pagepress

Confinia Cephalalgica 2025; 35:15779

Effects of botulinum toxin type A in a migraine-specific animal model

Chiara Demartini,[1] Rosaria Greco,[1] Sara Facchetti,[1,2] Miriam Francavilla,[1,2] Anna Maria Zanaboni,[1,2] Daniele Martinelli,[1] Cristina Tassorelli[1,2]

[1]Section of Translational Neurovascular Research, IRCCS Mondino Foundation, Pavia; [2]Department of Brain and Behavioral Sciences, University of Pavia, Italy

ABSTRACT

Background: OnabotulinumtoxinA (BoNT/A) is an established treatment for chronic migraine, although the detailed molecular mechanisms underlying its efficacy remain unclear. In this study, we examined the anti-hyperalgesic effects of BoNT/A using an animal model of migraine induced by nitroglycerin (NTG) administration associated with the orofacial formalin test, aiming to enhance our understanding of the modulatory effect of the drug on migraine pain pathways.

Methods: Male rats weighing 235-240 g (n=7 per group) were used. BoNT/A (10 U/kg) was administered unilaterally as a 25 µL bolus into the right upper lip. Rats in the control group received an injection of 25 µL of 0.9% saline. Seven days after BoNT/A injection, rats were administered NTG (10 mg/kg, i.p.) or its vehicle and were subjected to the orofacial formalin test 4 hours later. At the end of the behavioral test, the medulla-pons area and the trigeminal ganglia were collected and processed for RT-PCR analysis.

Results: At the orofacial formalin test, the NTG-treated rats had a more marked nocifensive behavior compared to vehicle-treated animals. BoNT/A pretreatment significantly reduced this behavior. In addition, calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating peptide (PACAP), and vasoactive intestinal peptide (VIP) mRNA levels were higher in the NTG-treated group in trigeminal ganglia on both sides compared to the control group, with CGRP and PACAP mRNA levels being higher on the side ipsilateral to BoNT/A injection. BoNT/A pretreatment in NTG animals reduced CGRP and VIP gene expression on both sides, while PACAP gene expression was reduced only on the trigeminal ganglion (TG) ipsilateral to BoNT/A injection. NTG treatment induced an increase in mRNA levels of all neuropeptides in the medulla-pons region, which was attenuated by BoNT/A pretreatment.

Conclusions: A single BoNT/A pretreatment attenuated mRNA upregulation of sensory neuropeptides induced by the NTG challenge in the trigeminal ganglia and medulla-pons regions.

Key words: migraine, trigeminal hyperalgesia, botulinum toxin A, neuropeptides.

Introduction

Migraine is a complex neurological disease characterized by a painful headache, gastrointestinal symptoms, and sensory abnormalities. The primary pathway involved in migraine pathophysiology is the trigeminovascular system, where trigeminal afferents innervating dural vessels transmit signals through the trigeminal ganglion (TG) to the trigeminocervical complex, a key center for nociceptive transmission to higher brain regions where pain is perceived. (1) Migraine sufferers exhibit widespread pain hypersensitivity both during and between attacks. Burstein *et al.* (2) first described increased pain sensitivity during migraine episodes, with cutaneous allodynia (3,4) and hyperalgesia. (5,6) Migraine attacks are also associated with the release of sensory neuropeptides, including calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP). (7)

The role of neuropeptides, such as CGRP and PACAP, in the pathophysiology of migraine is now widely accepted and likely involves elements of neurogenic dural inflammation. (8) Although PACAP and VIP are known as vasoactive molecules, cranial blood vessel dilation is no longer considered the basis of migraine. In addition, besides their role in inflammation, PACAP and VIP regulate innate and adaptive immune processes in the periphery. (9) The presence of VIP in neurons of the nucleus of the solitary tract suggests that VIP neurons may also contribute to central baroreceptor regulation. (10)

OnabotulinumtoxinA (BoNT/A) is an effective treatment for chronic migraine. (11) The molecular mechanisms by which BoNT/A reduces migraine pain are not well understood, but the simultaneous lowering of CGRP release, sensitivity to molecules that activate nociceptive meningeal C-afferents *via* transient receptor potential ankyrin type 1 (TRPA1) and vanilloid type 1 (TRPV1) channels, and pre-existing inflammation may be involved. BoNT/A interferes with neurotransmission through a four-step process: following the binding to the neuronal presynaptic membrane, the toxin is internalized *via* endocytosis and translocated into the cytosol, where it cleaves key proteins involved in exocytosis that modulate synaptic activity. BoNT/A inhibits synaptic vesicle trafficking, reducing neurotransmitter and inflammatory neuropeptide release, interfering with pain signaling. (12) In the sensory system, BoNT/A may specifically affect neuronal populations that mediate pain hypersensitivity, which could explain its effectiveness in certain types of chronic pain. The efficacy of BoNT/A may be due to its cellular localization, which allows for prolonged effects after a single treatment. (13)

Here, we sought to provide a deeper understanding of how the drug modulates pain pathways involved in migraine by investigating the mechanisms related to BoNT/A effects on an animal model of migraine induced by nitroglycerin (NTG) administration (14) associated with the orofacial formalin test. (15-17) More specifically, in such a model, we assessed the modulatory effect of a single BoNT/A administration on trigeminal nocifensive behavior and on the gene expression of CGRP, PACAP, and VIP in trigeminally related areas.

Results

Orofacial formalin test. NTG administration increased nocifensive behavior expressed as face-rubbing time in the second phase of the orofacial formalin test compared to the vehicle (CT group). No change was observed in the first phase of the test (**Figure 1A**), whereas pre-treatment with BoNT/A partially but significantly reduced the duration of NTG-induced nocifensive behavior in the second phase (**Figure 1B**). The time course of the test is reported in **Figure 1C**. Of note, pretreatment with BoNT/A (without NTG) did not induce changes compared to the control group (CT group) in either phase of the orofacial formalin test (**Figure 1**).

Gene expression. *mRNA expression*. Gene expression of CGRP, PACAP, and VIP was higher in the NTG-treated group compared to the vehicle-treated (CT) group in both TGs, *i.e.*, ipsi- and contralateral to BoNT/A injection and formalin administration (**Figure 2**). CGRP and PACAP mRNA levels were higher on the ipsilateral side compared to the contralateral TG (**Figure 2 A,B**). Pretreatment with BoNT/A in rats exposed to NTG (NTG+BoNT/A group) completely prevented the increase in CGRP mRNA levels in the contralateral TG and partially but significantly reduced them in the ipsilateral TG (**Figure 2A**). BoNT/A administration also reduced VIP mRNA levels in both TGs (**Figure 2C**). At variance, BoNT/A significantly reduced PACAP levels only in the ipsilateral TG (**Figure 2B**).

