

NMNAT1 AND ITS ROLE ON AGEING AND AGE-RELATED DISEASES

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Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except where specifically stated in the text. The work presented here is not substantially the same as any I have submitted for a degree or diploma or other qualification at any other University.

Abstract

NMNAT1 AND ITS ROLE ON AGEING AND AGE-RELATED DISEASES

NAD metabolism is increasingly implicated in a variety of biological functions, including regulation of gene transcription, lifespan, cell death, circadian rhythm and glucose homeostasis. The three mammalian isoforms of the central NAD biosynthetic enzyme, nicotinamide mononucleotide adenylyltransferases (NMNATs), have recently emerged as crucial players in neuronal maintenance and protection, in ageing-processes and in many neurodegenerative diseases, such as Alzheimer's disease. The biological basis of ageing and its related pathologies are not fully elucidated and there is an urgent need to develop valid therapeutic strategies to minimize the impact that these conditions have on the society.

In the present study, I used two mouse models, heterozygous *Nmnat1* knockout mice (*Nmnat1*^{+/-}) and mice overexpressing *Nmnat1* (*Nmnat1* tg), in order to assess the role of the nuclear isoform NMNAT1 in ageing and age-related diseases.

First, I asked whether modulating the expression of NMNAT1 influences ageing-related mechanisms. To this aim, *Nmnat1* tg and *Nmnat1*^{+/-} mice were subjected to metabolic analysis and behavioral tests for measuring locomotor activity. Furthermore, NMNAT enzyme activity, NAD levels, gene expression and protein levels were analyzed with biochemical techniques at 6 and 24 months of age. I found that *Nmnat1* tg mice had a reduced body weight and increased locomotor activity during ageing, while *Nmnat1*^{+/-} weighed more at 12 months than their wild type littermates. NMNAT enzyme activity, significantly higher in *Nmnat1* tg mice at 6 months, did not change during ageing. In contrast, NMNAT enzyme activity in *Nmnat1*^{+/-}, which is already low in young mice, showed a trend of decrease with ageing.

Second, in order to investigate the role of the nuclear isoform NMNAT1 in age-related diseases, I analyzed the effect of modulating NMNAT1 levels on behavioral and neuropathological traits in a mouse line which expresses non-mutant human tau isoforms (htau mouse), representing a model of tauopathy relevant to Alzheimer's disease.

To this aim, htau were crossed to *Nmnat1* tg and *Nmnat1*^{+/-} mice to produce experimental mouse groups with four genotypes that were all heterozygous for murine tau (mtau^{+/-}): *Nmnat1* tg/htau, *Nmnat1*^{+/-}/htau and wild type littermates. Mice were subjected to a battery of specific tests to assess potential behavioral abnormalities that correlate with dysfunctions characteristic of AD. Furthermore, image analysis was performed to assess the

integrity of the brain areas mainly impaired in AD. Finally, biochemical studies were conducted in order to test whether modulating NMNAT1 levels caused changes in NMNAT activity and in NAD levels in htau mice.

I found that htau mice have an early, selective deficit in food burrowing, a behavioral task used to assess activities of daily living which are impaired early in Alzheimer's disease, and that overexpression of *Nmnat1* ameliorates this defect. Despite the behavioral abnormalities, htau mice did not show neurodegenerative impairments in cortex and hippocampus. Modulating NMNAT1 levels produced a corresponding effect on NMNAT enzymatic activity but it did not alter NAD levels in htau mice. My results suggest beneficial effects of NMNAT1 on the early behavioral deficits in this mouse model of tauopathy.

Finally, I asked whether modulation of *Nmnat1* can influence the ischemic cell death, which is at least in part caused by an overactivation of PARP1 and NAD depletion. I hypothesized that reducing NMNAT1 levels exacerbates the ischemic brain damage, whereas increasing these levels confers protection by respectively decreasing or increasing NAD availability within the nucleus.

To address this question, I used a stable NSC-34 clone expressing *Wld^s/NMNAT1*, as well as wild type NSC-34 cells. I measured NAD levels and then tested cell viability after genotoxic stress induced by *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), which causes DNA damage and activation of PARP1, mimicking some mechanistic aspects of ischemic cell death. I first confirmed that *Wld^s* was expressed in all cellular fractions in *Wld^s/NMNAT1* cells, but not in wild type cells. Furthermore, both wild type and *Wld^s/NMNAT1* cells showed lower NAD levels in the nuclear fraction compared to the cytoplasmic fraction. Paradoxically, NAD levels tended to be even lower in all cellular fractions of *Wld^s/NMNAT1* cells respect to the wild type cells. Correspondingly, they show higher susceptibility to cell death induced by MNNG. In addition to the *in vitro* experiments, I also started to set-up an *in vivo* model of cerebral ischemia. Even though time constraints did not allow analyzing the effects of *Nmnat1* modulation, my study will be useful for future investigations to test whether overexpression of *Nmnat1* or its downregulation may protect or exacerbate the effect of cerebral ischemia in an *in vivo* model.

Taken together, my results suggest that modulating *Nmnat1* correspondingly influences ageing and neurodegeneration processes and underline the utility of the *Nmnat1* tg and *Nmnat1*^{+/-} mice as a tool for future research in this field.

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List of abbreviations

Alzheimer's disease **AD**

AMP-activated protein kinase **AMPK**

Amyloid precursor protein **APP**

Apolipoprotein E **APOE**

Apoptosis inducing factor **AIF**

Caloric restriction **CR**

Cerebral amyloid angiopathy **CAA**

Common carotid arteries **CCA**

CRE binding protein **CREB**

Cyclin-dependent kinase **cdk5**

Forkhead box **FOXO**

Glycogen synthase kinase **GSK-3 β**

Heat-shock protein 70 **Hsp70**

Human tau **htau**

Hypoxia-inducible factor 1 α **HIF1 α**

Leber Congenital Amaurosis **LCA**

Microtubule-associated protein **MAP**

Middle cerebral artery **MCA**

Mono (ADP-ribosyl) transferases **ARTs**

NA mononucleotide **NAMN**

NA phosphoribosyltransferase **NAPRT**

NAD synthetase **NADS**

NAM mononucleotide **NMN**

NAM phosphoribosyltransferase **NAMPT**

Neurofibrillary tangles **NFTs** Nicotina-
mide Adenine Dinucleotide **NAD** Nicotin-
amide **NAM**

Nicotinamide riboside **NR** Nicotinic acid **NA**

Nitric oxide **NO**

Nitric oxide synthase **NOS**

NMN adenylyltransferase **NMNAT**

Olfactory receptor neurons **ORN**

Oxygen/glucose deprivation **OGD**

Paired helical filaments **PHF**

Peri-infarct depolarizations **PIDs**

Peroxisome proliferator-activated receptor γ coactivator 1 α **PGC-1 α**

Poly(ADP-ribosyl) polymerases **PARPs**

Protein phosphatase **PP-2A**

QA phosphoribosyltransferase **QAPRT**

Quinolinic acid **QA**

Ribosomal S6 protein kinase 1 **S6K1**

Sirtuins **SIRTs**

Straight filaments **SF**

Suppressor of MEK null **SMK-1**

Target of rapamycin **TOR**

Type 2 diabetes **T2D**

Ubiquitin proteasome system **UPS**

Ubiquitination factor E4B **UBE4B**

Valosin containing protein **VCP**

Vessel occlusion **VO**

Wallerian degeneration slow **Wld^S**

β cell-specific SIRT1-overexpressing **BESTO**

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Chapter 1

Introduction

1.1 Neurobiology of ageing

Ageing is a complex, irreversible process caused by several factors, involving most organs and tissues, as a result of alterations at a molecular level. Ageing mechanisms refer to all physiological alterations developed with age, occurring in a healthy organism, which are not considered as a disease [1].

1.1.1 Cognitive and structural impairment in ageing

Cognitive abilities gradually decline with ageing [2]. Problem-solving and actions independent on learning, known as fluid intelligence, may be impaired with time [3]. In contrast, crystallized intelligence, representing the abilities to accumulate information based on one's life experiences, seems not to be impaired in older adults [2].

An aspect of the fluid intelligence, which appears to be reduced with time, is the processing speed, i.e., the speed with which cognitive activities are performed as well as the speed of motor response [4]. One of the most important changes evident in older people is alteration in memory. In fact, older adults perform worse than younger adults on tasks involving working memory [5]. Furthermore, semantic memory, which refers to information, language usage and practical knowledge, as well as the episodic memory related to experienced events linked to specific place and time are also impaired over ageing [6]. Another skill which declines with ageing is the ability to put together individual parts to make a coherent whole (for example, assembling furniture from a box of parts), known as visual construction skill [7]. Concept formation, abstraction, and mental flexibility decline with age, especially after the age of 70 years [2]. Finally, executive abilities are also negatively affected by ageing, as well as actions requiring a speeded motor component and the ability to inhibit an automatic response in favor of producing a novel response [8, 9].

Cognitive impairments might be a consequence of the volume reduction in both grey and white matter. Indeed, grey matter volume initiates to decrease in the prefrontal cortex [10] and it is evident, but more moderated in the hippocampus [11]. The reduction in the grey matter volume with time is due to several reasons and one of these might be the death of neurons themselves [12]. Furthermore, accumulation of β -amyloid can occur in normal ageing people and contribute to the grey matter reduction [13]. However,

this reduction might be also explained with a reduction in synaptic density [10] as well as changes in dendrite arborisation, dendrite length and neuritic spines [14].

Furthermore, white matter volume is shown to be decreased with ageing, possibly to a much greater extent than grey matter [15]. Indeed, the volume of the parahippocampal white matter is strongly reduced in older adults, which in turn results in impairments in communication with hippocampal structures, suggesting a possible mechanism for age-associated memory decline [16]. Furthermore, loss of integrity of the central portion of the corpus callosum becomes evident in older people, and it may mediate age-related cognitive decline [17].

1.1.2 Pathophysiology of ageing

Balance between protein synthesis and degradation occurring during life will change over ageing [18, 19]. Several studies highlighted damages and changes of structural or functional proteins, occurring in ageing organisms [20-23]. Processes playing a fundamental role include the accumulation of free radicals, which leads to a damage in the cellular macromolecules due to oxidative stress [24, 25], as well as non-enzymatic glycosylation, affecting proteins such as collagen and elastin [1]. Furthermore, post-translational modifications such as isomerization, racemization, deamination, incorrect folding, crosslinking reaction, DNA methylation decline and might contribute to changes observed during ageing [22, 26]. However, it is not clear whether some of these modifications are the cause or the consequence of ageing processes [1]. Alterations at molecular level induce subsequent alterations in metabolic functions and, more generally, a perturbation in homeostasis. The way proteins work in controlling metabolic pathways, which allow an organism to adapt to the environment, plays a fundamental role in age-related mechanisms [27]. Indeed, the only intervention, which efficiently increases lifespan is a caloric restriction (CR) diet, as shown in various organisms including yeast, flies, worms, fish, rodents and rhesus monkeys [28-30]. Benefit of CR is probably due to both the absence of specific dietary amino acids in the diet, such as methionine [31] and to the involvement of specific metabolic sensors such as target of rapamycin (TOR), AMP-activated protein kinase (AMPK), and sirtuin proteins that detect changes in specific metabolites such as amino acids, AMP, and NAD. Such changes might induce metabolic adaptations, involving other regulators, including transcription factors (i.e., FOXO and FOXA

families), SMK-1 (suppressor of MEK null), and the peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) [27]. These proteins are involved in complex reactions, all contributing to the longevity (Fig.1.1).

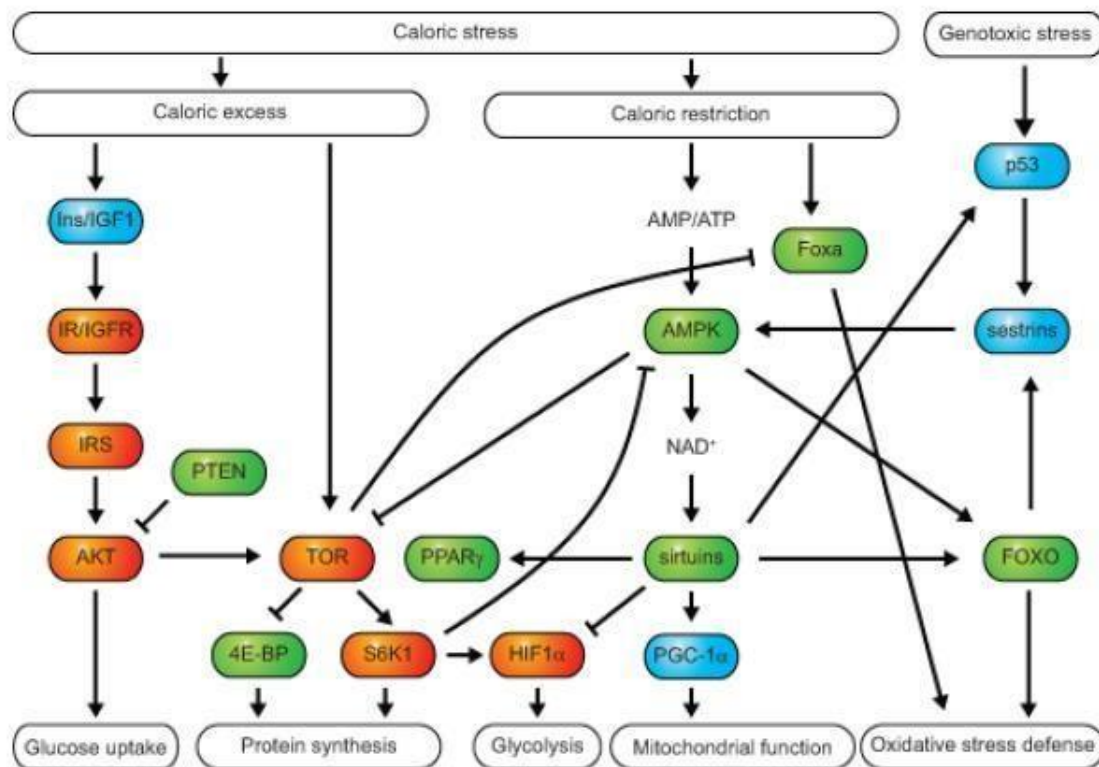


Fig.1.1 A metabolic network of ageing.

A simplified scheme for metabolic proteins that are involved in longevity. There are complex interactions among individual metabolic signaling pathways, such as the insulin/IGF1, TOR, AMPK, sirtuin and FOXO pathways. These interactions impact healthspan and longevity. For instance, the TOR pathway, which is activated in situations of nutrient excess, is not only linked to other nutrient-responsive pathways, such as the insulin/IGF1 pathway, but also to the CR-induced pathway involving AMPK and Foxa activation. Longevity promoting proteins are in green, proteins that when mutated increase longevity are in red, other proteins are indicated in blue; mammalian protein names are used [27].

- *Free radical oxidation*

Ageing as well as some neurodegenerative diseases are strongly influenced by the production of oxygen free radicals, which attack cellular constituents, such as chromosomes, mitochondrial DNA and connective tissues [32], causing apoptosis, ageing and cell death. In particular, oxygen free radicals induce the formation of pigment-like fluoro-

phores, similar to lipofuscin [33]. Age-related diseases, including cardio-vascular diseases, cancers, senile dementia, and diabetes, show a close connection with free radicals and related damages [1]. It is likely that consequences of oxidative stress, a reaction occurring during the whole life, are responsible for ageing-related changes.

Carbonyl compounds (biomolecules that contain a carbon– oxygen double bond, or carbonyl group, mainly aldehydes and ketones) are another molecular group that plays a crucial role as reactive species, leading to an age-related damage. Lipid peroxidation is mainly initiated by removing hydrogen from unsaturated fatty acids by oxygen-centered radicals, followed by the formation of hydroperoxides. Degradation of hydroperoxides results in a variety of derivatives, including various carbonyl products [34]. Such unsaturated carbonyls, including enals, dienals, trienals, hydroxylenals, 2-ketoaldehydes, deoxyones, are all very reactive and toxic to almost all cellular and extracellular biomolecules [34]. Production of these reactive species may cause depletion of glutathione, protein modification, disturbance of calcium homeostasis, retardation of respiration and glycolysis, cell membrane destruction, tissue injuries, enzyme inhibition and decreased DNA, RNA and protein synthesis [34].

- *Non-enzymatic glycosylation*

Glycosylation is another fundamental mechanism occurring during ageing, which is not-dependent on oxidation processes [35]. Previous evidences suggested that, under specific physiological conditions, glucose can react with amino acids, peptides and amino-groups in proteins [1], leading to the formation of secondary products similar to those produced in lipid oxidation [35]. Crosslinking and denaturation of proteins, including collagen and elastin caused by glycosylation are key factors for early ageing related alterations of blood vessel, kidney, lung and joint [36]. Those changes potentially alter protein structures, inducing damages of functional proteins, which in turn result in a decreased supply of energy and subsequent decline of metabolic functions [1].

- *Metabolic pathways*

One of the best characterized pathways involved in ageing mechanisms is the insulin/IGF1 network [27]. Insulin/IGF1 network is modulated by FOXO (forkhead box) transcription factors (Fig.1.1). When FOXO is phosphorylated by AKT (a downstream kinase

in this pathway), it translocates into the cytosol, causing inactivation of transcription factors for genes involved in defense against oxidative stress and genes encoding molecular chaperones [37].

Another important pathway in longevity includes TOR (target of rapamycin) signaling pathway, initially known for its involvement in the antifungal effects of the drug rapamycin, known to play a critical role in cellular growth [38]. Indeed genetic or pharmacological inhibition through rapamycin of TOR strongly increases life span in yeast, worms and flies as well as in mice [38]. Several mechanisms seem to be involved in the beneficial effect of rapamycin, such as autophagy, ribosomal S6 protein kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E binding protein (4EBP), which are downstream effectors of TOR [39]. Stimulation of autophagy using polyamine spermidine inhibits oxidative stress and increases lifespan [40], while genetic deletion of 4EBP decreases lifespan in *Drosophila* [41] and reduction of S6K1 increases lifespan in mice [42].

Sirtuins, NAD dependent deacetylases, are strongly linked to longevity. Lifespan-increase by CR is mediated by the sirtuin orthologous sir2/SIRT1 in yeast, fly, and worm. Indeed, overexpression of SIRT1 in the brain increases lifespan [43]. The mechanism by which SIRT1 increases lifespan seems to involve metabolic sensor AMPK, which may control longevity by raising NAD levels and activating SIRT1, leading to deacetylation and activation of specific targets, such as PGC-1 α and FOXO1, which in turn results in the induction of mitochondrial activity, respiratory metabolism, and oxidative stress responses [44, 45] (Fig.1.2)

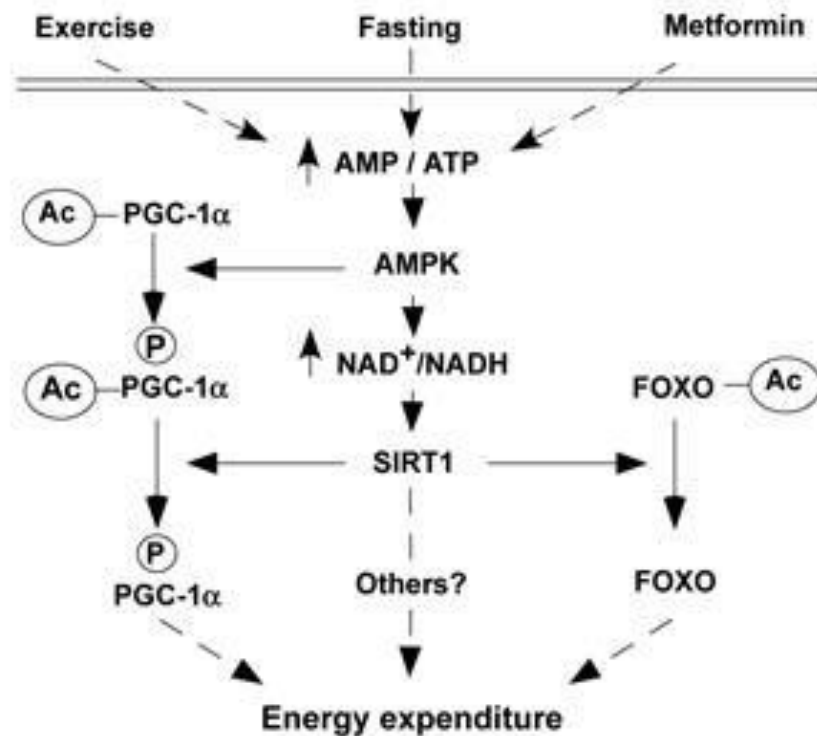


Fig.1.2 Mechanism by which SIRT1 increases lifespan.

Pharmacological (metformin) and physiological (fasting or exercise) activation of AMPK in muscle triggers an increase in the NAD/NADH ratio, which activate SIRT1. AMPK also induces the phosphorylation of PGC-1 α and primes it for subsequent deacetylation by SIRT1. The impact of AMPK and SIRT1 on the acetylation status of PGC-1 α and other transcriptional regulators, such as the FOXO family of transcription factors, will then modulate mitochondrial function and lipid metabolism [45].

Moreover, some of the health benefits of SIRT1 in ageing have been correlated to mitochondrial function. Changes in nuclear NAD levels during ageing cause a pseudo-hypoxia-driven imbalance between nuclear- and mitochondrially encoded oxidative phosphorylation subunits (OXPHOS). Deleting SIRT1 accelerates this process, whereas raising NAD levels in old mice restores mitochondrial function in a SIRT1-dependent manner [46], highlighting the pivotal role that NAD has on ageing mechanisms.

1.2 NAD metabolism

1.2.1 NAD biosynthesis

Nicotinamide Adenine Dinucleotide (NAD) is well known as co-enzyme in redox reactions. It acts as a hydride-accepting and hydride-donating molecule, which plays a

crucial role for both balance of cellular redox potential and synthesis of ATP. More recently, its action has been linked to other critical cellular processes, including energy metabolism, mitochondrial functions, Ca^{2+} homeostasis, gene expression, ageing, cell death [47]. Four different building blocks are known in NAD biosynthesis: quinolinic acid (QA) in the *de novo* pathway and nicotinamide (NAM), nicotinic acid (NA) and nicotinamide riboside (NR) in the salvage pathways [48, 49]. QA, NA and NAM are used by three distinct phosphoribosyltransferases [QA phosphoribosyltransferase (QAPRT), NA phosphoribosyltransferase (NAPRT) and NAM phosphoribosyltransferase (NAMPT)] for production of the respective mononucleotides, NA mononucleotide (NAMN) from QA and NA, and NAM mononucleotide (NMN) from NAM. NAMN and NMN are subsequently converted to NA adenine dinucleotide (NAAD) and NAD, respectively, by NMN adenylyltransferase (NMNAT). NAAD is converted to NAD by NAD synthetase (NADS) [50] (Fig.1.3).

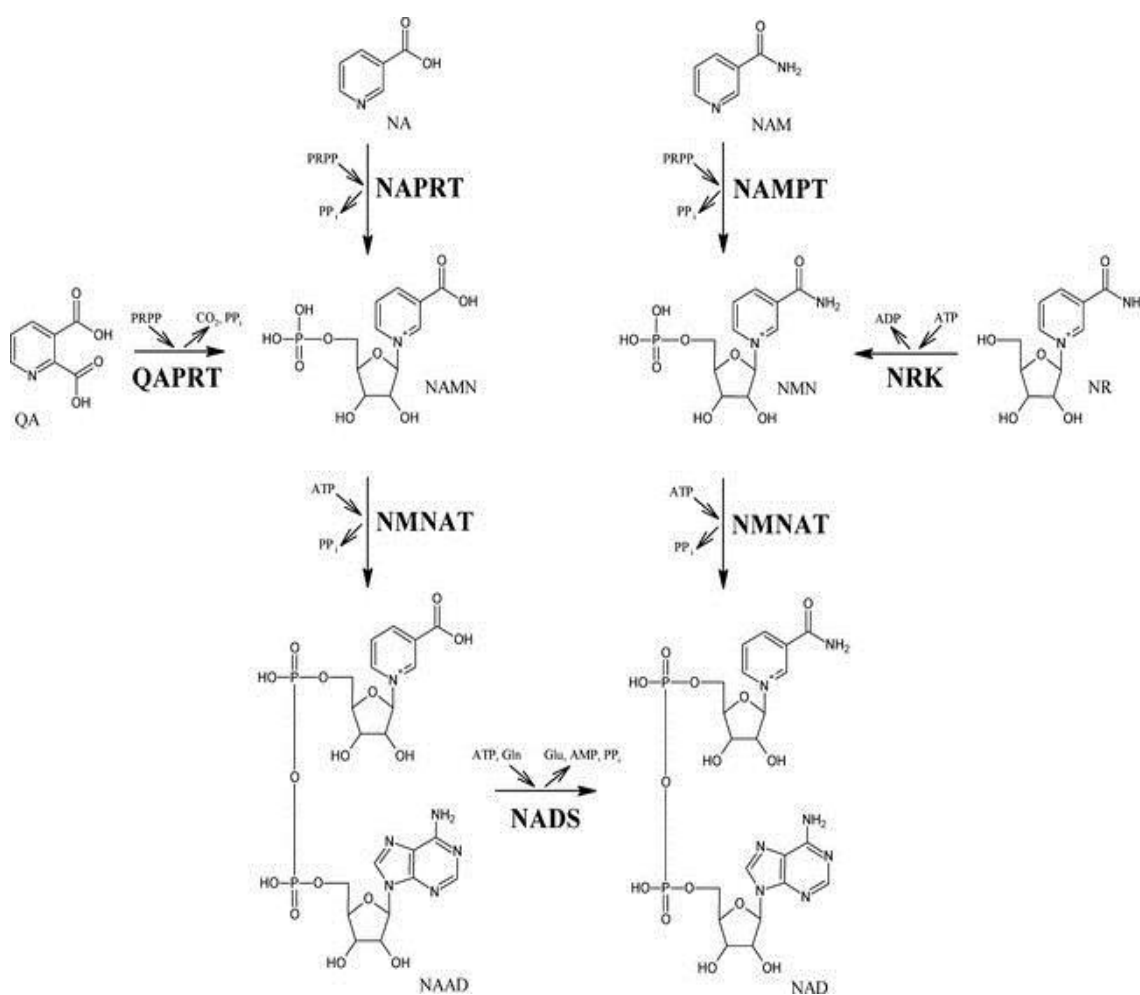


Fig.1.3 NAD biosynthetic pathway.

Four pathways allow NAD biosynthesis from four distinct sources of the pyridine ring: QA (quinolinic acid), NA (nicotinic acid), NAM (nicotinamide), and NR (nicotinamide riboside). The enzymes involved are QAPRT (QA phosphoribosyltransferase), NAPRT (NA phosphoribosyltransferase), NAMPT (NAM phosphoribosyltransferase), NRK (nicotinamide riboside kinase), NMNAT (NMN adenylyltransferase) and NADS (NAD synthetase) [50].

The NAD synthesizing enzymes nicotinamide mononucleotide adenylyltransferases (NMNATs) are central enzymes in NAD biosynthetic pathway and they catalyze the synthesis of NAD (or NAAD) from NMN (or NAMN) and ATP [51]. NMNATs are present throughout evolution. While in prokaryotes and in some eukaryotes, such as *Drosophila*, only one NMNAT isoform has been found, in higher eukaryotes, including mice and humans, more than one isoform has been characterized. In particular, in mammals there are three different isoforms, NMNAT1, NMNAT2 and NMNAT3, with specific tissue distribution and intracellular localization [52]. Human NMNAT1 is a 279 residue nuclear protein (31.9 kDa) containing two conserved ATP-recognition motifs [53, 54] and it is expressed in all tissues [55]; cytoplasmic and Golgi-associated NMNAT2 consists of 307 amino acids (34.4 kDa) [54] and it is mainly expressed in heart, skeletal muscle and brain [52, 56-59]. NMNAT3 is a 252 residue mitochondrial protein (28.3 kDa) and it is more strongly expressed in red blood cells, kidney, lung, spleen and placenta [60, 61].

1.2.2 NAD catabolism

The role of NAD in non-redox reaction has been recently elucidated. In particular, NAD is involved in post-translational modifications such as protein deacetylation [62] and mono- and poly(ADP-ribosyl)ation by mono (ADP-ribosyl) transferases (ARTs) and poly(ADP-ribosyl) polymerases (PARPs) [63, 64] (Fig.1.4). In these reactions, NAD glycosidic bond linking NAM to ribose is cleaved and the resulting ADPR is bound to an acceptor protein (ADP-ribosylation) or to an acetyl group (deacetylation) [50]. Protein deacetylation is catalysed by sirtuins [65]. These reactions play a fundamental role in transcriptional silencing, DNA repair, DNA recombination and life-span regulation [66, 67], DNA repair and modification, telomere length regulation, mitosis and apoptosis [68]. Mono (ADP-ribosyl)ation is catalysed by ART enzymes and it is involved in muscle cell development, actin polymerization and cytotoxic T-lymphocyte proliferation [69, 70]. In

addition, NAD is involved in calcium signaling through its derivative nicotinic acid adenine dinucleotide phosphate (NAADP) and degradation products ADP-ribose and cyclic ADP-ribose (cADPR)[71].

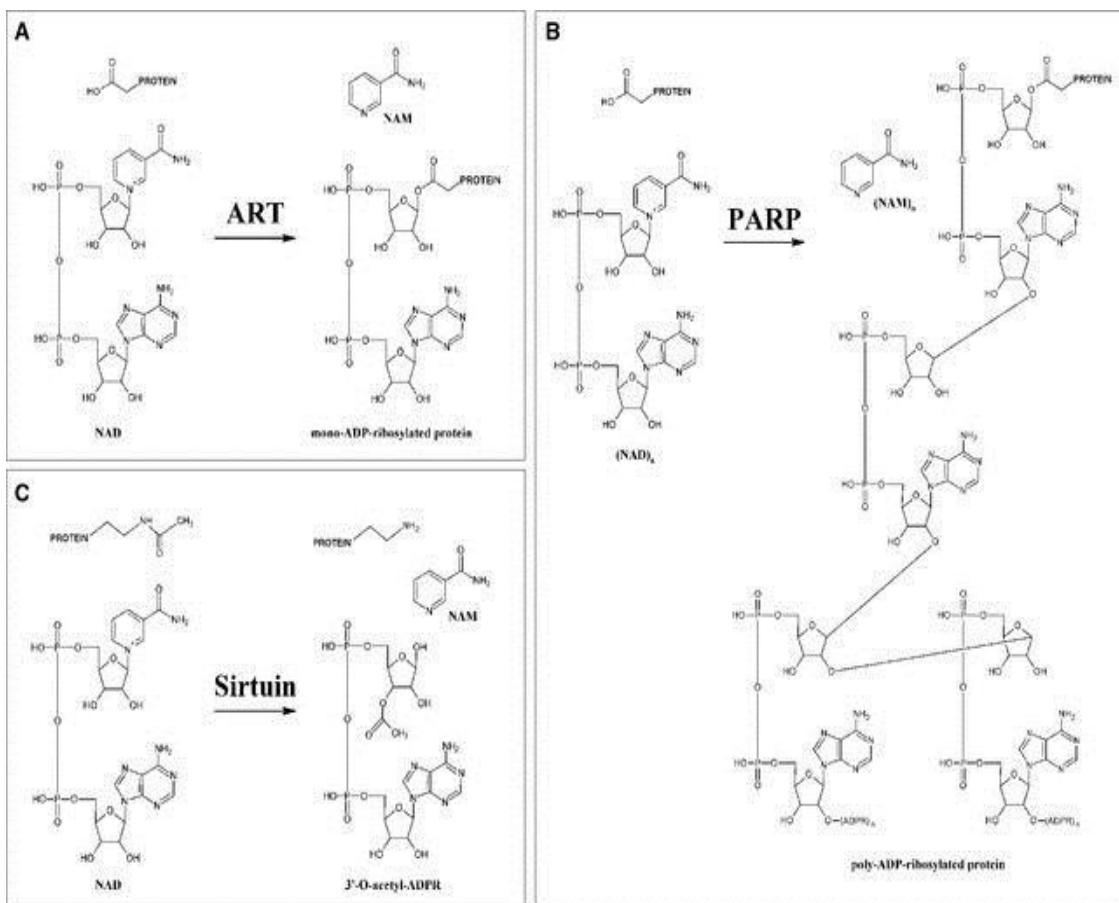


Fig.1.4 NAD-catabolic reactions.

(A) NAD is hydrolysed by ARTs, producing mono (ADP-ribosyl)ated protein and NAM. (B) NAD is hydrolysed by PARPs, producing poly(ADP-ribosyl)ated protein and NAM. (C) NAD is hydrolysed by Sirtuins, producing 3'-O-acetyl-ADPR and NAM [50].

■ PARP1

Poly-ADP-ribose polymerases (PARPs) belong to an ancient family of enzymes involved in DNA damage repair, cell death pathways, transcription and chromatin modification. In humans there are seventeen family members that share the PARP catalytic domain. However, only some of the family members have shown PARP activity. The first PARP purified and cloned is PARP1, which remains the best studied [68]. PARP1 works as a nick sensor for DNA break repair. It uses NAD as a substrate to synthesize polymers of

ADP-ribose (PAR). After that, PAR chains are covalently attached to many acceptor proteins, such as histones and PARP1 itself, activating a well-known mechanism called poly (ADP-ribose)ylation. PARP1 overactivation causes depletion of its nuclear substrate NAD, and subsequent cell death [78]. PARP1 overactivation also induces the nuclear translocation of substrates such as p65 and NF- κ B, causing abnormal gene transcription, leading to cell death [79]. Furthermore PAR can lead to PAR-dependent mitochondrial pore formation and membrane depolarization. This is accompanied by a release of apoptosis-inducing factor (AIF), its translocation into the nucleus and DNA degradation [80]. Recent studies showed that NAD repletion prevented glycolytic inhibition, mitochondrial depolarization and AIF translocation, even if PAR accumulation in the brain was increased [81].

