Modulatory effect of irisin on inhibitory afferent inputs to the suprachiasmatic nucleus from the arcuate nucleus and its medical implications

Alexey Inyushkin[1*], *Snezhanna* Pavlenko[2], *Laila* Khadzhieva[3]
[1]Samara National Research University, 443011 Samara, Russia
[2]Kadyrov Chechen State University, Grozny, Russia

Abstract. In vitro experiments on viable hypothalamic slices of male Wistar rats, the modulating effect of the myokine irisin on the parameters of inhibitory responses of neurons in the suprachiasmatic nucleus to stimulation of the arcuate nucleus was studied. In 25% of cases, applications of 4 nM irisin caused a qualitative change in reactions, expressed in the appearance of a new reaction phase, or in the disappearance of a pre-existing inhibitory phase. In remaining cases, there was a quantitative change in the inhibitory response to stimulation in the form of a decrease in its duration. The reactions were characterized by complete reversibility: 15 minutes after "washing" the slice from irisin with artificial cerebrospinal fluid, the parameters of inhibitory reactions did not differ from the initial ones. The results obtained show that in addition to directly influencing the spike activity of neurons of the suprachiasmatic nucleus, irisin has an indirect effect on the circadian biological clock, modulating the functional state of inhibitory afferent inputs from the region of the arcuate nucleus.

1 Introduction

In the mammalian body, the entire variety of circadian rhythms is regulated by the biological clock of the suprachiasmatic nucleus of the hypothalamus, which generates an endogenous circadian rhythm [1]. The rhythm period of the biological clock, however, does not correspond to 24 hours and needs to be synchronized with the external daily rhythm. In addition to the main, photic synchronization mechanism [2], the mammalian circadian system also uses other mechanisms for entraining the biological clock. In particular, information about the mode and intensity of physical activity plays the role of a zeitgeber. The signal carrier within this synchronization mechanism is the myokine irisin [3]. Produced in contracting skeletal muscles, irisin, formed from its precursor - membrane fibronectin type III domain-containing protein 5 (FNDC5), enters the systemic circulation, crosses the blood-brain barrier, and affects brain structures. Irisin is supposed to have a direct synchronizing effect on the clock function of the suprachiasmatic nucleus, however, the mechanisms of its

* Corresponding author: ainyushkin@mail.ru.

activity remain poorly understood. The main reason for this is the lack of reliable information about irisin receptors in the central nervous system [4].

The hypothesis about the direct effect of irisin on the suprachiasmatic nucleus received experimental confirmation in our recent study performed on hypothalamic slices of rats [5], where applications of irisin caused a change in the level of spike activity and parameters of spike coding of information by neurons of this nucleus. It was previously demonstrated that endogenous regulators, along with direct effects at the level of the suprachiasmatic nucleus, are able to modulate its function indirectly, influencing the functional state of afferent projections from the neighboring arcuate nucleus, which plays an important role in the regulation of appetite and metabolism. Such an indirect mechanism has been demonstrated, in particular, for neuropeptide Y and insulin [6, 7]. It is known that the ventromedial region of the arcuate nucleus is a source of projections into the suprachiasmatic nucleus [8]. At the same time, inhibitory projections are of particular interest due to the important role in the mechanisms of circadian rhythm generation of the local network of inhibitory neurons located here [9].

The purpose of this in vitro study was to study the possible modulating effect of the myokine irisin on the functional state of inhibitory afferent inputs to the suprachiasmatic nucleus from the arcuate nucleus.

2 Materials and methods

The experiments were performed on 12 male Wistar rats weighing 80-140 g. All procedures complied with ethical standards approved by the legal acts of the Russian Federation, the principles of the Basel Declaration and the recommendations of the Bioethics Committee of the Faculty of Biology of Samara National Research University (protocol No. 2018-41 dated October 3, 2018).

