EPIGENETIC REGULATION OF BACE1 IN ALZHEIMER’S DISEASE PATIENTS AND IN TRANSGENIC MICE

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Abstract—In Alzheimer’s disease (AD) the complex interplay between environment and genetics has hampered the identification of effective therapeutics. However, epigenetic mechanisms could underlie this complexity. Here, we explored the potential role of epigenetic alterations in AD by investigating gene expression levels and chromatin remodeling in selected AD-related genes. Analysis was performed in the brain of the triple transgenic animal model of AD (3xTg-AD) and in peripheral blood mononuclear cells (PBMCs) from patients diagnosed with AD or Mild Cognitive Impairment (MCI). BACE1 mRNA levels were increased in aged 3xTg-AD mice as well as in AD PBMCs along with an increase in promoter accessibility and histone H3 acetylation, while the BACE1 promoter region was less accessible in PBMCs from MCI individuals. Ncstn was downregulated in aged 3xTg-AD brains with a condensation of chromatin and Sirt1 mRNA levels were decreased in these animals despite alterations in histone H3 acetylation. Neither gene was altered in AD PBMCs. The ADORA2A gene was not altered in patients or in the 3xTg-AD mice. Overall, our results suggest that chromatin remodeling plays a role in mRNA alterations in AD, prompting for broader and more detailed studies of chromatin and other epigenetic alterations and their potential use as biomarkers in AD.

Key words: Alzheimer’s disease, Mild Cognitive Impairment, 3xTg-AD mice, histone acetylation, chromatin remodeling, peripheral blood mononuclear cells.

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of dementia in the elderly. The majority of cases (~99%) have complex etiology due to both environmental and genetic factors, which alone do not seem sufficient for causing disease (Alzheimer’s et al., 2011). The role of environmental factors on complex diseases such as cancer or neuropsychiatric disorders can, to some extent, be explained by epigenetic modulation, mediated by DNA methylation, miRNA or chromatin remodeling. Indeed, aberrant DNA methylation, miRNA regulation and histone modification profiles have just begun to be described for neurodegenerative disorders (Hebert et al., 2008; Urdinguio et al., 2009; Chouliaras et al., 2010; Nunez-Iglesias et al., 2010; Long and Lahiri, 2011; Marques et al., 2011).

Epigenetic regulation, involving changes in the micro- and macrostructures of chromatin, differentially alters access of the transcriptional machinery to some genes while leaving access to other genes intact. Chromatin regulation can be accomplished by covalent modification of histones or by the action of ATP-dependent remodeling complexes (Grewal and Jia, 2007). Most genes are subjected to chromatin-mediated transcription regulation and the degree of chromatin effects varies from gene to gene (e.g., gene clusters, tissue-specific genes, housekeeping genes and stress response genes). However, in most cases, acetylation of histones sets the positive epigenetic state for transcriptional activation. Cellular homeostasis is regulated by a fine balance between histone acetylation and deacetylation. Any imbalance in this process leads to aberrant acetylation patterns, with hypoacetylation at loci that should be transcriptionally active and hyperacetylation at genes that should be repressive which together result in disease manifestation (Selvi and Kundu, 2009). Nucleosome dynamics is important because it regulates DNA accessibility, which is a key to proper gene regulation.

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and transcription fidelity. The precise position of nucleosomes around the transcription start site (TSS) has an important influence on the initiation of transcription (Cairns, 2009). Nucleosome loss can occur as a specific response to environmental stresses or signals, leading to transcriptional reprogramming (Jiang and Pugh, 2009).

Several studies demonstrated alterations in the global level of histone acetylation in AD or reported protection induced by histone deacetylase inhibitors (HDACi) in disease models. Despite the evidence that histone modifications indeed occur in AD, the pattern of changes is complex and could entail both increases or decreases in histone acetylation at specific loci (Mastroeni et al., 2011). Numerous studies reported transcriptional deregulation of specific genes in AD (Kong et al., 2005; Papassotiropoulos et al., 2006; Wu et al., 2006) but whether this involves post-translational modifications of histones is still largely unknown.

