

R E V I E W

On: "Inotropic effect of obestatin in the heart of a frog (*Pelophylax ridibundus*). Role of autonomic innervation." – Dissertation Thesis of **Bilyana Marianova Ilieva, PhD student** at the Department of "Animal and Human Physiology" for the acquisition of the National Doctor of Science Degree in Scientific field 4.3. Biological Sciences (Animal and Human Physiology)

Reviewer: Prof. Dr. Habil. Rositza Konakchieva, Faculty of Biology, SU "St. Kliment Ohridski"

The review of the materials presented in this procedure is in accordance with the requirements of the Law of the Development of the Academic Staff in the Republic of Bulgaria, the Regulations for its Application, Order No. RD-38-632/02.12.2022 of the Rector of SU "St. Kl. Ohridski" for the appointment of a Scientific Jury and in compliance with the recommended criteria adopted by the Academic Council of SU "St. Kl. Ohridski" for acquiring scientific degrees and holding academic positions.

General presentation of the procedure and the PhD student

The presented set of materials on paper and electronic media is in accordance with the Procedure for the acquisition of the ONS "Doctor" at SU "St. Kliment Ohridski" and includes the following documents:

- CV
- Copies of diplomas for higher education
- Rector's orders for enrollment in doctoral studies and for dismissal with the right of defense
- Certificates of passed exams from the individual plan
- Dissertation thesis
- Summary

- List of scientific publications on the dissertation topic
- Copies of scientific publications
- List of participations in scientific forums
- Other documents related to the course of the procedure

Biographical data

Assistant Bilyana Ilieva graduated with honors in 2004 with a bachelor's degree in "Biology" at the Faculty of Science of the "St. Kliment Ohridski", and subsequently a master's degree in "Animal and human physiology" at the department of the same name at the Faculty of Biology.

In the period 2007 - 2008, Assistant Professor Bilyana Ilieva worked as a specialist biologist in the "Biological effects of natural and synthetic substances" section at the Institute of Neurobiology, BAS. His research work is in the field of mediator mechanisms underlying the medicinal effects of natural and synthetic substances.

From the end of 2008 to present, she worked as an Assistant with a full academic load at the Department of Animal and Human Physiology, Faculty of Biology of the University of St. Kliment Ohridski". She conducts laboratory exercises for 3rd and 4th year bachelor's students, master's students and carries out research work. She has participated in 13 scientific projects and 11 conferences, of which 4 are international. She is the author of 8 scientific articles, three of which with impact factor on the topic of the dissertation work.

Bilyana Ilieva has enrolled as a doctoral student of independent training in professional direction 4.3. Biological sciences, doctoral program "Animal and human physiology" for the period from 02.01.2019 to 02.01.2022 (Rector's order No. RD 20-322/02.6.2019). She was dismissed with the right of defense from 01.02.2022.

Relevance of the topic and appropriateness of the set goals and tasks

The topic is relevant from a scientific-theoretical and scientific-applied point of view. Obestatin is a new and still understudied hormone. Little is known about the mechanism of its action and it is reasonable to study its role in the complex regulation and modulation of the secretion of neurotransmitters from the axons of the sympathetic division of the autonomic nervous system.

The doctoral thesis of assistant professor Bilyana Ilieva was developed under the scientific supervision of Prof. Hristo Gagov in the Department of Animal and Human Physiology at the Faculty of Biology of the University of St. Kliment Ohridski". The dissertation is based on three scientific articles - all in international scientific journals with SJR and index Q3 and Q4. In all publications, the doctoral student is the first author, which is proof of her contribution to scientific works. Assistant Professor Bilyana Ilieva has participated in five scientific forums on the topic of the dissertation, two of which are at international conferences.

