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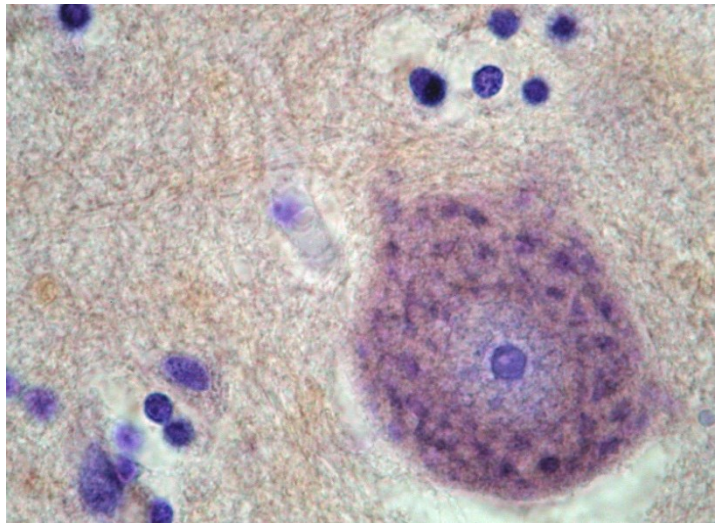
Oxidative stress and neuroinflammation effects in hippocampal development, ageing, and disease



Erica Cecilia Priori

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXIII – A.A. 2017-2020

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ABSTRACT

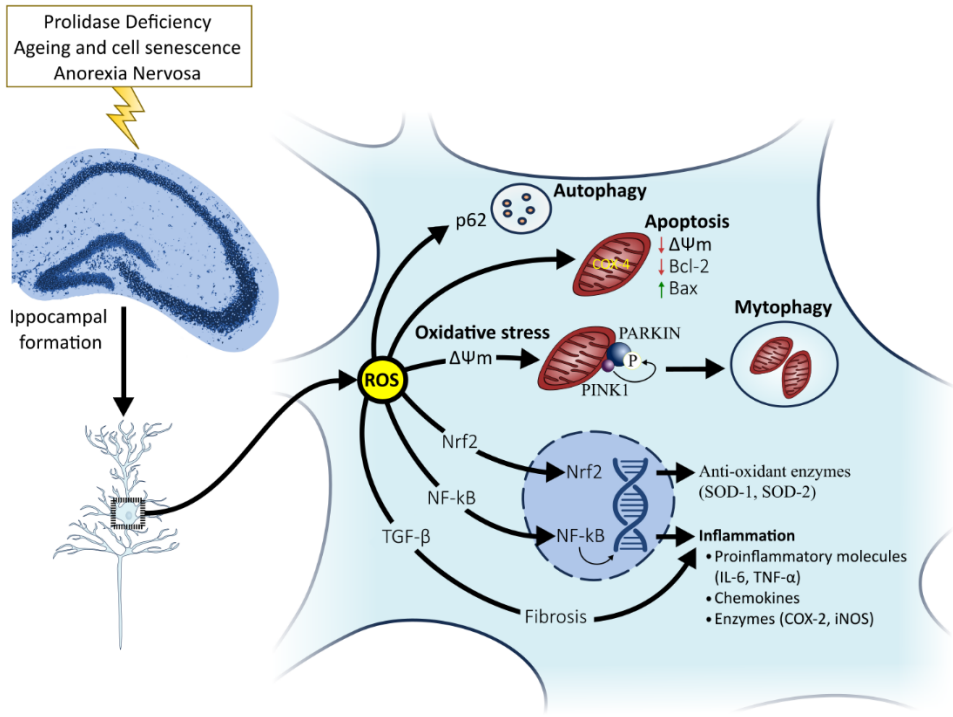
This PhD thesis aims to evaluate oxidative stress and inflammation pathways considering three pathological models such as Prolidase Deficiency (PD), physiological ageing and frailty and Anorexia Nervosa (AN). Furthermore, preliminary experiments were performed to better understand the involvement of different cell death pathways i.e. apoptosis, autophagy and mitophagy.

Prolidase deficiency is a rare genetic disorder caused by a mutation in the PEPD gene that codes for the prolidase enzyme. This enzyme can selectively cut proline dimers located at the carboxy-terminal end of a polypeptide and participates in the turnover of collagen. Recently published data have demonstrated the presence of cerebellar morphological alterations in PD mice, extracellular matrix disorganization, and consequently tissue damage and anomalies in cell migration [1]. In addition, some researchers have pointed out a correlation between oxidative stress and inhibition of collagen synthesis. Moreover, in the brain, it has been observed that an increase in proline levels lead to glutamate-mediated excitotoxicity which, in the end, could generate cytoarchitectural and functional alterations in the cerebral tissue [2–4]. To our knowledge, there is no information about the involvement of oxidative stress and subsequent inflammation in PD.

Ageing is a condition closely related to oxidative stress and inflammation that physiologically affects everyone. These events in several cases do not impair the quality of life of the individual but increases the susceptibility to the onset of disabilities and death [5]. Furthermore, ageing is characterized by the presence of a chronic inflammatory state called “inflammaging”. Ageing has a great impact on the hippocampus because it inhibits adult neurogenesis. The loss of neuronal proliferation could lead to a reduction in synaptic plasticity which is highly dependent on the neurons produced by progenitor cells. Even the hippocampal dependent cognitive abilities lose effectiveness during ageing, although it is not yet clear which is the role of adult neurogenesis in the regulation of this aspects, at least in humans [6–8]. Recently, it has been shown that the medicinal mushroom *Hericium erinaceus* (He1) has antioxidant activity and partially reactivates cell proliferation in some brain areas of physiologically aged mice and animals subjected to a diet supplemented with the mushroom [9].

Eating Disorders are common, complex, and difficult-to-cure diseases that mostly arise during adolescence. Among the various disorders, Anorexia Nervosa (AN) was characterized by an insufficient energy supply for survival with consequences that affect the whole organism. The reduced caloric intake in AN patient inevitably affects the energy state of the tissues. It is known that an energetic dysfunction within the cells generates an imbalance in the mitochondrial activity that causes the production of ROS. To our knowledge, there are not several studies explaining a precise correlation between AN and oxidative stress and the results are often very controversial. Hence the need to conduct further experiments to better understand the role of oxidative stress and its effects in AN.

Oxidative stress and consequently inflammation seem to be a common thread in these three pathological models and could trigger several types of cell death.
The purpose of this thesis was to evaluate oxidative stress and inflammation, as a common thread, in three different pathologies. By using mouse animal models and a model of human biopsies we obtained results that could be useful for the improvement of antioxidative and antiinflammation treatment. When oxidative stress and inflammation become chronic in the organisms, can trigger "suicide" cell death events to restore a damaged microenvironment. The persistence of a high oxidative and inflammatory state in the brain can cause irreparable neuronal damage which results in cognitive decline, neurodegeneration and damage to brain functions essential for the survival of the individual.
To investigate differences in the expression of antioxidant proteins, inflammatory cytokines and molecules and cell death markers in the hippocampal formation, mice affected by PD (*dal*) at 10 days after birth (P10), P21 and P60, aged mice supplemented for 2 months with 1 mg/day of standardized extracts of He1 and human brain biopsies of a control and AN patient were studied.
Morphological and immunohistochemical reactions were conducted on mouse and human hippocampal sections considering the following antibodies: superoxide dismutase 1 and 2 (SOD1, SOD2), cyclooxygenase 2 (COX2), nitric oxide synthase 2 (NOS2), interleukin 6 (IL-6), and transforming growth factor β (TGF-β). In addition, for the cell death pathways Bax, Bcl-2, p62, PINK1 and PARKIN were evaluated. Haematoxylin and eosin staining, and Nissl staining were also performed for cytoarchitectural analyses. The results were obtained by using microtome cutting techniques, immunohistochemistry and immunofluorescence experiments and optical microscope analysis followed by statistical evaluations.
Thanks to these data we can propose a model in which, in the presence of ROS and oxidative stress, the cells undertake different response mechanisms aimed to cell survival or death. In the presence of ROS-induced levels lysosomes engulf damaged mitochondria and subsequently a complex called mitophagosome is formed. After these events, the cells could attempt two pathways. In the presence of a severe accumulation of ROS p53 phosphorylation and the autophagosome formation are induced and cells are directed to perform autophagy. When the oxidative stress levels are lower the cells seem to prefer mitophagy cell death. We can consider this event as a challenge to cell survival triggered also by the fusion and fission events induced by several molecules such as Drp1. In conclusion, we think that these data could provide novel viewpoints which may further enrich our insight to antioxidative and anti-inflammation treatment through activation of programmed cell death for cell survival.



Graphical abstract illustrating the main topics investigated.

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ABBREVIATIONS

Δψm: Mitochondrial transmembrane potential

•NO: Nitric Oxide Anion

AA: Arachidonic Acid

AD: Alzheimer Disease

ADP: Adenosine Diphosphate

AI: Anterior Insula

AIF: Apoptosis-Inducing Factor

ALS: Amyotrophic Lateral Sclerosis

AMBRA1: Activating Molecule Beclin-1-Regulated Autophagy Protein 1

AM-S: Adhesion Molecules

AN: Anorexia Nervosa

ANOVA: Analysis of Variance

APAF: Apoptotic Peptidase Activating Factor

APP: Amyloid Precursor Protein

ARFID: Avoidant/Restrictive Food Intake Disorders

AR-JP: Autosomal Recessive Juvenile Parkinsonism

ATG: Autophagy-Related Genes

Barkor: Beclin 1-associated autophagy-related key regulator

Bax: Bcl-2-associated X protein

BBB: Blood-Brain Barrier

Bcl-2: B-cell lymphoma 2

BDNF: Brain-Derived Neurotrophic Factor

BECN1: Beclin-1

BED: Binge-Eating Disorders

BIS: Behaviour Inhibition System

BMI: Body Mass Index

BN: Bulimia Nervosa

BSA: Bovine Serum Albumin

CA: *Cornus Ammonis*

CARD: Caspase recruitment domain

CAT: Catalase

CBT-E: Cognitive Behaviour Therapy

CCK: Cholecystokinin

CMA: Chaperone-Mediated Autophagy

CNS: Central Nervous System

COX: Cyclooxygenases

Cu/Zn SOD: copper-zinc superoxide dismutase

Dal: Dark-like

DAMP: Damaged-Associated Molecular Pattern

DCX: Doublecortin

DD: Death Domain

DED: Death Effector Domain

DG: Dentate Gyrus

DISC: Death Inducing Signaling Complex

DMAPP: Dimethylallyl Diphosphate

DRP1: Dynamine-Related Protein 1

DSM: *Diagnostic And Statistical Manual Of Mental Disorders*

ECM: Extracellular Matrix

EC-SOD: Extracellular Superoxide Dismutase

ETC: Electron-Transport Chains

ED: Eating Disorders

EMA: *European Medicines Agency*

eNOS: Endothelial NOS

EOP: Early-Onset Parkinsonism

ER: Endoplasmic Reticulum

FADD: Fas Receptor-Associated Death Domain

FAK: Focal adhesion kinase

FBT: Family-Based Treatment

FGF: Fibroblastic Growth Factors

FI: Frailty Index

FIP200: focal adhesion kinase family interacting protein of 200 kD

FPT: Focal Psychodynamic Psychotherapy

G-TsF: Glioblastoma derived T-cell suppressor factor

GABARAP: Gamma-Aminobutyric Acid Receptor-Associated Protein

GAD67: Glutamic Acid Decarboxylase 67

GAL: Galanin

GFAP: Glial Fibrillar Acid Protein

GL: Granular layer

GPX4: Glutathione Peroxidase 4 Protein

GSH: Glutathione

H_20_2: Hydrogen peroxide

H&e: Haematoxylin and eosin

HD: Huntington Disease

HE: *Hericium Erinaceus*

HGMD®: Human Gene Mutation Database Professional

HO: Hydroxyl Radical

HSPA8/HSP70: Heat Shock protein

IAPs: Inhibitor of apoptosis proteins

ICD-11: International Classification Of Diseases

IFN-γ: Interferon-γ

IL-1: Interleukin-1

IL-10: Interleukin-10

IL-6: Interleukin-6

IL-8: Interleukin-8

iNOS: Inducible NOS

IPP: Isopentenyl Diphosphate

KA: Kainic Acid-Induced Convulsions

KIR: KEAP1 interacting region

LAMP2A: Lysosomal-Associated Membrane Protein 2a

LAP: Latency-Associated Peptide

LC3: Microtubule-associated protein 1A/1B-light chain 3

LIR: Lc3b Induced Receptor

LPS: Lipopolysaccharide

LTP: Long-Term Potential

MANTRA: Maudsley-Anorexia Nervosa Treatment For Adults

miRNAs: MicroRNAs

ML: Molecular layer

MLKL: Mixed Lineage Kinase Domain-Like Pseudokinase

MM: Medicinal Mushroom

MMP: Matrix Metallo-Proteinase

MnSOD: Manganese Superoxide Dismutase

MRI: Magnetic Resonance Imaging

mTORC1: Target Of Rapamycin Complex 1

NCCD: Nomenclature Committee On Cell Death

NDP52: Nuclear Domain 10 Protein 52

NF-kB: Nuclear Factor k chain transcription in B cells

NGF: Nerve Growth Factor

NLR: Nucleotide-Binding Oligomerization-Domain Protein-Like Receptor

NLS: Nuclear Localization Signal

nNOS: Neuronal NOS

NO: Nitric Oxide

NOD: Nucleotide-Binding Oligomerization-Domain Protein

NOS: Nitric Oxide Synthase

NPY: Neuropeptide Y

Nrf2: Nuclear factor erythroid 2–related factor 2

$O_2 \bullet^-$: Superoxide Anion

OD: Optical Density

OFC: Orbitofrontal Cortex

OH: Hydroxyl radical

OMM: Outer Mitochondrial Membrane

ONOO-: Peroxynitrite

OPA1: Optic Atrophy 1protein

PAMP: Pathogen-Associated Molecular Patterns

PAR: Poly (ADP-Ribosome)

PARKIN: Parkin Rbr E3 Ubiquitin-Protein Ligase

PARP-1: Polymerase 1

PAS: Phagophore Assembly Site

PB: Phox and Bem domain

PBS: Phosphate Buffered Saline

PCD: Programmed cell death

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PD: Prolidase Deficiency

PDGF: Platelet-Derived Growth Factor

PE: Phoshatidylthanolamine

PEPD: Peptidase D

PG: Prostaglandins

PGE2: Prostaglandin E2

PI3K: Phosphatidylinositol 3-Kinase

PINK1: Pten-Induced Kinase 1

PKB: Protein Kinase B (Also Called Akt)

PL: Polymorphic layer

PND: Post Natal Day

PRR: Pattern recognition receptor

PS-1: Presenilin

PSA-Ncam: Polysialylated-neural Cell Adhesion Molecule

PTM: Post-Translational Modifications

PTSD: Post-Traumatic Stress Disorder

PUFAs: Polyunsaturated Fatty Acids

RCD: regulated cell death

RIP: Receptor-Interacting Protein

RNS: Reactive Nitrogen Species

RONS: Reactive Oxygen and Nitrogen Species

ROS: Reactive Oxygen Species

RT: Room Temperature

SGZ: Subgranular Zone

SIRTs: Sirtuins

SLE: Systemic Lupus Erythematosus

Smac/DIABLO: Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding with low pI protein

SOD-1: Superoxidedismutase 1

SOD-2: Superoxidedismutase 2

SSCM: Specialist Supportive Clinical Management

SVZ: Subventricular Zone

TGF-β: Transforming Growth Factor-β

TGF-βR: Transforming Growth Factor-β Receptor

TIM: Translocase Of The Inner Membrane

TLR: Toll-Like Receptor

TNF: Tumour Necrosis Factor

TOM: Translocase Of The Outer Membrane

TOR: Target Of Rapamycin

TRADD: TNF Receptor-Associated Death Domain

TRAF2/5: Tnf Receptor Associated Factor 2/5

TRAF2: Enzyme-1α-Tnf Receptor-Associated Factor 2

TRAIL: Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand

UBA: Ubiquitin-Associated Domain

UBL: Ubiquitin-Like Proteins

ULK1/2: Unc-51-Like Autophagy Activating Kinase 1 and 2

UVRAG: UV Radiation Resistance Associated

VIP: Vasoactive intestinal peptide

ZZ: Zinc Finger

1. INTRODUCTION

1.1 Oxidative stress in cell and tissues

Oxygen is one of the most abundant elements on Earth and the use of this molecule by aerobic organisms leads to the formation of radical species known as reactive oxygen species (ROS). Free radicals were first described more than a century ago but were not considered to belong to biological systems due to their high instability and short half-life. In the mid-1900s, free radicals were found in various living organisms and their involvement in various pathologies, especially in ageing, was proposed. In recent years, the idea that free radicals are not only harmful agents but also participate in physiological processes in all living organisms has gained increasing strength.

Often the terms oxygen free radicals and ROS are used in the same way but do not always indicate the same molecules. ROS are produced in mitochondria by complexes that make up the electron transport chain (ETC), located on the inner membrane of the mitochondrion. In living organisms, more than 90% of oxygen consumed is completely reduced to water by the complex IV of the ETC called cytochrome oxidase without production of reactive species [10, 11]. Less than 10% of the oxygen consumed is reduced in a pathway that converts molecular oxygen into superoxide anion ($O2^{\bullet -}$) resulting in the production of hydrogen peroxide (H_2O_2). H_2O_2 by accepting an electron produces a hydroxyl radical (HO) and a hydroxyl anion (OH^-). Finally, the HO reacts with another electron to form water molecules. Cells can also produce reactive nitrogen species (RNS) such as a nitric oxide (NO), peroxynitrite ($ONOO^-$), nitrogen dioxide radical (NO_2) and molecules related to them [12]. In addition, less volumes of ROS are produced by ETC located on the plasma membrane of endoplasmic reticulum (ER) since, in this organelle, the production of ROS is strictly related with the protein folding process [13]. The accumulation of misfolded proteins triggers to the so-called “ER stress” that induces the formation of dysregulated disulfide bonds between cysteine residues and H_2O_2, which further increases the ROS levels and the oxidative stress condition [14].

To maintain low ROS levels, cells adopt several antioxidant defence mechanisms to protect organisms from oxidative damages. Besides, numerous components derived from dietary sources also possess antioxidant activities. Three major antioxidants systems directly eliminate ROS already formed, inhibit the formation of ROS and remove or repair the damage caused by these instable molecules [12].

Also, based on molecular mass, the antioxidant molecules can be divided into two groups: low molecular mass (below 1 kDa) and high molecular mass. The first group includes molecules supplied by food or supplements such as acid ascorbic, tocopherol, carotenoids, anthocyanins, polyphenols, and uric acid. Regarding high molecular mass antioxidant, the first line of defence against the effects of both reactive oxygen and nitrogen species (RONS) consists of the enzymes superoxide dismutase (SOD), catalase (CAT) and different types of peroxidase [13, 15].

However, if there is an excessive increase in free radicals and the balance with the antioxidant defences is lost, a phenomenon known as oxidative stress is generated, which causes oxidative damage to several types of biomolecules, and, in the end, exert deleterious effects for tissues and organs. A chronic oxidative state can result in pathological conditions, some of which are related to age, such as neurodegenerative diseases and geriatric frailty [16, 17]. ROS are believed to be generated primarily by leukocytes, although other systems, such as the xanthine/xanthine oxidase system, may also be important, and may exert a range of toxic effects that have been demonstrated in several biological systems. Specifically, these reactive species can determine a modification in receptor activity, in the signalling and release of endogenous inflammatory mediators, or they can be implicated in vascular damage. Furthermore, at high levels, ROS react with proteins, lipids, carbohydrates, and nucleic acids, often inducing irreversible functional alterations or full damage [18]. There is substantial evidence that ROS act as second messengers, essential in innate and adaptive immune cells [19, 20]. Nonetheless, the increase in their levels within immune cells can cause the hyperactivation of inflammatory responses resulting in tissue damage [21].
ROS are generated by the metabolism of oxygen which is balanced by the rate of formation and elimination of the oxidant. An imbalance in oxidant/antioxidant activity leads to a state of oxidative stress, which is associated with endothelial dysfunction, inflammation, hypertrophy, apoptosis, fibrosis, and tumour angiogenesis. It has also been observed that ROS can increase the migration of tumour cells, increasing the risk of invasion and metastasis. This state of stress is responsible for many other diseases, first ischemia and asthma [22]. Finally, it has been shown that prolidase, an intracellular enzyme necessary for collagen replacement, matrix remodelling and cell growth, is in some way correlated with the presence of oxidative stress; depending on the enzymatic levels, more or less serious situations can arise [23].

Several studies suggest that an oxidative imbalance and the resulting neuronal damage may play a fundamental role in the etiopathogenesis and progression of neurodegenerative diseases such as Alzheimer Disease (AD) [24]. Indeed, it seems that the accumulation of β-amyloid plaques increases oxidative stress causing mitochondrial dysfunction and alteration of the energy balance already in the early stages of the disease [25]. Furthermore, this evidence is corroborated by some *in vivo* studies. In transgenic mouse models expressing mutant forms of the precursor protein of β-amyloid (APP) and of presenilin-1 (PS-1), an increase in the levels of H_2O_2 and peroxidation of proteins and lipids was observed [26, 27]. Although the exact mechanism remains to be clarified, oxidative stress is now considered one of the main pathophysiological mechanisms also underlying Parkinson Disease (PD). From literature, it has been demonstrated that, in the neurons of affected subjects, there is a reduced activity of Complex I respiratory chain, with the generation of an excessive quantity of ROS, and consequent apoptosis [28, 29].

1.1.1 Superoxide dismutase

Superoxide dismutase (SOD) is a homodimer that acts as the first line of defence against ROS within cells. The protective mechanism implemented by this enzyme consists of catalysing the dismutation of the superoxide ion (●$O2^-$) into molecular oxygen and H_2O_2. Different types of SODs include copper-zinc superoxide dismutase (Cu/ZnSOD), manganese superoxide dismutase (MnSOD) and extracellular superoxide dismutase (EC-SOD), play a crucial role in destruction ●$O2^-$. The three types of SOD can be recognized based on the ions present in their active site, their distribution in organisms and the molecular mass: the Cu/ZnSOD is present principally in the cytosol and the mitochondrial membrane of eukaryotic cells, the MnSOD is connected with the mitochondrial matrix of some prokaryotes and eukaryotes and the EC-SOD which is found extracellularly in prokaryotes and a few species of eukaryotes [30, 31].

SOD1 or Cu/ZnSOD is a dimeric protein similarly expressed in rodents [32], bovines [33] and humans [34]. The human *sod1* gene is located on chromosome 21 in position q22 and consists of five exons and four introns; this structure is similar in all the species [34]. The reaction catalysed by Cu/Zn SOD (SOD1) is a dismutation that follows second order kinetics. During the catalytic reaction of SOD1, the Cu^{2+} ion is cyclically reduced and oxidized during subsequent interactions with the superoxide substrate in the active site. In a first phase, an electron from the radical superoxide ion is donated to the catalytic centre with the formation of molecular oxygen and Cu^+ which donates an electron to a second superoxide ion that subsequently join two protons, generating H_2O_2 [35]. The enzyme reacts with the superoxide ion with a mechanism that involves the use of only half the catalytic site, in which the dismutation activity is carried out mainly by the Cu^{2+} ion; however, the removal of the Zn^{2+} ion decreases the redox potential of Cu^+ by reducing the catalytic capacity of the enzyme. The Zn^{2+} ion also seems to have been incorporated into the enzyme to increase the stability of the active site. In all three subtypes of enzymes, the rates of oxidation and reduction reactions are equal [36].
Several post-translational modifications (PTM) occurred in SOD1 and plays a central role in the regulation of enzyme activities. No less than 12 phosphorylation sites have been revealed on SOD1 and a few seem to be implicated in regulating the activity of SOD1 in ROS elimination, preservation of cytoskeletal rearrangements and transcription rates [37–40]. In a recent paper, it has been demonstrated that the treatment of hepatocytes with nodularin, a phosphatase inhibitor, increased SOD1 phosphorylation. In this state, SOD1 loses its canonical cytoplasmic localization and appear in the apoptotic bodies characteristic of the early stage of apoptosis [38]. Another study published by Leitch et al. showed phosphorylation on yeast SOD1 at Ser38 (Thr39 in humans) residue and, afterwards suggesting that this event can control the mammalian target of rapamycin complex 1 or mechanistic target of rapamycin complex 1 (TORC1/mTORC1) conversion and consequently the SOD1 activity in yeast and mammals linking SOD1 activity to nutrients levels via the mTORC1 pathway [39–41]. Other types of PTMs that can be occur in SOD1 structure

are acetylation and succinylation of lysine residues and are generally referred to as acylation. These events could modify the functions of proteins by changing the net charge of the molecules.
The first studies conducted on SOD1 in *Xenopus laevis* revealed a strong correlation between this protein and proteins belonging to the sirtuins (SIRTs) family, particularly SIRT1 [42]. Subsequent studies have shown that SIRT1 regulates the acetylation of a lysine 70 residue in SOD1 which could consequently inactivate the formation of the homodimer and therefore the correct functionality of the molecule. Furthermore, it is thought that the activity of SOD1 may also depend on the levels of NAD^+ since the sirtuins use this cofactor as a substrate for their enzymatic activity. Given the role of SIRT1 in DNA deacetylation, its involvement in PTM on lysine residues of SOD1 could promote the antioxidant and transcriptional activity of this enzyme [37, 43]. SOD1 activity was inhibited in the absence of another sirtuin, SIRT5. Depletion of SIRT5 increases the succinylation of a lysine residue which in turn remove of the inhibition of complex I, thus promoting cellular respiration. These studies suggest that SIRT5 controls cellular respiration, by maintaining SOD1 in the desuccinylated state [40, 44].
SOD1 lysine is also modified by SUMOylation and ubiquitination. The SOD1 homodimers could be degraded by proteasomal degradation or by autophagy. A study conducted on murine and human neuroblastoma cells revealed that wild-type and mutant SOD1 cultures die for macroautophagy pathway suggesting an interconnection between oxidative stress and lysosomal cell death [40, 45]. SOD1 can also play an important role in the prevention of damage to the Central Nervous System (CNS). Mutations in the SOD1 gene are responsible in part for damage to the mitochondria leading to the onset of the progressive neurodegenerative disorder such as Amyotrophic Lateral Sclerosis (ALS) [46–48]. In addition, a variation of Cu/ZnSOD expression or catalytic activity has been identified in numerous physiological conditions such as ageing, in age-related diseases and calory restriction circumstances [49–52].

SOD2 is the Mn-dependent superoxide dismutase form. The human *sod2* gene is situated on chromosome 6 in position q25.3, it consists of five exons and 4 introns and produces two transcripts with no substantial differences [30]. SOD2 is located in the mitochondrial matrix where catalyse the dismutase of superoxide free radical firstly in oxygen and then in H_2O_2 [53]. This protein plays an important role in promoting cellular differentiation and in cellular resistance to cytotoxicity by Tumour Necrosis Factor (TNF) [54, 55]. Furthermore, SOD2 seems to be essential for life as confirmed by several studies conducted on MnSOD knock out mice in which very short life-span has been observed [56, 57]. The transcription of SOD2 is triggered by several molecules of the inflammatory pathway such as interleukin (IL)-IL-1, IL-4, IL-6, TNF-α, lipopolysaccharide (LPS), and interferon-γ (IFN-γ) [55, 58–62]. In cancer cells, overexpression of MnSOD is involved in the inhibition of primary tumour and metastasis proliferation since ROS can produce mutations in DNA. This event can be due to the increase in the basal levels of H_2O_2because of the increased activity of MnSOD. SOD2 overexpression can also modulate the activity

of nitric oxide (•NO) by preventing its reaction with oxygen [63, 64]. The enzymes seem to be implicated also in different cell death pathway and in preventing apoptosis. The protein overexpression protects from mitochondrial dysfunction and loss of mitochondrial membrane potential caused by ionizing radiation, Fe(II), amyloid β-peptide, NO generating agents, and TNF-related apoptosis-inducing ligand (TRAIL) [65–67]. MnSOD also prevents the release of various proteins from mitochondria that carry out apoptosis, such as cytochrome c and the Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding with low pI protein (Smac/DIABLO) [65, 67].

The human *sod3* gene is localized on chromosome 4 and consists of 3 exons and 2 introns [30, 68]. It codifies for the EC-SOD protein also called SOD3. This enzyme is located in the extracellular matrix (ECM) in most tissues and, even though it is not situated in the cytoplasm, it participates in the metabolic regulation of cells by altering blood flow [69, 70]. SOD3 is principally present in lung, cerebrospinal fluid, fibroblasts and vascular smooth muscle cells [71]. The catalytic activity of the enzyme has been associated with cardiovascular, pulmonary diseases and neurological disorders. It has been shown that EC-SOD is the main regulator of NO bioavailability and bioactivity by modulating generation of the toxic product peroxynitrite ($ONOO^-$) in the vasculature [72]. Several studies reported an internalization of SOD3 from ECM mediated via receptors or via endocytosis [73–75]. Furthermore, other studies have shown that this protein is expressed in the intracellular vesicle of macrophages and neutrophils [76]. SOD3 could not only be endocytosed but also secreted by macrophages and neutrophils in response to proinflammatory proteins for example by LPS stimulation [77]. Altogether these results suggest an implication of SOD3 not only in ECM but also in a temporal response to oxidative stress by creating a pool of ready-enzyme in the cytoplasm [71].

1.1.2 Nitric oxide Synthase

Nitric oxide synthase (NOS) is an enzyme belonging to the class of oxidoreductases, able to catalyse the production of NO from the oxidation of L-arginine. It is expressed in a wide range of mammalian cells, including macrophages, hepatocytes and endothelial cells, and represents a versatile signalling molecule that regulates many processes including the synthesis of collagen, matrix remodelling, neurotransmission, vascular function, host defence and regulation of the immune system [78]. High concentrations of NO are found in tissues undergoing repair and are associated with greater biosynthesis and modification of collagen [79, 80]. The radical putatively regulates the activity of prolidase, a particular type of metalloproteinase (MMP) that catalyses the terminal phase of the ECM turnover and plays an important role in collagen turnover and cell growth [81]. In particular, the levels of oxidative stress are directly related to the degradation of collagen, mediated firstly by the activation of various proteolytic enzymes and, at a later stage, by the increase in the activity of the prolidase [82]. The great reactivity of NO allows it to act both as a neurotransmitter and as a toxic substance causing the death of pathogens,

together with other ROS. Likewise, when produced at higher concentrations and combined with O2 • - superoxide radical anion, NO produces peroxynitrite, $ONOO^-$. This molecule could trigger several responses such as oxidative damage, nitration, and S-nitrosylation of proteins, lipids, and DNA [83, 84]. Nitrosative stress by $ONOO^-$ has been associated with DNA single-strand breakage, followed by poly-ADP-ribose polymerase (PARP) activation and cell death [85].
To date, in mammals, three enzymatic isoforms of the protein have been isolated: neuronal NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII) and endothelial NOS (eNOS or NOSIII) [86]. nNOS and eNOS are constitutively expressed in cells while NOSII is inducible by pro-inflammatory molecules [87]. Additionally, nNOS and eNOS are mainly expressed in neurons and epithelial cells, respectively, and are calcium dependent.

The NOSI isoform is responsible for the production of NO in the PNS and CNS and here it is implicated in neurons communication and signalling. The human NOSI gene is located on chromosome 12, in position 12q24.2 and has homologous genes in mouse and rat on chromosome 5 and 12, respectively [88–91]. Two different sizes of nNOS were detected in brain tissues spanning from 120 to 160 kDa; the protein has an inactive monomeric form that dimerizes to become catalytically functional [92–94]. nNOS is detected in immature and mature nervous cells and brain blood vessels [95, 96]. The subcellular localization of this enzyme varies from the cytoplasm to nucleus during cell development at least in cultured cells. In some cases, because NO is not storable in the cytoplasm, it seems to be anchored to the plasma membrane in a particulate form while in other conditions is a soluble protein [97, 98]. The highest levels of nNOS mRNA have been found in cerebellum of rat and mouse while, in humans, the highest expression was found in caudate and putamen nucleus and, in smaller quantities, in the cerebellum [99, 100]. Several studies have shown that NOSI is also expressed in many brain areas during ageing such as cerebral cortex, cerebellum, hippocampus and striatum [101–103]. The changes in the amount of nNOS may play a crucial role in a wide range of physiological and pathological conditions. NO is demonstrated to be an inhibitor of neurogenesis in the subventricular zone (SVZ) and the olfactory bulb. Nevertheless, in the dentate gyrus (DG) the inhibition of nNOS promote progenitor cells proliferation, migration and differentiation suggesting that nNOS could be implicated in neurogenesis [104]. An excess in NO production leads to neurotoxic effects and could give rise to neurodegenerative diseases even if the correlation remains poorly understood [105]. NO in CNS is involved in memory and cognitive functions in a retrograde way by stimulating the Long-Term Potential (LTP), a form of synaptic plasticity, to increase the release and the effects of neurotransmitter. These results suggest that the different expression of nNOS in brain areas could be associated with different rate of LTP induction in those regions [93, 104].

The iNOS isoform is well-known to be expressed after immunological stimulation with cytokines. Surprisingly, it was also expressed constitutively in almost all brain regions such as the cerebral cortex and some peripheral human tissues in physiological conditions [93]. The human NOSII gene is located on chromosome 17 in position q11.2 and codifies for a protein of 125-135 kDa. Many researchers have described NOSII as a harmful player in the development of diseases. Under pathological conditions, iNOS is expressed in macrophages and microglia, as well as in neurons, astrocytes and endothelial cells [106]. The NO produced by NOSII kills neurons through a variety of mechanisms including the inhibition of mitochondrial respiration, the inhibition of glycolysis and the activation of both apoptosis and parthanatos [107].

The eNOS protein is expressed constitutively in endothelial cells and in all the brain areas and peripheral tissues. The NO produces by eNOS acts as a vasodilator and induces muscle relaxation [93]. The human NOSIII gene is located on chromosome 7 in position q36.1 and codifies for a polypeptide of 4.2 kDa. Like nNOS, eNOS activity depends on the Ca^{2+} sensor protein calmodulin. Endothelial NOS synthesizes NO when intracellular Ca^{2+} rises [108]. Alongside the role of vasodilator, eNOS have several physiological functions such as vasodilation and inhibition of platelet aggregation and adhesion, inhibition of leucocytes adhesion and vascular inflammation, stimulation of angiogenesis and activation of endothelial progenitor cells [108].

1.1.3 Cyclooxygenase

Cyclooxygenase (COX) is a key oxidoreductase enzyme that catalyses the conversion of arachidonic acid (AA) into prostaglandins (PG). Prostaglandins E2 (PGE2) synthesis begins with the activation of AA by the enzyme phospholipase A2. Then, AA is oxygenated by COX enzymes to form prostaglandin G2 (PGG2) and finally, PGG2 is converted to PGE2. The PG molecules are primary regulators of inflammation but are also involved in ovulation, fertilization, platelet aggregation and renal function [109]. COX expression occurs in tissues in two isoforms, COX1 and COX2, which are homodimer membrane proteins sharing 65% of amino acid homology; each isoform is responsible for the production of preferential prostanoids. The human gene that codifies for COX1 is on chromosome 9 and the gene for COX2 is located on chromosome 1 [110, 111].

COX1 is constitutively expressed in many cell types, including vascular endothelium, platelets, and renal collecting tubules. In the platelets, COX1 is responsible for the recruitment of precursor for thromboxane synthesis that when produced, lead to the formation of aggregates. COX1 is also involved in physiological circumstances by inducing vasodilation during contracting conditions [112]. In the CNS, the protein is distributed throughout all the brain region with a particular concentration in the forebrain in which it could contribute to integrative and cognitive functions [113, 114].

COX2 is the major source of inducible PGE2 production and mediates inflammatory symptoms including fever, loss of appetite and hyperalgesia. COX-2 expression is stimulated by inflammatory signals such as bacterial lipopolysaccharide (LPS), cytokines and environmental stress and is the molecular target for analgesic and anti-inflammatory drugs [115]. COX2 is expressed only in a particular region of the brain such as cortex, hippocampus, hypothalamus, and spinal cord and is involved in nervous transmission [116, 117]. The relevant expression of COX2 in CNS and the consequent high production of PG increase the goal to investigate for inhibitory molecules to avoid excessive inflammatory activation [113, 114].

1.2 Inflammation in cell and tissues

Inflammation is a phenomenon that affects all living beings and was originally identified by the Greek scientist Hippocrates, in the 4th century B.C. The first description of this process is instead attributed to the 1st century B.C. when Celsius, in the essay *De Medicina*, assigns specific names to the symptoms of inflammation: *rubor* (redness), *tumor* (swelling), *color* (heat) and *dolor* (pain). Subsequently, during the 2nd century AD, Galen presented a fifth sign called *functio laesa* (loss of function) and introduced the term phlogosis. He also explained the concept of vital humours, namely *sanguis* (blood), *pituita* (phlegm), *chole* (yellow bile) and *melaine chole* (black bile) and proposed that the inflammation is due to an imbalance in the relationships between these components. In the second half of the nineteenth century Virchow discovered that the presence of a damaged cell could trigger inflammatory processes while, in the same period, Metchnikoff and collaborators characterized the process of phagocytosis in inflamed tissue [118–120]. Until now, only one more symptom of inflammation has been added, called acidosis because of the hypoxic condition of the affected tissue [121].

Thanks to the development of innovative study techniques in recent years it has been possible to investigate and understand the molecular mechanisms governing inflammation pathways. Inflammation is generated by biochemical events that change depending on the cause and the tissue involved; although it is not the primary cause, it often plays an important role in the development of various pathologies that affect also the CNS.

There are two types of inflammatory response: acute and chronic. The acute inflammatory response is triggered by a reaction to damage, wounds, or infections while the chronic one is characteristic of non-resolving diseases such as asthma, type II diabetes, neurodegenerative diseases, cancer, obesity, and ageing.

The chronic inflammatory response is triggered by infection or tissue damage and is mediated by a series of molecules physiologically detected in the blood that reaches the site of damage. Some components of innate immunity also participate in the inflammatory response.

Pathogens express on the plasma membrane the so-called Pathogen Associated Molecular Pattern (PAMP) molecules such as LPS, peptidoglycans, and glycolipids. Instead, the endogenous cells can release the Damage-Associated Molecular Pattern

(DAMP) molecules as a signal of cellular dysfunction that alerts the immune system of the presence of uncontrolled cell death, DNA damage, and stressful situations [122]. Both signal molecules are recognized by the Pattern Recognition Receptors (PRRs), e.g. Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization-domain protein)-like receptors (NLRs), a type of specialized receptors found on the membrane of some sentinel cells such as resident macrophages, dendritic cells or mast cells. The activation of these receptors triggers a cascade response that invokes the inflammatory mediators in damaged or infected tissue [123].

Among these pro-inflammatory molecules, we can mention IL-1, IL-6 and TNF. These components act on the endothelium of blood vessels to provoke vasodilation and selective release neutrophils. Subsequently, they reach the inflamed tissue where they recognize the pathogen, activate themselves and kill those responsible for the damage, releasing toxic molecules and RONS. Furthermore, some cytokines can act systemically by triggering the production of other components of the immune response. In particular, they induce hepatocytes to produce C-reactive protein and coagulation factor which in turn act in the brain by stimulating the production of prostaglandins such as PGE2 which trigger the onset of fever, fatigue, and loss of appetite [124, 125]. Parallelly, anti-inflammatory cytokines (i.e. Platelet-Derived Growth Factor (PDGF), Fibroblast Growth Factor (FGF), Transforming Growth Factor-β (TGF-β) and IL-10 participate in the maintenance and in the recovery of tissue homeostasis and after damage. Generally, the acute inflammatory response is resolved with the intervention of macrophages that eliminate infectious agents and induce the production of anti-inflammatory molecules such as prostaglandins but, if the inflammation does not resolve and the pathogen is not eliminated, the process persists, and chronic inflammation sets in [124, 126].

The CNS has always been considered as an immune-privileged site; nowadays, numerous experimental evidence affirm that it actively participates in the body's immune and anti-inflammatory defence.

The term neuroinflammation indicates a state of inflammation that affects the nervous tissue, and which may be due to viral, bacterial, infections, stroke, brain injury or physiological processes such as synaptic pruning and ageing. When neuroinflammatory responses are excessive, as in the case of, for example, haemorrhagic stroke, they are associated with diffuse cerebral oedema, white matter lesions, increased neuronal apoptotic phenomena and neuronal depletion, resulting in the onset of neurological deficits [127, 128].

In the CNS, the inflammatory response is driven by glial cells, macrophages, and dendritic cells. Glial cells include astrocytes, microglia, oligodendrocytes, and NG2 cells.

Astrocytes are considered the most abundant cell type in the CNS involved in inflammatory pathways. These cells bring TLRs on their plasma membrane and produce, after stimulation, pro and anti-inflammatory cytokines [129]. Astrocytes are not only involved in inflammatory responses but perform numerous other functions in the CNS. They actively regulate the relationship between blood vessels and Blood-

Brain barrier (BBB) thus indirectly acting on the accessibility of some neurotrophic molecules in the neurogenic niche [130, 131]. Furthermore, during neurogenesis, they secrete molecules capable of supporting the formation of synapses or eliminating, through the so-called pruning event, unnecessary contacts to form what will be the characteristic circuits of the adult individual [132]. For example, they secrete thrombospondins, a member of glycoproteins that participate in synaptogenesis. A deficiency in the production of these molecules leads to a reduced synaptic density which results in an impairment in the recovery of motor function after stroke [133–135].

Microglia cells develop during embryogenesis from circulating monocytes that colonize the CNS in the early stages of development and subsequently play the role of resident macrophages forming part of innate immunity. The turnover of these cells seems very limited during life and probably this low rate of regeneration may be a contributing cause of the pro-inflammatory effects of ageing and neurodegenerative diseases. Microglia cells appear in brain tissue in a resting form that contributes to survey all the brain parenchyma. When activated by PAMPs or DAMPs microglia appear in two different polarized forms: activated (M1) type and non-activated (M2) type.

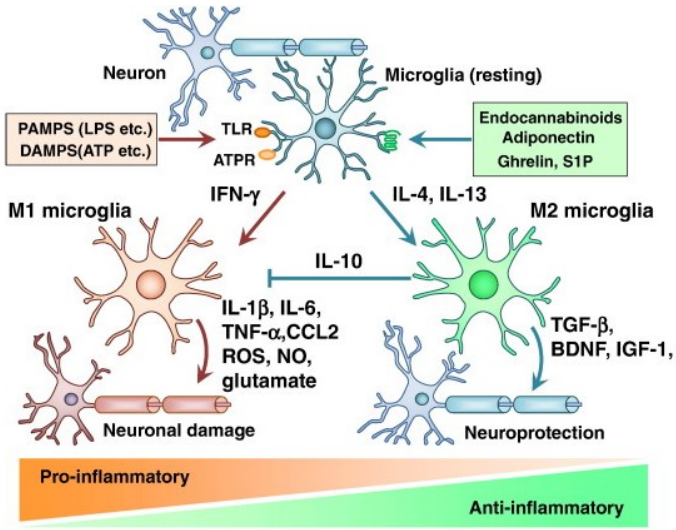


Figure 1. Different role of M1 and M2 microglia their immunoregulatory molecules [136].

The M1 phenotype promotes the inflammation response through the involvement of ROS, NO and proinflammatory cytokines i.e. IL-1, IL-6, and TNF-α while the M2 phenotype is involved in the anti-inflammatory response, leading to the production of IL-4, IL-10, and TGF-β [137–140]. Once activated, these cells modify the transcriptional profile by triggering the production of proinflammatory

cytokines and chemokines, modifying the expression pattern of some receptors and reorganizing the cytoskeleton to allow greater motility at the damage site [141]. Moreover, they increase the ability to phagocytize the pathogens or molecules that triggered their activation [142, 143, 136]. Microglia synthesize and secretes TGF-β in response to the inflammatory cytokines IL-1, IL-6 and TNF-α (**Fig. 1**). Studies conducted on TGF-β1 -/- mice have shown microgliosis in the cerebral cortex and hippocampal formation suggesting that this protein acts as an anti-inflammatory cytokine by inactivating microglia [144].

As reported in the previous paragraph, M1 microglia can produce pro-inflammatory cytokines that induce leakage of the BBB which allows the infiltration of cells belonging to the inflammatory pathways. This evidence could support the idea that M1 microglia contribute to aggravating neuronal damage and dysfunction in the brain tissue affected by the injury. On the contrary, the M2 microglia produces anti-inflammatory cytokines but interestingly, it also expresses molecules involved in restoring the tissues and growth factors such as TGF-β and Brain-Derived Neurotrophic Factor (BDNF) [136]. Nowadays, this classification is much debated. Some researchers indeed have proposed to refer to the different microglia state by using the term "resting" and to attribute the distinction between M1 and M2 types rather to infiltrating monocyte-derived macrophages [145].

1.2.1 Transforming Growth Factor

The Transforming Growth Factor beta (TGF-β) proteins are homodimeric peptides belonging to the cytokine family. At least 23 genes coding for the TGF-β superfamily molecules and, to date, 5 isoforms with very conserved aminoacidic sequences among species have been identified. Indeed, only TGF-β1, -β2 and -β3 are expressed in mammalian tissues [146]. It is synthesized as a precursor, the pro- TGF-β, which is split intracellularly and then secreted in the inactive form. The cleavage consists in the proteolysis between the C-terminal portion of the pro-peptide, called latency-associated peptide (LAP), and the N-terminal portion which will instead represent the mature TGF-β. Some exceptions may cause LAP to be present also in mature TGF-β even if in these cases the molecule is unable to interact with its receptor and is biologically inactive. For TGF-β to become biologically active, the LAP must be released or undergo conformational changes, such that, although it remains bound to the complex, it is still able to expose the appropriate site of the TGF-β receptor [147]. The molecular weight of the proteins belonging to these families is very heterogeneous, but generally, the bioactive molecule has a molecular weight of 25 kDa [146].

