CHARLES UNIVERSITY IN PRAGUE

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

EFFECTS OF CHRONIC GROWTH HORMONE AND MELATONIN ADMINISTRATION ON EEG DELTA POWER IN OLD RATS

Diploma thesis

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Declaration

I hereby declare I have worked on this project solely by my own with the use of referenced literature and I am presenting my own original data.

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Abstrakt

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Spánek pomalých vln (slow wave sleep), který se ve spektrální analýze EEG vyznačuje zvýšenou delta aktivitou (obvykle 0.1-4 Hz), je považován za hlavní složku hlubokého spánku a je spojován s jeho anabolickými a regeneračními vlastnostmi, jež jsou nezbytné pro dobré duševní i fyzické zdraví. Úbytek delta aktivity představuje jednu z charakteristických změn ve spánku při stárnutí. Abychom zjistili účinky podávání růstového hormonu a melatoninu na EEG spektrum, implantovali jsme elektrody pro EEG a EMG mladým (3-4 měsíčním) a starým (22-23 měsíčním) samcům potkana kmene Wistar. Po dobu jednoho měsíce byl jedné skupině starých zvířat podáván růstový hormon intraperitoneálně, druhé skupině starých zvířat melatonin v pitné vodě. Další dvě skupiny neléčených mladých a starých potkanů sloužily jako kontrolní. Po skončení podávání látek bylo nahráno a analyzováno EEG a EMG. Následně bylo použito Rychlé Fourierovy transformace k výpočtu EEG spektrální aktivity pro REM a NREM spánek. Naše výsledky ukazují velké rozdíly mezi mladými a starými kontrolními potkany v EEG aktivitě delta, i v některých dalších frekvencích. U starých zvířat, kterým byl podáván růstový hormon nebo melatonin, jsou hodnoty delta aktivity a celkově celé EEG spektrum frekvencí porovnatelné s hodnotami u mladých potkanů. Tyto výsledky naznačují, že alespoň některé změny ve spánku spojené se stárnutím mohou být napraveny podáváním růstového hormonu nebo melatoninu.

Abstract

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Title of diploma thesis: Effects of chronic growth hormone and melatonin administration on EEG delta power in old rats.

Slow wave sleep (SWS), characterized by an increase in delta power (typically 0.1-4 Hz) of EEG spectral analysis is considered to be a core component of deep sleep and is associated with sleep's anabolic restorative properties necessary for good mental and physical health. The reduction of delta power represents one of the hallmarks of sleep alterations with age. In order to study effects of chronic growth hormone (GH) and melatonin administration on sleep EEG power spectra, young (3-4 months) and old (22-23 months) male Wistar rats were implanted with EEG and EMG electrodes. During one month a group of old animals was treated with GH administered intraperitonealy (i.p.), second group of old animals was treated with melatonin diluted in drinking water. Additional two groups of young and old rats with no treatment served as controls. After the treatment, EEG and EMG were recorded and analyzed. Then Fast Fourier transform was used to compute spectral power of REM and NREM sleep. Our results show rapid differences in sleep EEG delta power as well as in some other frequencies between young and old control rats. Old animals treated with GH or melatonin exhibit values of delta power and generally whole EEG power spectra comparable with young rats. These results suggest that at least some of the age-related changes in sleep can be positively adjusted by GH or melatonin treatment.

CONTENT

ABBREVIATION

AA-NAT	Arylakylamine *N*-acetyltransferase
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
FFT	Fast Fourier transform
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
IGF-1	Insulin-like growth factor-1
NREM	Non-rapid eye sleep
PSG	Polysomnography
REM	Rapid eye movement
SCN	Suprachiasmatic nucleus
SD	Sleep deprivation
SWA	Slow-wave activity
SWS	Slow-wave sleep
TST	Total sleep time

1. INTRODUCTION

The overall ageing of population is one of the most distinctive demographic events of current era. The worldwide prolongation of the mean life expectancy has resulted in rapid rise in number of the elderly population. Increasing number of potential beneficiaries of health and social funds causes many social and economic problems since these individuals are supported by a relatively smaller number of potential contributors (United Nations, 2001a).

Therefore there is a need for any therapeutic agent improving the quality of life of the elderly, maintaining high cognitive and physical function, and continued engagement with life. The main goal of gerontological research and geriatric care should be adding more life to our years rather than adding more years to our live. The most ambitious aim of improving the well-being of the growing elderly population in our societies would be a delay in the onset of age-related diseases and slow down of their progression (Poeggeler, 2005).

A series of studies of the sleep characteristics in healthy older adults suggest the importance of sleep quality as a marker of overall health, well-being, and adaptability in later life (Hoch et al, 1994; Dew et al 1994; Dew et al 2003). Epidemiological studies have documented an increased prevalence of sleep complains with advancing age. Nearly half of older adults experience difficulty initiating and maintaining sleep. Several changes occur during ageing that can be the cause of sleep disturbance, including increased prevalence of various medical conditions, increased use of medication, changes in circadian rhythms, and environmental and lifestyle changes (Foley et al, 1995). Sleep complaints frequently mentioned in relation to ageing are sleep interruptions, early morning awakening, difficulty falling asleep connected with waking not refreshed, daytime napping and sleepiness (Foley et al, 1995; Kamel and Gammack, 2006; Groeger et al, 2004).

This correlates with high rate of prescriptions for hypnotics in aged, although the effectiveness of current hypnotics remains unsatisfactory (Glass et al, 2005). Moreover, the side-effects that are associated with use of hypnotics and sedatives, such as day time fatigue, cognitive impairments, falls and fractures are particularly dangerous for older people. Certain classes of sedatives such as benzodiazepines and nonbenzodiazepines

can also cause tolerance and physical dependence after long-term treatment (Barbone et al, 1998; Neutel et al, 2002).

Physiological studies of sleep have documented marked age-related changes in sleep including diminished slow-wave sleep (SWS), rapid eye movement (REM) sleep, increased number of awakenings and decreased total sleep time (TST), along with the proportion and distribution of sleep stages and the circadian sleep-wake cycle. Each of these age-related sleep changes has led to the characterization of the sleep of the elderly as lighter or more "fragile" than that of younger adults (Crowley, 2011). Many of these age-related changes have been also documented in rats, including reductions in high voltage non-rapid eye movement (NREM) sleep, REM sleep, mean duration of sleep episodes, a decrease in the length and an increase in the number of sleep bouts (Mendelson and Bermann, 1999a; Mendelson and Bermann, 1999b; Roumen and Moyanova, 2002). These changes in sleep can be interpreted within the framework of the homeostatic and circadian regulation of sleep (Dijk et al, 1999; Niggemayer et al, 2004; Buysse et al, 2005).

Age-related changes in sleep represent only a small piece of the complex array of chronobiological changes in physiological systems that accompany the ageing process. There are bidirectional interactions between sleep and endocrine system. The plasma concentrations of many hormones show sleep-related changes, suggesting that sleep influences hormone secretion. Some hormones are even directly affected by sleep. But daily rhythm in sleep/wake and other behaviors cannot solely fully explain the existence of a day/night rhythm in hormone levels. Hormones are also affected by an endogenous timing system. Sleep and the endogenous timing system actually interact in order to regulate secretion of many hormones. In healthy individuals and under normal circumstances, the behavioral cycle and endogenous timing system are synchronized at an appropriate phase angle. However, when the sleep/wake cycle and endogenous timing system are disturbed or desynchronized, normal day/night variations in numerous hormones are altered which may have adverse health consequences (Morris et al, 2012). Along with the changes in sleep and desynchrony in circadian rhythms, ageing is also accompanied by overall decline of neuroendocrine functions. That could be a potential factor in the development of several age-related diseases. Although the ageing process itself may not have a neuroendocrine basis, identification and correction of the related neuroendocrine dysfunction may be important for improving the quality of life (Rehman and Masson, 2001).

