Л.Н. Гумилев атындағы Еуразия ұлттық университетінің ХАБАРШЫСЫ.
BULLETIN of L.N. Gumilyov Eurasian National University.
ВЕСТНИК Евразийского национального университета имени Л.Н. Гумилева.
ISSN: 2616-7034. eISSN: 2663-130X

IRSTI 34.39
Research article

https://doi.org/10.32523/2616-7034-2025-150-1-70-82

A novel non-paralytic botulinum neurotoxin type A for chronic pain management in animal models

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Abstract. Botulinum neurotoxin is one of the most potent biological toxins known, capable of causing severe paralysis by blocking neurotransmitter release. The use of botulinum neurotoxin (BoNT) has grown beyond its traditional application for muscle overactivity disorders, now being explored for the treatment of various chronic pain conditions such as chronic migraine (CM) and painful diabetic peripheral neuropathy (PDPN). This article aimed to assess the therapeutic potential of a newly engineered botulinum neurotoxin molecule, el-iBoNT, in animal models of CM and PDPN pain. Utilizing the innovative SpyCatcher–SpyTag protein conjugation method, we successfully produced functional botulinum neurotoxin molecules with significantly reduced paralytic effects compared to the native toxin. In both CM and PDPN models, a single administration of el-iBoNT resulted in substantial pain relief, alleviating both mechanical and thermal hypersensitivity. The findings demonstrate that el-iBoNT holds promise as an effective therapeutic agent for managing chronic pain in these conditions. Additionally, the reduced paralytic activity of el-iBoNT suggests a safer profile compared to traditional BoNT therapies. Overall, this research supports the potential of el-iBoNT as a novel treatment option for chronic pain, offering a promising alternative to existing pain management strategies, particularly those that rely on opioids, which often carry the risk of dependency and severe side effects.

Keywords: non-paralytic botulinum neurotoxin, el-iBoNT, chronic migraine, painful diabetic peripheral neuropathy, mechanical sensitivity, temperature sensitivity, rat grimace scale

Received: 04.03.2025. Accepted: 27.03.2025. Available online: 04.04.2025

Introduction

Botulinum neurotoxin type A (BoNT/A) is the longest-acting serotype due to its unique mechanism of action, involving the cleavage of the SNAP25 target protein (9 amino acids). This results in prolonged inhibition (approximately 6 months) of SNARE complex function in presynaptic nerve terminals [1-3]. In neuromuscular terminals, BoNT/A blocks acetylcholine exocytosis, leading to muscle relaxation. This property has found applications in cosmetic procedures and the treatment of conditions such as strabismus, blepharospasm, and hemifacial spasm [4-6]. When acting on sensory nerve endings associated with pain, BoNT/A exhibits additional mechanisms of action. These include inhibiting the release of nociceptive neurotransmitters at peripheral terminals [7-8], modulating the expression of ion channels and pain receptors [9], and exerting effects within the central nervous system [10-12].

Despite its therapeutic potential, the paralytic effects of native BoNT/A limit its broader application. To address this issue, several laboratories have developed modified, non-paralytic botulinum molecules using advanced technologies. These include SNARE-stapling constructs such as Binary Toxin (BiTox), Binary Toxin/AA (BiTox/AA), Tetanus Toxin (TetBot), Substance P Toxin (SP-Bot), Dermorphine Toxin (Derm-Bot), and isopeptide-bonded molecules like iBoNT and elongated iBoNT (el-iBoNT), developed using the SpyCatcher–SpyTag system [13]. This study aims to evaluate the analgesic potential of the newly engineered el-iBoNT molecule as a non-paralytic treatment for chronic pain in animal models.

Materials and research methods

Botulinum Preparations

The experiments utilized native incobotulinumtoxinA, Xeomin® (Merz Pharma GmbH & Co. KGaA, Germany), and a novel non-paralytic botulinum toxin, el-iBoNT, prepared at the University of Sheffield (Sheffield, UK). To produce el-iBoNT, represented as (1) Light chain-Translocation domain–syntaxin 1A–SpyCatcher – (2) SpyTag–Heavy Chain, two recombinant proteins – (1) and (2) – were expressed in the BL21(DE3) Escherichia coli strain. Competent cell transformation was performed via heat shock using the pGEX-KG vector. Recombinant proteins fused with glutathione S-transferase (GST) were purified on glutathione-Sepharose beads (GE Healthcare, USA), followed by thrombin cleavage. Further purification was achieved using affinity chromatography on a Superdex 200 10/200 GL column (GE Healthcare, USA). The protein yield was approximately 200 μg per liter of bacterial culture. The el-iBoNT complex was assembled by mixing LC-Td–syx–SpyCatcher and SpyTag–HC for 2 hours at 4°C in Buffer A. Purified proteins were aliquoted and stored at -80°C for subsequent experiments.

