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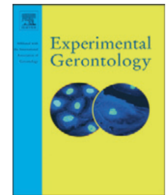

Aging modifies daily variation of antioxidant enzymes and oxidative status in the hippocampus

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ABSTRACT

Background: Aging is a complex and multifactorial biological process that leads to the progressive deterioration of physiological systems, including the circadian system. In addition, oxidative stress has been associated with the aging of the normal brain and the development of late-onset neurodegenerative diseases. Even though, functional weakening of circadian rhythms and antioxidant function has been observed during aging, the mechanisms by which the circadian system signaling and oxidative stress are interrelated have not yet been elucidated. The objectives of this study were to evaluate the consequences of aging on the temporal organization of the antioxidant defense system and oxidative status as well as to analyze the endogenous clock activity, in the hippocampus of aged rats.

Methods: Young adults (3-month-old) or older (22-month-old) male Holtzman rats were maintained under constant darkness conditions, during 15 days before the sacrifice. Levels of catalase (CAT) and glutathione peroxidase (GPx) mRNA and activity, reduced glutathione (GSH), lipoperoxidation (LPO) and BMAL1 protein were analyzed in hippocampus samples isolated every 4 h during a 24-h period. Locomotor activity was recorded during 20 days before the experiment.

Results: Our results show that aging modifies temporal patterns of CAT and GPx expression and activity in the hippocampus in a different way. On the one hand, it abolishes the oscillating CAT expression and specific enzymatic activity while, on the other, it increases the mesor of circadian GPx activity rhythm ($p < 0.01$). Additionally, we observed increased GSH ($p < 0.05$) and reduced LPO ($p < 0.01$) levels in the hippocampus of aged rats. Moreover, the nocturnal locomotor activity was reduced in the older animals in comparison to the young adult rats ($p < 0.01$). Interestingly, the 22 month-old animals became arrhythmic and showed a marked fragmentation as well as a significant decline in daily locomotor activity when they were maintained under constant darkness conditions ($p < 0.05$). Aging also abolished circadian rhythms of the core clock BMAL1 protein.

Conclusion: The loss of temporal organization of the antioxidant enzymes activity, the oxidative status and the cellular clock machinery could result in a temporally altered antioxidant defense system in the aging brain. Learning about how aging affects the circadian system and the expression of genes involved in the antioxidant defense system could contribute to the design of new strategies to improve the quality of life of older people and also to promote a healthy aging.

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1. Introduction

Aging is a complex and multifactorial biological process that leads to the progressive deterioration of organisms. Normal aging is often associated with cognitive decline, and the hippocampus is particularly vulnerable to it (Shankar, 2010).

Several studies over the years have associated the oxidative stress with the aging of the normal brain and the development of late-onset neurodegenerative diseases, such as Alzheimer and Parkinson's diseases (Finkel and Holbrook, 2000; Balaban et al., 2005; Wang et al., 2010; Dias

Abbreviations: CAT, catalase; GPx, glutathione peroxidase; GSH, reduced glutathione; LPO, lipid peroxidation; SCN, suprachiasmatic nucleus; TBARS, thiobarbituric acid reactive substances; ROS, reactive oxygen species.

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et al., 2013; Zhao and Zhao, 2013). Oxidative stress is generated by a combination of increased production of free radicals and oxidant agents with decreased antioxidant levels and dysregulation of the antioxidant defense system (Wang et al., 2010; Gilca et al., 2011; Dias et al., 2013; Zhao and Zhao, 2013). The main free radical is the superoxide anion, which is converted to hydrogen peroxide by the superoxide dismutase (SOD) enzyme. The hydrogen peroxide is then decomposed into oxygen and water by catalase (CAT) and glutathione peroxidase (GPx) enzymes, which are part of the cellular antioxidant defense system (Balaban et al., 2005). Furthermore, GPx also reduces lipid peroxides; in both cases GPx activity depends on the levels of reduced glutathione (GSH), since the reduction of hydrogen and lipid peroxides is coupled to the oxidation of this compound. GSH is the most abundant endogenous antioxidant in cells and besides its participation as substrate in enzymatic reactions, it exerts a powerful antioxidant effect by itself in the elimination of free radicals; therefore, this metabolite is particularly important in the regulation of the cellular redox state and protection against oxidative stress (Wu et al., 2004; Lu, 2009). Several studies have demonstrated that the activity of antioxidant enzymes decreases with the age in rat (Tsay et al., 2000; Cao et al., 2004; Rodrigues Siqueira et al., 2005; Wang et al., 2010). Additionally, the intracellular GSH concentration was also found to decrease with the age in the rat brain (Suh et al., 2004; Suh et al., 2005). Given the brain is rich in polyunsaturated fatty acids, it is highly susceptible to lipid peroxidation (LPO). It has been reported the levels of LPO increase with aging in several organs, including the brain (Radak et al., 2011). Particularly, and as mentioned before, GSH and GPx are especially important protecting lipids against oxidative stress (Radak et al., 2011). Investigations revealed an age-associated decrease in the activity of CAT, SOD and GPx as well as the GSH level, along with an increase in the level of lipid peroxidation during aging and Alzheimer's disease in the brain (Haddadi et al., 2014; Casado et al., 2008).

