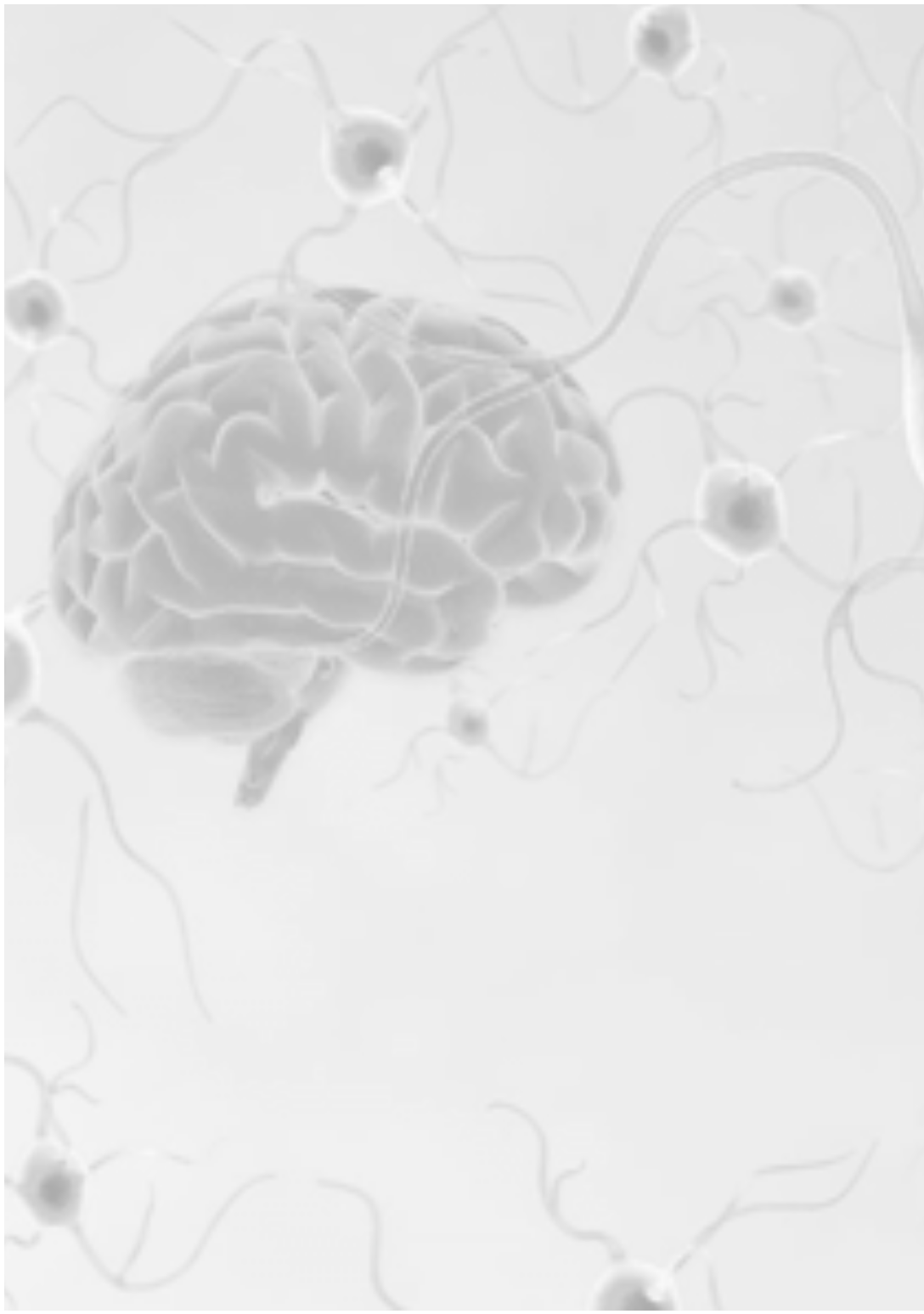

Universidade de Coimbra

Chromatin remodeling in Alzheimer's disease pathogenesis

Sueli C.F. Marques

Thesis for doctoral degree (Ph.D.) 2007-2011

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Chromatin remodeling in Alzheimer's disease pathogenesis /Remodelação da cromatina na patogénese da doença de Alzheimer

Sueli C.F. Marques

Thesis for doctoral degree (Ph.D.) 2007-2011

Dissertação apresentada à Faculdade de Ciências e Tecnologia, Universidade de Coimbra para prestação de provas de Doutoramento em Biologia, na especialidade de Biologia Molecular.

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COMPLICAÇÃO

As ondas indo, as ondas vindo — as ondas indo e vindo sem parar um momento.

As horas atrás das horas, por mais iguais sempre outras.

E ter de subir a encosta para a poder descer.

E ter de vencer o vento.

E ter de lutar.

Um obstáculo para cada novo passo depois de cada passo.

As complicações, os atritos para as coisas mais simples.

E o fim sempre longe, mais longe, eternamente longe.

Ah mas antes isso!

Ainda bem que o mar não cessa de ir e vir constantemente.

Ainda bem que tudo é infinitamente difícil.

Ainda bem que temos de escalar montanhas e que elas vão sendo cada vez mais altas. Ainda bem que o vento nos oferece resistência

e o fim é infinito.

Ainda bem.

Antes isso.

50 000 vezes isso à igualdade fútil da planície.

Mário Dionísio, in "Poemas" 1941, Coimbra

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Summary

Alzheimer's disease (AD) is the most common form of dementia in the elderly and is clinically characterized by a progressive loss of cognitive abilities. At present, this neurodegenerative disorder affects millions of people worldwide and the number of individuals with AD is expected to triple by mid-century with immense economic and personal tolls. Although considerable progress has been made in recent years towards better understanding the pathogenesis of AD, this knowledge has not yet been successfully translated into new and effective disease-modifying drugs. This can result from the fact that AD is a complex disorder in which both genetic and environmental factors play a significant role. Several environmental risk factors have been associated with increased risk to develop the sporadic form of the disease that accounts for the majority of AD cases, (~90%) but the underlying mechanisms are still unclear.

Recent epigenetic studies brought some light to understand the age-related loss of plasticity, monozygotic twin divergences and the molecular mechanisms implicated in other complex diseases, such as cancer. The role of epigenetics in neurodegenerative disorders such as AD has only recently started to be investigated but it could lead to novel and key findings to the development of truly effective therapeutic strategies.

Therefore, the aim of the present thesis was to investigate chromatin remodeling, one of the main epigenetic processes which involves nucleosome repositioning and histone

modifications, as a mechanism underlying changes in transcriptional regulation of AD-related genes, focusing on *BACE1*, *NCSTN*, *SIRT1* and *ADORA2A*. To achieve this purpose, we took advantage of two models of AD, the triple transgenic mice model of AD (3xTg-AD) and peripheral blood mononuclear cells (PBMCs) obtained from AD patients and from subjects with mild cognitive impairment (MCI), which represents a prodromal stage of the disease, as well as age-matched wild type mice and PBMCs from non-demented control individuals.

We observed an increase in *Bace1* messenger RNA (mRNA) in 15-months-old 3xTg-AD mice in both cortex and hippocampus, which was accompanied with an increase in histone 3 (H3) acetylation of the promoter. In PBMCs, the gene was also upregulated with a significant increase in DNA accessibility of the promoter. We also found a uniform decrease in DNA accessibility of the gene in PBMCs from MCI subjects. This occurred in the presence of a slight increase in *BACE1* mRNA at this stage, which could represent a compensatory mechanism.

Nucleosome displacement may also underlie *Ncstn* mRNA downregulation observed in older 3xTg-AD mice. This effect could represent a protective mechanism against an exacerbated γ-secretase activity that would result from *PSEN1* mutation in these animals. In human samples, no significant alterations in *NCSTN* transcription were observed between patients and healthy subjects.

The mRNA levels of *Sirt1* decreased in the brain of older 3xTg-AD mice despite histone tail acetylation in the promoter tended to increase. Other mechanisms may thus be involved in down-regulation of *Sirt1* mRNA, concordant with what was reported in AD brains. In PBMCs, the mRNA levels and DNA accessibility of the gene were not altered between patients and controls.

ADORA2A gene was not altered in both models of AD, suggesting that A2A receptors overexpression observed in the pathology may be caused by other regulatory mechanisms.

With this study it became clear that chromatin remodeling has an important role in mRNA alterations of specific loci in AD, providing a starting point to more detailed search for chromatin alterations in these and other AD-related genes. The fact that such alterations are present in peripheral tissues also opens new promising prospects for the use of these epigenetic modifications as biomarkers for AD.

Resumo

A doença de Alzheimer (DA) é a causa mais comum de demência nos idosos e é clinicamente caracterizada por uma perda progressiva de capacidades cognitivas. Actualmente, esta doença neurodegenerativa afeta milhões de pessoas em todo o mundo e prevê-se que o número de indivíduos com a DA triplique até 2050, apresentando assim um grande impacto socioeconómico. Apesar de nestes últimos anos se terem feito avanços consideráveis na investigação da patogénese da DA, o conhecimento obtido ainda não se traduziu em terapias novas que previnam de forma efetiva a progressão da doença. Tal resulta do facto da DA ser uma doença complexa em que fatores genéticos e ambientais desempenham um papel essencial. Vários fatores ambientais têm sido associados a maior risco de desenvolver a forma esporádica da doença que compreende ~90% dos casos. No entanto, os mecanismos subjacentes ao papel dos factores ambientais na DA continuam por esclarecer.

Estudos recentes epigenéticos permitiram desvendar parte dos mecanismos envolvidos na perda de plasticidade relacionada com o envelhecimento, nas divergências fisiológicas e patológicas entre gémeos monozigóticos e naqueles implicados noutras doenças complexas, como o cancro. O papel das alterações epigenéticas em doenças neurodegenerativas como a DA só começou a ser investigado recentemente mas poderá conduzir a novas descobertas-chave no desenvolvimento de estratégias terapêuticas verdadeiramente eficazes.

O objetivo desta tese foi assim investigar a remodelação da cromatina, um dos principais processos epigenéticos que envolve reposicionamento dos nucleossomas e modificações das histonas, como um mecanismo subjacente à desregulação da transcrição de genes relacionados com a DA, focando-nos no *BACE1*, *NCSTN*, *SIRT1* e *ADORA2A*. Para tal utilizámos dois modelos da DA, o modelo animal triplo transgénico (3xTg-AD) e células mononucleares do sangue periférico (PBMCs) de pacientes com DA e Defeito Cognitivo Ligeiro (DCL), o qual representa uma fase pré-clínica da DA, assim como ratinhos da estirpe selvagem 'wild-type' com a mesma idade e indivíduos controlo sem demência. Observámos um aumento nos níveis de RNA mensageiro (mRNA) da *Bace1* nos ratinhos 3xTg-AD com 15 meses, quer no córtex quer no hipocampo, acompanhado de um aumento na acetilação da histona 3 (H3) na região do promotor. Nas PBMCs, o gene também se encontrava mais expresso e a acessibilidade do ADN estava significativamente aumentada nos pacientes com DA. Também observámos uma diminuição uniforme na acessibilidade do gene em pacientes com DCL apesar do ligeiro aumento no seu mRNA, o que poderá representar um mecanismo compensatório.

O reposicionamento dos nucleossomas também poderá estar associado ao decréscimo no mRNA da *Ncstn* nos ratinhos 3xTg-AD com 15 meses de idade. Este efeito poderá indicar um mecanismo de compensação contra uma atividade exacerbada da γ-secretase resultante da mutação na *PSEN1* nestes animais. Nas amostras humanas, não observámos

alterações na transcrição da *NCSTN* entre pacientes e controlos.

Os níveis de mRNA da *Sirt1* encontram-se diminuídos no cérebro dos transgénicos aos 15 meses apesar da tendência para um aumento da acetilação da H3. Outros mecanismos deverão por isso estar envolvidos no decréscimo da expressão da Sirt1 nestes animais. Nos PBMCs, os níveis de mRNA do gene e a sua acessibilidade não diferiram entre pacientes e controlos.

O gene *ADORA2A* não se encontra alterado em nenhum dos modelos de DA sugerindo que a sobre-expressão dos recetores A2A para a adenosina durante a patologia poderão ser causados por outros mecanismos regulatórios.

O nosso estudo evidencia que a remodelação da cromatina tem um papel importante nas alterações do mRNA na DA, contribuindo assim com os alicerces para uma investigação mais aprofundada das modificações na cromatina nestes e noutros genes associados à DA. Adicionalmente, o facto de tais alterações estarem presentes em tecidos periféricos abre novos caminhos para o uso destas modificações epigenéticas como biomarcadores na DA.

Abbreviations

3xTg-AD - Triple transgenic mice model of AD

α2M - α2-macroglobulin

aMCI - Amnesic Mild Cognitive Impairment

ADORA2A – Adenosine 2A receptor gene (*Homo sapiens)*

Adora2a – Adenosine 2A receptor gene (*Mus musculus*)

ACE - Angiotensin-converting enzyme

AD – Alzheimer's disease

ADAMS -The Aging, Demographics, and Memory Study

Aβ - Amyloid-β

APOE - Apolipoprotein E

APP - Amyloid-β protein precursor

APP CTFβ - APP β-C-terminal fragment

BACE1 – Beta-site APP-cleaving enzyme 1 gene (*Homo sapiens*)

Bace1 – Beta-site APP-cleaving enzyme 1 gene (*Mus musculus*)

CA1 - *Cornu Ammonis* 1

CA3 - *Cornu Ammonis* 3

CBS - Cystathionine-Beta-Synthase

CBP - CREB-binding protein or CREBBP

CDGE - Common disease genetic and epigenetic

CDR - Clinical Dementia Rating Scale

cHD1 - Chromodomain-helicase-DNA-binding 1

CHIP – Chromatin immunoprecipitation

CREB - cAMP response element-binding

CT - Computed tomography

CSF - Cerebrospinal fluid

DNMT – DNA methyltransferase

DSM – Diagnostic and Statistical Manual of Mental Disorders

ER – Endoplasmic reticulum

FAD – Familial Alzheimer's disease

GSK3beta – Glycogen synthase kinase 3beta

HAT – Histone acetyltransferases

HCY – Homocysteine

HDAC – Histone deacetylases

HDACi – Histone deacetylases inhibitors

hTERT – Human Telomerase Reverse Transcriptase

IDE – Insulin degrading enzyme

IA – Inhibitory avoidance

LOAD – Late-onset Alzheimer's disease

LRP – Low-density lipoprotein receptor-related protein

MBD2 – Methyl-CpG binding domain protein 2

MCI – Mild cognitive impairment

miRNAs– microRNAs

MMSE – Mini Mental State Examination

MNase – Micrococcal nuclease

MRI – Magnetic resonance imaging

mRNA – Messenger RNA

MTHFR – Methylenetetrahydrofolate reductase

MWM – Morris water maze

NCSTN – Nicastrin gene (*Homo sapiens*)

Ncstn – Nicastrin gene (*Mus musculus*)

NMR – Nuclear magnetic resonance

PBA – Phenylbutyrate

PBMC – Peripheral blood mononuclear cells

PET – Positron emission tomography

PHF-1 – PHD finger protein 1

PPMT – PP2A methyltransferase

PSEN1 – Presenilin 1 gene (*Homo sapiens*)

PSEN2 – presenilin 2 gene (*Homo sapiens*)

RSC – Chromatin structure remodeling

SAH – S-adenosylhomocysteine

SAHA – Suberoylanilide hydroxamic acid

SAM – S-adenosylmethionine

SBA – Sodium butyrate

SWI/SNF – Switch/Sucrose Non Fermentable

SIRT1 – Sirtuin 1 gene (*Homo Sapiens*)

Sirt1 – sirtuin 1 gene (*Mus musculus*)

Tg – Transgenic

TSA – Trichostatin A

TSS – Transcription start site

VPA – Valproic acid

WT – Wild type

1. INTRODUCTION

1.1. Alzheimer's Disease

1.1.1. Epidemiology and clinical features

Alzheimer's disease (AD) is the most common form of dementia (60 to 80 % of cases) in the elderly and the fifth leading cause of death for those aged >65. In the absence of novel therapeutical interventions that efficiently delay its onset, the number of AD victims is expected to reach 11 to 16 million by the year 2050 since the number of cases doubles every 5 years after 65 years old. AD prevalence, that is a number of existing cases of a disease in a population at a given time and AD incidence, that is a number of new cases of a disease in a given time period, are depicted in Figure 1.1 (Qiu, C. et al. 2009).

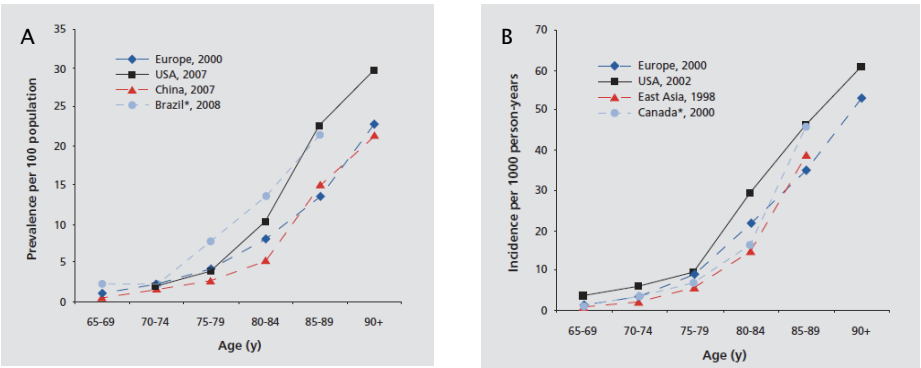


Figure 1.1. Age-specific prevalence (per 100 population) (A) and incidence (per 1000 person-years) (B) of AD across continents and countries. * all types of dementia. Adapted from (Qiu, C. et al. 2009).

Concerning gender, the prevalence of AD and other dementias is higher in women than in men. Based on estimates from *The Aging, Demographics, and Memory Study* (ADAMS), 16% of

women above 71 years old have AD or other dementia compared with 11% of men (Alzheimer's, A. et al. 2011).

The major clinical hallmarks of AD are progressive impairment in memory, judgment, decision-making, orientation and language. These characteristics are indistinguishable between the rare familial early-onset cases (before 60 years old) and the most common sporadic late-onset form of the disease (after 60 years old). Diagnosing AD with 100% certainty requires a detailed *post-mortem* microscopic examination of the brain to detect the neuropathological hallmarks of the disease. However, AD can be diagnosed nowadays with more than 95% accuracy in living patients, with the concomitant exclusion of other causes of dementia, by using a combination of tools including a careful history from patients and their families, assessing cognitive function by neuropsychological tests, brain imaging and tests of cerebrospinal fluid (CSF) and blood to identify changes that signal AD or other forms of dementia. Certain routine laboratory tests are recommended to rule out other conditions that can cause cognitive dysfunction, such as vitamin B12 deficiency or hypothyroidism. Routine brain imaging using computed tomography (CT) or magnetic resonance imaging (MRI) is also recommended for patients with suspected dementia, but neither of these can be used for definitive diagnosis. Rather, their primary role is to investigate or exclude other potential causes of cognitive dysfunction such as brain tumors. Patients with AD typically show brain atrophy on magnetic resonance images due to shrinkage of regions involved in learning and memory (hippocampus and cerebral

cortex). Furthermore, decreased glucose metabolism and increased uptake of radioligands that detect abnormal protein deposits (amyloid) on positron emission tomography (PET) scans can be detected in AD brains. CSF abnormalities include low levels of amyloid-β (Aβ) peptides and increased levels of the tau protein (Mucke, L. 2009). Despite promising results from recent research such biomolecular tests require significant additional research before they became part of the usual diagnosis procedures, being now accepted as complementary tools.

1.1.2. Pathological hallmarks

The histopathological features of the disease include senile plaques, composed by insoluble Aβ peptide fibrils that accumulate extracellularly, intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein and selective neuronal loss (Figure 1.2). These plaques and tangles are found predominantly in susceptible brain regions, the hippocampus and cerebral cortex. Initially, the neuropathological alterations are detected in the frontal and temporal lobes and in more advanced cases, the pathology extends to cortical regions in other brain areas, including the parietal and occipital lobes (Minati, L. et al. 2009).

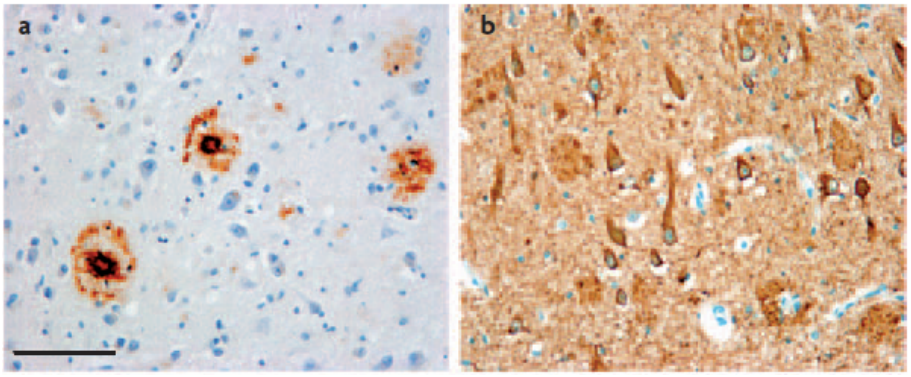


Figure 1.2- Pathological hallmarks of AD. a) Insoluble Aβ-containing plaques. b) Neurofibrillary tangles containing hyperphosphorylated tau. The scale bar represents 100 μm. From (Haass, C. and Selkoe, D.J. 2007).

The cleavage of amyloid-β precursor protein (APP) by β- and γ-secretase (amyloidogenic processing pathway) can produce several Aβ isoforms, of which the 40 and 42 amino-acid forms are the most common. Once released in a monomeric form, Aβ undergoes complex conformational changes, transitioning from small soluble fragments and oligomers into large fibrils, which in turn form plaques. In recent years, oligomeric species of Aβ have entered the main stage, receiving much of the attention due to their increased synaptotoxicity and neurotoxicity when compared to other forms of the peptide, as revealed by studies in animal and cellular models (Resende, R. et al. 2008) (Figure 1.3).