Here, we investigated whether a selected group of four AD-related genes was transcriptionally deregulated since variations in their normal expression are predicted to have a profound impact on the pathology (Table 1). To understand if the chromatin environment in the promoter of the selected genes is altered in AD we also evaluated the epigenetic regulation (Table 1).

**Table 1. Epigenetic regulation of the AD-related genes selected for analysis**

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**EXPERIMENTAL PROCEDURES**

**Animal brain samples**

Colonies of 3xTg-AD mice and wild-type (WT) background strain mice (kind gift from Frank La Ferla, University of California, Irvine, USA) were bred and maintained on 12-h light/dark cycles and provided *ad libitum* access to food and water, in accordance with the European Communities Council Directive (86/609/EEC) on this subject. 6- and 15-month-old WT or 3xTg-AD female mice were sacrificed by cervical dislocation and the brain was removed and divided in the two hemispheres. One half of the cortex and hippocampus were isolated for histone analysis and the other half was used for RNA analysis. All the samples were fast-cooled in liquid N₂ and then kept at –80 °C.

**Human PBMCs**

A total of 69 subjects (AD patients, MCI individuals and age-matched controls) participated in this study and were recruited at the dementia outpatient clinics, Hospitals of the University, Coimbra, and Hospital Santa Maria, Lisbon. Inclusion for MCI and AD was based on previously reported criteria (APA, 2000; Portet et al., 2006) and in order to implement these criteria, patients were subjected to clinical history, neurological and cognitive examination, laboratorial evaluation and brain imaging (CT-Scan or MRI). Cognitive evaluation included a comprehensive neuropsychological assessment using a normalized battery for the Portuguese population and the Mini Mental-State Evaluation (MMSE) (Folstein et al., 1975). Furthermore, severity of disease was classified according to the Clinical Dementia Rating Scale (CDR) (Morris, 1993) where a rating of zero indicates no cognitive abnormality, score 0.5 questionable or suspected deterioration and scores of 1, 2 or 3, respectively mild, moderate and severe dementia. Control subjects did not present evidence of cognitive deterioration or cognitive complaints, had a MMSE above cut-off for the Portuguese population and their value in the CDR 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 protocol investigation was approved by the Ethics Committees of both Portuguese clinical institutions and all participants, or respective caregivers, signed an informed consent before any procedure. Demographic and clinical characteristics of the participants are shown in Table 2.

Peripheral blood was withdrawn into EDTA-vacuum tubes and PBMCs were extracted using Ficol-Paque (GE Healthcare, Waukesha, WI, USA). PBMCs pellets were stored at –80 °C until use.

**Reverse transcription-polymerase chain reaction**

RNA was extracted by means of the Trizol® reagent. cDNAs were then obtained by reverse transcription reaction using the iScript Superscript in a My Cycler equipment (Bio-Rad Laboratories Headquarters, Hercules, CA, USA) and were measured by real-time

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PCR using SYBR Green Jumpstart Taq Ready mix’s instructions (Sigma-Aldrich, St. Louis, MO, USA) in a Rotor Gene Q equipment (Qiagen Sciences Inc., Germantown, MD, USA). cDNA levels were normalized for different primer efficiencies (Pfaffl, 2001) and versus Cyclophilin A (Ppia) for mice studies or Actin B (ACTB) for human samples as internal controls of basal, constitutive expression. Primer sequences are available in Table 3.

Formaldehyde-assisted isolation of regulatory elements (FAIRE)

Chromatin was extracted and used for FAIRE analysis (Giresi and Lieb, 2009). Samples were sonicated 15 × 20 seg in a MSE SoniPrep 150 (Thermo Fisher Scientific, Waltham, MA, USA). Input material was obtained from 5 μg of chromatin-DNA decrosslinked prior to DNA extraction. The same quantity was directly subjected to phenol–chloroform DNA extraction.