Previously, we and others have reported a well-orchestrated temporal expression and activity of the antioxidant defense system in different tissues, such as liver and brain (Pablos et al., 1998; Baydas et al., 2002; Fonzo et al., 2009; Ponce et al., 2012; Navigatore-Fonzo et al., 2014). It has been demonstrated that most living organisms have a circadian system that synchronizes internal events to the environmental time. In mammals, the circadian system has a hierarchic architecture. It is constituted by a master clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, which synchronizes several peripheral and subordinated clocks in the rest of the body, through a wide variety of mechanisms (Mendoza and Challet, 2009; Albrecht, 2012; Bollinger and Schibler, 2014). Thus, the SCN regulates the circadian rhythmicity of peripheral clocks by a direct way, through neural and humoral signals, as well as through an indirect way, by controlling the daily activity/rest patterns and, consequently, the body temperature and food cycle signalling (Dibner et al., 2010; Buhr and Takahashi, 2013). The persistence of rhythms when an individual is isolated from environmental cues, for example light for mammals, and kept under constant darkness conditions, is indicative of the endogenous clock control (Wollnik, 1989; Golombek and Rosenstein, 2010). The molecular clock machinery comprises: 1- transcriptional-translational feedback loops that encompass interconnected positive and negative mechanisms, 2- oscillating post-translational modifications, and 3 -epigenetic changes. In the positive loop of the mammalian cellular clock, the transcriptional activator protein, BMAL1 (from *Brain and Muscle ARNT Like protein 1*) dimerizes with CLOCK (*Circadian Locomoter Output Cycles Kaput protein*) and binds to E-box (CANNTG) promoter sequences to activate the transcription of other clock and clock-controlled genes (Reppert and Weaver, 2002; Buhr and Takahashi, 2013). Thus, the BMAL1:CLOCK heterodimer drives the transcription of three clock *Period* (Per1, Per2, and Per3), two *Cryptochromes* (Cry1 and Cry2) and other clock and clock-controlled genes. As Per and Cry mRNAs are translated and proteins accumulate in the cytoplasm, they form PER-CRY heterodimers, which, once phosphorylated, translocate into the nucleus to negatively interfere with BMAL1:CLOCK-dependent transcription (Mendoza and Challet, 2009; Reppert and Weaver, 2002; Buhr and Takahashi, 2013). It has been shown in *in vitro* models, that the reduced forms of the redox NADH and NADPH cofactors, strongly stimulates the binding of BMAL1:CLOCK heterodimers to the E-box sites in the promoter of the target genes, whereas the oxidized forms thereof (NAD^+ and $NADP^+$) inhibit it (Rutter et al., 2001). These observations suggest the possibility that oxidative stress and cellular redox imbalance observed in the senescence may have some effects on the activity of the circadian clock, and its target gene expression, probably, by modulating the binding activity of BMAL1:CLOCK to the DNA. This might constitute the biochemical and molecular basis and probably explain the altered temporal organization of behavioral and physiological parameters in older individuals.

To date, at least in our knowledge, there is no report on the aging consequences on circadian patterns of the antioxidant system in a peripheral clock such as the hippocampus. Taking into account above background information, the objectives of this study were: 1) to evaluate the consequences of aging on the temporal organization of the antioxidant defense system and the oxidative status in the aged hippocampus and 2) to analyze the endogenous clock activity in the same brain area of aged rats.

2. Materials and methods

2.1. Animal model

Male Holtzman rats bred in our animal facilities (LABIR, National University of San Luis, San Luis, Argentina), were weaned at 21-day old and immediately assigned randomly to each group: young adults (3-month-old, $n = 24$) or older rats (22-month-old, $n = 24$). Animals were maintained in a 21–23 °C controlled environment, with ad libitum access to food and water and under a 12 h-light:12 h-dark (LD) cycle (lights on at 07:00 a.m.). In order to analyze the endogenously-driven circadian rhythms, each group of animals was maintained under constant darkness (12 h-dark:12 h-dark, DD) condition during fifteen days before the experiment. After the DD period, four rats from each group were euthanized every 4 h during a 24-h period, at the circadian times (CT): CT0, CT4, CT8, CT12, CT16 and CT20 (with CT0 at the beginning of the subjective day). Rats were killed under dim red light to avoid acute effects of light. All experiments were repeated at least twice. They were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and the National University of San Luis Committee's Guidelines for the Care and Use of Experimental Animals (Protocol N° B-83/14 approved by Res. RD-2-1782/15).

2.2. Daily locomotor activity analysis

Locomotor activity of individually housed young adults and older rats was recorded during 5 days in LD, followed by 15 days in DD, using the ArChron® Data Acquisition System (Simonetta System, National University of Quilmes, Buenos Aires, Argentina). Activity counts were sampled and stored on a computer hard disk using time frames of 5 min. Data from an ASCII files were graphed in double-plotted actograms at modulo 24 h.

2.3. Hippocampus dissection

Hippocampus samples were isolated every 4 h starting at CT0, from young adults and older rats groups. Hippocampi isolation was carried out as described in Babu et al. (2011). Briefly, following animal decapitation, the head was recovered and the skull was opened with sterile scissors. Brain was carefully removed, quickly washed in ice-cold sterile saline solution and put on an ice-chilled plate. Immediately, it was cut along the longitudinal fissure to divide both hemispheres. The diencephalon was removed with sterile microsurgical forceps and scissors

and the exposed hippocampus was resected from the neocortex, immediately frozen under liquid nitrogen and stored under −80 °C.

2.4. Western blot

Protein extracts were prepared in 30 mM phosphate buffer, pH 7.4, with 120 mM KCl, from hippocampi obtained from each group of rats at the different CTs during a 24-h period. Aliquots containing 25 μg of total protein were subjected to electrophoresis on a SDS-polyacrylamide 15% gel. Resolved proteins were transferred to a PVDF transfer membrane (Thermo Scientific, Waltham, MA) by electroblotting. Briefly, membranes were blocked overnight at 4 °C in a blocking solution with 3% non-fat dry milk dissolved in TBS (10 mM Tris–HCl, pH 7.3, with 150 mM NaCl). The membranes were next incubated overnight at 4 °C with either rabbit monoclonal anti-BMAL1 (1:10,000, EPR8355(2)-ab140646, Abcam, Cambridge, MA) or polyclonal anti-ACTIN (1:3,000, I-19 sc-1616-R, Santa Cruz Biotechnology, Santa Cruz, CA) antibodies in TBS containing 0.05% Tween 20 (TBST). After incubation with primary antibody, the membranes were washed four times with TBST, before incubation with peroxidase-goat anti-rabbit IgG (Invitrogen, Thermo Scientific, Waltham, MA) diluted 1:7,000 in TBST for 2 h at room temperature. After washing, antibody/protein complexes on the membranes were detected using the Bio-Lumina detection system as directed by the manufacturer (Kalium Technologies, Buenos Aires, Argentina). The membranes were then covered with a plastic wrap and exposed to X-ray film (CL-X Posure™ Films, Thermo Scientific, Waltham, MA). Film was developed manually using Kodak GBX Developer and Fixer in a dark room. The mean of intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). BMAL1 protein levels were normalized against ACTIN (endogenous control).

2.5. mRNA isolation and RT-PCR

Total RNA was extracted from the hippocampus using the Trizol® reagent (Life Technologies, Carlsbad, CA) as directed by the manufacturers. Agarose gel electrophoresis and GelRed staining (Biotium Inc., Hayward, CA) confirmed the integrity of the samples. Quantification of total RNA was based on spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse-transcribed with 200 units of M-MLV reverse transcriptase (Promega, Madison, WI) using random primer hexamers (Biodynamics, Buenos Aires, Argentina) in a 25-μl reaction mixture and following the manufacturer's instructions.

