Epigenetic Regulation of *BMAL1* in Alzheimer's Disease Linking Neuropathology, Cognitive Decline and Sleep Quality

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A thesis submitted in fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science

(By Research)

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Declaration:

With the exception of any statements to the contrary, all the data presented in this report are the result of my own efforts. In addition, no parts of this report have been copied from other sources. I understand that any evidence of plagiarism and/or the use of unacknowledged third-party data was be dealt with as a very serious matter.

Signed ..... Date .....

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Abbreviations

Description	Abbreviation
Alzheimer's Disease	AD
Amyloid precursor protein	APP
Arginine Vasopressin	AVP
Aryl Hydrocarbon Receptor Nuclear Translator-like 1	*ARNTL/BMAL1*
Beck Depression Inventory	BDI
Bed Nucleus of the Stria Terminalis	BNST
Beta-amyloid	Aβ
Clock Circadian Regulator	*CLOCK*
Creb-Binding Protein	CBP
Cryptochrome *Clock* Gene	CRY1 & CRY2
Dopamine	DA
Dorsolateral Sectors	dlPFC
Major Depressive Disorder	MDD
Mild cognitive Impairment	MCI

Description	Abbreviation
Neurofibrillary Tangles	NFT
Non-rapid Eye Movement	non-REM
Obstructive Sleep Apnoea	OSA
Period Clock Gene	*PER1, PER2 & PER3*
Personal Details Questionnaire	PDQ
Pittsburgh Sleep Quality Index	PSQI
Polymerase Chain Reaction	PCR
Rapid Eye Movement	REM
REV-ERB	Nr1d1 & Nr1d2
Sleep Disordered Breathing	SDB
Slow Wave Sleep	SWS
Suprachiasmatic Nucleus	SCN
Vasoactive Intestinal Peptide	VIP
Ventromedial Prefrontal Cortex	vmPFC
Ventrolateral Preoptic Area	VLPO

Abstract

Introduction: Alzheimer's disease (AD) is a neurodegenerative condition that is prevalent in today's society and is on the rise. A disturbance of the circadian rhythm is an early symptom and is associated with disrupted sleep/wake cycles. *CLOCK* genes, important in regulating the circadian rhythm, have been hypothesised to regulate important factors in neurodegeneration. These *CLOCK* genes control circadian timekeeping by regulating their own expression over 24hours via a series of interacting positive & negative feedback loops; with *BMAL1* being a key gene that drives the circadian cycle. Sleep disturbances are observed in AD and the reasons for this may be multifactorial and involve beta-amyloid (Aβ), light exposure and sleep disordered breathing (SDB), which are all related to the circadian cycle. Circadian dysfunction is hypothesised to contribute to AD pathogenesis and previous studies show a link between the circadian clock, AD neuropathology, sleep regulation and cognition; particularly fluid intelligence, processing speed, memory and vocabulary. However, it is unclear if circadian dysfunction within AD is causal or consequential to the development of neuropathology.

Aim: To investigate if *BMAL1* methylation is epigenetically regulated in brains in relation to AD neuropathology, longitudinal changes in cognition, sleep quality and depressive symptoms. This study is designed to assess the hypothesis that *BMAL1* methylation will directly affect neuropathology, cognition, sleep quality and depression.

Methods: Prefrontal cortex (n=96) samples were acquired from Manchester Brain Bank. DNA methylation at six individual CpG sites on *BMAL1* was determined using bisulphite pyrosequencing that was statistically tested for associations with AD neuropathology, longitudinal changes in cognition, sleep quality and depressive symptoms (BDI score).

Results: Methylation across all the CpGs strongly correlated with each other. We found increased CpG2 methylation with higher Braak (F (1, 92)=6.1, p=0.015) stages. No significance was found between longitudinal fluid intelligence, processing speed and memory tests, but methylation at CpG1 (r=0.20, p=0.05) and CpG4 (r=0.20, p=0.05) positively correlated with vocabulary. When testing for age-adjusted cross-sectional data, CpG2 positively correlated with cross-sectional fluid intelligence (r=0.20 p=0.05) and vocabulary (r=0.22 p=0.03). Though longitudinal analysis revealed no significance between sleep duration, midsleep and efficiency for any of the CpG sites, CpG3 (B=0.03, 95%CI=0.00/0.06, p=0.03) and CpG5 (B=0.04, 95%CI=0.01,0.07, p=0.01) significantly correlated with night wake. CpG4 correlated with depressive symptoms (B=-0.27, 95%CI=0.49/-0.05, p=0.02).

Discussion: The results of this study indicate that DNA methylation of *BMAL1* is positively associated with AD neuropathology, longitudinal changes in cognition, sleep quality and depression; suggesting that the circadian cycle plays a linking role in regulating these key factors in the development of AD. Further research is needed to understand the dynamics of this relationship.

Keywords: Alzheimer's disease, circadian cycle, *BMAL1*, cognition, sleep quality, depressive symptoms.

1.0 Introduction

1.1 Epidemiology of Dementia/Alzheimer's Disease

Dementia is the umbrella term used to describe a set of symptoms that affect memory, behaviour, thinking and emotion and currently in the UK, 1 in 14 people aged over 65 have dementia (Prince et al., 2014). There are over 100 different forms of dementia, with Alzheimer's Disease (AD) being the most prevalent and accounting for 60-80% of all cases of dementia (Alzheimer's, 2016). AD is a neurodegenerative condition which is clinically characterised by a gradual, insidious onset of memory loss which then expands into multi-domain cognitive impairment (Musiek, 2017). Pathological hallmarks of AD are beta-amyloid plaques (Aβ) and neurofibrillary tangles (NFT), formed from the aggregation of misfolded tau protein. Aβ plaques occur when the two major enzymes neprilysin (NEP) and insulin degrading enzyme (IDE) decrease with normal ageing and in disease-affected regions (Caccamo et al., 2005). Additionally, NEP has been shown to decrease in cerebral spinal fluid (CSF) in early AD (Maruyama et al., 2005).

Amongst the symptoms listed above, disturbances to sleep and day-night rhythms are also very common. Sleep problems in AD patients are observed with over 60% of individuals with mild cognitive impairment (MCI) or dementia experiencing sleep disturbances (Guarnieri et al., 2012), as well as circadian rhythm disruption and misalignment (Phan & Malkani, 2019). Sleep and circadian rhythms are very closely correlated, but they have separate neuroanatomical and molecular substrates. Circadian dysfunction may contribute to AD pathogenesis and this will be discussed further in this study.

1.2 The Circadian Clock

Circadian rhythm, also known as sleep/wake cycle, is a biological system that acts as a 24-hour internal clock and regulates feelings of sleepiness and wakefulness throughout this time. Circadian rhythms exist in the majority of living organisms and are responsible for many behavioural and biochemical processes. The primary aim of the circadian system is to synchronise internal functions with the external environment; with particular focus on the light-dark cycle (Musiek, 2017). The circadian system that generates circadian rhythms in mammals, which consists of a central clock, is located in

the suprachiasmatic nucleus (SCN) of the hypothalamus with peripheral clocks in majority of other cells (Tomita & Onishi, 2018). The SCN sends signals to other brain areas, including sleep-wake centres, the pineal gland to regulate melatonin and to regions that regulate autonomic function and hormone secretion (Colwell, 2011).

The circadian clock consists of a transcription-translation feedback loop, which is regulated by clock genes. These genes include, brain and muscle Aryl Hydrocarbon Receptor Nuclear Translator-Like 1 (*ARNTL*, also known as *BMAL1*), clock circadian regulator (*CLOCK*), cryptochrome (*CRY1* & *CRY2*) and period circadian clock (*PER1*, *PER2* & *PER3*) and these are the core circadian genes in peripheral or central tissues (Kelsbeek et al., 2014). It is the interlocked transcriptional and post-translational negative feedback loops which are responsible for both the generation and preservation of circadian rhythms. *BMAL1* and *CLOCK* are transcription factors that act as positive regulators of circadian gene expression, which in turn activates the expression of the negative regulators *CRY1* and *CRY2*, *PER1*, *PER2* and *PER3* and REV-ERB (Nr1d1 & Nr1d2) (Kelsbeek et al., 2014). Nr1d1 & Nr1d2, which is a nuclear receptor that regulates lipid metabolism and adipogenesis, directly and indirectly represses *BMAL1/CLOCK*-mediated transcription. Additionally, Retinoic Acid Receptor-related Orphan Receptors (RORs) which are made up of RORα, RORβ and RORγ are all involved in the functioning of circadian rhythm and RORα is a positive regulator of *BMAL1*. A new transcriptional cycle starts through the proteolytic degradation of *PER* and *CRY*, when the *CLOCK:BMAL1* complex becomes de-repressed (Figure 1).

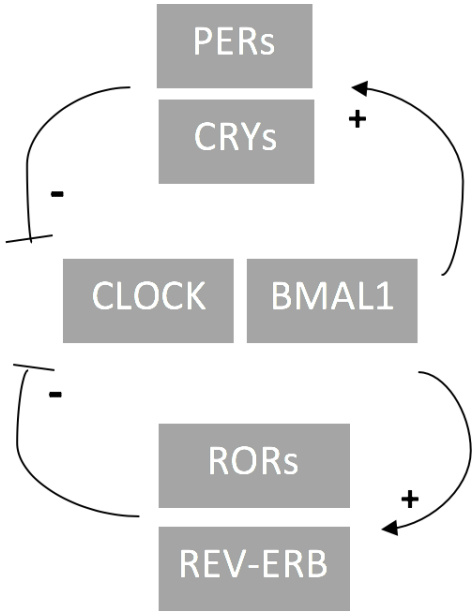


Figure 1: Molecular core clock mechanism. CLOCK:BMAL1, PER1-3 & CRY1&2 form the core loop with CLOCK:BMAL1 heterodimers driving PER1-2 & CRY1&2 transcription. PERs & CRYs heterodimers move to the nucleus which inhibits CLOCK:BMAL1 activity; thus the transcriptional activity of CLOCK:BMAL1 drops. This reduces the transcription of PER and CRY genes, which in turn activates CLOCK:BMAL1. REV-ERB and RORs enhance the efficiency of the core loop. Adapted from Spek et al., (2012).

The circadian system has a large influence on multiple aspects of physiology with the rhythmic clock gene expression being characterised in peripheral tissues, such as skin (Bjarnason et al., 2001), bone marrow (Kusanagi et al., 2008) white blood cells (Archer et al., 2008) and the heart (Leibetseder et al., 2009). However, due to the nature of the human brain and only being able to examine these oscillators in post-mortem, it is very difficult to look at the circadian rhythm in the brain. As the circadian system is implemented in many physiology processes, a severe disruption in this regulation is associated with the heightened and worsening of many disease states; including neurodegeneration in mice, (Musiek et al., 2013), coronary heart disease (Vetter et al, 2016) and breast cancer (Davis et al., 2001) in humans, and other metabolic problems (Cribbet et al, 2016).