Chromatin immunoprecipitation (ChIP)

After sonication, chromatin was pre-cleared with Protein A agarose–salmon Sperm DNA beads (Millipore, Billerica, MA, USA) for half an hour at 4 °C. Anti-total and anti-acetylated H3 antibodies (Abcam, Cambridge, UK), at a concentration of 0.5 μg/μg chromatin, were added to pre-cleared chromatin and incubated overnight with rotation at 4 °C. Beads were pooled down at 1000g for 2 min at 4 °C and washed with a sequence of buffers with rotation for 5 min at 4 °C or room temperature (RT) (1 × Low Salt Immune Complex Wash Buffer; 1 × High Salt Immune Complex Wash buffer; 1 × LiCl Immune Complex Wash buffer and 2 × TE). Beads were then incubated for 15 min at RT with elution buffer (1% SDS, 0.1 M NaHCO3) and samples were decrosslinked with 0.2 M NaCl at 65 °C during 6 h to overnight and then treated for 20 min at 37 °C with 0.01 μg/μl RNase A. Finally, samples were digested for 1 h at 45 °C with 0.0125 M EDTA, 0.05 M Tris–HCl, pH 6.5 and 0.5 g/l Proteinase K. Digested samples were then processed for DNA extraction by the phenol/chloroform DNA extraction, ethanol-precipitated and resuspended in dEPC-water.

Real-time quantitative PCR

Extracted DNA from FAIRE or ChIPs were used for qPCR with Power SYBR green following the manufacturer’s instructions in a 7500 Fast Real time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). FAIRE samples were normalized versus the input fraction by the comparative CT method (2^ε/ΔCT method) (Schmittgen and Livak, 2008). Enrichment levels of ChIP samples were obtained by normalization over the input fraction and normalized to an intergenic region using the equation % input = 2^(ΔCT target − ΔCT input). The value of H3 acetylation enrichment was finally normalized in relation to the total H3 enrichment. Primers designed around the TSS are shown in Table 4.

Table 2. Clinical evaluation of the sample population participating in this study. CDR values represent the average of individual scores according to the scale by Morris (1993). Statistical significance was achieved by Kruskal Wallis with Dunn’s multiple comparison post test or by Chi-square for % women. *p < 0.05; **p < 0.01; ***p < 0.001, in comparison to CT; $p < 0.01$, in comparison to MCI

<table>
<thead>
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<th>CT (n = 16)</th>
<th>MCI (n = 22)</th>
<th>AD (n = 31)</th>
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<tr>
<td>Age (years)</td>
<td>67.5 ± 9.2</td>
<td>74.4 ± 7.7</td>
<td>76.7 ± 7.7</td>
</tr>
<tr>
<td>Education (score)</td>
<td>8.9 ± 5.9</td>
<td>5.5 ± 4.7</td>
<td>4.1 ± 3.7</td>
</tr>
<tr>
<td>% Women</td>
<td>56.3</td>
<td>54.5</td>
<td>80.6</td>
</tr>
<tr>
<td>MMSE (score)</td>
<td>29.5 ± 0.6</td>
<td>25.8 ± 3.4</td>
<td>16.9 ± 5.6</td>
</tr>
<tr>
<td>CDR (score)</td>
<td>0.5*</td>
<td>1.7 ± 0.6**$</td>
<td>$</td>
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For \textit{Bace1} gene, no significant alteration on DNA accessibility was observed in its promoter (Fig. 1 C, D), despite being upregulated. On the other hand, for the downregulated genes, \textit{Ncstn} and \textit{Sirt1}, the promoters were significantly less accessible in both or at least in one of the affected brain areas, respectively (Fig. 2 C, D and Fig. 3 C, D).

We then analyzed by ChIP the enrichment of histone H3 acetylation in the promoter regions of the selected genes as a marker of active transcription (\(n = 6\) animals per group). For the \textit{Bace1} promoter, despite the absence of obvious alterations in nucleosome density, we found increased acetylation in 15-month-old transgenic mice, which correlates with the increased mRNA levels (Fig. 1 E, F). \textit{Ncstn} and \textit{Sirt1}, both of which presented decreased accessibility in one or both areas, did not exhibit a significant change in H3 acetylation although the tendency was toward an increase (Fig. 2E, F and Fig. 3E, F).

