# Alterations of Clock Gene RNA Expression in Brain Regions of a Triple Transgenic Model of Alzheimer's Disease

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**Abstract**. A disruption to circadian rhythmicity and the sleep/wake cycle constitutes a major feature of Alzheimer's disease (AD). The maintenance of circadian rhythmicity is regulated by endogenous clock genes and a number of external Zeitgebers, including light. This study investigated the light induced changes in the expression of clock genes in a triple transgenic model of AD ($3 \times$ Tg-AD) and their wild type littermates (Non-Tg). Changes in gene expression were evaluated in four brain areas–suprachiasmatic nucleus (SCN), hippocampus, frontal cortex and brainstem–of 6- and 18-month-old Non-Tg and $3 \times$ Tg-AD mice after 12 h exposure to light or darkness. Light exposure exerted significant effects on clock gene expression in the SCN, the site of the major circadian pacemaker. These patterns of expression were disrupted in $3 \times$ Tg-AD and in 18-month-old compared with 6-month-old Non-Tg mice. In other brain areas, age rather than genotype affected gene expression; the effect of genotype was observed on hippocampal *Sirt1* expression, while it modified the expression of genes regulating the negative feedback loop as well as *Rorα*, *Csnk1ε* and *Sirt1* in the brainstem. In conclusion, during the early development of AD, there is a disruption to the normal expression of genes regulating circadian function after exposure to light, particularly in the SCN but also in extra-hypothalamic brain areas supporting circadian regulation, suggesting a severe impairment of functioning of the clock gene pathway. Even though this study did not demonstrate a direct association between these alterations in clock gene expression among brain areas with the cognitive impairments and chrono-disruption that characterize the early onset of AD, our novel results encourage further investigation aimed at testing this hypothesis.

Keywords: Aging, Alzheimer's disease, clock genes, light exposure, suprachiasmatic nucleus

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## INTRODUCTION

Biological processes exhibit daily rhythms that enable organisms to coordinate their physiological activities with changes in demand over a day. In mammals, these rhythms exhibit a periodicity of about

24 h and are endogenously generated by the master circadian pacemaker located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus [1]. These rhythms are synchronized by regularly recurring environmental cues or "Zeitgebers". Circadian biological clocks are entrained to a 24 h day/night cycle through the influence of light, the main Zeitgeber [2, 3]. Light is transduced into a neural signal by the retina and conveyed to the core of the SCN [4], which responds to light/dark cycles and co-ordinates circadian function, including locomotor activity, hormone secretion, body temperature maintenance, and feeding. The molecular and cellular basis of circadian rhythmicity consists of a complex interaction between specialized transcription factors, commonly described as the clock pathway, which represents an internal timekeeping system that interacts with these Zeitgebers [5, 6]. The generation and modulation of circadian rhythms rely on interconnected, self-sustained transcriptional/translational feedback loops, which result in the activation or repression of gene expression.

Alzheimer's disease (AD) is the most frequent form of dementia and one of the most devastating psychiatric disorders [7] and is characterized by the accumulation of extracellular amyloid-β (Aβ) plaques, neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau, neuronal loss, and neuroinflammation [8, 9]. As well as severe cognitive deficits, patients with AD exhibit profound disruptions to their circadian rhythms in sleep–wakefulness and other processes. In recent years, increasing efforts have been made to better understand the molecular and cellular basis of the sleep disturbances and disrupted circadian rhythms observed frequently in AD patients [10, 11]. The clock pathway is crucially involved in the maintenance of such rhythmicity, and studies linking this molecular family to brain and body processes in AD patients can further elucidate the etiopathogenesis of this common dementia [12, 13]. Therefore, the investigation of the interactions of Aβ and NFTs with the clock gene pathway represents an interesting challenge for researchers.

A distributed network of extra SCN brain regions contains autonomous oscillators, which support circadian function [3, 14]. In common with the SCN, these secondary oscillators also rely on feedback loops comprising clock genes and proteins. However, the role of the clock pathway in the SCN and extra-SCN regions is thought to be distinct and reflective of the particular function of each brain region [14]. Therefore, the disruption to the expression of clock genes in specific areas of the brain might contribute to the clinical phenomenology of AD although it cannot address the question as to whether this disruption is a cause or an effect of the neurodegenerative processes.

The diversity of secondary clocks in the brain, their specific sensitivities to temporal cues, as well as their differential coupling to the master SCN clock, allows the circadian timing system to integrate and plastically respond to a wide range of temporal information. This raises the possibility that pathophysiological alterations of internal timing that are deleterious for health may result from desynchronization within this network of cerebral clocks [15]. The main brain areas affected by AD pathology are the cortex and the hippocampus, and recently morphological changes have been also reported in the brainstem [16]. Information on the molecular function of the clock pathway in different areas of AD afflicted brains is still limited, therefore this study aimed to evaluate the expression of the core genes comprising the clock pathway of the SCN and three other brain areas, such as hippocampus, frontal cortex and brainstem in a well-characterized triple transgenic murine model of AD ($3 \times$ Tg-AD) [17–19].

The $3 \times$ Tg-AD mice develop amyloid plaques and neurofibrillary pathology in a hierarchical manner in AD-relevant brain regions, develop age-related cognitive decline, and closely mimic the disease progression in humans [17–19]. For the purpose of our study, we have evaluated the clock gene expression at two different stages of the disease in the $3 \times$ Tg-AD mice. In particular, mice have been studied at 6 and 18 months of age, which corresponds to mild and severe pathology of AD-like symptoms, respectively. The control animals were age- and sex-matched, cognitively normal non-transgenic (Non-Tg) mice. Based on our earlier work [18–21], the youngest group of animals (6 months of age) was expected to show intraneuronal Aβ, somatodendritic tau accumulation and was named the mild pathology group. The second age group (18 months of age) was expected to develop extensive Aβ plaque burden, mature NFTs, along with signs of activated microglia, strong signs of plaque-associated inflammation, and was named the severe pathology group [18]. Although this study was not designed to demonstrate a direct association between clock gene alteration and circadian rhythms or sleep-wake cycle, our findings open new perspectives on the chronopathology of AD.

## MATERIALS AND METHODS

### Animal model

All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985), as well as with Italian laws on animal experimentation. All efforts were made to minimize the number of animals used in the study.

6-month-old (adult) and 18-month-old (aged) $3 \times$ Tg-AD male mice and their wild type male littermates (Non-Tg) were housed at the animal facility of the Puglia and Basilicata Experimental Zooprophylactic Institute (Foggia, Italy), according to the procedures described previously [18, 19]. Genotypes were confirmed by polymerase chain reaction (PCR) after tail biopsies as previously described [18, 19].

Fresh food and water were freely available, and the housing conditions were controlled, with temperature 22°C, humidity 50–60%, a 12 h light (L)-12 h dark (D) cycle, with light on at Zeitgeber Time (ZT) 0 corresponding to Circadian Time (CT) 07:00 h and light off at ZT12 corresponding to CT 19:00 h. A full-spectrum white fluorescent lamp (526 lux) served as a daytime light source. On the day of the experiment, mice were decapitated at CT 07:00 h (after exposure to D) or at CT 19:00 h (after exposure to L); the brains were rapidly removed and placed on a cold surface to dissect the suprachiasmatic nucleus, frontal cortex, hippocampus, and the brainstem following a procedure obtained by modifying a previous published protocol [22]. All the tissues were then snap frozen in liquid nitrogen and stored at –80°C. Because of the small amount of tissue available, each sample was obtained by pooling tissues from 2 animals (for a total of 12 mice per group).