In the medulla-pons region, NTG administration caused an increase in mRNA levels of all the neuropeptides assessed compared to the CT group; such increase was significantly attenuated by BoNT/A treatment (NTG+BoNT/A group) (**Figure 3**). The injection of BoNT/A alone in animals treated with the NTG vehicle (BoNT/A group) did not induce changes in the gene expression of any neuropeptides assessed, either in TGs or the medulla-pons, when compared to the CT group (**Figures 2 and 3**).

Discussion

Migraine pain is mediated by peripheral and central sensitization within the trigeminal system (18) and by the release of sensory neuropeptides such as CGRP, VIP, and PACAP. (19) In migraine, both hyperalgesia and central sensitization are closely linked to dysfunction within the trigeminal system. (20) It is known that BoNT/A inhibits the release of glutamate and CGRP from primary nociceptive fibers (21-24) and thus attenuates peripheral sensitization, thereby resulting in a reduction of central sensitization. (25) Previous studies have suggested that BoNT/A may also inhibit the release of other neuropeptides, such as substance P, (26) but to the best of our knowledge, this is the first study to suggest that BoNT/A administration interferes with PACAP and VIP mRNA expression in trigeminally related areas in a rodent model of migraine that combines the administration of NTG, which mimics the central and peripheral sensitization observed in migraine patients, (27,28) with the orofacial formalin test that specifically stimulates trigeminal nociception. (14-17)

This study shows that BoNT/A reduces hyperalgesia in the trigeminal area and inhibits mRNA expression of CGRP, PACAP, and VIP in the TGs and the medulla-pons area in rats exposed to NTG. These findings expand on the already hypothesized involvement of CGRP in the BoNT/A-mediated effects in migraine. We had previously reported an upregulation of CGRP mRNA in the TGs and in the medulla-pons areas associated with trigeminal hyperalgesia assessed with the orofacial formalin test in rats treated with NTG. (29,30) More recently, we showed the involvement of PACAP and VIP in the changes caused by NTG. (30) The anti-hyperalgesic effect of BoNT/A in the orofacial formalin test and the concomitant downregulation of the CGRP, PACAP, and VIP mRNA levels in trigeminal areas suggest that the responses to BoNT/A may result from reduced release of these neuropeptides.

Although the mechanisms underlying the changes in VIP and PACAP expression in the central areas evaluated remain unclear, it is plausible to hypothesize that NTG administration induces a co-change in gene expression of neuropeptides, as reported in recent studies. (30,31) However, the observed changes in neuropeptide gene expression relate to the medulla-pons *in toto* and not to specific brain nuclei. Therefore, at present, we cannot exclude that different nuclei in the area may respond differently to BoNT/A. (32,33) Further research on smaller areas is required for a more conclusive view.

Contrary to previous findings in which BoNT/A pretreatment attenuated plantar or orofacial formalin-induced hyperalgesia (34-36) and reduced expression of CGRP mRNA, (37-39) here we did not find any difference between the BoNT/A group and the control group in the absence of NTG administration. This discrepancy may be due to the lower concentration of formalin used in our study (1.5% *versus* 2%, 2.5%, or 3% in the other studies) or to the different injection site (upper lip *versus* vibrissa pad). (40-43) Notably, diverse doses of formalin may stimulate distinct primary sensory neurons (nociceptors and non-nociceptive afferents), although the relationship between formalin dose, type of fiber involved, and behavior is unclear. (44)

Our results suggest that the antinociceptive effect of BoNT/A specifically targets the hyperalgesia of the second phase of the formalin test, amplified by NTG. If we assume that the changes induced by NTG are characteristic of migraine, (14) we can infer that BoNT/A's mechanism of action is, at least partly, specific for migraine-like symptoms, further supporting the rationale for its use for migraine management. (45)

These findings support a role of BoNT/A as a means to better understand migraine pathophysiology. Retrograde axonal transport of BoNT/A from the sensory nerve endings to the central terminals of the primary afferent fibers may reduce nociception by modulating neuropeptide expression and release. Release inhibition may be due to a direct toxin proteolytic action on the SNARE complex, specifically by cleaving SNAP-25, and preventing the fusion of the synaptic vesicles with the membrane. (26) A reduced release of neuropeptides, especially of CGRP, may be caused by the downregulation of TRPV1 and TRPA1 expression in peptidergic primary sensory neurons. (46-48) Notably, increased TRPV1 and TRPA1 expression within the trigeminal structures was reported to increase migraine-like pain. (49-51) Finally, BoNT/A may reduce pain and inflammation by blocking local neurotransmitter release from peripheral sensory nerves. (52,53)

BoNT/A may also affect inflammatory and immune cells and glial cells in the central nervous system. In a neuropathic pain model (chronic constriction injury to the rat sciatic nerve), Gui *et al.* (54) reported that subcutaneous injection of BoNT/A into the metatarsal surface stimulated the polarization of microglia towards an anti-inflammatory phenotype. In a rat model of complete Freund's adjuvant-induced arthritis, the intra-articular administration of BoNT/A reduced the expression of the pro-inflammatory cytokines, interleukin-1β and tumor necrosis factor-α in synovial fluid, indicating its anti-inflammatory effects. (55) BoNT/A may regulate key analgesic genes and molecules associated with specific analgesic pathways. (56) However, other studies challenged the association between the anti-inflammatory and antinociceptive effects of BoNT/A, as no anti-inflammatory effects were observed at doses of BoNT/A that reduced pain caused by capsaicin and carrageenan. (57-59) However, BoNT/A doses used in animal models are usually higher than therapeutic doses for humans,

(60) likely due to the difference in BoNT/A sensitivity between rodents and humans. (61)

In NTG-challenged rats, BoNT/A reduced CGRP and VIP mRNA levels in both TGs, while PACAP expression was reduced only on the TG ipsilateral to BoNT/A and formalin injection. Because of the potential long-distance retrograde effects of BoNT/A, (62) we cannot exclude the possibility of a diffusion of the toxin to the contralateral side. The difference between the