Transcript levels of CAT and GPx1 were determined by RT-PCR and normalized to 28S as an endogenous control. Fragments coding for those genes were amplified by PCR in a 25 μl reaction mixture containing 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.625 U of Taq polymerase (Productos Biológicos, Buenos Aires, Argentina), 500 nM or 125 nM of CAT (IDT, Coralville, CA) and GPx1 (Invitrogen, Thermo Scientific, Waltham, MA) rat specific oligonucleotide primer, respectively, and RT-generated cDNA (1/30 of RT reaction). The sequences of the specific primers are shown in Table 1.

The samples were heated in a thermalcycler (MultiGene™ Gradient PCR Thermal Cycler, Labnet, Edison, NJ). For CAT cDNA amplification, samples were heated at 94 °C during 2 min followed by 27 cycles of: (1) denaturation, 94 °C for 1 min; (2) annealing, 59 °C for 1 min; (3) extension, 72 °C for 1 min. After 28 reaction cycles, the extension reaction was continued for another 5 min. In the case of GPx1, samples were heated under the following conditions: 94 °C during 2 min, followed by 32 cycles of: (1) denaturation, 94 °C for 1 min; (2) annealing, 61 °C for 1 min; (3) extension, 72 °C for 1 min. After 32 reaction cycles, the extension reaction was continued for another 5 min. PCR products were then electrophoresed on 2.5% (*w*/*v*) agarose gel with 0.004% (*v*/v) GelRed. The amplified fragments were visualized under ultraviolet (UV) transillumination and photographed using a Cannon PowerShot A75 3.2 MP 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.

2.6. Tissue homogenates and enzyme activity assays

The hippocampi extracted from young adults and older rats at the time points CT0, CT4, CT8, CT12, CT16 and CT20, were homogenized in 1/5 (*w*/*v*) dilution of 30 mM phosphate buffer, pH 7.4, with 120 mM KCl, at 4 °C. Suspensions were centrifuged at 3,500 rpm for 10 min at 4 °C to remove nuclei and cell debris. The pellets were discarded and supernatants were used to determine antioxidant enzyme activities. CAT activity was assayed spectrophotometrically according to Aebi (1984). Briefly, decomposition of H_2O_2 was monitored at 240 nm, after the addition of hippocampus homogenate supernatant. Enzymatic activity was expressed as International Units (IU)/mg of protein (1 IU decomposes 1 μmol H_2O_2/min at pH 7, at 25 °C). GPx total activity was measured following NADPH oxidation rate, according to Flohé and Günzler (1984) and expressed as IU/mg of protein (1 IU oxidizes 1 μmol NADPH/min at pH 7.7, at 30 °C).

2.7. GSH levels

GSH levels were determined in acid extracts of hippocampi isolated from young adults and older rats at CT0, CT4, CT8, CT12, CT16 and CT20, following Akerboom and Sies (1981). Briefly, GSH and the 5.5′-dithiobis(2-nitrobenzoic acid) (DTNB) react to generate 2-nitro-5-thiobenzoic acid (TNB), measurable at 412 nm. GSH values were expressed as nmoles/mg of tissue.

2.8. Lipoperoxidation levels

The hippocampi extracted from young adults and older rats at the time points: CT0, CT4, CT8, CT12, CT16 and CT20, were homogenized in 1/10 (*w*/*v*) dilution in 30 mM phosphate buffer, pH 7.4, with 120 mM KCl, at 4 °C. Suspensions were centrifuged at 3,500 rpm for 10 min at 4 °C to remove nuclei and cell debris. The pellets were discarded and supernatants were used to determine the thiobarbituric acid reactive substances (TBARS). Lipid peroxidation was quantified spectrophotometrically by determining MDA levels as TBARS according to Draper and Hadley (1990).

2.9. Statistical analysis

Time point data were expressed as means ± standard errors of the mean (SEM) and pertinent curves were drawn. Time series were computed first by one-way ANOVA followed by Tukey's post-hoc test for specific comparisons; a $p < 0.05$ was considered to be significant.

Table 1
Primer pairs used for RT-PCR.

Gene name	GeneBank Accession N°	Forward primer 5′-3′	Reverse primer 5′-3′	Fragment size
CAT	NM_012520	CGACCGAGGGATTCCAGATG	ATCCGGGTCTTCCTGTGCAA	175 bp
GPx1	NM_030826	CGGTTTCCCGTGCAATCAGTT	ACACCGGGGACCAAATGATG	245 bp
28S	NR_046246	GTGAAAGCGGGCCTCACGATCC	GTACTGAGCAGGATTACCATGGC	284 pb

When mesor, amplitude or phase was required, a fitting technique was applied. Data were fitted by the following function: Baseline + Amplitude*cos (Frequency*X + PhaseShift), where *baseline* is the mesor, *Frequency* is 2π/24, *X* is time in hours, and *PhaseShift* is the phase in hours from CT0. The fitting was performed using Nonlinear Regression from GraphPad Prism 5.0 software (CA, USA). Note that the frequency was taken as the 1 cycle per 24 h of the light regime. Given the classical ANOVA evaluates whether one or more means differ significantly from the others, but it does not confirm circadian rhythmicity, we included chronobiological analysis in order to validate the observed temporal changes as circadian rhythms (Refinetti et al., 2007). Thus, each series was analyzed with the Chronos-Fit 1.06 software (Zuther et al., 2009) and with the Cosinor method (S.E.P.T.M.R, 2000), an inferential statistical method that fits one (or several) cosine curve(s) by least squares to the data, yielding estimates for the mesor (a rhythm-adjusted mean), and for the amplitude and acrophase (measures of the extent and timing of predictable change within a cycle). Based on the residual sum of squares, a *P* value was derived from the zero-amplitude (no rhythm) test and from the computation of confidence intervals of 95% for the parameters (Thaela et al., 1998). A $p \leq 0.05$ or less was taken as indicative of the presence of a rhythm with the 24-h (anticipated) period. Percentage of rhythm is a chronobiological term for the coefficient of determination, i.e. the squared coefficient of correlation times 100 (% rhythm = $r^2 * 100$). It represents the percentage of variation in the data that is explained by the fitted model. Student's *t*-test was used for comparison of mesor, amplitude or acrophase between young adults vs older groups, with $p < 0.05$ for significant differences.