- *SIRT1*

Among the three nuclear sirtuins (SIRT1, SIRT6 and SIRT7), SIRT1 is the best studied [82]. Nuclear sirtuins control the expression of a number of genes by deacetylating histones [50]. It has been demonstrated that Sir2 (silencing information regulator protein 2), the yeast homologue of human sirtuins, controls lifespan [82]. In mammals, SIRT1 over-expression reduces the adverse effects of a high-fat diet or ageing on glucose metabolism, and correlates with a reduced incidence of metabolic diseases [67, 83]. SIRT1 plays a role in age-related diseases such as Alzheimer's disease. In particular, SIRT1 over-expression reduces the amount of amyloid β plaques in brains of APPswe, PSEN1dE9 mouse model of Alzheimer's disease [84]. Furthermore, SIRT1 decreases tau accumulation by reducing the levels of acetylated tau [85]. Increasing NAD synthesis by over-expressing *Nampt* has similar effects, indicating that SIRT1 activity is strongly dependent on the availability of its substrate, NAD [75, 86]. It has been shown that SIRT1 improved also mitochondrial function [87-90]. In particular, a decline in oxidative phosphorylation is evident in skeletal muscle over age and the same impairment is also evident by knocking down *Sirt1*. During ageing, a reduction of nuclear NAD levels decreases the activity of SIRT1, inducing a disruption in nuclear- mitochondrial communication, causing a decline in oxidative phosphorylation [46].

1.2.3 Role of NAD at different subcellular compartments

NAD is involved in several biological processes through its specific compartmentalization. In particular, NAD is present at extracellular level and, more abundantly, at intracellular level [72, 73].

Extracellular NAD

Extracellular NAD and its metabolites, as well as the enzymes involved in NAD biosynthesis, mediate several biological functions. In human mesenchymal cells, NAD is able to activate a cAMP/cADPR/ $[Ca^{2+}]_i$ signaling cascade by binding the purinergic receptor P2Y₁₁, inducing cellular responses such as proliferation [74]. NAD metabolites can also bind several receptors and induce cellular responses. For example, ADPR, the product of NAD glycohydrolases and ARTs, after being converted to adenosine binds to P2Y₁ receptors, causing an increase in cAMP levels. Extracellular NAMPT (eNAMPT), the rate limiting enzyme in NAD biosynthesis, is involved in several biological functions such as in the immune response and the regulation of glucose-stimulated insulin secretion in pancreatic β cells [75]. Furthermore, eNAMPT is up-regulated in obesity. In visceral fat, it is secreted by adipocytes and acts as an adipokine [76].

Intracellular NAD in the nucleus

Nuclear NAD regulates specific biological functions mainly through the two nuclear enzymes PARP1 and SIRT1. The presence of a specific enzyme located into the nucleus, suggests that a localized NAD pool must be maintained in close proximity to its site of action. Thus, nuclear NMNAT1 has an essential role in cellular metabolism, its presence is fundamental for organism survival [50]. Deletion of NMNAT1, is not compatible with organism survival, suggesting that it cannot be compensated by the other two NMNAT isoforms [77].

Intracellular NAD in the mitochondrion

Mitochondrial NAD plays a fundamental role as electron donor in several processes such as oxidative phosphorylation and ATP synthesis. NAD is also involved in other mitochondrial functions such as regulation of the activity of SIRT3, SIRT4 and SIRT5, which play a role in controlling the energy status of the cell [91] and modulates the activity of mitochondrial proteins including glutamate dehydrogenase [92]. In addition to

these functions, it has been demonstrated that mitochondrial DNA and proteins are subjected to both mono (ADP-ribosyl)ation, as for glutamate dehydrogenase and Poly- (ADP-ribosyl)ation, during oxidative stress and brain injury [93-97].

Cytoplasmic NAD

In addition to redox cofactor, cytoplasmic NAD can act as signaling molecule and induces an increase in Ca^{2+} , once it is converted into cADPR and NAADP [98]. NAD is also an agonist of Ca^{2+} channels on its own. In the cytoplasm, NAD is also responsible for stabilizing microtubules through deacetylation mediated by SIRT2 [99].

1.2.4 NAD and its role on ageing

Over the past years, NAD and its metabolic enzymes have emerged as crucial players in ageing and in age-associated neurodegenerative diseases. β cell-specific *Sirt1*- overexpressing (BESTO) transgenic mice showed an enhanced glucose-stimulated insulin secretion and improved glucose tolerance at 3-8 months. In contrast, those improvements disappeared in aged BESTO mice. However, SIRT1 activity remains high in *Sirt1* overexpressing mice, suggesting that glucose-stimulated insulin secretion and glucose tolerance reduction in ageing mice may be due to a decline in NAD levels over ageing. To prove this, administration of NAD precursor NMN restored the phenotype in old mice overexpressing *Sirt1*, highlighting the essential role that NAD plays during ageing [100]. Indeed, administration of NAD precursors, by restoring NAD levels, was shown to prevent ageing-induced diabetes in wild-type mice [101]. Importantly, overexpression of *Sirt1* in the mouse brain increases lifespan in both females and males. In particular, females showed ~16% extension for median life, whereas males showed 9% extension in median life, compared to their controls [43]. Consistently with its role on ageing, SIRT1 has also shown a neuroprotective effect in age-related diseases such as Alzheimer's, Huntington's, and Parkinson's diseases through its overexpression [84, 102, 103]. SIRT1 activity can be regulated by several mechanisms, including changes in NAD levels [104]. An important hallmark of ageing is the progressive mitochondrial function decline [105,106] and the benefits induced by SIRT1 include the improvement in mitochondrial function [88]. During ageing, decline in nuclear NAD levels reduces the activity of SIRT1 in

the nucleus, inducing hypoxia-inducible factor 1 (HIF-1 α) stabilization. This program becomes chronically activated in old mice, inducing a pseudohypoxic state, disrupting nuclear-mitochondrial communication.

Knockout mice for *Sirt1* showed the same decrease in mitochondrial, but not nuclear-encoded, components of oxidative phosphorylation, as observed in the skeletal muscle of aged mice [46]. NAD, which is fundamental for SIRT1 activity, was reduced in skeletal muscle of aged mice and this reduction could be restored by NMN supplementation. Knocking down *Nmnat1*, responsible for NAD synthesis at the nuclear level, mimicked the decline in mitochondrial function observed during ageing, while overexpression of *Nmnat1* increased both NAD levels in the skeletal muscle of 12-months-old mice and the expression of mtDNA-encoded genes [46]. The mechanism by which NAD and SIRT1 control this mechanism appears to be mediated by a pathway involving hypoxia-inducible factor 1 α (HIF1 α), c-Myc and von Hippel-Lindau (VHL) E3 ubiquitin ligase, which recognizes a hydroxylated proline residue on HIF1 α for degradation by proteasome [46].

The reason why NAD declines with ageing is still controversial. One reason could be a possible change in the NAD/NADH ratio [107]. Furthermore, the ability of NMN to restore mitochondrial function suggests there might be a defect during ageing in NAD salvage pathway, in particular the reaction catalyzed by NAMPT, responsible for NMN synthesis [108]. In addition to this, NMNAT, through the SIRT1-dependent pathway mentioned above, plays a pivotal role in mitochondrial function over ageing and it is able to restore NAD level, suggesting a possible impairment in NAD biosynthetic pathway in the final step of NAD synthesis [108]. Importantly, NAD decline over ageing may be due to changes in the activity of its consuming enzyme. In particular, PARP1, which is stimulated upon genotoxic stress, might lead to NAD depletion due to accumulation of DNA damage in the cells during ageing [109]. Consequently, reduced levels of NAD impair SIRT1 activity, causing mitochondrial dysfunctions.

1.2.5 NAD in axon degeneration and neurodegenerative diseases

Consistently with its fundamental role in ageing mechanisms, NAD and its biosynthetic enzymes are also involved in many neurodegenerative diseases. The three mammalian isoforms of the central NAD biosynthetic enzyme, NMNATs, have recently emerged as crucial players in neuronal maintenance and protection [110-113].

The first evidence showing the involvement of nuclear NMNAT1 in neurodegenerative mechanisms can be dated to the discovery of the spontaneous mutant mouse *Wld^S* (Wallerian degeneration slow) [114]. In the *Wld^S* mouse, axon degeneration after acute injury is delayed by 10-fold and a stable synaptic activity is recorded for more than 2 weeks after separation of axons from their cell bodies [115]. The spontaneous mutation responsible for this phenotype has been identified as an 85 kb tandem triplication located on distal mouse chromosome 4 [116]. This mutation leads to the formation of a chimeric gene encoding a fusion protein not present in wild type mice [116, 117]. The chimeric protein contains the amino (N)-terminal 70 amino acids (N70) fragment of the ubiquitination factor E4B (UBE4B), the complete sequence of the nuclear *Nmnat1* and an 18 AA sequence originally belonging to the 5' untranslated region of the *Nmnat1* gene [117] (Fig.1.5). In *Wld^S*, the full sequence of *Nmnat1* is fused to a N-terminal N70 domain which contains 16 amino acids (N16) region, a binding site for the valosin containing protein (VCP), a ubiquitous cytoplasmic AAA-ATPase with several roles including one in the ubiquitin proteasome system (UPS) [118]. It has been demonstrated that *Wld^S* is able to protect injured axons in transgenic rats [119], injured olfactory receptor neurons (ORN) in *Drosophila* [120, 121] and axotomised trigeminal neurons in zebrafish [122]. Furthermore, it was shown *Wld^S* protection in several models of disease such as in Parkinson's disease mouse models [123, 124], in a rat glaucoma model [125] and in a gracile axonal dystrophy mouse model [126], suggesting that similar mechanisms regulate axon degeneration in both acute injuries and chronic diseases. It has been demonstrated that both N70 and NMNAT1 are required for the protecting phenotype *in vivo* [127], highlighting the important role of NMNAT1 in the mechanism of neurodegeneration. In particular, the binding of VCP protein to the N16 region of N70 allows the relocation of nuclear NMNAT1 to the axon, a site where NMNAT1 is able to confer its protective effect. NMNAT1 enzymatic activity and its localization to the axon are both essential for the protective phenotype [127].

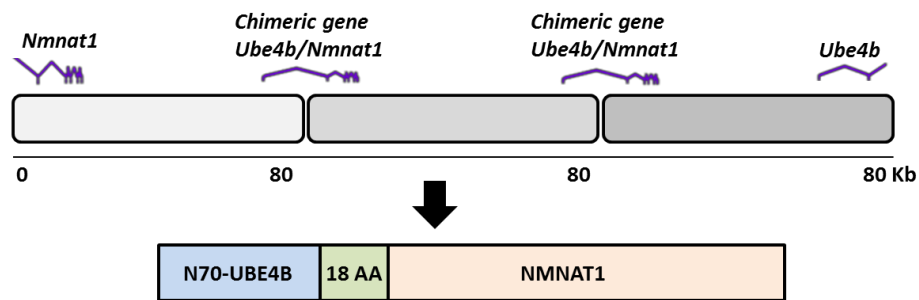


Fig.1.5 Schematic representation of *Wld^S* gene.

An 85Kb tandem triplication results in a chimeric gene that encodes for a chimeric protein, a fusion of the N-terminal 70 amino acids of the ubiquitination factor E4b (UBE4B), the full sequence of the enzyme NMNAT1 and a linking 18 AA sequence originally belonging to the 5' untranslated region of the *Nmnat1* gene.

The protective role of *Wld^S* in axon degeneration involves the cytoplasmic isoform NMNAT2 [113]. NMNAT2 is an essential axon survival factor, with a half-life of less than an hour. Its rapid depletion after an acute injury induces axon degeneration in mouse superior cervical ganglia (SCG) neurites. Similarly, its downregulation by specific RNAi in the absence of acute cut is sufficient to trigger axonal blebbing and fragmentation [113]. Under physiological conditions, NMNAT2 is constantly transported along the axons [113, 128], but after injury and interruption of NMNAT2 delivery from the cell body, NMNAT2 is rapidly depleted before signs of degeneration. When in the injured axon is present *Wld^S*, capable of enzymatic NAD synthesis by its NMNAT1 activity and much more stable compared to NMNAT2, this substitutes for loss of endogenous NMNAT2 maintaining its activity for a prolonged period [111, 113]. *Wld^S* mice, rats and *Drosophila* crossed with disease models are a unique experimental tool to investigate how axon degeneration contributes to neurodegenerative pathology and symptoms, ultimately leading the way to new therapeutic approaches.

In addition to the protective effect of NMNATs enzymes in axon degeneration, many evidences highlighted the crucial role that these enzymes have on several neurodegenerative diseases.

The discovery that loss-of-function mutations in the nuclear isoform NMNAT1 cause

Leber Congenital Amaurosis (LCA), a human nervous system disorder characterized by degeneration of retinal neurons and blindness [129-131], directly linked NMNAT1 to neuron survival. These evidences support the idea that NMNAT1 seems to be an essential protein for photoreceptor neuron function and survival. Indeed, the protective effects of NMNAT1 are correlated with NAD production, suggesting that LCA-causing *NMNAT1* mutations reduce enzyme activity and lower NAD levels in affected individuals [131].

NMNATs have also been associated to the mechanisms of proteotoxicity in yeast models of proteinopathies after heterologous expression of either mutant PolyQ domains (103Q) or α -synuclein fused to green fluorescent protein (GFP), which displayed evident growth defects. Indeed, overexpression of the *NMNAT* yeast homologs, *NMA1* and *NMA2* suppressed polyglutamine (PolyQ) and α -synuclein- induced cytotoxicities [132] through a mechanism similar to that of heat-shock protein 70 (Hsp70), displaying a chaperon-like activity [133]. In *Drosophila* a single, homogenously distributed NMNAT isoform has been reported, whose loss causes neuronal and axonal degeneration [110]. Studies in *Drosophila* and mice also highlight NMNAT protective function in models of tauopathies, a group of neurodegenerative diseases that includes Alzheimer's disease (AD) [134, 135]. Furthermore, NMNAT1 has been associated with neuroprotection in models of hypoxia-ischemia when it is redistributed to the cytoplasm (cyt-NMNAT1), with a mechanism which has been correlated to enzymatic activity [136].

All these evidences highlight the importance that NAD biosynthetic pathway has not only in ageing processes, but also in neurodegenerative diseases and ageing-related pathologies. Indeed, age is the main risk factor for several classes of either chronic or acute conditions, including neurodegenerative disorders, diabetes and cardiovascular diseases [137, 138]. Importantly, ageing is the largest risk factor for neurodegenerative diseases, such as Parkinson's disease [139] and Alzheimer's disease [140]. Furthermore, many acute health problems, such as stroke and heart attack, strongly increase with ageing [141].

In my study, I investigated the role of the nuclear NAD-biosynthetic enzyme NMNAT1 in age and in age-associated diseases, focusing on two pathological conditions: Alzheimer's disease and stroke, where a potentially protective role of NAD metabolism had been previously suggested.

1.3 Tauopathies including Alzheimer's disease.

Tauopathies, including AD, are characterized by the presence of hyperphosphorylated tau protein that aggregates and forms neurofibrillary tangles (NFTs)[142]. A list of tauopathies is illustrated in table 1.1 [143]. Tau is a microtubule-associated protein (MAP) expressed in neurons and normally localized in axons, where it promotes microtubule assembly and stabilization, depending on its level of phosphorylation [144, 145]. When tau is hyperphosphorylated, it is unable to stimulate microtubule assembly, leading to disruption of microtubules and axonal transport, finally resulting in retrograde degeneration and cell death. Furthermore, the association between abnormally hyperphosphorylated tau and normal tau leads to the formation of NFTs, which are accompanied by cell death and dementia [143].

• Alzheimer disease, including tangle-only form of the disease
• Down syndrome, adult cases
• Guam parkinsonism dementia complex
• Dementia pugilistica
• Pick disease
• Dementia with argyrophilic grains
• Fronto-temporal dementia
• Cortico-basal degeneration
• Pallido-ponto-nigral degeneration
• Progressive supranuclear palsy
• Gerstmann–Sträussler–Scheinker disease with tangles

Table 1.1 Tauopathies characterized by abnormal hyperphosphorylation of tau.

1.3.1 Epidemiology and main risk factors

Among the tauopathies, AD is the best-known. In this pathology, tau is accumulated in neurons, forming NFTs. AD, characterized by progressive decline in cognitive functions, is the first cause of dementia [146]. By 2005, 24.2 million people worldwide had dementia and 4.6 million new cases were arising every year and about 70% of all cases are attributable to AD [147] and the incidence is predicted to double every 20 years until at least 2040 [148]. 60 years-old population from North America and Western Europe show the highest prevalence and incidence of dementia, followed by those from Latin

America and China and its western-Pacific neighbors [147]. The prevalence of AD appears to be much higher in the US rather than Africa, Asia and Europe and higher among African-American and Hispanic populations living in the US, but lower for Africans in their homelands, for reasons that remain uncertain [149]. The prevalence rates for AD increase significantly with age, particularly after 65 years. There is almost a 15-fold increase in the prevalence of dementia, predominately AD, between the ages of 60 and 85 years [150]. Furthermore, incidence rates increases with age from approximately 0.5% per year among individuals aged 65–70 to approximately 6–8% for individuals over age 85 [148]. Early onset of AD (onset < 65 years), accounting for 1-5% of all cases, compared to late onset (onset $\geq$ 65 years) which accounts for >95% of affected, are associated with a Mendelian pattern of inheritance. In particular, rare mutation of three genes encoding proteins involved in amyloid β precursor protein (APP) breakdown and A β generation (*APP*, *PSEN1* and *PSEN2*) have been associated with early onset of AD [146, 151]. *APP* encodes a ubiquitously expressed transmembrane protein and most mutations cluster around or within the A β domain; mutation in this gene accounts for about 15% of early-onset autosomal dominant cases of AD. *PSEN1* and *PSEN2* are part of the γ - secretase protein complex responsible for APP cleavage and A β generation. Mutations in *PSEN1* accounts for 80% of all case of early-onset AD, whereas approximately 5% have been identified in *PSEN2*.

Furthermore, other common variants such as the apolipoprotein E (APOE) haplotypes are mostly associated to the risk for late onset AD, which accounts for more than 95 of total cases [151]. A detailed list of all mutations involved in AD is illustrated in table 1.2.

Gene	Location	Protein	Function
Rare variants			
<i>APP</i>	21q21.3	Amyloid β (A β) precursor protein	β-amyloid production - Synaptic formation and repair - Neural plasticity
<i>PSEN1</i>	14q24.3	Presenilin 1	β-amyloid production - Notch signaling pathway - Wnt signaling pathway - Endoplasmic reticulum calcium ion homeostasis
<i>PSEN2</i>	1q42.13	Presenilin 2	β-amyloid production - Notch signaling pathway
<i>ADAM10</i>	15q22	ADAM metalloproteinase domain 10	β-amyloid production - Notch signaling pathway
<i>TREM2</i>	6p21.1	Triggering receptor expressed on myeloid cells 2	Inflammatory response
<i>PLD3</i>	19q13.2	Phospholipase D family, member 3	Lipid metabolism
<i>AKAP9</i>	7q21-q22	A kinase (PRKA) anchor protein 9	Cell signaling
<i>GRN*</i>	17q21.32	Granulin	- Signal transduction - Growth factor activity
<i>MAPT*</i>	17q21.1	Microtubule-associated protein tau	Microtubule cytoskeleton organization
<i>PRNP*</i>	20p13	Prion protein	- Synaptic transmission - Neuronal copper metabolism
Common variants			
<i>APOE</i>	19q13.2	Apolipoprotein E	Lipid metabolism
<i>BIN1</i>	2q14	Bridging integrator 1	- Synaptic vesicle endocytosis - Cytoskeletal dynamics
<i>CR1</i>	1q32	Complement component (3b/4b) receptor 1	Regulation of complement activation
<i>ABCA7</i>	19p13.3	ATP-binding cassette, sub-family A (ABC1), member 7	- Lipid metabolism - Phagocytosis
<i>FERMT2</i>	14q22.1	Fermitin family member 2	- Cell-cell adhesion - Cell spreading - Cell shape modulation
<i>HLA-DRB5-DRB1</i>	6p21.3	Major histocompatibility complex, class II, DR β 5, β 1	Immune response
<i>CD2AP</i>	6p12	CD2-associated protein	Regulation of actin cytoskeleton reorganization
<i>PTK2B</i>	8p21.1	Protein tyrosine kinase 2 β	- Calcium-induced regulation of ion channels - MAP kinase signaling pathway
<i>INPP5D</i>	2q37.1	Inositol polyphosphate-5-phosphatase, 145 kDa	Immune response
<i>CUGBP-CELF1</i>	11p11	CUGBP, Elav-like family member 1	Regulation of RNA splicing
<i>CD33</i>	19q13.3	CD33 molecule	Cell signaling
<i>SLC24A4-RIN3</i>	14q32.12	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 4- Ras and Rab interactor 3	- Ion transmembrane transport - Cell signaling - Endocytosis
<i>ZCWPW1</i>	7q22.1	Zinc finger, CW type with PWWP domain 1	Zinc ion binding
<i>MEF2C</i>	5q14.3	Myocyte enhancer factor 2C	Myogenesis
<i>NME8</i>	7p14.1	NME/NM23 family member 8	Ciliary function
<i>MS4A cluster</i>	11q12.2	Membrane-spanning 4-domains, subfamily A	Signal transduction
<i>EPHA1</i>	7q34	Ephrin type-A receptor 1	- Immune response - Neural development
<i>PICALM</i>	11q14	Phosphatidylinositol binding clathrin assembly protein	Clathrin-mediated endocytosis
<i>CLU</i>	8p21-p12	Clusterin	- Lipid metabolism - Immune response
<i>CASS4</i>	20q13.31	Cas scaffolding protein family member 4	- Cell-cell adhesion - Cell spreading
<i>SORL1</i>	11q23.2-q24.2	Sortilin-related receptor, L(DLR class) A repeats containing	- Endocytosis - Sorting
<i>DSG2</i>	18q12.1	Desmoglein 2	Cell-cell adhesion

**GRN, MAPT, and PRNP mutations have been found in some individuals with clinical phenotypes indistinguishable from AD (Lee et al., 2014). Variants of genes associated with Alzheimer's disease (AD) can be classified according to their frequency as rare or common variants. Rare variants are commonly associated with early onset AD, while common variants are associated with late onset Alzheimer's disease (LOAD).*

Table 1.2 List of AD-associated genes [151].

Among the non-genetic risk, cerebrovascular changes, such as large ischemic cortical infarcts, increase the risk of dementia probably due to a direct damage of brain regions involved in memory function, but also increasing the deposition of A β [146]. Furthermore, several data linked high blood pressure in mid-life (40–60 years of age) and late-life

cognitive impairment, suggesting that elevated blood pressure in mid-life does increase the risk of later-life cognitive impairment, dementia and AD [152-156]. Hypertension may increase the risk of AD by affecting the integrity of the blood brain barrier, which may cause protein extravasation into brain tissue, resulting in cell damage, reduction in neuronal function, apoptosis, and an increase in A β accumulation and subsequent cognitive impairment [157, 158]. Furthermore, type 2 diabetes (T2D) has been found to nearly double the risk of AD, although this is still controversial [159-161]. Body weight has been linked to an increased risk of cognitive impairment and AD, but evidences showed that both low and high body weight may be related to AD and the mechanism is not clear [162]. In addition to these, other pathological conditions such as metabolic syndrome [163] and traumatic brain injury [164] have been correlated with an increased risk of AD. Finally, life-style plays a fundamental role in reducing the risk of AD and cognitive impairments. Indeed, diet and intellectual activity might have been correlated with lowering the risk to develop the pathology [165, 166]. Furthermore, it has been reported that physical exercise may have a role in limiting the progression of neurodegenerative diseases. Physical activity increases cerebral blood flow, which probably leads to an increase in angiogenesis, neurogenesis, synaptogenesis, and neurotransmitter synthesis in the different cerebral areas involved in cognition, suggesting that it can either protect against the onset of AD or slow down the progression of the pathology [167].

1.3.2 Pathophysiology of Tauopathies and Alzheimer's disease

The most established cause of tau pathology, characteristic of AD and other tauopathies is the abnormal hyperphosphorylation of tau [168]. However, the neuropathological changes in AD include other important features briefly described below.

- *Tau pathology*

Tau is a microtubule-associated protein (MAP) expressed in neurons and normally localized in axons, where it promotes microtubule assembly and stabilization, depending on its level of phosphorylation [144, 145]. Normal tau contains 2–3 mol phosphate/mol of the protein [169]. Human tau is a family of six proteins derived from a single gene by alternative mRNA splicing (Fig.1.6). These proteins differ in whether they contain three or four [143] tubulin binding domains (repeats, R) of 31 or 32 aminoacids each near the

C-terminal and two, one or no inserts of 29 amino acids each in the nN-terminal portion of the molecule [170, 171].

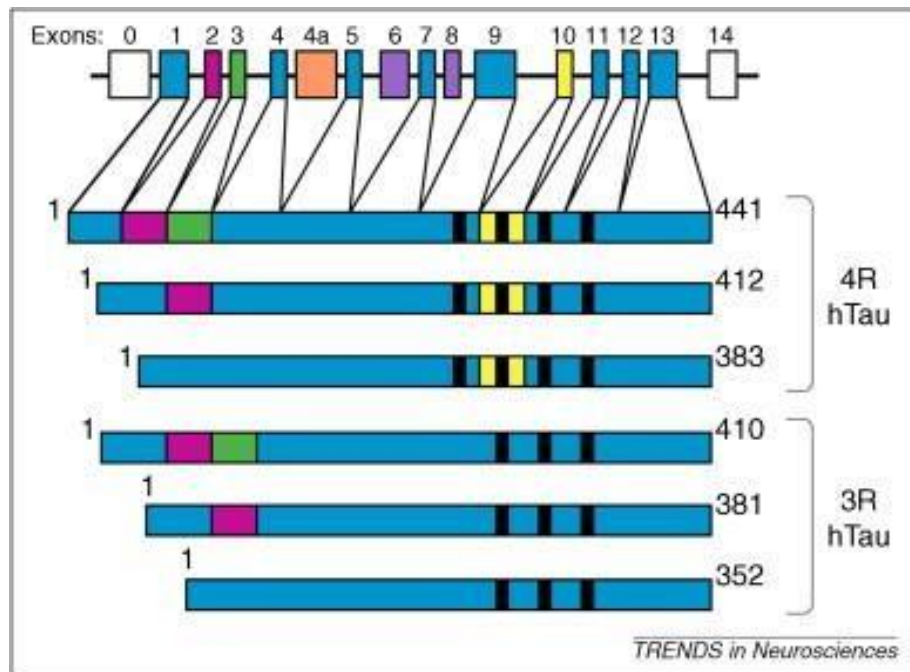


Fig.1.6 Schematic representation of the human tau gene and the six tau isoforms expressed in adult brain.

The human tau gene consists of 16 exons (E). Alternative splicing of E2 (red), E3 (green) and E10 (yellow) gives rise to the six tau isoforms (352-441 amino acids). The constitutively spliced exons (E1, E4, E5, E7, E9, E11, E12, E13) are indicated in blue. E0, which is part of the promoter, and E14 are non-coding (white). E6 and E8 (violet) are not transcribed in human brain. E4a (orange) is only expressed in the peripheral nervous system. Black bars indicate the microtubule-binding repeats of tau, with three isoforms having four repeats each (4R hTau) and three isoforms having three repeats each (3R hTau). Each repeat is 31 or 32 amino acids in length. Similar levels of 4R and 3R tau isoforms are expressed in normal human brain [172].

The evidence that mutations in tau gene co-segregate with the inherited disease FTDP-17 proved the critical role that tau protein plays in inducing neurodegenerative processes and subsequent dementia [173-175]. Hyperphosphorylated tau, as seen in tauopathies, is unable to stimulate microtubule assembly and sequesters normal tau, MAP1A/MAP1B and MAP2, the other microtubule-associated proteins, leading to

disruption of microtubules and axonal transport, finally resulting in retrograde degeneration and cell death [143].

Tau in AD brain is abnormally hyperphosphorylated and is accumulated not only in intraneuronal tangles of paired helical filaments (PHF), which forms NFTs [176] but also in the cytosol [169] (Fig.1.7B, C and E). Abnormal hyperphosphorylation of tau precedes its accumulation into NFTs [169]. In AD, tau protein structure is normally intact. However, immunostainings studies revealed that tau in NFTs is often truncated both at Glu 391 and Ser-42[177], but what percentage of tau is truncated has not been reported [143]. Truncated tau might be involved in the formation of NFTs. A transgenic rat model overexpressing truncated human tau has been shown to produce a significant number of neurofibrillary tangles [143].

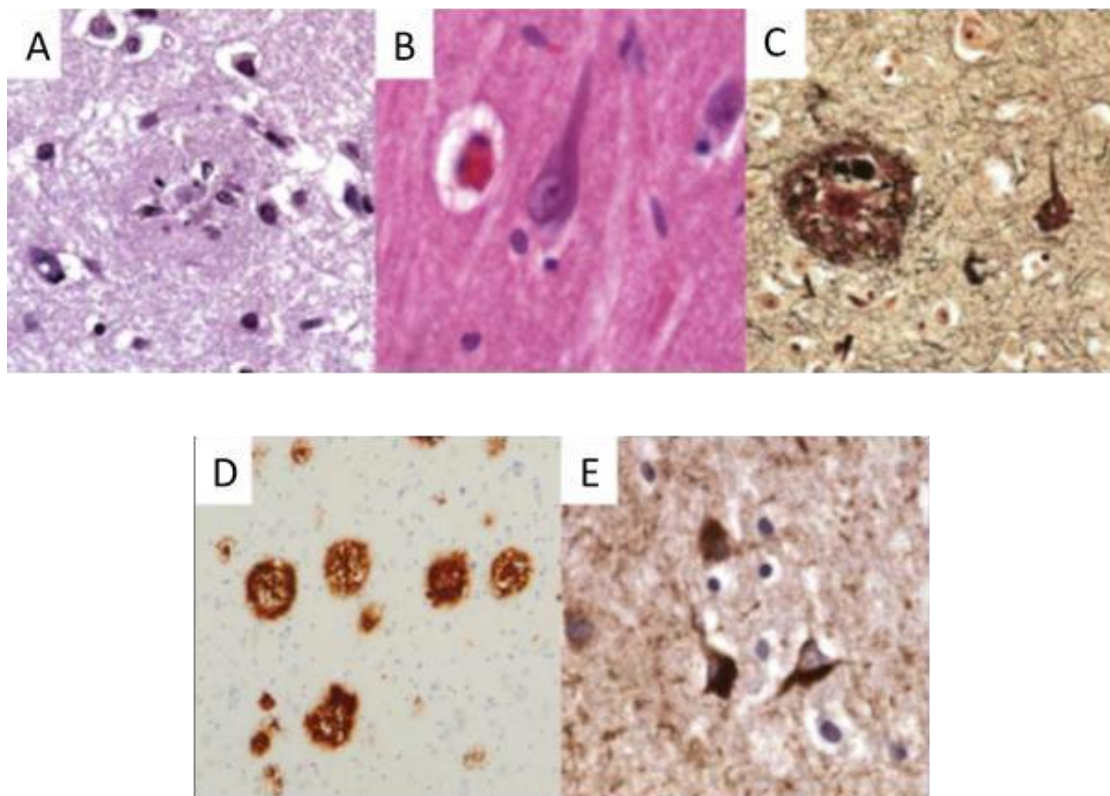


Fig.1.7 Pathological lesions observed in Alzheimer's disease.

(A) Plaque evident on H&E stained section of frontal cortex; (B) tangle in a hippocampal pyramidal neuron on H&E stained section; (C) silver stain highlights both a plaque and a tangle; (D) immunohistochemistry against Ab highlights plaques; (E) immunohistochemistry against tau highlights tangles [168].

Formation of NFTs can be divided into three different stages: (1) Pre-NFTs or diffuse NFTs consist of diffuse tau staining within the cytoplasm of neurons, with well-preserved dendrites and nucleus; (2) Mature or fibrillar intraneuronal NFTs (iNFTs) consist of cytoplasmic filamentous aggregates of tau that displace the nucleus toward the periphery of the soma and often extend to distorted-appearing dendrites and to the proximal segment of the axon; (3) extraneuronal NFTs (eNFTs) resulting from the death of the tangle-bearing neurons [178, 179].

NFTs start to appear in the entorhinal cortex, followed by the CA1 region of the hippocampus. Next, NFTs accumulate in limbic structures such as the subiculum of the hippocampus and the amygdala, then in thalamus, and claustrum. Finally, NFTs spread to all isocortical areas, affecting first and more severely associative areas and, after that spreading to the primary sensory, motor, and visual areas and at a later stage striatum and substantia nigra [180].

Unlike normal tau, the cytosolic hyperphosphorylated tau from AD brain (AD P-tau) contains 5 to 9 mol of phosphate per mole of the protein [169]. It has been demonstrated that abnormally hyperphosphorylated tau polymerizes into tangles of PHF in vitro at pH 6.9, under reducing conditions at 35°C during 90 minutes and these are named as self-assembly conditions [181]. Tau is able to self-assemble at the sites that flank the microtubule-binding domain. Tau self-assembles probably by intermolecular hydrophobic interaction and through its microtubule binding repeat R3 (in the case of 3R taus), and R2 and R3 (in the case of 4R taus). These inhibitory regions appear to be neutralized by abnormal hyperphosphorylation in AD and other tauopathies [182].

More than one mechanism is involved in the abnormal hyperphosphorylation of tau (Fig.1.8). Tau protein in AD is N-glycosylated, and this glycosylation makes tau a more favorable substrate for phosphorylation by protein kinase A (PKA), glycogen synthase kinase (GSK-3 β), and cyclin-dependent kinase (cdk5), and less favourable for dephosphorylation by protein phosphatase (PP-2A)[183, 184]. Furthermore, levels of protein O-GlcNAcylation, which regulates phosphorylation, is reduced in AD brain [185].

