Capsaicin protects neuromuscular junctions from the inhibitory effects of botulinum neurotoxin A

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Running title: capsaicin antagonizes botulinum neurotoxin A

No. of words in text: 6325

No. of figures: 9 (Panels: 32)

No. of supplemental figures: 1

No. of tables: 1

ABSTRACT

Within 24 hrs after injecting botulinum neurotoxin A (BoNT/A) into the hindlimb, mice lost the toe spread reflex and developed progressive muscle weakness. At the same time, the compound muscle action potential amplitude decreased. Injection of capsaicin before BoNT/A significantly reduced these affects and protected the muscle twitch tension of the *Extensor digitorum longus* (EDL) nerve muscle preparation. Acute *in vitro* exposure of isolated nerve muscle preparations, as well as Neuro 2a cells, to capsaicin prevented uptake of Alexa 647 BoNT/A. Motor nerve endings as well as Neuro 2a cells express the capsaicin receptor, a transient receptor potential channel of the vanilloid family (TRPV1). Capsaicin as well as disruption of clathrin coated pits (CCPs) reduced Neuro 2a cell uptake of BoNT/A. FM1-43 uptake indicated that exocytosis persists for BoNT/A treated Neuro 2a cells pretreated with capsaicin. Pre-injection of wortmannin (WMN), a PI3Kinase inhibitor, also protected mice from the paralytic effects of BoNT/A. When applied alone, either WMN or capsaicin selectively reduced stimulus-evoked transmitter release from motor nerve endings. We hypothesize that TRPV1 activation reduces $PI(4,5)P_2$ level within the membrane. This prevents CCP formation and uptake of BoNT/A.

INTRODUCTION

Neurotoxins produced by C*lostridium botulinum* that target peripheral cholinergic nerve endings are the most potent biological toxins. The seven serotypes of the toxin, designated BoNT/A to G (1), selectively cleave the SNARE complex proteins and inhibit acetylcholine release. Inhibition of neuromuscular transmission leads to paralysis (2). This action involves a cascade of cellular events including toxin binding to specific receptors (3,4), internalization by endocytosis, and translocation of the toxin light chain from endosomes to cytosol in a pH dependent process. The light chain metalloendoprotease of serotypes A and E cleave SNAP-25. Light chains of serotypes B, D, F and G cleave synaptobrevin II. Serotype C light chain cleaves primarily syntaxin I, as well as SNAP-25 in some cells (5,6).

Vesicular release of acetylcholine from motor nerve endings is an exocytic process which adds synaptic vesicle (SV) membrane to the nerve terminal membrane (7-10). This process of exocytosis is associated with compensatory endocytosis to replenish SV membrane and to maintain nerve terminal size. SV membrane retrieval from the terminal membrane may utilize clathrin coated pit (CCP) mediated endocytosis which requires phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$, 11). Increasing data indicate that the interaction of $PI(4,5)P_2$ with components of the endocytotic machinery is critical to CCP mediated endocytosis (12-16).

In mammalian cells, $PI(4,5)P_2$ results from phosphatidylinositol-4-phosphate-5-kinase (PIP5K) phosphorylation of phosphatidylinositol-4-phosphate PI(4)P (17,18). $PI(4,5)P_2$ interacts with several proteins that regulate exo-endocytosis of SVs. For example, binding of $PI(4,5)P_2$ to synaptotagmin at concentrations of Ca^{2+} typically found in the presynaptic terminal of a stimulated neuron modulates docking of SVs (19). $PI(4,5)P_2$ also binds to rabphilin 3 (20) and controls the formation of the SNARE complex. Other interactions that are relevant to vesicular secretion includes $PI(4,5)P_2$ inhibition of the synaptic vesicle protein casein kinase I which phosphorylates several proteins including SV2 (21). Also, $PI(4,5)P_2$ is required for the ATP-dependent priming step preceding exocytosis (22-25). The role of $PI(4,5)P_2$ in the endocytotic pathway is critical and equally complicated. For example, $PI(4,5)P_2$ is required during early and late stages of clathrin-mediated endocytosis (26). Furthermore, $PI(4,5)P_2$ interacts with proteins of the endocytotic machinery including AP2, epsin and AP180. These interactions are crucial for CCP formation (12-14, 32). Binding of $PI(4,5)P_2$ to dynamin (27,28), which is recruited to the neck of the invaginating clathrin-coated vesicle, enables vesicle closure by endophilin I (29). Activity of synaptojanin, a PI5-phosphatase that dephosphorylates $PI(4,5)P_2$ to PI(4)P (30), is important for uncoating of endocytic vesicles (31). These observations provide a compelling model of $PI(4,5)P_2$ in the regulation of CCP dependent endocytosis.

