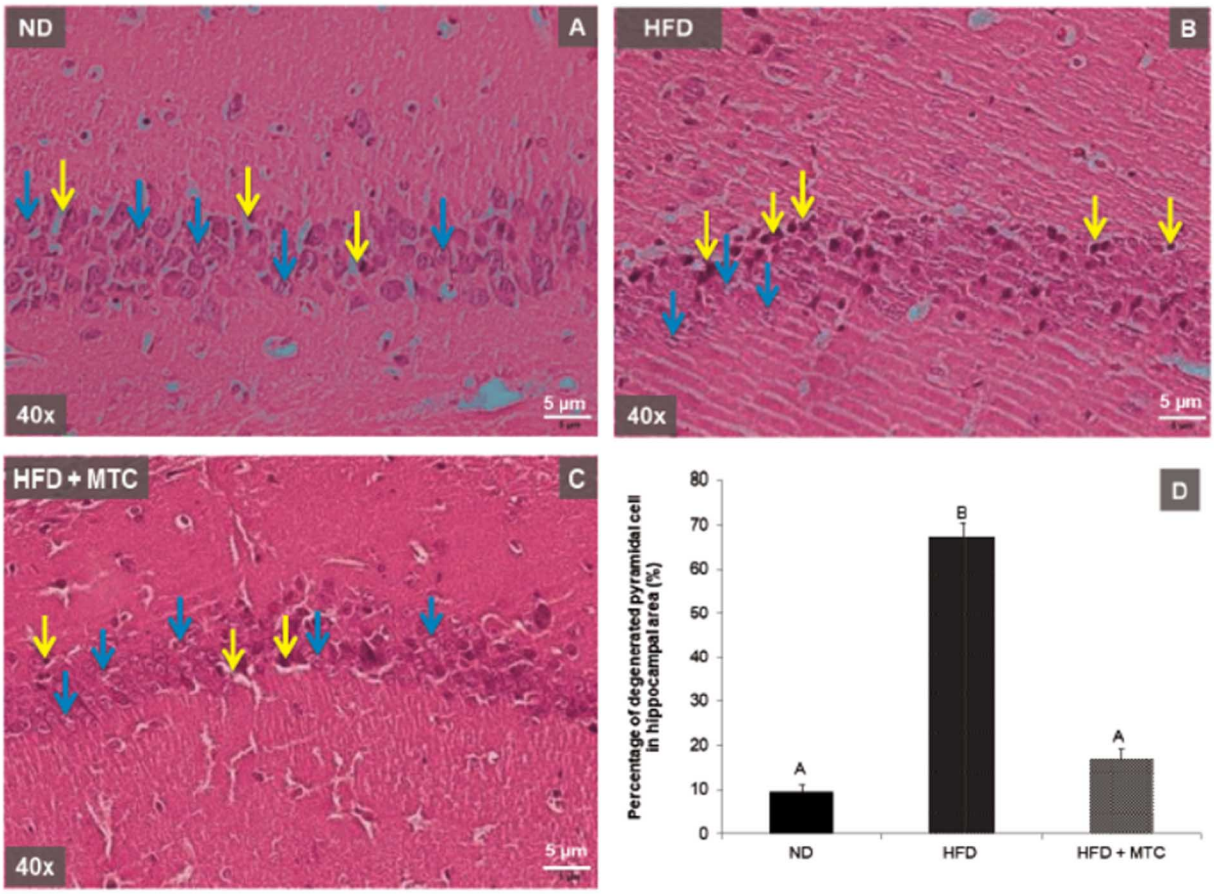
E-ISSN: 2149-7222 (Online)

Journal Cellular Neuroscience and Oxidative Stress



OPEN ACCESS and NO PUBLICATION FEE

http://dergipark.org.tr/jcnos

Former name; Cell Membranes and Free Radical Research

Volume 16, Number 1, 2024

Journal of Cellular Neuroscience and Oxidative Stress

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BSN Health Analyses, Innovation, Consultancy, Organization, Industry and Trade Limited Company

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Formerly known as:

Cell Membranes and Free Radical Research (2008 - 2014)

Volume 16, Number 1, 2024

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Volume 16, Number 1, 2024
E-ISSN Number: 2149-7222 (Online)
Indexing: Scopus (Elsevier), CAS (Chemical Abstracts Service), Citation Index Database, EBSCOhost Research Database, Google Scholar

AIM AND SCOPES
Journal of Cellular Neuroscience and Oxidative Stress is an online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+- K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals)

C- Interaction Between Oxidative Stress and Ion Channels in Neuroscience
(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels in neurodegenerative diseases such Parkinson's and Alzheimer's diseases)

D- Gene and Oxidative Stress
(Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

READERSHIP

Biophysics	Biochemistry
Biology	Biomedical Engineering
Pharmacology	PhysiologyGenetics
Cardiology	Neurology
Oncology	Psychiatry
Neuroscience	Neuropharmacology

Keywords
Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide, ageing, antioxidants, neuropathy, traumatic brain injury, pain, spinal cord injury, Alzheimer's Disease, Parkinson's Disease.