The activity of PP-2A is reduced in AD brain and leads to abnormal hyperphosphorylation of tau not only by a decrease in dephosphorylation, but also by an increase in the

activities of CaMKII, PKA and MAP kinases, which are negatively regulated by the phosphatase [184]. In FTDP-17, tau mutations G272V, P301L, V337M and R406W as well as mutations that lead to increase 4R/3R ratio make this protein a more favorable substrate for phosphorylation than the corresponding wild-type tau [186]. In AD brains abnormally hyperphosphorylated tau can be found as polymerized into NFTs of PHF mixed with straight filaments (SF) or as non-fibrillated form in the cytosol [169, 181, 187]. Tau forming NFTs is apparently inert whereas cytosolic tau is able to sequester normal tau, MAP1A/MAP1B and MAP2, inducing a disassembly of microtubules in vitro [188, 189]. The inhibition and disassembly of the microtubules compromises axonal transport, leading to retrograde synapse and axon degeneration, which, in turn, results in dementia. The association between the abnormally hyperphosphorylated tau and normal tau in the presence of glycosylation results in the formation of neurofibrillary tangles. The tangles are ubiquitinated through degradation by the non-lysosomal ubiquitin-proteasome system [143].

Tau Oligomerization and Neurofibrillary Degeneration

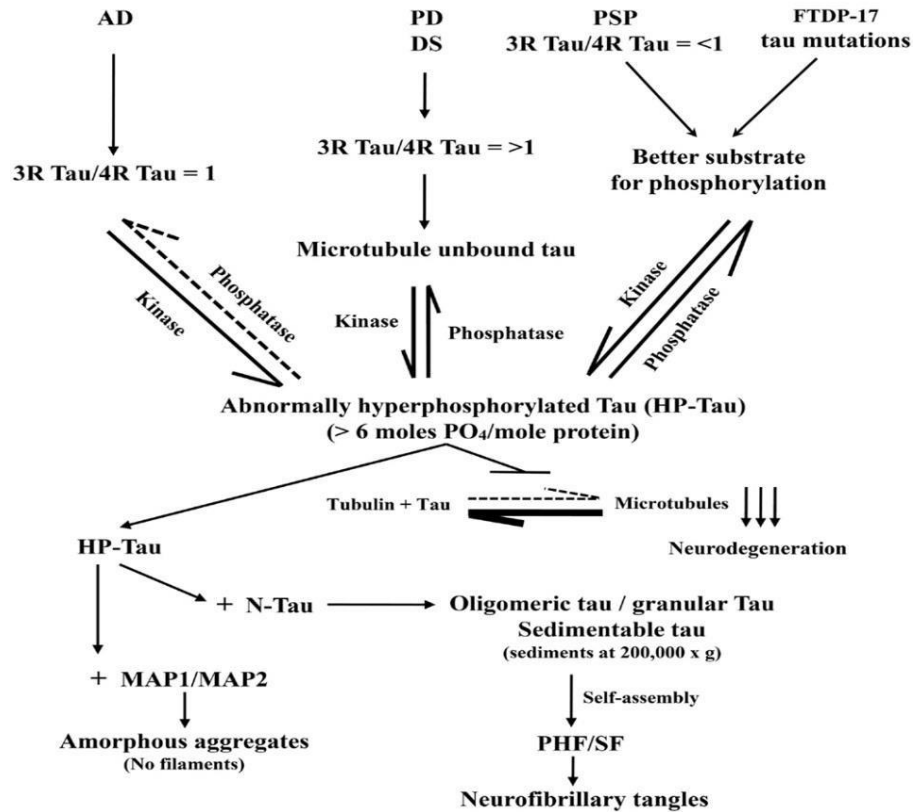


Fig.1.8 Abnormal hyperphosphorylation of tau promotes its oligomerization and self- assembly into paired helical filaments, forming neurofibrillary tangles.

A protein phosphorylation/dephosphorylation imbalance apparently caused by a decrease in protein phosphatase 2A (PP2A) activity leads to abnormal hyperphosphorylation of tau in AD brain. The abnormally hyperphosphorylated tau binds to normal tau (and not to tubulin) and this sequestration leads to the disruption of microtubules and the formation of oligomers which can sediment at $200,000 \times g$; the tau oligomers show up as granular structures by negative stain electron microscopy. The abnormally hyperphosphorylated tau isolated from AD brain cytosol readily self-assembles into paired helical filaments in vitro under polymerizing condition [190].

- *Amyloid plaques*

The senile plaques result from the abnormal extracellular accumulation and deposition of the amyloid- β peptide ($\alpha\beta$) with 40 or 42 amino acids ($\alpha\beta$ 40 and $\alpha\beta$ 42), two normal

products of the metabolism of the amyloid precursor protein (APP) after its sequential cleavage by the enzymes β - and γ -secretases in neurons [168] (Fig.1.7A,C and D). $A\beta$ regulates synaptic and neuronal activities, and $A\beta$ accumulation in the brain leads to a combination of synaptic depression and aberrant network activity [191]. Diverse lines of evidence suggest that $A\beta$ and APP contribute to the pathogenesis of AD causally [191]. Two types of amyloid plaques can be distinguished: diffuse plaques and dense-core plaques. Dense-core plaques are responsible for deleterious effects including dystrophic neurites, synaptic loss, neuron loss, and activation of both astrocytes and microglial cells [192-197]. Dense-core plaques cause the formation of abnormal neuronal processes, characterized by a central mass of extracellular filaments that radially extend toward the periphery, where they are intermingled with neuronal, astrocytic, and microglial processes.

These dystrophic neurites may contain paired helical filaments, abnormal mitochondria and dense bodies that probably include mitochondria and lysosomes [198-200]. Amyloid plaques can be detected mainly in the isocortex and usually involve the six layers of the isocortex, although layers I and VI are usually relatively more spared than layers II-V [168]. Cerebral amyloid angiopathy (CAA) is present in 80% of AD patients. In CAA, deposits of amyloid β that can be detected in the tunica media of leptomeningeal arteries and cortical capillaries in posterior areas of the brain. In some cases, CAA can weaken the vessel causing hemorrhages [168].

- *Neuroinflammation*

Amyloid- β plaques and neurofibrillary tangles are the main neuropathological hallmarks in AD. However, inflammation plays a significant role in the pathophysiology [191]. $A\beta$ plaques are frequently associated with the activation of microglia and astrocytes. $A\beta$ induces the expression of inflammatory cytokines and inflammatory enzymes such as COX-2 and iNOS. Inflammatory cytokines (such as TNF- α and IL-1 β), in turn, enhance APP production and the process of APP proteolytic cleavage to increase the production of $A\beta_{42}$ peptide. $A\beta$ is able to stimulate NF- κ B and MAPK signaling pathways [191]. Both these signaling pathways are associated with the transcription of inflammatory mediators. $A\beta$ can disrupt gliotransmitter release and astrocytic calcium signaling, and alter synaptic plasticity, which are vital processes for astrocyte–neuron communication [201]. Reactive

astrocytes and activated microglial cells are commonly associated to dense-core amyloid plaques, indicating that amyloid- β is a major trigger of this glial response [192, 194, 197]. A highly significant positive correlation between both astrocytosis and microgliosis and NFT has been observed, suggesting that glial responses are also related to neurofibrillary degeneration [191]. Indeed, repeated immunization with phosphorylated-tau peptides causes neuroinflammation in NFT and wild-type mice [202].

1.3.3 Symptoms and therapies

In AD and other tauopathies, tau is abnormally hyperphosphorylated and is accumulated as intraneuronal tangles of paired helical filaments (PHF), which directly correlates with dementia in these patients [143]. AD is characterized by specific neuropsychological symptoms that can be detected at a late stage of the pathology. However, research is now mainly focused on earlier stages of the pathology, as biological markers of AD can precede cognitive and behavioral symptoms by years. For this reason, a new diagnostic criterion has been established, which includes not only dementia, but also an intermediate stage of mild cognitive impairments (MCI) that precedes dementia and a pre-clinical AD stage [203], characterized by the presence of biomarkers, such as brain amyloid deposition and CSF tau and amyloid, that can be detected in asymptomatic individuals years before the onset of cognitive decline [180].

The earliest detectable changes in AD usually occur in medial temporal lobe structures, including hippocampus and entorhinal cortex, where NFTs start to accumulate, critical areas for episodic memory functions [180]. Indeed, the ability to learn and remember new information is a characteristic deficit in AD patients. Impairments in language and semantic knowledge such as difficulty in object naming and semantic categorization are also evident in mildly demented AD patients as well as in executive functions responsible for the mental manipulation of information, concept formation, problem solving and cue-directed behavior. Furthermore, deficits in visuospatial abilities may occur in the pathology, even in preclinical stages [203].

Up to date, available medications for AD only provide symptomatic benefits. These medications lead to an improvement in cognitive functions by increasing acetylcholine levels (acetylcholinesterase inhibitors) and blocking glutamatergic receptors (N-methyl- D-aspartate receptor antagonist, such as memantine) [204].

The first line of therapeutic strategy was inhibition of A β formation through the inhibition of α -, β - and γ -secretases, and enzymes responsible for the production of amyloid- β peptide. The β -secretase, also known as BACE1, might be a prime therapeutic target for AD, because inhibition of this enzyme may decrease production of all forms of A β . BACE1 is a transmembrane aspartic protease, highly expressed in neurons of the brain. Indeed, potent BACE1 inhibitors showed efficacy in lowering cerebral A β levels in animal models of AD. A potential candidate BACE1 inhibitor drug has already completed the first phase I clinical trial, and other candidate BACE1 inhibitors might soon be entering clinical trials [205]. Another enzyme, which had shown promising results in the pharmacological treatment of AD is γ -secretase, which is a multi-subunit aspartyl protease that cleaves APP and many other type 1 transmembrane proteins within their transmembrane domains. Indeed, brain-penetrant γ -secretase inhibitors (GSIs) decrease A β production in human and mouse brains, and chronic administration decreases A β deposition in APP mouse models [206]. These drugs are now being tested in clinical trials and, in particular, LY450139 (a nonselective GSI), is currently being tested in a phase III study [205]. Since inflammation plays a critical role in AD, immune system is another therapeutic target in the pathology. Cytokines and other neurotoxic adducts secreted by immune-related cells were suspected as possible mediators of neuronal degeneration and cell death [207]. Immunotherapy through targeting amyloid- β and tau is one of the most promising therapeutic strategy. Indeed, several strategies for A β peptide immunotherapy have been tested, including passive immunization with monoclonal antibody against different regions of A β 42 as well as active immunization using synthetic A β 42. Preclinical trials for both active and passive immunotherapies against A β showed beneficial effects of ameliorated brain A β burden, prevention of memory loss and improved cognitive function in different animal models of AD [208]. Preliminary test on patients exerted promising outcomes of reduced plaque burden and cognitive benefit [209]. However, unavoidable side effects found in clinical trials into AD therapeutic treatment on human and clinical evaluation in human AD patients failed to replicate the identical results as in the animal AD model [210].

Furthermore, hyperphosphorylated tau forming NFT is another fundamental hallmark of AD. Kinases responsible for tau phosphorylation (GSK-3 β , cdk5 and microtubule affinity-regulating kinase) and phosphatase (protein phosphatase 2A, PP2A) are possible

targets to achieve tau-aiming therapeutics, altering tau phosphorylation by modulating activity of the enzymes. In particular, GSK-3 β inhibition (by lithium, valproate and caffeine), which is also implicated in A β formation, has been tested. However, preclinical and clinical studies showed inconsistent results in different study and for this reason, the efficacy of these treatments needs to be confirmed [211].

In addition, other metabolic targets have emerged as crucial in AD. In particular, glucose metabolism and insulin signaling are impaired in AD brain [186] and insulin-degrading enzyme (IDE) induces A β degradation [187]. Indeed, Metformin, a peripheral insulin sensitizer drug, was reported to sensitize brain insulin action and it was recognized as a drug that can prevent pathological alteration in *in vitro* AD model [188].

Finally, mitochondrial dysfunction occurring in AD has been considered as a possible therapeutic target in the pathology. Piracetam has been found to improve membrane potential, ATP generation and decrease apoptotic vulnerability in ageing and AD model, suggesting that this drug can play a pivotal role in restoring mitochondrial impairments characteristic of AD [189].

1.3.4 Models of Tauopathies relevant to AD

Many models of Alzheimer's disease have been developed and have greatly advanced the understanding of AD pathogenesis. However, it is very difficult to develop a unique model exhibiting all AD features. Currently, the most used animal models are based on known genetic mutations associated to the pathology. In the next paragraph, I will focus on models of human tauopathy that reproduce one of the main features characteristic of AD.

- *In vitro models*

Non-neuronal cell line. Several cell lines have proven useful for tauopathy models, including fibroblast lines such as 3T3 and L cells, Cos cells, and CHO cells. These cell lines are very well characterized and easy to use in studies. Since tau is mainly localized to the nervous system, they allow examining the properties of exogenous human tau in the absence of endogenous tau. Such models can be used to test the effects of coding sequence tauopathy mutations on tau/microtubules interactions. Finally, non-neuronal cell lines are suitable for examining the effects of overexpressing exogenous human tau isoforms [212].

Neuronal cell lines. The most exploited and well-characterized cell lines are PC12 and SY5Y cells. These cells allow to study specific structural alteration such at the level of synapses, dendrites, axons, dendritic/axonal MTs, structures greatly impaired in the pathology, as well as interactions between overexpressed human tau and neuronal functions [212].

- *In vivo models*

Invertebrate transgenic models. Nematode *C. elegans* and the fruit fly *D. melanogaster* are widely used to reproduce tau pathology by creating transgenic models using normal and mutant human tau isoforms. Transgenic flies neuronally overexpressing either wild-type or mutant human tau, htau^{R406W}, associated with FTDP17, showed adult onset, progressive neurodegeneration with compromised lifespan and accumulation of hyperphosphorylated tau [134]. These models are easy to manipulate even at the single cell level and present advantages related to their rapid and detailed genetic manipulations [212].

Lower vertebrate models. Zebrafish have been used as a model to reproduce tau features. This model easily allow genetic manipulation. Most zebrafish neurons exhibit a typical vertebrate multipolar morphology, making this a particularly good system for studying tau phosphorylation [213].

Transgenic mouse models. Murine transgenic models have been used for expression of either wild-type or mutant isoforms of human tau in mice, which in turn results in development of NFT, reproducing a pathological condition similar to that of humans. Furthermore, these models present clear behavioral signs of progressive neurodegeneration. However, these transgenic mouse models need to account for the characteristic distribution, morphology and isoform content of neurofibrillary lesions that can vary in different pathologies. In particular, mice expressing mutant (P301L) tau protein provide the most complete mouse models of neurofibrillary tau pathology. However, the pathology observed in these mice mainly occurs in the hindbrain and spinal cord, areas of less relevance to AD [214]. These transgenic mice provide good models for inherited tauopathies, but less valuable to the study of pathogenesis in AD, where tau is not mutated. In contrast, a mouse model where wild type human tau (htau mice) is overexpressed reproduces AD-type spatial distribution of early tau pathology, as abnormal tau is mainly located to the neocortex and hippocampus, the most susceptible areas in AD [214].

1.3.5 NAD and tauopathies

Studies in *Drosophila* and mice highlight NMNAT protective function in models of tauopathies. Overexpression of either wild-type or enzyme-inactive *Drosophila* NMNAT reduces hyperphosphorylated tau load. Furthermore, it rescues cognitive and locomotor deficits. Importantly, NMNAT neuroprotection was also confirmed in a mouse model of tauopathy. NMNAT2 was downregulated in both cortex and hippocampus of mice overexpressing the FTDP-17 associated mutant tau_{P301L}, prior to the onset of neurodegeneration or cognitive deficits. Two functional cAMP-response elements (CREs) have been identified in the *nmnat2* promoter region. Both the total amount of phospho-CRE binding protein (CREB), which plays a relevant role in cognitive tasks and is critical for synaptic plasticity, learning and memory, and the pCREB bound to *nmnat2* CRE sites in the cortex and the hippocampus of rTg4510 mice are significantly reduced, suggesting that NMNAT2 is a direct target of CREB under physiological conditions and

that tau_{P301L} overexpression down-regulates CREB-mediated transcription. Indeed, hippocampus-specific overexpression of either NMNAT1 or NMNAT2 significantly reduced hippocampal neuronal loss [135].

It has been suggested that the ability of NMNAT to induce neuroprotection in the *Drosophila* and mouse models of tauopathy was related to its chaperone-like function, which enable the enzyme to reduce the amount of tau aggregates. Molecular chaperones have been implicated in many neurodegenerative diseases, including Parkinson's disease, AD and Huntington's disease [215]. They prevent improper protein folding and aggregation and facilitate formation of proper conformation by recognizing and binding to exposed hydrophobic residues of unfolded proteins [216-218]. Stress-regulated chaperones as Hsp70 and Hsp90, were shown to prevent aggregation in a cellular model of tauopathy [219]. NMNAT is capable to work as stress-responsive chaperone [133] and, similarly to Hsp70 and Hsp90 [219], is able to reduce toxic tau aggregates by binding tau oligomers and promoting their ubiquitination and clearance [134].

Another mechanism by which NMNATs confer neuroprotection in tauopathies could involve SIRT1. SIRT1 levels correlate negatively with the amount of neurofibrillary tangles in AD brains, suggesting a possible pathogenic role of SIRT1 deficiency in tau accumulation [220]. Indeed, tau was acetylated in the early and late stages of tauopathies. SIRT1 deacetylates tau protein promoting polyubiquitination and subsequent proteasomal degradation [220-221]. SIRT1 plays a role in the control of stress-regulated proteins. Indeed, through heat shock factor-1 deacetylation, SIRT1 increases expression of Hsp70 [222] and this mechanism may contribute to the reduction of toxic tau aggregates. Importantly, in 2004 it has been highlighted the importance of mitochondrial dysfunction in AD that could explain the correlation between advancing age and AD risk [223, 224]. This theory is called “AD mitochondrial cascade hypothesis” and it is based on three main points. First, the mitochondrial cascade hypothesis maintains gene inheritance, which defines an individual's baseline mitochondrial function, dependent on both parents. Second, inherited and environmental factors determine the rate at which age-associated mitochondrial changes develop and manifest. A greater mitochondrial durability should associate with slower brain aging and lesser mitochondrial durability should associate with faster brain ageing. Third, an individual's baseline mitochondrial function influences their AD chronology. Those with low baseline function and fast

rates of mitochondrial decline will develop symptoms and AD histology changes at younger ages than those with high baseline function and slow rates of mitochondrial decline (Fig.1.9) [224].

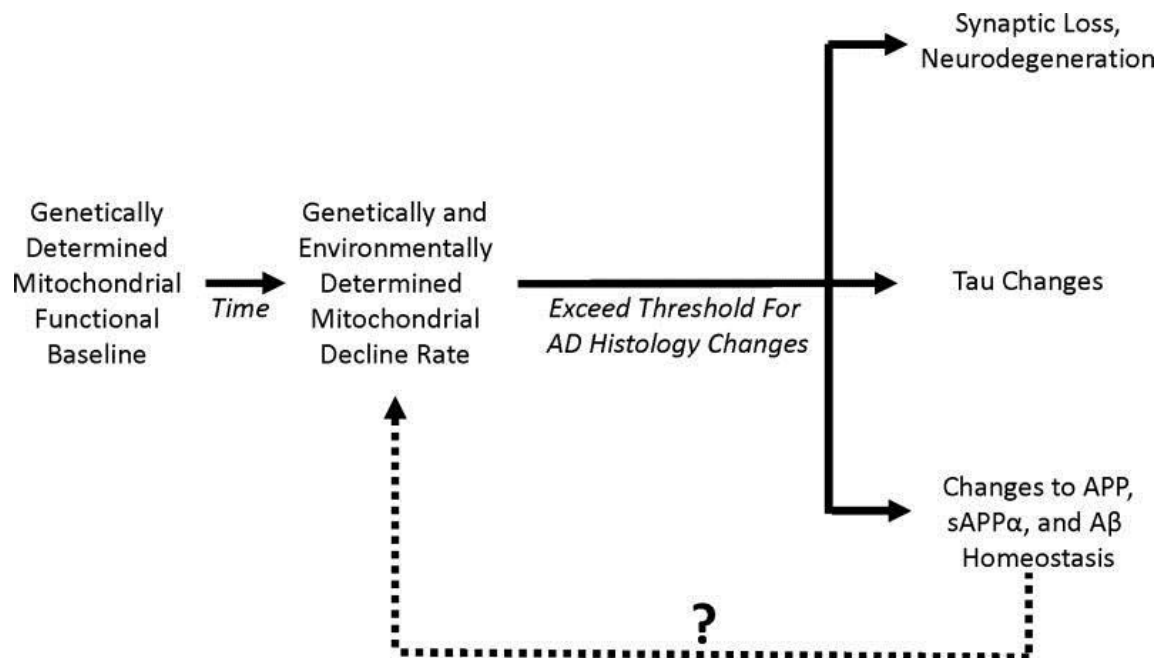


Fig.1.9 AD mitochondrial cascade hypothesis.

This hypothesis maintains individuals start out with a particular level of mitochondrial function, and that each individual's mitochondrial function declines at a particular rate. Eventually, mitochondrial decline surpasses a threshold and triggers the histologic changes associated with AD. The question mark indicates changes in APP, sAPP α , or A β may or may not further influence mitochondrial function. [224].

The maintenance of intracellular NAD levels may be fundamental for preserving cellular viability during conditions of chronic oxidative stress and mitochondrial dysfunction, characteristic of the ageing brain, by promoting ATP production [225]. SIRT1 is capable to restore ATP levels through a pathway involving HIF1 α [46-226].

1.4 Cerebral ischemia.

Cerebral ischemia is defined as a reduction of cerebral blood flow, resulting in insufficient amount of glucose and oxygen to support the homeostasis of the brain. It can involve the entire brain (global ischemia) or only a selective region (focal ischemia). Focal cerebral ischemia is characterized by an ischemic core with severe cerebral blood flow reduction and a distal ischemic penumbra, which includes marginal regions that may be served by collateral vascular channels. The ischemic insult results in two different processes of cell death, which differently affect the ischemic penumbra and the ischemic core: apoptosis, or programmed cell death is mainly activated in the ischemic penumbra; conversely, necrosis, a not regulated cell death, occurs in the ischemic core [227] (Fig. 1.10)

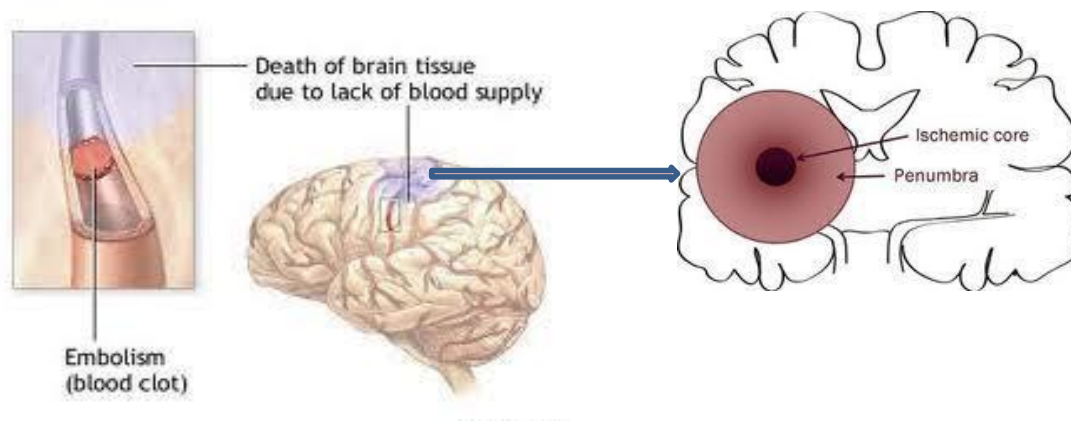


Fig.1.10 Brain ischemic injury.

One of the main causes of brain ischemia is a pathophysiological thrombosis or thromboembolism, which leads to a cerebral arterial occlusion and a subsequent ischemic area in the portion of the brain supplied by that artery. Two different injured areas can be recognized after an ischemic attack: the ischemic core, where neurons degenerate by necrosis and the surrounding area or penumbra, where apoptotic cell death-mechanisms are activated.

Thrombosis and thromboembolism are the most common cause of brain ischemia. These pathological conditions cause a cerebral arterial occlusion and a subsequent ischemic area in the portion of the brain supplied by that artery. The blood flow may be spontaneously and immediately restored so that the neurological symptoms are recovered; this episode is called transient ischemic attack. If blood flow is not restored there is a risk of infarction in the brain tissue, with a permanent damage and permanent neurological consequences and the event is called ischemic stroke. The term stroke can also be referred to other cerebrovascular events such as intracerebral hemorrhage and subarachnoid hemorrhage leading to a hemorrhagic stroke. Ischemic stroke accounts for approximately 80-85% of all cases of stroke, while hemorrhagic stroke for the remaining 15-20% [228].

1.4.1 Epidemiology and main risk factors

Cerebral ischemia is one of the major causes of disability and death worldwide. It has a profound impact not only on the affected individuals, but also on those who care for them. Stroke-associated long-term disabilities pose a substantial cost on the society, considering the direct cost of acute healthcare, rehabilitation and secondary prevention [228]. In the UK the cost to National Health Service for every patient experiencing a stroke episode

was around £15,000 per event over 5 years in 2001-2002 [229]. The prevalence of vascular diseases such as cerebral ischemia rises with increasing age for both sexes, so that older people are at main risk. Since the proportion of the population over 65 years of age is rapidly increasing, it has been estimated that there will be a 33% increase in 2020 of patients who experienced an ischemic insult [141]. The incidence of stroke varies widely depending on the study. Highest rate was in Japan and lowest in United Kingdom, Germany and New Zealand [230]. Stroke follows a circadian rhythm, and nearly a quarter of strokes occur during sleep; a peak incidence has been observed between 6 am and 12 noon, when the 55% of ischemic strokes occur [231].

Age is the most determinant risk factor of stroke. Its incidence becomes double in each successive decade from 55 years old. Prevalence for individuals older than 80 years is 27%, while for individuals 60-70 years is 13% [232, 233]. Recent analysis showed that stroke is more incident in men rather than in women. However, since stroke incidence is more elevated in older ages, women are more affected than men because of their longer life expectancy [234]. Several studies have found that stroke incidence can vary depending on the ethnicity. In fact, incidence is 2.4 times higher among black patients than in white patients, and that among Hispanic patients is 1.6 times than that of white patients [235]. It is still unclear what causes those differences.

Stroke incidence can depend on the family history and increases when there is a family history of stroke. This is probably due to a familiar association with other risk factors such as cholesterol or diabetes [233].

High blood pressure is the most important modifiable risk factor for both ischemic and hemorrhagic stroke. In particular, a high blood pressure accelerates the atherosclerosis, a predictor of ischemic stroke [236]. Indeed, high blood pressure has been found in 53-68% of patients affected of stroke [237]. Diabetes mellitus is also considered as a risk factor because it is a predisposing factor for the development of large and small-vessel atheroma [238].

The tobacco consumption has a clearly established relationship with atherosclerosis, for this reason it increases the risk of ischemic stroke. In addition, alcohol abuse can be associated to a higher risk of ischemic stroke. Finally, diet and physical activity can

influence the incidence of stroke. In particular it has been seen that women, but not men, who have a Mediterranean diet are at lower risk and a physical activity may reduce the risk of ischemic attack [239].

1.4.2 Pathophysiology of cerebral ischemia

The ischemic stroke can be due to several pathological conditions, all of which result in a reduction of blood flow, with subsequent insufficient glucose and oxygen. This in turn results in multiple processes leading to cell death: excitotoxicity, acidotoxicity and ionic imbalance, peri-infarct depolarisations, inflammation, apoptosis, oxidative and nitrative stress [240].

- *Excitotoxicity, acidotoxicity and ionic imbalance*

The brain requires a large amount of oxygen to produce sufficient ATP in order to maintain and restore ionic gradients into the cell. In particular, the Na^+/K^+ ATPase pump consumes 70% of the energy supplied to the brain [241]. This pump maintains high intracellular K^+ levels and low intracellular Na^+ levels fundamental for the propagation of action potentials. Furthermore, the Ca^{2+} -ATPase maintains low concentrations of Ca^{2+} within the cells. After an ischemic insult, ATP is depleted as a result of mitochondrial inhibition of ATP synthesis, causing neuronal plasma membrane depolarization, extracellular release of K^+ and entry of Na^+ into the cell [242]. Therefore, intracellular levels of Ca^{2+} raise leading to a massive activation of Ca^{2+} dependent protease, lipases and DNases. Since the cell is not able to supply ATP necessary to re-synthesise cellular constituents, the activation of these enzymes causes degradation of all cellular structures [242]. Due to the ionic imbalance (Fig.1.11), the neuronal plasma membrane remains in a depolarised state, increasing the release of excitatory neurotransmitter glutamate, which plays a fundamental role in the mechanisms of ischemic injury. The glutamate gradient is controlled by Na^+ dependent transporters located on presynaptic and postsynaptic membranes, which maintain a higher glutamate concentration inside the cells. The accumulation of Na^+ during an ischemic insult reverts the glutamate transporters, so that a massive glutamate amount accumulates in the extracellular compartments, activating AMPA receptors and NMDA receptors, which are Ca^{2+} permeable. NMDA receptors activation leads to a greater Ca^{2+} influx, responsible for the mechanism of excitotoxicity [242].

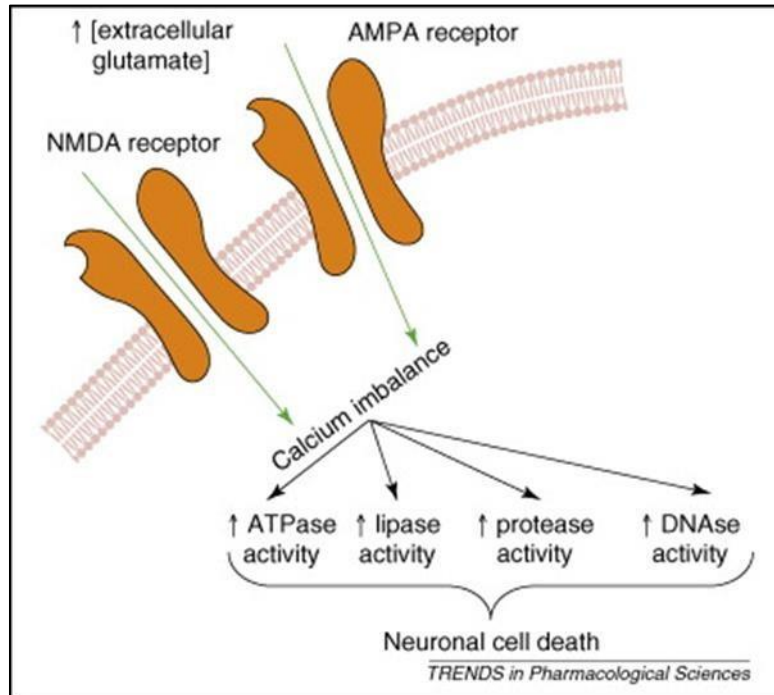


Fig.1.11 Mechanism of neuronal cell death due to ionic imbalance.

After an ischemic insult, due to the ionic imbalance, the neuronal plasma membrane remains in a depolarised state, increasing the release of glutamate. The glutamate gradient is controlled by Na^+ dependent transporters that are responsible for maintaining a higher glutamate concentration inside the cells. The accumulation of Na^+ reverts the glutamate transporters, causing a massive glutamate amount accumulation in the extracellular compartments. This, in turn, results in an activation of AMPA receptors and NMDA receptors, leading to a greater Ca^{2+} influx and a massive activation of Ca^{2+} dependent protease, lipases and DNases [243].

- *Peri-infarct depolarization*

Peri-infarct depolarizations (PIDs) are spontaneous waves of depolarization that propagate through the penumbra following focal stroke. Recurrent PIDs may be due to the massive release of K^+ and glutamate from the ischemic core and are associated with increased ischemic injury. These waves of depolarization allow Ca^{2+} to accumulate into the neurons causing tissue damage [242].

- *Role of inflammation in cerebral ischemia*

Inflammation is necessary for the clearance of the large amount of debris caused by brain cell necrotic death after an ischemic insult [244]. However, it contributes to stroke-related brain injury and may exacerbate vascular dysfunction leading to severe neuronal

cell death [245]. The distinct features of ischemic stroke are not only a large amount of necrotic neuronal death but also extreme infiltration of immune cells [246]. Ischemic neurons and glia release inflammatory cytokines, such as interleukin and tumour necrosis factor alpha, leading to a generation of adhesion molecules in the cerebral vasculature [242]. Inflammation cascade occurring during stroke is illustrated in figure 1.12

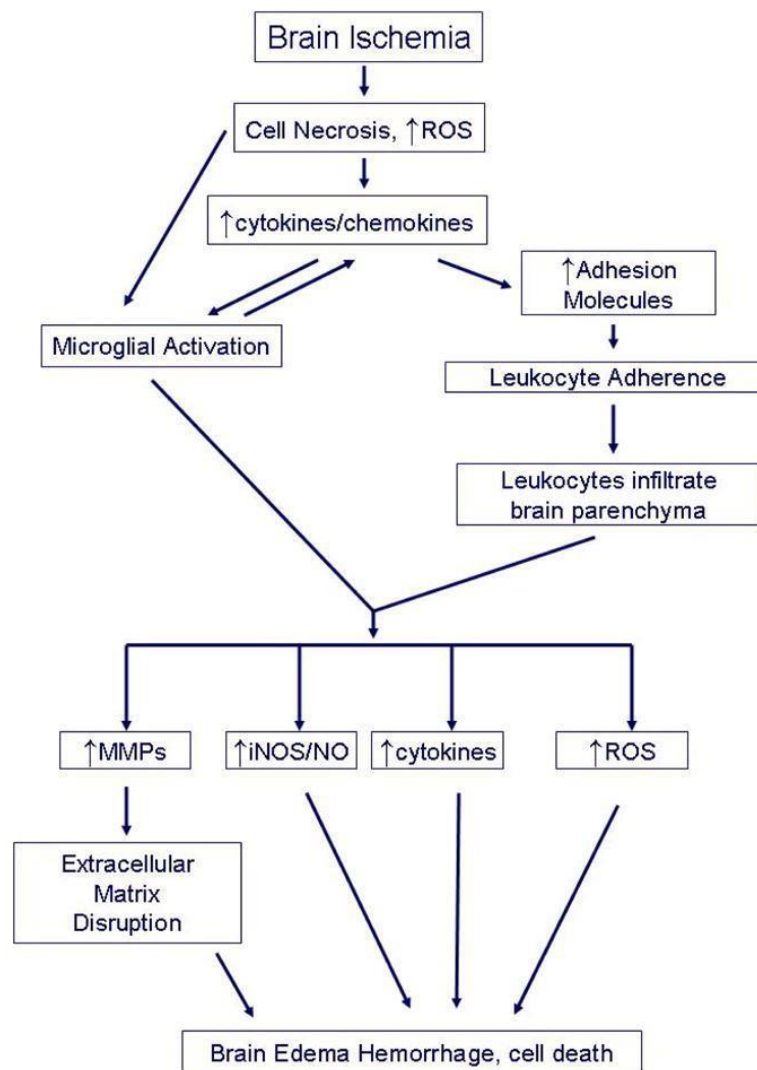


Fig.1.12 Inflammatory cascade following stroke.