Experimental Animals

The study subjects were sexually mature male laboratory rats bred and raised under the controlled conditions of the academic and research laboratory facility at Al-Farabi Kazakh National University (Almaty, Kazakhstan).

Chronic Pain Models

A nitroglycerin (NTG)-induced chronic migraine model was established via five intraperitoneal NTG injections (10 mg/kg) administered every two days over nine days (Figure 1A) [14]. A streptozotocin (STZ)-induced model of diabetic peripheral neuropathic pain was created using a single intraperitoneal STZ injection (45 mg/kg) (Figure 1B) [15].

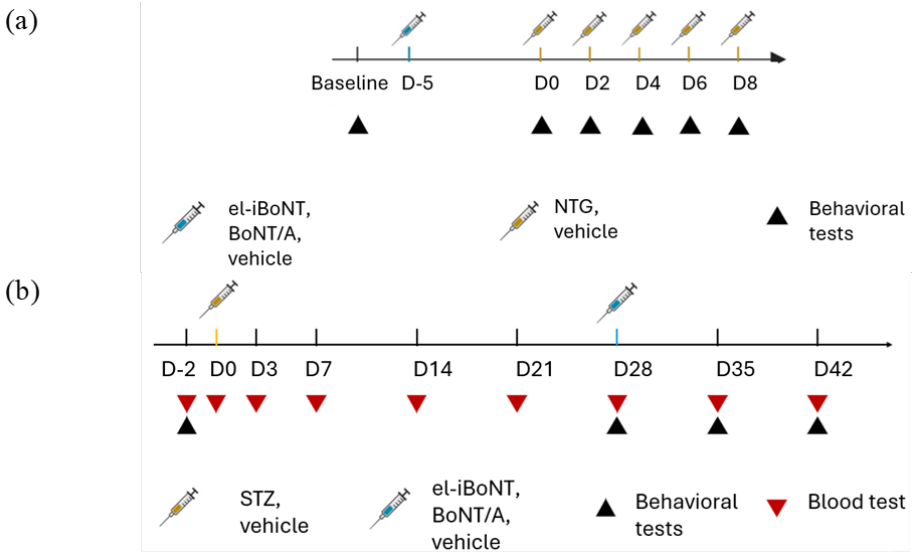


Figure 1. Timeline of behavioral testing: **(a)** CM model; **(b)** DPNP model

Behavioral Tests

The Rat Grimace Scale (RGS) was used to quantify pain by assessing specific facial features across four distinct action units: orbital tightening, nose/cheek flattening, ear changes, and whisker changes. Each action unit was scored on a 3-point scale (0 = no change, 1 = moderate change, 2 = obvious change) [16]. The von Frey Test involved placing rats in plexiglass chambers on an elevated grid and allowing them to acclimatize. Following acclimatization, the plantar surface of the paw was stimulated with a von Frey filament (BioSeb, France). The pain threshold was defined as the force (in grams) at which the rat withdrew its paw. The Hargreaves Test involved placing rats in plexiglass chambers on an elevated platform. After acclimatization, the plantar surface of the paw was stimulated with a heat beam (Hargreaves Apparatus, Ugo Basile, Italy). The pain threshold was defined as the time (in seconds) before the paw was withdrawn.

Injections

In the NTG model, prophylaxis was performed via bilateral periorbital injections of 2.5 U Xeomin®/10 ng el-iBoNT seven days before the first induced migraine episode. In the STZ model, pain control was administered on day 28 following STZ injection. This involved plantar injections of 5 U Xeomin®/20 ng el-iBoNT. Additionally, el-iBoNT (50 ng, 100 ng, and 150 ng) was injected into the gastrocnemius muscle to visually assess the in vivo paralytic activity of the molecules.

Statistical Analysis

Statistical analyses were performed 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).

