Received: 14 March 2024 | Revised: 24 May 2024 | Accepted: 6 June 2024

DOI: 10.1111/bph.17367

RESEARCH ARTICLE

Excitation–contraction coupling inhibitors potentiate the actions of botulinum neurotoxin type A at the neuromuscular junction

Mickaël Machicoane[1] | Marika Tonellato[2] | Marica Zainotto[2] | Paul Onillon[1] | Marco Stazi[2] | Mattia Dal Corso[2] | Aram Megighian[2,3] | Ornella Rossetto[2,4,5] | Jean-Marc Le Doussal[1] | Marco Pirazzini[2,5]

[1]Fastox Pharma SA, Lausanne, Switzerland

[2]Department of Biomedical Sciences, University of Padova, Padova, Italy

[3]Padova Neuroscience Center, University of Padova, Padova, Italy

[4]Institute of Neuroscience, Italian Research Council, University of Padova, Padova, Italy

[5]Interdepartmental Research Center of Myology CIR-Myo, University of Padova, Padova, Italy

Correspondence
Ornella Rossetto and Marco Pirazzini, Department of Biomedical Sciences, University of Padova, Via Ugo Bassi 58/B, Padova 35131, Italy.
Email: ornella.rossetto@unipd.it and marco.pirazzini@unipd.it

Present address
Marco Stazi, Cancer Neuroscience Laboratory, The Francis Crick Institute, London, UK.

Funding information
Fastox Pharma, Grant/Award Numbers: PIRA_COMM22_01, ROSS_COMM21_01; Università degli Studi di Padova, Grant/Award Numbers: Progetto DOR 025271, Progetto DOR 205071; Italian Ministry of University and Research, Grant/Award Number: PNRR DM 352/2022

Abstract

Background and Purpose: Botulinum neurotoxin type A1 (BoNT/A) is one of the most potent neurotoxins known. At the same time, it is also one of the safest therapeutic agents used for the treatment of several human disorders and in aesthetic medicine. Notwithstanding great effectiveness, strategies to accelerate the onset and prolong BoNT/A action would significantly ameliorate its pharmacological effects with beneficial outcomes for clinical use.

Experimental Approach: Here, we combined BoNT/A with two fast-acting inhibitors of excitation–contraction coupling inhibitors (ECCI), either the μ-conotoxin CnIIIC or dantrolene, and tested the effect of their co-injection on a model of hind-limb paralysis in rodents using behavioural, biochemical, imaging and electrophysiological assays.

Key Results: The BoNT/A-ECCI combinations accelerated the onset of muscle relaxation. Surprisingly, they also potentiated the peak effect and extended the duration of the three BoNT/A commercial preparations OnabotulinumtoxinA, AbobotulinumtoxinA and IncobotulinumtoxinA. ECCI co-injection increased the number of BoNT/A molecules entering motoneuron terminals, which induced a faster and greater cleavage of SNAP-25 during the onset and peak phases, and prolonged the attenuation of nerve-muscle neurotransmission during the recovery phase. We estimate that ECCI co-injection yields a threefold potentiation in BoNT/A pharmacological activity.

Conclusions and Implications: Overall, our results show that the pharmacological activity of BoNT/A can be combined and synergized with other bioactive molecules and uncover a novel strategy to enhance the neuromuscular effects of BoNT/A

Abbreviations: ABO, abobotulinumtoxinA; α-BTX, α-bungarotoxin; BoNT/A, botulinum neurotoxin type A; DAS, Digit Abduction Score; ECCI, excitation–contraction coupling inhibitors; EPP, endplate potential; INCO, incobotulinumtoxinA; NMJ, neuromuscular junction; ONA, onabotulinumtoxinA; SNAP-25, synaptosomal-associated protein 25; VAChT, vesicular acetylcholine transferase.

Mickaël Machicoane, Marika Tonellato and Marica Zainotto contributed equally as first authors.

Jean-Marc Le Doussal and Marco Pirazzini contributed equally as senior authors.

without altering the neurotoxin moiety or intrinsic activity, thus maintaining its exceptional safety profile.

KEYWORDS
abobotulinum, botulinum neurotoxin, conotoxin, dantrolene, excitation–contraction coupling, incobotulinum, neuromuscular junction, onabotulinum, spasticity

1 | INTRODUCTION

Botulinum neurotoxin type A1 (BoNT/A) is a Janus-faced clostridial exotoxin that is one of the most powerful neurotoxins known (Rossetto et al., 2014; Rossetto & Montecucco, 2019). At the same time, BoNT/A is one of the safest and most effective therapeutic treatments used to attenuate peripheral nerve hyperactivity in an ever-growing number of clinical conditions spanning from human neuromuscular, autonomic and dermatological disorders to dentistry and surgery indications (Jabbari, 2021; Jankovic, 2017; Martina et al., 2021; Pirazzini et al., 2017). In addition, BoNT/A has been commercialized for a variety of cosmetic applications becoming one of the most popular and effective treatments ever used in aesthetic medicine (Carruthers & Carruthers, 2001; Maio & Rzany, 2007).

The vast majority of therapeutic and aesthetic actions are based on the extreme selectivity of BoNT/A for peripheral cholinergic nerves, mainly the motor axon terminals, where the toxin potently, yet reversibly, blocks neurotransmitter release causing neuroparalysis (Pirazzini et al., 2017; Rossetto et al., 2014). The molecular structure and the mechanism of action of the neurotoxin have been comprehensively defined over the last few decades, contributing significantly to the understanding of the pharmacological actions of BoNT/A (Pirazzini et al., 2022, 2017; Rossetto et al., 2001).

BoNT/A consists of a di-chain structure encompassing a 50-kDa catalytic light chain (LC/A) disulfide-linked to a 100-kDa heavy chain (H), which folds into three distinct domains fulfilling specific tasks during the intoxication of the motor axon terminal (Dong et al., 2019; Lacy et al., 1998; Montal, 2010; Rossetto et al., 2014). The C-terminal domain of H (HC, 50 kDa) mediates an extremely selective binding and entry into neurons (Binz & Rummel, 2009; Dolly et al., 1984; Montecucco, 1986; Pirazzini et al., 2022), while the N-terminal domain of H (HN, 50 kDa) is responsible for the low pH-driven translocation of LC/A into the presynaptic cytosol (Fischer & Montal, 2013; Pirazzini et al., 2016). LC/A is a Zn^{+}-dependent metalloprotease that cleaves the synaptosomal-associated protein 25 (SNAP-25) (Blasi et al., 1993; Schiavo et al., 1992, 1993), a protein essential for the correct functioning of the 'soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors' (SNARE) complex, that is, the machinery responsible for the fusion of synaptic vesicles (SV) with the presynaptic membrane (Pantano & Montecucco, 2014; Rizo, 2022). As a result, BoNT/A-mediated cleavage of SNAP-25 at the neuromuscular junction (NMJ) prevents acetylcholine release causing neuromuscular paralysis, which is the basis of BoNT/A use in human therapy. Neither degeneration nor neuronal death occur in the intoxicated neurons, making the myorelaxant effect of BoNT/A fully reversible (Duregotti et al., 2015; Meunier et al., 2003; Pirazzini et al., 2017; Rossetto et al., 2014). Its duration mainly correlates with the lifetime of LC/A inside the axon terminals, reflecting the number of LC/A molecules reaching the synaptic cytoplasm that in turn depends on the injected dose (Pantano & Montecucco, 2014; Pirazzini et al., 2017; Shoemaker & Oyler, 2013).

Three commercial BoNT/A preparations, namely, OnabotulinumtoxinA (ONA), AbobotulinumtoxinA (ABO) and IncobotulinumtoxinA (INCO), are available worldwide. Several other BoNT/A products are marketed in specific areas of the world (Brin et al., 2014; Choudhury et al., 2021; Jankovic, 2017; Pirazzini et al., 2017; Scaglione, 2016), and novel preparations are continuously released or under development (Choudhury et al., 2021; Dressler & Johnson, 2022). Despite different manufacturing processes, the many BoNT/A preparations

What is already known?

- BoNT/A is a potent neurotoxin safely used for treating hyperactive motor peripheral nerves.
- BoNT/A is administered via local injections providing long-lasting pharmacological effects characterized by a slow onset.

What does this study add?

- Co-injection with fast-acting muscle blockers, such as ECCI, accelerates onset of pharmacological effects of BoNT/A.
- Co-injection with ECCI potentiates BoNT/A pharmacological activity, further prolonging its duration.

What is the clinical significance?

- Accelerated onset and prolonged duration can maximize BoNT/A effectiveness in conditions requiring frequent re-injections.
- Faster onset and longer-lasting effects can improve both the therapeutic and aesthetic use of BoNT/A.

display a similar pharmacological action reflecting comparable efficacy and safety profiles in therapeutic and aesthetic indications (Field et al., 2019; Frevert, 2015).

Due to the extreme potency of the neurotoxin, the clinical use of BoNT/A preparations is based on local injections and is restricted to the administration of extremely low doses ranging from picograms to a few nanograms of toxin (depending on the application). These are nonetheless able to provide a pharmacological effect persisting for months (Anandan & Jankovic, 2021; Dressler, 2012; Eleopra et al., 2020; Hallett et al., 2013; Ledda et al., 2022; Naumann et al., 2013), at the same time side limiting effects to nearby off target tissues and decreasing the possibility of developing anti-toxin antibodies (Carli et al., 2009; Pirazzini et al., 2017).

On the other hand, the injection of such minute amounts of toxins leads to a relatively slow onset of the pharmacological effect of BoNT/A. A lag phase, lasting from a few to several days, precedes the onset of myorelaxation and, depending on the injected dose, the treatment takes up to 2–3 weeks to reach its maximum effect (Anandan & Jankovic, 2021; Dressler, 2021; Eleopra et al., 2020; Ledda et al., 2022).

We tested whether a combined injection of BoNT/A with fast-acting muscle blockers could accelerate the onset of the myorelaxant action of BoNT/A, thereby improving its clinical value in neuromuscular applications. Among the many natural and synthetic compounds causing a rapid and effective muscle blockage, we considered two inhibitors targeting the excitation–contraction (e–c) coupling, that is, the series of functional events responsible for muscle contraction linking sarcolemma depolarization to the release of Ca^{2+} from the sarcoplasmic reticulum to trigger myofiber contraction (Franzini-Armstrong, 2018).

We report that the co-injection of BoNT/A with either the μ-conotoxin CnIIIC (CnIIIC), an inhibitor of muscle **$Na_V1.4$ sodium channels**, or **dantrolene** (DAN), an inhibitor of the **ryanodine receptor** 1 (RyR) Ca^{2+} channel, strongly accelerated the myorelaxant action of ONA, ABO and INCO in a rat model of hind limb paralysis. Unexpectedly, we also found that these combinations strongly potentiated and markedly prolonged the duration of the pharmacological activity of BoNT/A. Mechanistically, we show that the co-injection with these e-c coupling inhibitors (ECCI) increased the number of BoNT/A molecules entering motor axon terminals, which mirrored a faster and greater cleavage of SNAP-25 and a prolonged attenuation of neuromuscular transmission at the NMJ. Considering the current attempts aimed at ameliorating the performance of BoNT therapy, our work describes an innovative strategy to maximize the pharmacological effects of BoNT/A without altering the neurotoxin molecule or its intrinsic toxicity and safety profile.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures complied with the Italian and French laws and policies and with the guidelines established by the European Community Council (Directive 2010/63/UE) and were approved by the veterinary services of the University of Padova (Organismo Preposto al Benessere degli Animali) and by the French and Italian Ministries of Health. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Swiss-Webster female adult CD1 mice (24–26 g) were purchased from Charles River Laboratories Italia (Calco, Italy) and maintained under 12-h light/12-h dark cycles with water and food ad libitum. Female Sprague–Dawley Rats (200 g) were purchased from Janvier-Labs (Le Genest-Saint-Isle, France) and kept two per cage with a 12-h light/12-h dark cycles at around 21°C and 70% humidity.

2.2 | Preparation of BoNT/A solutions for intramuscular injection

CnIIIC was solubilized in saline solution (0.9% NaCl) at a concentration of 1 $mg{\cdot}ml^{-1}$.

DAN was prepared as a saturated solution in cyclodextrin as previously described (Chen et al., 2017). This saturated solution was maintained in agitation for 15 min at 37°C, then centrifuged at 180 x *g* for 45 s at room temperature for 45 s and filtered with a 0.22-μm filter. The concentration was then determined with a spectrophotometer at 394 nm.

ONA, ABO and INCO BoNT/A preparations were reconstituted in a working solution according to the manufacturer's recommendations and diluted in saline solution to an injection solution containing 50 $pg{\cdot}ml^{-1}$ of 150-kDa neurotoxin, split in aliquots and supplemented with either bioassay solution (0.9% NaCl 1-$mg{\cdot}ml^{-1}$ human albumin) or the indicated amount of CnIIIC or DAN to reach the indicated concentration of BoNT/A. The content of 150 kDa neurotoxin in ONA, ABO and INCO was determined as previously reported (Field et al., 2019).