### RNA extraction from fresh frozen tissue and first-strand cDNA synthesis

Tissues from brain areas stored at –80°C were homogenized and total RNA was extracted by RNeasy Lipid Tissue Mini Kit (QIAGEN GmbH, Germany) as recommended by the manufacturer. All RNA samples were quantified by measuring the optical density (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies). Quality and purity of the samples were analyzed by gel electrophoresis. Complementary DNA was generated from these samples using M-MLV Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

### Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time quantification of mRNA was performed with a SYBR Green I assay, and evaluated using an iCycler detection system (Bio-Rad Laboratories). The threshold cycle (CT) was determined, and the relative genes expression subsequently was calculated as follows: fold change $= 2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT$ target $-$ CT housekeeping and $\Delta(\Delta CT) = \Delta CT$ treated $- \Delta CT$ controls. All PCR primer pairs, purchased from Sigma-Aldrich (KiCqStartTM SYBR® Green qPCR ReadyMix TM, iQ, Sigma-Aldrich), are listed in Supplementary Table 1.

### Immunohistochemistry

Mice ($n = 3$ per group) were intra-cardioventricularly perfused with saline followed by fixation solution (4% paraformaldehyde in 0.1 M phosphate buffer, PB, pH 7.4). Free-floating coronal sections of 50 µm thickness were obtained using a vibratome slicing system (microM, Walldorf, Germany). Immunohistochemistry was performed using peroxidase-based detection system. Endogenous peroxidase activity was quenched for 30 min in 0.3% $H_2O_2$. The brain sections were blocked with 10% normal goat serum/PBS with 0.3% Triton X-100 and then incubated overnight at 4°C with the following antibodies: mouse monoclonal 6E10 antibody (1:3000 dilution, Signet Laboratories) for total amyloid; and human-specific anti-tau antibody HT7 (1:1000 dilution, Pierce Biotechnology) [20]. After removing the primary antiserum in excess, sections were incubated with secondary antibody (Biotin-SP-conjugated fragment donkey anti rabbit IgG) for 1 h at room temperature. After washing excess of antibody, sections were treated with avidin–biotin–peroxidase complex (ABC, 1:200 dilution, Vector Laboratories) and then developed with diaminobenzidine substrate using the avidin-biotin horseradish peroxidase system (Vector Laboratories). Immunolabeled sections were viewed using a Nikon 80i Eclipse microscope equipped with a DS-U1 digital camera, and NIS-elements BR software (Nikon, Tokyo, Japan). Semiquantitative analyses of the signal of Aβ

and tau immunostaining were performed using free-ware software from the National Institutes of Health (Scion Image software) and were expressed as optical densities.

*Statistics*

All data were expressed as mean ± standard error of the mean (SEM) of fold change values. Within-group variability was analyzed through Levene's test for homogeneity of variances. Data were analyzed using three-way analysis of variance (ANOVA), to test the main effects of D/L cycle (Dark versus Light), genotype (G) ($3 \times$ Tg-AD versus Non-Tg), age (A) (6 months versus 18 months of age) as between-subject factors; the interactions D/L × G, D/L × A, G × A and D/L × A × G were studied and a Tukey test was used as a *post-hoc* test for multiple comparisons. The correlation analysis between Aβ or tau protein levels and clock gene expression was performed by using the Pearson correlation test. The threshold for statistical significance was set at $p < 0.05$. The SPSS Statistics version 19 (IBM, Armonk, NY, USA) and GraphPad Prism 6 for Windows (GraphPad Software Inc., San Diego, CA, USA) were used to perform all the statistical analyses and to represent graph data, respectively.

## RESULTS

*Effect of D/L exposure on gene expression in the clock pathway in the suprachiasmatic nucleus (SCN) of 6- and 18-month-old $3 \times$ Tg-AD and Non-Tg mice*

Since the major circadian pacemaker in mammals is located in the SCN, we first determined whether the variations in clock gene expressions occurring after a 12 h D/L cycle was observed in the 6- and 18-month-old $3 \times$ Tg-AD and Non-Tg mice littermates, referred as the mild and severe pathology group, respectively (Fig. 1). To this aim, animals were sacrificed at CT 07:00 h (after exposure to D) or at CT 19:00 h (after exposure to L) and the variability of core clock gene expressions was analyzed. The results obtained by the statistical analysis aimed to test the main effects of D/L, G, A and the interactions D/L × G, D/L × A, G × A, and D/L × A × G are shown in Table 1.

The main effect of D/L cycle was significant for the expression of the core clock genes *Bmal1* and *Clock*, as well as the component of the negative feedback loop *Cry2*. The main effect of genotype was significant for the expression of *Cry1*, *Cry2*, and *Per1*, all coding for proteins of the negative feedback loop; moreover, the main effect of age was significant for the expression of all the genes studied, except for *Clock*. The interaction between the D/L cycle and genotype was significant only for *Clock* expression, while the interaction between the D/L cycle and age was significant for *Cry2* expression; interestingly, the interaction between genotype and age was significant for *Bmal1*, *Cry1*, *Cry2*, and *Per1* expression. Finally, the interaction of the three variables was significant for the expression of *Bmal1*, *Clock*, and *Sirt1*.

*Post-hoc* analysis showed an interesting picture of how D/L cycle, genotype and age affected the expression of clock genes in the SCN (Fig. 1). *Bmal1* was significantly upregulated after a 12 h exposure to L in both 6- and 18-month-old Non-Tg mice (+86% and +73%, respectively; Fig. 1a), while it was not modified or significantly downregulated (–50%) after 12-h L exposure in 6- and 18-month-old $3 \times$ Tg-AD mice, respectively. Interestingly, *Bmal1* expression in 18-month-old $3 \times$ Tg-AD mice was significantly higher (+111%) compared to 18-month-old Non-Tg mice after a 12 h exposure to darkness.

The expression of *Clock* was significantly higher (+115%) only in 6-month-old Non-Tg mice after 12 h exposure to L, but no significant differences were observed in the 6-month-old $3 \times$ Tg-AD and both 18-month-old Non-Tg and $3 \times$ Tg-AD mice (Fig. 1b).

Expression in the negative feedback loop (composed by *Per-Cry* genes) was also disrupted in $3 \times$ Tg-AD mice. We observed a lower expression of *Cry1* and *Per1* (–61% and –72%, respectively) after a 12 h exposure to L in 6-month-old Non-Tg mice. In the SCN of 6-month-old $3 \times$ Tg-AD, *Cry1* was significantly upregulated (+77%), while *Per1* was not modified. Moreover, the RNA expression of both genes was not significantly modified after D/L exposure in 18-month-old mice of both genotypes (Fig. 1c, e). Interestingly, *Cry1* expression in 6-month-old $3 \times$ Tg-AD mice was significantly higher (+345%) compared to 6-month-old Non-Tg mice after 12 h exposure to L (Fig. 1c).

