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AGE RELATED CHANGES IN THE CIRCADIAN RHYTHM OF MELATONIN AND SOME NEUROTRANSMITTERS IN THE MALE RATS

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ABSTRACT
Ageing refers to a series of time-dependent anatomical and physical changes that reduce physiological reserve and functional capacity. Age leads to important circadian rhythm changes in some physiological parameters like hormonal secretion and neurotransmitters contents. The present work aimed to study the age-related changes in the circadian rhythm of melatonin, acetylcholine Ach, serotonin 5-HT, and norepinephrine NE. Rats were divided into two groups, adult and aged rats, that maintained under 12: 12 LD. Ach, 5-HT, and NE levels were measured in the cerebral cortex and hippocampus by enzyme-linked immunosorbent assay (ELISA) and the fluorimetric method respectively. Melatonin levels in the serum were assayed by radioimmunoassay. Acetylcholine levels in the cerebral cortex of adult rats were peaked at the dark phase of the light-dark cycle. On the contrary, its level in aged rats peaked at the light phase of the light-dark cycle. Moreover, cortical acetylcholine levels in aged rats were decreased nearly at all time intervals as compared to the adult group. Serotonin contents of both the cerebral cortex and the hippocampus of aged rats were also decreased. Its rhythm was phase delayed in the cerebral cortex and exhibit a biphasic pattern in the hippocampus with peaks at light and the dark phases. NE contents in the cortex and hippocampus of adult rats peaked during the light phase. While, its rhythm displayed a biphasic pattern in the aged rat cortex. Melatonin levels of aged rats were decreased at most time intervals and exhibit an advance in the beginning of increment and decrement but, the peak was demonstrated at the same time as adults. In conclusion, the neurotransmitters in the studied brain areas as well as the melatonin levels were declined during the aging process and exhibit a shift or disturbances in its circadian rhythm which may result from atrophy of the brain tissue and abnormalities of the circadian system respectively.

KEYWORDS: Melatonin, Acetylcholine, Serotonin, Norepinephrine, Circadian Rhythm, Aging, Rats.

INTRODUCTION
Ageing is a series of time-dependent anatomical and physical changes that reduce physiological reserve and functional capacity.[1] Processes of decline that are associated with growing old can be divided into primary and secondary ageing.[2] Factors of decrement in primary ageing are determined by hereditary influences. Secondary ageing refers to defects and disabilities that are caused by hostile factors in the environment, including trauma and acquired diseases.[3] One of the important changes during ageing is the loss of irreplaceable cells, most noticeably in the skeletal muscles, heart, and brain. In the brain, neurons shrink and disappear, and alterations occur in neuronal synapses and networks. The loss of neurons, particularly those in the vulnerable areas of hypothalamus, may contribute to certain physiological changes including altered metabolism and circadian rhythm, and are associated with mental and emotional aberrations in the elderly.[3]

Most physiological processes in our body have a circadian rhythm. These include cerebral activity (sleep-wake cycles), metabolism and energy homeostasis, body temperature, heart rate, blood pressure, renal activity, and hormone as well as cytokine secretion.[4] This rhythm is generated by a molecular clock in the suprachiasmatic nucleus (SCN) located in the ventral hypothalamus, which is the circadian pacemaker,[4] Moreover, circadian cellular oscillators (peripheral clocks) have been described in almost all cell types. The cellular circadian oscillators in all cells have the same molecular mechanism.[5,6] The SCN and the peripheral clocks form the circadian system, in which the SCN synchronizes peripheral clocks through a wide range of mechanisms.[4]

Ageing is characterized by circadian timekeeping abnormalities.[7] These abnormalities result from the loss of synchronization between the multiple internal and external oscillators. This desynchronization associated

with poor neurobehavioral performance.[8] In fact, it has long been known that cognitive function is disturbed if there is a temporal misalignment between the sleep and clock-driven mechanisms. Therefore the present study aimed to investigate the changes in the daily profiles of some neurotransmitters as an indication of the neurophysiological state during aging process as well as the changes in the circadian rhythm of melatonin, as an important factor in the circadian rhythm disruption.

