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An intracerebroventricular injection of amyloid-beta peptide (1-42) aggregates modifies daily temporal organization of clock factors expression, protein carbonyls and antioxidant enzymes in the rat hippocampus

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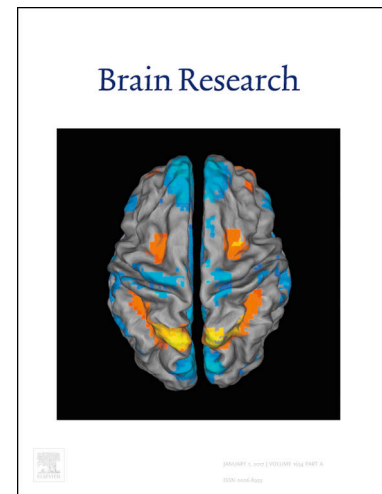
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AN INTRACEREBROVENTRICULAR INJECTION OF AMYLOID-BETA PEPTIDE (1-42) AGGREGATES MODIFIES DAILY TEMPORAL ORGANIZATION OF CLOCK FACTORS EXPRESSION, PROTEIN CARBONYLS AND ANTIOXIDANT ENZYMES IN THE RAT HIPPOCAMPUS

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Highlights

- CAT and GPx expression and activity display daily rhythms in the rat hippocampus.
- Protein oxidation levels vary rhythmically in this brain area. -Ror α and Rev-erb β mRNA expression varies throughout a day in the rat hippocampus.
- The A β group showed a phase shift in protein carbonyls and GPx rhythms.
- An injection of A β aggregates phase-shifted daily rhythms of clock genes.

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ABSTRACT

Alzheimer disease (AD) is the most frequent form of dementia in the elderly. It is characterized by the deterioration of memory and learning. The histopathological

hallmarks of AD include the presence of extracellular deposits of amyloid beta peptide, intracellular neurofibrillary tangles, neuron and synapse loss, in the brain, including the hippocampus. Accumulation of A β peptide causes an increase in intracellular reactive oxygen species (ROS) and free radicals associated to a deficient antioxidant defense system. Besides oxidative stress and cognitive deficit, AD patients show alterations in their circadian rhythms. The objective of this work was to investigate the effects of an intracerebroventricular injection of amyloid beta peptide A β (1-42) aggregates on temporal patterns of protein oxidation, antioxidant enzymes and clock factors in the rat hippocampus. Four-month-old male Holtzman rats divided into the groups control (CO) and A β -injected (A β), were maintained under 12h-light/12h-dark conditions and received water and food *ad-libitum*. Hippocampus samples were isolated every 6 h during a 24h period. Our results showed daily patterns of protein carbonyls, catalase (CAT) and glutathione peroxidase (GPx) expression and activity, as well as Rora and Rev-erba mRNA, in the rat hippocampus. Interestingly, an intracerebroventricular injection of A β aggregates modified daily oscillation of protein carbonyls levels, phase-shifted daily rhythms of clock genes and had a differential effect on the daily expression and activity of CAT and GPx. Thus, A β aggregates might affect clock-mediated transcriptional regulation of antioxidant enzymes, by affecting the formation of BMAL1:CLOCK heterodimer, probably, as a consequence of the alteration of the redox state observed in rats injected with A β .

Key Words: Alzheimer disease, antioxidant enzymes, protein oxidation, clock genes, daily rhythm, stress oxidative

1. Introduction

Alzheimer's disease (AD) is the main cause of dementia in the elderly (Forlenza et al., 2010). The main pathological characteristics observed in the brain of Alzheimer's disease patients are senile plaques (composed mostly of A β), neurofibrillary tangles (NFTs, composed of hyperphosphorylated tau protein), neural and synapse loss. (Bloom., 2014).

Numerous evidences support that oxidative stress plays a key role in the pathogenesis and progression of AD (Butterfield et al., 2001; Sayre et al., 2005; Sultana et al., 2012). It is known that oxidative stress is produced by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of the cell.

The antioxidant system, either enzymatic, such as glutathione peroxidase (GPx) and catalase (CAT), and non-enzymatic, such as uric acid, vitamin E and glutathione, neutralizes the deleterious effects of ROS (Baek et al., 1999; Glantzounis et al., 2005).

It has been shown that A β (1–42) oligomers induces oxidative damage in neuronal cultures and in brains from both animal models of AD and subjects with AD (Butterfield et al., 2007; 2010; Sultana et al., 2006; 2012). Specifically, the interaction of Met-35 within the lipid bilayer is a requirement for A β (1–42) mediated lipid peroxidation followed by oxidative damage to proteins and other biomolecules (Butterfield et al., 2010). Indeed, elevated levels of biomarkers of protein oxidation, lipid peroxidation, DNA and RNA oxidation in the brain were observed in postmortem studies of patients with AD (Liu et al., 2005; Markesbery et al., 2005, Yao et al., 2003). A recent research conducted by Balmus et al. (2017) showed increased levels of malondialdehyde (MDA), a lipid peroxidation marker and low activity of antioxidant enzymes (superoxide dismutase (SOD) and glutathione peroxidase) in Alzheimer's disease patients, compared to age-matched healthy subjects.

A growing body of evidence showed that oxidative stress plays a key role in neurodegenerative events which lead to a gradual impairment of memory observed in the AD (Selkoe., 2011; Walsh et al., 2007). AD is clinically characterized by a slowly progressive cognitive decline, accompanied by circadian rhythm alterations. Examples of them include disturbances in sleep-wake cycles, thermoregulation, activity and melatonin and cortisol secretion (Coogan et al., 2013; Hatfield et al., 2004; Harper et al., 2005; Skene and Swaab., 2003).