*Nr1d1* and *Rorα* are both targets of the heterodimer CLOCK-BMAL1 and regulate, in turn, the expression of *Bmal1* [23]. In our study, *Nr1d1* expression was upregulated (+56%) after 12 h L exposure in 6-month-old Non-Tg mice, while no significant difference was observed after D/L exposure in both 6-month-old $3 \times$ Tg-AD and 18-month-old mice of both genotypes (Fig. 1h).
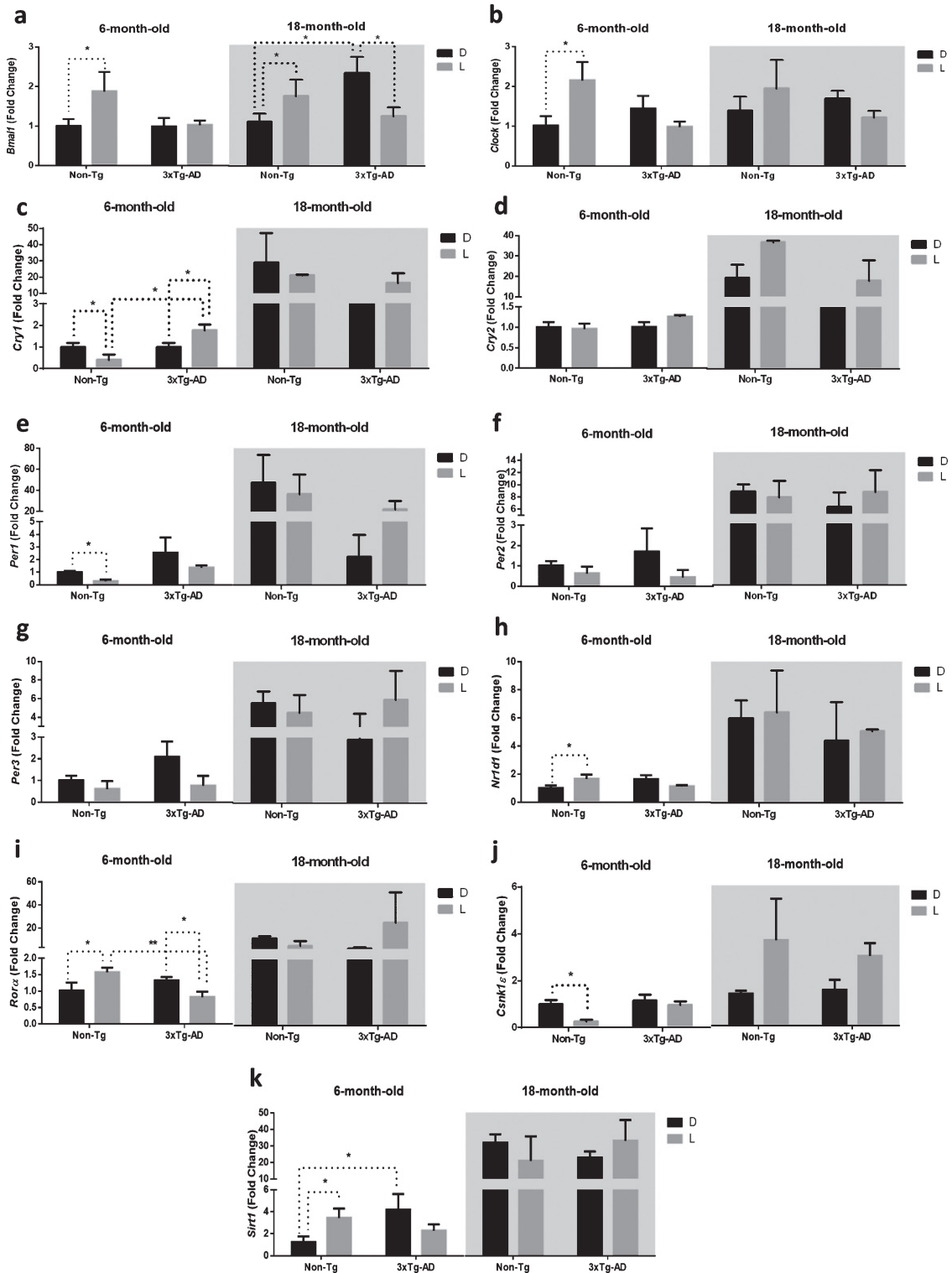
Fig. 1. Clock gene expression level in the suprachiasmatic nucleus (SCN) of 6-month-old and 18-month-old Non-Tg and 3 × Tg-AD mice after 12 h exposure to darkness (D) or light (L). Data are expressed as mean ± SEM of fold change values and were analyzed by 3-way ANOVA. Tukey's *post-hoc* test was used where appropriate to perform multiple comparisons. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 1

Results from the three-way ANOVA of clock gene expression in the suprachiasmatic nucleus of 6-month and 18-month-old Non-Tg and 3 × Tg-AD mice after a 12 h D/L cycle ($n = 12$ per group). D, darkness; L, light; G, genotype; A, age; F, Fisher's test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

| | | D/L | G | A | D/L × G | D/L × A | G × A | D/L × G × A |
|---|---|---|---|---|---|---|---|---|
| *Bmal1* | F | **27.78**\*** | 0.09 | **8.54**\** | 0.61 | 2.75 | **10.11**\** | **6.72**\* |
| *Clock* | F | **7.96**\* | 3.64 | 1.87 | **7.65**\* | 0.26 | 0.29 | **6.44**\* |
| *Cry1* | F | 0.18 | **5.60**\* | **30.47**\*** | 3.45 | 0.16 | **6.84**\* | 2.65 |
| *Cry2* | F | **16.78**\** | **19.76**\*** | **100.2**\*** | 0.21 | **16.35**\** | **20.49**\*** | 0.29 |
| *Per1* | F | 0.19 | **7.49**\* | **31.64**\*** | 1.85 | 0.11 | **9.05**\** | 2.01 |
| *Per2* | F | 0.00 | 0.14 | **33.86**\*** | 0.72 | 0.84 | 0.42 | 1.76 |
| *Per3* | F | 0.01 | 0.00 | **34.64**\*** | 1.66 | 2.21 | 1.04 | 4.15 |
| *Nr1d1* | F | 0.08 | 0.76 | **42.30**\*** | 0.02 | 0.32 | 1.80 | 0.12 |
| *Rorα* | F | 1.00 | 0.42 | **6.78**\* | 2.93 | 0.99 | 0.49 | 3.40 |
| *Csnk1ε* | F | 2.49 | 0.10 | **12.24**\** | 0.01 | 2.97 | 0.12 | 0.20 |
| *Sirt1* | F | 0.14 | 0.01 | **152.1**\*** | 2.69 | 0.15 | 0.10 | **6.52**\* |

As far as *Rorα*, we observed an upregulation (+68%) in the 6-month-old Non-Tg mice and, conversely, a downregulation (–38%) in the 6-month-old 3 × Tg-AD mice, while no significant difference was observed after D/L exposure in 18-month-old mice of both genotypes (Fig. 1i). Interestingly, *Rorα* expression in 6-month-old 3 × Tg-AD mice was significantly lower (–48%) compared with 6-month-old Non-Tg mice after 12 h exposure to L (Fig. 1i).

Casein kinase 1 epsilon (CSNK1ε) phosphorylates PER proteins promoting their degradation through a proteasomal pathway [24]. Interestingly, *Csnk1ε* was downregulated (–75%) after 12 h L exposure in 6-month-old Non-Tg mice, while no significant difference was observed after D/L exposure in both 6-month-old 3 × Tg-AD and 18-month-old mice of both genotypes (Fig. 1j).

The NAD-dependent deacetylase SIRT1 activates the transcription of both *Bmal1* and *Clock* in the SCN [25]. In this context, we observed a significant upregulation (+221%) of *Sirt1* in 6-month-old Non-Tg mice after 12 h L exposure, while no

significant difference was observed after D/L exposure in both 6-month-old 3 × Tg-AD and 18-month-old mice of both genotypes (Fig. 1k). Interestingly, *Sirt1* expression in 6-month-old 3 × Tg-AD mice was significantly higher (+240%) compared to 6-month-old Non-Tg mice after a 12 h exposure to darkness.

*Effect of D/L exposure on gene expression in the clock pathway of the hippocampus of 6- and 18-month-old 3 × Tg-AD and Non-Tg mice*

Clock genes are expressed in various parts of the central nervous system constituting a distributed circadian network with different degrees of autonomy and SCN-dependence [26]. Therefore, we first investigated the expression of clock genes in the hippocampus, which is one of the brain regions mainly affected by the neuropathological alterations of AD [27].

