QuantiFast SYBR Green RT-PCR Master Mix (proprietary composition), 6 µl of RNA sample containing 30 ng of RNA, 2.5 µl of QuantiTect® Primer Assay, 0.25 µl of QuantiFast RT Mix (proprietary composition) and 4.75 µl of nuclease-free water (cat# W086-050, GenDepot). The reaction was performed in Mastercycler epgradient S (Eppendorf, 261 Hamburg, Germany) with the following cycler conditions: Reverse Transcription for 10 minutes at 50 °C, PCR initial activation step (activation of DNA polymerase) at 95 °C for 5 minutes, then a two-step cycling, repeated 40 times, consisting of Denaturation for 10 seconds at 95 °C and Combined annealing/extension for 30 seconds at 60 °C. The levels of mRNA from TNF-α, IL-1β, IL-10, BDNF were normalized to those of β-actin. The relative fold change in expression of target miRNAs was determined using the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 8.4.3 software (GraphPad Software, San Diego, CA, USA). One-way ANOVA with Tukey's post hoc test was used to detect significant differences between groups. Data were then expressed as means ± SD and for all statistical analyses $p< 0.05$ was considered as statistically significant.

RESULTS

Effect of NIR light on inflammatory cytokine expression levels in the different brain areas of DIO mice

In neuroinflammation, along with glia activation, there is higher expression of pro-inflammatory cytokines (Park *et al.*, 2016; Jo *et al.*, 2017; Song *et al.*, 2018): this characteristic is also present in animal models of obesity, in particular in both hippocampus and cortex (Vinuesa *et al.*, 2016; Jena *et al.*, 2018; Wu *et al.*, 2018), therefore I investigated

whether NIR light had an effect on cytokine expression levels in our model, by performing quantitative real-time PCR. I analyzed mRNA levels from two areas of the brain, hippocampus and frontal cortex; I chose to study these two areas following the immunohistochemistry results already described, that showed opposite response to both HFD and NIR light treatment. Specifically, I investigated the expression levels of interleukin-1-beta (IL-1β), tumor necrosis factor-alpha (TNF-α), as examples of typical pro-inflammatory cytokines, and interleukin-10 (IL-10) as a typical anti-inflammatory cytokine (Kany, Vollrath and Relja, 2019).

Hippocampus. HFD induced significant increase of TNF-α (Figure 4.1A) and increasing trends of IL-1β (Figure 4.1B) mRNA levels compared to the RC SHAM group. On the other hand, NIR light reduced expression levels of both cytokines to levels close to controls. As far as regards the anti-inflammatory cytokine IL-10 (Figure 4.1C), interestingly it resulted increased in HFD SHAM animals, however, IL-10 levels returned to normal levels upon NIR light treatments, in line with the data from TNF-α. This evidence shows that NIR light can reduce the expression levels of pro-inflammatory cytokines.

Frontal cortex. In the FCTX, I did not observe any change for any group for any cytokine (Figure 4.2), thus agreeing with the immunofluorescence data showed in the previous chapter that show no change in glia activation with either HFD or NIR light.

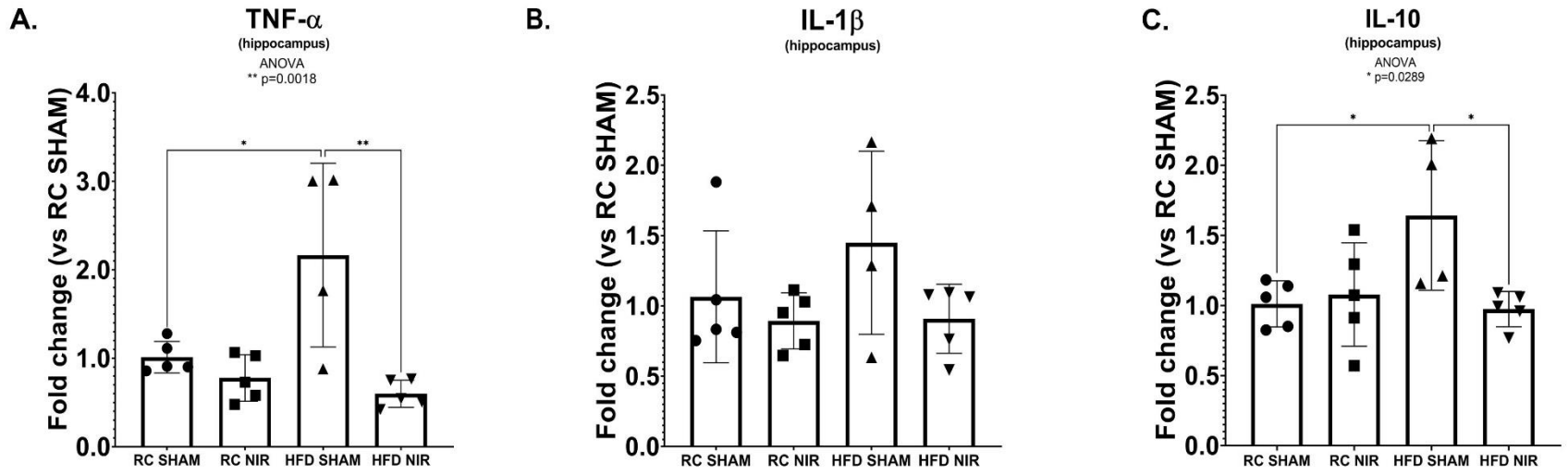


Figure 4.1: Analyses of inflammatory cytokines in the hippocampus of RC- and HFD-fed mice ± light treatment

The two analyzed pro-inflammatory cytokines, TNF-α (A.) and IL-1β (B.), were upregulated in the hippocampus after HFD, compared to control mice (although the increase of IL-1β is not significant). NIR light reduced the expression levels of TNF-α and IL-1β. Interestingly, the anti-inflammatory cytokine IL-10 (C.) shows the same trend seen for TNF-α and IL-1β. n= 4-5 animals/group; 2 replicates/animal; 2 technical replicates. Graphs presented as mean ± SD. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$.