2.3 | Digit abduction score assay

Female Sprague–Dawley rats were injected with the indicated commercial toxin preparations alone or in combination with either CnIIIC or DAN using a volume of 40 μl in the hind limb at the level of the tibialis anterior muscle, as previously described (Broide et al., 2013). This procedure was performed on animals anaesthetised with isoflurane (2% in oxygen). The mice were injected in the hind limb at the level of the ankle using a Hamilton micro syringe with a 28-gauge needle with a maximal volume of 25 μl.

The degree of hind limb paralysis was evaluated by monitoring the local paralysis of digits' abduction according to the Digit Abduction Score assay (DAS) scoring system on a five-point scale where 0 corresponds to a normal abduction of all digits and 4 indicates absence of toe abduction, as previously described (Aoki, 2001). DAS was assessed immediately before injection and after injection at 30 min, 1, 2, 4 h on day of injection (Day 0); 24 h (Day 1) and every following day until two consecutive DAS = 0 were measured.

2.4 | Immunofluorescence and quantification of cl-SNAP-25 in nerve muscle preparations

Mice were injected with ONA alone or in combination with either CnIIIC or DAN in the hind limb as described above. At the indicated time points, the animals were humanely killed by cervical dislocation and the soleus muscle was dissected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature. The samples were then quenched using 50-mM NH_4Cl in PBS, permeabilized and saturated for 2 h with a blocking-permeabilizing solution (PBS containing 15% goat serum, 2% bovine serum albumin, 0.25% gelatine and 0.2% glycine with 0.5% Triton X-100) and incubated with primary antibodies specific for vesicular acetylcholine transferase (VAChT; 1:500) and anti cl-SNAP-25 (1:200) for 72 h at 4°C. Subsequently, the muscles were washed with PBS and incubated with the appropriate secondary antibodies (1:200) supplemented with α-bungarotoxin (α-BTX) conjugated to Alexa-647 (1:200), in order to identify **nicotinic acetylcholine receptors**.

Images were collected with a Zeiss LSM 900 confocal with Airyscan 2 (Zeiss, Oberkochen, Germany) equipped with 63x/1.4 Plan-Apochromat oil immersion objective with the appropriate laser lines used at the same intensity and with non-saturating settings. A pseudocolor scale indicates colour associations with pixel intensity levels, where white represents the highest intensity, while black represents the lowest. Cleaved-SNAP-25 signal was quantified using ImageJ2 Fiji 2.15.1 software.

2.5 | BoNT/A-chase assay

Rats and mice were injected as described above with ONA alone or in combination with ECCI. After the indicated time points, 0.5 μg of the human monoclonal antibody C25 diluted in bioassay solution was injected in the same limb (mice) or intravenously at the level of the tail vein (rats). Neuroparalysis in rats was monitored over time and scored according to the DAS scale, while in mice the soleus muscle was collected and processed for immunostaining and cl-SNAP-25 quantification, as described above.

2.6 | Preparation and intoxication of primary neuronal cultures

Primary cerebellar granule neurons (CGN) from 4- to 6-day-old rats were prepared as previously described (Tehran & Pirazzini, 2018). Briefly, cerebella were mechanically disrupted, treated with trypsin and collected by centrifugation. Neurons were then plated onto 24-well plates coated with poly-L-lysine at a cell density of 3×10^5 cells per well and maintained at 37°C and 5% CO_2 in Basal Medium Essential (BME) (ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 25-mM KCl, 2-mM glutamine and 50-μg·ml^{-1} gentamycin. After 18–24 h, 10-μM cytosine arabinoside (Sigma) was added to arrest the growth of non-neuronal cells. After 6–8 days in vitro (DIV), neurons were incubated overnight with a lab grade BoNT/A (5 pM) alone or in combination with the indicated concentrations of either CnIIIC or DAN premixed for 1 h with the neurotoxin in complete BME. At the end of the experiment, neurons were lysed directly into the well with Laemmli Sample Buffer supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany) and β-mercaptoethanol, or fixed with paraformaldehyde 4% in PBS. Samples were then collected and prepared for western blotting.

Primary spinal cord motor neurons (SCMN) from 13.5–14.5 mouse embryos were prepared as previously described (Zanetti et al., 2019), and seeded in 24-well plates coated with laminin and poly-L-ornithine in Neurobasal medium (Thermo Fisher Scientific) supplemented with the appropriate growth factors. SCMN at 6 DIV were incubated for 48 h with ONA (0.5 pM) alone or in combination with the indicated concentrations of either CnIIIC or DAN premixed for 1 h with the neurotoxin in complete culture medium. The experiment was stopped by cell lysis directly on the well and samples were eventually prepared for western blotting.

2.7 | Western blotting

Proteins in total lysates from CGN and SCMN were separated on NuPAGE 4%–12% SDS-polyacrylamide gels (ThermoFisher) and then transferred onto a nitrocellulose membrane. After 2 h of saturation with 5% milk in PBS 0.1% Tween (PBS-T), the membranes were incubated overnight with antibodies against β3-tubulin, that was used as a loading control, and against SNAP-25 (clone SMI-81) that recognizes both the intact and the BoNT/A cleaved form of the protein (Pirazzini et al., 2014). Membranes were then extensively washed with PBS-T and incubated for 2 h with the appropriate secondary antibodies conjugated to HRP. After extensive washes, the membranes were developed using Luminata Crescendo (Merck Millipore, Burlington, MA, USA) using a Uvitec gel doc system (Uvitec, Cambridge, UK). SNAP-25 cleavage was estimated by densitometry and reported as a ratio with respect to intact SNAP-25.

2.8 | Electrophysiological recordings

Electrophysiological recordings of evoked end-plate potentials (EPP) were performed on the soleus at the level of single muscle fibres in oxygenated Krebs-Ringer solution using intracellular glass microelectrodes (0.86 i.d., 1.50 o.d., 20 MΩ resistance) (Science Products, Hofheim, Germany) filled with 3-M KCl and 3-M CH_3COOK in a 1:2 ratio, as previously described (Zanetti et al., 2018). Evoked neurotransmitter release was recorded in current-clamp mode, and resting membrane potential was adjusted with current injection to −70 mV. EPP were elicited by supramaximal nerve stimulation at 0.5 Hz using a suction microelectrode connected to a stimulator (S88, Grass Instruments, Quincy, MA, USA). After the dissection, samples were incubated for 10 min with 1 μM μ-Conotoxin GIIIB (Alomone Labs, Jerusalem, Israel) to prevent muscle action potential and contraction.

Signals were amplified with intracellular amplifier (BA-01X, NPI Electronics, Tamm, Germany), sampled at 10 kHz using a digital interface (NI PCI-6221, National Instruments, Austin, TX, USA) and recorded by means of electrophysiological software (WinEDR, Strathclyde University, Glasgow, UK). EPP measurements were analysed offline with Clampfit software (Molecular Devices, San Jose, CA, USA).

2.9 | Data and statistical analysis

The number of animals used in each experiment is either indicated by the data distribution displayed in the graph or reported in the figure legend. All experiments were performed blindly. Data are shown as means ± SEM, unless otherwise indicated. Statistical significance was calculated with GraphPad Prism 8 (GraphPad Software, Boston, MA, USA) for experiments with a minimum group size of $n = 5$ using the statistical tests indicated in the Figure legends. For comparisons between two treatment groups, a two-tailed Student's *t* test was employed. In instances involving three or more treatment groups, a one-way ANOVA or a two-way ANOVA accompanied by post-hoc multiple comparisons test was utilized. Values of $P < 0.05$ were considered to show statistically significant differences between group means. The data and statistical analysis adhere to the recommendations outlined by the *British Journal of Pharmacology* concerning experimental design and analysis in pharmacology (Curtis et al., 2022).

2.10 | Materials

The laboratory grade BoNT/A was prepared and purified as previously described (Schiavo & Montecucco, 1995). OnabotulinumtoxinA was purchased from Allergan Aesthetics, now AbbVie Inc. (North Chicago, IL, USA); AbobotulinumtoxinA was purchased from Ipsen France (Boulogne-Billancourt, France). IncobotulinumtoxinA was purchased from Merz Pharma (Frankfurt am Main, Germany). The conotoxin CnIIIC was synthetized by solid-phase synthesis as previously reported (Favreau et al., 2012). Dantrolene sodium (D9175) was purchased from Sigma Aldrich, now Merck KGaA (Darmstadt, Germany). Recombinant C25 antibody was produced in Chinese hamster ovary cells as previously described (Garcia-Rodriguez et al., 2007). The following primary antibodies were used: antibody for VAChT (Cat# 139105, RRID: AB_10893979), β3-tubulin (Cat# 302302, RRID: AB_10637424) from Synaptic Systems (Goettingen, Germany); anti-SNAP-25 (clone SMI-81) (Cat# 836303, RRID: AB_2715864; BioLegend, San Diego, CA, U.S.A.). The primary antibody for cleaved SNAP-25 (cl-SNAP-25) was polyclonal and generated in rabbit as previously described (Antonucci et al., 2008). The following secondary antibodies conjugated to Alexa fluorochromes were used (all from Thermo Fisher Scientific, Waltham, MA, USA): Goat anti Guinea Pig IgG (H + L) Secondary Antibody Alexa Fluor 488 (Cat# A-11073, RRID: AB_2534117), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody Alexa Fluor Plus 555 (Cat# A-21428, RRID: AB_2535849), α-Bungarotoxin (α-BTX) Alexa Fluor 647 conjugate (Cat# B35450). The following secondary antibodies conjugated to horseradish peroxidase (HRP) were used: Goat Anti-Mouse IgG H & L Chain Antibody Peroxidase Conjugated (Cat# 401215, RRID: AB_10682749; Millipore, Merck KGaA, Darmstadt, Germany), Anti-rabbit IgG HRP-linked Antibody (Cat# 7074, RRID: AB_2099233; Cell Signalling Technology, Danvers, MA, U.S.A.).

2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander et al., 2023).

3 | RESULTS

3.1 | Co-injection with ECCI potentiates the pharmacological activity of BoNT/A

BoNT/A interferes with SV fusion causing a potent and persistent, but slowly increasing, paralysis. To test whether this action can be complemented by a fast-acting myorelaxant, we combined BoNT/A injection with two ECCI (Figure 1a): (i) CnIIIC, a conopeptide from the venom of the marine cone snail *Conus consors*, which inhibits $Na_v1.4$ channels responsible for the generation and propagation of the post-synaptic action potential in the skeletal muscle (Markgraf et al., 2012), or (ii) DAN, a small molecule inhibitor of RyR that causes myorelaxation by blocking Ca^{2+} release from the sarcoplasmic reticulum into the myofibre cytosol (Krause et al., 2004).

Using ONA, a BoNT/A product commonly used worldwide, we injected the neurotoxin alone or combined with either CnIIIC or DAN into the tibialis anterior muscle of rats. We then assessed the paralytic effect through the DAS assay, a method specially designed for evaluating the pharmacological potency of botulinum neurotoxins in rodents by scoring the reduction in the animal's ability to abduct the toes of the injected hind limb (Aoki, 2001; Broide et al., 2013) (Figure 1b, bottom panel). We focused on two main parameters: (i) the time-resolved curve of DAS severity, to examine onset, peak effect and overall duration of neuroparalysis, and (ii) the area under the DAS curve after 24 h (AUC^{24h+}), to assess the overall pharmacological activity from the peak onward (Figure 1b, top right panel).

The black traces in Figure 1c show the DAS curves observed in rats injected with 1-U·kg^{-1} ONA, roughly corresponding to 10 pg·kg^{-1} (Field et al., 2019), and that the toxin alone caused a neuroparalysis rising between 4 and 8 h and taking almost 2 days to peak, yet never reaching the maximum severity. The onset of neuroparalysis was faster when ONA was combined with either CnIIIC or DAN, as shown in Figure 1d, which compares the DAS values 4 h after the injection. Of note, CnIIIC produced a much quicker onset than DAN.

