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Disruption of the Choroid Plexus Circadian Rhythm's in Alzheimer's Disease

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Dedication

I would like to dedicate this work to my family. Without them this wouldn’t be possible, and because of them, this was all worth it.

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Lastly, I thank my family, the most import people in my life.

Resumo

A doença de Alzheimer (AD) é uma doença neurodegenerativa caracterizada pela deposição do péptido beta-amilóide (Abeta) e proteína Tau no parênquima e vasos sanguíneos do cérebro. A acumulação destes, quando ocorre em áreas do cérebro responsáveis pelo controlo de ritmos circadianos, pode conduzir a alterações nos ritmos de atividade. O ritmo circadiano é coordenado por um marcador de atividade circadiana central que está localizado no núcleo supraquiasmático (SCN) do hipotálamo. No plexo coroide (CP), um oscilador extra-SCN recentemente caraterizado como tal, também se verificam alterações morfológicas na AD que são representadas pela acumulação de Abeta nas suas células epiteliais . A melatonina é uma hormona secretada pela glândula pineal, cujos níveis se encontram diminuídos na AD. Em condições normais, a melatonina tem uma função neuroprotetora na AD, e mesmo quando a doença já está instalada, esta ação continua presente, embora ainda se desconheça parcialmente os mecanismos responsáveis por esta neuroproteção. A melatonina funciona também como "Zeitgeber", sincronizando os ritmos de expressão dos diferentes genes relógio, regulando desta forma os diversos relógios circadianos distribuídos pelo organismo. Neste estudo, foi utilizado um modelo de murganhos da AD (APP/PS1), de ambos os sexos e com idades de 6 e 12 meses, para determinar alterações na expressão dos genes relógio no CP, em diferentes momentos ao longo do tempo. Para os estudos *in vitro* foi utilizada a linha celular Z310 tratada com Abeta e melatonina de modo a avaliar se a melatonina tinha um efeito modulador na ritmicidade do *Bmal1*. Demonstramos que apenas o *Bmal1* sofreu alterações na expressão circadiana e que esta ocorre em modelos APP/PS1 com 12 meses de idade. Contrariamente, a expressão do *Per2* e *Cry2* não é afetada no modelo APP/PS1. Também foi observado que a melatonina tem a capacidade de modular diversos parâmetros na ritmicidade do *Bmal1*. Estes resultados sugerem uma desregulação do ritmo circadiano da expressão do *Bmal1* num modelo da AD, bem como um efeito modulador da melatonina na expressão dos genes relógio do CP.

Palavras-chave

Amiloide beta, Doença de Alzheimer, Ritmo Circadiano, Melatonina.

Resumo Alargado

Em mamíferos, diversos processos fisiológicos, como ciclos de sono e vigília, secreção hormonal, entre outros, ocorrem associados a uma ritmicidade circadiana. Estes processos com ritmo circadiano associado apresentam dimorfismo entre sexos em várias espécies.

O processo responsável pelo controlo temporal envolve diversos genes denominados de genes relógio. Estes incluem genes como *Clock*, *Bmal1*, *Per1*, *Per2*, *Cry1*e *Cry2* que interagem entre si de forma a criar sistemas de retroação transcricionais autorregulatórios que constituem o relógio molecular circadiano. Um sistema de retroação positivo é acionado pela formação do heterodimero CLOCK/BMAL1 e promove a transcrição do PER e CRY. Outro sistema de retroação negativo termina quando o complexo PER/CRY, após se deslocar para o núcleo da célula, se liga ao CLOCK/BMAL1 inibindo a sua própria transcrição. O principal relógio em mamíferos é o núcleo supraquiasmático (SCN)do hipotálamo. No entanto, existem outros relógios extra-SCN, tanto no sistema nervoso central como no periférico. Um relógio recentemente descoberto foi o existente no plexo coroide (CP). Os CPs são estruturas vascularizadas localizadas no sistema ventricular do cérebro. O CP é formado por uma camada de células epiteliais cuboides que se encontram interconectadas por junções apertadas. O lado basal está em contacto com uma vasta rede de capilares sanguíneos e o lado apical está em contacto com o líquido cefalorraquidiano (CSF). O CP, entre outras funções, atua como barreira à passagem de substâncias nocivas do sangue para o CSF, e é ainda responsável pela produção deste mesmo líquido. O CP também destoxifica o CSF de produtos tóxicos resultantes do metabolismo cerebral.

A demência é descrita pela organização mundial de saúde como sendo “uma síndrome no qual existe uma deterioração da memória, pensamento e na capacidade para realizar tarefas comuns do dia-a-dia”, sendo que 50 milhões de pessoas sofrem com esta condição em todo o mundo. A demência pode ser causada por diversas patologias sendo que a mais comum é a doença de Alzheimer (AD). A acumulação de beta amiloide (Abeta) e de formas anormais de proteína tau estão descritas como sendo duas das principais alterações que contribuem para o desenvolvimento da AD. Na AD estão descritas diversas alterações que ocorrem no CP, tanto a nível morfológico como fisiológico. Umas dessas alterações é a deposição de Abeta que ocorre nas células epiteliais do CP, descrita como sendo tóxica para as mesmas. A acumulação de Abeta nas células epiteliais do CP ocorre tanto em indivíduos com AD como em indivíduos saudáveis. Isto ocorre porque o CP é responsável por retirar o Abeta do CSF e o transportar para a corrente sanguínea de modo a ser eliminado. Este mecanismo de limpeza do Abeta do CSF parece estar associado a um ritmo circadiano, uma vez que os níveis de Abeta aumentam durante o ciclo de vigília e diminuem durante o sono. A este processo está também associado um maior ritmo de limpeza do Abeta durante o sono. Como se sabe, os ciclos de vigília e sono estão associados ao SCN. Os genes relógio, quando mutados no SCN levam a alterações do sono

e na AD está provado que a expressão dos genes relógio está afetada, levando consequentemente a alterações do sono. Uma vez que a limpeza de Abeta está associada a uma ritmicidade circadiana, poderá a AD estar a provocar alterações na expressão dos genes relógio no CP?

A melatonina é uma hormona secretada pela glândula pineal. A sua produção está associada a uma ritmicidade circadiana, atingindo níveis máximos no plasma durante a noite. A melatonina não é a "hormona do sono" sendo que atua mais como um sincronizador endógeno dos ritmos circadianos dos diferentes relógios existentes no corpo. O CP é uma das estruturas descritas como contendo recetores para a melatonina. Níveis diminuídos de produção desta hormona estão diretamente relacionados com o avançar da idade sendo que este pode ser um dos fatores que contribui para o desenvolvimento de doenças neurodegenerativas como a AD. A suplementação com melatonina provou ser eficaz em indivíduos com AD, aumentado a quantidade de sono e melhorando a sua qualidade. Ainda possui diversas capacidades relacionadas com a melhoria da cognição, antiamiloidogénicas, antioxidantes, antidepressivas, entre outras.

De modo a verificar os efeitos da AD sobre a expressão dos genes relógio no CP, utilizamos murganhos APP/PS1 que são um modelo duplamente transgénico da AD. Estes murganhos, de ambos os sexos tinham 6 e 12 meses de idade. Como controlo foram utilizados animais wild-type (WT) que variavam de igual forma em idade e sexo. Com o objetivo de verificar se a melatonina exercia algum efeito modulador na expressão de *Bmal1* no CP, utilizaram-se 4 grupos constituídos por uma linha celular de CP à qual foram feitos diferentes tratamentos de melatonina previamente à adição de Abeta.

Para analisar a expressão circadiana dos genes relógio no CP dos modelos animais, os dados de *Real-Time reverse transcriptase polymerase chain reaction* (RT-PCR) foram analisados com recurso ao *software* CircWave. A análise de Circwave revelou que em machos WT, apenas o *Bmal1* apresentava ritmicidade de expressão, enquanto que em fêmeas WT, para além do *Bmal1*, também o *Per2* apresentava ritmicidade. Estes dados estão de acordo com outros estudos que também verificaram a expressão diferencial dos genes relógio entre sexos, reforçando a ideia de uma expressão dependente da ação das hormonas sexuais. Em animais APP/PS1 de ambos os sexos, o *Bmal1* é expresso de forma rítmica aos 6 meses de idade, mas perde essa ritmicidade em animais APP/PS1 de ambos os sexos com 12 meses de idade. Estes resultados claramente sugerem um efeito da AD sobre a expressão rítmica dos genes relógio no CP. De acordo com a literatura este efeito pode ser explicado pela desregulação dos ciclos de metilação do *Bmal1* provocada pelo Abeta. Enquanto em machos APP/PS1 o *Per2* nunca apresentou expressão rítmica, em fêmeas o *Per2* apresentou expressão rítmica tanto em animais APP/PS1 com 6 meses, como em animais APP/PS1 com 12 meses. A literatura sugere que este resultado se deve a um efeito modulatório do estrogénio sobre a expressão do *Per2*. O *Cry2* não apresentou ritmicidade de expressão em nenhuma das condições testadas.

Tendo a confirmação que a AD altera a expressão rítmica do gene relógio *Bmal1* no CP, fomos testar se a melatonina tinha capacidade modulatória sobre a expressão deste mesmo gene na

presença de Abeta numa linha celular de CP. O grupo com tratamento contínuo de melatonina e o grupo com tratamento descontínuo de melatonina, ou seja na presença da mesma aquando da adição do Abeta apresentaram alterações nos parâmetros de expressão rítmica do *Bmal1* em comparação com um grupo controlo que não teve tratamento com melatonina e que apenas teve exposição ao Abeta. Estes resultados estão de acordo com as funções sincronizadoras que já foram descritas para a melatonina. Também se verificou que o grupo tratado apenas com Abeta não perdeu a ritmicidade na expressão do *Bmal1,* o que sugere que a concentração de Abeta utilizada neste estudo não tem capacidade para desregular a expressão dos genes relógio nas células utilizadas. O grupo tratado com estímulos descontínuos de melatonina, mas que não tinha melatonina aquando da adição do Abeta, perdeu completamente a ritmicidade na expressão do *Bmal1*. Admitimos que possivelmente a melatonina estará a influenciar o equilíbrio *redox* existente nas células e que a sua remoção aquando da exposição ao Abeta facilita a ação do Abeta sobre a ritmicidade das células.
Estes resultados demonstram que estes modelos de murganhos duplamente transgénicos para AD apresentam desregulação da expressão dos genes relógio no CP e que a melatonina apresenta uma possível capacidade modulatória sobre a expressão dos genes relógios no CP.

Palavras-chave

Amiloide beta, Doença de Alzheimer, Ritmo Circadiano, Melatonina.

Abstract

Alzheimer´s disease (AD) is a neurodegenerative disorder, characterized by amyloid beta (Abeta) and tau protein deposition in the brain parenchyma and blood vessels. Abeta accumulation in areas of the brain controlling circadian rhythms can delay or shift activity rhythms. The circadian rhythm is coordinated by the master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The choroid plexus (CP), a recent characterized extra-SCN circadian oscillator, is also known to exhibit morphological changes in AD which are exacerbated by the presence of Abeta deposits in CP epithelial cells. Melatonin is a hormone secreted by the pineal gland and there's some evidence that, in AD patients, the circulating levels of this hormone are diminished. Under normal circumstances, melatonin acts as a neuroprotector against AD, but how this protection occurs is still to be fully comprehended. It also acts as a Zeitgeber, synchronizing the rhythms of the circadian genes, regulating the body's circadian clocks. In this study we addressed the question whether Abeta contributes to CP's circadian clock disruption and if melatonin modulates circadian clock genes expression therein. Using an AD mouse model (APP/PS1), we investigated changes in the expression of CP clock genes at different time points, in female and male animals, aged 6 and 12 months old. In addition, *in vitro* studies using Z310 cell line treated with Abeta and melatonin were used to examine if melatonin modulated *Bmal1* circadian expression. We demonstrated that only *Bmal1* circadian expression is altered in AD mice model 12 months old. Contrarily, *Cry2* and *Per2* expression were not affected in the APP/PS1 model. In addition, we found that melatonin modulated several parameters in the circadian expression of *Bmal1*. These results indicate that Abeta deposition on the CP disrupted the rhythmic circadian expression of *Bmal1* and that melatonin modulates CP clock gene circadian rhythms in the presence of Abeta.

Keywords

Amyloid beta, Alzheimer's Disease, Circadian Rhythm, Melatonin.