At the beginning of the experiment, rats were anesthetized with urethane (Sigma-Aldrich, USA, 1.2 g/kg of body weight intraperitoneally), decapitated, and sagittal slices of the hypothalamus with a thickness of 300 μm, including suprachiasmatic and arcuate nuclei, were prepared using a vibratom (NVSL, World Precision Instruments, USA). The artificial cerebrospinal fluid contained 124 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 1.5 mM CaCl2, 1 mM MgSO4, 0.5 mM NaH2PO4, 10 mM glucose. To register the spike activity of neurons, the slices were transferred to an organic glass chamber. The chamber was perfused with artificial cerebrospinal fluid saturated with carbogen at a constant rate of 1.5 ml/min at a temperature of 27-30 °C. The spike activity of neurons of the suprachiasmatic nucleus was recorded extracellularly using glass microelectrodes filled with artificial cerebrospinal fluid of the same composition. The signal from the microelectrode was amplified (Dagan 2400A, USA), noise was eliminated with a frequency of 50 Hz (Hum Bug; Quest Scientific, Canada), digitized (Micro 1401, CED, UK) and sent to a personal computer. The Spike 2 software package (CED, UK) was used for signal visualization, storage and primary data processing.

In order to study the ability of irisin to modulate the functional state of inhibitory afferent inputs to neurons of the suprachiasmatic nucleus from the hypothalamic arcuate nucleus, reactions of spike activity of neurons of the suprachiasmatic nucleus (n = 20) to electrical stimulation of the ventromedial region of the arcuate nucleus, neurons of which are the source of projections into the suprachiasmatic nucleus were recorded [8]. A bipolar stainless steel electrode (diameter 100 μm, interelectrode distance 200 μm) was used for stimulation. Stimulation was carried out by single two-phase rectangular pulses with a frequency of 1 Hz, an amplitude of 200 μA and a duration of 1 ms. The stimulating pulses were generated using the Model 2100 electrical stimulator (A-M Systems, USA). The distance between the stimulating electrode and the area of registration of spike activity was 2.5–3 mm.

After the detection of stable spike activity, the neuron's response to electrostimulation of the arcuate nucleus was recorded in its initial state (under conditions of perfusion of the slice with artificial cerebrospinal fluid). Then irisin was applied to a perfusion solution at a concentration of 4 nM for 10 minutes, after which electrostimulation of the arcuate nucleus was performed again during the action of irisin. Finally, the perfusion was switched back to artificial cerebrospinal fluid to "wash" the slice from irisin for 15 minutes and a final test was performed with stimulation of the arcuate nucleus. Based on the data obtained, peristimulus time histograms (PSTH) were constructed and analyzed, which determined the nature of neuron responses to stimulation. The histogram was constructed by summing up data on individual moments of spike generation within every 1 s interval from the previous to the next stimulus for the entire stimulation period [10], which, depending on the level of spike activity of the cell, ranged from 100 to 400 s. The moments of the beginning of 1-second cycles during the construction of histograms were determined based on the information contained in the channel of stimulation artifacts. The study included only those neurons that showed a pattern characteristic of short-latency orthodromic inhibition: with a "gap" at the PSTH, located at a short distance from the moment of the stimulus, and a gradual restoration of activity to a control level.

The experimental data obtained were subjected to statistical processing. To compare the values of the studied parameters in the initial state, against the background of the action of irisin and after "washing" the slice with artificial cerebrospinal fluid, the ANOVA on ranks was used, since the data in the samples did not correspond to the normal distribution. The normality of data distribution in the samples was checked using the Shapiro-Wilk test, and the homogeneity of variances was checked using Levene's test. When processing peristimulus time histograms, a cumulative summation procedure was used [10], which makes it possible to identify small differences in the probability of generating spikes in the poststimulus period relative to the control period of 200 ms immediately preceding the stimulus. Inhibitory reactions were identified by a statistically significant decrease in the total number of spikes in each of the 2 ms intervals located after the moment of the stimulus, compared with the control. When analyzing peristimulus time histograms, only those reactions where the p level was $p < 0.02$ were considered statistically significant [10].

3 Research results

In this study, we characterized inhibitory responses to stimulation of the ventromedial region of the arcuate nucleus of neurons in the suprachiasmatic nucleus. In the initial state (before irisin application), statistically significant reactions to stimulation ($p < 0.02$) in the form of short-latency (< 20 ms) orthodromic inhibition were recorded in 20 neurons. The inhibitory orthodromic reaction was expressed in the characteristic form of a peristimular time histogram with a "gap", which was located at a short distance from the moment of the stimulus, after which a gradual restoration of activity to the control level was observed. The area of a "gap" corresponds to a period of reduced probability of spike generation by the studied neurone of the suprachiasmatic nucleus.

