

DYNAMICS OF CHANGES IN THE DENSITOMETRIC DENSITY OF MELATONIN RECEPTORS IN MAGNOCELLULAR NEURONS OF THE HYPOTHALAMUS UNDER STRESS*Ahranov O.S., Bulyk R.Ye.**Bukovinian State Medical University, Chernivtsi, Ukraine*

Key words: supraoptic nucleus, hypothalamus, neurons, receptors, melatonin, immobilisation stress, rats.

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Abstract. *The aim of this study was to characterise the optical density of MT1 melatonin receptors in neurons of the supraoptic nucleus of the hypothalamus in aged rats under conditions of immobilisation stress, using an immunohistochemical technique combined with computerised microdensitometry.*

Material and methods. *The experimental studies were conducted on outbred male white rats aged 19–23 months and weighing 300–320 g. The animals were divided into two study series (each further subdivided into two groups), in which biomaterial was collected at 14:00 and 02:00. The animals in the first series (control) were maintained for 30 days under standard lighting conditions (light from 08:00 to 20:00, with a light intensity of 1000 lux at cage level provided by fluorescent lamps). Animals in the second series, which were kept under the same lighting conditions as the rats in the first series, were subjected to experimentally induced prolonged immobilisation stress by being confined to special plastic restraining cages for 6 hours daily for 30 consecutive days.*

For the immunohistochemical procedure, polyclonal antibodies against the MT1 melatonin receptor from Abcam (UK) and the LSAB2 streptavidin-biotin visualisation system (peroxidase label + diaminobenzidine) from Chemicon International Inc. (USA) were used. Given the need to perform multiple statistical comparisons of means in statistical samples, the Newman-Keuls criterion was used to determine differences between populations.

Results. *Using an immunohistochemical technique combined with computerised microdensitometry, we detected distinct positive immunohistochemical staining, appearing as granules varying in size and optical density. It was found in the neurons of the supraoptic nucleus of the hypothalamus in aged rats. The granules observed were concentrated mainly at the periphery of all cells, suggesting a transmembrane localisation of the MT1 (also known as MTNR1A or Mel1a) – high-affinity membrane receptors that play a key role in regulating circadian rhythms (the biological clock) and the sleep-wake cycle in humans and other mammals. They belong to the family of G-protein-coupled receptors (GPCRs). At the same time, no immunohistochemical staining of the nuclei was observed. They stained only with haematoxylin and exhibited the typical morphological characteristics of neurons in the rat hypothalamic supraoptic nucleus. The highest density of MT1 melatonin receptors in the neurons of the supraoptic nuclei of the hypothalamus in aged rats was detected at 02:00 compared with 14:00 (in a field of view measuring 1600 μm^2 according to the Newman-Keuls criterion, $p = 0.003$). Under immobilisation stress, the number of neurons in the supraoptic nuclei of the hypothalamus that were positively stained for the MT1 melatonin receptor within a 1600 μm^2 field of view was 0.219 ± 0.0018 at 02:00, at 14:00 – 0.217 ± 0.0022 optical density units, and was significantly lower ($p < 0.001$) compared with control values during the selected observation periods.*

Conclusions. *1. The density of MT1 melatonin receptors in neurons of the supraoptic nucleus of the hypothalamus in aged rats is highest at 02:00, but at 14:00 it is significantly lower. 2. Modelling of prolonged immobilisation stress leads to a reduction in the optical density of MT1 melatonin receptors in the neurons of the supraoptic nuclei of the hypothalamus in animals.*

ДИНАМІКА ЗМІН ДЕНСИТОМЕТРИЧНОЇ ЩІЛЬНОСТІ РЕЦЕПТОРІВ МЕЛАТОНІНУ У ВЕЛИКОКЛІТИННИХ НЕЙРОНАХ ГІПОТАЛАМУСА ПРИ СТРЕСІ

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Ключові слова: надзорове ядро, гіпоталамус, нейрони, рецептори, мелатонін, іммобілізаційний стрес, щури.

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Резюме. Мета роботи – охарактеризувати оптичну щільність рецепторів мелатоніну MT1 у нейронах надзорного ядра гіпоталамуса старих щурів на тлі іммобілізаційного стресу, на підставі імуногістохімічної методики, поєднаної з комп'ютерною мікроденситометрією.

Матеріал і методи. Експериментальні дослідження проведено на нелінійних білих щурах-самцях віком 19-23 міс. й масою 300-320 г. Тварин розподілено на дві серії досліджень (кожна ще на дві групи), у яких забір біоматеріалу проводили о 14.00 і 02.00 год. Тварини першої серії (контроль) перебували впродовж 30 діб в умовах стандартного режиму освітлення (світло з 08.00 до 20.00 год, освітленість люмінесцентними лампами на рівні кліток 1000 Лк). Тваринам другої серії, які перебували за таких умов освітлення як і щури першої серії, здійснювали моделювання тривалого іммобілізаційного стресу шляхом їх утримання впродовж 6 год щоденно 30 діб поспіль у спеціальних пластикових клітках-пеналах.

