INVOLVEMENT OF NESFATIN AND MELANIN-CONCENTRATING HORMONE IN THE REGULATION OF VIGILANCE; COMPARISON WITH THE EFFECT OF ESCITALOPRAM, THE SSRI ANTIDEPRESSANT

PhD thesis

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1. INTRODUCTION

Hypothalamus, the key integrator area of several physiological functions in the brain, has a crucial role also in the regulation of sleep. Thus, the importance of hypothalamic neuron populations expressing different neuropeptides in the regulation of sleep-wake cycle is an intensively investigated area with countless new questions.

Several data support the involvement of melanin-concentrating hormone (MCH), a 19-aminoacid peptide, in the regulation of sleep, besides other important physiological functions, such as food consumption, energy homeostasis, although its role has also been suggested in pathophysiological conditions, like depression and obesity. MCH neurons are localized in hypothalamic/thalamic structures intermingled with the orexin/hypocretin-containing neurons, which neuron populations together are considered to be crucial in the stabilization of the switch between different vigilance stages.

The implication of MCH in sleep regulation is shown by the fact that centrally administered MCH elevated the time spent in rapid eye movement (REM) sleep (~200%), and to a lesser degree, the amount of non-REM sleep, moreover, antagonists on the MCH receptor 1 (MCHR1) dose-dependently decreased deep slow wave sleep (SWS2), as well as REM sleep, while the amount of active and passive wake increased.

Former data of our laboratory in rat showed a positive correlation between the neuronal (Fos) activation of MCH-containing neurons and the time spent in REM sleep during the 3 h-long 'REM sleep rebound' after a 72 h-long selective REM sleep deprivation. During this 'REM sleep rebound' period, when the amount of REM sleep is approximately 6-7-times higher than in controls, MCH-containing neurons show a highly increased activation. However, it was not investigated yet whether MCH

populations of different hypothalamic/thalamic structures reveal differences in this activation.

Though the role of MCH in the regulation of sleep is known, the involvement of nesfatin-1/NUCB2 (nesfatin), the co-expressing neuropeptide, and their expression in different hypothalamic/thalamic nuclei has not been examined until the start of this work. Nesfatin-1, the 82-amino acid protein is a product of posttranslational cleavage of the prohormone NEFA/nucleobinding-2 (NUCB2). Nesfatin has a wide distribution in the central nervous system (CNS), although the largest nesfatin-containing neuron population has been localized in the perifornical area (PFA), the lateral hypothalamic area (LH) and the zona incerta (ZI) in rats. All hypothalamic/thalamic MCH neurons co-express nesfatin, but a relative small population of nesfatin-positive neurons do not contain MCH. While MCH has been shown to increase food intake and induce energy conservation, nesfatin has been reported as a satiety neuropeptide, reducing food intake in active (dark) phase. Beyond regulating food consumption, accumulating evidence proves that nesfatin plays an important role in the regulation of other physiological functions, like body temperature, blood pressure, stress response and reproduction. Its involvement has also been suggested in the pathophysiology of stress-associated mood disorders such as anxiety and depression.

To study the association between MCH and nesfatin neuropeptides and REM sleep, we applied the classic ‘flower pot’ selective REM sleep deprivation method followed by ‘REM sleep rebound’ to provoke an increased neuronal activation of structures possibly involved in the regulation of REM sleep. It was also pending whether an elevated serotonergic-tone, known to suppress REM sleep even during the ‘REM sleep rebound’ is able to modify the (Fos) activation of MCH neuron

populations. The activation of MCH- and nesfatin-positive neurons as a result of 'REM sleep rebound' was investigated in different hypothalamic/thalamic structures, such as zona incerta (ZI), lateral hypothalamic area (LH) and perifornical area (PFA). As no literature data was available about the role of nesfatin in the regulation of vigilance, we aimed to investigate this issue in rat using immunohistochemistry and electrophysiology.

2. OBJECTIVES

(1) We intended to investigate if 'REM sleep rebound' causes any difference in the activation of MCH neurons of different hypothalamic/thalamic nuclei, such as ZI, LH and PFA.

(2) Considering that escitalopram, the extracellular serotonin level-increasing antidepressant, suppresses REM sleep, even during 'REM sleep rebound', how escitalopram treatment influences the neuronal activation of MCH-neuron population during 'REM sleep rebound' in the investigated hypothalamic/thalamic nuclei?

