ANNOTATION

of the dissertation for the degree of Doctor of Philosophy (PhD)
in the educational program "8D05109 – Neuroscience"
by Aisha Kanatovna Zhantleuova
"Investigation of Non-Paralysing Botulinum Molecules on Nerve Function"

General characteristics of the work. The dissertation research focuses on studying the antinociceptive potential of a novel non-paralysing botulinum neurotoxin (BoNT), elongated isopeptide-bonded BoNT (el-iBoNT), in animal models of chronic pain.

Relevance of the topic. Chronic pain (CP) can be described as pain persisting for an extended period (more than 3–6 months) after the resolution of the initial cause (injury or event). CP poses a significant global burden, affecting more than 20% of the population according to some studies. Unlike acute pain, which serves a survival function, CP is more appropriately viewed as a disease. Globally, it results in adverse consequences such as increased healthcare costs, reduced population productivity, and social maladaptation. These outcomes are primarily due to the lack of specific, safe approaches for managing chronic pain conditions. Current pharmacological treatments for CP rely on combinations of antiepileptic drugs, anti-inflammatory agents, antidepressants, and opioids to manage pain. Recently, major organizations, such as the National Institutes of Health and the U.S. Food and Drug Administration, have concluded that this approach only increases healthcare costs and may lead to opioid dependence, higher rates of disability, and mortality. The high prevalence and refractory nature of CP, combined with the adverse effects of dependence on painkillers, have increased interest in developing novel pain management approaches.

The use of botulinum neurotoxin represents one such direction. Due to its unique property of long-term nerve transmission blockade, BoNT has become an effective means for treating and managing various conditions, including pain syndromes. However, the use of BoNT in its native form has a major drawback: the neurotoxin exhibits paralytic activity by blocking acetylcholine neurotransmission at neuromuscular synapses. This limits the maximum allowable dose of BoNT that can be safely administered to induce analgesia. Consequently, various protein reengineering methods aimed at manipulating the neurotoxin's structure have been developed to eliminate BoNT's paralytic activity. These methods are used to enhance BoNT's effectiveness as an analgesic and improve its safety profile. Using SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein) stapling technology, constructs such as Binary Toxin (BiTox), Binary Toxin/AA (BiTox/AA), Tetanus

Toxin (TetBot), Substance P Toxin (SP-Bot), and Dermorphine Toxin (Derm-Bot) have been created. Isopeptide-bonded BoNT (iBoNT) and elongated isopeptide-bonded BoNT (el-iBoNT) were created using SpyCatcher–SpyTag technology. In this dissertation, elongated isopeptide-bonded botulinum neurotoxin (el-iBoNT), distinguished by a more stable bond between its components and its elongated structure, which reduces potential side effects associated with paralytic action, was employed.

This research was conducted within the framework of the targeted funding program BR27198099, "Development of Integrative Scientific Research in Neuroscience," implemented by Al-Farabi Kazakh National University.

Research aim. To evaluate the potential of novel botulinum molecules (el-iBoNT) as non-paralysing analgesics in animal models of chronic pain.

Research objectives:

1. To produce the newly developed botulinum preparation el-iBoNT using SpyCatcher–SpyTag technology.
2. To compare the paralytic activity of native and novel neurotoxin using visual assessment.
3. To evaluate el-iBoNT's ability to correct behavioral hypersensitivity in a chronic migraine model.
4. To evaluate el-iBoNT's ability to correct behavioral hypersensitivity in a model of painful diabetic peripheral neuropathy.

Research object. Adult male white laboratory rats bred and housed at the educational and scientific laboratory base of Al-Farabi Kazakh National University (KazNU, Almaty, Kazakhstan).

Research subject. The effectiveness of the novel non-paralysing botulinum preparation el-iBoNT in controlling pain symptoms in animal models.

Research methods. Molecular-genetic, biotechnological, behavioral, biochemical, and statistical research methods were used. Molecular-genetic methods included the expression of recombinant proteins, their purification, assembly of el-iBoNT, and gel electrophoresis. Biotechnological methods involved culturing transformed *E. coli* cells in various nutrient media. Behavioral testing included the rat grimace scale, the Hargreaves test for thermal sensitivity, and the von Frey test for mechanical sensitivity. Blood glucose concentrations (a biochemical indicator) were measured using a glucometer. Statistical analysis was conducted using Prism 10.1.1 (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics for Windows, Version 29.0.1.0 (Armonk, NY, USA).