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Abbreviations and Acronyms list

AD	Alzheimer's disease
Abeta	Amyloid beta
APP	Amyloid precursor protein
AANAT	Aryalkylamine-N-acetyl transferase
Bmal1	Brain and muscle ARNTL-1
CNS	Central nervous system
CSF	Cerebrospinal fluid
CP	Choroid plexus
Clock	Circadian locomotor output cycles kaput
cDNA	Complementary deoxyribonucleic acid
Cry	Cryptochrome
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
Pgp	Glycoprotein-P
HIOMT	Hydroxyindole-O-methyl transferase
LRP	Lipoprotein receptor-related protein
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
ROR	Nuclear retinoic acid receptor-related orphan receptor
Per	Period
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
TRI-reagent	Reagent for the isolation of high quality total ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SIRT1	Sirtuin 1
SCN	Suprachiasmatic nucleus
tRNA	Total ribonucleic acid
TTR	Transthyretin
WT	Wild-type
ZT	Zeitgeber time

I. Introduction

1. Circadian Rhythms

An organism behavior, physiology, and biochemistry changes during the day, exhibiting circadian rhythmicity [1,2]. Robust circadian rhythms can be found governing diverse physiological processes in mammals, from sleep-wake cycles, to hormone secretion and many other functions [3]. The alignment of the circadian rhythm to the environmental stimuli or "Zeitgebers" to maintain periodicity is named "entrainment" [4]. Light is the strongest Zeitgeber, and knowing that a day on earth has a duration of 24 hours, the circadian clock entrains to a 24 hour light-dark cycle resulting in diurnal, nocturnal or crepuscular behaviors [4,5]. Maintaining both biochemical and behavioral rhythms aligned with the circadian time is essential for survival since it enables all species to match their physiological and behavioral processes in synchrony [6]. Even without external temporal cues, circadian rhythms maintain their period of 24 hours, approximately, if the conditions remain constant [7]. This supports the hypothesis that circadian rhythms are generated by internal clocks instead of external cues such as light or feeding [7].

Differences in the circadian rhythms between sexes are evident and seen across a multitude of species like mice, rats, hamsters and even humans [8,9]. Sexually dimorphic differences are seen in the distribution of daily activity [10], time spent sleeping [11], phase responses to light pulses [12,13], rates of re-entrainment [14], susceptibility to splitting [15] and free-running period [8,11,12,16,17]. A sexually dimorphic circadian period in adults, is only achieved in post-gonadal puberty completion, highlighting a significant impact of gonadal hormones on circadian period [18]. More recently, it was described that during the perimenopausal period, changes in the circulating hormones occur, and there is evidence that lower levels of estradiol and increasing follicular stimulating hormone levels are related to poorer sleep quality [19]. This shows the importance of understanding the connection between hormones and sleep, improving sleep quality and well-being.

1.1 Molecular Circadian Clock

The process of timekeeping involves a set of circadian clock regulated genes, which include *circadian locomotor output cycles kaput (Clock)*, *brain and muscle ARNTL-1 (Bmal1)*, *Period (Per1* and *Per2)* and Cryptochrome (*Cry1* and *Cry2)* that interact between them, forming transcriptional autoregulatory feedback loops that constitute the molecular circadian clock (Figure 1) [20-23].

The positive feedback loop is powered by the heterodimerization of CLOCK protein with BMAL1. In certain tissues or cells where CLOCK is not expressed, neuronal PAS domain-containing protein 2 can take its place in the heterodimer [24]. CLOCK/BMAL1 promotes the transcription of clock controlled genes which include the ones encoding for PER and CRY proteins [20,21,23]. The negative feedback loop closes at the point where PER/CRY after shuttling to the nucleus binds

with CLOCK/BMAL1, inhibiting their own gene transcription [20,21,23]. Nuclear retinoic acid receptor-related orphan receptor (ROR) and REV-ERB, a nuclear receptor that acts as a ligand-dependent suppressor of gene transcription [25], are all involved in the auxiliary loop, with CLOCK/BMAL1 mediating activation through E-boxes in their promoters [26]. REV-ERB represses [27] while ROR regulates positively *Bmal1* expression. [22,28].

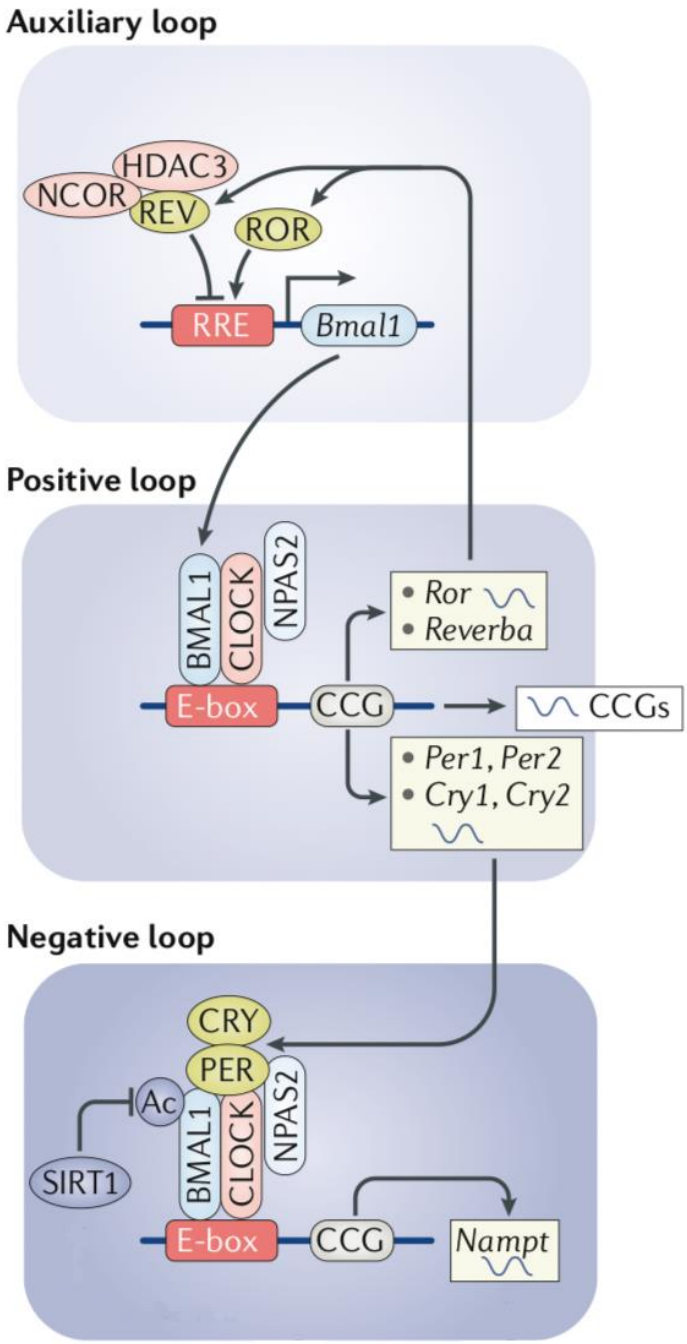

Figure 1. The mammalian molecular clock. The mammalian molecular clock oscillates within a 24 hour cycle, in a series of transcriptional and translational feedback loops. It can be divided in different loops: the positive, the negative and the auxiliary feedback loop. Adapted from *Logan and McClung, 2019* [22].

1.2 Suprachiasmatic Nucleus

The suprachiasmatic nucleus (SCN) in the hypothalamus is the primary circadian pacemaker in mammals [29-33]. Grafts of SCN tissue restore circadian rhythmicity in arrhythmic animals that had their own nucleus destroyed [34-37]. In mammals, the SCN exerts direct control over many physiological and neuroendocrine rhythms [38,39].

The SCN is essential for synchronization of the internal circadian time with the external environmental time that surrounds the organism, integrating different stimuli from internal and external sources [6,39]. Synchronization ("entrainment") of the SCN to the light/dark cycle is essential for the synchronization of SCN clock genes expression [23]. This entrainment involves the retinohypothalamic tract which sends time-of-day specific signals from the retina to the SCN using different combinations of peptides and thus modulating the SCN activity [40,41]. The sensitivity of the SCN to photic inputs can be modulated by extra visual stimuli [42]. The core of the SCN is essential for the production of the rhythmic output signal for the central nervous system (CNS) [43,44]. Within the SCN core there are small-world neuronal networks which are a robust and redundant way of maintaining circadian synchronization [45].

The SCN is not the only circadian clock present in the organism. Several extra-SCN clocks have been described in the CNS as well as in extra-CNS tissues, receiving not only SNC originating inputs, but also sending their own outputs (neurotransmitters or other modulators like steroid hormones) to the SCN, modulating it [46]. Extra-CNS oscillators, denominated as peripheral oscillators, are present in the retina, lung, liver, ovary and even fibroblasts [47-51]. This peripheral oscillators are all synchronized by the SCN [52] but they are also entrained by different non-SCN cues like food intake, one of the strongest entrainment cues in these tissues [49,53,54]. Other cues such as temperature [55,56], oxygen [57] and glucocorticoids [58] also have influence over the entrainment of these peripheral oscillators circadian clocks.

1.3 Extra-SCN Clocks

Desynchrony between SCN and extra-SCN clocks is suggested as a precipitating factor leading to disease [59]. It is a SCN responsibility to synchronize all the clocks present in the organism. Other synchronizing cues exist, and their continuous action over the body's circadian clocks might overrule SCN's synchronizing action leading to internal conflicts between different clocks and the associated functions [59]. Cellular oscillators located centrally at the CNS include the pineal and pituitary glands, the arcuate nucleus, median eminence and the retrochiasmatic and supraoptic nucleus of the hypothalamus [60]. In this list we also have the olfactory bulb [61], considered for many years the most robust extra-SCN circadian clock and the recently discovered choroid plexus (CP) [62-64]. More details concerning this new clock will be given in the next section.

1.3.1 The CP as a New Extra-SCN Clock

The CPs are vascularized structures located in the cerebroventricular system: third, fourth and lateral ventricles (Figure 2) [65]. On the ventricles, the ependymal epithelium that comprises the

CP is a modified version of the ependyma that coats the inside of those same ventricles [66]. Thus, the CP is constituted by a single layer of cuboidal epithelial cells connected to each other by tight junctions (Figure 3) [66,67]. On the basal side of the CP there's a dense network of capillary which are fenestrated [68,69]. This allows for molecules and fluids to exit the blood stream and integrate the interstitial fluid [70], with the CP acting as a barrier to the passage of polar compounds from the blood to the cerebrospinal fluid (CSF), [71]. Together with the blood-brain barrier, the blood-CSF barrier are essential in protecting the CNS from pathogens, toxins and inflammatory molecules circulating in the blood stream [72]

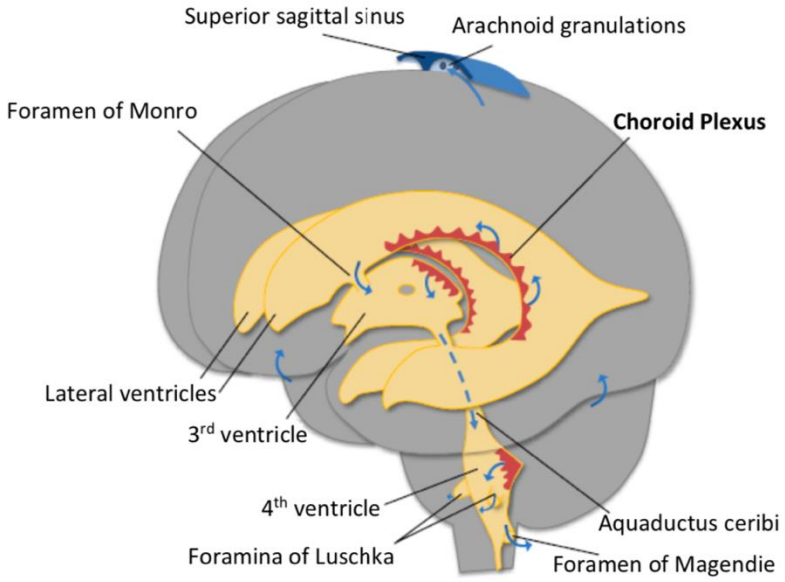

Figure 2. The CP localization in the CNS. The CPs are located in the third, fourth and lateral ventricles which are all interconnected to enable the circulation of the CSF. Adapted from *Damkier, Brown and Praetorius, 2013* [73].

Besides other functions, the CPs are responsible for the production of CSF [71,73]. The side of the CP in contact with the CSF presents numerous microvilli which are thought to mix the CSF maintaining and blending the different compounds present in this fluid [71]. The CP is also responsible for detoxifying the CSF from toxic products resulting from brain metabolism [74] and producing numerous proteins that have nutritional and neuroprotective properties in the brain [75]. It may use olfactory-like chemosensing systems to detect alterations to the CSF chemistry in order to respond to those alterations maintaining brain homeostasis [76–78]. Therefore, the CP has a crucial role in the brain's metabolism, neuronal function, neuro-signaling, immunological and inflammatory processes, neuroprotection and neurodegeneration [79]. As reported previously, the CP is now considered a strong circadian clock. The presence in CP of clock genes (*Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, *Per2* and *Per3)* messenger ribonucleic acid (mRNA) expression and corresponding proteins was first reported in 2015 [64]. The existence of a functional CP circadian oscillator was further confirmed with bioluminescence assays using PER2::LUC and

Bmal1-ELuc mice [62,63], showing that the CP is able to generate circadian rhythmicity with a mean period around 24 hours.