The results obtained by the statistical analysis aimed to test the main effects of D/L cycle, G, A, and the interactions between D/L × G, D/L × A, G × A,

Table 2

Results from the three-way ANOVA of clock gene expression in the hippocampus of 6-month and 18-month-old Non-Tg and 3 × Tg-AD mice after a 12 h D/L cycle ($n = 12$ per group). D, darkness; L, light; G, genotype; A, age; F, Fisher's test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

| | | D/L | G | A | D/L × G | D/L × A | G × A | D/L × G × A |
|---|---|---|---|---|---|---|---|---|
| *Bmal1* | F | **11.40**\** | 0.69 | **7.49**\* | 2.27 | **10.39**\** | 0.40 | 1.47 |
| *Clock* | F | **5.91**\* | 0.73 | 2.49 | 0.01 | **9.92**\** | 0.22 | 0.06 |
| *Cry1* | F | 0.64 | 0.01 | **16.18**\** | 0.02 | **19.51**\*** | 0.12 | 0.35 |
| *Cry2* | F | **7.44**\* | 0.38 | **8.06**\* | 0.45 | **6.42**\* | 0.37 | 0.20 |
| *Per1* | F | 3.32 | 1.57 | **24.20**\*** | 0.78 | 4.13 | 2.45 | 1.06 |
| *Per2* | F | 1.89 | 0.58 | **12.34**\** | 0.24 | 2.94 | 0.87 | 0.53 |
| *Per3* | F | 0.39 | 0.36 | **14.02**\** | 4.02 | 1.04 | 2.00 | **4.44**\* |
| *Nr1d1* | F | 3.87 | 0.02 | **13.21**\** | 0.55 | **5.93**\* | 0.24 | 0.61 |
| *Rorα* | F | 2.59 | 0.37 | **6.60**\* | 1.54 | 2.98 | 0.33 | 1.71 |
| *Csnk1ε* | F | **13.96**\** | 0.98 | **33.13**\*** | 0.02 | **12.14**\** | 0.90 | 0.29 |
| *Sirt1* | F | **11.36**\** | **12.21**\** | **22.40**\*** | **4.40**\* | **11.35**\** | **11.73**\** | **4.70**\* |

and D/L × A × G are shown in Table 2. Similarly to the SCN, we observed a significant main effect of D/L cycle on the expression of core clock genes *Bmal1* and *Clock*, as well as the component of the negative feedback loop *Cry2*; moreover, the main effect of D/L cycle was significant for the expression of *Csnk1ε* and *Sirt1* in the hippocampus. The main effect of genotype was significant only for the expression of *Sirt1*, while the main effect of age was observed for the expression of all the genes studied, except for *Clock*. The interaction between D/L and genotype was significant for *Sirt1* expression, while the interaction between D/L and age was significant for *Bmal1*, *Clock*, *Cry1-2*, *Nr1d1*, *Csnk1ε*, and *Sirt1*. The interaction between genotype and age was significant for Sirt1 expression. Finally, the interaction among the three factors was significant for the expression of Per3 and Sirt1 (Table 2). As reported in the Fig. 2, the *post-hoc* analysis showed a significant downregulation (–49%) of *Cry1* after 12 h L exposure in 6-month-old Non-Tg mice, while no significant difference was observed after D/L exposure in both 6-month-old 3 × Tg-AD and 18-month-old mice of both genotypes (Fig. 2c).

Moreover, the exposure to D/L cycle caused the upregulation (+59%) of *Sirt1* only in 18-month-old Non-Tg mice, while no significant difference was observed after D/L exposure in both 18-month-old 3 × Tg-AD and 6-month-old mice of both genotypes (Fig. 2k). Interestingly, *Sirt1* expression in 18-month-old 3 × Tg-AD mice was significantly lower (–80%) compared to 18-month-old Non-Tg mice after a 12 h exposure to L (Fig. 2k).

*Effect of D/L exposure on expression of genes of the clock pathway in the frontal cortex of 6- and 18-month-old 3 × Tg-AD and Non-Tg mice*

Gene expression in the clock pathway was then analyzed in the frontal cortex.

The results obtained by the statistical analysis aimed to test the main effects of D/L cycle, G, A and the interactions D/L × G, D/L × A, G × A, and D/L × A × G are shown in Table 3. The main effect of D/L cycle was significant for the expression of *Bmal1*; overall the main effect of genotype was not significant, while the main effect of age was significant on the expression of all the genes studied, except for *Sirt1*. The interaction between the D/L cycle and age was significant for *Bmal1*, *Clock*, *Cry1-2*, and *Per3*; no further significant interactions were observed (Table 3).

As reported in the Fig. 3c, the *post-hoc* analysis showed a significant downregulation (–51%) of *Cry1* after 12 h L exposure in 6-month-old Non-Tg mice, while no significant difference was observed after D/L exposure in both 6-month-old 3 × Tg-AD and 18-month-old mice of both genotypes.

Moreover, after 12 h L exposure we observed a significant downregulation of *Per1*, *2*, and *3* in 6-month-old 3 × Tg-AD mice (–51%, –52%, and –44%, respectively), while no significant difference was observed after D/L exposure in both 6-month-old Non-Tg and 18-month-old mice of both genotypes (Fig. 3e-g). Interestingly, *Per1* and *Per3* expression in 6-month-old 3 × Tg-AD mice was significantly higher (+85% and +82%, respectively) compared to 6-month-old Non-Tg mice after 12 h exposure to D (Fig. 3e, g).

*Effect of D/L exposure on gene expression of the clock pathway in the brainstem of 6- and 18-month-old 3 × Tg-AD and Non-Tg mice*

We then evaluated the expression of clock genes in the brainstem, which is connected with the SCN by the neural circuits regulating sleep-wake rhythms [28].

The results obtained by the statistical analysis aimed to test the main effects of D/L cycle, G, A and the interactions D/L × G, D/L × A, G × A, and D/L × A × G are shown in Table 4. We observed a significant main effect of the D/L cycle on the expression of *Bmal1*, *Cry2*, *Per1*, *Rorα*, and *Csnk1ε*. The main effect of genotype was significant on the expression of *Cry1-2* and *Per1*, as well as *Rorα*, *Csnk1ε*, and *Sirt1*. The main effect of age was a significant factor in the expression of all the genes studied, except for *Clock* and *Cry1*. The main effect of an interaction between the D/L cycle and genotype was significant for *Bmal1* and *Cry1-2* expression, while the interaction between the D/L cycle and age was significant for *Bmal1*, *Cry1-2* and *Per1* expression; moreover, the main effect of interaction between genotype and age was significant for *Cry1-2*, *Per1*, *Rorα*, *Csnk1ε*, and *Sirt1* expression. The main effects of interaction among the three factors was significant for the expression of *Cry2*, *Per1*, and *Csnk1ε* (Table 4).