TGF-β1 is the most abundant isoform and is expressed ubiquitously in the body. It has been identified in cartilage and bone during the ossification process and in the skin thus suggesting a pivotal role in regulating cell growth and differentiation in mouse models. Besides, it is also able to perform a chemotactic action for macrophages and lymphocytes, stimulate angiogenesis, fibrogenesis and synthesis of ECM in numerous tissues [148].

Regarding the other two isoforms, TGF-β2, known as glioblastoma derived T-cell suppressor factor (G-TsF), is expressed during development on neurons and astrocytes while, in adults, it participates in tumorigenesis by inducing cell proliferation and suppressing the immune response against tumour cells.

TGF-β3 is involved in the development of organs such as lungs and in the healing process of skin wounds. This molecule is secreted and stored in the ECM where it exists in a latent form. Several molecules participate in the activation of this molecule such as plasmins, MMPs, thrombospondin 1, low pH values and ROS.

Proteins belonging to the TGF-β family are involved in several functions both during the development and ageing of the nervous system. Indeed, during embryogenesis, proteins participate, among other in the formation of the neural tube, cell migration, axon development and synaptogenesis. It has been observed that TGF-β also acts as a neurotrophic factor although it does not strictly have this function; treatment with TGF-β in neuronal cultures promotes the neutrophil signal while the blockade of endogenous TGF-β lowers survival and neurotrophic rate [149, 150]. The TGF-β levels are important for the regulation of neurogenesis and neurodegeneration in CNS. In particular, the impairment in TGF-β signalling and a decrease in neuronal expression of Transforming growth factor II receptor (TGF-βRII) are both traits of AD [151–154].

1.2.2 IL-6

Interleukin-6 (IL-6) is a proinflammatory cytokine that has a molecular weight of 21 kDa in its non-glycosylated form. The IL-6 gene contains 4 introns and 5 exons and is located on human chromosome 7 at position p21. This molecule was discovered in the 1980s and is produced by a great variety of cells, including monocytes, fibroblasts, endothelial cells and various types of CNS cells; it is characterized by redundancy and pleiotropic activity, with a diversified set of actions that vary according to the type of target cell [155]. This small signalling glycoprotein plays a central role in the defence against environmental stress, infections, injuries, but it also appears to be involved in tumour metastases. IL-6 is mainly produced by astrocytes and microglia thanks to the induction of various factors; neurons produce this cytokine only in conditions of CNS damage or during intense neuronal activity [156–158]. IL-6 exerts biological actions through interactions with its specific receptor, a complex consisting of a ligand-binding subunit, IL-6Rα, and a signal-transducing subunit. The IL-6Rα component exists in two forms, a transmembrane and a soluble form. The levels of this molecule in the CNS are constitutively very low as they rise in pathological conditions [159]. Numerous studies have shown elevated levels of this protein in patients with diseases characterized by dementia and cognitive decline. In individuals affected by AD, IL-6 is highly expressed in association with high levels of TNF-α and IL-1β and TGF-β [160–162]. An increase in IL-6 concentrations was also observed in a random based study conducted on aged and control subjects ranging from 45 to 90 years old [163]. Studies conducted on the

hippocampus have reported evidence that IL-6 plays a very crucial role in the control of memory formation mechanisms and synaptic plasticity. A high IL-6 exposure has been shown to reduce long term potential (LTP) in Schaffer collateral fibres to Cornus Ammonis (CA1) hippocampal pyramidal neurons in proportion to the administered dose [164, 165].

1.3 Cell death pathways

The cell death phenomenon is a fundamental process for the homeostasis and growth of multicellular organisms. An imbalance between the number of new and dying cells lead to several human pathologies such as neurodegenerative diseases and cancer. To date, many types of cell death are known with differences based on the molecules involved and cell morphology.
The current classification of cell death has been updated by the Nomenclature Committee on Cell Death (NCCD) which states the existence of two types of cell death: accidental and regulated. Accidental cell death occurs when tissue is suddenly injured and eludes all control. In contrast, regulated or programmed cell death (RCD or PCD) involves a series of well-regulated cascade events [166].
In 1972 the term apoptosis was coined to indicate a phenomenon of cellular suicide with morphological changes of the cell in contrast to necrosis, a form of uncontrolled cell death [167]. Over the years, different types of non-apoptotic cell death have been identified such as necroptosis, pyroptosis, ferroptosis, entotic cell death, netotic cell death (NETosis), parthanatos and autophagy [168].
Each type of cell death is characterized by specific morphological traits that have made it possible to identify three different forms of cell death: type I or apoptosis, type II autophagy and type III or necrosis.

Type I cell death or apoptosis is characterized by cytoplasmic wrinkling, nucleus fragmentation, blebs formation and apoptotic bodies containing cytoplasmic material to be degraded.

Type II or autophagy is a form of cell death characterized by the formation of vacuoles which will subsequently be phagocytosed and degraded by lysosomes.

Type III of cell death or necrosis is instead associated with inflammation phenomena and is characterized by the fragmentation of the plasma membrane with consequent loss of cytoplasmic material in the microenvironment [168].

1.3.1 Apoptosis

Apoptosis is a type of defined PCD that has very specific morphological characteristics. In the initial stages, this event is characterized by cell wrinkling and compression of the organelles between them. Subsequently, a progressive loss of the selective permeability of both plasma and mitochondrial membrane occurs, causing, in the latter case, the loss of the mitochondrial transmembrane potential [169]. Nuclear fragmentation and chromatin condensation begin, the plasma membrane is extroverted to form the typical blebs and numerous vesicle-like structures, the

apoptotic bodies, containing organelles, nuclear fragments and cytoplasm and which are phagocytosed by macrophages to avoid the dispersion of the cytoplasmic contents that will further prevent the starting of possible inflammatory response [170, 171]. The molecular pathways of apoptosis have been identified and characterized in vertebrates, insects and nematodes and are evolutionarily conserved albeit with different roles in different species.

Caspases (*C*ysteine-*asp*artic prote*ases*) belong to a family of cysteine-dependent endoproteases that play an important role in PCD. Their function was discovered in the late 1980s in *Caenorhabditis elegans* where the first caspase, called *ced-3,* was studied and subsequently renamed caspase-1 and the Nedd2 molecule, the current caspase-2 [172, 173].
To date, 18 different caspases are known, and expressed differently between the different species. Based on their function they are divided into initiator, effector, and inflammatory caspases and all consist of an amino-terminal domain of variable length and a catalytic domain of about 30 kDa which exerts the protease function.
The caspases involved in apoptosis are divided into initiators and effectors and are present in the cells in the form of inactive enzymes. The N-terminal domain of the initiatory caspases 1, 2, 4, 5, 9, 11 contains a caspase activation and recruitment domains (CARD) domain while the same portion of the polypeptide of the caspases 8 and 10 contains the death effector domain (DED) domain that promotes the activation of multiprotein complexes. The effector caspases, on the other hand, do not possess an extended N-terminal domain and require to be cleaved by the initiating caspases to carry out their activity [174, 175].
Recently it has been shown that caspases are not only involved in apoptotic processes, but also in the suppression of necroptosis, a type of kinases receptor-interacting protein (RIP) RCD. Furthermore, proinflammatory caspases trigger another type of cell death, pyroptosis, which is associated with the release of inflammatory cytokines such as IL-1 and IL-18. A dysregulation in the proteolytic functionality of caspases triggers pathological mechanisms involved in tumorigenesis, autoimmunity, and infectious diseases.

The mechanisms of apoptosis are finely regulated, and two main pathways are highlighted: the extrinsic and the intrinsic pathway (**Fig. 2**). Experimental studies stated that the two pathways are not separated from each other but indeed they share common biological pathways and substrates, they can influence each other's activity and converge in the same final phase of execution.

The intrinsic pathway

The intrinsic apoptotic pathway (mitochondrial or Bcl-2 regulated) is triggered by intracellular stimuli such as irreversible DNA damage, hypoxic conditions, ion cytotoxicity, and ROS accumulation. These stimuli cause the activation of pro and anti-apoptotic proteins belonging to the Bcl-2 family. This family is composed by three groups of proteins: the anti-apoptotic such as Bcl-2, Bcl-x_L and Bcl-w, the pro-apoptotic such as Bax, Bak and Bok and the BH3-only proteins which include Bim,

Bad, Bid and Puma. All these proteins contain BH domains and they can be further divided in BH1, BH2, BH3 and BH4 [176, 177]. The pro-apoptotic molecules Bax and Bak in response to a stimulus form homodimer on the outer mitochondrial membrane (OMM) by recruiting proteins of the BH3 family. This reaction induces the formation of a pore in the OMM that consequently alters the permeability of the mitochondrial membrane and the dissipation of the transmembrane potential [178, 179]. Through these processes, pro-apoptotic molecules such as cytochrome c and the Smad/DIABLO protein are released into the cytoplasm. Specifically, cytochrome c will trigger the activation of caspases while the Smad/DIABLO complex will promote this activation. This event is considered the point of no return that pushes the cell to take the path of apoptosis. Cytochrome c in the cytosol triggers the formation of the apoptosome by interacting with the apoptotic peptidase activating factor (APAF-1) protein. This multiprotein complex recruits the initiating pro-caspase 9 that, after its transformation in the activated caspase 9 recalls the effector caspase 3. This protein catalyses the final phases of the enzymatic cascade causing the fragmentation of the nucleus and the rupture of the plasma membrane of the cell [180, 181]. The intrinsic pathway can also be induced by endoplasmic reticulum (ER) stresses such as calcium increase, excessive unfolded, or misfolded protein accumulation in the ER, and nutrient deprivation. This type of cell death involves caspase-12, an ER-resistant caspase that, in the active form, activated caspase-12, translocates in the cytosol and cleaves caspase-9 [182, 183]. The molecular mechanism of activation of caspase- 12 during ER stress include the formation of a complex with the inositol requiring enzyme-1α-TNF receptor-associated factor 2 (TRAF2) complex, or by calpains, a family of Ca^{2+}-dependent intracellular cysteine proteases [184–186]. In the end, caspase-9 activates caspase-3 promoting the PCD execution.

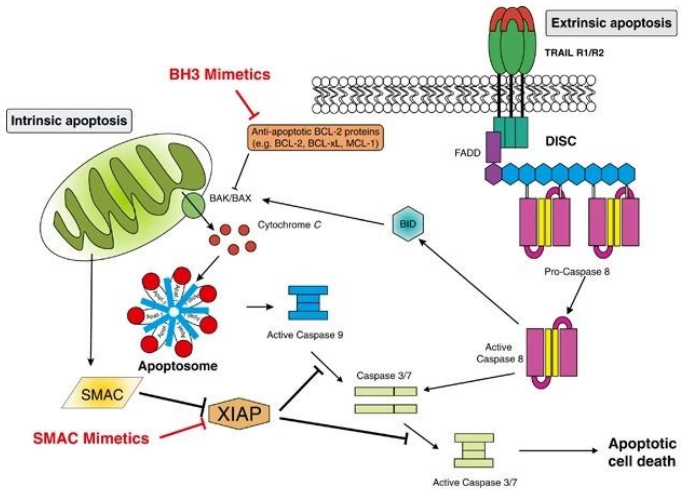


Figure 2. Intrinsic and extrinsic apoptosis pathways, modified by Fox and MacFarlane [187].

The extrinsic pathway

The extrinsic pathway (mediated by the death receptor) is triggered by extracellular signals that stimulate receptors present on the plasma membrane belonging to the TNF family. The interaction between ligand and receptor promotes the formation of complexes and, among the most known it can be mentioned the Fas / FasR, TNF / TNFR or TNF-related apoptosis-inducing ligand (TRAIL) R. The binding to the receptor triggers intracellular cascade events that induce cell suicide.

The death receptor generally consists of a cytosolic death domain (DD). The activated receptor exposes the death domains that call for the binding of adaptive molecules such as FADD (for the Fas/FasR complex) and TRADD (for the TNF/TNFR complex). These molecules in turn possess two domains: the death domain and the death effector domain. The DD of FADD or TRADD binds the DD of the respective receptor. For example, the TNF/TNFR complex recruits TRADD, RIP1, and Inhibitor of apoptosis proteins (IAPs) to form the TRADD-dependent complex I. The removal of residues of polyubiquitin on RIP allows the dissociation of the complex and interaction with the procaspase 8 generating complex II or DISC. In the DISC complex, the formation of caspase 8 occurs, which stimulates the activation of the effector caspases 3 and 7 [174, 181, 188]. Caspase 8 is also involved in the cleavage of the Bid protein, a pro-apoptotic molecule of the Bcl-2 family. Once activated, this protein translocates on the mitochondrial membrane thus activating the intrinsic apoptosis pathway and further increasing the effects of apoptosis [181, 189].

1.3.2 Autophagy

Autophagy is an energy-dependent RCD process that uses the components of the autophagic machinery to function. The autophagic response is under strict transcriptional and post-transcriptional control and is often triggered during protective rather than harmful mechanisms [168]. Among the signs that trigger autophagy, the most common are various types of stress, nutrient poverty and restrictive diet, damage to organelles, hypoxia, and ROS. Autophagy can also be triggered by signals that occur during development and embryogenesis or, during ageing, it can intervene in the removal of senescent cells [168, 186, 190]. There are three types of autophagy in mammals: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (**Fig. 3**). Among them, macroautophagy is the most studied process; in cells, it is induced by stress stimuli such as lack of energy or nutrients, and it triggers the demolition of cytoplasmic material which will subsequently be used as an energy source for cell survival. For this reason, macroautophagy is considered a protective cell death mechanism even if an excessive activation of it causes tissue damage [191].

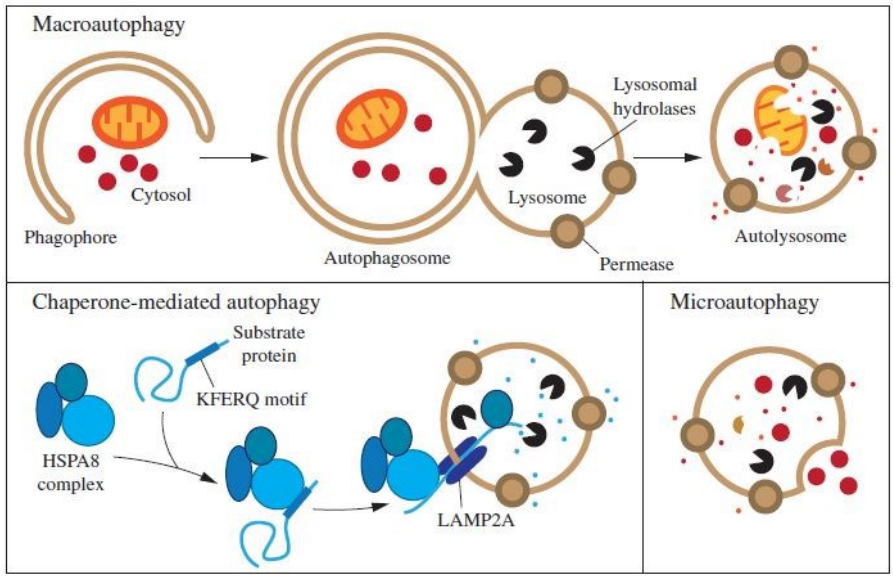


Figure 3. Three types of autophagy [191].

Macroautophagy is the best known and studied autophagic process. What differentiates this typology from microautophagy and CMA is that the degradation vesicle is generated *de novo*, by expansion and apposition of a multiprotein complex. In yeasts, autophagosome formation begins at a site called the phagophore assembly site (PAS) [192]. In mammals, on the other hand, it occurs at different sites in the cytoplasm such as the ER [191, 193]. The autophagy process can be divided into 4 phases: initiation, nucleation, fusion of the autophagosome with a lysosome and degradation. During these steps, the autophagy-related genes (Atg) proteins play a fundamental role. After the initiation phase, the phagophore membrane begins to expand probably starting from membrane portions of the cytoplasmic organelles. As the phagophore grows, the so-called spherical autophagosome is formed that surrounds the material to be digested. In yeasts, the induction of autophagosome is regulated by the complex of protein kinases Atg1-Atg13-Atg17-Atg31 and Atg29 [194]. In mammals, this complex consists of proteins belonging to the family of Unc-51-like kinases (ULK1 and ULK2), homologues of Atg1, Atg13, Atg101 and the scaffold focal adhesion kinase (FAK) family interacting protein of 200 kDa (FIP200). The association of mTORC1 with the induction complex is influenced by nutrient-rich conditions [191, 195]. When mTORC1 is complex associated, it phosphorylates ULK1/2 and ATG13, inactivating them. On the contrary, when cells are under rapamycin effects or in poor-nutrients conditions, mTORC1 dissociates from the complex, dephosphorylates these sites and induces macroautophagy [191]. In the nucleation step, the assemble of the autophagosome requires the involvement of the ATG14-containing class III phosphatidylinositol 3-kinase (PI3K) complex and the action of Beclin-1 (BECN1) factor, which acts binding cofactors, such as Barkor

(Beclin -1-Associated Autophagy-Related Key Regulator), p150 and UVRAG (UV Irradiation Resistance-Associated Gene) [196]. The ATG14 complex is positively regulated by the Activating Molecule in Beclin-1-Regulated Autophagy Protein 1 (AMBRA1) [197] and downregulated by BCL2 that can bind BECN1 therefore preventing the complex formation [198]. In the literature controversial data are reported about the role of the PIK3 complex: on the one hand, it seems to be involved in the formation of the autophagosome while on the other some data show involvement in the last stages of this process. The subsequent step is the elongation of the phagophore by ubiquitin-like proteins (UBL). Two conjugation systems are involved: the Atg12-Atg5 and the LC3-PE pathways. The first ubiquitin-like conjugation system is Atg12-Atg5-Atg16 complex; it functions very similar in yeast and mammals. Firstly, the UBL protein Atg12 conjugates with Atg5 in a reaction catalysed by Atg7 and Atg10. After the formation of the Atg12-Atg5 complex, the Atg16 protein binds Atg5 and dimerizes to form a larger complex of about 350 kDa [199, 200]. The second system involves the Atg8/LC3 complex. In this pathway, Atg8 is conformationally changed by the Atg4 protease to expose a C-terminal glycine residue; subsequently, Atg7 activates Atg8 and transfer it to Atg3 [201]. Finally, Atg8 is covalently bound to phosphatidylethanolamine (PE) with the help of Atg12-Atg5 complex [202]. Mammals have four different isoforms of ATG4 and various types of Atg8 proteins, such as microtubule-associated protein 1A/1B-light chain 3 (LC3) and gamma-aminobutyric acid receptor-associated protein (GABARAP) families. LC3 is the best characterized Atg8-like protein [191]. At this point, the autophagosome fuses with the lysosomes to form autolysosomes, triggering the degradation of the vacuole content by hydrolases.

p62 is a multifunctional stress induced protein ubiquitously expressed in all tissues. In humans it is encoded by a highly conserved gene called SQSTM1 which is located on chromosome 5 and translates for a complex protein of 440 amino acids characterized by several functional domains such as Phox and Bem1 (PB1) domain, Zinc finger (ZZ) domain, nuclear localization signal (NLS) domain, nuclear export signals (NES) domain, a region of interaction with LC3B (LIR), KEAP1 interacting region (KIR) domain, and an ubiquitin-associated domain (UBA) [203, 204]. In particular, the PB1 domain mediates the oligomerization of the p62 protein and thus interacting with some protein kinases such as PKC. The ZZ domain is located just after the PB1 domain, in proximity to the NLS and NES domains and together they are able to bind an Atg8/LC3B autophagy regulatory complex that directs ubiquitinylated proteins in the autophagosome or in the ubiquitin-proteasome system [205, 204]. In cells, therefore, it can be expressed either in the cytoplasm, where it is linked to the autophagosome or lysosomes, or in the nucleus. Additionally, as above mentioned, p62 can be expressed on vesicles or organelles that must be degraded by the proteasome and which are recognized thanks also to the interaction with the UBA domain [206]. For example, it can be observed in ubiquitinated protein aggregates or in damaged mitochondria. In the cytosol p62 also participates in the decrease of oxidative stress thanks to the binding of the KEAP1 domain with the Nrf2 molecule.

p62 is also involved in many other processes such as adipogenesis through the interaction with the ERK protein, the signal mediated by NF-kB, in apoptosis thanks to the interaction with caspase 8 and in the monitoring of nutrients with the interaction with the mTORC1 complex [207–209]. SQSTM1 controls adipogenesis and body weight thanks to the interaction with the ERK1 signal and leptin. Several studies report that the loss of SQSTM1 causes a hyperactivation of ERK1 which results in a state of insulin resistance. Moreover, SQSTM1 KO mice show leptin resistance and hyperphagia [210]. A further study showed that p62 is also involved in inflammatory states of the adipose tissue. In obese individuals, the decrease in p62 in visceral adipose tissue leads to dysregulation of mitochondrial functioning thus inducing anti-inflammatory respects or programmed cell deaths [211]. Besides, p62 phosphorylation seems to contribute to the onset of some forms of hepatocellular cancer [212]. The phosphorylated form of p62 can accumulate in the cytoplasm and it has been observed to be associated with the typical inclusions such as α-synuclein, Huntingtin and β-amyloid of Parkinson (PD), Huntington (HD), Alzheimer (AD) diseases respectively suggesting an involvement of this protein in these diseases. Furthermore, SQSTM1 is considered one of the first markers to appear in the inclusions of the tau protein which are then directed towards autophagic degradation preventing their spread in the brain [213, 214]. A decrease in p62 in these conditions seems to be related to the increase in age-related neurodegenerative diseases in close association with an increase in oxidative and inflammatory status [215].

In recent years, the involvement of p62 in the activation of a particular type of autophagy affecting the mitochondria, called mitophagy, has become increasingly clear (**Fig. 4**).

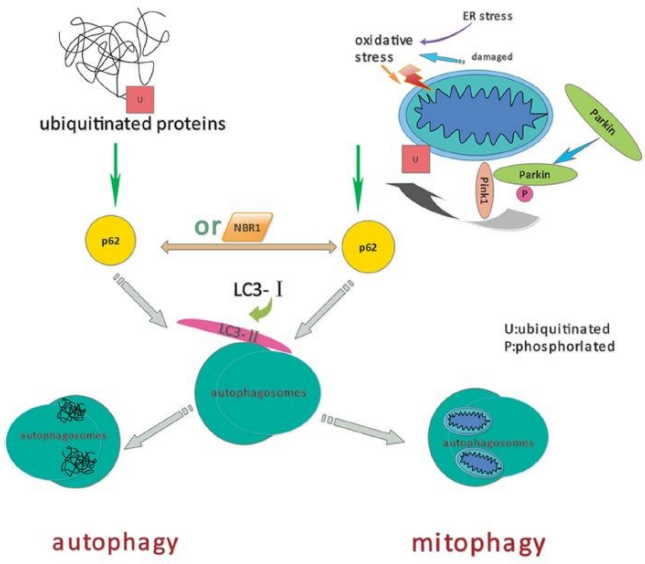


Figure 4. The role of p62 in autophagy and mitophagy [204].

Mitochondria are organelles that have always been considered crucial for maintaining the energy homeostasis in the cells [216]. Alongside this function, mitochondria are also involved in controlling the production and demolition of ROS that can be produced during stressful situations. Furthermore, these organelles are implicated in physiological processes such as cell differentiation, proliferation, and apoptosis. Finally, mitochondria can participate in innate immune responses, recruiting molecules belonging to the inflammatory pathways and inducing conformational changes in their structure. Stress events affecting the mitochondria can be generated by various degenerative diseases, nutrient deprivation, or physiological events such as ageing leading to the onset of neurodegenerative pathologies and neuroinflammation. The first line of defence adopted by mitochondria is the production of antioxidant enzymes and molecules such as SOD1, SOD2 and vitamins E, A and C. Subsequently, dynamic mechanisms of mitochondrial fission and fusion are triggered to segregate damaged mitochondria (fission) or promoting the exchange of proteins involved in mtDNA repair (fusion) and thus avoiding a worsening of intracellular damage [217]. Finally, if the implemented mechanisms are not conclusive, the ubiquitin proteasome system intervenes for the degradation of mitochondrial membranes and proteins in the mitophagic process [218]. Mitophagy is a process that therefore intervenes in the maintenance of cellular health.

The PTEN-induced kinase 1 (PINK1) and Parkin RBR E3 ubiquitin-protein ligase (PARKIN) signal path intervenes first. The PINK1 protein has the physiological role of protecting cells from mitochondrial dysfunction due to stresses. In humans, the gene that codes for this protein is located on chromosome 1 and produces a protein of 581 amino acids while the gene that codes for PARKIN is located on human chromosome 6 and consists of 465 amino acids [219]. Under normal conditions, mitochondria have an optimal transmembrane potential that allows to import, thanks to the presence of the proteins translocase of the outer membrane (TOM) and translocase of the inner membrane (TIM), PINK1 which is cleaved and retro-translocated into the cytosol to be finally degraded thus preventing the uncontrolled activation of mitophagy. Harmful situations that persist in the cells lead to a decrease in the transmembrane potential which causes the accumulation of PINK1 on the OMM. Here this molecule forms a super complex with TOM which triggers the autophosphorylation and therefore the conversion of PINK1 into its activated form. Once activated, PINK1 is capable of ubiquitinylating and phosphorylating OMM proteins that will act as receptors for the binding of PARKIN to the membrane itself, which will be recalled from the cytosol to the OMM, promoting the ubiquitination of mitochondrial proteins that will subsequently be degraded with mitophagy. In this way PINK1 and PARKIN create a feedback system that leads to covering the damaged mitochondrion with phosphor-ubiquitin chains. Ubiquitinylated OMM proteins are isolated and degraded in the proteasome while, subsequently, the phospho-ubiquitinylated chains are linked by two adapter molecules nuclear domain 10 protein 52 (NDP52) and optineurin which in turn promote the autophagy of damaged mitochondria. Furthermore, these two molecules recruit the formation of the autophagosome thanks to the bond with LC3 thus

concluding the degradation of the damaged mitochondrion [218].

Many studies have suggested a role of mitophagy in ageing and in the predisposition to neurodegenerative diseases and the absence/decrease of PINK1 and PARKIN seems to be involved in the accumulation of damaged mitochondria, therefore in an aggravation of the pathological scenario [220, 221]. In two papers it has been demonstrated that mutations in PARKIN and PINK1 cause respectively the autosomal recessive juvenile parkinsonism (AR-JP) and the sporadic early-onset parkinsonism (EOP) [222, 223]. These proteins are also important in fission/fusion mechanisms because they are able to interact with the fission dynamine-related protein 1 (Drp1) and the fusion optic atrophy 1 protein (OPA1). In a study conducted on rats, the overexpression of PINK1 or PARKIN in hippocampal neurons demonstrated increased fission and suppression of a mitochondrial elongation phenotype caused by Drp1 downregulation. A similar phenotype is caused by the inactivation of PINK1, which leads to greater fusion. Furthermore, the same researchers demonstrated that PINK1/PARKIN also had a comparable influence on mitochondrial dynamics in dopaminergic neurons by tilting the fission/fusion balance towards greater fission [218, 224].

Very often neurodegeneration and neuroinflammation are processes that share many pathologies. For example, humans with mutations in the PARKIN gene show higher serum levels of IL-6, IL-1β, CCL2 and CCL4, while the serum levels of these molecules in PINK1 heterozygotes were the same to controls [225]. Moreover, in another study brain sections from PINK1 knockout mice exhibited improved amounts of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6 [226]. Furthermore, many studies have shown that in pathological conditions the expression of PINK1 and PARKIN is increased in activated astrocytes, indicating an involvement of these molecules in the glia-dependent immune responses [227, 228]. In addition, in a study published by Sun and colleagues it was found that the absence of PINK1 promoted glial cell-mediated apoptosis in NO dependent neuroblastoma models, suggesting a role of this protein in exerting a protective effect on neurons. In this work it was also found that PINK1 ablation influences inflammation-induced gene expression and NO production in astrocytes and microglia. Defects in proliferation, elevated NO production, reduced mitochondrial function and increased levels of cytoplasmic and mitochondrial ROS were found in astrocytes in which low PINK1 levels were present, and exposure to LPS and IFNγ promoted iNOS expression, NO and TGFβ1 [229]. Altogether these studies suggest that the lack of PARKIN and PINK1 upon mitochondrial stress triggers a vigorous inflammatory phenotype and promotes reactive microglia survival contributing to neuroinflammation [218].

Microautophagy occurs when the cytoplasmic content is invaginated by the lysosome. It is a process little known in mammals both for its mechanisms and for its roles in human pathologies while it has been well characterized in yeasts [191, 230, 231]. In the initial phase of microautophagy, the GTPase Vps1p regulates the invagination of the lysosome membrane swells by separation of phospholipids and exclusion of transmembrane proteins [231, 232]. The invagination not only moves laterally, but it also grows and shrinks rapidly. Hunger induces the onset of susception

which turns out to be an ATP dependent event. In addition, there is a decrease in the density of proteins in the upper part of the invaginated membrane and this, together with the high density of lipids, allows a deeper invagination which tends to form a vesicle. The vesicle then closes on itself with the help of SNARE proteins. Microautophagy is induced by the target of rapamycin (TOR) signalling pathway, a protein involved in the stimulation of cell proliferation and survival in yeast and mammals in presence of growth factors and nutrients. The TOR pathway is controlled by a second regulator, the EGO complex that is composed of three proteins: Ego1, Ego3 and GTPase Gtr2 and is located on the vacuolar membrane [233]. Another complex, called VTC, plays a central role in microautophagy, this complex is located on the vacuolar membrane, but also in other cell membranes [232].

Chaperone-mediated autophagy (CMA) is a type of autophagy that has only been characterized in mammals. It is a very specific process that affects those proteins that contain the KFERQ pentapeptide. About 30% of the cytosolic proteins contain this sequence; these proteins undergo a conformational change that directly induces them to be phagocytosed by the lysosomes and to be degraded in their lumen. During CMA, the KFERQ domain is recognized by heat shock 70 kDa protein 8 (HSPA8/HSC70) and by several chaperones. HSPA8 induces the substrate towards the lysosome membrane where it binds the CMA receptor monomers, the lysosomal-associated membrane protein 2A (LAMP2A) proteins. This binding leads to the multimerization of LAMP2A and this complex is stabilized on the lysosomal membrane by the HSP90 protein. After translocation of the substrate into the lumen of the lysosome, HSP8 disassembles the translocation complex and LAMP2A returns to monomeric form so that a new substrate can be bound.

1.3.4 Necrosis

Necrosis has always been considered an uncontrolled and accidental cell death [234]. This process consists of the sudden rupture of the plasma membrane, loss of intracellular homeostasis and dispersion of the cytoplasmic content in the tissue microenvironment. Unlike apoptosis, necrosis is a cell death that is not dependent on energy because the cell is damaged by events such as heat, radiation, physical or chemical agents [235]. During necrosis, damage to the plasma membrane alters the electrochemical potential within the cell causing an influx of Na^+ and Ca^{2+} ions and the consequent loss of cell volume; organelles are damaged and Ca^{2+} dependent enzymes such as hydrolases, phospholipases and proteases are activated which rapidly degrade proteins, DNA and RNA. The fragments thus released diffusely into the extracellular space causing inflammatory responses and tissue damage [236].

1.3.5 Different types of cell death

For several years, necrosis was contrasted with apoptosis and was considered a completely uncontrolled cell death. It has recently been understood that the mechanisms of PCD and necrosis can overlap, giving rise to a multitude of pathways that can be undertaken by the cell to complete the programmed death process [237] Several forms of regulated necrosis had been identified *i.e*. necroptosis, ferroptosis, parthanatos, and entosis [168].

Necroptosis

In 2005, a new form of cell death very similar to necrosis was identified by the group of Degterev and colleagues [238]. Unlike necrosis, however, necroptosis is an event finely regulated by proteins called receptor-interacting proteins (RIPs) that work in conditions defined as apoptosis-deficient [239].
Necroptosis is activated by both extra and intracellular signals that are picked up by death receptors such as TNFR1 and, albeit in smaller numbers, by TRAIL and FasR receptors. The best-characterized necroptosis pathway is that of TNFR1 [240, 241].
The binding of a ligand to TNFR1 allows for the recruitment of complex I, consisting of TRADD, FADD and E3-ligase proteins such as TNF Receptor Associated Factor 2/5 (TRAF2/5). This complex also recalls the RIP protein, which is polyubiquitinated in a lysine residue 63 by TRAF2/5. The removal of the polyubiquitin residue allows the release of RIP1 in the cytoplasm and the formation of complexes IIa and IIb consisting of the molecules TRADD, FADD, the protein kinase RIP3 (RIPK3) and caspase 8. Complex IIa activates caspase-8 which consequently triggers apoptosis while complex IIb triggers necroptosis [242, 243].
In particular, the activation of RIPK3 allows the formation of the necrosome. In this complex RIPK1 and RIPK3 perform a series of self-phosphorylation which promote the involvement of the mixed lineage kinase domain-like pseudokinase (MLKL). This protein is phosphorylated and undergoes conformational changes that allow it to migrate to the plasma membrane, where it binds to the phosphatidylinositol phosphate (PIP). The binding of MLKL to the plasmalemma induces an increase in its permeabilization, ultimately promoting cell necrosis [235, 244].

Ferroptosis

Ferroptosis is a type of iron-dependent RCD described for the first time by Dixon and collaborators in 2012 [245]. This form of cell death is due to the process of lipid peroxidation, i.e. the addition of oxygen molecules to lipids such as, for example, membrane phospholipids and polyunsaturated fatty acids (PUFAs) [246]. Ferroptosis causes morphological changes in cells such as wrinkling of the mitochondria, condensation of chromatin, rupture of the plasma membrane and the outer mitochondrial membrane [245, 247].
Iron is a key molecule for cell proliferation and function, but it can accumulate in the cytoplasm of cells leading to cytotoxicity, damage, and cell death. The iron concentration must therefore be constantly regulated, and an incorrect metabolism of this ion can cause ferroptosis via two pathways. The first modality involves the

production of iron-induced ROS through the Fenton reaction [245]. The second way involves the activation of enzymes containing iron such as the lipoxygenases [248, 249]. However, excessive activation or malfunction of ferroptosis increases the risk of developing various diseases such as neurodegenerative diseases, cancer, tissue damage and infections [250, 251]. There are several mechanisms in the cell capable of counteracting oxidative damage from iron accumulation. Through the synthesis of antioxidant molecules such as glutathione (GSH), coenzyme Q10 (CoQ10) and tetrahydrobiopterin (BH4) the cells can defend themselves against this type of cell death [252]. GSH's anti-ferroptosis activity is mainly related to the glutathione peroxidase 4 protein (GPX4) which acts by reducing the production of hydroperoxide phospholipids. GPX4 is also involved in antioxidant defence in apoptosis, necroptosis and pyroptosis, suggesting a large role of this enzyme in the regulation of cell death [252, 253].

Parthanatos

Parthanatos is a form of PCD caused by an overactivation of the poly(ADP-ribosome) polymerase 1 (PARP-1) protein, one of the components of the DNA damage response pathway, in response to oxidative stress and chromatolysis [254]. The term was coined in 2009 by Dawson and colleagues [255]. PARP-1 is an enzyme associated with chromatin essential for cellular homeostasis that participates in the repair of DNA damage such as single-strand and double-strand breaks. PARP1 recognizes these breaks in DNA and triggers poly(ADP-ribose)-sylation (also called PARylation) using NAD^+ and ATP. During pathological conditions such as inflammation, oxidative stress, ROS and RNS production, and cytotoxicity there is an excessive activation of this protein which causes: i) decrease of ATP and NAD^+ which causes loss of cellular energy and ii) accumulation of polyproteins leading to dissipation of membrane potential and MOMP [256, 257].
PARP1 synthesizes poly(ADP-ribosome) (PAR) polymer from NAD^+ that subsequently binds to apoptosis-inducing factor (AIF) promoting its release in the cytosol by the mitochondria and its translocation into the nucleus where it induces DNA fragmentation and chromatin condensation [168, 258].

Entosis

Entosis is a type of cell death in which a cell engulfs and kills another cell. The term was coined in 2007 by Brugge and collab [259]. This cell death occurs mainly due to the loss of adhesion of cells from the ECM. The literature also reports alternative mechanisms that induce entosis and that involve the alteration in the expression of myosin during the formation of cell-cell contacts and differences in metabolic response and mechanical properties of the tissue involved [168, 254, 260–262]. The cellular mechanisms underlying this type of death are not yet well known but it is known that rearrangements occur in the cytoskeleton of cells and their adhesion capacity. In fact, in this process, it is essential to form adherent junctions and it seems that the cadherin 1 protein plays a central role in initiating entotic cell death [254].

1.4 Hippocampal formation

The hippocampal formation is an extension of the temporal lobe and is responsible for cognitive, emotional and motivational functions since it constitutes the major component of the limbic system [263]. It also plays roles in learning, memory, social behaviour and spatial navigation [264–267]. Impairment in the hippocampal formation is associated with numerous neurological damage and diseases such as neurotoxicity, epilepsy, AD and schizophrenia [268–271]. In humans, it is located in each hemisphere of the brain and has a volume of about 3-3.5cm^3 in the temporal lobe while in the rodent is located just under the cerebral cortex [272] (**Fig. 5**).

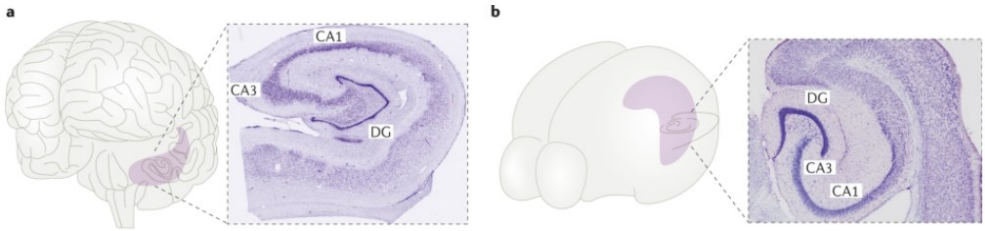


Figure 5. Human (a) and mouse (b) brain and Nissl- stained hippocampal sections, modified from Hainmueller & Bartos [272], abbreviations: Dentate Gyrus (DG), Cornus Ammonis 1 and 3 (CA1 and CA3).

The hippocampus anatomical structure was firstly identified in the 16th century by Giulio Cesare Aranzi (1530 – 1589), an anatomist who studied different anatomical region of the human body. He originated the term hippocampus from the Greek word seahorse (*hippos* [horse] and *kampos* [sea monster]) and he described it in his work entitled *De Humano Foetu Liber*. Different names of this region, *i.e.* "ram's horn", Ammon's horn and "Cornus Ammonis" were given during the latest years by respectively Winslow, Croissant de Garengeot and Rafael Lorente de Nò [273, 274]. Nowadays, the term hippocampus has become the most widely used in the literature and with hippocampal formation is indicated the dentate gyrus (DG), hippocampus proper or Cornus Ammmonis (CA), subiculum and entorhinal cortex. [275]. All the hippocampal formation fields differ for the cytoarchitectural structure. The members of each group vary between the authors and the works present in the literature. The most supported idea is to consider the DG, hippocampus, subiculum, and entorhinal cortex under the same term of hippocampal formation for the reason why these areas are linked to each other through unidirectional connections which seem to group them as a functional unit. Each of the fields of the hippocampal formation can be considered as parallel rows of cerebral tissue [276]. All three divisions of the hippocampus have a three-layered cortex which is called the allocortex.

1.4.1 Hippocampal development

The progenitor cells of pyramidal and granular neurons of the hippocampal formation derive from the germinal area of the lateral ventricles [277]. The hippocampal neuroepithelium is made up of three morphologically different components: i) the ammonic neuroepithelium, ii) the dentate neuroepithelium and iii) the fimbrial glioepithelium which generate respectively the pyramidal neurons of the hippocampus, the granular neurons of the DG and the glial cells of the fimbria. Pyramidal neurons of the hippocampus are generated between embryonic day 10 (E10) and E18 in the mouse [278].

These two waves of formation also decide the fate of pyramidal cells: the neurons generated in the early embryonic days will form the pyramidal layer of the CA3 area while the pyramidal cells will subsequently be located in the CA region. It should be emphasized that during development the formation of the CA1 area takes place first and only later than of the CA3 area despite the latter's neurons differing first. This event happens because pyramidal cells need the presence of the DG, that develops last in the hippocampal formation, to stabilize. DG granular neurons begin to form from day E16, and the typical structure of this hippocampal portion is not well distinguishable until day E21 [277]. The formation of the DG appears to be more complex. The area from which the cells of the DG derive are distinguished by shape and organization and are in the dentate notch [277, 278]. Up until E18, this epithelium is surrounded by a secondary germinal matrix which gradually thins until day E21 when the cells that make up this matrix migrate towards the crest of the DG and pack. From day E22 the innermost portion of the DG begins to be differentiated from the outermost one and the different components of cell migration begin to be distinguished. The first migratory wave follows a subpial path and will affect the innermost part of the DG. This migration will end around the 10th postnatal day (P10) and will remain only in a small area called the subgranular zone (SGZ) which will be the only site of adult neurogenesis. The second cell migration instead affects areas near the pyramidal cells of the CA and will make up the remaining part of the granular layer of the DG. The projections of the entorhinal cortex reach the DG and the hippocampus starting from day E17 using the Cajal-Retzius cells as a scaffold for migration and then stabilize during postnatal development [279].

1.4.2 Dentate gyrus

The dentate gyrus (DG) is a well identifiable structure of the hippocampal formation thanks to its typical C-shape conformation. Recent evaluations have estimated the presence of about 10 million granular neurons in the human dentate gyrus and about 1 million in the rodent hippocampal formation [280]. Overall, the DG consists of a three-layered cortex: i) an outer molecular layer (ML) characterized by the presence of few neurons and almost completely occupied by granular cells dendrites; ii) an intermediate granular layer (GL) containing the glutamatergic granular cells; iii) a polymorphic layer (PL) constituted by different cell types, the main ones being mossy neurons (**Fig. 5**) [281].

The DG in rodents has the three previously mentioned canonical layers. The GL and ML form a characteristic "V" shape which includes the PL also called the hilum. The main cells that compose this area are granular cells which in mice are estimated to reach about 390,000 units in each of the two hemispheres. These neurons are tightly packed together and do not possess a basal dendrite while the apical dendrite emerges from the layer branching off into the ML. The dendrites of these neurons have numerous dendritic spines that increase in number as one move to the most distal positions of the dendrite concerning the cell body. Often the neurons located in the most superficial portion of the layer have somatic spines especially in those areas of the membrane facing the ML [282, 283]. The DG also has numerous GABAergic neurons. Over the years these cells have been characterized through studies with specific markers that have shown a strong increase in GAD67 positive neurons in the dorsal hippocampus compared to the ventral hippocampus. Furthermore, neurons called basket are located in the deeper portions of the GL in contact with the hilum, a name that refers to the fact that the axons of these cells envelop the soma as if to form a plexus. These inhibitory neurons were also found to be parvalbumin-positive and are thought to have the role of sending signals to the innermost portion of the ML by modulating the activity of granular neurons [284]. The GL also contains positive NOS neurons [285]. The ML is mainly occupied by the dendrites of granular cells, basket cells and various polymorphic neurons. The few cells present appear to be vasoactive intestinal peptide (VIP) positive neurons that participate in the circuits that regulate the basket cells around the soma of granulated neurons and the “chandelier” cells that innervate only the initial segments of the granular cell axon. The hilum contains a different number of somatostatin and neuropeptide positive GABAergic neurons, but certainly, the most represented cells are the mossy cells. These neurons are glutamatergic and calretinin positive in mice with expressions ranging from the dorsal portion (where only 24% of neurons are CR^{+}) to the ventral portion (where approximately 85% of positivity is found). The soma of these cells has an oval shape and is about 21-25 μm. Numerous thin dendrites originate from the cell body and extend long distances into the PL, while others can traverse the GL and ascend to the ML. The peculiarity of these cells is that on the proximal dendrites there are growths which are the termination site of the axons of the cells themselves. These neurons exhibit a pattern that varies from dorsal to more caudal sections. First, in the most ventral portion of the hilum, there are more mossy neurons than in the dorsal area, while the cells of the latter area are characterized by a higher number of growths. However, the data on the number of mossy neurons present in the hilum must be interpreted considering that in the most ventral portion there is a greater area in which these cells can develop [286–288].

Even in humans, the DG consists of three layers. Its cytoarchitecture can be well appreciated in the most rostrocaudal portion of the brain where it forms a typical "C" -shaped structure separated ventrally from the CA3 area of the CA and the subiculum. For humans too, the most represented layer is the GL, composed of granular neurons with a single spiny apical dendrite only in the distal portions. The human DG is estimated to contain about 9 million cells, although this number is probably

underestimated. Surprisingly, some authors report a particular asymmetry in this data by proposing that the right side contains 20% more granular neurons than the contralateral hemisphere [289]. Human granular neurons have a developed basal dendrite which is not present in rodents and which extends into the thickness of the PL. The data relating to the hippocampal hilum are controversial since in the human hippocampus it is not always easy to distinguish the neurons of the PL from the rest of the hippocampal formation [290].

1.4.3 Cornus Ammonis

Cornus Ammonis (CA) or hippocampus properly is a voluminous semi-cylindrical structure, about 5 cm long, which is positioned above the DG.

It can be divided into three fields, indicated as CA, accordingly to the nomenclature of Lorente de Nò [291]. The CA3 field starts from the hilus of the DG and extends up to the CA2 area, a small transition zone between CA3 and CA1. The CA1 field occupies most of the upper region of the hippocampus reaching the subiculum. The Cornus Ammonis is made up of different cortical layers: i) the stratum *alveus* is the most superficial layer that contains the axons of pyramidal cells; ii) the stratum *oriens* that contains interneurons and the basal dendrites of pyramidal neurons; iii) the pyramidal stratum that comprises the soma of pyramidal cells; iv) the stratum *lucidum* that is composed by mossy fibres synapses with the proximal dendrites of pyramidal cells; v) the stratum *radiatum* that contains the axons of pyramidal cells, the axons of associate fibres in CA3 and the Schaffer collaterals in CA1; vi) the stratum *lacunosum*-molecular that contains fibres from the Schaffer collaterals [276, 281]. The boundaries separating the CA3 and CA2 areas are not always clearly distinguishable due to the presence of cells from the CA3 that also deepen into the CA2 zone. The absence of input from the mossy fibres in the latter area gives compactness and make the PL thinner, a feature that allows distinguishing between the two adjacent fields. The CA1 field seems to have the most complex anatomical subdivision because it contains a wider heterogeneity of neurons than the other hippocampal fields and a composition that varies along with its rostrocaudal development. Also, the boundaries between the CA2 and CA1 fields are not well distinct due to the presence of neurons belonging to the CA2 area which extend into the PL from the CA1 area.