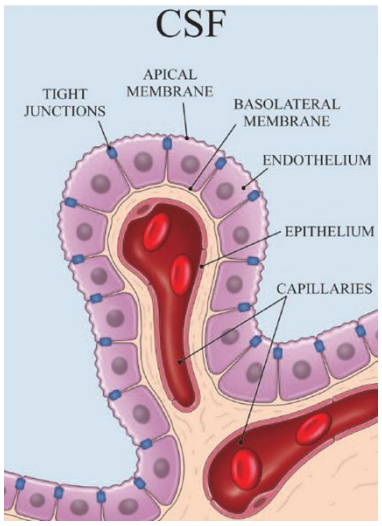

Figure 3. The CP structure. The CP is constituted by a single layer of cuboidal epithelial cells connected to each other by tight junctions. Adapted from *Nakada and Kwee, 2019* [80].

Moreover, it was demonstrated that clock genes are under circadian regulation in a sex dependent way in the rat CP [64]. Bmal1, Cry2 and Per2 are rhythmically expressed in the CP of female rats while only *Cry2* and *Per2* express rhythmic expression along the day in males [64]. *Clock* seems to be constitutively expressed throughout the day in both sexes [64]. In the males' CP, Bmal1 is downregulated when compared to females', whereas Per2 and Per3 are upregulated, suggesting that sex hormones control the CP clock gene expression [81]. Thus, the male CP clock has a less pronounced circadian variation of the clock genes than females' evidencing the sex hormones influence and their regulatory effect [64]. The CP possesses estrogen receptors [82] but these are scarce in the SCN [83]. Thus, the CP may act as an extra-SCN site of action for estrogen, communicating with the SCN and adjusting the SCN circadian rhythmicity [84] The variations that occur on the expression of the CP clock genes , resulting from sex hormone action, can be part of that regulatory mechanism. This regulatory effect might be supported by the fact that the circadian rhythms of expression of CP clock genes are more robust than the ones exhibited by the SCN [62].

2. Alzheimer's disease

Dementia is described as being "a syndrome in which there is a deterioration in memory, thinking, behavior and in the ability to perform everyday activities" by the World Health Organization (14, May 2019) , that also reports that, worldwide, 50 million people suffer from this condition. Dementia can be caused by several pathologies but none has the impact of Alzheimer's disease (AD). Between 75% to 80% of dementia cases are caused by AD [85].
AD was first reported in 1907 [86] and today it's considered by the world health organization as a priority regarding public health [87].

Accumulation of both Amyloid beta (Abeta) and of an abnormal form of tau protein are thought to be two of the major alterations occurring in the brain tissue contributing for AD development [88]. It can also encompass dystrophic neurites, microglial activation, neuropil threads and associated astrogliosis and also cerebral amyloid angiopathy [89]. All these pathological processes have consequences, culminating in neurodegeneration characterized by synaptic and neural loss, to the point where macroscopic atrophy is visible [87] being that the first pathological signs can appear approximately 15 years earlier than the onset of cognitive impairment [90]. In AD, the CP suffers some anatomical changes that are an exacerbation of the ones occurring with normal ageing [91]. Height of the epithelial cells can be reduced in 22% in relation to age matched controls [92]. The basement membrane becomes thicker and irregular and the villi stroma becomes fibrotic with extensive vascular thickening [92,93]. There is a greater intracellular distribution of pathological entities like lipofuchsin vacuoles and Biondi Ring Tangles, and these are present in a larger number of epithelial cells [94,95]. CSF production is also reduced in AD patients when compared to age matched controls [96]. Deposition of Abeta is neurotoxic for CP epithelial cells [69], which might be one of the factors contributing to the alterations that occur in the CP in AD and in normal ageing.

2.1. AD and Circadian Rhythms

The CP epithelia presents Abeta accumulation not only in AD patients [97] but also in healthy subjects [98]. The CP is responsible for the uptake of Abeta, and this mainly occurs from the CSF to the blood stream [98,99]. Several transporters are involved in this clearance process like the low-density lipoprotein receptor-related protein (LRP), megalin mediated transcytosis and glycoprotein P (Pgp) [99]. In normal aging, there's a decline in megalin mediated transcytosis [100,101], which is balanced with an increase in LRP and Pgp [101].

The CP synthesizes several Abeta scavenger proteins like transthyretin (TTR) and clusterin [102]. TTR is a protein known to bind Abeta [103] preventing the formation of Abeta plaques [104], while clusterin after binding to Abeta facilitates its transport across the blood-CSF barrier into the blood stream [102,105]. The Abeta clearance mechanism seems to be associated with a circadian rhythmicity. In amyloid precursor protein (APP) transgenic mice and in young healthy men volunteers, Abeta levels increase during wakefulness and decrease during sleep [106,107]. The mice model was also subjected to chronic sleep deprivation and this lead to greater Abeta plaques deposition when compared to their age-matched littermate controls [106]. Abeta plaques formation was reported to disrupt the sleep/wake cycle in a mouse model of AD amyloidosis [107]. Abeta clearance from the interstitial fluid to the CSF and from the CSF to the blood stream is greatly enhanced during sleep time [108]. This highlights the tight relation between sleep and Abeta formation and clearance.

As described above, circadian rhythms of sleep-wake cycles are driven by the master circadian clock [109]. The SCN core clock genes when mutated, lead to circadian rhythm sleep disorders in humans [110]. Changes in the clock genes expression can have different outcomes since, for example, Per1 and Per2 cooperation is responsible for confining the circadian rhythm to a 24

hour period [111]. Per1 expression shortens the period and Per2 lengthens it [111]. AD was found to have an effect over the clock genes, disrupting their expression in the SCN and in other brain regions such as the hippocampus, frontal cortex and the brainstem [112]. These changes, eventually result in desynchrony between various brain circadian oscillators which can also explain the sleep-awake cycle disruptions [113] associated with Abeta plaques formation [107]. Increasing severity of dementia in AD was also associated with disorganization and decreased amplitude of the daily pattern of activity [114].

If the clearance of Abeta is linked with the circadian rhythm mechanism, and the development of AD is associated with the disruption of the clock genes expression, the disruption of the CP circadian clock might lead to a deficient Abeta clearance and an aggravation of the disease and patient's condition. In addition, sex hormones also have a significant impact on the prevalence of the disease. The Framingham study found that there is a higher risk of developing the disease in women [115]. At the age of 65 years, the risk of developing AD is almost two times higher in women [115]. A faster decline and greater deterioration of cognition occurs in elderly women compared to elderly men suffering from the disease [116]. We believe that understanding the link between the dysregulation of the CP circadian clock and AD, bearing in mind the influence of sex hormones on the process, might represent an interesting target to prevent AD.

3. Melatonin

3.1 Biosynthesis

Melatonin is a hormone secreted by the pineal gland. There, tryptophan is absorbed from the blood stream and converted into serotonin, which is then transformed into melatonin by a two step process involving two enzymes that act in a sequential fashion [117]: Arylalkylamine-N-acetyl transferase (AANAT), which is the limiting enzyme, and hydroxyindole-O-methyl transferase (HIOMT) [118]. Melatonin rhythmic secretion has its origin in the SCN [119]. The SCN sends neural projections to the superior cervical ganglia [120] which then projects to the pineal gland. Norepinephrine is the main neurotransmitter regulating the pineal gland activity [117]. This neurotransmitter is released during the night in response to signals coming from the SCN [117]. Norepinephrine activates adenylate cyclase which promotes the biosynthesis of AANAT [121]. Light inhibits the secretion of melatonin, and darkness enhances it [122].

The retina, bone marrow cells, platelets, skin, the gastrointestinal tract of vertebrate species, lymphocytes, Harderian gland, cerebellum and the CP are all extra pineal sites for melatonin secretion [123-131].

3.2 Mechanisms of Action

Due to its high solubility in lipids [132], melatonin is able to reach the brain, getting through the blood-brain barrier, as well as reaching other body tissues [133].

Melatonin has two membrane bound receptors: MT1 and MT2 [134,135]. Both of these receptors belong to the G-protein couple family, seven transmembrane receptors [136]. Melatonin receptors

are present in a variety of tissues: blood vessels, heart, gonads, liver, kidneys, adrenal cortex, pancreas, spleen, breast and mammillary glands, placenta, skin, immune system, pituitary gland, adipose tissue, gastrointestinal tract and several brain structures [137]. The CP is one of the brain structures expressing melatonin receptors [138]. Melatonin also exerts action over nuclear receptors, one of which is ROR [139], an important piece of the circadian clock's network [22], suppressing RORalpha's transcriptional activity [140]. Some investigators defend the idea that melatonin binds directly to nuclear receptors [141] while others think that melatonin's effect is mediated by MT1 membrane receptor [140].

Melatonin is not the "hormone of sleep" but acts more as a night period indicator to the different circadian rhythmic physiological processes that occur in the body, like body temperature variations [142]. Melatonin may act as an endogenous synchronizer of the body's circadian rhythms, maintaining the different clocks working in synchrony [143].

3.3 AD and Melatonin Action

After birth, melatonin levels increase until puberty, where they reach a peak [144]. Later in life, melatonin synthesis [145] and consequent circulating levels, start to decline [146]. Decreasing levels of melatonin production in older individuals [147] can be a major point contributing to the development of neurodegenerative diseases [148]. Melatonin levels seem to be directly correlated with AD. Decrease of melatonin levels in the CSF occur at the same time as AD neuropathology progresses [149] and AD patients show lower levels of melatonin when compared to their healthy counterparts [150,151]. As described earlier, AD was found to disrupt the expression of circadian clock genes in the SCN and in other brain regions such as the hippocampus, frontal cortex and the brainstem [112]. Some reports also propose that poor sleep quality and quantity is directly related with an higher risk of developing dementia [152]. So circadian rhythms disruption, as we can see, can be interconnected to AD at the different levels of the development of the disease, both as a risk factor and as a consequence [112,152].

Melatonin supplementation improves total sleep time at night in AD patients [153]. Sirtuin 1 (SIRT1) is known as the "longevity protein" and in cellular and mouse models of AD it was described to attenuate Abeta production [154]. This protein expression is promoted by melatonin signaling, and SIRT1 overexpression may be a protection factor in AD phenotypes [154]. Melatonin stimulates antioxidant defense systems in the brain [155,156] and is itself a free radical scavenger [157,158]. Tau hyperphosphorylation is reduced in the presence of melatonin, this reduction can be partially due to melatonin's antioxidant properties preventing phosphorylation [159]. It also presents anti-amyloidogenic capabilities [159,160] (Figure 4). By binding to Abeta, melatonin prevents Abeta aggregation [161] and could have a regulatory effect over APP expression, diminishing beta-APP levels [160]. This anti-amyloidogenic properties might also present an anti-inflammatory effect since Abeta plaque formation is responsible for neuroinflammation [162]. Melatonin also presents the ability to prevent the formation of Abeta fibrils [163] by forming non-covalent complexes with Abeta itself [164]. Cognitive impairment was reduced in a mouse model of AD after prophylactic administration of melatonin, this effect was independent of an

antioxidant pathway [165]. Again, using a mouse model of AD treated with melatonin and subjected to behavioral tests, as for example elevated plus maze test and forced swimming test, it was demonstrated that melatonin prevented anxiety and depression-like behaviors [166]. This effect was associated to an augment in glutathione S-transferase P-1 (an anxiety-associated protein) and complexin-1 (a depression-associated protein) in the hippocampus [166]. A commonly observed condition in AD patients is "Sundowning" [167]. This condition is characterized by disorganized thinking, reduced focus, wondering, agitation, perceptual and emotional disturbances, and is associated to a circadian rhythmicity, manifesting late in the afternoon or in the early evening [167]. Melatonin treatment is associated with improvements in sundowning [167]. Together, melatonin seems to have a very powerful protection effect against AD development and the understanding of how this protection occurs might lead to better options regarding treatment of AD.