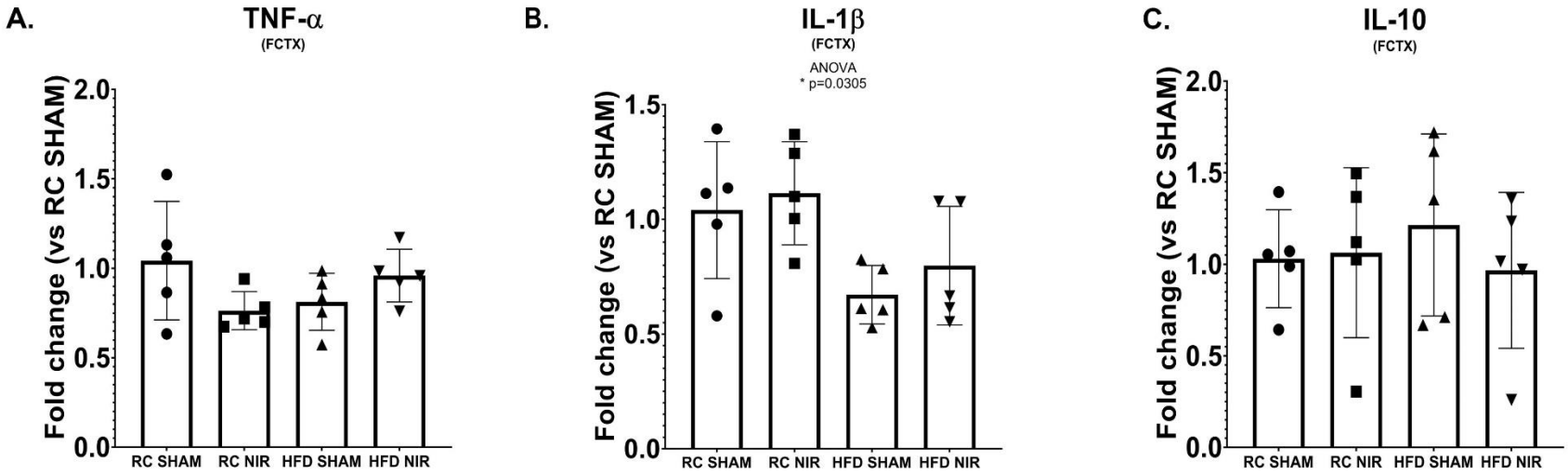


Figure 4.2: Analyses of inflammatory cytokines in the FCTX of RC- and HFD-fed mice ± light treatment.

There is no significant difference for the three analyzed cytokines, TNF-α (A.), IL-1β (B.), IL-10 (C.) in the FCTX.
n= 5 animals/group; 2 replicates/animal; 2 technical replicates. Graphs presented as mean ± SD. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$.

Effect of NIR light on BDNF expression levels in the different brain areas of DIO mice

In view of the well described downregulation of BDNF during neuroinflammation (Sona *et al.*, 2018; Wu *et al.*, 2018, 2019; Lim *et al.*, 2020; Sharma, Saini and Nehru, 2021; Tiwari *et al.*, 2021) and the increased levels of the neurotrophic factor after NIR treatment (Meng, He and Xing, 2013; Xuan *et al.*, 2015; Heo *et al.*, 2019), I tested the expression levels of BDNF in the DIO mouse model, considering its documented role as regulator of energy metabolism and food intake (Stranahan *et al.*, 2008; Abidin *et al.*, 2018; Sona *et al.*, 2018; Crispino *et al.*, 2020). Also in this case, I separately analyzed mRNA levels of BDNF in hippocampus and FCTX with q-RT-PCR.

Hippocampus. In the hippocampus (Figure 4.3A) I observed a decreasing trend of BDNF after HFD. Interestingly, NIR light treatment led to an increase of this neurotrophic factor, compared to HFD SHAM, suggesting that the anti-inflammatory effect of NIR light might involve BDNF activation as a mechanism influencing neuroinflammation that will be explored in future studies.

Frontal cortex. Not surprisingly, BDNF did not show significant changes in FCTX (Figure 4.3B); although the statistical analysis that was performed on the FCTX data revealed an overall significant p-value (*p=0.0308), Tukey's multiple comparison did not show any difference among the groups, thus confirming that the FCTX reacts differently to the HFD challenge, confirming what was observed in immunofluorescence analyses.

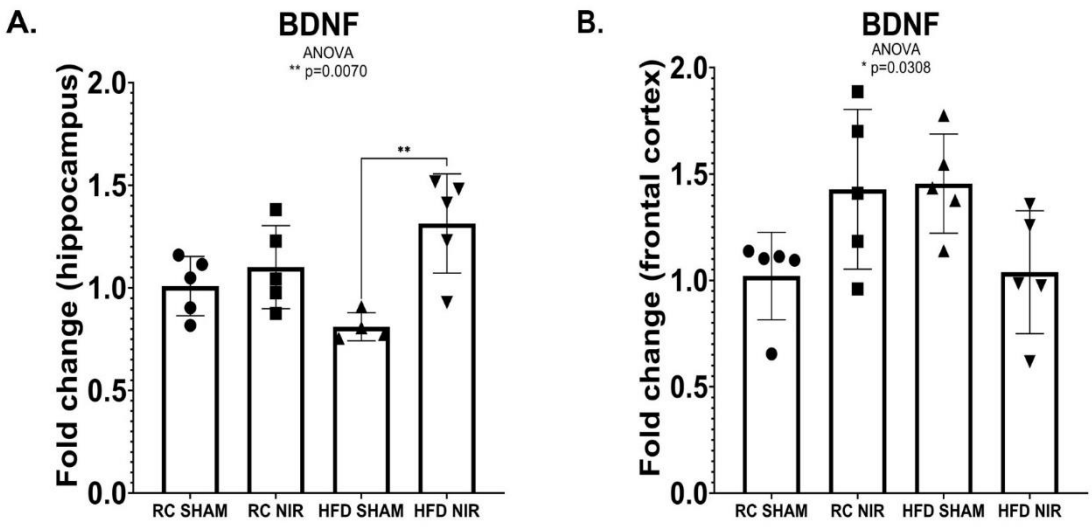


Figure 4.3: Analyses of BDNF in hippocampus and FCTX of RC- and HFD-fed mice ± light treatment