MATERIALS AND METHODS

Experimental animals and design

The experimental animals used in this study were male albino rats (*Rattus norvegicus*) that divided into two groups. Adult rats weighing (100-120 g) served as control and aged rats weighing (340- 360g). They were purchased from the breeding unit of the animal house of National Research Center (Giza, Egypt). Adult and aged rats were maintained under the normal light-dark cycle with 12 h of light (from 0700h to 1900h) and 12 h of darkness (from 1900h to 0700) per day with free access of food and water.

Methods and Techniques

After one month at a four-hour- intervals throughout 24 h cycle (at 0700h, 1100h, 1500h, 1900h, 2300h and 0300h), six male rats from each group were sacrificed by sudden decapitation. Blood samples were collected and the brains were removed.

Brain sampling

Heads of sacrificed rats were immediately dissected after decapitation and brains were rapidly excised from skulls, blotted and chilled. Brains were rapidly wiped dry with filter paper, and dissected on an ice cooled glass plate according to Glowinski and Iversen.[9]

Cerebral cortex, and hippocampus were removed, weighed, wrapped in plastic films then in aluminum foils and quickly frozen in a deep freezer at (-70^{o}C) till used.

Estimation of Acetylcholine

Acetylcholine (Ach) levels were assayed by enzyme-linked immuno sorbent assay (ELISA) using the rat acetylcholine ELISA kit according to the manufacturer instructions (Catalog. No: WAR-421).

Estimation of Serotonin and Norepinephrine

In the present study NE and 5-HT were extracted and estimated in the brain areas by the fluorimetric method according to the method of Chang[10] modified by Ciarlone.[11] The fluorescence was measured by fluorimeter model 6200.

Estimation of melatonin

Melatonin levels were assayed in the serum by radioimmunoassay using melatonin direct Serum/Plasma/Saliva RIA (RE29301) In Radioisotopes Department. Nuclear Research Center. Atomic Energy Authority. Egypt.

Statistical analyses of data

The data obtained in the present study were represented as mean ± S.E.. The statistical analyses were carried out between the means of control and aged group. As well as between different time intervals of the same group according to one way analysis of variance (ANOVA). **Duncan's test**[12] was performed using the statistical package for the social science (SPSS) version 16.

RESULT

Acetylcholine rhythm in the cerebral cortex and the hippocampus of adult and aged rats

Acetylcholine levels in the cerebral cortex of adult group showed a significant increase at 0700h, 1100h, 1500h and 1900h ($P < 0.05$) compared to its minimum value at 0300h.The maximum value of acetylcholine was observed at 2300h. In Aged rats acetylcholine levels peaked at 1500h then decreased significantly to its minimum value at 1900h ($P < 0.05$) compared to different time intervals of the same group (Fig.1). By comparing adult and aged groups, significant decreases were observed ($P < 0.05$) in acetylcholine levels of aged rats at 0700h, 1100, 1900h and 2300h.

In hippocampus, the acetylcholine levels of adult group peaked significantly at 0700h then sharply decreased reaching its minimal value at 1500h ($P < 0.05$). Oppositely, the minimum value of acetylcholine in the hippocampus of aged rats was observed at 0700h. Its levels increased sharply to the maximum value at 1100h then decreased significantly thereafter ($P < 0.05$) Fig.(2).

Significant increases of acetylcholine levels ($P < 0.05$) in hippocampus of aged rats were detected at 1100h,1500h, 1900h, 2300h and 0300h, as compared to adult group (its corresponding control). While, a significant decrease was observed at 0700h.

Serotonin rhythm in cerebral cortex and hippocampus of adult and aged rats

In adult group, the cerebral cortex serotonin content showed gradual and significant increase that began at 0700h, until reaching its maximum value at 1500h ($p<0.05$) as compared to the minimum decline at 0300h (Fig. 3) in the same group. Similarly, serotonin contents in the cerebral cortex of aged rats increased significantly ($p<0.05$) at 0700h, 1100h, 1500h with a peak at 1900h as compared to the minimum values of the same group at 2300h, 0300h (Fig. 3). The cerebral cortex serotonin contents in aged group decreased significantly comparing to adult group at 0700h, 1100h, 1500h and 2300h ($P < 0.05$). In contrast, a significant increase was observed at 1900h (Fig.3).