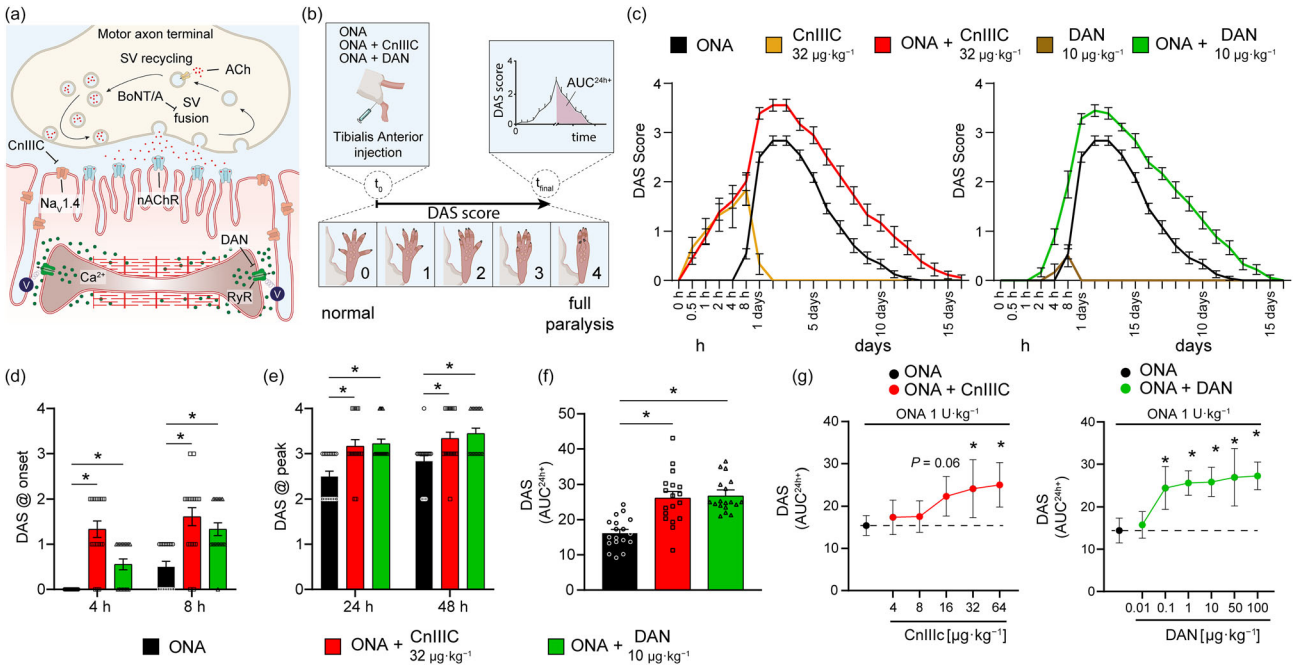

FIGURE 1 Co-injection of botulinum neurotoxin type A1 (BoNT/A) with excitation–contraction coupling inhibitors (ECCI) enhances its pharmacological action by accelerating the onset of myorelaxation, increasing peak potency and extending toxin duration at motor nerve terminals. (a) Scheme of neurotransmitter release and excitation-contraction coupling at the vertebrate neuromuscular junction with the site of action of BoNT/A at the presynaptic motor axon terminals, CnIIIC on $Na_V1.4$ channels at the sarcolemma and of DAN on RyR at the sarcoplasmic reticulum. (b) Scheme showing the experimental procedure to assess the pharmacological effect of BoNT/A injected in the rat tibialis anterior muscle alone or in combination with ECCI monitoring the severity of limb paralysis via the DAS scoring system over time and by measuring the AUC^{24h+}. (C) Time course of the DAS severity upon intramuscular injection of ONA (1 $U{\cdot}kg^{-1}$) alone or in combination with ECCI (CnIIIC 32 $\mu g{\cdot}kg^{-1}$ or DAN 10 $\mu g{\cdot}kg^{-1}$) or ECCI alone (CnIIIC 32 $\mu g{\cdot}kg^{-1}$ or DAN 10 $\mu g{\cdot}kg^{-1}$). Traces shown are means ± SEM; $n = 18$. (d–f) Severity of limb paralysis at relevant stages of BoNT/A pharmacological action injected alone or in combination with ECCI (CnIIIC 32 $\mu g{\cdot}kg^{-1}$ or DAN 10 $\mu g{\cdot}kg^{-1}$) assessed by DAS score at onset (4 and 8 h, panel d), peak (24 and 48 h, panel e) and for the duration by comparing the AUC^{24h+} (panel e). Data shown are means ± SEM; $n = 18$ animals. * $P < 0.05$, significantly different as indicated; two-way ANOVA with Tukey's test for multiple comparisons (panels d and f) and one-way ANOVA with Dunnett's test for multiple comparisons (panel e). (g) Enhancement of BoNT/A pharmacological effect in rats treated with intramuscular BoNT/A alone (1 $U{\cdot}kg^{-1}$, dotted line) or in combination with the indicated concentrations of CnIIIC (left panel) or DAN (right panel). Values are the AUC^{24h+}; data shown are means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; one-way ANOVA for multiple comparisons with Dunnett's multiple comparisons test.

Overall, these data confirmed our initial hypothesis that the BoNT/A-induced neuroparalysis could be accelerated by the specific pharmacological activity of ECCI.

At the same time, this experiment allowed two additional interesting observations. First, the maximal DAS score at 24–48 h was significantly higher upon co-injection of ONA with ECCI (Figure 1e), indicating a stronger peak effect. Second, the DAS curves of ONA in combination with ECCI took longer to return to normal levels, indicating a prolonged duration of neuroparalysis. Consistently, co-injections with CnIIIC and DAN produced a marked increase in the AUC^{24h+} (Figure 1f), indicating a potentiation of ONA that almost doubled the overall pharmacological performance of the neurotoxin. CnIIIC and DAN injected alone provided only a mild and short-lasting paralysis, becoming negligible after 24 h (see Figure 1c), ruling out the possibility that ONA potentiation was caused by a merely additive mechanism. Of note, ONA potentiation was dependent on the ECCI dose (Figures 1g and S1a). Although CnIIIC was more efficient in the first hours after injection (Figure S1b), as indicated by the AUC^{24h+} parameter, DAN elicited ONA potentiation in the $ng{\cdot}kg^{-1}$ range compared to the $\mu g{\cdot}kg^{-1}$ range for CnIIIC (Figure 1g). Similar results were obtained in mice (Figure S1c), indicating that ONA potentiation occurred, regardless of the animal model used.

Altogether, these results indicate that the co-injection of ONA with ECCI strongly enhanced the pharmacological activity of BoNT/A by decreasing the time of onset, increasing the peak potency and extending the duration of muscle relaxation.

3.2 | Potentiation does not depend on a physical interaction between BoNT/A and ECCI and does not occur in isolated neurons

The unexpected potentiation of ONA by ECCI prompted an investigation into how CnIIIC and DAN could alter the pharmacological profile of BoNT/A. Co-injection implies that BoNT/A and either CnIIIC or DAN molecules were pre-mixed in the same vial. Published evidence

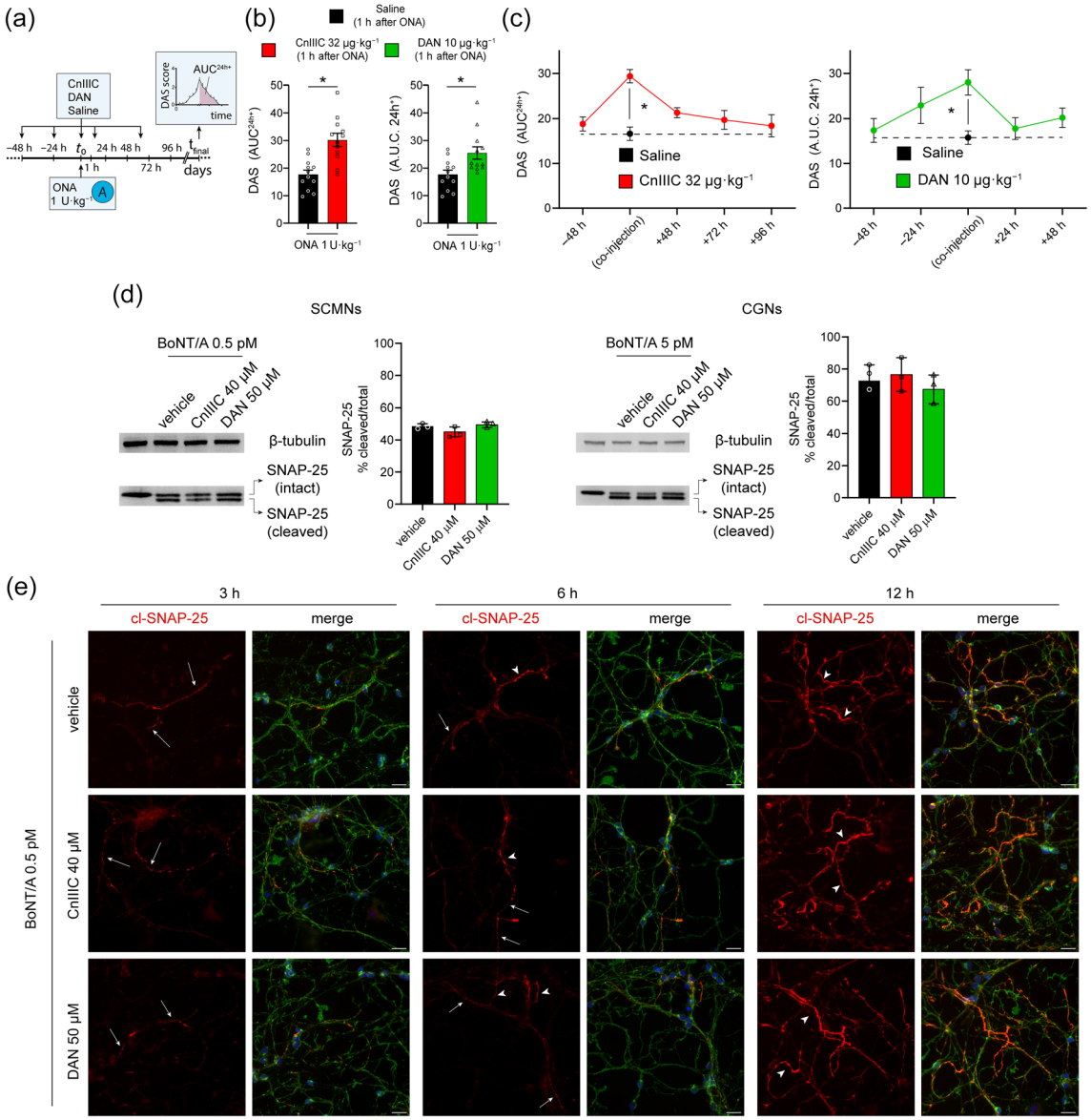

FIGURE 2 The enhancement of botulinum neurotoxin type A1 (BoNT/A) pharmacological action is not due to molecular interactions with the excitation–contraction coupling inhibitors (ECCI) or direct action on neurons. (a) Scheme showing the time course of the staggered injection of BoNT/A and ECCI in the rat hind limb. ECCI were injected before (48 or 24 h), together or after (1 h or 96 h) ONA (1 U·kg^{-1}) (b) ONA effect is still potentiated after the staggered injection of the neurotoxin alone (1 U·kg^{-1}) and then of the ECCI (CnIIIC or DAN) 1 h after the neurotoxin, as assessed by the AUC^{24h+}. Data shown are means ± SEM; $n = 12$. * $P < 0.05$, significantly different as indicated; Student's t test. (c) ONA potentiation is lost when the staggered injection of CnIIIC or DAN is performed at longer time points, either before or after neurotoxin injection alone. Data shown are means ± SEM; $n = 6$. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's multiple comparisons test. (d) A lab grade BoNT/A was pre-mixed for 1 h with either the vehicle (DMSO) or CnIIIC or DAN and then the mixtures were added to SCMN (left) for 48 h or to CGN (right) for 12 h in complete culture medium. Representative western blot showing SNAP-25 cleavage assessed with an antibody recognizing both the intact and the cleaved form of the protein and using β-III-tubulin as a loading control. Graphs showing the quantification of SNAP-25 cleavage assessed by densitometry analysis expressed as the percentage of cleaved on total SNAP-25. Data are from three independent experiments. (e) Time course of SNAP-25 cleavage in CGN treated with BoNT/A (0.5 pM) pre-mixed with the vehicle (top panels) or the indicated concentrations of either CnIIIC (central panels) or DAN (bottom panels). At the indicated time points, neurons were fixed and stained with an antibody specific for cl-SNAP-25 (red) and N-terminal SNAP-25 (green) to spot the axonal compartment, and with Hoechst to for the nuclei (merge). Images are representative of one experiment performed three times. Arrows indicate SNAP-25 cleavage within varicosity-like structures; arrow heads indicate SNAP-25 cleavage in axonal compartments. Bars indicate 20 µm.

indicated that chemical or protein compounds mixed with BoNT/A could alter the potency and pharmacological properties of the neurotoxin (Kutschenko et al., 2019; Stone et al., 2011). Therefore, we examined whether the physical interaction between the neurotoxin and ECCI could affect BoNT/A activity by performing both in vivo and in vitro experiments.