RESEARCH ARTICLE

J Cell Neurosci Oxid Stress 2024;16(1): 1183-1194

Aging Disrupts Circadian Rhythms of Antioxidant Enzymes, Clock, and Inflammatory Factors in The Rat Prefrontal Cortex. Potential Targets for Therapeutic Strategies

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Received: 9 May 2024; **Accepted:** 28 June 2024

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List of Abbreviations;

BMAL1, *brain and muscle ARNT-Like 1 protein;* ***CAT,*** *catalase;* ***CLOCK,*** *circadian locomotor output cycle kaput protein;* ***CT,*** *circadian time;* ***GPX,*** *glutathione peroxidase;* ***GSH,*** *glutathione;* ***H_2O_2,*** *hydrogen peroxide;* ***LPO,*** *lipid peroxidation;* ***MDA,*** *malondialdehyde;* ***NR,*** *nuclear receptor;* ***PFC,*** *prefrontal cortex;* ***ROR,*** *retinoic acid-related orphan receptor;* ***SCN,*** *suprachiasmatic nucleus;* ***TBARs,*** *thiobarbituric acid reactive substances;* ***TNF-α,*** *tumor necrosis factor alpha*

Abstract

Age impairs cognitive functions and antioxidant defenses, for example, by increasing oxidative stress and inflammation in the brain. However, to date, there are no reports on the consequences of aging on temporal patterns of lipid and protein oxidation, antioxidant enzymes activity, the endogenous clock, and proinflammatory cytokines in the prefrontal cortex (PFC). Therefore, our objectives were 1) to investigate the endogenous nature of 24-h rhythms of lipid peroxidation, protein carbonyl levels, catalase (CAT) and glutathione peroxidase (GPX) activity as well as retinoic acid-related orphan receptor a (RORa) and tumor necrosis factor alpha (TNF-α) levels, in the rat PFC, and 2) to study the consequences of aging on the circadian organization of these factors in this area. To do

that, we used male Holtzman rats, maintained under constant darkness conditions for 15 days before reaching 3- and 22-mo-old, respectively. PFC samples were isolated every 4 h under dim-red light for a 24 h period. Our results revealed circadian patterns of antioxidant enzymes activity, oxidative stress, RORa and TNF-α protein levels in the PFC of young rats. The circadian distribution of the rhythms' acrophases suggests reciprocal communication among antioxidant defenses, endogenous clock, and inflammation. Such circadian organization disappears in the PFC of aged rats. Increased oxidative stress modifies the cellular redox environment and, consequently, alters the endogenous clock activity and disrupts the circadian organization of antioxidant defenses and TNF-α in the PFC. These results might highlight novel chronobiological targets for the design of therapeutic strategies for healthy aging patients.

Keywords: circadian rhythm, oxidative stress, retinoic acid-related orphan receptor a, tumor necrosis factor alpha, prefrontal cortex.

Introduction

Normal aging is often associated with a chronic low-grade inflammatory state and cognitive decline, and several brain areas are particularly vulnerable (Permpoonputtana et al., 2018). Over the years, many studies have associated oxidative stress with aging and the development of neuroinflammation and neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Yildizhan & Naziroğlu, 2019; Qaid et al., 2021).

It is widely accepted that oxidative stress arises from a combination of an exaggerated increase of free radicals and oxidant agents, with lower levels of antioxidants and dysregulation of the antioxidant defense system (Sies et al., 2017; Ifeanyi, 2018). Thus, lipid peroxides, protein carbonyls, and antioxidant capacity as well as catalase (CAT) and glutathione peroxidase (GPX) enzymatic activities, among others, are good markers for defining the oxidative balance in cells and tissues (Sies et al., 2017).

A high content of polyunsaturated fatty acids makes the brain very susceptible to lipid peroxidation (LPO). Increased LPO has been reported in different organs and tissues, including the brain, during aging (Radak et al., 2011). Several studies have also shown age-dependent alterations in cellular redox homeostasis by increasing oxidative stress and decreasing antioxidant enzyme activity in the brain (Lacoste et al., 2017; Meng et al., 2017). However, until now, we found a scarce investigation on the consequences of aging on temporal (circadian) patterns of protein and lipid oxidation and/or antioxidant enzyme activity in the prefrontal cortex. Previously, we reported a very well-orchestrated daily expression and activity of antioxidant defenses in other brain regions, such as the hippocampus and temporal cortex of young rats (Navigatore-Fonzo et al., 2014; Lacoste et al., 2017; Coria-Lucero et al., 2023).

It is known that most living organisms have an endogenous timing system that synchronizes internal events with environmental cues, such as the light:dark cycle, among others. In mammals, a hierarchical circadian timing system orchestrated by a master clock located within the suprachiasmatic nuclei (SCN), synchronizes the peripheral clocks located in other brain areas and the rest of the body through neural and humoral signals (Bollinger & Schibler, 2014). Once synchronized, free-running rhythms observed in constant conditions (e.g., constant darkness) in isolation experiments provide strong evidence for the existence of an endogenous circadian oscillator (Golombek & Rosenstein, 2010).

The molecular and cellular bases of endogenous circadian rhythms consist of transcriptional-translational feedback loops that involve interconnected positive and negative factors. In mammals, the positive loop of the cellular clock consists of the transcriptional activator Brain and Muscle ARNT-Like 1 (BMAL1) protein, which heterodimerizes with the Circadian Locomotor Output Cycles Kaput (CLOCK) protein. The BMAL1:CLOCK heterodimer binds to clock-responsive, E-box (CANNTG) enhancing sequences in the regulatory regions of target genes to activate the transcription of other clock genes (the negative regulators Per1-3 and Cry1-2, RevErb, Rora, etc.) as well as clock-controlled genes (Buhr and Takahashi, 2013). A reduced cellular redox environment strongly stimulates the BMAL1:CLOCK binding to E-box sites in the target genes, whereas oxidized forms inhibit the clock heterodimer binding (Wilking et al., 2013; Gillette and Wang, 2014).

The main positive loop of the cellular molecular clock also depends on an accessory loop composed of the retinoic acid-related orphan receptor a (RORa) and nuclear receptor subfamily 1 group D member 1 (NR1D1 or REV-ERB) in brain. It has been shown that RORa monomer binds to ROR-responsive sites (ROREs) in the promoter of

target genes at the central 5-AGGTCA-3 motif of the monomeric half-site or as a homodimer at Rev-DR2 sites of direct repeats with a half site separated by 2 bp (Harding and Lazar, 1995). Thus, RORs are circadian clock proteins involved, among others, in diurnal rhythm regulation (He and Chen, 2016). BMAL1:CLOCK activates Rora and Rev-Erbα gene transcription, and, in turn, products of these genes, the RORa and REV-ERBα proteins, interact with RORE sites in the BMAL1 promoter. Thus, RORa, in the central nervous system, and RORc, in peripheral oscillators such as the liver, activate BMAL1 transcription directly through two RORE sites in the latter promoter (Cho et al., 2012).