The dissertation consists of 115 pages, 18 of which are cited literature. The paper is structured in accordance with accepted standards and contains all necessary sections as follows:

The literature review is detailed and thorough. The anatomy and physiology of the heart in a frog (*Pelophylax ridibundus*) are discussed in 11 pages, and the regulation of cardiac activity with an emphasis on that in amphibians - in another 7. The section "Obestatin" is of interest, where the data on this is presented in detail on 28 pages a hormone discovered in 2005. Obestatin is a degradation product of the preproghrelin polypeptide, resulting in two active products – ghrelin and obestatin. The development of scientific knowledge about obestatin is described, starting with the assumption that it suppresses eating, hence its name - from the Latin "obedere" - to swallow and "statin" - to suppress. There is little evidence for the specific mechanism of action and signaling pathway used by obestatin to initiate intracellular signaling in target cells. GPR39, originally identified as its receptor, was later dismissed as a possibility. Subsequently, glucagon-like peptide 1 has been proposed as a receptor, and data so far suggest the possibility of its activation in at least some tissues, while in others the mechanism may involve other receptor molecules. The picture is further complicated by its degradation into shorter peptides, which are also suggested to have a biological effect. The tissue-specific effects of obestatin with emphasis on the gastrointestinal tract, CNS, adipose tissue and cardiovascular system, its neuroprotective

effect and its role in metabolic syndrome are described in detail. The text in the Literature Review section is illustrated with nine figures and diagrams.

Purpose and tasks. The goal is clearly formulated - to investigate the role of autonomic innervation for the positive inotropic effect of obestatin and the mechanism by which this is realized. To achieve this goal, five experimental tasks are set.

Material and Methods section is five pages. The same experimental setup and methodology are used in all studies. Considerable space is devoted to historical notes on the isolated heart experimental model, which might find a more appropriate place in a literature review. The description is informative, proves the existence of a routine and independent experimental experience.

Results are presented on 16 pages. They are illustrated with one table and 12 figures, one of which (Fig. 8) is repeated with a different content. The PhD student used an original experimental in-vitro model and a pharmacological approach to identify the involvement of the varicose projections of sympathetic axons regulating the contractions of the frog heart chamber. The effects of obestatin and adrenaline on the amplitude of ventricular contractions of a frog heart were investigated using inhibitors for: 1) the rate-determining reaction of catecholamine synthesis, i.e. of tyrosine hydroxylase by 3-iodo-L-tyrosine (3-IT); 2) the catecholamine transporter in the neuron's secretory vesicles by reserpine and 3) the adrenaline reuptake transporter by the tricyclic antidepressant desipramine. In addition, oxidative stress was induced in axonal extensions by 6-hydroxydopamine. The data obtained are interesting and in some ways difficult to interpret. So e.g. the preliminary introduction of 3-IT completely removes the effect of obestatin, and the introduction of 3-IT in the preparation in vitro - only partially. Like 3-IT, desipramine completely abolished the effect of obestatin, but in the presence of the combination of 3-IT and obestatin, cardiac contractions were of significantly higher amplitude than controls. Reserpine has a weak positive inotropic effect that persists in the presence of obestatin, and the oxidative stress-inducing 6-OHDA increases contraction amplitude. In the presence of 6-OHDA, the direction of the obestatin effect is opposite – to a decrease in the amplitudes of ventricular contractions, although this is statistically unreliable.

Discussion section interprets the obtained data in detail in light of what is known to date about the signaling circuits and physiological effects of obestatin, as well as its effects on the

central and peripheral nervous system. In general, the results show a range from a very significant influence to complete elimination of a positive inotropic effect of obestatin in the presence of the investigated enzyme inhibitors, and the oxygen radical generator 6-OHDA, in addition to a toxic effect on axons, has a direct effect on the ventricular muscles. For an overview of the pharmacological approach used, a scheme of the studied pathways for the synthesis and secretion of catecholamines with the target molecules of the inhibitors used is included. Unfortunately, it is not clear whether the scheme is the author's original or borrowed from a literary source. .