The hippocampus of rodents as already mentioned is divided into three areas: CA3, CA2 and CA1. These three areas are distinct for the circuits they generate and the molecular markers they express. The identification of the CA2 area remains much debated due to the discordant data on connections and functions but, at least in the mouse, thanks to gene and protein expression data it is recognized as a separate area from the rest of *Cornus Ammonis*. Furthermore, Lorente de Nò in 1934 identified many subtle differences regarding the dendritic organization of pyramidal cells in the CA3 and CA1 areas and, based on these data, divided these areas into further subareas (CA3a, b, c; CA1a, b, c) [291]. However, all areas maintain a so-called laminar structure. The main cell layer is represented by the PL which is present in all areas of

the *Cornus Ammonis*. A peculiarity is represented by the *lucidum* layer which appears to be present only in the CA3 area, up to its most distal portion, thus marking the boundaries with the CA2 area. Pyramidal cells have a very branched basal dendrite that extends into the *oriens* layer and an apical dendrite that instead branches out to the hippocampal fissure. Basket neurons can also be found in the PL, among cells very different in shape and size, as for the granular cells of the DG, extending transversally to form a plexus around the cell soma of the pyramidal cell. Scattered throughout the various layers of the hippocampus there are numerous interneurons, mostly GABAergic, with a variable number from dorsal to caudal areas. These cells have been well characterized from the point of view of protein expression and very often are found to be parvalbumin-positive (especially in the CA1 area), calbindin-positive (for the CA3 area), neuropeptide Y (NPY), Cholecystokinin (CCK), and NOS positive [284].

The cytoarchitecture of the human CA horn follows the same subdivision already discussed above. In the CA3 and CA2 areas it is estimated that there is a total of about 2,800,000 pyramidal cells with an asymmetry between the left and right hemisphere; in the right hippocampus, an increase of 14% of pyramidal neurons is estimated. The PL also contains the mossy fibres that come from the hilum of the DG and accumulate in the stratum *lucidum*. The CA2 field is distinguished by the fact that it has a much thinner PL and, lacking the input of the mossy fibre, it is much less developed. The CA1 field is the most complex. It is certainly populated by a more heterogeneous variety of neurons and its composition varies following the rostrum-caudal progression. The boundaries of this area are not identifiable because both the CA2 cells and the cells of the subiculum encroach on the CA3 layer. Often resorting to the use of Nissl staining, the CA areas can be distinguished more easily. In humans, it is estimated that CA1 is composed of about 4,600,000 neurons and these about 10% are interneurons, but the numer could be underestimated. More recently, a cell number of about 2% of interneurons has been estimated. No distinctions were found between the two hemispheres [290, 292].

1.4.4 Hippocampal circuits

The afferent information reaches the hippocampus through the entorhinal cortex, a portion of the cerebral cortex that participates in the formation of the so-called trisynaptic circuit of the hippocampus. Neurons located in the different cortical layers send signals to specific areas of the hippocampus formation. Axons of those neurons located in layer II of the entorhinal cortex enter the hippocampal formation through the subiculum and travel to the DG and CA3 region of the hippocampus [293]. The projections from the entorhinal cortex to the DG give rise to the perforant pathway. The medial entorhinal cortex sends inputs to the central zone of the DG molecular layer while the lateral portion transmits direct signals to the outer third of the DG ML. Furthermore, these two pathways can also terminate in the *lacunosum*-molecular layer of CA3 and CA2 areas. Neurons in layer III of the entorhinal cortex direct their projections to the CA1 area and the subiculum. The axons originating

from the medial entorhinal cortex terminate in the CA1 *lacunosum*- molecular layer, very close to the CA3 area, and those deriving from the lateral area of the entorhinal cortex terminate in the portion of the *lacunosum*-molecular layer at the border between CA1 area and the subiculum. Subsequently, the DG is involved in the formation of the hippocampal circuit. Granular neurons of the DG generate axons called mossy fibres which terminate on the proximal dendrites of CA3 pyramidal cells [294]. The mossy fibres can also contact mossy cells and interneurons located in the hilum of DG. The pyramidal cells of CA3 participate in local circuits within the area itself or can send projections to the CA1 area generating the Schaffer collaterals. In turn, CA1 pyramidal cells project to the subiculum and deeper layers of the entorhinal cortex closing the circuit [295]. Therefore, sensory information converges on the entorhinal cortex from specific cortical areas, reaches the hippocampal formation where they undergo integration of information and then returns to specific cortical areas (**Fig. 6**) [279].

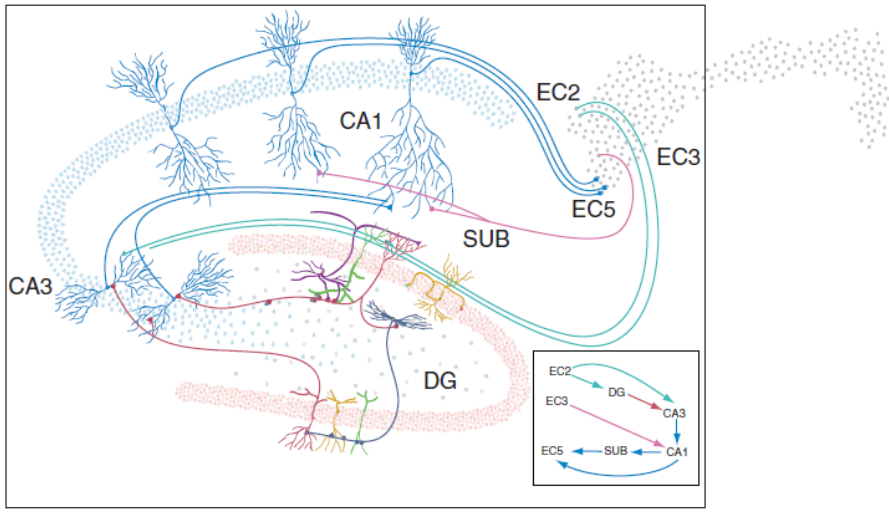


Figure 6. Hippocampal synaptic circuits [279].

1.4.5 Hippocampal functions

Recently, numerous studies have suggested a certain functional heterogeneity along the longitudinal axis of the hippocampal formation. Behavioural studies suggest that the dorsal regions are those responsible for spatial navigation and cognitive functions, while the ventral areas are involved in emotional and affective behaviour and constitute a fundamental area of the limbic system relative to the classical Papez circuit [296]. The DG can also be considered as the first step in the processing of information that ultimately leads to the formation of episodic memories [297]. The hippocampal formation is also involved in declarative memory related to facts and events. Within this structure, short-term memories are selected to keep only

those that will become long-term, which will then be stored in other brain areas. In particular, the hippocampal formation plays a fundamental role: in episodic memory, correlated with unique and personal events that have a precise location in space and time [298]. The importance of this area in space navigation was defined thanks to the discovery in CA3 of the so-called place cells, neurons that generate an action potential when an animal passes through a specific portion of the environment [299]. Subsequent studies have demonstrated the presence of other cells dedicated to the coding of different aspects of space, through the final creation of a cognitive map composed of associated areas that form a system dedicated to space navigation such as grid cells and [300, 301]. Numerous animal studies suggest a possible role of the hippocampus in the regulation of anxiety, as it is part of the Behaviour Inhibition System (BIS). It is argued that the hippocampal volume is somehow correlated with the cognitive and affective dimensions of the anxiety index [302].

It has been known for several years that the hippocampus is one of the areas in which adult neurogenesis occurs. This event attracted a lot of attention because it appears to be involved in cognitive functions, memory processes and behaviour regulation. Adult neurogenesis has so far been extensively described and characterized in animal models such as rodents, but it is also supposed to occur in humans whose study on the actual functional role is limited to the analysis of post-mortem samples. For this reason, it is still unclear how this phenomenon can participate in normal brain functions and how dysregulation can lead to the onset of diseases in humans [303, 304]. The proliferation of new neurons occurs in an area of the DG called the subgranular zone (SGZ), a term introduced by Altman in 1975 to indicate a thin strip of tissue located between the granular layer and the hilum in the DG [305]. SGZ possesses a microenvironment conducive to neuronal proliferation which is called neurogenic niche. The neurogenic niche consists of precursor cells, immature neurons, glial and endothelial cells, immune system cells, microglia, macrophages, and extracellular matrix proteins (**Fig. 7**) [306]. The proliferative surge generates only one type of neuron in the hippocampus, the granular DG cell. To date, there is no certain evidence that other neuronal types are generated under physiological conditions, although some researchers have reported many results about this aspect [307, 308]. Astrocytes appear to play a central role in promoting neurogenesis and, *in vivo*, a close interdependence between astrocytes and precursor cells has been observed. The SGZ receives various synaptic inputs from different brain areas such as the septal nuclei of the raphe nuclei and local inputs from interneurons of the DG and commissural fibres from the contralateral hippocampus [309, 310]. The role of interneurons actually remains unclear. What is certain is that GABA and inhibitory signals seem essential to stimulate the proliferation of the neurogenic niche [303]. Adult neurogenesis can be divided into four phases: a precursor cell phase which has the task of keeping the progenitor cell pool active, an early survival phase which determines the exit of the cells from the cell cycle, a postmitotic maturation phase in which they develop cytoplasmic extensions and establish connections and, and a late survival phase in which the cells establish themselves in their final location.[303]. It is estimated that the entire neurogenesis process takes about 7 weeks to be completed.

At each stage of proliferation, cells acquire different phenotypes and express different protein markers. In the first proliferative phase, precursor cells with radial glia characteristics (also called glia-like type I cells) divide, generating intermediate type II progenitors. Type I cells are stem cells that have an unlimited proliferative capacity and during conversion into transient amplifying cells (type II cells) they acquire various neuronal markers such as nestin, NeuroD1, Prox1 (specific transcription factor expressed by granular cells in progress development), doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM). Type II cells receive GABAergic inputs from local interneurons which in this phase control both the maintenance of radial glial cell quiescence and the proliferation of progenitors when activated. During the phase following cell division, cells acquire postmitotic markers such as the splicing factor NeuN and the calcium-binding protein calretinin. During this phase, the only input remains the GABAergic one and it remains excitatory. The neuronal progenitors in this phase begin to generate cytoplasmic extensions but many of them are eliminated by apoptosis before reaching the final target in the CA3 area. The subsequent postmitotic phase is characterized by the completion of the formation of dendrites and axons. Simultaneously with the development of the dendrites, there is also the appearance of dendritic spines. This phase occurs about 10 days after the start of the proliferation of radial glia cells. In the final stages, the maturation of the dendritic spines and the increase in synaptic plasticity occurs. The axons or mossy fibres reach the cells of the CA3 and here they establish synapses that will then take part in the hippocampal circuit. Mature neurons express proteins such as calbindin and the transcription factor NeuroD1 and become glutamatergic [303].

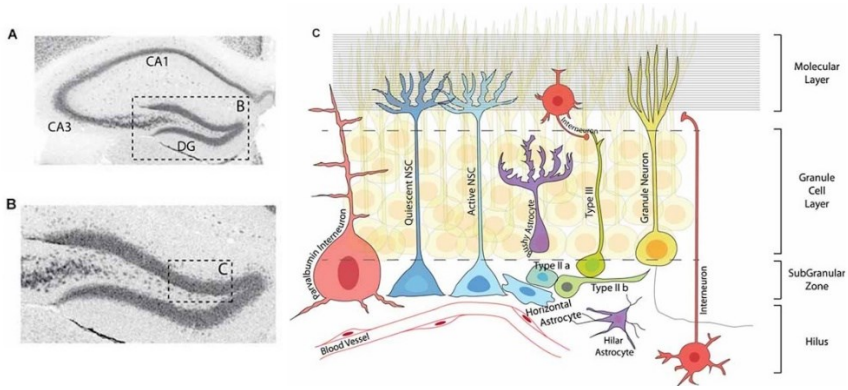


Figure 7. The microenvironment of hippocampal adult neurogenesis [311].

The process of adult neurogenesis in the hippocampus seems to be regulated by environmental stimuli and by the psychological state of the individual both positively and negatively. An environment rich in stimuli, e.g. open spaces, new objects, physical activity seems to significantly increase the number of neurons generated during the adult phase and to increase spatial learning in rodents [304, 312]. For example, optogenetic studies conducted on mice have shown how silencing the

activity of the DG in the presence of a stimulating environment inhibits the proliferation of immature neurons [313]. Another interesting aspect is that an environment rich in stimuli can modify the connections of the new neurons in the DG [314]. On the contrary, stress and ageing reduce adult neurogenesis through the stimulation of corticosteroids. Traumatic and adverse experiences during young age also seem to have effects on adult individuals, probably through epigenetic modifications that trigger a slowdown in neuronal proliferation [304, 315]. Also, it has been observed that hormonal stimulation, as operated for example by estrogen and thyroid hormones, can impact neurogenesis in adults and is involved in the phenomenon of synaptic plasticity and discrimination between different stimuli, creating recognition maps within the hippocampus. The role of adult neurogenesis in the formation of a recognition pattern derives from behavioural studies conducted on knockdown animals for neurogenesis in which the ability to discriminate between very similar fear stimuli was assessed [316, 317]. The newly generated neurons appear to enhance these discriminatory abilities and increase the performance of these animals. Furthermore, it has been proposed the involvement of adult neurogenesis in learning and memory albeit with controversial and distant results [304].

1.5 Pathologies induced or correlated to oxidative stress and inflammation

1.5.1 Prolidase Deficiency (PD)

Proline is an amino acid belonging to the nonpolar cyclic R group. The secondary amino group in the pyrrolidine ring, also called imino group, confers an intrinsic structural rigidity to the polypeptide chain in which it is present. This peculiarity translates in several important biological and structural functions in the molecules in which it is contained, such as cytokines, growth factors, neuro and vasoactive peptides [318, 319]. Furthermore, proline is a precursor of glutamate, a molecule that acts both as an excitatory neurotransmitter in the CNS, and as an amino acid in all other tissues. A specific transporter is located on the membrane of neurons belonging to the glutamatergic pathway, which allows the accumulation of this neurotransmitter in synaptosomes with a Na^+/Cl^- dependent mechanism [320, 321].

Prolidase

Prolidase or Peptidase D (PEPD, EC 3.4.13.9) is a ubiquitous manganese-dependent metalloenzyme isolated from Archaea, Bacteria and Eukarya. In Bacteria and Archaea, it might be involved in protein recycling alongside other peptidases. On the contrary, the primary role of prolidase in humans is well known. It is a cytosolic enzyme able to selectively cleave iminodipeptides containing C-terminal proline or hydroxyproline residues. It works with an optimum activity between pH 6.0 and 8.0 and 37-50°C dependent on the species [322–325]. The crystallographic structure of prolidase was first studied by Maher et al in *Pyrococcus furiosus* and was classified as a pita-bread fold protein due to the close structural similarity with other peptidases

such as aminopeptidase P, creatinase and methionine aminopeptidase. This conformation is made of a pair of domains with two α-helices and an antiparallel β-sheet that create a deep cavity between the two layers where the bond and the bimetallic catalytic centre are located [326, 327]. In humans, prolidase is an Mn (II)-dependent homodimer consisting of 493 amino acids polypeptides. In each monomer, two domains can be identified: a N-terminal domain from 1 to 184 residues and a C-terminal domain comprises between 185 and 493 residues and the latter containing the pita-bread fold (**Fig.8**) [328]. X-ray studies have demonstrated that two Mn (II) ions per subunit are required for the complete catalytical activity of the enzyme [329, 330].
Enzymatic purification assays conducted on erythrocytes, leukocytes and fibroblasts isolated from mice and humans lead to identifying two isoforms of prolidase, PDI and PDII [331]. The two isoenzymes differ in molecular weight, substrate specificity, response to manganese ion, and heat [332, 333]. In more detail, the PDI isoform has a molecular weight of 56 kDa in contrast to the 95 kDa of the PDII enzyme (data from SDS-PAGE). Regarding the substrate affinity, in rats, PDI had shown a high activity towards Serine-Proline (Ser-Pro) and Alanine-Proline (Ala-Pro) dimers, while the analysis of human PDI had shown a maximal activity ratio towards residues of Glycine-Proline (Gly-Pro). PDII, on the other hand, was found to be much more active against Methionine-Proline (Met-Pro) dimers than the other residues with a decrease in the activity of about 20%. The two isoforms are expressed in almost all organs while maintaining a different rate of activity. Indeed, the catalytic activity of PDI is high in the renal cortex (with an activity twice as high as in the medulla) and small intestine, while PDII does not show significant differences between the various organs [334–336].

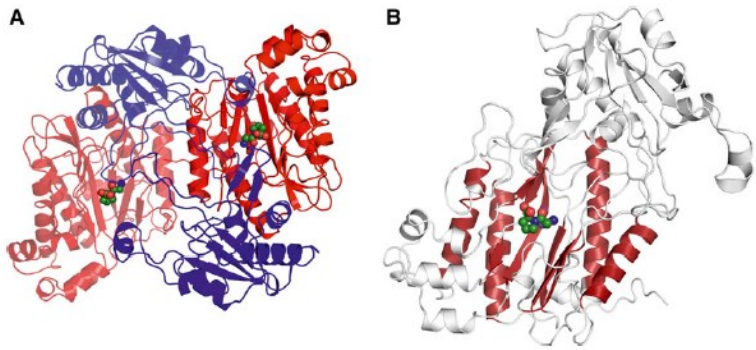


Figure 8. Human prolidase structure [328].

In the brain, prolidase is significantly functional in all areas, with the highest levels in the cerebellum. The enzymatic activity is detectable in all brain regions while remaining lower than in other tissues such as the kidney. Furthermore, it has been shown that in specific brain regions the activity of the enzyme is higher in young rats

(28 days) than in adult mice and it is concentrated more in cortical areas [337]. In detail, a study found that PDI activity in the cerebellum is higher than in other areas, while PDII is more catalytically active in the cerebellum and hippocampus than in other brain regions [336].

The enzymatic activity of the prolidase undergoes changes during pre-and postnatal development: PDI reaches a positive peak two days before birth, then decreases immediately after birth to go back up to a plateau at P21. The PDII curve also shows perinatal changes considerably similar to those of PDI [336, 337].
As previously reported, prolidase is an enzyme widely distributed in organisms and tissues and is involved in numerous functions. Given its principal role in the hydrolysis of dipeptides, it actively participates in the last steps of the catabolism of both endogenous and dietary proteins, particularly rich in proline residues such as collagen. Collagen is the main component of the ECM and represents about a third of the total body proteins with the greatest number of Gly-Pro imino acid residues. Cells are in close contact with the ECM thanks to the presence of integrins, a family of membrane proteins that participate in gene regulation, cell differentiation and growth.
The degradation of collagen involves MMPs that split the protein into oligopeptides which are cleaved into tripeptides and dipeptides by endonucleases and exonucleases, including prolidase. Therefore, this enzyme plays a fundamental role in the turnover not only of collagen but also of the ECM [338]. Excessive collagen turnover is in close correlation with a high activity of the prolidase characterizing many disorders and pathologies.
Proline acts as a substrate for stress and participates in metabolic signalling mechanisms. The degradation of collagen typically occurs during stressful metabolic conditions such as insufficient blood supply during tissue damage [338].
High activity of the prolidase has been observed in hypertension and erectile dysfunction in which collagen deposits in the walls of blood vessels have been observed [339–341]. The close correlation between prolidase activity and collagen turnover is supported by several studies conducted for example on human fibroblasts, during induced cell ageing, in Osteogenesis Imperfecta and numerous other highly malignant pathologies [342–347].

Prolidase deficiency (PD)

Prolidase Deficiency (PD, OMIM 170100) is a rare disease caused by several types of mutations in the PEPD gene (**Fig. 9**). This gene is located, in humans, on chromosome 19 in position q13.11 spanning 134 kb; it contains 15 exons and it transcribes an mRNA of 2-3 kb [348]. It is an autosomal recessive pathology with an incidence, probably underestimated, of 1-2 cases in 1,000,000 people [323, 349]. Over the years, fewer than a hundred cases have been diagnosed with PD. They belong to different geographical and ethnic backgrounds [350–352] and, from several studies, it seems to be more frequent in some Israeli populations [353, 351, 354]. A recent paper reported an epidemiological update resulting from the analysis of data published in PubMed and Human Gene Mutation Database Professional (HGMD®,

Qiagen, Aarhus C, Denmark). From this study, 75 patients underwent molecular analysis of the PEPD gene, of which 34 were males and 37 females with ages ranging from 3 months to 47 years (the gender of 4 patients is not available). 8 patients with PD died between two months and 36 years of age and, for two families, prenatal analyses were performed [348, 351, 352, 355–357]. However, since the actual number of patients is very limited, it is not possible to identify affected regions or geographic areas.

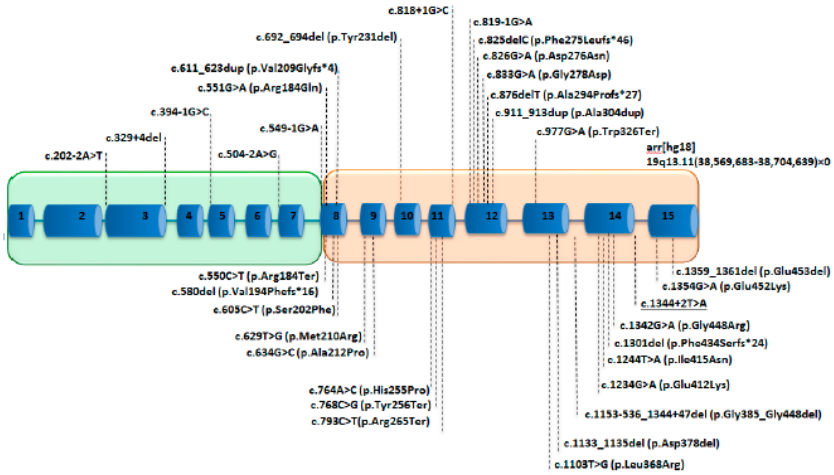


Figure 9. Schematic representation of the PEPD gene [358].

In all the patients considered 35 mutations, principally in C-terminal encoding region, were found: 16 missense/nonsense variants, 9 splice variants, 9 insertions/deletions and 1 copy number variation. In the N-terminal domain, a total of seven splicing variants were observed; the 27 other variants localized in the C-terminal domain, and a large deletion encompassed the entire PEPD gene [350, 358]. Additionally, an in-silico study revealed 85 single nucleotide polymorphisms and, by high-resolution analysis of eight single amino acid variants, four inactivation mechanisms were hypothesized: i) disruption of the catalytic MnII (OH^-) centre, ii) introduction of chain disorder along with the displacement of important active site residues, iii) rigidification, iv) flexibilization of the active site but the correlation between the variants type, enzymatic deficiency and clinical signs has not yet been investigated [325, 352, 359]. The age of onset and the severity of the pathology show interfamilial variability. PD could affect individuals during early postnatal days or in adult life starting under 22 years. Most of the patients develop symptoms before 10 years despite a case of PD appearing around 30 years old has been detected [351, 352, 360, 361]. The subjects present various degree of clinical phenotypes but some symptoms appear to be in common among patients. Particularly, recurrent leg ulcers, respiratory tract infections, facial dimorphism and a mild to moderate types of mental retardation are present. Among all the PD patients known, 61% of individuals have cutaneous

lesions. They are chronic, recurrent, irregular, bilateral, painful, and mainly located on the lower limbs. The ulcers appear early in childhood and are not always considered the first symptoms appearing; they are usually preceded by skin rashes, telangiectasia, photosensitivity, impetigo, eczematous lesions, and necrotic papules during the lifetime. Additionally, skin lesions are often present in PD patients, but their absence does not exclude the pathology. These lesions are intractable and, in the most severe cases, lead to extremities amputation [80, 358]. Respiratory infections and pneumonia are present in 76% of patients and are considered as major complications for PD, which can compromise the survival [351, 362, 363]. In some cases, pulmonary fibrosis, cytoarchitectural impairment and collagen deposition were found as well [364, 365]. Facial dysmorphism is frequent in PD individuals (among 93%) which present low hairline, hirsutism, saddle nose, hypertelorism, micrognathia and mandibular protrusion [80, 351]. A percentage of 71 subjects show developmental delay or intellectual disability (from moderate to severe) with motor and speech impairment [351, 355, 360]. Furthermore, in a four-year-old PD child, slight cortical and cerebellar atrophy were detected [366]. Anaemia and immunological disorder have been reported in 76% and 64% of patients, respectively. Particularly, high serum levels of IgE are detected in parallel with elevated expression of mRNA belonging to the inflammatory pathway such as IL-23 and TNF α [352, 367–370]. In addition, Systemic Lupus Erythematosus (SLE) and SLE-like syndromes occur in 26% of individuals [360, 354, 356, 371].

To date, there is no definitive treatment for this pathology and numerous approaches are used to alleviate the dermatological symptoms. For example, the oral supplementation of ascorbic acid or glycine/proline is used to enhance collagen metabolism, manganese chloride for improving prolidase activity and stability and corticosteroids or antihistaminic to decrease immunological reactions [358, 372, 371]. In addition, topical application of proline or glycine ointment seems to give relief to PD patients [369, 370, 373]. Blood transfusion and allogeneic hematopoietic stem cell transplantation improve ulcers quality and prolidase activity but unfortunately are not resolutive and patients die from secondary infections [355, 374–377]. Other different approaches have also been considered, i.e. Mg^{2+} activated erythrocytes transfusion, adenovirus-mediated gene transfer, liposome-encapsulated prolidase and pharmacological chaperons [378–381].

Prolidase and oxidative stress

Several studies have shown a strong interconnection between oxidative stress and prolidase activity in different diseases such as diabetic neuropathy and pulmonary pathologies, cancer, gastric disease, cognitive impairment, and different types of intellectual disability [382–387]. In the brain, proline and its peptides play a major role and the increases of prolidase levels directly elevate proline concentration [388]. Several studies affirm that NO is a powerful regulator of the activity of prolidase because it is involved in the regulation of the activity [79, 389]. Furthermore, the level of oxidative stress is directly related to the inhibition of

collagen synthesis. Recent studies in rats have shown that proline induces oxidative stress in the brain of rats and therefore can be involved in neurological dysfunctions [2]. Recent reports suggest a possible involvement of oxidative stress in the regulation of prolidase in patients with depressive disorders [3]. Selek and collaborators investigate the role of prolidase levels in patients with bipolar affective disorder and found that the serum enzyme level was significantly higher in affected patients [388]. These findings support the idea that prolidase activity may play a role in psychiatric disorders. In another article, the author investigates the role of prolidase activity in patients with dementia. The results obtained showed an increase in the activity of the prolidase and the intracerebral release of proline seems to selectively alter the formation of memory while in a further study researchers demonstrated that elevated proline levels activate glutamate NMDA receptors, leading to glutamate excitotoxicity and neurological dysfunction [4, 390].

Prolidase and hippocampus

To our knowledge, in literature, there are no studies concerning the effects of prolidase deficiency in the hippocampus. The only brain area that has been investigated in PD models is the cerebellum, an organ in which an alteration of the organization of the ECM and neuronal migration has been demonstrated during the postnatal development. Furthermore, in this work, dysregulation in the expression of some calcium-binding proteins such as parvalbumin and calbindin and consequent impairment in the expression of some markers of the glutamatergic and GABAergic pathway was demonstrated [1, 391]. Over the years, however, the role of different peptidases in hippocampal formation has been investigated because proline is a highly expressed amino acid in the brain and a precursor of glutamate. For example, an *in vivo* and *in vitro* study in rats showed that elevated proline levels reduce the reabsorption of glutamate in sections of the hippocampus and cerebral cortex. A high concentration of proline also seems to influence the activity of the Na^+/K^+ ATPase pump, leading to various brain dysfunctions [392]. Furthermore, another study has shown that chronic exposure to proline leads to an increase of membrane phospholipids, in particular gangliosides, in the hippocampus and cortex suggesting that these modifications could lead to neurological and cognitive dysfunctions found for example in patients with hyperprolinemia [393]. The concentrations of proline normally present in the human brain seem to be implicated in the enhancement of the transmission of Schaffer collateral fibres on CA1 pyramidal neurons in the rat hippocampus. It has been shown that proline acts by enhancing this circuit, thus not impacting the release of glutamate from the presynaptic terminals, but rather acting at the postsynaptic level. Proline acts by inhibiting the release of glutamate in physiological conditions and this, once again, could cause cognitive dysfunctions in the presence of pathologies in which this molecule is involved [4].

1.5.2 The ageing model

Ageing is a physiological process that produces changes in an organism, with a variable rate from individual to individual [394]. These modifications are very different from each other and, in some cases, do not affect the quality of life of the individual. Ageing, however, is also characterized by an increased risk of the onset of diseases, disability, or death and for this reason, the ageing process is often referred to as senescence [5]. Senescence represents the progressive deterioration of the body that causes the loss of complete organ function and tissue integrity, thus increasing the risk of mortality for individuals. These effects often lead to hospitalization of persons and hence the need to develop a universal term that describes the clinical symptoms of ageing which today are referred to as frailty.
Frailty in the elderly can be diagnosed based on the following criteria developed by Fried and collaborators in 2001: i) low grip strength, ii) lowering of energy, iii) slowing of the speed of movements, iv) low physical activity and v) involuntary weight loss [395]. Furthermore, frailty is often identified through the assessment of the frailty index (FI) considering the number of accumulated deficits in the individual including disability, physical and cognitive decline, and previous pathologies [396]. To date, the concept of frailty is still in evolution and there is not a universal definition of this condition. Recently, the relationship between physical weakness and cognitive deterioration is well documented in the literature. During the last years, the term "cognitive frailty" was coined and indicates a "heterogeneous clinical manifestation characterized by the simultaneous presence of both physical frailty and cognitive impairment". Two main aspects must be detected to define this condition: the presence of physical frailty and cognitive impairment; and exclusion of concurrent AD dementia or other types of dementia [397, 398]. Over the years, numerous theories have been proposed to better understand the bases of ageing which can be classified into two broad categories: the theory of programmed ageing and the theory of ageing due to the accumulation of errors within the cell. The cause of ageing has often been attributed to damage induced by ROS, a decline in the immune system, a shortening of telomeres and the presence of senescence genes in DNA. To date, scientists agree that it is a multifactorial mechanism that includes multiple interdependent and interactive processes [5]. An accumulation of damage or a chronic oxidative state can affect all organs and contribute to the onset of neurodegenerative diseases, aggravating the geriatric frailty of an individual [399, 400]. At the end of the early 2000s, about 8% of the world population was 65 years old and it is estimated that by 2050, 1.5 billion people aged over 65 years old will live in the world, about 16% of the entire population. The constant increase in the average age and life expectancy has led to a higher incidence of neurodegenerative diseases associated with ageing and has therefore made it necessary to study mechanisms that improve the quality of life of patients to age healthily. Neurodegenerative diseases are primarily age-related disorders characterized by memory loss, cognitive dysfunction, neuronal damage, and death. The *Diagnostic and Statistical Manual of Mental Disorders* (DSM)-IV defines dementia as a condition characterized by memory loss and progressive cognitive decline that can involve motor, linguistic or executive

functions [401, 402]. Cognitive and dementia syndromes can be reversible, such as in cases of behavioural disturbances or transient brain injury, or irreversible such as Alzheimer's syndrome, Parkinson's disease, vascular dementia, head trauma dementia and dementia due to multiple aetiologies [401]. Thanks to the discovery of new molecular mechanisms underlying cellular functioning, the study of ageing has found great success in the scientific community. The first study related to ageing date back to 1939 when it was observed that a reduced caloric intake increased the lifespan of mice and rats [403]. More recently, investigations conducted on primates show how, alongside the increase in lifespan, caloric restriction seems to delay ageing and the onset of age-related diseases [404–407]. In the mid-1900s, it was proposed the theory that ageing is the result of negative natural selection and the lifespan has a genetic basis. In those years, studies on *Drosophila* and *C. elegans* were conducted and a gene, called *age-1*, implicated in the lifespan of organisms, was identified [408–410].
Over the past 30 years, together with genetic studies, phenotypic characteristics associated with ageing have also been identified and complex interaction of intracellular signals were revealed [411].

Ageing in hippocampus

Ageing has a great impact on the hippocampus because it inhibits adult neurogenesis in rodents, primates, and humans. The reduction of neuronal proliferation following ageing could lead to a reduction in synaptic plasticity which is highly dependent on the neurons produced by progenitor cells. Even the cognitive abilities related to the hippocampus lose effectiveness during ageing, although it is not yet clear which is the role of adult neurogenesis in the regulation of cognitive abilities, at least in humans [6–8]. Furthermore, during ageing, there is an increase in gliogenesis, probably due to a change in the fate of neural progenitor cells [412, 413]. It has been observed that these cells after a certain number of cell divisions are no longer able to generate neurons but rather generate astrocytes and glial cells. In any case, several studies seem to show that even in models of ageing the positive stimuli of the environment such as physical activity or new spaces stimulate the resumption of the proliferative wave again suggesting that cognitive decline can be reversed by stimulating adult neurogenesis in the hippocampus. Not only the external environment but also the tissue microenvironment seems to intervene in the relationship between ageing and adult neurogenesis. For example, the presence of some chemokines such as CCL11 typical of ageing seems to decrease adult neurogenesis and contribute to the loss of hippocampus-dependent spatial learning [304].

Ageing and oxidative stress

The ROS particles accumulate in the CNS during ageing and very often several studies have been carried out on antioxidant molecules to reduce the onset of age-related neurodegenerative diseases. Therefore, ROS are often associated with a

negative idea aimed at triggering inflammation and a pathological state. In a broader view, it has been proposed that ROS trigger inflammation in those cases in which the repair of damage must be activated and therefore are associated with a positive aspect [414, 415]. The response of antioxidant enzymes often draws cells with phagocytic capacities such as macrophages or microglia into the CNS. In a work, by Little and collaborators it was shown that neurodegeneration is present even in the absence of inflammation and that the induction of inflammation by an inflammatory response does not induce neurodegeneration [416]. This could suggest that glial activation in response to damage during ageing may serve as a neurotrophic rather than degenerative factor. Recent studies have also observed a correlation between glial fibrillar acid protein (GFAP) expression and the onset of diseases. Therefore, the activation of astrocytes and other glial components in the brain of elderly people could promote neuronal plasticity and the restoration of tissue homeostasis [417, 418]. In support of this hypothesis, there is also the fact that astrogliosis is a highly conserved event during evolution, suggesting a positive effect of this cellular response in the survival of individuals [419].

Inflammaging

One of the main changes that occurs during ageing is the impairment of the immune response, which can lead to a chronic systemic inflammatory state that could be widely and deeply involved in several age-related chronic diseases, such as atherosclerosis, diabetes, obesity, sarcopenia, Alzheimer's disease and geriatric frailty. Furthermore, a recent study conducted a cohort of subjects over a hundred years old, showed that the inflammatory state is able to successfully reveal the possibility of ageing up to a considerably advanced age [420]. Additional evidence indicates that an increase in systemic inflammation is strongly correlated with ageing and with age-related chronic diseases [421, 422]. The systemic onset of a chronic inflammatory state with characteristics that differ from the acute condition, due to the high and sustained levels of pro-inflammatory mediators and an aberrant increase in the migration phenomena of pro-inflammatory factors in various tissues occurs during ageing. The macrophage infiltrate induces the production of various pro-inflammatory cytokines which act both locally and, as in the case of adipose tissue, at a systemic level [423].

One of the principal factors involved in the immune response during age-related inflammation is the nuclear factor (NF) κB. Several studies have shown an increase in the expression of pro-inflammatory genes in response to the age-related NF-κB activity such as (i) Tumour Necrosis Factor (TNF) - α / β; (ii) different interleukins (IL-1β, IL-2, IL-6, IL-13, IL-18); (iii) chemokines (IL-8); (iv) adhesion molecules (AMs), which in physiological conditions are usually present at low concentrations, while during ageing undergo an up-regulation probably due to the increase in oxidative stress and vascular pathologies [424–433]. In particular, a continuous dysregulation of IL-6 synthesis leads to a state of chronic inflammation and autoimmunity [434]. Furthermore, numerous evidence indicate that ageing and chronic inflammation are closely associated with the increase in the expression of

Toll-Like Receptors (TLRs), a family of receptors able to recognize specific patterns and involved in the first phase of the innate immune response; the expression of TLR5 and the TLR5-induced the production of IL-8 that is higher in monocytes of elderly subjects compared to younger individuals [435, 436]. In another clinical study, an elevated expression of TLR-4 was observed in the muscle tissue of elderly subjects, in association with loss of muscle tone and reduced insulin sensitivity [437]. Several evidence denote a significant role in ageing and age-related inflammatory diseases of a particular group of microRNAs (miRNAs), called inflamma-miRs (miR-155, miR-21 and miR146a), capable of modulating specific pathways of signalling (NF-κB, SIRT and TGF-β) related to inflammation, senescence and age-related diseases [438, 439].

The inflammatory response, both acute and chronic, involves coordinated humoral and cellular responses. The cellular response first recruits macrophages and subsequently leukocytes and lymphocytes. At the same time, the production of soluble mediators, proteins, interleukins, and cytokines is also triggered. ROS and RNS are involved in the formation of chronic inflammation (**Fig.10**). In elderly, the mechanisms that regulate inflammation are altered, leading the elderly to be more vulnerable to age-related infections and chronic diseases such as cardiovascular disease, type 2 diabetes, and dementia. This impairment is partly due to the observation of an increase in circulating pro-inflammatory cytokines even in the absence of infections, suggesting the presence of a chronic and latent age-related inflammation. This basal inflammatory state induces tissue damage which in turn leads to greater inflammation, creating a vicious circle that predisposes to age-related diseases [433, 440, 441]. The overproduction of ROS is at the basis of severe inflammatory processes while, on the contrary, NO has a dual function. Indeed, NO is not only a positive factor involved in the paracrine signalling but also an essential messenger involved in the permeability of the vascular endothelium. However, the result of an accumulation of ROS and RNS is also to be correlated to the microenvironment in which these reactive species accumulate [425, 442].

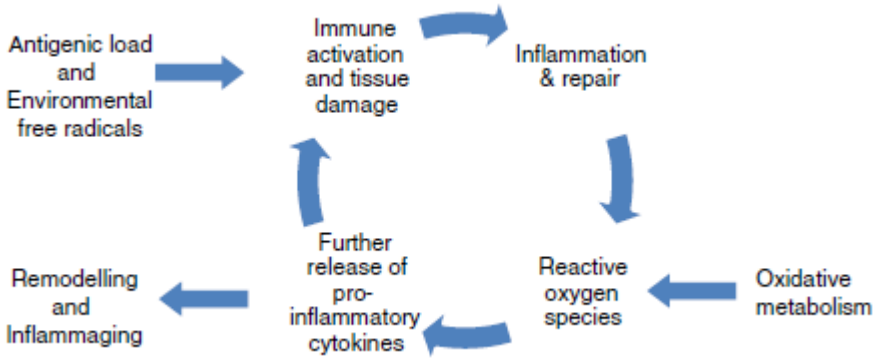


Figure 10. The inflammaging reactions [441].

Medicinal therapies

The classic therapies, typically used in the treatment of the cognitive decline and dysfunctions that affect patients, are often ineffective and involve a high number of side effects, guaranteeing only a short-term reduction in the progression of the disease. Recently, many herbal medicines have been used as a coadjuvant of canonical treatment to improve the patient's quality of life. For example, the dietary supplementation with the so-called "superfoods" or treatments with extracts of medicinal plants and mushrooms with potentially positive effects on the bases of their nutritional properties could be beneficial [443]. In Europe, the use of vegetable raw materials for pharmaceutical use are regulated by the *European Medicines Agency* (EMA). In Italy, the Legislative Decree of 24 April 2006, n. 219, in implementation of the European directive EU 2004/24 / EC, defines "medicinal product of plant origin or phytotherapic" any medicine that contains exclusively as active substances one or more plant substances or one or more plant preparations, or one or more plant substances in association with one or more herbal preparations.
Regarding phytotherapy, for example, *Taraxacum officinale* extracts have long been used in traditional oriental medicine thanks to its diuretic, antiangiogenic, antirheumatic, antidepressant and anti-inflammatory effects [444–446].

Mushrooms have also been traditionally used for millennia for their beneficial properties in foods and medicines, but only in recent years researchers have started to study the molecular mechanisms with positive effects of their constituents. According to the above-mentioned definition, even "medicinal mushrooms (MM)" can be considered "medicinal plants" [447]. For the mycotherapy, many mushrooms have been considered for these purposes such as *Agaricus subrufescens*, *Ganoderma sichuanense*, *Grifola frondosa*, *Lentinula edodes*, *Phellinus linteus*, *Pleurotus ostreatus* and *Polyporus umbellatus* [448, 449]. From this evidence and the well-known anti-inflammatory properties, MM has been used to reduce inflammation typic of many age-related chronic diseases, including neurodegenerative syndromes and dementias [450]. MM has been also used as adjuvants in the treatment of various tumour pathologies, chronic viral infections from hepatitis B, C and D, different types of anaemia, in AIDS patients, and individuals suffering for Alzheimer's disease [451]. These positive effects have therefore generated great interest in analysing the mechanisms underlying the properties of these organisms. Furthermore, many studies have shown that mushrooms have no side effects and present antimicrobial properties useful to overcome antibiotic resistance in human pathogenic microorganisms [449, 452–455]. MM have also neuroprotective and anticonvulsant properties, as emerged from an *in vivo* study on the mycelium of *Ganoderma lucidum* that induced the inhibition of kainic acid-induced convulsions (KA) in rats, reducing CA3 degeneration process, glial reactivity and KA-induced expression of IL-1β and TNF-α [456]. A subsequent *in vitro* study showed that the extract of *Lignosus rhinocerotis* can stimulate neuritogenic activity in rat pheochromocytoma (PC12) cells, comparable to that of NGF (50 ng / mL) [457]. Additionally, another study found that treatment with cordycepin, a bioactive component of the fungus *Cordyceps*

militarisa, attenuates lipopolysaccharide-induced microglial activation and significantly down-regulates iNOS mRNA levels and COX2, thus generating a neuroprotective activity [458]. Although numerous studies have already supported the effects of MM on brain and cognition, further experimental and clinical evidence is needed, to elucidate the cellular mechanisms through which these mushroom act [459]. Among the most known and studied mushroom for adjuvant treatment in conventional therapies are *Hericium erinaceus*, and *Ganoderma lucidum*.

Hericium erinaceus

Hericium erinaceus (Bull.) Pers. 1797, also known as "Lion's Mane" or Yamabushitake, is one of the most known edible MM that has long been used in traditional Chinese medicine but it has been firstly identified in North America. The fungus is also detectable in Europe, Australia but, it was not found in Africa and, surprisingly, in Asia, where the only source of this mushroom is by cultivation [460]. This mushroom is not commonly easy to find in nature and since 2003 it is included in the European species at risk of extinction due to the increasing disappearance of its habitat. *H. erinaceus* is a basidiomycete of the Hericiaceae family, order Russulales and class Agaricomycetes. It can be easily individuated by the extended basidiomes organized in several long and hanging spines which are white in the early stage of life, becoming yellow and then brown over time. Furthermore, the hymenophores could appear branched or unbranched with different length of the spines. Microscopically, the presence of amyloid-reach basidiospores helped to identify the species [461, 462]. However, it is possible to discriminate it from the other closest mushrooms by Polymerase Chain Reaction (PCR) analysis using primers specific for *H. erinaceus* [463, 464]. *H. erinaceus* is classified as a saprotroph and a weak parasite; it grows mostly on dead trees, but sometimes the fruiting body can protrude from a hole in the bark [449, 465] (**Fig. 11**). Recently, it has been shown that *H. erinaceus* also grows on plants traditionally used as medicinal herbs. Hence the idea that the mushroom is a "bio transformer" of active material, acquiring and using it for its beneficial properties. An example can be given by a molecule present in the *Artemisia capillaries* plant, the scoparone, which has beneficial properties in the kidneys of murine models. An extract of *H. erinaceus* grown on this plant can protect the gastric mucosa from damage, prevent lipid deposition in the liver and exert antioxidant properties *in vivo* studies. It is not clear whether the effects of this extract are due to the fungus itself or to the acquisition of particular molecules from the plant on which it was grown and further investigations on this topic will certainly need to better explain these properties.

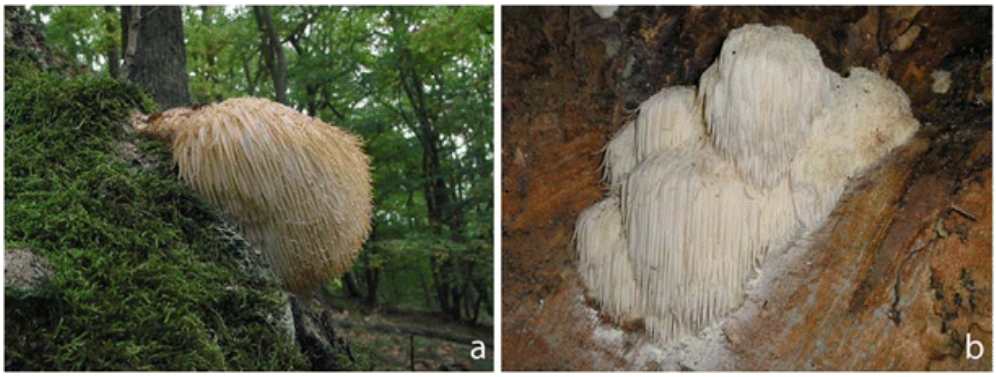


Figure 11. The macroscopical structure of *Hericium erinaceus* [462].