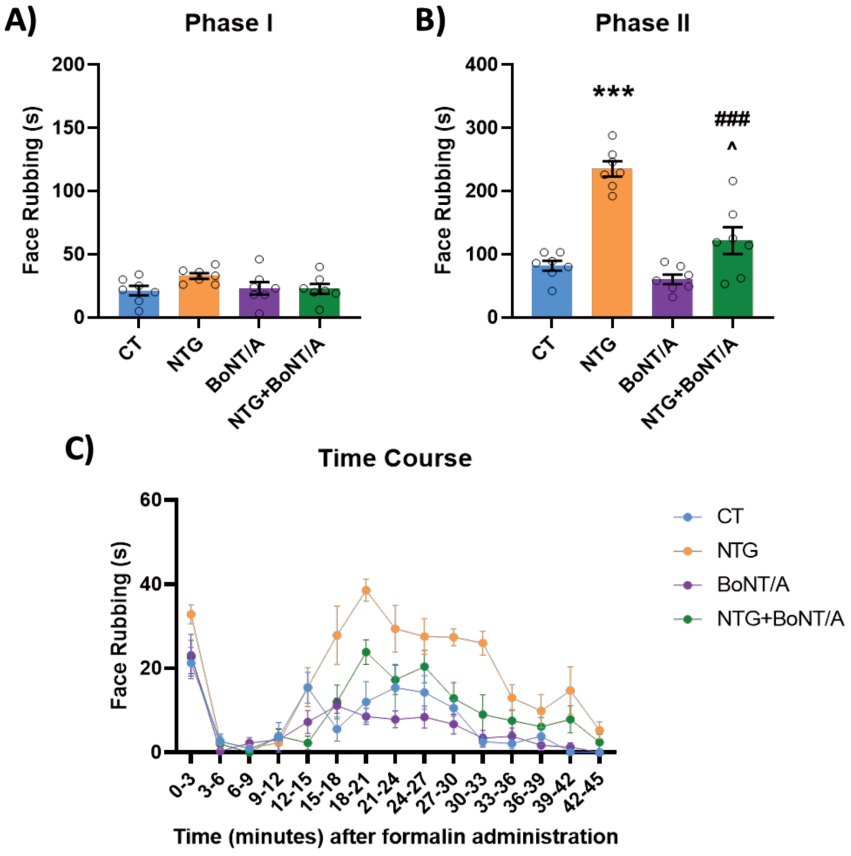


Figure 1. *Orofacial formalin test*. Time spent in face-rubbing behavior (seconds) during Phases I (A) and II (B) and time course (C) of the orofacial formalin test.
Data are expressed as mean ± SEM. one-way ANOVA followed by Tukey's multiple comparisons test: ***p<0.001 *vs.* CT and BoNT/A; ^p<0.05 *vs.* BoNT/A; ###p<0.001 *vs.* NTG. n=7.

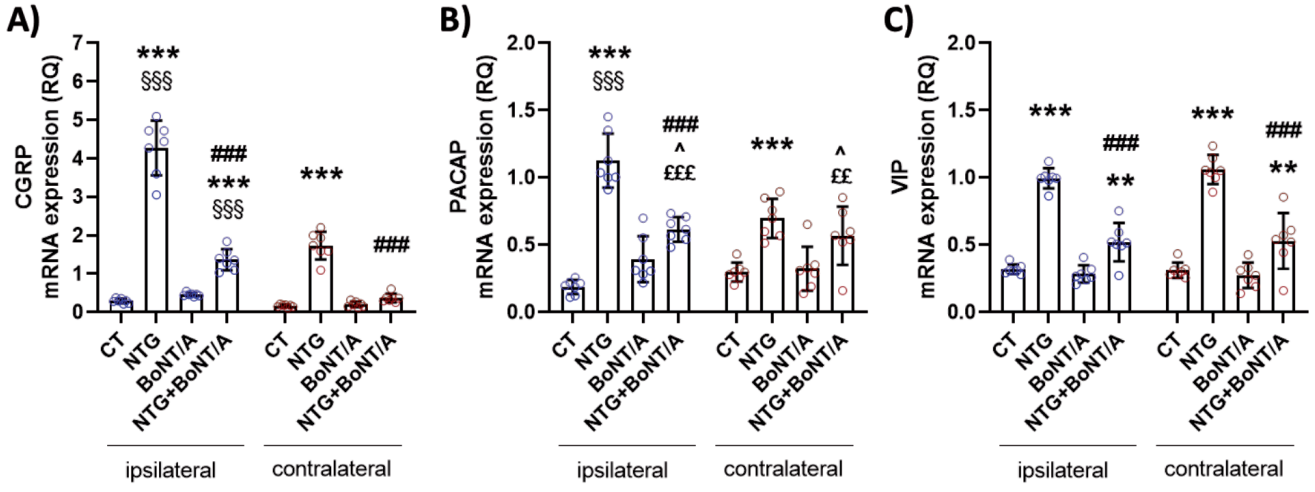


Figure 2. *Gene expression*. mRNA expression levels of CGRP (A), PACAP (B), and VIP (C) in the trigeminal ganglion ipsilateral and contralateral to the BoNT/A/vehicle and formalin injections. BoNT/A and formalin were administered unilaterally, on the same side. Data are expressed as mean ± SEM. Two-way ANOVA followed by Tukey's multiple comparisons test: **p<0.01 and ***p<0.001 *vs.* CT and BoNT/A; ££p<0.01 and £££<p0.001 *vs.* CT; ^p<0.05 *vs.* BoNT/A; ###p<0.001 *vs.* NTG; §§§p<0.001 *vs.* contralateral side. n=7.

expression of PACAP and CGRP or VIP may be related to the transport properties of BoNT/A (13) or to the different pattern of expression of these neuropeptides in the diverse neuron subpopulations. For instance, in TGs, most sensory neurons express CGRP, (63-65) while only a few express PACAP. (63) Additionally, using PACAP, but not CGRP, can be detected immunohistochemically in satellite glial cells, (66) where a direct interaction with BoNT/A may affect vesicle release. (67) Thus, BoNT/A may not have reached the contralateral trigeminal neurons expressing PACAP nor the satellite glial cells where PACAP is expressed. (68)

CGRP and PACAP exhibit different actions in humans and rodents, indicating the involvement of separate intracellular pathways and targets. (69) Unfortunately, although VIP presence has been reported in the rat TG, lesser data are available on neuropeptide distribution and function at this level. (70) Although BoNT/A's ability to target both neurons and glial cells in the central nervous system has been reported, (71) further studies are needed to identify the mechanisms and the mediators relevant for migraine in these different cell types.