(3) How the activation of nesfatin-positive neuron populations is influenced by REM sleep deprivation and the following 'REM sleep rebound' in the investigated hypothalamic/thalamic nuclei? Is there any difference between the activation of MCH-positive and -negative nesfatin neuron populations?

(4) How the exogenously (intracerebroventricularly, icv) administered nesfatin-1 peptide, injected in the passive phase, affects the architecture of sleep-wake cycle?

(5) Does the icv-injected nesfatin-1 alter the quantitative EEG spectra in different vigilance stages in passive phase?

(6) Is there any similarity in the EEG spectral effect of icv-injected nesfatin-1 vs. 2 or 10 mg/kg escitalopram, suggesting a possible serotonergic component in the action of nesfatin-1 on vigilance?

3. MATERIALS AND METHODS

Experiments and housing conditions were performed according to the international regulations of the EU [European Communities Council Directive of 24 November 1986 (86/609/EEC)] and the NIH "Principles of Laboratory Animal Care" (National Institutes of Health). All experiments were approved by the National Scientific Ethical Committee on Animal Experimentation, and permitted by the government (Food Chain Safety and Animal Health Directorate of the Central Agricultural Office, Permit Number: 22.1/1375/7/2010).We used male Wistar rats weighing 300–350 g.

3.1. *Experiment 1* and *Experiment 2 - Immunohistochemistry (IHC)*

3.1.1. REM sleep deprivation ('flower pot') method

This type of REM sleep deprivation method utilizes one of the key features of REM sleep, namely the absence of muscle tone. During the REM sleep deprivation procedure, rats are kept on single small platforms ('small pots') surrounded by water, and when they switch to REM sleep, they fall into the water and awaken. Following the 72 h-long REM sleep deprivation on 'small pots' (SPD group), one group of the REM sleep deprived rats were replaced into their singe cages and kept undisturbed for a 3 h-long 'REM sleep rebound' ('small pot rebound', SPR group). Following the REM sleep deprivation (SPD group) or the 'REM sleep rebound' period

(SPR group), rats had been sacrificed by transcardial perfusion using 4% paraformaldehyde solution to fix the brain tissue and prepare for immunohistochemical procedure.

3.1.2. Immunohistochemistry

The neuronal activation of MCH- and nesfatin-containing neuronal populations was investigated by MCH/Fos double *(Experiment 1)* as well as MCH/nesfatin/Fos triple *(Experiment 2)* IHC on paraformaldehyde-fixed 50 μm-thick coronal sections. In case of MCH/Fos IHC, we applied DAB (3,3'-diaminobenzidine)/NiDAB staining and light microscope, while the MCH/nesfatin/Fos IHC was performed using fluorescent technique. Neuronal activation was detected using the Fos early gene product. During the morphometrical analysis, we calculated the ratio of the activated (Fos-positive) portion of the MCH-positive neurons *(Experiment 1)* as well as the activated portion of both the MCH-positive and MCH-negative nesfatin-neurons *(Experiment 2)*.

3.1.3. Experimental groups

In *Experiment 1,* to investigate the effect of increased serotonergic tone on the neuronal activation of MCH cells, we applied escitalopram, a selective serotonin reuptake inhibitor (SSRI) antidepressant in 10 mg/kg (or vehicle in the control group) intraperitoneal (ip) injections.

The following groups were used for IHC experiments:

Experiment 1: vehicle- or escitalopram (SSRI) -treated small pot 'REM sleep rebound' groups (SPR-veh and SPR-SSRI, respectively), vehicle- or escitalopram-treated home cage groups (HC-veh and HC-SSRI, respectively).
Experiment 2: small pot REM sleep deprived (SPD), small pot REM sleep rebound (SPR) and home cage (HC) controls.

3.2. *Experiment 3* and *Experiment 4 - Electroencephalography (EEG)*

In order to investigate the effect of exogenously (icv) administered nesfatin-1 on the EEG pattern of sleep-wake cycle and the quantitative EEG power spectra (*Experiment 3)*, as well as to compare the quantitative power spectra of nesfatin-1 and ip-injected escitalopram (*Experiment 4)*, we performed EEG study.