Growth hormone (GH) and melatonin are one of the best documented hormones strongly associated with sleep and ageing. Most of total GH secretion occurs in correlation with the SWS, the deepest stage of NREM sleep. GH secretion progressively decreases with age, following a pattern very similar to that of SWS decrease (Van Cauter et al, 2004). Moreover some of the symptoms of ageing, including decrease in muscle mass, increased adiposity, reduced libido and energy, resemble symptoms of GH deficiency. Therefore it is assumed that age-related decrease in GH secretion accounts for or contributes to the development of many of these symptoms (Rudman et al, 1990).

Melatonin, also called the "hormone of darkness", is secreted from pineal gland during night in both day-active (diurnal) and night-active (nocturnal) animals. Probable function of melatonin secretion is to adjust the phase and synchronize internal circadian rhythms with day/night cycle (Challet, 2007). Many important physiological processes such as the sleep–wake cycle, the core body temperature, alertness, performance and secretion of many hormones show a regular circadian rhythmicity in healthy young organisms. These rhythms play an important role in maintaining health and well-being. With advancing age pineal synthesis of melatonin decreases significantly in various species, including humans and rats. The decrease of melatonin levels in advanced age may lead to disturbances in the circadian pacemaker, which causes internal temporal desynchronization causing a variety of chronopathologies and leading to generalized deterioration of health. Therefore, exogenous melatonin might have beneficial effects in terms of ageing due to its association with circadian timing system (Armstrong and Redman, 1991; Karasek, 2004; Poeggeler, 2005). Moreover, other studies have demonstrated very potent gerontoprotective actions of melatonin, most of them related to its powerful antioxidant activity, chronobiology influence and immunomodulation, which could all contribute to the observed anti-ageing potential of this natural agent (reviewed by Poeggeler, 2005).

Based on these findings we decided to use GH and melatonin treatment on aged rats in present sleep study. Usefulness of a rat species in neurophysiology research has been previously described. Their smaller size, easy caring and lower costs are benefits for the use of this animal. Moreover, compared to human studies, the variance produced by external factors is minimized in laboratory animals (Datta and Hobson, 2000).

Using spectral power analysis for EEG recordings quantification provides important information about sleep depth and intensity that is normally not captured by

sleep-stage scoring. Spectral analysis is sensitive to the amplitude of EEG recording. The EEG signal is digitalized, filtered and the spectral power density is calculated in standard frequency bands using fast Fourier transform (FFT). The low-frequency waves that are apparent during SWS of NREM are showed as an increase in spectral power in the delta range (typically 0.5-4 Hz) (Van Cauter, 2004). The delta power of computer detected slow-wave activity (SWA) in the EEG is considered to be an indicator of sleep intensity and is associated with sleep's anabolic restorative properties necessary for good mental and physical health. The reductions in delta frequencies of SWS have been thought to reflect a biological marker of the progressive sleep changes connected with ageing (reviewed by Crowley, 2011). SWA delta power is increased in cortical areas that have been most active during the day and also after sleep deprivation (SD), so it seems to reflect a form of restorative process for the brain cells of cortical local circuits (Mignot and Huguenard, 2009).

2. THEORETICAL PART

2.1. Sleep

Sleep is characterized by the absence of consciousness, an increased arousal threshold to sensory functions and reduction of motor output. Humans spend approximately one third of their lives sleeping. Although sleep has been intensively studied and discussed, its biological function is still not well understood. Commonsense and subjective observations clearly suggest some energy restoration effects of sleep. The important role of sleep and its physiological function has been better documented as a negative, by experiments what happens without sleep. Experiments with SD have demonstrated the necessity of sleep. It is known that sleep is essential, and that SD either resulting from lifestyle or a sleep disorder, causes various adverse effects and can ultimately lead to death. Short-term SD leads to impaired attention and concentration, reduced productivity and overall decrease in quality of life. Long-term consequences of SD include increased morbidity and mortality from coronary artery disease, heart failure, high blood pressure, obesity, type 2 diabetes mellitus, stroke and memory impairment as well as depression. Sleep is considered to have restorative, conservative, adaptive, thermoregulatory and memory consolidative functions (Chokroverty, 2009; Cirelli and Tononi, 2008). There are also findings indicating a specific role in immune responses, since sleep and the circadian system are strong regulators of immunological processes (Besedovsky et al, 2012)

The normal sleep of mammalian species including human is divided into two main distinct states based on three physiological measurements (EEG, EOG and EMG), including NREM sleep and REM sleep. In humans, NREM and REM sleep normally alternate or cycle between these states over approximately 90-min intervals during the night. Although this NREM-REM cycle length remains largely stable over the whole time of sleep, the ratio of NREM to REM changes within each 90-min cycle. Brain activity in NREM sleep and REM sleep is remarkably different. The characteristics of REM sleep are rapid eye movements, muscle atonia and desynchronous brain waves. Sawtooth wave pattern in EEG is often present. In this sleep state most memorable dreaming occurs (reviewed by Carskadon and Dement, 2011).

NREM sleep is further divided into 3 sub-stages (N1, N2, N3), based on EEG criteria and corresponding with increasing depth of sleep. Stages N1 and N2 are considered light sleep and stage N3 as a deep sleep. Stage N1 sleep is characterized by

relatively fast-wave EEG. It refers to the transition of the brain from alpha waves (8-13 Hz) common in the wake state to theta waves (4-7 Hz). In stage N2 EEG frequency is further slowing down while EEG amplitude increases. Stage N2 NREM is characterized by the presence of mixed frequency theta activity, presence of phasic electrical events including K-complexes (large electrical sharp waves in the EEG) and sleep spindles (short synchronized bursts of EEG electrical activity in the 11-15 Hz range). The deepest stage N3 of NREM sleep is often referred to as SWS. It is characterized by computer detected SWA, a low-frequency and high-amplitude delta waves (0.5-4 Hz) on EEG (reviewed by Carskadon and Dement, 2011).

SWS is considered to be the main component of the deep restorative sleep necessary for normal physical and intellectual performance and behavior. The delta power of computer detected SWA in the EEG is considered to be an indicator of NREM sleep intensity. Delta power is increased in cortical areas that have been most active during the day and also after SD, so it seems to reflect a form of restorative process for the brain cells of cortical local circuits. Imageing techniques also discovered that there is a localized decrease in metabolic activity at night in areas that have been activated during the day (Mignot and Huguenard, 2009).

2.2. Age related changes in Sleep

Both subjective and objective measures of sleep indicate that ageing is associated with some significant changes in sleep structure across entire human life span from early childhood to the very old age. These changes eventually often lead to problems sleeping and decline in overall satisfaction with quality of sleep, both during the night and during daytime. With age, several changes occur that can cause a risk for sleep disturbance including increased prevalence of medical conditions, increased medication use, age-related changes in various circadian rhythms, and environmental and lifestyle changes. Although sleep complaints are common among all age groups, older adults have increased prevalence of many primary sleep disorders. Nearly half of older adults report difficulty initiating and maintaining sleep (Ohayon et al, 2004; Foley et al, 1995).