A. In the hippocampus, BDNF is downregulated in HFD SHAM vs. RC mice, even if the decrease is not significant. However, NIR light induces upregulation of this gene in HF-fed mice, compared to untreated ones. B. Although the statistical analyses suggest differences of BDNF expression in the FCTX, the Tukey's multiple comparison did not show any significant difference among the groups.
n= 4-5 animals/group; 2 replicates/animal; 2 technical replicates. Graphs presented as mean ± SD. Statistical analyses: one-way ANOVA with Tukey's post-hoc test. * $p<0.05$; ** $p<0.01$.

DISCUSSION

The increased expression of inflammatory cytokines is typical of neuroinflammation. These molecules (chemokines, interleukins, TNFα, and many others) are produced by microglia and astrocytes in consequence of the presence of pathogens or following injuries that may threaten the homeostasis of the CNS (DiSabato, Quan and Godbout, 2016; Morales *et al.*, 2016; Shabab *et al.*, 2017; Rahman, Bhusal, *et al.*, 2018; Giovannoni and Quintana, 2020). The production of these molecules has positive effects when it lasts for short periods and lead to repair or immune surveillance, however an excessive production of inflammatory cytokines leads to chronic inflammation that may prodromal to neurodegeneration (DiSabato, Quan and Godbout, 2016). Also, in HFD-induced obesity there is an increased expression of inflammatory cytokines in CNS that sustains the neuroinflammation, starting in the hypothalamus, then spreading out in the hippocampus and other brain areas (Miller and Spencer, 2014; Guillemot-Legris and Muccioli, 2017; Kacířová *et al.*, 2020), which may lead to neurodegeneration (Erion *et al.*, 2014; Hao *et al.*, 2016). The experiments here presented show that NIR light can attenuate the expression of such inflammatory cytokines, thereby alleviating a major feature of neuroinflammation that could lead to neurodegeneration.

Notably, while some studies show increased expression levels of inflammatory cytokines in the cortex of HFD-fed mice (Pistell *et al.*, 2010; Jayaraman, Lent-Schochet and Pike, 2014; Cavaliere *et al.*, 2019), other ones show no significant change, in line with the observations here presented (Boitard *et al.*, 2014; Guillemot-Legris *et al.*, 2016; Almeida-Suhett *et al.*, 2017; Taga *et al.*, 2018; Wu *et al.*, 2019). Therefore, one plausible conclusion is that FCTX responds differently to HFD, compared to the hippocampus, at least in the conditions here proposed, as already seen with the analyses from the previous chapter. This differential response to HFD does not induce in the FCTX any increase on the expression of inflammatory cytokines, to the contrary of the hippocampus. It is also possible that

longer hypercaloric dietary regimens would induce signs of neuroinflammation in the FCTX, as well; perhaps, the structural differences between FCTX and hippocampus may underlie different responses to the HFD challenge, as already hypothesized from the immunohistological data.

I observed an increase of the anti-inflammatory cytokine IL-10 upon HFD in the hippocampus, that was lowered after NIR light treatment. I hypothesize that the levels of IL-10 may have increased as an attempt by the hippocampal glia to counteract the stress due to overload of fats; a similar mechanism was proposed to explain the increase of this anti-inflammatory cytokine in the hypothalamus of DIO mice after 8 weeks of HFD (Baufeld *et al.*, 2016). Mice fed a HFD show an initial increase of pro-inflammatory molecules, followed by a switch to an anti-inflammatory profile for prolonged HFD regimen, most likely in an attempt to reduce a defective or rather maladaptive microglia response (Baufeld *et al.*, 2016). In the present study, it was observed an increase of IL-10, in the hippocampus, following HFD, alongside the expected increase of the pro-inflammatory cytokines (especially TNF-α), similarly to what Han and colleagues demonstrated in their study (Han, Leem and Kim, 2019). On the other hand, NIR light lowers not only the levels of the pro-inflammatory cytokines, but also those of IL-10, thus suggesting that the hippocampal upregulation of IL-10, albeit likely triggered as an attempt to cope with the chronic stimuli coming from high-fat feeding, is rather an excessive response by the altered regional glia that NIR light is able to counteract and normalize. The ability of NIR light to normalize microglia activity has been previously proposed in *in vitro* and *in vivo* models (Song, Zhou and Chen, 2012; Song *et al.*, 2017). Researchers have showed that microglia cell cultures treated with NIR light show higher phagocytic activity and efficiency, especially when it is delivered to cells previously exposed to lipopolysaccharide (LPS) (Song, Zhou and Chen, 2012). This observation may suggest that the light treatment improves efficiency of microglia when the chronic presence of toxic molecules and stressful conditions likely induce exaggerated inefficient responses by

microglia. Therefore, the decrease of all the inflammatory cytokines by NIR light could be the result of an enhanced efficiency of microglia, that in turn reduces neuroinflammation. Likewise, in a rat model of spinal cord injury, the application of NIR light induces microglia to acquire an anti-inflammatory phenotype and to reduce the secretion of TNF-α, seven days post-injury (Song *et al.*, 2017), thus suggesting that NIR light triggers protective mechanisms aimed at preventing the glia activation and the release of inflammatory cytokines, in order to neutralize threatening conditions that may alter homeostasis.