Neuronal internalization of BoNT/A involves CCP mediated endocytosis (33,34,35). That is, disruption of CCP by incubating cells with potassium depleted buffer followed by hypotonic shock inhibits BoNT/A

uptake into Neuro 2a cells (35,36). Thus, it is of importance to test whether disruption of CCP by depletion of membrane bound $PI(4,5)P_2$ reduces the uptake of BoNT/A. Here, we report that the mouse NMJ expresses the capsaicin receptor TRPV1. *In vivo* and *in vitro* activation of TRPV1 inhibited BoNT/A uptake into motor nerve endings. Furthermore, we report that wortmannin (WMN) inhibits the effects of BoNT/A *in vivo*. Our data suggest that capsaicin inhibits BoNT/A uptake by depleting $PI(4,5)P_2$ and disrupting CCP mediated endocytosis. The net effect is capsaicin-induced protection of the NMJ from the inhibitory affects of BoNT/A.

METHODS

Animals

Adult Swiss Webster mice were anesthetized with ketamine (100 mg/kg) and xylazine (9 mg/kg) mixture (KXM) intraperitoneally. Three µl of 6.67 pM BoNT/A in HEPES Ringer Solution (HRS) was injected bilaterally with a 26 Gauge Hamilton syringe into the space surrounding the EDL innervation region. The skin incision was then surgically closed. Capsaicin was either bilaterally coinjected (3 µl of 1 mM stock solution) immediately before BoNT/A or preinjected at 4 or 8 hrs prior to BoNT/A. Wortmannin (3 µl of 1 mM) was injected bilaterally 6 hrs prior to BoNT/A. All procedures were approved by the Institutional Animal Care and Use Committee.

Cell culture and FM1-43 loading

Mouse cholinergic neuroblastoma (Neuro 2a) cells were cultured in DMEM-F12 medium supplemented with 10% Fetal Bovine Serum and antibiotics. Briefly, cells seeded on poly-L-lysine coated coverslips were incubated with either 40 mM KCl HRS alone (control) or in combination with 10 pM BoNT/A for 30 min at room temperature. Subsequently, cells were washed twice with PBS and further incubated with FM1-43 (1 µg/ml) in 40 mM KCl HRS for 10 min. In studies of capsaicin, cells were incubated with 10 µM capsaicin in HEPES for 15 min prior to incubation in 10 pM BoNT/A plus 40 mM KCl. Cells were

washed twice with HRS pH 7.4 and observed with a Zeiss (Oberkochen, Germany) LSM-510 confocal microscope equipped with an argon laser (488 nm). Images were analyzed with the ImageJ software.

Disruption of CCP

CCPs were disrupted as described previously (35).

Immunoblotting

Cultured cells or diaphragm muscles dissected from anesthetized mice were flash frozen in liquid nitrogen and homogenized in phosphate buffered saline (PBS) containing 1% NP-40 and complete protease inhibitor cocktail (Amersham, USA). After centrifuging the homogenate in the cold at 14,000 rpm for 10 min, the lysate (40 µg) was resolved *via* SDS-PAGE after boiling in 1X Laemmli buffer. Immunoblotting was performed with anti-TRPV1 antibody (Santa Cruz Biotechnology, Inc., USA).

Immunohistochemistry

Preparations were fixed with 4% paraformaldehyde, permeabilized with 1% Triton, blocked with 2% BSA, incubated with TRPV1 antibody in PBS (1:200) followed by FITC labeled secondary antibody (1:5000), washed with PBS, mounted with vectashield on a glass slide and imaged. All treatments after fixing were performed at 4 ºC.

Two electrode voltage clamping

Triangularis sterni nerve-muscle preparations (TS; 37) were dissected from isoflurane anesthetized adult Swiss Webster mice and superfused in HRS (22-25 °C) containing (mM): NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), HEPES (10, pH 7.4), glucose (5.5). To activate transmitter release, the TS nerve was drawn into a suction electrode and stimulated supramaximally. Spontaneous and stimulus evoked transmitter release were recorded by two electrode voltage clamping (Axoclamp 2B, Molecular Devices Inc., USA). For controls, the HRS contained 0.75 to 4 µM µ Conotoxin GIIIB to prevent

mechanical responses to nerve stimulation. Control recordings were made before application of 100 μM capsaicin or 100 μM wortmannin. Collected data were analyzed with pCLAMP 9.0 software.

Muscle twitch tension

EDL nerve-muscle preparations were dissected and mounted in a glass chamber (Rodnoti Glass Technology, Inc., USA) filled with oxygenated (95% O_2 - 5% CO_2) normal Ringer solution (pH 7.4, 37 °C) containing (mM) NaCl (135), KCl (5), $MgCl_2$ (1), $CaCl_2$ (2), Na_2HPO_4 (1), $NaHCO_3$ (15), glucose (5.5). The EDL nerve was drawn into a suction electrode for indirect activation of muscle twitches. One tendon of the muscle was tied to a Grass Force transducer connected to a Digidata 1440A (Molecular Devices, USA) for acquisition of the mechanical response to nerve stimulation. Muscles were stretched to optimal length for force generation and equilibrated for 15 min prior to stimulation at 0.1 Hz for data acquisition. Collected data were analyzed with pCLAMP 9.0 software.