The presence of necrotic cells, ROS and production of inflammatory cytokines even within neurons initiate the inflammatory cascade, leading to microglial activation which produce more cytokines causing upregulation of adhesion molecules in the cerebral vasculature. Chemokines lead to inflammatory cell chemotaxis to ischemic brain. Adhesion molecules mediate adhesion of circulating leukocytes to vascular endothelia and infiltration into the brain parenchyma.

Once in the brain, activated leukocytes and microglia produce a variety of inflammatory mediators such as matrix metalloproteinases (MMPs), inducible nitric oxide synthase (iNOS) which generates nitric oxide (NO), cytokines and more ROS which lead to brain oedema, haemorrhage and ultimately, cell death. MMPs are thought to mediate extracellular matrix disruption, a key event in brain oedema and haemorrhage [247].

- *Neuronal cell death in cerebral ischemia*

In neuronal cell death mechanisms, two distinct phases or processes are evident, based on molecular and morphological features: necrosis is not regulated and it is due to energy failure caused by mitochondria dysfunction, with membrane lysis, inflammation and oedema formation; in contrast, apoptosis (programmed cell death) leads to DNA fragmentation and chromatin aggregation, without inflammation and the surrounding tissue is preserved, also main feature of apoptosis is that it is ATP dependent so it cannot occur when ATP is low. Although necrosis and apoptosis are well-characterised and distinct processes, many studies suggest that these mechanisms coexist in injured tissue (necrosis is evident in the core while apoptosis in the penumbra) depending on the duration of the ischemic insult. A mild ischemic injury preferentially induces an apoptosis-mediated cell death. Since the ischemic penumbra preserves ATP, apoptosis predominates in this area. Apoptosis is activated by oxygen free radicals, DNA damage, protease activation and ionic imbalance. The release of cytochrome c from the mitochondrial membrane plays a central role in apoptosis during an ischemic injury and several mechanisms take part in this release. As a consequence of the DNA damage p53 is activated, inducing the transcription of Bax, a protein that promotes the mitochondrial pore formation and subsequent release of cytochrome c. Once present in the cytosol, cytochrome c binds to Apaf-1 and procaspase 9, forming a complex, the apoptosome, which in turn is able to activate other caspases. Apoptosis inducing factor (AIF) can also be released from the pore formed in the mitochondria, leading to DNA damage AIF release is induced by DNA damage following PARP1 activation [227].

- *Oxidative and nitrative stress*

Increased levels of intracellular Ca^{2+} , Na^{+} and ADP lead to the production of reactive oxygen species. Neurons have low levels of endogenous antioxidants such as glutathione, so that the brain is particularly susceptible to reactive oxygen species [248]. These radicals are responsible for the destruction of cellular macromolecules [249]

Ischemic insult leads to the activation of nitric oxide synthase (NOS) increasing the production of nitric oxide (NO). NO combines with superoxide producing peroxynitrite. These reactive species causes DNA damage, which in turn results in an over-activation of poly (ADP-ribose) polymerase-1 (PARP1), an enzyme activated after DNA damage [240]. PARP1 has been shown to play a fundamental role in neuronal cell death following excitotoxicity, ischemia-reperfusion and oxidative stress [250]. An over- activation of PARP1 leads to impaired NAD dependent processes such as anaerobic glycolysis and mitochondrial respiration, leading to ATP depletion and neuronal cell death [240].

- *PARP1 mediated cell death*

PARP1 works as a nick sensor for DNA break repair (Fig.1.13). It uses NAD as a substrate to synthesize polymers of ADP-ribose (PAR). After that, PAR chains are covalently attached to many acceptors proteins, such as histones and PARP1 itself, activating a well-known mechanism called poly (ADP-rybosil)ation. During an ischemic insult, reactive oxygen and nitrogen species induce DNA damage, leading to PARP1 hyper activation.. Furthermore PAR can lead to PAR-dependent mitochondrial pore formation and membrane depolarization. This is accompanied by a release of apoptosis-inducing factor (AIF), its translocation into the nucleus and DNA degradation [80].

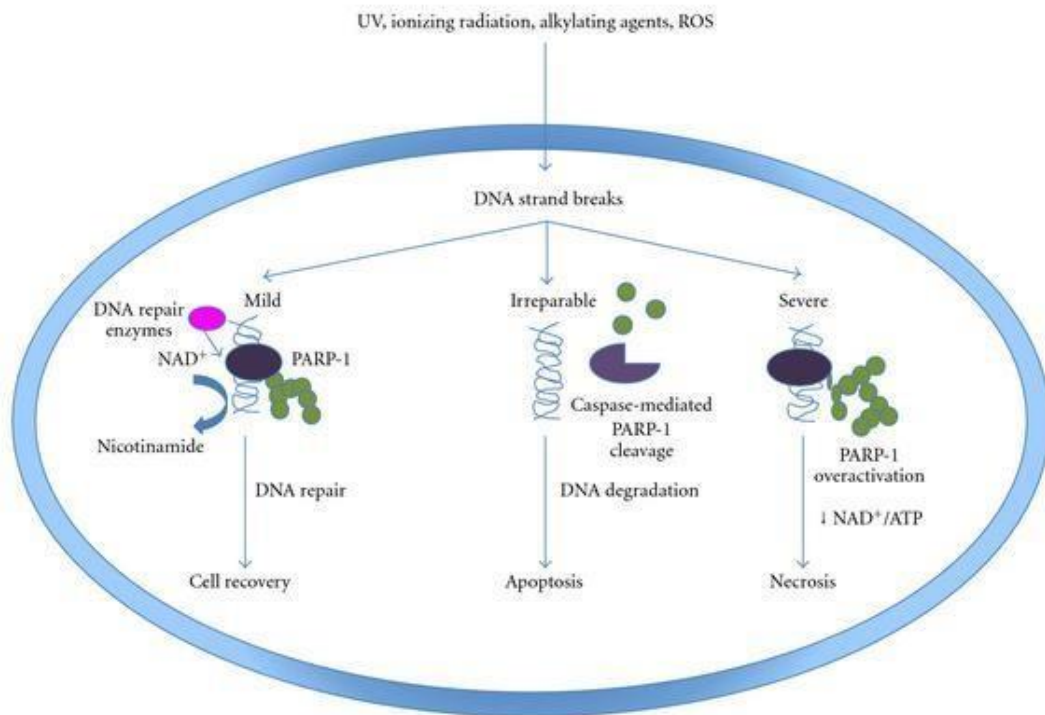


Fig.1.13 PARP1 and cellular responses to DNA damage.

The intensity of DNA damage determines cellular pathways: survival, apoptosis, or necrosis. In the case of mild DNA damage, poly(ADP-ribosylation) enhances DNA repair and thus cell survival. When the damage is beyond repair, PARP1 facilitates apoptosis, preventing ATP depletion and DNA repair through PARP1 caspase-mediated cleavage. Severe DNA damage leads to PARP1 overactivation, cellular energy depletion, and necrotic cell death [251].

To summarise, ischemic cell death is triggered by NAD depletion and/or by pro-apoptotic factors induced by PARP-1 activation. Recent studies showed that NAD depletion prevented glycolytic inhibition, mitochondrial depolarization and AIF translocation, even if the PAR accumulation in the brain was increased [81]. Furthermore, it was also shown that AIF translocation occurred in *Parp1*^{-/-} animals (which have less PAR polymers) as a result of NAD depletion [250]. On the other hand, it has been found that direct injection of PAR polymers is directly cytotoxic [252]. However, NAD levels were not measured and it is possible that the PAR polymers accumulation caused NAD depletion which in turn results in AIF translocation and subsequent cell death [81]. All these evidences highlight the crucial role that NAD plays in this cell death mechanism mediated by PARP1.

1.4.3 Symptoms and therapies

Cerebral ischemia and the cerebral infarct it causes is responsible for around 20% of all cases of dementia, which is a cause of morbidity [253]. Alzheimer's dementia shares several risk factors with cerebral ischemia. The vascular event due to an ischemic attack can play an important role on the development of this form of dementia. The neurological consequences of an ischemic attack are extremely serious. Studies have shown that even after 6 months following a stroke, the affected individuals still suffered with hemiparesis (paralysis on one side of the body), aphasia (difficult with language), hemianopia (loss of vision on one side), dysphagia (difficulty in swallowing) difficult walking, incontinence, depressive symptoms. Furthermore approximately 25% were institutionalised. After an ischemic insult only 45% of patients have a complete recovery, while the remaining suffer mild, moderate and severe disability [254]. Individuals who experienced an ischemic stroke can have not only direct neurological consequences, but also indirect consequences such as a higher risk of epilepsy. Neuropathic pain and fatigue may also occur in stroke survivors. All of these severe disabilities that affect stroke patients have a profound impact on the quality of their lives, especially on those who were employed and unable to continue working. So that, even the non-institutionalised patients will live in a condition of social isolation [228].

The most important intervention for limiting damages following an ischemic insult consists of a quick restoration of blood flow that occurs naturally or with thrombolytic drugs. Indeed, the only approved agent used for the treatment of stroke is intravenous recombinant tissue plasminogen activator (rtPA). Tissue plasminogen activator (tPA) is a serine protease that catalyzes the activation of the zymogen plasminogen by converting it to the broad-specificity, active protease, plasmin [255]. Furthermore, different approaches target single processes involved in the ischemic-cascade (calcium overload, glutamate excitotoxicity, oxygen radical production, apoptosis and inflammation) [256].

PARP1 inhibitors as a therapeutic target in stroke

Since PARP1 activation is one of the major causes of neuronal death in brain ischemia [250], PARP1 inhibition could potentially be used as a therapeutic strategy in ischemic stroke patients and PARP1 inhibitors are already in clinical trial. PARP1 inhibitors in

ischemic conditions may have protective effects by preserving cellular level of NAD and consequently ATP, since NAD biosynthesis is an ATP consuming process [257]. PARP1 inhibitors have a neuroprotective effect in a rat model of cerebral infarction induced by middle cerebral artery occlusion [258]. PARP1 inhibitors can also have a neuroprotective role in models of mild oxidative stress, as it occurs in neurodegenerative diseases [259]. However, several studies have shown an important role played by PARP1 for long-term memory formation in mammals. In behavioural studies, injection of PARP-1 inhibitors in mice prevented the formation of long-term memory [260]. This therapeutic strategy has the potential to reduce one of the main causes of cell death involved in cerebral ischemia.

1.4.4 Models of cerebral ischemia

In vitro models of cerebral ischemia

Several *in vitro* models are available to study mechanisms of cerebral ischemia. In particular, hippocampal slices, and primary neuronal/glial cultures from cortex, hippocampus and cerebellum of embryonic or perinatal rats and mice are widely used. Ischemia-like conditions can be obtained by replacing the normal O₂/CO₂ equilibrated medium with N₂/CO₂ equilibrated medium. Two different situations can be distinguished: hypoxia condition, when glucose is maintained in the anoxic buffer, and *in vitro* ischemia or oxygen/glucose deprivation (OGD), when glucose is absent. Glucose deprivation (GD) alone can also induce neuronal death with some features similar to those observed in the *in vivo* ischemia models. Chemical hypoxia can also be induced through the use of cyanide (NaCN or KCN) or by incubating in an anoxic atmosphere. However, this technique results in more free radical generation than does anoxia [261].

An important *in vitro* approach that can be reproduced is based on the use of organotypic brain slice culture, in which network connections are maintained. Brain slices can be obtained from neonatal (P0-P10) animals, commonly mice or rats. In these models, after reproducing the ischemic-like condition through OGD, cell death and apoptotic changes in neurons can be studied by looking at cellular uptake of propidium iodide and Fluro-jade staining, TUNEL staining and immunofluorescent staining for cleaved caspase-3 [256]. Primary neuronal cultures, obtained from mice or rat embryos at day fifteen to seventeen of gestation can also be used. Furthermore, the primary glial cultures

are considered a valid technique to study the ischemic cell death. Astrocytes account for about 50% of the cell population in adult mammalian brains. They provide structural, metabolic and trophic support to neurons and protect neurons from injurious stimuli. For this reason, understanding their role in ischemic condition may be crucial [256].

- *In vivo models of cerebral ischemia*

In vivo animal models of stroke can be divided into three main categories: global ischemia, focal ischemia, and hypoxia/ischemia. To study stroke, small laboratory species, such as rodent, are usually preferred as their cranial circulatory anatomy is similar to humans [256].

Global ischemia in rat. This model can be obtained by four-vessel occlusion (4-VO), particularly used to study the effectiveness of potential therapeutic agents. It consists of permanent coagulation of the vertebral arteries and temporary ligation of the two common carotid arteries. Alternatively, two-vessel occlusion (2-VO) can be performed by bilateral occlusion of the common carotid arteries (CCA, Fig.1.14) and a further reduction of the cerebral blood flow that can be obtained through controlling exsanguination and/or administering peripheral vasodilators. The main difference between the two types of occlusion depends on the fact that 4-VO is not performed under anaesthesia [262].

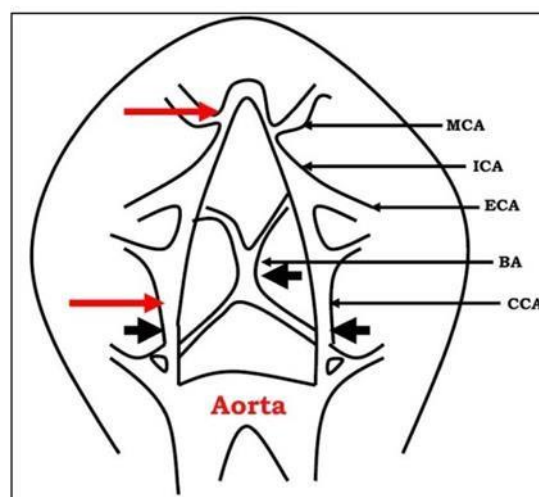


Fig.1.14 Schematic illustration of arteries

MCA: middle cerebral artery; ICA: internal carotid artery; ECA: external carotid artery; BA: basilar artery; CCA: common carotid artery [256].

Global ischemia in gerbil. This model can be obtained by temporary ligation of the carotid arteries, with no reduction in blood pressure [256].

Global ischemia in mouse. This model is similar to that in rats, (2-VO), consisting of bilateral occlusion of the common carotid arteries. However, it is difficult to obtain uniform injury to hippocampal CA1 neurons with high chance of survival because of the variability of collateral flow [263].

Focal ischemia. This model can be performed either in larger mammals such as cats, dogs or non-human primates, or in rodents. The technique used involves the middle cerebral artery (MCA, Fig.14). Focal ischemia can be differentiated from global ischemia because the blood flow is higher than during global ischemia, so to cause a damage it requires a longer insult. Furthermore, the insult is much more complex than in global ischemia due to its duration. However, it is considered a model that realistically reproduce human stroke. Focal ischemia is obtained by transient MCA occlusion through the insertion of a nylon suture into the carotid artery, past the point at which the MCA branches, so that the latter is occluded at its origin [256]. Furthermore, focal ischemia can also be obtained by permanent MCA occlusion. In addition to this, embolic model of focal ischemia is another procedure, similar to the transient focal ischemic model, based on the use of a catheter filled with bovine thrombin injected into the MCA [264]. Finally, another in use-technique is the photothrombotic distal MCA occlusion, in which vascular thrombosis is induced by transcranial illumination with a filtered light source in combination with intravenous injection of a photosensitive dye [265].

1.4.5 NAD and cerebral ischemia

Several studies highlighted the role of NAD biosynthesis in the ischemic cell death pathways. NAD replenishment in primary cultures of hippocampal and cortical neurons can confer protection against oxygen glucose deprivation (OGD)-induced cell death, which is an in vitro model of ischemic cell death. At a concentration range of 3 to 15 addition to that, several studies showed that nicotinamide, a vitamin precursor of NAD, can confer protection in a model of hypoxic-ischemic injury both in new born rats [267] and in adult rats [268]. This modulation can be achieved not only by exogenous administration of NAD and its precursors, but also acting on its synthesizing enzymes. In fact, it has

been shown that heterozygous knock-out mice for NAMPT, when subjected to photothrombosis-induced ischemia, an *in vivo* model of cerebral ischemia exhibit more severe brain damage, a larger infarct volume and neuronal degeneration than do wild type mice accompanied by decreased NAD level [269]. There are more evidences of NAMPT as a protective factor in cerebral ischemia. In addition, modulation of NMNAT1 could be a valid alternative strategy. NAMPT is the rate-limiting enzyme in the NAD biosynthetic pathway and it has only one isoform distributed ubiquitously within cell [50], therefore modulation of this enzyme is likely to affect all NAD intracellular pools. On the other hand, modulating NMNAT1 should enable to modify NAD synthesis only at a nuclear level, leaving the mitochondrial and cytoplasmic fractions undisturbed. A more recent study showed that *cyt-Nmnat1* transgenic mice, in which NMNAT1 is overexpressed and redistributed into the cytoplasmic fraction, are protected from the brain injury in a neonatal model of hypoxia-ischemia (H-I) [136]. Of note, NMNAT1 has been reported to interact with PARP1 at target gene promoters in MCF-7 cells, where it enhances PARP1-dependent PARylation. Therefore, PARP1 recruits NMNAT1 to promoters where it produces NAD to support PARP1 catalytic activity [52, 270]. All these evidences support the role that NMNAT1 may have in the mechanisms of cell death related to cerebral ischemia.

1.5 Aims and Objectives

The present study aims to understanding the role of the nuclear NAD biosynthetic enzyme NMNAT1 on the ageing mechanism and age-related pathologies.

Thus, the present thesis aims to:

1. To clarify the role of NMNAT1 on ageing mechanisms, particularly in brain.

To this aim, I pursued the following specific objectives:

- I used *Nmnat1* tg and *Nmnat1*^{+/-} mice at 6 and 24 months of age. Using this tool, I investigated whether overexpressing NMNAT1 ameliorated the metabolic parameters, including physical activity, impaired over ageing. Furthermore, I also asked whether reducing the expression of NMNAT1 exacerbated the metabolic dysfunction occurring in ageing.

- I analyzed how modifying the expression of NMNAT1 influenced NMNAT enzyme activity and NAD levels in the brain in young and old mice, which have been linked to ageing in skeletal muscle. In addition to this, I also investigated possible changes in gene expression and protein levels induced by the overexpression and reduction of NMNAT1 during ageing.

2. To study the role of NMNAT1 in a model of tauopathy relevant to Alzheimer's disease.

To this aim, I pursued the following specific objectives:

- I crossed *Nmnat1* tg and *Nmnat1*^{+/-} mice with a mouse line which expresses non-mutant human tau isoforms (htau mouse), representing a model of tauopathy relevant to Alzheimer's disease. In this mouse cross:
- First, I analyzed behavioral abnormalities using a battery of motor and cognitive tests that correlate with dysfunctions characteristic of Alzheimer's disease.
- Secondly, *in vivo* and post-mortem imaging analyses were performed to assess the integrity of the brain areas mainly impaired in Alzheimer's disease.
- Finally, biochemical studies were conducted in order to test whether modulating NMNAT1 levels caused changes in NMNAT activity and in NAD levels in htau mice.

3. To study the role of NMNAT1 in an *in vitro* model of cell death relevant to Cerebral Ischemia.

To this aim, I pursued the following specific objectives:

- I used stable NSC-34 clones expressing Wld^s/NMNAT1 and tested for cell viability after genotoxic stress induced by *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG).
- In addition to the *in vitro* experiments, I also started to set-up an *in vivo* model of cerebral ischemia, using wild type, *Nmnat1* tg and *Nmnat1*^{+/-} mice.

Chapter 2

Materials and Methods

2.1 Mice and genotyping

All experimental animals used were bred and housed in groups at the University of Nottingham Biomedical Service Unit. The animal work was carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986, under project licenses: PPL 40/3482 and 40/3601 and approved by the University of Nottingham Ethical Review Committee.

All mice were maintained at the University of Nottingham. Heterozygous *Nmnat1* knock-out (KO) mice, mice overexpressing NMNAT1 and human-tau mice were previously described [77, 127, 214]. Mice were genotyped by PCR using the following primers:

***Nmnat1* heterozygous KO (*Nmnat1*^{+/-})**

Forward 5'-CCCAGTCACTAAGACATTCAA-3'

Reverse 5'-CCTTCTTGCTTCCCACGAGG-3'

***Nmnat1* transgenic (*Nmnat1* tg)**

Forward 5'-ACTTCGGCTCACAGCGCG-3'

Reverse 5'-TCCTTGGCCAGCTCGAACA-3'

Human tau (*htau*)

Forward 5'-ACTTTGAACCAGGATGGCTGAGC-3'

Reverse 5'-CTGTGCATGGCTGTCCCTACCTT-3'

Murine tau (*mtau*^{+/-})

Forward 5'-CCAGTTGTGTATGTCCACCC-3'

Reverse 5'-CTCAGCATCCCACCTGTAAC-3'

Disrupted murine tau

Forward 5'-AAGTTCATCTGCACCACCG-3'

Reverse 5'-TGCTCAGGTAGTGGTTGTCG-3'

2.2 Study design

2.2.1 Study design for chapter 3

In order to study how modulating *Nmnat1* influences behavioral and biochemical changes occurring during ageing *Nmnat1*^{+/-} and *Nmnat1* tg mice and their relative littermates controls were used.

Mice of each genotype were subjected to a test measuring locomotor activity at the age of 4, 6, 12, 15 and 18 months. At the age of 15 months, metabolic analysis was also studied in mice of all genotypes. At the age of 24 months, mice were humanely killed by cervical dislocation and post-mortem analysis for NAD levels and NMNAT enzyme activity through Liquid chromatography–mass spectrometry (LC-MS) and western blotting were conducted (see table 2.1).

TESTS	Behavior	Metabolic analysis	NAD level	NMNAT enzyme activity	Western Blotting	qPCR
AGE (months)	from 4 to 18	15	6 and 24	6 and 24	6 and 24	6 and 24
N/GENOTYPE	12 (6/gender)	6 (6/gender)	6 (3/gender)	6 (3/gender)	5 (3 females, 2 males)	5 (3 females, 2 males)
SAMPLES			half brain, cortex, hippocampus, striatum, eye	half brain, cortex, hippocampus, striatum, eye	half brain	half brain

Table 2.1 Mice used for experiments illustrated in chapter 3.

2.2.2 Study design for chapter 4

To assess the role of NMNAT1 in a model of tauopathy relevant to AD, *Nmnat1*^{+/-} and *Nmnat1* tg were crossed with htau mice producing four genotypes htau, *Nmnat1* tg/htau, *Nmnat1*^{+/-}/htau and wild type littermates. All mice were heterozygous for murine tau (mtau^{+/-}): 12 mice of each genotype (n=6/gender) were subjected to a range of tests relevant to AD measuring activities of daily living, learning and memory. Locomotor activity and anxiety-related behavior were also tested. Activities of daily living were measured using the food burrowing test. Memory was assessed using spontaneous alternation in the Y-maze, novel object location/discrimination test and contextual fear conditioning. Open-field was used to test locomotor and anxiety-related behavior. Spontaneous alternation and open-field tests were repeated at the age of 4 and 6 months; food burrowing test was repeated from two to six months of age in a monthly interval and contextual fear conditioning was performed at the age of six months only. Behavioral assessment was followed by *in vivo* magnetic resonance spectroscopy (MRS) and magnetic resonance imaging (MRI). After imaging analysis at 6 months, mice were humanely killed and post-mortem analysis for NAD levels and NMNAT enzyme activity through HPLC-based method was conducted. Immunostainings and western blotting were also conducted (see table 2.2).

TESTS	Behaviour	Food-test	MRI/ MRS	Immunostaining analysis	NAD level	NMNAT enzyme activity	Western Blotting
AGE (months)	4 and 6	4 and 6	6	6	6	6	6
N/GENOTYPE	12	12	10	5	4	4	4
	(6/gender)	(6/gender)	(5/gender)	(3 females, 2 males)	(2 females, 2 males)	(2 females, 2 males)	(2 females, 2 males)
SAMPLES				Hippocampus	cortex, hippo- campus	cortex, hippo- campus	cortex, hippo- campus

Table 2.2. Mice used for experiments illustrated in chapter 4.

2.2.3 Study design for chapter 5

PARP1-mediated cell death, which is one of the main mechanisms of cell death that occurs during an ischemic insult, was analysed in a motoneuronal cell line NSC-34. Wild type (wild type cells) and cells stably transfected with the neuroprotective factor Wld^s, containing full length NMNAT1 (Wld^s/NMNAT1 cells) were used.

Cells were treated with *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) to induce overactivation of PARP-1 and cell death. Following subcellular fractionation, NAD levels was then measured through HPLC-based method. MTT assay was performed in wild type and in wld^s/NMNAT1 cells after MNNG treatment.

Middle cerebral artery occlusion (MCAO) was performed in *Nmnat1*^{+/-} and *Nmnat1* tg mice and their relative wild type littermates at three different time points in order to evaluate the ideal time of occlusion (see table 2.3).

MCAO	5 min occlusion	10 min occlusion	15 min occlusion
AGE (months)	4-5 months	4-5 months	4-5 months
N/GENOTYPE	3/group	3/group	3/group

Table 2.3 Mice used to set up a model of Cerebral Ischemia illustrated in chapter 5.

2.3 Behavioral analysis

2.3.1 Food-burrowing test

The protocol was adapted from Deacon et al [271]. Mice were individually placed for one night in a novel cage with *ad libitum* access to food and water. A glass jar containing 30 or 50 g of food pellet broken into small pieces was added to the cage. The amount of food displaced from the jar was measured. Data were transformed by square root in order to conform them to a normal distribution [272] and analyzed using 3-way ANOVA with gender and genotype and repeated measures over age, followed by post-hoc planned comparisons.

2.3.2 Spontaneous alternation task

Spontaneous alternation was used to test spatial working memory [273]. The Y-shaped maze comprised of three identical transparent Plexiglas® arms at a 120° angle from each other (41.5 cm in length and 6 cm in width surrounded by 15 cm high transparent Perspex walls). The start point (6 cm x 7.5 cm) was located in the center of the maze, and the animal was allowed to freely explore the three arms over five minutes. The number of alternations was recorded and expressed as percentage of alternation, as a measure of spatial working memory [274]. Data were analyzed using 3-way ANOVA with gender and genotype and repeated measures over age, followed by post-hoc planned comparisons.

2.3.3 *Open-field*

Open field was used to assess locomotor and anxiety-related behavior as previously described [274]. Mice were placed in Perspex activity boxes (30cm x 35 cm x 25 cm) for 30 minutes during which total distance moved (cm) and percentage of time spent in the centre (s) were recorded using Ethovision software (Noldus, Wageningen, Netherlands). Data were analyzed using 3-way ANOVA with gender and genotype and repeated measures over age, followed by post-hoc planned comparisons.

2.3.4 *Novel object location and recognition*

Novel object location and novel object recognition were used to measure spatial and recognition memory respectively, as previously described [274]. In the acquisition trial each mouse was allowed to explore two identical objects next to each other along one arena wall. After a 10-minute-long interval each mouse was placed into the same arena for the location trial, with one of the two objects being moved across to the opposite wall. After a 20-minute break each mouse was allowed to explore the objects for a third time (discrimination trial) in which one object had been exchanged. Total object exploration times, object location and object discrimination times were recorded using Ethovision software (Noldus, Wageningen, Netherlands). Data were analyzed using 3- way ANOVA with gender and genotype and repeated measures over age, followed by post-hoc planned comparisons. For each group, preference indices in the object tests were compared to chance levels (50%) using one-sample t-tests, in order to determine if the mice were able to discriminate the novel location or novel object.

2.3.5 *Contextual fear conditioning (CFC)*

Associative learning and memory were assessed using contextual fear conditioning following a previously described protocol [274]. Briefly, this test consisted of three trials conducted on three consecutive days using the same chamber (25cm x 25cm x 38cm): (1). In the acquisition trial, mice were exposed to a series of five foot-shocks (1s-long, 0.4 mA), each separated by a 60-s interval. (2) In the retention trial, mice were placed in the same chamber for one minute without the application of foot shocks. (3) In the one-minute-long extinction trial foot shocks were also omitted. Analysis of the time that the mice spent immobile was conducted in blind twice, and the data were averaged

for statistical analysis. Data from the acquisition trial were analyzed using 3-way ANOVA with gender and genotype and repeated measures over time, followed by post-hoc planned comparisons; data from retention and extinction trials were analyzed using two-way ANOVA, followed by post-hoc planned comparisons.

2.4 Metabolic analysis

Metabolic parameters and feeding behaviour parameters were measured using a comprehensive lab animal monitoring system (CLAMS) (Linton Instrumentation, Linton, UK, and Columbus Instruments, Columbus, OH) The apparatus of eight mouse chambers, where mice were individually housed, with dropper-style water bottles and food hoppers in the centre of each chamber. Metabolic parameters measured included oxygen consumption (V_{O_2}) and CO_2 production (V_{CO_2}) and food consumption. Locomotor activity was then defined as successive linear infrared beam breaks recorded in 9-minute bins. All measurements were taken at an ambient temperature of 21–22°C. All parameters were measured for 68–72 h, and after this period, mice were removed and put back into their home cages. Raw data were collected from the CLAMS using OxyMax software (ver. 4.2, Columbus Instruments). The first 16 h of data were considered as a period of time necessary for the habituation to the apparatus and for this reason were discarded. Data over the next 48 h were collected and used for analysis. Average values over the 48-h period were calculated for locomotor activity (breaks/9 min), V_{O_2} (ml/h), V_{CO_2} (ml/h) and food-intake for each mouse at each CLAMS session [275]. Data were analysed using two-way ANOVA, followed by Bonferroni post-hoc test.

2.5 *In vivo* MRS and MRI

Animals were anaesthetized with 1% - 1.75% isoflurane in a nitrous oxide:oxygen = 2:1 gas mixture and placed in a custom built holder inside the MR scanner to minimize head motion. Throughout the study, breathing rate and body temperature were monitored continuously and kept constant through adjustment of anesthesia levels and warm water circulation.

In vivo MRI and MRS data were acquired from mice of all four genotypes (male and female, age = 6 months, Table 1) using a horizontal 7T MR imaging system (Avance

Biospec 70/30 USR; Bruker, Karlsruhe, Germany) equipped with a volume transmit / surface receive radiofrequency coil.

MR spectroscopy data were acquired from a $2 \times 2 \times 2 \text{ mm}^3$ voxel (volume of interest) centered on the left hippocampus and positioned with multi slice rapid acquisition with relaxation enhancement (RARE) images [276] (Fig. 4A). A line-width of 10-15 Hz for the water peak was obtained after adjustment of first- and second-order shims with FAST-MAP [277]. A Point-Resolved Spin echo sequence (PRESS) [278] was used to acquire the MR spectra (repetition time, TR = 2000 ms, echo time, TE = 14 ms, 1024 signal averages) after water suppression with variable pulse power and optimized relaxation delays (VAPOR) [279]. The total acquisition time was 34 minutes. All spectra processing was conducted blinded to group assignment. Spectra were analysed using LCModel (Version 6.3-0I) [280]. Resonances corresponding to myo-inositol (Ins), taurine (Tau), glutamate (Glu), N-acetylaspartate (NAA), glutamine (Gln), glutathione (GSH), glycerophosphocholine and phosphatidylcholine (PCh+GPC), and creatine and phosphocreatine (Cr+pCr) were quantified relative to the total metabolic content (Glu+PCh+GSH+Ins+NAA+Gln+Tau+Cr+pCr). Only results with the Cramer– Rao lower bounds $\leq 15\%$ were included in the analysis. Data were further analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

For anatomical MRI, 30 coronal slices were acquired using a RARE sequence (256 x 256 matrix size, field of view, FOV = 1.66 x 1.66 cm, 0.3 mm slice thickness; TR = 7500 ms, effective TE = 29.4 ms, RARE factor = 8, number of averages = 8). The total acquisition time per volume was 32 minutes. What is referred to as hippocampal areas (covered in 10 $\pm$ 1 coronal slices) in the context of this work included fields CA1, CA2 and CA3, the pyramidal cell and lacunosum molecular layers, the stratum lucidum and radiatum of the hippocampus as well as the dentate gyrus including its molecular, polymorph and granular layers as well as the subiculum relating to figures 39-67 in The Mouse Brain Atlas by Paxinos & Franklin [281]. Hippocampal volumes were determined by manually drawing regions of interest in blind using a software [Medical Image Processing, Analysis and Visualization (MIPAV); v. 7.0.1; Center for Information Technology (CIT), National Institutes of Health (NIH)]. The mean of the hippocampal volume of the left and right hemispheres was expressed as percentage of the total brain volume as outlined in The Mouse Brain Atlas. Since the hippocampal

and whole-brain volume did not differ across the two sexes they were combined within each genotype for the statistical analysis of genotype differences. Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

2.6 *in vivo* Cerebral Ischemia

MCAO was performed in *Nmnat1*^{+/-} and *Nmnat1* tg mice and their relative wild type controls with the help of Dr Rebecca Trueman.

The intraluminal filament model was used to produce MCAO. A blunted filament is inserted into the internal carotid artery (ICA) with advancement until it reaches the origin of the MCA in the Circle of Willis.