\textbf{\textit{BACE1} gene is altered in PBMCs of AD patients}

To assess the transcriptional regulation of the four genes selected for our study, we analyzed their levels in human PBMCs of healthy controls, AD patients, and MCI subjects.

The mRNA levels and their promoter accessibility were analyzed by the same approaches utilized for the mouse model studies. However, due to limited availability of these samples we were not able to perform H3 acetylation analysis by ChIP.
In PBMCs from control, MCI and AD, only BACE1 showed a significant upregulation. BACE1 mRNA levels were significantly increased in AD patients and only slightly elevated in MCI compared to controls subjects. NCSTN, SIRT1 and ADORA2A mRNA levels were not significantly altered between the three groups although a trend toward an increase of NCSTN levels in AD could be observed.

In agreement with its upregulation, BACE1 promoter accessibility was also significantly higher in comparison with controls (Fig. 5A, B). In MCI samples, although a slight increase in mRNA levels was detected, a uniform and significant closing of chromatin in the BACE1 promoter was detected.

The accessibility of NCSTN, SIRT1 and ADORA2A promoter accessibility also did not differ significantly between controls, MCI subjects and AD patients, although for NCSTN the tendency was also toward an increase in AD patients (Fig. 5C–G).

DISCUSSION

Epigenetic alterations are responsible for gene deregulation in several human disorders, but their role in AD is still poorly understood (Esteller et al., 2000; Nguyen et al., 2001; Dempster et al., 2011). The present study was aimed at identifying epigenetic mechanisms underlying transcriptional alterations in AD-associated genes.

For this purpose we took advantage of the widely used 3xTg-AD mouse model that includes familial mutations in APP, PSEN1 and MAPT (encoding for Tau protein). Importantly, these mice develop both pathological hallmarks of AD (plaques and tangles) and display time- and tissue-dependent synaptic dysfunction and cognitive deficits. Therefore, we analyzed the transcriptional regulation of four genes directly or indirectly associated with AD (Bace1, Ncstn, Sirt1 and Adora2a) in the cortex and hippocampus of 6- and 15-month old 3xTg-AD compared to age-matched controls.
Both BACE1 protein levels and activity are elevated in AD brains and cerebrospinal fluid (Roberson et al., 2007; Stozicka et al., 2007). Despite these alterations, deregulation at the mRNA level is still controversial since no significant differences had been found in BACE1 mRNA in AD pathology (Whalley et al., 2006) until the recently described upregulation of the gene in AD post-mortem brains (Coulson et al., 2010). Our results are consistent with this upregulation, since Bace1 mRNA levels were significantly increased in later stages of the pathology in the 3xTg-AD mice, both in the cortex and hippocampus. In addition, the promoter region of the gene was significantly more acetylated without significant alterations in the promoter accessibility, which should translate in the observed Bace1 mRNA increase. These results indicate that H3 acetylation is a major mechanism involved in Bace1 upregulation at later stages of the pathology in 3xTg-AD mice.

NCSTN is critical for γ-secretase stabilization (Kong et al., 2005). Its overexpression increases Aβ production indicating that the strict regulation of its expression may play a fundamental role in AD pathogenesis (Papassotiropoulos et al., 2006). However, to our best knowledge, the mRNA levels of this gene during AD pathology had not been previously investigated. The only described alterations are the presence of polymorphisms that increase the risk to develop AD (Fukumoto et al., 2002; Yang et al., 2003). Here, we observed a downregulation in the brain of aged transgenic mice accompanied by a decreased accessibility in Ncstn promoter. This result supports that nucleosome remodeling may lead to a decreased accessibility of transcription machinery and consequently to gene downregulation. However, since the region analyzed included a CpG island, the gene could also be hypermethylated, which would compact chromatin and consequent decrease of DNA accessibility. Together, these two epigenetic mechanisms could have a greater effect on mRNA regulation explaining its decrease. Being part of the γ-secretase complex, in which PSEN1, (gene