The most common opinion supported by evidence suggests that older people have a decreased ability to sleep, rather than a decreased need for sleep. The total sleep time over 24 h either remains stable or may decrease with older age, and there is a decrease in sleep efficiency (i.e., the amount of time asleep over the time in bed). Common subjective characteristics of poor sleep quality and quantity are trouble falling asleep, waking up in the early morning hours, disturbed or "light" sleep, multiple nocturnal awakenings, decreased total time asleep and excessive daytime sleepiness connected with napping during the day. Using measurements such as polysomnography (PSG) and particularly EEG analysis, we can find objective evidence of changes in sleep structure, deficits in total sleep and sleep efficiency, as well as increased sleep latency and awakenings during the night. In addition, there is an age-related reduction in specific sleep stages. Studies comparing sleep across different age groups found that the percentage of lighter stages of sleep (N1 and N2) increases with age, while the percentage of deeper stages of sleep (N3 or SWS) decreases. In fact, stages 3 sleep may be completely absent in very old people. Sleep becomes more fragmented and less efficient as we age. Other changes in older people include an earlier onset of REM sleep during the night, a slight decrease in total REM sleep, and more equal distribution of REM sleep throughout the night, as compared to the relative increase in amount of time spent in REM as the night progresses in younger people. The most dramatic age-related change in sleep structure documented during sleep studies is a significant reduction of

SWS during NREM sleep stage. This change is characterized by reduction of delta power, reflecting in both amplitude and incidence of delta waves during NREM sleep EEG. In young people SWS is main component of sleep and is associated with sleep's anabolic restorative properties necessary for good mental and physical health. Therefore the reductions in SWS have been thought to reflect a biological marker of the progressive sleep changes connected with age. Studies suggest that some of these age-related changes in sleep are already evident in middle aged participants (Ohayon et al, 2004; Lapid et al, 2010; Crowley, 2011).

2.3. Sleep regulation

In mammals, including rats and humans, the alternation between wakefulness, sleep and the structure of sleep itself are regulated by the interaction of two physiological processes: outputs of the endogenous circadian pacemaker, that is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and a homeostatic process (reviewed by Cajochen et al, 2006).

2.3.1. Homeostatic regulation

The homeostatic process is thought to reflect the need or pressure for sleep, which builds up during sustained wakefulness and wears off during sustained sleep. One of the best established facts in sleep regulation in mammals is that SWS and computer detected delta activity of EEG increases as function of previous wakefulness in cortical areas that have been most active during the day and progressively decreases in the course of sleep. Therefore such slow-wave activity, which is the most pronounced EEG feature of NREM sleep, seems to reflect a form of restorative process. It is also considered to be marker of this homeostatic process and a reliable predictor of sleep depth and intensity (Borbély, 2001).

There are several hypotheses addressing how metabolic pathways interact with homeostatic sleep/wake regulation. Most significant of recent findings demonstrates involvement of adenosine in interaction between sleep and metabolic homeostasis. An accumulation of adenosine is supplied from Adenosine triphosphate (ATP) (Chikahisa and Séi, 2011***)***. ATP has been recognized as an intracellular energy source in the brain and multiple other tissues. The complex regulation of mitochondrial ATP synthesis is triggered within the human brain in response to daily synaptic activity (Kagawa, 2010). In recent study it was observed in rats that ATP levels in several parts of brain are stable during waking but exhibit a surge during the initial hours of sleep. This surge is dependent on sleep but not on time of day and positively correlates with the intensity of EEG delta activity during spontaneous NREM sleep. These findings suggest that ATP levels drastically change during sleep in several parts of brain and directly relate to SWA of NREM sleep (Dworak et al, 2010).

2.3.2. Circadian regulation

As a consequence of the Earth's rotation, almost all organisms experience day and night cycles. To adapt and synchronize biological rhythms to external daily cycles, organisms have evolved an internal time-keeping system. This daily time-keeping system is referred to as the circadian clock from the Latin *circa diem*, literally meaning 'approximately one day'. The mammalian circadian oscillator, located in the SCN of the anterior hypothalamus, serves as the principal source of rhythmic temporal information for virtually all physiological processes in the organism, including the alternation of sleep and wakefulness. This circadian system is characterized by endogenous rhythmicity with a period of approximately 24 h (Moore, 1997; Reppert and Weaver, 2002).

A proper entrainment of this endogenous clock mechanism to the outside world is ensured by a number of input signals, of which light, temperature, food intake and activity are the most important ones (Moore, 1996). The SCN takes the information about the lengths of the day and night from the retina, interprets it, and passes it on to the pineal gland located in the epithalamus. In response, the pineal gland secretes the hormone melatonin. Secretion of melatonin occurs at night and its presence provides information about night-length. Several studies have indicated that pineal melatonin secretion further affects SCN rhythmicity to modulate circadian patterns of activity and other processes (Reiter, 1991; Perreau et al, 2004). This circadian process alternates in cycles, affecting hormonal secretion, electrophysiology and behavioral variables, such as the propensity to sleep (Wehr et al, 2001).

The circadian pacemaker is important agent in the timing and consolidation of wakefulness and sleep by providing a signal that grows stronger during the daytime hours, peaks at approximately 21.00–22.00 h and dissipates rapidly after the onset of nocturnal melatonin secretion (Shochat et al, 1997). This signal opposes the homeostatic (wake-dependent) increase in sleep propensity. In the absence of this signal (after SCN lesion or during forced desynchrony, when wakefulness is scheduled out of phase with the endogenous circadian rhythm), sleep/wake episodes become fragmented and alertness is compromised (Dijk et al, 1992).

The demonstration that the circadian pacemaker plays a major role in the regulation of sleep timing, structure and consolidation suggests that age-related changes

in the characteristics of sleep could be caused by age-related changes in the circadian timing system and melatonin secretion. A review of available research shows a predominance of circadian rhythm-related changes with age, but these findings are not consistent. Most studies, report a decline in the amplitude of circadian markers, such as core body temperature, melatonin, and cortisol levels (reviewed by Cajochen et al, 2006).

The decrease in nocturnal secretion of melatonin during ageing can result in circadian rhythm disruption, most notably a phase advance. Individuals with an advanced phase rhythm would typically go to sleep around 19.00-21.00 corresponding to a drop in body temperature but then wake approximately 8 hours later between 3.00-5.00 in the morning. That is coherent with findings that older adults are usually much more likely to go to sleep and wake up somewhat earlier than younger adults. Such behavior leads to less total sleep time and significant daytime sleepiness. Examination of the relationship between circadian period and wake time, circadian phase, and diurnal preference (morningness/eveningness) in older subjects found no significant correlation between those measures, in contrast to findings in young subjects. These results provide further evidence that the circadian rhythms of older people are in some way dysfunctional or altered, but whether this is related to reduced sleep consolidation, altered sleep-wake timing, or reduced SWS with age remains unclear (Duffy and Czeisler, 2002). Age-related changes in the retina, the SCN, a decrease in the number of pinealocytes and the impairment of melatonin secretion have all been suggested as possible reasons for the disruption of the sleep/wake rhythms seen in the elderly (Van Someren et al, 2002).

2.4. Growth hormone

2.4.1. The relationship between nocturnal growth hormone secretion and sleep

There are bidirectional interactions between endocrine system and sleep. Sleep is one of important modulators of endocrine function, particularly of pituitary-dependent hormonal release. A number of hormones can affect sleep but the physiological significance of this hormonal modulation is still not fully understood. The plasma concentrations of many hormones show sleep-related changes, suggesting that sleep influences hormone secretion. But sleep and hormone levels may also correlate without causal relationship between them. For example, circadian or homeostatic regulation may synchronize these events. Marked sleep-related hormonal rhythms may also develop as secondary phenomena caused by changes in the activity of the autonomic nervous system during sleep (Obal and Krueger, 2004).

