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Alzheimer's & Dementia ■ (2016) 1-12

Alzheimer's & Dementia

Featured Article

Circadian alterations during early stages of Alzheimer's disease are associated with aberrant cycles of DNA methylation in BMAL1

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Abstract

Introduction: Circadian alterations are prevalent in Alzheimer's disease (AD) and may contribute to cognitive impairment, behavioral symptoms, and neurodegeneration. Epigenetic mechanisms regulate the circadian clock, and changes in DNA methylation have been reported in AD brains, but the pathways that mediate circadian deregulation in AD are incompletely understood. We hypothesized that aberrant DNA methylation may affect circadian rhythms in AD.

Methods: We investigated DNA methylation, transcription, and expression of *BMAL1*, a positive regulator of the circadian clock, in cultured fibroblasts and brain samples from two independent cohorts of aging and AD.

Results: DNA methylation modulated rhythmic expression of clock genes in cultured fibroblasts. Moreover, rhythmic methylation of *BMAL1* was altered in AD brains and fibroblasts and correlated with transcription cycles.

Discussion: Our results indicate that cycles of DNA methylation contribute to the regulation of *BMAL1* rhythms in the brain. Hence, aberrant epigenetic patterns may be linked to circadian alterations in AD.

Keywords: DNA methylation; Circadian rhythms; Methylation cycles; Alzheimer's disease; BMAL1; Neurodegeneration; Epigenetics; Fibroblasts; Circadian clock; Brain

1. Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder of aging worldwide. AD is characterized by accumulation of neuritic plaques and neurofibrillary tangles in the brain, accompanied by cognitive impairment and memory loss [1]. Disturbances in circadian rhythms affect almost all patients with AD, evidenced by altered sleep/wake cycles, thermoregulation [2], and exacerbations of cognitive impairment and confusion during the evening ("sundowning") [3,4], and represent a major cause of hospitalization and morbidity in AD [5]. Recent basic and clinical studies have revealed correlations in circadian regulation with amyloid-β (Aβ) production and clearance [6]; therefore, disturbance of this pathway may contribute to AD pathogenesis [6] and provide a novel therapeutic target in slowing or reversing the progression of AD.

In mammalian cells, circadian rhythms are generated by the transcriptional/translational oscillation of the core

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http://dx.doi.org/10.1016/j.jalz.2016.10.003

components of the biological clock, including the positive regulators *BMAL1*, *CLOCK*, and *NPAS2*, and the negative regulators *CRY1/2* and *PER1/2/3* [7]. These rhythms subsequently regulate the expression of >10% of the transcriptome, synchronizing metabolic and physiological functions to predictable changes in the environment [8]. At the organism level, circadian rhythms are coordinated by the main pacemaker located in the suprachiasmatic nucleus (SCN) that signals to peripheral oscillators throughout the body [9]. Recently, transcriptome-wide analysis in postmortem human brains revealed rhythmic patterns of gene expression in regions outside the SCN, including prefrontal cortex and hippocampus [10,11].

Epigenetic mechanisms contribute to the regulation of the circadian clock. Transient changes in histone modifications modulate clock gene transcription [12], and rhythmic methylation cycles have been uncovered in the SCN master clock in rodents [13] and in secondary oscillators in the human frontal cortex [14]. Although significant changes in methylation have been reported in AD brains [15] and altered circadian methylation rhythms have been noted in the human frontal cortex in the context of AD [14], the pathways that mediate circadian deregulation in AD are yet not fully elucidated, and nothing is known about epigenetic mechanisms as they pertain to the clock in AD.

We investigated whether aberrant DNA methylation contributes to circadian deregulation. Using fibroblasts and postmortem brain samples from two large AD cohorts and matched control subjects, we provide strong evidence for a role of DNA methylation cycles in the regulation of rhythmic *BMAL1* transcription in the brain and gene expression rhythms in cells, providing a link between epigenetic and circadian alterations associated with AD.

2. Methods

2.1. Study populations

We evaluated 66 postmortem frontal cortex samples (Brodmann's area 9) from the Shiley-Marcos Alzheimer's Disease Research Center (ADRC) at the University of California, San Diego (UCSD) (Supplementary Table 1). The parent study was reviewed and approved by the UCSD Human Research Protections Program. All subjects provided written informed consent before participating and donating tissue. We analyzed DNA 5mC methylation and RNAseq data from 396 participants from the Religious Orders Study and Rush Memory and Aging Project (ROS/MAP) (Supplementary Table 1). All participants provided written informed consent and signed an Anatomic Gift Act. The studies were approved by the institutional review board of Rush University Medical Center. Recruitment and assessment of these studies are reported elsewhere [16,17].

2.2. BMAL1 gene expression

RNA was extracted from frozen brain samples or from 1×10^6 fibroblasts using the RNeasy Lipid Tissue Mini kit (Qiagen). Total RNA (1 μg) was reversed-transcribed with SuperScript VILO complementary DNA synthesis kit (Life Technologies). Quantitative real-time polymerase chain reaction (qPCR) was run in duplicate samples with a probe that detects three human *BMAL1* isoforms (Taqman Hs00154147; Life Technologies) or the corresponding mouse Bmal1 transcripts (Taqman Mm00500226; Life Technologies). Probes detecting species-specific β-actin transcripts, known to be stably expressed across the 24 hours of the day [18], were used as internal control for both human and mouse assays. Relative transcript abundance was calculated as the inverse ratio to threshold cycle (1/dCt) and normalized using the lowest and highest levels as 0% and 100%, respectively.

Detailed RNAseq methods and analysis for ROS/MAP cases are reported elsewhere [14].

2.3. BMAL1 protein levels

Protein analysis of brain BMAL1 was performed on $n = 10$ subjects per group, selected according to their time of death (TOD) to include representative samples distributed across the 24 hours of the day. Extracts were obtained from 200 mg of cortical tissue according to the nuclear fractionation protocol (Abcam). Western blotting was performed as described [19] using anti-BMAL1 antibody (ab140646, 1:1000; Abcam) and anti β-actin (ab8227, 1:1000; Abcam). Quantity One (v.4.6.9; BioRad) was used for densitometry analysis. Data were expressed as the normalized ratio of BMAL1/ACTIN densitometry levels (normalization was performed using the lowest and highest values per group as 0% and 100%, respectively). Detection of BMAL1 on fixed human fibroblast cultures was performed as described previously [19] using anti-BMAL1 antibody (ab93806, 1:250; Abcam) and FITC-anti rabbit secondary (1:75; Vector).

2.4. DNA methylation

DNA methylation in brain samples was assessed in the ADRC cohort using the Illumina Infinium Human Methylation 450k BeadChip (Illumina) as described [20]. Briefly, genomic DNA extracted from frozen cortical tissue (DNeasy Blood & Tissue Mini kit; Qiagen) was bisulfite converted (EZ DNA Methylation kit; Zymo) and used for genome-wide methylation profiling at the UCSD IGM Genomics Core, under standard protocols (Illumina). The methylation status of a specific CpG site was expressed as β values, calculated as the ratio of the fluorescence intensity signals of the methylated (M) and unmethylated (U) alleles, using GenomeStudio Software (Illumina). For each individual subject, we calculated average β values for each of the 24 probes