3. Results

3.1. Consequences of aging on circadian rhythms of antioxidant enzymes expression and activity in the rat hippocampus

First, we were aimed to test whether antioxidant enzymes expression and activity follow a circadian pattern in the hippocampus of young adults and older rat. We found CAT mRNA levels oscillate significantly in the absence of light in the young adults rat hippocampus (ANOVA: $p < 0.01$; Chronos-Fit: $p = 0.00006$, % rhythm = 73), peaking at the second half of the subjective day (rhythm's acrophase at CT 10:07 ± 00:23; Fig. 1A and Table 2). Consistently with that, CAT enzymatic activity follows its oscillating expression and varies significantly throughout the day (ANOVA: $p < 0.01$) displaying also a circadian rhythm with its acrophase occurring at CT 11:26 ± 01:00, in the same brain area (Chronos-Fit: $p = 0.003$, % rhythm = 42; Fig. 1C and Table 2).

On the other hand, we observed GPx1 mRNA expression also varies significantly throughout the day (ANOVA: $p < 0.05$) and display a circadian rhythm in the young adults rat hippocampus (Chronos-Fit: $p = 0.008$, % rhythm = 47; Fig. 2A and Table 2), with maximal levels occurring at the first half of the subjective night (rhythm's acrophase at CT 16:13 ± 01:06). We also observed, GPx enzymatic activity oscillate significantly (ANOVA: $p < 0.05$; Chronos-Fit: $p = 0.004$, % rhythm = 51), peaking at the beginning of the subjective night (rhythm's acrophase at CT 12:13 ± 00:53; Fig. 2C and Table 2).

In addition, we did not observe circadian rhythmicity when analyzed superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2) and superoxide dismutase 3 (SOD3) gene expressions in the hippocampus of young adults rats (Supplementary Fig. 1).

Interestingly, the circadian rhythmicity of antioxidant enzymes in the hippocampus was differentially affected by age. On the one hand, aging abolishes the circadian rhythmicity of CAT mRNA expression and enzymatic activity (Chronos-Fit: $p > 0.05$; Fig. 1A–C and Table 2). On the other hand, even though GPx1 mRNA expression varies significantly throughout the day (ANOVA: $p < 0.05$), the data didn't fit to a cosine curve in older rats (Chronos-Fit: $p > 0.05$). Although GPx mRNA levels show no circadian oscillation in older animals, the enzyme activity continues oscillating in a circadian way (ANOVA: $p < 0.05$; Chronos-Fit: $p = 0.005$, % rhythm = 51). It shows an increase in the rhythm's mesor (0.103 ± 0.006 vs. 0.146 ± 0.003, $p < 0.01$) without changes in the rhythm's amplitude nor acrophase parameters in comparison with the young adults rats (Fig. 2A–C and Table 2).

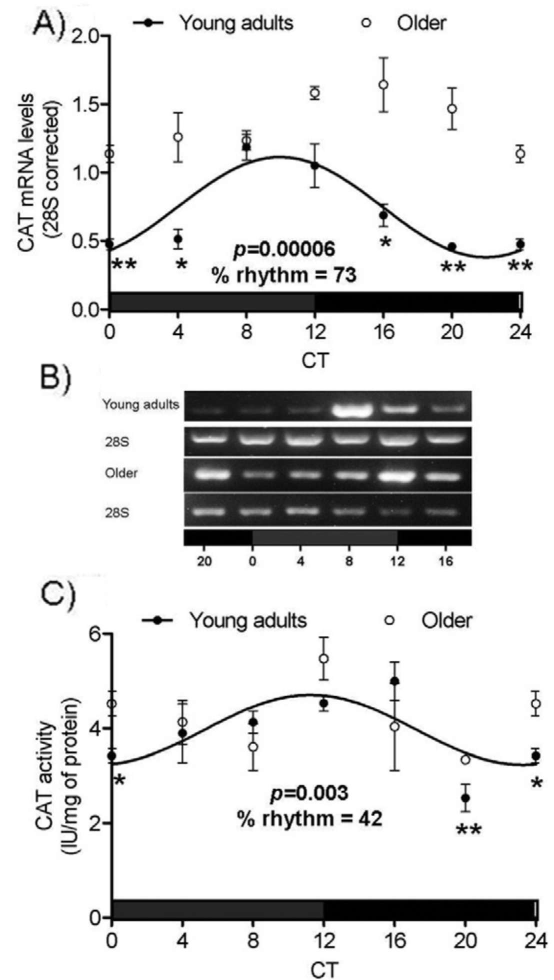

Fig. 1. Circadian rhythms of CAT mRNA expression and enzymatic activity in the hippocampus of young adults and older rat. (A) Cosine fitting curves represent normalized CAT mRNA levels. Each point represents the mean ± SEM of three hippocampus samples at each given CT. B) Representative patterns of PCR products at different CTs in a 24-h cycle. (C) Cosine fitting curves represent CAT enzymatic activity versus CT. Each point represents the mean ± SEM of four hippocampus samples at each given CT. Horizontal bars represent the distribution of dark-dark (DD) phases of a 24-h period (CT0-CT24). Statistical analysis was performed using one-way ANOVA followed by Tukey test with $^{*}p < 0.05$ and $^{**}p < 0.01$ when indicated means were compared to the corresponding maximal value in each group. The *p* and % rhythm in the figure indicates the detection of a rhythm from analysis by the Chronos-Fit method. Values at CT0 were repeated at CT24 on graphs to a better visualization of rhythms.

3.2. GSH levels in the hippocampus of young adults and aged rats

In order to evaluate the effect of aging on the temporal variation of the cellular redox state, we investigated whether GSH levels fluctuate throughout the day in the hippocampus of young and older rats. We found GSH levels do not oscillate rhythmically in the hippocampus of young and older rats (Chronos-Fit: $p > 0.05$, Supplementary Fig. 2). Given that, we decided to group samples and, on the one hand, to analyze the overall variation (including all CTs samples) between young and old groups, while on the other, to evaluate the day/night differences (grouping CT0 + CT4 + CT8 for subjective day, and CT12 + CT16 + CT20 for subjective night) among groups. Thus, we observed aging increases GSH levels (3525 ± 104.2 vs 3793 ± 50.29,

Table 2
Rhythm's parameters of circadian CAT and GPx expression and activity in the hippocampus of young adults and older rats.

Rhythm parameters	Mesor (mean ± SEM)			Amplitude (mean ± SEM)			Acrophase (hh:mm)		
	Young	Older	*p*	Young	Older	*p*	Young	Older	*p*
CAT mRNA	0.73 ± 0.03	N/A	–	0.38 ± 0.07	N/A	–	10:07 ± 00:23	N/A	–
CAT Activity	3.90 ± 0.10	N/A	–	0.91 ± 0.05	N/A	–	11:26 ± 01:00	N/A	–
GPx1 mRNA	0.53 ± 0.00	N/A	–	0.04 ± 0.01	N/A	–	16:13 ± 01:06	N/A	–
GPx Activity	0.10 ± 0.00	0.15 ± 0.00	*< 0.01*	0.06 ± 0.01	0.07 ± 0.00	n/s	12:13 ± 00:53	9:48 ± 00:38	n/s

Note: N/A: it does not apply, since mRNA expression or enzymatic activity became arrhythmic.
p-levels were obtained from the corresponding young adults vs. older group comparisons.
n/s: not significant.