Fig. 2. Ncstn transcriptional regulation. The mRNA level of Ncstn was analyzed by RT-PCR in the cortex (A) and hippocampus (B) of 6- and 15-month-old WT and 3xTg-AD mice (n = 5). Nucleosome repositioning (C, D) and histone H3 acetylation (E, F) in the promoter region surrounding the TSS of Ncstn were analyzed by FAIRE and ChIP, respectively in the same groups (n = 6). *p < 0.05 and **p < 0.01, significantly different from WT mice.
mutated in the 3xTg-AD mice), is the main effector, we were not expecting to observe a downregulation of Ncstn in the 3xTg-AD mice. This surprising decrease in Ncstn mRNA levels could represent a compensatory mechanism against the PSEN1 mutations. Recent evidences suggest that organisms have plastic compensatory responses against mutations in both specific and more promiscuous genetic interaction partners that vary among individuals. It is the combination of this variation that determines the outcome of each mutation in each individual (Burga

![Fig. 3. Sirt1 transcriptional regulation. A. The mRNA level of Sirt1 was analyzed by RT-PCR in the cortex (A) and hippocampus (B) of 6- and 15-month-old WT and 3xTg-AD mice (n = 5). Nucleosome repositioning (C, D) and histone H3 acetylation (E, F) in the promoter region surrounding the TSS of Ncstn were analyzed by FAIRE and ChIP, respectively in the same groups (n = 6). *p < 0.05 and **p < 0.01, significantly different from WT mice.](image)

![Fig. 4. Adora2a transcriptional regulation. The mRNA level of Adora2a was analyzed by RT-PCR in the cortex (A) and hippocampus (B) of 6- and 15-month-old WT and 3xTg-AD mice (n = 5).](image)
et al., 2011). Therefore, mutant human PSEN1 in transgenic mice could increase the γ-secretase activity, which the organism could try to counteract through Ncstn down-regulation, then leading to the destabilization of the complex. Nevertheless, at this stage this compensatory mechanism may not be sufficient to avoid the exacerbation of this pathway leading to the observed phenotype of disease progression in these mice. This could also explain why, despite Ncstn gene has a slight increase of H3 acetylation at both ages in the cortex, the mRNA does

![Fig. 5. Transcriptional regulation of BACE1, NCSTN, SIRT1 and ADORA2A in PBMCs. mRNA levels and DNA accessibility of promoters of BACE1 (A, B) NCSTN (C, D) SIRT1 (E, F) and ADORA2A (G) were analyzed by RT-PCR and FAIRE. *p < 0.05 and **p < 0.01 for significantly different groups.](image-url)
not mirror this increase due to a closed chromatin state in the promoter (by decreased DNA accessibility).

SIRT1 was reported as significantly reduced in the parietal cortex and hippocampus of AD patients, both at mRNA and protein level. However, this alteration was not observed in MCI patients (Julien et al., 2009). Here, we found that Sirt1 levels were downregulated in 15-month-old 3xTg-AD mice, a stage when tangles and plaques, as well as cognitive deficits, are present. The DNA accessibility of the Sirt1 promoter was decreased in the cortex and followed the same trend in the hippocampus. Since SIRT1 is a neuroprotective factor in AD (Donmez et al., 2010), the slight increase of acetylation observed could represent a strategy of the cells to produce more Sirt1 in a stage of the disease when Aβ production is highly increased. However, since the gene is significantly downregulated in both brain areas, even without the presence of chromatin modulation, other putative mechanisms may be responsible for its decrease, such as mRNA or the transcription factor CREB (Rouaux et al., 2003; Yamakuchi et al., 2008; Strum et al., 2009; Noriega et al., 2011).

Adenosine 2A receptors (A2AR) are highly expressed in animal models of AD (Arendash et al., 2006) and in the AD brain (Albasanz et al., 2008). However, the levels of the gene encoding the receptor (ADOR2A) were not yet described in AD. In our study, Adora2a mRNA was not altered in the 3xTg-AD mice suggesting that other transcription-independent mechanisms (such as increased recycling or post-translational modifications) may be responsible for its overexpression in AD.

