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Influence of Circadian Rhythmicity on Some Neurobiochemical Markers on Prefrontal Cortex Areas and Plasma of Male Wistar Rats

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Abstract: *The human body has the intrinsic ability to respond to day/night rhythm. The transition, from night to day or from day to night is marked by some already known physiological responses. The design of the present study examined the changes in brain oxidative and nitrosative stress markers and some biochemical parameters in the prefrontal cortical areas of male wistar rats in response to different circadian rhythm intervals. Twenty-four (24) male wistar rats weighing 80-120g were sampled in 4 groups of 6 rats each. Group 1 was control, and groups 2, 3, and 4 were test groups, exposed to short, mid, and prolonged periods of darkness, respectively. The study lasted for 21 days and the markers; Cortisol, Growth Hormone, Nitric Oxide, Nitric Oxide Synthase, Malondialdehyde, Protein Carbonyl, Isoprostanes, Glutathione, Glutathione Disulfide, Superoxide Dismutase and Glycated Hemoglobin, were assayed. The confidence interval for this study was 95%. There was a significant increase in; GH, NO, NOS, MDA, PC, F2isoP, GSH, GSSG, and HbA1C when compared with the control group, and a significant decrease in the following biochemical markers; Cortisol, PC, GSH, GSSG, and SOD when compared with the control group. The outcome of this study revealed some changes in brain oxidative and nitrosative stress markers and selected biochemical parameters in response to different intervals of circadian rhythm.*

Key words: *Circadian rhythm, Stress; Hormones; Biochemical; Brain.*

1. INTRODUCTION

Chronobiology is the branch of biology that studies the natural physiological rhythms of organisms, including people. One example of a type of natural cycle that is studied by chronobiologists is the circadian rhythm that governs our sleep schedule (Hülya Çakmur, 2018). Over the past decade, the field of chronobiology has rapidly expanded as researchers have begun unraveling the complex and integral role the circadian clock plays in most physiological processes. The field of chronobiology is not new, however. Since the 18th century, scientists have observed that many physiological processes in plants and mammals exhibited apparent 24-h rhythms (Richards & Gumz, 2012). Circadian rhythm is sometimes called the "body's clock." It is the natural cycle of physical, mental, and behavior changes that the body goes through in a 24-hour cycle (Man, 2020)". Circadian Rhythm is basically a biological clock that is built in our brain throughout the functionality of everyone's day and night processes, as per the 24-hour clock. The circadian rhythm of human body is a self-control system to regulate our eating habits, activities and body functionality, like getting hungry and food digested, passing urine and maintaining blood pressure, sleeping and awakening process, and temperature (Samithamby Senthilnathan & Kanthasamy Sathiyasegar, 2019). Circadian rhythms are entrained by light, and their 24-h oscillation is maintained by a core molecular feedback loop composed of canonical circadian ("clock") genes and proteins (Korshunov *et al.,* 2017). Circadian rhythms in organisms are involved in many aspects of metabolism, physiology, and behavior. In many animals, these rhythms are produced by the circadian system consisting of a central clock located in the brain and peripheral clocks in various