First, we repeated the DAS assay by staggering ONA and ECCI injection to avoid their direct mixing in the test tube (Figure 2a). When injected 1 h after ONA, both CnIIIC and DAN still markedly increased the AUC^{24h+} to levels similar to those when they were pre-mixed, indicating that the potentiation did not rely on physical interactions between BoNT/A and ECCI (Figure 2b). Interestingly, when the time was extended to longer intervals, both before or after ONA injection, CnIIIC and DAN did not cause any potentiation, indicating that ECCI synergize with the neurotoxin within a relatively restricted timeframe (Figure 2c).

Second, we performed a co-intoxication experiment on primary neurons, which offer a convenient model to study the mechanism of action and potency of clostridial neurotoxins by monitoring SNAP-25 cleavage (Kiris et al., 2014; Pellett, 2013; Tehran & Pirazzini, 2018) and to test whether their effect is altered by agents acting either on BoNT/A itself, on the neurons or on both (Pellett et al., 2007; Pirazzini et al., 2014, 2021; Seki et al., 2015). To this end, we used CGN and SCMN, two neuronal cultures highly susceptible to BoNT/A and expressing Na_V and RyR channels (Favreau et al., 2012; Jahn et al., 2006; Kuwajima et al., 1992; Schaller & Caldwell, 2003). The BoNT/A concentration was calibrated to achieve approximately 50% of SNAP-25 proteolysis allowing for a better estimation of possible modulatory effects on toxin activity. The cleavage was examined via western blotting with an antibody recognizing both intact and cleaved SNAP-25 to have a ratiometric measurement (Tehran & Pirazzini, 2018), and by immunofluorescence with an antibody selectively recognizing cleaved-SNAP-25 but not the intact protein (Duregotti et al., 2015; Pirazzini et al., 2014). Figure 2d shows that BoNT/A cleavage was unaffected by CnIIIC or DAN, regardless of their concentration (Figure S2a,b). In addition, the immunofluorescence analysis in Figure 2e shows that the localization of SNAP-25 cleavage, at any given time point, was very similar between neurons treated with BoNT/A alone or in combination with ECCI: It occurred first in varicosity-like structures, suggestive of BoNT/A entry at presynaptic level, and then spread over time at axonal level, suggesting that BoNT/A subcellular activity was not altered by the presence of the ECCI in the culture medium. Altogether, these results indicated that ONA potentiation at the NMJ did not rely on direct molecular interactions between the neurotoxin and ECCI, and ruled out the possibility that CnIIIC and DAN could directly alter BoNT/A activity in an isolated neuronal system.

3.3 | ECCI enhance SNAP-25 cleavage and prolong the blockade of neurotransmitter release at the NMJ

The results described above show that BoNT/A potentiation by ECCI did not occur in cultures of neurons. Consequently, we reasoned that BoNT/A potentiation must derive from a synergy taking place between the ECCI and the neurotoxin in the more complex context of the NMJ. Given that the factors responsible for BoNT/A pharmacological activity at the NMJ are the cleavage of SNAP-25 and the ensuing blockade of neurotransmission, we examined whether co-injection with ECCI causes any changes to these molecular and physiological parameters.

We injected ONA alone or in combination with ECCI in the mouse hind limb and collected the soleus muscle at specific time points corresponding to the onset, peak and recovery phases (Figure 3a). Using the cleaved-SNAP-25 specific antibody, we found that at the injected dose ONA yielded a signal barely visible at 3 h, but becoming progressively stronger at 6 and 12 h after injection (Figure 3b). Remarkably, co-injection with CnIIIC or DAN elicited a clear staining for cleaved-SNAP-25 as early as 3 h, which further increased at 6 and 12 h yielding a stronger signal, compared to ONA alone, at any considered time, as shown by the quantification (Figure 3c). Co-localization with VAChT, a SV marker, proved that the signal originated at presynaptic level within the motor axon terminals (Figure S3a). This result indicates that both CnIIIC and DAN accelerated the cleavage of SNAP-25 during the onset of ONA myorelaxant effect.

Next, we focused on the peak phase at 18 and 24 h to monitor SNAP-25 cleavage when the neurotoxin elicits its maximum activity. In keeping with the results of the DAS assay, muscles treated with the ONA-ECCI combinations raised stronger cleaved-SNAP-25 signals compared to ONA alone, thus indicating that the higher peak effect was accompanied by a greater proteolysis of SNAP-25.

We then analysed the recovery phase. As the long-lasting blockade of neurotransmission causes an intense neuronal sprouting and a remodelling of motor axon terminals that complicate the analysis of cleaved-SNAP-25 (Meunier et al., 2002; Pamphlett, 1989), we quantified NMJ neurotransmission by measuring the evoked EPP (Figure 3e). This electrophysiological method provides an accurate assessment of NMJ neurotransmission, that is, the physiological event disrupted by BoNT/A via SNAP-25 cleavage, at synaptic resolution (Del Castillo & Katz, 1954; Zanetti et al., 2018). At 45 days after injection, the average EPP amplitude in myofibres of muscles treated with the ONA-ECCI combination was markedly lower compared to animals treated with ONA alone (Figure 3f), attesting the longer recovery of ONA-ECCI treated muscles.

Taken together, these results indicate that the potentiation of BoNT/A activity by CnIIIC and DAN relies on a faster and greater SNAP-25 proteolysis during the onset and peak phases and accounts for the prolonged attenuation of neurotransmission during the recovery phase.

3.4 | ECCI elicit a faster and higher accumulation of LC/A within nerve terminals

At this point, we wondered how ECCI co-injection could lead to a faster and more intense cleavage of SNAP-25 immediately after BoNT/A injection and to a prolonged neurotransmission impairment during the recovery phase. While the first could be merely due to a faster BoNT/A uptake after injection, the peak and duration are strongly affected by the number of LC/A inside the nerve terminals (Binz, 2013; Pantano & Montecucco, 2014; Shoemaker &

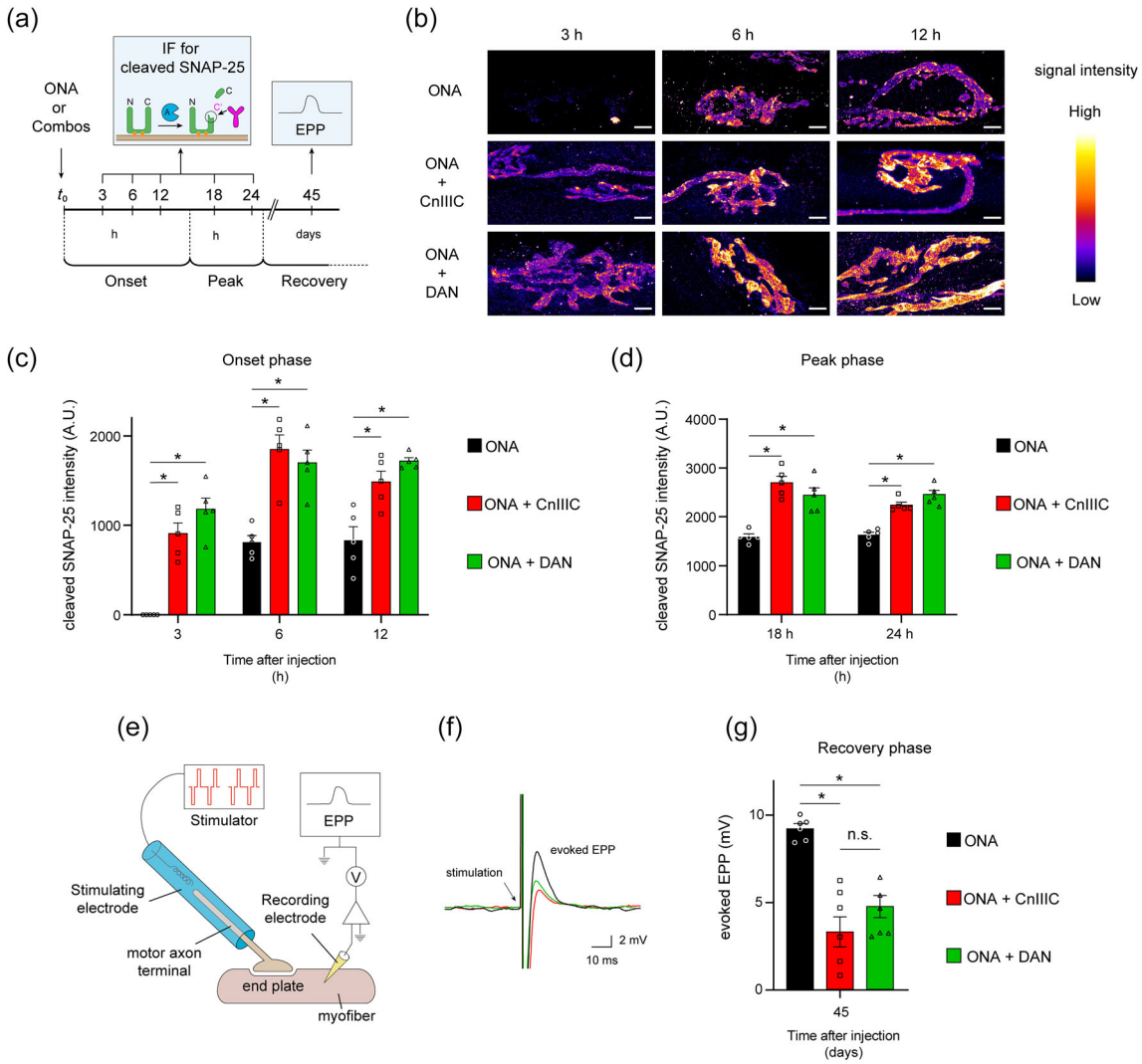

FIGURE 3 The combined injection with excitation–contraction coupling inhibitors (ECCIs) accelerated the onset of SNAP-25 cleavage and prolonged the impairment of neurotransmission into motor axon terminals. (a) Scheme showing the experimental design of the time course of SNAP-25 cleavage at the mouse nerve terminals using immunofluorescence (IF) imaging of cl-SNAP-25 and electrophysiology. At the indicated time points after botulinum neurotoxin type A1 (BoNT/A) injection (1 U·kg^{-1}) alone or in combination with ECCI (CnIIIC 50 μg·kg^{-1} or DAN 30 μg·kg^{-1}) in the mouse limb, the soleus muscle was collected and used for immunostaining of the C-terminal epitope newly generated in SNAP-25 by BoNT/A cleavage (onset and peak) and for assessing neurotransmission. (b) Representative images acquired at 3, 6 and 12 h after injection showing cl-SNAP-25 signal via pseudocolor scale (panel on the right). Scale bars are 10 μm. (c,d) Quantification of SNAP-25 cleavage at the NMJ at the indicated time points in the onset phase and at the peak. Data shown are individual values with means ± SEM; $n = 5$ animals. * $P < 0.05$, significantly different as indicated; two-way ANOVA for multiple comparisons with Tukey's multiple comparisons test. (e) Scheme showing the experimental setup to measure neurotransmitter release at the neuromuscular junction via the evoked endplate potential (EPP) assessing neurotransmission with single myofiber resolution. (f) Representative traces of evoked EPP measurements after treatment with ONA, ONA in combination with CnIIIC, and ONA in combination with DAN. (g) EPP values measured in the soleus muscles 45 days after the injection of BoNT/A alone or in combination with ECCI (CnIIIC 50 μg·kg^{-1} or DAN 30 μg·kg^{-1}) in the mouse hind limb. Data shown are individual values with means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's multiple comparisons test.

Oyler, 2013). Accordingly, we hypothesized that the higher peak and longer duration elicited by ECCIs could reflect, beyond a faster uptake of BoNT/A, also a higher number of BoNT/A molecules entering the NMJ presynaptic terminal. As there are no techniques available to directly measure the minute amounts of LC/A inside nerve terminals, we devised an experiment whereby BoNT/A uptake at the NMJ was chased by the 'C25' neutralizing antibody injected at specific time points after ONA alone or in combination with either CnIIIC or DAN (Figure 4a). As this antibody binds to an epitope in the HC that fully prevents the interaction of the neurotoxin with the motor axon terminals (Smith et al., 2005), the activity of BoNT/A after C25 injection reflects the number of molecules that have escaped neutralization and are thus able to enter the NMJ.