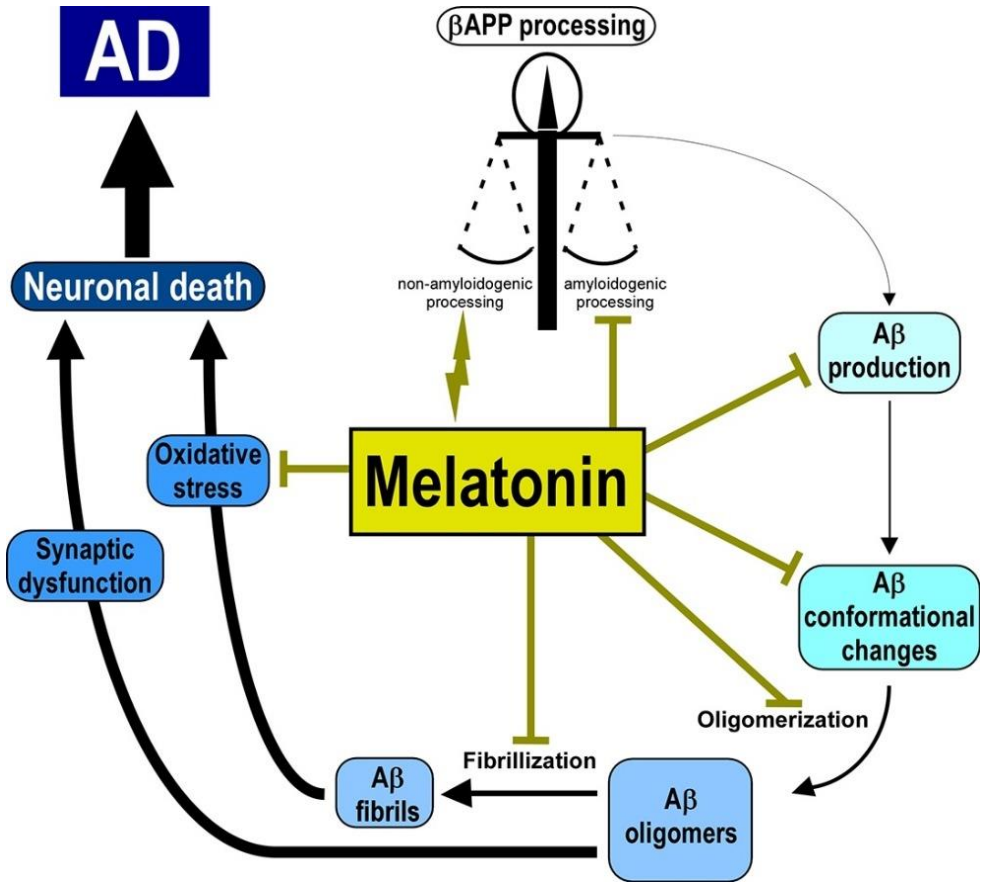

Figure 4. Melatonin's protective role in AD. Melatonin inhibitory effect over the different pathological processes that occur during the development of AD. *Vincent, 2018* [168].

II. Aim

The clearance of Abeta from the CSF is tightly associated with the CP and this process occurs with a circadian rhythmicity. There is some evidence relating clock gene's rhythmicity disruption to neurodegenerative diseases, particularly AD. There's also proof that AD has a sex dependent prevalence suggesting an effect of sex hormones in the development of the disease. Melatonin is a hormone secreted by the pineal gland and there is some evidence that, in AD patients, the circulating levels of this hormone are diminished. Under normal circumstances, melatonin acts as a neuroprotector against and during AD, but how this protection occurs is still to be fully comprehended. It also acts as a Zeitgeber, synchronizing the rhythms of the circadian genes, regulating the body's circadian clocks.

In this work we aim to evaluate AD effects on the rhythmicity and expression of several clock genes in the CP. We also plan to verify if melatonin is able to modulate the expression of CP's clock genes in the presence of Abeta.

III. Materials and Methods

1. Animal Samples

In this work, we used samples of mouse's CP that were collected by our collaborators of the research group located in the Research Institute Hospital 12 de Octubre (i+12) in Madrid, Spain. The samples were obtained from female and male, 6- and 12-month-old APP/PS1 transgenic and wild-type (WT) mice, at different time points during a 24 hour period (Table 1). The APP/PS1 strain of double transgenic mice results from a cross between Tg2576 (overexpressing human APP695) and mutant PS1 (M1462) [169]. The time points at which the samples were collected are denominated of Zeitgeber Time (ZT). Lights are turned on at ZT0 (7 a.m.) and off at ZT12 (7 p.m.).

Table 1. Number of samples collected for each condition used during the experiment.

		Males		Females	
		APP/PS1	WT	APP/PS1	WT
6-month-old	ZT 1	5	5	5	9
	ZT 7	6	6	6	5
	ZT 13	3	4	9	7
	ZT 19	5	4	5	5
12-month-old	ZT 1	4	4	5	6
	ZT 7	4	6	5	3
	ZT 13	4	2	4	5
	ZT 19	3	2	5	5

2. Cell Culture

The cell line used was an immortalized rat choroidal epithelium (Z310 cell line), donated by Dr. Wei Zheng [170]. The *in vitro* studies were performed using Dulbecco's Modified Eagle Medium (DMEM (1x) ref. 11880.028, Gilbco®) cell culture medium, supplemented with 3500 mg of D(+)-Glucose Anhydrous (Fisher Scientific®) and 584 mg of L-Glutamine (Sigma-Aldrich®) per every liter of culture medium used. To the culture medium was also added 10% fetal bovine serum (Sigma-Aldrich®) and 1% penicillin/streptomycin (Sigma-Aldrich®). The cell culture was maintained in a t-flask and kept in a LEEC® Culture Safe Precision P190 incubator.

At approximately 90% of confluence, a cellular passage was performed, allowing for continuous cell growth. For that, culture medium was removed, and the cells washed with phosphate buffered saline (PBS) 1x. PBS was then removed and trypsin-EDTA 0.25% added in a volume that would ensure total coverage of the cell layer. A 3- to 5-minute incubation at 37 ºC would follow and when most of the cells had detached, cell culture medium (double the amount of trypsin used) was added. The suspension was then collected to a falcon and subjected to centrifugation for 3 minutes at 301 RCF. The supernatant was rejected, and the pellet of cells resuspended in culture medium, ready to be used in an essay, cultured and/or cryopreserved.
For cell counting, 20µL of trypan blue were added to 20µL of cellular suspension. From this suspension, 10µL were transferred to a Neubauer chamber for viable cells counting. After counting the number of cells by quadrant, the total number of cells in the t-flask and the number of cells/mL were estimated using the following mathematical formula:

$$\frac{number\ of\ cells}{mL} = \frac{total\ number\ of\ cells}{number\ of\ quadrants}\ x\ dilution\ factor\ x\ 10^4$$

2.1 Cells Synchronization

In order to replicate what happens *in vivo*, cells in culture need to be synchronized. Synchronization allows for the internal clock of all the cells in culture to be in synchrony with each other [171]. The selected synchronization compound was dexamethasone (Sigma-Aldrich®). For that, $3x10^4$ cells were seeded per well and cultured in 12 well culture plates. After 72 hours the culture medium was discarded and culture medium supplemented with dexamethasone at a final concentration of 100nM, was added. A control condition with vehicle (water) was also included. After 2 hours, the culture medium was discarded and substituted by new complete cell culture medium. Cells were then collected at different time points (6 hours, 12 hours, 18 hours and 24 hours after synchronization). For that, the cell culture medium was removed and the wells washed with room temperature PBS. Ready to use reagent for the isolation of high-quality total ribonucleic acid (tRNA) (TRI-reagent) was added to each well and the cells transferred to a microtube. The microtubes were maintained at -80º C until RNA extraction.

2.2 Treatment of the Z310 cell line with Abeta and Melatonin

In order to study if melatonin treatment has any modulatory effect in *Bmal1* circadian expression in the presence of Abeta, the epithelial cell line was treated with these two compounds. We used a low non-apoptotic concentration of Abeta (AnaSpec®;3ug/mL) already tested by our group [172]. For that, $1.5x10^4$ cells were seeded per well and cultured in 24 well culture plates. After 24 hours, the cells were synchronized according to the protocol described above. Four different groups were established, and each group received a different melatonin treatment ("group 1" (+/-/+/-), "group 2" (-/+/-/+), "group 3" (+/+/+/+) and "group 4" -/-/-/-)) (Table 2). The culture medium was removed and to the "group 1" and "group 3" complete

culture medium supplemented with melatonin (Calbiochem®) at 10nM was added, while culture medium with 0,002% ethanol (vehicle) was added to "group 2" and "group 4". After 12 hours the culture medium was removed and to the "group 2" and "group 3" was added complete culture medium supplemented with melatonin at 10nM and to "group 1" and "group 4" was added culture medium with the vehicle. After 12 hours the culture medium was removed and complete culture medium supplemented with melatonin at 10nM was added to the "group 1" and "group 3" and culture medium with the vehicle was added to "group 2" and "group 4". After 12 hours the culture medium was removed and complete culture medium supplemented with melatonin at 10nM was added to the "group 2" and "group 3" and culture medium with the vehicle was added to "group 1" and "group 4". At this point Abeta was added to the cells at a final concentration of 3µg/mL. Scheme of these stimuli done during the three 12 hours cycles of melatonin treatment are represented in Table 2. Cells were then collected at different time points (6 hours, 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, 48 hours, 54 hours, 60 hours, 66 hours and 72 hours after treatment with Abeta). For that, the cell culture medium was removed and the wells washed with room temperature PBS. TRI-reagent was added to each well and the cells transferred to a microtube. The microtubes were maintained at -80º C until RNA extraction.

Table 2. Scheme of stimulus done to the different groups of the assay.

	2 hours after synchronization		14 hours after synchronization		26 hours after synchronization		38 hours after synchronization	
	Culture medium	Culture medium + Melatonin	Culture medium	Culture medium + Melatonin	Culture medium	Culture medium + Melatonin	Culture medium + Abeta	Culture medium + Melatonin + Abeta
"group 1"		+	+			+	+	
"group 2"	+			+	+			+
"group 3"		+		+		+		+
"group 4"	+		+		+		+	

3. tRNA collection

3.1 *In vivo* Assays: tRNA extraction

Samples were thawed and 300µL of TRIzol™ added to each microtube. With the aid of a pestle, the CP samples were homogenized, followed by 5 minutes incubation at room temperature to enable for complete dissociation of nucleoprotein complexes. 60µL of chloroform (200µL

chloroform for each 1mL of TRIzol™) were then added to the samples followed by vortex homogenization. Samples were left to incubate at room temperature for 15 minutes before undergoing centrifugation (4ºC, 12000g, 15 minutes). The supernatant (transparent phase) was carefully collected for a new microtube and 150µL of isopropyl (500µL of isopropyl for each 1mL of TRIzol™) were added, and homogenization by inversion performed enabling the ribonucleic acid (RNA) to precipitate. The microtubes were left at room temperature for 10 minutes before undergoing another centrifugation (4ºC, 12000g, 10 minutes). After centrifugation the supernatant was discarded and 200µL of 75% ethanol in diethylpyrocarbonate (DEPC) water was added to the pellet followed by another centrifugation (4ºC, 7500g, 5 minutes). The supernatant was discarded and, after total removal of the excess ethanol, the tRNA pellet was rehydrated in DEPC water. This was followed by tRNA quantification using a nanospectrophotometer (NanoPhotometer™, Implen). The equipment displays tRNA concentration (ng/µL) and also the 260nm/280nm ratio. tRNA is considered to be pure with a 260nm/280nm ratio between 1.8 and 2.1. If the ratio is <1.8 the tRNA is probably contaminated with protein or phenol and if the ratio is >2.1 tRNA is probably contaminated with genomic deoxyribonucleic acid (DNA).

3.2 *In vitro* Assays: tRNA extraction

Samples were thawed and 200µL chloroform for each 1mL of TRIzol™/ TripleXtractor (GRiSP®) were added. The microtubes were then subjected to vortex homogenization. They were left to incubate at room temperature for 15 minutes before undergoing centrifugation (4ºC, 12000g, 15 minutes). The supernatant (transparent phase) was carefully collected for a new microtube and 150µL of isopropyl (500µL of isopropyl for each 1mL of TRIzol™) were added, and homogenization by inversion performed. The microtubes were left at room temperature for 10 minutes before undergoing another centrifugation (4ºC, 12000g, 10 minutes). After centrifugation the supernatant was discarded and 200µL of 75% ethanol in DEPC water was added to the pellet followed by another centrifugation (4ºC, 7500g, 5 minutes). The supernatant was discarded and, after total removal of the excess ethanol, the tRNA pellet was rehydrated in DEPC water. This was followed by tRNA quantification using a nanospectrophotometer (NanoPhotometer™, Implen) as reported above.

4. cDNA Synthesis

Complementary DNA (cDNA) synthesis is done using a reverse transcriptase enzyme, using RNA as a template. This enzyme has the capacity of producing an exact copy of the expressed genes but without the intron segments. NZY M-MuLV Reverse Transcriptase (NZYTech®) was chosen and used according to fabricant's recommendations. A mix denominated MIX1 (n+2 reactions) composed by 2µL of Random Primers (NZYTech®) and 1µL of DNTPs (NZYTech®) for each sample, was prepared in a microtube. Polymerase chain reaction (PCR) tubes were prepared with approximately 500ng of tRNA extracted of each sample plus sterile water performing a final volume of 14µL. Next, 3µL of MIX1 were added to each PCR tube and the tubes were placed in

the MultiGene™ OptiMax Thermal Cycler (Labnet®) at 65ºC for 5 minutes, and immediately after deposited in ice. Meanwhile, in a new microtube, MIX2 (n+2 reactions) was prepared with 2µL of Reverse Transcriptase Buffer (5x buffer) and 1µL of MMLV for each sample. 3µL of MIX2 was added to each PCR tube, followed by a 10 minutes incubation at 25ºC and 50 minutes at 37ºC. To stop the reaction, samples underwent 15 minutes at 70ºC. cDNA was then stored at -20ºC until use.