Abbreviations Alzheimer's disease=AD, neurofibrillary tangles=NFTs, reactive oxygen species=ROS, glutathione peroxidase=GPx, catalase=CAT, MDA=malondialdehyde, SOD= superoxide dismutase, i.c.v =intracerebroventricular,

On a cellular level, circadian rhythms are generated by a cellular clock transcriptional machinery composed of two interacting transcription/translation-based feedback loops, a positive (BMAL1:CLOCK) and a negative (PER:CRY). The heterodimer BMAL1:CLOCK drives the expression of clock (PER1, 2 and 3 and CRY1 and 2) and clock-controlled genes by its binding to E-box enhancers on the target genes promoters (Panda and Hogenesch, 2004). REVERBs and ROR transcription factors complete the molecular clock machinery and drive the rhythm of BMAL1

transcription (Sato et al., 2004). Some studies have shown that the DNA-binding activity of BMAL1:CLOCK is regulated by the redox state of NAD cofactors (Rutter et al., 2001; Yoshii et al., 2015).

Above observations raise the possibility that A β aggregates might modulate the daily expression of clock genes, for example, by modifying cellular redox state. Considering: 1) A β induce oxidative stress 2) redox state -mediated effect has been observed on cellular clock activity.

Thus, we aimed to evaluate the consequences of an intracerebroventricular (i.c.v) injection of aggregated β -amyloid peptide (1-42) on temporal patterns of antioxidant enzymes activity, protein oxidation levels and Rora and Rev-erb β expression in the rat hippocampus, a peripheral oscillator with a relevant function in memory to learning processes.

2. Results

2.1 Daily patterns of protein oxidation levels in the hippocampus of A β -injected rats

Protein oxidation levels were analyzed in the hippocampus of control rats, throughout a 24-h period. We observed that protein carbonyl levels vary throughout a 24h-period ($F(3,12)=4.83$ $p\leq 0.05$), following a significant daily rhythm (Chronos-Fit: $p\leq 0.01$) with maximal levels occurring at ZT 10:51 \pm 00:33. The i.c.v. injection of A β aggregates phase delated daily rhythm of protein carbonyl (from ZT 10:51 \pm 00:33 to ZT 22:48 \pm 00:05, $t(6)=-21.11$, $p\leq 0.001$) and increased the rhythm's amplitude (1.47 \pm 0.17 vs 4.9 \pm 1.34, $t(6)=-2.54$, $p< 0.05$) in the rat hippocampus (Figure 1 and Table 1).

2.2 Daily expression and activity of CAT in the hippocampus of A β -injected rats

The results revealed that CAT expression oscillates significantly in a 24h cycle ($F(3,8)=8.38$ $p\leq 0.01$; Chronos-Fit: $p\leq 0.01$) with the highest mRNA levels occurring at ZT 08:29 \pm 00:36 (Figure 2A; Table 2). Consistently, we found that temporal variation of CAT enzymatic activity also display a daily rhythm in the rat hippocampus ($F(3,8)=9.94$ $p\leq 0.01$; Chronos-Fit: $p\leq 0.01$) with its acrophase occurring at ZT 03:14 \pm 01:15 (Figure 2C, Table 2). Interestingly, we observed that CAT mRNA and enzymatic activity also follow diurnal rhythms in the hippocampus of A β -injected rats ($F(3,8)=4.59$ $p\leq 0.05$; Chronos-Fit: $p\leq 0.01$ and ($F(3,8)=10.47$ $p\leq 0.01$; Chronos-Fit: $p\leq 0.001$) with their acrophases occurring at ZT 8:25 \pm 00:56 and ZT 04:53 \pm 00:45, respectively. The i.c.v. injection of A β aggregates did not modify the rhythms' parameters (Figures 2A-C; Table 2).

2.3 Daily rhythms of GPx mRNA expression and enzymatic activity in the hippocampus of A β -injected rats

GPx mRNA expression and activity also follow robust diurnal rhythms in the hippocampus of control rats ($F(3,8)=69.01$ $p\leq 0.01$; Chronos-Fit: $p\leq 0.05$; $F(3,8)=46.8$ $p\leq 0.001$, Chronos-Fit: $p\leq 0.05$) with their acrophases occurring at ZT 02:58 \pm 00:18 and 01:08 \pm 00:04, respectively (Figure 3A-C, Table 3). The i.c.v. injection of A β aggregates reduced the mesor (1.64 \pm 0.00 vs 1.32 \pm 0.04, $t(4)=7.46$, $p\leq 0.01$), increased the amplitude (0.10 \pm 0.00 vs 0.23 \pm 0.04, $t(4)=-3.08$, $p\leq 0.05$) and phase shifted (from ZT 02:58 \pm 00:18 to ZT 06:53 \pm 01:14, $t(4)=-3.46$, $p\leq 0.05$) GPx expression rhythm, in the rat hippocampus (Figure 3A, Table 3). The A β -injected group showed a phase delay in GPx activity rhythm, from ZT 01:08 \pm 00:04 to ZT 22:29 \pm 00:05, $t(4)=-172.6$ $p\leq 0.001$) and an increase in the mesor (0.56 \pm 0.02 vs 0.66 \pm 0.00, $t(4)=-5.68$ $p\leq 0.01$), compared with controls (Figure 3C, Table 3).