З метою виконання імуногістохімічної методики використані поліклональні антитіла до рецепторів мелатоніну MT1 виробника Abcam (Велика Британія) та стрептавідинбіотинову систему візуалізації LSAB2 (пероксидазна мітка + діамінобензидин) виробника Chemicon International Inc. (США). Враховуючи необхідність виконання множинних статистичних порівнянь середніх величин у статистичних вибірках, для визначення відмінностей між сукупностями використаний критерій Ньюмена-Кейлса.

Результати. Використовуючи імуногістохімічну методику, поєднану з комп'ютерною мікроденситометрією, нами виявлено чітке позитивне імуногістохімічне забарвлення, яке візуалізувалося у вигляді гранул, які різнилися за розмірами та оптичною щільністю. У нейронах надзорного ядра гіпоталамуса старих щурів виявлені гранули, які зосереджувалися здебільшого по периферії усіх клітин, що свідчить на користь трансмембранного розташування рецепторів мелатоніну MT1 (також відомих як MTNR1A або Mel1a). Це високоафінні мембранні рецептори, які відіграють ключову роль у регуляції циркадних ритмів (біологічного годинника) та циклу сну-неспанья в людини та інших ссавців. Вони належать до родини рецепторів, спряжених із G-білком (GPCR). Водночас, імуногістохімічного забарвлення ядер не спостерігали – вони забарвлювалися тільки гематоксиліном і мали типову морфологічну характеристику для нейронів надзорного ядра гіпоталамуса щурів. Найвищу щільність рецепторів мелатоніну MT1 у нейронах надзорних ядер гіпоталамуса старих щурів виявлено о 02.00 год доби порівняно з 14.00 год (у полі зору площею 1600 мкм² за критерієм Ньюмена-Кейлса $p=0,003$). В умовах іммобілізаційного стресу кількість позитивно забарвлених на рецептори мелатоніну MT1 нейронів надзорних ядер гіпоталамуса в полі зору площею 1600 мкм² перебувала в межах о 02.00 год – $(0,219 \pm 0,0018)$, о 14.00 год – $(0,217 \pm 0,0022)$ в. о. опт. щільності і була вірогідно нижчою ($p < 0,001$) порівняно з контрольними величинами в обрані періоди спостереження.

Висновки. 1. Щільність рецепторів мелатоніну MT1 у нейронах надзорного ядра гіпоталамуса старих щурів найвища о 02.00 год, проте о 14.00 год вона є вірогідно нижчою. 2. Моделювання тривалого іммобілізаційного стресу призводить до зниження оптичної щільності рецепторів мелатоніну MT1 у нейронах надзорних ядер гіпоталамуса досліджуваних тварин.

Introduction. The rapid development of science and technology has brought not only positive changes but also

several adverse factors into human life, including physical inactivity, excessive mental and physical strain, and

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occupational and everyday stress. A common situation today is a reduction in physical activity – physical inactivity – which leads to disturbances in the body's metabolism and energy balance, cardiovascular diseases, gastrointestinal disorders, excess weight, premature ageing, a reduced ability of the body to resist various diseases, and changes in the body's general condition, among other things. Such stress-related effects are observed at the cellular and molecular levels. As stress increases, metabolic disorders arise, and the activation of free radical oxidation leads to disruption of the primary (barrier, receptor, catalytic) functions of biological membranes [1, 2].

Melatonin plays a key role in the body's adaptation to environmental conditions, with the pineal gland (epiphysis) being the primary site of its production. It has been shown that melatonin secretion follows distinct circadian variations, with a minimum during the daytime and a maximum around 02:00 [3–5]. Via melatonin receptors (membrane, cytosolic and nuclear), the hormone regulates the state of the hypothalamic-pituitary system and the activity of the endocrine glands [6, 7]. Furthermore, via a feedback mechanism, it influences the activity of the magnocellular neurosecretory supraoptic nuclei of the hypothalamus [8–10].

Given the important role played by the hypothalamic supraoptic nuclei in the body's adaptive responses, it is of particular relevance to investigate the density of melatonin receptors in these brain structures when experimental animals are subjected to immobilisation stress [11, 12].

The aim of the study is to use immunohistochemical techniques combined with computerised microdensitometry to quantify the density of MT1 melatonin receptors in neurons of the supraoptic nucleus of the rat hypothalamus under conditions of immobilisation stress.