Results

The non-paralytic botulinum neurotoxin el-iBoNT differs from the native toxin in its structural characteristics (Figure 2). The length and mass of el-iBoNT are 22.8 nm and 182,255 Da, respectively, which are higher than those of the native toxin, which measures 12.5 nm and 149,425 Da.

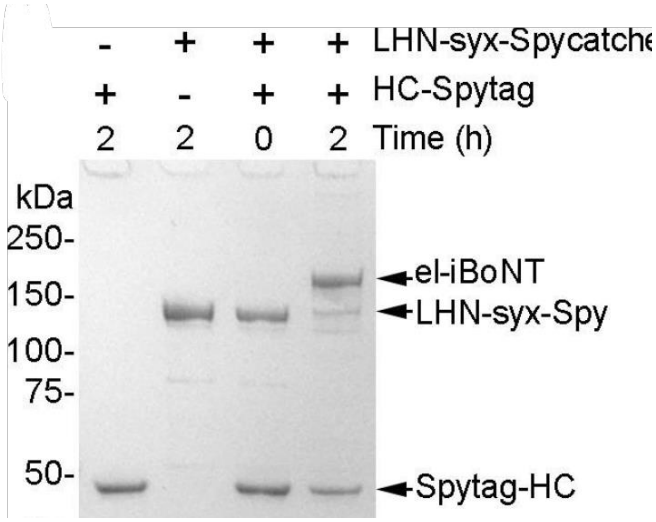


Figure 2. SDS-PAGE of el-iBoNT

It was demonstrated that when up to 150 ng of the new preparation was injected into the right gastrocnemius muscle of the rat, no motor dysfunction symptoms were observed, and the rats maintained normal mobility (Figure 3). However, rats exposed to 20 U of BoNT/A showed a characteristic response: the toes of the ipsilateral paw were clenched, the leg was extended, and the rat could not bear its weight. The animals exhibited jumping movements and had difficulty balancing while standing on their hind legs.

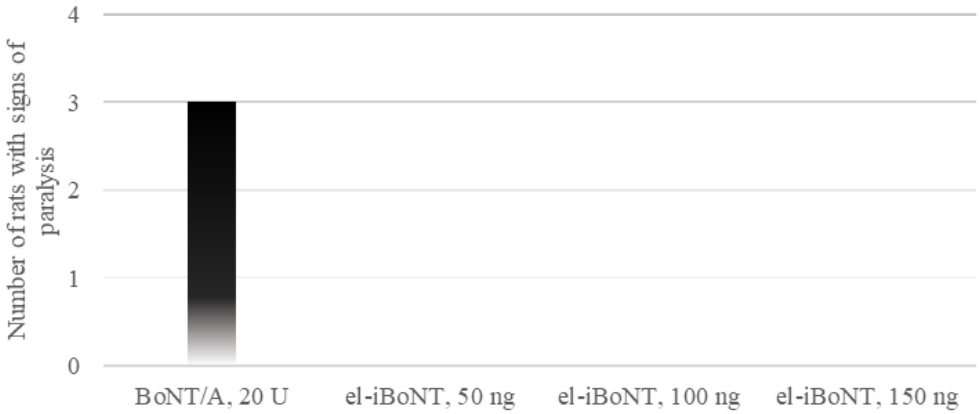
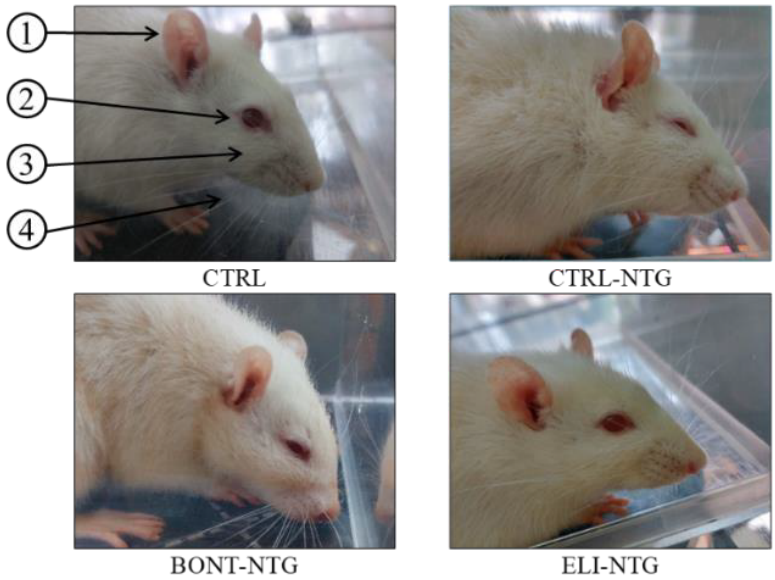