2.4 Daily variation of clock genes expression in the rat hippocampus of control and A β -injected rats

Previously, we observed BMAL1 protein levels varies rhythmically throughout a day in the rat hippocampus. The injection of A β aggregates phase shifted BMAL1 oscillating expression without affecting the rhythm's amplitude or mesor parameters (Castro et al., 2019). Additionally, here, we analyzed the daily patterns of other molecular clock components, Rora and Rev-erb β mRNA levels, in the hippocampus of control and A β -injected rats. As expected, Rora and Rev-erb β mRNA expression varies throughout a day in the rat hippocampus with their acrophases occurring at ZT 00:18 \pm 00:28 and 17:07 \pm 00:19, respectively ($F(3,8)=28.37$ $p\leq 0.001$; Chronos-Fit: $p\leq 0.05$; and $F(3,8)=69.07$ $p\leq 0.05$; Chronos-Fit: $p\leq 0.001$)(Figure 4A-C, Table 4). Injection of A β aggregates (1-42) phase shifted daily patterns of Rora mRNA levels (from ZT 00:18 \pm 00:28 to ZT 14:49 \pm 00:27, $t(4)=13.03$ $p\leq 0.01$), decreased the mesor and increased the rhythm's amplitude (1.73 \pm 0.01 vs 1.64 \pm 0.01, $t(4)=6.07$ $p\leq 0.01$ and 0.07 \pm 0.01 vs 0.12 \pm 0.01, $t(4)=-4.48$ $p\leq 0.05$, respectively) in the rat hippocampus (Figure 4A-C, Table 4). The A β -injected group showed a phase delay in Rev-erb β rhythm's, from ZT 17:07 \pm 00:19 to ZT 23:53 \pm 00:19, $t(4)=-15.26$ $p\leq 0.01$) and an increase in the rhythm's mesor and amplitude (1.03 \pm 0.01 vs 1.13 \pm 0.01, $t(4)=-12.24$ $p\leq 0.01$ and 0.08 \pm 0.00 vs 0.14 \pm 0.01, $t(4)=-7.88$ $p\leq 0.01$) compared with controls (Figure 4A-C, Table 4).

3. Discussion

Recent studies have reported that A β (1–42) oligomers can lead to oxidative damage of proteins and other biomolecules (Butterfield and Sultana., 2011). In this study, we observed 24-hour rhythms of oxidative stress and antioxidant related markers as well as of clock gene expression in the rat hippocampus. Interestingly, we found that an intracerebroventricular injection of A β aggregates modifies daily oscillation of protein carbonyls levels and daily rhythms of clock-related genes and has a differential effect on the oscillating CAT and GPx expression and activity.

We and others have observed daily rhythmicity of protein oxidation levels and antioxidant enzymes activity in several mammals' tissues (Baydas et al., 2002; Coto-Montes et al., 2001; Fonzo et al., 2009; Jiménez-Ortega et al., 2010). Recently, we showed rhythms of protein oxidation in the prefrontal cortex of rat during the light phase of the day (Ledezma et al., 2020). Similarly, here, we observed protein carbonyls levels vary throughout a 24h period, peaking at the end of the day in the hippocampus of control rats (Figure 1, Table 1).

In addition, we also found that both CAT and GPx expression and activity exhibit a daily rhythmicity in rat hippocampus. Lowest levels of protein oxidation concurs with the highest GPx and CAT activity in the hippocampus of control rats (Figures 1, 2 and 3; Tables 1, 2, and 3). This observation might indicate a neuroprotective effect of the antioxidant enzymes at the beginning of the day. Thus, knowing that sleep plays a role in the memory consolidation (Rasch and Born., 2013), the highest CAT and GPx activities during the light period, would have a proper timing for protecting hippocampus against oxidative damage, maintaining protein oxidation at lower levels.

It is known, the molecular clock machinery drives the expression of clock-controlled genes by the BMAL1:CLOCK heterodimer binding to E-box enhancers on target promoters (Reppert and Weaver, 2002). Previously, we observed BMAL1 protein expression varies rhythmically throughout a day in the rat hippocampus and that an i.c.v. injection of A β aggregates phase shifts BMAL1 oscillating expression (Castro et al., 2019). It has been demonstrated that ROR α and REVERB transcription factors regulate BMAL1 rhythmic expression and, at the same time, are target of the BMAL1:CLOCK heterodimeric transcription factor, constituting the accessory loop of the mammalian circadian clock, and contributing to keep the proper timing of the clock (Preitner et al., 2002; Emery and Reppert, 2004; Guillaumond et al., 2005). In addition, it has been reported that ROR α or its agonist, cholesterol sulfate, reduce oxidative stress in primary cultures of hepatocytes by inducing the mRNA level of antioxidant enzymes, superoxide dismutase 2 (SOD2) and glutathione peroxidase 1 (GPx1), through the ROR α response elements (RORE) located in the upstream promoters of

Sod2 and Gpx1 (Han et al., 2014); therefore, the analysis of Rora and Rev-erb β expression carried out in this study, contributes, directly and indirectly, to the understanding of the temporal organization of redox state in the hippocampus, in control and A β injected rats.

Recently, we observed BMAL1 protein oscillates throughout a 24h-period, peaking at the middle of the day, in the rat hippocampus (Castro et al., 2019). It is known that transcription factors ROR α and REVERB drive the rhythm of BMAL1, transcription (Guillaumond et al., 2005; Ni et al., 2019; Paganoni et al., 2010), and also it has been reported that ROR α induces the antioxidant, SOD2 and GPx, enzymes expression (Han et al., 2014). In this study, we show that transcript levels of Rora and Rev-erb β exhibit a daily rhythmicity, peaking at the beginning of the day and the middle of the night, respectively, in the rat hippocampus (Figure 4, Table 4). As expected, Rora maximal expression precedes the highest levels of ROR α protein showed previously in Castro et al., (2019). Consistently, ROR α expression precedes BMAL1 rhythm's acrophase and this heads Rev-erb β expression in the hippocampus of the control group. Thus, a well orchestrated temporal organization of the molecular clock is present in the rat hippocampus.