As shown in Figure 4b, the AUC^{24h+} parameter was abolished when C25 is injected within 1 h after ONA, indicating that the number of BoNT/A molecules that escaped neutralization by the antibody

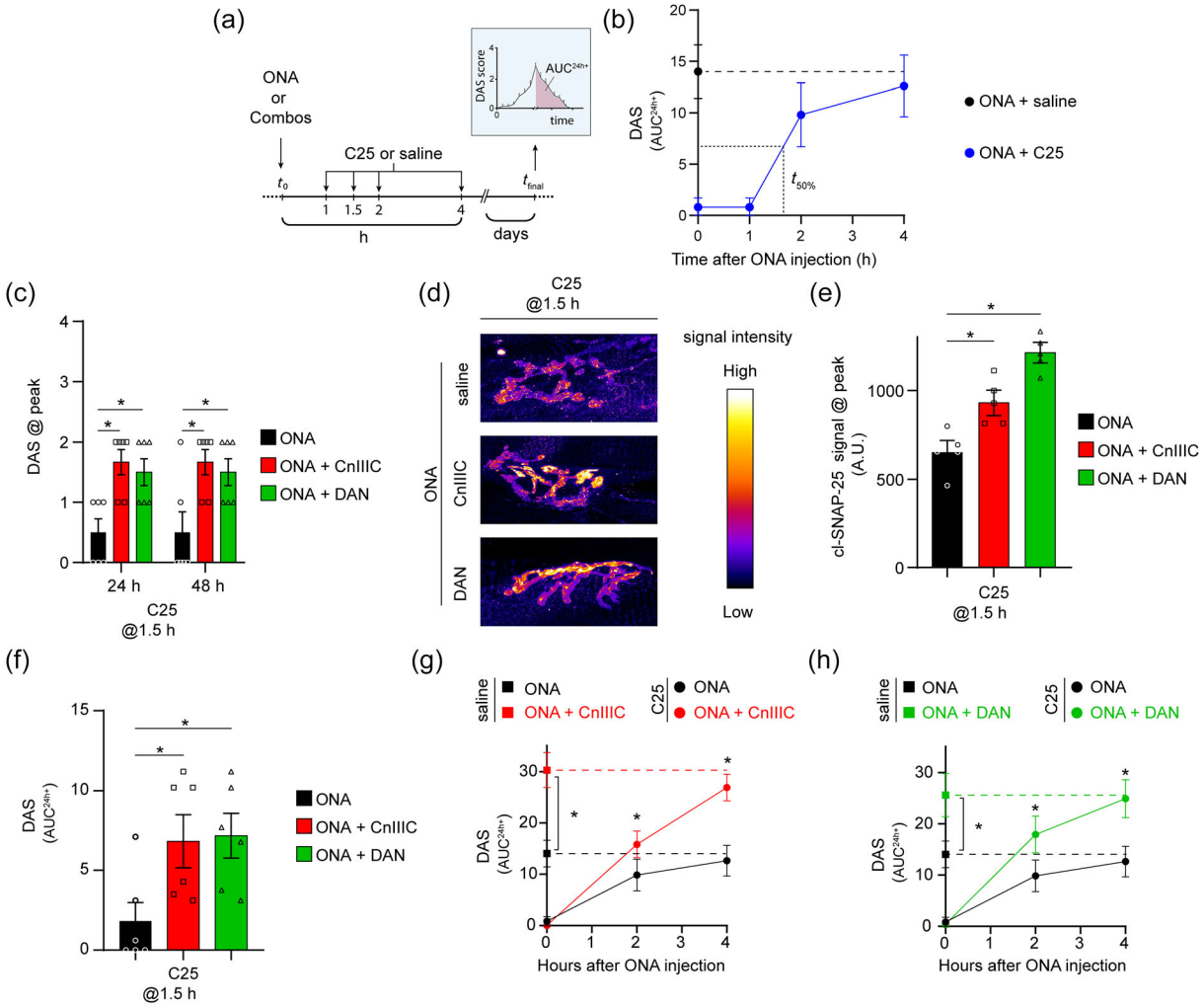

FIGURE 4 The combined injection with excitation–contraction coupling inhibitors (ECCI) increases botulinum neurotoxin type A1 (BoNT/A) uptake within motor axon terminals. (a) Scheme showing the time course of BoNT/A chasing with the C25 neutralizing antibody to monitor toxin entry into the motor axon terminals. BoNT/A alone or in combination with ECCI was injected in the rat limb; at the indicated time points, the human monoclonal antibody C25 specific for BoNT/A binding was injected in the same limb and then neuroparalysis duration was monitored with the DAS assay. (b) Time course of BoNT/A (1 U·kg^{-1}) entry upon neutralization by C25 antibody assessed by the AUC^{24h+}. Data shown are means ± SEM; $n = 6$. (c) Severity of limb paralysis at the peak phase after ONA injection alone or in combination with ECCI (CnIIIC 32 µg·kg^{-1} or DAN 10 µg·kg^{-1}) assessed by DAS score at onset (24 and 48 h). Data shown are individual values with means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; two-way ANOVA with Tukey's test for multiple comparisons. (d,e) Representative images and quantification of cl-SNAP-25. Data shown are individual values with means ± SEM; $n = 5$ animals. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's test for multiple comparisons. (f) AUC^{24h+} after injection in the rat limb of ONA (1 U·kg^{-1}) alone or in combination with ECCI (CnIIIC or DAN) and later (1.5 h) of either saline or C25 antibody. Data shown are individual values with means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's test for multiple comparisons (panel e). (g,h) Extended time course of BoNT/A chasing with C25 or saline after injection in the tibialis anterior of ONA alone (1 U·kg^{-1}) or in combination with CnIIIC 32 µg·kg^{-1} (d) or DAN 10 µg·kg^{-1} (e). Values are the AUC^{24h+}; Data shown are individual values with means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; two-way ANOVA with Tukey's test for multiple comparisons.

were not sufficient to elicit any evident neuroparalysis. Conversely, C25 was only partially effective when injected 2 h after ONA and, interestingly, neutralization was practically negligible at 4 h. These results indicate that, at the used doses, all the BoNT/A molecules responsible for paralysis are no longer accessible to the C25-antibody in about 3–4 h. We thus performed the same experiment at 1.5 h, a time roughly corresponding to the $t_{50\%}$ of C25 inhibition, comparing the ONA-ECCI mix with ONA alone. We found that the DAS values at the peak phase, when the effects of CnIIIC and DAN are negligible, were clearly higher for the combinations, indicating that the co-injection provided ONA with some protection from C25 neutralization (Figure 4c). In keeping with a larger number of BoNT/A molecules escaping neutralization, we found a stronger cleaved-SNAP-25 signal in muscles treated with the ONA-ECCI combination, compared with ONA alone, indicating that also substrate cleavage was less susceptible to C25 chasing (Figure 4d,e). To further test our hypothesis, we

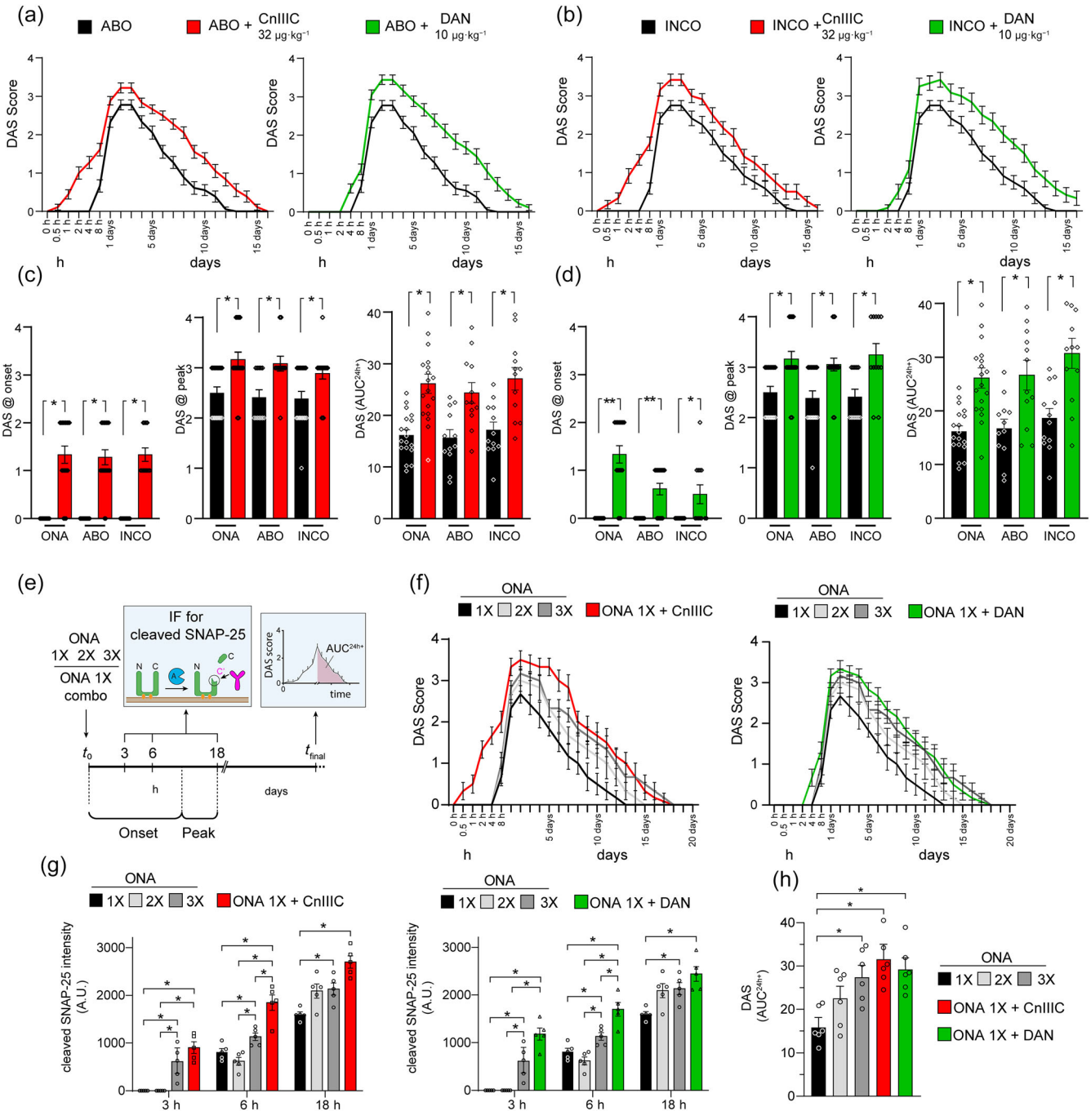

FIGURE 5 Legend on next page.

focused on the AUC^{24h+}. This parameter accounts for the overall effect of ONA paralysis, including the peak effect and the duration, but not the onset phase, and thus depends on the number of BoNT/A molecules escaping C25 neutralization and reaching the motor axon terminal in their active form. Figure 4f shows that CnIIIC and DAN co-injection almost tripled the AUC^{24h+} of ONA alone when C25 was injected 1.5 h after the toxin, and similar results were obtained when C25 injection was extended to the entire time course of BoNT/A chasing (Figure 4g,h). These results strongly indicate that the ECCI co-injection causes the entry of a larger number of BoNT/A molecules at the NMJ, which accounts for a faster and more potent pharmacological action of the neurotoxin.

3.5 | BoNT/A potentiation by ECCI is extended to several commercial preparations and equals a triple dose escalation

Our results provided a mechanistic explanation for the unexpected potentiation of BoNT/A by CnIIIC and DAN, yet they did not clarify whether these co-injections have any translational potential. For example, all the experiments were carried out with ONA, which is one out of many BoNT/A-containing commercial products routinely used in humans (Choudhury et al., 2021; Pirazzini et al., 2017). Therefore, we tested whether other products, such as ABO and INCO which have distinct manufacturing processes and excipients and are characterized by the presence (ONA and ABO) or absence (INCO) of non-toxic progenitor toxin complexes (Pirazzini et al., 2017), were also susceptible to ECCI potentiation. We therefore injected ABO and INCO alone or in combination with CnIIIC or DAN in the rat hind limb with doses adjusted to elicit a neuroparalytic effect comparable to that previously obtained with ONA. Consistently, the several parameters of DAS severity at the onset and peak phases (Figure 5a,b) and the AUC^{24h+} (Figure 5c,d) showed that ABO and INCO were also susceptible to ECCI potentiation.

Next, since a strategy commonly used in the clinic to enhance BoNT/A effects and prolong its duration is to increase the dose (Fabi et al., 2021; Kaufman-Janette et al., 2021), we compared the effects of the BoNT/A-ECCI combinations with BoNT/A alone injected at higher doses (Figure 5e). As previously reported (Šoštarić et al., 2022), DAS severity is proportional to the injected dose of toxin: a double (2X) and a triple (3X) dose of ONA elicited higher peak effects and longer durations, yet similar onset, compared to the single dose (1X). Strikingly, the pharmacological effect of 1X ONA in combination with either CnIIIC or DAN elicited an effect similar to ONA 3X, but with an earlier onset. Consistently, the amount of cl-SNAP-25 at the onset phase (Figure 5g) and the AUC^{24h+} (Figure 5h) for the combinations were higher than ONA 1X and ONA 2X while appearing similar to ONA 3X (Figure 5g).

Collectively, these results suggest that the combined injection with ECCI increased the pharmacological effects of BoNT/A with a two to threefold power effect, and that this mechanism is independent of the commercial source of BoNT/A and the presence of non-toxic accessory proteins.