HFD and obesity also induce downregulation of BDNF in the hippocampus and cortex of rodent models of obesity (Molteni *et al.*, 2002; Stranahan *et al.*, 2008; Park *et al.*, 2010; Pistell *et al.*, 2010; Karimi, Motamedi and Ranjbar, 2018; Ramalho *et al.*, 2018; Sona *et al.*, 2018; Cavaliere *et al.*, 2019; de Souza *et al.*, 2019; Zhuang *et al.*, 2019). BDNF, in addition to its known role in synaptic plasticity and maintenance of synaptic transmission and structure, plays an important role as regulator of energy metabolism by modulating food intake and weight gain, and increasing locomotor activity (Abidin *et al.*, 2018; Sona *et al.*, 2018; Crispino *et al.*, 2020). In animal models that are known to develop neuroinflammation (Luo *et al.*, 2019; Kotagale *et al.*, 2020; Lim *et al.*, 2020; Sharma, Saini and Nehru, 2021; Tiwari *et al.*, 2021), BDNF levels are decreased, thus suggesting that neuroinflammatory conditions on their own may contribute in reducing the levels of the neurotrophic factor. Interestingly, NIR light can restore BDNF levels in *in vitro* (Meng, He and Xing, 2013; Yan *et al.*, 2017), *ex vivo* (Heo *et al.*, 2019) and *in vivo* models (Xuan *et al.*, 2015; Ghanbari *et al.*, 2017) in which the neuroinflammation induces BDNF downregulation. In the present study, it was observed a decreasing trend for BDNF in the hippocampus following HFD, that is consistent with the immunostaining analyses and cytokines evaluations. NIR light treatment increased BDNF levels dramatically. On the other hand, in the FCTX, there was no significant differences among the groups of animals. Once again, I observed different outcomes among hippocampus and FCTX, thus further

suggesting that these two areas act differentially in response to HFD. However, it is important to note here that it cannot exclude the possibility that different protocols of hypercaloric food might lead to different outcomes. Whether the upregulation of BDNF induced by NIR light either determines decreased neuroinflammation or is an effect of the diminished neuroinflammation, remains to be established. At the same time, it is possible that the NIR light activates protective mechanisms for the CNS somehow involving BDNF, that necessitates future studies.

In this study the mechanistic pathways underlying the pathology were not explored in order to ascertain the involved anti-inflammatory effect of NIR light, but rather the aim was to establish whether NIR light can be proposed as a preventative approach for those conditions known to pose serious threats for CNS integrity. Pro-inflammatory stimuli cause NF-κB translocation to the nucleus with consequent activation of the expression of pro-inflammatory genes. NF-κB translocation can be induced by generation of ROS, especially in conditions of oxidative stress induced by several stressors (Hamblin, 2017). Paradoxically, in normal cells, NIR light causes an increase of ROS, that in turn activates NF-κB translocation, with consequent transcription of pro-inflammatory genes. Nonetheless, the increase of ROS induced by NIR light is modest and is accompanied by an increase of the antioxidant defenses, in order to promptly remove the transient burst of ROS (Ramezani *et al.*, 2021). Also, it has been demonstrated that cells already impacted by neuroinflammation and oxidative stress show reduction of NF-κB, thus leading to reduced neuroinflammation, when treated with NIR light (Hamblin, 2019).

In this study it was showed that NIR light is able to modulate the levels of inflammatory cytokines, consistently with previous studies (Salehpour, Farajdokht, *et al.*, 2018; Hamblin, 2019; Salehpour, Farajdokht, Cassano, *et al.*, 2019; Ramezani *et al.*, 2021): since NF-κB pathway can be activated in microglia through TLRs receptors, which in turn can be bound by lipids and cytokines, it is conceivable that the modulation of inflammatory cytokines levels occurs by regulating the cytosolic NF-κB in order to prevent the translocation of its

p65 subunit to the nucleus, as suggested by previous studies (Hashmi *et al.*, 2010; Song, Zhou and Chen, 2012; Lee, S.-W. W. Lee, *et al.*, 2017; Salehpour, Farajdokht, Cassano, *et al.*, 2019; Salehpour, Farajdokht, Mahmoudi, *et al.*, 2019).

NIR light decreases, in the brain, the levels of the main anti-inflammatory cytokines (IL-1β and TNF-α), as well as decreases glia activation, in different models of TBI and AD (De Taboada *et al.*, 2011; Zhang *et al.*, 2014; Lee, S.-W. W. Lee, *et al.*, 2017; Salehpour, Mahmoudi, *et al.*, 2018). Likewise, NIR light upregulates BDNF in rodent injury-models, thus suggesting that this effect may provide neuronal and synaptic protection (Meng, He and Xing, 2013; Ghanbari *et al.*, 2017; Yan *et al.*, 2017; Heo *et al.*, 2019). One of the possible mechanisms by which NIR light can lower the levels of inflammatory cytokines and microglia activation is the change of morphology induced by light. Microglia can in fact acquire different phenotypes, depending on the prevalence of pro- or anti-inflammatory stimuli: classically, the microglia cells are divided in two major phenotypes, M1 pro-inflammatory and M2 anti-inflammatory, and this balance shifts towards M1 in obesity conditions (Mendes *et al.*, 2018). NIR light is known to induce polarization towards an M2 phenotype, namely to an anti-inflammatory condition (Von Leden *et al.*, 2013; Song *et al.*, 2017; Hamblin, 2019), which is consistent with the results here reported showing reduction of inflammatory cytokines in the hippocampus and reduction of glia activation in hippocampal CA1 and DG and POCTX. These effects may be mediated through modulation of the protein Src, which in turn regulates many other pathways, such as NF-κB activation, PI3K/Akt to favor survival of the cells, and Rac to induce F-actin polymerization that induces higher motility (Song, Zhou and Chen, 2012). Also, BDNF levels are upregulated following NIR light treatment in animal models that develop neuroinflammation, and this effect seems to be mediated by the upregulation of the ERK/CREB pathway, and by the increasing effect on the levels of calcitonin gene-related peptide (CGRP), involved in inflammation, stress, injury and pain (Meng, He and Xing,

2013; Hamblin, 2019; Heo *et al.*, 2019; Enengl, Hamblin and Dungel, 2020; Ramezani *et al.*, 2021).