Figure (4) indicated that hippocampus serotonin contents of adult rats increased significantly from 0700h and 1100h to reach its significant peak at 1500h as compared to its minimum decline at 0300h ($P<0.05$). While in aged rats, hippocampus serotonin contents peaked twice significantly at 0700h and 2300h as compared to the

minimum declines that were observed at 1100h and 1900h. A significant decrease was shown in the hippocampus serotonin content of aged rats as compared to the adult group at 1100h, 1500h and 1900h (P<0.05); while at 0700h, 2300h and 0300h there were a non significant differences. Fig.(4).

Norepinephrine rhythm in cerebral cortex and hippocampus of adult and aged rats

Norepinephrine content in the cerebral cortex of adult group increased significantly at 0700h and 1100h until reaches its peak at 1500h then decreased gradually to reach its minimum value at 0300h (P<0.05%). On the other hand, the norepinephrine contents in the cerebral cortex of aged rats showed a significant increase at 1500h, 1900h, with a peak at 2300h as compared to the minimum decline that recorded at 1100h.Fig.(5). By comparing the cerebral cortex norepinephrine contents of aged rats to the adult group; it was clear that there were significant increases in norepinephrine levels of aged group at 0700h, 2300h and 0300h and significant decreases at 1100h, 1500h and 1900h (P<0.05%). Fig. (5).

Norepinephrine content in hippocampus of adult group (control group) showed its maximum significant peak at 1100h then decreased gradually to reach its significant minimum decline at 1900h compared to different time intervals of the same group(P<0.05%). Where in aged group; the hippocampus norepinephrine content showed its maximum significant peak at 0700h compared to its minimum decline that recorded at 1900h in the same group. Fig. (6). On the other hand, the significant decreases were observed at 1100h, 1900h and 2300h and 0300h (P<0.05%) as compared the hippocampus norepinephrine content of aged rats to its corresponding control (adult group). Fig. (6).

It was clear that in adult group the serum melatonin levels showed gradual and significant increases started at 1900h, until reaches its maximum elevation at 0300h. However, the minimum decline was observed at 1500h compared to different time intervals of the same group at P < 0.05 (Fig.7). In aged rats, melatonin levels began to increase significantly at 1500h until reach to the maximum values at 1900h, 2300h, and 0300h as compared to the minimum value at 1100h (P < 0.05). Although melatonin levels of aged rats decreased significantly at 0700h, 1100h, 2300h and 0300h, there were significant increases at 1500h and 1900h (P < 0.05) (Fig.7).

Figures

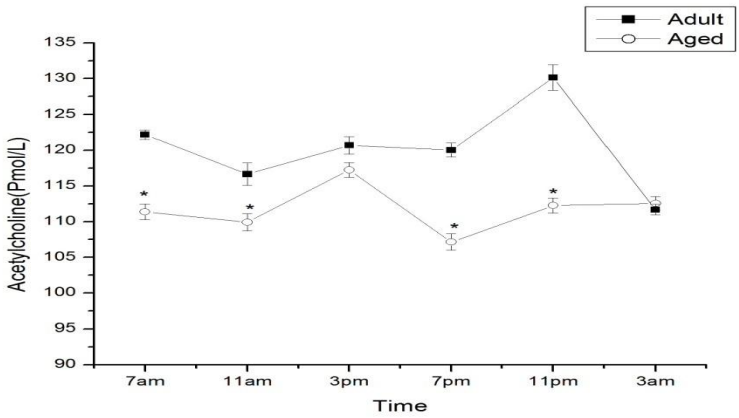

Figure (1): Daily profile of acetylcholine levels in cerebral cortex of adult and aged rats.

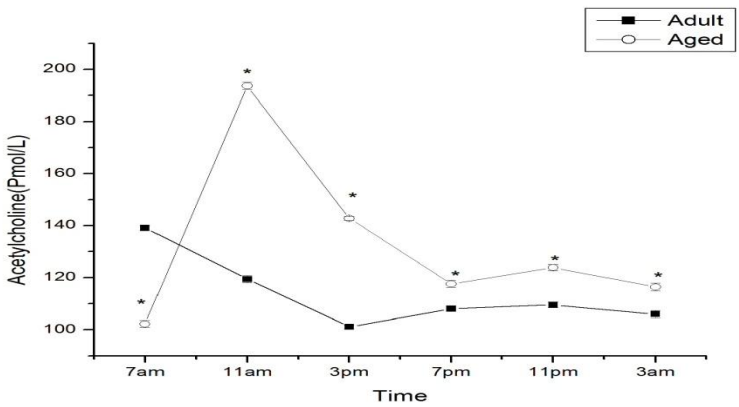

Figure (2): Daily profile of acetylcholine levels in the hippocampus of adult and aged rats.