5. Conventional PCR

Using conventional PCR, the expression of *Bmal1*, *Cry2* and *Per2* was confirmed in the *in vivo* samples and in the cell line Z310. NZYTaq II 2x Green Master Mix (NZYTech®) was selected for DNA fragments amplification, following the fabricants recommendations. For each sample it was prepared a mix containing 10µL of NZYTaq II 2x Green Master Mix, 0.6µL of both Forward and Reverse Primers at 0,25µmol each, 6.8µL of sterile water and finally 2µL of cDNA, with the exception of the negative control to which was added 2µL of sterile water. The oligonucleotides initiators (Primers) were chosen using Primer-Blast-NCBI-NIH program (Table 3). The amplification was performed in the MultiGene™ OptiMax Thermal Cycler (Labnet®). The process consisted of 5 minutes at 95ºc followed by 40 cycles of amplification. These amplification cycles consisted in 95ºC for 30 seconds, 45 seconds at the optimal annealing temperature, and 30 seconds at 72ºC. After the completion of the 40 cycles of amplification, a final 5 minutes at 72ºC were programed. PCR products were run on a 1.5% agarose gel in the presence of GreenSafe (GRiSP®) to stain DNA. 10µL of each PCR product were deposited in individual wells in the gel. In the first well was deposited 3µL of GRS Ladder 50bp (GRiSP®).

Table 3. Primers and respective annealing temperatures used on the Conventional PCR and for Real-Time RT-PCR.

Origin of the cDNA	Gene accession no.	Fragment Size (bp)	Annealing Temperature (ºC)	Primer Forward (5'-3')	Primer Reverse (5'-3')
Animal Samples	GAPDH XM_017321385.1	169	58	TCACCACCAT GGAGAAGGC	GCTAAGCAGT TGGTGGTGCA
	mBmal1 NM_007489.4	201	58	GCAGTGCCACT GACTACCAAGA	TCCTGGACAT TGCATTGCAT
	mCry2 NM_009963.4	151	58	AGGGCTGCCA AGTGCATCAT	AGGAAGGGACA GATGCCAATAG
	mPer2 NM_011066.3	75	58	CAACACAGAC GACAGCATCA	TCCTGGTCC TTCAACAC
Z310 Cell Line	GAPDH XM_017593963.1	169	60	TCACCACCAT GGAGAAGGC	GCTAAGCAGT TGGTGGTGCA
	Bmal1 NM_024362.2	100	60	ACACTGCACC TCGGGAGCGA	CGCCGAGCTC CAGAGCACAA
	Cry2 NM_133405.2	189	60	GCCCAGGAGC CACCAAGCAA	GCATGCACAC GCAAACGGCA
	Per2 NM_031678.1	177	60	CGCACACGCA ACGGGGAGTA	AACGCTGGGG TGCGGAGTCT

6. Real-Time RT-PCR

Real-Time RT-PCR allowed for relative quantification of the different circadian genes of interest in the samples. The assay was optimized for each individual gene and also for each different cDNA origin (animal sample or cell line). Primer efficiency was tested using different dilutions of cDNA (1:1 stock; 1:2; 1:4 and 1:8). After each cycle, SYBR™ Green's I fluorescence was detected and quantified by the CFX Connect™ Real- Time System (Bio-Rad®) software. The melting curves that were generated at the end of each assay, allowed for a strict control of possible contaminations and/or dimer primer formation. To normalize the level of expression of the genes of interest, the GAPDH gene was used as a housekeeping gene. Each mix and conditions used on the different assays are described on Table 4. The primers used are referred in Table 3.

Table 4. Each assay's specific Real-Time RT-PCR specifications and reaction mixes.

Assay	Mix Components		Set-up qPCR cycling		
			Nº cycles	Temperature (ºC)	Time
Animal Samples	NZYSpeedy qPCR Green Master Mix (2x) (NZYTech®)	5 µL	1x	95	5 min
	Forward Primer	0.25 µmol (0.4 µL)	40x	95	15 sec
	Reverse Primer	0.25 µmol (0.4 µL)		58	45 sec
	Sterile Water	3.2 µL		72	10 sec
	cDNA	1 µL	Dissociation/Melt Analysis	According to manufacturer's guidelines	
Cells Synchronization	NZYSpeedy qPCR Green Master Mix (2x) (NZYTech®)	5 µL	1x	95	5 min
	Forward Primer	0.25 µmol (0.4 µL)	40x	95	15 sec
	Reverse Primer	0.25 µmol (0.4 µL)		60	45 sec
	Sterile Water	3.2 µL		72	10 sec
	cDNA	1 µL	Dissociation/Melt Analysis	According to manufacturer's guidelines	
Treatment of Z310 cell line with Abeta and Melatonin	Xpert Fast SYBR (Uni) 2x Mastermix (GRiSP®)	5 µL	1x	95	3 min
	Forward Primer	0.25 µmol (0.4 µL)	40x	95	5 sec
	Reverse Primer	0.25 µmol (0.4 µL)		60	30 sec
	Sterile Water	3.2 µL		72	10 sec
	cDNA	1 µL	Dissociation/Melt Analysis	According to manufacturer's guidelines	

7. Statistical Analysis

To analyze the resulting data of the different assays four different statistical analysis were done. Animal samples Real-Time RT-PCR data was analyzed using CircWave to check for rhythmicity in clock genes expression. *In vitro* cells synchronization Real-Time RT-PCR data was evaluated using one-way ANOVA to determine significant differences in the clock genes expression between the different ZTs. CircWave analysis was done to verify if the clock genes expression presented circadian rhythmicity after cells synchronization protocol. Finally, in the treatment of Z310 cell line with Abeta and melatonin, the Bmal1 circadian expression was analyzed using Harmonic Regression Analysis with R software allowing for rhythmicity, amplitude, period and phase to be assessed. Data were considered statistically significant at $p<0.05$.

IV. Results

1. Changes in CP Clock Genes Expression in AD

WT 6-month-old male mice have rhythmical expression of *Bmal1* ($p<0.0001$; Figure 5), and the same happens in WT 12-month-old male mice ($p<0.05$; Figure 5). The peak of expression of this gene in these samples happens approximately between ZT13 and ZT15. In WT 6-month-old females, *Bmal1* is rhythmically expressed ($p<0.05$; Figure 5) just like in WT 12-month-old females ($p<0.0001$; Figure 5). In females *Bmal1* peak expression occurs approximately between ZT13 and ZT16. In APP/PS1 male mice, *Bmal1* expression is rhythmic at 6-months of age ($p<0.001$; Figure 6), with peak expression between ZT13 and ZT15, but at 12-month-old Bmal1 rhythmicity is lost (Figure 6). APP/PS1 female mice present *Bmal1* rhythmicity at 6-months of age ($p<0.001$; Figure 6), with peak expression between ZT13 and ZT14, but at 12-months of age they lose its rhythmic expression (Figure 6), also. *Per2* is rhythmically expressed in WT 6-month-old female mice ($p<0.001$; Figure 7), and in WT 12-month-old female mice ($p<0.01$; Figure 7), and the peak of expression is visible between ZT1 and ZT3 for 6-month-old and ZT0 and ZT2 for 12-month-old animals. In APP/PS1 female mice, *Per2* is expressed with a circadian rhythmicity at 6-months of age ($p<0.05$; Figure 7) and at 12-months of age ($p<0.01$; Figure 7), and the peak of expression occurs between ZT0 and ZT2. *Cry2* never presented rhythmic expression in any of the conditions analyzed.

Additional data in Attachment 1.

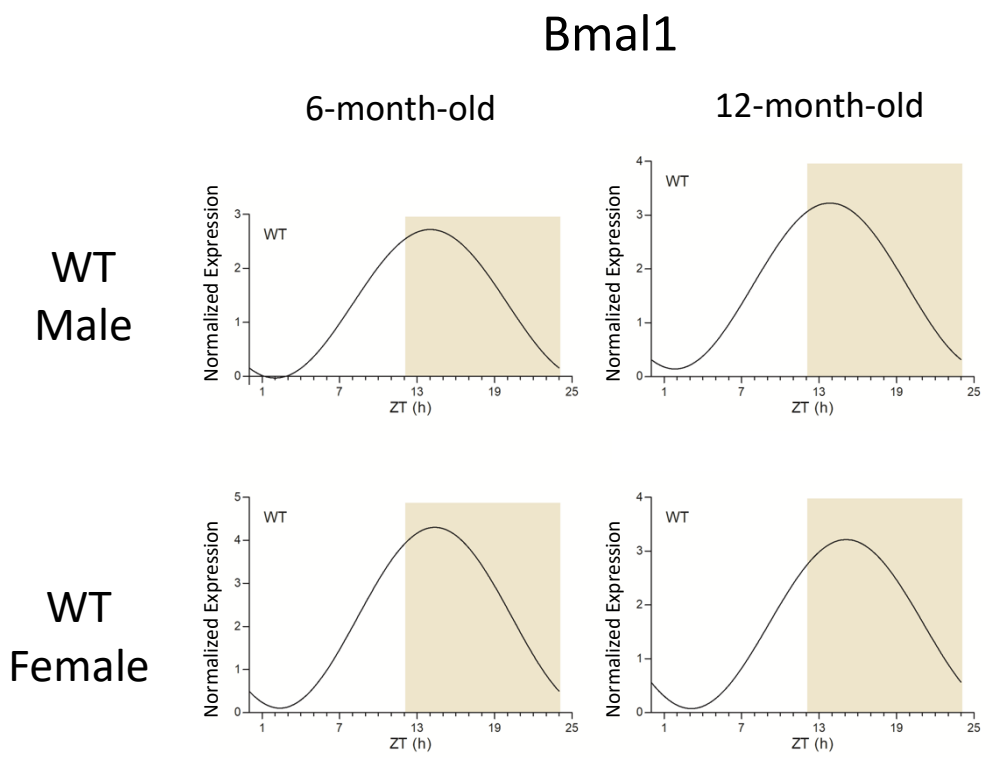

Figure 5. CircWave curves of *Bmal1* expression in the CP of WT animals. ZT1 and ZT25 are double plotted.

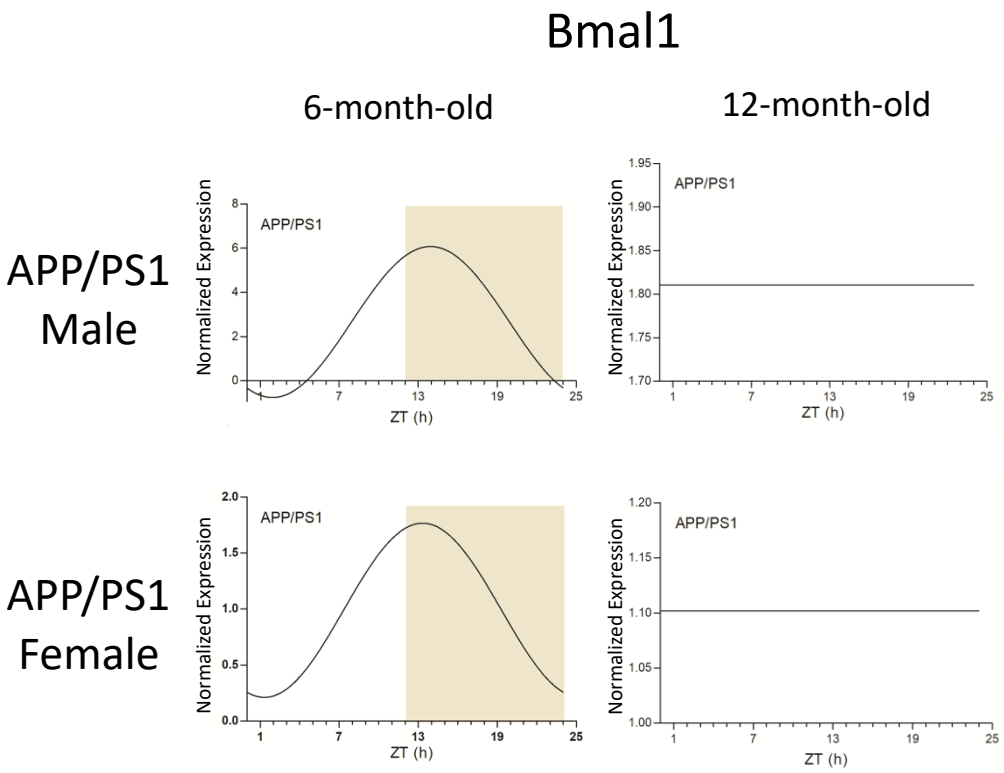

Figure 6. CircWave curves of *Bmal1* expression in the CP of APP/PS1 animals. Absence of the CircWave curve shows absence of significant rhythmicity in *Bmal1* expression. Both male and female APP/PS1 12-month-old mice don't present rhythmic expression of Bmal1. ZT1 and ZT25 are double plotted.