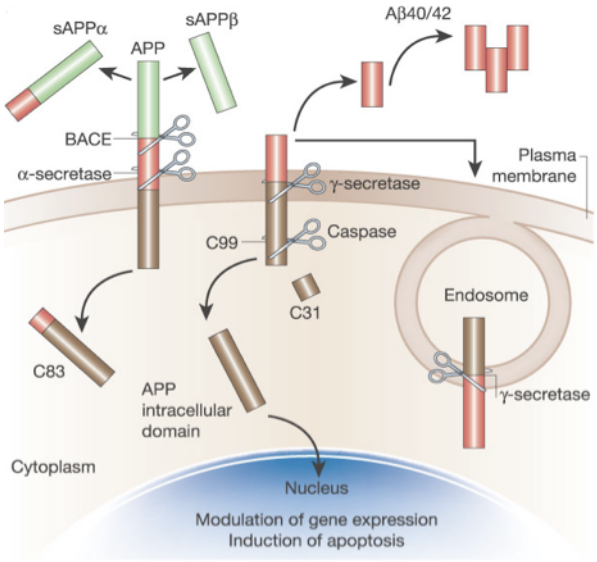


Figure 1.3- APP processing. APP can be cleaved in two independent pathways: 1) The non-amyloidogenic pathway is mediated by α-secretase, which cleaves APP in the extracellular portion of the protein to form the sAPPα fragment and then an intramembranar cleavage of the carboxy terminal (α-CTF) by γ-secretase produces the C83 fragment; 2) The amyloidogenic pathway comprises first the activity of β-secretase, which cleaves APP extracellularly to produce sAPPβ, and then γ-secretase that gives rise to Aβ and APP Intracellular domain (AICD) by cleaving the intramembranar portion of APP. AICD can go the nucleus where it modulates gene expression and induces apoptosis. Secreted Aβ can form oligomers, which are the most toxic forms, and later aggregates in senile plaques that ultimately contribute to cell death. From (Gandy, S. 2005).

Using *post-mortem* brain tissue, a significant correlation was found between the levels of soluble Aβ oligomers and cognitive decline (Tomic, J.L. et al. 2009). These small intermediates in the aggregation process can lead to synaptic dysfunction, whereas large, insoluble deposits might function as reservoirs of the bioactive oligomers (Haass, C. and Selkoe, D.J. 2007). Several evidences suggest that the

increased accumulation of Aβ in AD brains arise from enhanced production in familial cases. In contrast, what determines Aβ accumulation in sporadic AD is still under discussion, although some evidences point out to decreased degradation or clearance mechanisms. Research advances from pathological, neurochemical and genetic studies give increasing support to the "amyloid cascade hypothesis", which states that an imbalance between the production and clearance or degradation of Aβ in the brain is the initiating event in AD, ultimately leading to synaptic and neuronal dysfunction and neurotransmission deficits with subsequent cognitive disturbances (Hardy, J. and Selkoe, D.J. 2002) (Figure 1.4).

Tau, the principal component of neurofibrillary tangles, is believed to be essential for the pathological process leading to neurodegeneration in AD (Hanger, D.P. et al. 2009). The abnormal phosphorylation of tau protein, a normal axonal protein that binds to microtubules, significantly compromises their assembly and stability and has a negative impact on cell survival. Recently, tau has been implicated in Aβ toxicity, since the reduction or absence of tau was associated with neuroprotection against Aβ toxicity (Rapoport, M. et al. 2002; Roberson, E.D. et al. 2007). In addition, Aβ was demonstrated to induce tau hyperphosphorylation at epitopes characteristically phosphorylated in AD brain. Soluble Aβ oligomers were shown to stimulate tau phosphorylation in mature cultures of hippocampal neurons and in neuroblastoma cells. Tau hyperphosphorylation was also induced by a soluble aqueous extract from AD brains

containing Aβ oligomers, but not by an extract from non-AD brains (De Felice, F.G. et al. 2008).

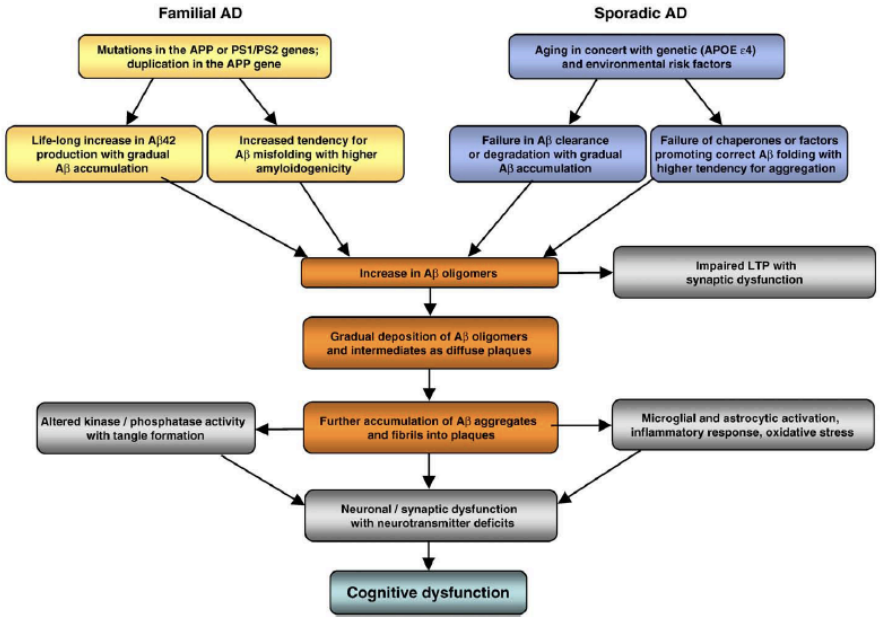


Figure 1.4- Amyloid cascade hypothesis. Accordingly to this theory, Aβ accumulation is considered the central event in AD pathology. In familial AD, mutations in the APP and/or PS genes can lead to increased production and accumulation of Aβ1-42 and also to enhanced misfolding, which will lead to its oligomerization and aggregation. These processes will be responsible for synaptic and neuronal dysfunction with neurotransmitter deficits, inflammatory processes in response to glia activation, oxidative stress, tau phosphorylation due to an unbalance between kinases and phosphatases activity and ultimately cognitive dysfunction. In sporadic AD, aging and other genetic and/or environmental risk factors may lead to a failure in clearance or degradation processes and also to impaired folding of Aβ leading to its accumulation and subsequent oligomerization and aggregation that will promote the same pathological mechanisms described for FAD. From (Hampel, H. et al. 2010)

1.1.3. Genetics

Early-onset familial AD (FAD) accounts for only 5-10% of all AD cases. First-degree relatives of FAD patients have a 50% lifetime risk of developing the disorder while for the general population, the risk is about 2% by the age of 65. Autosomal-dominant forms of FAD result from mutations in one of three genes: the gene encoding for APP, located on chromosome 21q21, the gene encoding for presenilin 1 (PSEN1), located on chromosome 14q24.3, and that encoding for presenilin 2 (PSEN2), on chromosome 1q31-q42 (Ertekin-Taner, N. 2007). Mutations in *APP* are located near the cleavage sites of the protein resulting in increased production of Aβ. The average age of onset for this mutation is between mid 40s and 50s but can be modified by the apolipoprotein E (*APOE*) genotype. Missense mutations within the *PSEN1* gene account for 18-50% of the early-onset autosomal dominant forms of AD and lead to a particularly aggressive form of the disease with an age of onset between 30 and 50 years, which is not influenced by the *APOE* genotype. The majority of *PSEN* mutations are single-nucleotide substitutions, but small deletions and insertions have been described as well. All mutations within *PSEN1* increase production of the Aβ1-42. Mutations within *PSEN2* have a variable age of onset (40-80 years), are not influenced by *APOE* genotype and result in increased Aβ1-42 production (Ray, W.J. et al. 1998; Bettens, K. et al. 2010)

The *APOE* gene, on chromosome 19q13, and its variants, is recognized as a major risk factor for late-onset AD (LOAD). *APOE* ε3 is the most frequent form (78%), *APOE* ε4 makes up

15% and *APOE* ε2 approximately 7%. The *APOE* ε4 allele is the main genetic risk factor for the development of sporadic AD, whereas the ε3 and ε2 alleles provide relative protection for this neurodegenerative disorder. Nevertheless, only less than 50% of non-familial AD cases are carriers of the *APOE* ε4 allele. Therefore, *APOE* ε4 is not a deterministic factor for the development of the disease and other genes must confer susceptibility to AD (Tanzi, R.E. and Bertram, L. 2001). Some candidate susceptibility genes include the ones encoding α2-macroglobulin (*α2M*), low-density lipoprotein receptor-related protein (*LRP*), angiotensin converting enzyme (*ACE*) and insulin degrading enzyme (*IDE*) (Rocchi, A. et al. 2003) (Table 1.1).

Genes	Chromosomal location	AD onset	Type of AD
ACE	17q23	Late	Sporadic
APOE	19q32.2	Late	Familial/sporadic
APP	21q21.3-q22.05	Early	Familial
BChE	3q26.1-q26.2	Late	Sporadic
catD	11p15.5	Late/early	Sporadic
CST3	20p11.2	Late	Sporadic
GAB2	11q14	Late	Sporadic
IDE	10	Late/early	Sporadic
LRP	10q23-q25	Late	Sporadic
PSEN1	14q24.3	Early	Familial
PSEN2	1q31-q42	Early	Familial
SORL1	11q23	Late	Sporadic
TGF-b1	19q13.1-q13.3	Late	Sporadic
α2M	12p	Late	Sporadic

Table 1.1 - Some genes involved in AD pathology. Adapted from (Wang, X.P. and Ding, H.L. 2008)

1.1.4. Risk factors

The majority of AD cases (>90%) typically occur after the age of 60-65 years. These sporadic cases have complex etiology due to both environmental and genetic factors, which alone do not seem sufficient for causing disease. Although the primary causes for this neurodegenerative disorder are unknown, aging is recognized as the main risk factor.

In addition to the previously described polymorphisms or the susceptibility genes that increase the risk for developing AD, multiple non-genetic environmental factors may influence the onset and progression of the disease. The main risk-increasing factors examined in epidemiological studies are associated with lifestyle, such as hypertension (as a long-term stress of the blood vessel endothelium and walls), diabetes (by vascular changes or insulin deregulation itself), inflammation, obesity, or head injury (Stozicka, Z. et al. 2007).

Family history is another risk factor for AD. Individuals with a parent, brother or sister with AD are more likely to develop the disease than those who do not have a first-degree relative with AD. First degree relatives of a person with sporadic late-onset AD (LOAD) have an approximately 2.5 times greater risk of developing sporadic late-onset AD than those who are not firstdegree relatives (Ertekin-Taner, N. 2007).

People with fewer years of education are at higher risk for AD and other dementias than those with more years of education. Some researchers believe that a higher level of education provides a "cognitive reserve" that enables individuals to better compensate for changes in the brain that could result in AD or another dementia. However, others believe that these differences in educational attainment and dementia risk reflect factors as increased risk for disease in general and less access to medical care in lower socioeconomic groups (Whalley, L.J. et al. 2006).

Another established risk factor for AD is the occurrence of mild cognitive impairment (MCI), which is characterized by problems in memory, language or another essential cognitive

ability that are severe enough to be noticeable to others and show up on cognitive tests, but do not interfere with daily life. Studies indicate that as many as 10-20% of people aged over 65 have MCI. MCI may in some cases represent a transitional state between normal aging and the earliest symptoms of AD. It's estimated that about 15% of these individuals progress from MCI to dementia each year but it is unclear why some people with MCI develop dementia while others do not (Morris, J.C. et al. 2001). Understanding the reasons behind this distinction between converting and non-converting MCI may allow deciphering the pre-clinical cellular and molecular alterations that could be used to develop novel therapeutic strategies.

1.1.5. Genes dysregulation

Numerous studies report specific gene dysregulation events associated with AD and have suggested important mechanisms whereby neuronal cells die in this disorder (Kong, L.N. et al. 2005; Papassotiropoulos, A. et al. 2006; Wu, Z.L. et al. 2006). Two of the genes selected in this study, encoding for β-secretase (*BACE1*) and sirtuin1 (*SIRT1*) are among the best described. BACE1 (for β-site APP-cleaving enzyme) mediates the primary amyloidogenic cleavage of APP and generates a membrane-bound APP C-terminal fragment (APP CTFβ), which is the immediate precursor for the intramembraneous γ- secretase cleavage. Several evidences exist that BACE1 expression is significantly enhanced in brains derived from patients with sporadic AD (Fukumoto, H. et al. 2002; Holsinger, R.M. et al. 2002; Yang, L.B. et al. 2003). Abnormal BACE1 trafficking and maturation also contribute to

the AD pathogenesis in Down's syndrome (Sun, X. et al. 2006). Recently, it was discovered that BACE1 activity could be measured in CSF. A first pilot study showed increased BACE1 activity in CSF from AD cases (Holsinger, R.M. et al. 2004), being consistent with the observation of BACE1 upregulation in the AD brain and has been confirmed in subsequent studies, using different assay formats (Holsinger, R.M. et al. 2006; Verheijen, J.H. et al. 2006). Other studies also showed elevated BACE1 activity and protein levels in CSF of MCI patients (Truong, A.P. et al. 2010), and BACE1 activity in MCI cases that progress to AD (Zetterberg, H. et al. 2008). These results suggest that upregulation of BACE1 may be an early pathogenic factor in AD and that up-regulated *BACE1* gene expression at the level of transcription or translation could contribute to AD pathogenesis in sporadic cases.

Silent information regulator 2 (Sir2) proteins, or sirtuins, are protein deacetylases found in organisms ranging from bacteria to humans. Sirtuins deacetylate histones and other substrates, being this capacity essential to the regulation of various cellular functions. SIRT1 has recently been shown to suppress γ-secretase activity in different *in vitro* models, thereby decreasing the production of Aβ. Reduction of γ-secretase activity has been replicated *in vivo* using transgenic mice that overexpress SIRT1, thus providing the first evidence linking sirtuins and AD (Donmez, G. et al. 2010). Significant reduction of SIRT1 was found in the parietal cortex, in the CA1 and CA3 hippocampal regions of AD patients, but not in the cerebellum, and was not observed in MCI. SIRT1 decrease paralleled the accumulation of tau and a significant linear

relationship between SIRT1 levels and *ante mortem* indices of global cognition was also found (Julien, C. et al. 2009).

The other two genes focused in this work are under some controversy regarding their levels in AD. Nicastrin (NCSTN) is a type I transmembrane glycoprotein and an essential component of the γ-secretase complex. Overexpression of wild type NCSTN increases Aβ production, indicating that the strict regulation of *NCSTN* expression may play a fundamental role in the pathogenesis of AD (Zhong, L. et al. 2009). Indeed, several polymorphisms in the promoter of the gene have been associated to AD (Helisalmi, S. et al. 2004; Ma, Z. et al. 2009). However, there is no study looking at the levels of the gene in AD brains or models.

Adenosine 2A receptors (A2AR) are excitatory receptors, whose effects are exacerbated in situations of compromised brain homeostasis due to the increase in adenosine levels. Despite the low levels of A2AR in the hippocampus, they became relevant in noxious brain conditions (Cunha, R.A. 2005). This was also observed in animal models of AD (Arendash, G.W. et al. 2006), following Aβ exposure (Canas, P.M. et al. 2009) and, more importantly, it was confirmed in the AD brain (Albasanz, J.L. et al. 2008). However, the levels of the gene encoding the receptor (*ADORA2A*) were not yet described in the disease.

It is therefore essential to characterize the dysregulation of the transcription in AD and understand if epigenetic modulation could play a determinant role in this phenotype.

1.2. Epigenetics

1.2.1. Definition

The epigenetic regulation of gene expression is required for many cellular events and for the proper development of an organism. The term “epigenetic” was first used by Conrad Waddington in the 1940s, who defined it as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being”. Nowadays, the epigenome is defined as the heritable but potentially reversible changes in gene expression that occur in the absence of changes to the DNA sequence itself. These changes are brought about by DNA methylation and modifications of chromatin, such as acetylation, methylation, phosphorylation or ubiquitylation of histones (Dolinoy, D.C. and Jirtle, R.L. 2008). Environmental exposure to nutritional, chemical, and physical factors can alter gene expression, and affect adult phenotype by changes in epigenetic modifications at labile genomic regions (Kovalchuk, O. 2008). The epigenotype shows far greater plasticity than the genotype, since it varies among tissues and throughout life, whereas the DNA sequence remains essentially the same.

1.2.2. Chromatin remodeling

For a long time, DNA methylation was believed to be the only epigenetic mechanism (Holliday, R. and Pugh, J.E. 1975; Riggs, A.D. 1975). Subsequently, chromatin remodeling was identified as another major epigenetic mechanism via posttranslational modifications of histone proteins (Hebbes,

T.R. et al. 1988; Landsberger, N. and Wolffe, A.P. 1997) (Figure 1.5).

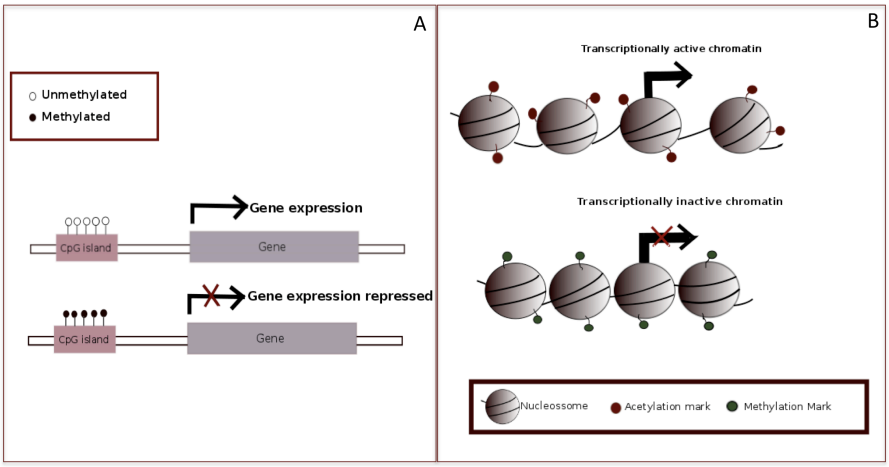


Figure 1.5- Epigenetic mechanisms. A. Genes with a CpG island in the promoter region can be methylated which will repress gene transcription. B. Histone tails can be chemically modified by enzymes, which will alter their conformation. Histone acetylation has been described as activator and histone methylation, at least in some residues, as repressor of transcription. Adapted from (Marques, S.C. et al. 2010)

1.2.2.1. Chromatin structure

Histones are important players in epigenetics. The core histones H2A, H2B, H3 and H4 group into two H2.A-H2.B dimers and one H3-H4 tetramer forms the nucleosome in which a 147-bp segment of DNA is wrapped around. Neighboring nucleosomes are separated by ~50 bp of free DNA (Figure 1.6). The core histones are predominantly globular except for their N-terminal tails, which are unstructured (Kouzarides, T. 2007). In this way, eukaryotic DNA is packaged into highly compacted chromatin.

Chromatin is also organized into well-defined domains. The chromosomes themselves occupy distinct sub-volumes of the nuclear space, termed chromosome territories and the position of gene loci within each chromosome is also subject to strict regulation. The nuclear positioning of both chromosome territories and gene loci within territories are nonrandom and are tissue, cell-type and developmental-stage specific (Takizawa, T. and Meshorer, E. 2008).

Chromatin can be divided in euchromatin that is less condensed, more accessible and generally more easily transcribed, and heterochromatin that is highly condensed, inaccessible and less transcribed. The regions with repetitive DNA, the centromers and telomeres, are the main targets of heterochromatin formation, although also present at developmentally regulated loci (Grewal, S.I. and Jia, S. 2007).

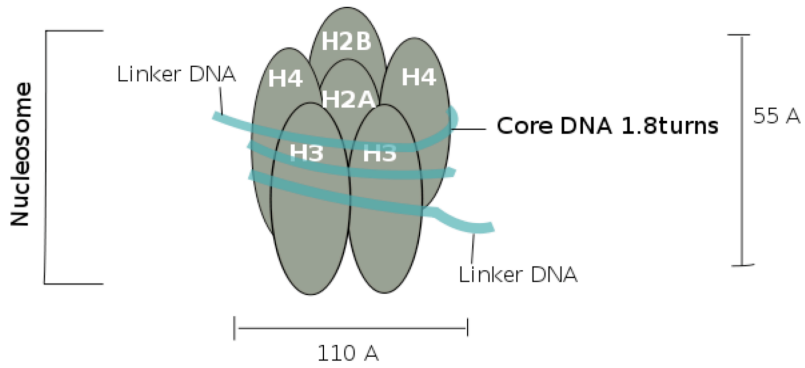


Figure 1.6 – Chromatin structure. The core histones H2A, H2B, H3 and H4 group into two H2.A-H2.B dimers and one H3-H4 tetramer forms the nucleosome in which a 147-bp segment of DNA is wrapped around. Neighboring nucleosomes are separated by ~50 bp of free DNA.

1.2.2.2. Histone modifications

All histones are subject to post-transcriptional modification, some of which occur in histone tails: acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation and ADPribosylation, among others (Figure 1.7). Histone modifications have important roles in transcriptional regulation, DNA repair, DNA replication, alternative splicing and chromosome condensation (ref).

In what concerns its transcriptional state, the human genome can be divided into actively transcribed euchromatin and transcriptionally inactive heterochromatin. Euchromatin is characterized by high levels of acetylation and trimethylated H3K4, H3K36 and H3K79. On the other hand, heterochromatin is characterized by low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation. Recent studies have demonstrated that histone modification levels are predictive for gene expression. Actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5ac and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body (Li, B. et al. 2007).