The mushroom produces several bioactive molecules that have been isolated and deeply studied for potential pharmaceutical applications. The bioactive compounds from *H. erinaceus* are classified in high molecular weight, such as polysaccharides, and low molecular weight, such as terpenoids [449, 466]. Considering the high molecular weight polysaccharide compounds, they are mainly found in the cell wall of fungi and represent about 20% of the biomass of the fruiting body and mycelium of these organisms [449, 467, 468]. The main isolated molecules have two properties: anticancer and immune system activators. Substances with anticancer effects have been isolated from the basidiomes of the fungus and are represented by xylans, glucoxylans and more complex molecules such as heteroxyloglucans. The biomolecules capable of attracting macrophages and triggering an immune response belong to the β-glucan family and have been isolated from the fruiting body and mycelium.[469, 470].

The low molecular weight molecules have been isolated from mycelium and fruiting bodies of *H. erinaceus*. Among the isolated molecules are erinacins, ericenones and erinacerins.
Regarding erinacerins, no significant data on their biological activities are reported in the literature [471]. Other substances have been isolated from fungal cultures and belong to the pyranones family. Among them, two molecules, erinapyrones A and B showed cytotoxicity in an *in vitro* study conducted on HeLa cells [462, 472].

Hericenones and erinacines are the most bioactive molecules isolated respectively from the fruiting bodies and the mycelia of the mushrooms. Erinacins are the main components extracted from the mycelium of *H. erinaceus*. 15 erinacins have been identified (erinacine A-K and P-S) and different studies have shown that 8 of these have numerous biological activities, including some neuroprotective, enhancing the release of NGF or alleviating neuropathic pain [473]. Among this, Erinacin A (HE-A), Erinacin C (HE-C) belong to the class of diterpenoid cyathans, while Erinacin S (HE-S) to the class of sesterterpenes [474]. Despite their structural differences, all terpenes are synthesized from compounds with 5 carbon atoms (isoprene) dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). In mushrooms, the two isomers are synthesized from acetyl-CoA through the

mevalonate pathway. The condensation of DMAPP and IPP results in the formation of linear hydrocarbons of various lengths that undergo a cascade of dephosphorylation and cyclization by terpene synthase (or terpene cyclase). In this way, monoterpenes, sesquiterpenes, diterpenes, sesterpenes and triterpenes are formed [475, 476]. In literature, it is reported that, of all the active compounds contained in *H. erinaceus*, only HE-A has a confirmed pharmacological action on the Central Nervous System [473]. Indeed, it has been shown that HE-A induces not only the increase of Nerve Growth Factor (NGF) synthesis *in vitro* but is also able to increase the content of NGF and catecholamines in the *locus coeruleus* and hippocampus of the rat after administration (8 mg/kg of body mass) [477]. Besides, the administration of *H. erinaceus* mycelium enriched in HE-A may improve stress-induced depressive-like symptoms by reducing the concentration of monoaminergic neurotransmitters in the hippocampus and the plasma concentration of pro-inflammatory cytokines such as IL-6 and TNF-α. It was also observed that nuclear factor κB (NF-κB) and IκB levels in the cytosolic fraction of hippocampal tissue, which is reduced in animals subjected to immobilization stress protocol, are normalized in animals treated with H. erinaceus. NF-κB is believed to be a crucial transcription factor; it translocates to the nucleus where it promotes the transcription of a high number of genes that code for pro-inflammatory cytokines and the activation of inducible enzymes such as inducible nitric oxide synthase (iNOS) or cyclo-oxygenase 2 (COX2) [478, 479]. Moreover, the Ericerin molecule can inhibit the growth of tumour cells by inducing programmed cell death through inhibition of the serine-threonine dependent protein kinase AKT (also known as protein kinase B, PKB) [480]. AKT activation contributes to cell survival and growth through the regulation of *c-myc* [481]. More recently, two-month oral supplementation with H. erinaceus reversed the decline in recognition memory of frail mice. In particular, immunohistochemistry studies demonstrated increased expression of proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) in the hippocampus and cerebellum in mice treated with H. erinaceus thus suggesting positive effects on adult neurogenesis of this fungus [9].

1.5.3 Eating disorders

Eating disorders (ED) are serious psychiatric diseases characterized by an altered perception of hunger and appetite that leads to an unbalanced consumption of food, compromising, in the most extreme cases, physical and psychological health. Altered habits regarding body weight, body shape and lifestyle also contribute to the onset of these disorders. These pathologies arise primarily in adolescents and young adult women but, in recent years, it is well-known that men are also affected. Different aspects characterize these diseases in the two genders: women seem more focused on weight loss while men seem more towards fitness and muscles [482, 483]. ED could affect people of all ages, genders and geography [483].
According to the DSM V and the International Classification of Diseases (ICD-11), the category of Feeding and Eating Disorders includes anorexia nervosa (AN), bulimia nervosa (BN), binge-eating disorders (BED), pica, avoidant/restrictive food

intake disorders (ARFID) and rumination. The DSM also indicates certain sub-categories and other behaviours associated with nutrition such as purging disorder, atypical anorexia and bulimia nervosa and night eating syndrome [484]. All these pathologies seem to have a common thread such as calorie restriction, binge eating, excessive and sometimes extreme control of weight and body, induced vomiting, and uncontrolled intake of laxatives and diuretics. This evidence suggests that eating disorders are very complex pathologies that also involve the psychology of the subject and self-esteem [485–488]. About 70% of people with eating disorders have psychiatric comorbidities such as anxiety, mood disorders, neurodevelopmental disorders, personality disorders and problems with alcoholism or substance of abuse [489, 490]. Several studies also document a predisposition to the development of ED in diabetic patients [491, 492]. Likewise, it is supposed to be a correlation between autoimmune diseases and ED [493]. Along with psychological factors, in recent years there has been an increase in awareness that neurobiological aspects are also present in patients with ED. Genetic factors have been highlighted between 50% and 80% of cases at risk of ED. Very often subjects present alterations in eating habits that could not only be associated with psychiatric disorders but which necessarily must have biological bases in pathways of appetite regulation [494–496].

Before investigating the biological aspects that regulate food intake, it is interesting to point out the difference between hunger, appetite, and satiety. Hunger is a need mainly dictated by the body's homeostasis and is, therefore, to be considered as a physiological force that guides the individual in search of food. Appetite, on the other hand, is an aspect more related to satisfaction and psychological desire for a specific food, driven by various stimuli such as social factors or very restrictive diets. When the request for food is satisfied, a sense of satiety is established. All those factors contribute to the onset of ED and are attributable to specific brain areas [497]. The physiological aspect of hunger is regulated by areas belonging to the hypothalamus and the brainstem, two regions that integrate psychological stimuli from the cortical regions [498]. Specifically, food is introduced into the body through the mouth. The tongue perceives a certain taste and transmits a nervous impulse to the receptors present on it, the to cranial nerves up to the nucleus tractus solitarius in the bulb, and to the ventroposteromedial thalamic nucleus reaching finally the primary gustatory cortex which, in humans, includes the orbitofrontal cortex (OFC) and the anterior insula (AI). From here the impulse is transmitted to the central nucleus of the amygdala and subsequently to the lateral nucleus of the hypothalamus and to the dopaminergic neurons of the midbrain. The hypothalamus and the regions of which it is composed play central roles in the pathways of appetite: the lateral nucleus is considered the centre of feeding that triggers the initiation of movements that allow the search for food; the ventromedial nucleus plays a primary role in the control of satiety; the arcuate nucleus receives stimuli from hormones that are released in the gastrointestinal tract and from the adipose tissue to regulate the supply of food. In fact, the hypothalamus has receptors for neurotransmitters that carry information about: i) stomach-filling, ii) satiety signals from the blood nutrients, iii) signals from gastrointestinal hormones; iv) hormonal signals from adipose tissue and v) signals

from the cerebral cortex. The main molecules involved are orexigenic substances that stimulate feeding such as NPY, Orexin A and B, endorphins, galanin (Gal), glutamate and GABA, ghrelin and cortisol or molecules defined as anorexigenic that inhibit feeding such as leptin, serotonin, norepinephrine, insulin, and CCK. Some molecules such as ghrelin and CCK act in the short term in regulating the supply of food while other molecules such as leptin act by inhibiting the storage of fats and, directly in the hypothalamus, inhibiting the orexinergic neurons in favour of anorexinergic activity. In humans, food intake is more complex and can also take place in the absence of hunger for a mere decision dictated by the will. Various brain areas mainly intervene in this aspect, including the AI and OFC, amygdala, hippocampus, and striatum. These areas are responsible for integrating physiological stimuli with stimuli such as the pleasure of eating, learning, reward, satisfaction [496, 499]. Regarding some epidemiological data, as mentioned above, adolescent, and young adults have a high risk to be affected by AN, BN and BED while adults are more frequently affected by BED [500, 501]. In recent years, the incidence of ED has increased by about 25% but only 20% of them are treated [483].

Anorexia nervosa

Anorexia nervosa (AN) is a mental disorder characterized by one of the highest mortality rates (between 5 and 15%). It is a disease characterized mainly by morbidity towards the control of body weight and an altered perception of the shape of one's body which lead to very restrictive low-calorie diet and significant weight loss. These people often have cognitive and psychological disorders [483, 502]. Individuals with AN can have above-average physical abilities and endure extreme weight loss. To ensure that they constantly lose weight, they develop an exaggerated passion for sports and physical training to burn calories, preferring to stand rather than sit. In addition, they practice self-induced vomiting and take substances such as laxatives and diuretics in sufficient quantities to ensure effective weight loss. Patients also develop an obsession with balance and fitness control to ensure that they are increasingly lean [503]. The aetiology of this disorder is unknown and probably multifactorial. It seems that various neurobiological, psychological, and environmental factors play a concomitant role in determining AN [495]. A genetic predisposition also seems to participate in the onset of this disease, which however is not a sufficient condition for developing the disease. Studies in twins and family members studies of affected and non-affected subjects of AN have reported that this pathology appears to be more present in families with obsessive compulsive, perfectionist and competitive behaviours [503]. Despite research into the genetic picture, the results have so far been inconclusive. As far as environmental factors are concerned, a distinction can be made between early environmental factors, i.e. those factors that interfere with the early stages of development of the central nervous system and with the maturation and programming of the stress response systems. The late factors are instead represented by all the relationships and events that are established from childhood to adulthood. The increase in AN in less developed countries could be associated with lifestyle changes, socio-cultural factors,

urbanization, globalization, and an attempt to adapt to more developed countries that often dictate fashions [502]. Despite numerous studies, research on environmental risk factors for AN has not been able to definitively determine why some individuals are more prone than others to environmental pressures towards thinness. In recent years it has been shown that hunger-induced by AN can be associated with profound alterations in the intestinal microbiota. The gut microbiota plays a role of considerable interest given its important interactions with the host metabolism in terms of weight regulation, hormonal, immunological and inflammatory processes, together with a direct influence on the brain and behaviour, being a key component gut-brain axis [504]. This pathology can affect all individuals of any sex, gender, and age although it is well documented to be prevalent in adolescent women and young adults no older than 30 years. In high-income countries, the prevalence of AN is about 1% in women and less than 0.5% in men. Furthermore, 46% of the population affected by AN manages to recover completely, a third shows partial remission and only in 20% of cases AN becomes a chronic disease [505, 506]. The rates of complete recovery and lowering of mortality are much higher in adolescents than in young adults [502]. The diagnostic criteria for the AN are: i) limited nutritional intake resulting in weight below the normal minimum for age and height; ii) presence of a strong fear of gaining weight, even when underweight; iii) altered body perception and evaluation that exaggeratedly affects self-esteem and denial of the severity of the current underweight condition. The DSM-5 classifies two subtypes of anorexia nervosa: restrictive and compulsive. The restrictive type is present in an individual who does not have recurrent binges or purging such as self-induced vomiting or misuse of laxatives, diuretics, or enemas. This subtype describes those cases in which weight loss is mainly achieved through diet, excessive exercise and/or fasting. The compulsive type is found in an individual who has recurrent binges or purging behaviours [507]. The DSM-5 also specifies the severity of the disease based on the Body Mass Index (BMI). The BMI of a normal weight person is between 18.5 and 24.9 kg / m2. Mild BMI ≥ 17 kg / m2; Moderate: BMI 16-16.99 kg / m2; Severe: BMI 15-15.99 kg / m2; Extreme: BMI <15 kg / m2 [508]. About three quarters of patients with an have suffered or suffer from mood disorders, often depression. Between 25 and 75% of anorexic patients developed anxiety disorders during childhood and obsessive-compulsive disorders were observed in 15-29% of cases. Between 9% and 25% of cases the presence of alcohol or substance dependence was found [502]. Over the years, several studies have been conducted to better understand the neurobiological basis of this pathology. First, it has been observed that anorexic patients have neurocognitive disorders and difficulties in processing emotions and relationships with society, show attention deficits, altered expressions and emotion regulation. Neuroimaging studies have shown a general reduction of white and grey matter which is more conspicuous in the hypothalamus and in brain regions related to reward such as the nuclei of the base and the sensorimotor cortex [502, 509]. Currently, in most cases, the treatments consist of nutritional rehabilitation and body weight restoration [495]. One of the first proposed approaches involves not only the individual but also the family unit and is called Family-Based Treatment (FBT). This

therapy constitutes an intervention model that integrates the different aspects of the cognitive-behavioural approach with those of the systemic-relational intervention. Family members must be included in the dietary education or meal planning of adolescent and young adults [510]. Two remedy models were proposed in a clinical trial: the Focal Psychodynamic Psychotherapy (FPT) and the Enhanced Cognitive Behaviour Therapy (CBT-E). FPT appears to be effective in healing within 1 year of the onset of the disease while CBT-E has more rapid effects on restoring body weight and improving eating behaviour [511]. Another psychotherapy with promising potential is represented by Specialist Supportive Clinical Management (SSCM), which has two distinct objectives: clinical management, which involves the alleviation of the AN symptoms, focusing in particular on increasing of weight through food consumption and a psychotherapeutic approach to support the problems identified by the patient as important. A very effective new therapy is the Maudsley-Anorexia Nervosa Treatment for Adults (MANTRA), an approach that is often preferred in cases of severe anorexia where it guarantees greater weight gain [512, 513, 502]. Pharmacological treatments with antidepressant or anxiolytic drugs have often been used but with very various and, often, ineffective results in body weight gaining. Weight restoration and diet must be well regulated and planned to avoid a sudden transition from a low calorie situation to a diet rich in fat that could lead to rapid electrolyte and ionic imbalances in the body [502, 503].

Anorexia nervosa and oxidative stress

The reduced caloric intake that characterizes patients with AN inevitably affects the energy state of the cells. It is known that an energetic dysfunction of the cells generates an imbalance in the mitochondrial activity that causes the production of ROS [514]. In a study conducted on leukocytes isolated from anorexic patients, it was observed that there is an increase in markers of oxidative stress which depends precisely on mitochondrial dysfunctions [515]. In the literature, there are not many studies on the correlation between AN and oxidative stress and the results are often very contrasting. This is the case, for example, of the levels of NO which in some papers seem to decrease while other researchers have found an increase in the production of this molecule in AN patient [516–518]. Hence the need to carry out further studies to better understand the role of oxidative stress in AN.

Anorexia nervosa and hippocampus

Generally, the most observed finding in AN patients is the bilateral reduction of hippocampal volume which, however, does not seem to depend on the age of onset, duration or severity of the disease [519]. The hippocampal formation is well known for its involvement in learning and memory, but it also plays an important role in emotional regulation [520]. Reduction in the volume of hippocampal formation has been reported in several studies of adults and adolescents with AN [519, 521–524]. Hippocampal atrophy is evident in other serious mental health disorders, such as major depression, schizophrenia, bipolar disorder, and post-traumatic stress disorder

(PTSD) [525–528]. Patients with AN often experience concomitant symptoms of depression and anxiety [496, 529]. The link between hippocampal volume reduction and comorbid symptoms has not been studied. Significant learning and memory deficits have been demonstrated in AN, with some evidence of an association between memory deficits and structural brain abnormalities [519, 530]. It is unclear to what extent hippocampal atrophy may reflect a cause or effect of the disease. In a recent experiment conducted on AN and healthy women, by the evaluation of hippocampal volume using magnetic resonance imaging (MRI), no correlation was found between the hippocampal volume reduction and AN and, furthermore, no hippocampal-dependent cognitive decline was evidenced [519]. Additionally, another study showed that anorexia seems to reduce the density of astrocytes in CA2, CA3 and DG, while no significant changes were observed in CA1. On the other hand, the decrease in $GFAP^+$ cell density resulted in a reduced expression of the GFAP protein [531].

The bulimia nervosa (BM) is a pathology that can arise both in normal or high weight and is characterized by several recurrent episodes of the so-called binge eating behaviour. This conduct is described as uncontrolled eating and then a repaying behaviour to avoid weight gains such as vomiting, excessive exercise and use of laxatives [532, 483].

The binge eating disorder (BED) is a disease characterized by regular and constant binge eating episodes along with a sense of loss and stress [533]. From 30% to 45% of BN and BED cases lead to obesity [483]. This pathology has a high prevalence in humans, but the aetiology remains poorly understood. Primarily, BED co-occurs with several psychiatric and anxiety disorders. BEDs are pathologies that have not yet been treated. Psychological therapy is often an effective treatment in the case of mild symptoms and the absence of psychiatric comorbidities. CBT treatments and pharmacological treatment with antidepressants, antiepileptic drugs, and obesity drugs can also be applied for this pathology [534].

The avoidant/restrictive food intake disorders (ARFID) are defined by the DSM-V as a failure to meet nutritional needs leading to low weight, vitamin deficiency, dependence on supplemental feedings, and/or psychosocial impairment. The symptoms could be due to a decrease in the interest in food and eating and fear for negative consequences of eating such as vomiting. It is a recently defined disorder so in literature there are not so many studies on this topic. Therefore, there are no epidemiological data or a common definition for this pathology [483, 535].

2. AIM

The purpose of this thesis was to evaluate oxidative stress and inflammation as a common thread in three different pathologies using mouse animal models and a model of human biopsies to provide insights for the improvement of antioxidative and antiinflammation treatment. When oxidative stress and inflammation become chronic in the organisms, can trigger suicide and cell death events to restore a damaged microenvironment. The persistence of a high oxidative and inflammatory state in the brain can cause irreparable neuronal damage which results in cognitive decline, neurodegeneration and damage to brain functions essential for the survival of the individual.

In this thesis work, it was therefore decided to evaluate by immunohistochemical analysis the expression of oxidative stress and inflammation markers by evaluating the enzymes SOD1, SOD2, COX2, COX4 and the inducible form of NOS. In addition, the analysis focused on evaluating the expression of two proinflammatory molecules, IL-6 and TGF-β that participate in inflammatory responses mediated by cellular damage. Based on the data obtained, the attention was then focused on which cell survival mechanisms occur in granular and mossy hippocampal neurons, through the investigation of different markers of programmed cell death. Proteins belonging to apoptosis (Bax and Bcl-2), autophagy (p62) and mitophagy (PINK1 and PARKIN) were analysed with preliminary studies.

The three experimental projects were based on different collaborations:

- Prolidase Deficiency (PD) project was conducted in collaboration with Prof. Forlino of the Molecular Medicine Dept., University of Pavia;

- Ageing and micotherapy was conducted in cooperation with Prof. Rossi, Neurobiology and Integrated Physiology Laboratory, University of Pavia;

- the Anorexia Nervosa and control human sample were provided by the archive of Public Health, Experimental and Forensic Medicine, "Antonio Fornari" Dept., Unit of Forensic Medicine and Forensic Sciences of the University of Pavia. This work is in collaboration with the research team of Prof. Osculati.

3. MATERIALS AND METHODS

3.1 Mouse models

3.1.1 Prolidase deficiency

The prolidase deficiency dark-like mice (*dal*) were provided by Dr Gunn TM (Great Falls, MT) and C3H wild- type (wt) mice were purchased from Charles River. The animals were maintained under standard experimental animal care protocol following the Italian Laws in the animal facility of the Department of Molecular Medicine of the University of Pavia (Italy). Water and food were provided ad libitum. The use of mice has been approved by the municipal authorities of the Province of Pavia (reference no. 7287/00) and by the Ministry of Health according to art. 12, D.L. 116/92. Every effort has been made to minimize the number of animals to be used and their suffering. Heterozygous, homozygous *dal* mice and WT littermate were sacrificed at three different ages: 10 days after birth (P10), 21 (P21) and 60 days after birth (P60) by decapitation after an i.p. injection of 35% chloral hydrate (100 µl/100 g b.w.; Sigma). The murine model for PD, called dark-like (*dal*) was initially discovered thanks to the presence of dal mice with a mutation in chromosome 7. Subsequently, the researchers identified other mice with dark coloured hair, and the gene mapping revealed a mutation on the PEPD gene. The *dal* genotype presents a deletion of 4 bp in exon 14 of the gene that codes for prolidase (PEPD). Thanks to this morphological association, these mice were called dark like. The *dal* mutants, in addition to the dark colour of the hair, are small in size, have anomalies affecting the gonads, cells rich in vacuoles near the cortical medullary junction of the adrenal gland, mild hydrocephalus and dark-coloured urine [536]. PD mice also show marked cardiac hypertrophy. This latter phenotypes is probably due to the fact that collagen is the main ligand of integrins and is the target molecule of prolidase, thus has been hypothesized that it causes an alteration in the growth of cardiomyocytes [537]. However, the animal model, unlike humans, does not have the skin ulcers typical of the disease. The prolidase activity in *dal/dal* mice was strongly reduced concerning WT animals both in the cerebrum (4.3±3.7%) and in the cerebellum (3.7±1.4%). Interestingly prolidase activity in both tissues was less than 50% in *dal/+* mice compared to controls, specifically 36.7±21% in the cerebrum and 30.0±8.8% in the cerebellum [538].

3.1.2 Ageing

Fifteen WT male mice (strain C57BL-6J), starting at 11 months old, were maintained in single cages in the Animal Care Facility at the University of Pavia on a 12-h light/dark cycle. Water and food were provided ad libitum. All experiments were carried out following the guidelines laid out by the institution's animal welfare committee, the Ethics Committee of Pavia University (Ministry of Health, License

number 774/2016-PR). Seven out of fifteen mice, starting from 21.5 months old, received for two months a drink made by a mixture of He1 mycelium and sporophore as ethanol extracts solubilized in water, in such a way that every mouse received 1 mg of supplement per day. This amount was chosen to mimic the oral supplementation in humans (about 1g/day). Daily consumption of water and supplements was monitored for each mouse. At each experimental time, mice were weighed, and no statistically significant change was recorded either during ageing or between the He1 and control groups. Mice at 23.5 months old were sacrificed by decapitation after an i.p. injection of 35% chloral hydrate (100 µl/100 g b.w.; Sigma Aldrich, Milan, Italy); brains were cautiously removed and hemi-dissected. Each half of brains was post-fixed in Carnoy's solution for 48h a room temperature (RT), dehydrated through a graded series of ethanol and finally embedded in Paraplast X-tra (Sigma Aldrich, Milan, Italy).

3.1.3 Tissue isolation

At each time point and for each experimental project, eight micrometer-thick sections were cut serially in the coronal plane with a microtome and collected on silane-coated slides for the histological, immunohistochemical and immunofluorescence analysis. Experiments were conducted simultaneously for all different mouse strains.

3.2 Anorexia Nervosa human samples

The AN and control tissue samples of the hippocampal formation were selected from the archive of the Department of Public Health, Experimental and Forensic Medicine, "Antonio Fornari", Unit of Forensic Medicine and Forensic Sciences of the University of Pavia. These samples were taken during judicial autopsies for diagnostic purposes, fixed in formalin and embedded in paraffin. From control and AN samples, eight micrometer-thick sections were cut serially in the coronal plane with a microtome and collected on silane-coated slides for the histological, immunohistochemical and immunofluorescence analysis. Experiments were conducted simultaneously for all different conditions.

3.3 Histological analysis

3.3.1 Haematoxylin/eosin staining

The sections included in Paraplast X-tra (Sigma) were spun for 20 minutes in xylol (Carlo Erba, Italy) and subsequently rehydrated according to a descending alcoholic scale protocol that includes: five steps, of 5 minutes each, as follows: xilol/absolute ethanol, absolute ethanol, ethanol 95%, ethanol 80%, and finally ethanol 70%. Then the sections were immersed in haematoxylin for 10 minutes and then washed with running water for about 3 minutes. Subsequently, the sections underwent two washes

in distilled water and then the sections were dehydrated following an ascending alcoholic scale protocol consisting of 80% ethanol (fast), 95% ethanol, absolute ethanol, xylene/absolute ethanol and xylene (3 minutes each). Finally, the slides were mounted with Eukitt (Kindler gmbH) for the bright-field microscope analysis.

3.3.2 Nissl staining

To determine the neuronal morphologies of the hippocampal section histological staining of Nissl was performed. This reaction allows to highlight the endoplasmic reticulum, the ribosomes contained in the cytoplasm, in the nucleus and in the densrites of nervous cells. The sections included in Paraplast X-tra (Sigma) were spun for 10 minutes in xylene and subsequently rehydrated according to the same protocol for the H&E experiments. After being washed in distilled water for 3 minutes (2 times), the sections were incubated in an oven at 58°C with a solution ofToluidine blue for 20 minutes. Subsequently, the sections underwent two washes in H_20_d, the first of a few seconds, the second of 3 minutes and then the sections were dehydrated following an ascending alcoholic scale protocol. Finally, the slides were mounted with Eukitt (Kindler gmbH).

3.3.3 Immunohistochemistry

After deparaffination in xylene (Carlo Erba, Cornaredo, Italy), the sections were rehydrated in decreasing ethanol series and rinsed in phosphate-buffered saline (PBS, SigmaAldrich, Milan, Italy). The hippocampal slides were incubated, at RT, for 7 min in a blocking buffer for the suppression of the endogenous peroxidases (3% H_2O_2 in 10%methanol in PBS), then for 20 min in foetal calf serum in order to block non specific antigen binding sites. Immunohistochemistry was performed using commercial antibodies on mice and human hippocampal sections, to localize presence and distribution of different specific markers indicative of oxidative stress, inflammation and apoptotic pathways: (i) Cu–Zn superoxide dismutase-1 (SOD1), (ii) Mn superoxide dismutase 2 (SOD2); (iii) nitric oxide synthase 2 (NOS2), and (iv) cyclo-oxygenase-2 (COX2), (v) Interleukin-6 (IL-6), (vi) Transforming Growth Factor-β1 (TGF-β1), (vii) Bcl2, and (viii) Bax (details and dilutions are reported in Table 1). The sections were incubated at 4°C overnight in a dark chamber. Subsequently, the slides were incubated with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 30 minutes and horseradish peroxidase conjugated avidin-biotin complex (Vector Laboratories, Burlingame, CA, USA) for 30 minutes at RT. Then, 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB;Sigma Aldrich, Milan, Italy) with 0.01% H_2O_2 in Tris–HCl buffer (0.05 M, pH 8) was used as a chromogen followed by nuclear counterstaining with Haematoxylin. Then, sections were dehydrated in ethanol, cleared in xylene (Carlo Erba Reagents, Cornaredo, Italy) and finally mounted in Eukitt (Kindler, Freiburg, Germany). For control staining, the primary antibody was omitted in some sections incubated with phosphate-buffer saline only. No immunoreactivity was observed in this condition.

The slides were observed with an Olympus BX51 microscope, images were acquired with an Olympus Camedia c-2000 Z digital camera and stored on a PC running Olympus software.

	Antigen	Species, Manufacturers	Dilution
Primary antibodies	Anti-**Superoxide Dismutase-1** (FL-154)	Rabbit polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
	Anti-**Superoxide Dismutase-2** (D3X8F)	Rabbit monoclonal IgG, Cell Signaling Technology (Cell Signaling Technology, Danvers, USA)	1:100
	Anti-**Nitric Oxide Sinthases-2** (M19)	Rabbit polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
	Anti-**Cyclooxygenase-2** (M-19)	Goat polyclonal IgG Santa Cruz Biotechnology, (Santa Cruz, CA, USA)	1:100
	Anti–**Cyclooxygenase-4**	Mouse monoclonal IgG, Abcam, Cambridge, USA)	1:100
	Anti-**Interleukin-6** (M-19)	Goat polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
	Anti-**Transforming Growth Factor β1** (V)	Rabbit polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
	Anti-**Bcl-2-associated X protein** (P-19)	Rabbit polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
	Anti-**B-Cell Leukemia/Lymphoma 2 protein** (N-19)	Rabbit polyclonal IgG, Santa Cruz Biotechnology (Santa Cruz, CA, USA)	1:100
Secondary antibodies	Biotinylated goat anti-rabbit IgG	Goat, Vector Laboratories (Burlingame, CA, USA)	1:200
	Biotinylated rabbit anti-goat IgG	Rabbit, Vector Laboratories (Burlingame, CA, USA)	1:200

Table 1. Primary and secondary antibodies and respective dilution used for immunocytochemical experimental procedures.

3.3.4 Immunofluorescence

For the immunofluorescence analysis the sections were deparaffinated, rehydrated and rinsed in PBS for 5 minutes. After this, the sections were incubated for 7 minutes at RT with a blocking solution (100mg BSA, 10µl Tween 20, 3.3ml glycine 0.3M in 7ml PBS) to block nonspecific binding sites. The slices were then placed overnight in a humid chamber for the incubation with primary antibodies reported in Table 2. The next day, the sections were incubated for 30 minutes with secondary antibodies and then nuclei were counterstained with 0.1 µg/ml Hoechst 33258 for 5 minutes. For control staining, some sections were incubated with PBS instead of the primary antibodies. No immunoreactivity was present in these sections. After washing in PBS twice for 10 minutes each, the sections were reacted with Hoechst 33258 (0.1 μg / ml) for 5 minutes to mark the nuclei, further rinsed in PBS and then the slides were sealed with Mowiol (Calbiochem, Inalco, Italy). The sections were then observed in fluorescence microscopy with an Olympus BX51 microscope equipped with a 100 W mercury lamp. The following equipment was used: 330 to 385 nm excitation filter (excf), 400 nm dichroic mirror (dm) and 420 nm barrier filter (bf), for Hoechst 33258; 450 to 480 nm excf, 500 nm dm and 515 nm bd for Alexa 488; and 540 nm excf, 580 nm dm and 620 nm bf for Alexa 594. Images were recorded with an Olympus Magnifier camera system and stored on PC running Olympus software for processing and printing.

3.4 Qualitative evaluations

After assembly, the slides were dried and then analysed under an Olympus BX51 microscope. A first panoramic observation, at 4x and 10x magnifications, was made to define the state of integrity of the slice and the encephalic area of interest. Larger magnifications, 20x and 40x, were used to evaluate cytoarchitecture and cell population; for this last aspect, higher magnifications (100x) have also been used for inserts.

	Antigen	Species, Manufacturers	Dilution
Primary antibodies	Anti-**p62**	Mouse monoclonal IgG, Abcam, Cambridge, USA)	1:100
	Anti-**PINK1**	Rabbit polyclonal IgG, Abcam, Cambridge, USA)	1:100
	Anti-**PARKIN**	Mouse monoclonal IgG, Abcam, Cambridge, USA)	1:100
	Mitochondria	Human autoimmune serum recognizing the 70 kDa E2 subunit of the pyruvate dehydrogenase complex[b]	1:200
Secondary antibodies	Alexa 594-conjugated anti-mouse antibody	Thermo Fisher Scientific (Waltham, MA USA)	1:200
	Alexa 488-conjugated anti-mouse antibody	Thermo Fisher Scientific (Waltham, MA USA)	1:200
	Alexa 488-conjugated anti-rabbit antibody	Thermo Fisher Scientific (Waltham, MA USA)	1:200
	Alexa 594-conjugated anti-human antibody	Thermo Fisher Scientific (Waltham, MA USA)	1:200

b [539]

Table 2. Primary and secondary antibodies and respective dilution used for immunofluorescence experimental procedures.

3.5 Quantitative analysis

In this study, particular attention has been paid to granular and polymorphic layer of the murine and human hippocampal dentate gyrus (DG).

3.5.1 Optical density

For several markers it was useful to measure the density of pixel here referred to optical density (OD) of the immunopositive areas after immunohistochemical reactions. After the acquisition, in the bright field immunoreactions the colour was inverted to have the positive areas lighter than the background or the non-expressed

neurons. The background and the haematoxylin staining were always subtracted with a plugin of the ImageJ (ImageJ 1.46p, NIH, Bethesda, MA, USA) program.

Depending on the area evaluated, the OD measurements were carried out using different tools of the ImageJ program. For the analysis of the GL, it was initially thought to use a circular mask with a fixed shape and size which, when positioned on a cell, allowed to measure the pixel density (**Fig. 1**). Since the neurons in this layer were densely packed and difficult to discriminate, it was subsequently decided to use a rectangular mask with a fixed area and size that would allow to analyse the entire layer under examination (**Fig. 2**). The results obtained from the analysis with a circular and rectangular mask were very similar to each other, confirming the validity of the method used. For what concerns the measurement of OD in mossy cells, a circular screen of fixed area and size was used. The dimensions of the masks, the number of measurements, the number of sections analysed, and the magnification were kept constant throughout the quantitative analysis.

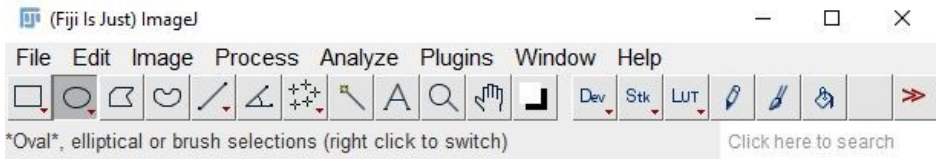


Figure 1. ImageJ toolbar with "Oval selection"

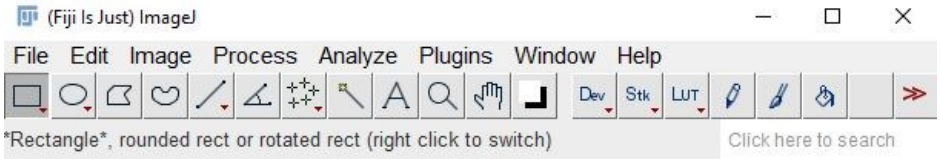


Figure 2. ImageJ toolbar with "Rectangular selection"

3.5.2 Statistical evaluations

All results were reported in histograms as mean ± SD. Three brain sections (n=3) per slide per animal (N = 3) were used for quantitative optical density analysis and statistical evaluations. After evaluating the normality of data, to verify statistically significant differences, the results were analysed through one-way analysis of variance (ANOVA), followed by Bonferroni post hoc analysis using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA, USA) for PD results. The statistical analysis for ageing results was carried out using an Unpaired Student's t-test. The differences are considered statistically significant for $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

4. RESULTS

The studies reported in this thesis made it possible to compare various physiological and pathological conditions that can affect humans throughout life. The results chapter has been divided into sub-chapters in which the results are reported in the same order for each individual pathology examination.

In all the considered conditions, were first reported the findings of morphological analysis carried out with Haematoxylin and eosin (H&E) staining. This technique is the most widely used method in histology and has allowed us to highlight the general morphology of the tissue, distinguishing between cells, cytoplasm, and extracellular matrix (ECM). However, staining with H&E does not fully allow studying the organization of the brain tissue, since for the most part only the nucleus of neurons is stained, and the cytoplasm appears as a homogeneous structure. Alongside this approach, a Nissl stain was also performed. This method allows to study the neuroanatomical organization of the brain and, for this reason, it is considered the election technique for investigations on nervous tissue. The colouring highlights the so-called Nissl zone or tigroid substance, that are cytoplasmic areas rich in aggregates of rough endoplasmic reticulum and ribosomes located in the soma and in the dendrites of nervous cells. These components are instead absent in the axon which is not coloured with the Nissl method. In addition, this stain also allows to highlight the RNA contained in the chromatin and in the nucleolus of the cells under examination.

Afterwards, for each pathology considered, bright-field immunohistochemistry studies were carried out to reveal the expression of oxidative stress, inflammation, and apoptosis proteins, while the molecules belonging to the autophagy and mitophagy pathways were investigated through immunofluorescence analysis. This difference is since their labelling very often colocalized with the nucleus of neurons and, thanks to the possibility of splitting the fluorescence channels under the microscope, their expression was more distinguishable with this type of technique. Furthermore, for the AN result it was possible to perform a double immunofluorescence to detect the colocalization with marker of mitophagy and mitochondria.

The figures in the results section show the most representative anomalies and differences of control and pathological (for Prolidase Deficiency and Anorexia Nervosa) or supplemented (for Ageing model) samples.

4.1. Prolidase Deficiency

In the study of Prolidase Deficiency (PD), animals at different ages were selected to analyse the expression of the proteins in correlation to the progression of the disease. Therefore, it was decided to evaluate hippocampal dentate gyrus (DG) sections of animals at 10, 21 and 60 postnatal days (the ages in the text are indicated as P10, P21 and P60, respectively). At P10, while the gross morphology of DG is already fully developed, the proliferation and migration of neurons within the Granular Layer (GL) and their ability to form synaptic contacts with other hippocampal cells are still present. On the other hand, P60 is considered the adult stage of development in rodents, and so most of the proliferative processes in the DG have stopped and remain only in a thin area of the GL, called the subgranular zone (SGZ). In addition, it was decided to investigate an intermediate age, the P21 timepoint, to follow in a more detailed approach the evolution of the disease.

4.1.1 *The dentate gyrus morphology*

Recent studies conducted in our laboratory have demonstrated the presence of morphological alterations in the cerebellum of PD mice with defects in cerebellar lobulation, in the organization of the extracellular matrix (ECM) and in neuronal migration. In cerebellum, the granular cells of the external granular layer (EML) make use of the ECM together with fibers of the radial glia to migrate into the inner GL (IGL) which will remain in adulthood. At P60, in *dal* mice, these cells were found to be ectopic and immersed in the ML, suggesting an impair in the migration process to reach the canonical site [391, 1].

In **Figure 1, 2 and 3** are shown the results obtained by Haematoxylin and eosin staining in P10, P21 and p60 PD mice, respectively. As shown, the morphology of the DG-GL revealed no evident malformations between the three genotypes. The presence of a thinner GL layer in **Figure 1c** could be attributable to the section level rather than to an actual difference between the homozygous genotype and the WT. In the GL of these young mice, the presence of two sub-layers, one localized in the outer two thirds of the layer facing the ML and one more internal, bordering the polymorphic layer (PL), can be clearly distinguishable. In WT mice, there were several immature neurons and mature granular cells displaying the characteristic rounded morphology. This difference at P10 confirms the presence of proliferative located in the SGZ that in addition were intensely coloured by haematoxylin, suggesting a strong nuclear activity. By evaluating the subsequent postnatal ages, the proliferative layer of GL in WT mice became very thin and was detectable only in the SGZ (**Fig.2 and 3**). However, unlike WT, the presence of wrinkled and irregularly shaped cells (black arrows) was found in the GL of both P21 and P60 *dal* animals (**Fig. 2 b, c, e, f, h, i** and **Fig. 3 b, c, e, f, h, i**) presumably still immature neurons that have not completed the maturation and proliferation due to defects in ECM organizations previously found in PD mice.

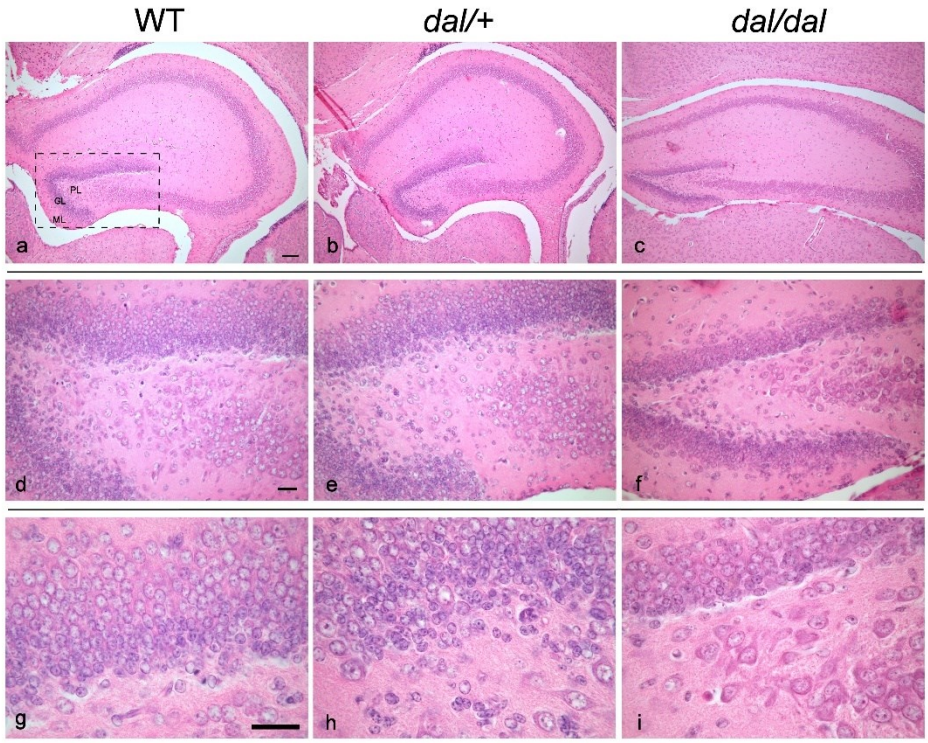


Figure 1. Haematoxylin and eosin (H&E) staining of hippocampal dentate gyrus (DG) at P10. (a, d, g), WT; (b, e, h), *dal*/+; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). All the analyses were conducted in the tissue area included in the dotted lines (a). Abbreviations: WT=wild type; *dal*=prolidase deficient mice; ML: Molecular layer; GL: Granular layer; PL: Polymorphic Layer.

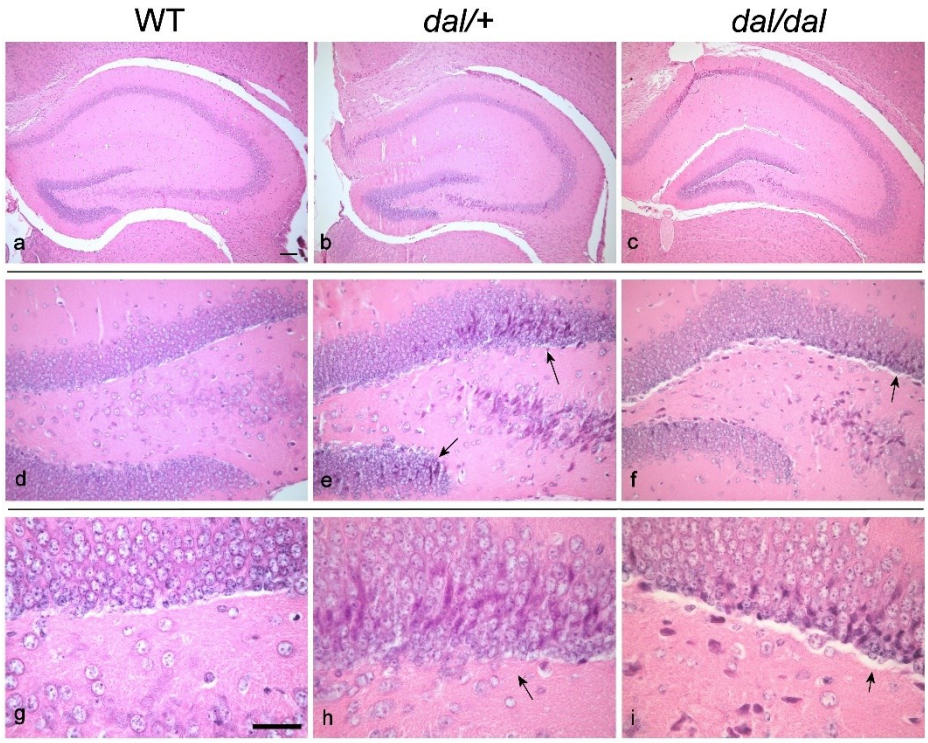


Figure 2. Haematoxylin and eosin (H&E) staining of hippocampal dentate gyrus (DG) at P21. (a, d, g), WT; (b, e, h), *dal/+*; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

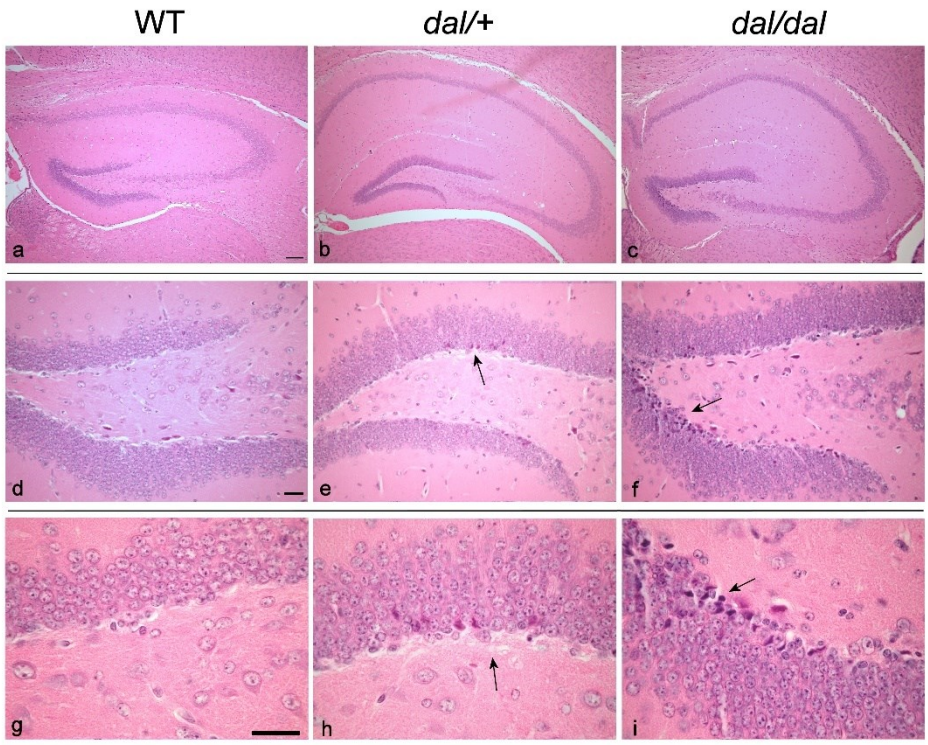


Figure 3. Haematoxylin and eosin (H&E) staining of hippocampal dentate gyrus (DG) at P60. (a, d, g), WT; (b, e, h), *dal*/+; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

In **Figure 4, 5 and 6** are shown the results obtained by toluidine blue staining in P10, P21 and p60 PD mice, respectively. As shown, at P10, the staining allowed to highlight a substantial homogeneity in the cellular organization of the DG between the three genotypes (**Fig. 1**). As for the H&E results shown in **Fig.1c, f, i**, the morphological differences highlighted can be ascribed to the section rather than to a variation between experimental conditions. At P21, the GL neurons appeared circular and well organized throughout the layer in all three genotypes while, again, in *dal/+* and *dal/dal* mice it was possible to find the presence of irregularly shaped neurons (black arrows) between the SGZ and the remaining part of the GL which are absent in the control animals (**Fig. 5 b, c, e, f, h, i**). Some neurons with similar morphology were also found in the thickness of the PL of the same genotypes. At P60, the neurons in the GL did not show considerable differences as regards the morphology between control animals and heterozygotes but, in the analysis of the *dal/dal* picture the irregularly shaped neurons already highlighted with H&E staining can be found (**Fig. 6 b, c, e, f, h, i**).

