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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.

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AUTHOR CONTRIBUTIONS

P.C. performed brain and fibroblast methylation, gene transcription and protein analysis. M.M. carried out circadian analysis on fibroblasts and contributed with data analysis and interpretation. A.L. analyzed transcription and methylation data from ROS/MAP cohorts. D.S. contributed with clinical analysis and case selection criteria. E.M. performed neuropathological analysis and advised on case selection. D.G. performed clinical studies. P.C., M.M., A.L., E.M., P.D.J., D.B. and P.D. contributed to manuscript writing. P.D. designed and supervised the study and performed data analysis and interpretation.

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 *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 of circadian rhythms affect almost all AD patients, 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 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 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 physiologic 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 post-mortem 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 controls, 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 at UCSD (Supp. Table 1). The parent study was reviewed and approved by the UCSD Human Research Protections Program. All subjects provided written informed consent prior to 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) (Supp. 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 μ g) was reversed-transcribed with SuperScript VILO cDNA synthesis kit (Life Technologies). Quantitative real-time PCR (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 hr 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 subject per group, selected according to their time of death to include representative samples distributed across the 24 h of the day. Extracts were obtained from 200 mg cortical tissue according to the nuclear fractionation protocol (Abcam). Western blotting was performed as described [19] using anti-BMAL1 antibody (Abcam, ab140646, 1:1000) and anti β -Actin (Abcam, ab 8227, 1:1000). 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 (Abcam, ab93806, 1:250) and FITC-anti rabbit secondary (Vector, 1:75).

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 interrogated by the array that map onto the *BMALI* gene. Methods of DNA isolation, array processing and data analysis on the ROS/MAP cohort are described in detail elsewhere [14].

2.5-Fibroblast analysis

Primary human fibroblast cultures were established as reported [21] from dermal punch biopsies taken from healthy control individuals and patients diagnosed with probable AD participating in a longitudinal study at the UCSD ADRC (approved by UCSD IRB). Biopsies (4 mm punch) were taken from the back of the forearm. After collection, biopsy material was immediately processed by a technician to establish cell lines. Human fibroblasts were cultured in DMEM containing 15% fetal bovine serum (FBS), L-glutamine, NAA and penicillin/streptomycin. At the time of experiments, cell lines had been passaged 3–10 times. NIH3T3 cells stably expressing the *Per2::luc* reporter were developed in our laboratory as reported previously [22] using a construct reported in detail elsewhere [23]. NIH3T3 cells were cultured under similar conditions as the human cells, but with the addition of Hygromycin B (200 $\mu\text{g}/\text{mL}$) as selection marker for the *Per2::luc* reporter. In order to make lumicycle experiments comparable to PCR time course assays, cells were synchronized in both experiments by changing the growth media to serum free DMEM, conditions that produce similar results to those employing serum shock or other stimuli [24].

2.6-Luminometry and circadian analysis

Case-control matching was maintained during cell culture procedures and experiments. Human fibroblast cells were split and grown in 35 mm plates to 70% confluence. Cells were then transduced with a *Per2::luc* lentiviral reporter construct (generously provided by Andrew Liu, University of Memphis). Viral particles were produced by the UCSD viral vector core facility. Additional details of the lentiviral construct have been published previously [24]. Infections were performed using 1×10^7 infectious units/plate and polybrene 0.1% for the 48 h prior to starting rhythm measurements. At the time of luminometry, cells were fully confluent (approximately 1.2×10^6 cells/plate). To synchronize rhythms, growth medium was replaced with HEPES-buffered, serum-free recording media containing 1 mM luciferin (Biosyth) immediately before luminometry [24]. Rhythm measurements were conducted in a luminometer (Actimetrics) as described [25]. Photoemissions from each cell line were measured in duplicate for 70 s, every 10 min over 5 days at 35°C. For drug experiments employing 5-Aza-2'-deoxycytidine (5-Aza-dC, Cayman Chemical;) or S-Adenosyl methionine (SAM from Cayman Chemical), drug or vehicle (PBS) was added to

the recording media, and administered through a single media change at the onset of luminometer recording and remained present in the growth medium over the course of the experiment. None of the drug concentrations used significantly decreased viability. At the conclusion of the experiment, background subtracted luminometry data were fit to a damped sine wave by the least squares method using commercial software (Lumicycle Analysis). To reduce signal artifacts, the first 14 h (human cells) or 24 h (mouse cells) were excluded. Rhythm parameters (amplitude, phase, period) were then estimated for each trace and averaged across replicates.

2.7-Quantification of DNA methylation in fibroblasts

Global methylation levels were determined by ELISA (MethylFlash methylated DNA quantification kit, Epigentek) on 100 ng genomic DNA isolated from NIH3T3 fibroblasts treated with 50 μ M 5-Aza-dC; 1 μ M SAM or vehicle for 48 h, as per manufacturer's instructions. Methylation at *BMAL1* cg13286116 was determined on synchronized human fibroblast cultures from 2 control and 4 AD lines, sampled at the indicated times. Bisulfite-converted DNA was amplified using primers 5'-atggagaagtattatagaagattgg-3' (sense) and 5'-acctatatccctaactccaac-3' (antisense). PCR products were subcloned into TOPO-TA vector (Life Technologies). Finally, 6 clones per cell line were sequenced. Analysis was performed using the Quantification Tool for Methylation Analysis (QUMA [26]) and expressed at each time point as the percentage of clones in each line that were fully methylated at cg13286116.