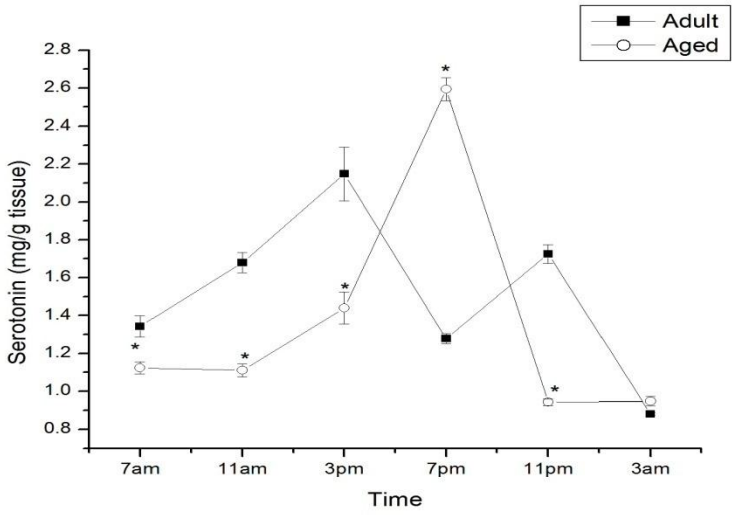

Figure (3): Daily profile of serotonin content in the cerebral cortex of adult and aged rats.

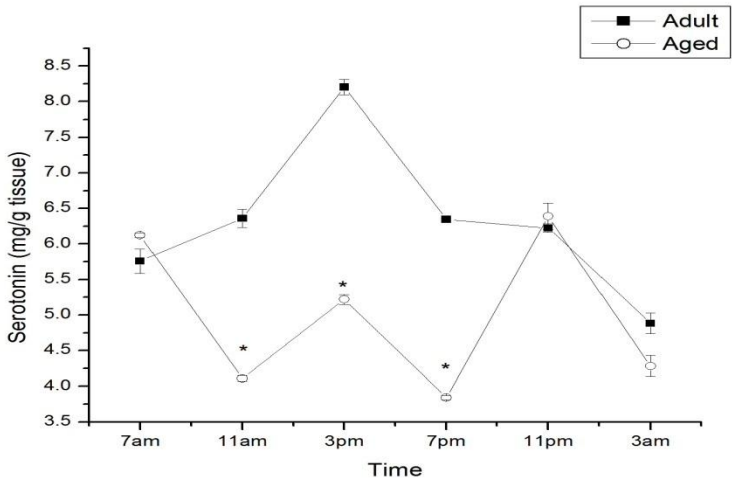

Figure (4): Daily profile of serotonin content in the hippocampus of adult and aged rats.

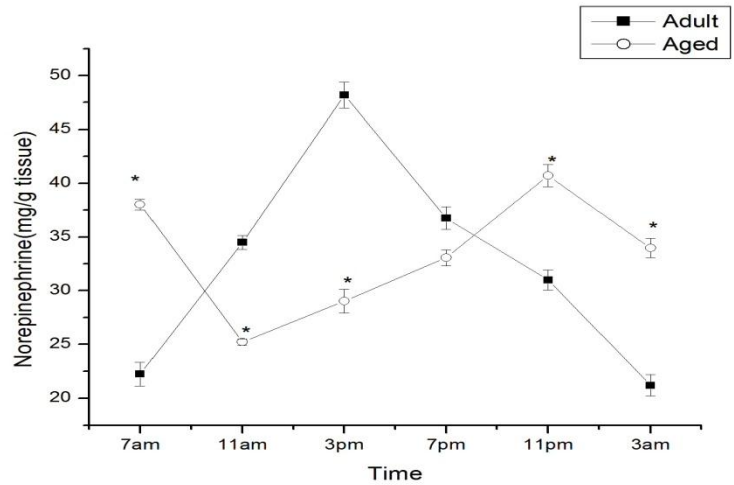

Figure (5): Daily profile of norepinephrine content in the cerebral cortex of adult and aged.