peripheral tissues (Ito & Kenji Tomioka, 2016). The suprachiasmatic nucleus (SCN) is the primary circadian pacemaker in mammals. For proper functioning of the circadian timing system, all the circadian clocks in the body must be kept synchronized with one another and to the 24-hr day; this is the function of the master circadian pacemaker, the suprachiasmatic nucleus (SCN) (Welsh *et al.,* 2010). The main function of the SCN is its work as a calibrator: it entrains the body to the environmental 24-h cycle of light and darkness. Light exposure impacts many physiological and metabolic processes including circadian rhythm, mood, metabolism, and eye growth. Light contributes to circadian rhythm by synchronizing diurnal activity of intrinsically photosensitive retinal ganglion cells, which modulate the release of the hormone melatonin (Ashutosh Jnawali *et al.,* 2017). The suprachiasmatic nucleus (SCN) of the hypothalamus functions as the master of the clock, synchronizing 24-h rhythms in the body's physiological behavior, including other brain regions and peripheral tissues. Identical clock oscillators to the SCN clock have been established in peripheral tissues such as gastrointestinal tract, liver, muscle, adipose tissue, and cardiovascular tissue. The clock induces sleep and other related anabolic functions at night when synchronized according to the environment, such as immune function and hormone release, and wakefulness and its associated catabolic functions throughout the day, i.e., food intake and metabolism and physical activity. Circadian fluctuations in the brain can lead to changes in the levels of these neurotransmitters (Estrada-Rojo *et al.,* 2020). Several hormones were shown to have daily oscillations, and among these the best characterized are melatonin, cortisol, gonadal steroids, prolactin, thyroid hormone and growth hormone (GH). The so-called nutrient-sensitive hormones, namely insulin, leptin, ghrelin and adiponectin also oscillate on a circadian basis (Davide Gnocchi & Giovannella Bruscalupi, 2017). Brain monoamines - such as noradrenaline (NA), dopamine (DA) and serotonin (5-HT) - regulate several important physiological functions, including the circadian rhythm (Matsumura *et al.,* 2015). Recently there has been an increase in the diagnosis and treatment of circadian disruptions and sleep disorders (Liu *et al.,* 2022). The aim of this study is to investigate the neurochemical changes in response to disruption in circadian rhythm that occur in the prefrontal cortex of male Wistar rats. This study will contribute to a better understanding of the underlying mechanisms of the circadian rhythmic changes and its impact on brain functions and behavior with potential implication for the diagnosis and treatment of circadian rhythm disorders.

2. Materials and Methods

Ethical approval

This study was approved by the Ethical Considerations Committee, Directorate of Research and Human Development, Madonna University, Elele, Rivers State, Nigeria. The reference Number is MUECC/20230148.

Materials used for this study include;

Chemical agents

Distilled water, normal saline, methylated spirit and lithium heparin.

Feed

Pelleted rat feed. The brand was Top Feed®.

Apparatus and Equipment

Metabolic cages and beddings, feeding troughs, trampoline, surgical gloves, orogastric tubes, micro capillary tube, plain sample bottles, lithium heparin sample bottles, test tubes of various sizes, dissecting set, dissecting board, water bath, cotton wool, medicated soap, desiccator bottle, slides and cover slips, animal weighing balance and electronic weighing scale.

Animal collection

A total of 24 male wistar rats weighing 80-120g were used for this study. The rats were purchased and housed in the Research Animal House in Madonna University, Elele Campus, River State, Nigeria. The animals were allowed to acclimatize for a period of one (1) week and exposed to 12/12 hours light/dark cycle. The animals had access to feed and water, *ad libitum*. Acclimatization lasted for two (2) weeks prior to the onset of this study.

Table1. *Study design*

Groups	Exposure
1	Normal
2	Darkness till 10am
3	Darkness till 12pm
4	Darkness till 2pm

Study duration

After one (1) week of acclimatization, this study lasted for four (4) weeks.

Biochemical assay

The biomarkers assayed include: Cortisol (CORT), Growth hormone (GH), Nitric oxide (NO), Nitric Oxide Synthase (NOS), Malondialdehyde (MDA), Protein Carbonyl (PC), Isoprostane (F_2isoP), Superoxide dismutase (SOD), Reduced glutathione (GSH), Oxidized glutathione (GSSG) and Glycated Hemoglobin (HbA1C)

Assay for cortisol

The principle of the following enzyme immunoassay test follows the typical competitive binding scenario (Ilochi, et al, 2018). Competition occurs between an unlabeled antigen (present in standards, control and patient samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibodies binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed is inversely proportional to the concentration of cortisol in the sample. A set of standards is used to plot a standard curve from which the amount of cortisol in patient samples and control can be correctly read.