2.8-Statistic analysis

Statistical analysis of fibroblast-based assays was performed using one-way ANOVA adjusted for multiple observations and followed by Tukey's post-test with a significance of $p < 0.05$ or non-parametric Mann Whitney *t* test (unpaired; two-tailed) with a significance of $p < 0.05$. Pearson's correlation was used to analyze the association between DNA methylation levels and transcription at *BMAL1*. Fisher *r*-to-*z* transformation was used to investigate the significance of the difference between correlation coefficients across groups. Analyses were performed in Prism 6 (GraphPad, San Diego). Results are shown as average with S.E.M. as indicated. To investigate the oscillatory pattern of methylation, average β values were plotted as a function of time of death and non-linear fitting to sinusoidal curves with a fixed period of 24 h was performed as indicated.

We characterized 24-hour rhythms of DNA methylation separately in each of the diagnostic subgroups of the ROS/MAP cohort (Supp. Table 1) using cosine functions; described in detail in Supp. Methods.

3. RESULTS

3.1-Circadian cycle abnormalities in fibroblasts from individuals with AD correlate with aberrant *BMAL1* expression

To understand the circadian alterations associated with AD pathology, we started by studying human fibroblasts cultures derived from 13 patients diagnosed with probable AD and 7 age-matched healthy controls (Fig. 1A). Time-course analysis in synchronized

cultures showed a delayed expression peak (acrophase) of *BMAL1* transcription in AD compared to control cells (Fig. 1B). Quantification of nuclear BMAL1 content at the times of transcript peak and nadir also showed significant differences between AD fibroblasts and control cells, even when the effect of transcriptional changes on protein abundance may appear later and may also be affected by protein stability and turnover (Fig. 1C–D).

To evaluate whether these changes were associated with the core transcriptional clock, we used a bioluminescent reporter (*Per2::Luc*) to record circadian oscillations longitudinally in real time [24, 25]. We transduced cultured fibroblasts with the *Per::Luc* reporter construct and recorded emissions during five days in culture. Exogenous reporter constructs usually contain a small fragment of the promoter region and may not undergo epigenetic changes resulting from AD. Therefore, in order to study the effects of epigenetic modification of *BMAL1*, it is necessary to use a reporter of *BMAL1* output, like *Per2::Luc*. Since these clock genes are tightly linked, epigenetic modifications of *BMAL1* that impact transcription, should be reflected in *Per2* expression. Notably, AD cells showed a significant phase advance and changes in cycle amplitude and period compared to controls (Fig. 1E–F), suggesting an association between molecular circadian alterations with AD. Period length, amplitude and phase were not significantly correlated with gender or age (Supp. Fig. 1). However, we observed a significant correlation between circadian phase (Tmax) measured in fibroblasts and the morningness scores determined in a subset of participants who completed the Basic Language Morningness Scale (BALM) [27] questionnaire, suggesting that individual differences in donor chronotype may be reflected in cell culture (Supp. Fig. 2).

3.2-DNA methylation changes modulate circadian cycles in cultured fibroblasts

Recently, 24 h methylation rhythms were described in the human frontal cortex, cycles that appear to be correlated with age and dementia [14]. Moreover, changes in DNA methylation were also reported to mediate the effects of day length on clock plasticity [13]. To directly test whether DNA methylation modulates circadian rhythms in cells, we treated NIH3T3 fibroblasts with 5-Aza-2' deoxycytidine (5-AZA-dC), a cytidine analog that inhibits DNA methyl-transferases, to decrease global DNA methylation; or exposed fibroblasts to the methyl donor S-adenosyl-methionine (SAM), to increase methylation (Fig. 2I). We observed concentration-dependent alterations in circadian rhythms in response to both treatments (Fig. 2A–B). Reduced methylation resulted in shorter period length, advanced phase and increased amplitude (Fig. 2C–E). Higher methylation elicited the opposite effects, with increased period length, delayed phase and decreased rhythm amplitude (Fig. 2F–H). Importantly, these drug-induced changes in methylation had specific effects on *Bmal1* transcription (Fig. 2J). To determine if methylation changes may also be involved in the alteration of circadian cycles in AD fibroblasts, we performed preliminary studies on a subset of the human fibroblasts lines (n=3 control and 3 Prob. AD). Treatment with 50 μ M 5-Aza-dC induced similar changes in phase, amplitude and period as those observed for NIH3T3 cells, although the magnitude of the changes was lower (Fig. 2K). These results support a role for DNA methylation in modulating the circadian clock, likely involving transcriptional changes in *Bmal1*, and provide a link between epigenetic disruption and circadian alterations in AD.

3.3-Rhythmic DNA methylation patterns correlate with transcription of *BMAL1* and are significantly dysregulated in the brains from patients with early AD

Based on these *in vitro* results, we performed a cross-sectional analysis in midfrontal cortex human postmortem samples. We included 66 samples from the Shiley-Marcos Alzheimer's Disease Research Center (ADRC) that were stratified according to ante-mortem cognitive evaluation and postmortem neuropathological burden (Supp. Table 1). To investigate the role of DNA methylation on *BMAL1* expression we interrogated a genomic region of >90 Kb covered by the Illumina 450k Methylation array, that corresponded to the promoter; 5'-untranslated region (5'UTR), and a portion of the coding sequence (CDS) of *BMAL1* (Fig. 3A). Overall, CpG sites at the promoter, including those at the CG island, appear unmethylated, while CpGs located at the 5'-UTR and CDS show intermediate and high methylation levels respectively. This pattern is preserved in control and AD brains (not shown). To evaluate rhythmic patterns of variation at specific sites, methylation levels from each subject were examined as a function of time of death (T.O.D.) and fit to cosinor models as previously reported [14]. We centered the analysis on the 5'UTR sequences of *BMAL1*, which we hypothesized were most likely to be involved in the circadian modulation of transcription given their intermediate levels of methylation. We observed oscillatory patterns with significant differences in methylation at peak times between AD cases (early and late) compared to controls. These alterations may reflect underlying changes in phase and amplitude (Fig. 3B). Notably, the five CGs showing the highest methylation amplitude reached peak values at similar times, suggesting coordinated epigenetic activity in this region. Indeed, combined analysis of cg13286116; cg10435282; cg19536902; cg14794298 and cg11619602 replicated their individual patterns of methylation, with rhythms from early AD patients being nearly completely antiphase to brains from controls, whereas brains from late AD cases showed lower amplitude and dampened rhythms (Fig. 3C).