Figure 3. Histogram showing the number of rats affected by botulinum neurotoxin injections (n = 3)

The CM model involved 24 animals, which were divided into 4 groups (6 animals in each group) depending on the administered substances: 1. CTRL (n=6): saline + saline; 2. CTRL-NTG (n=6): saline + nitroglycerin (NTG), 10 mg/kg; 3. BONT-NTG (n=6): botulinum toxin type A (BoNT/A), 5 U + NTG, 10 mg/kg; 4. ELI-NTG (n=6): modified botulinum toxin (el-iBoNT), 20 ng + NTG, 10 mg/kg.

A total of 144 images were obtained, which were evaluated by two independent researchers using the rat grimace scale. Representative photographs are shown in Figure 4. The overall Cronbach's alpha coefficient was 0.81, with the highest score for orbital narrowing (0.93).

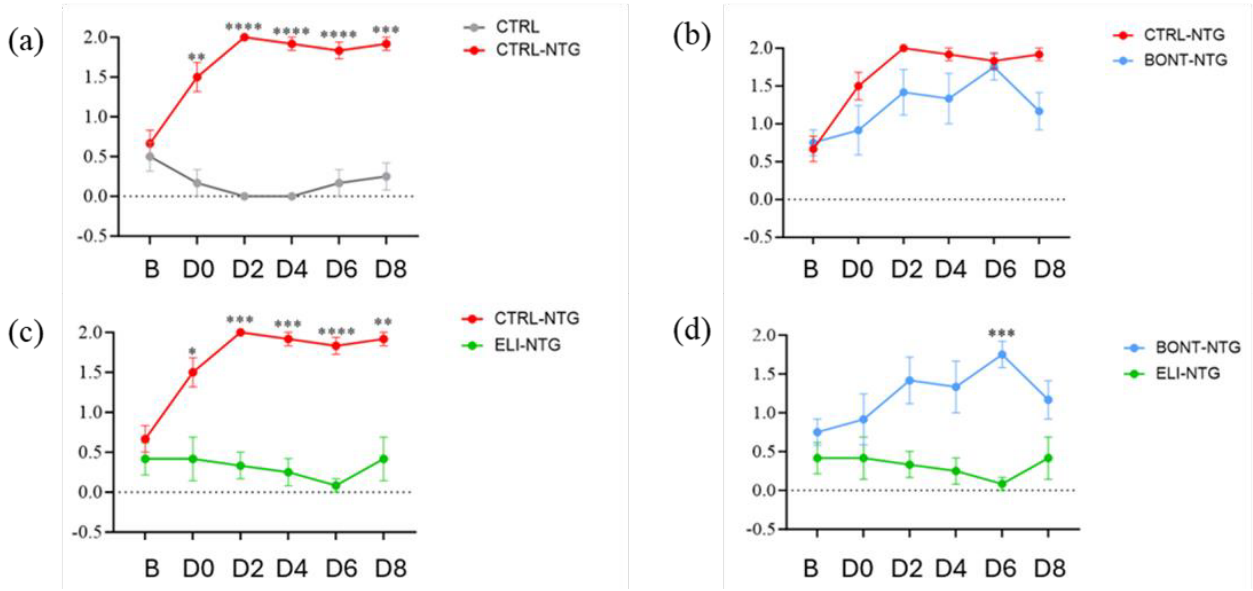


Note: 1 – ear change, 2 – orbital narrowing, 3 – smoothing of nose/cheeks, 4 – vibrissae change