As reported in the Fig. 4a, the *post-hoc* analysis showed a significant upregulation of *Bmal1* expression after 12 h L exposure only in the 6-month-old Non-Tg mice (–321%), while no significant difference was observed in both the 6-month-old
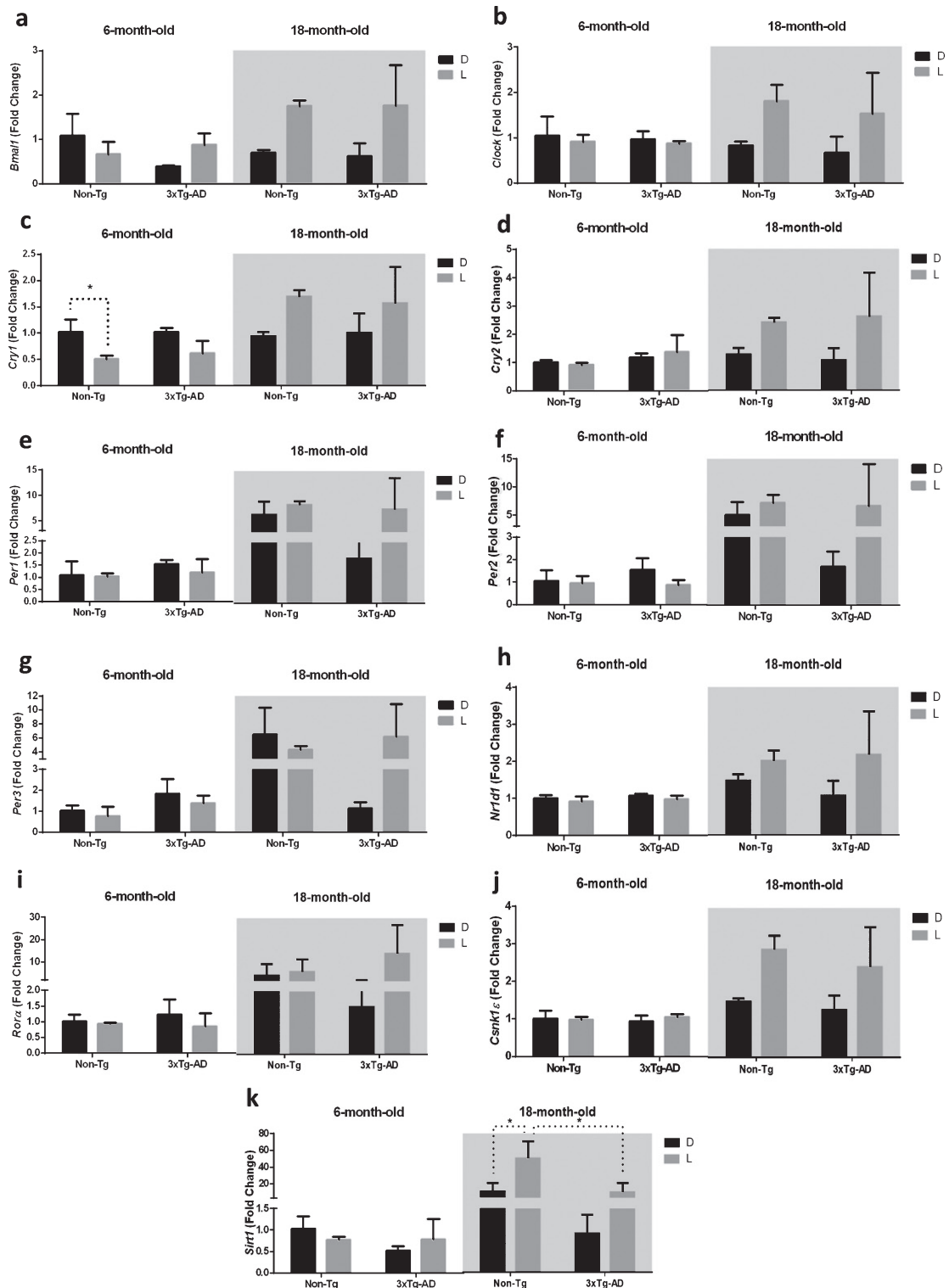
Fig. 2. Clock gene expression level in the hippocampus (H) of 6-month-old and 18-month-old Non-Tg and $3 \times$ Tg-AD mice after 12 h exposure to darkness (D) or light (L). Data are expressed as mean ± SEM of fold change values and analyzed by 3-way ANOVA. Tukey's *post hoc* test was used where appropriate to perform multiple comparisons. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 3
Results from the three-way ANOVA of clock gene expression in the frontal cortex of 6-month and 18-month-old Non-Tg and $3 \times$ Tg-AD mice after a 12 h D/L cycle ($n = 12$ per group). D, darkness; L, light; G, genotype; A, age; F, Fisher's test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

|  |  | D/L | G | A | D/L $\times$ G | D/L $\times$ A | G $\times$ A | D/L $\times$ G $\times$ A |
|---|---|---|---|---|---|---|---|---|
| *Bmal1* | F | **11.40**$^{**}$ | 0.02 | **12.84**$^{**}$ | 0.29 | **5.58**$^*$ | 0.45 | 1.17 |
| *Clock* | F | 1.95 | 0.01 | **6.92**$^*$ | 0.22 | **4.23**$^*$ | 0.51 | 0.01 |
| *Cry1* | F | 0.02 | 0.03 | **26.46**$^{***}$ | 0.18 | **5.24**$^*$ | 0.02 | 0.37 |
| *Cry2* | F | 1.88 | 0.08 | **16.64**$^{**}$ | 0.15 | **4.71**$^*$ | 0.02 | 0.14 |
| *Per1* | F | 1.70 | 0.45 | **7.06**$^*$ | 0.02 | 3.68 | 0.01 | 0.13 |
| *Per2* | F | 0.28 | 1.47 | **19.50**$^{***}$ | 0.62 | 2.81 | 0.59 | 0.31 |
| *Per3* | F | 0.88 | 1.43 | **17.23**$^{**}$ | 0.04 | **4.97**$^*$ | 0.01 | 0.17 |
| *Nr1d1* | F | 0.27 | 0.38 | **16.77**$^{**}$ | 0.69 | 0.52 | 0.03 | 0.98 |
| *Ror$\alpha$* | F | 2.59 | 1.81 | **4.72**$^*$ | 0.25 | 2.27 | 0.23 | 0.27 |
| *Csnk1$\varepsilon$* | F | 2.60 | 0.88 | **22.45**$^{***}$ | 0.07 | 3.79 | 0.01 | 0.13 |
| *Sirt1* | F | 2.04 | 0.85 | 1.61 | 0.00 | 2.76 | 0.37 | 0.93 |

$3 \times$ Tg-AD and 18-month-old mice of both genotypes.

*Cry1* expression in 6-month-old $3 \times$ Tg-AD mice was significantly higher (+212%) compared to 6-month-old Non-Tg mice after a 12 h exposure to D (Fig. 4c).

Moreover, *Cry2* was upregulated (+471%) in 18-month-old Non-Tg mice after a 12 h L exposure (Fig. 4d). Interestingly, *Cry2* expression in 18-month-old $3 \times$ Tg-AD mice was significantly lower (−74%) compared to 18-month-old Non-Tg mice after a 12 h exposure to L (Fig. 4d).

*Per1* was downregulated (−54%) in 6-month-old $3 \times$ Tg-AD mice after a 12 h L exposure (Fig. 4e). Interestingly, *Per1* expression in 6-month-old $3 \times$ Tg-AD mice was significantly higher compared to 6-month-old Non-Tg mice after a 12 h exposure to D and L (+444% and 467%, respectively, Fig. 4e).

As far as *Nr1d1*, the expression in 6-month-old $3 \times$ Tg-AD mice was significantly higher (+131%) compared to 6-month-old Non-Tg mice after a 12 h exposure to D (Fig. 4h).

Moreover, the expression of *Csnk1$\varepsilon$* was significantly higher (+237%) in 6-month-old $3 \times$ Tg-AD mice compared to 6-month-old Non-Tg mice after a 12 h exposure to L (Fig. 4j).

Finally, after a 12 h L exposure we observed an upregulation (+989%) of *Sirt1* expression in 6-month-old Non-Tg mice, while no significant differences were observed after D/L exposure in both 6-month-old $3 \times$ Tg-AD and 18-month-old mice of both genotypes (Fig. 4k). Interestingly, *Sirt1* expression in 6-month-old $3 \times$ Tg-AD mice was significantly higher (+347%) compared to 6-month-old Non-Tg mice after a 12 h exposure to L (Fig. 4k).

*Alterations of clock gene expression and neuropathology*

To verify whether variations in the clock pathway were related to brain pathology in the transgenic model, immunohistochemical analysis of both A$\beta$ and tau was performed in all the areas where the clock gene expression was investigated. As previously reported, no immunostaining was detected in the SCN and in the brainstem (data not shown) [17].

As the $3 \times$ Tg-AD mice age, they gradually accumulate A$\beta$ and tau pathology in their brains. Specifically, 6-month old $3 \times$ Tg-AD mice showed a significant increase in A$\beta$ immunostaining compared to age-matched Non-Tg mice, while no difference was found in tau immunoreactivity at this age in both the frontal cortex and the hippocampus. Finally, we found dense A$\beta$ deposits and extensive human tau immunoreactivity in the frontal cortex and hippocampus of 18-month-old $3 \times$ Tg-AD mice. None of these immunoreactive structures were detected in the Non-Tg brains (Fig. 5a).

Moreover, the application of a Pearson correlation test did not find any relationship between modification of clock gene expression and A$\beta$ deposition or tau immunoreactivity in the frontal cortex. Nevertheless, direct correlations between A$\beta$ and *Per3* variation ($\rho = 0.6223$, $p = 0.0307$), tau and *Per3* variation ($\rho = 0.6445$, $p = 0.0237$), as well as tau and *Ror$\alpha$* ($\rho = 0.6901$, $p = 0.0130$) were found in the hippocampus (Fig. 5b-d).