Confocal microscopy

TS nerve-muscle preparations were bathed in 667 pM Alexa 647 labeled BoNT/A. Nerve stimulation (1 Hz) or addition of 40 mM KCl (NaCl reduced to 100 mM to maintain osmolarity) to the BoNT/A-containing HRS initiated toxin uptake. After 90 min, preparations were fixed and exposed to Alexa 488 α-Bungarotoxin (α-BTX; 1 ng/ml, 6 hrs at 4°C) to label endplate acetylcholine receptors. The endplate region of the muscle was cut out, mounted on a slide with vectashield and observed with a NIKON LSM-410 confocal microscope equipped with argon and HeNe lasers at the Confocal Imaging Facility of the New Jersey Medical School.

Muscle strength assessment

Mice were adapted for 1 week to 300 sec episodes on a rotating (15 rpm) rod (Rotamex-5, Columbus Instruments, Inc., USA) prior to study. To evaluate motor strength and coordination after BoNT/A injected alone or in combination with capsaicin, duration on the rotarod was tested biweekly. Mean

duration on the rotarod was averaged for 3 runs 30 min apart. The *in vivo* muscle strength of legs and waist was assessed and scored using criteria of table 1.

Electromyography (EMG)

Animals anesthetized with KXM intraperitoneally were taped prone to a polystyrene foam board. A heated water pad maintained body temperature between 32 and 38° C. Stimulating electrodes (0.7 mm needles) were insulated with polytef (Dantec sensory needle, Skorlunde, Denmark); cathode was placed close to the sciatic nerve and the anode was placed subcutaneously 1 cm proximally. Motor responses were recorded with a ring electrode placed distal to the site of BoNT/A injection. The reference ring electrode was placed 1.2 cm distal to the recording electrode. Ground electrode was placed in the contra-lateral limb. Stimuli delivered through a stimulator were recorded by Dantec amplifiers connected to a computer. Filter settings were 500 Hz/5KHz. Stimulus intensity was increased until the compound muscle action potential (CMAP) was maximized. CMAP amplitude was recorded for both hind limbs.

Chemicals and drugs

BoNT/A and Alexa 647 BoNT/A were from Metabiologics Inc (WI, USA) and BB Tech (MA, USA), respectively. Wortmannin was from Calbiochem, USA. Other chemicals and drugs were from Sigma (USA).

Data analyses

Data were expressed as mean ± S.E.M. Student's *t* test evaluated the statistical significance for differences of population means; ** and *** indicate $p < 0.001$ and < 0.0001, respectively.

RESULTS

The right hind limb of the mouse in Figure 1 A was injected with capsaicin 4 hrs prior to bilateral injection of BoNT/A. Within 24 hrs, the toe spread reflex (TSR) was abolished in the left limb. In

contrast, TSR remained normal for the capsaicin pre-injected limb. Figure 1 A summarizes data from 24 mice injected with BoNT/A either immediately or at 4 or 8 hrs after capsaicin injection into the same hind limb. While BoNT/A abolished the TSR for all control mice (n=16), TSR was not altered for all capsaicin pre-treated mice. To further test *in vivo* muscle function and coordination after BoNT/A, mice were challenged with a rotating rod. All non-treated mice walked on the rod for at least 300 sec. Walk time significantly declined at 24 hrs and reached a minimal value of 127±48 sec at 4 days after BoNT/A. Walk time recovered to 300 sec at 20 days after BoNT/A alone. In contrast, walk time did not decline when BoNT/A was injected at 4 hrs after capsaicin (Figure 1 B). Furthermore, scoring for motor strength, by a blinded investigator using the criteria of table 1, suggested that capsaicin protected against the weakness caused by BoNT/A. That is, motor strength score was significantly less than control at 2 and 3 weeks after BoNT/A alone. In contrast, capsaicin pre-injected mice retained a normal score (Figure 1 C). Protection of muscle function may explain the larger weight of capsaicin pretreated *versus* BoNT/A alone treated mice (Figure 1 D).