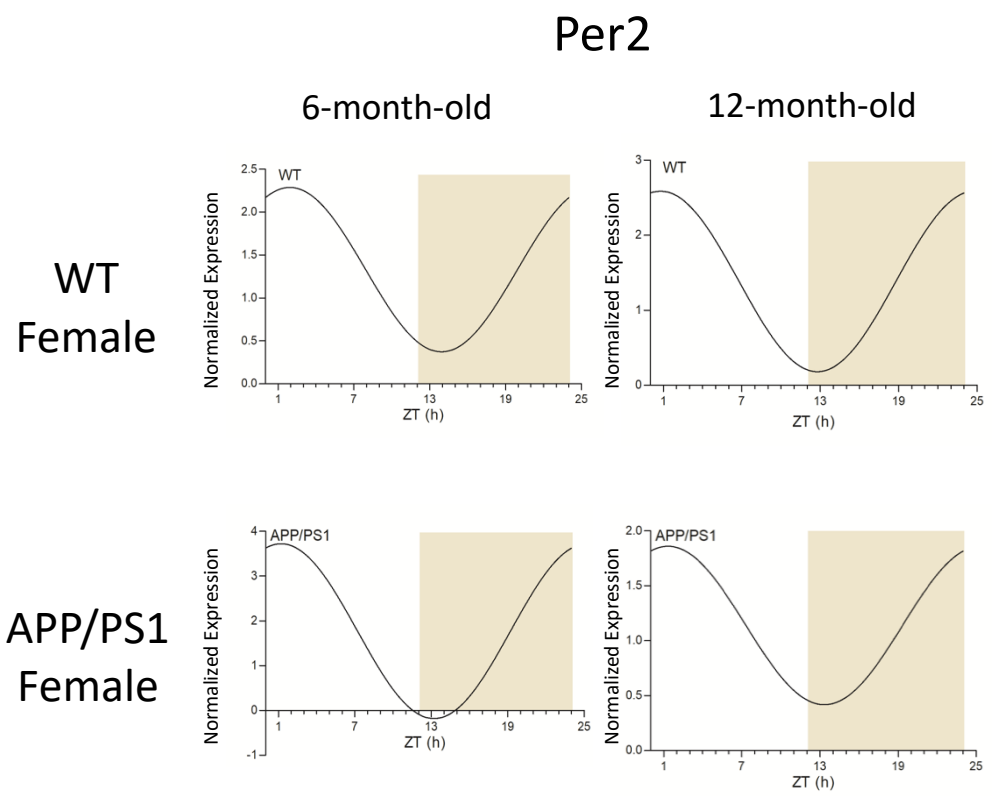

Figure 7. CircWave curves of *Per2* expression in the CP of females. Male mice, both APP/PS1 and WT at 6 and 12 months of age don't show rhythmicity and the lack of Circwave graphics is representative of that. Female mice *Per2* expression presents a circadian rhythmicity in all the conditions. ZT1 and ZT25 are double plotted.

2. *In vitro* Assays

2.1 Dexamethasone is a Synchronizer Agent for the Z310 cell line

In order to study clock genes expression *in vitro*, cells' synchronization was performed using a brief treatment with dexamethasone. The cells treated evidenced several significant differences between the time points tested (Figure 8). One-way ANOVA data analyses are showed in Table 5.For the control group, without the dexamethasone treatment, no significant differences in clock genes expression between time points were observed (Figure 8).

These data was also analyzed for clock genes expression circadian rhythmicity, using the CircWave software. Analysis of *Bmal1* revealed, in cells treated with dexamethasone, a rhythmic expression ($p<0.01$; Figure 8 A) with peak occurring approximately around 12h. *Per2* expression was also rhythmic ($p<0.001$; Figure 8 B) as well as *Cry2 expression* ($p<0.05$; Figure 8 C), with peak occurring approximately at 23h and 24h, respectively. No rhythmicity was detected in the control cells.

Table 5. Statistical differences between ZTs for each gene and condition tested in synchronized Z310 cell line.

	Gene	Statistical Significant Differences
Synchronized Z310 Cell Line	*Bmal1*	6h vs 12h ($p<0.05$) 12h vs 18h ($p<0.05$) 12h vs 18h ($p<0.05$)
	Per2	12h vs 24 ($p<0.05$)
	Cry2	6h vs 18h ($p<0.05$) 12h vs 24h ($p<0.05$) 18h vs 24h ($p<0.05$)

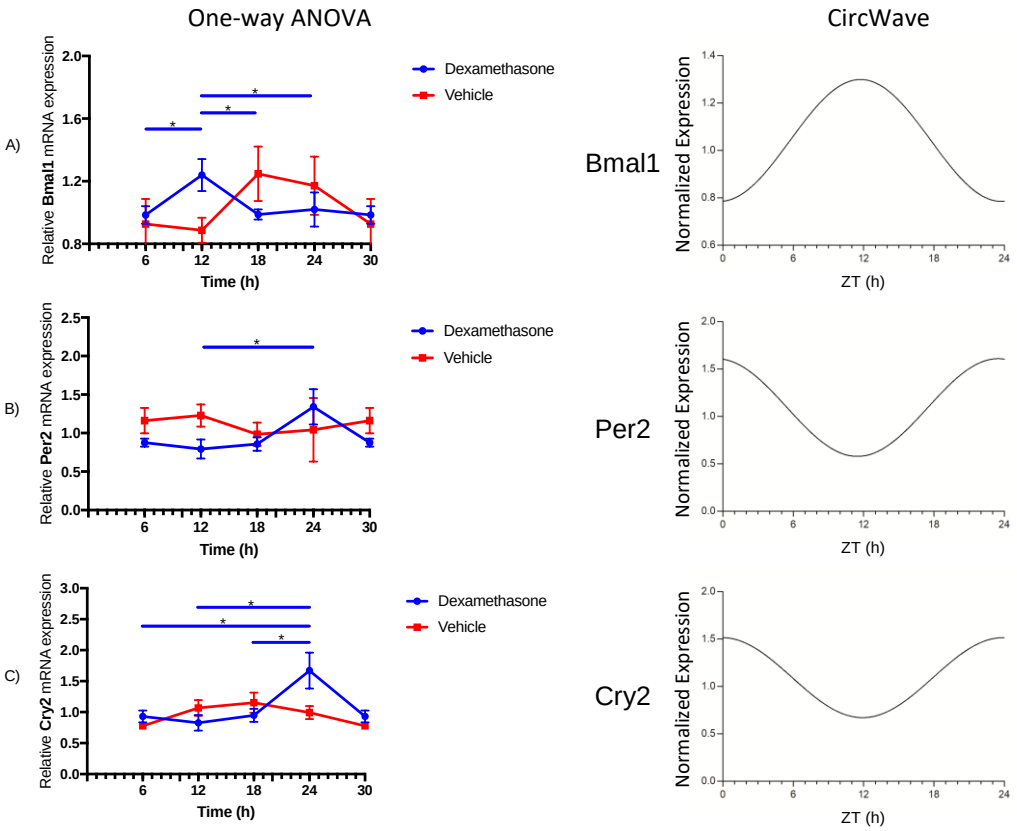

Figure 8. One-way ANOVA analysis of the clock genes treated with dexamethasone and vehicle, and CircWave curves of *Bmal1*, *Per2 and Cry2* expression in the Z310 cell line treated with dexamethasone. In one-way ANOVA each data set shows the mean of the target gene expression relative to control gene and error bars are ± standard error mean and 6h and 30h are double plotted. Presence of the CircWave curve shows significant rhythmicity in the expression of all the clock genes tested. A) One-way ANOVA reveals significant differences between: 6h vs 12h ($p<0.05$), 12h vs 18h ($p<0.05$) and 12h vs 24h ($p<0.05$); CircWave reveals *Bmal1* rhythmic expression ($p<0.01$). B) One-way ANOVA reveals

significant differences between: 12h vs 24h ($p<0.05$); CircWave reveals *Per2* rhythmic expression ($p<0.001$). C) One-way ANOVA reveals significant differences between: 6h vs 18h ($p<0.05$), 12h vs 24h ($p<0.05$) and 18h vs 24h ($p<0.05$); CircWave reveals *Cry2* rhythmic expression ($p<0.05$).

2.2 Melatonin Treatment Modulates *Bmal1* Expression in the Presence of Abeta

Real-Time RT-PCR results from cells treated with Abeta and melatonin were analyzed using Harmonic Regression Analysis. Like with the CircWave software, gene presents rhythmic expression if $p<0.05$ and this rhythmic expression is visually expressed by a sinusoidal curve. We choose this software since it enables the input of three continuous days of data while CircWave does not allow it. We only analyzed *Bmal1* expression since it was the only clock gene that lost its rhythmicity in both sexes in APP/PS1 animals. Each group represents a different previous melatonin treatment ("group 1" (+/-/+/-), "group 2" (-/+/-/+), "group 3" (+/+/+/+) and "group 4" -/-/-/-) (Table 2). In "group 1" Bmal1 presented no rhythmicity ($p=0.1404$; Figure 9). In "group 2", on the other hand, Bmal1 expression was rhythmic ($p<0.01$; Figure 9), with a period of 21 hours, a phase at 0.68 radians and with an amplitude of 0.52. In "group 3" Bmal1 also displayed rhythmic expression ($p<0.0001$; Figure 9), with a period of 28 hours, a phase at 5.09 radians and amplitude of 0.49. Finally, in "group 4", Bmal1 also presented rhythmicity ($p<0.01$; Figure 9), displaying a period of 28 hours, a phase at 4.72 radians and an amplitude of 0.45. The strongest rhythmicity (represented by the lowest p-value) was presented by "group 3" and the widest amplitude in *Bmal1* expression was presented by "group 2". The Abeta stimulus, at least in the concentration used, was not capable of deregulating the circadian rhythm of *Bmal1* expression.

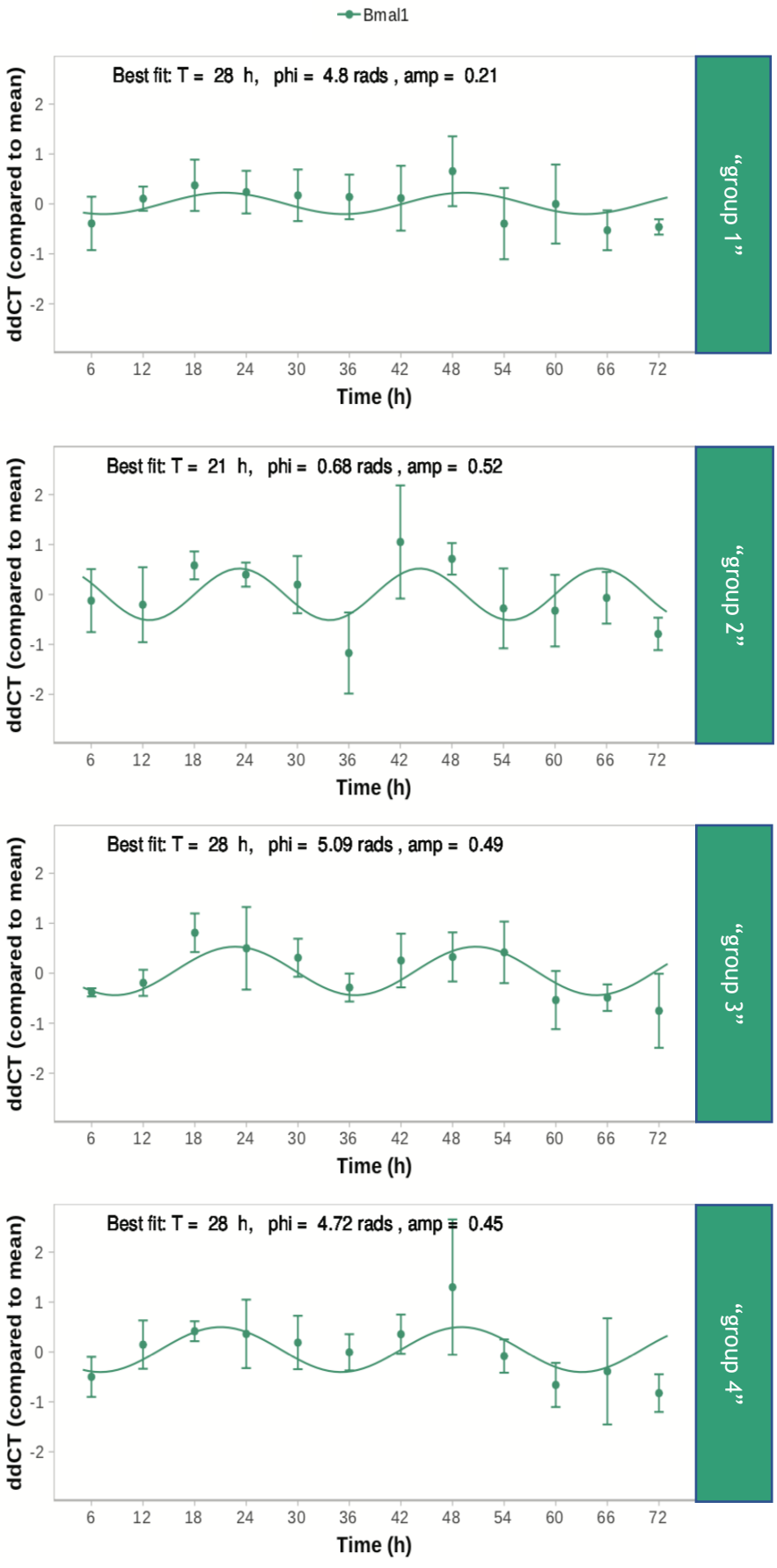

Figure 9. Harmonic Regression Analysis of *Bmal1* expression in cells treated with Abeta and melatonin.