Circadian dysfunction has been previously linked to neurodegeneration in AD, with different studies hypothesising it as both a consequence of and a potential contributor to the pathogenesis of AD (Hastings & Goedert, 2013; Musiek & Holtzman, 2016). This issue is the subject of this study.

1.3 Neuropathology of Circadian Dysfunction in Alzheimer's Disease

The brain changes associated with AD are thought to begin 20 years or more before clinical symptoms appear (Reiman et al., 2012). Circadian disruption in AD patients is partly mediated by changes and degeneration of the SCN. The SCN experiences neuronal loss during normal aging, however AD patients exhibit significantly higher neuronal loss (Stopa et al., 1999). Circadian rhythm is portrayed in which levels of a particular measure (*BMAL1* methylation), varies depending on the time. The difference between peak and trough values is called the amplitude of the rhythm. The phase of the rhythm is the timing of the referred to point in the cycle, for example the peak, relative to the fixed event, for example entering the night phase. The period of the rhythm is the time

interval between phase referred to points, for example between the two peaks (Vitaterna et al., 2000). Studies show a correlation between circadian rhythm amplitude of motor activity and SCN neuronal loss when comparing AD and cognitively healthy subjects (Wang et al, 2015). Wang *et al*., (2015) studied the loss of vasoactive intestinal peptide (VIP) and found it to be correlated with a decline in the amplitude of behavioural circadian rhythms. This is due to VIP and neuronal expression of Arginine Vasopressin (AVP) playing key roles in SCN synchronization and circadian rhythm output (Aton et al., 2005; Meida et al., 2016). Additionally, several studies show blunted circadian oscillations in melatonin secretion in AD patients (Mishima et al., 1999; Skene & Swaab, 2003). This is down to the pineal gland receiving output from the SCN which generates circadian oscillations in melatonin secretion (Wu et al., 2006). Furthermore, significant changes in methylation have been reported in AD patients (De Jager et al., 2014), particularly in the frontal cortex (Lim et al., 2014). However, the pathways that cause this still remain unexplored.

A hallmark of AD are deficits in executive functioning affecting skills such as working memory, fluid intelligence and processing speed and the frontal cortex has been widely associated with these functions. The frontal cortex is also highly sensitive to sleep changes (Wu et al., 2006). A PET study found that changes in this region, including the superior frontal gyrus, were present in patients with MCI that progressed to Alzheimer's disease (AD) compared to those that did not (Valdés et al., 2018). Accumulating evidence, especially in animal models, suggests that circadian clock dysfunction could promote neurodegeneration and contribute to AD pathogenesis. Studies in transgenic AD mice models have found that chronic sleep restriction and deprivation exacerbates AD pathology in brains, including increased amyloid-β (Aβ) and phosphorylated tau (Rothman et al., 2013; Qui et al., 2016; Di Meco et al., 2014).

To summarise, it is hypothesised that both the degeneration of the SCN and dysregulation of pineal melatonin secretion cause clock gene rhythms to be altered and in turn, disrupt the circadian clock. Not much is known about DNA methylation in AD brains and circadian dysfunction, however it is a possibility that DNA methylation contributes to the dysregulation of the circadian cycle.

1.4 Neuropathology and Braak Score

Braak & Braak, (1991) found that neurofibrillary tangles and neuropil threads exhibit a characteristic distribution pattern permitting the differentiation of six stages. Stages 1-2 were characterised by an either mild or severe change to the transentorhinal layer Pre-α. Stages 3-4 (limbic stages) were characterised by a conspicuous affection of layer Pre-α in both transentorhinal region and proper entorhinal cortex. Stages 5-6 (isocortical stages) refers to the destruction of the majority of the isocortical association areas.

1.5 Sleep and Alzheimer's Disease

Disturbances in sleep and disruptions in circadian rhythms are common in AD patients and studies report that up to 45% of patients have sleep disturbances (Moran et al., 2005). AD patients exhibit disturbances in sleep-wake cycles and rest-activity dysfunction which is primarily due to a higher level of wakefulness at night; caused by an increase in nocturnal awakenings (Hatfield et al., 2004). Consequentially, this further leads to an increase in sleep during the day, causing a disruption of day-night variation. Therefore, though the main cause of sleep disturbances in AD is hypothesised to be multi-factorial without a main effect, it appears sleep has some role.

There are several factors that could influence sleep disorders in AD. For example, lack of daylight exposure, which is common in AD patients, can directly affect circadian rhythms (Figueiro, 2017). AD patients have a higher risk for showing obstructive sleep apnoea (OSA) with an estimated ~70-80% of AD patients presenting with this (Wennberg et al., 2017). Sleep disordered breathing (SDB) which is also common in AD affects circadian rhythm (Hermann & Bassetti, 2016) and is associated with increases as dementia symptoms worsen. For example, SDB was found to be associated with an increased risk of developing cognitive impairment when compared against those without SDB (Yaffe et al., 2011). As many of these all are present in AD, it could pose the question whether these factors, particularly SDB, could contribute to the cognitive impairment that accompanies AD.

Additionally, it has been suggested, that one cause of disrupted sleep in AD patients, is Aβ accumulation. Studies in transgenic mice demonstrate that the amyloid precursor protein (APP) which is processed into Aβ deposition in the brain, causes an increase in wakefulness and an overall decrease in sleep. This initiates when the amyloid plaques began to accumulate in the cortex and hippocampus (~ 6 months of age), and then a significant disruption in sleep pattern was observed when the plaques became more widespread (~9 months of age) (Roh et al., 2012). Other Aβ studies in mice show an abnormality in altered nocturnal activity levels (Sterniczuk et al., 2010), phase delay (Duncan et al., 2012) and decreased non rapid-eye movement (REM) sleep (Jyoti et al., 2010). Hence, it could be hypothesised that these Aβ accumulations cause sleep disturbances in mice and could mimic potential aspects of AD in humans.

Another aspect to Aβ dynamics is the alterations and degradation of *BMAL1* and *PER2*. Song *et al*., (2015) found the changes in circadian rhythm caused by Aβ, correlated with the accelerated degradation of *BMAL1* in mice that express 5 familial AD mutations. Furthermore, the degradation of *BMAL1*, induced circadian rhythm disruption by dysregulating *PER2* expression. This would suggest that *BMAL1* is critically correlated with circadian rhythm and AD and thus is the focus of this study.

1.6 *BMAL1* Gene Methylation

Epigenetic regulation has been linked to the pathogenesis of neurodegenerative diseases due to the vital role it plays in regulating genes involved in neuronal function (Cholewa-Waclaw et al., 2016; Aarons et al., 2019). Epigenetics refers to potentially heritable and non-heritable changes in gene expression caused by environmental factors, independent of the DNA base sequences (Murgatroyd & Spengler, 2011).

DNA methylation is the most common epigenetic modification and consists of a covalent chemical modification of a base that plays a crucial role in many biological processes where alteration has been linked in AD pathology (Irier & Jin, 2012). In mammals, DNA methylation occurs almost entirely in the symmetric CG context and is estimated to occur at ~70-80% of CG dinucleotides throughout the genome (Ehrlich et al., 1982). The

remaining non-CG methylation is found in embryonic stem cells, and unmethylated CG dinucleotides which are mostly found near gene promoters in dense clusters; called CpG islands (Suzuki & Bird, 2008). CpG islands are short interspersed DNA sequences that deviate significantly from the typical genomic pattern by being CpG-rich, GC-rich and generally nonmethylated (Deaton & Bird, 2011). CpG islands are DNA methylations regions situated in promoters which are known to regulate gene expression by transcriptional silencing of the corresponding gene (Lim et al., 2019). CpG methylation is tightly regulated and any changes in methylation profiles are associated with diseases. This indicates a close relationship among DNA methylation sites, the mechanism of methylation and biological functions (Tomita & Onishi, 2018). 24-hour methylation rhythms have recently been described in the frontal cortex and these cycles appear to be correlated with age and dementia (Lim et al, 2013). Studies suggest that global methylation levels are higher in the prefrontal cortex in individuals with AD, when compared with control (Coppieters et al., 2014; Rao et al., 2012).

The *BMAL1* gene itself is a core gene in the circadian rhythm and has been linked to haematological malignancies. The CpG islands in the promoter of *BMAL1* are hypermethylated, which silences the expression in haematological malignancies, such as acute lymphocytic and myeloid leukaemia's (Taniguchi et al., 2009). Taniguchi *et al.,* (2009), found epigenetic inactivation of *BMAL1* prevented the activation of CLOCK protein to targeted areas; enhancing the disrupted circadian rhythm in malignant cells. These findings suggest the epigenetic inactivation of *BMAL1* contributes to disruption of the cellular circadian clock.

Additionally, altered circadian transcription of *BMAL1* in the mid frontal cortex and fibroblasts of humans, have been associated with abnormal *BMAL1* methylation in AD when compared with controls (Cronin et al., 2017). Cronin *et al.,* (2017) found that in post mortem mid frontal cortex samples, oscillatory patterns had significant differences in methylation at peak times when comparing between early and late AD cases; potentially representing underlying changes to the phase and amplitude to circadian

rhythm. This difference across the disease states suggest that this may be a molecule marker of progression.

The above studies suggest that the epigenetic regulation of *BMAL1* plays an important role in AD via deregulation of circadian rhythms, however the link between the two has yet to be fully explored.

1.7 Depression and Alzheimer's Disease

Depression occurs in up to 20% of patients with AD (Valkanova et al., 2017) but the relationship is still not fully understood. It has been hypothesised that depression is a risk factor for AD (Diniz et al., 2013; Gao et al., 2013), however the underlying molecular mechanisms have not been fully explored. Ganguli (2009), came to the conclusion that there was no single cause but more a series of factors that interact with each other in various ways at different points during the course of life. However, depression has been found to damage neurons and one way it does this is amyloid deposition and neurofibrillary formation (Rapp et al., 2006). In particular, a lifetime history of depression is linked to an increase in Aβ plaques. Disruptions in circadian rhythms has been associated with depression (Abarca et al., 2002) and individuals with an arrhythmic biological clock have been associated with a higher risk of developing depression (McClung, 2007).

Additionally, *BMAL1* was found to be hypermethylation in patients with bipolar disorder when compared against healthy control (Bengesser et al., 2016). It was also hypothesised that a decrease in *BMAL1* methylation may lead to an increase in *BMAL1* gene expression and increase in the dopamine (DA) breakdown seen in depression. Furthermore, DA levels in the brain are suspected to influence mood in human & mice (Andretic & Hirsh, 2000; Nestler & Carlezon, 2006; Roybal et al., 2007) and DA levels drop with aging. This supports the hypothesis that altered epigenetic regulation of *BMAL1* may provide a mechanistic basis for circadian rhythms and mood swings and depression (Hampp et al., 2008).

The relationship between *BMAL1* and neuropathology (Braak score), cognition, sleep and depression will be explored in this study using DNA methylation. It is hypothesised that *BMAL1* could be regulated differently in the brain of AD patients and this study will be looking at this by using AD brains and controls and observing any correlations between neuropathology, cognition, sleep and depression.