Assay for growth hormone

The principle of the following enzyme immunoassay test follows a typical one-step capture or 'sandwich' type assay (Ilochi, et al, 2019) . The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for hGH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of hGH is conjugated to horse radish peroxidase (HRP). hGH from the sample and standards are allowed to bind simultaneously to the plate and to the HRP conjugate. The washing and decanting steps remove any unbound HRP conjugate. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stopping solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed by the enzymatic reaction is directly proportional to the concentration of hGH in the sample. A set of standards is used to plot a standard curve from which the amount of hGH in subject samples and controls can be directly read.

Assay for nitric oxide and nitric oxide synthase

In aqueous solution, nitric oxide rapidly degrades to nitrate and nitrite. Spectrophotometric quantitation of nitrite using Griess Reagent is straightforward, but does not measure nitrate. This Nitric Oxide Synthase Colorimetric Assay kit employs the NADH-dependent enzyme nitrate reductase for conversion of nitrate to nitrite prior to the quantitation of nitrite using Griess reagent — thus providing for accurate determination of total NO production. This Nitric Oxide Synthase Colorimetric Assay kit can be used to accurately measure as little as 1 pmol/µL (~1µM) NO produced in aqueous solutions. Very little sample is required (5 to 85 µL depending on the [NO] in the sample. The completed reaction is read at 540 nm.

Assay for malondialdehyde

Under acidic condition, MDA produced from the peroxidation of fatty acid membranes react with chromogenic reagent, 2- thiobabituric acid to yield a pink colored complex with is measured at 532nm. The unit is mmol/g.

Assay for protein carbonyl

The reaction between 2,4 dinitrophenylhydrazine (DNPH) and Protein carbonyls form a Schiff base that produces the corresponding Hydrazone which can be analysed spectrophotometrically.

Assay for isoprostanes

The assay principle for measuring isoprostanes involves quantifying the levels of specific isoprostane molecules, such as 8-isoprostane or 15-F2t-isoprostane, in biological samples (Ilochi, et al., 2021). This kit is a competitive enzyme-linked immunoassay (ELISA) for determining levels of 15-F2t-Isoprostane in biological samples. Briefly, 15-F2t-Isoprostane in the samples or standards competes with 15-F2t-Isoprostane conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-F2t-Isoprostane coated on the microplate. The HRP activity results in color development when the substrate is added, with the intensity of the color proportional to the amount of conjugated15-F2t-Isoprostane bound and inversely proportional to the amount of unconjugated 15-F2t-Isoprostane in the samples or standards.

Assay for superoxide dismutase

The activity of SOD to inhibit the auto-oxidation of epinephrine at pH 10.2 make this reaction a basis for a simple assay for this dismutase.

Assay for reduced glutathione and glutathione disulfide

The low concentration of GSSG (high GSH/GSSG ratio) in tissues coupled with the need to prevent GSH oxidation during sample preparation, are important considerations for the accurate measurement of GSSG and GSH/GSSG ratios. Guntherberg and Rost first reported the use of N-ethylmaleimide (NEM) reacting with GSH to form a stable complex, therefore removing the GSH prior to the quantification of GSSG in tissues. Unfortunately, NEM inhibits GR. To overcome this problem, Griffith employed 2-vinylpyridine (2-VP) to derivatize GSH. Although 2-VP does not significantly inhibit GR, it reacts relatively slowly with GSH and is not very soluble in aqueous solutions.

Assay for glycated hemoglobin

The HbA1c test is based on a colorimetric, enzymatic method. The concentrations of HbA1c and total hemoglobin are determined separately. Calculation of the HbA1c ratio from total hemoglobin is done by the instrument automatically.

Statistical analysis

In other to properly translate the outcome of this study, data collected from this study was analyzed using One-Way analysis of variance (ANOVA) and Post Hoc analysis with the aid of IBM®SPSS Version 20.0. The Percentage change (*%c*) was also presented in standard charts and tables, adopting methods used by Ilochi, et al., 2024.