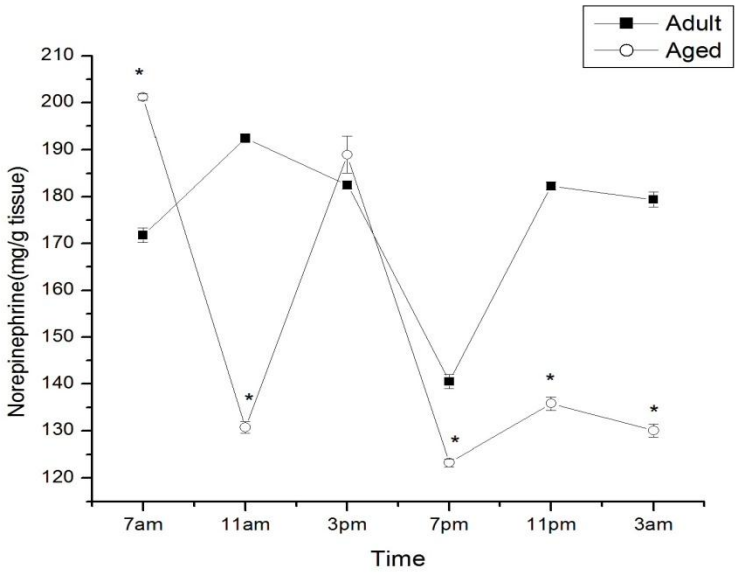

Figure (6): Daily profile of norepinephrine content in hippocampus of adult and aged rats.

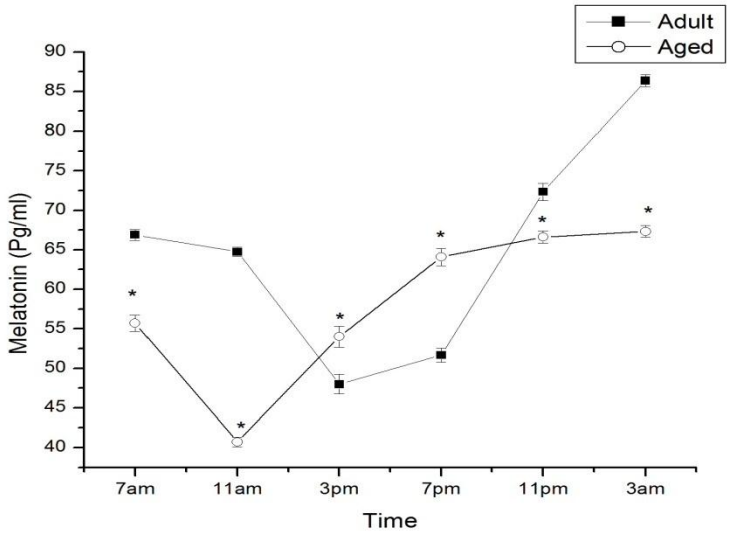

Figure (7): Daily profile of melatonin levels in adult and aged rat.

DISCUSSION

Aging is associated with a large variety of multi-organic changes that altogether produce characteristic behavioral patterns that are typically associated with old age and that are mistakenly viewed as a normal and predicable aspects of ageing.[13] One of these main changes concerns the circadian rhythms.[7] The important changes in the circadian rhythm include changes in the amplitude and the phase of the rhythm. The amplitudes of hormone secretion, sleep-wake cycles, body temperature, and other physiological parameters decline during the aging process of humans.[4] In extreme cases, old human subjects may become arrhythmic.[4]

In the present study, acetylcholine levels in the cerebral cortex of adult rats have peaked at the dark phase of the light-dark cycle. This result agreed with the study of Kametani and Kawamura[14] who reported that cortical acetylcholine exhibited clear circadian rhythmisity under (LD 12:12) with the highest peak in the dark phase and the lowest peak in the light phase. On the contrary, the level of cortical acetylcholine in aged rats of this study peaked at the light phase of the light-dark cycle. This advance in the circadian rhythm of acetylcholine may be due to circadian system disruption which are characteristic of the ageing process.[7]

Moreover, cortical acetylcholine levels in aged rats of this study were decreased nearly at all time intervals compared to adult group. Similarly,[15,16,17] stated that aging reduces acetylcholine and the integrity of the basal forebrain cholinergic neurons that project to cortical areas**.** Hippocampal acetylcholine level of aging rats in the present study was increased more than that of the

adult one. Its circadian rhythm was peaked at the light phase of the LD in both the adult and aged rats with a phase delay in the aged group.