Figure 4. Representative images of rat grimaces

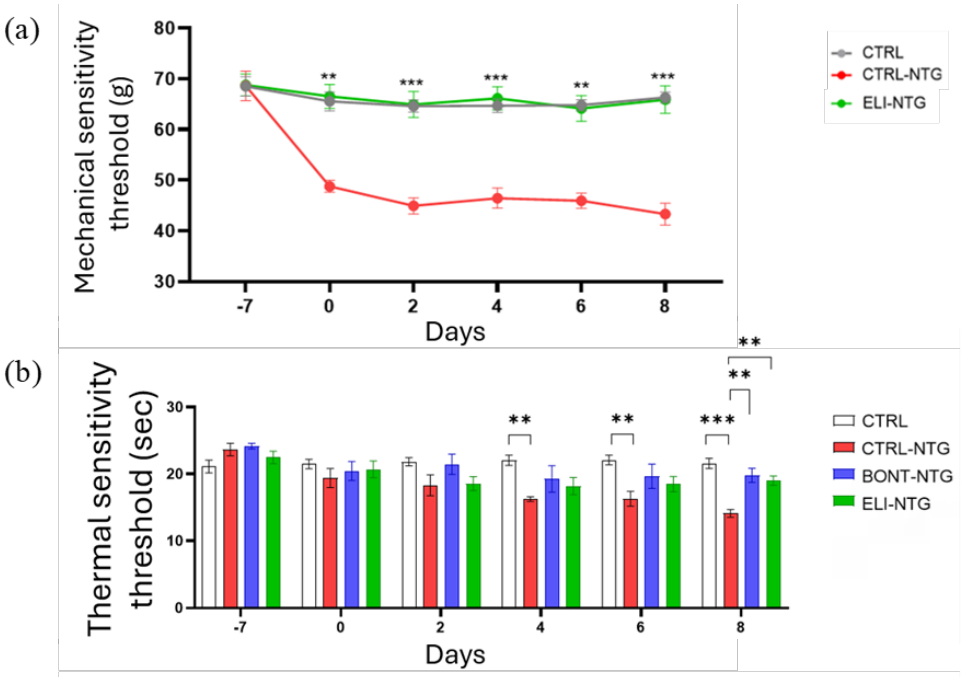
Groups CTRL and CTRL-NTG showed significant differences in the intergroup comparison of grimace scale (GS) and orbital narrowing (ON) on Days 0-8. No significant differences were found between the groups CTRL-NTG and BONT-NTG throughout the experiment. Significant differences between groups CTRL-NTG and ELI-NTG were observed in most cases (Days 2-8 for GS, Days 0-8 for ON). Animals in the ELI-NTG group also showed statistically significantly lower scores compared to BONT-NTG (Days 2, 6 for GS, Day 6 for ON; Figure 5).

On the days of nitroglycerin injection (10 mg/kg, i.p.), the animals developed mechanical hypersensitivity measured as a decrease in the mechanical threshold on the hind paws. Extracranial injections of el-iBoNT (20 ng) five days prior to CM induction maintained the mechanical threshold at levels comparable to native animals (Figure 6A). On the fourth, sixth, and eighth days of nitroglycerin injections, animals developed thermal hypersensitivity, measured by a decrease in the latency period in response to heating the medial surface of the hind paw. Extracranial injections of BoNT/A and el-iBoNT maintained the thermal threshold at levels comparable to native animals only on Day 8 (Figure 6B).



Note: B – baseline, D0 – session 1, D2 – session 2, D4 – session 3, D6 – session 4, D8 – session 5; CTRL (n=6), CTRL-NTG (n=6), BONT-NTG (n=6), ELI-NTG (n=6); asterisks indicate intragroup differences. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5. Intergroup comparison of orbital narrowing: **(a)** CTRL vs CTRL-NTG; **(b)** CTRL-NTG vs BONT-NTG; **(c)** CTRL-NTG vs ELI-NTG; **(d)** BONT-NTG vs ELI-NTG