2.0 Aims & Objectives

2.1 Aims

The experimental hypotheses are to determine epigenetic DNA methylation levels of *BMAL1* in the brain and relate to neuropathology, cognitive decline, sleep quality and depression.

2.2 Objectives

The molecular mechanisms surrounding the important clock gene *BMAL1* and its role in neuropathology, cognition, sleep and depression in regard to AD, still remains relatively unknown. A collection of prefrontal cortex brains (n=96) were assessed using their predetermined Braak score (determined by the brain bank), assessed longitudinally for both change in cognition, and using past sleep data (night wake, sleep duration, mid-sleep and sleep efficiency) and assessed cross-sectionally for depression (BDI score).

DNA methylation levels of *BMAL1* in the brains were determined using bisulphite pyrosequencing and these were tested for any correlations between the CpG sites on BMAL1 and neuropathology, cognition, sleep and depression data.

3.0 Methodology

3.1 Reagents and Supplies

All the kits, reagents, chemicals and any other laboratory equipment used in this project can be found in the appendix.

3.2 Study Population and Questionnaires

The prefrontal cortex samples used in this project were obtained from the Manchester Brain Bank which were from The University of Manchester Longitudinal Study of Cognition in Normal Healthy Old Age cohort (Rabbitt et al., 2004).

This study started in 1983 and 6375 participants were recruited from Greater Manchester and Newcastle upon Tyne for 10 years. Before the study started, written informed consent was gained from all participants and the self-report and questionnaire data was collected under the approval of The University of Manchester research ethics committee. The study consisted of five waves which were assessed between 1982 and 2010 and in which Personal Details Questionnaire (PDQ) were performed. The first PDQ questionnaire was performed in recruitment until 1995; along with a depression survey. The second PDQ questionnaire was performed between 1984 and 1996 and the third PDQ was performed between 2001 and 2003. The fourth and fifth PDQ were completed in 2007 and 2010, respectively (Didikoglu et al., 2019). The fifth wave also included validated sleep questionnaires, including Pittsburgh Sleep Quality Index (PSQI).

A total of 3477 participants attended a minimum of two test session whilst 212 people completed PDQs at all time points. The average age at the first visit was 65.19 ± 7.45 years and a total of 69.9% of the cohort was female. This project investigated n=96 of these samples; 64 females & 32 males with females making up 66.7%. Vocabulary, fluid intelligence, processing speed and memory cognitive tests were collected longitudinally biennial in waves and these are included in the analysis. The methods of these cognitive assessments were previously described by Rabbitt *et al.,* (2004).

The time at which the tests were completed was not noted so could not be accounted for during statistical analysis. Additionally, time of death and post mortem delay was also not noted or accounted for.

Brain samples were acquired from donors through the Manchester Brain Bank. Ethical approval was granted from the Manchester Brain Bank Committee. All participants had provided written consent to donating their post-mortem samples to the brain bank for research purposes.

COSHH forms and risk assessments were completed prior to the beginning of this study to ensure that all necessary precautions were taken to ensure safety and security, when in the laboratory.

3.3 Brain Pathology Assessments

The brain samples had been assessed and scored at the brain bank by experienced pathologists using the 'ABC score' which is recommended by National Institute on ageing – Alzheimer's Association (Hyman et al., 2012). Only the Braak – neurofibrillary tangles stage (Braak & Braak, 1991) score was used in this analysis. Braak is the assessment of AD-related neurofibrillary pathology that allows the differentiation between initial, intermediate and late stages of AD. This is done by looking at the gradual deposition of hyperphosphorylated tau protein within selected neuronal types in specific nuclei or areas central to the disease process.

3.4 DNA Extraction

Fresh, frozen tissue was taken from superior frontal gyrus (Brodmann area 8) of the frontal cortex from 96 donors. These were extracted using Bioline Isolate II genomic DNA kit (Bioline, UK), following the manufacturers protocol. Throughout the cutting process, the samples were kept on dry ice to ensure no thawing of the tissues.

Once DNA had been extracted, purity and concentration were both measured using Nanodrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). Purity ratios

of A260/A280 were used to assess DNA purity and to ensure the DNA was free from protein contamination. The mean ratio of the absorbance at 260 and 280 nm (A260/280) of the 96 samples was 1.89 (StdDev .118).

3.5 Bisulphite Conversion

The DNA samples were diluted with water in separate Eppendorf tubes, in order to achieve an equal concentration of 500 ng/µL and working mix of 40 µl, following the guidelines from Qiagen EpiTect Fast Bisulphite Conversion Handbook Table (table 1).

Table 1: Bisulphite reaction components

Component	Low Concentration samples (1-500ng) Volume per reaction (µl)
DNA	Variable (Maximum 40 µl)
RNase-free water	Variable
Bisulphite Solution	85
DNA Protect Buffer	15
Total Volume	140

The samples turned from green to blue when DNA Protect Buffer was added, indicating sufficient mixing and correct pH for the bisulphite conversion reaction. The Eppendorf tubes were then incubated in the Eppendorf Mastercycler, using thermal conditions specified according to the Handbook (table 2).

Table 2: Bisulphite conversion thermal cycler conditions

Step	Description	.Time	.Temperature [°C]
1	Denaturation	.5 minutes	.95
2	Incubation	10 minutes	60
.3	Denaturation	5 minutes	95
4	Incubation	10 minutes	60
.5	Hold	indefinite	20

After the PCR finished, the rest of the protocol was followed according to the Qiagen Handbook. A total of 15 µl of DNA was eluted and stored at -20° C until used for analysis.

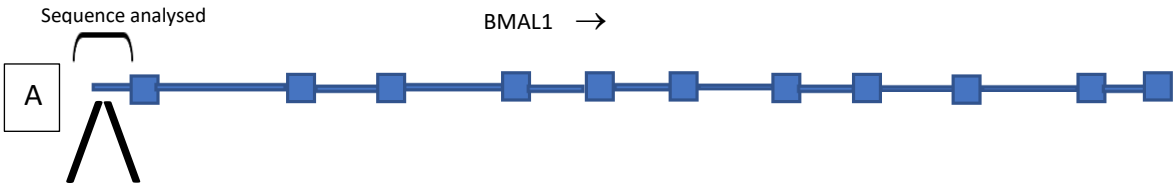
3.6 Designing of *BMAL1* primer

The genomic browser (https://genome-euro.ucsc.edu/index.html), was used to search the gene *BMAL1*. The primers were designed using Pyromark Assay Design SW 2.0 (Qiagen Pyromark Assay Design). The assay was shown with different sets of forward, reverse and sequencing primers with a score out of 100. The forward, reverse, sequencing primers and sequence to analyse are shown in table 3.

Table 3: Primer Information for *BMAL1* gene

	***BMAL1* gene**
Forward Primer	GTGGGATATTTGGAGGTTATGATG
Reverse Primer	ACAATTCCTAACTCCCTCTCT Biotin labelled
Sequencing Primer	ATATTTGGAGGTTATGATGA
Sequence to Analyse	AYGTAAAGAA YGTGAGAATA TTTGTAGTTT TYGGGGTGGA AATGTTTTTT AGAAATATTA AGTATTYGTT TTTTYGTTGA GATTTTGGTA AATTAGGGAT TTTAGGAAGG GTTTGGTATT TAAYGTTTTT AAAATTGGTT TTTTAGATG

3.7 *BMAL1* Schematic Diagram of Target Region



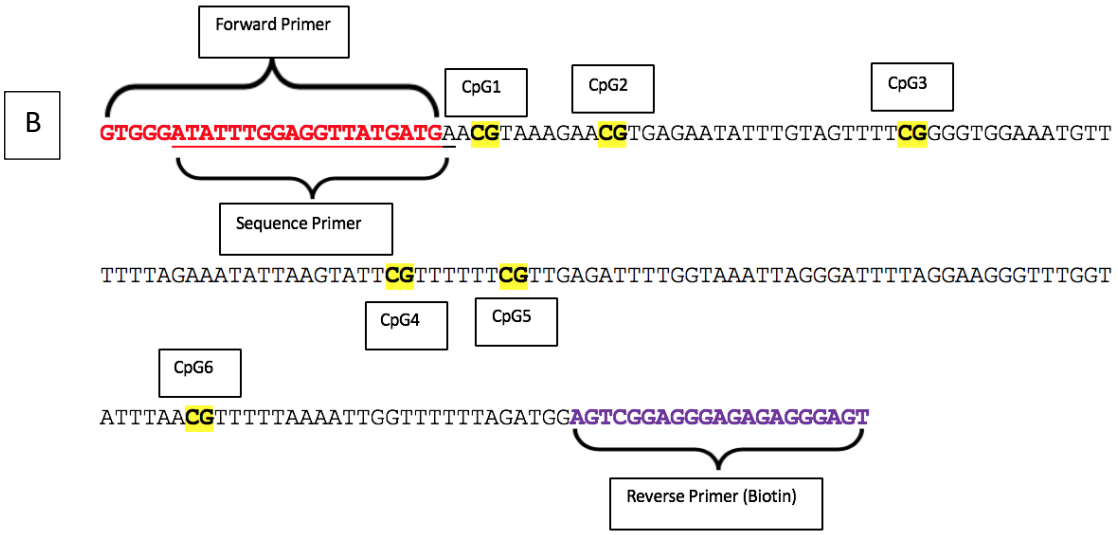


Figure 2: A - Genomic Map of *BMAL1*. *Squares represent gene exons. Transcription of gene occurs from left to right as shown by arrow. Schematic representation – not to scale.* **B- *BMAL1* sequence to analyse.** *Highlighted with the forward, sequence and reverse primers and each CpG site location in the sequence.*

3.8 Polymerase chain reaction (PCR)

To avoid foreign DNA contamination, the following was prepared in a PCR workstation. Master mix was prepared on ice using the reagents and volumes in table 4 and 18 µl added to a 96-well PCR plate. 2µl of DNA was then added but outside of the workstation to avoid risk of contamination and to create a total volume of 20 µl. The plate was covered with an adhesive seal to secure the contents and once secured, it was vortexed and then amplified using the Eppendorf Mastercycler thermal cycler with the steps shown in table 5.

PCR Reagents	**Amount (1 reaction)**
MyTaqHS (DNA Polymerase)	10 µl
Forward Primer	1 µl
Reverse Primer	1 µl

Nuclease free H_20	6 µl
DNA	2 µl
Total concentration	20 µl

Table 4: PCR for 1 reaction

Table 5: DNA amplification PCR thermal cycling conditions

Step	Description	Temperature (°C)	Time	Number of Cycles Per Step
1	Hot Start (DNA Polymerase Activation)	95	5 minutes	1 cycle
2	Denaturation	95	30 seconds	50 cycles
	Annealing	56	30 seconds	
	Extension	72	30 seconds	
3	Hold	4	Indefinite	1 cycle

The PCR products were stored at -20 °C until samples were used for analysis.