Applications of 4 nM irisin in 4 cases (25%) led to qualitative changes in the response of cells of the suprachiasmatic nucleus to stimulation of the arcuate nucleus. Qualitative changes manifested themselves in the form of the appearance of a new reaction phase, or in the disappearance of a pre-existing inhibitory phase. In one case, a new phase of the reaction was the appearance in the presence of irisin of long-latency (>20 ms) orthodromic excitation following the initial inhibitory phase (Fig. 1a). In two cases, a qualitative change in the response to stimulation was manifested in the disappearance of the pre-existing inhibitory reaction after application of irisin (Fig. 1b). Finally, in one more case, the appearance of

additional short-latency orthodromic excitation was observed before the inhibitory phase observed in the initial state.

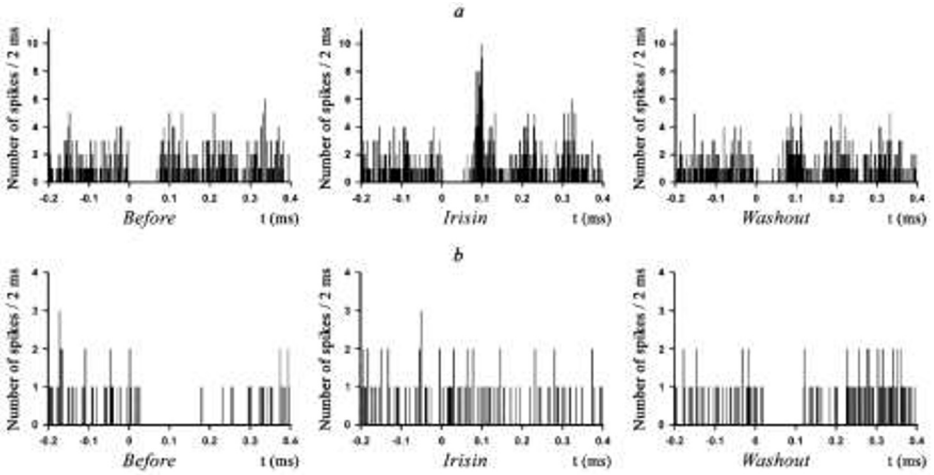

Fig. 1. The effect of 4 nM irisin on the peristimulus time histogram of neurons of the suprachiasmatic nucleus in the form of reactions of short-latency orthodromic inhibition. Examples of qualitative reactions to the effects of irisin are presented. In both cases (*a* and *b*), short-latency orthodromic inhibition was registered in the initial state (*Before*) in response to stimulation of the arcuate nucleus. In the presence of *Irisin*, the reaction of the first neuron (*a*) was transformed into a two-phase one due to long-latency excitation that appeared after the initial orthodromic inhibition. On the peristimulus time histogram of the second neuron (*b*) in the presence of *Irisin*, the initial reaction of orthodromic inhibition disappeared. In both cases, the reaction to irisin turned out to be reversible, since after 15 minutes of "washing" the slice from irisin (*Washout*), the original nature of the reaction to stimulation was restored. On the histograms, the x-axis is time in ms (the "0" mark corresponds to the moment of the stimulus); The y-axis is the total number of spikes for each 2 ms time interval.

The responses of the remaining 16 neurons of the suprachiasmatic nucleus to stimulation were characterized by quantitative changes in orthodromic inhibition. These changes were expressed in a change in the latency period or duration of the reaction. Statistical data on quantitative changes in the parameters of orthodromic inhibition are presented in Fig. 2. A group analysis of the change in the latency period of inhibition using the ANOVA on ranks showed that despite the tendency to decrease, the change in this parameter did not reach the level of statistical significance ($p = 0.169$). At the same time, a decrease in the duration of orthodromic inhibition was revealed ($p = 0.023$: ANOVA on ranks). A post hoc Student-Newman-Keuls test confirmed a statistically significant decrease in the duration of the reaction in the presence of irisin relative to the initial value. The qualitative and quantitative changes in orthodromic inhibition caused by irisin were completely reversible, since after a 15-minute "washing" of the slice with artificial cerebrospinal fluid, the initial nature of the reaction was restored, and the inhibition parameters did not differ from the initial ones ($p > 0.05$).