4 | DISCUSSION

The main finding of this study is that the pharmacological activity of BoNT/A preparations for neuromuscular applications can be accelerated and potentiated through the co-injection with the fast-acting muscle blockers CnIIIC or DAN. Despite its established efficacy, BoNT/A is well-known to have a slow onset of effect that, in humans, takes days to be perceived by the patients and up to several weeks to display its full pharmacological activity (Dressler, 2021; Kaufman-Janette et al., 2021). Here, we showed that CnIIIC or DAN can accelerate the onset phase inducing muscle relaxation very soon after administration, an effect mediated by a larger number of LC/A molecules reaching the motor axon terminals and causing a faster and more robust cleavage of SNAP-25. Consistent with the established

FIGURE 5 Excitation–contraction coupling inhibitors (ECCI) potentiate different types of botulinum neurotoxin type A1 (BoNT/A) preparations leading to threefold power effect. (a, b) Time course and severity of limb paralysis showing that ECCI can potentiate BoNT/A activity independently from toxin formulation. ABO (a, 1 $U{\cdot}kg^{-1}$) or INCO (b, 1 $U{\cdot}kg^{-1}$) were used as prototype toxin preparations injected in the rat tibialis alone or in combination with ECCI (CnIIIC 32 $\mu g{\cdot}kg^{-1}$ or DAN 10 $\mu g{\cdot}kg^{-1}$). Data shown are means ± SEM; $n = 12$. (c,d) Severity of limb paralysis upon injection of ONA, ABO or INCO (1 $U{\cdot}kg^{-1}$) alone or in combination with either CnIIIC (32 $\mu g{\cdot}kg^{-1}$) or DAN (10 $\mu g{\cdot}kg^{-1}$) as assessed by the DAS score at onset (4 h, left panel) and at peak effect (2 days, central panel), and for duration estimated with the AUC^{24h+}. Data shown are individual values with means ± SEM; $n = 12$ animals.* $P < 0.05$, significantly different as indicated: one-way ANOVA with Tukey's test for multiple comparisons. E) Scheme showing the experimental design and the readouts used to compare the pharmacological effects of BoNT/A injected alone as multiple doses or as a single dose in combination with ECCI. SNAP-25 cleavage at the indicated time points was assessed via immunofluorescence (IF) in the mouse soleus while the DAS severity and the AUC^{24h+} in the rat. (f) Time course of the severity of BoNT/A neuromuscular paralysis when injected alone as 1X dose (1 $U{\cdot}kg^{-1}$), 2X dose (2 $U{\cdot}kg^{-1}$) and 3X dose (3 $U{\cdot}kg^{-1}$) or injected as 1X dose (1 $U{\cdot}kg^{-1}$) in combination with ECCI (CnIIIC 32 $\mu g{\cdot}kg^{-1}$, left panel or DAN 10 $\mu g{\cdot}kg^{-1}$, right panel). Values are from 12 rats per conditions. Bars indicate the SEM. (g) Onset of SNAP-25 cleavage by BoNT/A when injected in combination with CnIIIC or DAN, compared to double and triple doses of the toxin. Data shown are individual values with means ± SEM; $n = 5$ mice. * $P < 0.05$, significantly different as indicated; two-way ANOVA for multiple comparisons with Tukey's multiple comparisons test. (h) Duration of the neuroparalytic effect of BoNT/A upon dose sale compared to the injection of 1X dose in combination with ECCI (CnIIIC 32 $\mu g{\cdot}kg^{-1}$, left panel or DAN 10 $\mu g{\cdot}kg^{-1}$, right panel) using as read out the AUC^{24h+}. Values are the AUC^{24h+}; Data shown are individual values with means ± SEM; $n = 6$ animals. * $P < 0.05$, significantly different as indicated; one-way ANOVA with Dunnett's test for multiple comparisons.

correlation between the duration of neuroparalysis and the lifetime of the LC/A molecules inside the motor axon terminals (Rossetto et al., 2021), a higher number of LC/A reflected a widened maximum effect and a prolonged duration of BoNT/A pharmacological activity at the NMJ.

Considering the great efforts of clinical and basic research aimed at improving BoNT therapy, these findings show for the first time that the pharmacological action of BoNT/A can be combined with other bioactive compounds acting on molecular targets different from that of the neurotoxin leading to a synergistic potentiation of its activity. This is at variance from the strategies attempted so far mostly oriented at modifying the neurotoxin molecule, such as testing alternative serotypes or subtypes, and/or engineering their structures (Choudhury et al., 2021; Elliott et al., 2019; Guo et al., 2013; Pirazzini et al., 2013; Steward et al., 2021; Zanetti et al., 2017). BoNT/E was considered for its rapid onset, but its use might be limited to indications requiring short duration of action (Hanna & Pon, 2020); BoNT/A2 has been tested in a small-sized clinical study showing a fast onset of action (Kaji et al., 2022), while different BoNT/A subtypes have been evaluated in animal models showing specific toxicological properties (Whitemarsh et al., 2013). Similarly, chimeric toxins and mutant toxins have been tested in preclinical models showing interesting properties (Steward et al., 2021). Alternatively, a change in formulation with different excipients has been considered and was found to improve BoNT/A pharmacological performance (Solish et al., 2021; Stone et al., 2011). Conversely, we found that BoNT/A effects can be potentiated by a simple co-administration with other bioactive substances, which complement and/or extend the pharmacological action of the neurotoxin without altering the molecule itself or its mechanism of action, therefore maintaining its valuable safety profile. Of note, potentiation was achieved with three different commercial preparations of BoNT/A and two ECCI specific for different molecular targets, which indicate a general mechanism behind the pharmacological synergy. These findings pave the way to the exploration of new combinations of BoNTs with the arsenal of different biomolecules targeting the neuromuscular system, and to the potential development of novel pharmacological approaches aimed at the fine tuning of BoNT pharmacological profile according to the specific clinical goals.

We estimated that ECCI co-injection elicits a pharmacological effect roughly corresponding to a triple dose of BoNT/A alone. This result is particularly relevant considering that onset and duration strongly depend on the injected dose and that the strategy normally used to accelerate and prolong BoNT/A effects is uniquely based on dose escalation, both in medical and cosmetic applications (Fabi et al., 2021; Kaufman-Janette et al., 2021). Indeed, this practice is associated with several challenges, including dose/volume adjustments, response variability, increased risk of off-target diffusion and increased possibility of immunization with formation of neutralizing antibodies (Fabi et al., 2021; Joshi & Joshi, 2011; Lange et al., 2009). In contrast, the co-injection with ECCI increases the pharmacological potential of the injected BoNT/A dose, thus minimizing the issues related to dose escalation.

The novel features acquired by BoNT/A co-injection with CnIIIC and DAN have direct translational implications both in medical and aesthetic medicine indications, especially when frequent injections or booster doses are needed to keep BoNT/A effects within its window of pharmacological efficacy. Pathological conditions like dystonia, tremors and spasticity (post stroke, traumatic brain injuries and cerebral palsy), which are disabling and painful, or conditions in which satisfactory outcomes are achieved with doses higher than the approved ones, could benefit significantly (Wissel, 2018). An accelerated onset and a heightened peak effect would provide a faster and more effective relief after each administration, while a longer duration would reduce the frequency of injections, two improvements long awaited by patients (Comella et al., 2021; Poliziani et al., 2016; Sethi et al., 2012). Likewise, a faster onset and a longer duration also meet the growing interest of aesthetic medicine patients to have faster and prolonged effects maximizing the effectiveness of treatments and, as a result, to reduce re-injections.

In conclusion, we report that the pharmacological effect of botulinum neurotoxin type A, a drug administered in millions of doses per year worldwide, can be combined with either CnIIIC or DAN leading to a significant improvement of the pharmacological actions of BoNT/A. Although the specific mechanism as to how a rapid blockade of muscle contraction leads to a potentiation of BoNT/A activity is still under investigation, the fact that CnIIIC belongs to the heterogeneous group of pharmacologically active conopeptides, a class of biomolecules already approved for pain therapy (Lewis et al., 2012; Safavi-Hemami et al., 2019), and that DAN has been used for several years in the treatment of malignant hyperthermia and spasticity, speaks in favour of the rapid testing of BoNT/A-ECCI combinations in clinical trials.

AUTHOR CONTRIBUTIONS

Jean-Marc Le Doussal and Mickaël Machicoane conceived the project. Jean-Marc Le Doussal, Mickaël Machicoane, Ornella Rossetto and Marco Pirazzini devised the study design with contribution of other authors. Paul Onillon performed and evaluated the experiments with rats. Marika Tonellato, Marica Zainotto, Marco Stazi, Mattia Dal Corso and Aram Megighian performed and evaluated the experiments with mice. Marco Pirazzini and Ornella Rossetto wrote the paper with contribution of Mickaël Machicoane. Conceptualization: Jean-Marc Le Doussal, Mickaël Machicoane, Ornella Rossetto and Marco Pirazzini; methodology: Paul Onillon, Mickaël Machicoane, Marika Tonellato, Marica Zainotto, M. S. and Aram Megighian; data curation: Paul Onillon, Mickaël Machicoane, Marika Tonellato, Marica Zainotto and Marco Stazi; original draft preparation: Marco Pirazzini and Ornella Rossetto; review and editing: Ornella Rossetto and Mickaël Machicoane with the contribution of all the authors; funding acquisition: Jean-Marc Le Doussal, Marco Pirazzini and Ornella Rossetto. All authors have read and agreed to the submitted version of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by grants from the University of Padova (Progetto DOR 025271 to M. P. and Progetto DOR 205071 to O. R.), from the Italian Ministry of University and Research (scholarship funded by PNRR DM 352/2022 to M. Z.) and by research grants from

Fastox Pharma (ROSS_COMM21_01 to O. R. and PIRA_COMM22_01 to M. P). We thank Prof. Cesare Montecucco, Prof. Giampietro Schiavo, Dr. Ivica Matak and Dr. Philippe Picaut for critical reading of the original manuscript and for providing insightful comments and suggestions. Open access publishing facilitated by Universita degli Studi di Padova, as part of the Wiley - CRUI-CARE agreement.

CONFLICT OF INTEREST STATEMENT

J. M. L. D., M. M. and P. O. are employees of Fastox Pharma and J. M. L. D. and M. M. hold shares in the company. J. M. L. D. and M. M. are named inventors of a patent application that describes the modulation of BoNT/A effects by CnIIIC and DAN.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article because no new data were created or analysed in this study.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

ORCID

Marco Pirazzini https://orcid.org/0000-0003-4127-254X

REFERENCES

Alexander, S. P. H., Mathie, A. A., Peters, J. A., Veale, E. L., Striessnig, J., Kelly, E., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Aldrich, R. W., Attali, B., Baggetta, A. M., Becirovic, E., Biel, M., Bill, R. M., Caceres, A. I., Catterall, W. A., Conner, A. C., … Zhu, M. (2023). The concise guide to pharmacology 2023/24: Ion channels. *British Journal of Pharmacology*, *180*(Suppl 2), S145–s222. https://doi.org/10.1111/bph.16178

Anandan, C., & Jankovic, J. (2021). Botulinum toxin in movement disorders: An update. *Toxins (Basel)*, *13*(1), 42. https://doi.org/10.3390/toxins13010042

Antonucci, F., Rossi, C., Gianfranceschi, L., Rossetto, O., & Caleo, M. (2008). Long-distance retrograde effects of botulinum neurotoxin a. *The Journal of Neuroscience*, *28*(14), 3689–3696. https://doi.org/10.1523/JNEUROSCI.0375-08.2008

Aoki, K. R. (2001). A comparison of the safety margins of botulinum neurotoxin serotypes a, B, and F in mice. *Toxicon*, *39*(12), 1815–1820. https://doi.org/10.1016/s0041-0101(01)00101-5

Binz, T. (2013). Clostridial neurotoxin light chains: Devices for SNARE cleavage mediated blockade of neurotransmission. *Current Topics in Microbiology and Immunology*, *364*, 139–157. https://doi.org/10.1007/978-3-642-33570-9_7

Binz, T., & Rummel, A. (2009). Cell entry strategy of clostridial neurotoxins. *Journal of Neurochemistry*, *109*(6), 1584–1595. https://doi.org/10.1111/j.1471-4159.2009.06093.x

Blasi, J., Chapman, E. R., Link, E., Binz, T., Yamasaki, S., De Camilli, P., Sudhof, T. C., Niemann, H., & Jahn, R. (1993). Botulinum neurotoxin a selectively cleaves the synaptic protein SNAP-25. *Nature*, *365*(6442), 160–163. https://doi.org/10.1038/365160a0

Brin, M. F., James, C., & Maltman, J. (2014). Botulinum toxin type a products are not interchangeable: A review of the evidence. *Biologics*, *8*, 227–241. https://doi.org/10.2147/btt.S65603