3.2.1. Electroencephalography

Rats were equipped with chronic electroencephalographic (EEG) and electromyographic (EMG) electrodes. During the EEG scoring (by Sleep Sign for animal software followed by visual supervision), the following vigilance stages were distinguished: active and passive wakefulness (AW and PW, respectively), light and deep slow wave sleep (SWS1 and SWS2, respectively), rapid eye movement (REM) sleep and intermediate stage of sleep (IS, also called pre-REM stage). We also performed quantitative EEG analysis, namely we calculated the EEG power of the relevant frequency ranges of each vigilance stage [eg. theta power (5-9 Hz) in REM sleep] using Fast Fourier Transformation (FFT). During the EEG spectral analysis the following EEG oscillations were separated: delta

(0.5-4 Hz), theta (5-9 Hz), alpha (9-13 Hz), sigma (12-14 Hz), beta (15-30 Hz) and gamma (>30 Hz).

In *Experiment 3*, 25 pmol nesfatin-1 was injected *via* chronically implanted intracerebroventricular (icv) cannula at the beginning of passive (light) phase. The first hour of the EEG recording was omitted due to the stress caused by the icv procedure, thus, EEG recording was scored during the 2^{nd}-6^{th} (passive phase) and 13^{th}-18^{th} hours (active phase). We evaluated the time spent as well as the number and average duration of episodes in different vigilance stages.

In *Experiment 4*, we compared the EEG spectra (in the summarized 2^{nd}-3^{rd} hours) of the nesfatin- and two doses (2 and 10 mg/kg ip) of escitalopram-treated groups using 'state space analysis technique' followed by a 'conventional' EEG spectral analysis.

3.2.2. Heat map spectra

To compare the spectral distribution of EEG power in different vigilance stages of the nesfatin- and escitalopram-treated groups in the summarized 2^{nd}-3^{rd} hours after treatment, we adapted 'state space analysis technique', a relative new method that – based on two ratio [Ratio 1 (6.5-9/0.5-9 Hz) on the abscissa and Ratio 2 (0.5-20/0.5-60 Hz) on the ordinate], created from the EEG spectral data (in 4 s intervals, i.e. epochs). These two ratios define 2-dimensional spectra separating the main vigilance stages as clusters, namely REM sleep, non-REM sleep and wake. On the heat map, one point represents one epoch. This technique enables a quick visualization of the 'topography' of sleep-wake cycle using heat map spectra. It can provide a way to observe general EEG spectral changes (eg. 'shifts') in the

density and distribution of the ‘clusters’, that are visible easily to the naked eye. Since the topography of non-REM, REM sleep and wake stages is based on their power spectral content, ‘state space analysis technique’ can be performed without sleep scoring. As we can calculate shifts in the centroids (arithmetic mean) of ‘REM, non-REM and wake clusters’ as a result of treatment, the technique is quantifiable, appropriate for statistical analysis.

4. RESULTS

4.1. *Experiment 1:* Activation of MCH neuron populations as a result of ‘REM sleep rebound’ in different hypothalamic/thalamic nuclei

We found markedly increased activation in the MCH neurons as a result of REM sleep deprivation followed by ‘REM sleep rebound’ in all the investigated thalamic/hypothalamic structures, such as ZI (65,69±6,98%), LH (64,99±5,47%) and PFA (56,24±7,15%), compared to controls (<2%).

The elevated activation of MCH-neurons as a result of ‘REM sleep rebound’ was significantly decreased by the prior (10 mg/kg) escitalopram treatment in the ZI (56.33±11.32%), the LH: (52.03±8.52%) and the PFA (46.08±9.79%). On the contrary, we did not find any difference between the HC-SSRI and HC-veh groups in any of the investigated structures.

However, the total number of MCH-containing neurons (Fos–positive and –negative together, relative to the unit area) was not affected by the experimental procedures.

4.2. *Experiment 2:* Activation of nesfatin-containing neurons in different hypothalamic/thalamic structures as a result of ’REM sleep rebound’

Based on the MCH/nesfatin/Fos triple immunostaining, the rate of co-localization of MCH and nesfatin in HC controls showed slight differences in the investigated areas: ZI: 81.9±1.7%, LH: 75.4±2.1%, PFA: 66.2±4.8%, and these ratios were not affected by the 'REM sleep rebound'.