In addition, RORs are involved in the regulation of redox homeostasis. Previous studies show that RORa plays a protective role against oxidative stress. Notably, RORa activates the expression of antioxidant enzymes such as GPx1 and peroxyredoxin 6 in cultured mouse neurons, thus exerting a neuroprotective effect against oxidative stress (Han et al., 2014).

Increased proinflammatory cytokine levels have been reported in the aged brain, and RORa has also been implicated in the control of inflammatory signaling (Permpoonputtana et al., 2018; Nejati-Moharrami et al., 2018). Nejati-Moharrami et al. (2018) showed that Rora gene deletion results in constitutive activation of transcription for a subset of NFκB-regulated genes, including tumor necrosis factor (TNF) and interleukin-1B (IL-1B). The circadian clock also controls the rhythmic expression of cytokines such as TNF-α, but several studies have shown that TNF-α induces the transcription of the clock, Bmal1, and Rora genes (Yoshida et al., 2018).

The above observations suggest that both oxidative stress and the cellular redox imbalance associated with aging may affect the circadian clock activity and its target genes expression, probably by modulating the activity of BMAL1:CLOCK and/or RORa transcription factors. These findings might constitute biochemical and molecular bases that explains the impaired temporal organization of both behavioral and physiological parameters observed in older individuals.

To our knowledge, there are no reports on the consequences of aging on circadian patterns of antioxidant defenses, oxidative stress, endogenous clock factors, and inflammatory cytokines in the prefrontal cortex. Considering the above background information, the objectives of our study were 1) to investigate the endogenous nature of 24-h rhythms of LPO, protein carbonyls, CAT and GPX activity, RORa, and TNF-α protein levels, in the rat PFC, and 2) to study the consequences of aging on the circadian organization of these factors in the same brain area.

Materials and methods

Animal model

Male Holtzman rats bred in our animal facilities at the National University of San Luis (San Luis, Argentina), were weaned at 21-day old and randomly assigned to the following groups: young adult (3-mo-old, n=24) and aged rats (22-mo-old, n= 24). Animals were maintained with *ad-libitum* access to food and water, in a 21–23 °C controlled environment, and under a 12 h-light:12 h-dark (LD) cycle (lights on at 07:00 a.m.). To evaluate the endogenous clock-driven circadian rhythms, both groups of rats were kept under constant darkness (DD) conditions during fifteen days before the experiment, following Aschoff's rules (1967). When animals reached the 3- and 22-mo-old, respectively, we euthanized them by decapitation under dim red light to avoid acute effects of lights that could interfere with the free-running condition. On the experiment day, n=4 rats from each group were euthanized every 4 h throughout a day, at the circadian times (CT): CT0, CT4, CT8, CT12, CT16 and CT20, being CT0 the beginning of the subjective day and CT12 the beginning of the night. All experiments were repeated at least twice. The experiments were carried out following 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 approved by Res. RD-2-296/17 y 01/19).

Tissue sample isolation

Prefrontal cortex (PFC) samples were isolated from young adult and aged rats at the CTs mentioned in 2.1 Animal model. Briefly, following animal decapitation, the head was recovered, and the skull was opened with sterile scissors. The brain was carefully removed, quickly washed in ice-cold sterile saline solution, and placed on an ice-chilled plate. The prefrontal cortex was obtained through a cut at 2.5 mm parallel to the longitudinal fissure that divides both cerebral hemispheres in the frontal lobe and another cut perpendicular to it, from bregma 6.12 to bregma 5.64 (Paxinos and Watson, 2006). Immediately

after it was cut, the PFC was frozen in liquid nitrogen and stored at −80 °C.

Western blot

Obtaining the protein extracts and the immunoblotting procedure were carried out mostly as in Lacoste et al. (2017). Aliquots containing 25 μg of total protein were subjected to electrophoresis on an SDS polyacrylamide 12% gel. The resolved proteins were transferred to a PVDF membrane (Thermo Scientific, Waltham, MA) by electroblotting. After blocking with 3% nonfat dry milk dissolved in TBS (pH 7.3), membranes were incubated overnight at 4 °C with either rabbit polyclonal anti-RORa (1:1000, H-65: sc-28612), goat anti-TNF-α (1:1000, N-19: sc-1350) or rabbit polyclonal anti-ACTIN (1:3000, I-19: sc-1616-R) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), in TBS containing 0.05% Tween 20 (TBST). After washing, membranes were incubated either with peroxidase-goat anti-rabbit IgG or HRP-rabbit anti-goat IgG (Invitrogen, Thermo Scientific, Waltham, MA) diluted 1:7000 in TBST, for 2 h at room temperature. Finally, antibody/protein complexes were detected by using the Bio-Lumina detection system as indicated by the manufacturer (Kalium Technologies, Buenos Aires, Argentina). The membranes were then exposed to X-ray film (CL-X Posure™ Films, Thermo Scientific, Waltham, MA). The film was developed using a Kodak GBX Developer and Fixer in a dark room. The mean intensity of each band was measured using NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/).