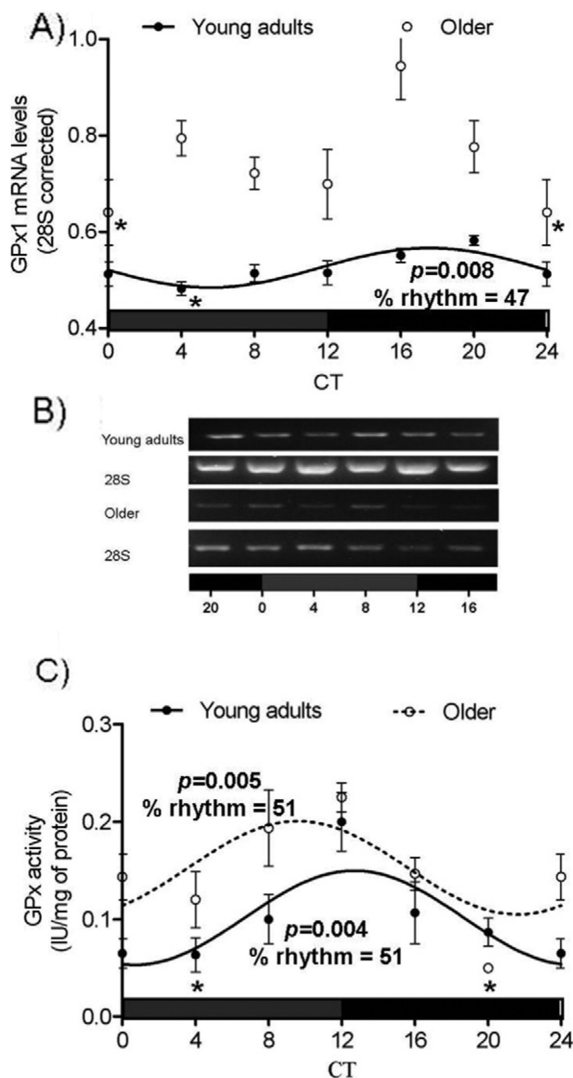

Fig. 2. Circadian rhythms of GPx1 mRNA expression and enzymatic activity in the hippocampus of young adults and older rat. (A) Cosine fitting curves represent normalized GPx1 mRNA levels. Each point represents the mean ± SEM of three hippocampus samples at each given CT. B) Representative patterns of PCR products at different CTs in a 24-h cycle. (C) Cosine fitting curves represent GPx enzymatic activity versus CT. Each point represents the mean ± SEM of three hippocampus samples at each given CT. Horizontal bars represent the distribution of dark-dark (DD) phases of a 24-h period (CT0-CT24). Statistical analysis was performed using one-way ANOVA followed by Tukey test with $^{*}p < 0.05$ when indicated means were compared to the corresponding maximal value in each group. The *p* and % rhythm in the figure indicates the detection of a rhythm from analysis by the Chronos-Fit method. Values at CT0 were repeated at CT24 on graphs to a better visualization of rhythms.

$^{*}p < 0.05$, Fig. 3A), particularly, during the subjective night (3460 ± 160.7 vs 3913 ± 49.88, $^{*}p < 0.05$, young adults vs older group, respectively; Fig. 3B).

3.3. Patterns of lipoperoxidation in the rat hippocampus

Once we had knowledge that aging modified circadian patterns of CAT and GPx expression and activity as well as GSH levels, we continued to test LPO patterns in the hippocampus of rats. We found TBARS levels do not oscillate rhythmically in the hippocampus of young and older rats (Chronos-Fit: $p > 0.05$, Supplementary Fig. 3). Given that, we decided to group samples and, on the one hand, to analyze the overall variation (including all CTs samples) between young and old groups, while

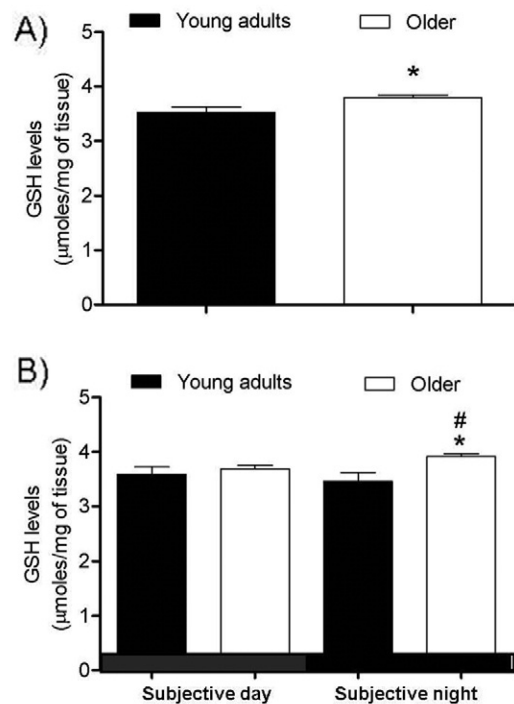

Fig. 3. GSH levels in the hippocampus of young adults and older rats. (A) GSH levels in the hippocampus of young adults and older rats. Each bar represents the mean + SEM of $n = 18$ samples. Student *t*-test was used to evaluate differences between young adults and aged groups with $^{*}p < 0.05$. B) Day/Night variation of GSH levels. Student *t*-test was used to evaluate differences between young adults and aged groups ($n = 9$) at each period, with $^{*}p < 0.05$ when young adults were compared to the older group in the subjective night and $^{\#}p < 0.05$ when GSH levels in the subjective day were compared to those in the subjective night in the hippocampus of older rats.