Altogether, these data support the view that the anti-hyperalgesic effect of peripherally administered BoNT/A may modulate central pathways (72) by targeting second-order neurons. (73,74) This hypothesis is corroborated by other studies showing the presence of BoNT/A-truncated SNAP-25 in several central nervous system regions, including the dorsal horn of the trigeminal nucleus caudalis following injection into the rat whisker pad, (75) the ipsilateral dorsal and ventral horns of the spinal cord following toxin injection into the sciatic nerve (76) and spinal astrocytes following peripheral sciatic nerve injection. (77) However, a reduction in pain-evoked c-Fos expression in areas where BoNT/A activity was not detected suggests that the indirect and distant effects in the central nervous system might be more extensive than those at the site where BoNT/A cleaves SNAP-25. (47)

Limitations of the study. The animal model based on NTG administration used in this study is widely accepted as a migraine-specific model because it reproduces many characteristics of the disease. (14,78) However, there are a few aspects that should be considered. Due to the lower bioconversion efficiency of NTG in rat liver compared to the human liver, (79) a high dose of NTG is required to induce migraine-like behavioral and neurobiological effects in rats. (14,78) The systemic NTG administration is considered a reliable animal model of migraine, (14-17,78,80) allowing comparisons with our prior studies with CGRP, with the effect of other anti-migraine compounds in the same model. (29,81) However, we acknowledge that other readouts could be used instead of the chemical hypersensitivity with formalin test, such as mechanical allodynia with the von Frey filaments. Finally, we investigated the effects of a single dose of BoNT/A when, in clinical practice, patients receive several BoNT/A administrations. Further evaluations will thus be necessary to investigate BoNT/A effects in an experimental paradigm that better reflects its preventive activity.

Conclusions

In the migraine-specific model of NTG-enhanced oral-facial formalin test, BoNT/A shows an anti-hyperalgesic effect that is associated with downregulation of the mRNA of pro-migraine neuropeptides in peripheral and central sites (trigeminal ganglia and medulla-pons, respectively). These findings provide additional information on the mechanisms through which BoNT/A modulates the pathways implicated in migraine pain.

Materials and Methods

Animals and experimental design. All experiments described in this study were conducted in accordance with the European Community Council Directive of 22 September 2010 (2010/63/EEC). The experimental protocol was approved by the Italian Ministry of Health (Document No. 1032/2015-PR). For this research, 28 adult male Sprague-Dawley rats (Charles River, Calco, Como, Italy), each weighing between 235 and 240 grams, were used. All rats were housed in pairs on a 12h/12h light/dark cycle with food and water available ad libitum at an ambient temperature of 22°C at the centralized animal husbandry facility of the University of Pavia. The animals were randomly allocated into four experimental groups and treated as follows. One hundred units of BoNT/A (Botox®, Allergan Inc., Irvine, CA, USA) were dissolved in 1 mL of 0.9% saline; 10 U/kg of BoNT/A was admin-

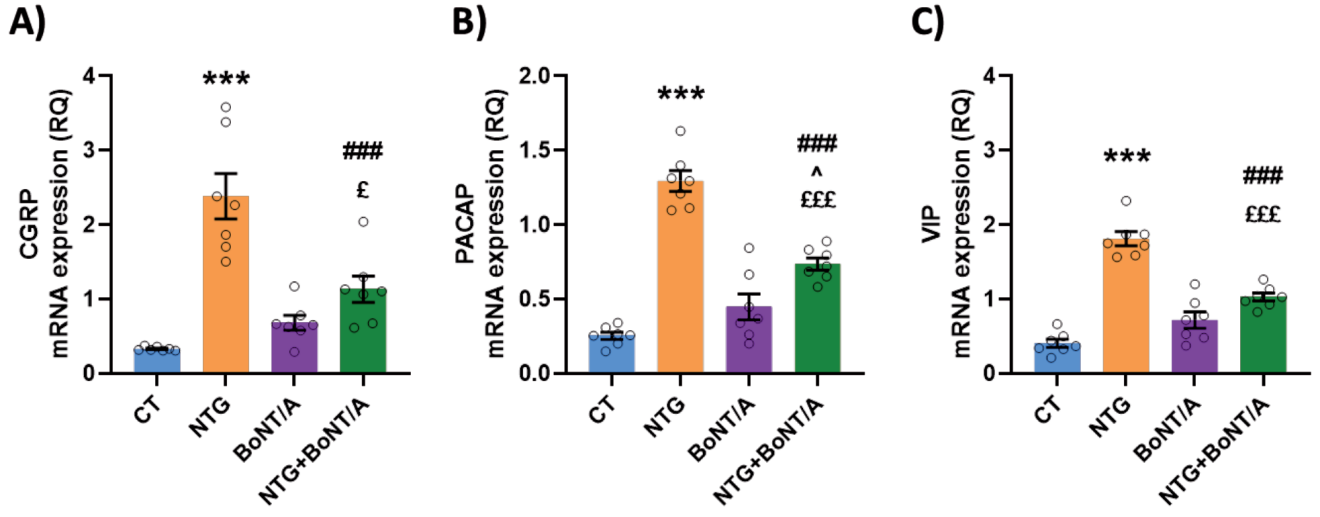


Figure 3. *Gene expression*. mRNA expression levels of CGRP (A), PACAP (B), and VIP (C) in the medulla-pons area. Data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test: ***p<0.001 *vs.* CT and BoNT/A; £p<0.05 and £££<p0.001 *vs.* CT; ^p<0.05 *vs.* BoNT/A; ###p<0.001 *vs.* NTG. n=7