GH is one of the best documented hormones with a strong sleep-related secretory pattern. GH is synthesized and secreted by the somatotroph cells of the anterior pituitary. This process is controlled by two hypothalamic neurohormones; growth hormone-releasing hormone (GHRH), which stimulates, and somatostatin, which inhibits GH secretion. The somatotropic axis is a fundamental anabolic system for the body. It stimulates tissue growth via cell division and via stimulation of protein synthesis. GH acts in part directly and in part via insulin-like growth factor-1 (IGF-1) on target tissues. IGF-1 is hormone produced by the liver and also a local paracrine/autocrine substance synthesized in the tissues (Obal and Krueger, 2004).

Well-documented studies involving analyses of polygraphically recorded sleep and GH plasma levels revealed that there is a consistent relationship between the NREM SWS on EEG and GH secretion during early sleep, as well as during the later part of the night. Deconvolution procedure was used to estimate GH secretory rates, which were mathematically derived from plasma GH concentrations by eliminating the effects of hormonal distribution and clearance. This deconvolution procedure provides an accurate estimation of the amount of GH released during each secretory pulse rather than plasma levels o GH. Calculation of the secretory rate instead of using plasma GH concentration was an important technical development in the analysis of the relationship

between sleep and hormone secretions. This technique revealed that GH secretion occurs predominantly during appearance of delta waves on EEG. In studies examining GH secretion in normal young men of similar constitution, approximately 70% of GH pulses were observed during stage N3 of NREM sleep. The amount of GH secreted during these pulses quantitatively correlated with the duration of the SWS episodes. Furthermore, the longer the SWS episode, the more likely it was to be associated with a GH pulse. In normal adult men, GH secretion on sleep-onset is generally the largest pulse in the 24-h cycle. There are gender differences in GH secretion in both animals and humans. The sleep-related GH secretion is a robust phenomenon in young males. In females GH secretory pulses are more frequent and the amount of GH secreted in association with deep NREM sleep is a much smaller fraction of the total daily GH secretion than in men. Studies have shown that pharmacological enhancement of SWS results in increased GH release. This correlative evidence suggests there is a common mechanism underlying SWS generation and GH release. Nevertheless, this relationship does not seem to be obligatory, since nocturnal GH secretion can also occur in the absence of SWS. Approximately one-third of the SWS periods are not associated with detectable GH secretion. Because GH secretion is under dual stimulatory and inhibitory control, involving GHRH and somatostatin, respectively, the variability of somatostatinergic tone may underlie dissociations between SWS and nocturnal GH release (reviewed by Van Cauter et al, 2004).

2.4.2. Growth hormone secretion and ageing

There is a strong correlation between chronology of ageing and GH secretion and it follows a pattern very similar to that of delta power of SWS. In males, GH secretion during sleep progressively decreases starting in the third decade and practically disappears above the age of 50 due to GHRH hypoactivity and somatostatin hyperactivity. In women, the decrease in sleep-related GH secretion occurs after menopause (Van Cauter, 2000). It has been suggested that this age-related decline in somatotropic function may impact quality of life, since many elderly show symptoms of GH deficiency such as muscle atrophy, central obesity, sleep disturbances, and depression (reviewed by Hull and Harvey, 2003).

Age-related changes in GH secretion correlate with the decline of deep SWS. Studying the link between sleep and plasma GH is more difficult in animals than in humans because of the short sleep-wake cycles in most laboratory animals. Nevertheless, correlation between GH secretion and sleep was also discovered in rats (Kimura and Tsai, 1984; Mitsugi and Kimura, 1985).

2.5. Melatonin

Melatonin, also known chemically as N-acetyl-5-methoxytryptamine, is a natural molecule with functional activity occurring in unicellular organisms, plants, fungi and animals. In most vertebrates, including humans, melatonin is synthesized primarily in the pineal gland. However, biosynthesis of melatonin also occurs in other body tissues, including the retina, bone marrow cells, platelets, the gastrointestinal tract, skin and lymphocytes. Melatonin pineal secretion is regulated by the environmental light/dark cycle via the SCN with a nocturnal maximum and low diurnal baseline levels, allowing the entrainment of the circadian rhythms of several biological functions. This increase of melatonin levels provides a signal to all body cells about the onset of night. The diurnal changes of light intensity provide the daily clock and the seasonal changes of day length provide the yearly clock (reviewed by Pandi-Perumal et al, 2006).

2.5.1. Secretion, synthesis

The secretion of pineal melatonin is regulated by the retinohypothalamic tract, which projects from the retina to the SCN, the major circadian oscillator. SCN controls the release of noradrenaline from the sympathetic, autonomic neurones innervating the pineal gland. This multisynaptic neural pathway from the SCN to the pineal is activated during darkness, resulting in an increase in melatonin synthesis responsible for the dramatic diurnal rhythm in plasma melatonin concentration. Melatonin in pinealocytes is biosynthesized from amino acid tryptophan. Tryptophan is taken up from the blood and converted, via 5-hydroxytryptophan, to serotonin. Serotonin is then acetylated to form *N*-acetylserotonin by arylakylamine *N*-acetyltransferase (AA-NAT), which, in most cases, represents the rate-limiting enzyme. *N*-acetylserotonin is converted into melatonin by hydroxyindole *O*-methyltransferase. There is no storage of melatonin in the pineal gland or elsewhere in the body and the circulating melatonin is degraded rapidly by the liver. Therefore plasma (or saliva) levels of melatonin as well as urinary levels of the main metabolite 6-sulfatoxymelatonin reflect appropriately the pineal biosynthetic activity. Melatonin has both hydrophilic and lipophilic character. Upon its release into capillaries it diffuses through biological membranes with ease and can

exhibit actions in almost every cell in the body within a very short time period (reviewed by Pandi-Perumal et al, 2006).

2.5.2. Melatonin secretion and ageing

With advancing age and the onset and progression of age-related diseases, there is a significant decrease in nocturnal synthesis of melatonin in various species, including humans. This decrease in melatonin secretion during ageing correlates with higher incidence of sleep disorders and disturbances. The exact mechanism by which melatonin levels decline with ageing has not been fully understood. Several possible explanations have been suggested based on documented age-related decrease in the number of beta-adrenergic receptors in the pinealocytes, decreased activity of AA NAT, the key enzyme responsible for melatonin synthesis, increased clearance of plasma melatonin and degenerative changes of the neural structures like SCN controlling the pineal gland. Changes in SCN neurons also seem to play a crucial role in age-related deterioration in circadian clock function (reviewed by Poeggeler, 2005; Pandi-Perumal et al, 2006).

2.5.3. Effects of melatonin

Melatonin contributes to a number of physiological functions like regulation of circadian rhythms, sleep and body temperature, sexual maturation, immune function, antioxidant mechanisms, regulation of mood and cardiovascular functions. Some effects of melatonin are receptor mediated, while others are receptor independent and probably connected with its powerful antioxidant properties. The actions of melatonin have been attributed to the two subtypes of human melatonin receptors (MT1 and MT2). MT1 mostly present in the SCN while MT2 in the SCN and other areas of the brain, as well as in the periphery. It is believed that the SCN MT1 receptors are related to amplitude of SCN circadian rhythmicity, while MT2 receptors are involved in entrainment of circadian rhythms. The SCN controls the circadian rhythmicity of pineal synthesis of melatonin and also express high-affinity to melatonin receptors, therefore this hormone is thought to play a feedback role on the SCN, by adjusting the phase and synchronizing internal rhythms with day/night cycle (reviewed by Pandi-Perumal et al, 2006).

Melatonin is one of the most potent antioxidants effective in scavenging highly toxic hydroxyl radicals and is several times more efficient than vitamin E in neutralizing the peroxyl radicals. Therefore, melatonin can prevent or minimize damage caused by free radicals in deoxyribonucleic acid (DNA) and other macromolecules. Therefore, according to Free-radical theory of ageing, melatonin can significantly influent this process (reviewed by Poeggeler, 2005; Pandi-Perumal et al, 2006).