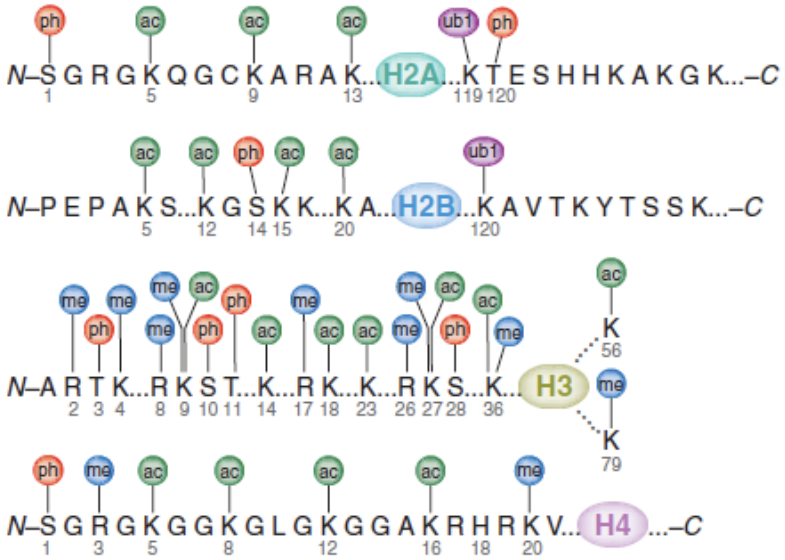


Figure 1.7 – Histone tails modifications. The different components of chromatin can be altered in their histone tails by acetylation, methylation, phosphorylation, ubiquitylation and addition of other chemical groups. From (Bhaumik, S.R. et al. 2007)

Histones can be modified at different sites simultaneously. The core histones forming the nucleosome can each have several modifications, giving rise to cross talk among the different marks. Communication among histone modifications can occur within the same site, in the same histone tail and among different histone tails. Thus, a single histone mark does not determine outcome alone; instead, it is the combination of all marks in a nucleosome or region that specifies outcome (Jenuwein, T. and Allis, C.D. 2001).

Histone code may be transiently altered by the cell environment. The existence of such transient histone codes is the result of the exact cell physiological state and surrounding signals, among others, that can vary over time. A heritable histone code is defined as an epigenetic code (Bird, A. 2007).

Many enzymes can catalyze covalent post-transcriptional modifications and because the modifications are dynamic, several enzymes can also remove these post-transcriptional modifications. Methyltransferases, histone demethylases and kinases are the most specific to individual histone subunits and residues. Conversely, most of the histone acetyltransferases (HATs) and histone deacetylases (HDACs) are not highly specific and modify more than one residue (Portela, A. and Esteller, M. 2010).

It has recently been reported that HDACs and HATs are both targeted to transcribed regions of active genes by phosphorylated RNA polymerase II. Thus, most HDACs in the human genome function to reset chromatin by removing acetylation at active genes, whereas HATs, by contrast, are mainly linked to transcriptional activation (Wang, Z. et al. 2009).

1.2.2.3. Nucleosome positioning

Nucleosomes block the access of activators and transcription factors to their sites on DNA and inhibit the elongation of the transcripts by engaged polymerases. The packaging of DNA into nucleosomes appears to affect all stages of transcription, thereby regulating gene expression (Li, B. et al. 2007). In particular, the precise position of nucleosomes around the transcription start sites (TSSs) has an important influence on the initiation of transcription. The 5′ and 3′ ends of genes possess nucleosome-free regions needed to provide space for the assembly and disassembly of the transcription machinery. The loss of a nucleosome directly

upstream of the TSS is tightly correlated with gene activation, whereas the occlusion of the TSS by a nucleosome is associated with gene repression (Cairns, B.R. 2009).

Nucleosome positioning not only determines accessibility of the transcription factors to their target DNA sequence but has also been reported to play an important role in shaping the methylation landscape (Chodavarapu, R.K. et al. 2010). Several mechanisms can make nucleosomal DNA more accessible to the cellular machinery: i) the transient dissociation and re-binding (breathing) of the ends of nucleosomal DNA; ii) nucleosome sliding and remodeling (spontaneous or catalysed); and iii) changes in chromatin higher-order structure (Figure 1.8). All of these processes are affected by modifications in the amino acid sequence of histones (either by posttranslational modifications or by the introduction of histone variants). Additionally, remodeling factors, histone chaperones, and chromatin-binding proteins all contribute in a combinatorial manner to the structural changes that are necessary to allow access to the DNA template (Luger, K. 2006).

Access to DNA sites that are internal to a nucleosome also require catalysed remodeling. Regulated nucleosome dynamics are driven by ATP-dependent chromatin remodeling complexes, such as SWI/SNF (Switch/Sucrose Non Fermentable) in many ways: i) SWI/SNF transiently exposes DNA regulatory sites by creating DNA loops on the nucleosome surface; ii) by nucleosome sliding, in which complexes move nucleosomes laterally to expose or cover DNA regulatory sites; iii) nucleosome removal and deposition by the Chromatin structure remodeling (Rsc) complex and

histone chaperones, for example, FAcT (facilitates chromatin transcription); iv) replacement of histone subunits, for instance by the cHD1 (chromodomain-helicase-DNA-binding 1) remodeling complex.

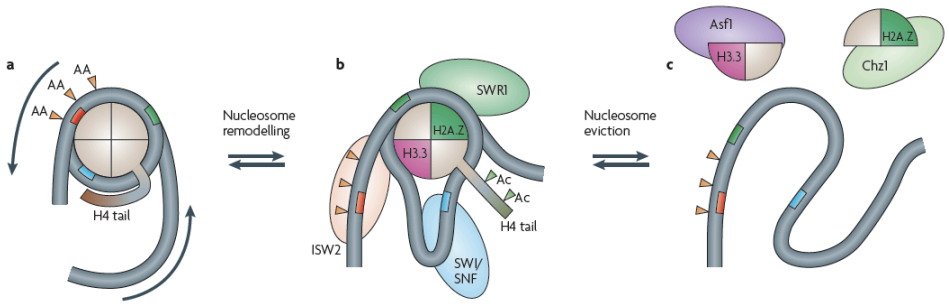


Figure 1.8 – Mechanisms involved in DNA accessibility. a | A stable nucleosome. b | A remodeled nucleosome. c | An evicted nucleosome. Three transcription factor binding sites are shown in red, green and blue, respectively. Some transcription factor binding sites (red and blue) become accessible only during remodeling, either by nucleosome sliding, as indicated by the arrows in a, or by chromatin remodeling complexes (for example, ISW2, SWR1 and SWI/SNF) that 'extract' DNA from the nucleosome surface, as shown in b. Rotational phasing can make a binding site (green site) always accessible in the various states. Nucleosome eviction (c) might be necessary to assemble a pre-initiation complex and to transcribe the underlying DNA. Anti-silencing function 1 (Asf1) and H2A.Z-specific chaperone (Chz1) are examples of histone chaperones. Ac, acetylation. From (Jiang, C. and Pugh, B.F. 2009).

Nucleosome dynamics are important because they regulate DNA accessibility, which is a key to proper gene regulation and transcription fidelity. In addition to shifting the contacts between DNA and histones, eviction of a nucleosome from a particular genomic location allows DNA-binding factors to access the DNA and can therefore affect gene

expression (Li, B. et al. 2007).

Nucleosome loss can occur as a specific response to environmental stresses or signals, leading to transcriptional reprogramming (Jiang, C. and Pugh, B.F. 2009).

The nucleosome remodeling machinery is influenced by DNA methylation and has been linked with specific histone modifications. For instance, the activity of SWI/SNF (and related complexes) can be enhanced by histone acetylation. Acetylation might reduce histone-DNA electrostatic interactions by neutralizing positively charged lysines, which might disrupt repressive chromatin structures and also provide acetyl-lysine binding sites for SWI/SNF and other complexes (Workman, J.L. 2006; Jiang, C. and Pugh, B.F. 2009).

1.2.3. Involvement in aging

The study of epigenetics in aging is an emerging discipline that promises exciting revelations in the near future, such as the definition of a DNA methylome and a histone modification map that will help to distinguish between a ‘‘young’’ and an ‘‘old’’ cell and to characterize all the chromatin modifying enzymes involved in the process (Fraga, M.F. and Esteller, M. 2007).

The aging process consists of a complex of anatomical, physiological, biochemical and genetic changes that all organisms undergo during their lifetime (Calvanese, V. et al. 2009). Epigenetics is probably only one of several components of aging, but its features make it a very strong candidate for explaining these changes. Although epigenetic factors are heritable (at least at the cellular level), they can be

modulated by external factors (Barros, S.P. and Offenbacher, S. 2009) and in this way represent a molecular link between environment and aging.

Epigenomic alterations are now increasingly recognized as part of aging. Several clues of the relationship between DNA methylation and aging arose as soon as 1973, with the evidences of a global loss of cytosine methylation during aging in rat brain and heart, to more recent studies that support the idea of intra-individual epigenetic variation over time in humans (Vanyushin, B.F. et al. 1973; Bjornsson, H.T. et al. 2008). In addition to this global DNA methylation, a number of specific loci have also been described as becoming hypermethylated during aging as well as some specific histone modifications (Table 1.2).

Epigenetic Hit	Species	Tissue or cells type	References
Global hypomethylation			
	Rat	Brain, heart	(Vanyushin, B.F. et al. 1973)
	Mouse	Brain, liver, small intestines	(Wilson, V.L. et al. 1987)
	Human	Bronchial epithelial cells	[135]
	Human	Leukocytes	(Fuke, C. et al. 2004)
	Mouse, hamster and human	Primary cult. fibroblast	(Wilson, V.L. and Jones, P.A. 1983)
Promoter -specific hypermethylation			
Ribosomal DNA	Rat	Liver and germ cells	(Oakes, C.C. et al. 2003)
ER	Human	Colon	(Issa, J.P. 2003)
MYOD1,N33	Human	Colon	(Ahuja, N. et al. 1998)
IGF2	Human	Colon	(Issa, J.P. et al. 1996)
MLH1 and p14ARF	Human	Colon	[139]
E-cadherin, c-fos and cytogen- alpha 1	Human	Various tissues	(Fraga, M.F. and Esteller, M. 2007)
Histone Marks and histone-modifying enzymes			
Global hystone acetylation	Human	Human diploid cells	(Ryan, J.M. and Cristofalo, V.J. 1972)
Histone methylation	Rat	Brain and liver	(Lee, C.T. and Duerre, J.A. 1974)
Senescence-associated-heterochromatin foci	Human	Fibroblasts	(Narita, M. et al. 2003; Braig, M. et al. 2005)
H3K9me, SUV39h1	Mouse	Splenocytes, lymphocytes	(Braig, M. et al. 2005)
H3K27me3 and EZH2	Human, mouse	HEF, MEF	(Bracken, A.P. et al. 2007)
SIRT1	Human, mouse	Human lung, MEF	(Sasaki, T. et al. 2006)
SIRT1	Mouse	--	(Sommer, M. et al. 2006)

Table 1.2- Epigenetic alterations associated with aging. Adapted from (Calvanese, V. et al. 2009)

The role of epigenetics in aging biology may be able to explain many of the phenotypic changes related to the aging process and simultaneously to age-related pathologies.

1.2.4. Involvement in complex disease

Complex disorders, such as cancer, schizophrenia, lupus, cardiovascular and neurodegenerative diseases, exhibit a heritable component but do not follow Mendel's laws of inheritance. These diseases are generally considered to derive from a combination of multiple heritable and environmental factors (van Vliet, J. et al. 2007). The contribution made by environmental factors may be mediated through epigenetic changes (Figure 1.9).

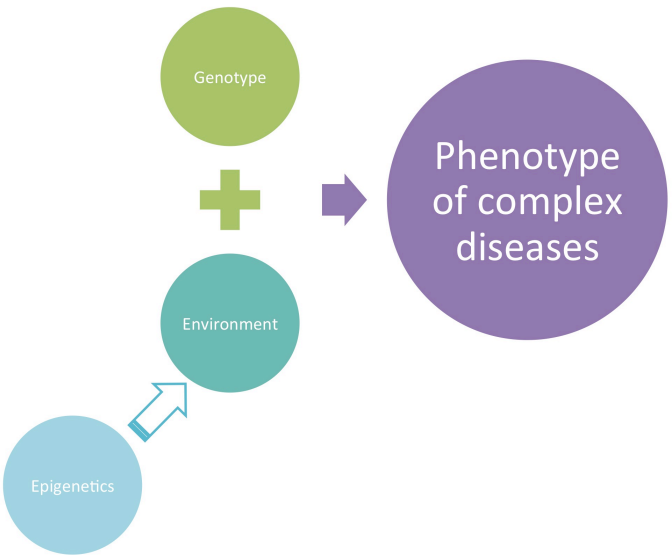


Figure 1.9– Components of complex disorders. The complexity of diseases, such as cancer, cardiovascular, neuropsychiatric and neurodegenerative diseases, is due to the combination of genetic susceptibility and environment factors, being the latter now accepted to be mediated by epigenetic modulation.

Feinberg *et al.* proposed in 2007 that in the etiology of these diseases, an epigenetic framework can help to provide an

explanation for three characteristics: (i) their age-dependence, which is not well explained by accumulated mutation; (ii) their quantitative nature; and (iii) the mechanism by which the environment might modulate genetic predisposition to disease. The resulting common disease genetic and epigenetic (CDGE) hypothesis argued that in addition to genetic variation, epigenetics provides an added layer of variation that might mediate the relationship between genotype and internal and external environmental factors (Feinberg, A.P. 2007; Feinberg, A.P. 2008).

Indeed, it is now possible to explain the marked increase in common diseases with age, as well as the frequent discordance of diseases between monozygotic twins (Dempster, E.L. et al. 2011) and several studies provided evidences for a link between epigenetic regulation and cancer, neuropsychiatric or neurological disorders (Table 1.3).

Disease condition	Gene	Biological process	References
Cancer			
Bladder	Multiple genes	Hypermethylation	(Esteller, M. et al. 2001)
Brain (glioma)	*RASSF1A*	Hypermethylation	(Gao, Y. et al. 2004)
Brain (glioblast)	*MGMT*	Hypermethylation	(Bello, M.J. et al. 2004)
Breast	*BRCA1*	Hypermethylation	(Mancini, D.N. et al. 1998)
Breast	Multiple genes	Hypermethylation	(Szyf, M. et al. 2004)
Colon	Multiple genes	Hypermethylation	[150]
Colorectal	L1 repeats	Hypomethylation	(Suter, C.M. et al. 2004)
Esophagus	*CDH1*	Hypermethylation	[150]
Head/neck	*p16*, *MGMT*	Hypermethylation	[150]
Kidney	*TIMP-3*	Hypermethylation	[150]
Leukemia	p15	Hypermethylation	[150]
Liver	Multiple genes	Hypermethylation	(De Zhu, J. 2005)
Lung	*p16*, *p73*	Hypermethylation	[150]
Lymphoma	*DAPK*	Hypermethylation	[150]
Myeloma	*DAPK*	Hypermethylation	(Chim, C.S. et al. 2004)
Ovary	*BRCA1*	Hypermethylation	(Esteller, M. et al. 2000)
Ovary	*Sat2*	Hypomethylation	(Widschwendter, M. et al. 2004)
Pancreas	*APC*	Hypermethylation	[150]
Pancreas	Multiple genes	Hypomethylation	(Sato, N. et al. 2003)
Prostate	*BRCA2*	Hypermethylation	(Li, L.C. et al. 2004)
Stomach	Cyclin D2	Hypomethylation	(Oshimo, Y. et al. 2003)
Uterus	*hMLH1*	Hypermethylation	[150]
Neurological diseases			
Schizophrenia	*RELN*	Hypermethylation	(Sharma, R.P. 2005)
Bipolar disorder	*11p*	Unknown	(Petronis, A. 2003)
Rett syndrome	*MECP2*	Mutation	(Van den Veyver, I.B. and Zoghbi, H.Y. 2001)
Other complex traits			
Lupus	Retroviral DNA	Hypomethylation	(Sekigawa, I. et al. 2003)
Atherosclerosis	Multiple genes	Hypo, hypermethylation	(Lund, G. et al. 2004)
Vascular endothelium	*eNOS*	Hypomethylation	(Chan, Y. et al. 2004)

Table 1.3- Associations between epigenetic modifications and complex human diseases. Adapted from (Rodenhiser, D. and Mann, M. 2006).

1.3. Epigenetics in AD

The etiology of neurodegenerative diseases implicates complex interactions between genes and environment, as stated earlier. However, not much is known about how environmental factors and the associated experience-dependent plasticity modulate pathogenesis and disease progression (Laviola, G. et al. 2008). According to the current knowledge, several authors propose that neurodegenerative diseases are not a result of a single-hit event, but rather a complex step-by-step process involving genetic, epigenetic,

and environmental events (Migliore, L. and Coppede, F. 2009). Indeed several evidences are now demonstrating a putative role of epigenetics in neurodegenerative diseases, and particularly in AD.

1.3.1. DNA methylation

Some cytosines in the promoter region of the APP gene are frequently methylated in cases ≤ 70 years old and significantly demethylated in cases >70 years old. These age-related modifications on DNA methylation alter APP expression and consequently can affect the progressive Aβ deposition with aging in the brain (Tohgi, H. et al. 1999).

Several studies demonstrate a correlation between dietary factors, the epigenome and AD pathology. It is hypothesized that nutritional deficits could lead to hyperhomocysteinemia due to alterations in the homocysteine/S-adenosylmethionine (HCY/SAM) cycle, which is involved in the transfer of methyl groups, subsequently decreasing SAM levels. Methyl donor decrease could, in turn, induce demethylation of DNA and this will result in activation and overexpression of genes involved in AD pathology (Obeid, R. and Herrmann, W. 2006). Indeed, folate, vitamin B12, and SAM are frequently reduced in the elderly (Wolters, M. et al. 2004; Park, L.K. et al. 2011). One of the first studies regarding this issue showed that the depletion of folate and vitamin B12 in culture medium could cause a reduction of SAM levels and an increase in Aβ production (Fuso, A. et al. 2005). The same authors also demonstrated that vitamin B deprivation could induce hyperhomocysteinemia and an imbalance of SAM and SAH, in association with *PSEN1* and

BACE1 up-regulation and Aβ deposition (Fuso, A. et al. 2007).

Vitamin B deficiency was also shown to induce hypomethylation of specific CpG moieties in the 5'-flanking region of *PSEN1* gene, and this was reverted by SAM supplementation (Fuso, A. et al. 2011). In line with the changes observed for *PSEN1* methylation patterns, DNA methylases (*DNMT1*, *3a* and *3b*) and a putative demethylase (methyl-CpG binding domain protein 2 - *MBD2*) were differently modulated in the same experimental conditions (Fuso, A. et al. 2011). This relation was further explored by developing a triple transgenic/mutant mouse model (APP*/PSEN1*/CBS*) showing both amyloid deposition and high serum levels of HCY resultant of deficient cystathionine-beta synthase (CBS) activity. The study showed that female APP*/PSEN1*/CBS* mice exhibited significant increases of Aβ1-40 and Aβ1-42 levels in the brain compared with APP*/PSEN1* double transgenic mice suggesting that hyperhomocysteinemia can be a risk factor for AD (Pacheco-Quinto, J. et al. 2006).

In line with this nutritional regulation of epigenetic alterations it was described a downregulation of the neuronal PP2A methyltransferase (*PPMT*), along with a decrease in PP2A methylation in affected brain regions from AD patients (Sontag, E. et al. 2004). Taking this into account, it was recently observed that hyperhomocysteinemia induced in mice by feeding a high-methionine, low-folate diet was associated with increased brain S-adenosylhomocysteine (SAH) levels, with a reduced *PP2A* methylation levels, and with tau and APP phosphorylation (Sontag, E. et al. 2007). These results supported the hypothesis that impaired HCY metabolism and

deregulation of critical methylation reactions can trigger the accumulation of phosphorylated tau and APP in the brain, a process that may favor neurofibrillary tangle formation and amyloidogenesis.

Dietary and environmental factors, which are present from as early as the prenatal phase, have also a profound impact on epigenome and may affect diseases developed later in life. Expression of AD-related genes (*APP*, *BACE1*) was found to be elevated in aged (23-year-old) monkeys exposed to lead (Pb) as infants, along with a decrease in DNA methyltransferase activity and higher levels of oxidative damage to DNA (Wu, J. et al. 2008). These data suggest that AD pathogenesis is influenced by early life exposures and argue for both an environmental trigger and a developmental origin of AD, being the intermediate DNA methylation a form of epigenetic imprinting.

The age-related epigenetic modifications in AD were further explored in two studies. The first one presented a straight evidence of epigenetic involvement in AD pathogenesis by showing an age-specific epigenetic drift in late-onset AD. *PSEN1* and *APOE*, which participate in APP processing, methylenetetrahydrofolate reductase (*MTHFR*) and *DNMT1*, which are responsible for methylation homeostasis, presented a significant inter-individual epigenetic variability in the brain and lymphocytes of these patients, which could contribute to late-onset AD predisposition (Wang, S.C. et al. 2008). Longevity-related genes were investigated with respect to promoter methylation in peripheral blood in relation to gender, age and AD. Only one of the genes, *HTERT*, was found to be hypermethylated in AD compared to aged normal

subjects and is, by opposite to the normal effect of methylation on gene expression, activated by this epigenetic modification (Silva, P.N. et al. 2008). These results suggest a higher telomerase activity, probably due to telomere and immune dysfunctions involved in AD pathogenesis.

1.3.2. Histone modifications

In contrast to DNA methylation studies, very few studies were aimed at characterizing chromatin modifications in AD, and these studies are mainly focused on HDAC inhibitors.