Mice were kept under anaesthesia with inhalable isoflurane in a 70:30 nitrous oxide:oxygen mixture and core body temperature was constantly maintained at 36.7 ± 1°C throughout the duration of the procedure. Mice were then shaved and the incision site cleaned with iodine scrub. MCAO was then performed as described [282]. Occlusion was performed at 5, 10 and 15 minutes in order to establish the time of occlusion necessary to induce a stroke and to ensure a proper post-surgery recovery of the mouse. Following the surgery, an adequate post-operative care was provided by re- hydrating the animal with subcutaneous injections of 2.5 mL of physiological saline and by administering 1 mg/mL of Paracetamol (Boots, UK) into the drinking water for pain relief. After 24 hours, post-mortem staining in coronal brain sections highlighted the ischemic region through the use of 2, 3, 5-triphenyltetra-zolium chloride (TTC).

2.7 Cell cultures

2.7.1 NSC-34-tTA cell cultures

A stable neuronal cell line, NSC-34, expressing the Tetracycline-controlled transactivator protein (tTA) [283] was used to generate a bidirectional promoter controlling the expression of a reporter gene (EGFP) and the gene of interest, the neuronal protective factor Wld^s, which contains the full length of NMNAT1 (L. Janeckova and L. Conforti, unpublished). Gene expression is switched off by the addition of tetracycline or its analogue doxycycline. Cells were cultured in high glucose Dulbecco's Modified Eagle's medium supplemented with 10% heat-inactivated foetal

bovine serum (FBS), 2 mM glutamine, 1 mM pyruvate, and antibiotics (100 IU penicillin, 100 µg/ml streptomycin and 0.5 mg/ml geneticin). Cells were allowed to reach 90% of confluency, then harvested by trypsinisation. Cell pellets were washed once in PBS then stored at -80°C.

2.7.2 MTT assay in NSC-34

Wild type and Wld^s/NMNAT1 cells were seeded in 24-well plates at a density of 10⁵ cells per well the day before the assay. Then cells were treated with 50 µM and 100 µM of *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG), a DNA methylating agent which induces a PARP1-overactivation and mediated cell death. Cell death was analysed at time 0, after 16 hours, 20 and 24 hours. After treatment with or without MNNG, for the indicated times, the medium was replaced with fresh medium containing 0.5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) which is then reduced to purple formazan in the mitochondria of living cells. After 2 hours of incubation, the medium was removed and DMSO was used to dissolve formazan crystals. Absorbance was measured at 690 nm (background) and 570 nm with a microplate reader as a measure of cell viability [284]. Data were analysed using two- way ANOVA, followed by Bonferroni post-hoc test.

2.8 Determination of NMNAT enzyme activity and NAD levels

2.8.1 High-Performance Liquid Chromatography (HPLC)

Brain areas from htau mice, and cell pellets were snap-frozen in liquid nitrogen and stored at -80 °C. On the day of use, tissues were homogenized in 0.5M HEPES buffer pH 7.5 and protease inhibitor cocktail (Roche Diagnostic) using a Teflon-glass pestle and immediately processed for NAD level and NMNAT enzyme activity determination as described previously [285]. For NAD levels measurement, homogenates were treated with 50 µL of HClO₄ 1.2 M. After 10 min of incubation on ice, samples were centrifuged at 13.4 RPM for 2 min and 37 µL of K₂CO₃ 0.8 M was added to the supernatant. After 1 hour at -20°C, NAD levels were detected with a High-Performance Liquid Chromatography [285]. NMNAT enzyme activity was performed using a mixture containing HEPES/KOH pH 7.5 50 mM, ATP 1mM, NMN 1mM, DTT (DL-Dithiothreitol) 1mM, MgCl₂ 20 mM, NaF (sodium fluoride) 10 mM, tissue lysate volume (add at the end to start the reaction)

corresponding to 40 µg of total protein and H₂O to a final volume of 100 µl. Mixture was then incubated at 37°C for 1 h and treated with HClO₄ 1.2 M. After 10 min of incubation on ice, samples were centrifuged at 13.4 RPM for 2 min and 37 µL of K₂CO₃ 0.8 M was added to the supernatant. Data were analysed using two-way ANOVA, followed by Bonferroni post-hoc test.

2.8.2 Liquid chromatography–mass spectrometry (LC-MS)

Brain areas, half brains and eyes from *Nmnat1* tg and *Nmnat1*^{+/-} ageing mice were snap-frozen in liquid nitrogen and stored at -80 °C. On the day of use, tissues were homogenized in 0.5M HEPES buffer pH 7.5 and protease inhibitor cocktail (Roche Diagnostic) using a Teflon-glass pestle and immediately processed for NAD level.

Homogenates were treated with 100 µl 0.5 M HClO₄. After 10 min of incubation on ice, samples were placed in filtered tubes and centrifuged at 13.5 RPM for 5 min. After 1 hour at -20°C, 50 µl of Formic acid were added to the supernatant and NAD levels were detected with a Liquid chromatography–mass spectrometry. NMNAT enzyme activity assay was performed as described in the previous paragraph. Following the assay, samples were processed for measuring NAD levels. Data were collected using MassLynx V4.0 software and analysed using two-way ANOVA, followed by Bonferroni post-hoc test.

2.9 Tissue processing and immunohistochemistry

Mice were humanely killed using an approved Schedule 1 method (cervical dislocation) at six-months-of-age. Half brains from htau mice, *Nmnat1* tg/htau, *Nmnat1*^{+/-}/htau and wild type mice were post-fixed in 4% PFA for a minimum of 48 hours at room temperature. Brains were dehydrated by an ascending alcohol series and then embedded in paraffin. Paraffin blocks were cut into coronal microtome sections (thickness 8 µm) and drawn up on microscope slides. After paraffin removal by xylene treatment and rehydration, sections were incubated with NeuroTrace fluorescent Nissl stain (Invitrogen-Molecular Probes, 1:300) diluted in phosphate-buffered saline (PBS) for 20 min and then washed in tris-buffered saline (TBS) for 2 h at room temperature before being mounted on slides (n=5/genotype). Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

For evaluation of gross hippocampal morphology, 8 μ m coronal sections were mounted on Superfrost slides (BDH), air dried and stained with Haematoxylin and Eosin (H&E). (n=5/genotype). Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

For immunohistochemistry, cut sections were deparaffinized in xylene and rehydrated through decreasing concentration of ethanol before the immunohistochemical procedure. Sections were then rinsed in water and subsequently incubated at room temperature in a solution, containing 0.3% H₂O₂ diluted in PBS (0.1 mol/L, pH 7.4) for 30 minutes in order to block endogenous peroxidases. After rinsing, sections were treated with 10mM sodium citrate buffer pH 6 for 20 minutes at 100°C for antigen retrieval. Sections were then incubated with mouse immunoglobulin G blocking reagent (mouse on mouse [MOM] Kit, Vector Laboratories, Burlingame, CA) for 1 hour in a humid chamber at room temperature, subsequently incubated with monoclonal antibody CP13 (1:25, generously provided by Prof. Peter Davies, Bronx, NY, USA [214]) and developed with 3,3'-diaminobenzidine (DAB). Sections were viewed using NanoZoomer digital slide scanner (Hamamatsu, Japan).

2.10 Subcellular fractionation

Brains from wild type and NMNAT1 heterozygous KO mice, or wild type and Wld^s/NMNAT1 cells were homogenised using a Teflon-glass pestle in ice-cold homogenization buffer containing 10 mM HEPES, 6 mM MgCl₂, 1 mM EDTA, 10% sucrose, pH 7.2 and protease inhibitor cocktail (Roche Diagnostic) and homogenates were fractionated according to Beirowski et al., 2009. Cells/brain tissue were centrifuged at 2000xg for 5 min, in order to obtain pellet A and supernatant B. Pellet A was repeatedly washed, resuspended in homogenization buffer containing 0.5 M NaCl and incubated on ice for 1 hour. After incubation the suspension was centrifuged at 8000xg for 10 min and the final supernatant was the nuclear fraction. Supernatant B was considered as the cytoplasmic fraction. For brain tissue only, supernatant B was centrifuged at 10000xg for 10 min, in order to obtain pellet C and supernatant D. Pellet C was washed and finally regarded as mitochondrial fraction, while supernatant D was considered as the cytosolic fraction. All fractions were then stored at -80°C until used for Western Blotting or NAD quantification.

2.11 Western blotting

Freshly frozen half brains (chapter 3) or hippocampi and cortices from all genotypes (chapter 4) were homogenized in RIPA lysis buffer containing protease inhibitors (Complete mini, Roche). Furthermore, subcellular fractions obtained from wild type and NMNAT1 heterozygous KO mouse brains, or wild type and Wld^s/NMNAT1 cells (chapter 5) were re-suspended in 10 mM HEPES homogenization buffer. The protein samples were then loaded on a 10% SDS polyacrylamide gel and subsequently transferred to nitrocellulose membrane using a semi-dry blotting apparatus (BioRad). Membranes were blocked for 1h in 5% BSA in PBS plus 0.2% Tween-20 (PBST), incubated overnight with primary antibody in 5% BSA at 4°C and incubated for 1h at RT with HRP-linked secondary antibody in 3% BSA in PBST. The primary antibodies used are illustrated in table 2.4. HRP-goat α -rabbit (1:3000) or HRP-goat α -mouse (1:5000) were used as secondary antibodies. Bands were visualized on film using a chemiluminescent detection kit (GE Healthcare) and analysed using imageJ gel analysis software. For each band, the relative density was measured and then the adjusted density value, referred to the loading control for each sample, was calculated. Data were analyzed using two-way ANOVA followed by Bonferroni post-hoc test.

Antibody name	Dilution	Company
Mouse anti-PSD-95	1:2000	Abcam
Rabbit anti-SIRT1	1:2000	Sigma
Mouse anti-synaptophysin	1:10000	Dako clone Y38
Mouse anti-NAMPT	1:2000	Enzo Life Sciences
Mouse anti Phospho-PHF-tau pSer202+Thr205	1:500	Pierce
Mouse anti- β -tubulinIII	1:5000	Sigma
Rabbit anti-Wld ^s (Wld 18)	1:2000	
Mouse anti- α -H1 histone	1:500	Millipore
Mouse anti-COX	1:5000	Abcam

Table 2.4 Primary antibodies used for western blotting experiments.

2.12 qPCR analysis

Total RNA from half brain was isolated with Trizol and reverse transcribed with Superscript III and random primers. qPCR was performed in triplicates with the SensiMix Plus SYBR Green PCR kit (Qiagen Ltd.) and a Rotor Gene 3000 cyclor (Corbett Research). PCR primers were designed with Primer3 (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>). qPCR validation was performed for genes encoding *Nmnat1*, *Nmnat2*, *Nampt* and *Sirt1*.

Gene expression was normalized to a control genes (PGK1) with the GenNorm software(<http://medgen.ugent.be/~jvdesomp/genorm>) [286].

2.13 Imaging and quantitative analysis

Brain sections were imaged with Leica DMRB fluorescence microscope using a 40X oil immersion objective. The number of neurons was measured in the CA1 and CA3 hippocampal areas using ImageJ software. Quantitative analyses were performed on 3 different sections per mouse. Comparable sections were chosen in different mice. H&E stained sections were imaged by low power (Plan-NEOFLUOAR 1.25X/0,035) light

microscopy (Zeiss Axioplan). Sections were matched between all genotypes and used for the analysis.

2.14 Statistical analysis

Data are presented as mean $\pm$ SEM. Student's t-test and One or Two-way ANOVA followed by Bonferroni post-hoc test were performed using Prism GraphPad 5.0 software; Three-way ANOVA followed by post-hoc planned comparison was performed using InvivoStat software for analysis of behavioural tests.

Chapter 3

NAD-biosynthetic enzyme NMNAT1 and its role in ageing mechanisms.

Note: I would like to thank David Tooth, who helped me with LS-MS experiments; Dr Maria Toledo and Helen Taylor, who helped me with qPCR experiments.

3.1 Introduction

The present study focuses on the role of NMNAT1 on ageing mechanisms, with the use of two mouse models: *Nmnat1* transgenic (*Nmnat1* tg) and *Nmnat1* heterozygous knock out (*Nmnat1*^{+/-}) mice.

NAD is a substrate of the nuclear enzyme, SIRT1, which plays a recognized role in ageing [287-289]. Indeed, overexpression of *Sirt1* in the brain increases lifespan [43]. In addition to this, SIRT1 plays a fundamental role for the regulation of physiological parameters, which are impaired during ageing. Moreover, 20-months-old mice overexpressing *Sirt1* showed significantly higher physical activity during the dark time, compared to age-matched control mice, whereas they showed no differences at 4 months of age. These mice also exhibited higher rectal body temperature and oxygen consumption, with a trend to increased energy expenditure during the dark time compared to control mice. These evidences suggest a pivotal role of SIRT1 in maintaining youthful physiology, contributing to the delay in ageing and to life span extension [43].

SIRT1 contributes to improve the mitochondrial functions that decline during ageing [88]. Knockout mice for *Sirt1* showed the same decrease in mitochondrial, but not nuclear-encoded, components of oxidative phosphorylation, as observed in the skeletal muscle of aged mice. NAD, which is fundamental for SIRT1 activity, was reduced in skeletal muscle of aged mice and this reduction could be restored by NMN supplementation. Knocking down *Nmnat1*, responsible for NAD synthesis at nuclear level, mimics the decline in mitochondrial function observed during ageing [46]. Moreover, during ageing, there is a specific loss of mitochondrial, but not nuclear, encoded OXPHOS subunits. A decline in nuclear NAD levels induces a disruption of nuclear-mitochondrial communication through a mechanism involving the activation of HIF-1 α . Deleting SIRT1 accelerates this process, whereas raising NAD levels in old mice restores mitochondrial function [46]. Consistently with its role on ageing, SIRT1 has also shown a neuroprotective effect in

age-related diseases such as Alzheimer's, Huntington's, and Parkinson's diseases through its overexpression [84, 102, 103].

Importantly, NMNAT1 loss of function has been directly linked to the neurodegenerative processes occurring in the retina, indicating the role that NMNAT1 has on cell maintenance. Indeed, mutations in NMNAT1, leading to a reduction in NMNAT1 activity, have been identified as responsible for degeneration of retinal neurons in Leber Congenital Amaurosis [290]. As previously described, NMNAT has also been associated with neuroprotection in models of proteinopathies, such as polyglutamine (PolyQ) and α -synuclein-induced cytotoxicities [132] and spinocerebellar ataxia 1-induced neurodegeneration [133, 291]. Furthermore, studies in *Drosophila* and mice highlighted NMNAT protective function in models of Tauopathies, a family of neurodegenerative diseases, which include Alzheimer's disease (AD) [142].

Although these studies support a crucial role of NMNAT1 both in the cellular ageing and in age-related pathologies, these mechanisms have not been investigated in *in vivo* models, where NMNAT1 is ubiquitously expressed or is downregulated. For this reason, I decided to use *Nmnat1* tg and *Nmnat1*^{+/-} mice to determine involvement of NMNAT1 on ageing and age-related diseases.

Nmnat1 tg and *Nmnat1*^{+/-} mice have been previously described. *Nmnat1* tg were healthy, fertile and appeared to be normal. Selected *Nmnat1* tg mouse lines showed NMNAT1 protein levels significantly greater than in the wild type. Furthermore, enzyme activity was strongly increased when compared to their controls. *Nmnat1*^{+/-} mice were generated by inactivating the gene through homologous recombination. Complete inactivation was embryonic lethal but *Nmnat1*^{+/-} born and developed normally. These mice showed reduced NMNAT1 mRNA, protein and enzyme activity [77, 127].

Nmnat1 tg and *Nmnat1*^{+/-} provide a model to investigate the role of modulation of NMNAT1 level and activity in ageing mechanism and allow to test the hypothesis that NMNAT1 might influence specific mechanisms occurring over ageing.

3.1.1 Aims and objectives of the chapter

This chapter aims to investigate whether modulating the expression of NMNAT1 influences ageing-related mechanisms. To this aim, I used *Nmnat1* tg and *Nmnat1*^{+/-} mice and performed metabolic analysis and behavioral tests for measuring locomotor activity. Furthermore, biochemical analysis in brains of young and old mice from both genotypes were performed first, to confirm changes in NMNAT enzyme activity; second, to test whether modifying the expression of NMNAT1 might influence NMNAT enzyme activity in old mice. Finally, I measured NAD levels, expression of genes involved in ageing mechanisms, in particular *Nmnat1*, *Nmnat2*, *Sirt1* and *Nampt* and their protein levels in old mice to correlate those changes to metabolism and locomotor activity.

3.2 Results

3.2.1 *Body weight-increase occurring during ageing is strongly delayed in Nmnat1 tg mice.*

NAD plays a pivotal role in metabolic regulation and in ageing mechanisms, particularly by modulating the activity of nuclear NAD-dependent SIRT1. Indeed, 20-months' old mice overexpressing SIRT1 in the brain (BRASTO mice) showed an increase in lifespan and metabolic parameters, such as locomotor activity and oxygen consumption. Furthermore, these mice exhibited a higher food intake, compared to their controls [43]. In the nucleus, NMNAT1 is responsible for synthesis of NAD, which is then used as substrate by SIRT1. I reasoned that *Nmnat1* tg mice could reproduce a similar phenotype to that of BRASTO mice. In contrast, *Nmnat1*^{+/-} mice, where NMNAT1 is downregulated, would exacerbate the impairments occurring over ageing. I used *Nmnat1* tg and *Nmnat1*^{+/-} mice and their controls. Interestingly, *Nmnat1* tg mice body weight increased significantly less during ageing than that of wild type and *Nmnat1*^{+/-} mice. Furthermore, the body weight in *Nmnat1*^{+/-} mice was higher compared to that of their littermates wild type controls at all age tested starting from 12 months (Fig.3.1 and 3.2).

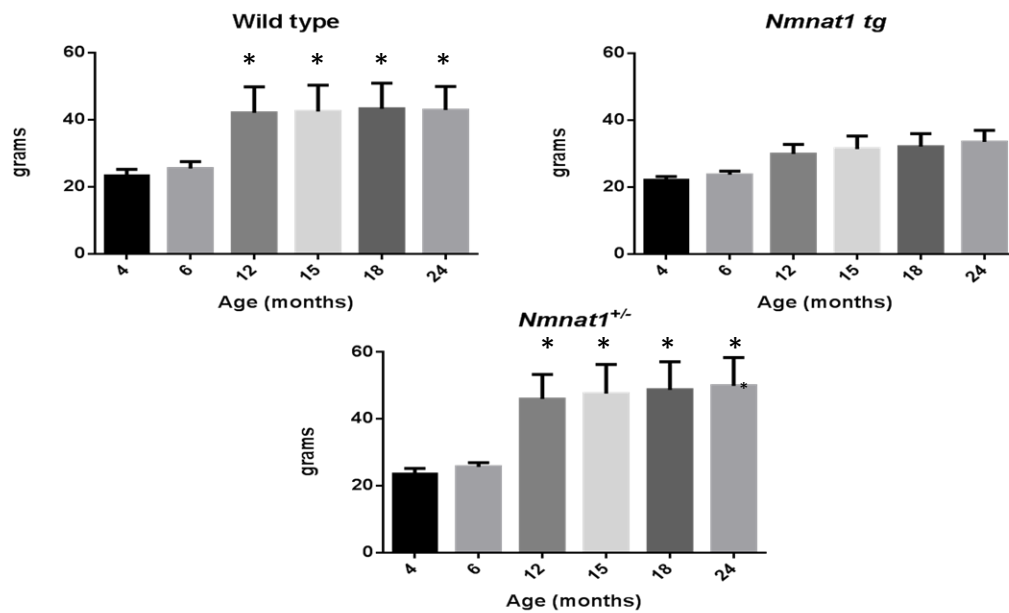


Fig. 3.1 Body weight was unaltered in *Nmnat1* tg mice during ageing.

Body weight was measured in *Nmnat1* tg, *Nmnat1*^{+/-} and wild type mice at the age of 4, 6, 12, 15, 18 and 24 months. Body weight in *Nmnat1* tg appeared to be unaltered during ageing. In contrast, body weight in *Nmnat1*^{+/-} mice and wild types was significantly higher at 12, 15, 18 and 24 months of age compared to that of 4 and 6 months of age ($n=20/\text{group}$, two way ANOVA followed by Bonferroni post-hoc test*, $P < 0.05$).

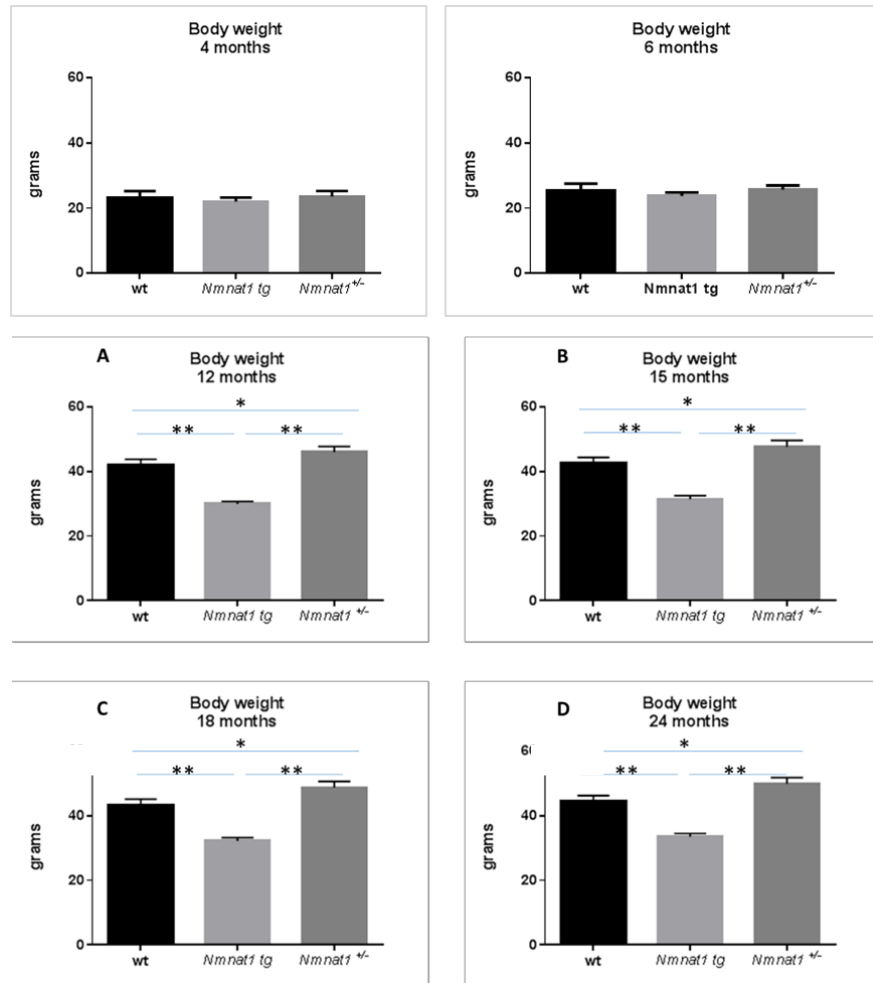


Fig. 3.2 Body weight in *Nmnat1* tg and *Nmnat1*^{+/-} mice.

Body weight was measured in *Nmnat1* tg, *Nmnat1*^{+/-} and wild type mice at the age of 4, 6, 12, 15, 18 and 24 months. *NMNAT1* tg showed a reduced body weight compared to the wild types and *Nmnat1*^{+/-} starting from 12 months of age. Furthermore, body weight in *Nmnat1*^{+/-} mice was significantly higher from 12 to 24 months of age compared to the controls (n=20/group, two way ANOVA followed by Bonferroni post-hoc test*, $P < 0.005$, **, $P < 0.001$)

3.2.2 *Nmnat1* tg mice showed a trend of increased oxygen consumption and locomotor activity.

To assess whether the differences in body weight observed in *Nmnat1* tg and *Nmnat1*^{+/-} mice might reflect changes in metabolic regulation, I analyzed the metabolic profile of these mice at the age of 15 months for a period of 68-72 h. Mice overexpressing NMNAT1 showed a trend of increased locomotor activity (Fig.3.3A) and oxygen consumption

(Fig.3.3B) compared to the controls and *Nmnat1*^{+/-}. Furthermore, food consumption appeared to be higher in *Nmnat1*^{+/-} mice compared to the other genotypes (Fig.3.3C), which might partially explain the increased body weight respect to the wild types.

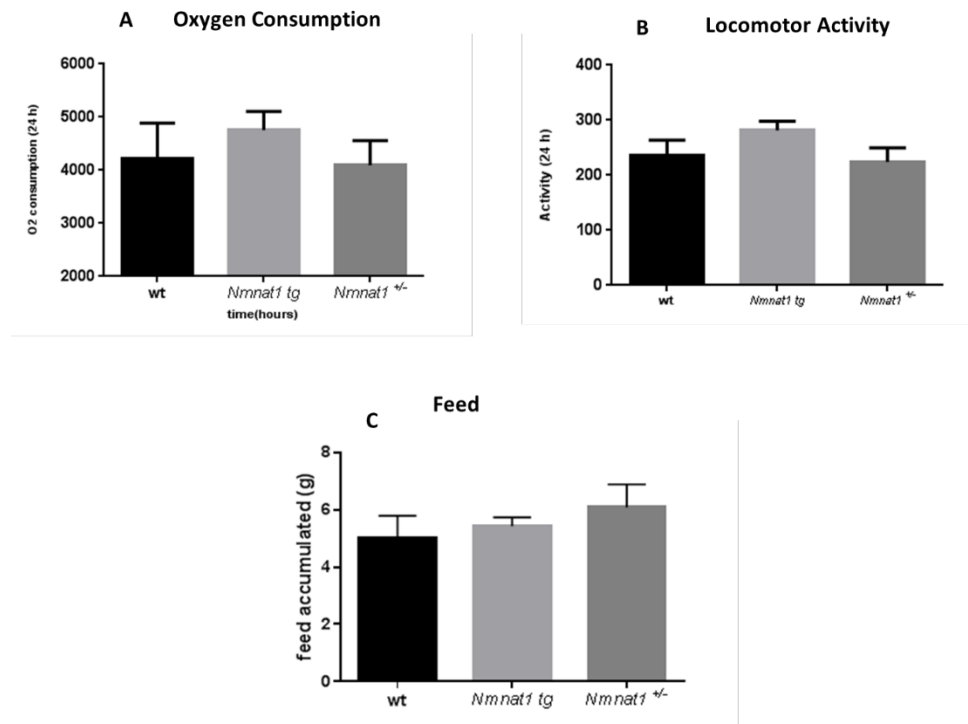


Fig. 3.3 *Nmnat1* tg mice showed a trend of increased oxygen consumption and locomotor activity.

Metabolic analysis was performed at 15 month of age in all genotype. Oxygen consumption (A) and locomotor activity (B) showed a non-significant trend to increase in mice overexpressing *Nmnat1*. Food intake was also measured (C) and a trend to increase was detected in *Nmnat1*^{+/-}, compared to the other genotypes (n=6/group).

3.2.3 Locomotor activity was strongly increased in *Nmnat1* tg mice and reduced in old *Nmnat1*^{+/-}.

Consistent with the trend to increase of locomotor activity detected in the metabolic analysis, *Nmnat1* tg mice showed a significant increase in locomotor activity compared to the wild type and *Nmnat1*^{+/-}, when tested in the Open Field for 30 minutes at the age of 12, 15 and 18 months. There was a significant genotype effect ($F_{(2,33)} = 10.22$; $p < 0.001$). Interestingly, the reduced locomotor activity in *Nmnat1*^{+/-} became more evident at 18

months, where its reduction was significantly lower respect to the controls. Furthermore, locomotor activity in mice overexpressing NMNAT1 was also significantly higher at the age of 6 months, but not at the age of 4 months, when compared to the wild type (Fig.3.4 and 3.5). There was a significant genotype effect ($F_{(3,64)} = 5.72$; $p = 0.002$) and age X genotype interaction at six months ($F_{(3,64)} = 2.94$; $p = 0.04$). However, at these two time points, NMNAT1^{+/-} mice did not differ from the other genotypes.

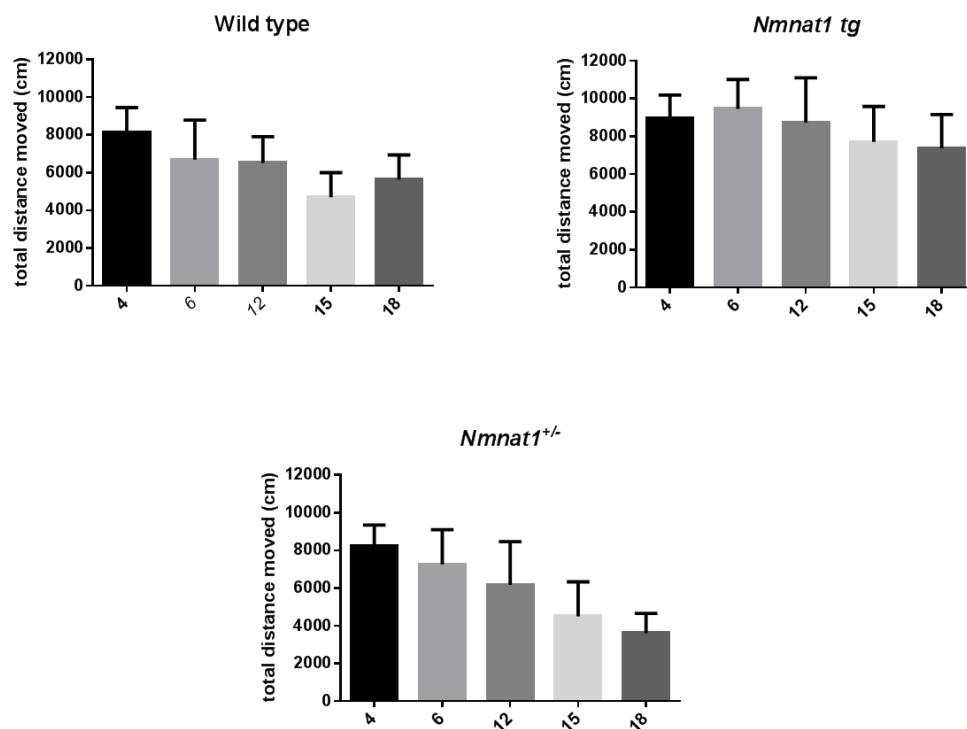


Fig. 3.4 *Nmnat1* tg mice showed an unaltered locomotor activity during ageing

Open-field test was performed at 12 15 and 18 months of age in all genotype. Mice overexpressing *Nmnat1* showed an unchanged locomotor activity over ageing, whereas *Nmnat1*^{+/-} and wild type mice reduce their locomotor activity ($n=12$ /group)...

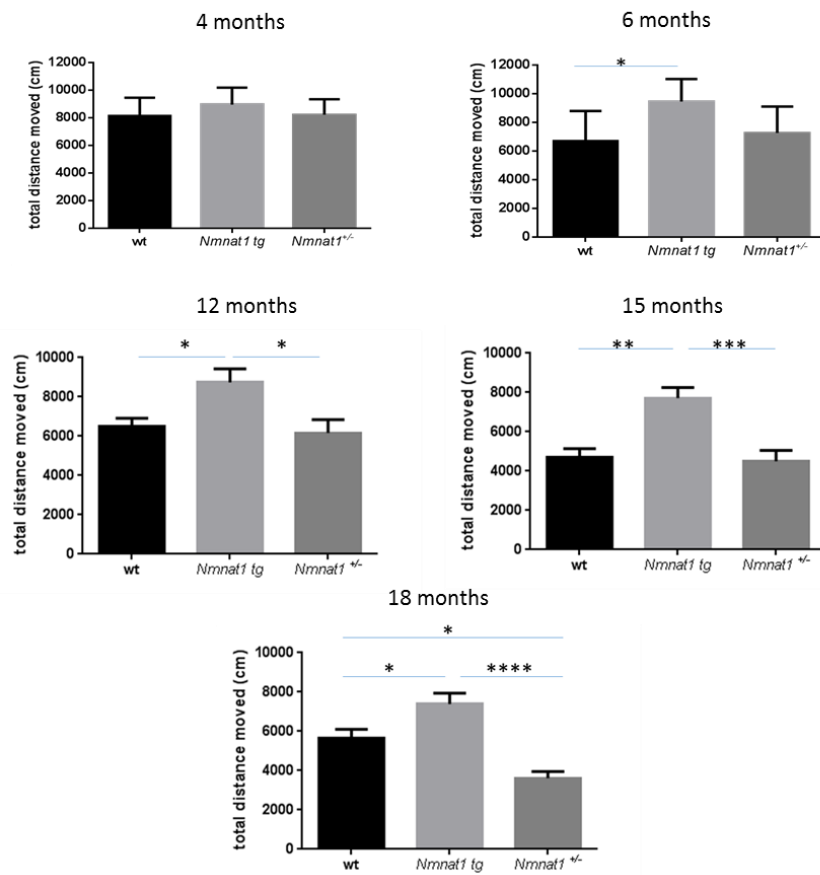


Fig. 3.5 Locomotor activity in wild type, *Nmnat1* tg and *Nmnat1*^{+/-} mice.

Open-field test was performed at 4 and 6 months of age in all genotype. Mice overexpressing *Nmnat1* showed an increase in locomotor activity at 6 months compared to the controls. (n=12/group, three-way ANOVA, followed by post-hoc planned comparison *, $P < 0.005$).

3.2.4 Total NAD levels in brains of old mice were not affected by changes in NMNAT enzyme activity

I first measured the NMNAT enzyme activity to detect changes over ageing that correlate to the phenotype observed. As shown in Fig. 3.6A, *Nmnat1* tg mice showed an increase in enzyme activity of the brain at both 6 and 24 months of age compared to the other genotypes, confirming the previous findings. Interestingly, the reduction in the enzyme activity of *Nmnat1*^{+/-} mice became evident at 24 months only, when compared to the wild type. However, enzyme activity of *Nmnat1*^{+/-} mice did not significantly differ to that of the wild type, which is in contrast with previous finding. This incongruence might be due

to technical issues or to a low number of samples tested. Enzyme activity in wild type and *Nmnat1* tg mice maintained similar levels during ageing. In contrast, enzyme activity of *Nmnat1*^{+/-} mice was significantly reduced at 24 months. Despite these differences, NAD levels in the total brain were unaltered in young and old mice (Fig. 3.6B). NAD levels in *Nmnat1* overexpressing mice appeared to be increased at 6 months of age. However, likely due to the high variability, this difference did not reach a statistical difference.