However, as a result of 'REM sleep rebound' after the selective REM sleep deprivation, the neuronal activation of MCH-positive and MCH-negative nesfatin neuron populations revealed significant differences. Investigating the MCH-positive nesfatin neuron population in the ZI, LH and PFA, we found that in HC controls and SPD group, neuronal (Fos) activation was minimal (less than 0.5%). However, 'REM sleep rebound' caused a significant elevation in the rate of neuronal activation regardless of the area investigated (ZI, 86.9±2.9%, LH: 79.0±1.8%, and PFA: 78.3±2.7%). However, the MCH-negative nesfatin neurons showed higher activity in HC and SPD groups, particularly in the PFA (24±8.1% and 39.4±7.4% in HC and SPD groups, respectively). 'REM sleep rebound' caused an increase in the Fos positivity, although it reached the level of statistical significance only in the LH (SPR: 37.6±4.8% vs. HC: 6±2.4%).

4.3. *Experiment 3*: The effect of exogenously (icv) administered nesfatin-1 on the architecture of sleep-wake cycle

The high neuronal activity of the MCH/nesfatin-co-expressing cell population as a result of 'REM sleep rebound', raised the question, whether nesfatin-1 neuropeptide, the N-terminal fragment of the NUCB2 protein, can influence sleep-wake cycle or not. To investigate this question, we injected 25 pmol nesfatin-1 icv at the beginning of passive phase, and analysed the architecture of sleep-wake cycle during the 2^{nd}-6^{th} h after injection, as well as the first 6 h of active phase (13^{th}-18^{th} h).

We found that icv nesfatin-1 significantly elevated sleep fragmentation, namely the number of awakenings, however, the amount of total sleep time showed only a tendency to decrease during the five investigated hours of passive phase.

Regarding the effect of nesfatin-1 on different stages of sleep-wake cycle, we saw the most apparent change in REM sleep. It decreased the time spent in REM sleep significantly, compared to controls, which reduction was approximately 60% in the 2^{nd} h, but in the next three hours, the decline in REM sleep reached an approximate value of 90%, while in the 6^{th} h it was ca. 70%. The amount of IS showed a similar diminution, with the lowest value in the 3^{rd} h. The REM sleep- and IS-declining effect of nesfatin-1 seemed to be also apparent in both the number and the average duration of episodes in both REMS and IS.

Considering the time spent in non-REM sleep, we differentiated light slow wave sleep (SWS1) and deep slow wave sleep (SWS2). In the time spent in SWS1, we found an elevation in the 2^{nd}, 3^{rd} and 4^{th} hours, while in SWS2, we detected no alteration in any of the investigated hours, compared to controls. Regarding wakefulness, the amount of PW elevated markedly in the 3^{rd}, 5^{th} and 6^{th} hours, while the time spent in AW was unchanged. Noteworthy, that the increase of SWS1 and PW was parallel to the elevation of episode-numbers generally, although the average episode-durations of these stages were unaffected.

In contrast to the alterations of sleep time in passive phase, we found no significant change in the active phase.

4.4. *Experiment 4*: Comparison of the EEG power spectral effect of nesfatin-1 and escitalopram using ‘state space analysis’

Based on the strong REM sleep-decreasing and PW-increasing effect of nesfatin-1 in our study, moreover, literature data have reported that icv nesfatin-1 elevates the neuronal (Fos) activation of the dorsal and median raphe nuclei (DRN and MRN, respectively) that provide the main serotonergic innervation of the brain, we raised the question whether the effect of icv nesfatin-1 can be associated with the serotonergic system or not. To address this issue, we investigated the possible similarities between the effect of nesfatin-1 and escitalopram, the SSRI antidepressant that is known to increase the serotonergic tone.

The distribution and density of 'state space clusters' shows that nesfatin-1 treatment significantly diminished the extent of the cluster corresponds to the REM sleep stage, compared to vehicle, reinforcing the REM sleep-inhibiting effect of icv nesfatin-1. We found a significant shift to the left on the *x*-axis in the centroid position of 'REM sleep cluster'. Nesfatin-1 also caused a significant shift to the left in the centroid position of the cluster corresponds to wake, compared to control. As 'Ratio 1' on the *x*-axis is sensitive to the high theta power content (6.5-9 Hz) of EEG spectra, shift to the left on the *x*-axis in the centroid positions of REM sleep and wake 'clusters' suggests a theta power-reducing effect of nesfatin-1 in these stages. Despite alterations in theta power in wake and REM sleep, icv nesfatin-1 caused no alteration in the centroid position of non-REM sleep 'cluster' according to the 'state space analysis' technique.