**DISCUSSION**

The present study demonstrates that the RNA expressions of clock genes are disrupted in both the SCN and three extra-hypothalamic brain areas that
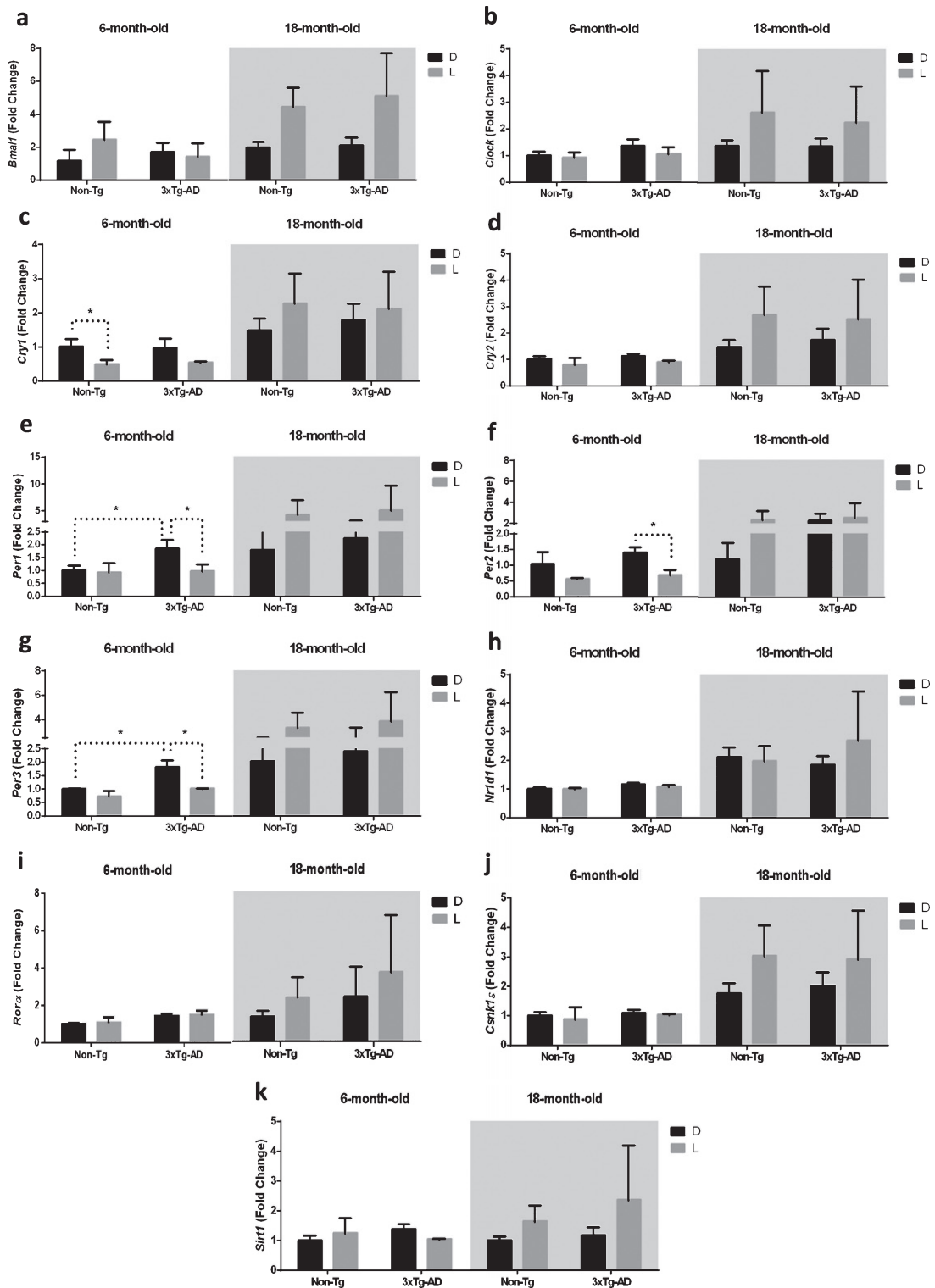
Fig. 3. Clock gene expression level in the frontal cortex (FC) of 6-month-old and 18-month-old Non-Tg and 3 × Tg-AD mice after 12 h exposure to darkness (D) or light (L). Data are expressed as mean ± SEM of fold change values and analyzed by 3-way ANOVA. Tukey's *post hoc* test was used where appropriate to perform multiple comparisons. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Table 4
Results from the three-way ANOVA of clock gene expression in the brainstem of 6-month and 18-month-old Non-Tg and 3 × Tg-AD mice after a 12-hour D/L cycle ($n = 12$ per group). D, dark; L, light; G, genotype; A, age; F, Fisher's test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

|  |  | D/L | G | A | D/L × G | D/L × A | G × A | D/L × G × A |
|---|---|---|---|---|---|---|---|---|
| *Bmal1* | F | **19.10**** | 0.29 | **19.49**** | **18.08**** | **11.66**** | 2.28 | 0.57 |
| *Clock* | F | 1.94 | 3.56 | 0.03 | 2.50 | 1.21 | 1.50 | 1.03 |
| *Cry1* | F | 0.00 | **5.24*** | 1.29 | **8.61*** | **6.70*** | **23.83***** | 2.08 |
| *Cry2* | F | **32.74***** | **20.53***** | **69.57***** | **19.51***** | **36.61***** | **34.32***** | **13.37**** |
| *Per1* | F | **10.43**** | **14.26**** | **25.90***** | 0.89 | **6.87*** | **57.84***** | **9.06**** |
| *Per2* | F | 0.05 | 0.66 | **98.86***** | 0.06 | 0.08 | 0.60 | 0.08 |
| *Per3* | F | 0.02 | 0.82 | **107.6***** | 0.24 | 0.00 | 0.66 | 0.05 |
| *Nr1d1* | F | 0.45 | 0.05 | **83.16***** | 1.00 | 0.80 | 1.95 | 2.65 |
| *Rorα* | F | **7.58*** | **6.00*** | **42.50***** | 0.91 | 2.85 | **8.09*** | 1.81 |
| *Csnk1ε* | F | **4.44*** | **14.57**** | **18.57***** | 0.08 | 0.51 | **21.90***** | **5.02*** |
| *Sirt1* | F | 2.60 | **24.03***** | **24.44***** | 0.05 | 1.24 | **27.02***** | 0.01 |

contribute to circadian regulation—the hippocampus, frontal cortex, and brainstem—in the 6-month-old 3 × Tg-AD compared to age-matched control Non-Tg mice. Differences between these genotypes were observed at 6 months (the stage of mild pathology), but then disappeared by 18 months at an increasingly severe stage of pathology. These data therefore demonstrate that, with the progression of AD, the increasing impact of Aβ and NFTs accumulation on gene expression in the clock pathway were obscured by an interaction with the normal aging process.

In the clock pathway, the clock gene product BMAL1 and its partner CLOCK are positive regulators of transcription, while others, such as CRY, PER, and RORα, are components of a negative feedback loop that attenuates activation of a range of endogenous clock genes [23]. During the day, CLOCK and BMAL1 heterodimerize and activate the rhythmic transcription of *Per1-3* and *Cry1-2*, which in turn form complexes in the cytoplasm and then translocate to the nucleus interacting with CLOCK-BMAL1, which inhibit their own transcription. In a second positive loop, the CLOCK-BMAL1 complexes activate the transcription of *Nr1d1* and *Rorα*, whose protein products repress and activate, respectively, *Bmal1* transcription influencing its rhythmic expression. While BMAL1 level falls, the expression of *Per* and *Cry* genes increases, proteins enter the nucleus inhibiting their own transcription and also *Rev-erb* α transcription, leading to a de-repression or activation of *Bmal1* expression, which completes and fine tunes this transcriptional feedback loop.