In the human, the 5-HTergic and NAergic systems, both appear to remain stable with aging. Most studies, thereby report that the levels of both 5-HT and NE remain unchanged in the cerebral cortex, hippocampus, basal ganglia and brainstem during the course of aging.[18, 19, 20] There are, however notable exceptions, with studies showing reductions of 5- HTin the hippocampal formation and globus pallidus[21], and increases in the mesencephalon, medulla oblongata and putamen[21,22], and reductions of NE in the cingulated cortex.[23]

The present study revealed a decrease in the serotonin contents of both the cerebral cortex and the hippocampus of aged rats compared to adult group. This finding is not only in agreement with Gozlan et al. and Lee et al.[24,25] who reported a decrease in 5-HT levels in the whole cortex of rats and mice, but also agreed with Koprowska et al.[26] who indicated a similar decrease in the rat hippocampus during the course of aging.

Previous studies revealed that the release of serotonin in the brain is highest during periods of activity and in nocturnal rodents, its peak occurs around the time of lights off.[27, 28] In this study, the circadian rhythm of serotonin in adult rats cerebral cortex and hippocampus peaked around afternoon. In the aged rats, the rhythm was phase delayed in the cerebral cortex and exhibit a biphasic pattern in the hippocampus with peaks at light and the dark phases.

Norepinephrine contents in the cerebral cortex of aged rats in the present study fluctuate between decrement and increment while the hippocampal NE content decreased with aging. Several studies indicated that levels of NE in the neocortex and hippocampus have both been shown to increase[29,30], remain unchanged[31,32] and decrease during aging.[25]

In adult rats of the present study, the NE contents of the cortex and hippocampus peaked during the light phase. This result can be confirmed by[33] who indicated that NE contents in the SCN in rats kept under LD or constant dark (DD) conditions displayed significant variations over one day with a peak during the subjective day and a minimum value during the subjective night. On the other hand, the NE rhythm displayed a biphasic pattern in the aged rat cortex of the present study.

Briefly, the neurotransmitters in the studied brain areas were declined during the aging process and exhibit a shift or disturbances in its circadian rhythm which may result from atrophy of the brain tissue[34, 35, 36, 37] and abnormalities of the circadian system[7] respectively.

Melatonin is synthesized by the pineal gland predominately in a circadian manner; however, there is also non-circadian production in other organs.[38] Its concentrations in the body are lower during the day and reach to maximal levels at night.[39] In the present study, the melatonin levels of adult rats have low values during the daytime and high values at night.

Melatonin system is one of the physiological mechanisms underlying the circadian rhythm disruption.[40] With aging melatonin nocturnal levels either decrease slightly with maintained circadian rhythmicity or display circadian arhythmicity with equal day and night time melatonin levels. In individuals with maintained circadian rhythmicity, it has been noticed that there is a consistent phase-advance of the nocturnal plasma melatonin peak compared to young individuals.[41] These changes in melatonin secretion during aging may be due to pineal gland dysfunction, impaired pineal innervation/interconnection between the suprachiasmatic nucleus and the pineal gland, generalized central nervous system (CNS) dysfunction or, degeneration of SCN and changes in its gene expression.[41]

In the present study, melatonin levels of aged rats were decreased at most time intervals and exhibit an advance in the beginning of increment and decrement but, the peak was demonstrated at the same time as adults. These results were in agreement with several studies that reported decreases in melatonin levels during aging process.[42,43,44]

In conclusion, aging process causes a decline in the levels of acetylcholine, serotonin, norepinephrine as well as the melatonin secretion. In the mean time, the circadian rhythm of the neurotransmitters and melatonin were phase shifted in aged rats. These effects may result from neurodegenerations and disruption of SCN clock.

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