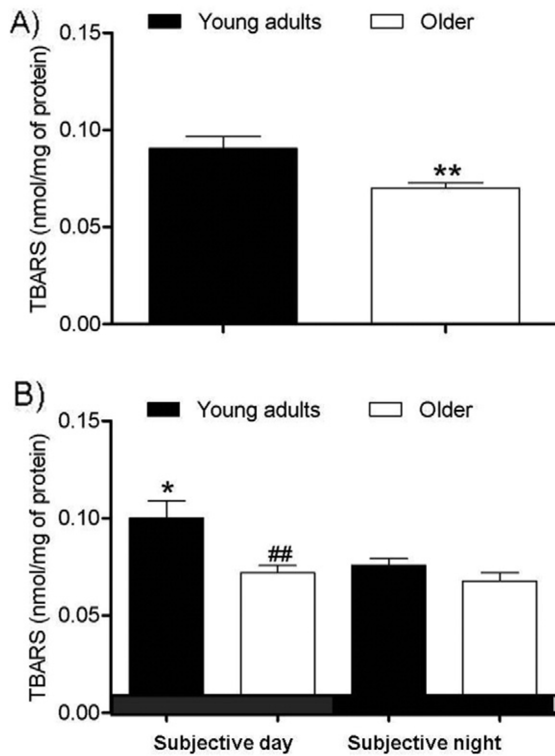

Fig. 4. Daily variation of LPO levels in the hippocampus of young adults and older rats. LPO was measured as thiobarbituric acid reactive substances (TBARS). (A) LPO levels in the hippocampus of young adults and older rats. Each bar represents the mean + SEM of $n = 18$ samples. Student t-test was used to evaluate differences between young adults and aged groups with $^{***}p < 0.001$. B) Day/Night variation of LPO levels. Student t-test was used to evaluate differences between young adults and aged groups ($n = 9$) at each period, with $^{\#\#}p < 0.01$ when older group was compared to young adults at subjective day and $^{*}p < 0.05$ when LPO levels in the subjective day were compared to those in the subjective night in the hippocampus of young adults rats.

on the other, to evaluate the day/night differences (grouping CT0 + CT4 + CT8 for subjective day, and CT12 + CT16 + CT20 for subjective night) among groups. Thus, we observed aging reduces TBARS levels in the hippocampus of older rats (0.090 ± 0.006 vs 0.070 ± 0.003, $p < 0.01$, Fig. 4A) particularly, during the subjective day (0.100 ± 0.008 vs 0.072 ± 0.003, $p < 0.01$; Fig. 4B).

3.4. Circadian locomotor activity

Given the central clock in the SCN controls the daily activity-rest cycles via a direct route, the continuous registry of a physiological circadian rhythm is fundamental in every study of circadian rhythmicity. As expected for animals with nocturnal habits, locomotor activity in LD was synchronized to the dark phase in the young adults rats (Fig. 5A, upper panel). When young animals were kept in constant darkness conditions (free running), we observed they continued showing a clear activity/rest pattern but shifted rightward, which is characteristic of animals an endogenous period than 24 h (Fig. 5B, upper panel). With respect to the 22-month-old group, they also showed an activity pattern confined to the dark period under LD conditions. However, the locomotor activity was reduced in comparison to the young adult rats (1178 ± 172 vs 259 ± 38 infrared beam interruptions per day, $p < 0.01$; Fig. 5A, lower panel and Fig. 6A). Interestingly, the older animals became arrhythmic and showed a marked fragmentation as well as a significant decline in daily locomotor activity (1124 ± 239 vs 361 ± 63 infrared beam interruptions per day, $p < 0.05$), when they were maintained under constant darkness conditions (Fig. 5B, lower panel and Fig. 6B).

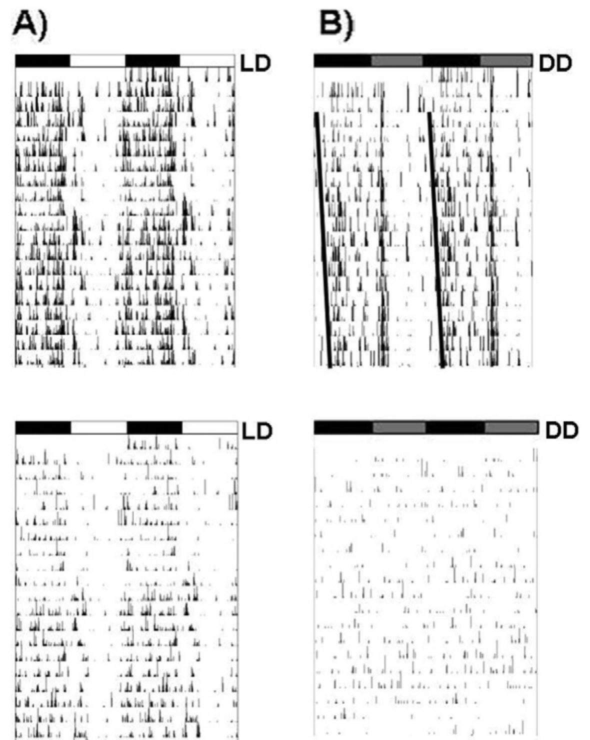

Fig. 5. Representative actograms of young adult and older rats. Double-plot representation of daily locomotor activity of young adults (upper panels) and older (lower panels) rats under a 12 h:12 h LD cycle (A) or a 12 h:12 h DD cycle (B). Subsequent days are plotted on rows from the top to the bottom.

3.5. Circadian rhythms of the core clock protein BMAL1 in the hippocampus of young adults and older rats

In order to assess whether aging modifies the circadian expression of a key core clock protein in the hippocampus, we analyzed the BMAL1

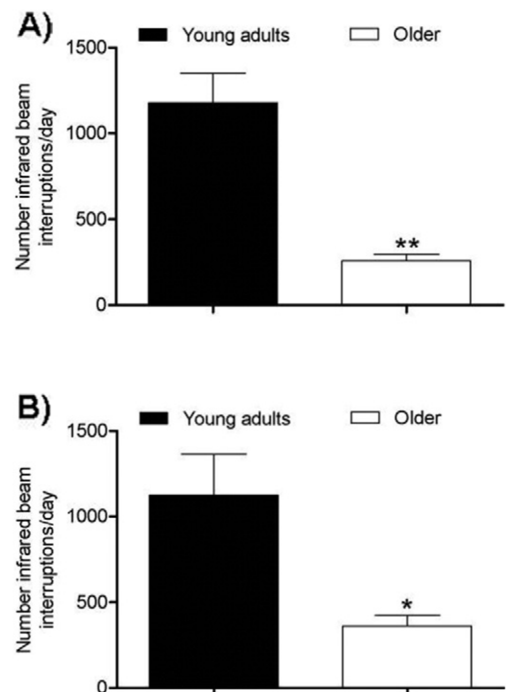

Fig. 6. Daily total locomotor activity of young adults and older rats in LD (A) and DD (B) conditions. Statistical analysis was performed by the Student t-test with $^{*}p < 0.05$ and $^{**}p < 0.01$ when older group was compared to young adults rats.