We next profiled the cycles of *BMAL1* transcription in the brain by qPCR (Fig. 3D). Circadian oscillation of *BMAL1* was evident in control subjects, with peak expression in the early evening (16:00–20:00), in agreement with previous reports [11]. By contrast, *BMAL1* transcription was significantly altered in both early and late AD brains, with lower amplitude and advanced phase. *BMAL1* transcript abundance significantly correlated with cg13286116 methylation in the control group, but this association was not found in AD brains (Fig. 3E–F). Pearson's correlation coefficients were significantly weaker in the AD samples compared to controls ($p=0.028$). Finally, to evaluate the impact of epigenetic and transcriptional alterations on the circadian clock machinery, we assessed the oscillation of nuclear *BMAL1* protein levels in cortical tissue. Although individual variability was high, *BMAL1* protein was elevated during PM hours in control cases (Fig. 3G–H). In contrast, early AD cases showed the highest *BMAL1* levels in the morning, while *BMAL1* accumulation shifted towards night hours in late AD cases (Fig. 3G–H). Taken together, these results show that aberrant methylation rhythms correlate with abnormal patterns of *BMAL1* transcription and protein expression in AD. These alterations differ across disease stages, and may represent a molecular marker of progression.

Next, we validated these results on an independent cohort. We performed secondary analysis using the comprehensive brain epigenomic and transcriptomic data from two cohort studies

of aging and AD, the Religious Order Study and the Rush Memory and Aging Project (ROS/MAP) [14], including 396 cases from the methylome analysis and 171 from the RNAseq that matched our criteria (Supp. Table 1).

Visual inspection of the best fit cosine curves showed quantitative differences in methylation at individual sites within the *BMAL1* promoter and the 5' UTR in early and late AD cases, similar to what we observed in the initial study (Fig. 4A–B). Cosinor analyses determined that 24 h methylation rhythms were present in the 5'UTRs of control subjects at cg22322535 ($p=0.042$); in early AD at cg07568015 ($p=0.012$) and in late AD at cg13286116 ($p=0.0026$). Moreover, there was a trend toward different methylation rhythms between early AD and controls for cg07568015 ($p=0.063$) and between late AD and controls for cg13286116 ($p=0.055$). *BMAL1* transcription correlated with methylation of 5'UTR CGs, including cg13286116, a site that showed significant alterations in AD, validating the results from the discovery cohort (Fig. 4C). Evaluation of the distribution of acrophase methylation timing across all CGs interrogated for *BMAL1*, showed significant changes in dispersion in early and late AD cases in comparison to controls, also illustrating changes in the acrophase timing across groups (Fig. 4D–F). We therefore hypothesize that dynamic methylation cycles at the 5'UTR have a role in the regulation of rhythmic transcription of *BMAL1*.

3.4-Similar methylation abnormalities at *BMAL1* are observed in AD cells and brains

Finally, we examined whether AD patient fibroblasts and brain samples presented similar alterations in *BMAL1* methylation. We quantified the methylation levels the site that showed most significant changes in AD brains, cg13286116, using bisulfite sequencing. DNA was obtained from control and AD fibroblasts at the circadian times when *BMAL1* mRNA reached its lowest abundance in control cells (C.T.30) and again at a later time when *BMAL1* transcripts peak (C.T.40; Fig. 5A). About 80% of the control clones were fully methylated at C.T.30, compared to 20 % of AD clones at the same time point. Ten hours later, 50% of control clones were fully methylated, while 80% of AD clones were methylated (Fig. 5B). These results, although limited by the small sample size, suggest that the differences observed in *BMAL1* methylation patterns in the brains of AD patients are indeed reflected in fibroblast cell lines, thereby supporting the validity of our cell-based functional studies.

4. DISCUSSION

In the present study we provide evidence that DNA methylation regulates *BMAL1* expression rhythms in human brain samples and fibroblasts from AD patients and controls. To the best of our knowledge, this is the first report describing the association between rhythmic methylation and cyclic clock gene expression, and its potential role in circadian alterations in the AD brain. Characterization of circadian rhythms in real-time using AD patient fibroblasts enabled us to directly compare *BMAL1* methylation changes in AD brains with functional differences in cultured cell lines. Moreover, by showing that pharmacological manipulation of DNA methylation leads to changes in rhythms and *BMAL1* expression, our data support a causal role for methylation as a modulator of circadian cycles. This suggests that the aberrant brain methylation patterns reported in AD

could alter clock gene expression and neuronal circadian rhythms, thereby contributing in part to the sleep and behavior alterations associated with pathology.

Circadian disruption is recognized as an important clinical feature in the course of AD [28–30]. However, the underlying molecular alterations linked to such deregulation are yet to be discovered. Epigenetic mechanisms are intimately linked with the regulation of circadian clocks. Chromatin modifiers SIRT1 and HDAC3 are recruited to clock-regulated genes in a circadian pattern [31, 32]. CLOCK-BMAL1 dimers induce histone modifications at the promoters of clock-controlled genes to activate transcription [33] [34]. Still, the potential role of DNA methylation on this system is underexplored [35–37]. In model organisms, like *Neurospora*, methylation at the *frq* locus is associated with modulation of circadian period and phase [38, 39]. A recent study reported that the effects of day length on clock plasticity are likely modulated by DNA methylation changes in the mouse SCN [13]. Altered circadian light cycles affected behavior, global transcription and DNA methylation, and older mice failed to entrain to light changes [13]. Our results support the hypothesis that similar epigenetic mechanisms may regulate circadian clocks in the human brain, and may be affected by AD pathology.