Previous bioinformatic analysis done in our lab revealed the presence of clock-responsive, E-box, sites in regulatory regions of CAT and GPx genes, as well as a ROR responsive site in the GPx regulatory region (Fonzo et al., 2009). In the present study, maximal expression of GPx is in phase with the previously reported BMAL1 protein peak, while maximal levels of CAT transcript occur following BMAL1 protein peak (Figures 2 and 3, Tables 2 and 3). In addition, the higher Rora expression precedes the peak of its molecular target GPx (Figures 3 and 4 and Tables 3 and 4). Thus, our findings would implicate the existence of a synchronization between the clock and the temporal organization of antioxidant defenses in the rat hippocampus.

A growing body of evidence supports a role for oxidative stress in the pathogenesis of AD (Barnham et al., 2004; Fang et al., 2017, Peizhong et al 2011). In addition, it has been shown that the A β -induced neurotoxicity is associated with oxidative stress and cognitive dysfunction observed in patients with AD (Murakami et al., 2005). In this study, our results reported that oxidation protein levels showed a daily oscillation in the hippocampus of A β -injected rats, maintained under 12h-light:12h-dark conditions (Figure 1, Table 1). Interestingly, we found, for the first time in our knowledge, that an i.c.v injection of A β aggregates caused a phase shift and increase the rhythm's amplitude of protein oxidation levels in comparison to controls (Figure 1, Table 1). Previously, we found that an injection of A β aggregates caused a phase shift in daily oscillations of lipoperoxidation levels (acrophase: 19:48 \pm 00:54 vs 10:16 \pm 01:42)

and increased their rhythm's amplitude (Navigatore Fonzo et al., 2017). Our previous results showed that A β proteins display a daily oscillation profile in the hippocampus of A β -injected rats, with maximal A β levels occurring at ZT 07:09 \pm 00:17 (Navigatore Fonzo et al., 2017). As expected, peaks of lipid peroxides and protein oxidation levels occur at the middle and at the end of the night, respectively, following A β expression rhythm's acrophase (ZT 07:09 \pm 00:17) in the hippocampus of A β -injected rats, in the context of reactive homeostasis. Although others have demonstrated that A β leads to lipid peroxidation and protein oxidation in *in vivo* and *in vitro* models of Alzheimer's disease (Butterfield et al., 1999; Yatin et al. 1999; Pratico et al. 2001), this would be, at least at our knowledge, the first published report on the effects of an i.c.v injection of aggregated A β (1-42) on the daily rhythmicity of protein oxidation.

In this study, we observed that A β aggregates exerted differential effects on the daily expression and activity of CAT and GPx. On one hand, A β aggregates have no significant effect on the temporal patterns of CAT expression and activity (Figure 2, Table 2). On the other hand, an i.c.v injection of aggregated A β (1-42) phase shifted the daily pattern of GPx mRNA and activity as well as decreased the rhythm's mesor of GPx expression, at transcript levels, in the rat hippocampus (Figure 3, Table 3). Similarly, we have observed changes in the mesor and phase of CAT and GPx rhythms, at protein levels, in the same animal model (Castro et al., 2019). Although other authors have demonstrated the effects of A β peptide on antioxidant enzymes activity in vivo models of Alzheimer's disease (Cioanca et al., 2013, Lin et al., 2019; Nisha et al., 2017), this would be, at least at our knowledge, the first published report on the effects of an i.c.v injection of aggregated A β (1-42) on the daily rhythmicity of CAT and GPx activity and its putative impact on temporal patterns of antioxidant defenses in the hippocampus.

Changes observed in the BMAL1 protein rhythm in the A β -injected animals (Castro et al., 2019), led us to continue studying the consequences of an i.c.v injection of aggregated A β (1-42) on daily rhythms of BMAL1 transcriptional regulators, ROR α and/or REVERB. Interestingly, we found, that A β peptide aggregates modify daily rhythmicity of ROR α and Rev-erb β mRNA in the rat hippocampus. Particularly, A β aggregates delayed both the ROR α and the Rev-erb β rhythms' acrophases (Figure 4, Table 4). Thus, the changes observed in the expression of BMAL1 and its target genes (CAT and GPx, for example) in the A β -injected rats could be a consequence of changes in the daily profiles of their regulatory factors, ROR α and Rev-erb β . Noteworthy, Boukhtouche and collaborators (2006) demonstrated that the neuroprotective effect of ROR α is predominantly mediated by GPx1 and peroxiredoxin 6. These results suggest a new role for ROR α in the control of neuronal oxidative

stress and thus, it represents a transcription factor of interest in the regulation of reactive oxygen species-induced neurodegenerative processes during ageing.

In conclusion, the results presented here show that antioxidant enzymes expression and activity is in phase with daily patterns of key molecular clock factors, however, such temporal organization would be affected by A β aggregates. An i.c.v. injection of A β aggregates changed the phase of Rora and Rev-erbb rhythmic expression in the rat hippocampus. These alterations in the daily rhythmicity of BMAL1 regulatory factors might be responsible for changes in the temporal patterns of the clock activating factor and its target genes (CAT and GPx) in this brain area.

Thus, we propose that alterations of daily rhythms of the molecular clock and, consequently, of temporal patterns of antioxidant enzymes, might be crucial signs in the pathogeny of AD.