Tissue homogenates and enzyme activity assays

Tissue samples were homogenized in a 1/5 (w/v) dilution of 30 mM phosphate buffer, pH 7.4, with 120 mM KCl at 4 °C. The homogenates were centrifuged at 3,500 rpm for 10 min at 4 °C to remove nuclei and cell debris. The pellets were discarded, and the supernatants were used to determine antioxidant enzyme activities. CAT activity was assayed spectrophotometrically according to Aebi (1984). Briefly, the decomposition of H_2O_2 was monitored at 240 nm after the addition of the prefrontal cortex homogenate supernatant. The enzymatic activity was expressed as International Units (IU)/mg of protein (1 IU decomposes 1 μmol H2O2/min at pH 7 and 25 °C). GPx total activity was measured following the 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.

Lipid peroxidation levels

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

Carbonyl concentration

Oxidative protein damage was analyzed by quantifying the carbonyl groups by ELISA following Winterbourn and Buss (1999) with modifications. Briefly, tissue homogenates were derivatized to 2,4-dinitrophenylhydrazone by reacting carbonyl groups in oxidized proteins with 2,4-dinitrophenylhydrazine in 2 M HCl. Ten (10) microliters of the derivatized or non-derivatized sample was added to 190 ml 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 a rabbit polyclonal anti-dinitrophenyl antibody (1:2000 dilution in washing buffer). The immunocomplexes were quantified using a goat anti-rabbit IgG-HRP conjugate (1/10000 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 nmol of carbonyl per milligram of total protein (nmol/mg of prot).

Total Antioxidant Capacity

The total antioxidant capacity (TAC) was measured by quenching the 2,20-azino-bis-(3-et hylbenzothiazoline-6-sulfonic acid) cation radical ($ABTS^{\bullet+}$) by both lipophilic and hydrophilic antioxidants contained in the tissue homogenates (Re et al., 1999). Cationic radicals were generated by the oxidation of 7 mM ABTS with 2.45 mM

K_2SO_8 24 h before the day of the test. The technique consisted of diluting this solution with 5 mM PBS, pH 7.4, to an absorbance of 0.700±0.02 at 734 nm and equilibrating it at 30 °C. Once obtained, the assay was performed by taking 990 µl of this dilution and measuring the initial absorbance (iAbs). Then, we added 10 µl of the sample and measured the final absorbance (fAbs) at 30 °C, exactly 1 min after initial mixing and up to 6 min. Finally, the calculations were performed using the following equation: % inhibition= ((iAbs-fAbs)/iAbs)x100. At the time of testing, a series of ascorbic acid standards were prepared and used as reference antioxidants. All measurements were performed in duplicate for each sample.

Statistical analysis

Data were analyzed first by one-way ANOVA followed by Tukey's post hoc test for specific comparisons within each group; a $p \leq 0.05$ was considered to be significant. Circadian rhythms were assessed by the Chronos-Fit 1.06 software, 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., 2009). The significance of rhythmicity, testing the null hypothesis of the amplitude being equal to zero, was performed by an F test ($F>3.5$; $p \leq 0.05$; Thaela et al., 1998). The percentage of rhythm is a chronobiological term for the coefficient of determination, i.e., the squared coefficient of correlation times 100 (% rhythm = r2*100). When the Chronos-fit analysis confirmed the presence of rhythm, a cosine-fitted curve was generated with GraphPad Prism 8.0 software (CA, USA), and the following parameters were calculated by the Cosinor method (Molcan, 2019): mesor (the rhythm adjusted mean), acrophase (span of time to reach the peak of the detected rhythm for the 24h-period), and amplitude (the difference found between the maximal value and the average value, mesor, of the adjusted of rhythm). Student's t-test was used for comparison of mesor (or daily mean), amplitude or acrophase between Young Adult and Aged groups, with $p < 0.05$ for significant differences.

Results

Oxidative stress and total antioxidant status

Given that most aging-related disorders are linked to oxidative stress and the cellular redox state and that the brain is particularly susceptible to oxidation, in this work, we analyzed lipid peroxidation, protein carbonylation and antioxidant capacity in the PFC of young adult and aged rats. We observed that lipid peroxidation increased significantly in the PFC of aged animals ($p<0.001$, Figure 1A). Similarly, carbonyl levels were significantly greater in the PFC of 22-month-old rats than in those of young adult rats ($p<0.05$; Figure 1B). However, when we analyzed the total antioxidant capacity, we observed that the percentage inhibition of ABTS oxidation did not change with age (Figure 1C).

Temporal variation of lipid and protein oxidation

When we explored whether lipids and proteins oxidation changes in a time-dependent manner, our results revealed that lipoperoxidation and protein carbonylation vary significantly throughout a day (ANOVA, $p \leq 0.05$ and $p<0.001$, respectively) and oscillate in a circadian and endogenous way (Chronos-fit: $p \leq 0.01$, %rhythm: 40.87, and $p<0.05$, %rhythm: 32.98, respectively), in the rat PFC. The rhythms' acrophases occur at CT 19:33±00:25 and at CT13:56±01:11, respectively (Figure 2A-B, Table 1). Aging overrides the circadian rhythms of oxidative stress in that brain area (Figure 2A-B).

Table 1: *Parameters of circadian rhythms of lipid peroxidation and protein carbonylation in the PFC.*

Rhythms' parameters	MDA		Protein Carbonyls	
	Young Adult group	Aged group	Young Adult group	Aged group
MESOR	1.55± 0.06	NA	6.75±0.44	NA
AMPLITUDE	0.90±0.12	NA	1.53±0.76	NA
ACROPHASE	19:33±00:25	NA	13:56±01:11	NA
% RHYTHM	40.87	NA	32.98	NA

Note: *Mesor, amplitude, and acrophase parameters are expressed as mean ± SEM (n=3). NA: Not applicable, as proteins levels in the aged animals do not display a circadian rhythm.*

Temporal patterns of CAT and GPx enzymatic activity

We analyzed the temporal patterns of antioxidant enzymes activity in the PFC of young adult and aged rats. We observed that both specific CAT and GPx activities vary significantly (ANOVA, $p<0.05$) on a circadian basis (Chronos-fit, $p<0.05$ in both cases, % rhythm: 50.32 and 42.07, respectively), peaking at CT 19:30±00:36 and CT 21:48±00:09, respectively, in the PFC of young adult rats. Aging abolishes the circadian rhythms of both antioxidant enzyme activities (Figure 3A and B, Table 2) and reduces the daily mean of GPx activity (11.56±0.99 vs 8.25±0.76, $p<0.05$).