3.9 Agarose Gel Electrophoresis

2% agarose gels were used in gel electrophoresis. To make these, 100 ml of 1 x TBE buffer (tris, boric acid and EDTA) is mixed with 2 g of agarose powder and heated in the microwave for 1-2 minutes, until completely clear; being mixed every 15-20 seconds to ensure the solution does not erupt. Once cool enough, 5 µl Midori Green Advance DNA Stain (NIPPON Genetics Europe) is added and mixed in the beaker. The gel was poured into a tray with combs already placed for the wells and waited to set for 20-30 minutes. The gel was placed into a Biorad tank with 1 x TBE buffer and a 50bp DNA hyperladder (Bioline), was added to the first well. The amplified samples were then prepared with 2 µl loading dye per 6 µl of PCR DNA sample and added into the wells and the gel was

electrophoresed at 90V for 30-60 minutes. Once finished, the gel images were viewed using the Odyssey® Fc imaging system (LI-COR Biotechnology, UK).

3.10 Bisulphite Pyrosequencing

DNA methylation analysis for *BMAL1* was carried out using the bisulphite pyrosequencing method on the Pyromark Q24 System (Qiagen, Hilden, Germany) following the manufacturer's protocol. The workstation was prepared, using 70% ethanol, denaturing solution, wash buffer and distilled water and placing them in the correct locations and the heat block (DB-2D, Dri-Block®, Techne) set to 85ºC with the Q24 plate holder placed on top. A master mix was made containing Streptavidin Sepharose High Performance Beads (GE Healthcare Biosciences), Pyromark Binding Buffer and PCR-grade water to the ratio of 1:40:29 respectively. In a 24-well PCR plate, 70 µL of this master mix was combined with 10 µL of PCR product in each well and covered with a film then placed on a TS-100 Thermo-shaker (Biosan) at 1400RPM for 10 minutes. The sequencing primer was diluted to 0.3µM by adding 2.2 µL of primer stock to 712.8µL of Annealing Buffer and vortexed. 25 µL of this was then added to each well of a Pyromark Q24 Plate and then placed in the appropriate place on the work station. After the 10 minutes of shaking, the samples were placed on the workstation to be processed with the lid removed carefully and the vacuum turned on. To process the samples, the vacuum was placed into the samples for 15 seconds to ensure all the liquid had gone and was in the probes filter. The vacuum was then placed into the 70% ethanol for 5 seconds, denaturing buffer for 5 seconds and then the wash buffer for 10 seconds. Next, the vacuum was tilted vertically for a few seconds to allow any excess wash buffer to pass through, then was placed onto the Q24 plate containing the diluted primer with the vacuum switched off and shaken from side to side to dislodge the beads. The Q24 plate was transferred onto the heat block (DB-2D, Dri-Block®, Techne) and incubated for 2 minutes at ~85^{o} C.

The cartridge was cleaned using distilled water to ensure the channels were clear and not blocked from previous use. The cartridge was then loaded with enzyme mix, substrate mix and PyroMark deoxy nucleotide triphosphates (dNTPs) in proportions to pre run information for the specific run determined by the PyroMark Advanced

software. Both this cartridge and the Q24 plate were loaded into the machine and the run started.

Once the run was completed, analysis of the methylation levels were determined using the PyroMark Q24 analysis feature.

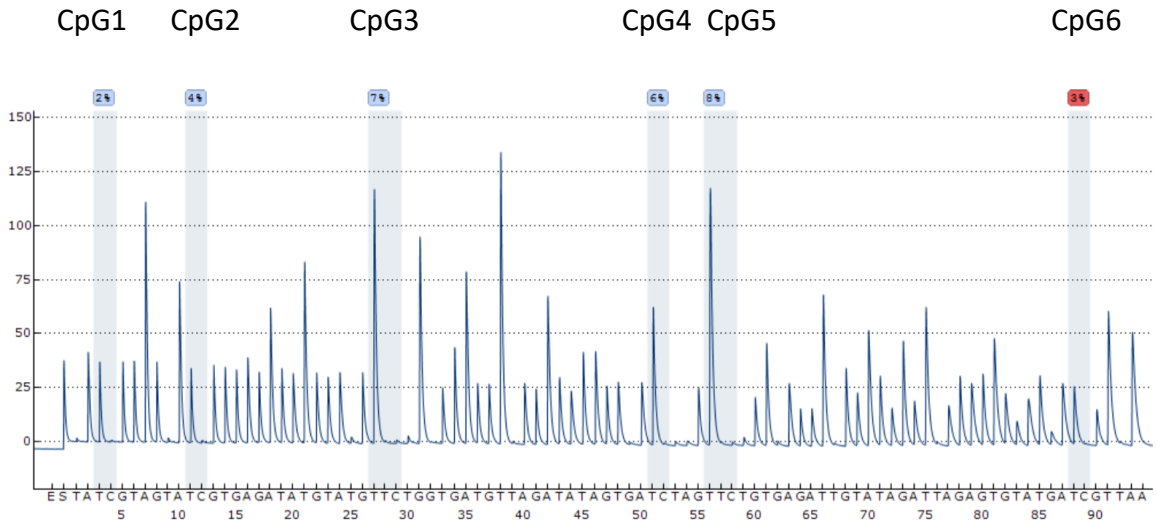


Figure 3: Pyrogram showing the methylation % of CpG sites analysed on *BMAL1.*

3.11 Pyrosequencing Assay Optimization & Troubleshooting

The main issue that was experienced with the pyrosequencing method was the initial signal strength at 0 which would cause a negative number for the rest of the sample. To troubleshoot this, the volume of PCR product added into the sequencing reaction was increased from 10 µL to 15 µL, and the volume of water was reduced accordingly; ensuring the concentration of other reagents remained constant. This increased the signal strength and stopped any negative numbers; allowing all samples to be sequenced. This was the case with 29 of the samples and all are included in this analysis.

For quality control purposes, five samples per each 24 samples sequenced, were chosen at random and sequenced twice. The mean was then taken for both results to get a final methylation result. Six CPG sites on the *BMAL1* gene were successfully assessed for methylation levels.

3.12 Cognitive Scores

All the cognitive data used for the analysis in this study were obtained previously by Rabbitt *et al.,* (2004). Longitudinal scores for fluid intelligence, processing speed, memory and vocabulary were used for analysis and each were taken biennially.

For fluid intelligence, the tests were Alice Heim Tests (1970), also called AH4-1 and AH4-2, and the Cattell and Cattell (1960) "Culture Fair" Test overall total correct score. AH4-1 consists of logic, arithmetic and completion of number series and verbal comparisons. AH4-2 consists of non-verbal problems in which participants must select alternative solutions to the correct completions of logical series that are defined by either progressive mental rotation, addition and subtraction or other comparisons of line-drawn shapes (Rabbitt et al., 2004) - both intelligence tests were 65 problems long and volunteers were encouraged to solve as many of the 65 problems as they could in 10 minutes. The score was calculated with the total number of correct answers and the total number of items attempted.

All four parts of the Cattell and Cattell (1960) "Culture Fair" non-verbal test of general fluid intelligence were included. This test incorporates 46 non-verbal problems with each part only lasting 2.5-4 minutes each. It involves a sequence of pictures with one missing and a specific question relating to the images. The participant must select which one is missing out of a possible 5 images. The score was the total correct score

For the processing speed factor, the contributing measures were the Visual Search task and Letter Search Tests. The Visual Search Task consisted of capital letters printed on pages appearing in random orders, with the participants being given 8 minutes to detect all occurrences of the letters I and O. Scores were the numbers of targets detected and omitted, for example to measure both scanning rate and a direct measure of accuracy also. The Letter Search Test was the Savage (1984) Alphabet Coding Task that is a letter/letter coding task. The participants encoded random sequences of 15 different letters of the alphabet as quickly as possible, using a guide printed as a heading on each score sheet. This was run 4 consecutive times with each lasting 2 minutes during which the code remained constant. This was scored with the numbers of letters correctly

coded during each of these runs and also, the numbers of code letters correctly recalled after all runs had been completed.

For the memory factor, there were 8 available measures: the 10-item free recall test, the coding/letter-letter substitution test, the propositions about people test, the memory circle test (total correct object and position), the 30-item free recall test, the verbal free recall test, cumulative free recall test and delayed recall test. All tests were designed specifically to test different aspects and areas of the brain and scores were recorded accordingly (Rabbitt et al., 2004).

For vocabulary, the Raven (1965) Mill Hill A and Raven (1965) B vocabulary tests were used. A vocabulary test required a selection of the most exact synonym for 33 words from a possible 6 and B vocabulary test required generation of an exact definition of the meaning of each 33 words and there were no time limits. The score was calculated with the total number of correct answers and the total number of items attempted. In addition to these tests, The Wechsler Adult Intelligence Scale (WAIS) (Wechsler, 1986), was also used. WAIS produces scores on four separate subsets of adult intelligence. These are: the Perceptual Reasoning Index (PRI), the Verbal Comprehension Index (VCI), the Working Memory Index (WMI) and the Processing Speed Index (PSI). Scores are calculated based on each of these and then combined to create a Full-Scale IQ (FSIQ).

Participants with severe auditory or visual handicaps were excluded from the longitudinal study.

3.13 Cognition Used Model

Cognitive g factors (vocabulary, fluid intelligence, processing speed and memory) derived from longitudinal biennial measures were included in the analysis. The methods of these cognitive assessments were previously described by Rabbitt *et al*., (2004); they included an intercept and slope per fluid, vocabulary, speed and memory which represent two different perspectives. Intercept is estimate of function at age 70 years from the longitudinal model while slope is the measure of change over time from model.

3.14 Sleep Data

Sleep questions within the PDQ included “Generally, at what time do you go to bed at night?”, “Generally, at what time do you get up in the morning?”, “On average, how many hours sleep do you get every night?”, “How many times during the night do you wake up?” and “Do you have any difficulty in getting to sleep?”. Sleep efficiency (%) was calculated as “sleep duration x 100/(getting up time-going to bed time)” – sleep efficiency above 100% was accepted as 100%. Before analysis, data was cleaned from outliners for sleep times (Didikoglu et al., 2019).

3.15 Depression Data

The Beck Depression Inventory (BDI) (Beck et al., 1988) was used to measure depression and the scores for this were continuous. The self-score questionnaire included “I do not feel sad”, “I am not particularly discouraged about the future”, “I don’t cry any more than usual” and “I have lost interest in other people”. Each has three options to how much the participant agrees with the statement and they must choose one.

3.16 Statistical Analysis

All statistical analyses were performed using Stata Statistical Software (Release 14. College Station, TX: StataCorp LP). The significance threshold was accepted at $p<0.05$. Data obtained was tested for normal distribution using the Kolmogorov-Smirnov test. Data considered to be normally distributed is presented as Mean ± Standard Deviation (SD) and data not normally distributed is presented as Median ± Range unless otherwise stated. T tests were used for normally distributed data and Mann Whitney U tests for not normally distributed data to determine differences in methylation levels between the genders. Data was analysed using Pearson Correlation to determine association between pathology group (Braak score) and methylation amount per CpG sites, and one-way analysis of variance (ANOVA) to determine differences between Alzheimer’s Groups. Pearson correlation was used to analyse methylation amounts and score of longitudinal cognition change; separately for men and women. For longitudinal sleep data analysis, linear mixed model was used to analyse methylation, adjusting for age

and sex. For cross-sectional depression data, linear regression was used to analyse methylation amounts adjusting for age and sex.