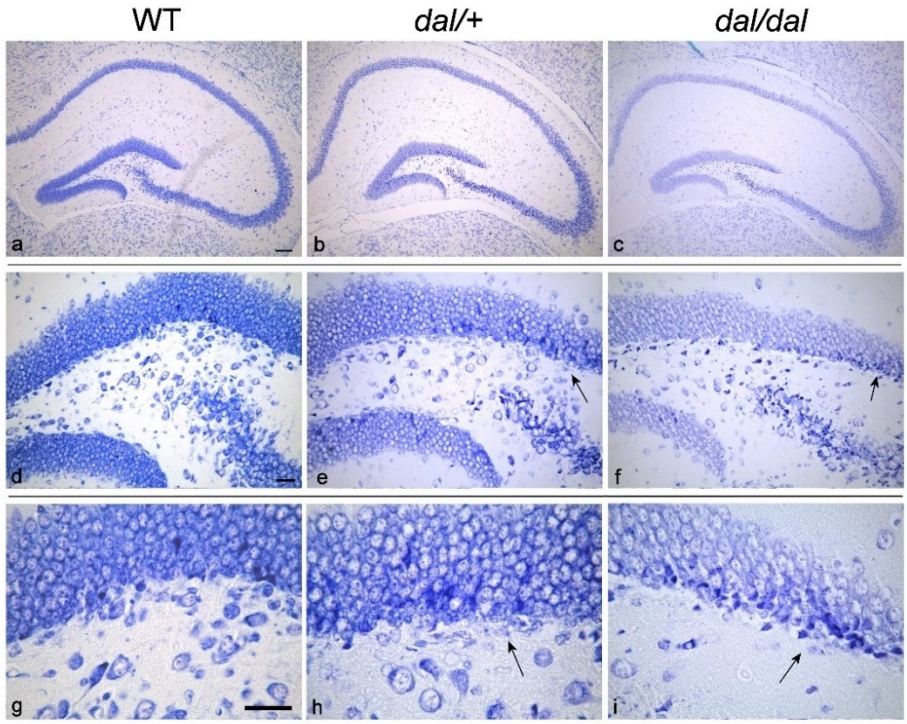


Figure 4. Toluidine blue staining of hippocampal dentate gyrus (DG) at P10. (a, d, g), WT; (b, e, h), *dal*/+; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

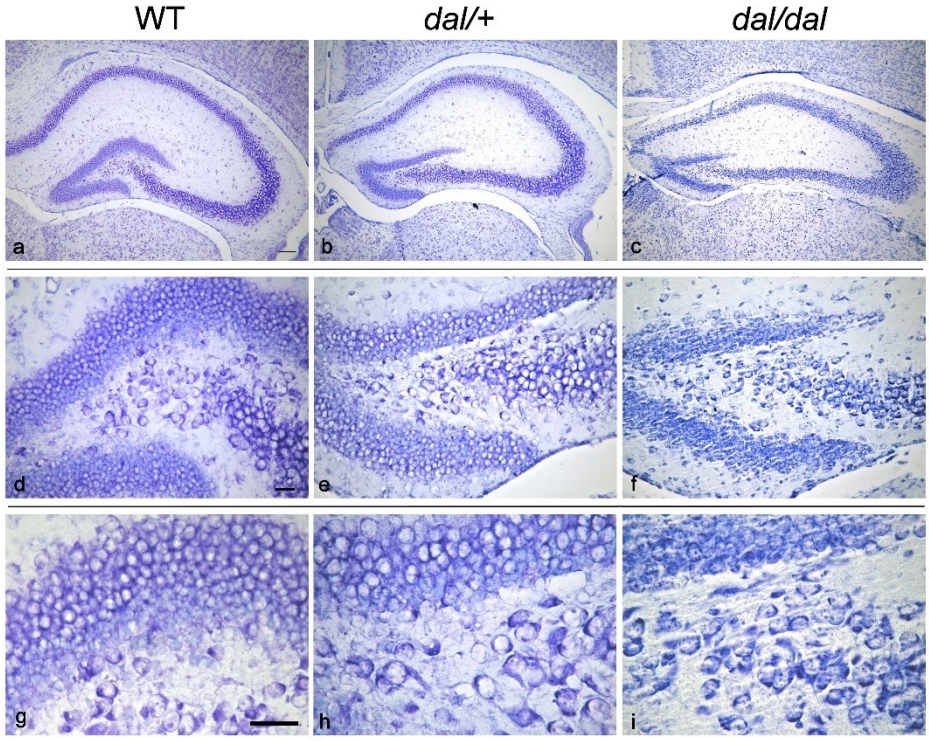


Figure 5. Toluidine blue staining of hippocampal dentate gyrus (DG) at P21. (a, d, g), WT; (b, e, h), *dal*/+; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

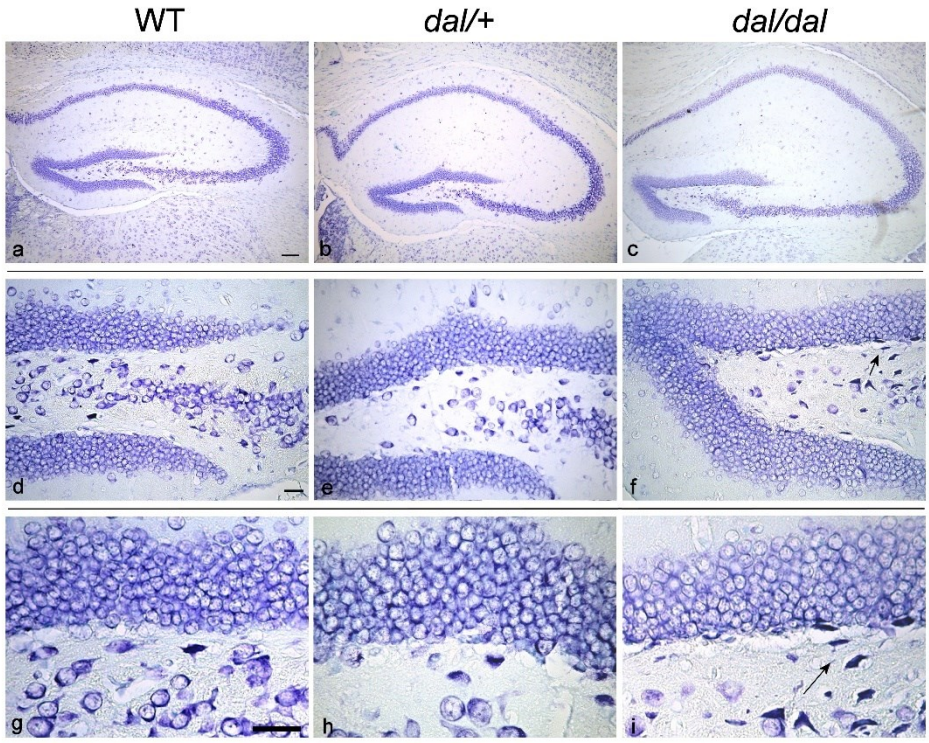


Figure 6. Toluidine blue staining of hippocampal dentate gyrus (DG) at P60. (a, d, g), WT; (b, e, h), *dal*/+; (c, f, i), *dal/dal*. Magnifications: 10x (a, b, c); 40x (d, e,f); 100x (g, h, i). Bars: 100µm (a); 20 µm (d, g). Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

4.1.2 The anti-oxidative stress enzymes

SOD1 constitutes the first line of defence against ROS generated by tissue and cellular damage, thus the protein was used as a specific marker of oxidative stress pathways. By comparing the results obtained in P10 mice, the expression of SOD1 was cytoplasmatic and appeared to be spread homogeneously throughout the GL in all the three conditions considered. Particularly, $SOD1^+$ neurons were mostly located in the outer two thirds of the layer rather than in the area closest to the SGZ. Similarly, the immunolabeling in polymorphic cells did not seem to vary between WT and *dal* genotypes (**Fig. 7a-c**). At PD21, on the other hand, an increase in positivity of granular neurons was observable in *dal/+* and *dal/dal* mice compared to control animals. Most of the labelled neurons was located, presumably, in the new-born cells proximal to the SGZ. In these animals there was also an increase in SOD1 expression in mossy neurons that, in the case of homozygous mice, appeared strongly positive (**Fig. 7d-f**). No differences were observed in the GL of P60 animals. In this case, SOD1 antibody was evident especially in the mossy neurons of *dal/dal* mice but it is important to highlight a strong heterogeneity in the immunolabelling (**Fig. 7g-i**). The OD analysis did not report remarkable changes in GL immunopositivity at PD10 (P10 GL OD values: WT 95.996 ± 2.74; *dal/+* 92.571 ± 2.51; *dal/dal* 94.394 ± 2.16). In the subsequent timepoints analysed regarding the GL, the quantitative analysis showed lower SOD1 intensity in control animals than in *dal* genotypes, confirming the high levels of SOD1 evaluated during the early stages of postnatal development. In both ages considered, the immunolabeling was significantly increased in heterozygous PD mice at P21 and in homozygous at P60 (P21 GL OD values: WT 84.198 ± 4.94; *dal/+* 98.818 ± 3.71; *dal/dal* 91.093 ± 3.63; P60 GL OD values: WT 88.572 ± 2.68; *dal/+* 89.153 ± 1.16; *dal/dal* 98.006 ± 1.53) (**Fig. 7j**). As for the analysis of granular cells at P10, the results relating to OD demonstrated a very slight and not significant increase in immunopositivity that became more evident at 21 days with statistically significant results in the comparison between homozygous and WT mice (P10 PL OD values: WT 87.356 ± 3.52; *dal/+* 88.981 ± 3.35; *dal/dal* 92.124 ± 4.175; P21 PL OD values: WT 89.573 ± 5.53; *dal/+* 96.452 ± 5.47; *dal/dal* 105.201 ± 5.93). At P60, the labelling was found to be various among the conditions and the values obtained were not statistically significant (P60 PL OD values: WT 93.945 ± 4.84; *dal/+* 90.538 ± 3.92; *dal/dal* 99.408 ± 3.73) (**Fig. 7k**).

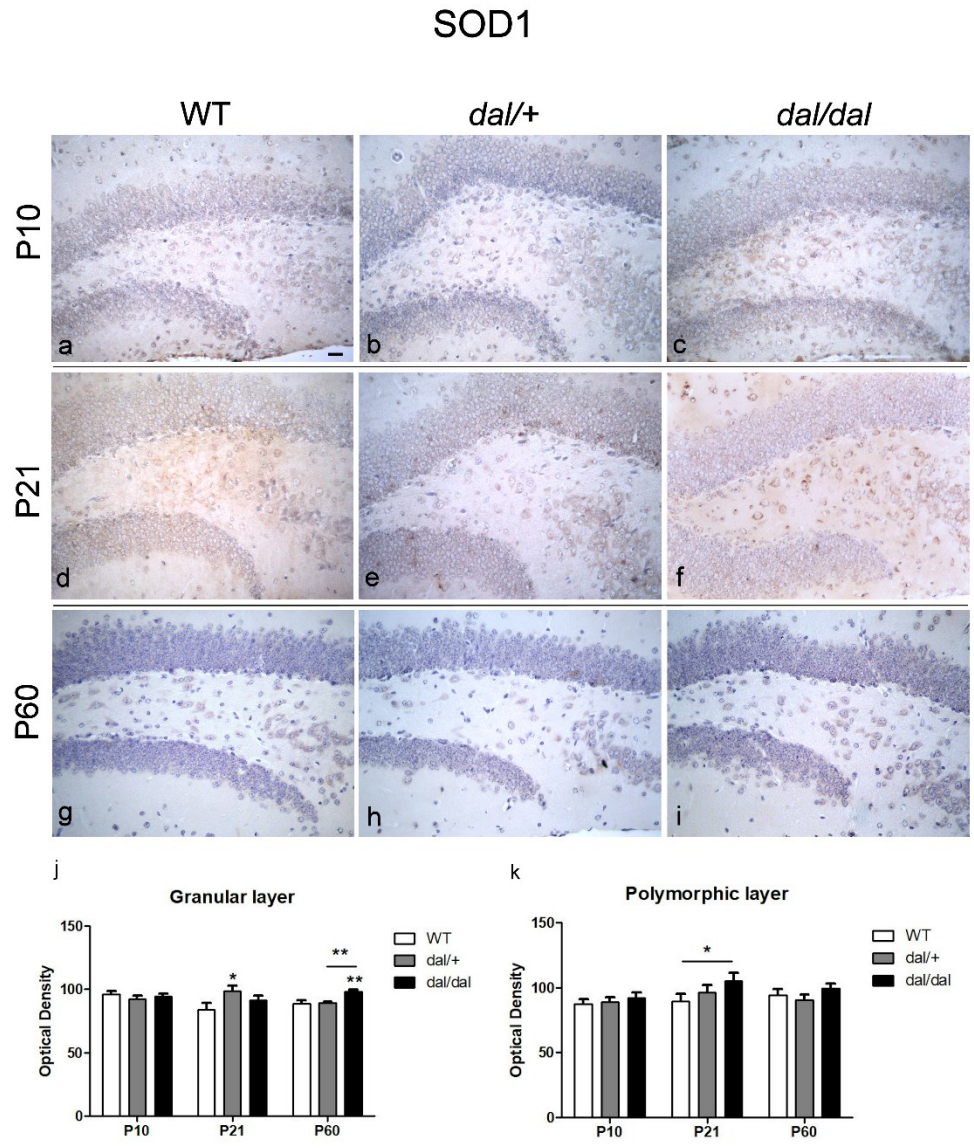


Figure 7. Immunohistochemical reaction for SOD1 (Superoxide dismutase 1) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); p values, GL P21: WT *vs dal/+* (*) p <0.05; GL P60: WT vs *dal/dal* (**) p <0.01, *dal/+* vs *dal/dal* (**) p <0.01; PL P21: WT vs *dal/dal* (*) p <0.05. The same statistical analysis performed on the other OD values does not show any statistically significant differences, p >0.05. Abbreviations: WT=wild type; *dal*=prolidase deficient mice.

SOD2 is the Mn^{2+}-dependent isoform of superoxide dismutase enzymes which, in physiological conditions is located in the mitochondrial matrix. In this microenvironment, it plays central role in cell development and appears to be involved in life span duration. In the GL at P10, the SOD2 labelling was more evident in the lower third of the layer itself, near the SGZ. In *dal/+* mice, these neurons showed a strong increase in immunopositivity which, after OD evaluations, was found to be statistically significant in comparison with both control animals and *dal/dal* mice (P10 GL OD values: WT 104.244 ± 2.46; *dal/+* 132.161 ± 5.49; *dal/dal* 107.472 ± 2.46). Interestingly, these latter mice were characterized by a different pattern of expression since most of the positivity could be detectable in the outer two thirds of the GL (**Fig. 8a-c, j**). At this stage of development, SOD1 was also expressed in neurons of the PL with a statistically significant increase in *dal/+* mice compared to WT and among *dal* genotypes (P10 PL OD values: WT 95.134 ± 2.19; *dal/+* 115.810 ± 2.57; *dal/dal* 99.964 ± 2.37) (**Fig. 8k**). At P21, a slight labelling of cells close to SGZ persisted in WT mice and this expression increased in both *dal* animals albeit to a lesser extent in homozygous mice (**Fig. 8d-f**). The quantitative analysis performed on GL showed statistically significant differences between the two genotypes and the control condition. Also in the PL evaluations, the OD results showed a statistically significant increase in heterozygous and homozygous mice compared to the control and among each other (P21 GL OD values: WT 95.073 ± 3.03; *dal/+* 116.705 ± 4.33; *dal/dal* 110.266 ± 1.60; P21 PL OD values: WT 88.097 ± 1.71; *dal/+* 96.100 ± 2.34; *dal/dal* 105.487 ± 0.58) (**Fig. 8k, j**). Lastly, as shown in **Figure 8g-i**, the positivity at P60 was quite similar in all three conditions but less intense compared to P10 and P21. Furthermore, most of the SOD2-expressing neurons were localized in the PL with an increase in positivity in homozygous mice compared to WT even if no significant differences were highlighted (P60 GL OD values: WT 87.724 ± 11.45; *dal/+* 85.978 ± 10.41; *dal/dal* 97.142 ± 5.48; P60 PL OD values: WT 89.542 ± 2.55; *dal/+* 83.550 ± 9.96; *dal/dal* 98.729 ± 5.58) (**Fig. 8a-i, j, k**).

Mitochondrial cytochrome c oxidase (COX, complex IV) is the terminal electron acceptor of the mitochondrial respiratory chain that catalyses the transfer of an electrons from cytochrome c to molecular oxygen, contributing to the electrochemical gradient used by ATP synthase to form ATP. **COX4** is the last enzyme complex involved in the electron transport chain, so its alteration could potentially lead to the accumulation of ROS and oxidative stress. Since COX4 was not detectable in the images, no evaluable results were obtained from the analysis of this marker in PD animals and control condition (**Fig. 9**).

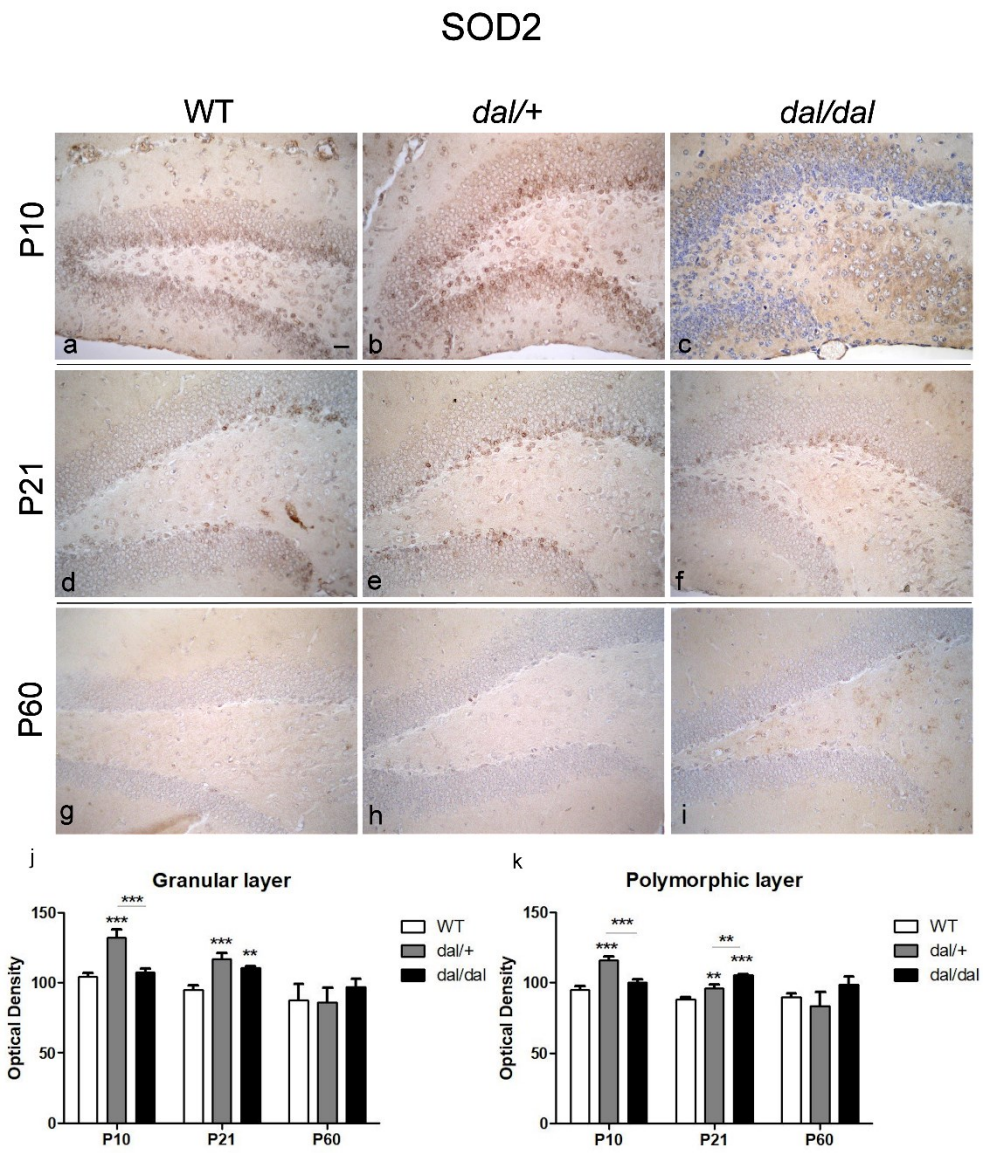


Figure 8. Immunohistochemical reaction for SOD2 (Superoxide dismutase 2) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); p values, GL P10: WT *vs dal/+* and *dal/+* vs *dal/dal* (***) p <0.001; GL P21: WT *vs dal/+* (***) p <0.001, WT vs *dal/dal* (**) p <0.01; PL P10: WT *vs dal/+* and *dal/+* vs *dal/dal* (***) p <0.001, PL P21: WT *vs dal/+* (**) p <0.01, WT vs *dal/dal* (***) p <0.001, *dal/+* vs *dal/dal* (**) p <0.01. The same statistical analysis performed on the other OD values does not show any statistically significant differences, p >0.05.

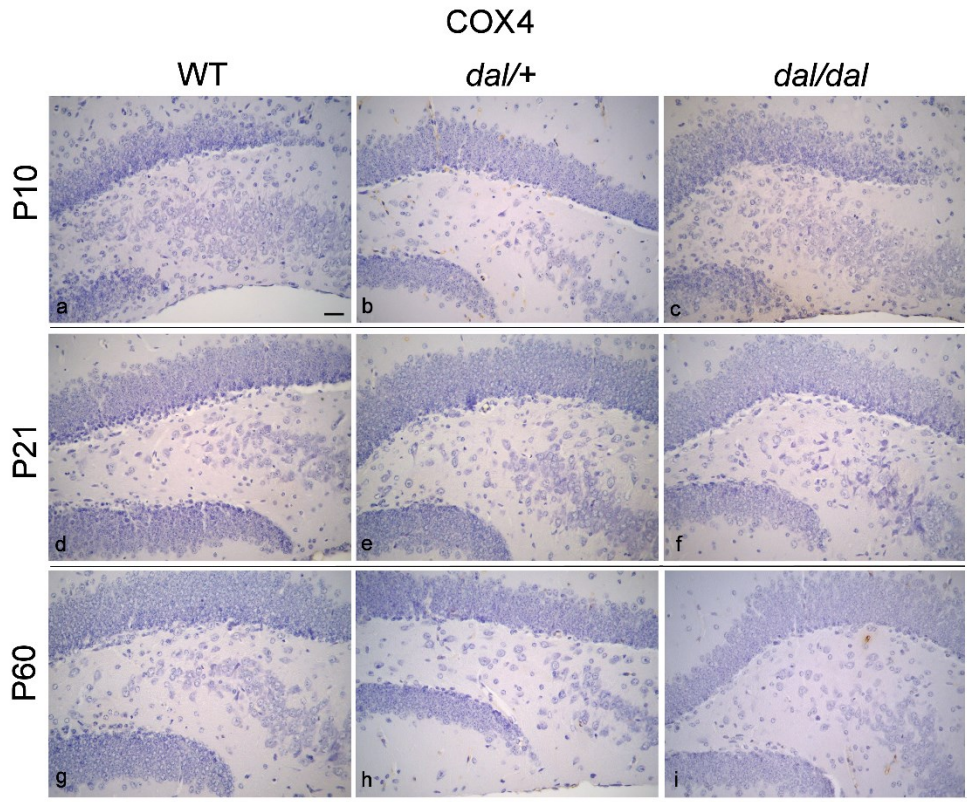


Figure 9. Immunohistochemical reaction for COX4 (Cytochrome c oxidase subunit 4) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm.

NOS2 is an inducible protein that already plays a pivotal role in inflammation mechanisms, through NO synthesis. This molecule, in the presence of ROS, gives rise to RNS, contributing to the increase of inflammation and oxidative stress. The results obtained from the analysis of NOS2 to P10 did not show significant differences when comparing WT and animals from the. The positivity was more visible in the control condition in granular neurons and mossy and decreased in homozygous and heterozygous genotypes (**Fig. 10a-c**). At PD21 and PD60 no positivity differences were shown between wt and dal (**Fig. 10b-f**). These results were confirmed by the analysis of the OD in which no statistically significant differences were shown both in GL and PL analysis (P10 GL OD values: WT 87.724 ± 11.45; *dal/+* 85.978 ± 10.41; *dal/dal* 97.142 ± 5.48; P10 PL OD values: WT 89.542 ± 2.55; *dal/+* 83.550 ± 9.96; *dal/dal* 98.729 ± 5.58) (**Fig. 10j, k**).

COX2 is a key enzyme in prostaglandin biosynthesis, significantly over-regulated during the immune response. Its expression is also closely related to oxidative stress phenomena. COX2 was used as a specific marker of the inflammatory and oxidative stress pathway [540]. At P10 the labelling for COX2 appeared very weak in WT while it increased considerably in the GL and PL of *dal/+* and *dal/dal* mice (**Fig. 11a-c**). The differences evaluated were statistically significant for both genotypes from compared to WT (P10 GL OD values: WT 69.778 ± 3.58; *dal/+* 85.340 ± 3.98; *dal/dal* 95.876 ± 9.70; P10 PL OD values: WT 67.631 ± 7.60; *dal/+* 87.950 ± 6.51; *dal/dal* 99.323 ± 3.30) (**Fig. 11j, k**). Interestingly, it is to be noted the diffuse positivity in the lower portion of the PL near the border with the pyramidal layer CA3. This positivity probably involves fibers that run through this layer and lead to the different areas of the *Cornus Ammonis*. The differences observed in mice at P21 were much lower than in P10 and the GL evaluations were not statistically significant while they became significant in the comparison of OD values measured in the PL layer of *dal/dal* mice (P21 GL OD values: WT 76.318 ± 6.86; *dal/+* 79.726 ± 2.44; *dal/dal* 85.647 ± 3.77; P21 PL OD values: WT 80.969 ± 4.42; *dal/+* 84.510 ± 3.68; *dal/dal* 94.559 ± 5.53) (**Fig. 11d-f, j, k**). In adult mice, on the other hand, the positivity was higher than in the other two ages and a statistically significant increase was observed between WT and heterozygous mice in the GL analysis alone (P60 GL OD values: WT 91.326 ± 4.570; *dal/+* 98.592 ± 3.43; *dal/dal* 81.659 ± 4.35; P60 PL OD values: WT 95.504 ± 4.34; *dal/+* 102.860 ± 3.72; *dal/dal* 105.744 ± 6.97) (**Fig. 11j, k**).

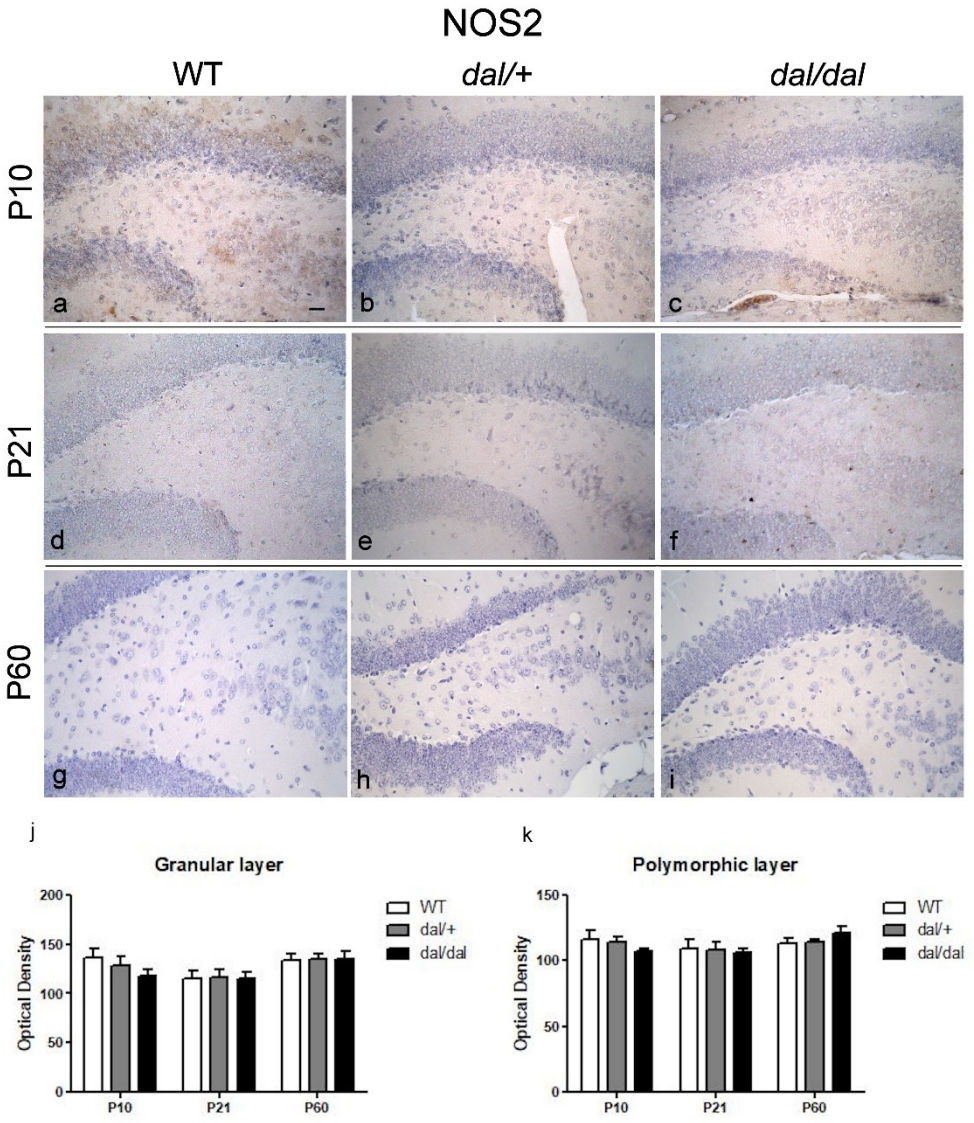


Figure 10. Immunohistochemical reaction for NOS2 (Nitric Oxide Synthase 2) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

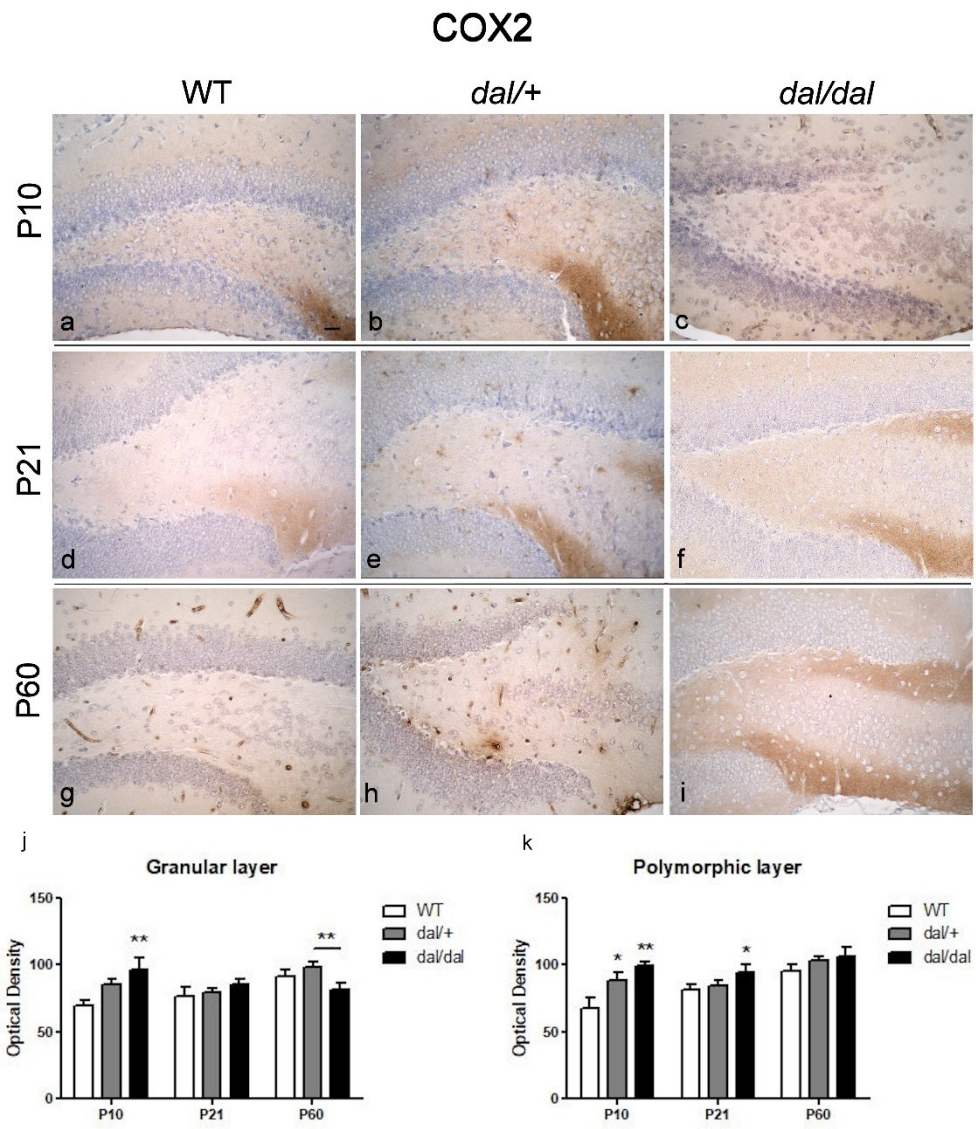


Figure 11. Immunohistochemical reaction for COX2 (Cyclooxygenase 2) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); p values, GL P10: WT *vs dal/+* and *dal/+* vs *dal/dal* (***) p <0.001; GL P21: WT *vs dal/+* (***) p <0.001, WT vs *dal/dal* (**) p <0.01; PL P10: WT *vs dal/+* and *dal/+* vs *dal/dal* (***) p <0.001, PL P21: WT *vs dal/+* (**) p <0.01, WT vs *dal/dal* (***) p <0.001, *dal/+* vs *dal/dal* (**) p <0.01. The same statistical analysis performed on the other OD values does not show any statistically significant differences, p >0.05.

4.1.3 The inflammatory pathway

IL-6 is a multifunctional cytokine with both pro-inflammatory and anti-inflammatory properties (Tanaka et al., 2014); in this thesis it has been used as a specific marker of the inflammatory pathway. The labelling for IL-6 showed no relevant differences when comparing the GL and PL of control and *dal* animals (**Fig. 12 a-i**). Furthermore, these results were confirmed by OD measurements with statistically no significant differences (P10 GL OD values: WT 112.555 ± 1.13; *dal/+* 117.113 ± 1.08; *dal/dal* 121.824 ± 9.28; P21 GL OD values: WT 110.649 ± 12.69; *dal/+* 116.531 ± 3.33; *dal/dal* 117.115 ± 0.40; P60 GL OD values: WT 119.321 ± 1.71; *dal/+* 122.936 ± 10.18; *dal/dal* 115.767 ± 6.15; P10 PL OD values: WT 113.766 ± 9.50; *dal/+* 98.508 ± 1.57; *dal/dal* 110.408 ± 8.56; P21 PL OD values: WT 97.511 ± 2.21; *dal/+* 106.347 ± 5.21; *dal/dal* 100.675 ± 9.71; P60 PL OD values: WT 108.142 ± 3.02; *dal/+* 109.251 ± 8.87; *dal/dal* 109.448 ± 4.77) (**Fig. 12j, k**).

TGF-β1 is the most abundant isoform of the TGF protein family and is ubiquitously expressed in organisms. This protein has numerous functions including regulating cell growth and differentiation during development. Furthermore, it can act as a chemotactic molecule for macrophages and lymphocytes thus participating in inflammatory responses. At P10, TGF-β expression appeared similar among the three genotypes. Following the postnatal development stages, the immunopositivity decreased at P21 to further increase at P60 while maintaining lower OD values than those detected in the previous timepoints evaluated (P10 GL OD values: WT 85.721 ± 1.69; *dal/+* 83.627 ± 5.83; *dal/dal* 89.139 ± 2.35; P21 GL OD values: WT 98.202 ± 25.74; *dal/+* 69.285 ± 4.01; *dal/dal* 64.335 ± 4.46; P60 GL OD values: WT 50.997 ± 2.73; *dal/+* 58.307 ± 2.96; *dal/dal* 74.303 ± 19.29) (**Fig. 13 a-j**). By analysing the PL, the WT OD values showed similarities between the three ages considered and, parallelly, a reduction in OD both of *dal/+* and *dal/dal* mice in the subsequent postnatal ages was highlighted (P10 PL OD values: WT 82.322 ± 1.43; *dal/+* 70.770 ± 7.22; *dal/dal* 76.210 ± 4.68; P21 PL OD values: WT 81.198 ± 11.97; *dal/+* 69.120 ± 4.01; *dal/dal* 70.310 ± 2.65; P60 PL OD values: WT 85.590 ± 3.02; *dal/+* 58.010 ± 7.02; *dal/dal* 71.792 ± 10.86) (**Fig. 13 a-i, k**).

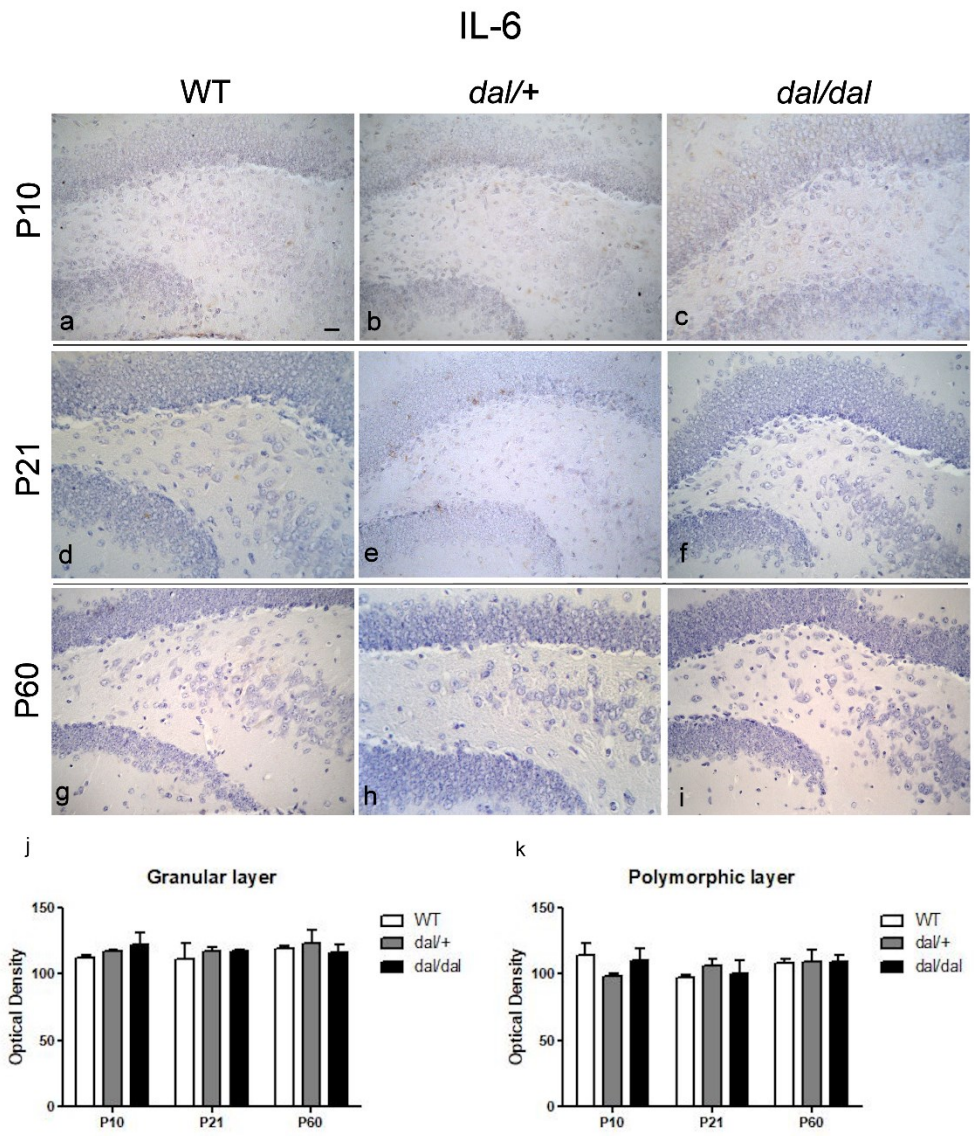


Figure 12. Immunohistochemical reaction for IL-6 (Interleukin 6) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

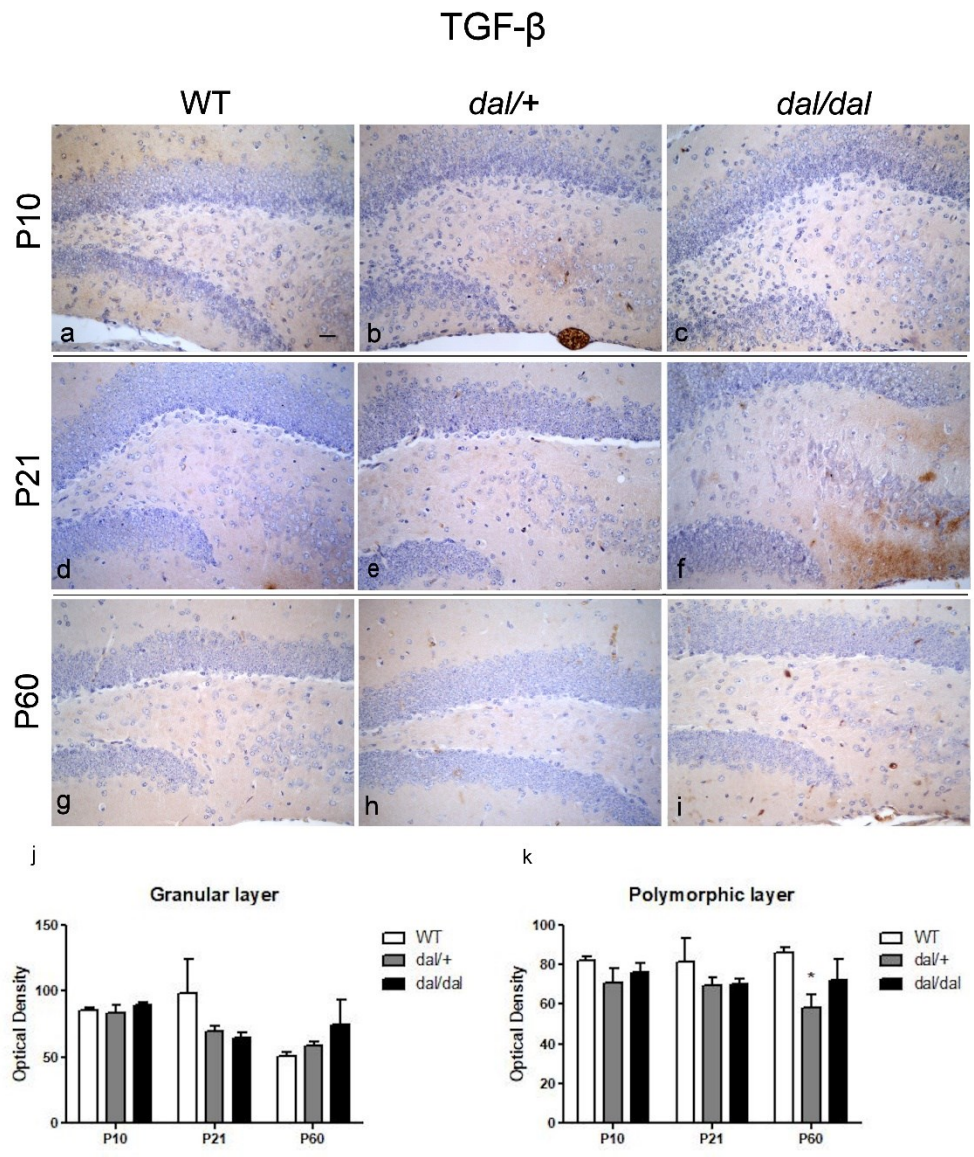


Figure 13. Immunohistochemical reaction for TGF-β (Transforming Growth factor β) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20μm. Histograms relative to the OD analysis of GL (j) and PL (k). Statistical analysis: WT vs each genotype and *dal/+* vs *dal/dal*; p values: (*) p <0.05, (**) p <0.01. Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); p values, PL P60: WT *vs dal/+* (*) p <0.05. The same statistical analysis performed on the other OD values does not show any statistically significant differences, p >0.05.

4.1.4 Evaluation of different cell death pathways

The persistence of a chronic oxidative state in the body can cause more extensive cell and tissue damage over time. Cells can activate defence mechanisms such as the production of antioxidant enzymes or the activation of programmed cell death pathways. The following sections will present the results obtained from the investigation of markers of apoptosis, autophagy and mitophagy.