Figure 1

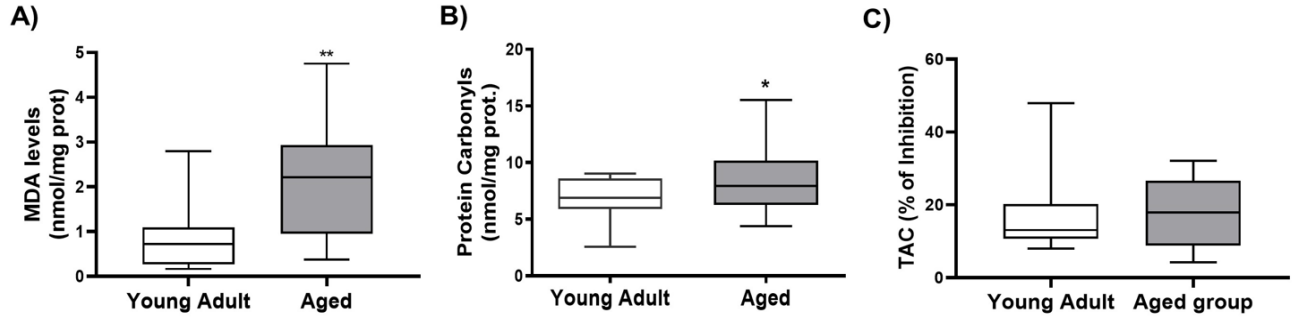

Figure 1. Lipid peroxidation (LPO) by protein carbonyl (PC) levels and total antioxidant activity (TAA) in the PFC of young adults and older rats*. A) LPO was measured as the MDA concentration (nmol/mg prot.). B) Protein oxidation levels as PCs (nmol/mg prot.). C) TAA is expressed as a percentage of the inhibition of ABTS radical. In all cases, box-and-whisker plots represent the 5th-95th percentiles of n=18 samples. An unpaired t test was used to evaluate differences between the young adult and aged groups, with p<0.05 indicating significant differences. A reliability degree of 95% was used. *p<0.05 and **p<0.01.*

Figure 2

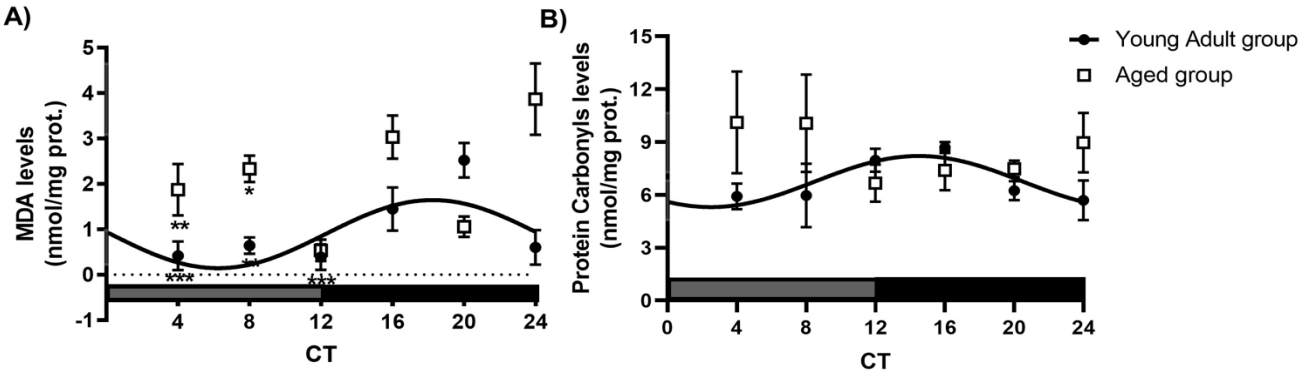

Figure 2: Circadian patterns of lipid and protein oxidation in the rat PFC*. Cosine fitting curves represent circadian rhythms of LPO (A) and PC (B) levels. Each point in the curves represents the mean ± SEM of n=3 PFC samples at every CT. The horizontal bars, gray and black, represent the distribution of the subjective day (CT0-CT12) and night (CT12-CT24) in a 24-h period. Statistical analyses were carried out using one-way ANOVA followed by Tukey's test, with *p<0.05, **p<0.01 and ***p<0.001, when the indicated means in the (A) figure were compared with the corresponding maximal values within each group. From the Chronos-fit analyses, F=5.03 and p<0.01 confirmed a circadian rhythm of LPO levels; in the same way, F=3.69 and p<0.05 confirmed a circadian rhythm of PC levels in the young adult PFC. For better visualization of the rhythms, the value at CT0 was repeated at CT24 when plotting; this value was not repeated in the statistical analyses.*

Table 2. *Parameters of circadian rhythms of CAT and GPx enzymatic activity in the PFC.*

Rhythms' parameters	CAT activity		GPx activity	
	Young Adult group	Aged group	Young Adult group	Aged group
MESOR	0.54±0.04	NA	11.56±0.99	NA
AMPLITUDE	0.22±0.06	NA	8.84±1.33	NA
ACROPHASE	19:30±00:15	NA	21:48±00:09	NA
% RHYTHM	50.32	NA	42.07	NA

Note: *Mesor, amplitude, and acrophase parameters are expressed as mean ± SEM (n=3). NA: Not applicable, as proteins levels in the aged animals do not display a circadian rhythm.*

Temporal profile of RORa and TNF-α protein levels

Levels of RORa protein vary significantly throughout a 24 h period (ANOVA, p≤ 0.01) oscillating on a circadian basis (Chronos-fit, p<0.05 and 32.37% rhythm), with an acrophase at CT23:00±02:06, in the young PFC. Although TNF-α levels do not vary significantly throughout the day (ANOVA, p>0.05), they do display a circadian rhythm (Chronos-fit, p=0.05, and 34.11% rhythm) with an acrophase at CT 09:14±01:32 in the same brain area. Aging abolishes the circadian rhythms of the RORa and TNF-α proteins in the PFC (Figure 4A-B

and Table 3).