The preceding effects of capsaicin were associated with partial protection of neuromuscular physiology *in vivo*. Although CMAP amplitude declined for BoNT/A alone as well as BoNT/A after capsaicin groups of mice, the magnitude of the decline was significantly less for the latter group. That is, CMAP amplitude was 11.3 ± 2.3 (n=4), 0.7 ± 0.28 (n=4) and 4.6 ± 0.92 (n=4) mV at 4 weeks after BoNT/A for control, BoNT/A alone and capsaicin pre-injected mice, respectively. At 8 weeks after BoNT/A, CMAP amplitude had partially recovered for the BoNT/A alone and capsaicin pre-injected groups to 2.6 ± 0.79 (n=4) and 6.7 ± 1.24 (n=4) mV, respectively. For both groups of mice, CMAP amplitude remained abnormally low as late as 14 weeks after BoNT/A (Figure 2 B). Capsaicin alone had no affect on muscle strength or CMAP amplitude (Figure 2 C).

The force generating capacity was tested for EDL preparations isolated from mice injected with BoNT/A alone or after capsaicin. As anticipated, the nerve-evoked muscle twitch tension was reduced at 24 hrs after BoNT/A alone. However, pre-injection of capsaicin significantly reduced this affect of

BoNT/A (Figure 3 A); tension was 140 ± 60.0 (n=4) and 500 ± 100 (n=4) mg for mice injected with BoNT/A alone and at 4 hrs after capsaicin, respectively. Thus, while BoNT/A continued to significantly reduce twitch tension after capsaicin, the reduction was significantly less than after BoNT/A alone (Figure 3 B). Capsaicin injected alone had no effect on force generation (Figure 3 C).

Since capsaicin is an agonist of the TRPV1 receptor, we assayed whole muscle for this protein. Immunoblots (Figure 4 A) revealed TRPV1 in NMJ as well as Neuro 2a cells. The TRPV1 antibody also detected this protein on motor nerve endings and Neuro 2a cells (Figure 4 B and C).

The protective role of TRPV1 was observed for Neuro 2a cells induced to take up FM1-43, a fluorescent marker of exocytosis (Figure 5 A). That is, exposure to BoNT/A prevented 40 mM KCl-induced uptake of FM1-43. This inhibitory effect of BoNT/A was not observed for Neuro 2a cells exposed to capsaicin prior to BoNT/A (Figure 5 B).

Capsaicin pretreatment reduced BoNT/A uptake. Figure 6 presents representative images of NMJs in the TS muscle labeled with Alexa 488 αBTX and Alexa 647 BoNT/A; neurotoxin uptake was activated by nerve stimulation (A) or depolarization with 40 mM KCl (B). Comparison of the upper and lower panels of Figure 6 A and B indicates that pre-exposure to 100 μM capsaicin reduced BoNT/A uptake. Similarly, capsaicin reduced 40 mM KCl stimulated Alexa 647 BoNT/A uptake into Neuro 2a cells. As expected (34,35), CCP disruption also reduced BoNT/A uptake into Neuro 2a cells (Figure 7).

Growth and nucleation of CCPs involves $PI(4,5)P_2$ (32). $PI(4,5)P_2$ depletion will affect the formation of CCP. To test this hypothesis, we examined the effect of WMN which inhibits phosphatidylinositol 3-kinase (PI3K) as well as PI5K to prevent $PI(4,5)P_2$ synthesis. The right hind limb of the mouse in Figure 8 A was injected with 3 μl of 1 mM WMN solution 6 hrs prior to bilateral injection of BoNT/A. Within 24 hrs, the TSR was abolished in the left limb. In contrast, TSR remained normal for the WMN pre-injected limb. This protective action of WMN was observed for all four mice

pretreated with WMN. Thus, WMN is similar to capsaicin in protecting neuromuscular function against BoNT/A.

Acute *in vitro* application of 100 μM WMN alone decreased the amplitude of endplate currents (EPCs) but not spontaneous miniature EPCs (Figure 8 B-E). Since the frequency of miniature EPCs (F) was unaltered, these data suggest that WMN reduces the quantal content of EPCs. The inhibitory action of WMN did not reverse with wash during the 4-6 hr time frame of our *in vitro* electrophysiological study.

Similarly to WMN, 2 hrs of acute *in vitro* application of 100 μM capsaicin reduced the amplitude of EPCs (Figure 9). Capsaicin changed neither the amplitude (2.2 ± 0.18 nA before and 2.5 ± 0.25 nA after capsaicin) nor the frequency (1.8 ± 0.2 sec^{-1} before and 1.6 ± 0.1 sec^{-1} after capsaicin) of miniature EPCs. In contrast, capsaicin reduced the quantal content of EPCs from 38 ± 1.2 to 8 ± 0.5.

DISCUSSION

Mechanism of capsaicin protection against BoNT/A

This study shows that pretreatment with capsaicin protected the mouse NMJ from the paralytic effects of BoNT/A. Our observations, when considered in light of current understanding of BoNT/A uptake, suggest the schematic of supplementary figure 1 to explain the protective action of capsaicin. According to this model, TRPV1 channels on cholinergic nerves mediate the action of capsaicin. We demonstrate that TRPV1 is expressed in motor nerve terminals and in Neuro 2a cells by western blotting and immunocytochemistry. Mammalian homologues of Drosophila transient receptor potential (TRP) channels are plasma membrane cation permeable channels that interact with a variety of intracellular proteins and macromolecular complexes (38). Thus, capsaicin protection against BoNT/A may involve ion flux and/or modulation of intracellular signaling cascades.