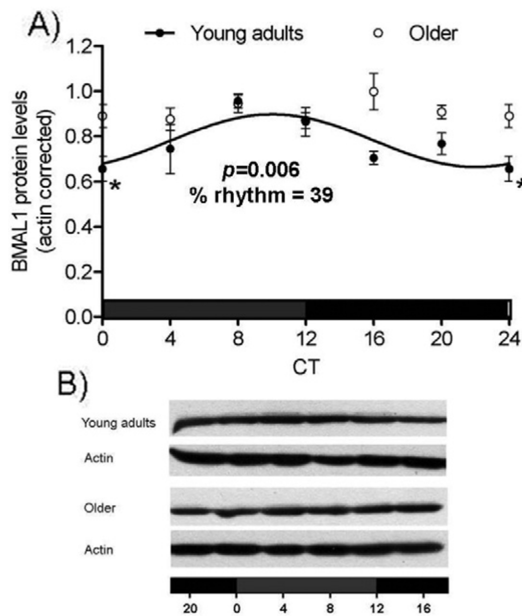

Fig. 7. Circadian rhythms of BMAL1 protein levels in the hippocampus of young adults and older rats. (A) Cosine fitting curves for rhythmic normalized BMAL1 protein levels throughout a day, obtained from the densitometric quantitation of the Western blot. Horizontal bars represent the distribution of dark-dark (DD) phases of a 24-h period (CT0-CT24). Each point represents the mean + SEM of $n = 4$ hippocampus samples at each given CT. Statistical analysis was performed using one-way ANOVA followed by Tukey test with $^*p < 0.05$ when indicated means were compared to the corresponding maximal value in each group. The p and % rhythm in the figure indicates the detection of a rhythm from analysis by the Chronos-Fit method. (B) Representative Western blot analysis of protein extracted from the hippocampus of young adults and older rats isolated at CT0, CT4, CT8, CT12, CT16, and CT20. Values at CT0 were repeated at CT24 on graphs to a better visualization of rhythms.

protein levels during a 24-h period, in this brain area of young adults and aged rats, maintained under constant darkness (DD) conditions. We found BMAL1 protein levels varies significantly throughout the day in the hippocampus of young adults rats (ANOVA: $p < 0.05$). Such temporal variation is rhythmic and circadian (Chronos-Fit: $p = 0.006$, % rhythm = 39) with maximal protein levels occurring on the second half of the subjective day (rhythm's acrophase at CT 09:16 ± 01:11; Fig. 7). Aging completely abolished endogenously-driven BMAL1 protein rhythmicity in the rat hippocampus (Fig. 7).

4. Discussion

Aging is a complex biological process that leads to the decline of the functionality of many physiological systems, including the circadian system. Likewise, alteration of the circadian system has a profound effect on the longevity of different organisms (Lee, 2005; Kondratov et al., 2006). Even though, functional weakening of circadian rhythms and antioxidant function has been observed during aging (Siqueira et al., 2005; Kondratova and Kondratov, 2012; Manikonda and Jagota, 2012; Farajnia et al., 2014), the mechanisms by which the circadian system signaling and oxidative stress are interrelated in the aging process is a very interesting point to be elucidated.

Here, and for the first time in our knowledge, we report that aging modifies circadian rhythmicity of the locomotor activity (a direct output of the central clock in the SCN) and the circadian rhythm of the molecular clock component, BMAL1, in the hippocampus of rats, probably, as a consequence of age-associated changes in the temporal organization of the antioxidant defense system, as well as in peroxidation levels, strongly suggesting a close relationship between aging, oxidative status and the circadian clock.

It is known, the molecular clock machinery regulates rhythmic target genes expression by the BMAL1:CLOCK heterodimer binding to specific (E-box) motifs in the genes regulatory regions (Reppert and Weaver, 2002). Previous bioinformatic analysis done in our lab revealed the presence of putative E-box sites in the regulatory regions of the CAT and GPx genes (Fonzo et al., 2009). Based on these antecedents, our goal was to evaluate the consequences of aging on the circadian expression and enzymatic activity of CAT and GPx, in the hippocampus of animals maintained under constant darkness (DD) conditions. We found CAT mRNA expression and activity display endogenous circadian rhythmicity, with the transcript levels peaking in the second half of the subjective day followed by the maximal CAT activity at the end of the subjective day, in the young rat hippocampus. On the other hand, the GPx1 mRNA levels also display a circadian oscillation, with maximal levels at the first half of the subjective night, that were in phase with its specific enzymatic activity, in the young group. These observations are consistent with our previous work in aged matched rats, where we analyzed the effects of a vitamin A-free diet on circadian rhythms of antioxidant enzymes expression and activity (Navigatore-Fonzo et al., 2014). In both cases, and as expected in terms of predictive homeostasis (a function of circadian rhythms) maximal antioxidant enzymes activity precedes the activity period (mainly catabolic and ROS generating) of nocturnal rats. Some other investigators have also shown circadian and daily rhythms of antioxidant enzymes in different brain areas and other tissues (Pablos et al., 1998; Baydas et al., 2002; Fonzo et al., 2009; Ponce et al., 2012). However, none of them have proven the endogenous character of those rhythms, nor have studied the aging consequences on them.

Interestingly, in this study, we observed that circadian rhythmicity of antioxidant enzymes is differentially modified in the hippocampus of older rats. On the one hand, aging abolished the oscillating CAT expression and specific enzymatic activity, while on the other; it increased the mesor of circadian GPx activity rhythm. The first observation is consistent with investigations which show that aging reduces the amplitude, or even flattens some circadian rhythms (Mirmiran et al., 1992; Manikonda and Jagota, 2012; Farajnia et al., 2014). The second observation could be interpreted as a protective response against oxidative stress, mainly during the subjective day (rest and anabolic period of nocturnal animals). Similarly, we have observed an increased GPx rhythm's mesor and amplitude in another model of oxidative stress, such as the vitamin A deficiency (Navigatore-Fonzo et al., 2014), and Manikonda and Jagota (2012) reported increased GPx activity in the liver during aging. Since GPx is often considered the most important antioxidant enzyme in the brain, therefore, we might expect that the increased GPx activity would have a neuroprotective effect in the hippocampus of aged rats.