HDAC inhibitors can be classified into four main chemical families, the short-chain fatty acids [e.g. sodium butyrate (SBA), phenylbutyrate (PBA), and valproic acid (VPA)], the hyroxamic acids [e.g. trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA)], the epoxyketones (e.g. trapoxin), and the benzamides. Of these, the most widely studied are SBA, PBA, TSA, and SAHA (Carey, N. and La Thangue, N.B. 2006). The initial interest in these inhibitors came from studies linking HDACs to a wide variety of human cancers. HDAC inhibitors arrest growth, induce differentiation and, in some cases, apoptosis and have potent anti-cancer activities, with remarkable tumor specificity (Minucci, S. and Pelicci, P.G. 2006). For this reason, inhibitors of class 1 and 2 HDACs are in phase I/II clinical trials for cancer therapy and potentially cancer prevention. In the nervous system, the anticonvulsant and mood-stabilizing drug VPA was identified as an inhibitor of HDAC1, thereby linking its antiepileptic effects to changes in histone acetylation (Rosenberg, G. 2007; Trinka, E. 2007).

More recent work has revealed that inhibitors of class 1 and 2 HDACs represent novel therapeutic approaches to treat neurodegenerative disorders, depression and anxiety, and the cognitive deficits that accompany many neurodevelopmental disorders (Ferrante, R.J. et al. 2003; Alarcon, J.M. et al. 2004; Weaver, I.C. et al. 2006; Schroeder, F.A. et al. 2007)

In AD, specifically, a recent study showed that acute treatment of the APP/PS1 mouse model of AD with TSA had an effect on memory impairment (Francis, Y.I. et al. 2009). After fear conditioning training, levels of hippocampal acetylated histone 4 (H4) in these mice were about 50% lower than in wild-type littermates. The acute treatment with TSA prior to training rescued both acetylated H4 levels, contextual freezing performance and CA3-CA1 LTP in slices from APP/PS1 mice (Francis, Y.I. et al. 2009). In APPswe/PS1dE9 mice, chronic HDACi (VPA, SB and vorinostat) injections (2-3 weeks) completely restored contextual memory. The newly consolidated memories were stably maintained over a 2-week period. All HDACi affected class I HDACs (HDAC1, 2, 3, 8) with little effect on the class IIa HDAC family members (HDAC4, 5, 7, 9) (Kilgore, M. et al. 2010). In another study, systemic administration of PBA reversed spatial learning and memory deficits in the Tg2576 mouse without altering Aβ burden. However, the phosphorylated form of tau was decreased in the brain after PBA treatment, along with an increase in the inactive form of the glycogen synthase kinase 3beta (GSK3beta) (Ricobaraza, A. et al. 2009). The same authors then showed that PBA administration reinstated fear learning in the same animal model of AD, independently of the disease stage: both in 6-month-old Tg2576 mice, at the onset of the

first symptoms, but also in aged, 12- to 16-month-old mice, when amyloid plaque deposition and major synaptic loss has occurred. Reversal of learning deficits was associated with a clearance of intraneuronal Aβ accumulation, and alleviation of endoplasmic reticulum (ER) stress. The expression of plasticity-related proteins was also significantly increased by PBA (Ricobaraza, A. et al. 2010).

Another type of chromatin modifiers, HAT such as CREB-binding protein (CBP), has also been implicated in AD pathology. In primary neurons, CBP is specifically targeted by caspases and calpains at the onset of neuronal apoptosis, and CBP was further identified as a new caspase-6 substrate. This ultimately impinged on the CBP/p300 HAT activity that decreased with time during apoptosis entry, whereas total cellular HAT activity remained unchanged. Consequently, histone acetylation levels decreased at the onset of apoptosis. Interestingly, CBP loss and histone deacetylation were observed in two different pathological contexts: APP-dependent signaling and amyotrophic lateral sclerosis model mice, indicating that these modifications are likely to contribute to neurodegenerative diseases (Rouaux, C. et al. 2003).

Aβ accumulation, which plays a primary role in cognitive deficits of AD, interferes with CREB activity. Restoration of CREB (cAMP response element-binding) function via brain viral delivery of CBP improved learning and memory deficits in a triple transgenic model of AD, in the absence of changes in Aβ and tau pathology, and were linked to an increased level of brain-derived neurotrophic factor (BDNF) (Caccamo, A. et al. 2010).

Despite the link between AD and histone deacetylation, there is no evidence that the expression of AD-related genes is affected by chromatin modulation in AD. To close this gap, it will be important to undertake a more global study looking at the epigenetic control of the expression of AD-related genes by histone modifications in AD samples and in cell and animal models. In this way, we would develop a better understanding of the role of chromatin remodeling in AD pathogenesis.

2. AIM OF THE THESIS

Given that changes in chemical modifications of histones lead to changes in chromatin structure, and in turn gene transcription, the aim of the present study was to investigate if altered nucleosome remodeling and acetylation of H3 are mechanisms underlying the transcriptional dysregulation observed in four AD-related genes- *BACE1*, *NCSTN*, *SIRT1* and *ADORA2A*. For this purpose, the following models of AD were used: (i) the triple transgenic AD mice model (3xTg-AD) versus age-matched wild type mice and (ii) human peripheral blood mononuclear cells (PBMCs) from subjects with Mild Cognitive Impairment (MCI) or AD patients versus age-matched non-demented controls.

2.1. *In vivo* and *ex vivo* models

2.1.1. Triple transgenic AD mice model (3xTg-AD)

Several AD animal models have been generated in order to better understand the predictive and causal factors of the disease (Sterniczuk, R. et al. 2010). Presently, the 3xTg-AD mice model is the most accurate in mimicking the behavioral and neuropathological changes (both amyloid and tau pathologies) that are observed in AD patients (Oddo, S. et al. 2003). This model is unique from previously generated models as it expresses three dementia-related mutant transgenes, namely APPSwe, PS1M146V, and tauP301L, and demonstrates a clear age-dependent onset of AD neuropathology. Rather than crossing three transgenic lines, Oddo et al. co-microinjected two independent transgenes

encoding human APPSwe and human tauP301L (both under control of the mouse Thy1.2 regulatory element) into single-cell embryos harvested from homozygous mutant PS1M146V knockin (PS1-KI) mice (Figure 2.1).

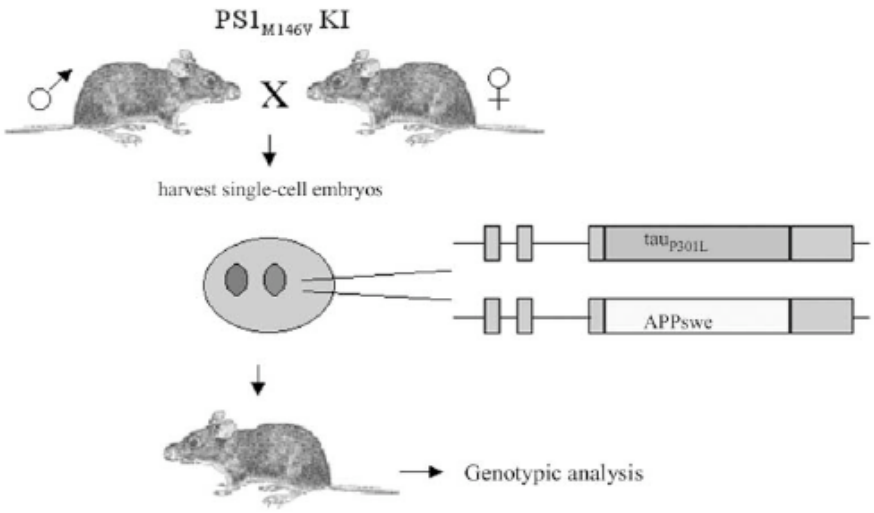


Figure 2.1- Strategy used to develop 3xTg-AD mice. Two independent transgene constructs encoding human APPSwe and tauP301L (4R/0N) were coinjected under the control of the mouse Thy1.2 regulatory elements, into single-cell embryos harvested from mutant homozygous PS1M146V knockin mice. The entire mouse Thy1.2 genomic sequence is shown with exons depicted as boxes and noncoding sequences as thin lines. The injected embryos were reimplanted into foster mothers and the resulting offspring genotyped to identify 3xTg-AD mice. From (Oddo, S. et al. 2003).

Rarely does a single copy of any transgene integrate into a particular locus, and indeed, tau and APP transgene cassettes co-integrated into the same site in the majority of

the founder lines. Due to this co-integration, these 3xTg-AD mice breed as readily as a "single" transgenic line, although containing three transgenes, which facilitates the establishment and maintenance of the colony. Another advantage of this strategy is that mice are of the same genetic background, reducing the biological variability.

The 3xTg-AD mice develop intracellular Aβ accumulation at 4 months, first in neocortical regions and then in CA1 pyramidal neurons. Extracellular Aβ deposits first appear in 6 month-old mice in the frontal cortex and are evident in the hippocampus by 12 months. These evidences suggest an age-related, regional dependent Aβ deposition. Moreover, tau pathology first appears in the hippocampus and then progresses to cortical structures. Tau is conformationally altered and hyperphosphorylated at multiple residues in an age-related and regional dependent manner. Somato-dendritic tau accumulation is evident at 6 months and PHF-1 positive tangles are visible at 15-18 months. At 6 months of age, synaptic dysfunction and impairment of long-term potentiation (LTP) are already apparent along with intracellular Aβ immunoreactivity, although extracellular Aβ is only evident 6 months later, suggesting that intraneuronal Aβ underlies synaptic dysfunction (Oddo, S. et al. 2003).

The behavior in these mice presents some sex-related differences. Male and female 3xTg-AD mice show comparable impairments on Morris water maze (MWM) and inhibitory avoidance (IA) at 4 months. Shortly thereafter, however, the cognitive performance varies between gender, with females performing worse than males. Female 3xTg-AD mice have

been shown to exhibit more aggressive Aβ pathology (Hirata-Fukae, C. et al. 2008), which is exacerbated in ovariectomized females (Carroll, J.C. et al. 2007). These behavioral differences are not attributable to differences in Aβ or tau levels and are transient as from 12 months onward, the disparity is no longer apparent. This disparity seems to be rather related to an enhanced corticosterone response of young female since they presented a markedly heightened corticosterone response after 5 days of MWM training compared to age-matched male 3xTg-AD mice that was no longer apparent in older mice (Clinton, L.K. et al. 2007).

Therefore, in order to characterize the role of chromatin remodeling in the transcription of specific AD-related loci, we performed the following analysis in the cerebral cortex and hippocampus of 3xTg-AD females:

a. Global H3 acetylation
b. mRNA dysregulation of 4 genes altered in human brains of AD.
c. DNA accessibility and H3 acetylation in the promoter of the transcriptionally dysregulated genes.

2.1.2. Peripheral blood mononuclear cells (PBMCs)

The assessment of tissues from patients with neurological disorders is limited by the inaccessibility of the organ and consequently, there is a long history of searching for peripheral markers capable of reflecting the pathology within the brain. PBMCs are particularly useful as a model for

genetic and proteomic markers in the brain and is now considered as a potential model for epigenetic gene regulation as well (Gavin, D.P. and Sharma, R.P. 2009).

PBMCs can provide a reliable means for studying the impact of environment/life experiences on chromatin structure and DNA methylation as observed by Fraga et al. (Fraga, M.F. et al. 2005). The authors examined several epigenetic parameters in lymphocytes from monozygotic twins aged between 3 and 70 years old and found differences in DNA methylation and acetylated H3/ H4 only in older twins. These results were consistent in subjects across at least 12 weeks, indicating that global measures of epigenetic parameters in lymphocytes are a reliable method to analyze chromatin state and also that chromatin from lymphocytes may be considered as a fingerprint of an individual's environment, life experience, and stochastic factors which would not be revealed through genetic testing.

Another important point is that PBMCs share much of the nonsynaptic biochemical environment of neurons, such as neurohormones, neuropeptides, cytokines, metabolites, and medication blood levels, which allows the study of the full evolution of the disorder, including response to pharmacological, metabolic and environmental events, representing the only approach for prospective longitudinal clinical research (Levine, J. et al. 2002; Sharma, R.P. et al. 2006).

PBMCs also contain the full complement of epigenetic enzymes and machinery found in most tissues including both

neurons and peripheral nucleated cells (de Ruijter, A.J. et al. 2003). Previous studies have shown that PBMCs are capable of reflecting overall abnormalities in epigenetic mechanisms also thought to be present in the brain. For example, in Huntington's disease, a disorder known to be associated with impaired histone acetyltransferase activity, a similar pattern of transcriptional repression across several chromosomes was found in blood and brain (Anderson, A.N. et al. 2008). Moreover, using peripheral markers it was possible to differentiate the chromatin structure in twins discordant for mental illness, as well as similarities in epigenetic parameters among individuals affected by the same illness (Petronis, A. et al. 2003; Kuratomi, G. et al. 2008).

Therefore, if PBMCs could serve as a reliable model of overall epigenetic mechanisms then this could lead to a "biomarker" approach able to reveal pathological chromatin state in neurological disorders (Gavin, D.P. and Sharma, R.P. 2009).

With the purpose of finding alterations in blood transcriptional profile during AD progression, which could be useful for diagnosis, PBMCs from control and MCI subjects and from AD patients were used. However, due to the limited amount of sample available, we were only able to analyze the following parameters:

a. mRNA dysregulation of the 4 selected genes.
b. DNA accessibility of the promoter of the transcriptionally altered genes.

3. METHODS

3.1. Samples

3.1.1. Animal model

A total of 12 wild type and 12 transgenic mice were used in this study. Colonies of 3xTg-AD and wildtype (WT) background strain (C57BL/6/129S) mice (La Ferla's lab, University of California, US) were bred and maintained at the Center for Neuroscience and Cell Biology, University of Coimbra. Female mice used in these studies were housed on 12 h light/dark cycles under temperature and humidity controlled conditions and provided *ad libitum* access to food and water. All animal experiments were carried accordingly to the principles and procedures outlined in the European Union (EU) guidelines (86/609/EEC).

3.1.2. Human samples

A total of 69 subjects participated in this study, including 31 AD patients, 22 MCI and 16 healthy subjects. Patients were recruited at the dementia outpatient clinics, University Hospital, Coimbra, and Hospital Santa Maria, Lisbon. Controls were volunteers, usually spouses or friends of patients who were requested to participate in the study.

At the participating centers, amnesic MCI and AD cases were subjected to clinical history, neurological examination, laboratorial evaluation and brain imaging (computed tomography or nuclear magnetic resonance scan). All participants performed the "Mini Mental State Examination" (MMSE) (Folstein, M.F. et al. 1975). MMSE is widely used for

brief evaluation of the mental state and screening of dementia; the normative cut-off values for the Portuguese population adjusted to education were used (Guerreiro M., S.A., Botelho MA 1994). Controls and MCI patients had to score above 22 if they had ≤11 years of education, or above 27 if they had >11 years of education.

All participants were also classified according to "Clinical Dementia Rating" (CDR) scale (De Mendonça A, G.M. 2008). The CDR is a structured-interview protocol that assesses the cognitive and functional performance in six areas: memory, orientation, judgment and problem solving, community affairs, home and hobbies and personal care, in order to quantify the severity of dementia symptoms. Inclusion criteria for AD were based on the 4th edition of "The Diagnostic and Statistical Manual of Mental Disorders" (DSM IV – TR) (APA 2000). MCI criteria were those proposed by the "European Alzheimer's Disease Consortium" (Portet, F. et al. 2006). Briefly, cases should present: i) cognitive complaints and cognitive decline during the last year, reported by the patient and/or family; ii) a MMSE above cut-off; iii) immediate free recall of story A from the "Logical Memory subtest of the Wechsler Memory Scales" at least 1 standard deviation below the norm for age and education; iv) maintained activities of daily living or slight impairment in instrumental activities of daily living, in other words, no more than one item from the "Lawton instrumental activities of daily living scale" suffered any changes and absence of dementia, according to the DSM IV – TR criteria. Control subjects included in the study: i) did not present evidence of cognitive deterioration or cognitive

complaints; ii) had a (MMSE above cut-off; iii) their value in the CDR scale was zero.

The exclusion criteria for all groups were the presence of other neurological, psychiatric or medical pathologies that could cause cognitive impairment, or a history of alcohol or drug abuse. The study was approved by the ethics committees of the University Hospital of Coimbra and Hospital Santa Maria of Lisbon. All participants signed an informed consent before any study procedure. For AD patients, informed consents from respective caregivers were also obtained.

Blood samples from 10 controls, 16 MCI and 25 AD subjects were used for mRNA analysis, and 12 controls, 16 MCI and 22 AD were used for the DNA accessibility assays. Demographic and clinical characteristics of the participants are shown in Table 4.1.

3.2. Samples extraction

3.2.1. Brain tissue

At 6 or 15 months of age, mice were sacrificed by cervical dislocation and the brain was removed and divided in the two hemispheres. One half of cortex and hippocampus were isolated for histone analysis and the other half was used for RNA (and protein) analysis. All the samples were fast-cooled in liquid N_2 and then kept at -80 ºC until assayed.

3.2.2. Peripheral blood mononuclear cells

Peripheral blood was withdrawn into EDTA-vacuum tubes. An equal volume of diluted blood was overlaid on Ficoll-Paque Plus (GE Healthcare Life Sciences) in a 1:1 ratio

and centrifuged at 1250 x g for 30 min at 22 °C without brake or acceleration. The PBMCs layer was harvested and washed with phosphate buffered saline (PBS) to remove plasma and Ficoll at 1000 x g for 10 min at 22 ºC. The pellet of PBMCs was then stored at -80 °C until assayed.

3.3. Western blot analysis

3.3.1. Protein extraction and quantification

The cerebral cortices and hippocampi isolated from the brain of 3xTg-AD or WT mice were weighted and an equal quantity of starting material (~15 mg) was diluted in 100 uL of Laemmli buffer (Karlsson, J.O. et al. 1994; Illi, B. et al. 2003) supplemented with 25 U benzonase (Sigma-Aldrich, St. Louis, MO, USA) was added. Tissues were sonicated 5 sec at medium power and boiled for 5 min. Protein extracts were then diluted 1:10 with a mixture of Laemmli buffer and water (2 uL sample + 1/4 volume of buffer + 13 uL water) and an equal volume of each sample (5 uL) was applied in a gel electrophoresis.

3.3.2. Protein electrophoresis

Samples were loaded onto a SDS-polyacrylamide gel (SDS-PAGE). To facilitate the identification of proteins, a pre-stained precision protein standard (Bio-Rad Laboratories Headquarters, Hercules, CA, USA) was used. Proteins were then transferred to nitrocellulose membranes and then assayed using The Reversible ATX Ponceau S red staining (Sigma-Aldrich, St. Louis, MO, USA) technique. The membrane was placed in Ponceau-S solution and incubated for 2 min at room temperature (RT) under agitation. To slightly de-stain

and enhance contrast after incubation, the membrane was soaked in dH_2O for 1 min. To completely de-stain the membrane, it was washed in TBS-T at RT with agitation for 3 min. To avoid unspecific binding, membranes were further blocked for 1 h at RT with 5% (wt/vol) milk in Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.6) supplemented with 0.1% (vol/vol) Tween 20 (TBS-T). The membranes were next incubated overnight at 4 ºC with the primary antibodies (see Table 3.1).

Membranes were washed three times for 10 min at RT in TBS-T and subsequently incubated with the appropriated secondary antibody diluted in 5 % (wt/vol) BSA in TBS-T. Membranes were then incubated for 2-3 h at RT under agitation and detection was performed by enhanced chemiluminescence (ECL -Millipore, Billerica, MA, USA). This procedure is based on the enzymatic conversion of a luminol-like molecule to a reactive molecule by horseradish peroxidase (HRP). This molecule generates light in the presence of hydrogen peroxide, which is detected manually using X-ray. In addition, the light produced by this method peaks after 5-20 min and decays slowly thereafter.

Antibody	**Dilution**	**Company**
Rabbit polyclonal to Histone H3 (acetyl K9 + K14 + K18 + K23 + K27)	1:2000	Abcam, Cambridge, UK
Rabbit polyclonal to Histone H3	1:5000	Abcam, Cambridge, UK
Goat alkaline phosphatase-linked anti-rabbit	1:15000	Amersham Pharmacia Biotech, Buckinghamshire, UK

Table 3.1- List of antibodies used.

3.4. Primers design

3.4.1. Primers used for Reverse-transcriptase qPCR

The genomic sequences were browsed (http://www.ensembl.org/index.html) and the transcription start site (TSS) of each gene was identified (http://www.fast-db.com/). A region surrounding the TSS, which comprises two exons flanking an intron, was used to design primers with a scientific tool from Integrated DNA Technologies website (http://eu.idtdna.com/scitools/Applications/RealTimePCR/).
The intron-flanking primers obtained were blasted to confirm gene specificity and analyzed for the existence of hairpins or primer dimers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Finally, the annealing temperature was determined using an online tool for PCR protocol optimization http://www.mutationdiscovery.com/md/MD.com/screens/optimase/OptimaseInput.html?action=none. Primer sequences and annealing temperatures are presented in the following table and were purchased to Eurofins MWG Operon, Huntsville, AL, USA.

Species	Gene	Primer	Annealing Temp.
Mouse	*Bace1*	Forward GTCTTTTCTCTCCCCTTTCTCTG Reverse CTTTCGGAGGTCTCGATATGTG	60°C
	Ncstn	Forward AATGGAGAAGCTGAAGGGAAC Reverse CCCGTAGGAGTTGGAGTAAATAC	60°C
	Sirt1	Forward GAGGGTTCTACATCTTGGTCTG Reverse CATTCAATCCTAGCCCCTCAG	60°C
	Adora2a	Forward CAGAGTTCCATCTTCAGCCTC Reverse CACCCAGCAAATCGCAATG	59°C
	Ppia	Forward CAAACACAAACGGTTCCCAG Reverse TTCACCTTCCCAAAGACCAC	59°C
Human	*BACE1*	Forward CAGTCAAATCCATCAAGGCAG Reverse GTTGGTAACCTCACCCATTAGG	60°C
	NCSTN	Forward CCCGCAATGTCATGTTTGTC Reverse AACTTGCCCTTCTCCATATCG	59°C
	SIRT1	Forward AGTATGTGCCTGTGCAGTG Reverse TTCCAGCGTGTCTATGTTCTG	59°C
	ADORA2A	Forward CAAGTCCATTCCTCTCCTTGG Reverse ACCTGTAATCCCAGCACTTTG	60°C
	ACTB	Forward CCACCCTTTCCCACATACTTC Reverse TCACGAGTTCAAGACCAGC	60°C

Table 3.2 – Primers for mRNA analysis.