In many studies exogenous melatonin was reported to improve subjective and/or objective sleep parameters in patients suffering from insomnia, having sleep promoting effects such as reduced sleep latency, induction of sleepiness, increased total sleep time and sleep efficacy. The hypnotic and chronobiotic properties of melatonin make it an optimal candidate for sleep disorders treatment. Finally, melatonin might be effective in the prevention of neurodegenerative diseases. In animals, prophylactically administered melatonin either reduced the beta amyloid toxicity or totally prevented death of cells in the experimental models of Alzheimer disease. Melatonin also reduced oxidative damage in several models of Parkinson's disease (Karasek, 2004; Pandi-Perumal et al, 2005; Bubenik and Konturek, 2011).

Among various functions, the hypnotic and sleep/wake rhythm regulating effects of melatonin received wide scientific attention resulting in numerous studies both in animals and in human subjects. The finding that melatonin is secreted primarily during night-time, the close connection between nocturnal increase of endogenous melatonin, and the timing of human sleep and the sleep-promoting effects of exogenous melatonin suggests that melatonin is highly involved in the physiological regulation of sleep. A number of studies have also correlated age-related disturbances in sleep/wake cycle and decrease in melatonin secretion. Therefore, it was proposed that melatonin might have beneficial effects on sleep in terms of ageing because of its association with circadian timing system. The rapid decrease of pineal melatonin secretion in advanced age leads to disturbances in the circadian pacemaker, which causes desynchronization of internal time system inducing a variety of chronopathologies. This can ultimately lead to generalized deterioration of health and may significantly contribute to ageing process and make the organism more susceptible to old age diseases. Therefore this decline in melatonin secretion with age has been suggested as one of the main reasons for increased sleep disruption in older adults and periodic administration of exogenous melatonin may readjust and reset the circadian phase, synchronizing internal rhythms

and increasing their amplitude to normal in aged individuals (Karasek, 2004; Pandi-Perumal et al, 2005; Poeggeler, 2005).

3. AIM OF THE WORK

1. To qualitatively analyze sleep EEG activity and compare the main differences and changes in power spectra between groups of young and old rats.

2. To find out if supplementary doses of growth hormone or melatonin administered chronically during period of one month have any positive effects on the sleep EEG power spectra in old rats.

4. EXPERIMENTAL PROCEDURES

4.1. Materials and Methods

4.1.1. Animals

Two different age groups of male Wistar rats (Harlan, Barcelona, Spain) were used in this study. The young, age 3-4 months (n=6), weighing between 300-350 g and the old, age 22-23 months (n=18), weighing between 450-600 g. The animals were housed individually in Plexiglas cages under constant temperature (22±1 ºC) and maintained on 12/12 hours of light/dark cycle (lights turned on from 08.00 h to 20.00 h). Standard Panlab (Barcelona, Spain) laboratory animal food and water was available *ad libitum*. All procedures were performed following the 'Principles of Laboratory Animal Care' (NIH Publication no. 85-23, revised 1996), Spanish law RD 1201/2005 and in accordance with the guidelines of the local Bioethical Committee of the University of the Balearic Islands (Spain). Every effort was made to reduce the number of animals used and to minimize their suffering.

4.1.2. Surgery

All surgical procedures were performed under isoflurane (Abbott®, The Netherlands) inhalation anesthesia, which was administered first using ventilated chamber for initiating dose. After this dose the still animal was placed on the surgery desk with a pad preheated to approximately 30 ºC. Atropin (Braun®, The Netherlands) was injected to avoid a rise in salivary secretion. From now on the anesthesia was continuously administered through inhalation mask. Anesthetic depth and condition of the operated animals were checked by monitoring physiological parameters (immobility, absence of stimulus response, body temperature, oxygen saturation, and heart and respiration rate). Two stainless steel electrodes (Plastics One Inc., The Netherlands) were placed into the frontal cortex (2.0 mm posterior and 2.5 mm lateral to the bregma) for recording of EEG with another electrode screwed in the skull (10.0 mm posterior to the bregma in the midline) serving as reference. Two additional electrodes with silver wires were placed over the dorsal neck muscle under the skin for EMG recording. One extra screw (2.0 mm anterior to the bregma) was used for better

resistance. Electrodes were fixed on the skull using dental acrylic cement (Duralay®, USA). 0.3 ml of metamizol (Nolotil®, Portugal) was injected i.p. immediately after surgery to ease the postoperative pain of the animals.

4.1.3. Drug Treatment

Animals were allowed to recover from surgery for at least 10 days before start of the experiment. Rats were divided into four groups with different treatment. Group of 7 old rats received daily dose 1 mg per kg of melatonin (Fagron® Ibérica, Spain) diluted in drinking water. Fresh melatonin solution was prepared every day and given to the animals in desired amount based on water drunk in previous days. Drinking bottles were coated in opaque foil to prevent light-induced degradation of melatonin. The second group of 6 old animals was treated daily with growth hormone (Saizen®, Serono, Spain) in total dose 1 mg per kg. GH solution (c=2 mg/l) was administered i.p. in two separate doses during each day, always at 09.00 h and 18.00 h. All rats were weighted every week and melatonin/growth hormone doses were adjusted to achieve desired 1 mg per kg per day. Last two groups of 6 young and 5 old rats received no treatment and served as control groups.

4.2. Working procedures

4.2.1. Recording

After the one month of drug administration period we proceeded with the experiment. Just before the recording, rats were softly anesthetized with isoflurane (Abbott®, The Netherlands) inhalation anesthetics for easy manipulation during connecting the EEG and EMG electrodes with the head plugs, and lessening overall stress caused to the animals. The EEG recording box in which were the animals placed measured 25x25x30 cm with three mirror walls and the front one from transparent glass. The top of the box was open to facilitate freely moving conditions for the rats while wearing the head plugs. The head plugs were connected with a rotating system preventing twisting of the wires. Signals were sent through the rotating system to an amplifier (Grass®, USA) with 3 channels, connected with a computer in another room. The EEG and EMG recordings data were processed and recorded in Axoscope® 10.2 (Union city, CA, USA). EEGs were filtered with a 1-100 Hz band pass filter and sampled at a frequency of 256 Hz with a notch filter at 50 Hz. EMG was filtered with a 10-500 Hz band pass filter.

Mild behavioral stress signs (chewing, exploratory movements, grooming) were observed after connection to the system in the experimental cage. Therefore every animal was allowed to fully recover from the soft anesthesia and adapt to a new environment for at least 30 minutes before start of recording. Recordings took place between 10 AM to 7 PM for duration of 2 hours. The rat was continuously observed on the computer monitor from another room to indentify the behavior of the animal. Visual observance of the behavior was possible through four different cameras positioned around the recording box to cover all the possible angles and positions of studied animal. The behavior was classified directly by observing researcher in 4 different states: (1) active waking, (2) grooming, (3) passive open eyes and (4) passive closed eyes. A keyboard was used to manually enter observed behavioral state and include it in the polygraph recordings. In this way, animal behavior, EEG and EMG recordings could later be assessed.