Table 3: *Parameters of circadian rhythms of RORa and TNFα in the rat PFC.*

Rhythm's parameters	RORa		TNFα	
	Young Adult group	Aged group	Young Adult group	Aged group
MESOR	0.57 ± 0.07	NA	0.77±0.12	NA
AMPLITUDE	0.24 ± 0.07	NA	0.13±0.04	NA
ACROPHASE	23:00 ± 02:06	NA	09:14±01:32	NA
% RHYTHM	32.37	NA	34.11	NA

Note: *Mesor, amplitude, and acrophase parameters are expressed as mean ± SEM (n=3). NA: Not applicable, as proteins levels in the aged animals do not display a circadian rhythm.*

Discussion

Morphological and physiological changes are consequences of aging. Such modifications include, among others, the inability of the organism to adapt and to respond to environmental changes (Buijink et al., 2020). This inability could be explained by a deterioration of the endogenous clock and defense systems (Lacoste et al., 2021; Buijink and Michel, 2021; Altamirano et al., 2021). Here, we evaluated lipid peroxidation, protein carbonylation and total antioxidant capacity in the PFC of young adult and aged rats; then, we studied the consequences of aging on the circadian patterns of oxidative stress, antioxidant enzymes activity, clock RORa levels, and TNF-α in the same brain area.

As expected, we first found that oxidative stress increased significantly in the PFC of aged animals; however, unexpectedly, we observed no change in cortical total antioxidant capacity (Figure 1). Increased oxidative stress underlies chronic inflammation and cellular damage in the aged brain (Miller et al., 2018).

It is known that, in response to increased oxidative stress, there is a pool of scavenger molecules and antioxidant defenses that protect cells against oxidative damage. Previously, we showed that oxidative stress and antioxidant defenses display clock-driven circadian rhythms in different brain areas, such as the hippocampus and temporal cortex (Lacoste et al., 2017; Coria-Lucero et al., 2023). Here, we investigated whether time and aging could also modulate oxidative stress levels, antioxidant enzymes activity, TNF-α, and a clock´s and anti-inflammatory factor, RORa, in the PFC of young adult and aged rats.

Notably, we found that lipid peroxidation and protein carbonylation display circadian rhythms, peaking at the middle and the first half of the subjective night, respectively, in the PFC of young adult rats (Figure 2 and Table 1). Increased oxidative stress at the end of the night is consistent with the end of the activity phase and mainly a catabolic and ROS-producer period in nocturnal animals. On the contrary, when we analyzed lipid peroxidation levels in the hippocampus of young adult rats maintained under DD conditions, we found MDA levels do not oscillate rhythmically in this brain area. However, we did observe a significant variation between samples isolated during the subjective day (CT0, CT4 and CT8) and the isolated during the night (CT12, CT16 and CT20), with higher lipid peroxidation levels occurring during the subjective day, in the hippocampus (Lacoste et al 2017). Either a circadian oscillation or a simple day-night variation, the temporal pattern of lipid peroxidation would be important to identify the time of higher sensitivity of tissues to the attack of reactive oxygen species.

Antioxidant CAT- and GPx-specific activities vary significantly over a 24 h period in the PFC of the young adult group, which was maintained under free-running conditions (Figure 3 and Table 2). As expected, the maximal levels of CAT activity are in phase with the peak of the lipoperoxidation rhythm, which precedes the acrophase of GPx activity rhythm, in the context of reactive homeostasis. Similarly, previously, we showed CAT and GPX activities also oscillate on a circadian basis in the hippocampus of young adult rats, with their acrophases occurring at the end-of-the-day-beginning-of-the-night, following the diurnal increase in lipid peroxidation (Lacoste et al, 2017).

Circadian rhythms of antioxidant enzymes might be consistent with the presence of clock-responsive Ebox (CACGTG or CANNTG) sites found previously in the regulatory region of the Cat and Gpx genes (Fonzo et al., 2009). In particular, the presence of a RORa-responsive site in the regulatory region of the GPx gene might explain why the GPx rhythm is in phase with the circadian oscillation of RORa protein levels, showed here (Figures 3B and 4A, Tables 2 and 3).

In addition, herein, we observed that the peak of the proinflammatory cytokine TNF-α precedes the anti-inflammatory, RORa, rhythm´s acrophase, in the PFC of young adult rats (Figure 4 and Table 3). This finding is consistent with the role of TNF-α as a positive regulator of