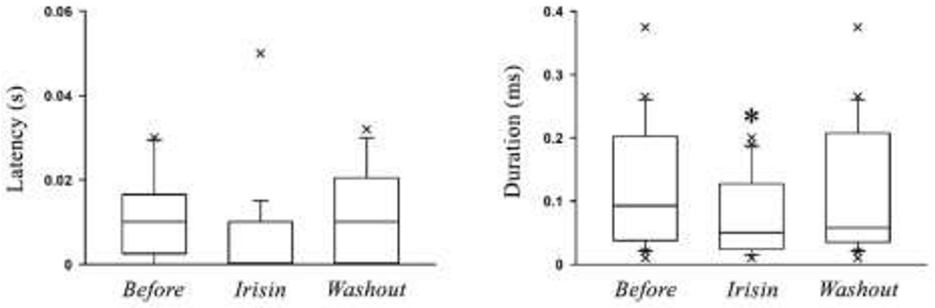

Fig. 2. The effect of 4 nM irisin on the latency period (Latency, s) and duration of orthodromic inhibition (Duration, ms) of neurons of the suprachiasmatic nucleus that responded with quantitative changes in response parameters to the effect of irisin (n = 16). The symbols under the diagrams: *Before* – the initial state; *Irisin* – during the action of irisin; *Washout* – after "washing" the slice with artificial cerebrospinal fluid. An asterisk indicates a statistically significant difference with the initial state: $*p < 0.05$.

4 Discussion of results

In this work, the effect of irisin on the functional state of inhibitory afferent inputs to neurons of the suprachiasmatic nucleus from the arcuate nucleus was studied in vitro using the electrophysiological technique of peristimulus time histogram analysis. Earlier, in electrophysiological and neurochemical studies, as well as using retrograde tracers, the existence of reciprocal connections between neurons of the arcuate and suprachiasmatic nuclei was proved [11, 12]. It has been established that reciprocal interaction between these nuclei is a necessary condition for the expression of circadian rhythms [13]. This caudal input is involved in the mechanisms of nonphotic entrainment of the circadian oscillator in accordance with the level of energy exchange, diet, and the severity of food motivation. Due to the special importance of inhibitory mechanisms for the generation of circadian rhythm [9], the object of this study was inhibitory afferent inputs to the suprachiasmatic nucleus.

The results obtained show that this mechanism of synchronization of the biological clock of the suprachiasmatic nucleus is also modulated by the myokine irisin produced by working skeletal muscles. Previously, we demonstrated the direct effect of irisin on the spike activity of neurons of the circadian oscillator of the suprachiasmatic nucleus [5]. Data obtained in the present study indicate an additional indirect mechanism of irisin's effect on the circadian biological clock, through which nonphotic entrainment of the biological clock may occur in accordance with the mode and intensity of physical activity.

The results of this work indicate the existence of a modulating effect of irisin on the functional state of inhibitory afferent inputs to neurons of the suprachiasmatic nucleus from the arcuate nucleus. In 25% of cases, under the influence of irisin, there was a qualitative transformation of the reaction of neurons of the suprachiasmatic nucleus to stimulation of the arcuate nucleus, which consisted in the disappearance of a pre-existing reaction or a phase of a complex reaction or in the appearance of an additional phase of the reaction. In addition, a statistical analysis of the reactions of the remaining 75% of neurons revealed their quantitative changes under the influence of irisin. These changes consisted primarily of reducing the duration of orthodromic inhibition. At the same time, it was not possible to detect statistically significant changes in the latency period, despite a pronounced tendency towards its shortening.

For an adequate assessment of the results obtained, the question of the place of application and the mechanisms of the modulating effect of irisin is of particular interest. At the same

time, modulation of the activity of neurons of the arcuate nucleus in the area of stimulation, modulation of the conduction of action potentials along axons traveling from the area of stimulation to the suprachiasmatic nucleus, and a modulating effect on the functional state of synapses through which information is transmitted from the endings of afferent fibers of neurons of the arcuate nucleus are potentially possible. Among the listed possibilities, the least likely is the effect of irisin on the mechanisms of action potentials propagation along axons; in any case, in the available literature there is no data on the direct effect of irisin on axonal conduction. The possibility of a modulating effect of irisin at the level of neurons of the arcuate nucleus or postsynaptic structures of the suprachiasmatic nucleus seems more likely. However, this possibility is the subject of a separate study, the implementation of which requires information about specific irisin receptors and their distribution in the central nervous system. Unfortunately, these receptors have not yet been identified [4], although evidence has been obtained of the interaction of irisin with unknown membrane receptors of hippocampal neurons and astrocytes, mediating the positive central effects of this myokine, such as improving memory, synaptic plasticity and adaptation to external influences [14]. Currently, the most likely candidates for the role of irisin receptors in the brain are integrin aVß5, while the combination of irisin with specific receptors in brain tissue can lead to the internalization of the latter into the cytoplasm [15]. There is evidence of several intracellular pathways mediating the effects of irisin in the central nervous system. The main ones among them are MAPK/ERK, PI3K/PKB и cAMP/PKA/CREB pathways [3]. Identification of irisin receptors in the central nervous system is a prerequisite for elucidating the specific mechanisms underlying the central effects of irisin.