4. Experimental Procedure

4.1 Rats and agents

Male Holtzman rats bred in our animal facility (National University of San Luis, Argentina) and maintained in a 21–23°C controlled environment with a 12h-light:12h-dark cycle, with ad libitum access to food and water. Four-month old rats were randomly assigned to the groups: control (CO) and A β -injected (A β) (n=12/group). First group received an intracerebroventricular (i.c.v) injection of 10 μ L of sterile saline solution, and the second group received an i.c.v injection of 10 μ L of aggregated A β (1-42). Lyophilized synthetic A β (1–42) which was purchased from Sigma-Aldrich (St Louis, MO, USA) was dissolved in sterile saline solution (final concentration, 2 g/L) and incubated at 37°C for 1 week in order to induce aggregation (Pike et al., 1993).

4.2 A β 1–42-Induced AD Rat Model

The rats were anesthetized with an intraperitoneal injection of 0.2 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine(10 mg/kg). They were then stereotaxically injected into the lateral ventricles (at coordinates AP:-1 mm, L: 1.5 mm, and DV: -3.5 mm according to Paxinos and Watson, 1997). Each injection was performed over 5 min and following injection, the needle remained in the target location for 10 min to avoid A β 1–42 reflux along the needle tract. Seven days after surgery, three rats from each group were sacrificed at different time points throughout a 24-h period. Those time points are referred to as zeitgeber (from German zeit: time and geber: giver) times (ZT) ZT2, ZT8, ZT14 and ZT20 (with ZT0 when light is on). Rats were killed under dim red light at ZT14 and ZT20 to avoid acute effects of light.

Hippocampi were removed on an ice chilled plate (Babu et al. 2011) and immediately placed in liquid nitrogen.

Experiments were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), and with approval from the Animal Subjects Committee at San Luis University (approved protocol No B-263/18). All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.3 Measurement of protein oxidation

As a marker of protein oxidation, protein carbonyls were determined as previously reported by using an enzyme-linked immune-sorbent assay (ELISA) (Winterbourn and Buss, 1999) with some modifications. Briefly, tissue homogenates (n=12/ group) were derivatized to 2,4-dinitrophenylhydrazone by reaction of carbonyl groups in oxidized proteins with 2,4-dinitrophenylhydrazine in 2 M HCl. Ten microliters of the derivatized or non-derivatized sample were added to 190 μ l of 0.1 M bicarbonate buffer, pH 9.6, in clear 96-well microplates (Corning Incorporated, Corning, NY) and incubated overnight at 4°C. After washing with 0.05% Tween 20 in PBS and blocking with 2.5% cold-water fish skin gelatin (Sigma) in PBS at 37 °C for 1 h, the microplates were incubated for 1 h at 37° C with the rabbit polyclonal anti-dinitrophenyl antibody (1:2000 dilution in washing buffer). The immunocomplexes were quantified using a goat anti-rabbit IgG-HRP conjugate (1/10,000 in washing buffer), and the oxidation of the HRP substrate tetramethylbenzidine was read at 450 nm using a TECAN microplate reader (Infinite M200 PRO, Research Triangle Park, NC). The results are shown as nmoles of carbonyl per milligram of total proteins (nmol/mg of prot).

4.4 mRNA isolation and RT-PCR

RNA isolation and RT reaction techniques were carried out as described in Navigatore-Fonzo et al. (2014). Briefly, total RNA was extracted from hippocampus samples by using the Trizol reagent (Invitrogen Co) as directed by the manufacturers. Gel electrophoresis and Gel-Red staining confirmed the integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse-transcribed with 200 units of MMLV Reverse Transcriptase (Promega) using random hexamers, in a 25- μ l reaction mixture and following manufacturer's instructions. Transcript levels of Cat, Gpx, Rora and Rev-erb β were determined by RT-PCR and normalized to 28S expression as endogenous control. Fragments coding for those genes were amplified by PCR in 50 μ l of reaction solution containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 1.25 U of Taq polymerase, 25 pmol

of each rat specific oligonucleotide primer and RT generated cDNA (1/5 of RT reaction). The sequences of the specific primers used were: for Cat forward primer 5'-CGACCGAGGGATTCCAGATG-3' and reverse primer 5'-ATCCGGGTCTTCCTGTGCAA-3' (fragment size 174 bp), for Gpx forward primer 5'-CGGTTTCCCGTGCAATCAGTT-3' and reverse primer 5'-ACACCGGGGACCAAATGATG-3' (fragment size 225 bp), for Rora, forward primer 5'-GAGACAAATCGTCAGGAATCCAT-3' and reverse primer 5'-CCACAGCCAGGCACTTCTG-3' (fragment size 180 bp), and for Rev-erb β , forward primer 5'-GGTGCCTAGAATCCTGATTGTGA-3' and reverse primer 5'-TCCGCTGGAGCCAATGTAG-3' (fragment size 200 bp). In the case of antioxidant enzymes expression, samples were heated in a thermalcycler (My Cycler, BioRad, Hercules, CA, USA) to 94°C for 2 minutes, followed by 32 cycles of (1) denaturation, 94°C for 1 minute; (2) annealing, 59°C during 1 minute; and (3) extension, 72°C for 1 minute. After 32 reaction cycles, the extension reaction was continued for another 5 minutes. For Rora and Rev-erb β genes the thermalcycling conditions were similar but following 40 cycles of denaturation-annealing-extension. PCR products were then electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) Gel-Red. The amplified fragments were visualized under ultraviolet (UV) transillumination and photographed using a Cannon PowerShot A75 3.2MP digital camera. The mean of gray value for each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from <http://rsb.info.nih.gov/ij/>) and the relative abundance of each band was normalized according to the housekeeping 28S gene, calculated as the ratio of the mean of gray value of each product to that of 28S.