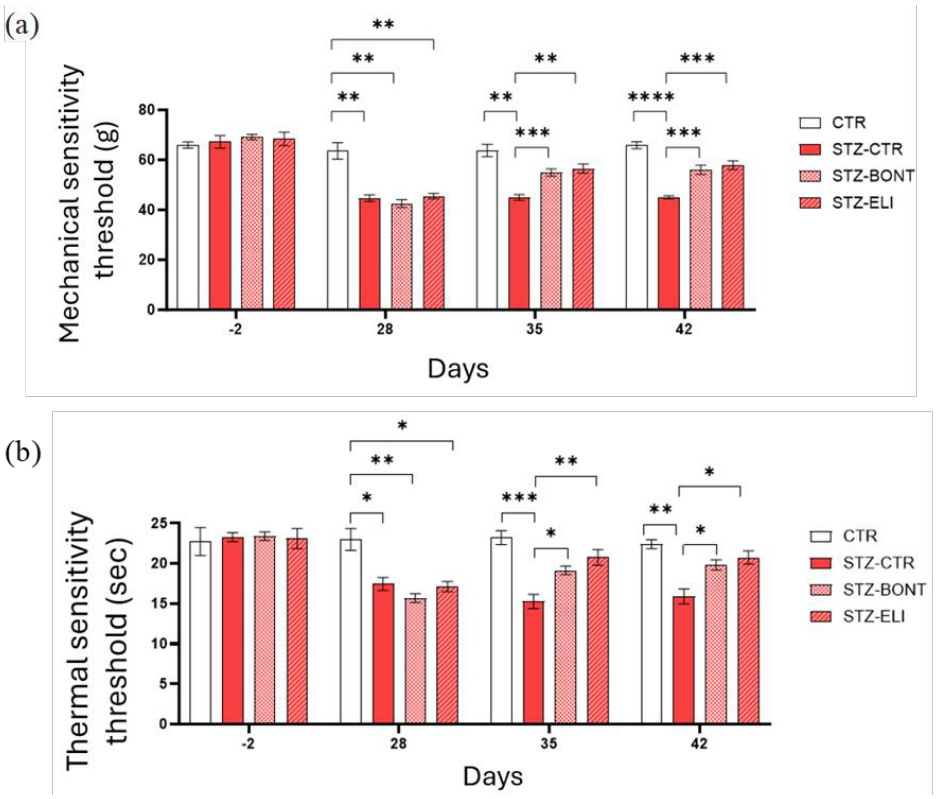


Note: -7 – seven days before CM induction, 0, 2, 4, 6, 8 – days of CM induction; CTRL (n=6), CTRL-NTG (n=6), BONT-NTG (n=6), ELI-NTG (n=6); asterisks indicate intragroup differences compared to baseline levels. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. Changes in **(a)** mechanical and **(b)** thermal hypersensitivity NTG model

The PDPN model involved 26 animals, which were divided into 4 groups as follows: 1. CTR (n=6): saline + saline; 2. STZ-CTR (n=6): streptozotocin (STZ), 45 mg/kg + saline; 3. STZ-BONT (n=7): STZ, 45 mg/kg + BoNT/A, 5 U; 4. STZ-ELI (n=7): STZ, 45 mg/kg + el-iBoNT, 20 ng.

The average blood glucose level in the STZ group was above 410 mg/dl (non-fasting) throughout the experiment. Other signs of diabetes were observed in this group, including weight loss, polydipsia, polyuria, and polyphagia (data not provided). In these animals, a single unilateral injection of BoNT/A (5 U) or el-iBoNT (20 ng) into the hind paw significantly reduced both mechanical (Figure 7A) and thermal hypersensitivity (Figure 7B).



Note: -2 – 7 days before the induction of diabetes, 0, 3, 7, 14, 21, 28, 25, 42 – days after STZ injection; CTR (n=6), STZ-CTR (n=6), STZ-BONT (n=7), STZ-ELI (n=7); asterisk indicates within-group differences compared to baseline levels. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 7. Changes in **(a)** mechanical and **(b)** thermal hypersensitivity in the PDPN model

Discussion

Attempts to develop non-paralytic variants of botulinum neurotoxin have been made previously. The protein stapling technique was used to generate BiTox [17, 18], BiTox/AA [19], TetBot [20], SP-Bot [21, 22], and Derm-Bot [21, 23]. However, the stapling method required the assembly of three polypeptides, and pain relief was only achieved at high nanogram doses, which

raises concerns about potential immune responses upon repeated administration. Moreover, the non-covalent nature of the stapling process means that dissociation of the components cannot be ruled out [24].

Unlike these stapled molecules, el-iBoNT was designed using a different approach: isopeptide bonding. Notably, the novel elongated botulinum neurotoxin has only been investigated in one study to date, where it was shown to treat nerve injury pain without causing muscle paralysis [24]. This technique allows for the spontaneous covalent linkage of the two botulinum components upon mixing, ensuring molecular stability. The results of our study show that el-iBoNT effectively reduces mechanical and thermal hypersensitivity in animal models of chronic migraine and painful diabetic peripheral neuropathy.