3.4.2. Primers used for chromatin analysis

The genomic sequences were browsed (http://www.ensembl.org/index.html) and the TSS of each gene was identified (http://www.fast-db.com/). A region of 1000bp containing a CpG island around the TSS was selected for primer design (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), except for human *ADORA2A*, which

didn't present CGI in the promoter region. The presence of CGI was analyzed by the free software MethylPrimerExpress (https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=602121&tab=DetailInfo). The promoter region sequence was analyzed for the presence of a 200-bp region of DNA with a high GC content (greater than 50%) and observed CpG/expected CpG ratio (ObsCpG/ExpCpG) of greater or equal to 0.6, according to the definition of CGI by Gardiner-Garden and Frommer in 1987 (Gardiner-Garden, M. and Frommer, M. 1987). Primers were evaluated for primer dimers, hairpin and blast as described previously. Primer sequences and annealing temperatures are presented in the following table and were purchased to Eurofins MWG Operon, Huntsville, AL, USA.

Species	Gene	Primer	Annealing Temp.
Mouse	*Bace1*	Forward GTCTTTTCTCTCCCCTTTCTCTG Reverse CTTTCGGAGGTCTCGATATGTG	60ºC
	Ncstn	Forward AATGGAGAAGCTGAAGGGAAC Reverse CCCGTAGGAGTTGGAGTAAATAC	60ºC
	Sirt1	Forward GAGGGTTCTACATCTTGGTCTG Reverse CATTCAATCCTAGCCCCTCAG	60ºC
	Adora2a	Forward CAGAGTTCCATCTTCAGCCTC Reverse CACCCAGCAAATCGCAATG	59ºC
	Intergenic	Forward CAAACACAAACGGTTCCCAG Reverse TTCACCTTCCCAAAGACCAC	59ºC
Human	*BACE1*	Forward CAGTCAAATCCATCAAGGCAG Reverse GTTGGTAACCTCACCCATTAGG	60ºC
	NCSTN	Forward CCCGCAATGTCATGTTTGTC Reverse AACTTGCCCTTCTCCATATCG	59ºC
	SIRT1	Forward AGTATGTGCCTGTGCAGTG Reverse TTCCAGCGTGTCTATGTTCTG	59ºC
	ADORA2A	Forward CAAGTCCATTCCTCTCCTTGG Reverse ACCTGTAATCCCAGCACTTTG	60ºC
	Intergenic	Forward CAATAGCACCAAATCCAGCATG Reverse TGGGCTAGACAATCGGAAAAC	60ºC

Table 3.3 – Primers for FAIRE and ChIP analysis.

3.5. RNA extraction

Trizol reagent (Invitrogen's Carlsbad Headquarters, California, USA) (1ml) was added to brain tissue (100 mg) or directly to the obtained PBMCs pellet. Tissues were homogenized using a Potter-Elvehjem homogenizer and PBMCs were resuspended in the correspondent volume. Cells were then centrifuged at 16000 x g for 10 min at 4 ºC. The supernatant was collected and 40 ug/mL of glycogen

(Invitrogen's Carlsbad Headquarters, California, USA) and 200 µl of chloroform were added. The mixture was vortexed for 10 sec and incubated at RT for 2-3 min, being centrifuged again at 16000 x g for 15 min at 4 ºC. The solution then appear separated in an upper colorless aqueous phase, which corresponds to ~60% of Trizol volume, an interphase containing proteins and a red phenol-chloroform phase. The colorless phase was transferred to a new tube and 500 µl of isopropanol was added, being the solution mixed for 5 sec and incubated at RT for 15-30 min. RNA was precipitated at 16000 x g for 15 min at 4 ºC and the pellet was washed once with 75% ethanol and then air-dried. Ethanol-free pellet was finally resuspended in 30 µl dEPC water. RNAs were quantified with Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and kept at -80 ºC. All the process was performed on ice in a hood with pipettes exclusive for RNA, filter tips and dEPC-treated tubes. All the space and materials were cleaned with RnaseZap (Ambion, Applied Biosystems, Foster City, CA, USA) reagent to avoid contamination with Rnase.

3.6. RNA analysis

3.6.1. Reverse transcription reaction

RNA (1 µg) was used to obtained cDNA by reverse transcription reaction with iScript Supermix (Bio-Rad Laboratories Headquarters, Hercules, CA, USA). Briefly, a reaction setup (20 µl final volume) was assembled on ice as followed:

Component	Volume per reaction
5x iScript reverse transcription Supermix	4 µl
1µg RNA template	Variable
Nuclease free water	Variable

The reaction mixes containing 1 µl of cDNAs were spined down and incubated in a MyCycler equipment (Bio-Rad Laboratories Headquarters, Hercules, CA, US) using the following protocol:

Priming	5 min at 25 ºC
Reverse transcription	30 min at 42 ºC
RT inactivation	5 min at 85 ºC

1 µL of cDNAs were used in real time PCR mixes.

3.6.2. Quantitative Polymerase Chain Reaction (qPCR)

Primers were diluted and aliquoted at a 20 µM concentration. Primer amplification efficiency was determined using a standard 10 times dilution of cDNAs from a WT tissue or control human PBMCs.

qPCR mastermixes were prepared according to SYBR Green Jumpstart Taq Ready mix's instructions (Sigma-Aldrich, St. Louis, MO, USA). Briefly, for each reaction (20 µl final volume) assembled on ice:

Component	Volume per reaction
SYBR Green Jumpstart Taq Ready	10 µL
cDNA template	1 µL of iScript product
Primers	0.5 µM of each
Mg^{2+}	2.4 µL
Nuclease free water	5.6 µL

The reaction mixes were spin down and incubated in the Corbett Rotor Gene 6000 equipment (Qiagen Sciences Inc, Germantown, MD, USA) using the equipment two-step protocol. Levels of mRNA were calculated using the PFAFFL method (Pfaffl, M.W. 2001), which is mostly used when primer efficiencies vary. The relative expression ratio is calculated only from the real-time PCR efficiencies of the target gene in relation to the reference gene, Ppia for mice and ACTB for human cDNA. The equation used is depicted below:

$$\text{Ratio} = \frac{(Etarget)^{Ct}target}{(E\ ref)^{Ct}ref}$$

The results were then transformed with Log2. For PBMCs mRNA, the graphs were produced with all the individual values and the inherent standard deviation. For the mouse model, the fold changes of mRNA in transgenic samples compared to WT were calculated and the average and standard deviation presented in the graphs .

3.7. Chromatin analysis

3.7.1. Chromatin extraction

Tissues <100 mg were homogenized 20 times with a potter and PBMCs pellets were resuspended in PBS to a 10 µg/ml concentration. DNA crosslink with associated proteins was performed in the presence of 1% (wt/vol) formaldehyde for 10 min at RT. The crosslink was stopped through incubation during 5 min at RT with glycine at a final

concentration of 0.125 M. Cells were pulled down and the pellet was washed twice with ice-cold PBS. The washed pellet was resuspended with 10 µl/mg SDS lysis buffer [1% (wt/vol) SDS, 10 mM EDTA, 50 mM Tris pH 8.1] and incubated on ice for 15-20 min. The lysate was sonicated with an optimized protocol (see below-"Optimization of sonication"), to shear DNA to an average length between 200-1000 bp, keeping samples on ice throughout the process. Sonicated samples were then centrifuged for 10 min at 16000 x g to discard cell debris. DNA in the supernatant was quantified with Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA) and 2mL of 10 ng/µl chromatin solution was prepared using ChIP dilution buffer [0.01% (wt/vol) SDS, 1.1% (vol/vol) Triton-X, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, 167 mM NaCl). From this, 5 µg was used to FAIRE analysis and 10.5 µg for ChIP, the remaining chromatin being kept at -80 ºC.

3.7.2. Optimization of sonication

Samples of mice brain and PBMCs were treated as mentioned before until the lysis step with SDS lysis buffer and then divided in two equal parts. One part was sonicated 10 times for 20 sec and the other part 15 times for 20 sec, both at 7 microns in Soniprep 150 (Sanyo MSE Ltd, London, UK). After centrifugation to discard cell debris, the lysates were decrosslinked with 0.2 M NaCl for 6 h, and DNA purified by phenol-chloroform extraction. DNA was loaded on a 1% (wt/vol) agarose gel to check for DNA extract size.

3.7.3. Formaldehyde- Assisted Isolation of Regulatory Elements (FAIRE)

The chromatin extract (5 μg=500 uL) was divided in two equal portions. One half (the FAIRE sample) was kept at -20 ºC until processed for DNA extraction and the other half (the input sample) was decrosslinked by incubation for 6 h to overnight at 65 ºC with 0.2 M NaCl. After decrosslink, DNA was extracted in both samples by phenol-chloroform extraction (described later on).

3.7.4. Chromatin Immunoprecipitation (ChIP)

The total volume of sonicated chromatin for ChIP (10.5 μg=1050 μL) was first pre-cleared with 60 μl Protein A agarose-salmon Sperm DNA beads (Millipore) for 30 min at 4 ºC. 1/3 volume was kept at -20 ºC for Input, and the remaining volume was divided for two immunoprecipitations. 0.5μl/μg of chromatin of anti-total H3 or anti-acetylated H3 antibodies (both from Abcam, Cambridge, UK) were added and the immunoprecipitation samples were incubated overnight with rotation at 4 °C. After that, the beads containing the complexes were washed with a sequence of buffers under rotation for 5 min at 4 ºC or RT (for TE wash): i) 1 time Low Salt Immune Complex Wash Buffer [0.01% (wt/vol) SDS, 1% (vol/vol) Triton-X100, 2 mM EDTA, 20 mM Tris, pH 8.1, 150 mM NaCl]; ii) 1 time High Salt Immune Complex Wash buffer [0.01% SDS (wt/vol), 1% (vol/vol) Triton-X100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl]; iii) 1 time LiCl Immune Complex Wash buffer [0.25 M LiCl, 1% (wt/vol) IGEPAL-CA360, 1% (wt/vol) deoxycholic acid, 1 mM EDTA, 10

mM Tris, pH 8.1]; iv) 2 times TE [10 mM EDTA, 1 mM Tris, pH 8.1]. After the last wash with TE buffer and removal of supernatant, complexes were eluted 2 times with 100 µl fresh made Elution buffer [1% (wt/vol) SDS, 0.1 M $NaHCO_3$] for 15 min at RT. The eluted samples were decrosslinked with 0.2 M NaCl at 65 ºC during 6 h to overnight and then treated for 20 min at 37 ºC with 2 mg/ml RNase A (Qiagen Sciences Inc, Germantown, MD, USA). Finally, samples were digested with 0.0125 M EDTA, 0.05 M Tris-HCl, pH 6.5 and 0.5 µg/µl of Proteinase K and incubate for 1 h at 45 ºC. Digested samples were then processed for DNA extraction.

3.7.5. Phenol-Chloroform DNA extraction

A volume of phenol/chloroform (phenol, chloroform, and isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA – Sigma-Aldrich, St. Louis, MO, USA) equal to the lysate volume was added, well vortexed, spun down at 12,000 x g for 1 min and the aqueous fraction was transferred to a fresh tube. To ensure that all protein has been removed, an additional extraction was performed by repeating the previous steps and then a similar protocol was done this time with an equal volume of chloroform. Two volumes of 95% ethanol supplemented with 2 M of sodium acetate and 0.1 µg/µL of glycogen (both from Sigma-Aldrich, St. Louis, MO, US) were added to the aqueous fraction, mixed by inverting and incubated on ice for 30-60 min. The precipitate was pelleted at 12,000 x g for 15 min at 0 °C, and the pellet was washed with ice cold 70% ethanol, centrifuged at 12,000 x g for 2 min at 4 ºC. The washed pellet was air-dried and resuspended in 40 µl of 10 mM Tris-HCl, pH 7.5.

3.7.6. Quantitative real-time PCR

Primers were diluted and aliquoted at a 20 μM concentration. Primer amplification efficiency was determined using a standard 10 times dilution of cDNAs from a WT tissue or control human PBMCs.

qPCR mastermixes were prepared according to Power SYBR Green mastermix's instructions (Applied Biosystems, Foster City, CA, USA). Briefly, for each reaction (20 μl final volume) assembled on ice:

Component	**Volume per reaction**
POWER SYBR Green mastermix	10 μl
cDNA template	3 μl of DNA
Primers	0.5 μM of each
Nuclease free water	6 μl

The reaction mixes were spin down and incubated in the 7500 Fast Real-time PCR system (Qiagen Sciences Inc, Germantown, MD, USA) using the equipment two-step protocol. FAIRE samples were normalized to the input fraction by the 2ΔCt method, being the results presented as % of input. Enrichment levels of ChIP samples were obtained by normalization over the input fraction and then normalized to an intergenic region using the equation:

$$\% \text{ input} = 2^{\wedge(\text{Ct target} - \text{Ct input})} / 2^{\wedge(\text{Ct interg} - \text{Ct input})}.$$

The resulting value of H3 acetylation enrichment was finally normalized in relation to the total H3 enrichment.

3.8. Statistical analysis

Data are expressed as mean ± standard deviation of at least 6 animals or 10 PBMCs samples. Statistical significance between two groups (WT/transgenic in both area or age) was achieved using non-parametric Mann Whitney U test or between PBMC samples by Kruskal-Wallis test (data non-parametric) and Dunn's multiple comparison post-test for PBMC samples. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, statistically significant.

4. RESULTS

4.1. Human subjects database

Data regarding age, gender, years of education, MMSE and CDR scores of human subjects enrolled in this study were obtained during clinical evaluation and are gathered in Table 4.1.

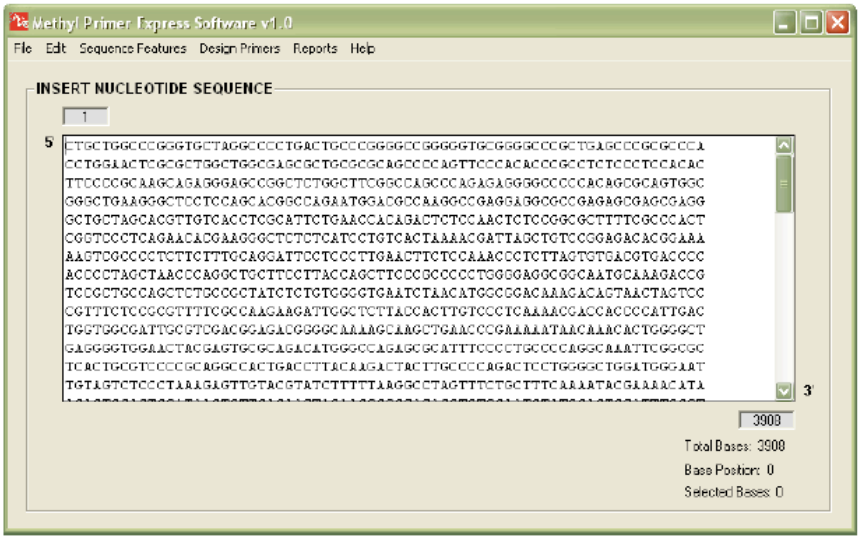
	CT (n=16)	MCI (n=22)	AD (n=31)	Significance
Age (years)	67.5 ± 9.2	74.4 ± 7.7	76.7 ± 7.7*	CT < AD
Education (score)	8.9 ± 5.9	5.5 ± 4.7	4.1 ± 3.7	NS
% women	56.3	54.5	80.6	NS
MMSE (score)	29.5 ± 0.6	25.8 ± 3.4*	16.9 ± 5.6**	CT; MCI > AD
CDR (score)	0	0.5*	1.7± 0.6**	CT < MCI < AD

Table 4.1- Age and gender distribution, education, MMSE and CDR of the sample population participating in this study. CDR scale was according to Morris J, 1993: 0- (none); 0.5 (suspect); 1 (mild); 2 (moderate); 3 (severe) (Morris, J.C. 1993). Statistical significance was achieved by Kruskal Wallis with Dunn's multiple comparison post test or by Chi-Square for % women.

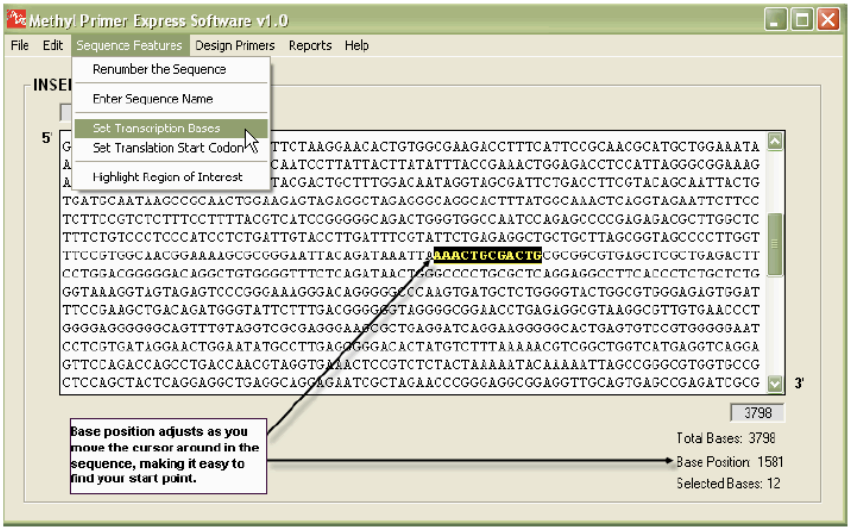
4.2. Bioinformatic analysis

4.2.1. CpG islands search

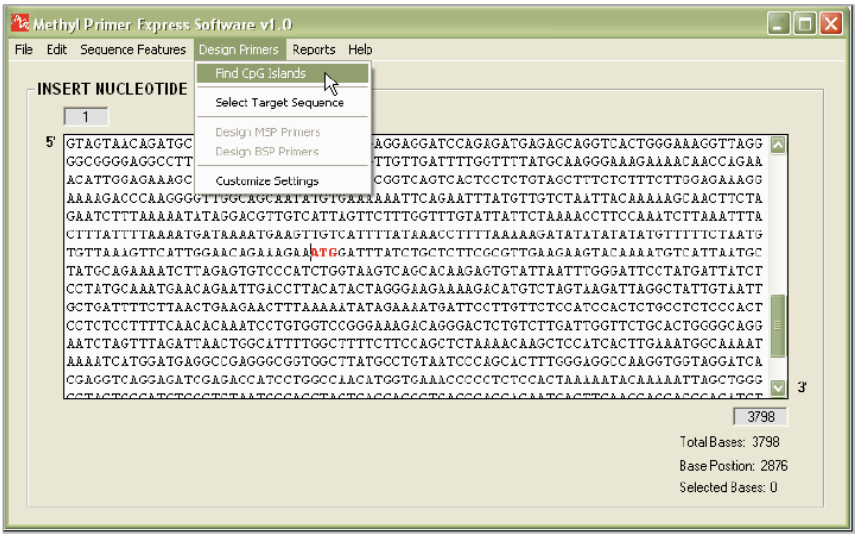
The sequence of the promoter region of each gene was applied to MethylPrimer Express software.

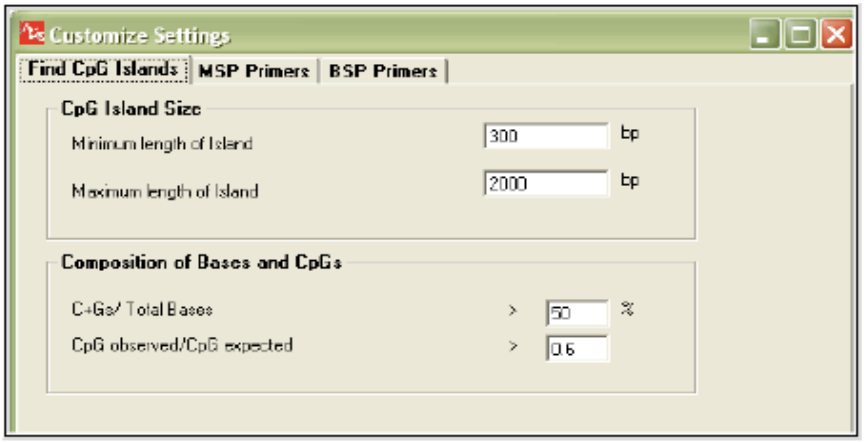


TSS bases were set as depicted below.



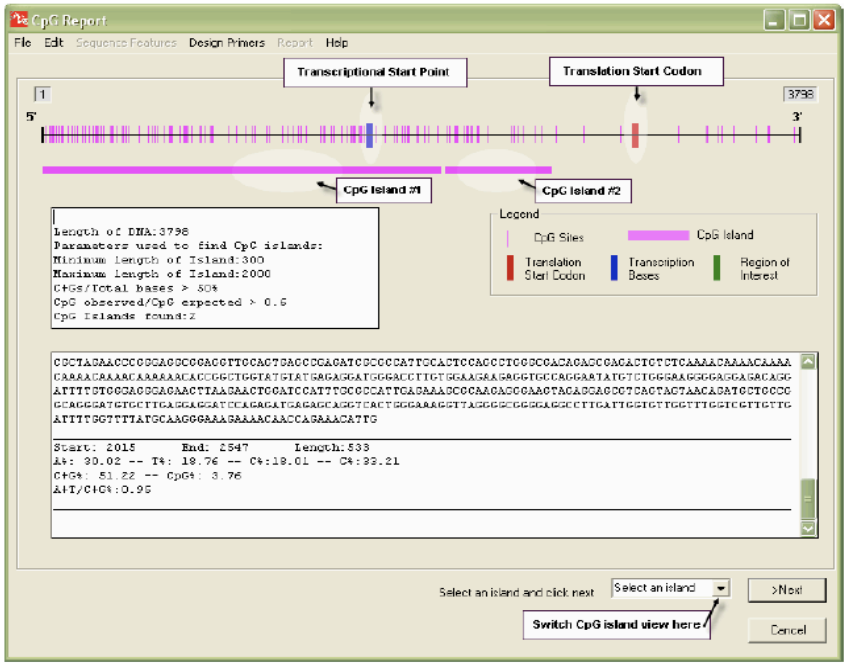
CpG islands were found by the algorithm, according to the criteria of Gardiner and Frommer (Gardiner-Garden, M. and Frommer, M. 1987).