4.5 Measurement of antioxidant enzyme activities

Tissue samples extracted from control and A β injected rats at every time point (ZT2, ZT8, ZT14, and ZT20), were homogenized in 1/5 (w/v) dilution in 120 mM KCl and 30 Mm phosphate buffer, pH 7.2 at 4°C. Suspensions were centrifuged at 800 \times g for 10 minutes at 4°C to remove nuclei and cell debris. The pellets were discarded and supernatants were used to determine antioxidant enzyme activities. CAT and GPX activity was determined according to the methods of Aebi (1984) and Flohé and Günzler (1984), respectively. Briefly, 1 ml of supernatant was added to 33 μ l of H₂O₂ and the decrease of the absorbance at 220 nm was registered every 5 seconds during a total time of 30 seconds. During this time the decomposition of the H₂O₂ follows a first order reaction kinetic. One CAT unit is defined as the amount of enzyme required to decompose 1 μ M of H₂O₂/min. GPx activity was determined following NADPH oxidation

at 340 nm in an reaction medium containing 0.2 mM GSH, 0.25 U/ml yeast glutathione reductase, 0.5 mMtert-butyl hydroperoxide and 50mM phosphate buffer (pH 7.2). Total protein concentration was measured by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard. All reagents were from Sigma-Aldrich (St Louis, MO, USA).

4.6 Statistical Analysis

Time point data were expressed as mean \pm standard error of the mean (SEM) and pertinent curves were drawn. Previous time series were computed by one-way analysis of variance, analyses of normality and variance homogeneity were performed using Shapiro-Wilk and Levene tests, respectively. P values ≥ 0.01 indicated our data were consistent with a normal distribution and equal variances. Time series were computed first by one-way analysis of variance followed by Tukey post hoc test for specific comparisons; a $p < 0.05$ was considered to be significant. The daily rhythm was assessed by the Chronos-Fit program, using a combination of a partial Fourier analysis and a stepwise regression technique, with a single fundamental period set to 24 h (Zuther et al., 1996). The Chronos-Fit, is a specialized, comprehensive program to analyze 24 h rhythm data from humans and animals. The following parameters were calculated for each of the fitted curves: mesor (24-hourrhythm-adjusted mean), amplitude and acrophase (measures of the extent andtiming of predictable change within a cycle). The percentage of rhythm (%rhythm, an index of the amount of variance accounted for) of the fitted curve, and the significance of rhythmicity, testing the null hypothesis of the amplitude being equal to zero, was performed using an F test (>3.5 ; $p < 0.05$). A cosine-fitted curve was generated with GraphPad Prism® 5.0 software (CA, USA). Student's t-test was used for comparison of mesor, amplitude or acrophase between control and A β experimental groups, with $p \leq 0.05$ for significant differences. Previous Student's t-tests, analyses of normality and variance homogeneity were performed using Shapiro-Wilk and Levene tests, respectively. P values ≥ 0.01 indicated our data were consistent with a normal distribution and equal variances.

Figure legends

Figure 1. Daily patterns of protein oxidation levels in the hippocampus of A β -injected rats Each value on the curves represents the mean \pm SEM of $n=3$ samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24 h (ZT0-ZT24) photoperiod. Statistical analyses were performed using one-way ANOVA for each time series data followed by Tukey post-hoc test with $*p \leq 0.05$ and

** $p \leq 0.01$ when indicated means were compared to the corresponding maximal value in each group. Rhythms were detected by the Chronos-Fit method (Zuther et al., 2009).

Figure 2. Temporal expression and daily activity of CAT in the hippocampus of A β -injected rats. Daily rhythms of A) mRNA CAT levels C) CAT activity. Each point on the graphs represents the mean \pm SEM of $n=3$ hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility. B) Representative patterns of PCR products at different ZTs throughout a day-night cycle. Statistical analyses were performed using one-way ANOVA for each time series data followed by Tukey post-hoc test with $*p \leq 0.05$ and $**p \leq 0.01$ when indicated means were compared to the corresponding maximal value in each group. Rhythms were detected by the Chronos-Fit method (Zuther et al., 2009).

Figure 3. Daily rhythms of GPx mRNA expression and enzymatic activity in the hippocampus of A β -injected rats Daily rhythms of A) mRNA GPx levels C) GPx activity. Each point on the graphs represents the mean \pm SEM of $n=3$ hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility. B) Representative patterns of PCR products at different ZTs throughout a day night cycle. Statistical analyses were performed using one-way ANOVA for each time series data followed by Tukey post-hoc test with $***p \leq 0.001$ when indicated means were compared to the corresponding maximal value in each group. Rhythms were detected by the Chronos-Fit method (Zuther et al., 2009).

Figure 4 Daily variation of clock genes expression in the rat hippocampus of control and A β -injected rats A-C) Each value on the curves represents the mean \pm SEM of $n=3$ hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24 h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility. B-D) Representative patterns of PCR products at different ZTs throughout a daynight cycle. Statistical analyses were performed using one-way ANOVA for each time series data followed by Tukey post-hoc test with $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ when indicated means were compared to the corresponding maximal value in each group. Rhythms were detected by the Chronos-Fit method (Zuther et al., 2009).

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Declaration of competing interest

There is no conflict of interest associated with the study or any of the authors.