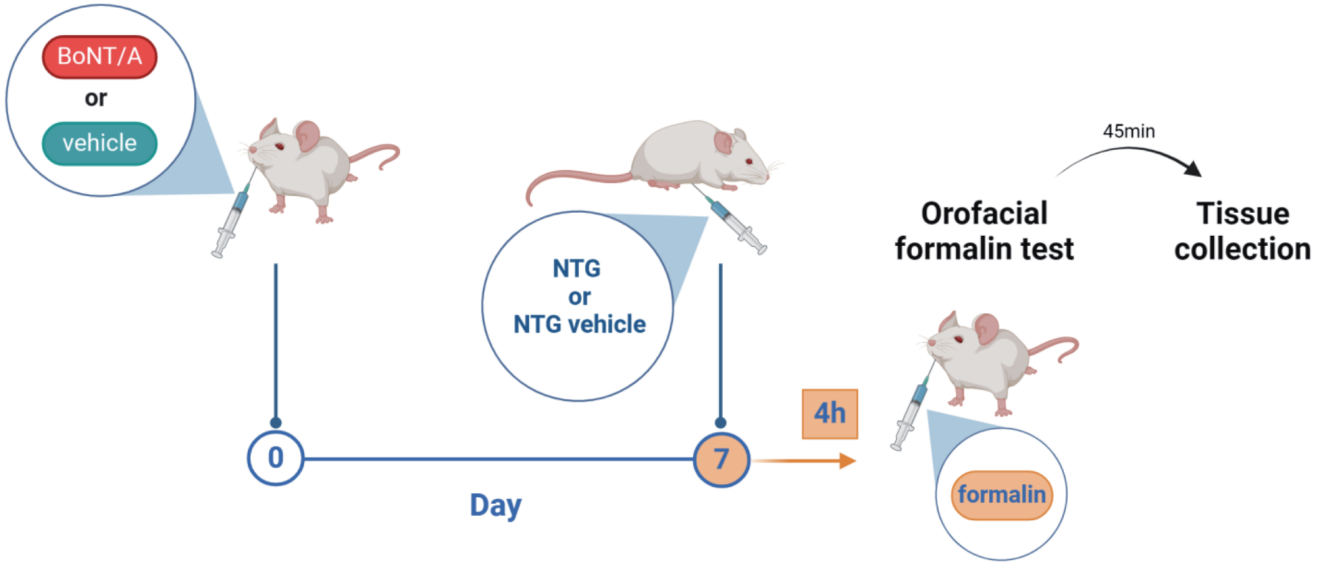
istered unilaterally as a 25 µL bolus into their right upper lip, using a 0.5 mL syringe with a 30-gauge needle, 7 days before NTG (NTG+BoNT/A group) and vehicle (BoNT/A group) administration, following the experimental design of a previous study, done in the same animal model but with a modified toxin. (82) The 10 U/kg dose of BoNT/A used in our study aligns with the dose range commonly employed for nociceptive evaluations in rats, including animal models such as the formalin test. (47,83) As a control group, 25 µL of a 0.9% saline solution was injected similarly to BoNT/A. Seven days after BoNT/A (or vehicle) injection, rats were treated with NTG (10 mg/kg, i.p.; Bioindustria L.I.M., Novi Ligure, Italy) or its vehicle (6% alcohol, 16% propylene glycol, and saline) and subjected to the orofacial formalin test on the same side of BoNT/A injection. **Figure 4** depicts the experimental design and groups.

Behavioral test. After an acclimatization period of 20-25 minutes in the test box (a 30 cm x 30 cm x 30 cm glass chamber with mirrored sides), all rats were treated subcutaneously with 50 µL of formalin 1.5% (v/v) into the right upper lip (ipsilateral to BoNT/A or vehicle injection). A camera was placed 50 cm from the box to record each animal's face rubbing for later examination. A researcher blinded to treatment evaluated the face-rubbing time by measuring the number of seconds the animal spent rubbing the injected area with the ipsilateral forepaw or hind paw 0-3 min (Phase I) and 12-45 min (Phase II) after formalin injection. The observation time was divided into 15 blocks of three minutes each for the time course analysis.

mRNA expression. At the end of the orofacial formalin test, rats were sacrificed under deep anesthesia (sodium thiopental, 150 mg/kg, i.p.). Following decapitation, the medulla pons *in toto* (bregma: -13.30 to -14.60 mm) and TGs were dissected, washed in cold 0.9% saline solution, placed in cryogenic tubes, and stored at -80°C until rt-PCR analysis for CGRP, PACAP, and VIP gene expression. RNA extraction was performed in an RNase-free environment, ensuring all RNA samples had an absorbance ratio (260/280 nm) between 1.9 and 2.0, indicating minimal protein contamination, including from blood. The primer sequences are detailed in **Table 1** and provided by

Table 1. Primer sequences.

Gene	Forward primer	Reverse primer
GAPDH	AACCTGCCAAGTATGATGAC	GGAGTTGCTGTTGAAGTCA
CGRP	CAGTCTCAGCTCCAAGTCATC	TTCCAAGGTTGACCTCAAAG
PACAP	CAAGACTTCTATGACTGGGAC	CTTCGTTAAGGATTTCGTGG
VIP	CAGGCATGCTGATGGAGTTT	TGCTTTCTAAGGCGGGTGTA



Experimental groups:

CT = BoNT/A vehicle, 25 µl s.c. lip + NTG vehicle, i.p.

NTG = BoNT/A vehicle, 25 µl s.c. lip + NTG 10mg/kg, i.p.

BoNT/A = BoNT/A 10 U/kg, 25 µl s.c. lip + NTG vehicle, i.p.

NTG + BoNT/A = BoNT/A 10 U/kg, 25 µl s.c. lip + NTG 10mg/kg, i.p.

Figure 4. *Experimental design and groups*. Rats were treated with either OnabotulinumtoxinA (BoNT/A) or its vehicle in the right upper lip. Seven days later, they received an intraperitoneal injection of nitroglycerin (NTG) or its vehicle. Four hours after the administration of NTG or the NTG vehicle, each rat was injected in the right upper lip with formalin and underwent the orofacial formalin test, which lasted 45 minutes. At the end of the behavioral test, the animals were sacrificed, and samples were collected.

Merck. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization, as its expression remained consistent across all experimental groups. All samples were assayed in triplicate, and the $2_{-\Delta\Delta Ct}$ method was used to investigate the differences in gene expression levels.

Statistical analysis. To calculate the minimum sample size required, we used the nociceptive response in Phase II of the orofacial formalin test (measured by face rubbing time), identified as the primary outcome of the present study. Based on a previous study (82) in which we tested the effect of a modified botulinum toxin in the orofacial formalin test, we assumed an effect size of 0.78 using the unmodified toxin under the same experimental conditions. An *a priori* power analysis was conducted to obtain a statistical power of 0.80 at an alpha level of 0.05 (GPower 3.1), obtaining a minimum of 6 animals per experimental group. However, due to the intergroup variability seen in the orofacial formalin test, we used a maximum of 7 rats per group. To confirm data normality, the Kolmogorov-Smirnov (K-S) test was applied. Differences among groups were compared by one-way or two-way analysis of variance (ANOVA) followed by the Tukey's multiple comparisons test. Statistical significance was set at $p<0.05$. All statistical analyses were performed using GraphPad Prism software (version 8). Data were expressed as mean ± SEM.