3. RESULTS

Table2. *Cortisol and Growth hormone levels in the blood*

Groups	CORT (U/ml)	GH (U/ml)
Control	1.13±0.01	0.02±0.01
Darkness till 10am	0.98±0.17	0.08±0.01
Darkness till 12pm	0.46±0.17*	1.14±0.01*
Darkness till 2pm	0.16±0.04*	1.44±0.08*
Total	**2.73**	**2.68**
Average	**0.68**	**0.67**

Key=asterisk (*) indicates statistical significance, p≤ 0.05 = significantly different compared to control

Table3. *Nitric oxide and Nitric oxide synthase levels in prefrontal cortex*

Group	NO (mmol/g)	NOS (mmol/g)
Control	0.03±0.01	0.04±0.01
Darkness till 10am	0.70±0.13*	1.54±0.46*
Darkness till 12pm	0.06±0.02	0.05±0.01
Darkness till 2pm	0.02±0.01	0.04±0.01
Total	**0.81**	**1.67**
Average	**0.20**	**0.42**

Key=asterisk (*) indicates statistical significance, p≤ 0.05 = significantly different compared to control

Table4. *Malondialdehyde, carbonyl proteins and F_2 Isoprostanes in prefrontal cortex*

Group	MDA (mmol/g)	PC (mmol/g)	F2isoP (mmol/g)
Control	0.05±0.00	2.38±0.07	0.05±0.01
Darkness till 10am	1.13±0.03*	2.66±0.09*	0.44±0.24*
Darkness till 12pm	0.04±0.01	1.42±0.12*	0.03±0.01
Darkness till 2pm	0.02±0.00	1.16±0.05*	0.01±0.00
Total	**1.24**	**7.82**	**0.53**
Average	**0.31**	**1.91**	**0.13**

Key=asterisk (*) indicates statistical significance, p≤ 0.05 = significantly different compared to control

Table5. *Reduced glutathione, oxidized glutathione and superoxide dismutase in prefrontal cortex*

Group		GSH (U/g)	GSSG (U/g)	SOD (U/g)
Control		2.33±0.10	0.94±0.08	2.60±0.13
Darkness till 10am		0.62±0.12*	2.60±0.11*	0.44±0.14*
Darkness till 12pm		1.98±0.06	0.94±0.12	2.66±0.16
Darkness till 2pm		3.76±0.27*	0.38±0.06*	2.86±0.06
Total		**8.69**	**4.86**	**8.56**
Average		**2.17**	**1.22**	**2.14**

Key=asterisk (*) indicates statistical significance, p≤ 0.05 = significantly different compared to control

Table6. *Glycated hemoglobin in plasma*

Group	HbA1c (%)
Control	4.28±0.06
Darkness till 10am	5.08±0.10*
Darkness till 12pm	4.22±0.02
Darkness till 2pm	4.26±0.04
Total	**17.84**
Average	**4.46**

Key=asterisk (*) indicates statistical significance, p≤ 0.05 = significantly different compared to control