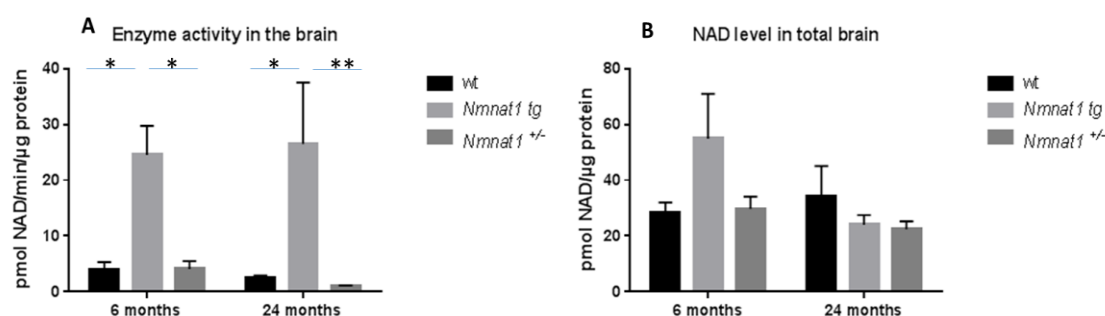


Fig. 3.6 NMNAT Enzyme activity and NAD levels in the brain extracts of *Nmnat1* tg and *Nmnat1*^{+/-} mice.

NMNAT enzyme activity and NAD levels were measured in the total brain homogenate at 6 and 24 months in all genotypes. The enzyme activity was increased in *Nmnat1* overexpressing mice compared to the other genotypes at both ages. The enzyme activity of wild type mice and *Nmnat1* tg mice was unaltered over ageing. In contrast, it was significantly reduced in old-*Nmnat1*^{+/-} mice compared to the young. Despite these changes, NAD levels were unaltered across the genotypes ($n=5/\text{group}$, two-way ANOVA followed by Bonferroni post-hoc test*, $P < 0.05$, **, $P < 0.001$).

3.2.5 Increased NAD levels in brain areas and eyes of old-*Nmnat1* tg mice

A modulation of NAD levels in specific brain areas of *Nmnat1* tg and *Nmnat1*^{+/-} mice could have been hindered while determining the nucleotide level in the total brain homogenate. To test this possibility, I measured NAD levels in the cortex, hippocampus and striatum of all genotype. In consideration of the crucial role that NMNAT1 has in the human retinal neurodegenerative disorder LCA, I also measured NAD levels in the eye of all mouse groups. As shown in Fig. 3.7, NAD levels were similar across the genotypes at both 6 and 24 months in all brain areas. Interestingly, NAD levels presented a trend to increase at 24 months in the cortex (Fig. 3.7A), in the hippocampus (Fig. 3.7B)

and in the striatum ((Fig. 3.7C) of *Nmnat1* tg mice. In contrast, the same trend was not consistently evident in the other groups. Crucially, NAD levels in the eye of 24-months-old *Nmnat1* tg mice were significantly increased compared to the wild type and *Nmnat1*^{+/-}. Furthermore, NAD levels presented a trend to increase in eyes of old-*Nmnat1* tg mice compared to their younger counterparts, following a similar trend to that observed in the brain areas (Fig.3.7D).

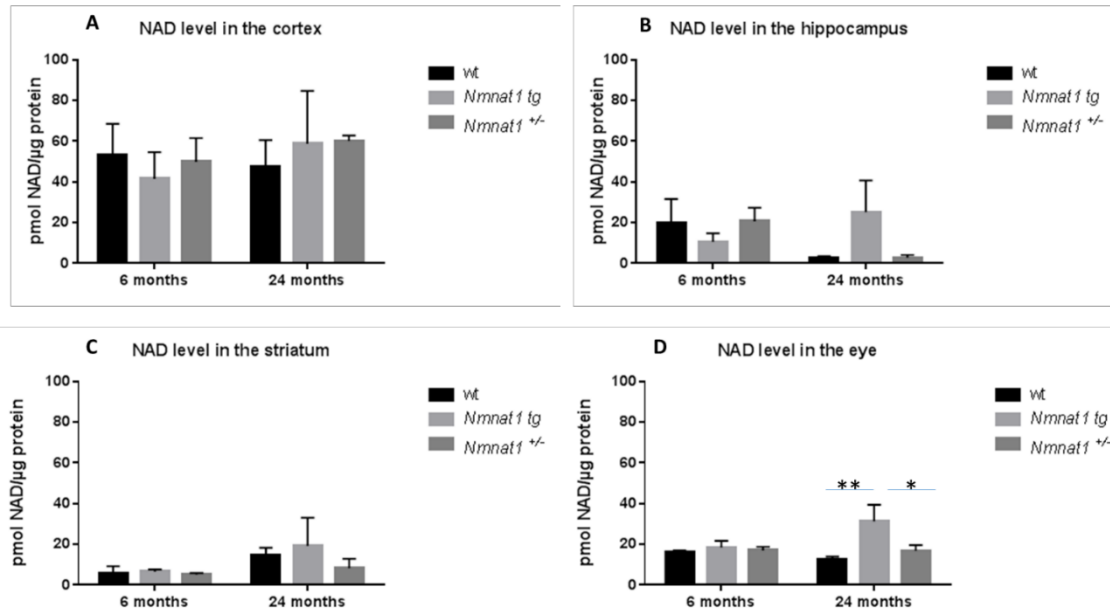


Fig. 3.7 NAD levels in specific brain areas and in eyes of 6 and 24 months-old *Nmnat1* tg and *Nmnat1*^{+/-} mice.

NAD levels were measured in cortex (A), hippocampus (B), striatum (C) and eyes (D) of wt, *Nmnat1* tg and *Nmnat1*^{+/-} mice. *Nmnat1* tg mice showed a trend in increased NAD levels in all brain areas considered and in the eyes over ageing, which was not evident in the other groups. NAD levels were significantly higher in eyes of *Nmnat1* overexpressing mice at 24 months compared to wild type and *Nmnat1*^{+/-} mice ($n=5/\text{group}$, two-way ANOVA followed by Bonferroni post-hoc test*, $P < 0.005$, **, $P < 0.001$).

3.2.6 Expression of NAD-metabolism related genes in *Nmnat1* tg and *Nmnat1*^{+/-} mice.

I then analyzed possible changes in expression of genes related to NAD-metabolic enzymes occurring during ageing and induced by overexpression or reduction of *Nmnat1*. I first measured the expression of *Nmnat1* gene by qPCR in young and old mice of all groups and analyzed changes across the genotypes at both 6 and 24 months

and how they change within the same genotype over ageing. As predicted, *Nmnat1* gene expression was significantly higher compared to the wild type and *Nmnat1*^{+/-} mice at 6 and 24 months (Fig. 3.8A). The changes observed were similar in young and old mice of all genotypes. However, surprisingly *Nmnat1* expression was not reduced in *Nmnat1*^{+/-} mice, as expected. These results might need further confirmation as expression in wild type mice is very low. I then asked whether a modulation of NMNAT1 levels in our mouse models could reflect on the levels of other enzymes involved in NAD metabolism and evaluated the expression of *Nmnat2* (Fig.3.8B), *Nampt* (Fig.3.8C) and *Sirt1* (Fig.3.8D) to test whether overexpressing or reducing *Nmnat1* might influence the expression of other genes involved in NAD biosynthesis or NAD-dependent genes strongly involved in ageing mechanisms and metabolism. No differences were detected across all genotypes neither in young nor old mice of all groups.

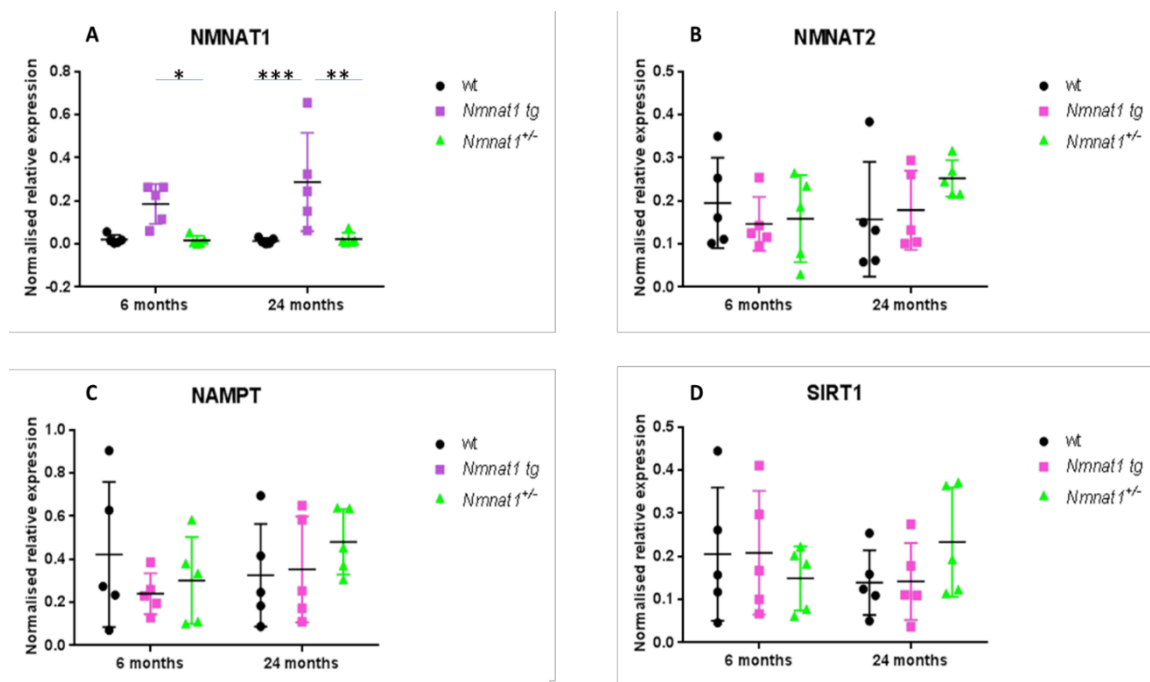


Fig. 3.8 Expression of *Nmnat1*, *Nmnat2*, *Nampt* and *Sirt1* in *Nmnat1* tg and *Nmnat1*^{+/-} Mice.

Expression of genes *Nmnat1* (A), *Nmnat2* (B), *Nampt* (C) and *Sirt1* (D) were measured by qPCR in total brain homogenates of wt, *Nmnat1* tg and *Nmnat1*^{+/-} mice at 6 and 24 months. Expression of *Nmnat1* was significantly increased in young and old *Nmnat1* tg mice compared

to the other groups. No differences were detected in the expression of *Nmnat2*, *Nampt* and *Sirt1* across the genotypes ($n=5/\text{group}$, two-way ANOVA followed by Bonferroni post-hoc test*, $P < 0.005$, **, $P < 0.001$).

3.2.7 Levels of SIRT1 and NAMPT were unaltered in young and old *Nmnat1* tg and *Nmnat1*^{+/-} mice

Then, I assessed levels of NAMPT and SIRT1, enzymes involved in the synthesis and catabolism of NAD respectively (Fig.1.3 and 1.4), through Western Blotting. As shown in Figure 3.9, levels of NAMPT (Fig.3.9A and C) and SIRT1 (Fig.3.9A and B) were unaltered in wild type mice at 6 and 24 months. Similarly, NAMPT and SIRT1 levels did not differ across the genotype and within the same genotype at different ages

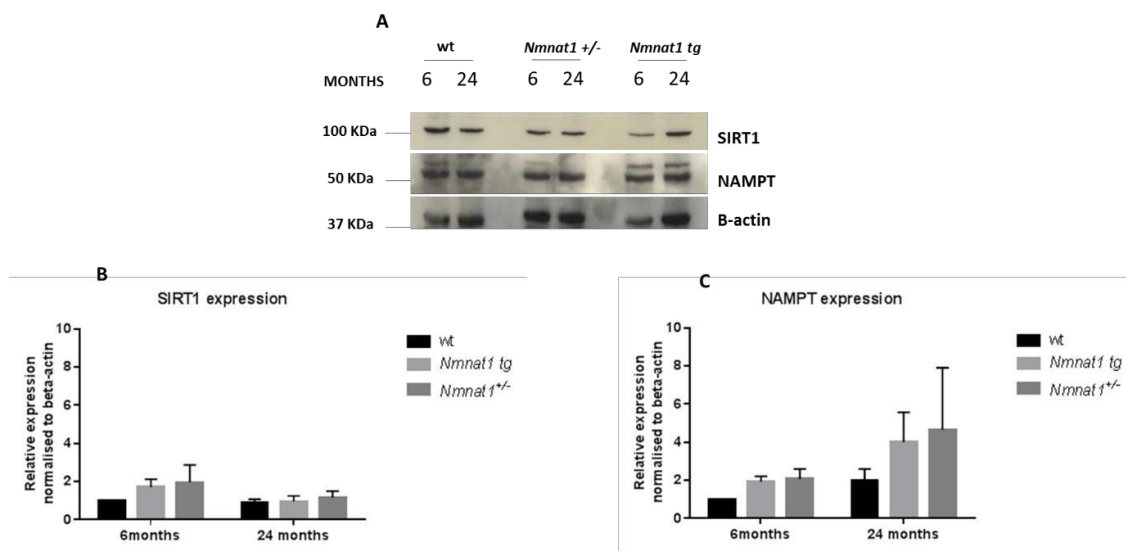


Fig. 3.9 SIRT1 and NAMPT levels were unchanged in wt, *Nmnat1* tg and *Nmnat1*^{+/-} mice.

Western blots of brain homogenates were probed with an antibody against SIRT1 and NAMPT (A) and with β -actin as a loading control. Quantification of the density relative to the loading control (B and C) showed no significant differences ($n=5/\text{group}$).

3.3 Discussion

NAD and its biosynthetic enzymes play a pivotal role in ageing and age-related mechanisms. NAD levels were reduced in skeletal muscle of aged mice and this reduction could

be restored by NMN supplementation. The NAD-nuclear biosynthetic enzyme NMNAT1 is crucially involved in the decline of mitochondrial function observed during ageing [46]. Knocking down *Nmnat1* mimics the decline in mitochondrial function observed during ageing [46]. Moreover, there is a specific loss of mitochondrial, but not nuclear, encoded OXPHOS subunits. A decline in nuclear NAD levels causes a disruption of nuclear-mitochondrial communication. Deletion of SIRT1 induces this process, whereas restoring NAD levels in old mice ameliorates mitochondrial function [46]. Furthermore, NAD is responsible for increasing lifespan through the nuclear enzyme, SIRT1 [287-289]. Therefore, modulating *Nmnat1* in *in vivo* mouse models should influence ageing through these mechanisms. I characterized behaviorally and biochemically mice where *Nmnat1* gene dosage is increased or downregulated. In these mice, corresponding changes in protein levels and enzymatic activity had previously been reported [77, 127].

To specifically investigate the direct role of NMNAT1 in ageing mechanism, I used *Nmnat1* tg and *Nmnat1*^{+/-} mice [77, 127]. These mice present increased or reduced NMNAT enzyme activity, respectively, allowing analyzing the impact of modulating this enzyme on ageing mechanisms. Although other studies highlighted the role of NMNAT1-synthesised NAD on ageing, via modulation of the activity the NAD- dependent deacetylase SIRT1 [43] a direct evidence linking NAD changes within the nucleus to a modulation of ageing is still missing.

I observed differences in the body weight induced by changes in *Nmnat1* expression. In particular, mice overexpressing *Nmnat1* presented a significantly lower body weight when compared to wild type and *Nmnat1*^{+/-} mouse. In contrast, body weight of *Nmnat1*^{+/-} was increased, in comparison to the other experimental groups. Interestingly, changes observed in the body weight were evident in mice starting from 12 months of age and not in mice at a younger age, indicating that possible metabolic regulation involving NMNAT1 occurred over ageing, but not at earlier time points. To explain this, it has already been shown the role that NAD plays in metabolism. Indeed, NAD- dependent deacetylase is responsible for the expression of the circadian transcription factors BMAL and CLOCK in the suprachiasmatic nucleus (SCN) of the hypothalamus via deacetylation of PGC-1 α [167]. Furthermore, NAD⁺ synthesis is regulated by the circadian machinery to provide a critical link from the clock oscillator to metabolic pathways [292] and one of the key

target genes of BMAL and CLOCK is the rate-limiting enzyme for NAD, NAMPT. Any modification in the circadian function with aging would reflect on the temporal order of metabolism, leading to a deterioration in health [206].

Based on these evidences and on the observed changes in the body weight, I decided to analyze specific metabolic parameters in *Nmnat1* tg and *Nmnat1*^{+/-} mice at 15 months of age. Feed-accumulated quantification revealed that *Nmnat1*^{+/-} mice assumed more food than the other mouse groups over the time considered, which might partially explain the increased body weight in these mice. Furthermore, a trend to increase in oxygen consumption and locomotor activity was evident in mice overexpressing *Nmnat1*, compared to the other mouse groups, consistently with the results observed in old-*Sirt1* overexpressing mice [43]. However, a more detailed analysis will be necessary, as an increased number in the experiment and additional time points, including younger mice, will further confirm these evidences. The increased locomotor activity was confirmed by subjecting mice to the open-field. *Nmnat1* tg mice showed a strongly increased locomotor activity compared to both wild types and *Nmnat1*^{+/-}, starting from 12 months of age. The increased locomotor activity started, but less markedly, at 6 months of age. In contrast, *Nmnat1*^{+/-} showed a reduced locomotor activity at only 18 months of age, compared to the wild types. However, a further analysis on the locomotor activity at late time points was extremely difficult, as *Nmnat1*^{+/-} were not able to move properly when placed to the apparatus and this was probably influenced by the high body weight.

To explain the differences observed in body weight and locomotor activity, I measured NMNAT enzyme activity of 6 and 24 months old mice from all experimental groups considered. It has already been shown that *Nmnat1* tg mice and *Nmnat1*^{+/-} presented an increased and reduced NMNAT enzyme activity in 6 months-old mice, respectively [77, 127]. There are no previous studies indicating how the already altered enzyme activity in these mouse models could be influenced over ageing. In my results, I first confirmed an increased NMNAT enzyme activity in 6-months-old *Nmnat1* overexpressing mice, when compared to the other groups. However, in contrast with previous findings, enzyme activity in *Nmnat1*^{+/-} did not differ to that in the wild types, indicating a possible technical issue occurred during the measurement, which will need to be addressed. Interestingly, levels of enzyme activity were maintained unaltered in wild type and *Nmnat1* tg mice

over ageing. In contrast, 24-months-old-*Nmnat1*^{+/-} mice showed a reduction respect to their younger counterpart; the reduction in the enzyme activity caused by lower levels of NMNAT1 could be further exacerbated over ageing.

Despite changes in NMNAT enzyme activity, levels of NAD in the total brain homogenate remained at a steady-state level in all genotypes. However, changes in NMNAT activity could induce localized changes in NAD availability or affect the activity of NAD-dependent enzymes. Furthermore, loss of function mutations in *NMNAT1* that cause retinal degeneration in humans have been shown to affect NAD biosynthetic activity and could modify local nuclear NAD pools [131].

Possibly, NAD levels might be altered in specific brain areas, which might not be detectable through the whole brain.

For this reason, I decided to measure NAD levels in some brain areas, specifically in the cortex, hippocampus and striatum. My results showed a trend to increase of NAD levels in mice overexpressing *Nmnat1* over ageing. In contrast, wild type and *Nmnat1*^{+/-} showed a variable pattern of increase and/or decrease between young and old mice, within the same genotype. This evidence might suggest the possibility for a selective change in NAD levels, induced by alteration in the NMNAT enzyme activity, which are detectable in specific brain areas, but not in the whole brain. Moreover, a measurement of NAD levels in more brain areas might be required. In particular, hypothalamus has been recently shown to be involved in systemic aging/longevity control in mammals through NF-κB signaling [208].

Interestingly, NAD level in *Nmnat1* tg mice was significantly higher compared to the other genotypes at 24 months in eyes, an organ where NMNAT1 seems to play a most important role. The fact that NAD levels also in this organ are unaltered at 6 months suggests that NAD and NMNAT enzyme activity change at an older age. Furthermore, the results observed in the eye appeared to be consistent with the pivotal role of NMNAT1 in LCA [131], where *NMNAT1* mutations reduced enzyme activity and lowered NAD levels in affected individuals [131]. In light of these evidences, *Nmnat1* tg and *Nmnat1*^{+/-} mice may provide a strong model to study this neurodegenerative disease and possible pharmacological therapies.

To further analyze possible changes induced by overexpressing or reducing *Nmnat1* and its impact on ageing, which could explain the metabolic changes observed, I considered a possible modification at gene-expression levels. Firstly, I confirmed with qPCR that, as expected and in agreement with previous data [127], expression of *Nmnat1* gene was significantly higher in *Nmnat1* overexpressing mice compared to all genotypes. Similar levels of *Nmnat1* were maintained during ageing. Unexpectedly, levels of *Nmnat1* were unchanged in *Nmnat1*^{+/-} mice compared to the wild types. Furthermore, the low level of *Nmnat1* observed in the wild types were not in agreement with previous findings [127]. However, a low expression of *Nmnat1* is sufficient for a significant enzyme activity, as NMNAT1 is catalytically more active than the other two NMNAT isoforms. For these reasons, further investigation will be required, as possible technical issues and low n number might have affected the analysis. Secondly, genes involved in NAD biosynthesis, specifically cytoplasmic isoform NMNAT2, the rate-limiting NAD biosynthetic enzyme NAMPT and NAD-dependent SIRT1, were not influenced by overexpressing or reducing *Nmnat1*. Similarly, protein levels were unchanged across the genotypes and within the same genotype over ageing. However, these data would require a more careful analysis by increasing the n number of the sample. These evidences suggested that modulating *Nmnat1* might not influence gene expression or protein levels of NAD-related enzymes and, consequently, it was not possible to correlate modification in the expression of those enzymes to changes occurring over ageing. However, NMNAT1 could influence SIRT1 enzymatic activity and this influence could have an impact on the expression of gene modulated by SIRT1. In support of this, it is known that, within the nucleus, NAMPT and NMNAT1 form a nuclear NAD salvage pathway that supplies NAD as a substrate for a variety of NAD- dependent enzymes, including SIRT1. Furthermore, NMNAT1 is recruited to target gene promoters through interaction with SIRT1. Both NAMPT and NMNAT1 regulate cellular NAD production as well as SIRT1 activity at the promoter of common target genes, highlighting the central role of NAD production in SIRT1-dependent transcriptional regulation [20]. Taken together, these results might provide evidences for a role of NMNAT enzyme activity, on the metabolic regulation and locomotor activity. How these could correlate, and whether NAD levels changes locally could cause this potentially by stimulating SIRT1 activity, still needs to be fully elucidated. Further analysis, as measuring the SIRT1 activity and how it could

be influenced by modulation of NMNAT1 over ageing will shed more light on the ageing mechanisms and metabolism.

In conclusion, my results indicate that modulation of NMNAT1 provides a good model for studying ageing mechanisms.

Chapter 4

NAD-biosynthetic enzyme NMNAT1 reduces early behavioral abnormalities in the htau mouse model of tauopathy.

Note: the following results have been used for writing a scientific manuscript that is now under journal submission. Some of the experiment were performed by me with a contribution from other co-authors:

Genotyping: Andrea Loreto; Immunohistochemistry: Weina Meng, Anna Herd-Smith; MRI/MRS: Malcolm Prior Philippine C. Geiszler, Weina Meng, Maria Yanez Lopez, Dorothee P. Auer and Henryk Faas.

4.1 Introduction

The present study aims to investigate the role of NMNAT1 in a mouse model of tauopathy relevant to AD.

Several studies showed the contribution that NMNATs have on this pathology. In particular, the neuroprotective function of NMNAT in a *Drosophila* model of tauopathy have been described [134], where overexpression of either wild-type or enzyme-inactive *Drosophila* NMNAT reduces the amount of hyperphosphorylated tau. Importantly, overexpression of NMNAT ameliorates cognitive and locomotor deficits characteristic of the pathology. NMNAT induces the protective effect through its ability to interact with phosphorylated tau, promoting clearance of toxic tau species with a mechanism independent of its enzyme activity. It has been suggested that the ability of NMNAT to induce neuroprotection in the *Drosophila* and mouse models of tauopathy was related to its chaperone-like function, which enables the enzyme to reduce the amount of tau aggregates [134].

NMNAT neuroprotection was also confirmed in a mouse model of tauopathy, overexpressing the FTDP-17 associated mutant tau P_{301L} . Indeed, hippocampus-specific overexpression of either *Nmnat1* or *Nmnat2* significantly reduced hippocampal neuronal loss in this model [135]. NMNATs could confer neuroprotection in tauopathies with a mechanism involving the NAD-dependent SIRT1. SIRT1 levels correlate negatively with the amount of neurofibrillary tangles in AD brains, suggesting a direct correlation of SIRT1 with tau accumulation [221]. SIRT1 plays a role in the control of stress-regulated proteins and this mechanism may contribute to the reduction of toxic tau aggregates [222].

Although these studies support a crucial role of NMNATs, these mechanisms have not been investigated in *in vivo* models, where the nuclear isoform NMNAT1 is ubiquitously expressed or is downregulated. For this reason, I decided to use *Nmnat1* tg and *Nmnat1*^{+/-} mice and investigate the effect that modulating NMNAT1 has in tauopathies, crossing these mice with a mouse model of tauopathy, that overexpresses the human tau wild type isoform (htau mouse).

Previous lines of transgenic mice expressing mutant human tau transgenes were developed and used to study tau pathology. These mice provide a good model for inherited tauopathies. However, most sporadic AD cases are not associated with tau mutations [214]. The htau mouse model expressing human, wild type tau and develops tau pathology following a time course and a distribution comparable to that of the early stage of AD; for these reasons it provides a good model to study the pathology.

The adult profile of tau isoforms differs between humans and mice. Six human tau isoforms are known, all derived from alternative splicing of a single tau gene. The isoforms are present as either 3 repeat (3R) or 4 repeat (4R), depending on the inclusion or exclusion of an imperfect repeat region coded for by exon 10. In the mouse brain, only 4R are present, while in humans, both 3R and 4R have been found. Htau mice express the human tau isoforms [214].

In htau mice, tau re-localization into the cell bodies and dendrites is evident in the cortex and the hippocampus of htau mice at the early age of 3 months and becomes more evident in older mice as shown in Fig. 4.1. Furthermore, accumulation of hyperphosphorylated tau begins by 6 months.

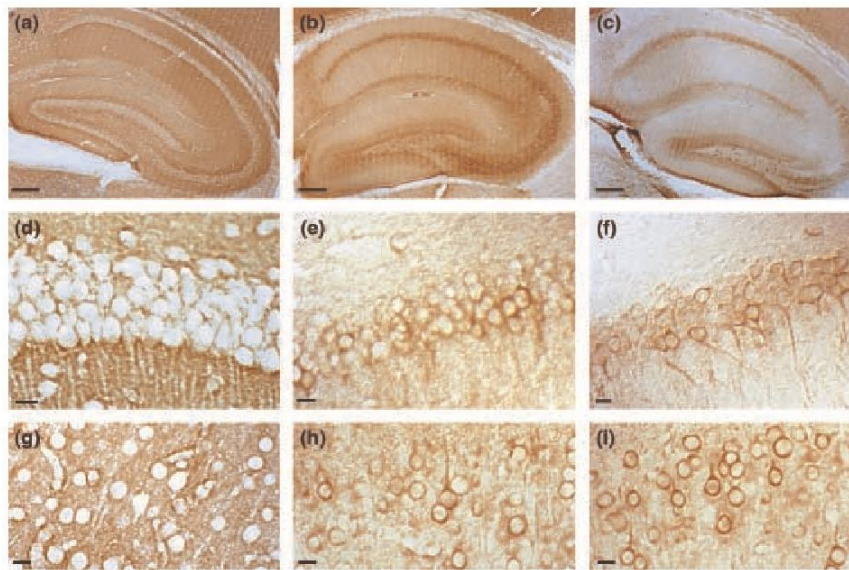


Fig.4.1 Abnormal accumulation of phosphorylated tau in cell bodies and dendrites of htau mice occurs with ageing.

Immunohistochemistry using the antibody CP13 (Ps202) revealed an absence of cell body staining in 6-week-old htau mouse brain hippocampus (a and d) and neocortex (g). Cell body accumulation of CP13 positive tau was detected in htau mice by 3 months of age in the hippocampus (b and e) and neocortex (h). CP13 staining was strong in the older htau mice, exemplified in the 13-month-old htau mouse hippocampal (c and f) and neocortical (i) sections. Scale bars: (a–c) 80 μ m; (d–i) 5 μ m [214].

In a model where mice express mutant tau, it was shown that tau pathology occurs predominantly in the hindbrain and spinal cord. In contrast, in htau mice overexpressing the human wild type tau isoform, the distribution of early tau pathology has been detected in the cortex and in the hippocampus. These evidences demonstrate that the htau model closely reproduce the human AD pathology, developing tau pathology in the brain areas relevant to AD [214].

4.1.1 Aims and objectives of the chapter

This chapter aims to investigate whether modulating the expression of the nuclear NMNAT1 influences early behavioral and neuropathological abnormalities in a mouse line, which expresses non-mutant human tau isoforms (htau mouse), representing a model of tauopathy relevant to AD. To this aim, I pursued the following objectives using four different genotypes that were all heterozygous for murine tau (mtau^{+/+}): htau, *Nmnat1*

tg/htau, *Nmnat1*^{+/-}/htau and wild type littermates. **Objective 1**-mice were subjected to a battery of specific tests to assess behavioral abnormalities that correlate with dysfunctions characteristic of AD. **Objective 2**- imaging analysis was performed to assess the integrity of the brain areas known to be impaired in AD. Finally, biochemical studies were conducted in order to test whether modulating NMNAT1 levels caused changes in NMNAT activity and in NAD levels in htau mice.

4.2 Results

4.2.1 *Distribution of tau in htau mice crossed with heterozygous KO murine tau mice*

Previous characterization of htau transgenic mice has been in the context of the homozygous deletion of murine tau and has shown that, starting from 3 months of age, hyperphosphorylated tau redistributes from its axonal position to the cell body and dendrites (see Fig.4.1) [214]. In order to assess whether a similar redistribution was also evident in a heterozygous KO murine tau background, immunohistochemistry was performed with CP13 antibody, which recognizes phosphorylated tau serine 202 [214]. As shown in fig.4.2, tau is more abundant in the axons of wild type mice (Fig.4.2A and C), while it redistributes to a perinuclear location in htau mice, which are heterozygous KO for murine tau (fig.4.2B and D), similar to what reported in htau mice homozygous for the murine deletion [214] (Fig.4.1). This observation supports the validity of our model in studying the pathology caused by overexpression of wild type htau, indicating that murine tau heterozygous KO is sufficient to develop the phenotype.

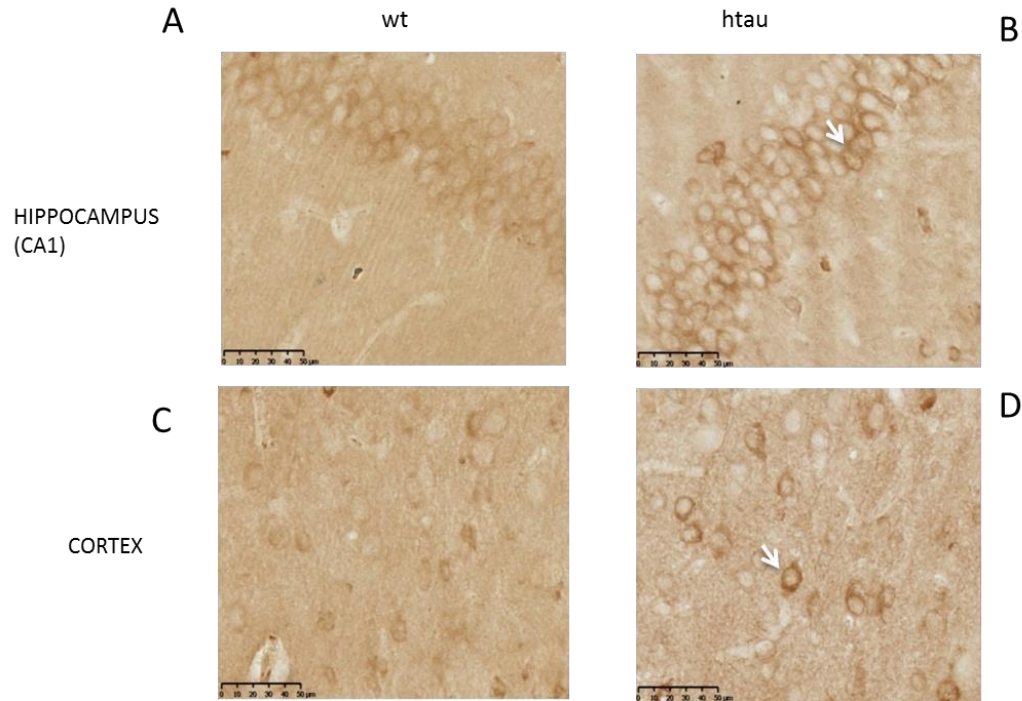


Fig. 4.2 Hyperphosphorylated tau distribution in htau mice at 6 months of age.

CP13 staining shows accumulation of phosphorylated tau in neurons of hippocampus and cortex in six-month-old htau mice. CP13 immunoreactivity appears higher in the neuronal cell bodies of htau mice (B and D, white arrows) compared to wild types (A and C). Scale bar: 100μm.