References

1. Puledda F, Silva EM, Suwanlaong K, Goadsby PJ. Migraine: from pathophysiology to treatment. J Neurol 2023;270:3 654-66.
2. Burstein R, Yarnitsky D, Goor-Aryeh I, Ransil BJ, Bajwa ZH. An association between migraine and cutaneous allodynia. Ann Neurol 2000;47:614-24.
3. Louter MA, Bosker JE, van Oosterhout WPJ, van Zwet EW, Zitman FG, Ferrari MD, et al. Cutaneous allodynia as a predictor of migraine chronification. Brain 2013;136:3489-96.
4. Lipton RB, Bigal ME, Ashina S, Burstein R, Silberstein S, Reed ML, et al. Cutaneous allodynia in the migraine population. Ann Neurol 2008;63:148-58.
5. Weissman-Fogel I, Sprecher E, Granovsky Y, Yarnitsky D. Repeated noxious stimulation of the skin enhances cutaneous pain perception of migraine patients in-between attacks: clinical evidence for continuous sub-threshold increase in membrane excitability of central trigeminovascular neurons. Pain 2003;104:693-700.
6. Nicolodi M, Sicuteri R, Coppola G, Greco E, Pietrini U, Sicuteri F. Visceral pain threshold is deeply lowered far from the head in migraine. Headache 1994;34:12-9.
7. Demartini C, Francavilla M, Zanaboni AM, Facchetti S, De Icco R, Martinelli D, et al. Biomarkers of Migraine: An Integrated Evaluation of Preclinical and Clinical Findings. Int J Mol Sci 2023;24.
8. Goadsby PJ, Holland PR, Martins-Oliveira M, Hoffmann J, Schankin C, Akerman S. Pathophysiology of Migraine: A Disorder of Sensory Processing. Physiol Rev 2017;97: 553-622.
9. Delgado M, Pozo D, Ganea D. The Significance of Vasoactive Intestinal Peptide in Immunomodulation. Pharmacol Rev 2004;56:249-90.
10. Palkovits M, Léránth Cs, Eiden LE, Rotsztejn W, Williams TH. Intrinsic vasoactive intestinal polypeptide (VIP)-containing neurons in the baroreceptor nucleus of the solitary tract in rat. Brain Res 1982;244:351-5.
11. Baraldi C, Lo Castro F, Ornello R, Sacco S, Pani L, Guerzoni S. OnabotulinumtoxinA: Still the Present for Chronic Migraine. Toxins (Basel) 2023;15.
12. Luvisetto S, Gazerani P, Cianchetti C, Pavone F. Botulinum Toxin Type A as a Therapeutic Agent against Headache and Related Disorders. Toxins (Basel) 2015;7:3818-44.
13. Matak I, Bölcskei K, Bach-Rojecky L, Helyes Z. Mechanisms of Botulinum Toxin Type A Action on Pain. Toxins (Basel) 2019;11.
14. Demartini C, Greco R, Zanaboni AM, Sances G, De Icco R, Borsook D, et al. Nitroglycerin as a comparative experimental model of migraine pain: From animal to human and back. Prog Neurobiol 2019;177:15-32.
15. Casili G, Lanza M, Filippone A, Campolo M, Paterniti I, Cuzzocrea S, et al. Dimethyl fumarate alleviates the nitroglycerin (NTG)-induced migraine in mice. J Neuroinflammation 2020.
16. Lanza M, Filippone A, Ardizzone A, Casili G, Paterniti I, Esposito E, et al. SCFA Treatment Alleviates Pathological Signs of Migraine and Related Intestinal Alterations in a Mouse Model of NTG-Induced Migraine. Cells 2021;10.
17. Tajabadi A, Abbasnejad M, Kooshki R, Esmaeili-Mahani S, Raoof M, Lobbezoo F. Repeated gentle handling or maternal deprivation during the neonatal stage increases adult male rats' baseline orofacial pain responsiveness. Arch Oral Biol 2023;151:105699.
18. Burstein R. Deconstructing migraine headache into peripheral and central sensitization. Pain 2001;89:107-10.
19. Tanaka M, Szabó Á, Körtési T, Szok D, Tajti J, Vécsei L. From CGRP to PACAP, VIP, and Beyond: Unraveling the Next Chapters in Migraine Treatment. Cells 2023;12.
20. Deodato M, Granato A, Martini M, Sabot R, Buoite Stella A, Manganotti P. Instrumental assessment of pressure pain threshold over trigeminal and extra-trigeminal area in people with episodic and chronic migraine: a cross-sectional observational study. Neurol Sci 2024;45:3923-9.
21. Durham PL, Cady R, Cady R. Regulation of Calcitonin Gene Related Peptide Secretion From Trigeminal Nerve Cells by Botulinum Toxin Type A: Implications for Migraine Therapy. Headache 2004;44:35-43.
22. Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. Toxicon 2000;38:245-58.
23. Purkiss J, Welch M, Doward S, Foster K. Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. Biochem Pharmacol 2000;59:1403-6.
24. Ashina M, Hansen JM, Do TP, Melo-Carrillo A, Burstein R, Moskowitz MA. Migraine and the trigeminovascular system—40 years and counting. Lancet Neurol 2019;18:795-804.
25. Durham PL, Cady R. Insights Into the Mechanism of OnabotulinumtoxinA in Chronic Migraine. Headache: The Journal of Head and Face Pain 2011;51:1573-7.
26. Burstein R, Blumenfeld AM, Silberstein SD, Manack Adams A, Brin MF. Mechanism of Action of OnabotulinumtoxinA in Chronic Migraine: A Narrative Review. Headache 2020;60: 1259-72.
27. Suzuki K, Suzuki S, Shiina T, Kobayashi S, Hirata K. Central Sensitization in Migraine: A Narrative Review. J Pain Res 2022;15:2673-82.
28. Dodick D, Silberstein S. Central sensitization theory of migraine: clinical implications. Headache 2006;46 Suppl 4:S182-91.
29. Greco R, Demartini C, Francavilla M, Zanaboni AM, Tassorelli C. Antagonism of CGRP Receptor: Central and Peripheral Mechanisms and Mediators in an Animal Model of Chronic Migraine. Cells 2022;11:3092.
30. Greco R, Francavilla M, Facchetti S, Demartini C, Zanaboni AM, Antonangeli MI, et al. Intranasal administration of