In the CpG Report, CpG sites are indicated by pink vertical bars along the horizontal axis. The transcription start site is indicated by a blue bar; the translation start codon is indicated by a red bar.

CpG islands are indicated by solid pink bars below the horizontal axis.



The sequence of the CGI containing the TSS was then applied in Primer Blast to search for primers for ChIP analysis. In Table 4.2. are presented the CGI chosen for each gene analyzed.

Gene	Start –End bases	Length	C+G (%)
Bace1	324 – 1236	913	64
Ncstn	1– 1331	1331	53.72
Sirt1	1122 – 3121	2000	64
Adora2a	9463 –10223	761	58.34
BACE1	39 – 1312	1274	66.17
NCSTN	1 – 1445	1445	54.05
SIRT1	1 – 2000	2000	58.4

Table 4.2– CpG islands containing the Transcription Start Site of each gene.

4.3. Optimization of techniques

4.3.1. Quantitative Real-time PCR

The efficiency of primers for real-time PCR was analyzed by the generation of a standard curve, by plotting a dilution series of template against the control for each dilution. The template matched the samples used in the experiments (cDNA for reverse transcriptase PCR or Input DNA for FAIRE/ChIP analysis). The slope of the curve was used to calculate the reaction efficiency, which was found to be between 85-100% for all tested primers.

The RNA integrity prior to cDNA conversion was also analyzed to confirm the efficiency of RNA extraction in a denaturing agarose gel stained with ethidium bromide (EtBr). Intact total RNA run on a denaturing gel has sharp, clear 28S and 18S rRNA bands (eukaryotic samples). A 2:1 ratio between the 28S and the 18S rRNA bands (28S: 18S) gives a good indication that RNA is intact.

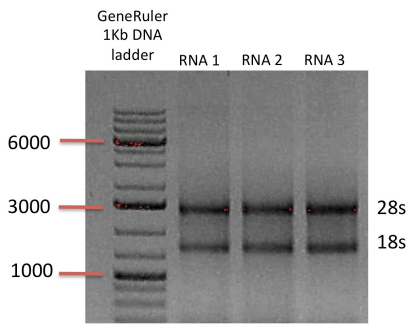


Figure 4.3- RNA integrity. Different samples of extracted RNA were loaded into an agarose gel, which was stained with ethidium bromide to confirm RNA integrity. The presence of the 28S and 18S RNA subunits, detected by comparing their size to a DNA ladder, as well as the ratio between the levels of both subunits were indicative of a completely intact RNA.

4.1.1. Chromatin-related experiments

The resolution obtained by the ChIP procedure is determined by the size of the chromatin fragments used as input material. Two methods are commonly used to fragment chromatin: sonication (hydrodynamic shearing) and micrococcal nuclease (MNase) digestion. When using formaldehyde crosslinking, sonication is the preferred method, since it restricts the access of MNase to chromatin. Optimal fragmentation is achieved by testing various sonication conditions on chromatin, followed by DNA isolation and electrophoresis on an agarose gel to estimate the sonication efficiency. Ideally, the bulk of the chromatin is sonicated to a length between 200 and 1000 bp. For efficient fragmentation, sonication at low power, in combination with several pulses, is preferred over sonication at high power and few pulses, but conditions vary with the sonication device used. It is essential to keep the chromatin sample cooled on ice during sonication, as heat released by the sonication probe can reverse the crosslinks. The presence of detergent (SDS) in the sonication buffer also improves sonication efficiency considerably but foam needs to be prevented since it would make the chromatin sample unsuitable for ChIP, probably as a result of the surface tension imposed by the foam, which can disrupt protein conformation (Haring, M. et al. 2007).

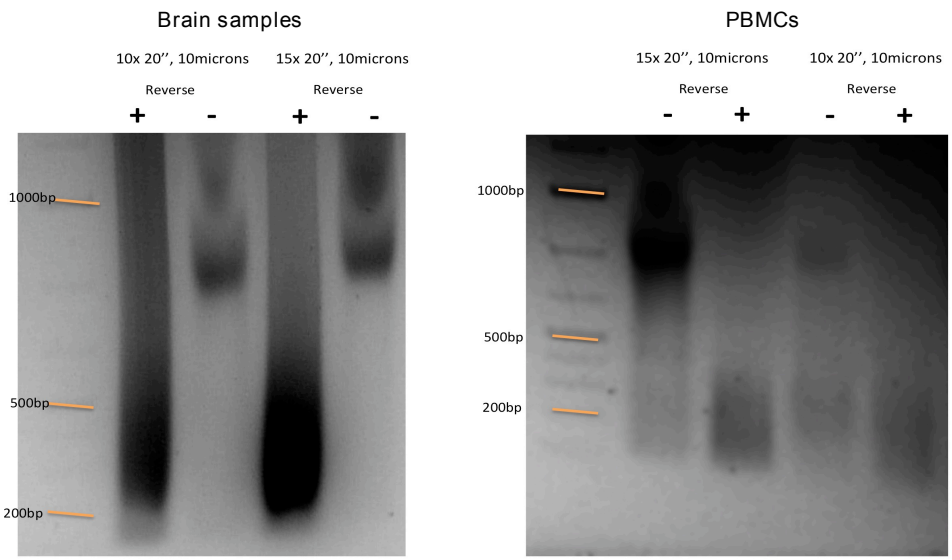


Figure 4.4 – Sonication optimization. Brain tissue extracts or PBMCs were crosslinked and lysed according to ChIP protocol (described in methods). The samples were then divided in 4 portions to optimize sonication conditions: 2 portions were sonicated 10 times during 20 sec at an intensity of 10 microns and the other two were sonicated 15 times for 20 sec also at 10 microns. In each group, one sample was decrosslinked prior to DNA extraction and in the other DNA was directly extracted without the decrosslink step. The resulting DNAs were run in an agarose gel to confirm appropriated shearing.

In brain tissue extracts and PBMCs, more than 10 pulses of 20 sec at a medium power were shown to originate chromatin fragments smaller than 1000 bp, after decrosslink and phenol-chloroform extraction with the protocol used for input samples. For brain tissue, 15 pulses were able to increase the level of DNA fragments within the range 200-1000 bp and therefore this protocol was subsequently applied.

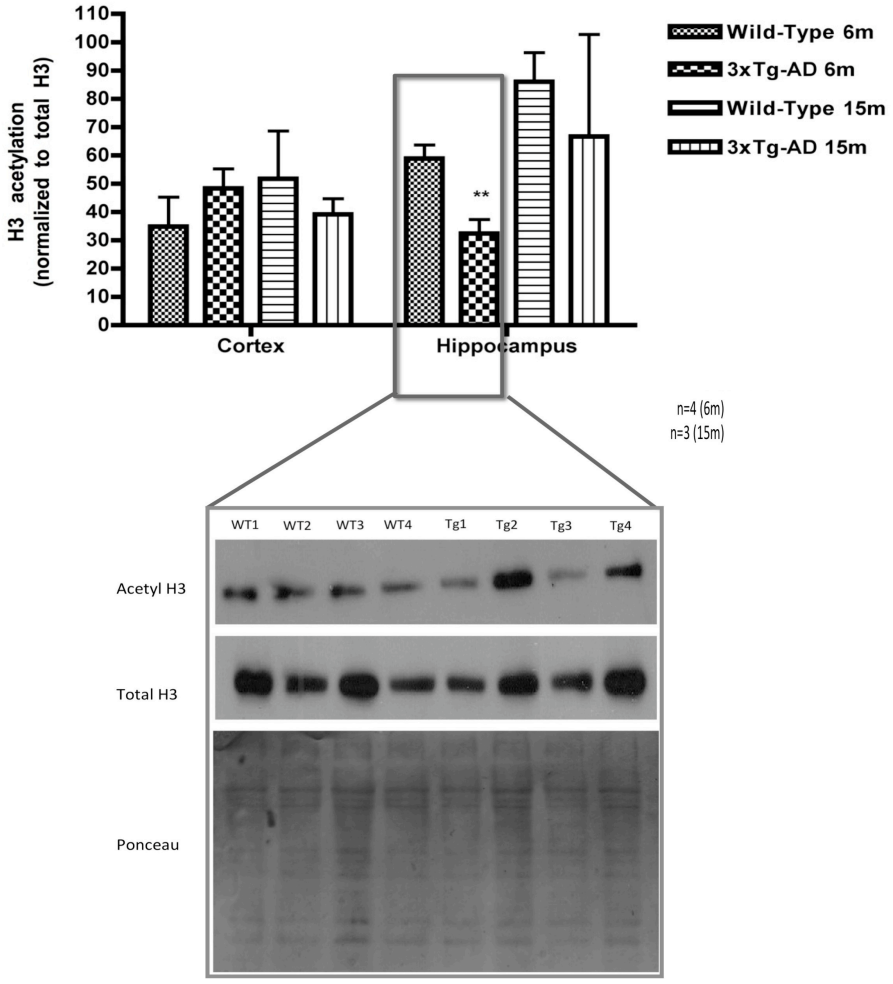
4.4. Epigenetic dysregulation in the brain of 3xTg-AD mice

Several studies reported an involvement of chromatin modifications in neurodegenerative disorders (Kontopoulos, E. et al. 2006; Saha, R.N. and Pahan, K. 2006; Sadri-Vakili, G. et al. 2007; Kim, M.O. et al. 2008) A recent work showed that the APP/PSEN1 mouse model of AD, characterized by the presence of amyloid pathology, presented 50% lower levels of hippocampal acetylated H4 than the wild type (WT) littermates after fear conditioning training. Treatment of these mice with the HDACi, TSA, prior to training, rescued both acH4 levels and contextual freezing performance to WT values (Francis, Y.I. et al. 2009). In the same model, chronic HDACi administration also had a profound effect in restoring contextual memory (Kilgore, M. et al. 2010). Recently, another HDACi, nicotinamide, was found to be effective in the 3xTg-AD mice and the effect was dependent on Sirt1 inhibition (Green, K.N. et al. 2008). However, few are the studies focused on chromatin alterations during the progression of the pathology in AD models. In the present study, we evaluated the age-dependent dysregulation of histone acetylation in AD susceptible brain regions (cerebral cortex and hippocampus) of the 3xTg-AD mice model. Our hypothesis was that there could be a global alteration in acetylation of histones or, alternatively, that dysregulated histone acetylation could occur only at specific genomic loci.

4.4.1. Global H3 acetylation levels

To assess the global alteration in chromatin, we used western blot analysis to determine the levels of H3 acetylation, which have been directly associated with activation of transcription (Li, B. et al. 2007). By doing this, we were able to test whether chromatin has been globally altered in the phenotype triggered by APP/MAPT/PS1 mutations.

Proteins were extracted from the cerebral cortex and hippocampus of 6- (n=4) and 15-month-old (n=3) WT and 3xTg-AD females and the levels of H3 acetylated and total H3 were analyzed. Ponceau S dye was used to make a rapid reversible staining of proteins to confirm equal loading of samples. Although the amount of protein didn't differ significantly, the observed variances in the loading are probably due to the inability to quantify proteins extracted in Laemmli buffer containing bromophenol blue. Acetylated H3 was normalized to total H3 in each sample in order to obtain a relative value for global H3 acetylation in each extract. There were no significant differences in acetylated H3 levels between Wt and 3xTg-AD mice, except in hippocampus at 6 months of age, where less immunoreactivity for acetylated H3 was found in Tg tissue. However, the variability between the 3xTg-AD hippocampi analyzed was evident. Therefore, the different patterns of global H3 acetylation may not be a good indicator of epigenetic modulation in AD and specifically in this animal model.



Figue 4.5 – Global H3 acetylation levels in wild type and 3xTg-AD brain extracts. Proteins were extracted from the cerebral cortex and hippocampus of 6- and 15- month-old wild type (WT) and 3xTg-AD mice with Laemmli buffer. Western blots were performed using antibodies reactive against acetylated and total H3, and the ratio H3ace/H3total was calculated for each sample to give the relative value of global H3 acetylation. ** $p<0.01$, significantly different from WT mice (Mann-Whitney U test).

4.4.2. mRNA of dysregulated genes

Since there were no evidences for a global dysregulation of H3 acetylation in transgenic brains, we then analyzed chromatin state at specific genomic loci. Two genes investigated in this study, *BACE1* and *SIRT1*, were previously shown to be transcriptionally altered in the brain of AD patients (Julien, C. et al. 2009; Coulson, D.T. et al. 2010). The transcriptional status of the two other selected genes, *NCSTN* and *ADORA2A*, was not yet described. However, both genes are involved in AD pathogenesis, being NCSTN a component of γ-secretase complex (Wolfe, M.S. 2008) and A2AR being increased in neuronal cells upon stress conditions and in AD brains (Albasanz, J.L. et al. 2008; Rahman, A. 2009). Therefore, both genes represent good candidates to search for epigenetic modulation of transcriptional regulation. Consequently, the first step, before the analysis of chromatin remodeling, was to determine *Bace1*, *Ncstn*, *Sirt1* and *A2a* mRNA levels. For this purpose, RNA was extracted from the cerebral cortex and hippocampus of 6- (n=4) and 15-month-old (n=6) 3xTg-AD and WT littermates. cDNA was then obtained by reverse transcriptase reaction and the amplification status of cDNA, which directly indicates mRNA levels for each gene, was analyzed by real time PCR using normalization over the reference gene *Ppia*.

Bace1 protein levels were previously shown to be increased in the brain of 3xTg-AD mice (Cai, Y. et al. 2011). As expected, *Bace1* was upregulated in the cerebral cortex and hippocampus of 3xTg-AD mice in an age-dependent manner,

with differences only reaching statistical significance in animals aged 15-months (Figure 4.6). *Bace1* dysregulation in the 3xTg-AD mice comparatively to WT mice could be responsible for an increased APP amyloidogenic processing and progressive deposition of Aβ plaques at older ages.

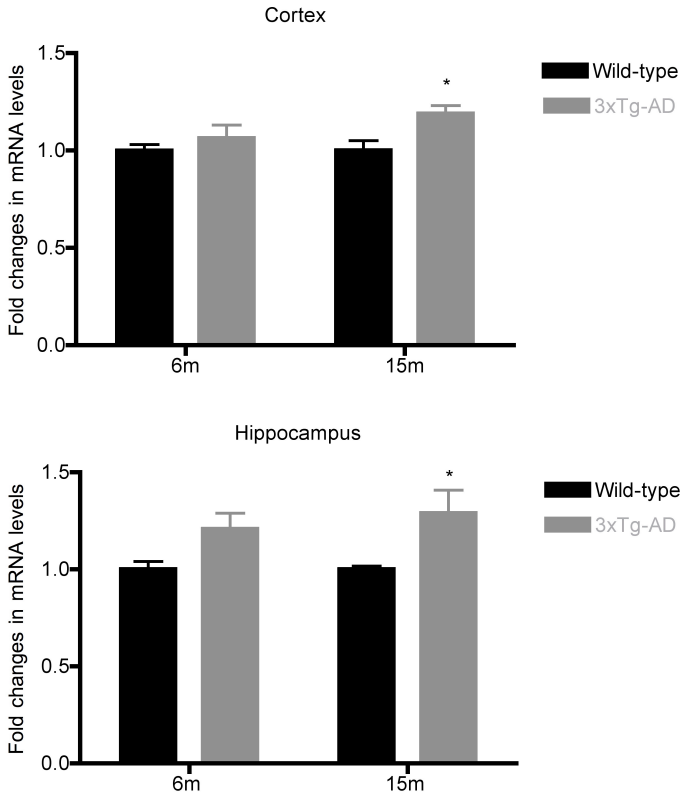


Figure 4.6 – *Bace1* mRNA levels in wild type and 3xTg-AD mice. Relative quantitative analysis of *Bace1* mRNA levels determined by RT-PCR in the cerebral cortex and hippocampus of 6- and 15-month-old WT or 3xTg-AD, normalized to cyclophilin A (*Ppia*). Results are presented as mean±SD. *p<0.05, statistically different from WT mice (Mann-Whitney U test).

Ncstn transcriptional regulation has not been studied in AD patients or in AD animal models. Despite its role in APP amyloidogenic processing, *Ncstn* appeared as downregulated at later stages of the pathology (15-month-old) in 3xTg-AD mice, in comparison with WT mice (Figure 4.7). In these mice, APP amyloidogenic processing pathway is dysregulated due to *APP* and *PSEN1* mutations, which will be translated in the presence of intracellular Aβ as soon as 4 months of age and deposition in plaques that occurs at 12-months in both the cortex and hippocampus.

NCSTN is one of the components of γ-secretase, which also comprises PSEN1, anterior pharynx-defective-1 (APH-1) and presenilin enhancer-2 (PEN-2) (Wolfe, M.S. 2008). Since *PSEN1* is altered in these mice, *Ncstn* downregulation could be a cellular defense to counteract the effect of *PSEN1* mutation on γ-secretase function. In addition, *Bace1* upregulation, as observed in our study, may increase the shift to the amyloidogenic APP processing, and it is therefore plausible to think that the decrease in *Ncstn* expression could be a compensatory mechanism.

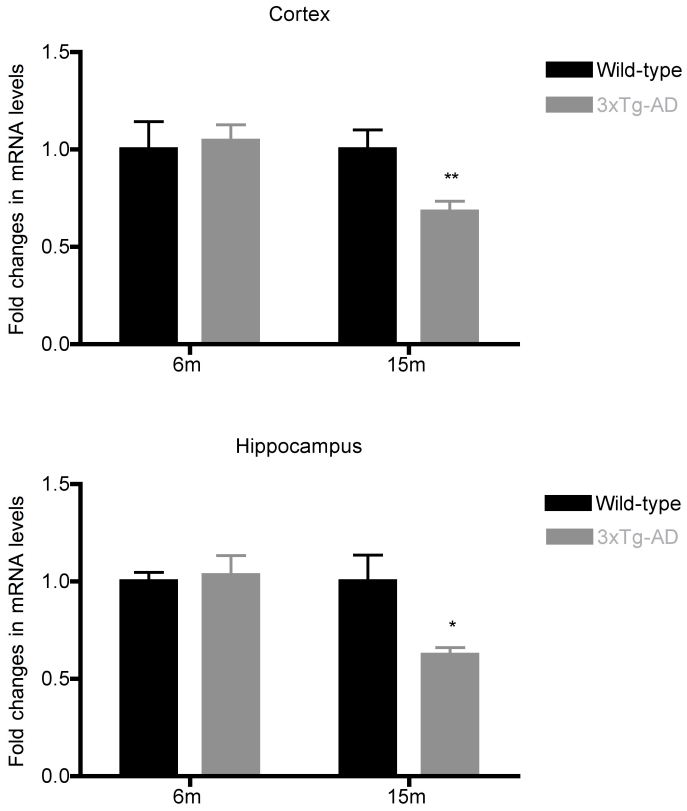


Figure 4.7 – *Ncstn* mRNA levels in wild type and 3xTg-AD mice. Relative quantitative analysis of *Ncstn* mRNA levels by RT-PCR in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice, normalized to cyclophilin A (*Ppia*). Results are presented as mean±SD. *p<0.05, statistically different form WT mice (Mann-Whitney U test).

SIRT1 was reported to be reduced in the parietal cortex and hippocampal regions of AD patients, but not in individuals with MCI (Julien, C. et al. 2009). In the 3xTg-AD mice, this gene was significantly downregulated in 15-month-old animals, both in the cerebral cortex and in the hippocampus (Figure 4.8), which correlates with the alterations described in

the brain of AD patients. In 6-month-old 3xTg-AD mice, when the first pathological changes occur, mRNA levels of *Sirt1* were not significantly affected (Figure 4.8), which is also in agreement with the previously mentioned study showing a correlation between *SIRT1* levels and AD severity (Julien, C. et al. 2009).

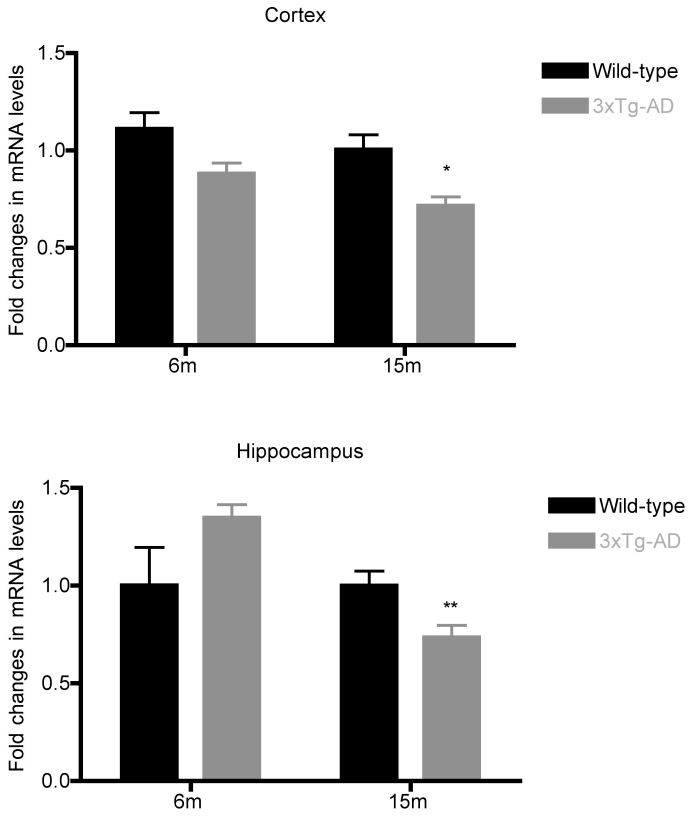


Figure 4.8 – *Sirt1* mRNA levels in wild type and 3xTg-AD mice. Relative quantitative analysis of *Sirt1* mRNA levels by RT-PCR in the cerebral cortex and hippocampus of 6- and 15-month-old WT or 3xTg-AD mice, normalized to cyclophilin A (*Ppia*). Results are presented as mean±SD. *p<0.05, statistically different from WT mice (Mann-Whitney U test).