One of the most notable advantages of el-iBoNT is the lower required dosage for effective pain relief. For example, while BiTox required hundreds of nanograms for analgesic effects [18], el-iBoNT was effective at 20 ng, suggesting improved potency. Moreover, SP-Bot and Derm-Bot [21] target specific neuronal populations (NK1R and μ-opioid receptors, respectively), whereas el-iBoNT retains a broader mechanism of action, making it applicable to multiple pain conditions.

Another key advantage of el-iBoNT is its significantly reduced paralytic activity compared to both native BoNT/A and other engineered variants. While traditional BoNT/A blocks neurotransmission at both sensory and motor nerve terminals, leading to pain relief but also muscle paralysis, el-iBoNT was specifically designed to avoid neuromuscular toxicity while maintaining analgesic effects. This distinction is particularly evident when comparing el-iBoNT with iBoNT. Electromyographic analysis has shown that animals injected with iBoNT exhibited significant motor deficits, whereas el-iBoNT-treated animals retained normal motor function. Furthermore, immunohistochemical studies demonstrated that iBoNT caused stronger cleavage of SNAP25 in neuromuscular junctions, whereas el-iBoNT had minimal activity at these sites, explaining its lack of paralytic effects [24].

While the full mechanisms underlying the analgesic effects of el-iBoNT remain to be elucidated, recent findings suggest that it may share common pathways with BoNT/A. The analgesic effects of BoNT/A are believed to involve inhibition of neurotransmitter release (CGRP, Substance P, glutamate), modulation of nociceptive ion channels (TRPA1, TRPV1, P2X3), and retrograde transport to central pain-processing areas [25, 26]. It was demonstrated that el-iBoNT shares at least some of the key features of BoNT/A action. Firstly, Leese et al. confirmed that el-iBoNT cleaves SNAP25 in TRPV1-positive sensory fibers, further supporting its role in modulating nociceptive transmission via ion channel regulation. Additionally, el-iBoNT has been shown to suppress microglial activation in the dorsal horn, which may contribute to its central antinociceptive effects [24].

Taken together, these findings confirm that el-iBoNT retains the key analgesic mechanisms of BoNT/A while offering a potentially improved safety profile due to its reduced paralytic activity. Further studies are warranted to explore its long-term effects on neurotransmitter release, ion channel expression, and microglial function to fully define its therapeutic potential. In addition, clinical trials will be necessary to confirm the safety and efficacy of el-iBoNT in human patients.

While we demonstrated the absence of paralysis, additional studies are required to evaluate potential off-target effects.

Conclusion

The new findings presented here can be divided into several key conclusions. First, it was demonstrated that the SpyCatcher–SpyTag protein conjugation approach allows for the creation of a functional botulinum neurotoxin. This method represents a safe approach to producing botulinum molecules for therapeutic use. Second, it was shown that the el-iBoNT molecule exhibits significantly lower paralytic activity compared to the native toxin, highlighting the importance of structure in the action of molecules derived from BoNT/A. Third, el-iBoNT effectively alleviates pain induced by systemic administration of nitroglycerin in the NTG-induced chronic migraine model. Fourth, the effectiveness of el-iBoNT in pain therapy was demonstrated in the STZ-induced diabetic peripheral neuropathy pain model. Together, our study suggests that the engineered el-iBoNT molecule could become a new therapeutic agent for individuals suffering from chronic migraine and diabetic peripheral neuropathy. This is crucial given the currently limited therapeutic options for treating chronic pain, which are often ineffective, cause intolerable side effects, and contribute to the opioid crisis.

Author Contributions

B.D., A.K. – concept and supervision of the work; **A.Z. –** conducting the experiments; **A.Z., B.D., A.K. –** discussion of the research results; **A.Z. –** writing the text; **A.Z., B.D.,** and **A.K.** – editing the text of the article. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan (Grant No BR27198099).

Acknowledgments

The software used in this study was provided by Anna Andreou, King's College London, UK.

Conflicts of Interest

The authors declare no conflicts of interest.

Compliance with ethical standards

The experiments were conducted with the approval of the local ethics committee of Al-Farabi Kazakh National University (Almaty, Kazakhstan), protocol №IRB-377 dated 24.02.2022. All procedures performed in studies involving animals complied with the ethical standards of the institution where the studies were conducted and the approved legal acts of the Republic of Kazakhstan and international organizations.