AD postmortem brains show an acceleration of age-related changes in methylation [40] and genes associated with increased disease susceptibility show abnormal methylation and transcription levels, even early in AD [15]. These observations, in addition to the results reported here suggest that DNA methylation may represent an accessory feedback loop that helps synchronize the expression of clock genes and maintain rhythmicity. Therefore, aberrant methylation may weaken rhythms and impact downstream rhythmic processes. At the molecular level, circadian clock function is based on interlocking transcriptional/translational mechanisms where positive regulators modulate the expression of their own repressors [7]. Therefore, while we provided evidence that DNA methylation modulates *BMAL1* transcription, BMAL1 in turn may directly regulate the enzymes that direct methylation, the DNA methyl transferases (DNMTs) and demethylases. Changes in *Dnmt* expression were observed in response to altered day length in mouse SCN [13], hence neuronal degeneration in the SCN reported in AD [29] may also initiate epigenetic alterations that will further impact circadian regulation in peripheral clocks. Additional mechanistic studies are needed to completely understand the potential complexity of this mechanism. With respect to other disease specific changes in AD, BMAL1 protein levels may be directly affected by accumulation of amyloid- β (A β) during the course of AD pathology. A recent study reported that A β enhances the degradation of Bmal1 and Cbp, another circadian clock regulator, reducing binding of transcription factors to the Per2 promoter, and altering the oscillation of Per2 mRNA and protein expression in cell culture models and in the SCN of the 5 \times FAD transgenic mice model of AD [41].

In humans, the phase of circadian behavior presents high variability among different individuals as a result of the interaction of different chronotypes, daily life schedules and metabolism that impact the clock function at a cellular level [42]. As our sample size increased in the validation cohort, we observed a higher dispersion in the methylation rhythms, evidenced by lower amplitudes and higher p-values for rhythmicity in controls than AD cases, an effect that may be associated to individual life-styles. In “late AD” cases with

severe dementia, exposure to tight schedules of care may contribute to entrain the clock to external clues, reducing the inter-individual variability. These confounding external factors limit the present study.

Functional longitudinal studies are not feasible in the brain of living human subjects, but circadian rhythms can be reliably measured from skin fibroblasts, and likely reflect the activity of non-SCN brain oscillators [42], since period length (of about 24 h) is genetically determined. Although we observed alterations in *BMAL1* transcription and protein levels in both, brain samples and fibroblast cultures, the changes are different. The post-mortem brain studies were conducted in subjects living in regular light-dark cycles that impose limits in the BMAL1 rhythm and affect the expression profile. Cell culture studies, on the other hand, were conducted under constant conditions and reflect the free running rhythm. Therefore, the two data sets are not directly comparable. Noteworthy, the ability of detecting alterations in fibroblasts that may resemble alterations in the human brain, beyond their expected differences in epigenotype, exemplifies the power of this cellular system for monitoring circadian rhythmicity *in vivo*.

The clinical continuum of AD includes three distinctive disease stages: preclinical; mild cognitive impairment (MCI); and dementia [43]. Circadian and sleep-wake dysfunction are well described in advanced AD, but less studied in individuals with MCI, although 60% of them report a prevalence of sleep disturbance [44] and are at higher risk to develop dementia [44, 45]. Circadian abnormalities have been associated with degeneration of SCN neurons [46] and decreased melatonin levels in the cerebrospinal fluid (CSF) of AD patients [47]. In our study, we detected the most striking alterations in the frontal cortex from “early AD” patients (i.e. a group intermediate between MCI and late AD), supporting the idea that epigenetic and circadian alterations may not only be a consequence of SCN cell loss, but deregulation of peripheral clocks in other brain regions at initial disease stages may also contribute to circadian impairment.

Sleep quality plays an important role in preserving memory and cognition, and reducing the risk of converting to dementia in susceptible individuals [48]. Here we show significant deregulation of *BMAL1* in association with early AD. BMAL1 not only controls the transcription of core clock genes, but also regulates cerebral redox homeostasis. Targeted deletion of *Bmal1* in mouse neurons and glia triggers degeneration of synaptic terminals and neuronal oxidative damage and impairs cortical functional connectivity [49]. Our findings suggest that epigenetic alterations early in AD could contribute to cognitive impairment and neurodegeneration via deregulation of circadian rhythms. If so, then early interventions, including epigenetic and chronotherapies, may prove effective in delaying disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- DNA methylation cycles associate with rhythmic *BMAL1* transcription in the brain.
- This epigenetic mechanism is altered early in Alzheimer's disease progression.
- Aberrant *BMAL1* methylation and transcription may contribute to circadian impairment observed in AD.

RESEARCH IN CONTEXT

1. **Systematic review:** No previous study has investigated the role of dynamic cycles of DNA methylation on cellular circadian rhythms in AD.
2. **Interpretation:** Our findings show that cycles of DNA methylation may contribute to the regulation of circadian rhythms in the brain via modulation of *BMAL1* transcription. This epigenetic mechanism is altered early in AD pathology, likely resulting in aberrant *BMAL1* expression and possibly circadian impairment.
3. **Future directions:** Future studies will focus on elucidating the mechanisms of cyclic DNA methylation and identifying how rhythms interact with specific pathological mechanisms in AD.