5 Conclusion

Thus, the results obtained indicate that the effects of irisin are not limited to a direct effect on the level of activity and parameters of spike coding of information by neurons of the suprachiasmatic nucleus, as demonstrated in our previous study [5]. An additional mechanism of influence of this myokine on the functional state of the circadian oscillator is the modulation of the parameters of inhibitory afferent inputs from the arcuate nucleus, due to which irisin can be involved in the synchronization of the circadian biological clock in accordance with the mode and intensity of physical activity within the functional muscle-brain axis [3].

The work was carried out with the financial support of the Russian Science Foundation (grant No. 23-25-00152).

References

1. M. H. Hastings, M. Brancaccio, E. S. Maywood, J Neuroendocrinol **26**, 2–10 (2014)
2. A. Ashton, R. G. Foster, A. Jagannath, Int J Mol Sci **23**, 729 (2022)
3. A. N. Inyushkin, V. S. Poletaev, E. M. Inyushkina, I. S. Kalberdin, A. A. Inyushkin J Biomed Res **38**, 1–16 (2024)
4. R. Waseem, A. Shamsi, T. Mohammad, M. I. Hassan, S. N. Kazim, A. A. Chaudhary, H. A. Rudayni, M. Al-Zharani, F. Ahmad, A. Islam, Molecules **27**, 1118 (2022)
5. A. N. Inyushkin, E. M. Inyushkina, S. I. Pavlenko, T. S. Isakova, A. T. Konashenkova, I. D. Romanova, R. A. Zainulin, A. A. Inyushkin, Curr Top Pept Protein Res **24**, 85–93 (2023)

6. A. N. Inyushkin, A. A. Petrova, M. A. Tkacheva, Neurosci Behav Physiol **48**, 511–520 (2018)
7. A. N. Inyushkin, K. A. Mistryugov, O. V. Ledyaeva, I. D. Romanova, T. S. Isakova, A. A. Inyushkin, J Evol Biochem Physiol **59**, 256–273 (2023)
8. C. X. Yi, J. van der Vliet, J. Dai, G. Yin, L. Ru, R. M. Buijs, Endocrinology **147**, 283–294 (2006)
9. M. Nygard, M. Palomba, Chronobiol Int **23**, 427–435 (2006)
10. L. N. Cui, K. Saeb-Parsy, R. E. J. Dyball, J Physiol **502**, 149–159 (1997)
11. K. Saeb-Parsy, S. Lombardelli, Z. F. Khan, K. McDowall, I. T. H. Au-Yong, R. E. J. Dyball, J Neuroendocrinol **12**, 635–648 (2000)
12. S. L. Padilla, J. G. Perez, M. Ben-Hamo, C. W. Johnson, R. E. A. Sanchez, I. L. Bussi, R. D. Palmiter, H. O. de la Iglesia, Curr Biol **29**, 592–604 (2019)
13. R. Mendez-Hernandez, C. Escobar, R. M. Buijs, Obesity **28**, 10–17 (2020)
14. M. V. Lourenco, R. L. Frozza, G. B. de Freitas, H. Zhang, G. C. Kincheski, F. C. Ribeiro, R. A. Gonçalves, J. R. Clarke, D. Beckman, A. Staniszewski, H. Berman, L. A. Guerra, L. Forny-Germano, S. Meier, D. M. Wilcock, J. M. de Souza, S. Alves-Leon, V. F. Prado, M. A. M. Prado, J. F. Abisambra, F. Tovar-Moll, P. Mattos, O. Arancio, S. T. Ferreira, F. G. De Felice, Nat Med **25**, 165 (2019)
15. Y. Luo, X. Qiao, L. Xu, G. Huang, Endocrine **75**, 663–671 (2022)