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Table 1 Rhythms' parameters of protein oxidation levels in hippocampus samples of control and A β -injected groups

PROTEIN OXIDATION LEVELS			
Rhythm Parameters	Control group (mean \pm SEM)	A β injected group (mean \pm SEM)	p
MESOR	5.42 \pm 0.48	7.27 \pm 1.07	N/S
AMPLITUDE	1.47 \pm 0.17	4.90 \pm 1.34	< 0.05
ACROPHASE	10:51 \pm 00:33	22:48 \pm 00:05	< 0.001

Note: Data are presented as mean \pm SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. A β -injected groups comparison using Student's t-test.

N/S = not significant

Protein oxidation levels (% rhythm Control group: 48.84 A β -injected group: 62.32)

F test values (Control group: 6.21 A β -injected group: 10.75)

Table 2 Rhythms' parameters of daily CAT oscillating expression and activity in the hippocampus of control and A β -injected groups

CAT mRNA

Rhythm Parameters	Control group (mean±SEM)	A β injected group (mean±SEM)	p
MESOR	1.42 ± 0.06	1.17 ± 0.09	N/S
AMPLITUDE	0.28 ± 0.04	0.31 ± 0.08	N/S
ACROPHASE	08:29 ± 00:36	08:25 ± 00:56	N/S

CAT activity			
Rhythm Parameters	Control group (mean±SEM)	A β injected group (mean±SEM)	p
MESOR	0.52 ± 0.01	0.56 ± 0.03	N/S
AMPLITUDE	0.21 ± 0.01	0.25 ± 0.04	N/S
ACROPHASE	03:14 ± 01:15	04:53 ± 00:45	N/S

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. A β -injected groups comparison using Student's t-test.

N/S = not significant

CAT mRNA levels (% rhythm Control group: 59.06 A β -injected group: 62.14)

F test values (Control group: 6.49 A β -injected group: 7.39)

CAT activity (% rhythm Control group: 67.46 A β -injected group: 78.46)

F test values (Control group: 9.33 A β -injected group: 16.39)

Table 3 Rhythms' parameters of daily GPx oscillating expression and activity in the hippocampus of control and A β -injected groups

GPx mRNA			
Rhythm Parameters	Control group (mean±SEM)	Aβ injected group (mean±SEM)	p
MESOR	1.64 ± 0.00	1.32 ± 0.04	< 0.01
AMPLITUDE	0.10 ± 0.00	0.23 ± 0.04	< 0.05
ACROPHASE	02:58 ± 00:18	06:53 ± 01:14	< 0.05
GPx activity			
Rhythm Parameters	Control group (mean±SEM)	Aβ injected group (mean±SEM)	p
MESOR	0.56 ± 0.02	0.66 ± 0.00	< 0.01
AMPLITUDE	0.47 ± 0.00	0.43 ± 0.06	N/S
ACROPHASE	01:08 ± 00:04	22:29 ± 00:05	< 0.001

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. Aβ-injected groups comparison using Student's t-test.

N/S = not significant

GPx mRNA levels (% rhythm Control group: 53.71 Aβ-injected group: 56.05)

F test values (Control group: 5.22 Aβ-injected group: 5.74)

GPx activity (% rhythm Control group: 72.44 Aβ-injected group: 94.40)

F test values (Control group: 10.51 Aβ-injected group: 67.37)

Table 4 Rhythms' parameters of daily *Rora* and *Rev-erb β* oscillating expression in the hippocampus of control and A β -injected groups

Rora mRNA			
Rhythm Parameters	Control group (mean\pmSEM)	Aβ injected group (mean\pmSEM)	p
MESOR	1.73 \pm 0.01	1.64 \pm 0.01	< 0.01
AMPLITUDE	0.07 \pm 0.01	0.12 \pm 0.01	< 0.05
ACROPHASE	00:18 \pm 00:28	14:49 \pm 00:27	< 0.01
Rev-erbβ mRNA			
Rhythm Parameters	Control group (mean\pmSEM)	Aβ injected group (mean\pmSEM)	p
MESOR	1.03 \pm 0.01	1.13 \pm 0.01	< 0.001
AMPLITUDE	0.08 \pm 0.00	0.14 \pm 0.01	< 0.01
ACROPHASE	17:07 \pm 00:19	23:53 \pm 00:19	< 0.01

Note: Data are presented as mean \pm SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. A β -injected groups comparison using Student's t-test.

N/S = not significant

Rora mRNA levels (% rhythm Control group: 61.78, A β -injected group: 90.75)

F test values (Control group: 6.20, A β -injected group: 39.65)

Rev-erb β mRNA levels (% rhythm Control group: 95.96, A β -injected group: 94.82)

F test values (Control group: 106.99, A β -injected group: 51.29)