4.0 Results

4.1 *BMAL1* Methylation Levels

The mean methylation percentages for all samples per each CpG site are presented in a bar graph (figure 4) to indicate the methylation % per each site CpG site. The mean methylation percentages for all samples per each CpG site were: CpG1 (5.77 ± 6.93), CpG2 (4.80 ± 6.18), CpG3 (7.76 ± 6.34), CpG4 (7.15 ± 5.69), CpG5 (8.40 ± 6.48) and CpG6 (3.48 ± 3.50). Methylation levels were not significantly different between males (n=32) and females (n=64) at any CpG site (p= > 0.05).

4.2 *BMAL1* Methylation and Neuropathology

In association with the Braak & Braak, (1991) criteria, control, intermediate and AD were split into groups by the Brain Bank defined by their Braak score. For control purposes, the highest score was used. For example, a Braak score of IV-V, meant V was taken and used. 2 samples could not be classified into a classification due to Braak score being unavailable, so these were omitted from the analysis.

A Pearson Correlation test was used to analyse Braak stage and methylation revealing that CpG site 2 positively correlated with Braak stage (r=0.26, p=0.01) but lost significance when accounting for multiple analysis. CpG sites 1, 3, 4, 5 and 6 did not significantly associate. Samples were grouped into AD (Braak stage V-VI) and Non-AD (Braak stage 0-IV). Mean methylation of each CpG between the groups are shown in Figure 4 with CpG site 2 again showing a significant difference (t (92)=-2.47, p=0.015) with higher methylation in the AD group. When samples were grouped into control, intermediate and AD by Braak stage, Control (Braak 0-II) (n=46), Intermediate (Braak III-IV) (n=36), AD (Braak V-VI) (n=12), AD group have more CpG2 methylation then intermediate and control groups (F(2,91)=3.02, p=0.05). This suggestive significance is still valid after adjusting for age of death and sex (B=4.60, 95%CI=-0.85/8.34, p=0.017).

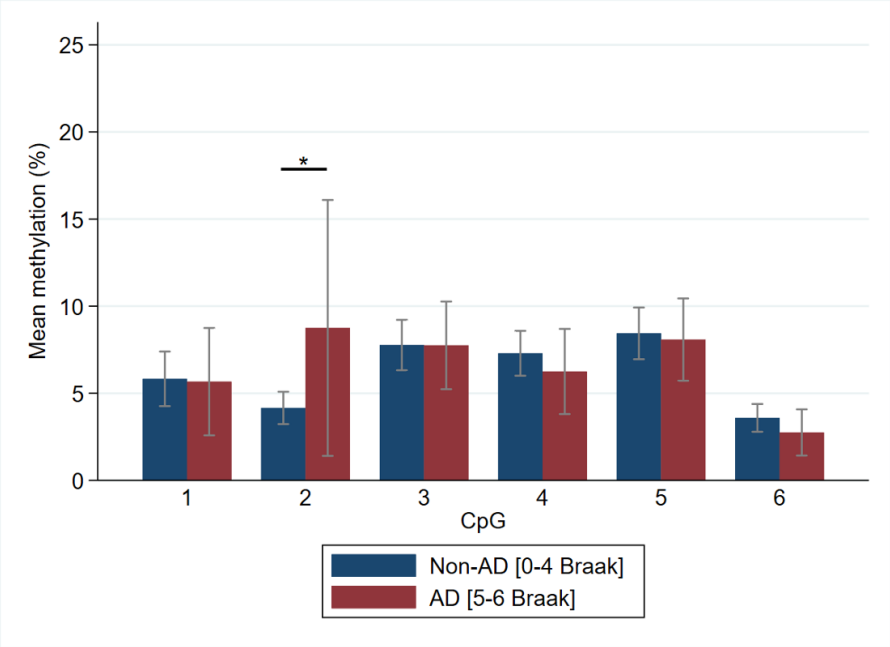


Figure 4: Methylation of CpG sites comparing Braak stage. Mean ± 95% CI is presented. n=94; Control (Braak 0-II) n=46, Intermediate (Braak III-IV) n=36, AD (Braak V-VI) n=12. *= significant at 0.05 level.

4.3 *BMAL1* Methylation and Longitudinal Change in Cognition

A Pearson Correlation test was used to analyse longitudinal cognition scores and *BMAL1* methylation. No significance was found between fluid intelligence, processing speed and memory cognitive tests, though for vocabulary CpG 1 (r=0.20, p=0.05) and CpG4 (r=0.20, p= 0.05) showed positive correlations that did not remain significant when accounting for multiple analysis. Average methylation across all CpGs showed a non-significant association (p=0.06) with vocabulary. When testing intercept (age-adjusted cross-sectional data), CpG2 showed positive correlations with fluid intelligence (r=0.20 p= 0.05) and vocabulary (r=0.22 p=0.03) that again did not remain significant when accounting for multiple analysis (table 6).

Table 6: A Pearson Correlation test to analyse cognitive measures and CpG methylation.

Variable	CpG1	CpG2	CpG3	CpG4	CpG5	CpG6
Longitudinal Fluid intelligence	0.05	0.06	0.06	0.14	0.10	0.15
Longitudinal Processing Speed	0.16	-0.01	-0.08	0.01	-0.04	0.05
Longitudinal Memory	0.06	0.08	0.11	0.13	0.10	0.12
Longitudinal Vocabulary	0.20*	0.13	0.12	0.20*	0.12	0.15
Intercept Fluid intelligence	0.11	0.20*	0.06	0.05	0.05	0.04
Intercept Processing Speed	0.10	0.08	0.01	-0.03	-0.03	0.03
Intercept Memory	0.10	0.07	0.09	0.09	0.07	0.08
Intercept Vocabulary	0.18	0.22*	0.11	0.09	0.05	0.07

Pearson correlation coefficients (r) *=correlation is significant at 0.05 level

4.4 *BMAL1* Methylation and Sleep

Sleep characteristics of the samples used in this study are shown in table 10. A cross-sectional analysis was performed using linear regression adjusted for age and sex to analyse methylation % per CpG and sleep data for the Pittsburgh Sleep Quality Index (PSQI). Data for PSQI was only available for 38 participants and there were no significant findings between any CpG island and PSQI total score (supplementary table 2). However, CpG4 showed a non-significant trend (F(3, 34)=1.92, p=0.06).

Table 7: Sleep Characteristics of the samples used in this study. PDQ (Personal Detail Questionnaire), SD: standard deviation, h: hour, m: minute. %: percent/100. Mean ages at each wave were, respectively 1- 62.66 ± 5.32, 2- 66.24 ± 5.49, 3- 77.90 ± 5.4, 4- 83.03 ± 5.25, 5- 85.0 ± 5.45 (mean ± SD). PDQ4 for night wake was not collected.

	Night wake (number of times)		**Sleep duration (h)**		**Midsleep (hh:mm)**		**Efficiency (%)**	
	n	**mean ± SD**	***n***	**mean ± SD**	***n***	**mean ± SD**	***n***	**mean ± SD**
PDQ1	91	1.14 ± 1.08	93	7.13 ± 1.16	94	3.58 ± 0.65	93	86.23 ± 12.49
PDQ2	70	1.24 ± 1.08	73	7.16 ± 1.18	73	3.6 ± 0.70	73	85.25 ± 12.37
PDQ3	42	2.01 ± 1.06	42	6.95 ± 1.29	44	3.28 ± 0.68	42	80.66 ± 12.83
PDQ4			70	6.64 ± 1.19	74	3.23 ± 0.74	69	77.38 ± 13.63
PDQ5	42	2.31 ± 1.07	52	7.01 ± 1.43	51	3.14 ± 0.7	51	77.91 ± 14.1

A longitudinal analysis was performed using linear mixed model adjusted for age and sex to analyse sleep data and methylation percent per CpG site. Mixed-effects ML regression was used for n=95. No significance was found between sleep duration, midsleep and efficiency for any CpG sites (supplementary table 3). However, night wake was significant for CpG3 (p= 0.03) (figure 5) and CpG5 (p=0.01) (figure 6).

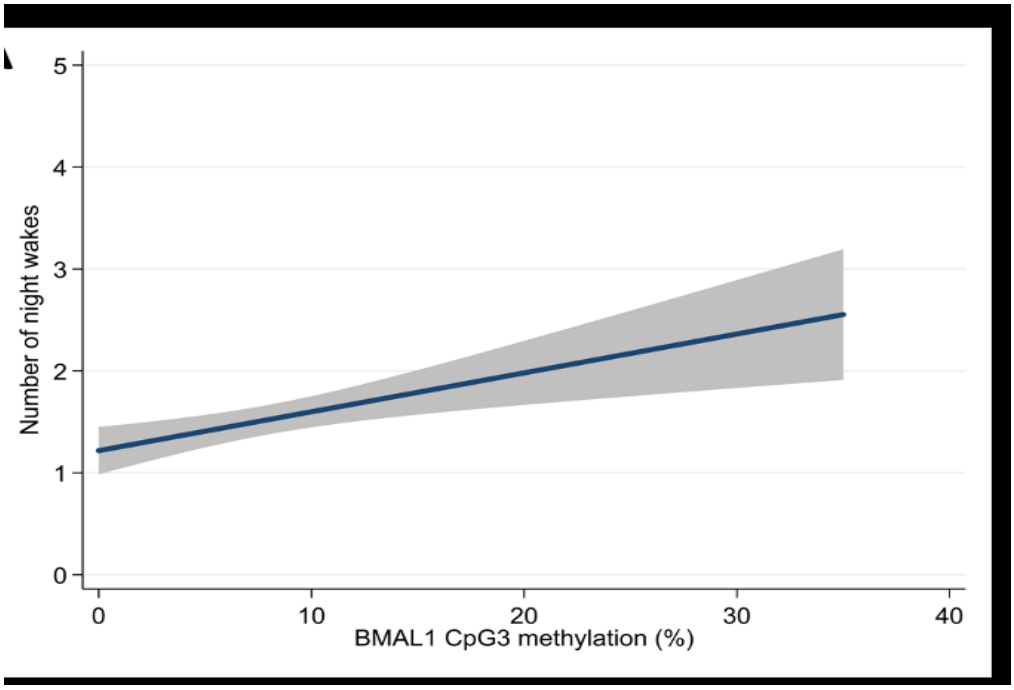


Figure 5: A positive correlation between number of night wakes and % of methylation of CpG site 3 in the linear model adjusting for age and sex*. The grey area represents 95% confidence intervals.*