Currently, we are still lacking an ideal biomarker for differential diagnosis, for tracking disease progression, and to measure treatment efficacy in AD. There is an urgent need to develop biomarkers that are sensitive and specific to AD pathology with positive and negative predictive value for the disorder. Therefore, we applied the same approaches described above for the 3xTg-AD model to blood samples (PBMCs) from AD patients in the hope of finding a pattern that could be validated as a biomarker. Indeed, PBMCs provide a reliable means for studying the impact of environment/life experiences on chromatin structure and DNA methylation. This cell type contains the full complement of epigenetic enzymes and machinery found in most tissues and can reflect overall abnormalities in epigenetic mechanisms present in the brain (de Ruijter et al., 2003; Fraga et al., 2005; Anderson et al., 2008). We compared the results from AD patients with PBMCs from control subjects and MCI patients. Since MCI is an important risk factor for AD and is thought to represent a transitional state between normal aging and AD, it will be crucial to understand the early molecular mechanisms implicated in AD.

The expression of BACE1 in PBMCs was studied previously, and reduced levels of BACE1 mRNA were found in PBMCs compared to the brain. However, to the best of our knowledge, no study had tried to identify changes in BACE1 mRNA levels in PBMCs from AD and non-demented subjects (Decourt and Sabbagh, 2011). Here, we showed for the first time an upregulation of BACE1 mRNA in PBMCs isolated from AD patients, and FAIRE analysis also clearly indicated that the promoter region of this gene is significantly more accessible in AD, which is consistent with a higher transcription of the gene. MCI subjects displayed a slight, non-significant increase in BACE1 mRNA levels, intermediate between controls and AD patients. Our results suggest that the expression of BACE1 is proportional to the severity of the cognitive impairment and that, therefore, BACE1 mRNA levels could be a biomarker for AD progression. Interestingly, the accessibility to the BACE1 promoter was dramatically decreased in MCI patients. This decrease could be caused by a compensatory mechanism to counteract the beginning of BACE1 upregulation. In fact, recent evidence points to the existence of a plateau in pre-clinical AD that coincides with several complementary models of compensation, such as neurotransmitter upregulation, recruitment of broader neural networks to maintain performance level, upregulation in cholinergic activity and presence of neurotrophic factors in regions that subserves memory function, particularly early in the disease course (Smith et al., 2007).

The increase of BACE1 mRNA levels in PBMCs may also indicate that a proportion of peripheral Aβ could result not only from the production in the brain but also from processing of APP in these peripheral cells, since Jung and collaborators presented evidences that this cell type displays cell-surface APP immunoreactivity (Jung et al., 1999).

Most importantly, the increase of BACE1 mRNA/DNA accessibility in AD patients and the homogenous closing of chromatin in MCI patients could pave the way to new potential biomarkers for MCI and AD.

In human samples, in contrast to 3xTg-AD, in which the downregulation may be compensatory, NCSTN mRNA levels and promoter accessibility showed a trend toward upregulation in AD PBMCs, in agreement with its function on the γ-secretase complex, although the variation did not reach statistical significance.

Although SIRT1 has been reported as significantly reduced in AD brains (Julien et al., 2009), no evidence of SIRT1 mRNA alterations in PBMCs from AD patients was observed in our study. This could indicate a tissue-specific alteration of this gene limited to the brain in AD. Similarly, no alterations in ADORA2A regulation or levels were observed in the peripheral blood of MCI or AD patients.

Taken together, our results strongly support the idea that specific gene deregulation in AD could be caused by epigenetic modulation. We presented new starting points for broader analysis of epigenetic regulation by chromatin modulators in AD pathology and the fact that such alterations are present in peripheral tissues also opens 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.

Acknowledgements—We are grateful to Dr. Frank M. LaFerla (UC Irvine Institute for Memory Impairments and Neurological Disorders, CA, USA) for providing 3xTg-AD mice.

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