Figure 1

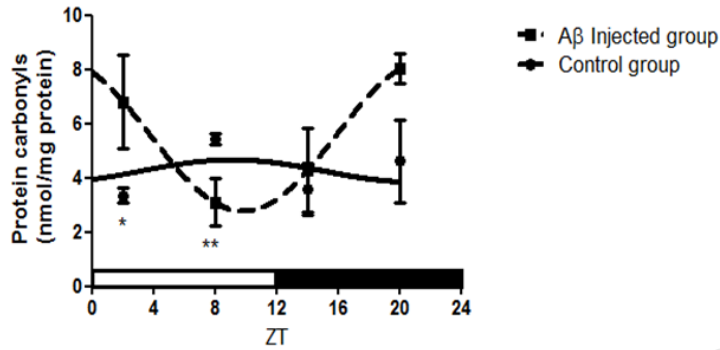


Figure 2

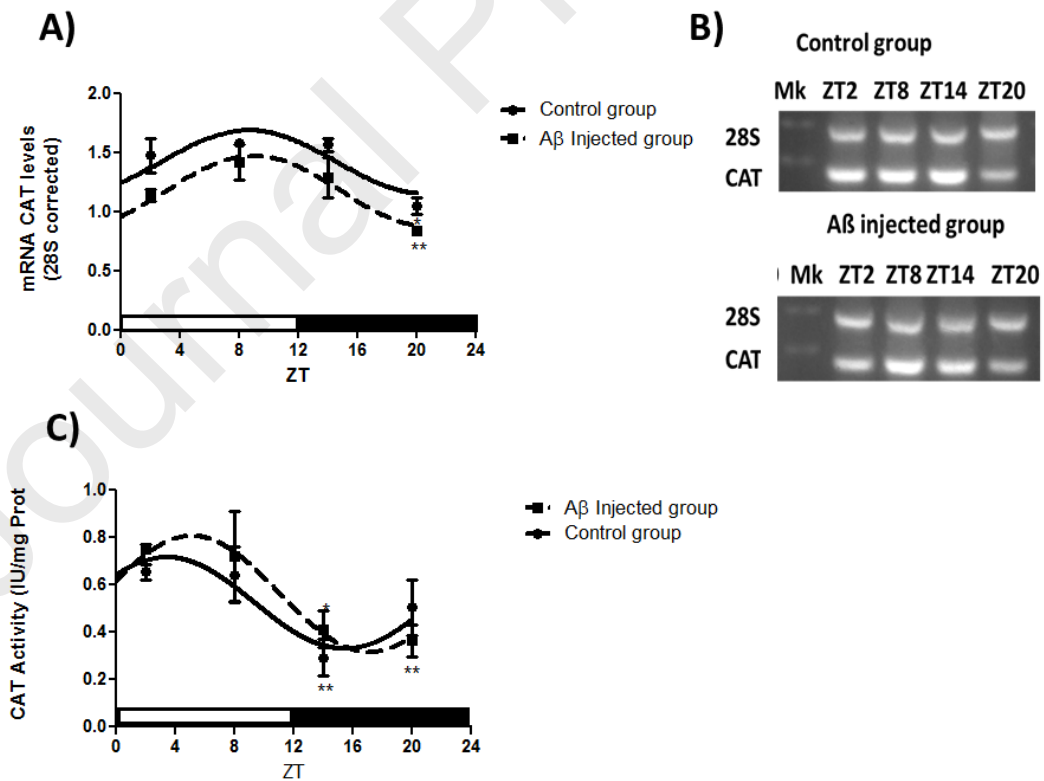


Figure 3

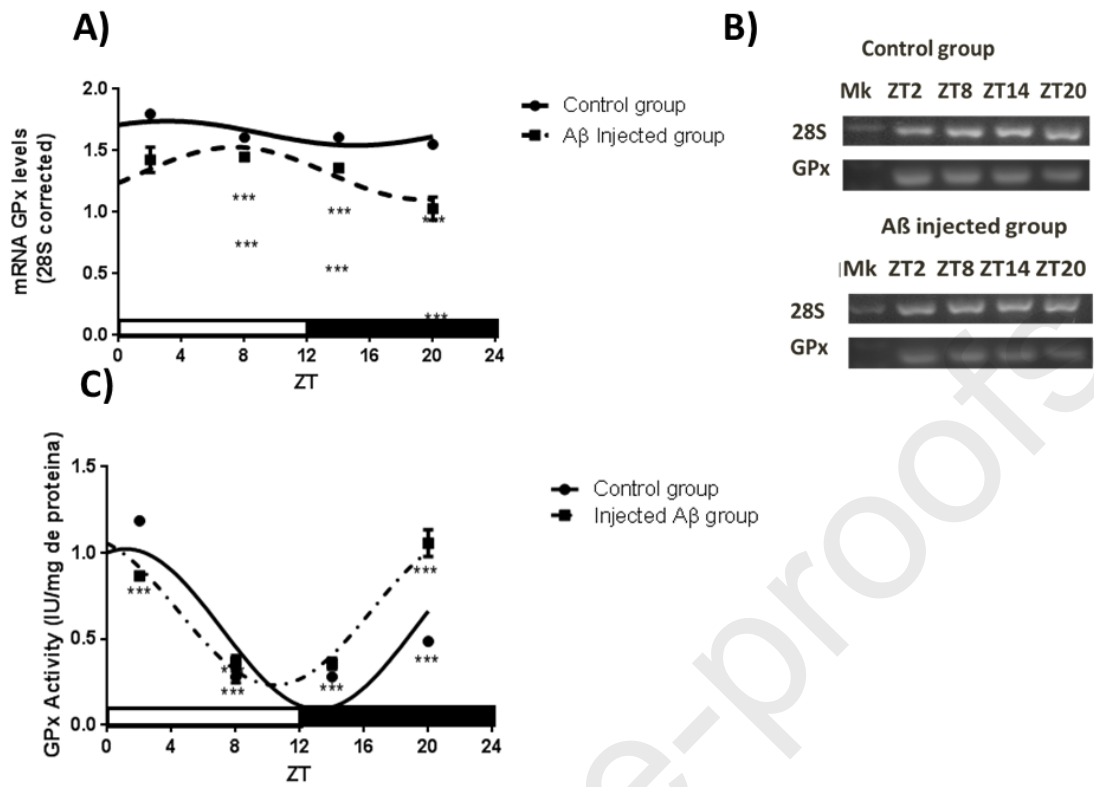


Figure 4