Material and methods. The experimental studies were conducted on male outbred white rats aged 19–23 months and weighing 300–320 g. The animals were divided into two study series (each further subdivided into two groups), in which biomaterial was collected at 14:00 and 02:00. The chosen times for the experiment were determined by the varying functional activity of the pineal gland during these periods of the day. The rats were housed under standard vivarium conditions with free access to food and water.

Animals of the first series (control group) were maintained for 30 days under a standard lighting conditions (light from 08:00 to 20:00; with a light intensity of 1000 lux at cage level provided by fluorescent lamps). Rats of the second series, kept under the same lighting conditions as those of the first series, were subjected to experimentally induced prolonged immobilisation stress by being confined to special plastic restraining cages for 6 hours daily for 30 consecutive days.

At the completion of the experiment, on the following day at 14:00 and 02:00, the animals were euthanised by decapitation under thiopental sodium anaesthesia (40.0 mg/kg intraperitoneally).

The scientific research was conducted in compliance with the key provisions of the Law of Ukraine No. 3447-

IV “On the Protection of Animals from Cruelty”, the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (18 March 1986), European Union Directive 2010/63/EU, and the orders of the Ministry of Health of Ukraine No. 690 dated 23 September 2009, No. 944 dated 14 December 2009, and the Order of the Ministry of Education and Science No. 249 dated 1 March 2012. The research protocol was approved by the BSMU Biomedical Ethics Committee on 15 February 2024.

For immunohistochemical analysis, sections of the cerebral hemispheres, including the supraoptic nucleus of the hypothalamus, were fixed in 10% neutral buffered formalin for 22 hours. It was followed by accelerated dehydration in a series of alcohols of increasing concentration, embedding in paraffin at 58°C, and the subsequent preparation of 5-micrometre-thick histological sections.

For the immunohistochemical procedure, polyclonal antibodies against the MT1 melatonin receptor from Abcam (UK) and the LSAB2 streptavidin-biotin visualisation system (peroxidase label + diaminobenzidine) from Chemicon International Inc. (USA) were used. The standardisation of the method protocol was adhered to as closely as possible across all sections. Nuclei were counterstained with Mayer's haematoxylin.

Quantitative analyses of staining intensity were performed as follows. First (using a 40× microscope objective), digital copies of the optical image were obtained, which were subsequently analysed using a licensed version of the software, specifically by performing computerised microdensitometry. The measurement results were analysed using the microprobe technique of regions showing positive staining according to the ‘Optical Density’ parameter (expressed as a relative value on a scale of 0–1, where ‘0’ corresponds to absolute optical transparency in the microprobe and ‘1’ to absolute optical opacity). The intensity of specific staining (the ‘Optical Density’ index) was equated with the degree of melatonin receptor density. Given the need to perform multiple statistical comparisons of means in statistical samples, the Newman-Keuls criterion was used to determine differences between populations.

Results. In the neurons of the supraoptic nucleus of the hypothalamus, we observed distinct positive immunohistochemical staining in the form of granules varying in size and optical density (Fig. 1).

The granules detected were concentrated mainly at the periphery of each neuron, clearly reflecting the transmembrane localisation of MT1 melatonin receptors. At the same time, no immunohistochemical staining of the nuclei was observed. The latter were stained only with haematoxylin and exhibited the typical morphological characteristics of neurons in the rat hypothalamic supraoptic nucleus. Based on the experimental results, microphotographs were taken at magnifications lower than the ‘working’ one. They are purely illustrative, and intended to demonstrate the uniformity of the response of the studied melatonin receptors in neurons of the supraoptic nuclei of

the hypothalamus at different locations.

We observed the highest density of MT1 melatonin receptors in the neurons of the supraoptic nuclei of the rat hypothalamus at 02:00 compared with 14:00 (in a field of view measuring $1600 \mu\text{m}^2$ according to the Newman-Keuls criterion, $p = 0.003$).

Under conditions of immobilisation stress, the number of neurons in the supraoptic nuclei of the hypothalamus that were positively stained for the MT1 melatonin receptor within a $1600 \mu\text{m}^2$ field of view was 0.219 ± 0.0018 at

02:00, and at 14:00 – 0.217 ± 0.0022 in terms of optical density. Differences between the study groups according to the Newman-Keuls criterion were not statistically significant ($p > 0.05$). At the same time, we recorded a significant decrease in this indicator during the observation periods ($p < 0.001$) compared with the indices of animals kept under standard lighting conditions. These patterns are illustrated in micrographs of immunohistochemical sections of the rat hypothalamus containing neurons of the supraoptic nuclei (Fig. 2).