For each condition and at each time point, the dots represent the average data of gene expression value over all replicates and shows error bars ± standard deviation. The sinusoidal curve was plotted with the best fitting parameters of each condition. T: period in hours, phi: phase in radians, amp: amplitude.

V. Discussion

Alzheimer's disease is known to disrupt circadian rhythms [112]. The CP is an extra-SCN clock [62-64] that cleans Abeta from the CSF with an associated circadian rhythmicity [99].

In order to see if AD had any effects over the CP's circadian clock, we used a double transgenic mouse model of AD. The APP/PS1 mouse model of AD varied in age and in sex, just like the WT control group. In male WT mice, *Bmal1* was rhythmically expressed while WT females had both *Bmal1* and *Per2* rhythmically expressed. This differential expression of rhythmic clock genes between sexes was also previously noted in the superior blade of the dentate gyrus [173]. In a previous study done by our research group, *Bmal1* was only rhythmic in female Wistar rats, while *Per2* was rhythmic in both sexes of Wistar rats [64]. In addition, in this previous report *Cry2* presented rhythmicity in both sexes while in this present study *Cry2* doesn't present rhythmicity in any of the conditions tested. This differences between both studies advocate for a species and age dependency. The species argument was suggested by Dibner C. *et al.* which concluded that pattern and phase of oscillating gene expression might have an influence of species-specific factors [174]. Age is a proven factor affecting clock genes expression [175] and while the animals used in this study had a minimum of 6-months of age, in Quintela T. *et al. study*, animals had between 8 and 10 weeks of age [64]. This age effect might explain the *Cry2* lack of rhythmicity in the animal models used. In APP/PS1 6-month-old males and females, *Bmal1* presents circadian rhythmicity, losing it in APP/PS1 12-month-old animals of both sexes. This age-dependent effect over rhythmicity in an APP/PS1 model is explained by the fact that in this mice model, at 6 months, Abeta plaque formation and deposition is at the beginning, whereas, at 12 months, the deposition of Abeta plaques is at an advanced state [176]. DNA methylation represses gene expression [177] and DNA methylation is affected by Abeta deposition [178]. Then, *Bmal1* loss of rhythmicity can be explained by the aberrant methylation cycles that occur in AD due to Abeta deposition [179]. While Bmal1 lost its circadian rhythmicity in females, Per2 managed to maintain it , suggesting a sex hormone dependent effect over this clock gene expression. The estrogen effect over *Per2* was previously described in different areas of the brain and in the uterus and was demonstrated to directly affect the timing of this clock gene expression [180,181]. From the analysis of the CircWave results, we are also able to evaluate which is the time point of peak expression. In WT and APP/PS1 mice, *Bmal1* peak expression occurred approximately between ZT13 and ZT16 while *Per2* expression peaked approximately between ZT0 and ZT3 for all samples. Altogether, this is a very good representation of the antiphase that exists between the expression of these circadian clock genes, with one being expressed at the opposite time of day of the other. In the mouse's SCN, Bmal1 peaks around ZT4 and *Per2* at around ZT16 reinforcing the idea of an antiphase between these two clock genes [182].

To proceed with *in* vitro studies, we needed to synchronize the Z310 cell line in culture to replicate what happens *in* vivo. The Z310 cell line synchronization went as planned, with the clock genes tested presenting circadian rhythmicity and *Bmal1* peaking at an opposite time of day than *Per2* and *Cry2* which is in accordance with the animal samples results [22].

Having the confirmation that AD alters and affects the circadian rhythmicity of clock gene *Bmal1* in the CP, and that the CP cell line Z310 synchronization was possible, we decided to see if melatonin could modulate *Bmal1* circadian parameters. We observed that treatment of Z310 cells with melatonin and Abeta demonstrated the melatonin's capacity to modulate *Bmal1*'s circadian expression. In "group 2", a previous and discontinuous melatonin treatment associated with the presence of melatonin when Abeta is added, seems to reinforce the amplitude of *Bmal1*'s rhythmic expression which was greater in this group than in "group 3" and "group 4". This reinforcement is explained by the melatonin's synchronizing capabilities [142]. Moreover, the period of "group 2" was of 21 hours, while in "group 3" the period was of 28 hours just like "group 4". "group 3" also presented the lowest p-value. These results demonstrate that there are different effects between a discontinuous previous treatment with melatonin ("group 2") and a continuous one ("group 3"). Melatonin amplitude reinforcement capabilities were also noted between "group 3" and "group 4" with the first group having a greater amplitude when compared to the second. This is mentioned as a consequence of a greater synchronization [183], and this greater synchronization can be attributed to the melatonin's action on the clock genes [142]. Nevertheless, all the other parameters such as period and phase are very similar. These results also suggest that a low Abeta concentration was not capable of disrupting *Bmal1*'s circadian rhythmicity in these three groups. This finding is very similar to what happened *in vivo* since at 6 months of age, APP/PS1 mice did not present alterations in circadian clock genes rhythmicity which was explained by the necessity for high concentrations of Abeta to form plaques and deregulate *Bmal1* expression. A previous treatment with melatonin and withdraw of this same compound before Abeta treatment ("group 1") led to a complete loss of rhythmicity which when compared to the other groups suggests that not only a previous and discontinuous treatment with melatonin does not protect against the desynchronization effects of Abeta, it also potentiated this same effects. Melatonin is an antioxidant compound with free radical scavenger functions [148]. This might lead to a reduction in endogenous production of antioxidants by the cells in order to maintain an equilibrium between oxidative and antioxidative molecules which is essential for redox signaling [184]. Abeta generates oxidative stress in the cells [185] and free radicals seem to be involved in nuclear DNA damage [186]. Damaged DNA leads to deficient protein synthesis which might include BMAL1. So, we hypothesize that a melatonin treatment that negatively regulated endogenous antioxidant production lead to an exacerbated effect of Abeta over *Bmal1* expression after melatonin withdrawal. This was not seen in the other groups either because melatonin was never added ("group 4"), thus never negatively regulating antioxidants production, or because melatonin was not withdrawn when Abeta was added ("group 2" and

“group 3”), thus continuing to exert its antioxidative function. Future studies are needed in order to support this theory.

VI. Conclusion and Future Perspectives

With this work, AD was acknowledged as a disruptor of the CP circadian clock, having an age dependent effect that is associated with the quantity of Abeta plaques in the tissue. We also demonstrate melatonin's ability to modulate circadian parameters of *Bmal1* expression in the presence of Abeta.

In the future, we aim to evaluate if the circadian rhythmicity of Abeta clearance by the CP is associated to CP's molecular clock and if this clock's disruption leads to impairment in Abeta clearance.

VII. Bibliography

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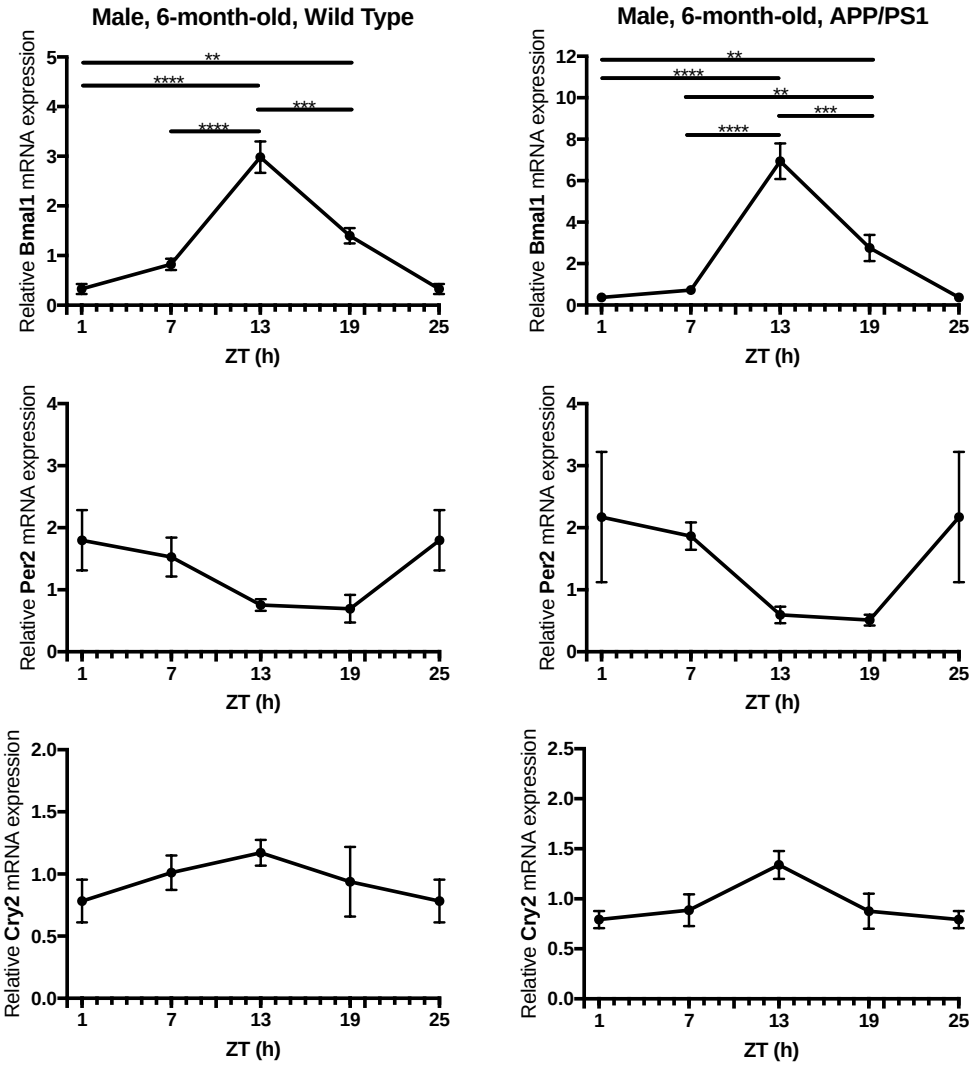
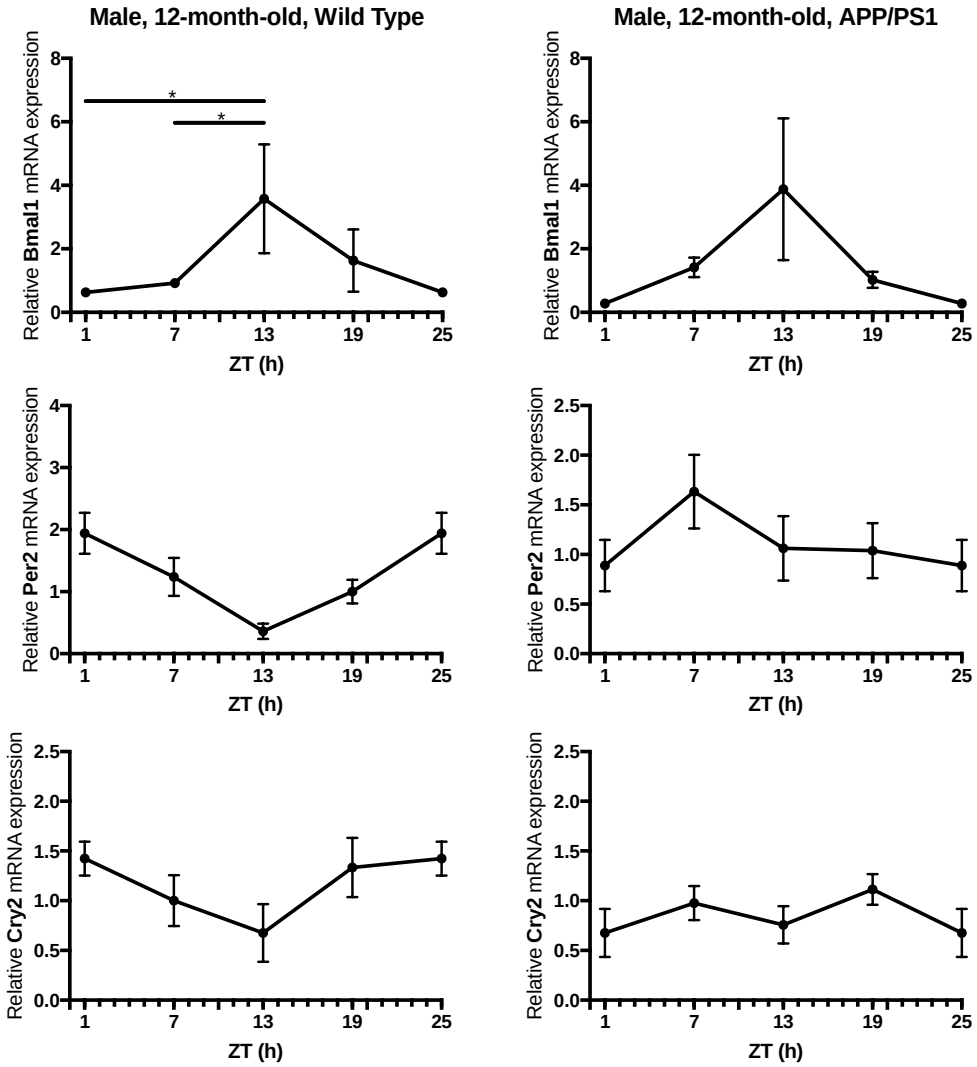
VIII. Attachments