Taken together, these observations suggest that the anti-inflammatory effect of NIR light on obesity-induced neuroinflammation may involve BDNF (and perhaps through CREB and ERK) in order to restore the proper control on food intake and metabolism by CNS. Moreover, given the paramount importance of BDNF in the maintenance of synapses functionality and integrity, I hypothesize that its upregulation in the hippocampus, induced by NIR light, may retrieve or improve synaptic transmission and neuronal plasticity, with beneficial effects on memory and learning processes. Besides, the previous work published by my laboratory showed that NIR light ameliorates the cognitive impairment of hTau mice models, while diminishing the concentration of toxic Tau oligomers at the hippocampal and cortical synaptosomes (Comerota *et al.*, 2018).

Chapter 5. Conclusions and Future Directions

CONCLUSIONS

The present study shows that a transcranial delivery of NIR light reduces microglia and astrocyte activation caused by HFD, in hippocampus and POCTX of diet-induced obese mice, normalizes the increased levels of inflammatory cytokines caused by HFD in the hippocampus, and upregulates the hippocampal levels of BDNF. Interestingly, my results show that FCTX is not impacted by obesity, at least in the dietary conditions that were offered in this study, albeit it cannot be ruled out that longer dietary treatment would have impacted this area, as well. However, this different reaction to the challenge offered by HFD and obesity suggests that the different brain areas may have different types of glia cells, compared to hippocampus and POCTX.

Notably, also within the hippocampus substantial differences are noticeable: while DG and CA1 show increased microglia and astrocyte activation upon HFD feeding, CA3 results not affected by HFD; likewise, NIR light treatment is more effective in DG and CA1, while does not exert any action in CA3.

Alternatively, the differences among the regions that were analyzed here can be explained hypothesizing that each hippocampal region reacts differently to the stressful conditions caused by HFD, perhaps due to differences in BBB permeability. Similarly, the differences reported between FCTX and POCTX may be explained in the same way, thus suggesting a different "sensitivity" to the overload of fatty acids that is able to cross BBB.

Moreover, the opposite trends regarding the levels of inflammatory cytokines in hippocampus and FCTX corroborate the hypothesis of a different permeability between the different areas of the brain. Also, it is reasonable to hypothesize that these differences may be due to the presence of distinct glia population between the areas taken into account, which would reveal the different reaction to the HFD. One possible explanation is that the

areas with increased glia activation upon HFD have an altered glia that sustain neuroinflammation, while the areas with no sign of neuroinflammation may be populated by a functional glia. Nonetheless, it is worthy to underline once again the possibility that these opposite results would eventually overlap in a neuroinflammatory condition if the dietary treatment lasted for longer times.

Regarding BDNF it must be noted that it exerts a crucial role in synapse maintenance and development, especially in the hippocampus, and it is also downregulated in neuroinflammatory states (Tong *et al.*, 2012; Lynch, 2015; Lima Giacobbo *et al.*, 2019; Crispino *et al.*, 2020; Lim *et al.*, 2020; Sharma, Saini and Nehru, 2021; Tiwari *et al.*, 2021). Animal models of injury, such as TBI and stroke, that were treated with transcranial illuminations of NIR light delivered within hours of the injury, showed increased levels of BDNF upon light treatment (Meng, He and Xing, 2013; Xuan *et al.*, 2015; Ghanbari *et al.*, 2017; Lee, S.-W. W. Lee, *et al.*, 2017; Heo *et al.*, 2019). Therefore, the upregulation of this neurotrophic factor in obese mice after light delivery confirms the capacity of NIR light of stimulating biochemical pathway aimed at protecting synapses and neuronal function through BDNF, in presence of stressors and conditions that may alter the homeostasis (Hamblin, 2016, 2017, 2019; Thunshelle and Hamblin, 2016; Enengl, Hamblin and Dungel, 2020).

Therefore, it can be concluded that NIR light reduces the impact of obesity on brain function, particularly the neuroinflammation, one of the early key-events underscoring dementia, including AD, and perhaps induces neuroprotective mechanisms that involve BDNF.

In this project, the treatment with NIR light was started 13 weeks after the beginning of HFD, therefore at a time when the neuroinflammation has already become chronic. That the approach used in this study was successful in lowering neuroinflammation suggests that can also be used in chronic pathologic situations. However, understanding the limits

of the time window within which such treatment would be still successful has to be investigated in the future.

Taken together, these observations suggest that NIR light approach has potential to be developed as a novel, preventative, and non-invasive treatment for those individuals with obesity, diabetes and metabolic syndrome, who are at high risk of developing dementia, including AD. The strategy to prevent cognitive decline should encompass the treatment and the prevention of those conditions that pose a serious risk of accelerating the occurrence of AD. For example, since obesity in mid-life is known to be a major risk factor for AD, in particular for people in the age range 45-64 (Pugazhenthi, Qin and Reddy, 2017; Crispino *et al.*, 2020; Kacířová *et al.*, 2020; Leigh and Morris, 2020), any treatment aimed at reducing the impact of obesity on brain function, could prevent the development of dementia, including AD. Besides promoting weight loss programs, physical activity, healthy diet in order to reduce adiposity and the damage that obesity may cause to the entire organism, including the onset of neurodegenerative diseases, NIR light treatment can be added as further ameliorative strategy aimed at preventing the neuronal impairments associated with obesity-induced neuroinflammation. It is conceivable that curbing neuroinflammation would reduce the risk of promoting neurodegeneration, considering that microglia and astrocytes activation and the excess of pro-inflammatory cytokines has been associated with promotion of protein aggregation and impaired synaptic and neuronal function.