Despite the obvious role of A2A receptors under conditions of compromised brain homeostasis (Rahman, A. 2009) and the increased expression in AD (Arendash, G.W. et al. 2006; Albasanz, J.L. et al. 2008), changes in *ADORA2A* mRNA levels were not yet reported in AD brains. The mRNA levels of *Adora2a* were not significantly affected in the cerebral cortex or hippocampus of 3xTg-AD in comparison with WT mice either in animals aged 6 or 15 months (Figure 4.9).

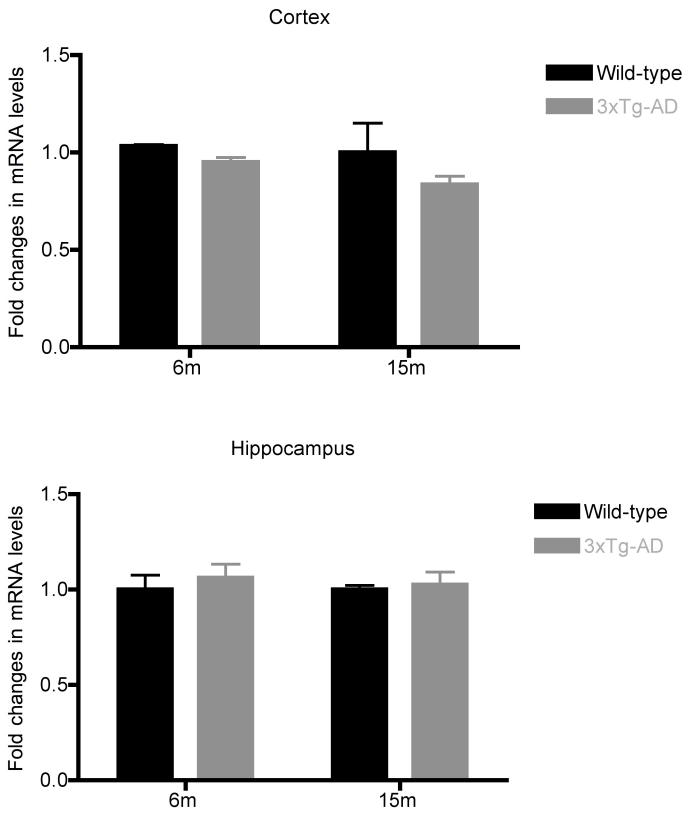


Figure 4.9 – *Adora2a* mRNA levels in wild type and 3xTg-AD mice. Relative quantitative analysis of *Adora2a* mRNA levels by RT-PCR in the cerebral cortex and hippocampus of 6- and 15-month-old WT or 3xTg-AD mice, normalized to cyclophilin A (*Ppia*). Results are presented as mean±SD.

4.4.3. DNA accessibility of promoter regions

After confirming the transcriptional state of the four selected genes in the 3xTg-AD brain areas affected by the pathology, chromatin remodeling was investigated and correlated with changes in mRNA levels in order to explain their transcriptional dysregulation. Chromatin remodeling by the repositioning of nucleosomes around genes alters the accessibility of the transcription machinery to DNA (Cairns, B.R. 2009). To evaluate the presence of nucleosome in the promoter region of the gene, we use FAIRE, in which the final sample enrichment corresponds to the amount of promoter region accessible to transcription factors.

Bace1 promoter accessibility was not altered significantly in the 3xTg-AD mice although its mRNA levels were significantly increased at 15months-old suggesting that the *Bace1* upregulation in aged 3xTg-AD mice was not a result of nucleosome repositioning per se (Figure 4.6 e 4.10).

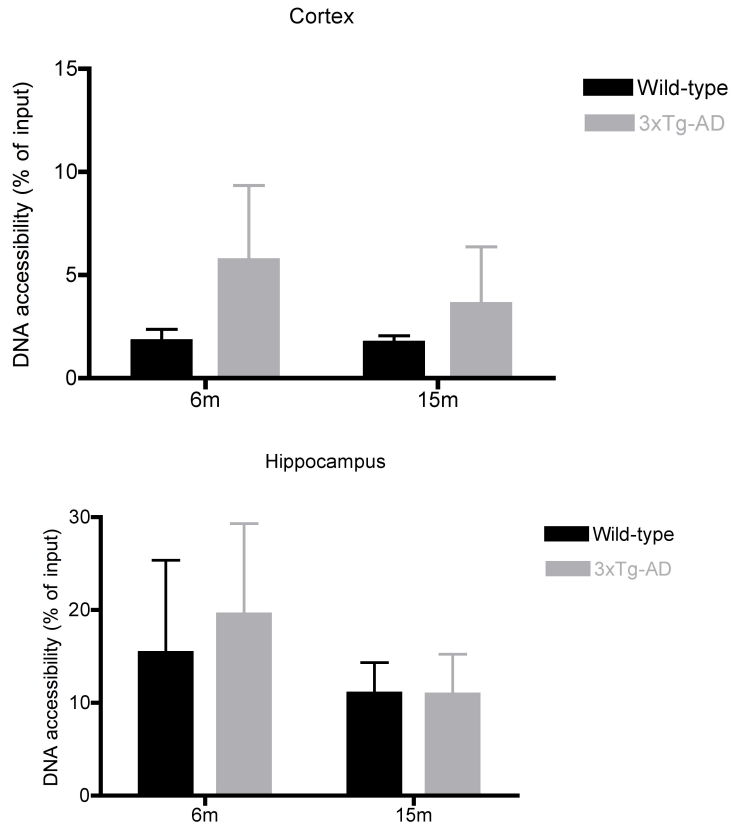


Figure 4.10 – DNA accessibility in the *Bace1* promoter region in wild type and 3xTg-AD mice. DNA accessibility in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice was calculated as the % of the respective input fraction, using the 2ΔCt formula for each condition. Results are presented as mean±SD.

Ncstn was downregulated in the cerebral cortex and hippocampus of 15-month-old 3xTg-AD mice (Figure 4.7), and this event was accompanied by a decrease in its promoter accessibility (Figure 4.11). Consequently, chromatin

remodeling may play a role in the decrease of *Ncstn* transcription at the later stages of AD pathology in this mouse model, and may represent a compensatory mechanism against the exacerbation of Aβ formation by β- and γ -secretases.

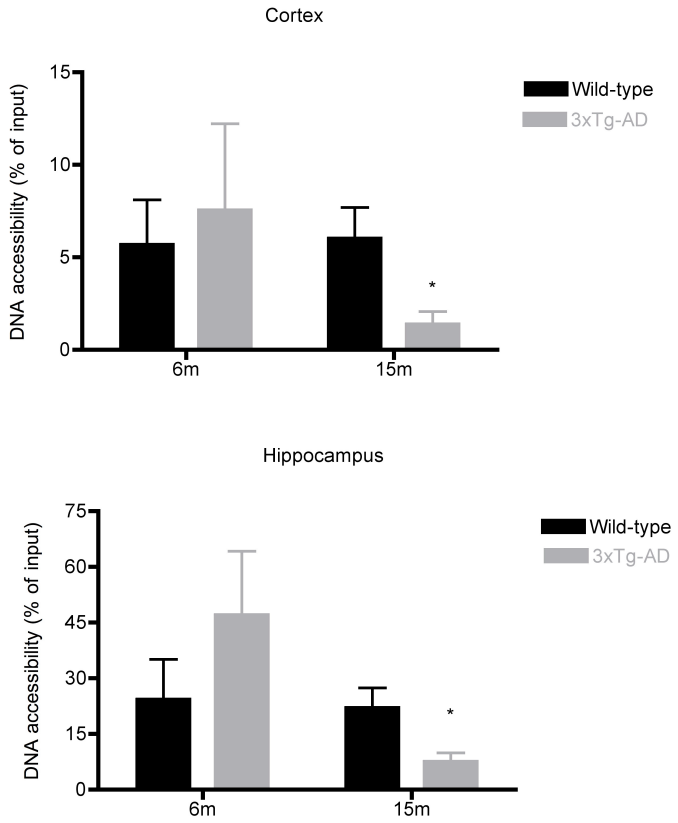


Figure 4.11 – DNA accessibility in *Ncstn* promoter region in wild type and 3xTg-AD mice. DNA accessibility in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice was calculated as the % of the respective input fraction, using the 2ΔCt formula for each condition. Results are presented as mean±SD. *$p<0.05$ significantly different from WT mice (Mann-Whitney U test).

Sirt1, which regulates several cellular functions (Chung, S. et al. 2010), has been shown to suppress γ-secretase activity in different models, thereby reducing Aβ formation (Donmez, G. et al. 2010). As previously reported in human AD brains (Julien, C. et al. 2009), the gene was shown to be downregulated in cortex and hippocampus in the 3xTg-AD brains (Figure 4.8). The accessibility to the *Sirt1* promoter also decreased in the cerebral cortex of older 3xTg-AD mice (Figure 4.12), which could translate into a decreased binding of the transcription machinery and may be part of the process behind the decrease in its mRNA levels in AD pathology. However, the decrease of mRNA observed in 15months-old transgenic hippocampi was not accompanied with alterations in DNA accessibility, implying that other mechanisms may be acting to the downregulation of the gene in the disease.

Adora2a gene did not present significant mRNA alterations in the brain of 3xTg-AD mice comparatively with WT mice, and therefore, we did not follow to the characterization of the chromatin in this gene promoter.

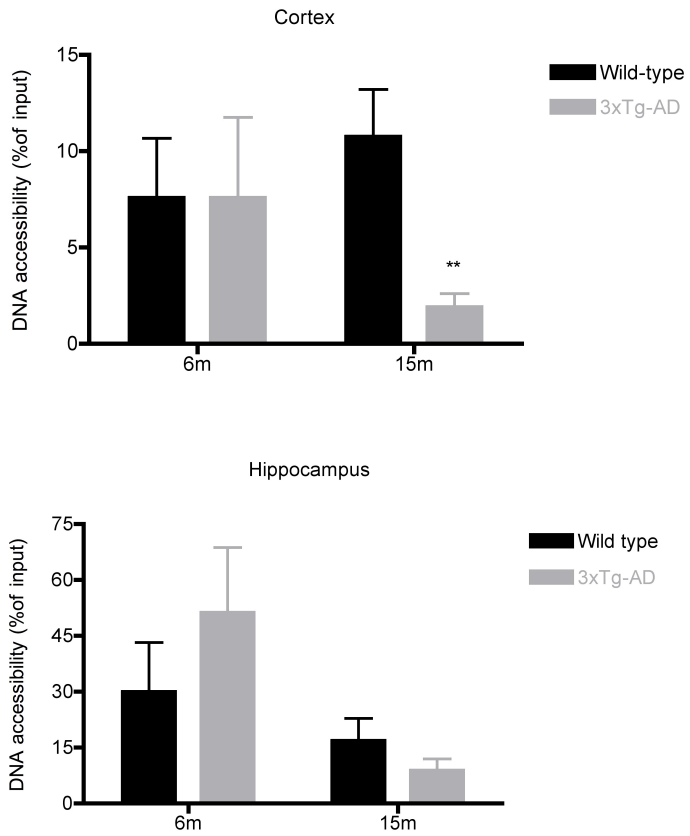


Figure 4.12 – DNA accessibility in *Sirt1* promoter region in wild type and 3xTg-AD mice. DNA accessibility in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice was calculated as the % of the respective input fraction, using the ΔΔ Ct formula for each condition. Results are presented as mean±SD. **p<0.01 significantly different from WT mice (Mann-Whitney U test).

4.4.4. Histone acetylation of promoter regions

In addition to chromatin remodeling processes, such as nucleosome positioning, histone tail modifications have also a profound impact in transcription, being histone acetylation in promoter regions an important marker of active genes (Li, B. et al. 2007). Therefore, we analyzed H3 acetylation in the promoter regions of *Bace1*, *Ncstn* and *Sirt1*, by ChIP, in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice.

Despite *Bace1* upregulation in 15-month-old 3xTg-AD mice in both brain regions, no further alterations in DNA accessibility was observed, indicating that the regulation of *Bace1* transcription in later stages of the disease may be due to mechanisms other than just nucleosome repositioning (Figure 4.6). In fact, we also found that *Bace1* promoter tended to be hyperacetylated in hippocampus and was significantly more acetylated in cortical regions in 15-month-old 3xTg-AD mice (Figure 4.13) in concordance with the mRNA increase. In this gene, evidences point out to a more refined regulatory mechanism of *Bace1* transcription that is histone tail chemical modifications, during the progression of AD.

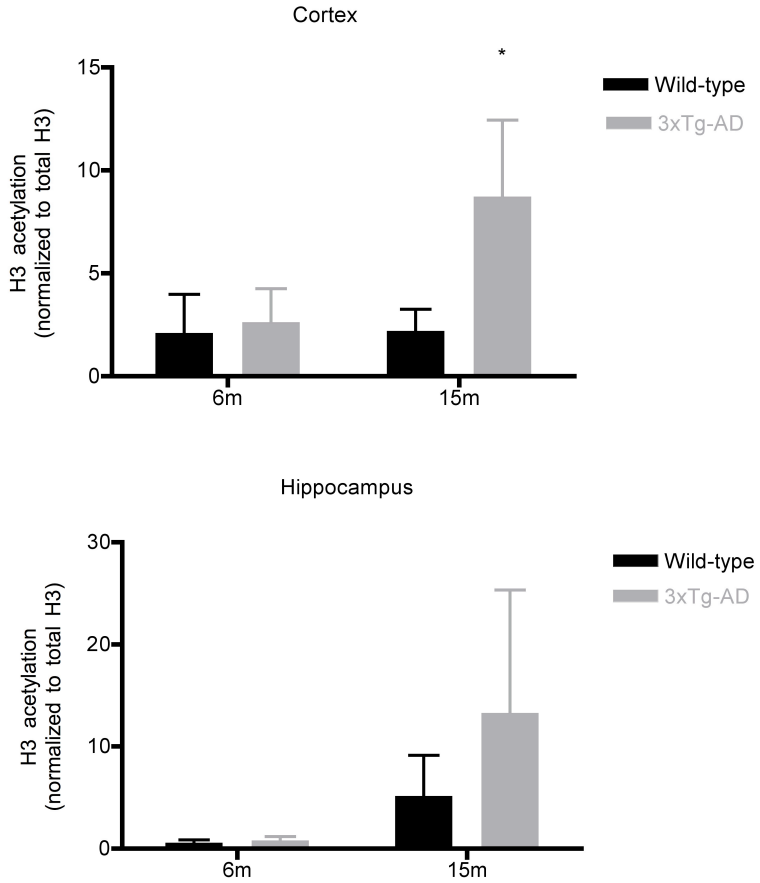


Figure 4.13 – Histone 3 acetylation in the *Bace1* promoter region in wild type and 3xTg-AD mice. Relative levels of H3 acetylation in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice were obtained calculating the % of input, using the ΔΔ Ct formula for each condition, and normalized to an intergenic region. Results are presented as mean±SD. *p<0.05, significantly different from WT mice (Mann-Whitney U test.

Ncstn was downregulated in the cerebral cortex and hippocampus of 15-month-old 3xTg-AD mice in comparison with age-matched WT mice (Figure 4.7) and this decrease was associated with a higher nucleosome positioning in the

promoter of the gene (Figure 4.11). However, H3 acetylation levels did not change significantly in these aged mice (Figure 4.14). Therefore, H3 acetylation does not seem to be the main responsible for the decrease in the transcription of the *Ncstn* gene that might otherwise arise from nucleosome repositioning in the promoter or from other processes such as CpG island methylation.

Similarly to *Ncstn*, *Sirt1* was downregulated in susceptible brain regions of 15-month-old 3xTg-AD mice (Figure 4.8) and could be partially due to nucleosome positioning since decreased DNA accessibility in *Sirt1* promoter occurred in the cortex of aged transgenic mice, (Figure 4.12). However, in both tissues, H3 acetylation tended to be increased in older 3xTg-AD mice (Figure 4.15). Therefore, other mechanisms, such as transcription factor or miRNA regulation might be responsible for the decrease in mRNA levels in the presence of acetylated *Sirt1*. This hypothesis will be further explored in the discussion section.

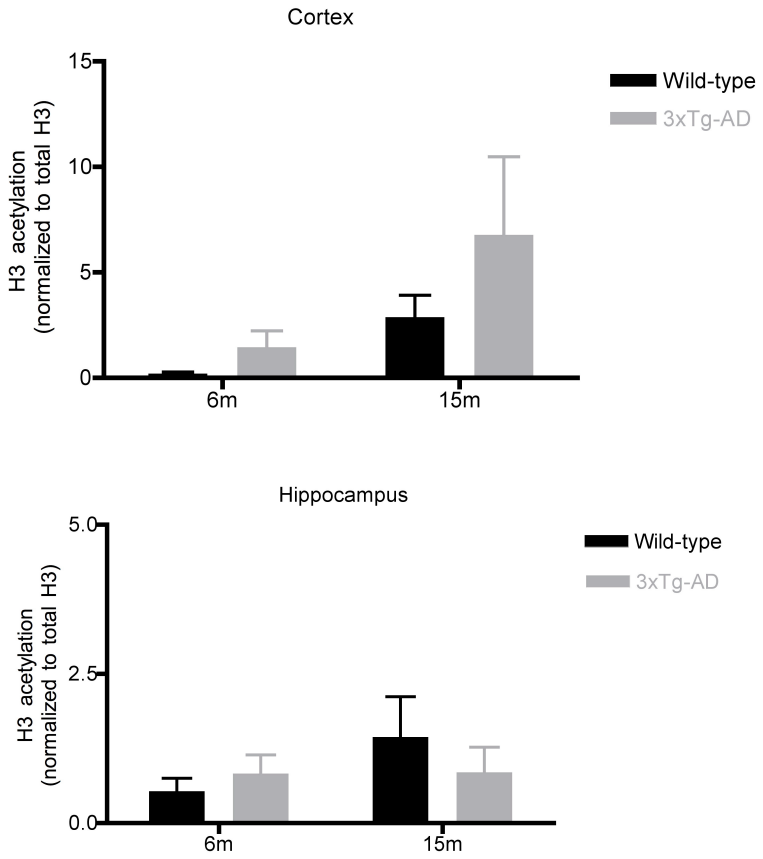


Figure 4.14 – Histone 3 acetylation in the *Ncstn* promoter region in wild type and 3xTg-AD mice. Relative levels of H3 acetylation in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice were obtained calculating the % of input, using the ΔΔ Ct formula for each condition, and normalized to an intergenic region. Results are presented as mean±SD.

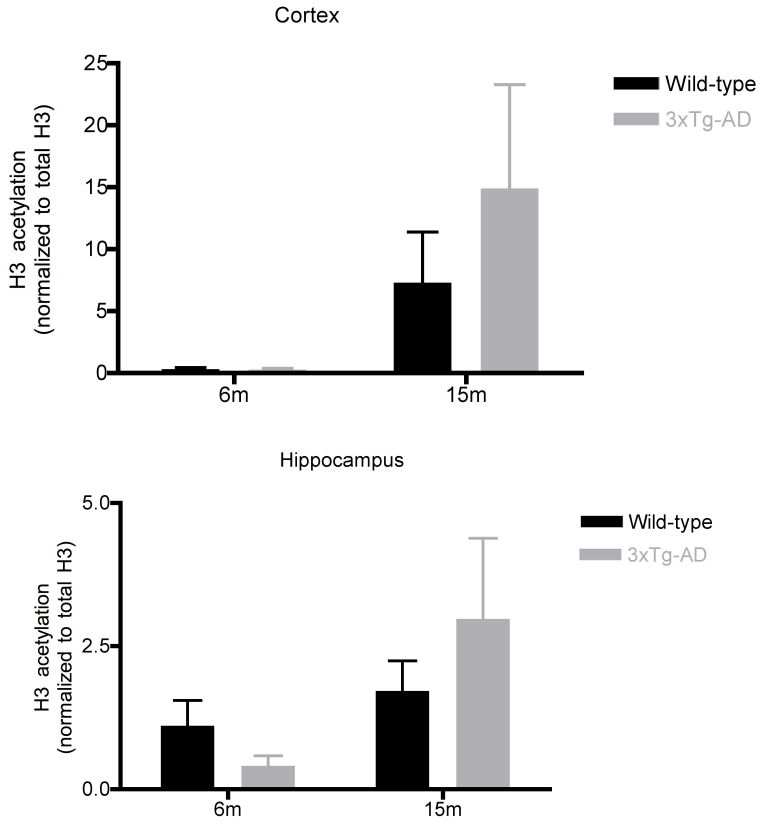


Figure 4.15 – Histone 3 acetylation in the *Sirt1* promoter region in wild type and 3xTg-AD mice. Relative levels of H3 acetylation in the cerebral cortex and hippocampus of 6- and 15-month-old WT and 3xTg-AD mice were obtained calculating the % of input, using the ΔΔ Ct formula for each condition, and normalized to an intergenic region. Results are presented as mean±SD.

4.5. Epigenetic dysregulation in Peripheral Blood Mononuclear Cells

The search for an effective blood-based biomarker for AD, as for other neurodegenerative diseases, where the primary affected tissue is unavailable, is a considerable challenge but also of great clinical value for an earlier and more accurate diagnostic (Shaw, L.M. et al. 2007).