Apoptosis is the most widespread type of programmed cell death used by cells to respond in a controlled manner to harmful situations.

First, the expression of **Bcl-2** was evaluated since its role as an antiapoptotic factor that prevents the activation of apoptotic death. At PD10, neither in the GL nor in the PL were evidenced differences in expression of the protein which appears to be scattered localized only in the cytoplasm of mossy cells (**Fig. 14a-c**). Similar results were obtained at PD21 where indeed it seems that the immunopositivity has further decreased compared to the previous assessed age. (**Fig. 14d-f**). At P60, however, a slight increase in positivity was noted in the mossy neurons of the PL of the mice compared to the control genotypes (**Fig. 14g-i**). The quantitative analysis conducted did not show statistically significant differences and the OD values were very close to each other in all genotypes and ages considered (P10 GL OD values: WT 130.326 ± 5.37; *dal/+* 131.069 ± 8.27; *dal/dal* 122.171 ± 6.54; P21 GL OD values: WT 123.970 ± 7.80; *dal/+* 120.839 ± 6.35; *dal/dal* 118.829 ± 8.06; P60 GL OD values: WT 127.752 ± 7.64; *dal/+* 137.075 ± 7.39; *dal/dal* 129.834 ± 6.15; P10 PL OD values: WT 116.050 ± 7.52; *dal/+* 108.013 ± 1.95; *dal/dal* 111.935 ± 4.14; P21 PL OD values: WT 112.230 ± 8.47; *dal/+* 92.117 ± 1.25; *dal/dal* 104.593 ± 8.73; P60 PL OD values: WT 118.552 ± 1.27; *dal/+* 109.393 ± 7.28; *dal/dal* 109.381 ± 2.94 (**Fig. 14j, k**).

After this analysis, the attention was focused on evaluating the expression of the pro-inflammatory molecule **Bax**. Similarly to Bcl-2 results, the immunopositivity of Bax did not provide very relevant and measurable differences among all the conditions examined. The measurements of the OD conducted confirmed the results shown in **Fig. 15 a-i** providing statistically no significant differences (P10 GL OD values: WT 118.820 ± 5.61; *dal/+* 130.225 ± 5.64; *dal/dal* 122.824 ± 4.87; P21 GL OD values: WT 131.330 ± 6.41; *dal/+* 129.873 ± 7.07; *dal/dal* 130.669 ± 7.16; P60 GL OD values: WT 132.528 ± 6.89; *dal/+* 132.844 ± 4.79; *dal/dal* 133.875 ± 7.26; P10 PL OD values: WT 114.010 ± 7.32; *dal/+* 115.637 ± 2.41; *dal/dal* 110.960 ± 5.93; P21 PL OD values: WT 110.177 ± 2.93; *dal/+* 103.814 ± 3.49; *dal/dal* 114.823 ± 2.09; P60 PL OD values: WT 118.922 ± 1.73; *dal/+* 112.921 ± 7.15; *dal/dal* 115.280 ± 7.27) (**Fig.15j, k)**.

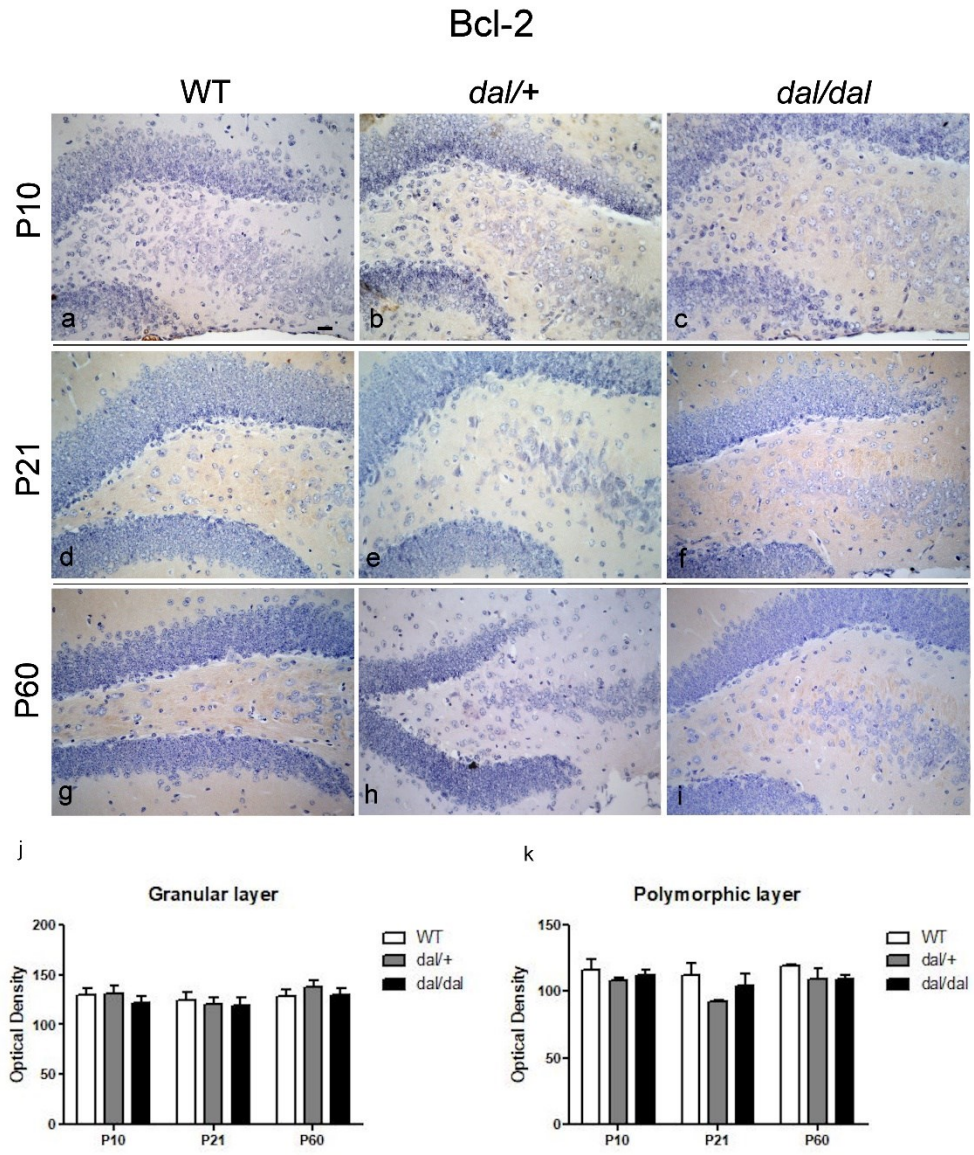


Figure 14. Immunohistochemical reaction for Bcl-2 in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

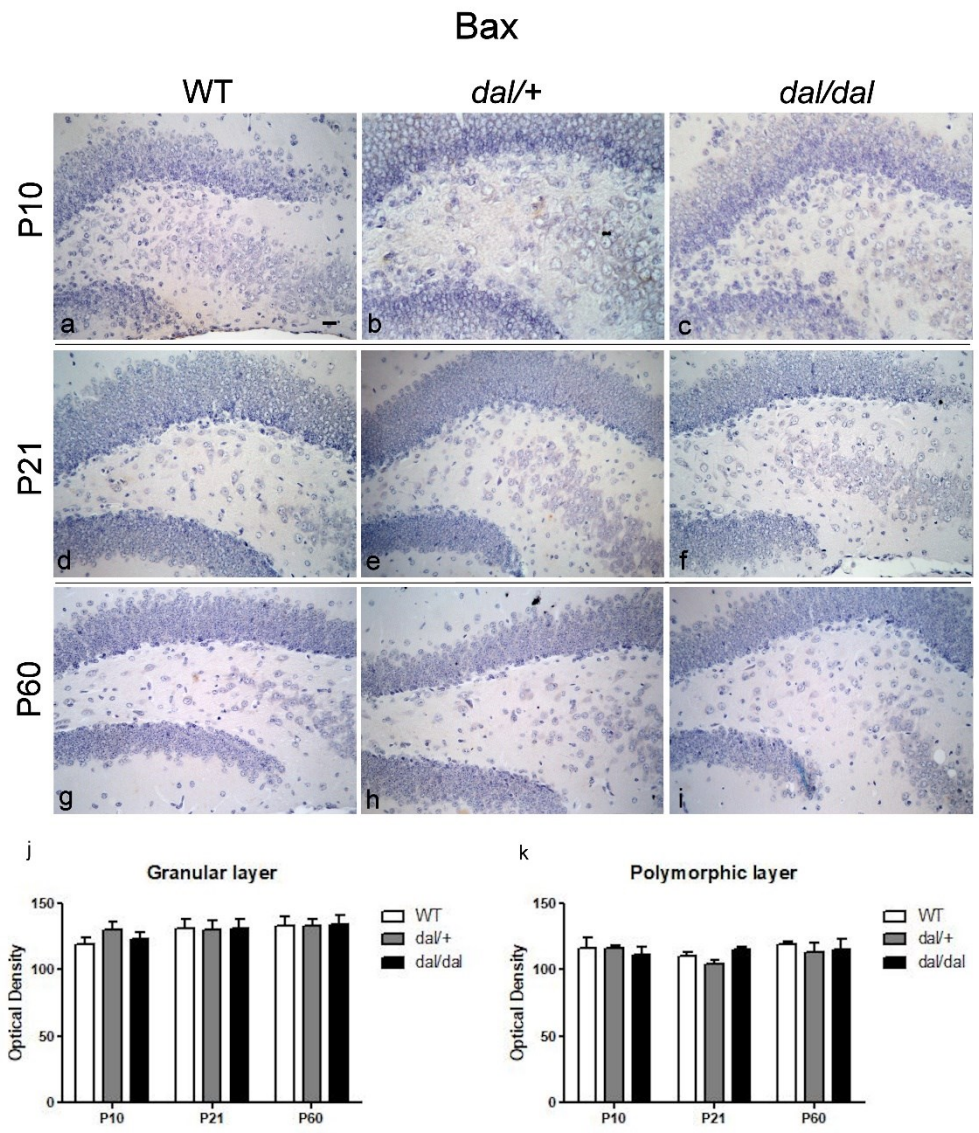


Figure 15. Immunohistochemical reaction for Bax in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20µm. Histograms relative to the OD analysis of GL (j) and PL (k). Significant differences between WT vs each genotype and *dal/+* vs *dal/dal* were determined using one-way ANOVA and Bonferroni's post-hoc test (N=3); the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

Autophagy is an energy-dependent programmed cell death process that is activated by different stress stimuli to ensure cell survival. This process occurs during rapid environmental changes such as, for example, nutrient deprivation, oxidative stress or during physiological mechanisms such as ageing [237]. SQSTM1 or **p62** is a protein commonly used as an autophagic marker that can be localized both in the cytoplasm and in the nucleus under normal conditions while translocate in the autophagosome during stressful conditions. In P10 mice, p62 labelling was mainly localized in neurons of the PL and to a lesser extent also in some areas of the GL. In **Fig. 16, 17a-c** the images obtained in WT P10 mice highlighted that a weak expression in DG neurons that increased in the cell cytoplasm of heterozygous and homozygous mice (panel c, white arrows). At P21 the findings were controversial. In fact, the immunofluorescence for p62 seems to highlight a high expression already in the WT animals that then increase in both genotypes affected by PD (**Fig. 16, 17d-f**). It should be noted that in both these timepoints the expression of p62 seems to involve more heterozygous mice than the other condition (**Fig. 16, 17b, e**). In panels **g-i**, the results obtained at P60 showed a considerable decrease in immunopositivity compared to the ages before considered, while maintaining the trend of differences already noted with higher expression in the GL and PL of the genotypes *dal* (white arrows) compared to the control animals (**Fig. 16, 17g-i**). For p62 marker the quantitative analysis was not performed (ongoing studies).

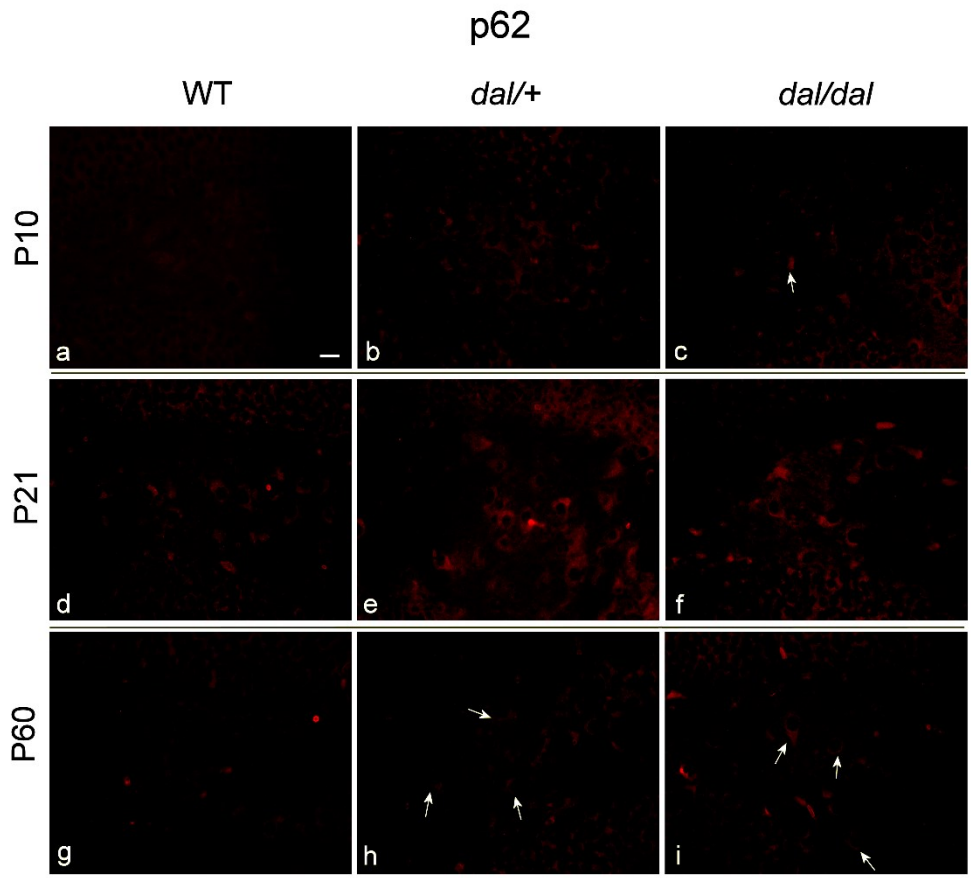


Figure 16. Immunofluorescence reaction for p62 (red fluorescence) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Magnification of the objective: 40x. Bar: 20 µm.

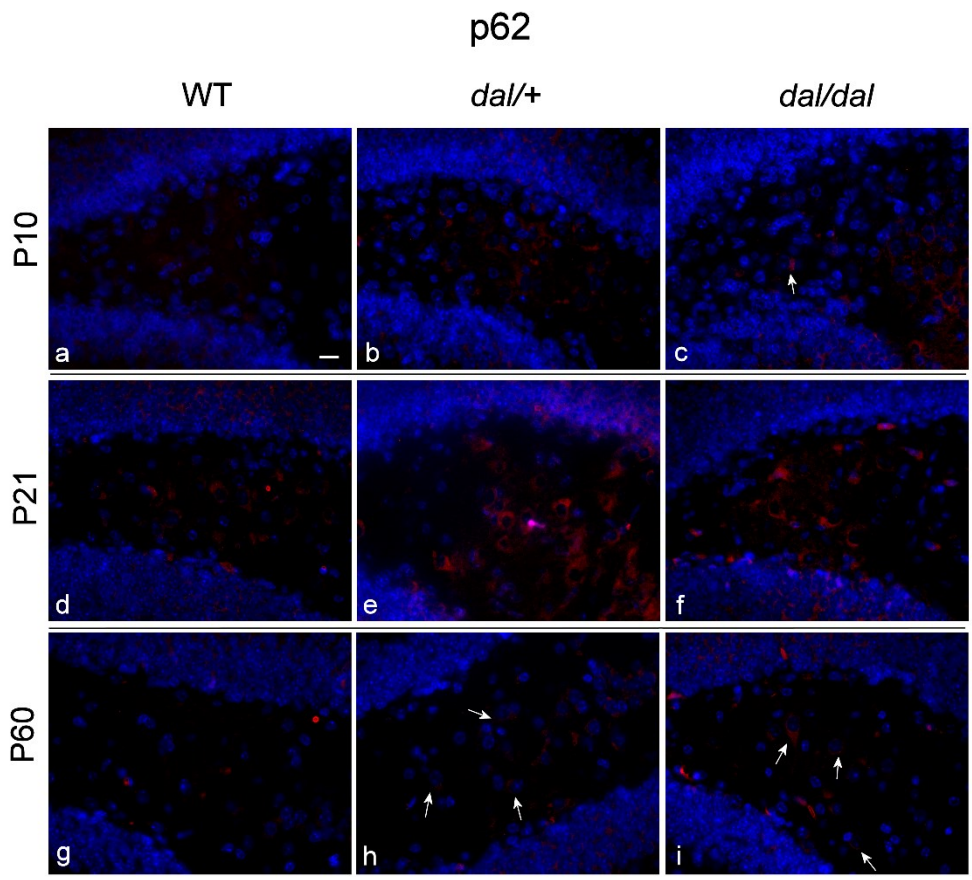


Figure 17. Immunofluorescence reaction for p62 (red fluorescence) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i). Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bar: 20 μm.

Mitophagy is a type of autophagy affecting the mitochondria and related to neuroinflammation and neurodegenerative diseases. The **PINK1** protein is recruited on the outer mitochondrial membrane (OMM) during ROS accumulation and mitochondrial damage. From the preliminary results obtained on PD mice at P10, the immunofluorescence for PINK1 was very weak in WT animals and from a preliminary analysis it did not seem to colocalize with the labelling of mitochondria (**Fig. 18d-f**). The PINK1 signal then appeared to be increased in heterozygous and homozygous mice. In particular, in *dal/+* mice some cells scattered in the GL and PL layers showed colocalization between the mitochondrial signal and the PINK1 protein (**Fig. 18d-f**, white arrow). In *dal/dal* animals the labelling did not provide evaluable results (**Fig. 18g-i**). At P60, the mitochondrial signal appears homogeneous across the three genotypes. Preliminary results for **PINK1** protein showed colocalization between mitochondria and protein already in WT mice (**Fig. 19a-c**, white arrows). Since we had not used a specific marker for OMM, it was not possible to understand whether the protein is localized on the membrane or is inside the mitochondrion. In control animals there was also a large immunopositivity for PINK1 in the cytoplasm of mossy neurons without colocalization with mitochondria. In *dal/+* and *dal/dal* mice there seems to be an increase in mossy neurons and PINK1 immunolabelling suggested a localization in the mitochondria (**Fig. 19d-f**). Furthermore, in **Fig. 19g-i** it is possible to observe an altered morphology of the mossy neurons which appeared shrivelled (**pictures h, i**, white arrows). For PINK1 marker the quantitative analysis was not performed (ongoing studies).

Once PINK1 is activated it inserts itself into the OMM and recruits **PARKIN** which in turn will trigger the addition of ubiquitin residues on specific mitochondrial targets and therefore mitophagy. In animals at P10, the immunofluorescence for PARKIN did not appear to colocalize with the mitochondrial signal in control mice, while the presence of double-labelled neurons was increased in heterozygous and homozygous mice (**Fig. 20**). At P60, the results obtained did not show noticeable differences with respect to the age previously analysed. Again, an increase in mossy neurons PARKIN expression could be observed and the mitochondrion and PARKIN signal colocalized (**Fig. 21**). For PARKIN marker the quantitative analysis was not performed (ongoing studies).

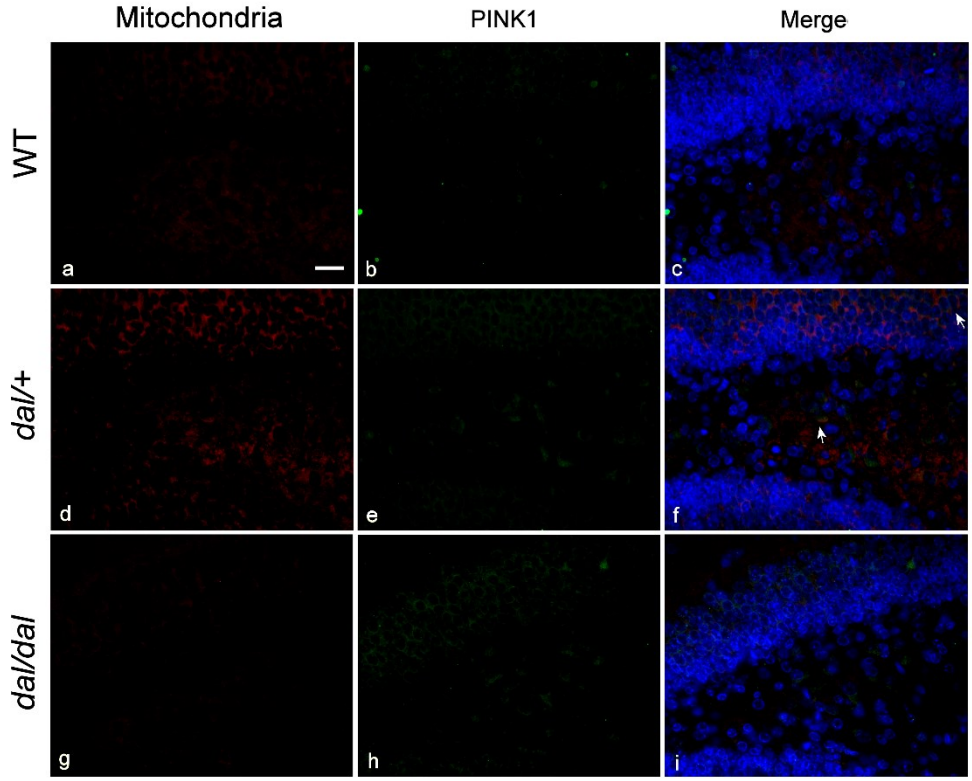


Figure 18. Double immunofluorescence reaction for PINK1 (green fluorescence) and mitochondria (red fluorescence) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i) at P10. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bar: 20 µm.

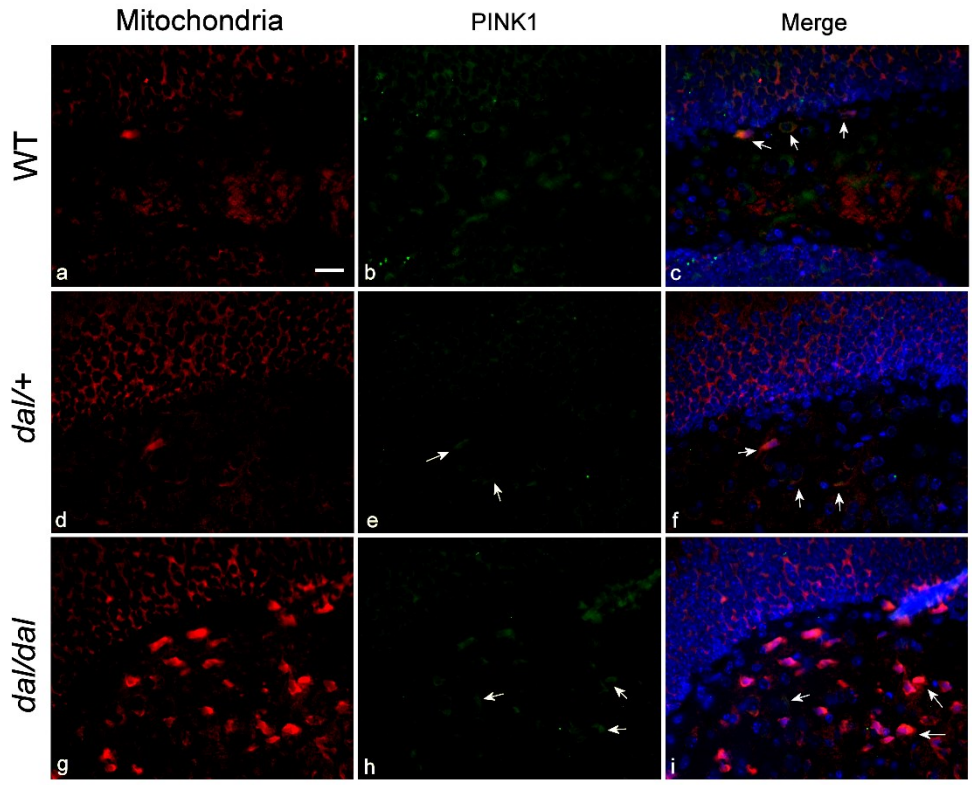


Figure 19. Double immunofluorescence reaction for PINK1 (green fluorescence) and mitochondria (red fluorescence) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i) at P60. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bar: 20 µm.

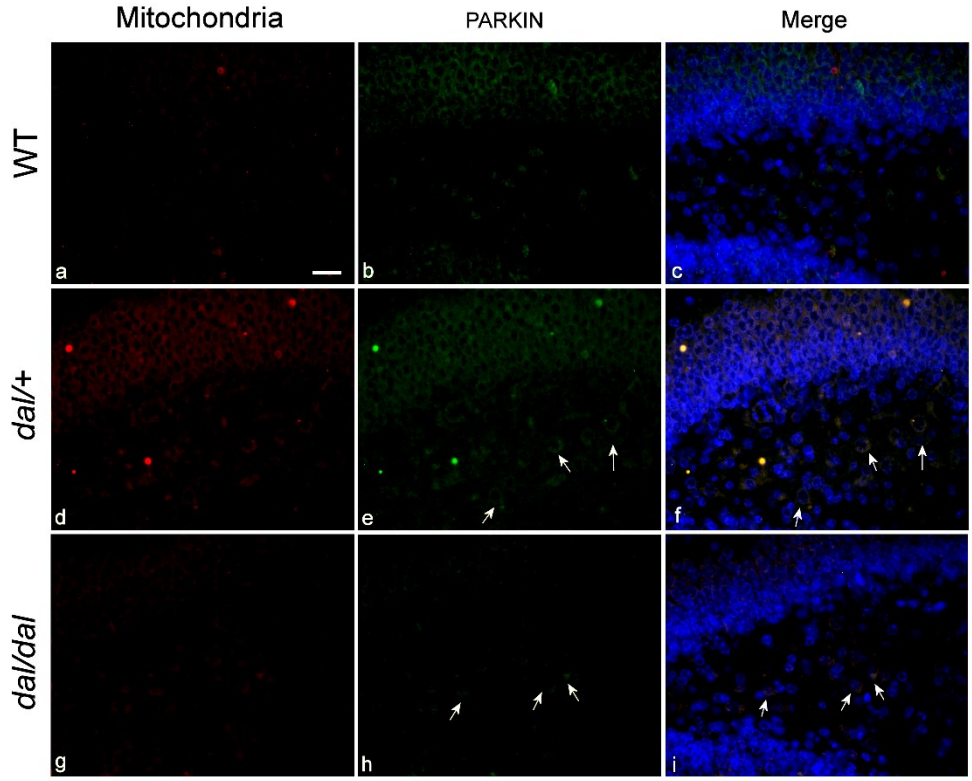


Figure 20. Double immunofluorescence reaction for PARKIN (green fluorescence) and mitochondria (red fluorescence) in WT (a, d, g), *dal/+* (b, e, h), and *dal/dal* (c, f, i) at P10. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bar: 20 µm.

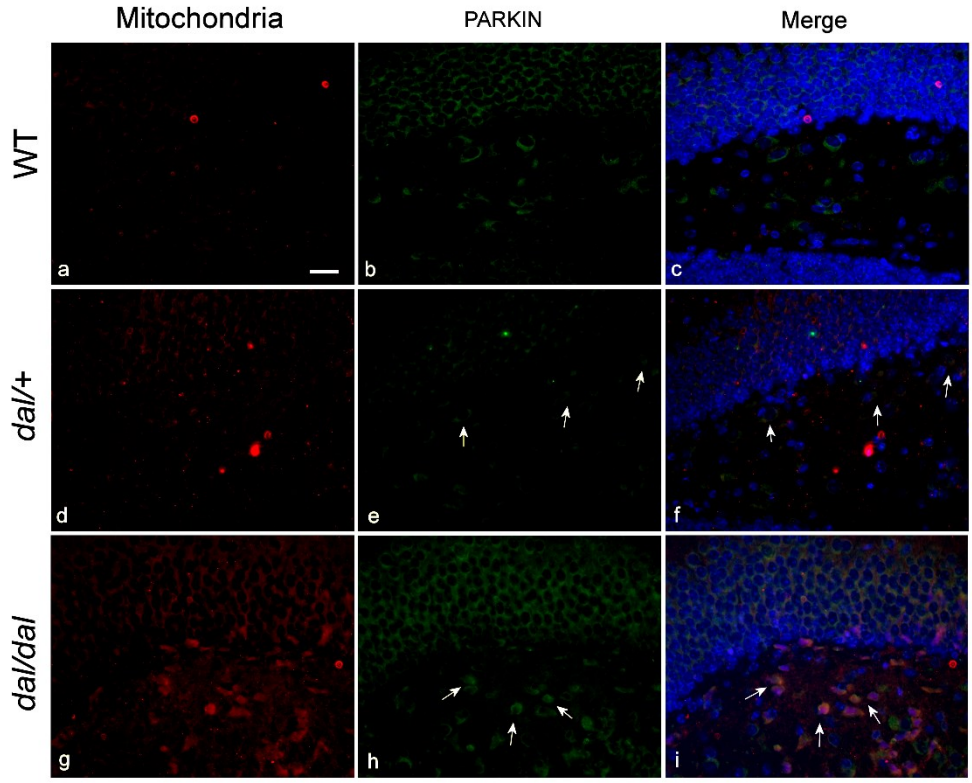


Figure 21. Double immunofluorescence reaction for PARKIN (green fluorescence) and mitochondria (red fluorescence) in WT (a, d, g), *dal*/+ (b, e, h), and *dal/dal* (c, f, i) at P60. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bar: 20 µm.

Marker	P10			P21			P60		
	WT	***dal/+***	***dal/dal***	**WT**	***dal/+***	***dal/dal***	**WT**	***dal/+***	***dal/dal***
SOD1	-	-	-	-	+	+	-	-	+
SOD2	-	+++	+	-	++	++	-	-	+
COX4	-	-	-	-	-	-	-	-	-
NOS2	-	-	-	-	-	-	-	-	-
COX2	-	+	++	-	+	+	-	+	-
IL-6	-	-	-	-	-	-	-	-	-
TGF-β	-	-	-	-	-	-	-	-	-
Bax	-	-	-	-	-	-	-	-	-
Bcl-2	-	-	-	-	-	-	-	-	-
p62	-	+	+	-	+	-	-	+	+
PINK1	-	-	-	/	/	/	-	+	+
PARKIN	-	+	-	/	/	/	-	+	++

Table 1. Summary of the immunohistochemical results considering the Granular Layer of PD mice. To express the expected results (increasing of oxidative stress, inflammation, and cell death molecules) the hyphen and + symbols were used, expressed from basic condition (-) to maximum (+++).

Marker	P10			P21			P60		
	WT	***dal/+***	***dal/dal***	**WT**	***dal/+***	***dal/dal***	**WT**	***dal/+***	***dal/dal***
SOD1	-	+	+	-	+	+	-	-	+
SOD2	-	++	+	-	+	++	-	-	-
COX4	-	-	-	-	-	-	-	-	-
NOS2	-	-	-	-	-	-	-	-	-
COX2	-	+	++	-	+	+	-	+	+
IL-6	-	-	-	-	-	-	-	-	-
TGF-β	-	-	-	-	-	-	-	-	-
Bax	-	-	-	-	-	-	-	-	-
Bcl-2	-	-	-	-	-	-	-	-	-
p62	-	+	+	-	+	+	-	+	+
PINK1	-	+	-	/	/	/	-	+	++
PARKIN	-	+	+	/	/	/	-	+	++

Table 2. Summary of the immunohistochemical results considering the Polymorphic Layer of PD mice. To express the expected results (increasing of oxidative stress, inflammation, and cell death molecules) the hyphen and + symbols were used, expressed from basic condition (-) to maximum (+++).

4.2 Evaluation of the adjuvant effects of mycotherapy in ageing

For the ageing project, the expression of antioxidant proteins, the inflammatory pathway and cell death molecules were investigated in 23.5 months old physiologically aged mice and mice whose diets were enriched for 2 months with standardized extracts of *H. erinaceus*, a MM with known anti-inflammatory and immunomodulators properties [449, 541]. Furthermore, studies recently conducted in our laboratory have highlighted the ability of *H.erinaceus* extracts to improve cognitive decline in frailty mice and to perform neurotrophic functions [9]. Investigations were conducted on sections of frailty mouse hippocampus and the analyses focused on the neurons that compose the dentate gyrus (DG).

4.2.1 *The dentate gyrus morphology*

The evaluation carried out through H&E did not show substantial differences in the cytoarchitecture of the entire DG and of the neurons belonging in the GL and PL (**Fig. 22a, b**). On the other hand, the results of Nissl staining have highlighted the presence of cytoplasmic extensions of the mossy neurons which in the mice supplemented with *H.erinaceus* extracts appeared more numerous and developed than in the control animals (**Fig. 22c, d**). These data agree with the beneficial and neurotrophic effects already reported in the literature.

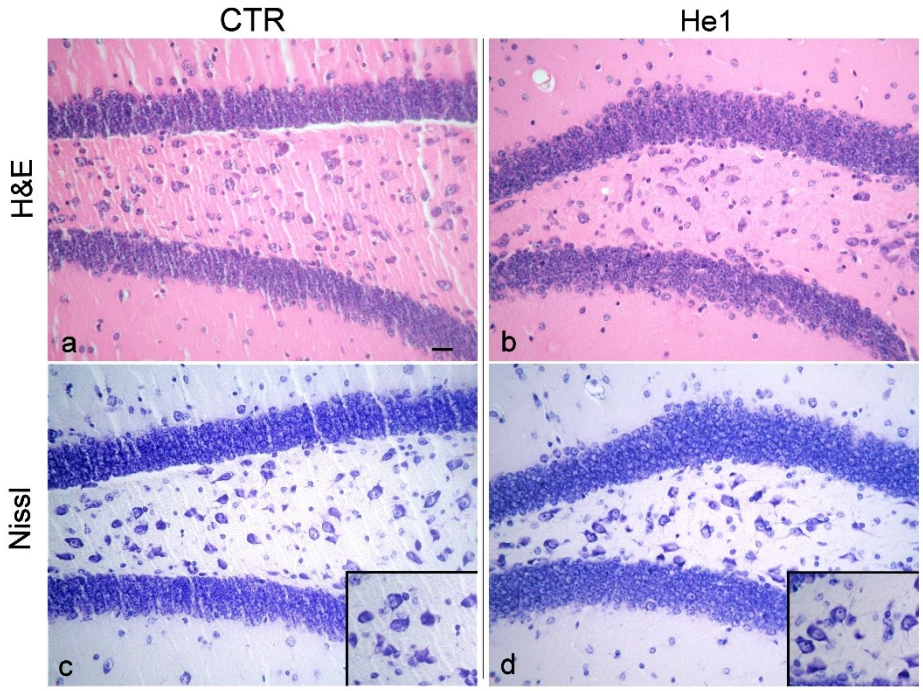


Figure 22. Haematoxylin and eosin (H&E) and Nissl staining of hippocampal dentate gyrus (DG) in aged mice (a, c) and supplemented animals (b, d). Bar: 20µm.

4.2.2 The anti-oxidative stress enzymes

Oxidative stress is one of the major factors involved in neurodegeneration associated with ageing. As in the case of PD, the expression of SOD1 as a specific marker of oxidative stress pathways was evaluated in this project.

The immunohistochemical reactions highlighted the **SOD1** expression in the GL and in the PL of the DG of both experimental conditions. The neurons of the GL showed a cytoplasmic immunopositivity that appear higher in He1 treated mice compared to the control and principally localized in an area attributable to the SGZ (**Fig. 23a, b**). Furthermore, a statistically significant increase of OD in the supplemented animals compared to the control conditions have been observed (GL OD values: CTRL 98.286 ± 1.92; *He1* 105.697 ± 3.25) (**Fig. 23e**). Similarly, the cytoplasm of polymorphic neurons expressed the protein with minor differences between control and treated conditions (**Fig. 23c, d**). The quantitative analysis performed on these cells reported a slight increase in SOD1 immunopositivity in He1 mice compared to controls with no statistical significance (PL OD values: CTRL 108.590 ± 3.48; *He1* 113.280 ± 2.52) (**Fig. 23f**).

SOD2 plays central role in cell development and appears to be involved in life span duration. The localization of SOD2 appeared to be absent in granular cells and is mostly observed, even if in low levels, in some neurons of the PL with slight differences between the two conditions (**Fig. 24a-d**). The quantitative analysis of granular and polymorphic DG layers correlates with the observed results and showed no significant variations in the SOD2-immunopositivity of He1 mice and control animals (GL OD values: CTRL 103.868 ± 2.51; *He1* 102.211 ± 1.11, PL OD values: CTRL 102.424 ± 4.72; *He1* 105.606 ± 2.35) (**Fig. 24e, f**).

Since **COX4** expression was not detectable, no evaluable results were obtained from the analysis of COX4 expression in He1 treated mice and control condition (**Fig. 25**).

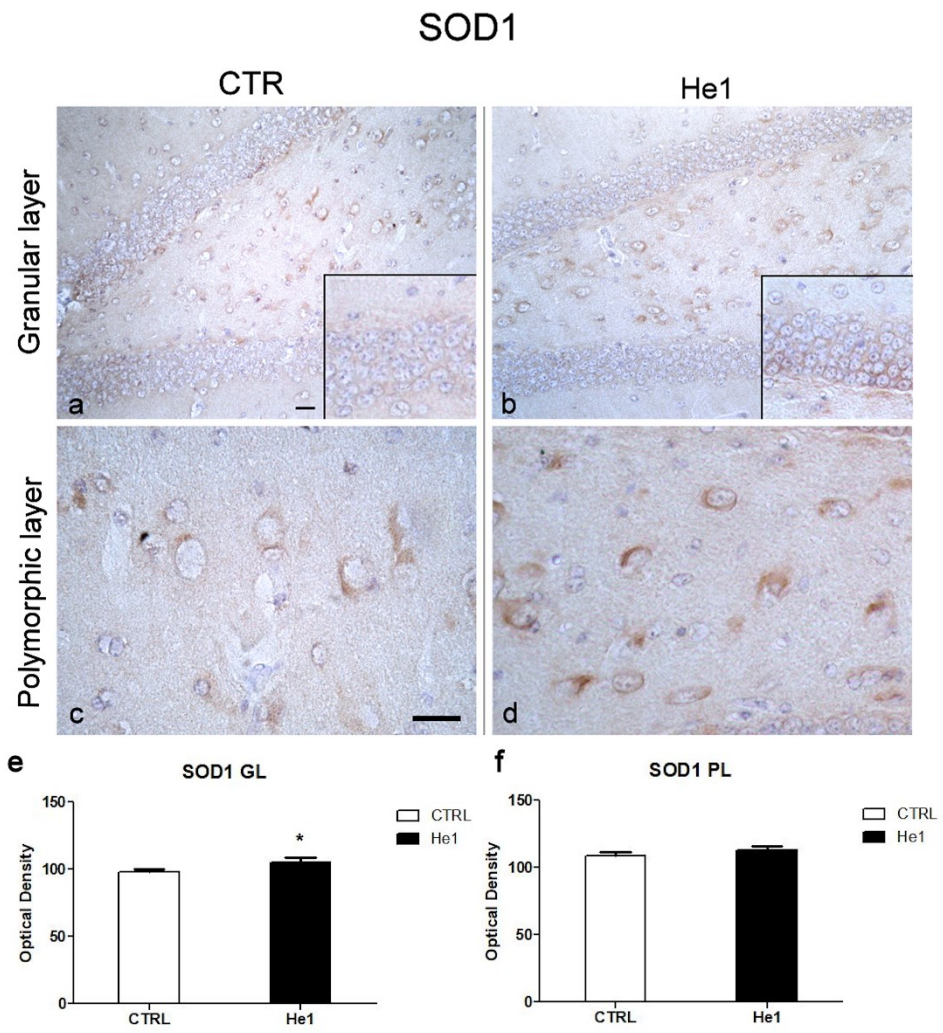


Figure 23. Immunohistochemical reaction for SOD1 (Superoxide dismutase 1) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for SOD1 in GL and PL (e, f; respectively); $p < 0.05$ (*). Significant differences between groups were determined using Student's t-test (N=3), p values, GL CTRL vs He1, (*) $p=0.027$. The same statistical analysis performed on the other OD values does not show any statistically significant differences, $p > 0.05$.

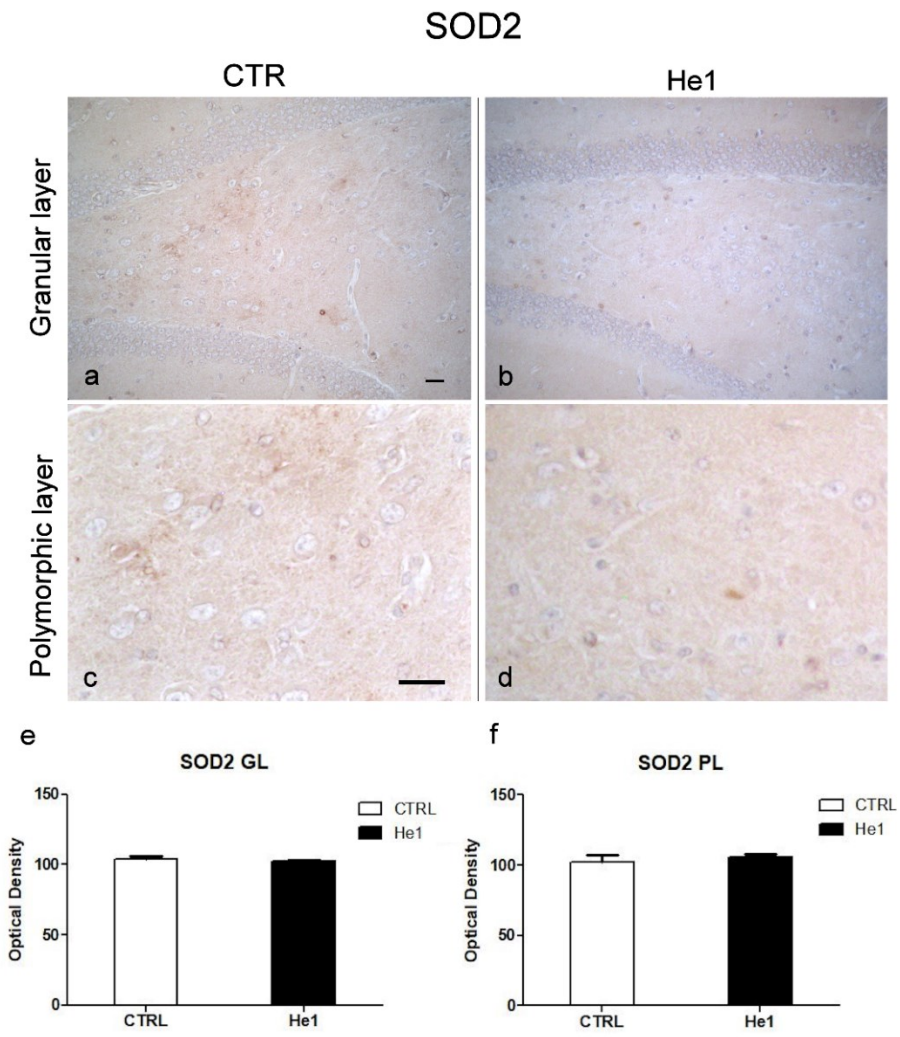


Figure 24. Immunohistochemical reaction for SOD2 (Superoxide dismutase 2) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for SOD2 in GL and PL (e, f; respectively). Significant differences between control and He1 treated mice were determined using Unpaired Student's t-test (N=3), the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

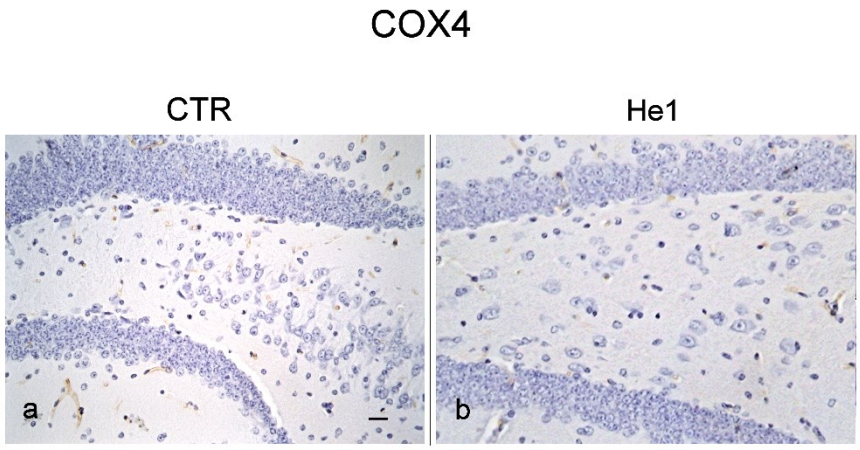


Figure 25. Immunohistochemical reaction for COX4 (Cytochrome c oxidase subunit 4) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b) and untreated control animals (CTR) (a). Magnification of the objective: 40x (a, b. Bars: 20 μm.

NOS2 was used as a specific marker of oxidative stress pathways in ageing conditions. As shown in Fig. 19, the immunoreactivity for NOS2 appears much more intense in control animals than in treated mice (**Fig.26a-d**). Furthermore, this expression was observed widespread in a thin strip of tissue located at the edge between the GL and PL and possibly interests the mossy fibers directed to the CA3 area of the hippocampus (**Fig. 26a, b**). The OD analysis showed a significant decrease in NOS2-immunopositivity of treated mice compared to healthy controls (GL OD values: CTRL 111.519 ± 1.87; *He1* 106.035 ± 2.46) (**Fig. 26e**). Regarding the PL analysis, on the other hand, the positivity does not seem to concern the mossy neurons in which significant OD differences were not observed (PL OD values: CTRL 107.002 ± 2.51; *He1* 108.171 ± 3.12) (**Fig. 26c, d, f**). However, in this layer it should be noted the presence of $NOS2^{+}$ fibers probably attributable to the cytoplasmic extensions of the granular cells that take part in the hippocampal circuit.