4.2.2 NMNAT1 overexpression rescues an early impairment in food burrowing performance in htau mice

Subtle behavioral abnormalities, preceding cognitive dysfunction, are among early signs in AD [294]. In htau mice, cognitive decline has been detected by 12 months of age, but earlier non-cognitive behavioral abnormalities have not been reported [295]. Therefore, I subjected the mice to the food burrowing test from 2 months to 6 months of age. This test has been used to assess activities of daily living and the inability to perform these activities is an early sign of AD [272]. Strikingly, I found that increasing NMNAT1 levels improved the food burrowing performance at 3 months and at all time later points (Fig.4.3). However, reducing NMNAT1 levels did not further decrease the htau mice's impaired performance (Fig. 4.3). Importantly, burrowing behavior of *mtau*^{+/-}, *Nmnat1* tg and *Nmnat1*^{+/-} mice was indistinguishable from the ones of wild-types (Fig. 4.4).

Thus, NMNAT1 overexpression restores food burrowing in htau mice.

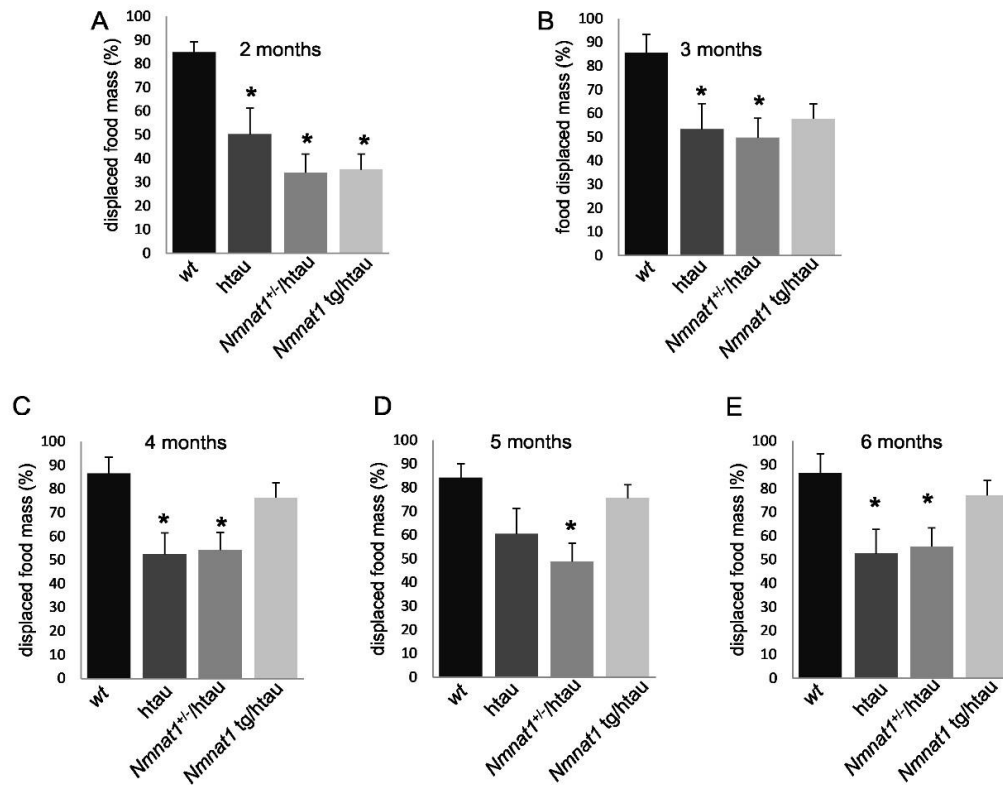


Fig. 4.3 Food burrowing performance in htau mice is improved by NMNAT1 overexpression.

Food-burrowing performance was determined as described in the methods in mice of two (A), three (B), four (C), five (D) and six (E) months of age. At the age of two months (A), burrowing behavior was strongly impaired in all genotypes compared to the wild type. However, from three- to six-months-of-age (B-E), increasing NMNAT1 levels in htau mice rescues their food burrowing deficit, whilst reducing NMNAT1 levels did not further decrease the low performance of htau mice. (n=12/group, three-way ANOVA followed by post-hoc planned comparison*, $P < 0.005$).

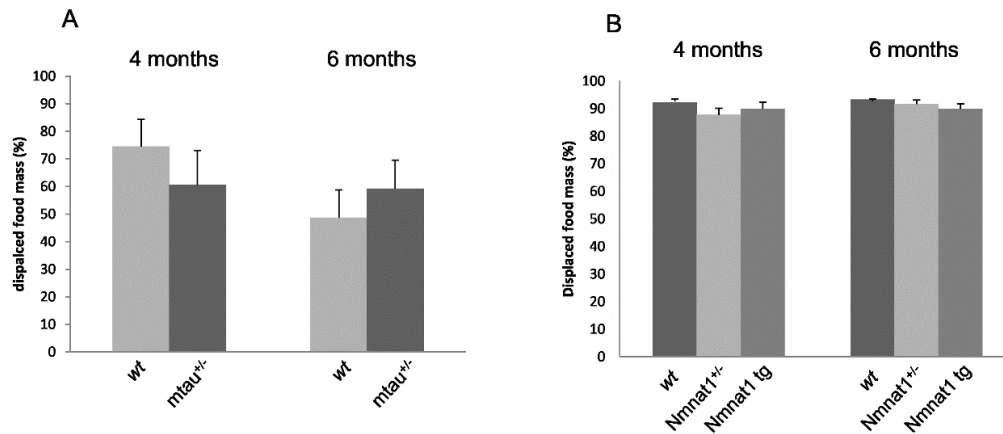


Fig. 4.4 Food-burrowing test in wild type, $Nmnat1^{+/-}$, $Nmnat1$ tg and murine $\tau^{+/-}$ mice.

Mice were tested at 4 and 6 months of age. Food burrowing performance was not altered neither in murine $\tau^{+/-}$ mice (A) nor in $Nmnat1^{+/-}$ and $Nmnat1$ tg mice (B) compared to their controls ($n=12/\text{group}$)

4.2.3 Locomotor activity is increased in htau mice overexpressing $Nmnat1$

I assessed locomotor activity and anxiety-related behavior in mice of all genotypes using the open field. Anxiety-related behavior did not differ between the genotypes at both four months nor at six months of age (Fig. 4.5A and B). There was no significant genotype or age X genotype interaction effects. Interestingly the locomotor activity in six month old htau mice which overexpressed NMNAT1 was higher than that of the other genotypes. There was a significant genotype effect ($F_{(3,64)} = 5.72$; $p = 0.002$) and age X genotype interaction at six months ($F_{(3,64)} = 2.94$; $p = 0.04$) (Fig. 4.5C-D). This higher activity was also observed in $Nmnat1$ tg mice but not in $Nmnat1^{+/-}$ or $\tau^{+/-}$ mice (Fig. 4.6).

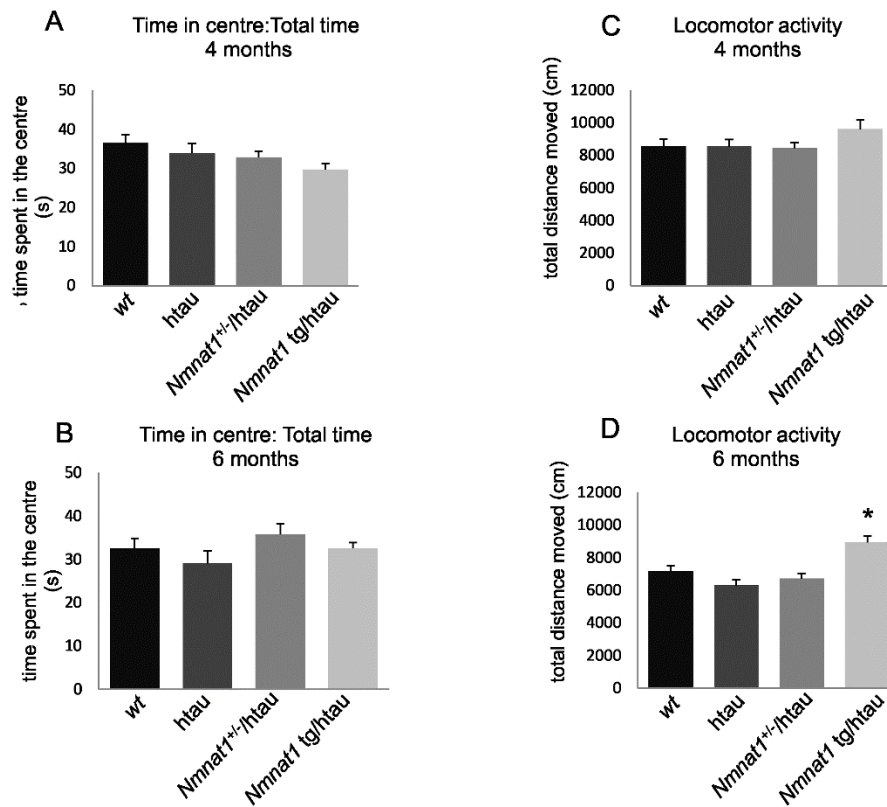


Fig. 4.5 Locomotor activity of *Nmnat1* tg/htau mice at six months is increased.

Locomotor activity and anxiety-related behavior were assessed using the open field test. As an index of anxiety-like behavior, the proportion of time the mice spent in the center was measured at four- (A) and at six-months-of-age (B). No difference was detected between the genotypes. Locomotor activity is represented as the total distance moved (cm) measured in mice of all genotypes at four- (C) and at six-months-of-age (D). Locomotor activity is higher in htau mice overexpressing NMNAT1 at six months-of-age (D) ($n=12/\text{group}$, three-way ANOVA followed by post-hoc planned comparison, *, $P < 0.005$).

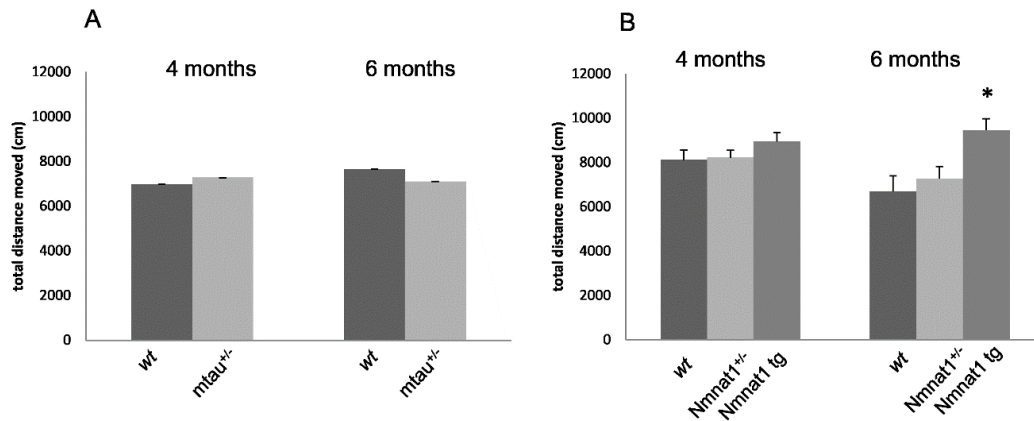


Fig. 4.6 Locomotor activity in wild type, *Nmnat1*^{+/+}, *Nmnat1* tg and murine tau^{+/+} mice.

Locomotor activity was unaltered in murine tau^{+/+} at 4 and 6 months of age compared to their wild type (A). *Nmnat1* tg mice showed a higher locomotor activity compared to the other genotypes at 6 months, but not at 4 months of age (B). (n=12/group, three-way ANOVA followed by post-hoc planned comparison, *, $P < 0.005$).

4.2.4 htau mice do not show learning and memory deficits at six-months-of-age

In the spontaneous alternation test, mice of all genotypes alternated arms with a frequency higher than chance without statistically significant differences across the genotypes (Fig. 4.7 A-B). Likewise, mice of all genotypes displayed a comparable performance in object recognition and location both at 4 months (Fig. 4.8A, B and C) and at 6 months (Fig. 4.8D, E and F), as tested by the novel object paradigm. Although the performance between groups did not differ, htau mice were unable to discriminate the two objects at 6 months in the discrimination trial. In the contextual fear conditioning test, during the acquisition trial, all mice were able to acquire contextual fear ($F_{(4;120)} = 135.69$; $p < 0.001$. Fig. 20 C). No significant differences were detected among the different genotypes in contextual memory retention and extinction (Fig. 4.7D-E).

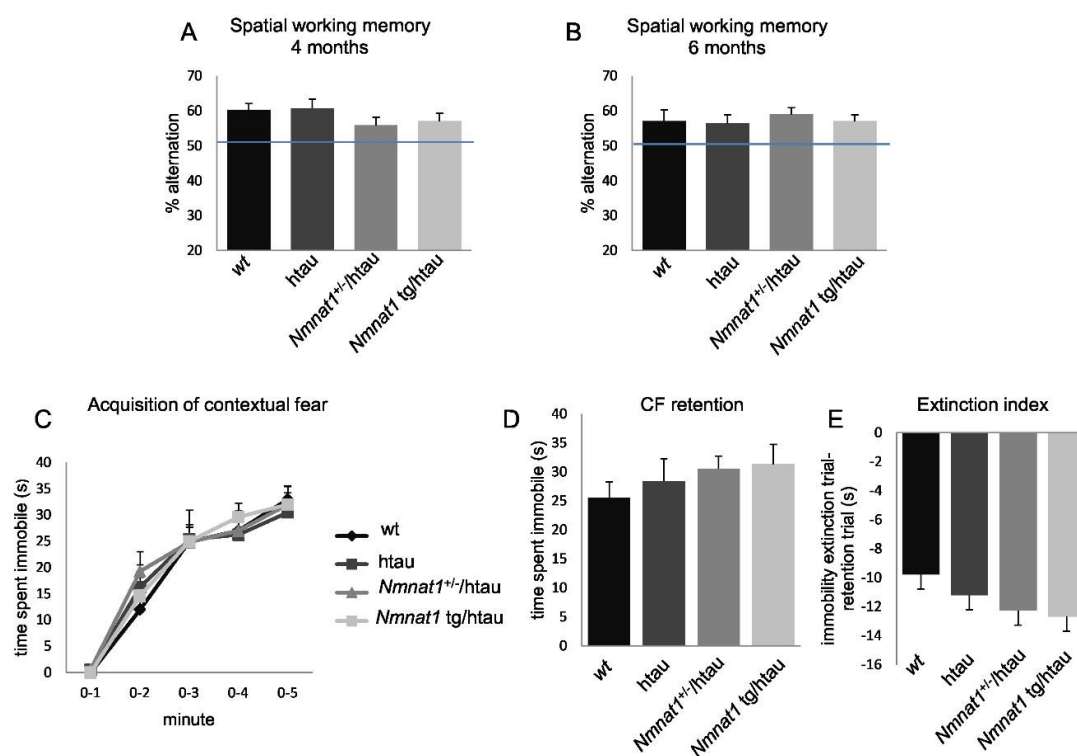


Fig. 4.7 Contextual fear conditioning and spontaneous alternation test in wild types, htau, *Nmnat1*^{+/-}/htau and *Nmnat1* tg/htau mice is similar.

The alternation rate [%] in the spontaneous alternation task was measured in mice of all genotypes at four- (A) and six-months-of-age (B). All mice were tested for contextual fear acquisition (C), retention (D) and extinction (E) as described in the methods. No differences were detected between the genotypes ($n=12/\text{group}$, two-way ANOVA followed by Bonferroni post-hoc test *, $P < 0.005$).

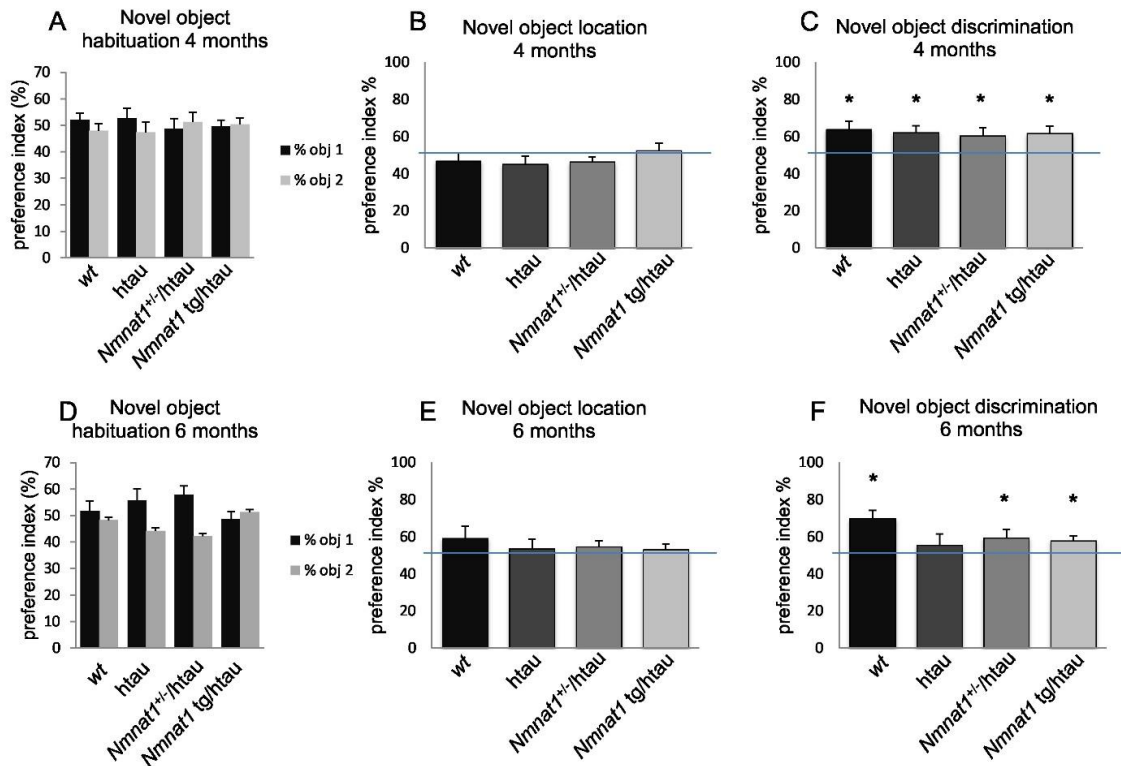


Fig. 4.8 Novel object paradigm in htau mice.

Mice were habituated to the objects and tested at 4 (A, B and C) and 6 months of age (D, E and F). No significant difference across genotypes was detected in memory location (B and E) and recognition performance (C and F). ($n = 12/\text{group}$, $* p < 0.005$ compared to chance level 50%, one sample t -test).

4.2.5 Absence of neurodegenerative changes in the htau mouse brain

I then asked whether any sign of neurodegeneration in the htau mouse brains could explain the specific defect seen in the hippocampal-dependent food burrowing test. First, we measured non-invasively the levels of Ins, Tau, Glu, NAA, Gln, GSH, PCh+GPC, and total creatine (Cr+PCr) *in vivo* using magnetic resonance spectroscopy (MRS) in the hippocampus of six-month-old mice. It has been previously shown that the levels of these metabolites change during neurodegenerative processes [296, 297].

I did not observe any statistically significant difference in metabolite levels, assessed by MRS across the four genotypes at the time point studied (Fig. 4.9 A and B). I also measured the volume of the hippocampus *in vivo* (Fig. 4.9C and D) and *post-mortem* (Fig.

4.10 A and B) and found no differences across the genotypes. Similarly, the number of hippocampal neurons was comparable across all genotypes (Fig. 4.10 C and D). Finally, I determined the levels of the synaptic marker synaptophysin in the hippocampus (Fig. 4.10 E and F) and cortex (Fig. 4.10 G and H) and no significant changes were detected in all genotypes.

Taken together, these data suggest that there are no obvious neuropathological abnormalities in htau mice up to six months of age and that the early behavioral abnormality, i.e. the reduced performance in the food burrowing test, precedes any obvious sign of pathology.

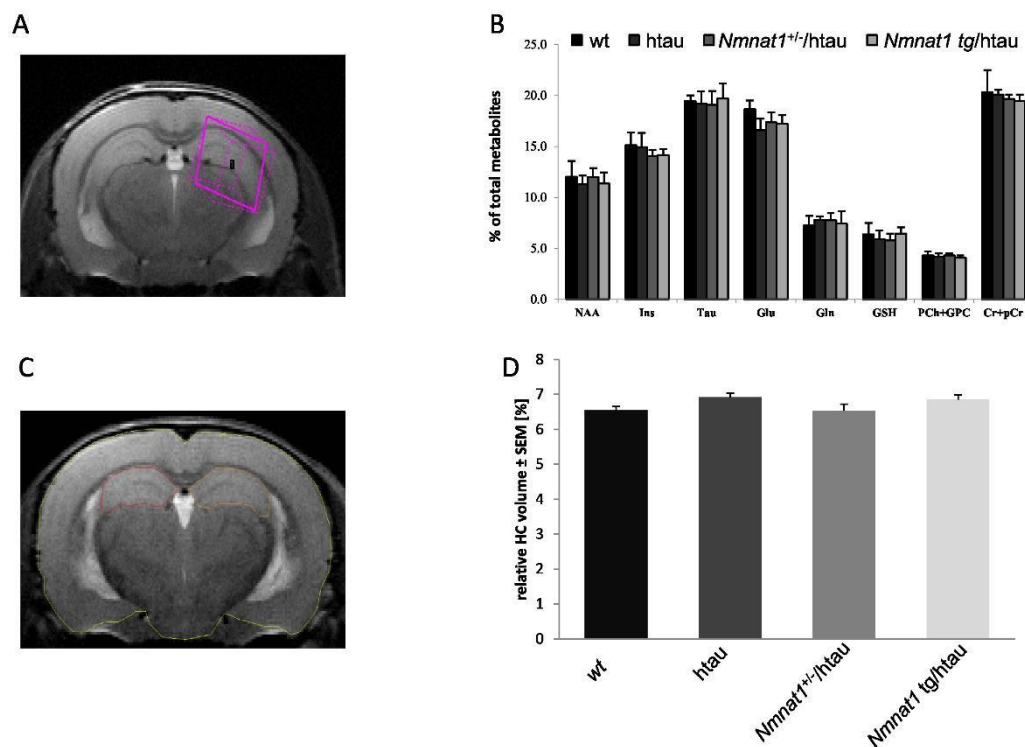


Fig. 4.9 Metabolite levels and hippocampal volume did not differ between genotypes.

Relative metabolite concentrations were assessed in vivo using MRS with the voxel of interest being positioned in the left hippocampus (2x2x2 mm³) as shown (A). Specific metabolite levels were quantified with respect to the total metabolite content as detailed in methods (B) and were unchanged across genotypes. For 3D anatomical MRI, coronal slices were acquired using a RARE sequence and hippocampal volumes were determined by manually drawing regions of

interest around the left and right hippocampus, as shown in (C), in blind using a software as described in methods. No differences were detected across the genotypes (D)(n= 10/group).

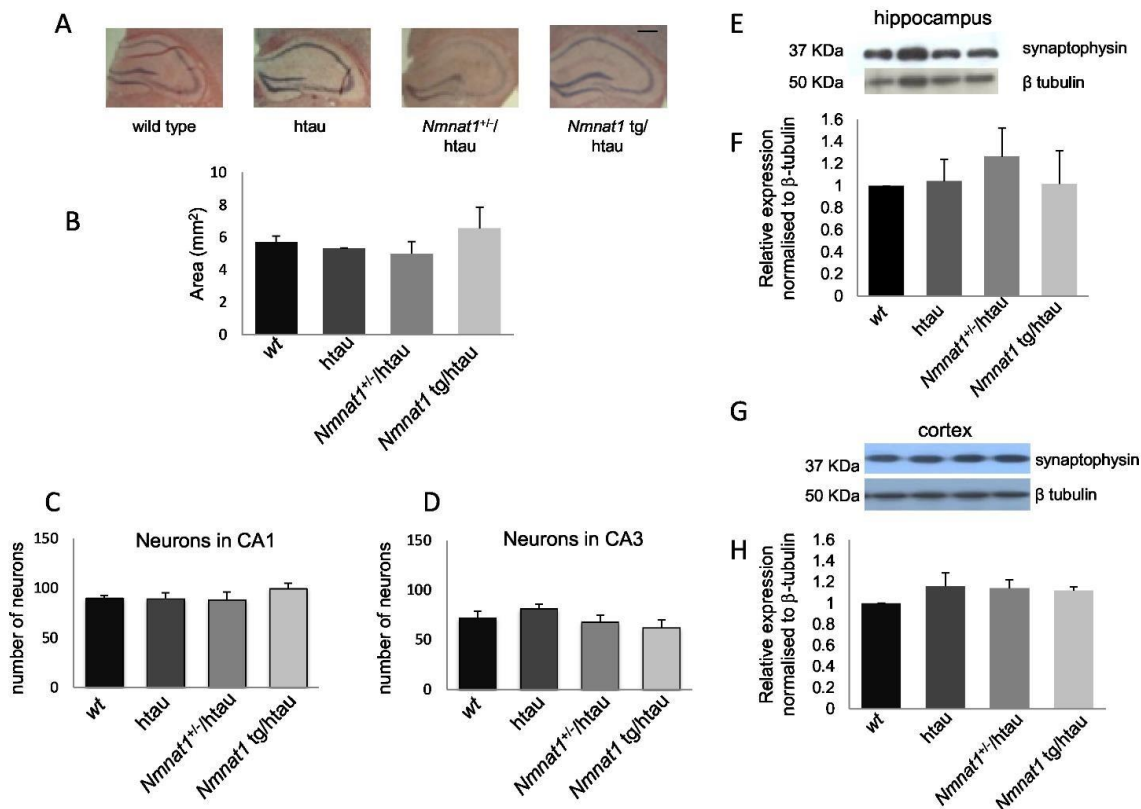


Fig. 4.10 Absence of neurodegenerative changes in the cortex and hippocampus of htau mice.

(A) Coronal sections of brains from all genotypes stained with H-E and (B) relative quantification of the hippocampal areas are shown. Scale bar, 500 μm. There were no differences in the hippocampal area across all genotypes (n=5/group, one-way ANOVA with Bonferroni post-hoc test, compared to the wild type). (C, D) The number of neurons in Nissl- stained coronal sections was quantified in CA1 and CA3 hippocampal regions. No differences were detected. (E-H) Western blots of homogenates from hippocampus (E) and cortex (G) were probed with an antibody against the presynaptic marker synaptophysin and with β-tubulin as a loading control. Quantification of the density relative to the loading control (F and H) showed no difference in the hippocampus (F) and in the cortex (H) (n=5/group).

4.2.6 NAD levels are not affected by changes in NMNAT enzyme activity

I determined NMNAT enzyme activity and NAD levels in both cortex and hippocampus of htau mice. Overexpression or downregulation of *Nmnat1* in htau mice had expected

corresponding effects on enzymatic activity, which was reduced in *Nmnat1*^{+/-}/htau mice and increased in *Nmnat1* tg/htau mice (Fig.4.11 A-B). Despite the difference in the enzymatic activity, steady-state NAD levels were not significantly different across all genotypes (Fig.4.11 C-D).

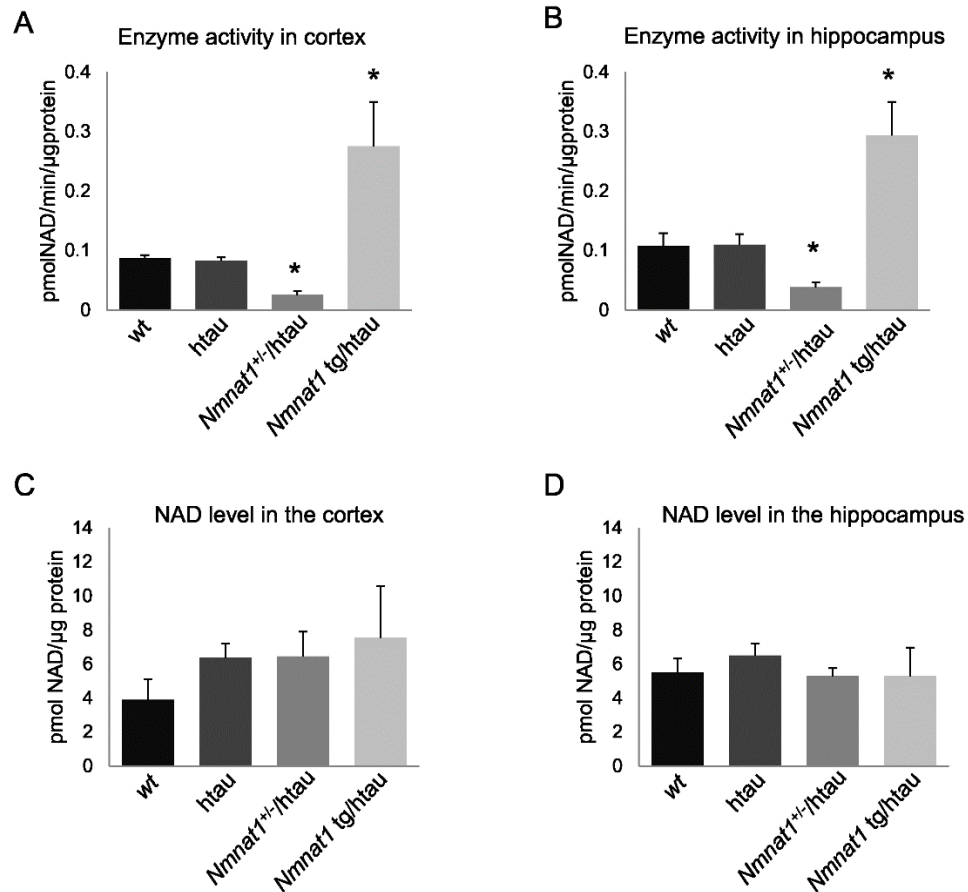


Fig. 4.11 NAD levels are not affected by changes in NMNAT enzyme activity.

NMNAT enzyme activity (A, B) and NAD levels (C, D) were measured in extracts of cortex (A, C) and hippocampus (B, D). NMNAT level modulation changes the enzyme activity but not the NAD levels. Enzyme activity is significantly reduced in *Nmnat1*^{+/-}/htau and increased in *Nmnat1* tg/htau mice in both hippocampus and cortex. However, NAD levels did not change by increasing or reducing *Nmnat1* in the cortex (C) and hippocampus (D). (n=4/group. One-way ANOVA followed by Bonferroni post-hoc test, * P < 0.05).

4.2.7 Levels of hyperphosphorylated tau in cortex and hippocampus of htau mice appear to be reduced by *Nmnat1* overexpression.

Finally, in order to test whether overexpression and downregulation of NMNAT1 may induce changes in the hyperphosphorylated tau, western blots were performed in cortex (Fig. 4.12 A) and hippocampus (Fig. 4.12 B) of htau mouse brains. As shown in Fig. 4.12, tau immunoreactivity, evident in htau and in *Nmnat1*^{+/+}/htau mice, tends to decrease in *Nmnat1* tg/htau mice. These results indicate that overexpressing *Nmnat1* in htau mice reduced the amount of hyperphosphorylated tau, whereas downregulating *Nmnat1* did not further increase the levels of hyperphosphorylated tau.

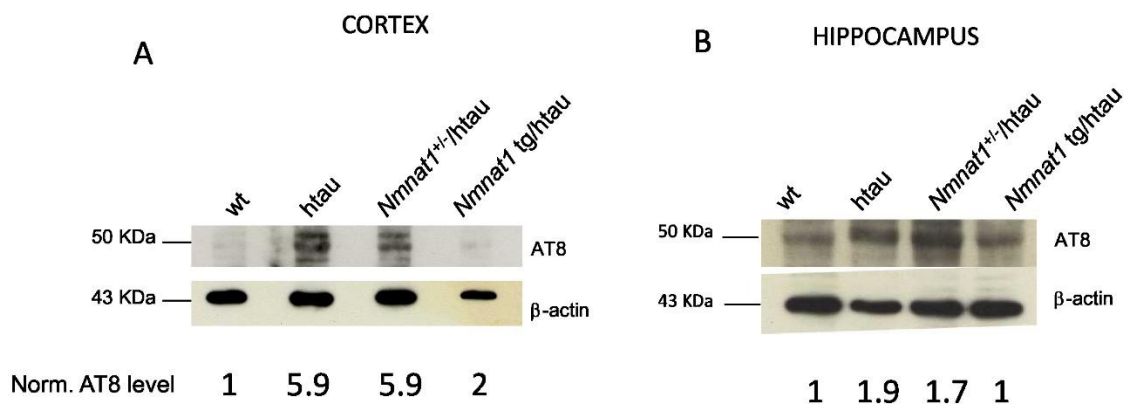


Fig. 4.12 Levels of hyperphosphorylated tau in cortex and hippocampus of htau mice appear reduced by *Nmnat1* overexpression.

Western blots of cortex (A) and hippocampus (B) homogenates probed with antibody AT8, indicating that tau immunoreactivity, evident in htau and in *Nmnat1*^{+/+}/htau mice, tends to decrease in *Nmnat1* tg/htau mice ($n=3$, one representative experiment shown).

4.3 Discussion

NMNAT1 plays an important role in neuron maintenance and its loss of function causes neurodegeneration [130]. In addition, NMNAT1 is neuroprotective in several animal models of neurodegenerative disorders [135]. My study extends these observations to a mouse model of tauopathy relevant to AD [214]. I found an early behavioral impairment

in htau mice, which overexpress wild type human tau and are reported to develop late cognitive deficits and neurodegeneration [295]. The behavioral deficit detected at an early age was remarkably ameliorated by NMNAT1 overexpression. However, cerebral morphometry and cognition appear to be unaltered at this stage of the pathology, indicating that early behavioral deficits precede obvious neurodegeneration and can be ameliorated specifically by *Nmnat1* overexpression.