4.2.2. Analysis of cortical EEG activity

All EEG, EMG and visual behavioral state recordings were analyzed using the recording in Axoscope® 10.2 (Union city, CA, USA). In order to analyze recorded data, two hours recordings of each rat were explored, excluding artifacts and manually selecting as many as possible of consistent 10 seconds epochs (examples showed in Fig. 1-3) of these three states: REM sleep, NREM sleep and vigilant/waking state based on following criteria:

REM sleep: low EMG and rapid, low-amplitude saw pattern EEG, state (4)

NREM sleep: low EMG, slow high-amplitude EEG, state (4)

Waking: high EMG, fast low-amplitude EEG, state (1-3)

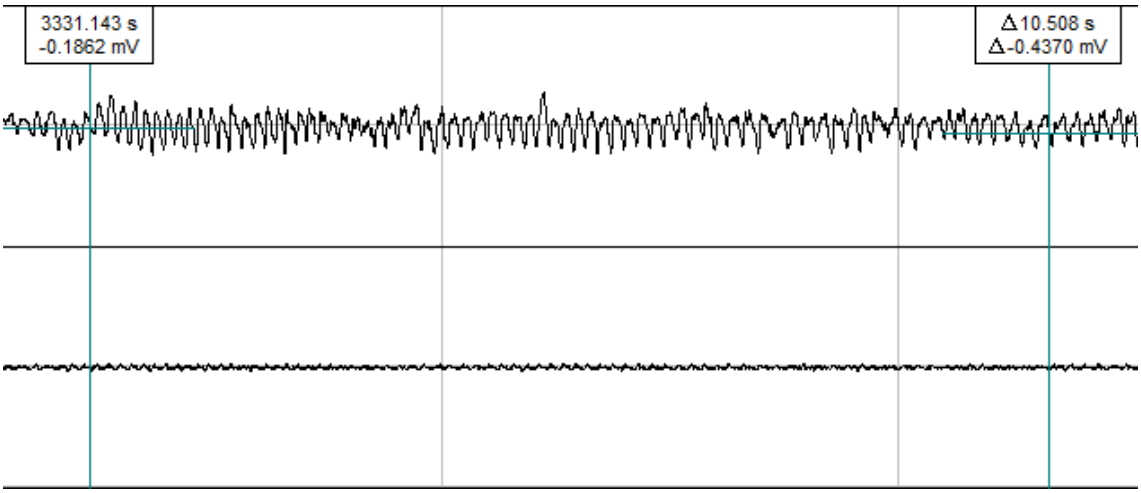

Fig. 1. 10 seconds example of one channel EEG (upper entry) and EMG (bottom) of REM sleep. EEG shows characteristic rapid, low-amplitude saw-tooth waves. EMG displays minimal activity.

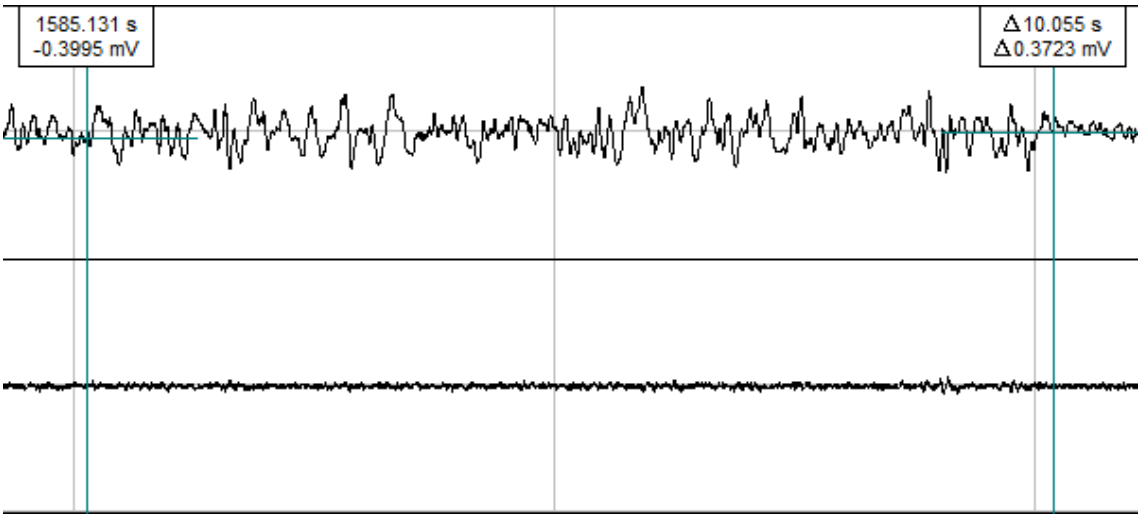

Fig. 2. 10 seconds example of one channel EEG (upper entry) and EMG (bottom) of NREM sleep. EEG shows characteristic slow and high-amplitude waves. EMG displays minimal activity.

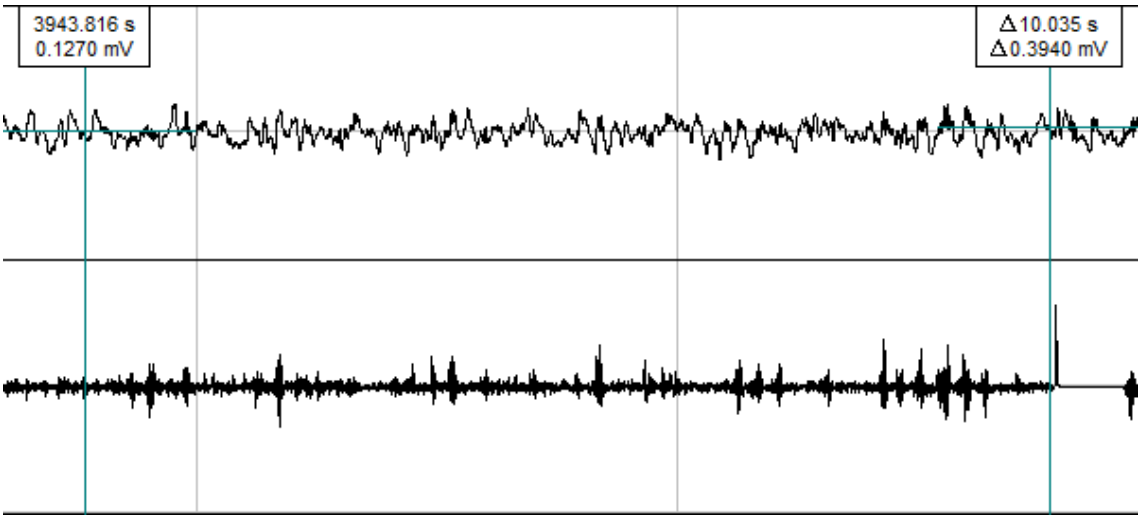

Fig. 3. 10 seconds example of one channel EEG (upper entry) and EMG (bottom) of waking. EEG shows characteristic fast and low-amplitude waves. EMG displays marked muscle activity.

4.2.3. Spectral power analysis

All artifact-free 10 seconds episodes of REM, NREM and waking EEGs for each rat were saved individually. Spectral power analysis was computed using FFT on these segments in software developed by member of our research team. Frequency bands were chosen commonly as follows for delta (0.1-4 Hz), theta (4-8 Hz), alpha1 (8-12 Hz), alpha2 (12-20 Hz), beta1 (20-30 Hz), beta2 (30-40 Hz).

4.2.4. Data standardization

Obtained spectral power data from all rats was sorted by different states, age group and treatment so we ended up having group of Young, Old, Old treated with GH and Old treated with melatonin for REM sleep, NREM sleep and waking across all EEG frequency bands. The absolute EEG power is quite variable between different age groups and even from rat to rat, which can be caused by anatomical differences or slightly different placement of electrodes during surgery (Trachsel et al, 1988). Therefore the data for each rat was normalized before statistical analyses. That was done by expressing power in a frequency band as a percent of total power in the entire spectrum for each rat and state.