The CLOCK-BMAL1 duet also drives the expression of output genes, the so-called clock controlled genes [29–32]. The mechanism of negative feedback loops is needed to establish and maintain circadian rhythms, but the clock has further levels of complexity including post-transcriptional regulation, phosphorylation, ubiquitination, post-translational modifications, and chromatin remodeling [33, 34]. Therefore, we designed our gene expression study as previous observations of the interactions between environmental cues and gene expression of clock pathway transcripts have yielded important insights into the regulatory mechanisms of circadian function [35].

The function of circadian clocks, in common with several physiological activities, is greatly affected by neurodegenerative diseases [36], as well as the aging process [37]. The most novel finding of our study is the demonstration that gene expression in the clock pathway is disrupted in 6-month-old 3 × Tg-AD mice, which is accompanied with the onset of mild pathology and cognitive impairments but these disruptions were masked by the normal ageing process and expression was not significantly disrupted in 18-month-old 3 × Tg-AD, compared with normal aging control. This hypothesis is supported by clinical observations demonstrating that changes in the circadian patterns of gene expression are significant during the normal aging processes, but less differences are observed when comparing elderly AD patients with age-matched controls [38, 39]. To our opinion, it is not conceivable that normal aging processes are stronger than AD pathology in the induction of circadian disruption, but rather that the lack of differences between 18-month-old Non-Tg and 3 × Tg-AD mice may reflect a ceiling effect.

In a recent postmortem study, significant differences in the RNA expression level of *Per1-2* and *Bmal1* were found in the bed nucleus of the stria terminalis, cingulate cortex, and the pineal gland [40]. However, there are significant methodological difficulties in accurately interpreting clock pathway gene expression in human samples, as a daytime death
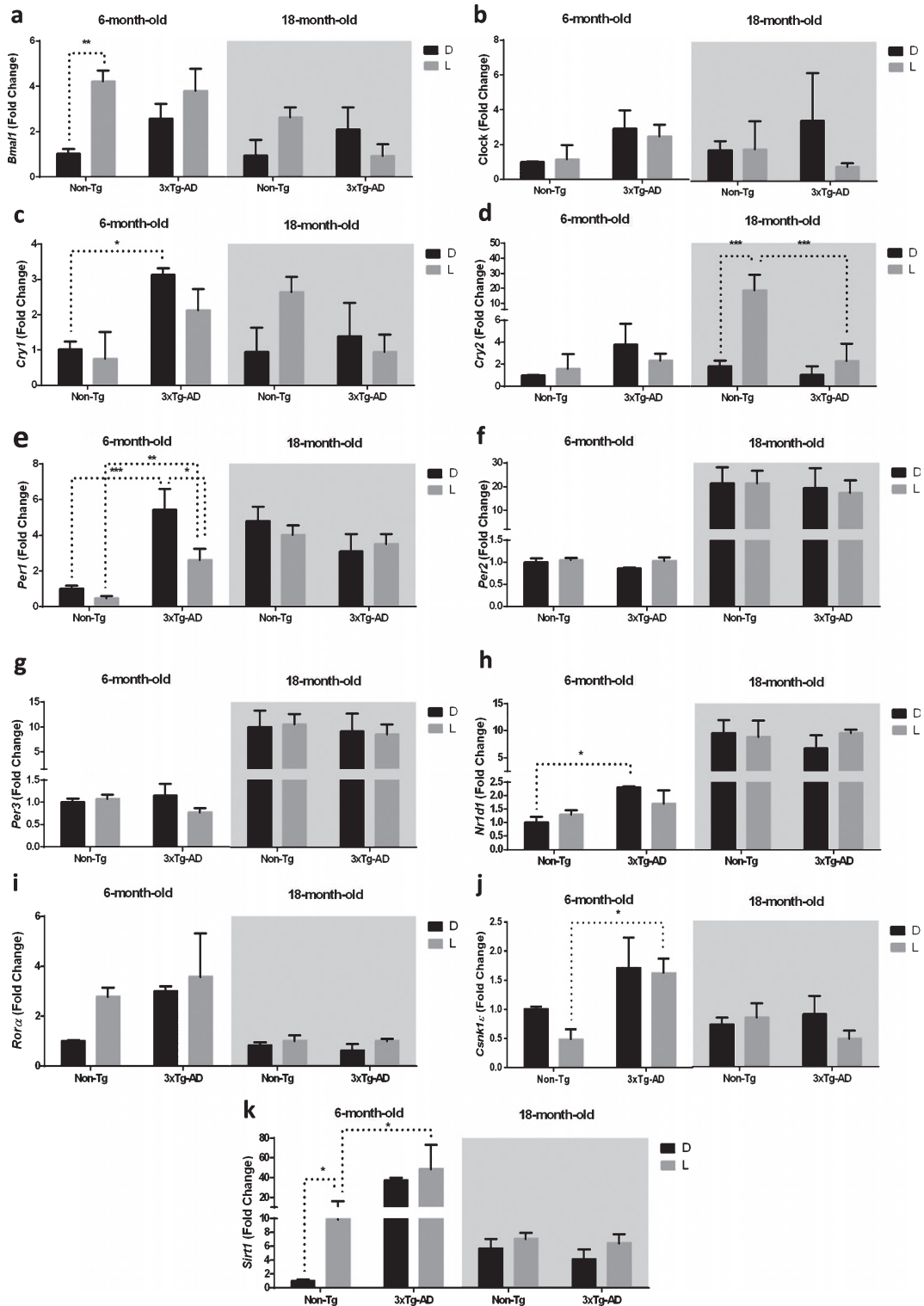
Fig. 4. Clock gene expression level in the brainstem (BS) of 6-month-old and 18-month-old Non-Tg and 3 × Tg-AD mice after 12 h exposure to darkness (D) or light (L). Data are expressed as mean ± SEM of fold change values and analyzed by 3-way ANOVA. Tukey's *post hoc* test was used where appropriate to perform multiple comparisons. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
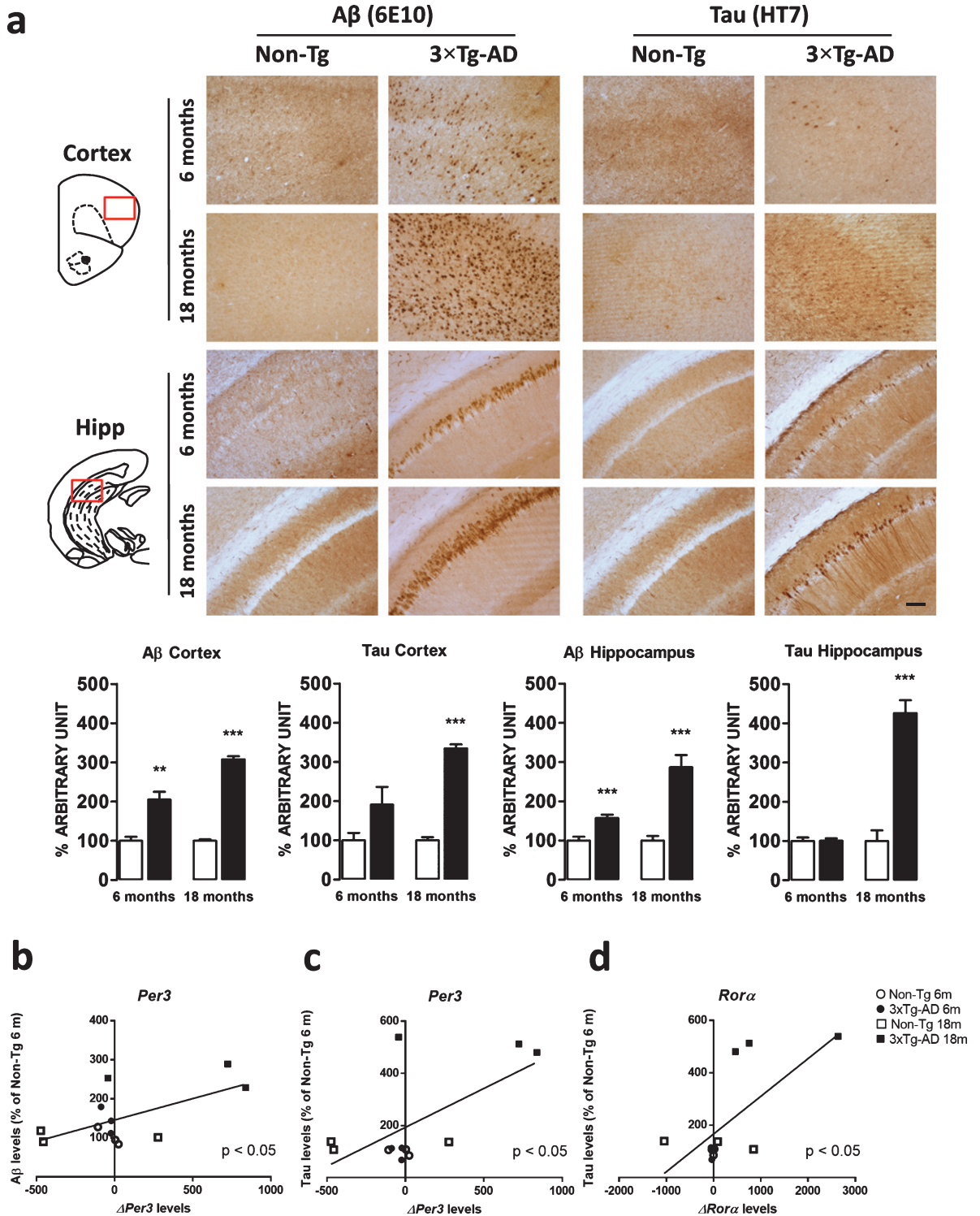
Fig. 5. a) Representative microphotographs (10 × magnification, scale bar 100 μm) and results obtained from the semi-quantitative analyses of Aβ (6E10 antibody) and tau (HT7 antibody) immunostaining from Non-Tg ($n = 3$, white bars) and 3 × Tg-AD ($n = 3$, black bars) mice. The red squares within the brain diagrams illustrate the sites where the representative microphotographs were taken. The data are mean ± SEM **$p < 0.01$ and ***$p < 0.001$ *versus* age-matched Non-Tg mice (Unpaired Student's $t$-test, $n = 3$). b-d) Scatterplot of Aβ or tau protein levels *versus Per3* and *Rorα* mRNA expression showing a direct correlation (Pearson test) in the hippocampus of 3 × Tg-AD mice.