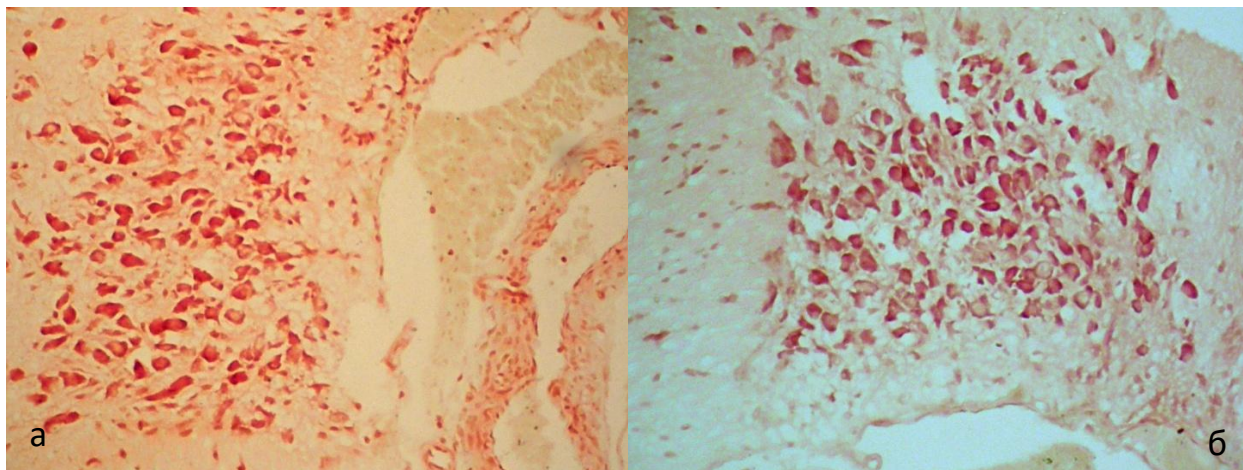


Fig. 1. Optical density of specific staining of melatonin MT1 receptors in neurons of the supraoptic nucleus of the rat hypothalamus: (a) at 02:00; (b) at 14:00 Obj. 40 \times , Oc. 10 \times

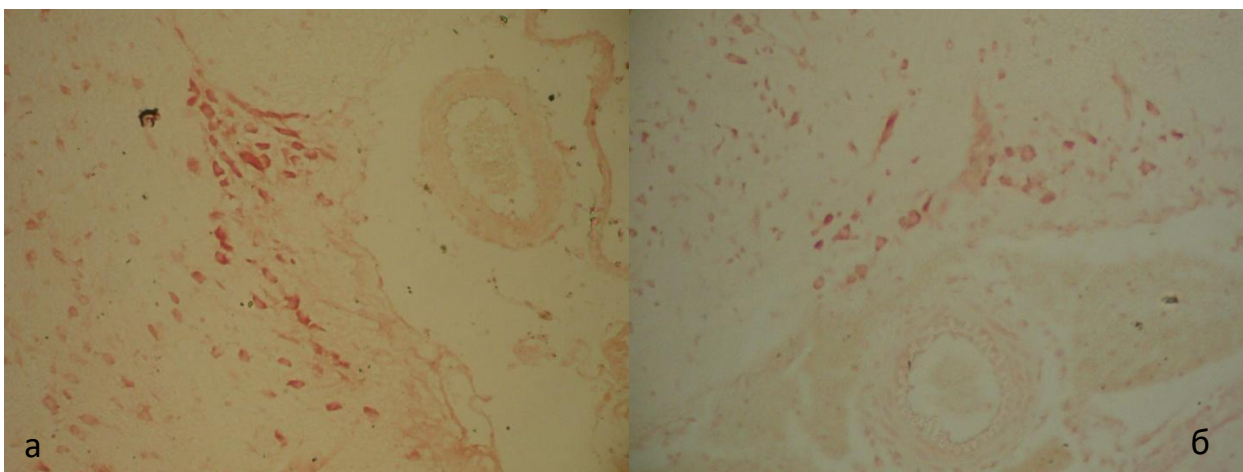


Fig. 2. Effect of immobilisation stress on the optical density of the specific staining of MT1 melatonin receptors in neurons of the supraoptic nucleus of the rat hypothalamus: (a) at 02:00; (b) at 14:00 Obj. 40 \times , Oc. 10 \times

Conclusions. 1. The density of MT1 melatonin receptors in neurons of the supraoptic nucleus of the hypothalamus in aged rats is highest at 02:00, and by 14:00 it has decreased significantly. 2. Modelling of prolonged immobilisation stress leads to a reduction in the optical density of MT1 melatonin receptors in the neurons of the supraoptic nucleus of the hypothalamus in animals.

Prospects for further research. In future studies, it is planned to perform pharmacological correction of the

observed changes in the density of melatonin MT1 receptors in neurons of the supraoptic nucleus of the hypothalamus in aged rats induced by immobilization stress.

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