Figure 3

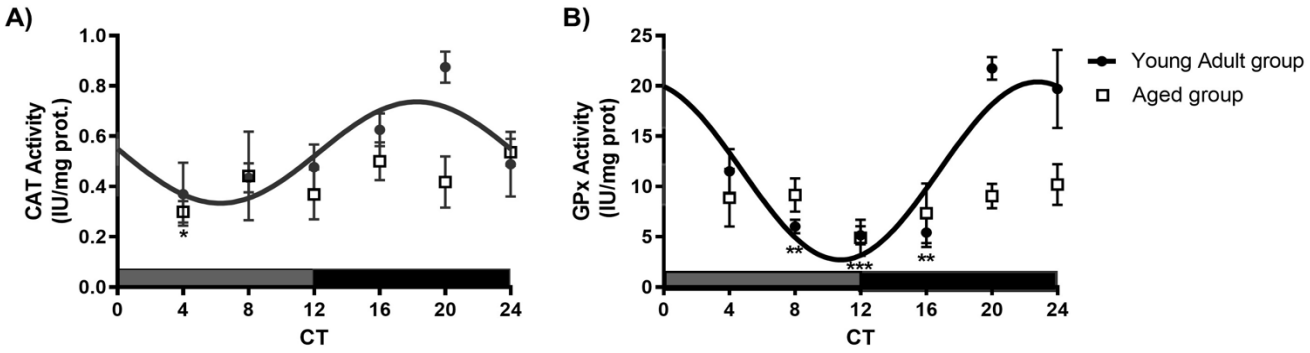

Figure 3. Temporal patterns of CAT- and GPx-specific activities in the PFC of young and old rats*. Cosine fitting curves represent circadian rhythms of CAT (A) and GPX1 (B) specific activity (IU/mg prot.). Each point in the curves represents the mean ± SEM of n=3 PFC samples at each CT. The horizontal bars, gray and black, represent the distribution of the subjective day (CT0-CT12) and night (CT12-CT24) in a 24-h period. Statistical analyses were carried out using one-way ANOVA followed by Tukey's test, with **p<0.01 when the indicated means in panel (B) were compared with the corresponding maximal value within the young adult group. From the Chronos-fit analyses, F=5.28 and p<0.01 confirmed that the temporal variation in CAT antioxidant activity depicts a circadian rhythm; in the same way, F=4.72 and p<0.01 confirmed a circadian rhythm of GPX activity in the young adult PFC. For better visualization of the rhythms, the value at CT0 was repeated at CT24 when plotting; this value was not repeated in the statistical analyses.*

Figure 4

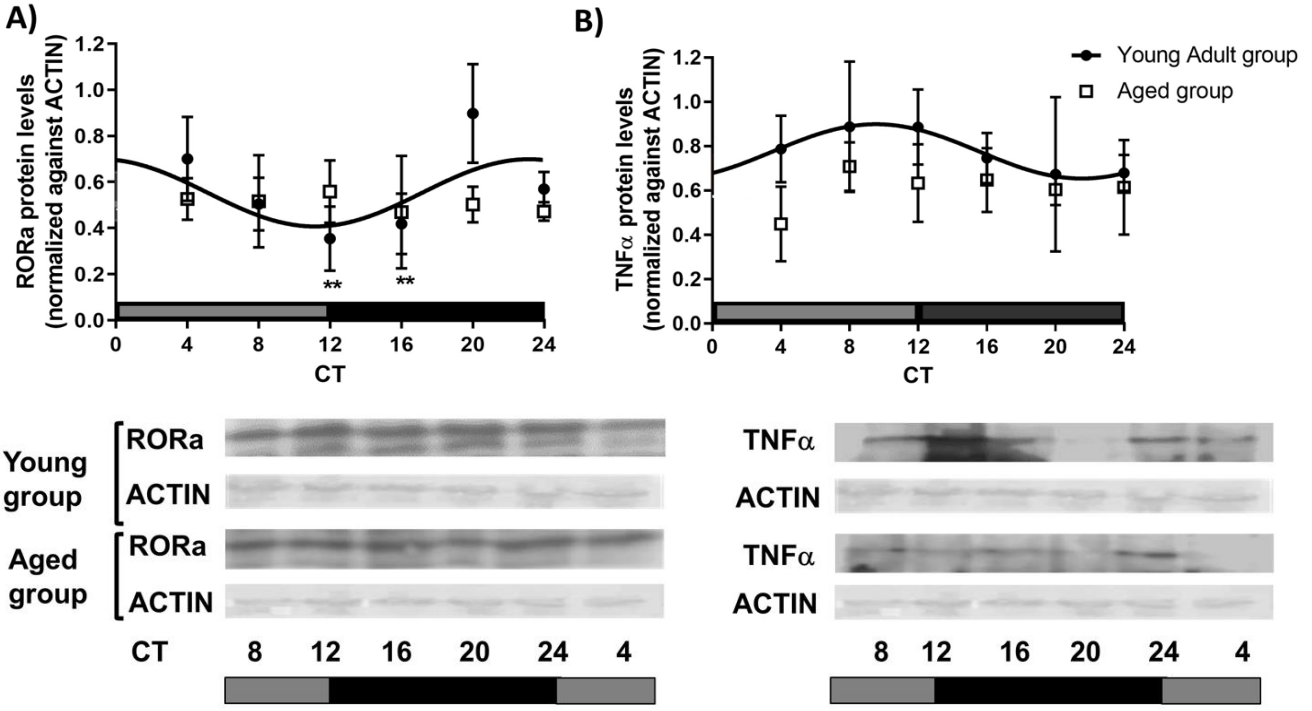

Figure 4: Temporal variation in RORa and TNF-α protein levels in the prefrontal cortex of young and old rats. *Cosine fitting curves represent circadian oscillations of RORa (A) and TNF-α (B) protein levels. Each point in the curves represents the mean ± SEM of n=3 PFC samples at each CT. The horizontal bars, gray and black, represent the distribution of the subjective day (CT0-CT12) and night (CT12-CT24) in a 24-h period. Statistical analyses were carried out first using one-way ANOVA followed by Tukey's test, with **p<0.01 when the indicated means in panel A were compared with the corresponding maximal value within the young adult group. According to the Chronos-fit analyses, F=5.70 and p<0.05 confirmed that the temporal variation of the RORa levels displays a circadian rhythm; similarly, F=3.62 and p=0.05 confirmed a circadian rhythm of TNF-α levels in the PFC of young adults. For better visualization of the rhythms, the value at CT0 was repeated at CT24 when plotting; this value was not repeated in the statistical analyses. C) and D) Representative Western blot analysis of protein extracts obtained from the PFC samples isolated at CT4, CT8, CT12, CT16, CT20, and CT24 from young adults and aged rats.*