Regarding **COX2**, in the GL, a substantial expression of the protein was not highlighted and widely expressed within the layer (**Fig. 27a, b**). Quantitative analysis however revealed a decrease in COX2 expression in treated mice compared to controls with no statistical differences (GL OD values: CTRL 101.928 ± 1.11; *He1* 98.796 ± 1.68) (**Fig. 27e**). In the hippocampus of the mice treated with He1, the immunolabeling for COX2 appeared localized in the soma of the mossy neurons within the PL, even if compared to the control animals, the treated mice had less widespread and marked immunoreactivity (**Fig. 27c, d**). Quantitative analysis showed a slight decrease in OD in supplemented animals compared to controls, but the data are not statistically significant (PL OD values: CTRL 108.511 ± 1.08; *He1* 109.181 ± 1.48) (**Fig. 27f**).

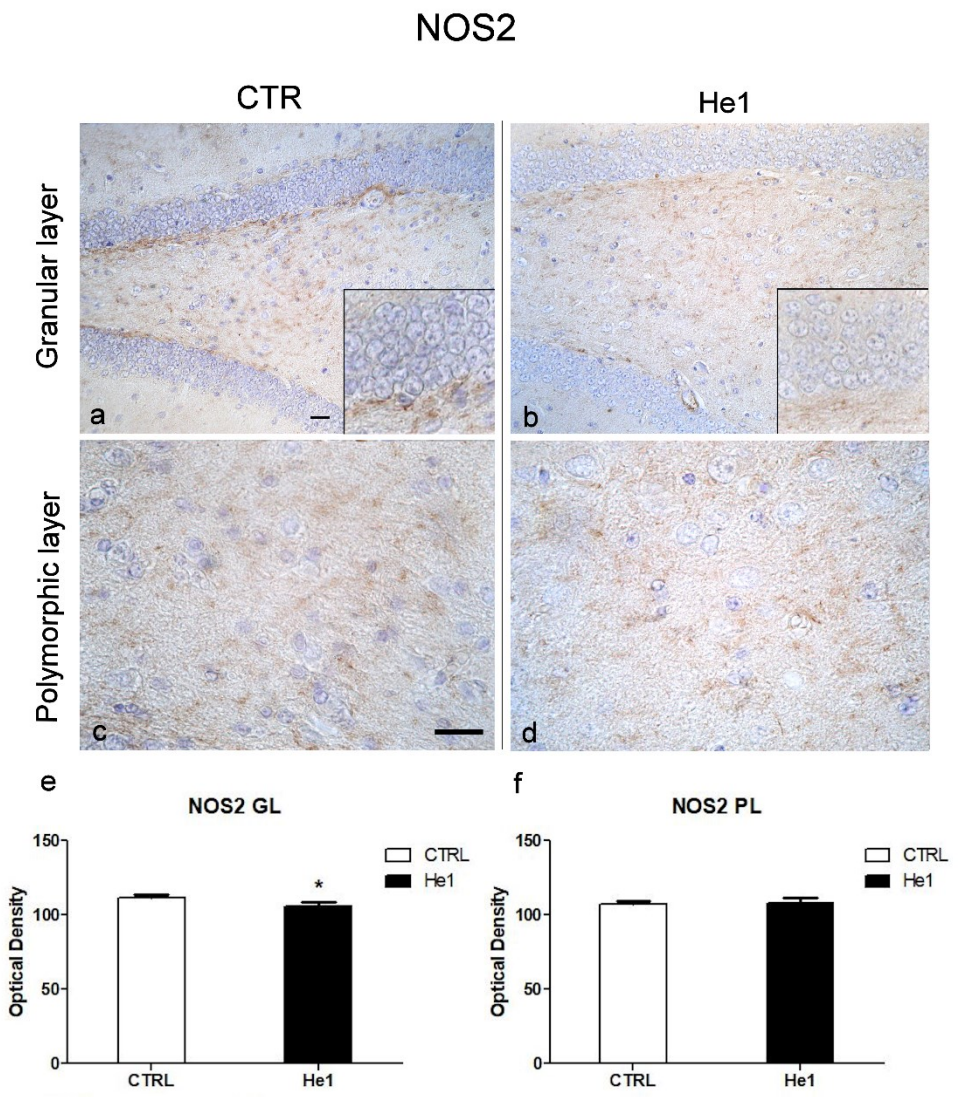


Figure 26. Immunohistochemical reaction for Nitric Oxide Synthase (NOS2) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for NOS2 in GL and PL (e, f; respectively), $p < 0.05$ (*). Significant differences between groups were determined using Student's t-test (N=3), p values, GL CTRL vs He1, (*) p= 0,037. The same statistical analysis performed on the other OD values does not show any statistically significant differences, $p > 0.05$.

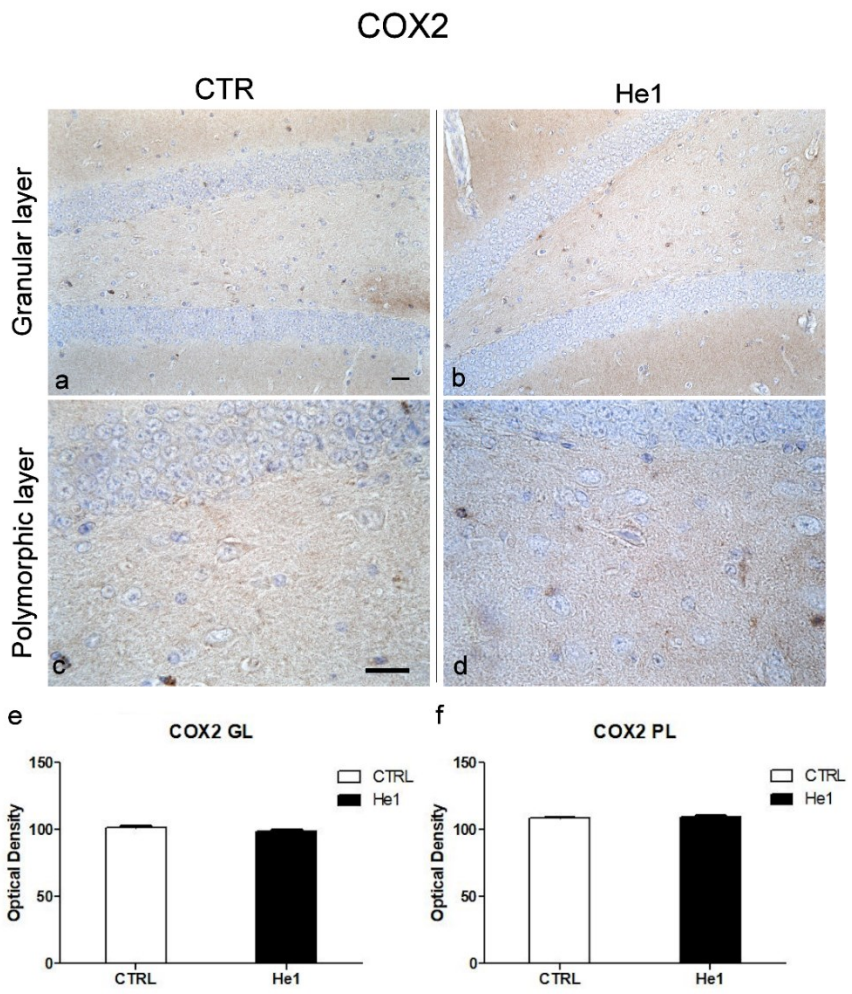


Figure 27. Immunohistochemical reaction for Cyclooxygenase 2 (COX2) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for COX2 in GL and PL (e, f; respectively). Significant differences between control and He1 treated mice were determined using Unpaired Student's t-test (N=3), the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

4.2.3 The inflammaging pathway evaluations

In He1-treated mice, the immunolabeling for **IL-6** and the OD appeared slightly decreased in the GL of the DG, compared to the control animals (GL OD values: CTRL 101.927 ± 2.44; *He1* 99.415 ± 1.69) (**Fig. 28a, b, e**). In the PL of the DG, the positivity of IL-6 is localized in the cytoplasm of some mossy neurons with a slightly lower expression regarding the supplemented animals (**Fig. 28c, d**). The quantitative values show a statistically significant decrease in IL-6 immunoreactivity in He1 mice respect to healthy aged conditions (PL OD values: CTRL 111.368 ± 1.40; *He1* 98.679 ± 4.05) (**Fig. 28f**).

For **TGF-β** analysis, as shown in **Figure 29**, in He1-treated GL, a slight non-significant decrease in the protein expression was observed compared to control animals (GL OD values: CTRL 110.859 ± 2.42; *He1* 109.876 ± 2.34) (**Fig. 29a, b, e**). The expression of TGF-β was also decreased in the PL of the mice that received the supplement compared to healthy aged mice with again no statistically significant differences among the conditions (PL OD values: CTRL 104.653 ± 1.92; *He1* 102.706 ± 0.953) (**Fig. 29c, d, f**).

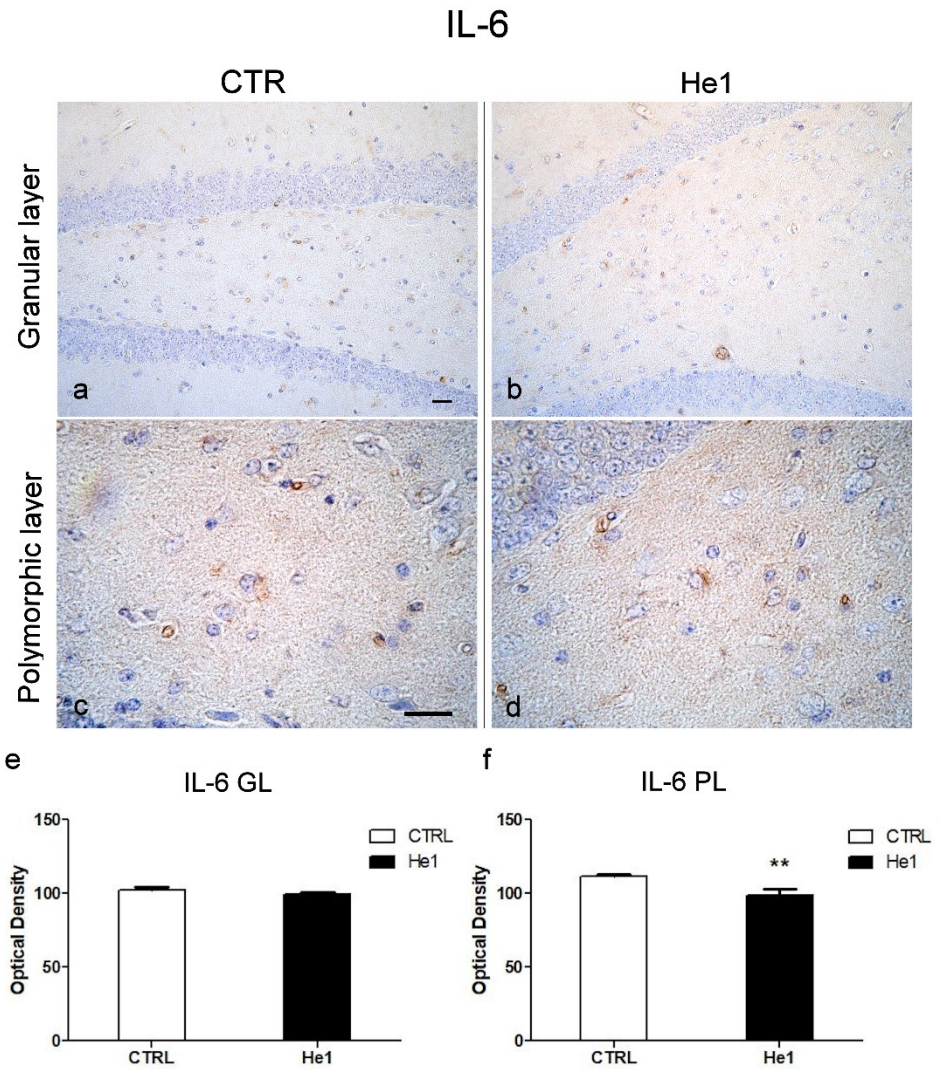


Figure 28. Immunohistochemical reaction for Interleukin 6 (IL-6) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for IL-6 in GL and PL (e, f; respectively), $p < 0.01$ (**). Significant differences between groups were determined using Student's t-test (N=3), p values, PL CTRL vs He1 (**) p= 0,007. The same statistical analysis performed on the other OD values does not show any statistically significant differences, $p > 0.05$.

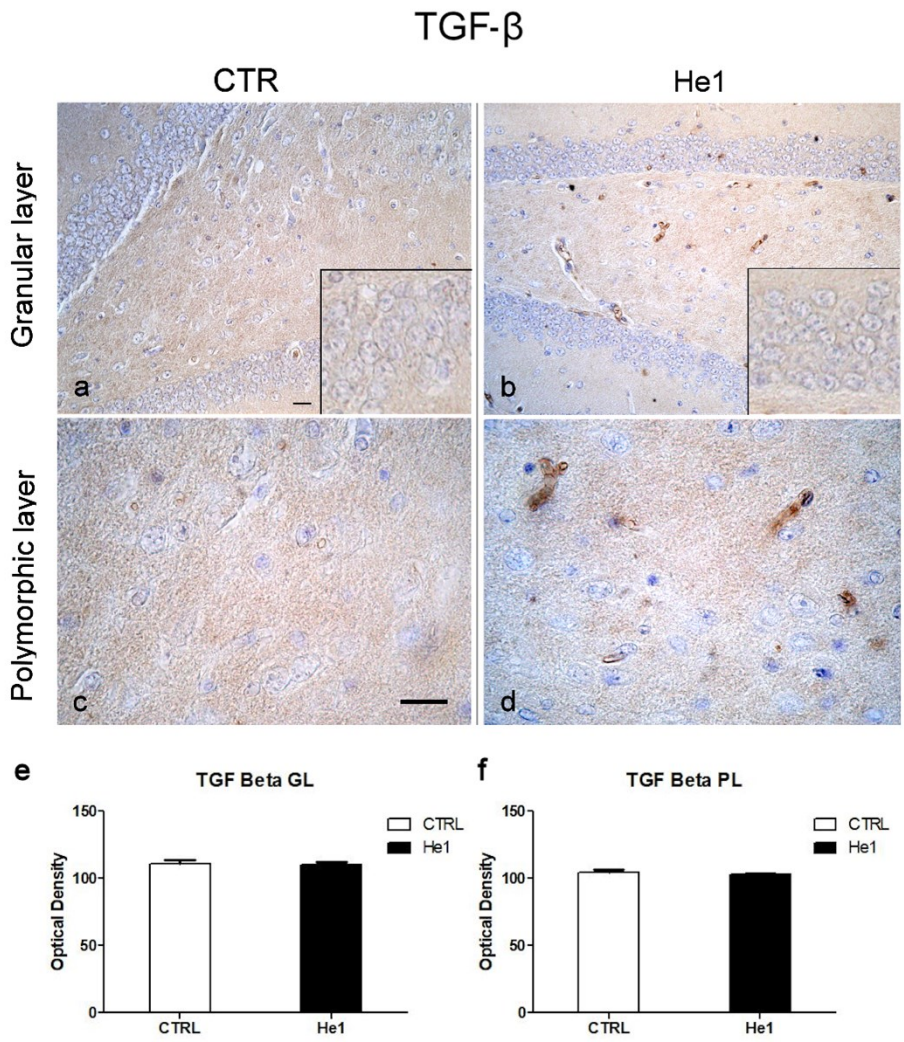


Figure 29. Immunohistochemical reaction for Transforming Growth Factor-β (TGF-β) after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 μm. Histograms relating to the analysis of the OD for TGF-β in GL and PL (e, f; respectively). Significant differences between control and He1 treated mice were determined using Unpaired Student's t-test (N=3), the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

4.2.4 Evaluation of different cell death pathways in ageing

To consider the role of cell death in ageing, it was decided to investigate the pathways of apoptosis, autophagy an mitophagy.

Regarding apoptosis, antibodies were used for the **Bax** proteins, pro-apoptotic factor and **Bcl-2**, with an anti-apoptotic effect. As shown in **Figure 30a, b**, Bax was widely expressed in the cytoplasm of neurons that were located throughout the GL and in mossy neurons especially in mice treated with the He1. The OD analysis showed a slight increase in immunopositivity in the treated condition compared to the control one with values that were not statistically significant (GL OD values: CTRL 108.590 ± 3.48; *He1* 113.280 ± 2.52, PL OD values: CTRL 120.769 ± 5.11; *He1* 123.300 ± 4.68) (**Fig. 30e, f**). From the analysis of the slices incubated for Bcl-2 no positive polymorphic neurons were highlighted (GL OD values: CTRL 128.469 ± 8.15; *He1* 118.822 ± 6.93, PL OD values: CTRL 115.114 ± 4.75; *He1* 116.508 ± 4.96) (**Fig. 30g, h**).

The **p62** protein was used as a marker of autophagy and was investigated by immunofluorescence analysis. In the control condition, p62 was localized in the cytoplasm of polymorphic neurons (**Fig. 31a-c**). After treatment with He1, the immunofluorescence of the protein increased and becomes evident also in the granular cells of the DG (**Fig. 31d-f**). For p62 marker the quantitative analysis was not performed (ongoing studies).

PINK1 and **PARKIN** were used as markers of mitophagy and were both investigated by immunofluorescence analysis. As for the p62 protein, PINK1 under control conditions was mainly expressed in the cytoplasm of mossy neurons. After supplementation with *H.erinaceus* extracts, the protein increased its expression and was also evident in the GL neurons (**Fig. 32**). The analysis of the immunopositivity for PARKIN showed the presence of the protein in polymorphic cells but, unlike PINK1, also in the granular neurons of control mice. In general, the immunopositivity for PARKIN is increased in mice aged with the supplement compared to mice that have not received treatment (**Fig. 33**). For PINK1 and PARKIN markers the quantitative analysis was not performed (ongoing studies).

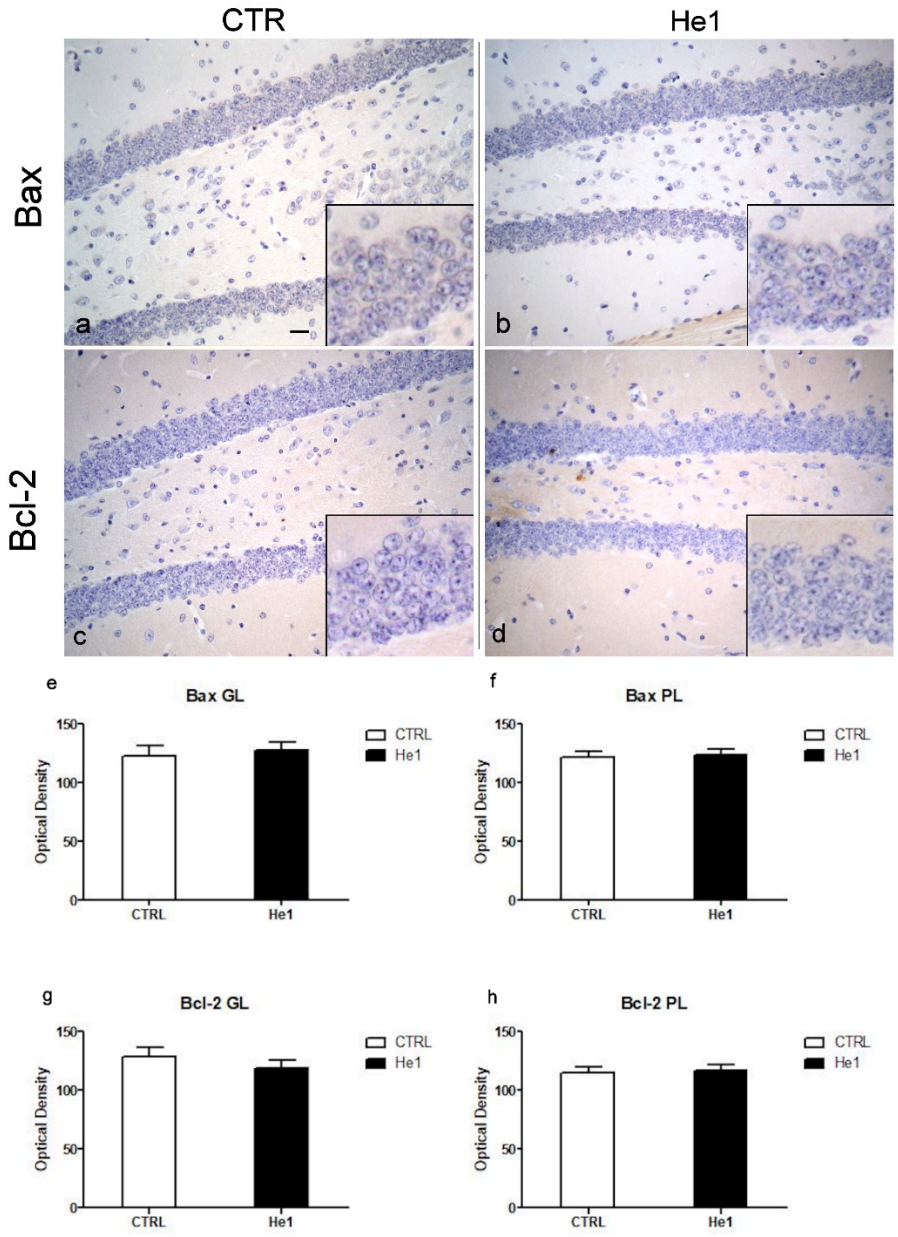


Figure 30. Immunohistochemical reaction for Bax and Bcl-2 after two months of oral supplementation with He1 extracts evaluated in DG of aged mice (b, d) and untreated control animals (CTR) (a, c). Magnification of the objective: 40x (a, b); 100x (c, d). Bars: 20 µm. Histograms relating to the analysis of the OD for BAX in GL and PL (e, f; respectively) and Bcl-2 (g, h). Significant differences between control and He1 treated mice were determined using Unpaired Student's t-test (N=3), the statistical analysis performed does not show any statistically significant differences, $p > 0.05$.

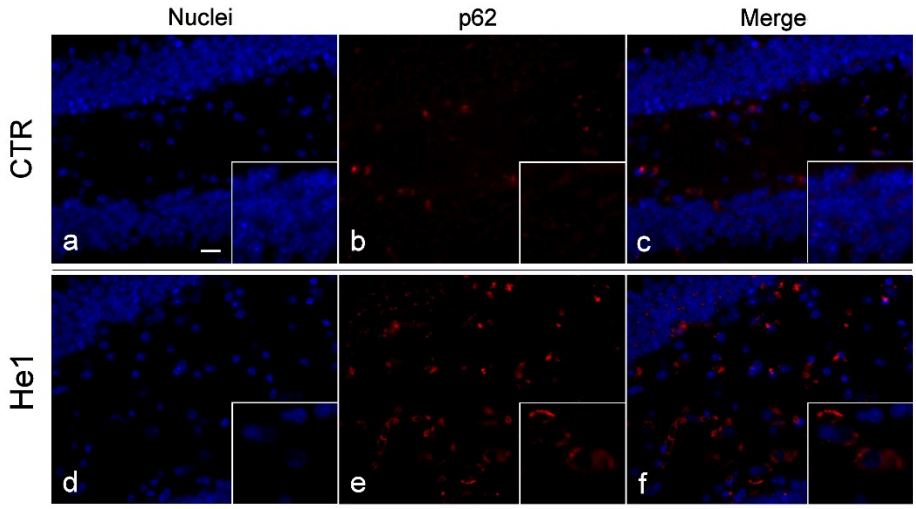


Figure 31. Immunofluorescence reaction for p62 (red fluorescence) after two months of oral supplementation with He1 evaluated in aged mice in the hippocampus (d-f) compared to untreated control mice (CTR) (a-c). Magnification of the objective: 40x. Bar 20 µm.

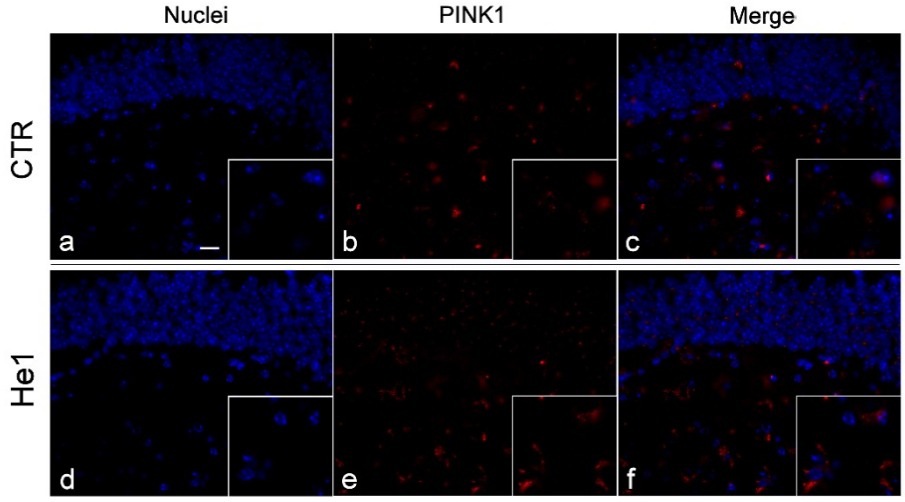


Figure 32. Immunofluorescence reaction for PINK1 (red fluorescence) after two months of oral supplementation with He1 evaluated in aged mice in the hippocampus (d-f) compared to untreated control mice (CTR) (a-c). Magnification of the objective: 40x. Bar 20 µm.

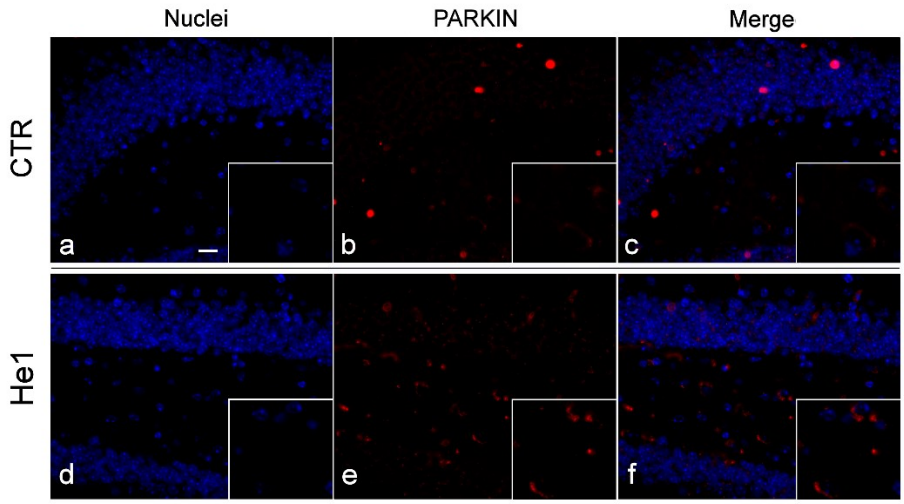


Figure 33. Immunofluorescence reaction for PARKIN (red fluorescence) after two months of oral supplementation with He1 evaluated in aged mice in the hippocampus (d-f) compared to untreated control mice (CTR) (a-c). Magnification of the objective: 40x. Bar 20 μm.

Marker	Granular layer		Polymorphic Layer	
	CTR	He1	CTR	He1
SOD1	-	++	-	++
SOD2	-	-	-	-
COX4	-	-	-	-
NOS2	-	++	-	-
COX2	-	-	-	-
IL-6	-	+	-	++
TGF-β	-	+	-	++
Bax	-	+	-	-
Bcl-2	-	-	-	-
p62	-	-	-	+
PINK-1	-	-	-	+
PARKIN	-	-	-	+

Table 3. Summary of the immunohistochemical results considering Granular and Polymorphic Layer of aged mice. To express the expected results (increasing of oxidative stress, and cell death molecules and decreasing of inflammation) the hyphen and + symbols were used, expressed from basic condition (-) to maximum (+++).

4.3 The Anorexia Nervosa in humans

In a situation of caloric restriction, there is an unbalanced energy supply which, in overtime, causes imbalances in the homeostasis of organisms and cells. Often these situations are associated with high levels of oxidative stress and inflammation in individuals and, based on this hypothesis and thanks to the collaboration with the Department of Public Health, Unit of Forensic Medicine and Forensic Sciences A. Fornari of the University of Pavia, it has been possible to investigate these aspects in human biopsies.

4.3.1 The human dentate gyrus

The human hippocampal formation and DG are much larger than those of rodents. All the reactions presented in the following pages were carried out on entire human hippocampal sections but, for reasons of space, the images refer only to a portion of the DG area. The structure of the human DG like that of rodents and is divided into three layers: an outermost molecular layer, a granular layer in which granular neurons reside and a polymorphic layer occupied by mossy neurons and in close connection with the CA3 area of the Ammon's Horn. No differences in hippocampus gross morphology and in neuronal cytoarchitecture were detected by H&E and Nissl evaluations (**Fig. 34**).

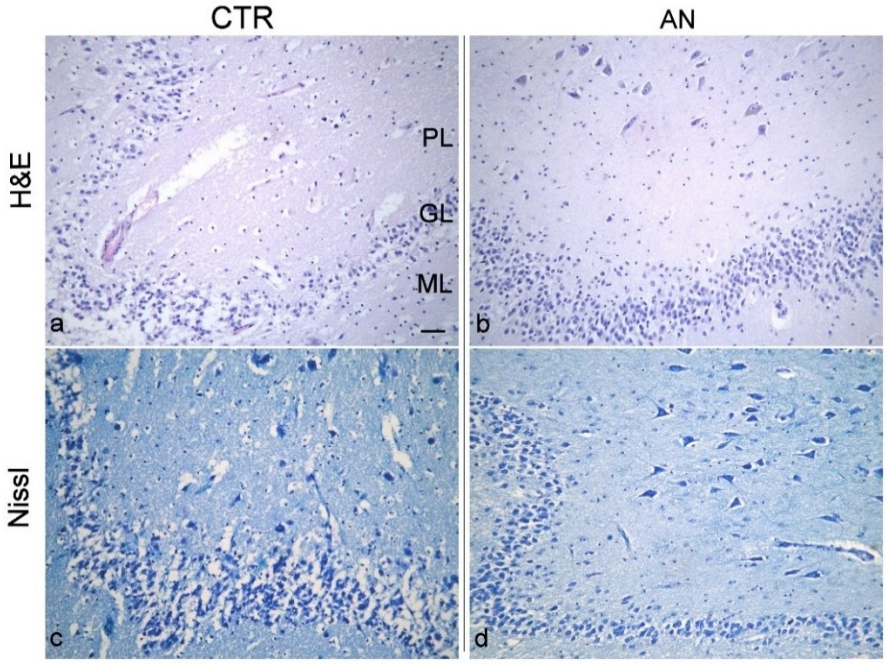


Figure 34. Haematoxylin and eosin (H&E) and Nissl staining of hippocampal dentate gyrus (DG) in AN (b, d) and control human brain (a,c). Magnification of the objective: 20x. Bar: 50μm. PL: Polymorphic Layer, GL: Granular Layer, ML: Molecular Layer.

4.3.2 Evaluation of oxidative stress in AN

Eating disorders lead to an imbalance of caloric intake and consequent energy expenditure in individuals. These variations are detected by the cells and by the mitochondria, organelles in which energy production takes place. If the mitochondria do not function properly, reactive oxygen species (ROS) are produced.

SOD1 is an enzyme that catalyses the demolition of the superoxide anion. The expression of this protein was evaluated by immunohistochemical reaction on hippocampal sections of post-mortem human brains. The results obtained did not show immunopositivity differences between the GL of human DG of control samples and individuals affected by AN (**Figure 35a, b**). The mossy cells of the PL showed a slight increase in immunolabeling in the cytoplasm of neurons of AN patient compared to the control (**Figure 35c, d**).

As highlighted in Figure 31, the expression of **SOD2** resulted to be increased in conditions of AN compared to control patients. The increase in immunopositivity interested not only the granular neurons (**Figure 36a, b**) but also the mossy cells of the PL (**Figure 36c, d**).

Regarding **COX4** immunolabelling, the expression of the enzyme was higher in AN neuron compared to control condition. The immunopositivity was found in granular cells of AN and seems to be concentrated around the nuclear envelop. The same localization was highlighted in polymorphic neurons of patients affected by AN compared to the control hippocampal formation in which no positive neurons were observed (**Fig. 37**).

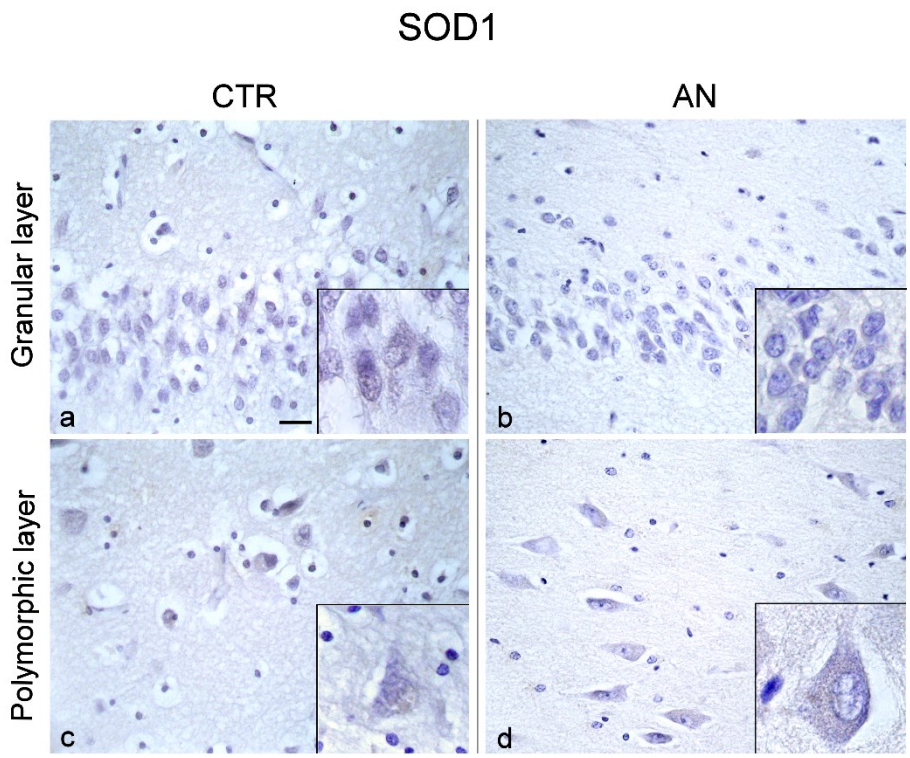


Figure 35. Immunohistochemical reactions for SOD1 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

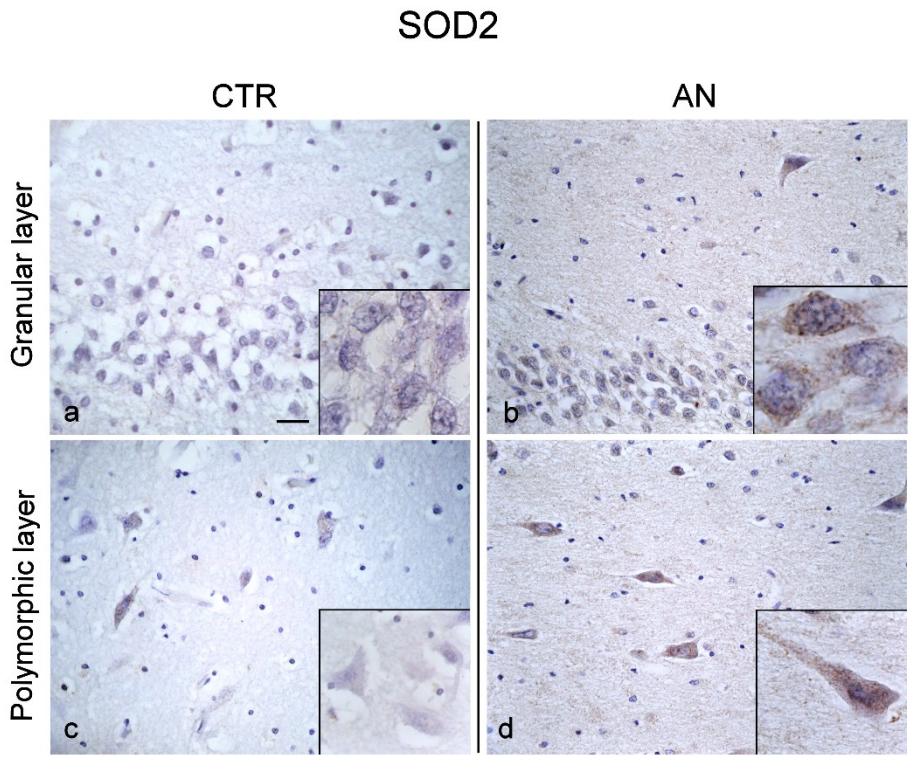


Figure 36. Immunohistochemical reactions for SOD2 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

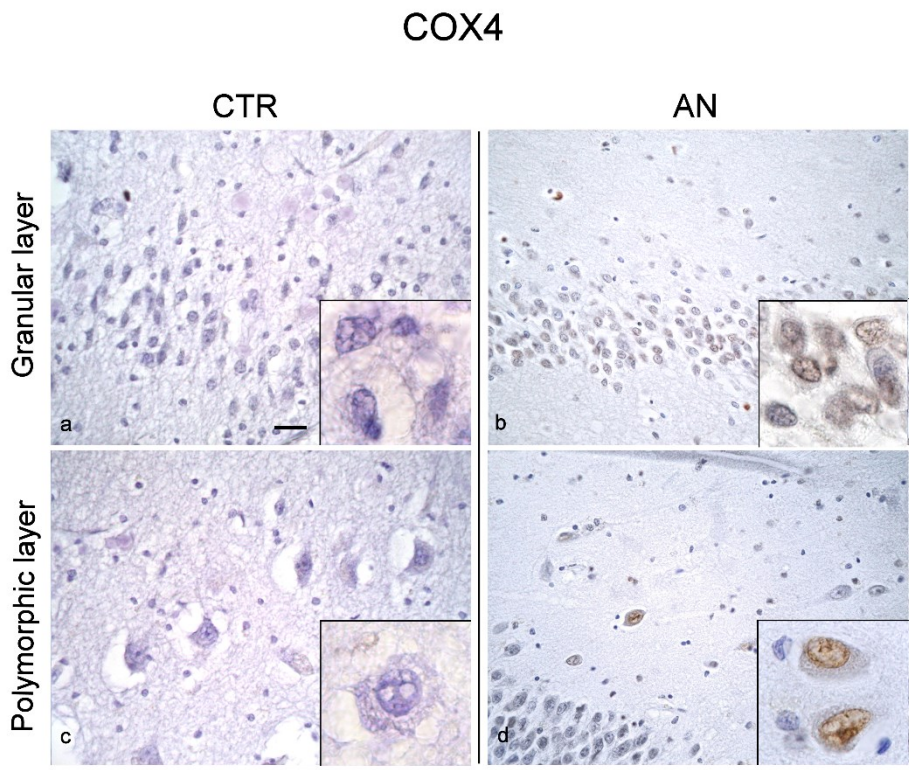


Figure 37. Immunohistochemical reactions for COX4 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

NOS2 is the inducible form of the enzyme that catalyses the production of NO from the oxidation of L-arginine. The results obtained from immunohistochemistry demonstrated an increase in NOS2 expression in the fibers that cross the PL of AN patients compared to the control and attributable to axonal extensions of the granular cells of DG. A small amount of this protein was also found in the cytoplasm of some neurons within the GL (**Figure 38a, b**). The analysis of the PL cells showed an increase in immunolabeled fibers in AN patient together with a high expression of the enzyme in the cytoplasm of polymorphic neurons of the same experimental condition (**Figure 38c, d**).

The **COX2** enzyme is one of the major producers of Prostaglandins (PG) in cells which regulate inflammation by attracting cytokines, chemokines, and macrophages in the tissues. As shown in Figure 34, COX2 is highly expressed in the cytoplasm of several granular neurons of AN patient compared to the control condition (**Figure 39a, b**). In the PL of the DG the mossy neurons appear to be immunopositive for COX2, a pathological condition with respect to the control (**Figure 39c, d**).

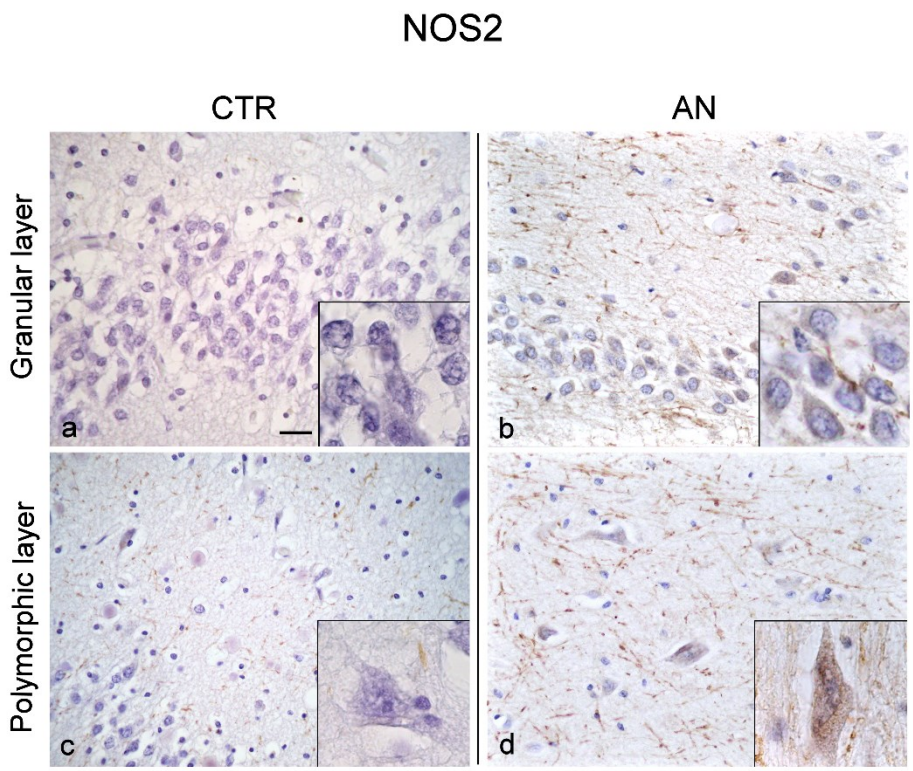


Figure 38. Immunohistochemical reactions for inducible NOS2 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

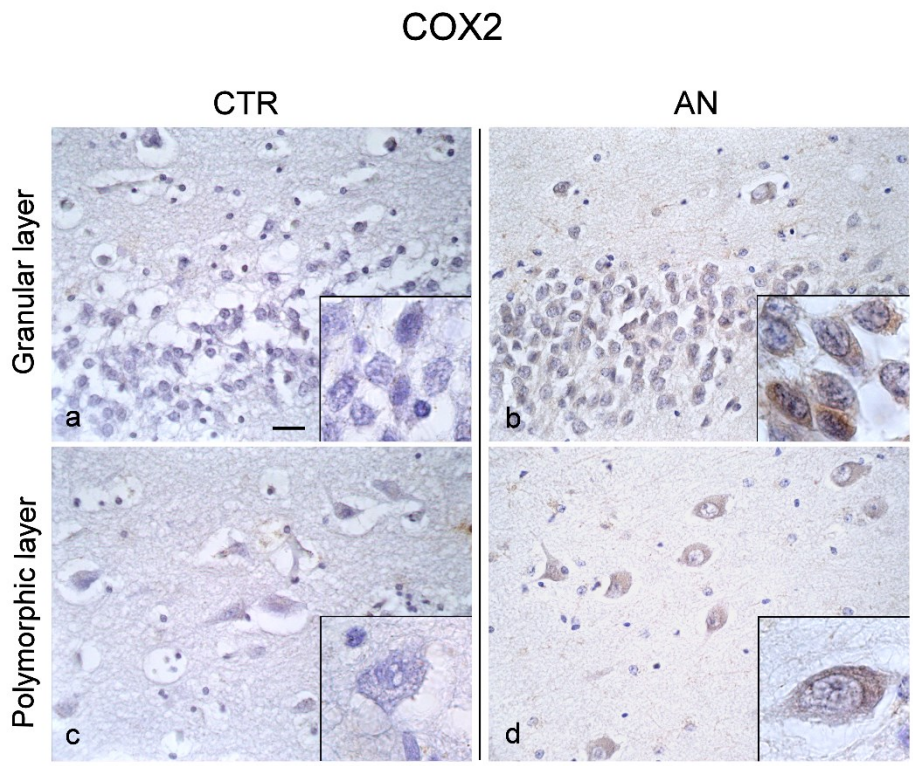


Figure 39. Immunohistochemical reactions for inducible COX2 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

4.3.3 Evaluation of inflammatory pathway in AN

Based on the results obtained by evaluating the proteins involved in oxidative stress, the analysis continued by evaluating two markers of the inflammatory pathway: IL-6 and TGF-β.

IL-6 is a proinflammatory cytokine that performs various functions in organisms such as defence against environmental stress, infections, and trauma. In patients affected by This cytokine appeared to be highly expressed in almost all granular neurons of the DG with respect to physiological conditions (**Figure 40a, b**). in the evaluation of cells belonging to the PL, an increase in immunopositivity was observed in the neurons of sick patients compared to controls. Furthermore, in pathological conditions, the expression of IL-6 was also observed in small cells attributable to the glia (**Figure 40c, d**).

TGF-β is a protein expressed ubiquitously in the body and plays an important role not only in pathological conditions, as an attractor of macrophages and lymphocytes, but also during physiological processes such as cell growth and differentiation. Under the experimental conditions evaluated, TGF-β appeared to be expressed in a very similar way by comparing sections of control DG granular cells with respect to the pathological condition, albeit with slightly higher levels in the latter case (**Figure 41a, b**). Also, the evaluation of the polymorphic layer showed a similar trend of the marking, with a quite relevant homogeneity of polarity by comparing the two analysed sections (**Figure 41c, d**).