Capsaicin reduced Alexa 647 BoNT/A labeling of motor nerve endings as well as Neuro 2a cells. Disruption of CCPs mimicked the later effect of capsaicin. The model of supplementary figure 1 suggests

that $PI(4,5)P_2$ regulates CCP-mediated BoNT/A endocytosis by depleting $PI(4,5)P_2$ which disrupts CCP-mediated BoNT/A uptake. A growing literature addresses the role of phosphoinositides in the regulation of TRP channels. For example, membrane bound $PI(4,5)P_2$ regulates TRPV1 (40,41). $PI(4,5)P_2$ also affects the nucleation and growth of CCPs (32). Hence, it is reasonable to hypothesize that Ca^{2+} flowing into the nerve terminal through capsaicin-activated TRPV1 channels activates phospholipase C which hydrolyzes $PI(4,5)P_2$. The resultant disruption of CCPs reduces endocytosis of BoNT/A.

The upper row of supplementary figure 1 depicts synaptic vesicles (SVs) which have fused with the motor nerve terminal membrane during exocytosis. In the absence of capsaicin pretreatment, fused SV membranes are coated with nucleated clathrin filaments (hexagons of red circles) in a $PI(4,5)P_2$ dependent manner. BoNT/A can bind to gangliosides and SV2 available on the exocytosed SV. When endocytosed *via* the CCP mediated process BoNT/A is ready for translocation into the nerve terminal cytosol. The second row of the right panel introduces capsaicin which activates TRPV1 protein on the motor nerve teriminal. Ca^{2+} influx *via* TRPV1 activates phospholipase C which hydrolyzes $PI(4,5)P_2$. The reduction of membrane $PI(4,5)P_2$ disrupts clatrin coating of the SV. As a consequence, less BoNT/A is endocytosed.

The protective effect of capsaicin on muscle performance *in vivo* (Figure 1) was associated with partial protection of the compound action potential (Figure 2) as well as the force of the nerve initiated twitches (Figure 3) for muscles nearby the site of BoNT/A injection. TRPV1, the capsaicin receptor, was found on native motor nerve endings as well as on cholinergic neuroblastoma Neuro 2a cells (Figure 4). Capsaicin preserved FM1-43 uptake, an indication of continued exocytosis, for Neuro 2a cells exposed to BoNT/A (Figure 5). Capsaicin reduced uptake of BoNT/A into motor nerve endings as well as Neuro 2a cells; disruption of CCPs also reduced BoNT/A uptake into Neuro 2a cells (Figures 6 and 7). Pretreatment with WMN, an inhibitor of $PI(4,5)P_2$ synthesis, also protected muscle behavior from BoNT/A (Figure 8). When applied alone WMN and capsaicin reduced the quantal content of EPCs (Figures 8 and 9).

Effects of capsaicin pretreatment

In vivo skeletal muscle performance was evaluated with performance on a rotating rod, hind limb strength evaluation, the TSR and electromyography. When 3 μl of 6.67 pM BoNT/A was injected into the innervation region of the EDL muscle, all of the preceding indicators of neuromuscular function declined within 24 hrs. Capsaicin injection 4 or 8 hrs as well as immediately before BoNT/A prevented these *in vivo* affects of BoNT/A. It is significant that electromyographic measurements *in vivo* and muscle mechanics *in vitro* showed no effect of capsaicin alone. This is discussed below in relation to the acute, *in vitro* inhibitory action of capsaicin, as well as WMN, on the quantal content of EPCs.

The *in vivo* electromyographic and *in vitro* mechanical measurements demonstrated that capsaicin does not produce 100% protection against BoNT/A. Despite the partial protection of EDL functions and CMAP amplitude inhibition, the capsaicin pretreated animals did not show any sign of BoNT/A induced paralysis. This is consistent with the observations of decreased internalization of fluorescently labeled BoNT/A into mouse NMJ *in vitro* (Figure 5). However, capsaicin did not totally inhibit BoNT/A uptake. Also, when the mouse NMJ was exposed to capsaicin at a concentration of 100 μM, it decreased EPC amplitude in 2 hours. In contrast, injection of capsaicin alone did not affect muscle function and performance. Thus, it appears that the *in vitro* tissue effects of BoNT/A which persist after capsaicin pretreatment are not sufficient to inhibit muscle performance. This suggests participation of *in vivo* compensatory mechanisms.