Statistical analysis was carried out using Microsoft Office Excel® (2007 for Windows®), SPSS® (v. 12.0 for Windows®) and one-way analysis of variance (ANOVA). Results in the graphs are expressed as mean ± S.E.M., and $p<0.05$ was considered statistically significant.

5. RESULTS

The main goal of present work is to compare the results of EEG spectral power analysis in delta frequencies of NREM SWS in young and old rats and old rats treated with GH or melatonin. Our results for other EEG frequencies in NREM, as well as results of REM sleep EEG power spectra analysis are also showed.

5.1. EEG power spectra for non-rapid eye movement sleep

In Fig. 4 we can see the summary results of REM sleep EEG spectral power analysis for the broad frequency bands. There are marked differences in standardized (relative) values of spectral power EEG in delta, alpha2, beta1 and beta2 frequencies within groups of young, old, treated and untreated rats. These differences are here further described in detail for each frequency band.

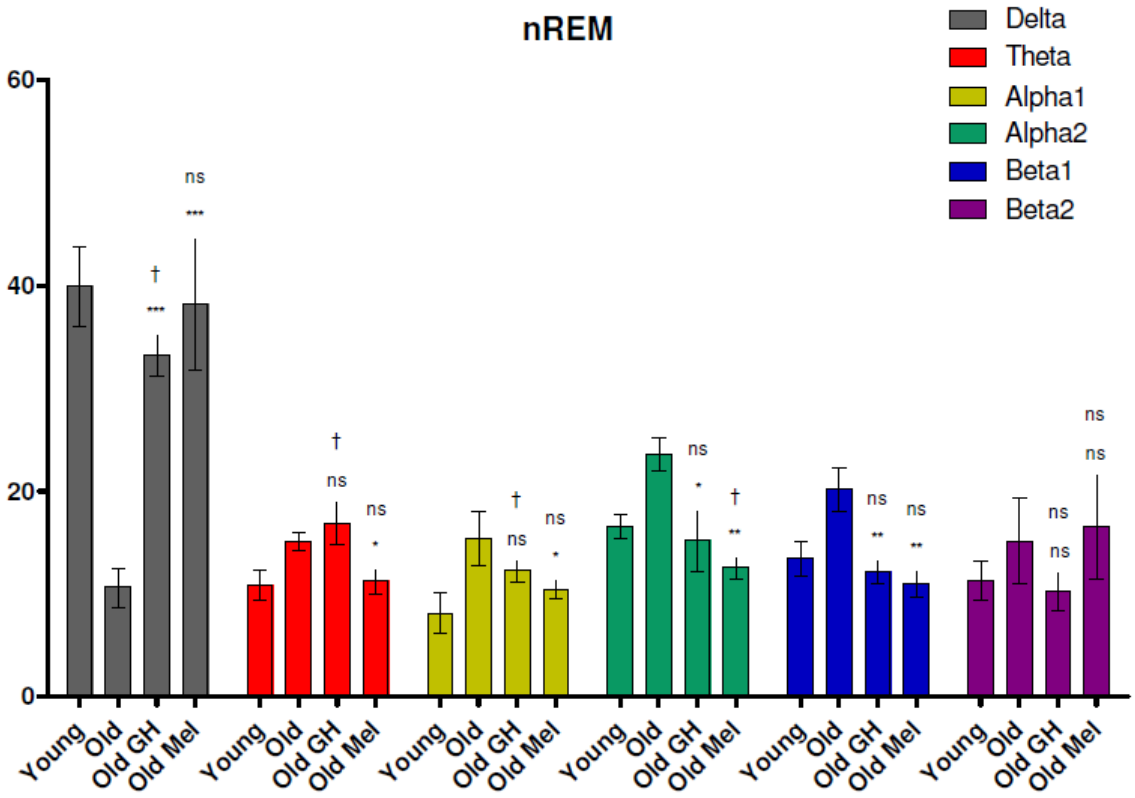

Fig. 4. Relative power of NREM sleep EEG in delta, theta, alpha1, alpha2, beta1 and beta2 bands in young rats (Young), old untreated rats (Old), old rats treated with growth hormone (Old GH) and with melatonin (Old Mel). Data are expressed as a percentage of a particular frequency band power in total power in the entire spectrum. Bars represent means ± SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to old untreated rats (Old) and † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ when compared to young animals (Young) (one way ANOVA analysis).

Delta

As expected, high value of relative NREM delta power in young rats (39.9±3.8) and low in old animals (10.6±1.9) was found. In old rats treated with GH, delta power (33.2±2.1) appears to have values much closer to the values of young animals. One-way ANOVA showed significant difference between old rats with no treatment and the old rats treated with GH ($p<0.001$). Values of delta power for animals treated with melatonin exhibit similar results (38.2±6.5). One-way ANOVA again showed significant difference between untreated old rats and the old rats treated with melatonin ($p<0.001$) along with no significance between old melatonin treated rats and the young animals ($p>0.05$).

Theta

NREM EEG power in theta frequencies is lower in young animals (10.9±1.5) than in old ones with no treatment (15.1±0.9). Old rats treated with GH exhibit even a little higher values (16.9±2.1). There is no significant difference in NREM theta power between old rats with no treatment and GH treated ($p>0.05$) as found with one-way ANOVA. On the other hand theta power in old animals treated with melatonin is much closer to the values of young rats (11.2±1.3) and shows significant difference from untreated old animals ($p<0.05$).

Alpha

Alpha1 and alpha2 powers of NREM EEG tend to follow similar direction of change, both with lower values in young (8.1±2.0 and 16.5±1.2, respectively) and higher in old untreated rats (15.4±2.7 and 23.6±1.6, respectively). In old rats treated with GH amount of alpha1 power (12.3±1.1) is not significantly different from old untreated animals ($p>0.05$). In alpha2 power (15.2±3.0) there is a significant difference compared to old rats with no treatment ($p<0.05$) as showed one-way ANOVA. Melatonin treatment resulted in lower values of alpha1 and alpha2 power (10.4±1.0 and 12.6±1.1, respectively) and shows significant difference compared to untreated old animals for both frequencies ($p<0.05$ and $p<0.01$, respectively).

Beta1

Relative value of NREM sleep beta1 frequencies in young rats is lower compared to the old untreated animals (13.4±1.7, 20.1±2.1, respectively). In old rats

treated with GH and melatonin, beta1 power (12.1±1.1 and 11.0±1.3) appears to have values much closer to the beta1 of young animals. One-way ANOVA showed significant difference in beta1 power between old rats after GH or melatonin treatment and the old rats without any treatment ($p<0.01$).

Beta2

Within relative beta2 power of NREM sleep no significant changes were found in young and old rats, neither in GH and melatonin treated old animals.

5.2. EEG power spectra for rapid eye movement sleep

In Fig.5 we can see the summary results of REM sleep EEG spectral power analysis for the broad frequency bands. There are marked differences in standardized (relative) values of spectral power EEG in delta, alpha2, beta1 and beta2 frequencies within groups of young, old, treated and untreated rats. These differences are here further described in detail for each frequency band.