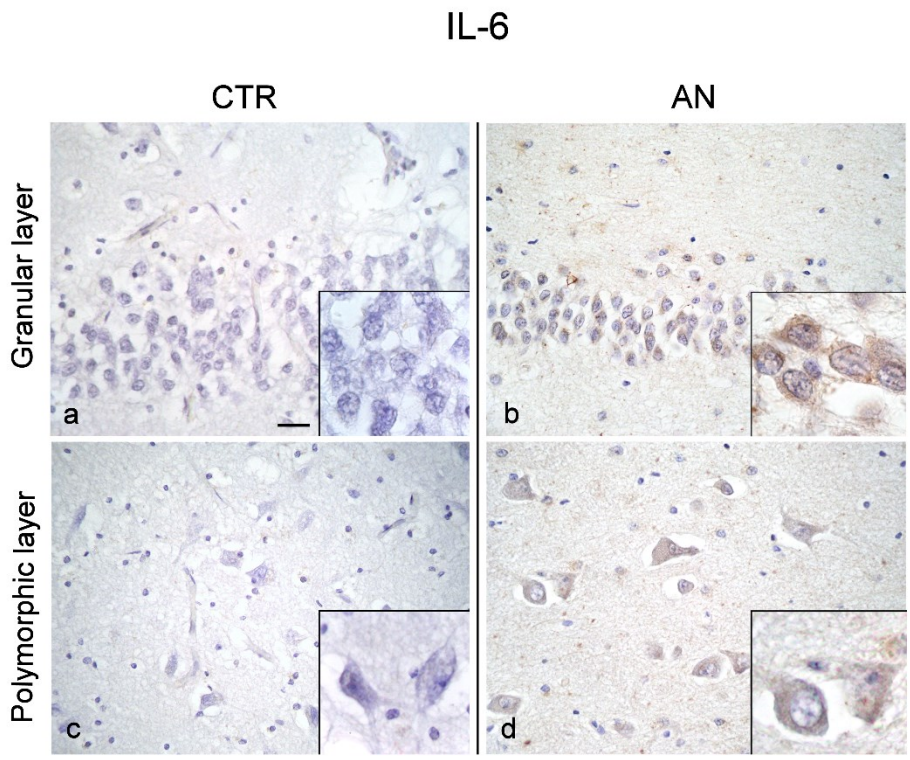


Figure 40. Immunohistochemical reactions for inducible IL-6 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

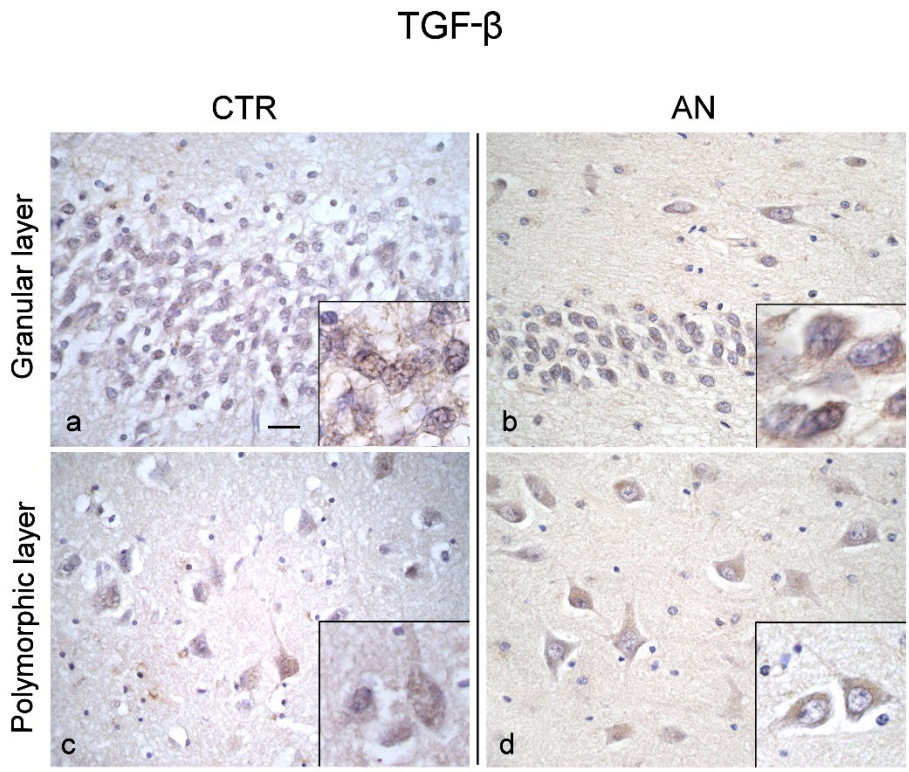


Figure 41. Immunohistochemical reactions for inducible TGF-β in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25μm.

4.3.4 Analysis of different cell death pathways

A state of chronic oxidative stress and inflammation can lead the cells to trigger programmed cell death mechanisms aimed at eliminating the damage or attempting to survive and restore physiological conditions.

The first investigated programmed cell death pathway is apoptosis and two molecules involved in the initial stages of this process, the pro-apoptotic molecule Bax and the antiapoptotic protein Bcl-2. A slight increase in **Bax** immunolabeling in granular cells of AN was demonstrated by immunohistochemical analysis compared to the control sample (**Figure 42a, b**). Mossy cells of PL showed a higher Bax levels than granular cells in the AN patient compared to the control (**Figure 42c, d**).

Regarding **Bcl-2**, this marker did not appear to vary in the expression when comparation sections of DG granular neurons with respect to the control condition were performed (**Figure 43a, b**), while it appeared slightly more expressed in polymorphic cells of the subject with AN than in the normal condition (**Figure 43c, d**).

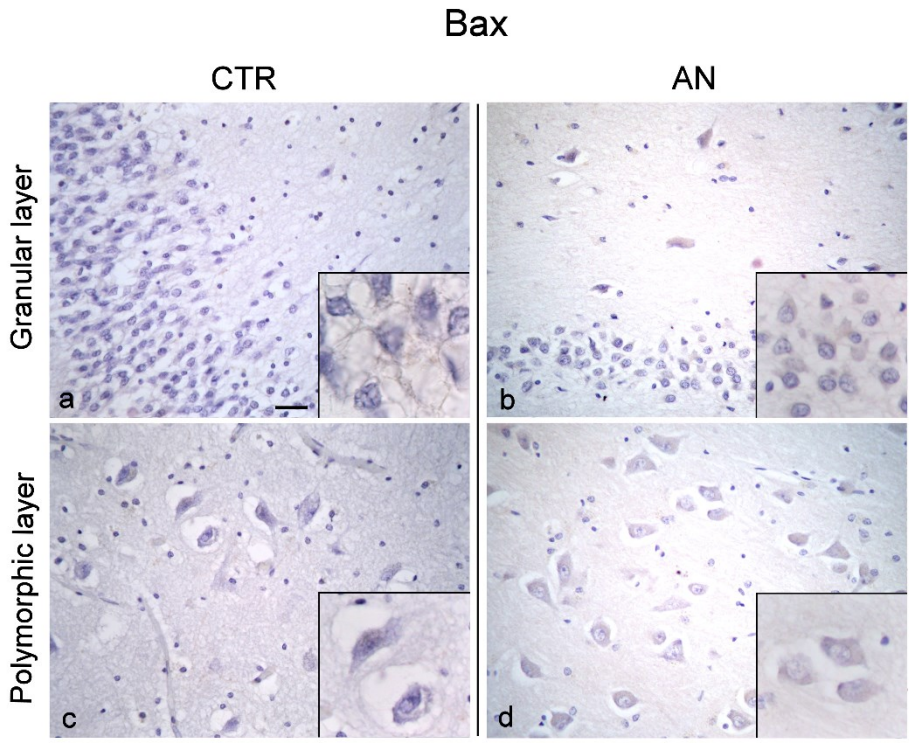


Figure 42. Immunohistochemical reactions for inducible Bax in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25µm.

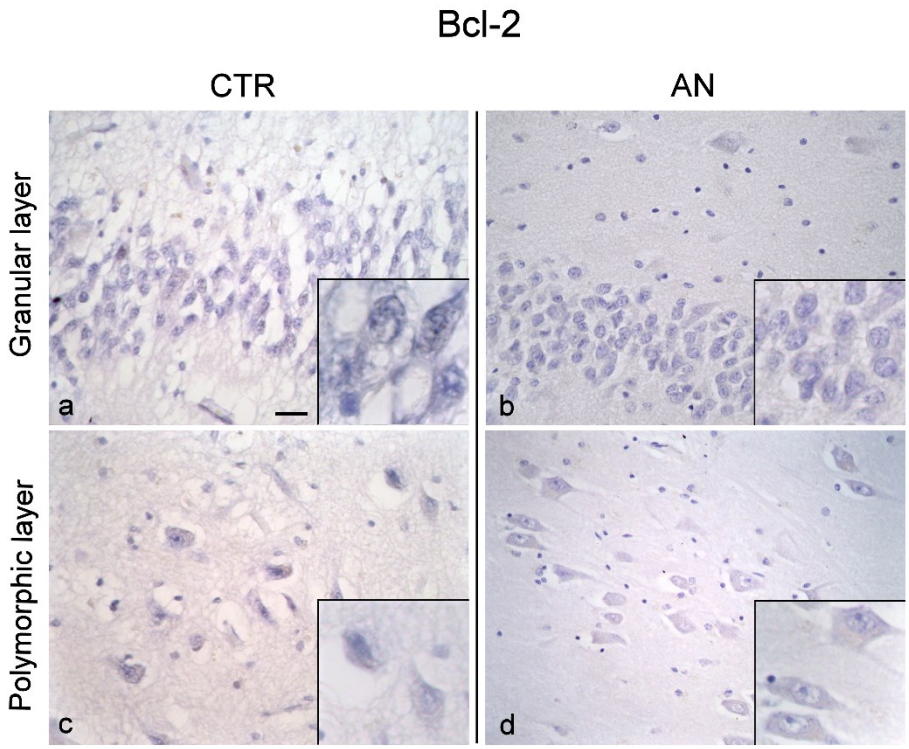


Figure 43. Immunohistochemical reactions for inducible Bcl-2 in human DG granular (a, b) and polymorphic layer (c, d). Magnification of the objective: 40x. Bar: 25μm.

For autophagy analysis the evaluation of **p62** was performed. As shown in figure 36, the p62 protein was mainly localized in the cytoplasm of the granular neurons of the DG with levels slightly higher in the pathological condition than in the control (**Figure 44, Panel A**). On the other hand, by analysing the polymorphic neurons of the DG, the immunolabeling interested many more neurons than those of the GL, while maintaining higher levels in AN than in control condition (**Figure 44, Panel B**).

Regarding the mitophagy pathway, the **PINK1** protein was not widely expressed in human DG granular neurons (**Fig. 45, Panel A**). Instead, the results obtained from the analysis of mossy neurons had highlighted the presence of the protein in the cytoplasm of these neurons and the signal in many cases was superimposed on the positivity of the mitochondria. Particularly, in the AN sample, the expression of PINK1 was increased and more clustered than in the control condition (**Fig. 45, Panel B**).

The expression of **PARKIN**, on the other hand, was also found in some neurons of the GL in the AN hippocampus in colocalization with the mitochondrial signal, suggesting a possible onset mitophagy cell death (**Fig. 46, Panel A, d-f**). In the control case, the signal of the protein did not colocalize with that of the mitochondria (**Fig. 46 a-c**). However, the PL cells, as for PINK1, were more affected by immunofluorescence and several mossy neurons were simultaneously PARKIN and mitochondrion positive in the case of AN compared to the control (**Fig. 46, Panel B**).

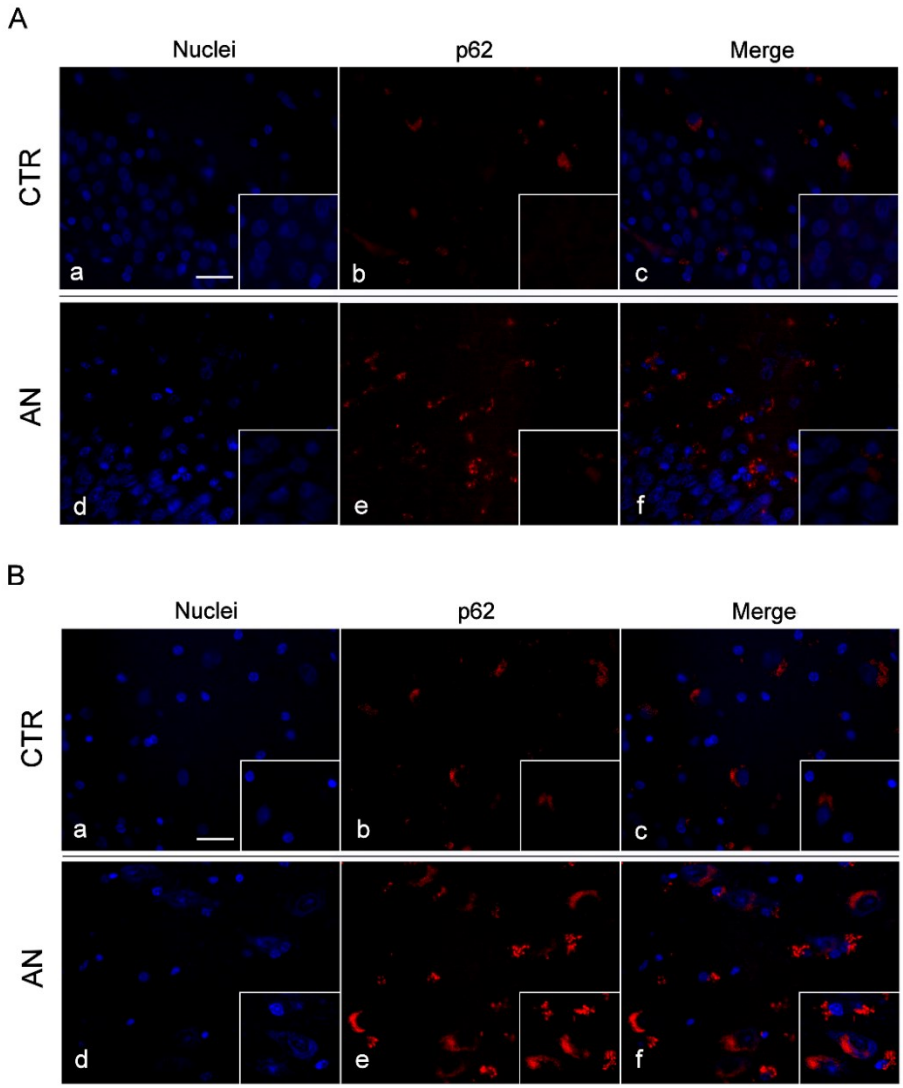


Figure 44. **A**) Immunofluorescence reactions for p62 (red fluorescence) in the GL of control and AN DG. **B**) Immunofluorescence reactions for p62 (red fluorescence) in the PL of control and AN DG. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bars: 20 μm.

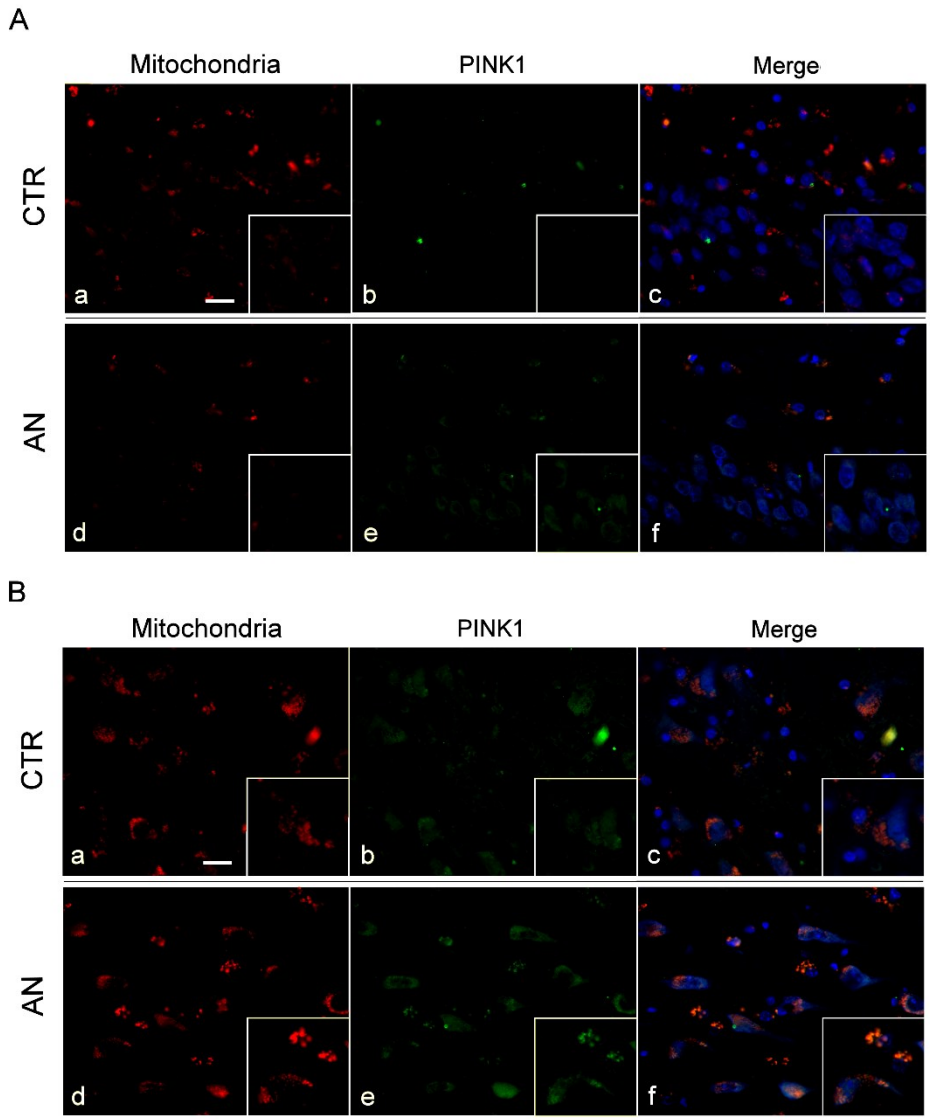


Figure 45. Double immunofluorescence reactions for PINK1 (green fluorescence) and mitochondria (red fluorescence) in the GL (Panel A) and PL (Panel B) of control and AN DG. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bars: 20 µm.

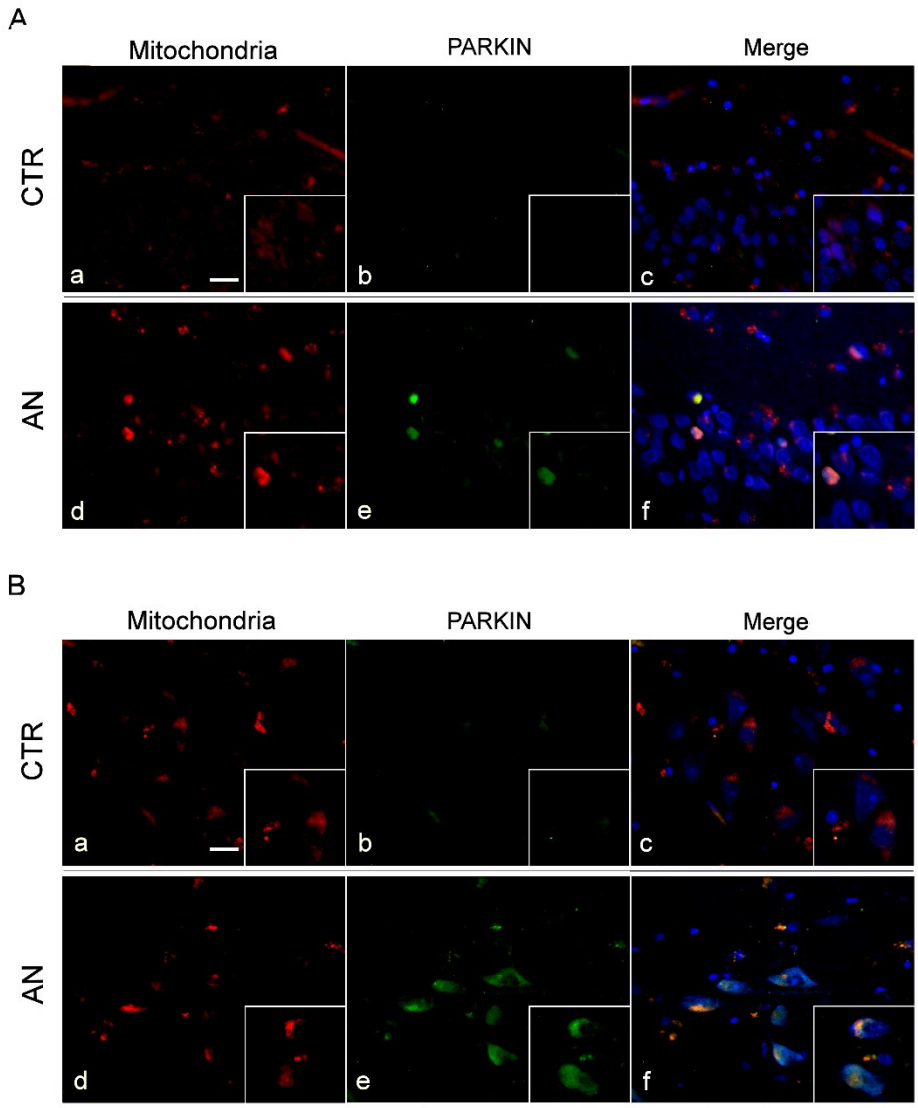


Figure 46. Double immunofluorescence reactions for PARKIN (green fluorescence) and mitochondria (red fluorescence) in the GL (Panel A) and PL (Panel B) of control and AN DG. Nuclei were counterstained with Hoechst 33258 (blue fluorescence). Magnification of the objective: 40x. Bars: 20 μm.

Marker	Granular layer		Polymorphic Layer	
	CTR	AN	CTR	AN
SOD1	-	-	-	+
SOD2	-	++	-	+++
NOS2	-	+	-	++
COX2	-	+	-	++
IL-6	-	+	-	++
TGF-β	-	+	-	+
Bax	-	+	-	+
Bcl-2	-	-	-	+
p62	-	+	-	++
PINK-1	-	-	-	+
PARKIN	-	+	-	++

Table 4. Summary of the immunohistochemical results considering Granular and Polymorphic Layer of AN and control human samples. To express the expected results (increasing of oxidative stress, inflammation and cell death molecules) the hyphen and + symbols were used, expressed from basic condition (-) to maximum (+++).

5. DISCUSSION

In this thesis it is possible to distinguish three major macro-arguments united by a single common thread: the oxidative stress. This aspect has been by far one of the most studied and its involvement is well documented in all the pathologies analysed. In addition, the study continued studying the effects of a possible chronic oxidative state in pathological and physiological conditions and investigating the involvement of signal molecules of an inflammatory state in the body. Chronic oxidative stress and inflammation could trigger mechanisms of programmed cell death that can be considered both beneficial and protective or harmful amplifiers of the damage already present in the individual.

Firstly, a genetic pathology caused by mutations in the gene that codify for the prolidase enzyme was considered. The project involved the analysis of control animals (WT), heterozygous (*dal/+*) and homozygous (*dal/dal*). In humans, this condition causes Prolidase Deficiency (PD), a rare autosomal recessive disorder characterised by variable manifestation onset and a wide range of clinical phenotype including different degrees of mental retardation [80]. To date, very few studies have been reported in the literature on the neurological aspect of patients with PD, including a paper recently published by our laboratory in which defects in cerebellar development and function were observed [1]. Based on these data, it was considered interesting to examine a different brain area, the hippocampus since it is the site of memory formation and cognitive behaviour regulation. The analysis were performed at different days of postnatal brain development: from P10 when the brain is still developing to P21, and lastly to the adult stage of P60 in order to monitor the possible alterations during this timepoints. Moreover, the study was performed considering the dentate gyrus (DG) since it is the area where neurogenesis, both in young and adult individuals, take place.

In parallel it was decided to also analyse a model of physiological ageing and what were the effects of an adjuvant mycotherapy on the brains of frailty mice. This study is part of a larger project that aims to find supportive medication for the canonical treatments that elderly patients undergo during their life. It is estimated that 1.5 billion people aged 65 and over will live in the world by 2050, about 16% of the world population [542]. The constant increase in the average age and life expectancy has led to a higher incidence of neurodegenerative diseases associated with ageing and has, therefore, made it necessary to study mechanisms that improve the quality of life of patients in order to age healthily. More recently, two-month oral supplementation with *Hericium erinaceus* standardizing extracts partially but significantly reversed the decline in recognition memory of frail mice. In particular, immunohistochemistry studies demonstrated increased expression of proliferating cell nuclear antigen (PCNA) and doublecortin (DCX) in the hippocampus and cerebellum in mice treated with *H. erinaceus* thus suggesting positive effects on adult neurogenesis of this fungus [9]. Based on these data and the known effects of *H. erinaceus* on the hippocampus, it was decided to focus attention on this brain area.

Lastly, in the last year, in laboratory we have begun to study the effects of some eating disorders by analysing human plasma. Thanks to the collaboration with the forensic medicine of our University, it was then possible to find biopsy samples of patients suffering from anorexia nervosa. In the area of eating disorders, the search for a potential correlation between elements appears of particular interest. The comparison could represent the starting point for their integration and the development of new concepts, as well as an opportunity for an enrichment of clinical practice and psychopathological reflections. The results showed were very preliminary, but it seemed interesting to us to be able to include them in this study.

Prolidase Deficiency

Alterations were also found in the hippocampus, as in the cerebellum. More than the gross morphology of the DG, these modifications seemed to affect single cells or portions of the ML and GL. Furthermore, it is known that the prolidase intervenes in the remodelling of the extracellular matrix (ECM) which is used by neurons during development for migration to the final site and for the formation of synaptic circuits [543, 79]. We have seen that in the early stages of development there were no evident alterations in the composition of GL and ML. In the later stages of development, on the other hand, neurons located in the GL did not show the classic rounded shape of the granular neuron but rather appeared wrinkled and crushed among the other cells. It has been hypothesized that these were developing neurons that, due to the damage to the ECM already observed in PD mice, failed to mature properly and therefore remained undifferentiated in the stratum. It must be considered that the ECM not only provides a scaffold for neurons but also acts as a real trophic support by transporting molecules and cytokines that cells use to differentiate. Thanks to the analysis with toluidine blue, it was also possible to highlight the cytoplasmic extensions of the mossy neurons which in the *dal* genotypes, at P21 and P60, did not appear well developed as could be observed in the control animals. These morphological data together could suggest that, in the late stages of development, the accumulation of defects in the ECM due to the absence or malfunction of the enzyme gradually damages the structure of the dentate gyrus and consequently the correct formation of synaptic circuits with repercussions on the transmission of nerve signals and on the cognitive aspects of hippocampal formation.

Several studies have shown a strong interconnection between oxidative stress and the activity of the enzyme prolidase [2]. The results of SOD1 demonstrated strong expression of the protein already at PD10 but with differences that are not so obvious and that require further investigation. At P21 and P60, on the other hand, the results showed an increase in SOD1. Already at 10 days after birth, the mossy neurons of hetero and homozygous animals seemed to accumulate SOD1 in their cytoplasm. This data, together with the evidence that oxidative stress inhibits collagen production, could be a sign of the presence of an inflammatory state in the PL layer as a defence mechanism against collagen turnover altered by the lack of the enzyme [2]. In subsequent ages, a gradual increase in the immunopositivity of SOD1 was observed in mice with PD compared to control animals, suggesting an increase in the

oxidative state which, however, affects both the granular and mossy neurons in a fluctuating way between *dal/+* and *dal/dal* animals. In the analysis of the data obtained from heterozygous and homozygous animals it must kept in mind that PD is a pathology in which the phenomenon of negative dominance is observed, i.e. the mechanism by which even just one mutated allele in the gene that codes for the protein carries impair the functioning of the protein encoded by the healthy allele. This could explain why some heterozygous mice have worse results than those obtained from animals homozygous for PD. Furthermore, the accumulation of SOD1 during the final stages of development could suggest an aggravation of the inflammatory state or an attempt to recover the cells by inducing the expression of more superoxide dismutase molecules. Continuing the analysis, the very high levels of SOD2 expression found in young mice correlates very well with the role of the enzyme in cell differentiation during development. In general, however, there is an increase in positivity in the *dal* genotypes, suggesting an inflammatory state in any case much higher than normal. SOD2 is still elevated in *dal* mice at P21 compared to control conditions while more contrasting differences are observed at P60 that need to be further clarified. The high expression of SOD2 affects both the cells belonging to the GL and the mossy neurons of the PL, indicating the presence of a widespread inflammatory state throughout the DG that could impact hippocampal nerve transmission.

Furthermore, it is known from the literature that nitric oxide (NO) participates in the regulation of prolidase activity [80]. The analysis therefore focused on the expression of NOS2 which, however, did not provide statistically significant differences in all the conditions analysed. Several hypotheses could be formulated regarding these results. The absence of NOS could correlate with the low expression of pro-inflammatory cytokines, such as IL-6 and the TGF-β factor found during the analysis conducted on the inflammatory pathway in PD mice. These results together could argue that there is an absence, at least in the conditions that have been evaluated, of nitrosative stress and inflammation. Furthermore, since NO is involved in the remodelling of ECM, in the turnover of collagen and in the repair of tissue damage, it is possible that the absence of this molecule together with the absence of the enzyme responsible for the degradation of ECM contributes to aggravate the pathological state and to impair the circuital dysfunction which may contribute to the different degrees of mental retardation observed in PD patients [79].

An interesting data was obtained from the COX2 analysis. The expression of COX2 under physiological conditions undergoes variations in which a negative peak is observed in the first 7 postnatal days, an increase that reaches a positive peak at P30 and subsequently a progressive decline in the expression of this enzyme in the entire brain [544]. In WT animals for both the GL and PL analysis this trend was confirmed but the results obtained at P60 still showed higher levels of COX2 compared to previous ages. Elevated levels of COX2 during physiological development act as positive factors for synaptogenesis and neurogenesis while in pathological conditions they act as toxic factors leading to ROS production and triggering prostaglandin-mediated inflammatory responses [544]. In any case, the

high expression of the protein observed in the DG of PD mice correlates well with the data obtained from SOD1 and suggests the presence of a very high oxidative state in this pathology. From these evidence one would also expect a high presence of proinflammatory cytokines i.e. IL-6 and TGF-β which, however, unlike the data found on the cerebellum in our laboratory (unpublished and not shown data), was not observed in the DG of PD mice. One possible hypothesis supporting these results could be that the proinflammatory molecules are not able to reach the hippocampal formation due to the damage already mentioned in the ECM but furthermore to alterations in the blood brain barrier (BBB) that could prevent transfer from the bloodstream. Based on these data, two putative approaches to validate these findings could be the use of Western Blot and ELISA method.

The accumulation of ROS in cytoplasm can lead to various dysfunctions and force cells to initiate programmed cell death pathways to limit damage or, in the worst cases, to start accidental death from necrosis. In this sense, two proteins belonging to the apoptotic pathway i.e. Bcl2 and Bax, the p62 protein to evaluate autophagy and two molecules involved in mitophagy called PINK1 and PARKIN were analysed as preliminary data. With regard to apoptosis, Bax and Bcl2 participate in the intrinsic pathway that is triggered by intracellular damage such as the accumulation of ROS [176]. In all the analysed condition no significant differences in the immunopositivity of these proteins was found in the DG. These results may suggest the presence of an inflammatory state not yet so advanced as to induce apoptosis or the cells may undertake a different death pathway. In addition. it could be that the number of neurons that undertake apoptosis were very small and therefore not correctly assessable with immunohistochemical techniques, given the presence of cells with wrinkled morphology, a typical trait of apoptosis, in *dal* animals compared to controls. However, these findings will be validated by investigating caspase protein expression and by using the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labelling (TUNEL) method, an assay that allows to detect DNA fragmentation for quantify the number of apoptotic cells [174, 545].

It was subsequently opted to evaluate whether neurons could undertake the autophagy pathway in this microenvironment rich in antioxidant enzymes. For this investigation it was decided to evaluate the p62 protein which is involved in the formation of the autophagosome. In the early stages of development in the PD genotypes an increase in immunofluorescence was found compared to control animals, indicating a possible activation of autophagic pathway in relation to the accumulation of ROS. Also at P60, an increase in $p62^{+}$ neurons was shown, especially in PL, which suggests that autophagic death could actually occur in mice with PD and thus excluding an involvement, in the early stages of postnatal development, of p62 as a signal molecule that intervenes in the development to remodel the nervous tissue.

Finally, given the large involvement of the mitochondrial enzymes SOD2 and COX2 in this pathology, it was decided to focus attention on the autophagy of the mitochondria called mitophagy. PINK1 in physiological conditions is internalized by the mitochondrion, processed, and in the end transferred to the cytoplasm where it

will be degraded preventing mitophagy. During mitochondrial stress, on the other hand, PINK1 is exposed on the outer mitochondrial matrix (OMM) to act as a signal for PARKIN. For this reason, it has been proposed to carry out immunofluorescence experiments to determine a possible colocalization of the mitochondrial signal with proteins. Preliminary studies conducted on mice at P10 demonstrated an increase in PINK1 expression in colocalization with mitochondria in heterozygous mice while, in the adult stage, the colocalization was observed in both genotypes since. The immunofluorescence analysis for PINK1 alone did not allow us to understand if the protein was associated with OMM or was in the mitochondrial matrix, so we proceeded with the evaluation of PARKIN expression. This molecule at both P10 and P60 was found to be in colocalization with the mitochondrion in both *dal* genotypes since, while this result was not observed in control mice. These preliminary results together may lead us to supposed that oxidative stress, stimulated by ROS accumulation, induces neuronal cells of PD mice to undertake mostly mitophagy to try to survive and to limit tissue damage.

The frailty model of ageing and micotherapy

Ageing is a physiological process characterized by various clinical symptoms that in the long run can become disabling such as cognitive decline and frailty. The DG morphology of frailty and aged supplemented mice did not show large different features that had instead been observed with Nissl staining. In fact, in the animals supplemented with *H. erinaceus* the mossy cells showed the classical morphology with somata of various shapes and highly developed cytoplasmic extensions which, on the other hand, could not be seen in physiologically aged animals. These results could suggest a positive effect on neuronal morphology and a recovery of the correct neuronal shape that certainly will impact the hippocampal circuitry.

ROS accumulate in cells during ageing and can trigger phenomena that lead to the onset of neurodegenerative diseases. The expression of the enzymes SOD1 and SOD2 had provided interesting data that showed an increase in isoform 1 and, on the contrary, a reduction in the expression of isoform 2 in the granular neurons of the treated mice compared to control animals. These data could suggest that in these animals, SOD has a beneficial effect in two different ways. The activity of SOD1 is regulated by sirtuins, that in high quantities act as protective molecules against neurodegenerative diseases [37, 43]. High levels of SOD1 expression could suggest an attempt by cells to buffer the oxidative stress triggered by superoxide anion while the lowering of the levels of SOD2, the mitochondrial isoform, in granular neurons, could exclude their involvement in oxidative stress. Instead, mossy neurons showed high levels of both isoforms in animals treated with the extracts and this could suggest that mycotherapy stimulates the expression of SOD and therefore increases the ability of cells to eliminate superoxide anion. In addition, given that the overexpression of SOD2 is implicated in the prevention of mitochondrial damage, loss of transmembrane potential and release of mitochondrial proteins, the increase observed in mossy cells, albeit minimal, could provide a good correlation with the data obtained on the cell death.

Furthermore, supplementation with *H.erinaceus* seemed to decrease the

expression of NOS2 which was very evident in the area considered the SGZ and however correlates with the decrease of SOD2 expression highlighted in the same layer, a sign probably that in this area the neurons do not have such a high oxidative state. NO activity is regulated by various factors including the increase in SOD2 which prevents it from reacting with oxygen to generate peroxynitrite which would increase the damage induced by ROS and RNS in the cells. Therefore, a decrease in inducible NOS in polymorphic cells in association with an increase instead of SOD2 expression could once again suggest that in these neurons the supplementation with the medicinal mushroom (MM) triggered an antioxidant response.

The expression of COX2 was also decreased in the animals treated with the MM compared to controls, confirming the hypothesis of a lower oxidative stress while the mossy neurons did not seem to be affected by the expression of the enzyme. These results may explain those obtained from the analysis of inflammatory pathways. In fact, both in the GL and in the PL of the treated animals, a decrease in the expression of IL-6 and TGF-β was observed, which, also probably from the evidence that *H.erinaceus* seems to decrease the expression of COX2, could suggest an anti-inflammatory effect of the supplementation confirming the data already reported in the literature [478, 479].

The preliminary data obtained by analysing the cell death pathways suggested the presence of cells that seem to trigger apoptosis in mice treated with erinacins extracts compared to controls which, when viewed in a perspective of controlled cell death to eliminate senescent cells, were positive because they can eliminate degraded cells which overtime would cause damage to brain tissue. The antiapoptotic Bcl2 protein was not widely expressed and this data supports even more the hypothesis formulated of the presence of apoptosis in DG neurons.

Furthermore, the expression of p62 in supplemented mice suggested an effect of the MM extracts in the activation of autophagy which, seen together with apoptosis, could amplify the beneficial and restorative effects of the treatment. It must be said that p62 was also evident in control mice. Probably in these conditions, the neurons were still trying to survive through the induction of death, but with effort.

Finally, the analysis of mitophagy showed promising results with an increase in the PINK1 and PARKIN markers in the supplemented mice compared to the control mice. In general, we can say that the MM extracts seem to activate antioxidant and anti-inflammatory pathways, but they also appear to induce cells to initiate three types of programmed and controlled cell death without triggering inflammation mechanisms. The mechanism of action of this mushroom exploit the ability to stimulate the release of neurotrophic factors. In the PNS, it stimulates the expression of Nervous Growth Factor (NGF) thus promoting the regeneration of peripheral nerve injury at least in the early stage of recovery [546]. This event occurs also in the CNS, where an *in vivo* administration of *H. erinaceus* extracts showed an increase in NGF mRNA expression and in glutamatergic synaptic in hippocampus [547, 541]. This behaviour could be considered a neuronal survival attempt to avoid the accumulation of senescent cells in brain tissue that in the end could aggravate the harmful state already evident in the elderly and thus prevent the onset of cognitive decline and

neurodegenerative diseases.

The Anorexia Nervosa in human brain

The results obtained in the Anorexia Nervosa project are very preliminary and are part of a larger project in which we are evaluating the effects of this pathology on oxidative stress and inflammation in human blood samples from controls and patients. Only an immunohistochemical analysis was carried out on these results since they were human biopsies that are immediately fixed in formalin at the time of collection to avoid degradation. Furthermore, precisely because human biopsies we could not find enough samples to be able to carry out a statistical analysis, but still interesting data were found in a general perspective of the topic of this thesis.

The H&E and Nissl histological stains allowed us to highlight the cytoarchitecture of the human dentate gyrus and to select an area that was kept constant in the analysis of immunohistochemical reactions. The evaluation of the expression of SOD1 allowed to highlight a slight increase in immunolabeling in the mossy neurons of the AN patient while regarding the results for SOD2, the increase was certainly more evident and localized both in the granular neurons and in the polymorphic cells of the pathological condition. These data confirm that a caloric restriction regimen affect the production of ROS in the cells and in this case on the neurons of the hippocampal formation.

In this patient, moreover, an increase in immunopositivity for NOS2 was observed which appears to be localized not only in the cytoplasm of mossy neurons but also highlights the mixed fibers in the PL. This data could suggest the presence, alongside the oxidative stress induced by ROS, also a high production of NO which, as previously reported, in the presence of high oxygen levels can produce harmful molecules such as peroxynitrite.

The COX2 enzyme was also very expressed in this subject compared to the control condition in both GL and PL neurons. Together these results on the one hand confirm the presence of a strong oxidative state in patients with AN, on the other hand they provide new information about the state of an important brain area such as the hippocampus, involved not in the formation of memory but also in emotions and in close connection with brain areas that regulate appetite and mood such as the hypothalamus and amygdala.

To this situation of cellular imbalance is also added a high inflammatory state evidenced by IL-6 expression analysis. In fact, the IL-6-labelled neurons were found to be more immunopositive in the AN sample than in the control and this labelling affected both granular cells and PL neurons. TGF-β, on the other hand, was already expressed in the control patient's neurons, but from a first analysis there seemed to be an increase in immunopositivity in the patient with AN. We can therefore say that in the case of AN there is a very altered oxidative and inflammatory state with a strong expression of antioxidant proteins, probably to buffer the harmful situation.

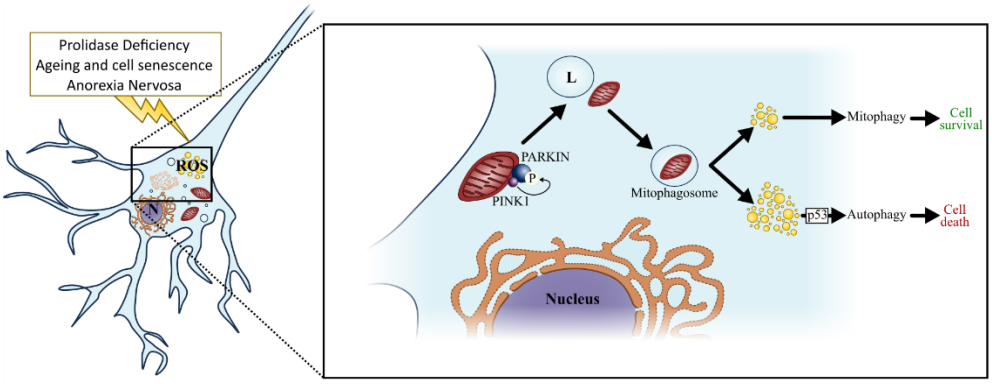
Based on this evidence, for this project too we decided to investigate different cell death pathways. The GL and PL cells of the AN patient showed a slight increase in immunopositivity for the pro-apoptotic molecule Bax compared to the control patient, a sign that these neurons probably begin to trigger reparative processes

inducing programmed cell death.

In addition, the analysis of p62 showed a strong increase in $p62^{+}$ cells in the patient with AN compared to the control, suggesting that multiple cell death pathways seem to act together to try to recover the cellular homeostasis present in the control. The positivity observed in the control sample may however be due to a physiological level of programmed cell death which also intervenes in health conditions to eliminate senescent or damaged cells.

The neurons of the subject AN also seem to take the path of mitophagy. In fact, an increase in the intensity of immunolabeling for PINK1 in colocalization with the mitochondrion was observed, especially in mossy neurons, i.e. those neurons that possessed much higher levels of anti-inflammatory enzyme expression. Also in this case a physiological level of protein expression is observed but, for the same reason already highlighted above, it is not possible to discriminate with certainty whether PINK1 is exposed on the OMM or resides in the mitochondrial matrix. In relation, a confirmation of the presence of mitophagy and therefore presumably of the fact that the positivity of PINK1 is found on OMM, comes from the results obtained by immunofluorescence for PARKIN. PARKIN in physiological conditions is present in the cytoplasm while, only when PINK1 is linked to OMM, it reaches the mitochondrion. Therefore, the colocalization observed in the GL neurons (to a lesser extent) and in the PL between this molecule and the mitochondrion allows us to hypothesize the possible involvement of mitophagy in the attempt of neuronal survival of this subject. Together these results lead us to conclude that indeed in the anorexic patient there is a relevant inflammatory state and an onset of inflammation, damage that the neurons try to overcome by triggering different cell death pathways regulated in order to eliminate the suffering cells and loaded with ROS and to improve the physiological state of the hippocampal area.

Thanks to these data we can propose a model in which, in the presence of ROS and therefore of oxidative stress, the cells undertake different response mechanisms aimed at cell survival or death. In the presence of ROS-induced levels lysosomes engulf damaged mitochondria and subsequently a complex called mitophagosome is formed. After these events, the cells could undertake two pathways. In the presence of a severe accumulation of ROS in the cytoplasm p53 phosphorylation and the autophagosome formation are induced; then cells perform autophagy. When the oxidative stress levels are lower the cells seem to prefer mitophagy cell death. We can consider this event as an attempt to cell survival preceded by the fusion and fission events triggered by several molecules such as Drp1.



Graphic representation of the cell death or survival model proposed by the data obtained in this thesis. The yellow circles in the figure represent ROS molecules.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The data obtained in this thesis provide a broad assessment of how oxidative stress can trigger cascade reactions that cause inflammatory relationships and especially how these tissue damage can be overcome, albeit sometimes with difficulty and failure, by cellular mechanisms such as the production of antioxidant enzymes and the induction of cell death. In a particular organ such as the brain, where there is no conspicuous cell renewal except in very limited locations and where the presence of BBB prevents all molecules from reaching the nervous tissue, it is important that neurons are able to activate mechanisms of survival to prevent the emergence of brain dysfunctions such as early cognitive decline, neurodegenerative diseases and changes in mood and emotions.

Based on the data obtained in PD, it will certainly be of great interest in the future to better understand the role of oxidative stress and inflammation in pathological models by analyzing further aspects of this pathway in strong correlation with other inflammation markers. In addition, it will be necessary to further investigate the mechanisms of induction of cell death with the use of new antibodies to investigate in more detail the aspects of cognitive disabilities observed in patients suffering from this pathology.

In addition, the ageing and mycotherapy project will continue with the analysis of the preoteins that make up the BBB to investigate how the extracts of the H. erinaceus fungus reach neurons and exert their beneficial properties. In addition, various neurotrophic factors will be investigated to understand the role of treatment as a possible aid in improving the quality of life of elderly patients in an era in which the average age of life is rising more and more.

The AN will then certainly be expanded with new molecules and with the results obtained from reactions conducted on human plasma of patients affected by the disease.

It would be interesting in the future to be able to combine ultrastructural studies with the transmission electron microscope (TEM) and molecular studies with Western Blotting to the immunohistochemical studies.

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