Attachment 1

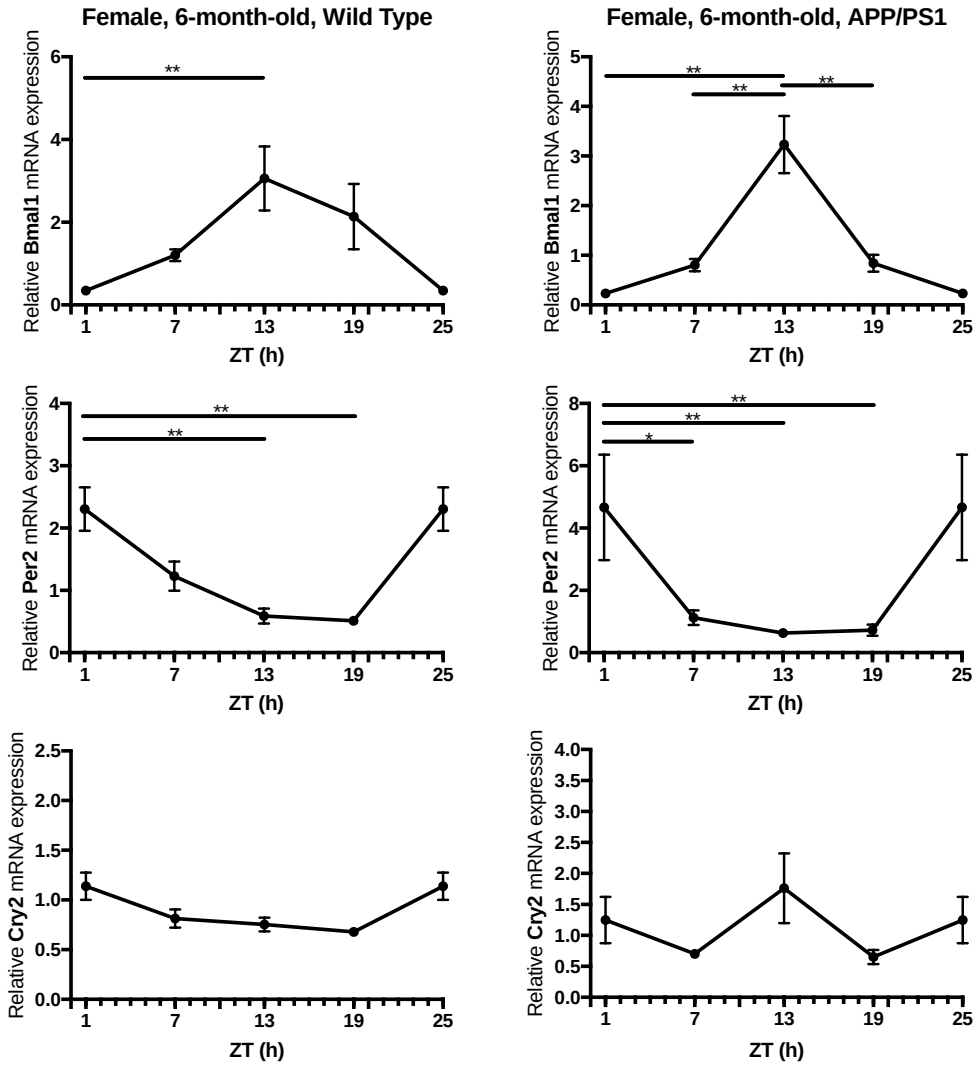
One-way ANOVA graphics and resume tables showing significant differences between ZTs for each *in vivo* condition analyzed.



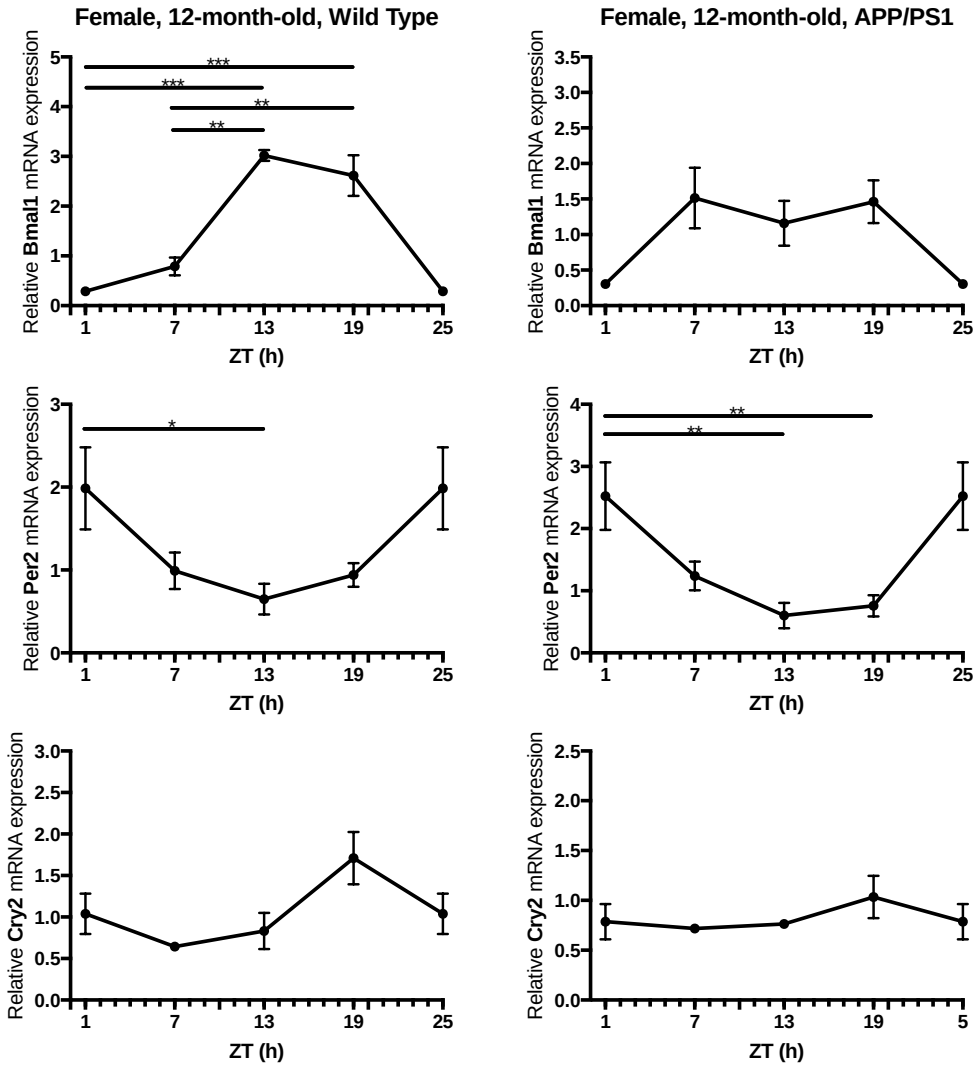
Expression of *circadian* clock genes in the male mice's APP/PS1 and WT CP at the age of 6 months. Each data set shows the mean of the target gene expression relative to one control gene and error bars are ± standard deviation. ZT1 and ZT25 are double plotted. Significant differences are present in: A) ZT1 vs ZT13 ($p<0,0001$), ZT1 vs ZT19 ($p<0,01$), ZT7 vs ZT13 ($p<0,0001$) and ZT13 vs ZT19 ($p<0,001$); B) ZT1 vs ZT13 ($p<0,0001$), ZT1 vs ZT19 ($p<0,01$), ZT7 vs ZT13 ($p<0,0001$), ZT7 vs ZT19 ($p<0,01$) and ZT13 vs ZT19 ($p<0,001$).



Expression of circadian clock genes in the male mice's APP/PS1 and WT CP at the age of 12 months. Each data set shows the mean of the target gene expression relative to one control gene and error bars are ± standard error mean. ZT1 and ZT25 are double plotted. Significant rhythms are present in: A) ZT1 vs ZT13 ($p<0,05$) and ZT7 vs ZT13 ($p<0,05$).



Expression of circadian clock genes in the female APP/PS1 and WT mice's CP at the age of 6 months. Each data set shows the mean of the target gene expression relative to one control gene and error bars are ± standard error mean. ZT1 and ZT25 are double plotted. Significant rhythms are present in: A) ZT1 vs ZT13 ($p<0,01$); B) ZT1 vs ZT13 ($p<0,01$), ZT7 vs ZT13 ($p<0,01$) and ZT13 vs ZT19 ($p<0,01$); C) ZT1 vs ZT13 ($p<0,01$), ZT1 vs ZT19 ($p<0,05$); D) ZT1 vs ZT7 ($p<0,05$), ZT1 vs ZT13 ($p<0,01$) and ZT1 vs ZT19 ($p<0,01$).



Expression of circadian clock genes in the female APP/PS1 and WT mice's CP at the age of 12 months. Each data set shows the mean of the target gene expression relative to one control gene and error bars are ± standard error mean. ZT1 and ZT25 are double plotted. Significant rhythms are present in: A) ZT1 vs ZT13 ($p<0,001$), ZT1 vs ZT19 ($p<0,001$), ZT7 vs ZT13 ($p<0,01$) and ZT7 vs ZT19 ($p<0,01$); C) ZT1 vs ZT13 ($p<0,05$); D) ZT1 vs ZT13 ($p<0,01$) and ZT1 vs ZT19 ($p<0,01$).

Statistical differences between ZTs for each gene and condition tested in WT mice.

Sex	Genotype	Age (months)	Gene	Statistical Significant Differences	p-value
Male	WT	6	*Bmal1*	ZT1 vs ZT13 ZT1 vs ZT19 ZT7 vs ZT13 ZT13 vs ZT19	p<0.0001 p<0.01 p<0.0001 p<0.001
		12	*Bmal1*	ZT1 vs ZT13 ZT7 vs ZT13	p<0.05 p<0.01
Female	WT	6	*Bmal1*	ZT1 vs ZT13	p<0.01
			Per2	ZT1 vs ZT13 ZT1 vs ZT19	p<0.01 p<0.01
		12	*Bmal1*	ZT1 vs ZT13 ZT1 vs ZT19 ZT7 vs ZT13 ZT7 vs ZT19	p<0.001 p<0.001 p<0.01 p<0.01
			Per2	ZT1 vs ZT13	p<0.05

Statistical differences between ZTs for each gene and condition tested in APP/PS1 mice.

Sex	Genotype	Age (months)	Gene	Statistical Significant Differences	p-value
Male	APP/PS1	6	*Bmal1*	ZT1 vs ZT13 ZT1 vs ZT19 ZT7 vs ZT13 ZT7 vs ZT19 ZT13 vs ZT19	p<0.0001 p<0.01 p<0.0001 p<0.01 p<0.001
Female	APP/PS1	6	*Bmal1*	ZT1 vs ZT7 ZT1 vs ZT13 ZT1 vs ZT19	p<0.05 p<0.01 p<0.01
			Per2	ZT1 vs ZT13 ZT1 vs ZT19	p<0.01 p<0.01
		12	Per2	ZT1 vs ZT13 ZT1 vs ZT19	p<0.01 p<0.01