4. DISCUSSION

Studies have shown that disruptions to the normal functioning of brain areas can lead to various health issues. For example, individuals with sleep disorders, such as insomnia or shift work disorder, often exhibit altered neurobiochemical profiles in brain areas involved in circadian rhythm regulation. Imbalances in neurotransmitters like serotonin, dopamine, and melatonin have been observed, which can contribute to sleep disturbances and mood disorders (Walker et al., 2020). Disturbances in circadian functions and sleep have long been associated with metabolic, psychiatric, and neurodegenerative disorders. However, increasing evidence suggests that such disturbances may not always be secondary symptoms but may in fact contribute to the etiology and severity of disease states (Joiner, 2018) The parameter assayed in this study include; Cortisol, Growth Hormone (GH), Nitric Oxide (NO), Nitric Oxide Synthase (NOS), Malondialdehyde (MDA), Protein Carbonyl (PC), Isoprostanes (F2isoP), Glutathione (GSH), Glutathione Disulfide (GSSG), Superoxide Dismutase (SOD), and Glycated Hemoglobin (HbA1C). After 2 weeks of exposure to different period of darkness (short, mid and prolonged) there was a significant alteration in the assayed parameters. The serum cortisol concentration decreased in the group subjected to mid period and prolonged period of darkness when compared to the control group. The synthesis and secretion of cortisol are controlled by the hypothalamic–pituitary–adrenal axis. In the evening and nighttime, reduced light exposure signals the brain to produce and release melatonin, a hormone that promotes sleep and relaxation. Melatonin, in turn, inhibits the release of cortisol, resulting in lowered levels during the dark period. Melatonin might act on the adrenal glands as an endogenous pacemaker. Glucocorticoids levels are low after the onset of darkness and rise after the middle of the night accompanied with a decrease in melatonin levels (Arendt & Aulinas, 2022). Cortisol follows, circadian rhythm, which are the body's internal clock. In human and rodents, cortisol levels peak in the morning (approximately 250–850 nmol/L between 8:00 and 10:00 a.m.) and gradually declines throughout the day (110–390 nmol/L) and the lowest levels at around 04:00 a.m. (Syahira et al., 2021). During the dark period, the suppression of light stimuli may have contributed to decease in cortisol levels as observed in Group 3 and 4. Cortisol being widely known as body's stress hormone

(Thau et al., 2022), cortisol is released in response to stress (Bastien Sadoul & Geffroy, 2019). Cortisol levels may also be affected by stress. Due to the absence of certain stressors and decrease in external stimulations and activities during dark periods, it might have resulted in the decreased level of cortisol production in Group 3 and 4. The decrease in cortisol levels during this period helps facilitate relaxation, promote sleep onset, and support the body's recovery processes. Therefore, this decrease in cortisol during the dark period can be considered beneficial for the body's overall well-being (Sherrell, 2020). The serum GH concentration increased in the group subjected to mid period and prolonged period of darkness when compared to the control group. The increase in the concentration of serum GH may be due to presence of internal biological clocks in the rat that regulate their behavior and physiology. Growth hormone is secreted from the adenohypophysis of the pituitary gland and is regulated by a final common pathway, consisting of inhibitory control of somatostatin (SST) and stimulatory control of hypothalamic GH-releasing hormone (GHRH) (Nassar et al., 2007). Human growth hormone (GH) secretion is subject to a circadian and diurnal rhythm that follows a sleep pattern. Specifically, the peak in hGH secretion occurs during sleep (deep/slow wave sleep) in the dark stage of the daily cycle (Ilochi et al., 2024). Research studies by Valcavi et al., and Nassar et al., provides evidence that melatonin, a hormone produced in response to darkness, plays a facilitatory role in the neuroregulation of growth hormone (GH) secretion at the hypothalamic level. Melatonin itself can therefore directly stimulate the release of GH, contributing to the increased levels observed during the dark period. The increase in human growth hormone during the mid and prolonged period may be as a result of the facilitatory role of melatonin in the neuroregulation of growth hormone (GH) secretion at the hypothalamic level which resulted in the elevated levels of growth hormone in the dark period. The increase of Growth hormone during this dark period is beneficial as GH plays a crucial role in stimulating growth, development, tissue repair, cell regeneration, and repair processes in the body (Admin, 2018). The level of NO and NOS concentration significantly increased in the group exposed to short period of darkness(5pm-10am), when compared to control. The significant increase in NO AND NOS in this group may be due to the increase in neuronal activations in the rats. Nitric oxide (NO) is an unorthodox messenger molecule, which has numerous molecular targets. NO controls servo regulatory functions such as neurotransmission or vascular tone (Ulrich Förstermann & Sessa, 2011). It plays various roles in neuronal signaling, synaptic plasticity, and cerebral blood flow regulation. Since NO is known to relax blood vessels and to increase blood supply to the brain, this action of NO can also be assessed to have a role in inducing neuronal activity (Paul & Ekambaram, 2011). NO is produced in the brain by specific neurons through the enzymatic action of nitric oxide synthase (NOS). Neuronal activity, particularly excitatory synaptic transmission, can trigger the release of NO. Calcium influx into neurons during synaptic activity activates neuronal NOS (nNOS), leading to the production of NO. Rats are nocturnal animals, meaning they are more active during the night and tend to sleep during the day. As a result, the neuronal activation in the rat Prefrontal cortex (PFC) is generally higher during their active nighttime phase (Andrea Rørvik Marti et al., 2020) which is the short period of darkness in this study. During the night, rats engage in various behaviors such as exploration, foraging, and social interactions. These activities require cognitive processing and decision-making, which are closely associated with PFC function. Therefore, the rat PFC exhibits increased neuronal activation during their active phase due to their activeness and engagement in these activities at night. NOS expression and activity can be upregulated in response to neuronal activation. When neurons are activated, particularly during synaptic transmission, it can lead to an increase in NOS expression and subsequent production of nitric oxide (NO). This activation-induced upregulation of NOS can occur through calcium-mediated signaling pathways. Glutamate, the primary excitatory neurotransmitter in the brain, can trigger calcium signaling and influence NO production in the PFC through glutamate receptor activation. The extreme increase of NO & NOS in this period could be detrimental as excessive production of nitric oxide (NO) can lead to the formation of reactive nitrogen species, such as peroxynitrite, which can induce oxidative stress. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the ability of cells to neutralize them. ROS can damage cellular components, including lipids, proteins, and DNA, and disrupt normal cellular functions (Lubos et al., 2008). In the context of the Prefrontal cortex, oxidative stress resulting from excessive NO production can contribute to neuronal injury, impair synaptic plasticity, and promote neurodegenerative processes. It can lead to cellular dysfunction, inflammation, and neuronal damage, ultimately affecting cognitive function and overall brain health (Paul & Ekambaram, 2011). Oxidative stress is also an important factor in human health and disease occurrence. In general, an appropriate balance is needed between the production and