Disruption of CCPs, a plausible mechanism for reduced BoNT/A uptake

Recent studies (33,35) demonstrate the role of CCP in BoNT/A uptake. When CCPs are disrupted, BoNT/A is less active on Neuro 2a cells. Our experiments with Neuro 2a cells reveal that disruption of CCPs inhibited activity independent uptake of BoNT/A (Figure 7). This reduced uptake might involve $PI(4,5)P_2$ since hypotonicity transiently reduces $PI(4,5)P_2$ level (42). Hence, we hypothesize that capsaicin disrupts CCPs by stimulating Ca^{2+} entry *via* TRPV1 channels. Ca^{2+} then activates phospholipase C (PLC) to hydrolyze $PI(4,5)P_2$. Our *in vivo* experiment with WMN supports this hypothesis. That is,

inhibition of $PI(4,5)P_2$ resynthesis by blocking PI3Kinase and PI5Kinase with WMN protects the mouse NMJ from the inhibitory effects of BoNT/A.

Capsaicin protects FM1-43 labeling of synaptic vesicles

BoNT/A (10 pM) significantly inhibited activity dependent (40 mM KCl depolarization mediated) labeling of the Neuro 2a secretory vesicles with FM1-43 (Figure 5). This is consistent with earlier observations showing that BoNT/A reduced FM1-43 labeling of nerve endings in the mouse hemidiaphragm (43). Pretreatment of Neuro 2a cells with capsaicin prior to BoNT/A exposure protected the labeling of secretory vesicles. These observations are consistent with our hypothesis that capsaicin pretreatment reduced uptake of BoNT/A and preserved exocytosis-dependent labeling with FM1-43.

Capsaicin inhibits CCP dependent endocytosis.

Our results suggest that chronic stimulation of motor neurons with capsaicin reduces clathrin dependent endocytosis of BoNT/A. In the absence of any interventions, BoNT/A is trafficked intracellularly *via* a clathrin dependent endocytotic pathway. Interruption of BoNT/A uptake follows hypo-osmotic or capsaicin-induced disruption of CCP formation. A similar mechanism for inhibition of clathrin dependent endocytosis of $GABA_B$ receptors has been suggested for sensory neurons (44).

To date, no studies have reported a direct effect of capsaicin on motor neurons. This study reveals that acute *in vitro* capsaicin application decreases stimulus-evoked transmitter release. In contrast, a dose of capsaicin which protects against BoNT/A *in vivo* lacked any detectable affect on muscle function. This discrepancy may be attributed to *in vivo* compensatory mechanisms that are not present in the *in vitro* experiments. Two critical factors that potentially regulate CCP formation are membrane bound $PI(4,5)P_2$ and extracellular K^+. Depletion of $PI(4,5)P_2$ by ionomycin or exposure of cells to K^+ depleting conditions followed by hypotonic shock inhibit clathrin dependent endocytosis.

Overall, our results provide evidence for the involvement of proteins that are critical to the regulation of BoNT/A endocytosis. Supplementary figure 1 presents a model where capsaicin affects the BoNT/A uptake by interfering with clathrin . However, capsaicin may have other direct or indirect effects on

binding of BoNT/A to its receptor complex. We suggest this is a remote possibility since recordings of EDL contractions indicate only partial protection from BoNT/A. Furthermore, BoNT/A endocytosis was not completely prevented by capsaicin. Another possibility is that capsaicin might hasten the process of BoNT/A clearance from the intracellular space. Our hypothesis of clathrin involvement and its regulation by PI(4,5)P2 is further supported by the finding that WMN (PI3K inhibitor) inhibited the effect of BoNT/A *in vivo*. WMN sensitive PI kinases are involved in clathrin-dependent endocytosis where AP2 and dynamin serve as potential effectors (45,46). The plekstrin homology domain of dynamin binds to phosphoinositides (46, 26) and cleaves clathrin-coated pits from the plasma membrane (47,48). Also, dynamin may preferentially bind to PI(4,5)P_2, a product of PI 4-kinase that is reduced at higher concentrations of WMN (49). WMN pretreatment prevented the paralytic effects of BoNT/A in the mouse. *In vitro* treatment of WMN alone, like capsaicin alone, also significantly decreased stimulus evoked transmitter release. However, this did not cause any failure of transmitter release in response to stimuli. This effect could be mediated by severe inhibition of PI(4,5)P_2 that is essential for the normal maintenance of endo-exocytosis. However, such an *in vitro* effect was not manifested with *in vivo* WMN injection; that is, mice injected with WMN alone showed normal muscle performance. Therefore, it is compelling to speculate that WMN mediated inhibition of PI(4,5)P_2 resynthesis could inhibit clathrin association and thereby reduce BoNT/A endocytosis.