Conclusions, although too briefly formulated, well summarize the results of the research conducted by the doctoral student. The new data obtained clearly show that the obestatin effect on the ventricular musculature is entirely due to stimulation of the sympathetic innervation, which leads to an adrenaline-dependent increase in the strength of contractions of the frog heart chamber. I also accept the formulated contribution because the interesting pharmacological approach used allows to separate the direct regulation of a certain mediator from the indirect paracrine effect carried out by adrenergic neuromediation.

Bibliography includes 284 citations, of which 282 are in English and 2 in Bulgarian. Beyond any doubt is the fact that the PhD student is well versed in the literature and skilfully handles it in interpreting the results, which is particularly evident in the Discussion section.

The Summary consists of 36 single-spaced pages and an additional 37th page of acknowledgments. It reflects the main highlights and achievements of the doctoral work, presents and discusses the obtained results, as well as contains 22 figures, 1 table, 5 conclusions, 1 contribution, a list of the 3 publications on the topic of the dissertation and the five participations in scientific forums of the PhD student on independent training Assistant Bilyana Ilieva. Summary of the thesis in English is also submitted according to the requirements. In addition, the results of this dissertation have been presented consecutively at four annual "Clementi Days" conferences, two of these conferences being international. The PhD student also personally participated in a conference organized by the Institute of Neurobiology of the BAS.

In addition to those already mentioned above, I allow myself to draw attention to technical shortcomings, the most important of which are:

- 1) The list of used abbreviations includes terminology both in Bulgarian and English. In my opinion, it is appropriate to use an English version that is generally accepted for scientific works;
- 2) The quality of the illustrations in the Literature review part is not of appropriate resolution and contrast - there is a need for professional reproduction of the figures and diagrams;
- 3) Literary sources are not numbered in the text. Using this way of presentation, the numbering of cited authors in the attached bibliography is redundant and makes it difficult for the reader.

These and other notes of a technical nature do not change my overall positive assessment of the research presented and the quality of the scientific results obtained.

The main merit of the dissertation work is the convincingly supported hypothesis that the hormone obestatin increases the force of ventricular contractions of a frog heart by an indirect mechanism related to the activation of adrenaline secretion from sympathetic nerve endings. This main conclusion was reached by developing an original approach to eliminate autonomic regulation by blocking the synthesis of catecholamines, their inclusion in secretory vesicles, their re-uptake to the axon for new secretion (ie, recycling by reuptake). The conducted research contains new knowledge and contributes to a better understanding of the regulatory effects of obestatin on the peripheral nervous system in vertebrates.

The three research papers related to the dissertation have been published in journals with SJR. In all of them, assistant professor Bilyana Ilieva is the first author, which testifies to her leading participation. Two of the publications – respectively in *Acta Zoologica Bulgarica* (SJR 0.211/2020; IF 0.354/2019; Q4) and *Current Topics in Pharmacology* (SJR 0.137/2020; Q4) include important parts of the literature review, and the third – in *Current Topics in Pharmacology* (SJR 0.121/2021; Q4) – the results of the conducted research. These 3 publications score 36 points (not 32, because two of the journals only have SJR, but are Q-indexed, which gives me a basis for awarding them 12 points, not 10 points) under criterion "D". Regardless of how the scientometric indicators of these publications are reported, they exceed the required minimum of 30 points.

Conclusion

The peer-reviewed dissertation thesis reflects an original independent scientific investigation, realized with modern methods of physiology and pharmacology. Using a consistent

and purposeful approach, the research has led to the obtaining of original data with fundamental importance and a medical-applied perspective. Bearing in mind the professional qualities of the PhD student and the scientific achievements in the present work, I express my positive assessment of the presented Thesis and confidently recommend it to the members of the Scientific Jury, appointed by Order No. RD-38-632/02.12.2022 of the Rector of SU " St. Kliment Ohridski", to award Bilyana Marianova Ilieva the educational and scientific degree "Doctor" in professional field 4.3. "Biological Sciences", scientific specialty "Animal and Human Physiology".

02/28/2023 Reviewer:

(Prof. Dr. Habil. Rositzza Konakchieva)