Previous results from my laboratory have shown that NIR light has proven successful in reducing the levels of toxic oligomers in different AD mouse model, thus limiting their deleterious impact on synapse and neuronal function (Comerota, Krishnan and Taglialatela, 2017; Comerota *et al.*, 2018). In addition, the results here presented show that NIR light treatment is effective in curbing events that may pave the way to neurodegeneration. Therefore, NIR light could be used either to slow down the progression of AD pathology or to alleviate the neuroinflammation that may accelerate the onset of

AD. Preventing the occurrence of dementia, through transcranial illumination of NIR light, can in fact be developed as a preventative treatment for AD; on the other hand, developing an approach that would be able to slow down and perhaps stop the progression of the disease may be an attractive avenue to pursue in the effort of fighting dementia, including AD. In the previous chapters, I mentioned that much research effort has focused on treatments, strategies and programs that would prevent cognitive decline and dementia, rather than finding a disease-modifying treatment (Imtiaz *et al.*, 2014; Crous-Bou *et al.*, 2017; Livingston *et al.*, 2017, 2020; Joe and Ringman, 2019; Bhatti *et al.*, 2020; Oxford, Stewart and Rohn, 2020; Vaz and Silvestre, 2020; Alzheimer's Association, 2021). Considering that the development of several drugs has led to disappointing outcomes and that the number of failed attempts has reached staggering levels, the researchers have started to develop preventative strategies that could be more efficient than a curative therapy, therefore NIR light transcranial delivery is a feasible, safe and effective strategy that can be further advanced. Although NIR light can potentially reduce the toxic effect of oligomers on synapse, thus slowing down the progression of neurodegeneration, is also prudent to propose such non-invasive method to support the prevention of AD and other neurodegenerative diseases through reducing the accompanying neuroinflammation. Limiting the discussion to obesity-related risk of AD, a potential novel treatment, such as the transcranial delivery of NIR light, must not be considered as a substitute of weight loss programs; likewise, since metabolic syndrome and T2DM often accompany obesity, NIR light cannot replace antidiabetic treatments or antihypertensive drugs, meant to control glycemia, blood pressure and heart functionality. However, the development of a treatment that protects brain function, such as the one presented here, shall be considered.

Recently, several studies and clinical trials based on the transcranial delivery of NIR light have proven successful in patients with AD and other neurologic and neurodegenerative disorder where neuroinflammation plays a crucial role in their progression, such as depression, PD and TBI (Maksimovich, 2012; Hamblin, 2016; Berman *et al.*, 2017;

Saltmarche *et al.*, 2017; Salehpour, Mahmoudi, *et al.*, 2018; Enengl, Hamblin and Dungel, 2020). At the same time, NIR light has been demonstrated effective in improving the cognitive performance in normal subjects (Gonzalez-Lima and Barrett, 2014; Blanco, Maddox and Gonzalez-Lima, 2017; Chan *et al.*, 2019; Holmes *et al.*, 2019). Furthermore, several studies conducted in animal models have demonstrated that NIR lowers crucial elements of neuroinflammation, namely glia activation and overexpression of pro-inflammatory cytokines, in models of injury, depression and neuropsychiatric disorders. Therefore, this strategy may be proposed for several other neurodegenerative diseases, besides AD, such as PD, MS, TBI, stroke and ischemia. On the other hand, the beneficial effects of NIR light can be useful for the treatments of these diseases, not only their prevention: the capacity of NIR light to promote angiogenesis can be useful to ameliorate the blood flow and the oxygen supply to the brain tissues in case of trauma or ischemia; at the same time, the upregulation of the neurogenesis may lead to the replacement of damaged neurons due to trauma (TBI, stroke, ischemia) or neurodegeneration, thus limiting the effect of neuronal loss; also, the stimulation of neurotrophic factors can help the maintenance of functional synapses, thus limiting the progression of neurodegenerative diseases, where neurotransmission is impaired; likewise, neurologic disorders such as depression could be potentially treated with NIR light deliveries, thus avoiding the use of antidepressant that often carry severe side effects that reduce the overall quality of life; in general, the beneficial effects against neuroinflammation can halt the evolution of the pathology, thus avoiding the accelerating effect of a detrimental microglia, as well as the continuous activation of pro-inflammatory pathways mediated by cytokines; finally, the improvements in mitochondrial activity, energy metabolism and antioxidant defense, on one hand reinstate a normal metabolic activity, while on the other, provide protective effects for neurons and glia (Von Leden *et al.*, 2013; Oueslati *et al.*, 2015; El Massri *et al.*, 2016; Gonçalves *et al.*, 2016; Thunshelle and Hamblin, 2016; Hamblin, 2016, 2017, 2019; Moro *et al.*, 2016; Song *et al.*, 2017; Lee, S.-W. W. Lee, *et al.*, 2017; Salehpour and Rasta,

2017; Salehpour, Farajdokht, *et al.*, 2018; Salehpour, Cassano, *et al.*, 2019; Salehpour, Farajdokht, Cassano, *et al.*, 2019; Salehpour, Farajdokht, Mahmoudi, *et al.*, 2019; Salehpour *et al.*, 2020; Vogel *et al.*, 2021). Therefore, the wide range of beneficial effects showed by NIR light poses this approach as a potential strategy for the prevention of several neurodegenerative disorders and neurologic pathologies.

It must be highlighted that NIR light delivery is non-invasive and does not cause major side effects. However, it is hard to define at this point standardized protocols for the administration of the light, considering that different parameters need to be assessed, such as duration of the treatment, wavelength, energy to deliver, power density (irradiance), energy density (fluence), energy absorption, distance travelled, light source (coherence). Given that the literature is discordant regarding these parameters, once a general consensus from research and clinical trials will be reached, this approach could become a groundbreaking strategy to prevent aging-associated pathologies, especially neurodegenerative disorders (Avci *et al.*, 2013; Salehpour, Mahmoudi, *et al.*, 2018; Ramezani *et al.*, 2021).