Broide, R. S., Rubino, J., Nicholson, G. S., Ardila, M. C., Brown, M. S., Aoki, K. R., & Francis, J. (2013). The rat digit abduction score (DAS) assay: A physiological model for assessing botulinum neurotoxin-induced skeletal muscle paralysis. *Toxicon*, *71*, 18–24. https://doi.org/10.1016/j.toxicon.2013.05.004

Carli, L., Montecucco, C., & Rossetto, O. (2009). Assay of diffusion of different botulinum neurotoxin type a formulations injected in the mouse leg. *Muscle & Nerve*, *40*(3), 374–380. https://doi.org/10.1002/mus.21343

Carruthers, J., & Carruthers, A. (2001). Botulinum toxin (Botox) chemodenervation for facial rejuvenation. *Facial Plastic Surgery Clinics of North America*, *9*(2), 197–204. https://doi.org/10.1016/S1064-7406(23)00395-4

Chen, M., Wu, Q., Jiang, J., Jin, X., Liu, S., Wang, M., & Zhao, C. (2017). Preparation, characterization and in vivo evaluation of a formulation of dantrolene sodium with hydroxypropyl-β-cyclodextrin. *Journal of Pharmaceutical and Biomedical Analysis*, *135*, 153–159. https://doi.org/10.1016/j.jpba.2016.12.022

Choudhury, S., Baker, M. R., Chatterjee, S., & Kumar, H. (2021). Botulinum toxin: An update on pharmacology and newer products in development. *Toxins (Basel)*, *13*(1), 58. https://doi.org/10.3390/toxins13010058

Comella, C., Ferreira, J. J., Pain, E., Azoulai, M., & Om, S. (2021). Patient perspectives on the therapeutic profile of botulinum neurotoxin type a in cervical dystonia. *Journal of Neurology*, *268*(3), 903–912. https://doi.org/10.1007/s00415-020-10217-7

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*(15), 3907–3913. https://doi.org/10.1111/bph.15868

Del Castillo, J., & Katz, B. (1954). Quantal components of the end-plate potential. *The Journal of Physiology*, *124*(3), 560–573. https://doi.org/10.1113/jphysiol.1954.sp005129

Dolly, J. O., Black, J., Williams, R. S., & Melling, J. (1984). Acceptors for botulinum neurotoxin reside on motor nerve terminals and mediate its internalization. *Nature*, *307*(5950), 457–460. https://doi.org/10.1038/307457a0

Dong, M., Masuyer, G., & Stenmark, P. (2019). Botulinum and tetanus neurotoxins. *Annual Review of Biochemistry*, *88*, 811–837. https://doi.org/10.1146/annurev-biochem-013118-111654

Dressler, D. (2012). Clinical applications of botulinum toxin. *Current Opinion in Microbiology*, *15*(3), 325–336. https://doi.org/10.1016/j.mib.2012.05.012

Dressler, D. (2021). Clinical Pharmacology of Botulinum Toxin Drugs. In *Botulinum toxin therapy* (pp. 93–106). Springer International Publishing. https://doi.org/10.1007/164_2019_273

Dressler, D., & Johnson, E. A. (2022). Botulinum toxin therapy: Past, present and future developments. *Journal of Neural Transmission (Vienna)*, *129*(5), 829–833. https://doi.org/10.1007/s00702-022-02494-5

Duregotti, E., Zanetti, G., Scorzeto, M., Megighian, A., Montecucco, C., Pirazzini, M., & Rigoni, M. (2015). Snake and spider toxins induce a rapid recovery of function of botulinum neurotoxin paralysed neuromuscular junction. *Toxins (Basel).*, *7*(12), 5322–5336. https://doi.org/10.3390/toxins7124887

Eleopra, R., Rinaldo, S., Montecucco, C., Rossetto, O., & Devigili, G. (2020). Clinical duration of action of different botulinum toxin types in humans. *Toxicon*, *179*, 84–91. https://doi.org/10.1016/j.toxicon.2020.02.020

Elliott, M., Favre-Guilmard, C., Liu, S. M., Maignel, J., Masuyer, G., Beard, M., Boone, C., Carre, D., Kalinichev, M., Lezmi, S., Mir, I., Nicoleau, C., Palan, S., Perier, C., Raban, E., Zhang, S., Dong, M., Stenmark, P., & Krupp, J. (2019). Engineered botulinum neurotoxin B with improved binding to human receptors has enhanced efficacy in preclinical models. *Science Advances*, *5*(1), eaau7196. https://doi.org/10.1126/sciadv.aau7196

Fabi, S. G., Carruthers, J., Joseph, J., Cox, S. E., Yoelin, S., Few, J., Kaufman-Janette, J., & Dayan, S. (2021). High-dose neuromodulators: A roundtable on making sense of the data in real-world clinical practice. *Aesthetic Surgery Journal Open Forum*, *3*(4), ojab036. https://doi.org/10.1093/asjof/ojab036

Favreau, P., Benoit, E., Hocking, H. G., Carlier, L., D'hoedt, D., Leipold, E., Markgraf, R., Schlumberger, S., Córdova, M. A., Gaertner, H., Paolini-Bertrand, M., Hartley, O., Tytgat, J., Heinemann, S. H., Bertrand, D., Boelens, R., Stöcklin, R., & Molgó, J. (2012). A novel μ-conopeptide, CnIIIC, exerts potent and preferential inhibition of NaV1.2/1.4 channels and blocks neuronal nicotinic acetylcholine receptors. *British Journal of Pharmacology*, *166*(5), 1654–1668. https://doi.org/10.1111/j.1476-5381.2012.01837.x

Field, M., Splevins, A., Picaut, P., van der Schans, M., Langenberg, J., Noort, D., Snyder, D., & Foster, K. (2019). AbobotulinumtoxinA (Dysport®), OnabotulinumtoxinA (Botox®), and IncobotulinumtoxinA (Xeomin®) neurotoxin content and potential implications for duration of response in patients. *Toxins (Basel)*, *10*(12), 535. https://doi.org/10.3390/toxins10120535

Fischer, A., & Montal, M. (2013). Molecular dissection of botulinum neurotoxin reveals interdomain chaperone function. *Toxicon*, *75*, 101–107. https://doi.org/10.1016/j.toxicon.2013.01.007

Franzini-Armstrong, C. (2018). The relationship between form and function throughout the history of excitation-contraction coupling. *The Journal of General Physiology*, *150*(2), 189–210. https://doi.org/10.1085/jgp.201711889

Frevert, J. (2015). Pharmaceutical, biological, and clinical properties of botulinum neurotoxin type a products. *Drugs in R&D*, *15*(1), 1–9. https://doi.org/10.1007/s40268-014-0077-1

Garcia-Rodriguez, C., Levy, R., Arndt, J. W., Forsyth, C. M., Razai, A., Lou, J., Geren, I., Stevens, R. C., & Marks, J. D. (2007). Molecular evolution of antibody cross-reactivity for two subtypes of type a botulinum neurotoxin. *Nature Biotechnology*, *25*(1), 107–116. https://doi.org/10.1038/nbt1269

Guo, J., Pan, X., Zhao, Y., & Chen, S. (2013). Engineering clostridia neurotoxins with elevated catalytic activity. *Toxicon*, *74c*, 158–166. https://doi.org/10.1016/j.toxicon.2013.08.055

Hallett, M., Albanese, A., Dressler, D., Segal, K. R., Simpson, D. M., Truong, D., & Jankovic, J. (2013). Evidence-based review and assessment of botulinum neurotoxin for the treatment of movement disorders. *Toxicon*, *67*, 94–114. https://doi.org/10.1016/j.toxicon.2012.12.004

Hanna, E., & Pon, K. (2020). Updates on botulinum neurotoxins in dermatology. *American Journal of Clinical Dermatology*, *21*(2), 157–162. https://doi.org/10.1007/s40257-019-00482-2

Jabbari, B. (2021). *Botulinum toxin treatment in surgery, dentistry, and veterinary medicine*. Springer International Publishing. https://doi.org/10.1007/978-3-030-50691-9_1

Jahn, K., Grosskreutz, J., Haastert, K., Ziegler, E., Schlesinger, F., Grothe, C., Dengler, R., & Bufler, J. (2006). Temporospatial coupling of networked synaptic activation of AMPA-type glutamate receptor channels and calcium transients in cultured motoneurons. *Neuroscience*, *142*(4), 1019–1029. https://doi.org/10.1016/j.neuroscience.2006.07.034

Jankovic, J. (2017). Botulinum toxin: State of the art. *Movement Disorders*, *32*(8), 1131–1138. https://doi.org/10.1002/mds.27072

Joshi, T. N., & Joshi, S. (2011). Adverse effects of botulinum neurotoxin a in spasticity management. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *1*(2), 126. https://journals.lww.com/ijnp/Fulltext/2011/01020/Adverse_effects_of_botulinum_neurotoxin_A_in.7.aspx, https://doi.org/10.4103/2231-0738.84202

Kaji, R., Miyashiro, A., Sato, N., Furumoto, T., Takeuchi, T., Miyamoto, R., Kohda, T., Izumi, Y., & Kozaki, S. (2022). A pilot study of A2NTX, a novel low-molecular-weight neurotoxin derived from subtype A2 for post-stroke lower limb spasticity: Comparison with OnabotulinumtoxinA. *Toxins (Basel)*, *14*(11), 739. https://www.mdpi.com/2072-6651/14/11/739, https://doi.org/10.3390/toxins14110739

Kaufman-Janette, J., Cox, S. E., Dayan, S., & Joseph, J. (2021). Botulinum toxin type a for glabellar frown lines: What impact of higher doses on outcomes? *Toxins (Basel)*, *13*(7), 494. https://www.mdpi.com/2072-6651/13/7/494, https://doi.org/10.3390/toxins13070494

Kiris, E., Kota, K. P., Burnett, J. C., Soloveva, V., Kane, C. D., & Bavari, S. (2014). Recent developments in cell-based assays and stem cell technologies for botulinum neurotoxin research and drug discovery. *Expert Review of Molecular Diagnostics*, *14*(2), 153–168. https://doi.org/10.1586/14737159.2014.867808

Krause, T., Gerbershagen, M. U., Fiege, M., Weisshorn, R., & Wappler, F. (2004). Dantrolene--a review of its pharmacology, therapeutic use and new developments. *Anaesthesia*, *59*(4), 364–373. https://doi.org/10.1111/j.1365-2044.2004.03658.x

Kutschenko, A., Bigalke, H., Wegner, F., & Wohlfarth, K. (2019). The role of human serum albumin and neurotoxin associated proteins in the formulation of BoNT/a products. *Toxicon*, *168*, 158–163. https://doi.org/10.1016/j.toxicon.2019.07.005

Kuwajima, G., Futatsugi, A., Niinobe, M., Nakanishi, S., & Mikoshiba, K. (1992). Two types of ryanodine receptors in mouse brain: Skeletal muscle type exclusively in Purkinje cells and cardiac muscle type in various neurons. *Neuron*, *9*(6), 1133–1142. https://doi.org/10.1016/0896-6273(92)90071-k

Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., & Stevens, R. C. (1998). Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nature Structural Biology*, *5*(10), 898–902. https://doi.org/10.1038/2338

Lange, O., Bigalke, H., Dengler, R., Wegner, F., deGroot, M., & Wohlfarth, K. (2009). Neutralizing antibodies and secondary therapy failure after treatment with botulinum toxin type a: Much ado about nothing? *Clinical Neuropharmacology*, *32*(4), 213–218. https://doi.org/10.1097/WNF.0b013e3181914d0a

Ledda, C., Artusi, C. A., Tribolo, A., Rinaldi, D., Imbalzano, G., Lopiano, L., & Zibetti, M. (2022). Time to onset and duration of botulinum toxin efficacy in movement disorders. *Journal of Neurology*, *269*(7), 3706–3712. https://doi.org/10.1007/s00415-022-10995-2

Lewis, R. J., Dutertre, S., Vetter, I., & Christie, M. J. (2012). Conus venom peptide pharmacology. *Pharmacological Reviews*, *64*(2), 259–298. https://doi.org/10.1124/pr.111.005322

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M. M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

Maio, M., & Rzany, B. (2007). *Botulinum toxin in aesthetic medicine*. Springer. https://doi.org/10.1007/978-3-540-34095-9

Markgraf, R., Leipold, E., Schirmeyer, J., Paolini-Bertrand, M., Hartley, O., & Heinemann, S. H. (2012). Mechanism and molecular basis for the sodium channel subtype specificity of μ-conopeptide CnIIIC. *British Journal of Pharmacology*, *167*(3), 576–586. https://doi.org/10.1111/j.1476-5381.2012.02004.x

Martina, E., Diotallevi, F., Radi, G., Campanati, A., & Offidani, A. (2021). Therapeutic use of botulinum neurotoxins in dermatology: Systematic review. *Toxins (Basel)*, *13*(2), 120. https://doi.org/10.3390/toxins13020120