the cellular clock via the transcriptional activation of RORa shown by Yoshida and collaborators (2018). Furthermore, in line with the studies of Moharrami et al. (2018), the maximal levels of RORa at the end of the night period are consistent with the nadir of the TNF-α rhythm. Therefore, importantly, a functional clock maintains high antioxidant and anti-inflammatory defenses at the end of the night (activity) period to reduce oxidative stress and inflammation, anticipating, in terms of predictive homeostasis, the diurnal anabolic and resting period of nocturnal rodents. Chaudhury and Colwell (2002) showed that mice acquire conditioning faster during the day than during the night. Furthermore, the recall for context and tone consistently peaked during the day for at least 3 days after training, irrespective of the time of training, and this temporal pattern persisted under constant darkness conditions. Thus, taking into account that those processes require a restoration of the synapses, reestablishing the cellular homeostasis (Tononi and Cirelli, 2014), a healthy (antioxidant) cellular environment during the subjective day might be favorable for sleep-dependent memory that occurs mainly during the day in nocturnal animals (Binder et al., 2014; Kersanté et al., 2023).

Notably, circadian rhythms of antioxidant defenses are lost, and enzymatic activity is reduced in the PFC of aged rats (Figure 3A-B). This observation is consistent with investigations showing that aging reduces the amplitude or even flattens circadian rhythms (Farajnia et al., 2014; Hood and Amir, 2017). The effects of aging on circadian patterns of antioxidant enzymes expression and activity, as well as on 24-h rhythms of oxidative stress and the cellular redox state, have been previously reported by our group and others in the hippocampus, liver and heart (Manikonda and Jagota, 2012; Lacoste et al., 2017; Altamirano et al., 2021). Particularly, we have shown that aging also abolishes the circadian rhythm of CAT activity and cancels the day-night difference of the lipid peroxidation levels in the rat hippocampus; however, it advances the phase and increases the rhythm's mesor of the GPx circadian activity (Lacoste et al, 2017). Thus, we might assume that aging modifies circadian rhythms in different ways, depending on the tissue. This finding is in line with a comprehensive analysis by Zhang and colleagues (2014), who reported that most transcriptionally regulated rhythms are organ specific. They concluded that such organ specificity indicates that although the molecular clock is active throughout the body, it regulates biological processes very differently in each organ (Zhang et al., 2014). Additionally, we suggest that such differential regulation might also depend on physiological and/or environmental conditions.

Reduced CAT and GPx enzymatic activities and loss of circadian rhythms explain the increased lipid peroxidation and carbonyl levels during the subjective day and the disruption of circadian patterns (Figure 2, Table 1). Increased and arrhythmic oxidative stress throughout the day creates a pro-oxidant environment that may alter, among other factors, the cellular redox state, which, as reported previously, is crucial for molecular clock activity (Gillette and Wang, 2014). This would underlie the loss of circadian oscillations in antioxidant enzymes activity as well as RORa and TNF-α levels observed in the PFC of our 22-month-old rats.

Loss of temporal patterns or reduced clock rhythms' amplitude have been observed by our group and others in different aging models. For instance, we showed that the BMAL1 rhythm is abolished in the PFC and hippocampus of 22-month-old rats (Coria-Lucero et al., 2016; Lacoste et al., 2017). Similarly, investigations carried out by Wyse and Coogan (2010) showed that aging modifies the diurnal expression of CLOCK and BMAL1 in the mouse brain, and Fonken et al. (2016) reported that the daily oscillation of clock gene expression is reduced in hippocampal microglia and may contribute to age-related neuroinflammatory sensitization. In this work, we observed that aging suppresses the endogenous circadian rhythm of RORa, a Bmal1 gene transcriptional activator, in the rat PFC. This might explain the loss of the BMAL1 circadian rhythm reported previously by our group (Coria-Lucero et al 2016), as well as the alterations in the temporal (circadian) organization of clock-regulated processes in the PFC of senile individuals. On the other hand, the circadian rhythm of TNF-α was also abolished in the PFC of aged rats, probably because of the loss of RORa oscillation.

In summary, increased oxidative stress leads to deterioration of the cellular clock and aging. In turn, loss of the clock's regulator, RORa, rhythm might cause increased oxidative stress and chronic inflammation. Thus, aging, together with, or, as a consequence of, an altered circadian clock, leads to the loss of the circadian organization of the studied parameters and increased oxidative stress in the PFC. Particularly, reduced antioxidant activity and increased oxidative stress during the subjective day, might underlie temporal alterations in

sleep-dependent memory consolidation in the PFC of aged individuals. We expect that our results may contribute to highlighting at least some of the biochemical, molecular and chronobiological targets of therapeutic strategies for the prevention and/or treatment of cognitive deficits associated with aging.

Acknowledgments and Funding

This study was supported by FONCyT, Préstamo BID, Argentina (Project PICT No: 2020-01575 by Dr. ACA), the National Council for Science and Technology (CONICET), Argentina (Project PIP No: 986 by Dr. ACA), the National University of San Luis (UNSL), and San Luis, Argentina (Project PROICO No: 2-0518 by Dr. ACA).

Authors' Contributions

ACA planned and designed the research. SMD, DCR, CMD and SEGM provided methodological support and advice; ITP, CCL, and MCDV carried out the experimental procedures; ITP and MGL obtained and analyzed the data. ITP and CMD performed the statistical analysis. ITP, ACA, SMD and SEGM wrote and revised the manuscript; SMD and ACA project administration and funding acquisition. All authors approved the final version of the manuscript.

Declaration of Conflicting Interests

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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