would result in differing patterns of gene expression than a nightly one [36]. To date, studies in gene-manipulated mice commonly used as models of AD have not provided evidence of a disruption to clock pathway gene expression in AD, or demonstrated the potential involvement of these genes in the neurodegenerative process. This potential disruption in gene expression is further complicated by the variety of transgenic mice used as AD models, which can harbor single, double, or triple gene mutations, and show different phenotypes. The $3 \times$ Tg-AD mice exhibit both Aβ and tau pathology characteristic of the human form of AD, and have been so far extensively characterized in the context of biochemical, circadian, behavioral and cognitive changes [19, 41, 42]. Nevertheless, a methodological limitation of this investigation consists in the absence of electrophysiological measurement of circadian rhythms and sleep-wake cycle, taking into account that behavioral studies are characterized by environment-gene interaction and researcher-associated bias [43, 44].

Interestingly, this present study shows that the effects of light exposure on the expression of clock pathway genes in the SCN were altered in the AD model. Similar results though related to fewer genes were observed in the brainstem, but not in the hippocampus and the frontal cortex, the most affected brain areas in AD. Changes pertaining to circadian rhythmicity that occur prior to and post-AD pathology have been previously characterized in $3 \times$ Tg-AD mice, demonstrating that abnormalities in circadian rhythmicity precede the expected onset of AD pathology [41]. It is interesting to note that these differences presented in relatively young (6 months of age) $3 \times$ Tg-AD mice who express mild pathology and cognitive impairments, but by 18 months of age as the pathology and cognitive impairments increase in severity, these differences become statistically insignificant due to an interaction with ageing. Other studies have suggested that alterations in the expression of clock pathway genes association with AD are only slightly observed in brain areas where pathology develops. One rodent AD-model showed altered expression of *Per2* in the SCN, but not in the hippocampus [45] and there is evidence that Aβ could induce degradation of BMAL1 and modulate *Per2* expression [46]. Other recent reports have demonstrated that AD-related morphological findings occur in the hypothalamus and brainstem as well [47, 48]. It has been previously considered that the intact circadian system may have neuroprotective functions, but disruption of the circadian clock seems to promote neurodegeneration [49]. This study showed that correlations between amyloid plaques/NFTs accumulation and altered expression of clock genes were confined to the hippocampus, which is a brain region particularly affected by AD pathology in the $3 \times$ Tg-AD mice. In this regard, further studies are needed to clarify the underlying molecular mechanisms.

Previous experiments in rodents have shown that the age-related deterioration in circadian pacemaker function occurs at the level of individual cells in the SCN, and it is correlated with an altered expression of several of the genes that support the molecular function of the circadian clock [50, 51]. Moreover, it has been described that the light-induced expression of clock genes is modified in the SCN of aging hamsters [52], and that phase-shifts of the D/L cycle are affected in the pineal gland and the arcuate nucleus of aged rats [53]. When we examined and compared expression of clock pathway genes after light exposure as a function of aging in 6-month-old and 18-month-old animals, the main changes found as a consequence of aging suggested that expression was increased in aged mice. This study did not address whether clock gene dysregulation during aging was dependent on an impairment of the retina; however, a recent study performed with cholera toxin tracing has concluded that aging rather than retinal degeneration is a more important factor in reducing the efficiency of L or D information to the SCN [54]. Another cause of the alteration of the circadian pacemaker may be linked to age-related loss of sensitivity to melatonin in the SCN [55]. A further comment should be addressed concerning the progression of brain pathology on altered clock gene expression as a consequence of the D/L cycle in the extra-hypothalamic brain areas. While SCN clocks are able to independently generate and maintain their circadian oscillations, secondary clocks do require external signals to sustain and/or synchronize their rhythms [56]. It has then been suggested that the age-associated changes in the SCN might account for the impairments in circadian clock synchronization in other peripheral clocks [35]. However, since the brain contains multiple circadian oscillators in extra-hypothalamic areas, it is also conceivable that these peripheral regions could adapt to changes related to the pathological process [57]. It is interesting to note that in our study we observed the effect of light exposure on *Bmal1* expression in all brain areas observed. However, this was not associated with an effect of light exposure on the negative feedback loop. The disruption of the

circadian system by targeted deletions of *Clock* or *Bmal1* significantly affects the rate of ageing, and is associated with increases in brain inflammation and neurodegeneration [37, 58]. CLOCK proteins may have other functions other than regulation of the circadian rhythm [49]. This is supported by evidence showing accelerated neurodegeneration in mouse models of brain-selective or conditional *Bmal1* knock out mice [59, 60]. It is then conceivable that upregulation of *Bmal1* and the altered activation of the negative feedback loop, which occurs in all the brain areas, would represent a counteracting mechanism to the neurodegenerative effect. However, these aspects need to be further investigated by using different circadian mutant models.

In conclusion, our results demonstrate that a mild AD-pathology in 6-month-old, $3 \times$ Tg-AD mice is associated with a disruption in the temporal patterns and quantitative levels gene expression in the clock pathway, particularly in the SCN but also in extra-hypothalamic brain areas associated with circadian function, suggesting a severe impairment of functioning of the clock gene machinery. Even though this study did not allow to establish a direct association between clock gene impairment and circadian behavioral changes, our novel findings encourage future studies testing the hypothesis that altered clock gene expression may account for the disruption of timely organization among brain areas, hampering the coordination between basic cellular metabolic and homeostatic processes, ultimately leading to altered functionality and connectivity of brain structures, cognitive impairment and chrono-disruption that characterize the early onset of AD in patients.

## ACKNOWLEDGMENTS

## SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/ 10.3233/JAD-160942.

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