elimination of oxidative stress. If this balance is broken, biochemical damage due to oxidative stress may occur, and disease may develop or worsen by this damage. It is related to the aging process and is known as a major risk factor for degenerative diseases in the elderly (Chi Eun Oh et al., 2022). Considering that both oxidative stress and circadian rhythm are involved in maintaining the homeostasis of the body, there is a possibility that oxidative stress and circadian rhythm are related to each other. Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or nonradical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides (Ayala et al., 2014). Lipid peroxidation or reaction of oxygen with unsaturated lipids produces a wide variety of oxidation products. The main primary products of lipid peroxidation are lipid hydroperoxides (LOOH). Among the many different aldehydes which can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA) (Ayala et al., 2014). It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids. The two most prevalent ROS that can affect profoundly the lipids are mainly hydroxyl radical (HO•) and hydroperoxyl ($HO•^2$). The hydroxyl radical (HO•) is a small, highly mobile, water-soluble, and chemically most reactive species of activated oxygen (Ayala et al., 2014). Lipid peroxidation is considered a marker of oxidative stress. Lipid peroxidation is a hallmark of oxidative stress, and excessive production of lipid peroxidation products has been implicated in the pathogenesis of a number of human diseases. Lipid peroxidation can cause damage to cellular membranes through disturbance of membrane organization and alteration of membrane integrity, fluidity, and permeability (Gaschler & Stockwell, 2017). Malondialdehyde has been widely used for many years as a convenient biomarker for lipid peroxidation of omega-3 and omega-6 fatty acids because of its facile reaction with thiobarbituric acid (TBA) (Hermann Esterbauer & Cheeseman, 1990). MDA is an end-product generated by decomposition of arachidonic acid and larger PUFAs, through enzymatic or nonenzymatic processes. The increase in MDA concentration in the short period of darkness may be as a result of an influence on the cumulative effect of oxidative stress and lipid peroxidation. A relatively shorter exposure to darkness may lead to a more concentrated and pronounced increase in MDA levels within the PFC. It may lead to a more rapid accumulation of ROS within the PFC. These ROS can react with unsaturated fatty acids in cell membranes, initiating lipid peroxidation and resulting in the production of MDA. The study by Krzysztof Michal Tokarz et al., shows that relatively pronounced increase of MDA were observed during shorter duration of exposure to darkness. In a shorter period of darkness, the antioxidant capacity of the PFC may be limited, leading to a reduced ability to scavenge and neutralize ROS. The imbalance between ROS production and antioxidant defense can contribute to increased lipid peroxidation and subsequent MDA formation. According to the changes in circadian rhythms in humans and animals, oxidative stress reactions and the expression of antioxidant enzymes show circadian changes. These studies showed that oxidative stress indicators increased mainly during periods of high activity among the circadian rhythms, and the expression of the antioxidant system increased simultaneously to reduce the occurrence of oxidative stress (Ayala et al., 2014). Therefore, the increase in MDA observed during the short period of darkness in the PFC may be considered unfavorable or potentially harmful. It indicates an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms, leading to increased lipid peroxidation and cellular damage. Protein carbonylation is a type of protein oxidation that can be promoted by reactive oxygen species. Oxidative stress is a major cause of post-translational changes in proteins and includes severe chemical modifications (Gonos et al., 2018). The formation of protein carbonyls typically responds to the oxidative deamination of alkaline amino acids such as lysine, arginine and proline (Ilochi, et al., 2021). Protein carbonylation is a post-translational modification that occurs when proteins are oxidatively damaged by reactive oxygen species (ROS) or reactive carbonyl species. It involves the addition of carbonyl groups (such as aldehydes and ketones) to specific amino acid residues in proteins, resulting in the formation of protein carbonyl groups. The increase in protein carbonyl content during the short period of darkness is typically associated with oxidative stress and reactive oxygen species (ROS) generation. The increase in protein carbonyl levels in short period of darkness may be as a result of the unavailability and inactivity of antioxidant defense mechanisms in the Prefrontal cortex during the short period of darkness which can influence the extent of protein carbonylation. During the short period of darkness, antioxidant systems may be overwhelmed or insufficient to counteract the increased ROS production, leading to a significant increase in protein carbonyl content. The decrease in protein carbonyl levels in mid and prolonged period of darkness may