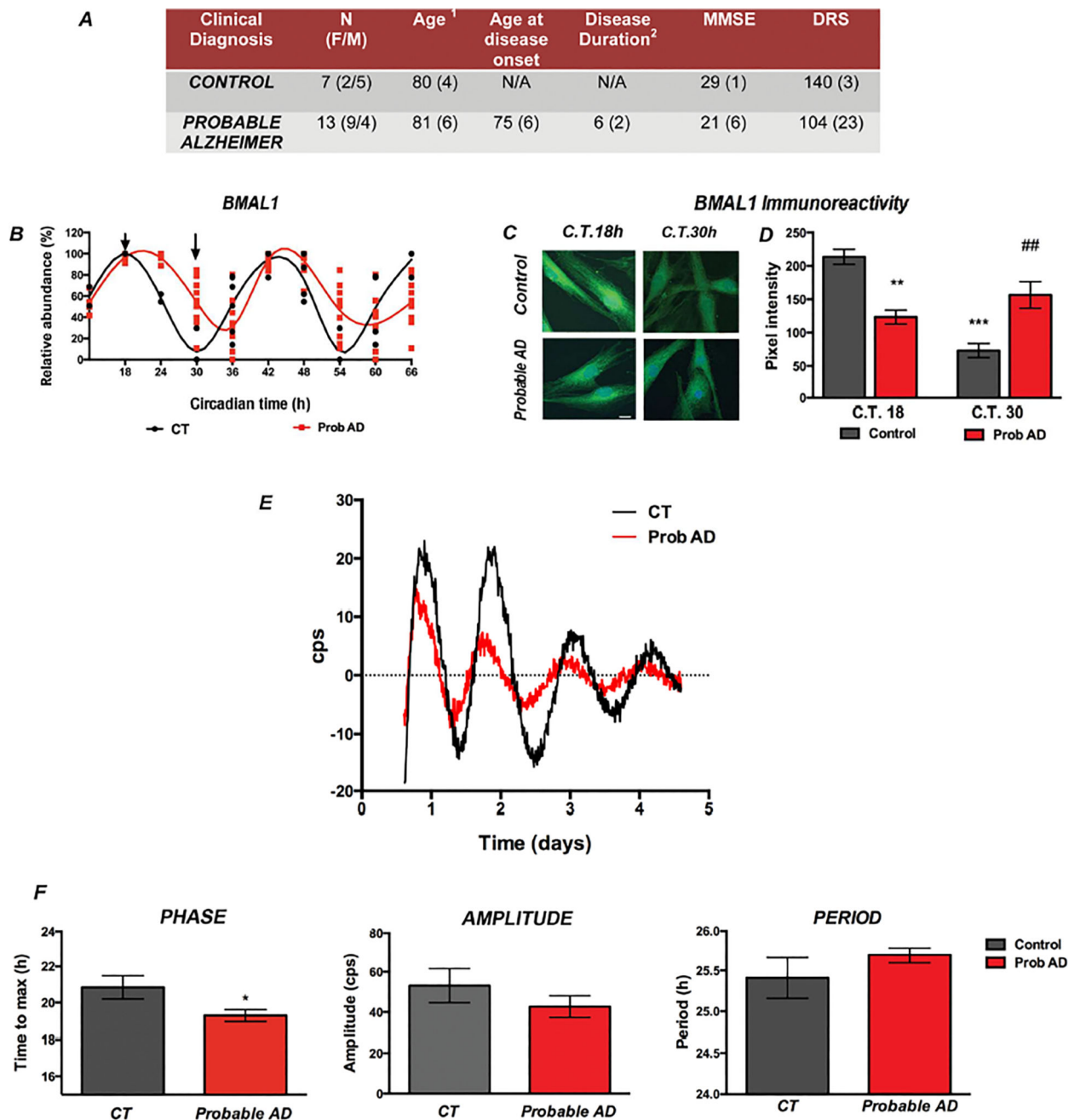


Figure 1. Altered circadian rhythms in AD patients' fibroblasts associate with aberrant *BMAL1* transcription

A. Clinical and demographic characterization of cell line donors. (¹Age at skin biopsy. ²Calculated in years from clinical diagnosis to biopsy date). **B.** Lowess curves representing *BMAL1* transcript abundance at different circadian times. Symbols represent duplicate determinations per cell line tested. **C.** Immunohistochemical detection of *BMAL1* comparing protein abundance in control and AD fibroblasts at the times when *BMAL1* transcripts reached peak and nadir values in control cells (indicated by arrows in Figure 1B). Imaged at 60 \times . Scale bar = 50 μ m. **D.** Quantification of pixel intensity of *BMAL1*

immunoreactivity. ** $p < 0.01$ and *** $p < 0.001$ in comparison to control cells at peak time; or nadir time ## $p < 0.01$ as per unpaired non-parametric Mann-Whitney t test. **E–F.** Rhythmic *Per2::luc* expression was measured from transduced control and AD-derived fibroblasts to determine circadian parameters. **E.** Representative data for *Per2::luc* rhythms obtained from a control subject and a patient with diagnosis of probable AD. **F.** Mean circadian rhythm parameters in AD case-control group analyses. * $p < 0.05$ probable AD (n=13) vs. control fibroblasts (n=7) as determined by unpaired, non-parametric Mann-Whitney t test.