In this study, it was utilized a light source of 670 nm, which differs somewhat from several studies that employ lights with wavelength in the range 800-810 nm. Athough CCO, the most important chromophore, absorbs mostly in the range 600-700 nm, a longer wavelength guarantees deeper penetration, especially in humans, where the barriers constituted by bones and hair are more difficult to overcome, compared to animal models (Salehpour, Cassano, *et al.*, 2019). Moreover, the absorption of water, that occurs at wavelength greater than 800 nm could even be a positive factor, thanks to the structural change of the IWL that favors higher activity from ATP synthase and ion channels activated by light delivery (Hamblin, 2019). In conclusion, considering the number of studies that successfully used the 670 nm light source (see Tables 1.3 and 1.4), it is safe to state that this wavelength (in addition to the type of light, the power density and the energy dose) is sufficient to generate a beneficial effect in the brain. However, it must be

acknowledged that in animal models, especially rodents, shorter wavelengths of light are absorbed in a substantial quantity, compared to human, therefore, to make this strategy translatable for use in human, there is the need to find the wavelength that guarantees sufficient penetration, sufficient energy and that would not cause any damage (such as tissue heating) to the brain. It is known that a range of wavelength show beneficial effects in both human and animal studies, therefore there is the need to find standardized parameters in order to develop a suitable and accessible device for the prevention of AD and other neurodegenerative disorders.

In summary, NIR light reduces the impact of neuroinflammation caused by obesity and HFD in brain of DIO mice by lowering glia activation in hippocampus and POCTX, modulating the levels of pro-inflammatory cytokines in the hippocampus, and upregulating BDNF in the hippocampus. The involvement of BDNF suggests a possible activation of neuroprotective mechanisms that need further investigations. Moreover, this strategy can be developed as a novel, non-invasive preventive strategy against events, such as neuroinflammation, that may accelerate or exacerbate the onset of cognitive decline and dementia, including AD. In addition, this approach can be proposed for the prevention of other neurodegenerative diseases and neurologic disorders, where neuroinflammation has been shown to play a central role. Finally, it is reasonable to consider people with familiar history of AD, obesity, insulin resistance as subjects to prioritize for a potential treatment, considering that this strategy has therapeutic potential against those obesity-induced CNS deficits that are known to concur to the AD neuropathological cascade, although further evidence is needed to make this strategy suitable for clinical testing.

FUTURE DIRECTIONS

Future investigations should follow two directions: 1) to establish standard criteria for the use of the light in order to translate it from the bench to the bedside; 2) to find the time

window within which such treatment might be effective. Investigations aimed at finding the most effective light source, wavelength, fluence, irradiance, length of the treatment, intervals between sessions are paramount to make this approach suitable. Another important aspect concerning the translatability of NIR light delivery is the construction of devices that can be used in domestic settings: interesting observations have been made by combining a transcranial with an intranasal device, with the latter suitable for use in personal households (Saltmarche *et al.*, 2017), while another study explored the possibility to deliver NIR light through intravascular catheters, obtaining promising results, thus opening new avenues not only for AD treatment, but also for vascular dementia (Maksimovich, 2012).

Therefore: 1) given the promising data shown in literature showing prevention of AD progression and amelioration of human overall brain function, 2) considering the data presented in this thesis, that are consistent with other results obtained from different animal models of neuroinflammation, and 3) once the paramount technical aspects mentioned in the previous paragraph will be completely fulfilled, the following step should be developing double-blind full-sized clinical trials to test whether NIR light deliveries are effective in AD prevention. Also, keeping in mind that obesity and T2DM are prominent risk factors for AD, these clinical trials must be directed at treating individuals with BMI > 25-30 Kg/m^2, obese, insulin-resistance (pre-diabetic) and anticipate follow-up observations throughout years to establish whether NIR light can be considered a preventative strategy against AD.

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Vita

Salvatore (Salvo) Saieva was born in Palermo (Italy) on August 7th, 1986 to parents Vincenzo Saieva and Giovanna Stassi. He was awarded a Master of Science in Pharmaceutical Chemistry and Technology from University of Palermo (Italy) *cum laude* on October 25th, 2013. After attending a post-degree High-Specialization Course in Mass Spectrometry Methodologies and Applications at the University of Palermo, in collaboration with the University of Catania (Italy), he was selected in November 2014 for the combined International Doctorate Program between the University of Palermo and the University of Texas Medical Branch. This is a highly competitive PhD program that leads to successful students conferring both a European and American PhD. Salvo successfully obtained his European PhD on March 12th, 2018. He joined the Neuroscience Graduate Program at the University of Texas Medical Branch in September 2015. While at UTMB he received several awards and scholarships including the GSBS Associates Endowment Scholarship and the Samuel N. Kolmen, PhD and Barbara Kass Kolmen, MD Travel Scholarship.

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Publications

Peer-Reviewed Manuscript

- **Saieva S**, Taglialatela G, Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brains of Diet-Induced Obese Mice, in preparation

- Krishnan B, Sallam HS, Tumurbataar B, **Saieva S**, Tuvdendorj D, Baymon DM; Micci MA, Abate N, Taglialatela G, ADIPOSE TISSUE-DERIVED STEM CELL TRANSPLANTATION IMPROVES HIPPOCAMPAL DYSFUNCTION IN AT-ENPP1 Tg MICE, Journal of Neurochemistry, 2019; e14915. doi:10.1111/jnc.14915

Proceedings and Symposia

- **Saieva S**, Near-Infrared Light Reduces Glial Activation in Hippocampus And Cortex Of Diet-Induced Obese Mice, December 3rd, 2020 Neuroscience Across Texas Virtual Symposium, College Station, TX, USA.
- **Saieva S**, Near-Infrared Light Reduces Glial Activation in Hippocampus And Cortex Of Diet-Induced Obese Mice, 5th Neuroscience Graduate Program Symposium, November 18th-19th, 2020, University of Texas Medical Branch, Galveston, TX, USA
- **Saieva S**, Adipose tissue-targeted stem cell transplantation for Type 2 Diabetes-related CNS dysfunction, 4th Neuroscience Graduate Program Symposium, July 22nd-23rd, 2019, University of Texas Medical Branch, Galveston, TX, USA
- **Saieva S**, Adipose tissue-targeted stem cell transplantation for Type 2 Diabetes-related CNS dysfunction, 3rd Neuroscience Graduate Program Symposium, July 15th-16th, 2018, University of Texas Medical Branch, Galveston, TX, USA
- **Saieva S**, Adipose tissue-targeted stem cell therapy for Type 2 Diabetes-related CNS dysfunction, 2nd Neuroscience Graduate Program Symposium, June 28th-29th, 2017, University of Texas Medical Branch, Galveston, TX, USA
- **Saieva S**, Prevention of Insulin Resistance in Brain through Peripheral Mesenchymal Stem Cell Transplantation as Potential Therapeutic strategy against Alzheimer's Disease, 1st Neuroscience Graduate Program Symposium, June 6th-7th, 2016, University of Texas Medical Branch, Galveston, TX, USA.