be as a result of the restoration of oxidative balance or an upregulation of antioxidant defense mechanisms in the prefrontal cortex. A significant increase in the percentage change of protein carbonyl content during the short period of darkness in the prefrontal cortex (PFC), followed by a decrease during the mid period and prolonged period, is generally indicative of oxidative damage and considered unfavorable or "bad". The observed significant increase in the percentage change of protein carbonyl content during the short period of darkness suggests an elevated oxidative stress level in the PFC during that time. This increase indicates that the balance between ROS production and antioxidant defense mechanisms is disrupted, leading to oxidative damage to proteins. The subsequent decrease in protein carbonyl content during the mid period and prolonged period may indicate a restoration of oxidative balance or an upregulation of antioxidant defense mechanisms. The isoprostanes (IsoPs) are a unique series of prostaglandin-like compounds formed in vivo via a nonenzymatic mechanism involving the free radical-initiated peroxidation of arachidonic acid induced by reactive oxygen species (ROS) (Milne et al., 2011). These can contribute to the impairment of the chemical and physical properties of cell membranes that give rise to the oxidative damage. In general, an increase in isoprostane levels indicates an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms in the PFC. The increase in the levels of isoprostane in the short period of darkness, may be as a result of an elevation in oxidative stress and lipid peroxidation in the brain region being studied. Elevated isoprostane levels are commonly observed in various pathological conditions associated with oxidative stress, including neurodegenerative disorders, cardiovascular diseases, and inflammation. Long-term or chronic elevation of isoprostane levels would be of greater concern, as sustained oxidative stress and lipid peroxidation can lead to tissue damage, inflammation, and contribute to the development or progression of various diseases (Miller et al., 2014). Superoxide is a primary oxygen radical that is produced when an oxygen molecule receives one electron. Superoxide dismutase (SOD) plays a primary role in the cellular defense against an oxidative insult by ROS (Fujii et al., 2022). SOD is an important enzyme that plays a crucial role in neutralizing and scavenging superoxide radicals, which are reactive oxygen species (ROS) generated during normal cellular metabolism.