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Жануарлардың ауырсыну үлгілеріндегі созылмалы ауырсынуды емдеуге арналған А типті жаңа ботулиннің паралич емес молекулалары

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Аңдатпа. Ботулиндік нейротоксин – нейротрансмиттерлердің бөлінуін бөгей отырып, ауыр салдануды тудыратын ең күшті биологиялық токсиндердің бірі. Ботулиндік нейротоксин (BoNT) дәстүрлі түрде бұлшықет гиперактивтілігін емдеуде қолданылып келген, алайда қазіргі уақытта ол әртүрлі созылмалы ауырсыну жағдайларын, оның ішінде созылмалы мигрень (CM) және ауыр диабеттік перифериялық нейропатия (PDPN) сияқты ауруларды емдеу үшін қолданылып келеді. Бұл зерттеу жаңа инжинирингтелген ботулиндік нейротоксин молекуласы, el-iBoNT, оның созылмалы мигрень мен ауыр диабеттік перифериялық нейропатия моделдеріндегі терапевтік әлеуетін бағалауды мақсат етті. SpyCatcher–SpyTag ақуызды байланыстырғыш әдісін пайдалана отырып, біз функционалды ботулиндік нейротоксин молекулаларын сәтті өндірдік, олар бастапқы токсинге қарағанда айтарлықтай төмен паралитикалық белсенділікке ие.

СМ және PDPN модельдерінде el-iBoNT бір рет енгізілгеннен кейін ауырсынуды айтарлықтай жеңілдетіп, механикалық және термиялық гиперсезімталдықты төмендетті. Бұл зерттеу нәтижелері el-iBoNT-тің созылмалы ауырсынуды басқару үшін тиімді терапевтік агент ретінде қолданылатынын көрсетеді. Сонымен қатар, el-iBoNT-тің төмен паралитикалық белсенділігі оның дәстүрлі BoNT терапияларына қарағанда қауіпсіз екендігін көрсетеді. Жалпы, бұл жұмыс el-iBoNT-ті жаңа емдеу нұсқасы ретінде дамытудың мүмкіндігін қолдайды, бұл созылмалы ауырсынуды жеңілдетуге және дәстүрлі ауырсынуды емдеу стратегияларына, оның ішінде опиоидтарға тәуелділікті азайтуға мүмкіндік береді.

Түйін сөздер: паралитикалық емес ботулиндік нейротоксин, el-iBoNT, созылмалы мигрень, ауыр диабеттік перифериялық нейропатия, механикалық сезімталдық, температуралық сезімталдық, егеуқұйрықтардың бет-әлпеті шкаласы

Новый непаралитический ботулинический нейротоксин типа А для лечения хронической боли в животных моделях боли

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Аннотация. Ботулинический нейротоксин – один из самых мощных биологических токсинов, способный вызывать тяжелый паралич, блокируя высвобождение нейротрансмиттеров. Использование ботулинического нейротоксина (BoNT) расширяется за пределы традиционного применения для лечения расстройств гиперсокращения мышц и активно исследуется для лечения различных хронических болевых состояний, таких, как хроническая мигрень (ХМ) и болевая диабетическая периферическая нейропатия (БДПН). Целью данного исследования было оценить терапевтический потенциал новой молекулы ботулинического нейротоксина, el-iBoNT, в моделях болевого синдрома при ХМ и БДПН. Используя инновационный метод конъюгации белков SpyCatcher–SpyTag, мы успешно создали функциональную молекулу ботулинического нейротоксина с существенно сниженной паралитической активностью по сравнению с нативным токсином. В обеих моделях после однократного введения el-iBoNT наблюдалось значительное облегчение боли, что проявилось в снижении как механической, так и температурной гиперчувствительности. Эти результаты демонстрируют, что el-iBoNT представляет собой эффективный терапевтический агент для лечения хронической боли при ХМ и БДПН. Кроме того, сниженная паралитическая активность el-iBoNT предполагает его более безопасный профиль по сравнению с традиционными препаратами BoNT. В целом данное исследование подтверждает потенциал el-iBoNT как новой терапии хронической боли, предлагая перспективную альтернативу существующим стратегиям лечения боли, особенно тем, которые основаны на применении опиоидов, часто сопряженных с риском зависимости и серьезных побочных эффектов.

Ключевые слова: непаралитический ботулинический нейротоксин, el-iBoNT, хроническая мигрень, болевая диабетическая периферическая нейропатия, механическая чувствительность, температурная чувствительность, шкала гримас крыс

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