Given the substrate of GPx, GSH, is one of the determining factors for maintaining optimal cellular redox state, we study the subjective-day/subjective-night variation of GSH levels, in the hippocampus of young adult and older rats. Although the levels of GSH do not oscillate in a circadian manner in the hippocampus, we observed aging increases overall GSH levels, especially, during the subjective night. On the other hand, since the brain is very sensitive to free radical attack and lipid peroxidation due to its high content of polyunsaturated fatty acids, we continue to study the levels of LPO throughout the day, in young adults and older rats hippocampus. As with GSH, we did not find circadian rhythmicity in the LPO levels, however, we were able to analyze subjective-day/subjective-night LPO patterns. Thus, we observed TBARS levels in young rats are higher during the subjective day, and precede the maximal GPx activity. Surprisingly, we found that older rats show lower levels of lipoperoxidation during the subjective day. Probably, this may be the result of the protective effect exerted by the highest levels of GPx observed in this group at the same phase of the day. Our results of GSH and TBARS levels are in contrast with a number of previous studies showing reduced GSH and increased lipid peroxidation during aging (Rodrigues Siqueira et al., 2005; Kunthavai Nachiyar et al., 2011; Manikonda and Jagota, 2012). However, this would be the first study, to our knowledge, showing day/night patterns of GSH and LPO levels

in the hippocampus of aged animals under constant darkness conditions. On the other hand, the observation of increased levels of LPO during the subjective day in young adult rats is consistent with another study that found the maximum level of MDA during rest span (light period) in young mice (Sani et al., 2007). In the recent years, the idea that lipid peroxides have only a destructive role is changing. It has been shown that both LPO and ROS can participate in signal transduction cascades and cause the removal of damaged cells, fulfilling a protective role (Gago-Dominguez et al., 2007). Likewise, recent data support the notion that ROS production at suitable levels can induce downstream processes which lead to an adaptive response, such as improved antioxidant capacity, metabolic health and extended longevity (Ristow and Schmeisser, 2011).

Temporal patterns described above and alterations observed in the aged animals, led us to continue studying the aging consequences on the endogenous clock. Given the central clock in the SCN controls the daily activity-rest cycles via a direct route, the continuous registry of a physiological circadian rhythm is fundamental in every study of circadian rhythmicity, since it is useful as a control of the circadian signalling pathway integrity and as a reference of the individual inner time (Schibler and Sassone-Corsi, 2002; Dibner et al., 2010). When we exposed young adult rats to constant darkness, as expected, their locomotor activity pattern persisted and was shifted rightward, indicating an endogenous period >24 h. Such observation was consistent with previous results obtained by Navigatore-Fonzo et al. (2014). Interestingly, we observed an alteration in the circadian pattern of locomotor activity in the 22-month-old rats, especially when they were kept in constant darkness, showing a marked fragmentation and decreased amplitude, with a clear absence of an endogenous rhythm throughout the day (arrhythmia). In agreement with other studies carried out in rodents subjected to different lighting conditions (Valentinuzzi et al., 1997; Kolker et al., 2003; Nakamura et al., 2011), our results suggests that aging affects the SCN function, as indicated by the decline in their output signals, such as the locomotor activity. Given communication between the central and peripheral clocks is essential to sustain the circadian rhythms in the body, alterations in the SCN could affect the molecular clock and the establishment of rhythms in peripheral tissues such as the hippocampus.

Thus, we evaluated the consequences of aging on the circadian variation of BMAL1. First, we observed that BMAL1 protein levels display a circadian rhythm in the hippocampus of young animals with maximal levels occurring on the second half of the subjective day. As expected, we found maximal CAT and GPx mRNA expression follow BMAL1 protein peak in this group. This result, as well as the presence of E-box responding sites found in the CAT and GPx promoters, suggests those antioxidant enzymes would be under the endogenous clock control in the hippocampus. Second and noteworthy, we observed aging abolished the BMAL1 protein circadian rhythm and, consequently, oscillating target CAT and GPx genes expression, in the aged hippocampus. Dampening oscillation of Bmal1 mRNA expression was previously observed in the SNC of aged mice (Bonaconsa et al., 2014) and the hippocampus of older hamsters (Duncan et al., 2013). As a result of the loss of mRNA rhythmicity, CAT enzymatic activity was also flattened in this brain area, however, GPx activity increased and continued oscillating in the hippocampus of aged animals. An explanation to this apparent controversy resides in the post-translational enzyme regulation. For example, Zhang et al. (2014) demonstrated that Ca^{2+} can modulate positively the activity of an artificial GPx enzyme. A similar mRNA-independent GPx regulation might be expected when the release of Ca^{2+} back to the cytoplasm increases upon ROS attack to the mitochondrial membrane, which could be the case of cellular aging.

GSH levels are higher during subjective night in the aged hippocampus, being in anti-phase with maximal GPx enzyme activity. As we said before, it has been shown in in vitro models, that cellular redox state modulates BMAL1:CLOCK-driven regulation of rhythmic gene expression (Rutter et al., 2001). Our observations suggest the possibility that alterations in temporal patterns of cellular redox state observed in the senescence could have some effects on the endogenous clock activity through the modulation of BMAL1:CLOCK binding activity to the DNA. At the same time, models of Bmal1 deficiency also result in acceleration of aging and increased levels of ROS in several tissues (Kondratov et al., 2006), suggesting that the alteration of cellular clock function can have important consequences in the regulation of oxidative stress during aging.

In summary, in the present work, we propose a model of physiological aging maintained under constant darkness condition to analyze the endogenous nature of temporal changes. Here, for the first time to our knowledge, we observed a disorganization of temporal patterns of locomotor activity, a loss of the BMAL1 circadian rhythmicity and alterations in the circadian expression and activity of antioxidant enzymes, in the aged rat hippocampus.

Our investigation incentives further inquiries and will be extended in future studies. Those will include temporal and mechanistic studies on epigenetic modifications on the CAT and GPx gene promoters and the expression of proteins involved in such regulation. In addition, other studies will be conducted to investigate whether and how the observed age-related changes in the hippocampus could be reverted by dietary manipulations.

5. Conclusions

Taken together, these results strongly suggest an interplay between circadian system, aging and the cellular oxidative status. The loss of temporal organization of the antioxidant enzymes activity, the oxidative status and the cellular clock machinery could result in a temporally altered antioxidant defense system in the aging brain. This could cause the organisms were not able to be adequately prepared for daily changes in metabolism, thereby favoring the aging process and age-related degenerative diseases. Taking into account ours and other's results, impairment of the circadian clock could be proposed as the tenth hallmark of aging, expanding the nine principles contributing to the aging process proposed by López-Otín and collaborators in 2013 (López-Otín et al., 2013).

Given life expectancy has increased enormously during the last several decades, aging and associated diseases have become a serious worldwide public health problem. Learning about how aging affects the circadian system and the expression of genes involved in the antioxidant defense system could contribute to the design of new strategies to improve the quality of life of older people and also to promote a healthy aging.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.exger.2016.12.002.

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