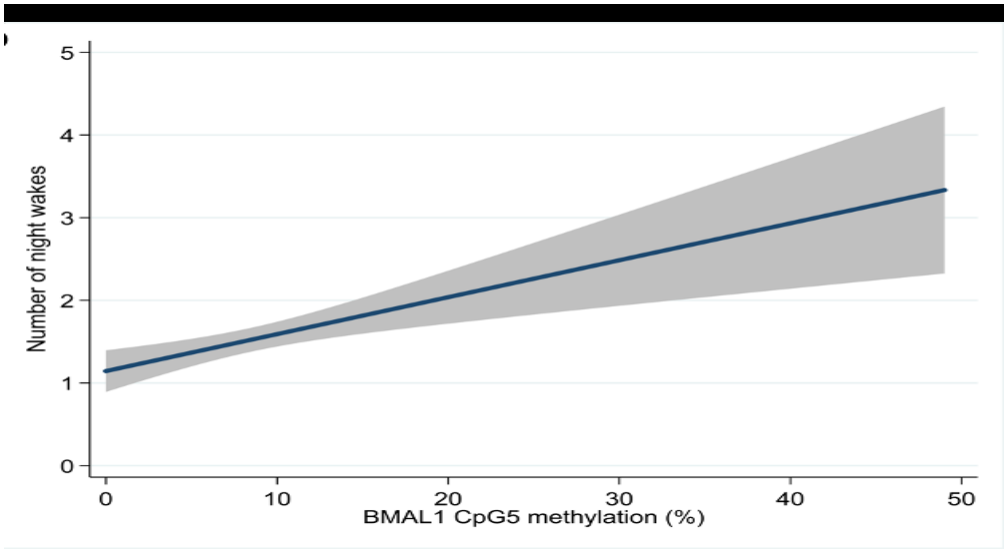


Figure 6: A positive correlation between number of night wakes and % of methylation of CpG site 5 in the linear mixed model adjusting for age and sex*. The grey area represents 95% confidence intervals.*

4.5 *BMAL1* Methylation and Depression

A cross-sectional analysis was performed using linear regression adjusted for age and sex to analyse methylation % per CpG and depression score using Beck Depression Inventory score (BDI). No significance was found between CpG site 2, 3, 5 or 6 and BDI score. However, CpG4 was positively associated with BDI score ($F(3, 89)= 5.64$, $p = 0.02$) and CpG1 showed a non-significant trend $F(3, 89)= 4.91$, $p = 0.06$).

Table 8: Methylation % and BDI score using linear regression adjusted for age and sex. **= significant at 0.05 level.*

BDI Score*CpG site (n=93)	F	P Value
BDI Score * CpG1	(3, 89)=4.91	0.06
BDI Score * CpG2	(3, 89)=3.50	0.96
BDI Score * CpG3	(3, 89)=3.52	0.82
BDI Score * CpG4	(3, 89)=5.64	0.02*
BDI Score * CpG5	(3, 89)=3.53	0.75
BDI Score * CpG6	(3, 89)=4.55	0.09

4.6 Overview & Summary of CpG sites

Table 9: Overview of CpG site results. *The arrows indicate a positive &/or negative correlation. F = females (n=64); M = males (n=32). Almost significant correlations are also shown.*

	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6
Braak score		↑				
Fluid intelligence				↑M		↑M
Processing Speed						
Memory						
Vocabulary				↑F		
PSQI				↑0.06		
Night wake			↑		↑	
Sleep Duration						
Mid-sleep						
Efficiency						
BDI score	↑0.06			↑		

This study found that: CpG1 associates with BDI score to a nearly significant level, CpG2 associates with Braak score, CpG3 associates with night wake, CpG4 associates with fluid intelligence in males, vocabulary in females and BDI score, CpG5 also associates with night wake and CpG6 associates with fluid intelligence in males.

5.0 Discussion

5.1 General findings

The study investigated the associations between methylation on 6 CpG sites on the *BMAL1* gene, with Braak score, cognition scores of fluid intelligence, processing speed, memory and vocabulary in males and females, sleep data including night wakes, sleep duration, midsleep and efficiency and BDI (depression) score. The results found that CpG2 was associated with Braak score, CpG3 was associated with night wake, CpG4 was associated with female vocabulary, male fluid intelligence and BDI score, CpG5 was associated with night wake and CpG6 was associated with male fluid intelligence.

5.2 Braak Score & Methylation

It was found in this study that CpG2 was positively correlated with Braak score ($p=0.01$). Additionally, CpG2 was also nearly significant ($p=0.05$), when comparing the differences between the pathology groups and CpG sites. These results suggest that *BMAL1* is regulated with Braak score to some extent and that levels of *BMAL1* methylation is associated with higher levels of Aβ.

The positive correlation between methylation with Braak stage suggests a reduced activity of *BMAL1* with increased AD pathology, specifically tau and neurofibrillary tangles. Tau pathology has been shown to be the earliest observable AD-like change in human brain, with abnormal tau phosphorylation and aggregation beginning as early as young adulthood and extending to other connected regions even before Aβ is detected (Braak et al., 2011). Several animal studies find mechanistic links between tau pathology and circadian clock gene disruption. Koss *et al*., (2016) showed that transgenic mice with forebrain mutant human tau expression show increased wake and decreased NREM sleep, as well as more robust changes in EEG power than observed in transgenic mice with both tau and Aβ. A transgenic mouse model that develop progressive tau pathology leading to formation of neurofibrillary tangles, show a long free-running period indicating a disruption in the circadian rhythm. They further show disruption in the cyclic

expression of *BMAL1* and other circadian clock genes in the hippocampus (Stevanovic et al., 2017).

Additionally, the methylation rhythm of the *BMAL1* promoter changes in the pre-frontal cortex of patients with AD. Lim *et al*., (2014) found that post-mortem human prefrontal cortex samples revealed attenuated methylation rhythms from samples with AD when compared with control. The rhythms of DNA methylation were then related with rhythms of RNA expression determined by RNA sequencing to support the evident of significant 24-hour rhythmicity of DNA methylation. These studies could explain why CpG2 was positively correlated with Braak score which could be due to the specific CpG islands tested on the *BMAL1* gene. However, distinguishing the cause from effect in epigenetic epidemiology is difficult due to disorders such as AD manifesting in tissues that are inaccessible and are not capable of collecting for longitudinal study.

Another study has shown that *BMAL1* expression peaks at night (Cermakian et al., 2011) and along with *PER1* & *PER2*, displayed significant 24-hour rhythmicity in the brains of AD patients. However, a desynchrony in oscillation was found between the cortex, Bed Nucleus of the Stria Terminalis (BNST) and pineal gland in AD patients (Cermakian et al., 2011); possibly due to the degeneration of the SCN cells in AD brains. This suggests that *BMAL1* methylation, as well as other *CLOCK* gene methylation, would have to be looked at in other parts of the brain and not just limited to the pre-frontal cortex. Due to the BNST and cingulate cortex being involved in decision-making, as well as the pineal gland being a major output of the SCN, the abnormal rhythms observed in these areas may contribute to cognitive and sleep-wake deficits in AD patients (Coogan et al., 2013).

5.3 Cognition & Methylation

AD is characterised by cognitive dysfunction. This includes thinking, reasoning and remembering. The tests used in this study were fluid intelligence, processing speed, memory and vocabulary and only longitudinal scores were used. It is widely reported that sleep deprivation is a major factor affecting cognitive performance. Multiple studies report this, with particular focus on tasks mediated by the prefrontal cortex function

(Harrison & Horne, 2000; Jones & Harrison, 2001). Additionally, it has been previously reported that memory and learning are regulated through the circadian timekeeper (Monk et al., 1997; Wright et al., 2002; Lyons et al., 2006). If memory and learning are regulated by the circadian clock and AD is characterised by disruption to the circadian rhythm, then it could be hypothesised and assumed that memory and learning would deteriorate as AD worsens and the circadian clock is further disrupted.

In this study, a positive correlation was found for male fluid intelligence and CpG4 methylation (p=0.04) and CpG6 methylation (p=0.04). Vocabulary and CpG4 methylation for females (p = 0.03) were also positively correlated. It is noted, within the central nervous system that clock timings were not restricted to the SCN, and ancillary oscillatory capacity has been detected in a variety of brain regions and cell types, including forebrain circuits that underlie complex cognitive processes (Snider et al., 2016). Previous research in mice, suggest that essential signalling events in the hippocampus required for memory, depend on *BMAL1* (Wardlaw et al., 2014). It was reported that mice who were completely arrhythmic in constant conditions, had impaired spatial learning and memory. This supports the theory that cognition performance is dependent on the circadian clock. Snider *et al,* (2016) also reported in mice, when *BMAL1* was selectively deleted from excitatory forebrain neurons but the SCN clock remained the same, deficits in both acquisition and recall were observed. These studies suggest that both the clock timings and *BMAL1*, play a critical role in cognitive performance and for both learning, and memory retrieval.

Only fluid intelligence and vocabulary were positively correlated to the CpG sites tested in this study. Due to testing for gender differences, this could be the reason why there are differences in correlations. It has been previously reported that men had relatively later rhythms of DNA methylation than women (Lim et al., 2014) and in particular, the expression of *BMAL1*, varied based on daily timings but was significantly earlier in women than in men (Lim et al., 2013). In addition to any gender differences, there are other aspects which could be very important. Burke *et al.*, (2015) found that attention, mood and reaction time showed circadian variation when tested at different times throughout the day. This would suggest that other factors would have to be included,

for example, time at which the cognition tests were undertaken, and this could be adjusted for when completing the statistical analysis.

5.4 Sleep Tests & Methylation

Sleep disturbances and lack of sleep are extremely common within the general population; with sleep disturbances and disorders in the elderly affecting more than 80% of people over 65 years old (Foley et al., 1995), but these numbers are even higher for people with AD (Van Someren, 2000). Severe lack of sleep contributes to increased risk of cardio-metabolic disorders like cardiovascular diseases and type 2 diabetes, as well as mental disorders (Porkka-Heiskanen et al., 2013). In this study, night wake had a positive correlation with CpG3 and CpG5 methylation, however, sleep duration, mid sleep and efficiency did not have a correlation to any of the CpG sites tested.

Multiple regions in the brain are responsible for the regulation of sleep/wake states, however, the ventrolateral preoptic area (VLPO) in the anterior hypothalamus and the SCN are implemented in age related changes (Sherin et al., 1996). The VLPO contains galaninergic and GABAergic neurons which send inhibitory signals to arousal areas whilst asleep (Wennberg et al., 2017). Lesions to the VLPO have been associated with long-lasting insomnia in rats (Lu et al., 2000) which could also explain the mechanism in humans, and of which is observed in AD.