- **Saieva S**, Peripheral Adipose Tissue Insulin Resistance Alters Lipid Composition and Function of Hippocampal Synapses, XI Conference of Italian Researchers in the World, February 25th-26th, 2016, Italian Consulate at Houston, Houston, TX, USA.

Abstracts

- **Saieva S**, Fracassi A, Zhang W, Marcatti M, Taglialatela G, "Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brains of Diet-Induced Obese Mice". July 26th-30th, Alzheimer's Association International Conference, AAIC 2021, Denver, CO, USA.
- **Saieva S**, Fracassi A, Zhang W, Marcatti M, Taglialatela G, "Near-Infrared Light Reduces Glia Activation and Modulates Neuroinflammation in Brains of Diet-Induced Obese Mice". March 9th-14th, 2021, 15th International Conference on Alzheimer's and Parkinson's Diseases and related neurological disorders, AD/PD™ 2021, Virtual Conference.
- **Saieva S**, Fracassi A, Zhang W, Marcatti M, Taglialatela G, "Near-Infrared Light Reduces Glial Activation in Hippocampus and Cortex Of Diet-Induced Obese Mice". January 28th, 2021, Texas Alzheimer's Research and Care Consortium (TARCC) Scientific Symposium, Austin, TX, USA (Virtual Conference).
- **Saieva S**, Fracassi A, Zhang W, Marcatti M, Taglialatela G, "Near-Infrared Light Reduces Glial Activation in Hippocampus And Cortex Of Diet-Induced Obese Mice". December 3rd, 2020 Neuroscience Across Texas Virtual Symposium, College Station, TX, USA (Virtual Conference).
- **Saieva S**, Fracassi A, Zhang W, Taglialatela G, "Near-Infrared Light Reduces Neuroinflammation in the Hippocampus of Diet-Induced Obese Mice". Meeting: 61st Annual National Student Research Forum, Galveston, TX, USA (*abstract accepted but meeting canceled due to COVID-19 pandemic*).

- **Saieva S**, Fracassi A, Taglialatela G, "Near-Infrared Light Reduces Neuroinflammation in the Hippocampus of Diet-Induced Obese Mice". January 30th, 2020, Texas Alzheimer's Research and Care Consortium, Austin, TX, USA.
- **Saieva S**, Krishnan B, Abate N, Taglialatela G, "Human-umbilical derived mesenchymal stem cell transplant in adipose tissue improve fear conditioning memory in a diet-induced obese mouse model". October 19th-23rd, 2019, Society for Neuroscience 49th Annual Meeting, Chicago, IL, USA.
- **Saieva S**, Krishnan B, Abate N, Taglialatela G, "Human-umbilical derived mesenchymal stem cell transplant in adipose tissue improve fear conditioning memory in a diet-induced obese mouse model". October 17th, 2019, 22nd Forum on Aging, Galveston, TX, USA.
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- **Saieva S**, Krishnan B, La Rocca G, Taglialatela G, Abate N, "Adipose tissue-targeted stem cell transplantation for Insulin Resistance-related CNS dysfunction". October 20th, 2018, 21st Forum on Aging, UTMB, Galveston, TX, USA.
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- **Saieva S**, Sallam HS, Krishnan B, Tumurbaatar B, Anzalone R, La Rocca G, Taglialatela G, Abate N, "Adipose tissue-targeted stem cell therapy for Type 2 Diabetes-related CNS dysfunction". November 11th-15th, 2017, Society for Neuroscience 47th Annual Meeting. Washington, DC, USA.
- **Saieva S**, Sallam HS, Krishnan B, Tumurbaatar B, Anzalone R, La Rocca G, Taglialatela G, Abate N, "Adipose tissue-targeted stem cell therapy for Type 2 Diabetes-related CNS dysfunction". October 21st, 2017, 20th Forum on Aging, UTMB, Galveston, TX, USA.
- **Saieva S**, Sallam HS, Krishnan B, Tumurbaatar B, Anzalone R, La Rocca G, Taglialatela G, Abate N, "Adipose tissue-targeted stem cell therapy for Type 2 Diabetes-related CNS dysfunction". March 22nd, 2017, Galveston Symposium on Alzheimer's Disease & Related Disorders: Basic, Translational & Clinical Advances. Galveston, TX, USA.
- **Saieva S**, Sallam HS, Krishnan B, Tumurbaatar B, Anzalone R, La Rocca G, Taglialatela G, Abate N, "Prevention of Insulin resistance in Brain through Mesenchymal Stem Cell Therapy Approach as Potential Therapeutic Strategy against Alzheimer's Disease". December 13th, 2016, 2016 Neuroscience and Cell Biology Departmental Retreat. Galveston, TX, USA.
- **Saieva S**, Sallam HS, Tumurbaatar B, Anzalone R, La Rocca G, Taglialatela G, Abate N, "Prevention of Insulin resistance in Brain through Mesenchymal Stem Cell Therapy Approach as Potential Therapeutic Strategy against Alzheimer's Disease". November 12th-16th, 2016, Society for Neuroscience 46th Annual Meeting. San Diego, CA, USA.

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