Research base. All research stages were conducted at the laboratory of the Department of Biophysics, Biomedicine, and Neuroscience at KazNU, except for the synthesis of the novel botulinum preparation el-iBoNT. This stage was

performed at the Faculty of Biomedical Sciences, University of Sheffield (Sheffield, UK) during a research internship.

Scientific novelty of the dissertation. The novel neurotoxin was applied for the first time in the nitroglycerin (NTG)-induced chronic migraine model and the streptozotocin (STZ)-induced painful diabetic peripheral neuropathy model. For the first time, the paralytic activity of el-iBoNT was studied after high-dose administration. Additionally, the ability of el-iBoNT to correct mechanical and thermal hypersensitivity was evaluated using the von Frey test and the Hargreaves test, respectively. The efficacy of el-iBoNT in preventing chronic migraine was also demonstrated using the rat grimace scale.

Theoretical and practical significance of the work. Currently, the potential of novel botulinum molecules as non-paralysing analgesic agents has not been fully explored in animal models. This study focuses on evaluating the therapeutic potential of el-iBoNT, an elongated botulinum neurotoxin type A (BoNT/A) with an isopeptide bond, in the NTG-induced chronic migraine and STZ-induced painful diabetic peripheral neuropathy models. The paralytic activity of the novel drug is also examined, as the strong paralytic effect of native neurotoxin is a major limitation to its current usage. It was established that the constructed botulinum preparation el-iBoNT possesses pronounced antinociceptive activity against chronic pain and reduced paralytic activity. This research contributes to understanding the molecular and cellular mechanisms of action of the modified botulinum neurotoxin. The findings provide a foundation for further studies of the developed molecule for treating other pain syndromes where the paralytic effect of the toxin is undesirable. In the long term, this research paves the way for creating a novel therapeutic molecule for treating chronic pain syndromes in humans.

Main statements to be defended:

1. An elongated botulinum neurotoxin type A with an isopeptide bond was obtained through recombinant synthesis with chromatographic purification.
2. Reduced paralytic efficiency of el-iBoNT was demonstrated following high-dose intramuscular administration.
3. The efficacy of el-iBoNT in pain therapy was shown in a chronic migraine model.
4. The efficacy of el-iBoNT in pain therapy was demonstrated in the painful diabetic peripheral neuropathy model.

Author's contribution. The author investigated the safe production process of botulinum molecules for chronic pain control using modern biochemical approaches, as well as molecular and cellular biology methods. The effectiveness of the novel botulinum neurotoxin el-iBoNT was studied in NTG-induced chronic migraine and STZ-induced painful diabetic peripheral neuropathy models using

behavioral tests such as the rat grimace scale, von Frey test, and Hargreaves test. The paralytic activity of the new preparation was also studied. Additionally, the author wrote abstracts, scientific articles, and the dissertation according to the established plan approved by academic advisors. The author independently conducted experiments, collected primary data, and performed statistical analysis and interpretation of the results.

Approval of the work. The materials of the dissertation were presented and published at the following conferences:

- International Scientific Conference of Students and Young Scientists "FARABI ALEMI" (Almaty, Kazakhstan, April 4–7, 2022);
- I International Scientific and Practical Conference "Integration of Sciences: Biophysics, Biomedicine, Neuroscience, and Biology" (Almaty, Kazakhstan, June 6, 2022);
- International Scientific Conference of Students and Young Scientists "FARABI ALEMI" (Almaty, Kazakhstan, April 6–8, 2023);
- II International Scientific and Practical Conference "Integration of Sciences: Biophysics, Biomedicine, Neuroscience, and Biology" (Almaty, Kazakhstan, May 25, 2023);
- Toxins 2024 7th International Conference (Berlin, Germany, January 17–20, 2024).

The main results of the dissertation were annually reviewed at the meetings of the Department of Biophysics, Biomedicine, and Neuroscience at KazNU and at doctoral research seminars.

Publications. The main content of the dissertation is reflected in 10 published works, including 2 articles in Q1 Scopus-indexed journals, 3 articles in journals recommended by the Committee for Quality Assurance in Education and Science of the Ministry of Science and Higher Education of Kazakhstan, and 5 abstracts in international conference materials, including 1 international abstract.

Volume and structure of the dissertation. The dissertation consists of an introduction, literature review, materials and methods, research results and discussion, conclusion, references, and appendices. The dissertation spans 103 pages of computer-generated text, includes 21 tables, 24 figures, and 2 appendices. The list of references comprises 183 sources, 91 of which were published within the last 10 years.