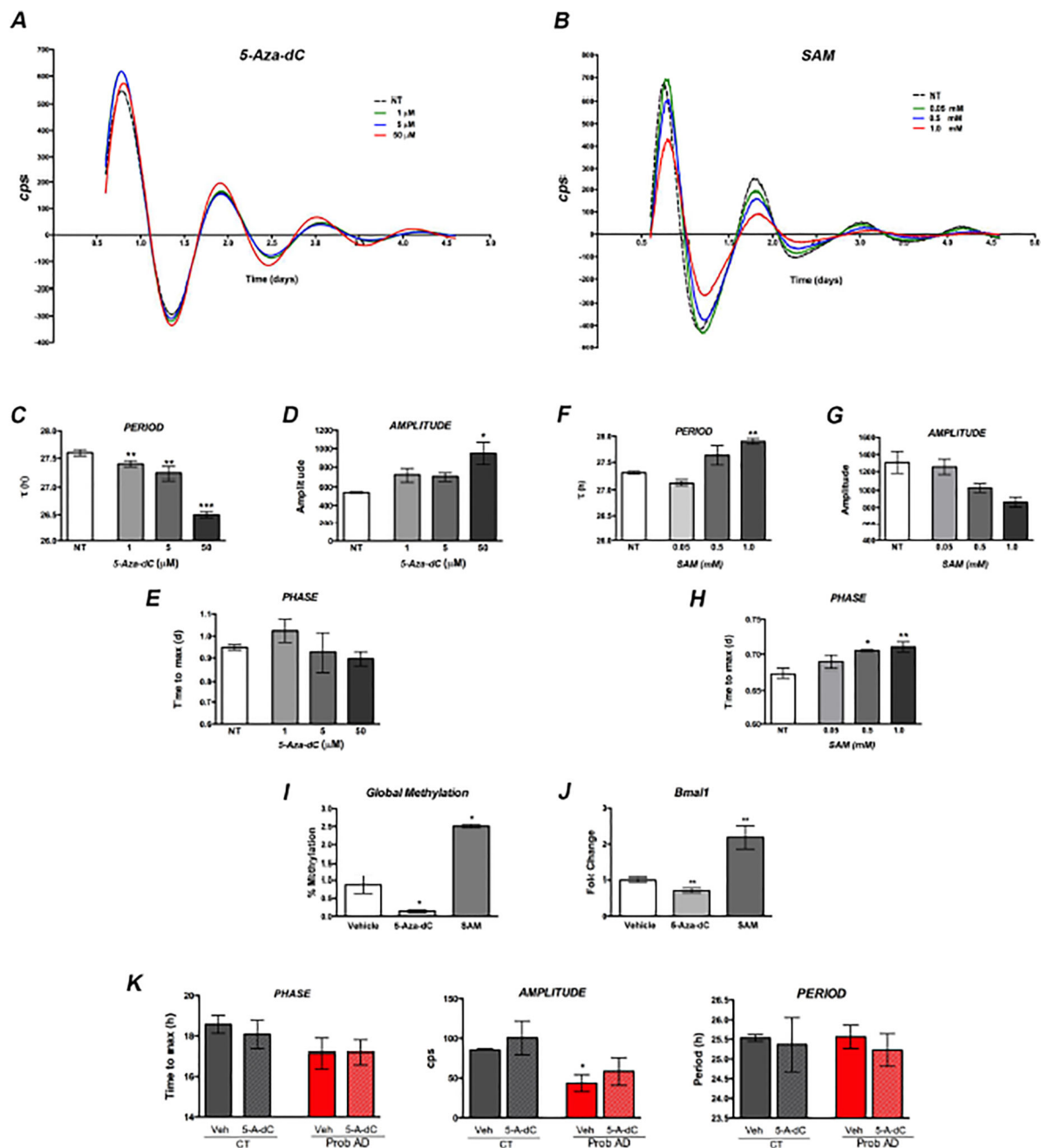


Figure 2. Pharmacological modulation of DNA methylation induces changes in circadian rhythms in cultured fibroblasts

Rhythmic *Per2::luc* expression in NIH 3T3 mouse fibroblasts treated with 5-Aza-dC; SAM or vehicle (NT). **A–B**. Concentration-dependent changes in circadian rhythms were observed in response to both compounds. **C–E**. Reduced global methylation shortens period; increases amplitude and advances phase. **F–H**. Increased methylation levels lengthen period; decreases amplitude and delays phase. **I–J**. Both treatments significantly changed global DNA methylation (**I**) and expression level of *Bmal1* transcripts (**J**). **K**. Circadian rhythms are modulated by changes in methylation in human fibroblasts treated with 50 M 5-Aza-dC.

Error bars represent S.E.M. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ in comparison to vehicle treated cells as per One Way ANOVA (C-I) or paired parametric t test (J-K).