Sleep deprivation on both the transcriptome and methylome has been studied and looked at in human samples and experimental animal models (Cirelli & Tononi, 2000; Benedict et al., 2014; Massart et al., 2014; Cedernaes et al., 2015). Cedernaes *et al.*, found that a single night of wakefulness, or missing one nighs sleep, altered the epigenetic and transcriptional profile of core circadian clock genes (including BMAL1) in a number of key metabolic tissues; *BMAL1* methylation in particular, decreased in skeletal muscle. When comparing against nightshift and dayshift workers, Bhatti *et al.,* (2015) found a significant decrease in overall average methylation in the clock genes. Additionally, a CpG island near the transcription start site of *BMAL1* was also hypomethylated, suggesting it be overexpressed amongst nightshift workers. An increase in *BMAL1* expression has also been found amongst shift workers when

compared with night work, supporting this theory (Bracci et al., 2014). This could suggest why night wake was significant in this study. Work history, if known, would also have to be adjusted for when performing the statistical analysis as this could have a very prominent effect on *BMAL1* methylation levels.

AD patients tend to have disrupted sleep patterns and sleep disorders, as well as suffering from OSA (Wennberg et al., 2017) and SDB (Hermann & Bassetti, 2016) and an estimated 70-80% of people with dementia potentially suffer (Wennberg et al., 2017). SDB has been associated with several negative health problems, including cardiovascular disease (Young & Peppard, 2000) and diabetes (Punjabi et al., 2004) and the most common form is OSA. OSA is characterised by episodes of upper airway closure during sleep which results in intermittent hypoxia, impaired gas exchange and arousal from sleep (Shashri et al., 2015). OSA has also been found to be associated with poor executive functioning (Saloria et al., 2002; Sutton, 2008), a decline in verbal episodic memory (immediate recall, delayed recall, learning and recognition) and visuo-spatial episodic memory (immediate and delayed recall) (Wallace & Bucks, 2012). Ayalon *et al.,* (2010) also found that performance for attention and recall was reduced with OSA and increasing age. Additionally, OSA has also been associated with MCI and dementia, with it being estimated in longitudinal studies that there is a 2-6 times greater risk of developing MCI or dementia (Yaffe et al., 2011; Chang et al., 2013). It has been suggested that OSA is associated with several brain changes, including loss of regional volume (Kumar et al., 2008), as well as white matter integrity in the cingulate cortex (Macey et al., 2008), hippocampus (Joo et al., 2013) and some cerebellar regions (Kim et al., 2013). As the majority of these are also symptoms of AD and OSA is also very common in AD, it is difficult to establish which is the cause and which is the consequence; OSA has associations with a decline in cognition which could be the decline that accompanies AD, or it could be the AD causing the OSA.

In order to regulate the circadian clock, the SCN receives input from various time cues such as daylight exposure. The level of light received is a very important factor in synchronising the circadian system and AD patients could exhibit reduced circadian rhythm amplitude due to the SCN becoming less responsive to light as it exhibits reduced

neuronal activity (Swaab et al., 2002). The first process of the light signals being converted into neural signals is naturally affected in the ageing population due to reduced optical transmission at short wavelengths (Turner & Mainster, 2008; Brondsted et al., 2013) resulting in less light reaching the back of the eye. Light exposure can either expand or compress the circadian period, potentially leading to a change in timing or phase, however, the degree of change varies depending on the intensity, timing and duration of light exposure (Phan & Malkani, 2018). Older people, particularly those institutionalised with AD, are more likely to lead an indoor lifestyle; resulting in a decrease in bright light during the day which could lead to circadian disruptions. Studies found that the light conditions in nursing or care homes were not sufficient for both visual and the non-visual aspects of light (Riemersma et al., 2008; Sloane et al., 2008). Figueiro et al., (2015) found that lighting intervention significantly increased circadian entrainment in AD, in regard to phasor magnitude and sleep efficiency, whilst also decreasing symptoms of depression. This disruption in circadian rhythm can lead to sleep problems with symptoms including daytime sleepiness, napping during the day and wandering during the night (Nolan et al., 2003); all of which are common in AD (Bliwise, 2004). Furthermore, studies also found Aβ deposits in the retina of AD mice (Ning et al., 2008; Koronyo-Hamaoui et al., 2011) and also in vivo in humans (Koronyo et al., 2017), suggesting that Aβ accumulation also affects the retina.

This suggestion also leads to the hypothesis that the sleep-wake cycle directly influences levels of Aβ in the brain due to sleep deprivation being shown to increase the concentration of soluble Aβ in mice; resulting in the accumulation of Aβ (Kang et al., 2009). In human cerebrospinal fluid, Huang *et al*., (2012) found that Aβ concentrations were correlated to total sleep time, providing the link between sleep and Aβ accumulation. There are also other aspects of sleep that links Aβ accumulation. For example, sleep is often characterised into two general states measured by polysomnography: non-rapid eye movement (non-REM) sleep and rapid eye movement (REM) sleep. Non-REM consists of three stages, N1, N2 and N3 with slow wave sleep (SWS) being the deepest point of N3 (Wennberg et al., 2017). The main physiological difference between SWS and being awake is down to changes in neuronal activity and these neuronal firings release Aβ into the brain interstitial fluid, thus leading to an

increase in concentration of Aβ in the interstitial fluid when awake (Cirrito et al., 2005). During SWS, the majority of the neurons are in the hyperpolarised silent state, which releases less Aβ than during other stages of sleep, or wakefulness (Ju et al., 2014). This means that if SWS is not reached during sleep, the cortical neurons will fire and depolarise, leading to an increase in the release of Aβ and thus, higher levels in the interstitial fluid. Kang *et al.,* (2009) also found the levels of extracellular Aβ in mice was ~25% higher during wakefulness compared with sleep, and persistently elevated Aβ levels in sleep deprivation conditions. These studies show the connection between sleep and Aβ accumulation, however it is difficult to establish whether the Aβ plaques seen in AD are the cause or consequence of sleep disturbances or if they exacerbate and accelerate the onset of AD pathology. If Aβ is accumulated due to lack of sleep, but AD is characterised by Aβ and lack of sleep, establishing the precursor remains difficult however, it is clear that they both influence each other to some extent.

Poor sleep has also been linked to an increase in cognitive decline, which again is a hallmark of AD. Both short sleep duration (Kronholm et al., 2009; Xu et al., 2011; Stenberg et al., 2013) and long sleep duration (Schmutte et al., 2007; Kronholm et al., 2009; Ramos et al., 2013) have been linked to poorer cognitive performance. Kronholm *et al.,* (2009) found that both short and long sleep duration, as well as tiredness and fatigue were associated with a decrease in self-reported and objectively assessed cognitive functioning. Additionally, sleep duration has also been linked to poorer cognitive performance (Loerbroks et al., 2010; Ferrie et al., 2011) and a risk factor for dementia. Loerbroks *et al*., (2010) found a sleep duration of ≥9hours was associated with the impairment of verbal memory. Additionally, it was also found that increasing sleep duration from 7-8 hours to ≥9 hours was also associated with an increase in cognitive impairment. This again provides the link between AD pathology and sleep; however, the cause or consequence question still remains but it could be hypothesised that poor sleep helps contribute to AD pathology.

The examples above are all factors of AD and sleep however for all it is hard to distinguish which is the cause and which is the consequence. As all are hallmarks of AD which lead to disruptions in sleep, it could be hypothesised that poor sleep is a result of

AD. However, there is significant evidence to support the hypothesis that poor sleep definitely contributes to AD pathology and potentially could lead to AD with a bidirectional link appearing between disturbed sleep and AD.

5.5 Depression & Methylation

Cognitive impairment is the hallmark of AD; however, depression is also common in a large number of AD patients with up to 50% of patients experiencing depression or clinically depressive symptoms (Starkstein et al., 2005). Additionally, between 80%-90% of depressed patients report insomnia, with insomnia also being a risk factor for developing depression (Reynolds & Kupfer, 1987). DNA methylation percentages were determined for each CpG site and tested for correlation against BDI score; CpG4 was positivity associated with BDI score (p=0.02) and CpG1 was nearly significantly associated (p=0.06). These results indicate that *BMAL1* plays some role in depression.

Disruptions in circadian rhythm have been previously associated with psychiatric illnesses such as depression (Sahar & Sassone-Corsi, 2012) and its different forms (Baird & Cauvin, 2000; Abarca et al., 2002); as well as Bipolar Disorder (Yang et al., 2008). The mechanism linking the two is likely represented by uncoupling of autonomous oscillators in the SCN or disruptions in the output from the SCN to other parts of the brain (Yang et al., 2008). Clock genes have been associated with depression and individuals with an abnormally-shifted or arrhythmic biological clock have been linked to a higher risk of developing depression (McClung, 2007). In animal models, Christiansen *et al*., (2016) found that *BMAL1* in particular, along with PER2, was more susceptible to stress. Landgraf *et al*., (2016) also found that SCN-specific *BMAL1*-knockdown mice exhibited depression-like behaviour. Polymorphisms in clock genes have also been investigated and reported to manifest in depressed patients (Partonen et al., 2007; Kovanen et al., 2013; Shi et al., 2016). These studies provide the link between the circadian cycle and depression; however, it is unclear if one or all are related. As *BMAL1* is the main driver of the circadian cycle, it could be assumed that polymorphisms and degradation of this would in turn affect the other clock genes, potentially resulting in a higher risk of developing depression.

Additionally, there are other aspects that circadian timing controls, for example neurotransmitters. Studies have implicated the presence of a large number of neurotransmitters in the SCN (Rusak & Bina, 1990; Reghunandanan et al*.,* 1991; Abrahamson & Moore, 2001). Dysregulated neurotransmission has been observed in AD (Selkoe, 2002; Martorana & Koch, 2014) and dopamine (DA) has been identified as a crucial neurotransmitter that is involved in long-term memory and motor activity. Martorana & Koch., (2014) found in AD-affected mice brains, that Aβ plaque-induced dopaminergic dysfunction was observed. The dopaminergic system has been studied as a key neurotransmitter system that is involved in cognition and emotion (Nardone et al., 2014) because of the changes that it undergoes during the neuropathological ageing process. It has also been suggested that DA plays a key role in synaptic plasticity mechanisms (Hagena & Manahan-Vaughan, 2016). The disarrangement of synapses, impairment of neurotransmissions and cell losses promotes the presence of extracellular deposits of amyloid protein, plaques and intracellular fibrillary tangles which in turn, prompts the symptoms of predementia, like a decline in cognition (Pan et al., 2019). However, it is unclear whether the changes seen in AD patients is the cause, or effect of the disease, due to the changes to the dopaminergic pathways that occur naturally with age. Furthermore, changes in DA levels in the brain are suspected to influence mood in both humans and mice (Andretic & Hirsh, 2000; Nestler & Carlezon, 2006; Roybal et al., 2007). As DA is an important neurotransmitter in depression and the DA system is disrupted with age and particularly with AD but is regulated with *BMAL1* and the circadian rhythm, then it could be hypothesised that depression is more a symptom of AD. However, it is unclear if depression comes before AD, or is rather a consequence of having AD.