Our results support a novel mechanism for the inhibition of clathrin dependent endocytosis of BoNT/A by capsaicin. This hypothesis promises new understanding of pathways in BoNT/A trafficking into the motor nerve terminal and the involvement of important regulatory cascades of BoNT/A endocytosis.

Figure legends

Figure 1. Capsaicin protects mice from the paralytic effects of BoNT/A. A inset illustrates the protective effect of capsaicin pretreatment on the mouse TSR. The histogram summarizes TSR score with BoNT/A alone (n=16) or capsaicin pretreatment (n=24); capsaicin was injected immediately (n=14) or at 4 (n=6) or 8 (n=4) hrs before BoNT/A. TSR was scored according to the number (0-5) of toes which the

mouse could extend and flex from the midline of the foot. B shows performance of mice on the rotarod. Four groups of mice (control, + CAP, + BoNT/A bilaterally and + CAP + BoNT/A bilaterally; 4 mice in each group) were studied. BoNT/A was injected on day 8. The time that each mouse walked on the rotarod was averaged for three trials at each time point and plotted against time. C summarizes motor strength (%) evaluated as per table 1.

Figure 2. Capsaicin preserves muscle function *in vivo*. A. Illustrative records of CMAP for control (a), 4 and 8 weeks after BoNT/A alone (b and d) or BoNT/A after pretreatment with capsaicin (c and e). B summarizes the effect of BoNT/A with and without capsaicin pretreatment. CMAP amplitude (%) is plotted against time in weeks after BoNT/A. Bars represent average of normalized values ± S.E.M. C summarizes the effect of capsaicin alone. Left axis represents the normalized muscle strength in (%). Right axis is the normalized CMAP amplitude (%) of untreated control and mice that received only capsaicin.

Figure 3. Capsaicin preserves EDL contractility. A. Represents twitch tension records for control, BoNT/A alone and BoNT/A plus capsaicin pretreated animals. B summarizes normalized twitch tension for EDL exposed to BoNT/A alone or after capsaicin *in vivo*. Bars are mean ± S.E.M. C summarizes normalized twitch tension for untreated control and capsaicin alone treated EDL.

Figure 4. Mouse NMJ and Neuro 2a cells express TRPV1. A. Immunoblot shows TRPV1 in lysates of HEK293 cells transfected with TRPV1 cDNA, mouse hemidiaphragm and Neuro 2a cells. Lower panels illustrate TRPV1 expression in the motor nerve and Neuro 2a cells (B, C).

Figure 5. Capsaicin pretreatement protects FM1-43 uptake. A. Images of KCl stimulated FM 1-43 labeling of control (left), BoNT/A alone (middle) and capsaicin pretreated (right) Neuro 2a cells. B. Average fluorescence intensity (arbitrary units; a.u.) ± S.E.M. Numbers in bars indicate the number of cells summarized in the mean value.

Figure 6. Capsaicin reduces BoNT/A uptake into motor nerve endings. A. Representative fluorescence labeling of endplate ACh receptors with Alexa-488 α-BTX and motor nerve endings with Alexa-647 BoNT/A for control (a, b) and capsaicin pretreated (c, d) preparations. BoNT/A was loaded with nerve stimulation. B. BoNT/A was loaded with 40 mM KCl; BoNT/A alone (e, f) or after capsaicin pretreatment (g, h).

Figure 7. Capsaicin effect on BoNT/A uptake into Neuro 2a cells mimics CCP disruption. Fluorescence labeling with Alexa 647 BoNT/A for: A) BoNT/A alone, B) BoNT/A after capsaicin, C) BoNT/A after CCP disruption. BoNT/A was loaded by incubating cells with Alexa 647 BoNT/A in 40 mM KCl. Right panels represent corresponding bright field images.

Figure 8. Wortmannin protects mice from BoNT/A *in vivo* but acutely decreases quantal content of end plate currents *in vitro*. A. Inset photo shows TSR in a mouse that received bilateral injection of BoNT/A 24 hrs earlier. The right limb was pretreated with WMN (3 µl of 1 mM) 6 hrs prior to BoNT/A; TSR was protected for the WMN pretreated limb. B and C. Representative EPC and MEPC recorded before and 2 hrs after *in vitro* WMN exposure respectively. D. The mean ± S.E.M. for EPCs. E. MEPC amplitudes before and after WMN. F. MEPC frequency.

Figure 9. Acute *in vitro* capsaicin treatment reduces quantal content of end plate currents. A and B. Representative traces of EPCs (A) and MEPCs (B) measured before and after 100 µM capsaicin application for 2 hrs. Histograms showing mean ± S.E.M. amplitudes for EPCs (C) and MEPCs (D) before and after capsaicin, MEPC frequency (E) before and 2 hr after capsaicin application and quantal content (F) before and after capsaicin.