Meunier, F. A., Schiavo, G., & Molgo, J. (2002). Botulinum neurotoxins: From paralysis to recovery of functional neuromuscular transmission. *Journal of Physiology, Paris*, *96*(1–2), 105–113. https://doi.org/10.1016/S0928-4257(01)00086-9

Meunier, F.r A., Lisk, G., Sesardic, D., & Dolly, J. O. (2003). Dynamics of motor nerve terminal remodeling unveiled using SNARE-cleaving botulinum toxins: The extent and duration are dictated by the sites of SNAP-25 truncation. *Molecular and Cellular Neuroscience*, *22*(4), 454–466. https://doi.org/10.1016/S1044-7431(02)00016-7

Montal, M. (2010). Botulinum neurotoxin: A marvel of protein design. *Annual Review of Biochemistry*, *79*, 591–617. https://doi.org/10.1146/annurev.biochem.051908.125345

Montecucco, C. (1986). How do tetanus and botulinum toxins bind to neuronal membranes? *Trends in Biochemical Sciences*, *11*(8), 314–317. http://linkinghub.elsevier.com/retrieve/pii/0968000486902823, https://doi.org/10.1016/0968-0004(86)90282-3

Naumann, M., Dressler, D., Hallett, M., Jankovic, J., Schiavo, G., Segal, K. R., & Truong, D. (2013). Evidence-based review and assessment of botulinum neurotoxin for the treatment of secretory disorders. *Toxicon*, *67*, 141–152. https://doi.org/10.1016/j.toxicon.2012.10.020

Pamphlett, R. (1989). Early terminal and nodal sprouting of motor axons after botulinum toxin. *Journal of the Neurological Sciences*, *92*(2–3), 181–192. https://doi.org/10.1016/0022-510X(89)90135-4

Pantano, S., & Montecucco, C. (2014). The blockade of the neurotransmitter release apparatus by botulinum neurotoxins. *Cellular and Molecular Life Sciences*, *71*(5), 793–811. https://doi.org/10.1007/s00018-013-1380-7

Pellett, S. (2013). Progress in cell based assays for botulinum neurotoxin detection. *Current Topics in Microbiology and Immunology*, *364*, 257–285. https://doi.org/10.1007/978-3-642-33570-9_12

Pellett, S., Tepp, W. H., Clancy, C. M., Borodic, G. E., & Johnson, E. A. (2007). A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. *FEBS Letters*, *581*(25), 4803–4808. https://doi.org/10.1016/j.febslet.2007.08.078

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLOS Biology*, *18*, e3000410. https://doi.org/10.1371/journal.pbio.3000410

Pirazzini, M., Azarnia Tehran, D., Leka, O., Zanetti, G., Rossetto, O., & Montecucco, C. (2016). On the translocation of botulinum and tetanus neurotoxins across the membrane of acidic intracellular compartments. *Biochimica et Biophysica Acta*, *1858*(3), 467–474. https://doi.org/10.1016/j.bbamem.2015.08.014

Pirazzini, M., Azarnia Tehran, D., Zanetti, G., Megighian, A., Scorzeto, M., Fillo, S., Shone, C. C., Binz, T., Rossetto, O., Lista, F., & Montecucco, C. (2014). Thioredoxin and its reductase are present on synaptic vesicles, and their inhibition prevents the paralysis induced by botulinum neurotoxins. *Cell Reports*, *8*(6), 1870–1878. https://doi.org/10.1016/j.celrep.2014.08.017

Pirazzini, M., Grinzato, A., Corti, D., Barbieri, S., Leka, O., Vallese, F., Tonellato, M., Silacci-Fregni, C., Piccoli, L., Kandiah, E., Schiavo, G., Zanotti, G., Lanzavecchia, A., & Montecucco, C. (2021). Exceptionally potent human monoclonal antibodies are effective for prophylaxis and therapy of tetanus in mice. *The Journal of Clinical Investigation*, *131*(22), e151676. https://doi.org/10.1172/JCI151676

Pirazzini, M., Henke, T., Rossetto, O., Mahrhold, S., Krez, N., Rummel, A., Montecucco, C., & Binz, T. (2013). Neutralisation of specific surface carboxylates speeds up translocation of botulinum neurotoxin type B enzymatic domain. *FEBS Letters*, *587*(23), 3831–3836. https://doi.org/10.1016/j.febslet.2013.10.010

Pirazzini, M., Montecucco, C., & Rossetto, O. (2022). Toxicology and pharmacology of botulinum and tetanus neurotoxins: An update. *Archives of Toxicology*, *96*(6), 1521–1539. https://doi.org/10.1007/s00204-022-03271-9

Pirazzini, M., Rossetto, O., Eleopra, R., & Montecucco, C. (2017). Botulinum neurotoxins: Biology, pharmacology, and toxicology. *Pharmacological Reviews*, *69*(2), 200–235. https://doi.org/10.1124/pr.116.012658

Poliziani, M., Koch, M., & Liu, X. (2016). Striving for more good days: Patient perspectives on botulinum toxin for the treatment of cervical dystonia. *Patient Preference and Adherence*, *10*, 1601–1608. https://doi.org/10.2147/ppa.S106560

Rizo, J. (2022). Molecular mechanisms underlying neurotransmitter release. *Annual Review of Biophysics*, *51*, 377–408. https://doi.org/10.1146/annurev-biophys-111821-104732

Rossetto, O., & Montecucco, C. (2019). Tables of toxicity of botulinum and tetanus neurotoxins. *Toxins (Basel).*, *11*(12), 686. https://doi.org/10.3390/toxins11120686

Rossetto, O., Pirazzini, M., Fabris, F., & Montecucco, C. (2021). *Botulinum neurotoxins: Mechanism of action* (pp. 35–47). Springer International Publishing. https://doi.org/10.1007/164_2020_355

Rossetto, O., Pirazzini, M., & Montecucco, C. (2014). Botulinum neurotoxins: Genetic, structural and mechanistic insights. *Nature Reviews. Microbiology*, *12*(8), 535–549. https://doi.org/10.1038/nrmicro3295

Rossetto, O., Seveso, M., Caccin, P., Schiavo, G., & Montecucco, C. (2001). Tetanus and botulinum neurotoxins: Turning bad guys into good by research. *Toxicon*, *39*(1), 27–41. https://doi.org/10.1016/S0041-0101(00)00163-X

Safavi-Hemami, H., Brogan, S. E., & Olivera, B. M. (2019). Pain therapeutics from cone snail venoms: From Ziconotide to novel non-opioid pathways. *Journal of Proteomics*, *190*, 12–20. https://doi.org/10.1016/j.jprot.2018.05.009

Scaglione, F. (2016). Conversion ratio between Botox®, Dysport®, and Xeomin® in clinical practice. *Toxins (Basel)*, *8*(3), 65. https://doi.org/10.3390/toxins8030065

Schaller, K. L., & Caldwell, J. H. (2003). Expression and distribution of voltage-gated sodium channels in the cerebellum. *Cerebellum*, *2*(1), 2–9. https://doi.org/10.1080/14734220309424

Schiavo, G., & Montecucco, C. (1995). Tetanus and botulism neurotoxins: Isolation and assay. *Methods in Enzymology*, *248*, 643–652. https://doi.org/10.1016/0076-6879(95)48041-2

Schiavo, G., Rossetto, O., Santucci, A., DasGupta, B. R., & Montecucco, C. (1992). Botulinum neurotoxins are zinc proteins. *The Journal of Biological Chemistry*, *267*(33), 23479–23483. https://doi.org/10.1016/S0021-9258(18)35863-0

Schiavo, G., Santucci, A., Dasgupta, B. R., Mehta, P. P., Jontes, J., Benfenati, F., Wilson, M. C., & Montecucco, C. (1993). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. *FEBS Letters*, *335*(1), 99–103. http://www.ncbi.nlm.nih.gov/pubmed/8243676, https://doi.org/10.1016/0014-5793(93)80448-4

Seki, H., Xue, S., Hixon, M. S., Pellett, S., Remes, M., Johnson, E. A., & Janda, K. D. (2015). Toward the discovery of dual inhibitors for botulinum neurotoxin a: Concomitant targeting of endocytosis and light chain protease activity. *Chemical Communications (Camb).*, *51*(28), 6226–6229. https://doi.org/10.1039/c5cc00677e

Sethi, K. D., Rodriguez, R., & Olayinka, B. (2012). Satisfaction with botulinum toxin treatment: A cross-sectional survey of patients with cervical dystonia. *Journal of Medical Economics*, *15*(3), 419–423. https://doi.org/10.3111/13696998.2011.653726

Shoemaker, C. B., & Oyler, G. A. (2013). Persistence of botulinum neurotoxin inactivation of nerve function. *Current Topics in Microbiology and Immunology*, *364*, 179–196. https://doi.org/10.1007/978-3-642-33570-9_9

Smith, T. J., Lou, J., Geren, I. N., Forsyth, C. M., Tsai, R., LaPorte, S. L., Tepp, W. H., Bradshaw, M., Johnson, E. A., Smith, L. A., & Marks, J. D. (2005). Sequence variation within botulinum neurotoxin serotypes impacts antibody binding and neutralization. *Infection and Immunity*, *73*(9), 5450–5457. https://doi.org/10.1128/IAI.73.9.5450-5457.2005

Solish, N., Carruthers, J., Kaufman, J., Rubio, R. G., Gross, T. M., & Gallagher, C. J. (2021). Overview of DaxibotulinumtoxinA for injection: A novel formulation of botulinum toxin type a. *Drugs*, *81*(18), 2091–2101. https://doi.org/10.1007/s40265-021-01631-w

Šoštarić, P., Vukić, B., Tomašić, L., & Matak, I. (2022). Lasting peripheral and central effects of botulinum toxin type a on experimental muscle hypertonia in rats. *International Journal of Molecular Sciences*, *23*(19), 11626. https://www.mdpi.com/1422-0067/23/19/11626, https://doi.org/10.3390/ijms231911626

Steward, L., Brin, M. F., & Brideau-Andersen, A. (2021). *Novel native and engineered botulinum neurotoxins* (pp. 63–89). Springer International Publishing. https://doi.org/10.1007/164_2020_351

Stone, H. F., Zhu, Z., Thach, T. Q., & Ruegg, C. L. (2011). Characterization of diffusion and duration of action of a new botulinum toxin type a formulation. *Toxicon*, *58*(2), 159–167. https://doi.org/10.1016/j.toxicon.2011.05.012

Tehran, D. A., & Pirazzini, M. (2018). Preparation of cerebellum granule neurons from mouse or rat pups and evaluation of Clostridial neurotoxin activity and their inhibitors by Western blot and immunohistochemistry. *Bio-Protocol*, *8*(13), e2918. https://doi.org/10.21769/BioProtoc.2918

Whitemarsh, R. C., Tepp, W. H., Bradshaw, M., Lin, G., Pier, C. L., Scherf, J. M., Johnson, E. A., & Pellett, S. (2013). Characterization of botulinum neurotoxin a subtypes 1 through 5 by investigation of activities in mice, in neuronal cell cultures, and in vitro. *Infection and Immunity*, *81*(10), 3894–3902. https://doi.org/10.1128/iai.00536-13

Wissel, J. (2018). Towards flexible and tailored botulinum neurotoxin dosing regimens for focal dystonia and spasticity - insights from recent studies. *Toxicon*, *147*, 100–106. https://doi.org/10.1016/j.toxicon.2018.01.018

Zanetti, G., Negro, S., Megighian, A., Mattarei, A., Lista, F., Fillo, S., Rigoni, M., Pirazzini, M., & Montecucco, C. (2019). A CXCR4 receptor agonist strongly stimulates axonal regeneration after damage. *Annals of Clinical Translational Neurology*, *6*(12), 2395–2402. https://doi.org/10.1002/acn3.50926

Zanetti, G., Negro, S., Megighian, A., & Pirazzini, M. (2018). Electrophysiological recordings of evoked end-plate potential on murine neuromuscular synapse preparations. *Bio-Protocol*, *8*(8), e2803. https://doi.org/10.21769/BioProtoc.2803

Zanetti, G., Sikorra, S., Rummel, A., Krez, N., Duregotti, E., Negro, S., Henke, T., Rossetto, O., Binz, T., & Pirazzini, M. (2017). Botulinum neurotoxin C mutants reveal different effects of syntaxin or SNAP-25 proteolysis on neuromuscular transmission. *PLoS Pathogens*, *13*(8), e1006567. https://doi.org/10.1371/journal.ppat.1006567

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Machicoane, M., Tonellato, M., Zainotto, M., Onillon, P., Stazi, M., Corso, M. D., Megighian, A., Rossetto, O., Le Doussal, J.-M., & Pirazzini, M. (2024). Excitation–contraction coupling inhibitors potentiate the actions of botulinum neurotoxin type A at the neuromuscular junction. *British Journal of Pharmacology*, 1–17. https://doi.org/10.1111/bph.17367