To compare the effect nesfatin-1 on 'state space' heat maps with a serotonergic-tone-increasing agent, we applied escitalopram in 2 and 10 mg/kg ip doses.

In both escitalopram-treated groups, the distribution of the 'cluster' corresponds to REM sleep demonstrated a REM sleep-inhibiting effect, however, the shift in the centroid position of 'REM sleep cluster' was

significant only in the 10 mg/kg escitalopram-treated group. On the contrary, in the 'wake-cluster' (TW), we observed a notable shift to the left in the centroid-position of both the 2 and the 10 mg/kg escitalopram-treated groups, compared to controls. Besides the changes in the heat map spectra of TW and REM sleep in the escitalopram-treated groups, in non-REM sleep stage, we did not find any alteration in the centroid positions, compared to control group.

However, the more detailed 'conventional' EEG spectral analysis revealed differences between nesfatin-1 and escitalopram. Beyond similarities in the effect of nesfatin-1 and escitalopram, namely, the reduction in theta power in REM sleep and TW, comparison of AW and PW data showed differences. While nesfatin-1 decreased theta power solely in PW, both doses of escitalopram affected theta power only in AW. Moreover, escitalopram caused a deceleration in the theta oscillation (a reduction in the peak of theta power). In TW and PW, both doses of escitalopram shifted the peak frequency of theta power from 7 Hz to 6 Hz, while in AW this shift was observed only in the 10 mg/kg-treated group. In the nesfatin-1-treated group, theta peak was unaffected.

5. CONCLUSIONS

The most important conclusions are the followings:

(1) 'REM sleep rebound' following REM sleep deprivation caused a strong neuronal activation of the MCH-positive nesfatin neuron populations in the ZI, LH and PFA, suggesting a close association between the regulation of REM sleep and the MCH/nesfatin-neuronal populations of these structures.

(2) This strong activation was reduced by increased serotonergic tone in all investigated areas, suggesting a direct or indirect inhibitory effect of

serotonin on these neurons, in line with the substantial REM sleep suppressing effect of escitalopram.

(3) MCH-negative nesfatin neurons can be associated with REM sleep regulation exclusively in the LH, suggesting that MCH-negative nesfatin neurons of the ZI and the PFA may be under a different regulation.

(4) Nesfatin can be a potential new element in the regulation of sleep-wake cycle. Possessing a mostly REM-suppressing and PW-increasing effect, without altering non-REM sleep considerably, nesfatin alters sleep pattern similarly to several antidepressant.

(5) The theta power reducing effect of nesfatin in PW and REM sleep, suggests that it may affect functions associated with theta oscillation, like attention or memory formation, however, it does not influence delta power in non-REM sleep, namely the resting aspect of sleep.

(6) Despite similarities in the EEG effect of nesfatin and escitalopram, differences in their theta-reducing effect suggests a disparate influence on the generation of theta power, however, increase in the serotonergic tone can be a potential component in this effect.

To clarify the mechanism how nesfatin influences sleep-wake cycle and EEG spectra, as well as to prove the involvement of a serotonergic and other components in the effect of nesfatin, further studies are needed. The REM sleep and theta power reducing effect of nesfatin can suggest its potential role in the pathomechanism of depression and anxiety.

6. PUBLICATIONS

6.1. *Publications related to the PhD thesis*

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6.2. *Publications not related to the PhD thesis*

1. Bergman P, Adori C, Vas S, Kai-Larsen Y, Sarkanen T, Cederlund A, Agerberth B, Julkunen I, Horvath B, Kostyalik D, Kalmar L, Bagdy G, Huutoniemi A, Partinen M, Hokfelt T Narcolepsy patients have antibodies that stain distinct cell populations in rat brain and influence sleep patterns. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 111:(35) pp. E3735-E3744. (2014)

2. Kostyalik D, Katai Z, Vas S, Pap D, Petschner P, Molnar E, Gyertyan I, Kalmar L, Tothfalusi L, Bagdy G Chronic escitalopram treatment caused dissociative adaptation in serotonin (5-HT) 2C receptor antagonist-induced effects in REM sleep, wake and theta wave activity. EXPERIMENTAL BRAIN RESEARCH 232:(3) pp. 935-946. (2014)

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