The htau mouse provides a model to study tau pathology relevant to AD. Through the expression of non-mutant human tau it develops late onset neuropathology, which is reminiscent of that occurring in the early stages of AD [214, 298]. The earliest abnormality detected in the htau mice is a remarkable impairment in the food burrowing performance. This abnormality was already present at the first time point tested (two months). The food burrowing test exploits a natural rodent behavior which requires the integrity of the hippocampus and frontal cortex, brain areas particularly sensitive to AD-like pathology, and is affected following lesions of these brain areas [271]. The first evident symptom in AD is a deterioration in the ability to perform ‘daily-life activity’ that in mouse models correspond to burrowing and nest construction [272]. Although behavioral screening in AD patients are mainly focused on cognitive tests [272], it may be important to assess more specific behaviors which, as suggested by our results, could be modified at early stage. I found that increasing NMNAT1 levels in htau mice rescued the specific food burrowing deficit at three, four and six months of age. Reducing NMNAT1 levels did not further decrease the food burrowing performance of htau mice; this may be due to the fact that the deficit of the htau mouse was already pronounced. Interestingly, we found that htau mice overexpressing *Nmnat1* display a significant increase in locomotor activity. Differences in locomotor activity were also evident in *Nmnat1* tg mice at six months compared to all other mice at the same age. Overexpression of *Nmnat1* has been proposed to delay ageing and improve metabolism ([293] and Rossi, Conforti et al, unpublished observations), an observation that could explain why *Nmnat1* tg mice are more active. *Nmnat1* overexpression also results in a higher activity of htau mice suggesting a possible beneficial effect.

The absence of learning and memory defects suggests that the burrowing behavior may be under the control of specific hippocampal circuits that are affected at a very early stage

of the pathology. A progressive, age-dependent decline in memory and learning, functions that are critically regulated by the hippocampus [299-301], characterizes AD symptomatology [302]. Htau mice did not show remarkable cognitive impairments at four months, even though their inability to discriminate novel object at six months may suggest that at this time point a memory deficit could begin to appear. This result agrees with previous findings on the htau mouse model [295].

To compare brain metabolite levels between genotypes *in vivo*, I used MRS. This method has been widely used to study cellular mechanisms preceding complex pathological conditions [303] such as in models of amyloidosis and Pick disease [304, 305]. Changes in specific metabolites may indicate the onset or the course of specific neurodegenerative processes, therefore measuring brain metabolism might be a sensitive method to screen for early biomarkers of pathology. For example, NAA, a neuronal marker, is reduced in the medial temporal lobe in AD patients that show only mild cognitive impairments [306]. Tau, which plays a role in osmoregulation and neuroprotection in the central nervous system, is also reduced in a murine mouse model of amyloidosis [307]. Several lines of evidence suggest that other metabolites, such as myo-inositol, a glial marker, Glu, Gly, and choline, are changed in AD [308, 309]. In the present study, the metabolic contents of the hippocampus in six month old htau mice remained unchanged. Likewise, no gross structural changes were detected in the presence of human tau as determined by *in vivo* MRI and *post-mortem* tissue analysis. However, these sensitive methods were able to detect changes in other more aggressive models of tauopathies [310]. Moreover, studies in human ApoE4 carriers have reported early changes that can be observed by assessing connectivity with functional resting state MR imaging methods [311]. These observations suggest that the age of six months is an early time point of pathology in the htau mouse and underline that behavioural abnormalities in this model long precede neurodegenerative changes.

NMNAT enzyme activity in htau mice was similar to that of wild type mice, while it was increased when NMNAT1 was overexpressed and decreased when NMNAT1 was downregulated, in agreement to corresponding changes in activity in *Nmnat1* tg and *Nmnat1*^{+/-} mice [77, 127]. Despite the modulation of NMNAT enzyme activity which correlated to its levels of expression, steady-state NAD levels were not significantly different in all genotypes. We cannot rule out that the increased NMNAT activity could cause

localized changes in NAD availability and therefore affect the activity of downstream NAD dependent enzymes. In support of this, recent observations correlate nuclear NAD changes sensitive to the levels of NMNAT1 with SIRT1 activity and mitochondrial dysfunctions [293]. Moreover, loss of function mutations in *NMNAT1* that cause retinal degeneration in humans have been shown to affect NAD biosynthetic activity and could modify local nuclear NAD pools [131].

NMNAT has been reported to act via a chaperone-like activity in models of polyQ toxicity [133], in a *Drosophila* model of tauopathy, where its overexpression reduced hyperphosphorylated tau load [134, 135], and when lentivirally expressed in the hippocampus of a tauopathy mouse model [135]. Therefore, it remains possible that NMNAT1 overexpression could influence the amount of hyperphosphorylated tau also in our htau mice. Indeed, using the AT8 antibody, which detects tau^{pSer202} and tau^{pThr205}, characteristic of the AD state [312-314] and increased in htau mice [214], I saw that *Nmnat1* overexpression tends to reduce phospho-tau reactivity in htau cortex and hippocampus at 6 months, an observation which will need further investigations, as the number of samples available was limited, due to technical issues.

In summary, results of this part of my study show that htau mice show an early, specific behavioral deficit in food burrowing that can be suppressed when *Nmnat1* is overexpressed. The capability of NMNAT1 to notably ameliorate the food burrowing performance in htau mouse model at very early stage highlights the potential for NMNAT1 to be a target for early stage AD and other pathologies, once its mechanism of action will have been fully elucidated.

Chapter 5

*The role of NAD In PARP1-mediated cell death
relevant to cerebral ischemia*

5.1 Introduction

The present study aims to investigate the role of NMNAT1 in an *in vitro* model of PARP1-mediated cell death relevant to Cerebral Ischemia.

Several studies demonstrated the role that NAD biosynthesis has in cerebral ischemia. NAD confers neuroprotection in primary cultures of hippocampal and cortical neurons after OGD-induced cell death, a model that reproduces cell death occurring after an ischemic insult [266]. Furthermore, several studies showed that administration of nicotinamide, a vitamin precursor of NAD, can confer protection in a model of hypoxic-ischemic injury both in new born rats [267] and in adult rats [268]. In addition to this, it has been shown that heterozygous knock-out mice for NAMPT, the rate-limiting enzyme in NAD biosynthesis, showed an exacerbation of brain damage in an *in vivo* model of cerebral ischemia, photothrombosis-induced ischemia. Importantly, these mice have decreased NAD levels, suggesting an important role of NAD in this pathological condition [269]. A more recent study showed that *cyt-Nmnat1* transgenic mice, are protected from brain injury in a neonatal model of hypoxia-ischemia (H-I). In this mouse model, NMNAT1 is redistributed to the cytoplasm [136]. Importantly, NMNAT1 has been reported to interact with PARP1 at target gene promoters in MCF-7 cells, where it enhances PARP1-dependent PARylation. Therefore, PARP1 recruits NMNAT1 to promoters where it produces NAD to support PARP1 catalytic activity [52, 270].

These studies support a role of NAD metabolism in cerebral ischemia. However, the *in vivo* model described focus on NAMPT, which is the rate-limiting enzyme in the NAD biosynthetic pathway and its modulation of this enzyme could affect all NAD intracellular pools. Whether modulation of NMNAT1 levels cause corresponding changes in NAD is debated [46, 86], but it is likely that a modulation of this enzyme could modify NAD availability within the nucleus [46, 50]. Furthermore, studies performed overexpressing *cyt-NMNAT1* showed the protective effect of NMNAT1 in a model of H-I only when NMNAT1 is re-localized to the cytoplasm, whereas it would be important to investigate the contribution of NMNAT1 in cerebral ischemia, when it is located into the nucleus, which reproduces the physiological condition.

Although these studies support a crucial role of NMNAT1 in cerebral ischemia, evidences on the effect of NMNAT1 ubiquitously expressed or downregulated in the entire organism are lacking. For this reason, I decided to use *Nmnat1* tg and *Nmnat1*^{+/-} mice.

This chapter describes the experiments I performed to analyze the role of NMNAT1 in cerebral ischemia. I started with an *in vitro* model that enabled to study a specific cell-death mechanism occurring during cerebral ischemia, followed by a series of experiments to set up an *in vivo* model of cerebral ischemia. However, due to technical reasons, the *in vivo* part contains only preliminary results and it does not allow drawing specific conclusion. A more completed study will be necessary and the present results will give useful preliminary information to address the role that NMNAT1 plays in this pathology.

5.1.1 Aims and objectives of the chapter

The principal aim of the study is to clarify the role of NAD in PARP1-mediated cell death, which is one of the main mechanisms of cell death that occurs during an ischemic insult. The question we aim to address is whether the modulation of the enzyme responsible for the nuclear biosynthesis of NAD, NMNAT1, can influence the ischemic cell death. We hypothesise that reducing NMNAT1 levels will exacerbate the ischemic brain damage, whereas increasing these levels may confer protection by respectively decreasing or increasing NAD availability within the nucleus.

First, I used an *in vitro* model of PARP1-mediated cell death in NSC-34 cell line overexpressing Wld^S, which contains the full-length *Nmnat1* (referred to as Wld^S/NMNAT1). Cell death was induced by administration of MNNG, which causes genotoxic stress.

In addition to the *in vitro* experiments, and as a preliminary to study the role of NAD metabolism in an *in vivo* model of cerebral ischemia, I have contributed to the set up of an MCAO model in the mouse. Despite technical difficulties and time restriction precluded the conclusion of this study, the data I obtained will be useful for future studies and will enable to test whether overexpression of NMNAT1 or its downregulation may protect or exacerbate the effect of cerebral ischemia in an *in vivo* model.

5.2 Results

5.2.1 *Wld^s is expressed in Wld^s-transfected cells but not in wild type cells*

Stable NSC-34 clones expressing Wld^s/NMNAT1 had been previously generated (L. Janeckova and L. Conforti). However characterisation of Wld^s/NMNAT1 expression and how this expression influences NAD levels is still missing. In order to perform this characterisation, I first analysed Wld^s/NMNAT1 expression in the total cell homogenate and in the nuclear and cytoplasmic fractions. Fig. 5.1 shows the expression of Wld^s/NMNAT1 in all cellular fractions of Wld^s/NMNAT1 cells, with enrichment in the nuclear fraction. Despite a similar amount of protein loaded, the band in the nuclear fraction is more intense than that in the cytoplasmic fraction. Considering the unavoidable presence of nuclear contaminant in the cytoplasmic fraction, this suggests an enrichment of Wld^s/NMNAT1 in the nucleus. In contrast, wild type, untransfected cells showed no expression of the protein.

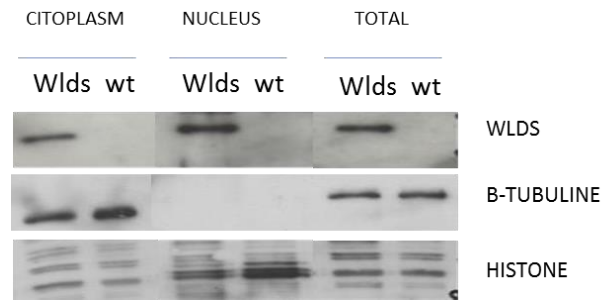


Fig. 5.1 Western blotting of Wld^S in NSC-34 cell line.

Samples were probed with Wld^S, β tubuline and histone antibodies, showing that Wld^S is expressed in all cellular fractions of Wld^S/NMNAT1 cells, with enrichment in the nuclear fraction. Wild type cells did not show Wld^S expression (Wld^S: 43 KDa; Tubuline: 50 KDa; Histone: 17 KDa).

5.2.2 NAD levels are decreased in Wld^S transfected cells compared to wild type cells

It is generally assumed that intracellular NAD is freely exchanged between the nucleus and the cytoplasm but a clear determination of the nucleotide subcellular levels is missing. First of all, in contrast to the general assumption, I found that NAD levels are not homogeneous in the various cellular fractions, but NAD level in the nucleus is lower than in the cytoplasm (Fig. 5.2). In addition and in contrast to what expected, I found that NAD levels in Wld^S/NMNAT1 cells were strongly decreased in the total, in the cytoplasmic and in the nuclear fraction, compared to the Wild type (Fig. 5.2). However, in the nuclear fraction, even if NAD levels tend to decrease, they did not reach a significant difference.

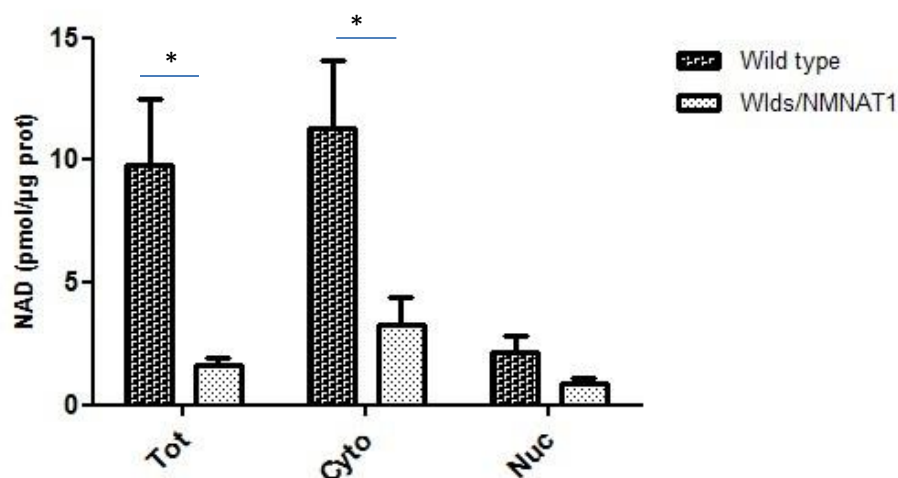


Fig. 5.2 NAD levels in Wld^S/NMNAT1 and wild type cells.

*Wld^S/NMNAT1 cells were decreased in the total, in the cytoplasmic and in the nuclear fraction, compared to the wild type (n=5, two-way ANOVA followed by Bonferroni post-hoc test, * $P < 0.05$).*

5.2.3 Cell viability in Wld^S/NMNAT1- cell clones after PARP1-mediated cell death induced by MNNG treatment.

Although my findings that in Wld^S/NMNAT1 cells NAD levels were decreased is surprising, I exploited this cell model to analyse the cell viability in Wild type and in Wld^S/NMNAT1 cells after MNNG treatment, which induces PARP-1 overactivation and NAD depletion with subsequent cell death. I reasoned that in cells containing Wld^S/NMNAT1 a further NAD depletion may lead to a higher cell death compared with the wild type. Indeed, the results of MTT assay showed that cell viability tend to be reduced in Wld^S/NMNAT1 cells after MNNG treatment in a dose-dependent manner (Fig.5.3). However, these differences did not reach statistical significance, perhaps due to the high variability of data collected with this assay.

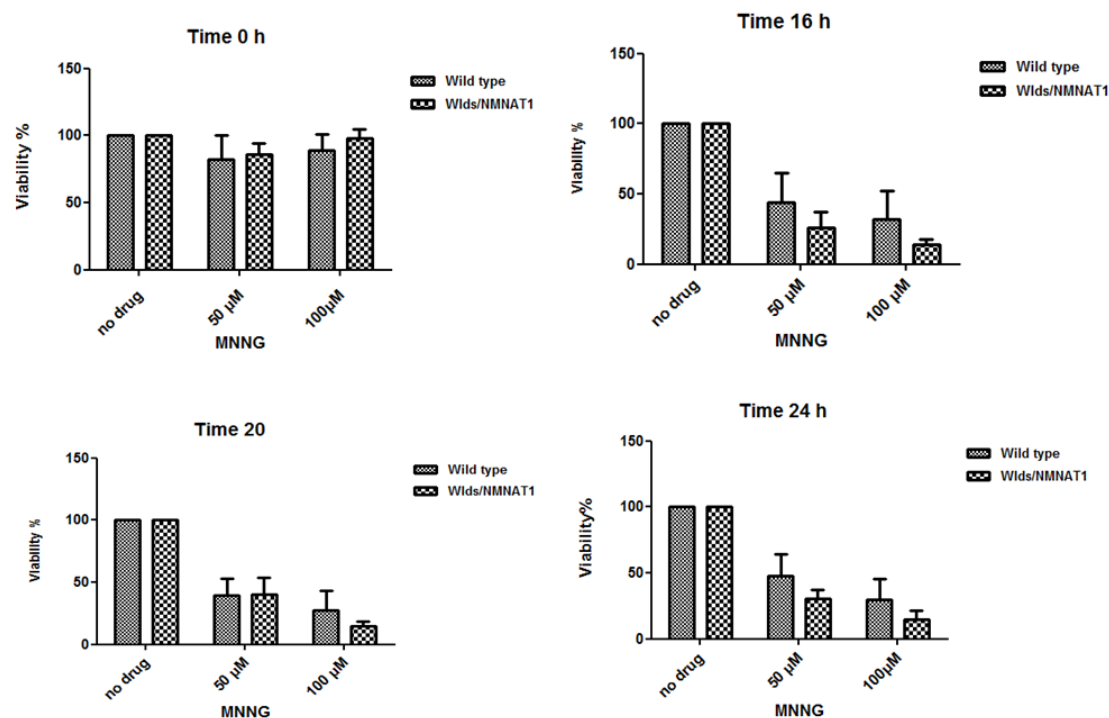


Fig. 5.3 MTT assay in wt and Wlds/NMNAT1 cells.

MTT assay showed reduced cell viability in Wlds/NMNAT1 cells compared to the Wild type cells after MNNG treatment in a dose-dependent manner ($n=5/\text{group}$).

5.3 Establishing an *in vivo* model of cerebral ischemia: MCAO mouse model

As a preliminary to the use of MCAO to determine the effect of NMNAT1 level modulation in cerebral ischemia, I performed an initial series of experiments on wt, *Nmnat1*^{+/-} and *Nmnat1* tg mice with the aim to verify the proper time of occlusion which allowed us to reproduce a consistent stroke model. All these experiments were done in collaboration with Dr. Rebecca Trueman, who performed all surgeries. We tested three different time of occlusion in all mice, 5, 10 and 15 minutes. During these tests, we encountered several issues mainly related to the anesthetic used and how the mice reacted on it. Table 5.1 summarizes the first set of trials we performed and the technical problems encountered while studying occlusion time lengths of 5 and 10 minutes.

Genotype	Occlusion length	Comments	Death under anaesthesia	Unexpected death	S. 1 due to welfare (following recovery)	S.1 during anaesthesia due to experimental reasons	S.1 at end of experiment 24 hrs post-stroke)	Stroke
(Long recovery >1hr to come round full)								
WT	5	ok					x	No stroke
WT	5	Long recovery					x	No stroke
WT	5	Long recovery					x	No stroke
WT	10	Long recovery	x					
WT	10	Long recovery					x	Large stroke
WT	10	Long recovery		x				
WT	10	Long recovery	x				x	
C57BL/6	10	Long recovery						No stroke
On advice from other people using the model, used NO and O2 for anaesthesia rather than O2 alone. Improved monitoring sheets.								
HETKO	5		x					
HETKO	5		x					
WT	5	ok					x	No stroke
HETKO	5	ok					x	No stroke
HETKO	5	ok					x	No stroke
HETKO	5	ok					x	No stroke
HETKO	10	Long recovery			x			
Reduced size of anaesthetic mask								
HETKO	5	ok					x	No stroke
HETKO	10		x*					
HETKO	10					x		
WT	10					x		
HETKO	10	ok					x	Small stroke
HETKO	10		x					
Spoke to others using model. New mouse specific mask and reduction of size of induction chamber								
HETKO	10	Long recovery					x	No stroke
NMNAT tg	5	good					x	No stroke
NMNAT tg	5	good					x	No stroke
NMNAT tg	5	Long recovery					x	No stroke
NMNAT tg	10	Long recovery					x	No stroke
NMNAT tg	10	Long recovery					x	No stroke
NMNAT tg	10	Long recovery					x	No stroke
*Suspected over heating due to rectal temperature probe moving								

Table 5.1 MCAO at 5 and 10 minutes occlusion.

At these time points, and before any change in the protocol, almost all mice presented a long recovery time from anaesthesia. This time was strongly reduced after the introduction of NO and the use of a smaller anaesthetic mask. As shown in table 5.1, *Nmnat1* tg mice needed a long recovery time at both time points. However, these mice seemed not to have any consequence and this difference may be due to high variability between transgenic mice and the other two genotypes used. This will need to be carefully considered for future experiments.

Most mice of all genotypes did not show signs of stroke neither at 5 nor 10 minutes (Fig. 5.4A). However, a *Nmnat1*^{+/-} and a wild type mouse presented a small (Fig. 5.4B) and a large (Fig. 5.4C) stroke respectively after 10 minutes occlusion. These results highlight the high variability of this surgical procedure, even within the same genotype.

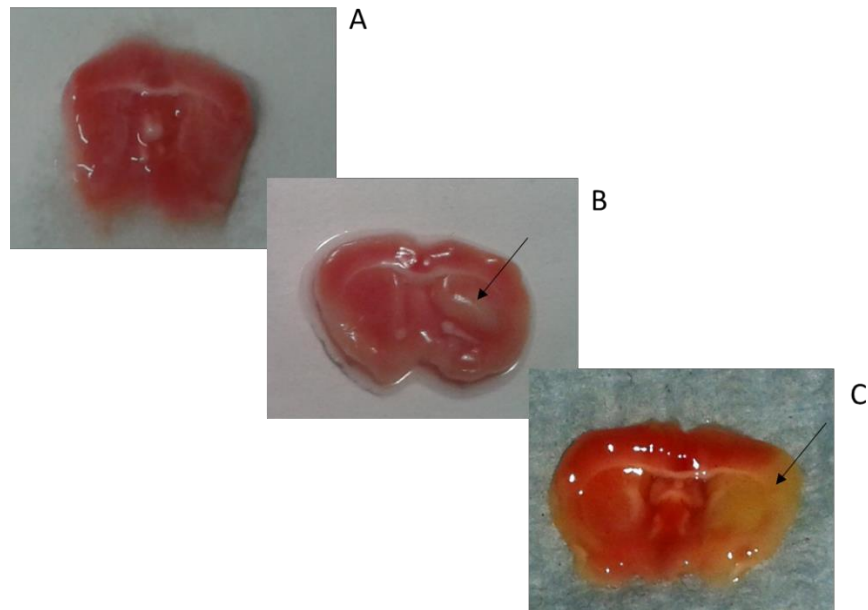


Fig. 5.4 MCAO in mice after 10 minutes-time occlusion.

Coronal mouse-brain sections stained with TTC (see methods), 24 hours after MCAO showing no stroke (A, wild type mouse 10 minutes-time occlusion), a small stroke (B, heterozygous KO mouse 10 minutes-time occlusion) and a massive stroke (C, wild type mouse 10 minutes-time occlusion).

We then tested the effect of a 15 minutes occlusion time on the development of stroke after MCAO. We did not detect any stroke in any mouse tested, independently of the genotype. Furthermore, all mice reacted well to the anaesthesia as the improvements in the protocol strongly reduced the recovery-time after surgery.

5.4 Discussion

In my results with NSC-34 cell line, I first confirmed that Wld^s is expressed in all cellular fractions in Wld^s/NMNAT1 cells but not in wild type cells. Furthermore, both wild type and Wld^s/NMNAT1 cells showed lower NAD levels in the nuclear fraction compared to the cytoplasmic fraction and in particular, NAD levels tended to be even lower in Wld^s/NMNAT1 in all fractions respect on the wild type cells. These reduced NAD levels in Wld^s/NMNAT1 may explain the higher susceptibility to cell death induced by MNNG.

It has already been established that NAD plays a crucial role in signaling pathways, which have an impact on cell survival. NAD is a substrate of PARP1, which is an important enzyme involved in processes of DNA break repair. Numerous evidences in literature show that a brain ischemic insult leads to PARP1 over-activation with subsequent decline of NAD levels in the brain and cell death [315]. Several mechanisms of cell death are activated as a consequence of an ischemic attack and the fact that PARP1 over-activation plays a major role is supported by the positive effect of PARP1 inhibitors which are now in clinical trials for the treatment of stroke [257].

A large body of literature highlighted the neuroprotective effect of NAD in the ischemic insult [315]. Previous evidence suggested that not only NAD itself can confer protection, but also the enzymes involved in the NAD synthesis, such as NAMPT [270]. In fact, heterozygous knock-out mice for *Nampt* showed an exacerbation of brain damage in an *in vivo* model of cerebral ischemia, photothrombosis-induced ischemia. Importantly, these mice have decreased NAD levels condition [269]. Furthermore, another study showed that overexpression of NMNAT1 redistributed to the cytoplasm (cyt-NMNAT1), confers protection from the brain injury in a neonatal model of hypoxia-ischemia (H-I). Mice overexpressing cyt-NMNAT1 also showed a significant increase in NAD levels compared to their controls, highlighting the crucial role of NAD in this pathological condition [136].

However there is no previous study which addresses the effect that the modulation of NAD at a nuclear level may have in PARP1-mediated cell death. To test this hypothesis, I first conducted an *in vitro* study, using NSC-34 cells stably transfected with Wld^s, containing full length NMNAT1, in order to have a model of PARP1-mediated cell death induced by MNNG. Surprisingly, I found that these cells have decreased NAD levels in physiological conditions, compared to those in the wild type cells. Since the reduced levels of NAD in Wld^s/NMNAT1 were not expected, it is possible that decreased NAD levels may be due to a peculiarity of the specific clone selected for our experiment. Analysis of different clones will shed more light on this aspect. Even if the reason of this decrease remains unexplained, I used these cells to test the hypothesis that these decreased NAD levels may exacerbate the PARP1-mediated cell death. Wld^s/NMNAT1 cells showed a lower percentage of viability after MNNG treatment, in comparison with wild type cells, suggesting that cells with higher levels of NAD may be protected against this

pathway of cell death. Even though the decreased cell viability in Wld^s/NMNAT1 cells did not seem significant from a statistical point of view, consideration on the variability of this method and the low n number make us believe these data could achieve a statistical significance if more experiments will be performed.

In parallel to the *in vitro* study, I have contributed to the set up of an MCAO model in the mouse to study the role of NMNAT1 in cerebral ischemia. The aim of this preliminary study was to establish the ideal time of occlusion to induce a stroke. The most common time of occlusion in a MCAO model usually starts from 30 minutes, where it produces striatal insult, or 60 minutes, where the insult is also evident in the cortex and in subcortical areas, but it can reach up to 200 minutes [282]. However, the desired time of occlusion needs to be tested before. In our preliminary experiments, we found that mice from all genotypes do not present signs of stroke at the time points tested, suggesting that a longer time of occlusion will be needed to induce a stroke. However, as few mice evidenced a stroke at 10 minutes of occlusion, a possible variability along mice from the same genotype has to be considered. Furthermore, unexpected death and death under anaesthesia occurred in our experiments. All this suggests that a setup of MCAO protocol is necessary to preserve the animals used and to obtain accurate results. Indeed, to reduce the variability observed in our preliminary data a large number of animals will be needed.

In summary, my results confirm an important role that NMNAT1 has in a PARP1-mediated cell death, likely by modulation of local nuclear NAD pools. Despite technical difficulties and time restriction precluded the conclusion of this study, the data I obtained will be useful for future studies and will enable to test whether overexpression of NMNAT1 or its downregulation may protect or exacerbate the effect of cerebral ischemia in an *in vivo* model.

Chapter 6

General discussion and future studies

In my thesis I have investigated the contribution that NAD biosynthetic enzyme NMNAT1 has on changes occurring during ageing and how a modulation of this enzyme could influence these mechanisms. Moreover, I have also studied the impact of this modulation in age-related pathologies, with a particular focus on Tauopathies relevant to AD and on a model of PARP1-mediated cell death relevant to Cerebral Ischemia. To address these aims, I have used a variety of behavioral, biochemistry, imaging and cell biology analysis. I have demonstrated that specific metabolic changes occurring over ageing might be restored when overexpressing *Nmnat1*, although these finding will need further investigation. In addition to this, I have also demonstrated a specific role of NMNAT1 in early behavioral deficits characteristic of AD.

The data illustrated in chapter 3 suggest that metabolic regulation, normally impaired during ageing, as body weight and locomotor activity, could be ameliorated when *Nmnat1* is overexpressed. In contrast, reducing the expression of *Nmnat1* does not influence body weight and locomotor activity in young mice, but it has a strong impact when *Nmnat1* is downregulated in old mice, as differences are only evident at later stages. Those changes might be related to differences observed in the enzyme activity. Indeed, overexpression of *Nmnat1* significantly increases enzyme activity, whereas downregulating NMNAT1 reduces the enzyme activity as already reported [77, 127]. Importantly, a more detailed metabolic analysis will be further required to test first, how the metabolism is influenced in young mice with increased and/or reduced levels of NMNAT1 and secondly, to check whether differences observed at 15 months of age will be reproduced at an older age.

The contribution of the NAD-dependent deacetylase SIRT1 in ageing mechanisms might be of interest, consistently with the protective effect that its overexpression has on ageing and metabolism [43]. Modulating NMNAT1 does not appear to influence gene expression and protein levels of SIRT1. However, NMNAT1 might influences expression of genes modulated by SIRT1, as NMNAT1 regulates not only cellular NAD production, but also SIRT1 activity at the promoter of common target genes. These evidences highlight the central role of NAD production in SIRT1-dependent transcriptional regulation [207]. For this reason, a close analysis of SIRT1-target genes expression could clarify the contribution of NMNAT1. Importantly, NMNAT1 is crucially involved in the decline of

mitochondrial function observed during ageing. A decline in nuclear NAD levels causes a disruption of nuclear- mitochondrial communication with a mechanism involving SIRT1 [46]. However, as modulation of *Nmnat1* does not alter expression of *Sirt1* gene and its protein levels, it is conceivable that the increased NMNAT enzyme activity may influence the activity of SIRT1.

In this regard, further experiment will shed more light on this mechanism and measuring SIRT1 enzyme activity when *Nmnat1* is either overexpressed or reduced will be the first step.

In addition to this, I have also investigated the contribution of NMNAT1 in a model of tauopathy relevant to AD (htau mice). NMNAT1 plays an important role in ageing mechanisms and it is reasonable that its contribution could be even more significant in age-related pathologies. I have observed a deficit in food burrowing performance at an early age of these mice that was remarkably ameliorated by *Nmnat1* overexpression. In contrast, reducing NMNAT1 does not further exacerbate the already low performance of these mice. Food burrowing analysis allowed to reproduce a behavior representing a daily life activity, which is the first activity impaired in early stages of AD in humans. However, volume of hippocampus and cognition appear to be unaltered at this stage of the pathology, indicating that early behavioral deficits precede obvious neurodegeneration and can be ameliorated by NMNAT1 overexpression. Interestingly, I have found that the amount of hyperphosphorylated tau protein, which is one of the main hallmarks in AD, was strongly reduced in htau mice, when *Nmnat1* was overexpressed. In contrast, NAD levels and NMNAT enzyme activity were not altered in htau mice. These evidences rise the conclusion that NMNAT1 acts ameliorating specific deficits at the early stage of the pathology and its mechanism appeared to be related to its ability to reduce the amount of tau loads, rather than through its enzyme activity. In fact, it has already been described in *Drosophila* and mouse models of tauopathy the ability of NMNAT of reducing the amount of hyperphosphorylated tau, acting as a chaperon-like protein [134, 135].

The capability of NMNAT1 to notably ameliorate the food burrowing performance in htau mouse model at very early stage highlights the potential for NMNAT1 to be a target for early stage AD and other pathologies, once its mechanism of action will have

been fully elucidated. However, it would be interesting to analyze the NMNAT1 contribution at later stages of the pathology, to test whether increasing NMNAT1 levels will have an impact on behavioural impairments occurring late in the disease, confirming the role that NMNAT1 has on ageing mechanisms.

I also investigated the role that NMNAT1 has in a mechanism of the NAD-dependent PARP1-mediated cell death characteristic of cerebral ischemia, an age-associated brain disease. I conducted *in vitro* experiments in a cell line overexpressing *Nmnat1* in the form of the chimeric fusion protein WLD^S [114] and tested whether its overexpression might have a protective role in cell death induced by genotoxic stress. To address this aim, I have used stable NSC-34 clones expressing Wld^S/NMNAT1 and tested for cell availability after genotoxic stress induced by *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG). Two interesting observations emerge from these *in vitro* experiments. First, both wild type and Wld^S/NMNAT1 cells showed lower NAD levels in the nuclear fraction compared to the cytoplasmic fraction. This is interesting in view of the assumption that NAD freely equilibrates between nucleus and cytoplasm [50] but it is consistent with other observations in primary neurons (Conforti L unpublished). Secondly, and paradoxically, NAD levels are lower in Wld^S/NMNAT1 in all cellular fractions compared to the wild type cells. Even though the reason of this paradoxical decrease is not clear, and it may be linked to the genomic integration locus of Wld^S/NMNAT1 in this stable cell clone, these cells could be used as a tool to understand the role of NAD levels in PARP1 activation induced cell death. I found that these cells have a higher susceptibility to cell death induced by MNNG, which may be explained, by the reduced NAD levels in this Wld^S/NMNAT1 cell clone. Although these preliminary data support that NAD levels correlate to the extent of cell death caused by PARP1 activation, specific conclusions will need further analysis. Investigation of different clones will shed more light on this aspect. Furthermore, analysis of cell viability and NAD levels will need to be further confirmed with increasing the sample size considered.

In parallel to the *in vitro* study, I have performed in collaboration with Dr. Rebecca Truman, a series of experiments aimed to set up an *in vivo* model of Stroke. I tested three different time points in mice overexpressing *Nmnat1* and *Nmnat1* heterozygous KO mice, together with their controls. During these tests, we encountered several issues mainly related to the anesthetic used and how the mice reacted on it. Furthermore, mice

presented a high variability in developing stroke. For this reason, this study contains only preliminary results and it does not allow drawing specific conclusion. However, as a set-up of an *in vivo* model is necessary, these observations will be useful to develop a model of stroke and investigate whether overexpressing or reducing *Nmnat1* in the whole organism will protect or exacerbate, respectively from an ischemic insult.

In conclusion, this work contributes to the understanding of the role that NMNAT1 plays on ageing and age-related diseases. Even though the mechanism of NMNAT1 action still remains to be fully elucidated before defining precisely the full potential of NMNAT1 as a possible therapeutic target, my work has identified a crucial involvement of NMNAT1 in these processes and poses the ground for future research.

Appendix

Original manuscript on CD enclosed

NAD-biosynthetic enzyme NMNAT1 reduces early behavioural abnormalities in the htau mouse model of tauopathy

Francesca Rossi, Philippine C. Geiszler, Weina Meng, Malcolm Prior, Anna Herd- Smith, Andrea Loreto, Maria Yanez Lopez, Dorothee P. Auer, Henryk Faas, Marie- Christine Pardon and Laura Conforti.

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