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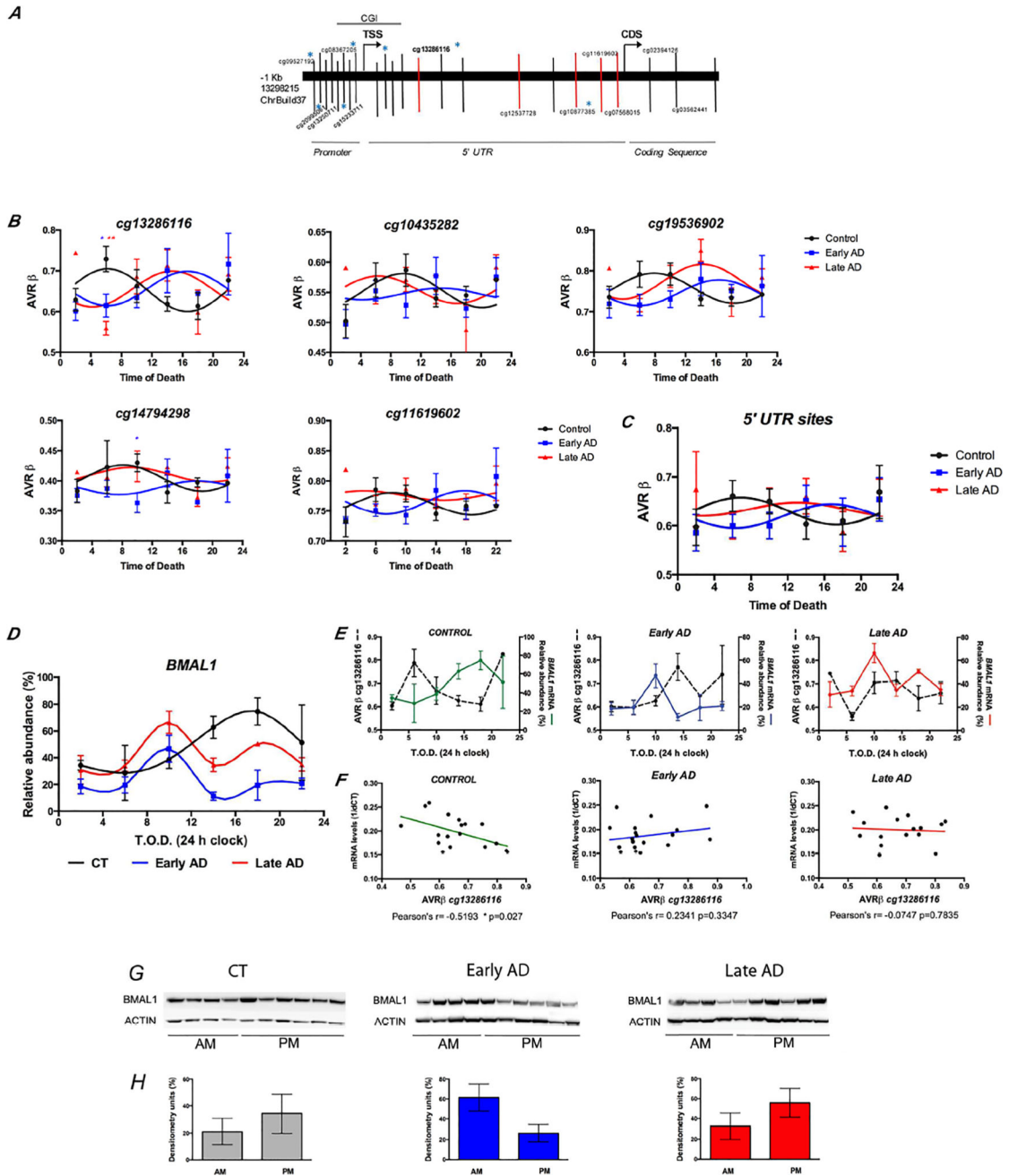


Figure 3. Rhythmic oscillation of DNA methylation correlate with BMAL1 transcription in AD brains

A. Schematic representation of *BMAL1* genomic region screened. Vertical bars indicate the location of probes in the Illumina 450k array. Blue * denotes probes showing significant rhythms with 24 h period. Red bars depict 5' UTR probes that showed altered methylation rhythms in AD brains. Gray bar indicates the position of CG island Chr11:13298796–13300735 in ChrBuild37. **B.** Comparison of methylation rhythms at 5'UTR CpGs between control, early and late AD cases. Panels show non-linear fit of methylation values to sinusoidal curves. Blue * $p < 0.05$; red ** $p < 0.01$ in comparison to controls at the same time

(one way ANOVA, with Tukey's posthoc test). **C.** Representation of fit-sinusoidal curves for the combined 5'UTR sites from (B). **D.** Locally weighted scatterplot smoothing (Lowess) curves representing *BMAL1* transcript abundance as a function of time of death as 4 h bins (T.O.D.). Symbols represent mean values \pm S.E.M. **E–F.** Analysis of cg13286116 methylation and *BMAL1* transcript abundance. Lower panel shows significant correlation (Pearson's R) for control cases, which is lost in AD brains. **G.** Immunohistochemical detection of BMAL1 protein in frontal cortex samples at different T.O.D. ACTIN immunoreactivity is showed as loading control. **H.** Densitometric analysis of BMAL1 protein comparing abundance during AM vs. PM hours (as indicated in **G**). Data represents normalized values of BMAL1/ACTIN ratios.