recombinant human BDNF as a potential therapy for some primary headaches. J Headache Pain 2024;25:184.
31. Park S, Jung H, Han S-W, Lee S-H, Sohn J-H. Differences in Neuropathology between Nitroglycerin-Induced Mouse Models of Episodic and Chronic Migraine. Int J Mol Sci 2024;25:3706.
32. Dong Y, Li J, Zhang F, Li Y. Nociceptive Afferents to the Premotor Neurons That Send Axons Simultaneously to the Facial and Hypoglossal Motoneurons by Means of Axon Collaterals. PLoS One 2011;6:e25615.
33. Jasmin L, Burkey AR, Card JP, Basbaum AI. Transneuronal Labeling of a Nociceptive Pathway, the Spino-(Trigemino-)Parabrachio-Amygdaloid, in the Rat. The Journal of Neuroscience 1997;17:3751-65.
34. Piovesan EJ, Leite L da S, Teive HG, Kowacs PA, Mulinari RA, Radunz V, et al. Botulinum toxin type-A effect as a preemptive treatment in a model of acute trigeminal pain: a preclinical double-blind and placebo-controlled study. Arq Neuropsiquiatr 2011;69:56-63.
35. Matak I, Stracenski I, Lacković Z. Comparison of analgesic effects of single versus repeated injection of botulinum toxin in orofacial formalin test in rats. J Neural Transm 2013;120:141-4.
36. Kim H-J, Lee G-W, Kim M-J, Yang K-Y, Kim S-T, Bae Y-C, et al. Antinociceptive Effects of Transcytosed Botulinum Neurotoxin Type A on Trigeminal Nociception in Rats. The Korean J Physiol Pharmacol 2015;19:349.
37. Lee W-H, Shin TJ, Kim HJ, Lee J-K, Suh H-W, Lee SC, et al. Intrathecal Administration of Botulinum Neurotoxin Type A Attenuates Formalin-Induced Nociceptive Responses in Mice. Anesth Analg 2011;112:228-35.
38. Reducha PV, Bömers JP, Edvinsson L, Haanes KA. The impact of the migraine treatment onabotulinumtoxinA on inflammatory and pain responses: Insights from an animal model. Headache 2024;64:652-62.
39. Edvinsson J, Warfvinge K, Edvinsson L. Modulation of inflammatory mediators in the trigeminal ganglion by botulinum neurotoxin type A: an organ culture study. J Headache Pain 2015;16:73.
40. Raboisson P, Dallel R. The orofacial formalin test. Neurosci Biobehav Rev 2004;28:219–26.
41. Clavelou P, Dallel R, Orliaguet T, Woda A, Raboisson P. The orofacial formalin test in rats: effects of different formalin concentrations. Pain 1995;62:295-301.
42. Abrahão Cunha TC, Gontijo Couto AC, Januzzi E, Rosa Ferraz Gonçalves RT, Silva G, Silva CR. Analgesic potential of different available commercial brands of botulinum neurotoxin-A in formalin-induced orofacial pain in mice. Toxicon X 2021;12:100083.
43. Filipović B, Matak I, Bach-Rojecky L, Lacković Z. Central Action of Peripherally Applied Botulinum Toxin Type A on Pain and Dural Protein Extravasation in Rat Model of Trigeminal Neuropathy. PLoS One 2012;7:e29803.
44. Shields SD, Cavanaugh DJ, Lee H, Anderson DJ, Basbaum AI. Pain behavior in the formalin test persists after ablation of the great majority of C-fiber nociceptors. Pain 2010;151:422-9.
45. Cernuda Morollón E, Martínez Camblor P, Ramón C, Larrosa D, Serrano Pertierra E, Pascual J. CGRP and VIP Levels as Predictors of Efficacy of Onabotulinumtoxin Type A in Chronic Migraine. Headache 2014;54:987-95
46. Luvisetto S, Vacca V, Cianchetti C. Analgesic effects of botulinum neurotoxin type A in a model of allyl isothiocyanate- and capsaicin-induced pain in mice. Toxicon 2015;94:23-8.
47. Matak I, Rossetto O, Lacković Z. Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons. Pain 2014;155:1516-26.
48. Moore AA, Nelson M, Wickware C, Choi S, Moon G, Xiong E, et al. OnabotulinumtoxinA effects on trigeminal nociceptors. Cephalalgia 2023;43:3331024221141683.
49. Meents JE, Neeb L, Reuter U. TRPV1 in migraine pathophysiology. Trends Mol Med 2010;16:153-9.
50. Benemei S, Dussor G. TRP Channels and Migraine: Recent Developments and New Therapeutic Opportunities. Pharmaceuticals 2019;12:54.
51. Demartini C, Tassorelli C, Zanaboni AM, Tonsi G, Francesconi O, Nativi C, et al. The role of the transient receptor potential ankyrin type-1 (TRPA1) channel in migraine pain: evaluation in an animal model. J Headache Pain 2017;18:94.
52. Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. Pain 2004;107:125-33.
53. Aoki KR. Review of a Proposed Mechanism for the Antinociceptive Action of Botulinum Toxin Type A. Neurotoxicology 2005;26:785-93.
54. Gui X, Wang H, Wu L, Tian S, Wang X, Zheng H, et al. Botulinum toxin type A promotes microglial M2 polarization and suppresses chronic constriction injury-induced neuropathic pain through the P2X7 receptor. Cell Biosci 2020;10:45.
55. Wang L, Wang K, Chu X, Li T, Shen N, Fan C, et al. Intra-articular injection of Botulinum toxin A reduces neurogenic inflammation in CFA-induced arthritic rat model. Toxicon 2017;126:70-8.
56. Li X, Ye Y, Zhou W, Shi Q, Wang L, Li T. Anti-Inflammatory Effects of BoNT/A Against Complete Freund's Adjuvant-Induced Arthritis Pain in Rats: Transcriptome Analysis. Front Pharmacol 2021;12:735075
57. Favre-Guilmard C, Auguet M, Chabrier P-E. Different antinociceptive effects of botulinum toxin type A in inflammatory and peripheral polyneuropathic rat models. Eur J Pharmacol 2009;617:48-53.
58. Bach Rojecky L, Dominis M, Lacković Z. Lack of anti inflammatory effect of botulinum toxin type A in experimental models of inflammation. Fundam Clin Pharmacol 2008;22:503-9.
59. Bach-Rojecky L, Lacković Z. Antinociceptive effect of botulinum toxin type A in rat model of carrageenan and capsaicin induced pain. Croat Med J 2005;46:201-8.
60. Luvisetto S. Botulinum Neurotoxins beyond Neurons: Interplay with Glial Cells. Toxins (Basel) 2022;14:704.
61. Pickett A. Animal Studies with Botulinum Toxins May Produce Misleading Results. Anesth Analg 2012;115:736.
62. Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. Long-Distance Retrograde Effects of Botulinum Neurotoxin A. J Neurosci 2008;28:3689-96.
63. Edvinsson JCA, Grell A-S, Warfvinge K, Sheykhzade M, Edvinsson L, Haanes KA. Differences in pituitary adenylate cyclase-activating peptide and calcitonin gene-related peptide release in the trigeminovascular system. Cephalalgia 2020;40:1296-309.
64. Lennerz JK, Rühle V, Ceppa EP, Neuhuber WL, Bunnett NW, Grady EF, et al. Calcitonin receptor like receptor (CLR), receptor activity modifying protein 1 (RAMP1), and calcitonin gene related peptide (CGRP) immunoreactivity in the rat trigeminovascular system: Differences between peripheral and central CGRP receptor distribution. J Comp Neurol 2008;507:1277-99.
65. Eftekhari S, Salvatore CA, Calamari A, Kane SA, Tajti J, Edvinsson L. Differential distribution of calcitonin gene-related peptide and its receptor components in the human trigeminal ganglion. Neuroscience 2010;169:683-96.
66. Frederiksen SD, Warfvinge K, Ohlsson L, Edvinsson L. Expression of Pituitary Adenylate Cyclase-activating Peptide, Calcitonin Gene-related Peptide and Headache