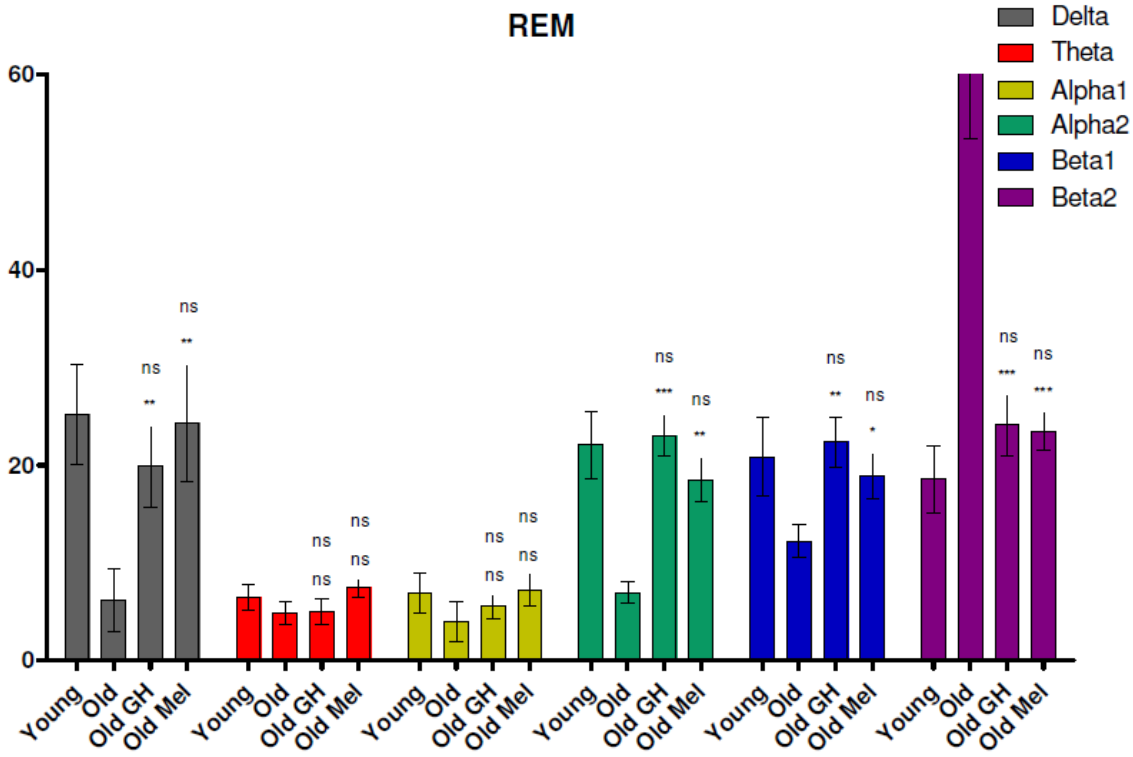

Fig. 5. Relative power of REM sleep EEG in delta, theta, alpha1, alpha2, beta1 and beta2 bands in young rats (Young), old untreated rats (Old), old rats treated with growth hormone (Old GH) and with melatonin (Old Mel). Data are expressed as a percentage of a particular frequency band power in total power in the entire spectrum. Bars represent means ± SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ when compared to old untreated rats (Old) and † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ when compared to young animals (Young) (one way ANOVA analysis).

Delta

REM delta power in Young rats (25.2±5.1) decreased in Old rats to less than a quarter (6.1±3.3). In old rats treated with GH, values of delta power (19.9±4.2) appear to be much closer to the values of young rats. One-way ANOVA showed significant difference between old rats without any treatment and the old rats treated with GH ($p<0.01$) along with no significance between GH treated and young rats ($p>0.05$). Very similar are the results of melatonin treated animals (24.3±6.1). One-way ANOVA again showed significant difference between untreated old rats and the old rats treated with melatonin ($p<0.01$) along with no significance between melatonin treated and the young animals ($p>0.05$).

Alpha2

REM EEG power in alpha2 frequencies shows similar characteristics as delta. Data in young animals (22.0±3.5) is markedly higher than in old ones with no treatment (6.9±1.1). Values in old rats treated with GH (23.0±2.1) are closer to young animals. There is a significant difference in alpha2 power between old rats with no treatment and GH treated ($p<0.001$) and no significance ($p>0.05$) between GH treated and young rats, as found with one-way ANOVA. Alpha2 power in old rats after melatonin treatment (18.5±2.3) also significantly differs from untreated animals ($p<0.01$) and shows no significant difference from young rats ($p>0.05$).

Beta

Beta frequencies in young rats, both beta1 (20.8±4.0) and beta2 (18.5±3.4) seems to be in balance. In old animals there is a marked shift in beta towards higher frequency, with beta1 (12.2±1.7) and beta2 (65.9±12.5). GH and melatonin treatment in old rats put the values back to balance with beta1 (22.4±2.6) and beta2 (24.1±3.2) for GH treatment and beta1 (18.9±2.4) and beta2 (23.5±2.0) for melatonin. One-way ANOVA of beta1 and beta2 power for both GH and melatonin treated old rats showed significant difference from old animals with no treatment ($p<0.001$).

Theta, alpha1

Changes in young and old rats in the relative frequency bands for theta and alpha1 in REM sleep EEG were not found. Also no significant differences in GH or melatonin treated animals were observed for these frequencies.

6. DISCUSSION AND CONCLUSIONS

The data presented in this work address two basic questions: 1) What are the main differences and changes in EEG spectral power activity between the group of young and old rats during sleep?; and 2) How does chronic growth hormone or melatonin administration in old rats during period of 1 month affect sleep EEG power spectra, especially in NREM delta frequencies? In summary, we have found marked decrease in delta power of NREM sleep in old rats compared to the young animals. This previously well-documented finding was expected and is coherent with other studies both in humans (Ohayon, 2004; Crowley, 2011) and rats (Mendelson, 1999b). Further age-related differences in NREM sleep power spectra are less striking. Our results show increase in relative power in theta, alpha1, alpha2 and beta1 frequencies in old rats. This overall increase in fast wave EEG activity with higher frequencies can be explained by the age-related increase in lighter stages of NREM sleep (N1 and N2) at the expanse of the deeper stages of sleep (N3 or SWS) (Ohayon, 2004). GH as well as melatonin treatment in old rats increased relative values of NREM delta power so they resembled values in young animals. Morris et al (2012) in their recent review report inconsistent results regarding the influence of GH on human sleep. Some researchers found that intravenous infusion of GH decreases SWS and increases REM sleep whereas others have observed no effect on sleep architecture. But in these studies only single dose of GH or very short-term GH administration was assessed, not the chronic treatment as in our work. Meta-analysis of effects of exogenous melatonin on sleep in humans documented that melatonin decreases sleep onset latency, increases sleep efficiency, and increases total sleep duration (Brzezinski et al, 2005). But there is not enough previous information about effects of chronic melatonin treatment on EEG spectral power composition.

Our spectral power analysis of REM sleep displays age-related differences in delta, alpha2, beta1 and beta2 frequencies. There is decrease in delta, alpha2 and beta1 and rapid increase in beta2 in old animals. These findings are difficult to compare, because most of other studies addressing age-related changes in sleep only provide information about decrease in total percentage of REM with age in humans (Ohayon, 2004), but no data concerning changes in power spectra frequencies. Previous studies of rats have indicated modest (Rosenberg et al, 1979; Zepelin et al, 1972), not-so-modest

(van Gool and Mirmiran, 1983) or even none (Mendelson, 1999b) age-related decline in total amounts of REM sleep. Our findings bring some novel information about age-related changes in REM sleep EEG frequencies. Even though total amount of REM sleep energy doesn´t have to demonstrate marked decrease, we found that distribution of power between different frequency bands changes with age. Moreover our work found, that both treatment with GH and melatonin seems to compensate this changes in spectral power. Old rats treated during 1 month with GH or melatonin exhibit similar REM sleep power spectra as young animals.

In conclusion our data demonstrate overall a positive effect of GH and melatonin administration on sleep EEG power spectra in old rats. Mechanism of this action remains to be fully revealed in next studies. It is probable, that these hormones somehow directly affect homeostatic and circadian regulation and/or structures and processes behind sleep regulation. Our data are based only on a small sample of tested animal subjects and there is for sure necessity for further research in this field.

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