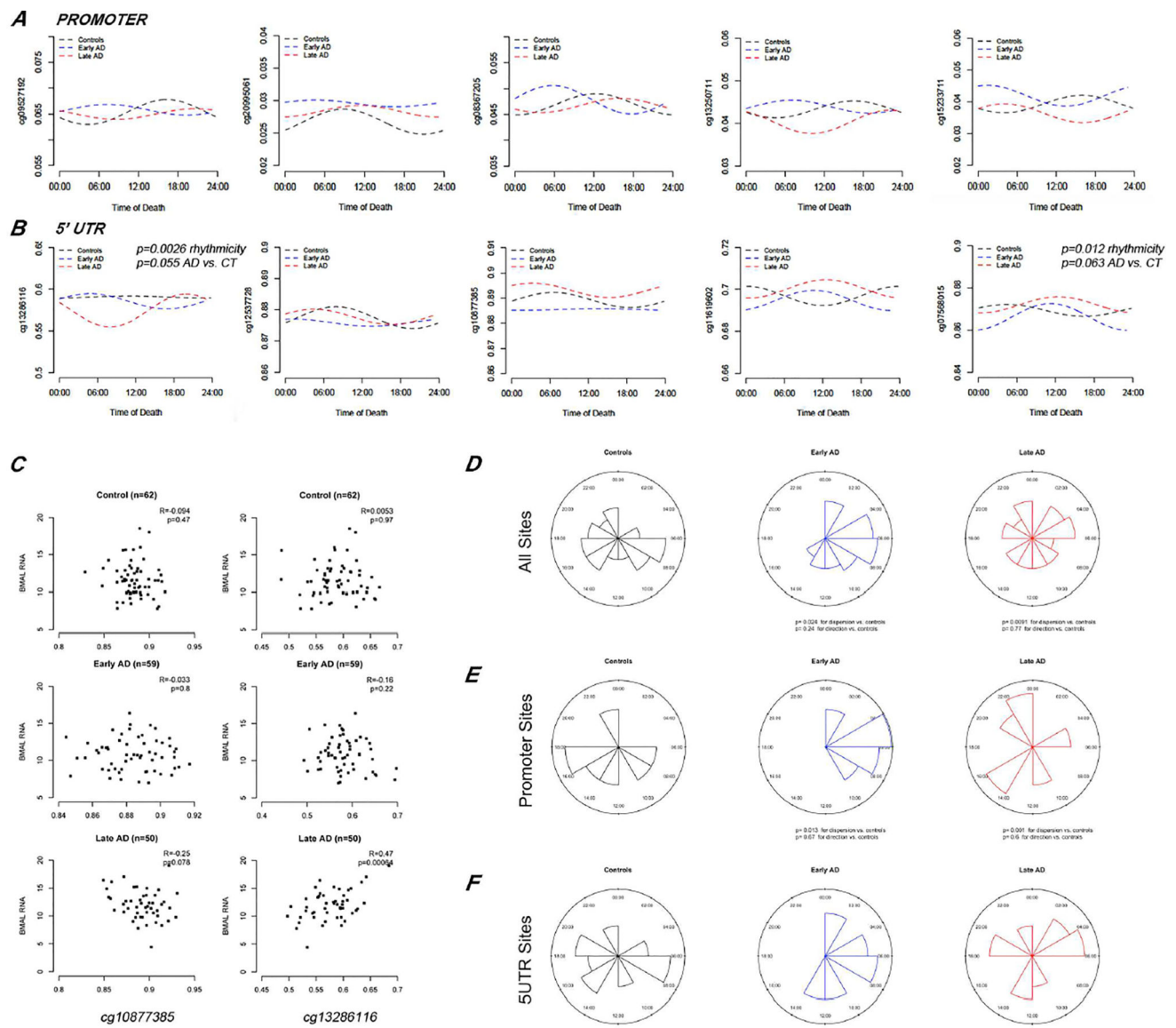


Figure 4. Validation of rhythmic DNA methylation, *BMAL1* transcription correlations and acrophase alterations in the ROS/MAP cohorts

A–B. Individual CpG site analysis on 396 ROS/MAP participants adjusting for age at death and sex. Lines represents best-fit cosine curve. Selected panels showing comparison of fitted cosinor curves per group for probes located at *BMAL1* promoter (**A**) and 5' UTR (**B**) regions. Top p value refers to rhythmicity in the group. Bottom p value refers to rhythmicity differences in comparison to the control group. **C.** Selected scatterplots showing association of the combined signal of all *BMAL1* isoforms detected by RNAseq with methylation levels at individual CpGs. Pearson's R coefficients and p values for the correlation are indicated. **D–F.** Distribution of methylation acrophase times for all 22 *BMAL1* probes detected (**D**); or for probes located at *BMAL1* promoter (**E**) or 5'UTR regions (**F**) in controls, early and late AD brains.

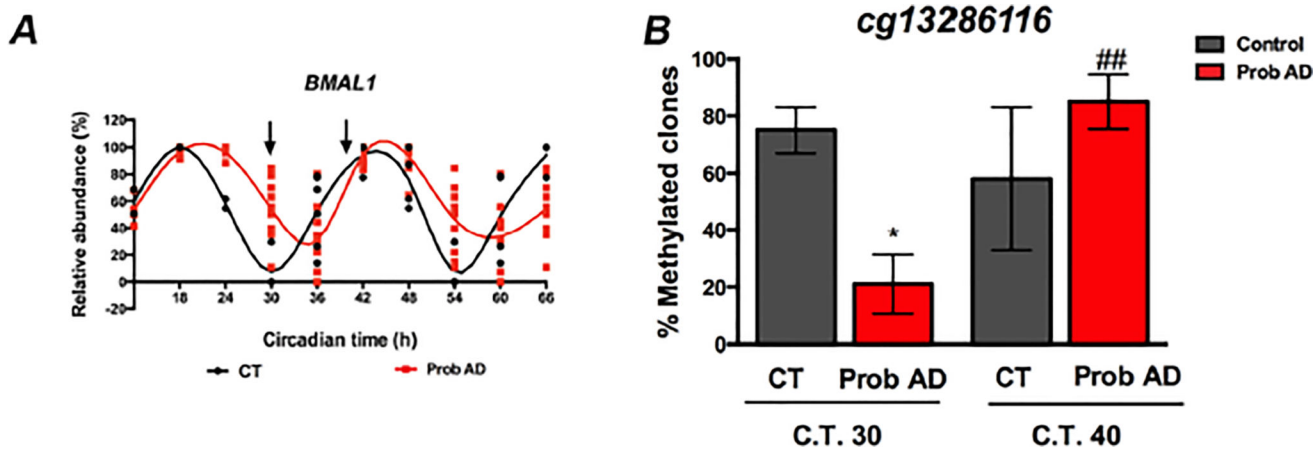


Figure 5. Differences in *BMAL1* methylation between AD patients and controls are also evident in fibroblast cells

Bisulfite sequencing was used to compare the levels of methylation at site *cg13286116* between control subjects and AD patients' cells at the times when *BMAL1* transcription is close to nadir (C.T.30) and peak (C.T.40) in control fibroblasts. **A.** Panel B from Fig. 1 is provided as a reference of *BMAL1* transcript levels at the time points tested. **B.** The graph shows the percentage of sequenced clones that showed fully methylated *cg13286116* at each time point. * $p < 0.05$ in comparison to control cells, ## $p < 0.01$ in comparison to Probable AD cells at C.T.30 as per unpaired Mann-Whitney t test.