In epigenetic studies, PBMCs seems to be a reliable source to search for chromatin alterations (Gavin, D.P. and Sharma, R.P. 2009). Consequently, and because until now no blood-based study in AD focused on the four previously discussed genes, we determined mRNA levels and nucleosome positioning on *BACE1*, *NCSTN*, *SIRT1* and *ADORA2A* in PBMCs isolated from controls (CT), MCI subjects and AD patients.

4.5.1. mRNA of dysregulated genes

An increase in *BACE1* mRNA levels was observed in PBMCs isolated from MCI subjects and AD patients in comparison with CT subjects, but this upregulation only reached statistical significance in the AD group (Figure 4.16). This result is concordant with the increased *BACE1* transcription reported in human AD brains (Coulson, D.T. et al. 2010) as well as in the cortex and hippocampus of aged 3xTg-AD (Figure 4.6).

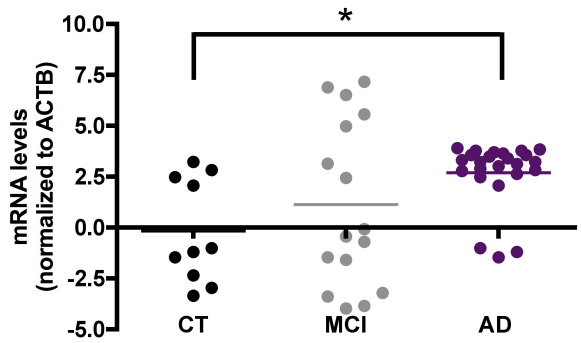


Figure 4.16 – Peripheral blood *BACE1* mRNA levels in Mild Cognitive Impairment and Alzheimer's disease. Relative quantitative analysis of *BACE1* mRNA levels by RT-PCR in PBMCs from CT, MCI or AD individuals, normalized to ActinB (*ACTB)*. *p<0.05 by Kruskal-Wallis test and Dunn's multiple comparison post-test.

NCSTN transcriptional state presented a tendency to increase in AD PBMCs, however without statistical significance. This tendency was interestingly opposite to the result obtained in 3xTg-AD brains, in which the gene was downregulated, which could be due to cell type specificity or to the fact that in the animal model, the γ -secretase complex may be affected by *PSEN1* mutation, and the *Ncstn* decrease could constitute a compensatory mechanism.

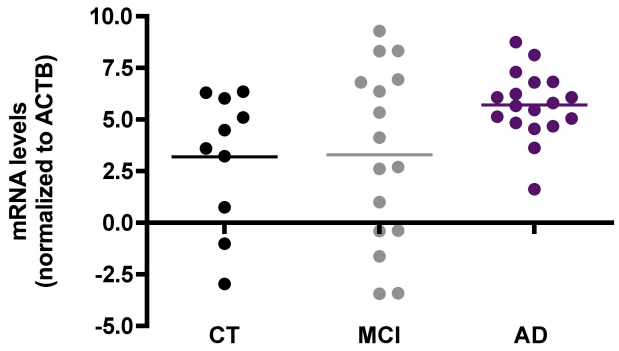


Figure 4.17 – Peripheral blood *NCSTN* mRNA levels in Mild Cognitive Impairment and Alzheimer's disease. Relative quantitative analysis of *NCSTN* mRNA levels by RT-PCR in PBMCs from CT, MCI or AD individuals, normalized to ActinB *(ACTB)*. Results are presented as mean±SD.

Although *SIRT1* was described to be decreased in the brain of AD patients (Julien, C. et al. 2009), as well as in brain of 15-month-old 3xTg-AD (Figure 4.8), *SIRT1* mRNA levels determined in PBMCs did not differ between CT, MCI subjects and AD patients (Figure 4.18). A similar pattern was observed for *ADORA2A* gene, for which the corresponding mRNA levels did not significantly changed in 3xTg-AD brains (Figure 4.9). These discrepancies could indicate tissue-specific dysregulation regarding these two genes.

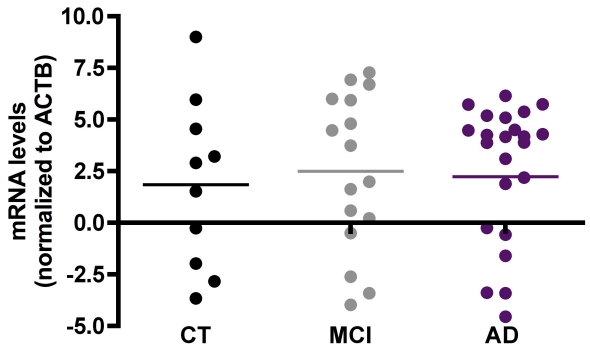


Figure 4.18 – Peripheral blood *SIRT1* mRNA levels in Mild Cognitive Impairment and Alzheimer's disease. Relative quantitative analysis of *SIRT1* mRNA levels by RT-PCR in PBMCs from CT, MCI or AD individuals, normalized to ActinB *(ACTB)*. Results are presented as mean±SD.

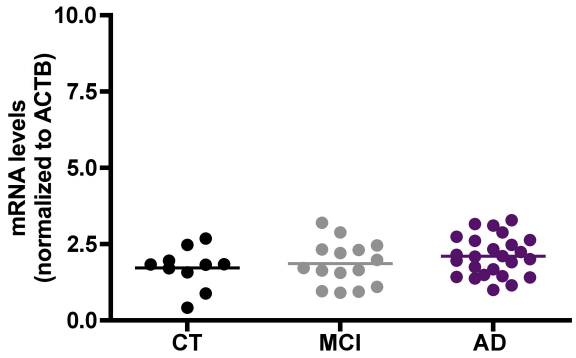


Fig 4.19 – Peripheral blood *ADORA2A* mRNA levels in Mild Cognitive Impairment and Alzheimer's disease. Relative quantitative analysis of *ADORA2A* mRNA levels by RT-PCR in PBMCs from CT, MCI or AD individuals, normalized to ActinB *(ACTB)*. Results are presented as mean±SD.

4.5.2. DNA accessibility of promoter regions

After the analysis of mRNA levels in peripheral blood samples of MCI subjects and AD patients versus non-demented healthy subjects, we characterized chromatin status in the promoters of the selected genes. However, due to the limited amount of blood that can be withdraw from each subject, the human PBMCs samples was used only for mRNA and FAIRE, since ChIP analysis needs a high quantity of input chromatin for the immunoprecipitation.

BACE1, that was upregulated in AD PBMCs (Figure 4.16) presented significantly less nucleosomes in its promoter regions comparatively with that determined in control subjects (Figure 4.20). Consequently, the access of promoters to transcription factors could be enhanced thus representing a mechanism underlying *BACE1* upregulation in AD. Surprisingly, despite the slightly increase in *BACE1* mRNA levels in MCI, the DNA accessibility was significantly decreased in comparison with controls (Figure 4.20). Since MCI represents a pre-clinical stage during AD progression, decreased DNA accessibility to *BACE1* promoter may be a compensatory mechanism triggered to avoid *BACE1* up-regulation and thus Aβ production and accumulation.

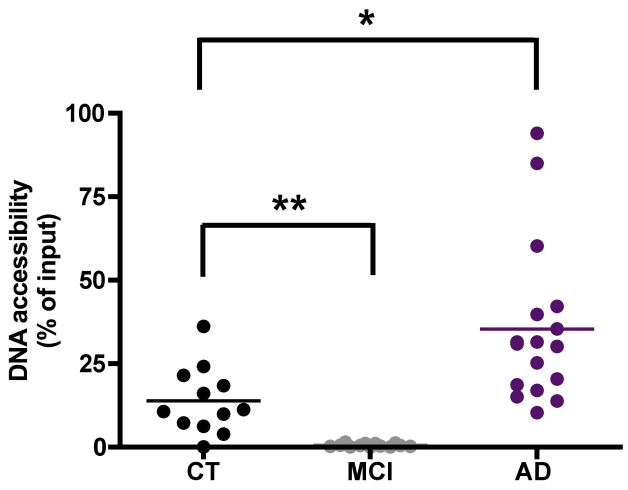


Figure 4.20 – DNA accessibility in *BACE1* promoter region in Mild Cognitive Impairment and Alzheimer's disease. DNA accessibility in PBMCs from CT, MCI and AD individuals was calculated as the % of the respective input fraction, using the ΔΔ Ct formula for each condition. Results are presented as mean±SD. *p<0.05, **p<0.01, significantly different from CT (Kruskal-Wallis test and Dunn's multiple comparison post-test).

Concordant with the *NCSTN* mRNA levels in AD (Figure 4.17), its promoter also presented the same tendency toward an increase of DNA accessibility (Figure 4.21). However, both parameters were not significant.

The mRNA levels of *SIRT1* and *ADORA2A* did not differ in PBMCs from controls, MCI subjects and AD patients (Fig. 4.18 and 4.19). Accordingly, the extent of DNA accessibility for *SIRT1* was not changed between these groups (Fig. 4.22). Regarding *ADORA2A*, it was not possible to detect accessible DNA in PBMCs from CT, MCI or AD individuals, probably indicating a closed chromatin status of *ADORA2A* promoter in this tissue.

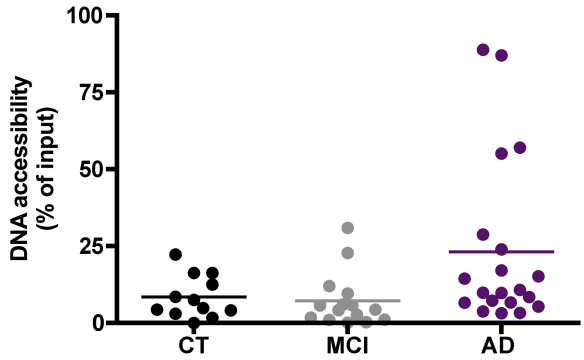


Figure 4.21 – Nucleosome positioning in *NCSTN* promoter region in Mild Cognitive Impairment and Alzheimer's disease. DNA accessibility in PBMCs from CT, MCI and AD individuals was calculated as the % of the respective input fraction, using the ΔΔ Ct formula for each condition. Results are presented as mean±SD.

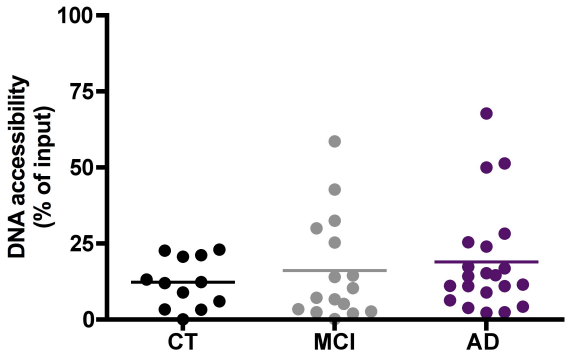


Figure 4.22 – Nucleosome positioning in *SIRT1* promoter region in Mild Cognitive Impairment and Alzheimer's disease. DNA accessibility in PBMCs from CT, MCI and AD individuals was calculated as the % of the respective input fraction, using the ΔΔ Ct formula for each condition. Results are presented as mean±SD.

5. DISCUSSION

AD is the leading cause of dementia in the elderly and due to its complexity, no effective treatment has been discovered yet (Alzheimer's, A. et al. 2011). Several risk factors have been described, being the aging process itself the most important (Yankner, B.A. et al. 2008). Epigenetics has provided possible mechanisms to the loss of plasticity observed in aging and a link between the environment and the pathology of complex disorders, such as cancer or neurodegenerative diseases (Fraga, M.F. and Esteller, M. 2007; Portela, A. and Esteller, M. 2010). Several evidences show that genetic dysregulation found in these complex processes, such as in cancer, is mediated at least in some extent by alterations in epigenetic modifications (Jones, P.A. and Baylin, S.B. 2007). In AD, the involvement of epigenetics in the pathology is starting to be evident, mainly by DNA methylation studies. The methylation status of several genes was found to be altered in AD brains in an age-specific pattern (Wang, S.C. et al. 2008). Furthermore, hyperhomocysteinemia was found to decrease methyl donor availability, which in turn, could induce demethylation of DNA and could result in overexpression of genes involved in AD pathology (Pacheco-Quinto, J. et al. 2006; Fuso, A. et al. 2011). Regarding the other main epigenetic component, chromatin remodeling, studies relating it to AD pathology are sparse, and involve basically HDAC inhibition, which seems to be neuroprotective (Kilgore, M. et al. 2010). However, these chemicals do not induce a gene-specific increase in histone acetylation, but rather a global

hyperacetylation of histones. This could give rise to activation of genes already upregulated and involved in AD pathology such as the case of *BACE1* or *APP* (Xu, W.S. et al. 2007).

In AD patients, several studies have described the presence of dysregulation of specific loci in AD (Papassotiropoulos, A. et al. 2006; Wu, Z.L. et al. 2006; Saetre, P. et al. 2011). Why these genes are affected is still under investigation but epigenetic modulators may play a role in the regulation of transcription in AD.

Taking these evidences into account, the aim of this study was to investigate the transcriptional alteration of four genes previously described to be involved in the pathogenesis of AD and to understand the role of chromatin remodeling as a mediator of this dysregulation. For this purpose, we used an animal model of AD (the triple transgenic mice model, 3xTg-AD *versus* age-matched wild type mice) and a human peripheral model (PBMCs from MCI subjects or AD patients versus age-matched controls).

BACE1 protein levels and activity is elevated in AD brains and CSF compared to controls (Yang, L.B. et al. 2003; Holsinger, R.M. et al. 2004). Despite this alteration, mRNA dysregulation is still under some controversy, since earlier studies did not find significant differences in *BACE1* mRNA levels in AD, but more recent works showed that the gene is indeed upregulated in AD *post-mortem* brains (Coulson, D.T. et al. 2010). Our results are concordant with this later study, since we found mRNA levels to be significantly increased in later stages of the pathology in the 3xTg-AD model and

progressively increasing in PBMCs isolated from CT to MCI and to AD. To understand the reason behind the transcriptional dysregulation of *BACE1*, we looked at the promoter chromatin status. In these older mice, that embody the pathological features present in later stages of AD and when the gene was upregulated in both areas, the promoter accessibility did not differ from that found in wild type mice but the level of H3 acetylated tended to be increased in hippocampi or was significantly increased in cortical regions. This result seems to indicate that the component of chromatin implicated in the transcriptional increase of the gene in later stages of the disease is the refined addition of chemical groups to histone, specifically acetyl groups, which are responsible for chromatin opening, allowing the access of transcription factors.

In PBMCs, we found a surprising pattern in nucleosome positioning in the MCI group. Despite the tendency to an increase of *BACE1* mRNA levels in MCI subjects, which correlates with the previously observed increased expression in the CSF (Zetterberg, H. et al. 2008), the DNA accessibility was decreased when compared to controls. In an applied point of view, this pattern could be further explored for the search of a MCI-specific biomarker, since there was very little variation between the MCI samples, indicating it is a consistent phenotype. In biological terms, this decrease in *BACE1* accessibility could represent a compensatory mechanism to counteract the gene upregulation. Indeed, several evidences now point to the existence of a plateau in a pre-AD period, which begins about 4 years prior to the

clinical diagnosis of AD and ends with a decline that probably contributes to the clinical diagnosis of AD (Smith, G.E. et al. 2007). This period seems to coincide with compensatory mechanisms involving redundant memory systems, up-regulation of neurotransmitters, or recruitment of other neural networks (Smith, G.E. et al. 2007). MCI, being a potential transitional state between normal aging and dementia, could occur during this plateau phase in which compensatory mechanisms seems to be occurring (Gigi, A. et al. 2010).

NCSTN is a component of the γ-secretase complex and was recently observed to be dispensable for its activity and cell surface transport although critical for its stabilization (Zhao, G. et al. 2010). Overexpression of wild type NCSTN increases Aβ production (Ma, Z. et al. 2009), indicating that the strict regulation of its expression may play a fundamental role in the pathogenesis of AD. However, alterations in its mRNA or protein levels during AD pathology have not been described until now. The only evidence of an altered *NCSTN* regulation is the presence of polymorphisms that increase the risk to developing AD (Helisalmi, S. et al. 2004; Zhong, L. et al. 2009) In our studies, we observed *Ncstn* downregulation in the cerebral cortex and in the hippocampus in 15-months-old 3xTg-AD mice, which was accompanied by a nucleosome repositioning around the promoter in the absence of changes in H3 acetylation. This finding supports that nucleosome remodeling may lead to a decreased accessibility of the transcription machinery and consequently a downregulation of the gene expression. Since NCSTN is part of the γ-secretase

complex, which will increase amyloidogenic processing of APP, we would expect the gene to be upregulated. Therefore, we hypothesize that this decrease in the *Ncstn* mRNA levels could represent a compensatory mechanism against the exacerbation of the γ-secretase activity caused by *PSEN1* mutation in these transgenic mice. Overexpression of mutant PSEN1 can make cells to respond to the affected γ-secretase activity by down-regulating *Ncstn* in an effort to destabilize the γ-secretase complex. *APP* and *PSEN1* mutations, together with the observed upregulation of *Bace1* in later stages, would contribute to the increased amyloidogenic processing of APP. The resulting production of Aβ would be so exacerbated in older mice that compensatory mechanisms, such as downregulation of *Ncstn*, could not be able to avoid its accumulation leading to the progression of the disease. In PBMCs, we only found a tendency to an increased transcription and nucleosome positioning in *Ncstn* promoter in the AD group compared to CT or MCI. However, these differences were not statistically significant.

SIRT1 was reported to be significantly reduced in the parietal cortex and hippocampus of AD patients, either at the mRNA and protein level. However, this alteration was not observed during the MCI stage (Julien, C. et al. 2009). This downregulation was shown to parallel the accumulation of tau and to be correlated with the decrease in cognitive scores (Julien, C. et al. 2009). In the 3xTg-AD mice, *Sirt1* mRNA levels were also demonstrated to be downregulated when tangles and plaques are present, concomitantly with cognitive deficits observed in 15-months-old animals (Oddo, S. et al.

2003). Interestingly, we found that the DNA accessibility of the gene decreased in cortical regions or was not altered in hippocampi while H3 acetylation of *Sirt1* promoter tended to be increased. SIRT1 is neuroprotective in AD, being important in Aβ clearance (Donmez, G. et al. 2010). Chromatin remodeling may be used to produce more Sirt1 by opening the chromatin through H3 acetylation of the gene promoter in a stage of the pathology when Aβ production should be highly increased (Oddo, S. et al. 2003). However, other mechanism may be responsible for the observed decrease in *Sirt1* levels. miRNA regulation may be a possible repressor mechanism for *Sirt1* since miR-34 and miR-132 were described as negative regulators of the gene (Yamakuchi, M. et al. 2008; Strum, J.C. et al. 2009). Another possibility, is the regulation of the gene by cAMP Response Element Binding (CREB) transcription factor. An increase of activated CREB helps to compete the residual Carbohydrate responsive element-binding protein (ChREBP) away from the *SIRT1* promoter, leading to *SIRT1* transcription (Noriega, L.G. et al. 2011). Since CREB phosphorylation and activity are described as decreased in AD patients and models or upon Aβ exposure (Yamamoto-Sasaki, M. et al. 1999), (Ma, Q.L. et al. 2007) this CREB shuttle out, could lead to ChREBP binding to the *SIRT1* promoter and its transcriptional repression. In PBMCs, *SIRT1* mRNA levels, as well as the nucleosome density in the *SIRT1* promoter, were not significantly altered between healthy controls and MCI subjects and AD patients, indicating that *SIRT1* dysregulation is limited to brain areas affected in AD.

ADORA2A gene was not yet described as dysregulated in AD, although A2AR levels are increased in animal models of AD and in AD human brains (Arendash, G.W. et al. 2006; Albasanz, J.L. et al. 2008). In our studies, *ADORA2A* mRNA was not altered in the 3xTg-AD mice neither in PBMCs of MCI or AD patients. This may indicate that the increasing levels of A2AR in AD should be caused by other regulatory mechanisms, such as post-transcriptional modifications or increased recycling of the receptors.

The 3xTg-AD model reproduces an interesting age-related progression of the disease and shows a similar pattern of brain areas specificity as in human cases (Oddo, S. et al. 2003). Consequently, it represents a good tool to analyze time and tissue- dependent molecular alterations and to characterize the pathological progression of AD. Despite the presence of the three mutations, which in some extent may influence the homeostasis of other genetic factors, such as the observed downregulation of *Ncstn* that may represent a compensatory mechanism against *PSEN1* increase, we can conclude from our studies that chromatin remodeling can underlie the transcriptional dysregulation of some genes, with a special emphasis for the late stages of the disease.

In peripheral samples of human patients, transcriptional regulation by chromatin effectors also seems to occur, showing that epigenetic modulation not only occurs in the affected brain tissues but also translates into alterations visible in AD PBMCs. This could represent a good starting point for the search of chromatin remodeling biomarkers in MCI and AD. The potential advantage of blood-

based biomarkers is obvious – obtaining blood is easier than almost any other body fluid, and blood-based tests lend themselves to high-throughput and cheap measurements. Despite some recent advances on blood-based markers for AD, the present biochemical blood tests included in clinical diagnostic are mainly to rule out other diseases, and so new markers that distinguish healthy subjects from patients with early cognitive deficits, such as MCI, and AD patients are of extreme value.

The implication of epigenetics in AD, especially by DNA methylation, is becoming increasingly evident. The results of this study obtained by single gene analysis show that chromatin remodeling is also important in the regulation of specific AD-related loci. Histone tail acetylation and/ or nucleosome positioning seem to be in part responsible for the transcriptional dysregulation of some genes in later stages of AD pathology, or to act as compensatory mechanisms for genes dysregulated by other modulators. Therefore, here were presented novel starting points for broader analysis of epigenetic regulation by chromatin modulation in AD pathology. The fact that such alterations are present in peripheral tissues, more accessible than the brain, also opens new promising prospects for the use of epigenetic modifications as biomarkers for AD stages and progression, especially the ever-present *BACE1* transcription upregulation in several AD models and the uniform closing of chromatin in this gene in MCI patients.

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