In addition to neurotransmitter dysfunction, another hallmark of AD is Aβ plaques and NFT, which have both also been linked to depression. It has been suggested that a lifetime history of depression is associated with Aβ deposition (Rapp et al., 2006; Wu et al., 2014; Chung et al., 2015). Rapp *et al*., (2006) found that AD patients with a lifetime history of depression corresponded to an increase in Aβ plaques and NFT when compared against AD patients without a history of depression. This suggests an

interaction between major depression and AD neuropathology. It has also been suggested that depressive symptoms in older age could be affected by Aβ pathology. Harrington *et al.,* (2016) found that elevated Aβ levels were associated with an increased risk of developing clinically significant depression symptoms during follow-up in preclinical AD. Furthermore, it has also been found that people with MCI and depression were more than twice at the risk of developing AD than those without depression (Modrego & Ferrandez, 2004). There are clear links between depression, Aβ and AD and it could be hypothesised that depressive symptomatology could be an early symptom of underlying AD neuropathology.

The prefrontal cortex has also been associated with depression (Treadway et al., 2015). The prefrontal cortex consists of two sub-regions, the ventromedial prefrontal cortex (vmPFC) and dorsolateral sectors (dlPFC) (Koenigs & Grafman, 2009), with each one being involved in different aspects of human physiology. The vmPFC is responsible for the regulation of affection, which includes the generation of negative emotions and the dIPFC is responsible for cognitive functions, including intention formation, attentional control and goal-directed action (Miller & Cohen, 2001) and both have been linked to depression. It has been found that in lesion models, loss of dIPFC can provoke depression, but loss of vmPFC can cause a decrease in the severity of depression (Ellenbogen et al., 2005; Sachdev & Sachdev, 2005). Additionally, it has been suggested that the mechanism in the prefrontal cortex that's associated with depression is due to lack of activation of oxygenated haemoglobin (Pu et al., 2015). As only the prefrontal cortex was looked at during this study, the positive correlation that was found between BDI score and *BMAL1* methylation could be due to the nature of the prefrontal cortex and it would be advised to investigate other brain regions to confirm the association.

Furthermore, DNA methylation itself has been shown to play an important role in the pathogenesis of various stress-related psychiatric disorders, such as depression due to its reaction to external stress (Januar et al., 2015). Byrne *et al.*, (2013) found in female monozygotic twins that a history of depression was associated with a decrease in global methylation levels when compared with control. Additionally, Numata *et al.,* (2015) found lower methylation levels in patients with MDD when compared against control in

393 CpG sites using an epigenome-wise approach. This would suggest that DNA methylation levels tend to be lower in depressed patients.

Despite the evidence mentioned which links alterations in circadian rhythms to behavioural disturbances and psychiatric diseases, it is challenging to determine whether circadian rhythm disturbances are the underlying cause of diseases or simply symptoms of the disease process (Benca et al., 2009).

6.0 Limitations

One limitation of this study is only *BMAL1* methylation of one brain region (prefrontal cortex) was looked at. However, it is possible that DNA methylation varies across different brain regions. Further research should consider this possibility and investigate other regions of the brains and compare their DNA methylation variability on AD pathology. Additionally, the pre-frontal cortex samples used were from Manchester Brain Bank which, along with the data, were from The University of Manchester Longitudinal Study of Cognition in Normal Healthy Old Age cohort. As the samples were from here, the post-mortem delay was not known and could not be adjusted for when performing the statistical analysis.

Another limitation to this study is sample size. Though 96 samples were investigated, all could not be used due to lack of relevant data. A larger sample size in which all the data could be used, would make for a more substantial statistical analysis. Furthermore, if the six CpG sites that were investigated were corrected for Bonferroni, as a conservative approach, significance would be lost due to limited samples with all the measures.

An additional limitation is reporting error during PSQI/PDQ which are both self-reporting scales. Sleep data may be improved through use of wrist actigraphy studies. However due to this being a large cohort study, the PSQI/PDQ were deemed effective.

A further limitation to this study would be the other factors that should be included. For example, time of death and time of tests undertaken. In the discussion, it was reviewed how these could have an effect on *BMAL1* methylation and how it could change throughout the day. Due to this, these should be accounted for in the statistical analysis if possible and known throughout the longitudinal data analysis.

However, the study also has several strengths. To my knowledge, this is the first study that has examined the association between *BMAL1* methylation in post-mortem pre-frontal cortexes' and related it to AD neuropathology (Braak score), cognition, sleep and depression.

Additionally, this is the first study to use the data obtained from The University of Manchester Longitudinal Study of Cognition in Normal Healthy Old Age cohort and relate them to *BMAL1* methylation and observe correlations with neuropathology, cognition decline, sleep data and depression scores.

7.0 Future Research

This study has shown that *BMAL1* methylation is linked to Braak score, cognitive decline, sleep waking and depression. However, the cause or consequence question still remains unanswered and it is still not known if *BMAL1* methylation is the cause of these correlations or rather a consequence instead.

Further research should consider longitudinal data if possible, for cognition tests, sleep and depression and relate to the different stages of AD. This would allow analyses to be conducted as AD progresses and relate to this. Additionally, further research needs to be conducted on gene regulation in cognitive decline and AD using human brain samples. This study should be repeated using another region of the brain, for example the hippocampus, to allow comparison to the prefrontal cortex and to assess if the levels are altered at all in different regions. Manipulating *BMAL1* and studying effects in other brain regions will shed light on circadian clocks in brain regions that play a direct role in cognition function, sleep and mood regulation.

Furthermore, this study has highlighted the importance of sleep on AD pathology and further work may be able to consider a longitudinal study of care home sleeping patterns against people in their own homes by looking at ways to help improve sleep. Also, a longitudinal study that focuses on sleep and AD but with light therapies would help support the data found in this study.

Other clock genes should also be looked at and this study should be repeated but with PER1, PER2, & PER3 and CRY1 & CRY2 with the use of the same data. This will help form a mechanistic view of the circadian cycle and the role it plays in AD neuropathology, cognition, sleep and depression.

8.0 Conclusion

The use of human brain samples from the prefrontal cortex in this study allowed us to develop a mechanistic view of how the DNA methylation of *BMAL1* methylation, AD neuropathology, cognition, sleep and depression may all be linked together (figure 11).

This study found that *BMAL1* methylation was positively associated with Braak score, longitudinal fluid intelligence score for males, longitudinal vocabulary for females, night wakes and depression (BDI score). However, surprisingly, no significance or associations were found between *BMAL1* and processing speed and memory tests, sleep duration, mid-sleep and sleep efficiency. These results suggest that *BMAL1* methylation does play a role in sleep and AD neuropathology but to what extent needs further research. As the circadian cycle is linked to sleep and sleep highly linked to the circadian cycle, it could only be hypothesised that all play a role in AD neuropathology.

Further studies should look at other important CLOCK genes and relate their methylation to sleep and AD. This will help create a more robust view of the circadian cycle and give insight into how much influence sleep has on CLOCK gene methylation and AD neuropathology.

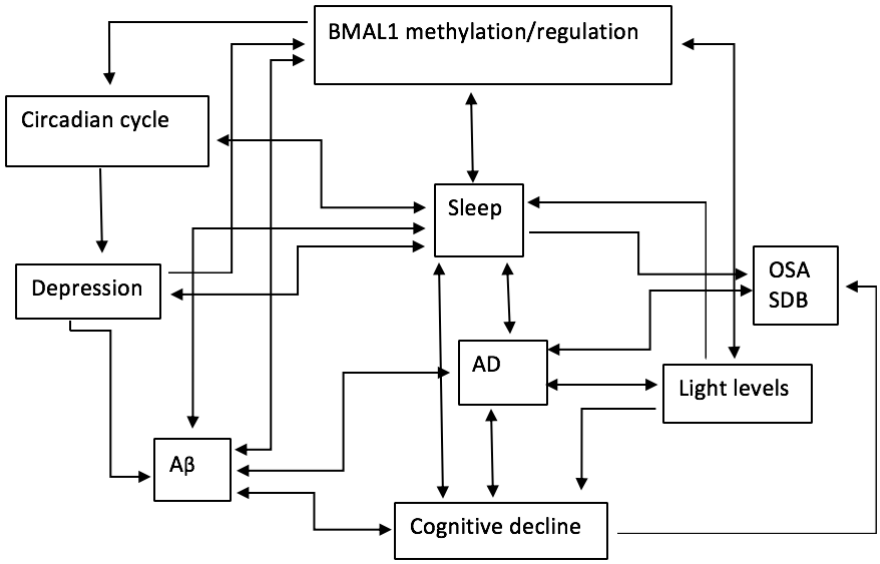


Figure 7: The proposed bidirectional relationship between *BMAL1* methylation, AD and sleep. Potential positive-feedback mechanisms exist between *BMAL1* methylation, Aβ accumulation, sleep quality, AD and cognitive decline. Aβ=Beta-amyloid; AD=Alzheimer's Disease; OSA=Obstructive sleep Apnoea; SDB=Sleep disordered breathing.

9.0 References

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10.0 Appendix: Supplementary Material

Table 1: Chemical, reagents and kits used:

96 well plate	**Starline**
Q24 sequence plate	Quigen
0.2ml PCR tubes	Starline
Pipette tips 10ul,20ul,200ul,1000ul	
PCR plate cover slips	
Nuclease free water	
PCR grade water	
Isolate 2 genomic DNA extraction kit	Bio line
EpiTec fast Bisulphite kit	Quigen
Pyromark q24 regents	Quigen
Agarose powder	.
Tris base	-
EDTA	
DNA ladder 100bp	Bioline
MyTaqHS	Bioline
70% ethanol	-
Denaturing buffer	
Washing buffer	.
BMAL1 forward primer	.Invergion
BMAL1 reverse primer	.Invergion
BMAL1 sequence primer	Invergion
Biorad RT PCR Machine	Biorad
Eppendorf master cycler	
Pyromark sequencer	Quigen
Biorad power pack and tank	Biorad

Table 2: PSQI Score and CpG site using linear regression adjusted for age and sex. *No CpG sites were significant at 0.05 level, however, CpG 4 was nearly significant. N=38.*

PSQI * CpG site (n=38)	F	P Value
PSQI * CpG1	(3, 34)=0.64	0.96
PSQI * CpG2	(3, 34)=0.64	0.94
PSQI * CpG3	(3, 34)=0.64	0.93
PSQI * CpG4	(3, 34)=1.92	0.06
PSQI * CpG5	(3, 34)=0.70	0.68
PSQI * CpG6	(3, 34)=1.79	0.08

Table 3: Sleep data and CpG site usng linear mixed model adjusted for age and sex. **= significant at 0.05 level; **=significant at 0.01 level. N=95.*

Sleep Data*CpG site (n=95)	Sleep Duration (P value)	Midsleep (P value)	Efficiency (P value)	Night Wake (P value)
CpG1	0.92	0.97	0.47	0.84
CpG2	1.0	0.28	0.89	0.86
CpG3	0.33	0.71	0.90	0.03*
CpG4	0.17	0.94	0.28	0.76
CpG5	0.21	0.99	0.69	0.01**
CpG6	0.35	0.49	0.40	0.82