The decrease in SOD concentration in the short period of darkness may be as a result of a decrease in the antioxidant defense mechanisms during the dark period, it could lead to oxidative stress and a subsequent decrease in SOD levels. The production of free radicals exceeded the body's antioxidant defenses and oxidative stress occurred. Prolonged or excessive oxidative stress in the short period of darkness depleted SOD levels and this enzyme became overwhelmed by the increased demand to neutralize free radicals. However, if the decrease in SOD levels is persistent or significant, it can have negative consequences. SOD plays a crucial role in the body's defense against oxidative stress and helps prevent cellular damage caused by free radicals. When SOD levels are depleted, there is a reduced ability to neutralize superoxide radicals, leading to an imbalance between ROS production and antioxidant defenses. This imbalance can contribute to increased oxidative damage to cellular components, including proteins, lipids, and DNA. GSH is highly abundant in all cell compartments and is the major soluble antioxidant. GSH/GSSG ratio is a major determinant of oxidative stress. GSH shows its antioxidant effects in several ways. It detoxifies hydrogen peroxide and lipid peroxides via action of GSH-Px (Esra Birben et al., 2012). The short period of darkness may lead to increased oxidative stress in the PFC of rats. During this time, the balance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms may shift towards higher ROS levels. As a consequence, GSH, which acts as a major antioxidant and plays a vital role in neutralizing ROS, may be depleted as it reacts with and neutralizes these harmful molecules. The decrease in GSH levels may also be linked to changes in the activity or expression of antioxidant enzymes involved in maintaining GSH levels. For example, decreased activity of enzymes like glutathione peroxidase (GPx) or glutathione reductase (GR) during the dark period may impair the regeneration of GSH from its oxidized form (glutathione disulfide or GSSG), leading to a decline in GSH levels. The prolonged dark period may result in a shift in the redox balance of the PFC, favoring an increase in GSH levels. This could be due to a decrease in the production of reactive oxygen species (ROS) during the dark period, resulting in less oxidative stress and allowing GSH levels to accumulate. The short period of darkness may lead to increased oxidative stress in the PFC. During this time, there might be an imbalance between reactive oxygen species (ROS) production and the antioxidant defense mechanisms, resulting in higher ROS levels. The accumulation of ROS can lead to the oxidation of reduced glutathione (GSH) to GSSG, causing an increase in GSSG levels. The decrease in antioxidant enzyme activity during the

short period of darkness may contribute to the increase in GSSG levels. Antioxidant enzymes, such as glutathione peroxidase (GPx) and glutathione reductase (GR), play a crucial role in converting GSSG back to GSH. If their activity is reduced during this period, the regeneration of GSH from GSSG may be impaired, leading to an accumulation of GSSG. Glycated hemoglobin (HbA1c) is a measure of long-term glucose control and is typically used as a marker for diabetes management. Hemoglobin becomes glycated when it is exposed to high blood glucose levels over an extended period (Ilochi et al., 2018). The short period of darkness may have disrupted glucose metabolism. Changes in glucose uptake, utilization, or regulation can lead to increased blood glucose levels, which may contribute to glycation. Insulin plays a crucial role in glucose regulation. Insulin is inhibited in the short period of darkness and glucagon is secreted, which may have resulted in reduced glucose uptake by tissues, leading to higher blood glucose levels and potential glycation.

5. CONCLUSION

In conclusion, the overall neurobiochemical changes in specific brain areas in response to circadian rhythm fluctuations in male wistar rats provides valuable insights into the intricate relationship between circadian rhythmicity and brain function. The findings of the study indicate that circadian rhythm fluctuations have a significant impact on the neurobiochemical composition of specific brain areas in male wistar rats.

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