Targets in the Trigeminal Ganglia of Rats and Humans. Neuroscience 2018;393:319-32.
67. Silva LB da, Poulsen JN, Arendt Nielsen L, Gazerani P. Botulinum neurotoxin type A modulates vesicular release of glutamate from satellite glial cells. J Cell Mol Med 2015;19:1900-9.
68. Jansen-Olesen I, Baun M, Amrutkar DV, Ramachandran R, Christophersen DV, Olesen J. PACAP-38 but not VIP induces release of CGRP from trigeminal nucleus caudalis via a receptor distinct from the PAC1 receptor. Neuropeptides 2014;48:53-64.
69. Kuburas A, Russo AF. Shared and independent roles of CGRP and PACAP in migraine pathophysiology. J Headache Pain 2023;24:34.
70. Buonvicino D, Urru M, Muzzi M, Ranieri G, Luceri C, Oteri C, et al. Trigeminal ganglion transcriptome analysis in 2 rat models of medication-overuse headache reveals coherent and widespread induction of pronociceptive gene expression patterns. Pain 2018;159:1980-8.
71. Marinelli S. BoNT/Action beyond neurons. Toxicon 2025;255:108250.
72. Bach-Rojecky L, Šalković-Petrišić M, Lacković Z. Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy: Bilateral effect after unilateral injection. Eur J Pharmacol 2010;633:10-4.
73. Restani L, Antonucci F, Gianfranceschi L, Rossi C, Rossetto O, Caleo M. Evidence for Anterograde Transport and Transcytosis of Botulinum Neurotoxin A (BoNT/A). J Neurosci 2011;31:15650-9.
74. Nemanić D, Mustapić M, Matak I, Bach-Rojecky L. Botulinum toxin type a antinociceptive activity in trigeminal regions involves central transcytosis. Eur J Pharmacol 2024;963:176279.
75. Matak I, Bach-Rojecky L, Filipović B, Lacković Z. Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A. Neuroscience 2011;186:201-7.
76. Matak I, Riederer P, Lacković Z. Botulinum toxin's axonal transport from periphery to the spinal cord. Neurochem Int 2012;61:236-9.
77. Marinelli S, Vacca V, Ricordy R, Uggenti C, Tata AM, Luvisetto S, et al. The Analgesic Effect on Neuropathic Pain of Retrogradely Transported botulinum Neurotoxin A Involves Schwann Cells and Astrocytes. PLoS One 2012;7 e47977.
78. Sureda-Gibert P, Romero-Reyes M, Akerman S. Nitroglycerin as a model of migraine: Clinical and preclinical review. Neurobiol Pain 2022;12:100105.
79. Sokołowska M, Bednarski M, Kwiecień I, Filipek B, Włodek L. Bioactivation of nitroglycerin to nitric oxide (NO) and S nitrosothiols in the rat liver and evaluation of the coexisting hypotensive effect. Fundam Clin Pharmacol 2004;18: 449-56.
80. Demartini C, Greco R, Francavilla M, Zanaboni AM, Tassorelli C. Modelling migraine-related features in the nitroglycerin animal model: Trigeminal hyperalgesia is associated with affective status and motor behavior. Physiol Behav 2022;256:113956.
81. Ernstsen C, Christensen SL, Olesen J, Kristensen DM. No additive effect of combining sumatriptan and olcegepant in the GTN mouse model of migraine. Cephalalgia 2021;41: 329–39.
82. Andreou AP, Leese C, Greco R, Demartini C, Corrie E, Simsek D, et al. Double-Binding Botulinum Molecule with Reduced Muscle Paralysis: Evaluation in In Vitro and In Vivo Models of Migraine. Neurotherapeutics 2021;18:556-68.
83. Shao Y-F, Zhang Y, Zhao P, Yan W-J, Kong X-P, Fan L-L, et al. Botulinum toxin type a therapy in migraine: preclinical and clinical trials. Iran Red Crescent Med J 2013;15:e7704.

Correspondence: Rosaria Greco, PhD, Section of Translational Neurovascular Research, IRCCS Mondino Foundation, Via Mondino 2, 27100 Pavia, Italy. E-mail: rosaria.greco@mondino.it

Contributions: RG, conceptualization; CD, methodology and analysis; CD, SF, MF, and AMZ, investigation; RG, CD, and DM, writing—original draft preparation; CT, final revision. All authors have read and agreed to the published version of the manuscript.

Conflict of interest: the authors declare no potential conflict of interest.

Ethics approval and consent to participate: the experimental protocol was approved by the Italian Ministry of Health (Document No. 1032/2015-PR).

Availability of data and materials: raw data are available upon reasonable request at www.zenodo.org (doi: 10.5281/zenodo.14941992).

Funding: this study was supported by a research grant from the Italian Ministry of Health to the IRCCS Mondino Foundation (RC 2018-2019).

Received: 15 January 2025. Accepted: 28 April 2025.

Confinia Cephalalgica 2025; 1:15779. doi:10.4081/cc.2025.15779