Supplimentary figure 1. Model of possible mechanism underlying the protective effect of capsaicin against BoNT/A. In the absence of capsaicin pretreatment (left panel), fused SV membranes are coated with nucleated clathrin filaments (red hexagons) in a $PI(4,5)P_2$ dependent manner. BoNT/A can bind to gangliosides (G) and SV2 available on the CCP. Upon endocytosis *via* the CCP mediated process

BoNT/A is ready for translocation into the nerve terminal cytosol. Right panel introduces capsaicin which activates TRPV1 protein on the motor nerve teriminal. TRPV1 inwardly conducts Ca^{2+} to activate phospholipase C which hydrolyzes $PI(4,5)P_2$. The reduction of membrane $PI(4,5)P_2$ disrupts clatrin coating of the SV. As a consequence, less BoNT/A is endocytosed.

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Figure 1

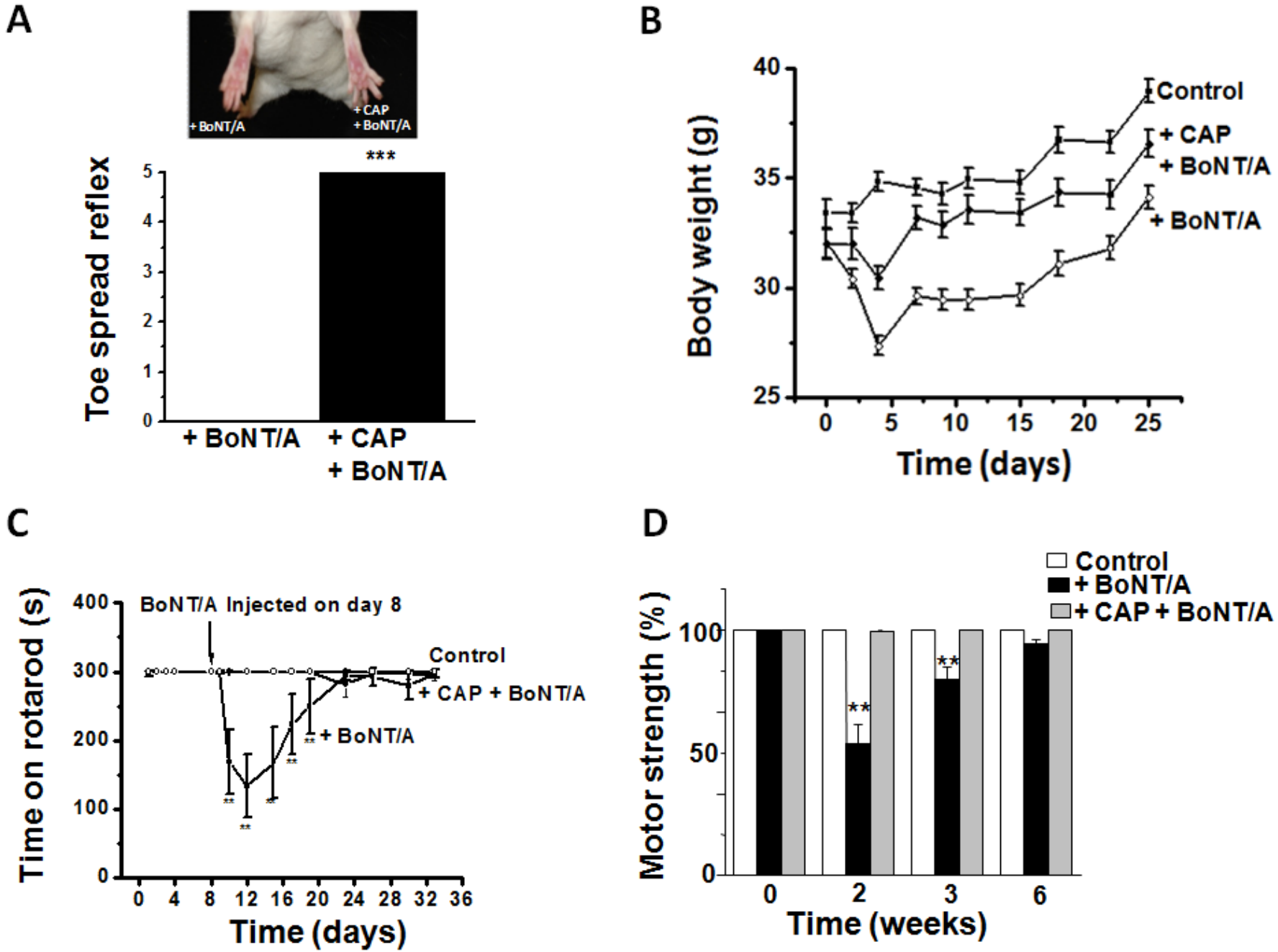

A
+ BoNT/A
+ CAP
+ BoNT/A
***
Toe spread reflex
0
1
2
3
4
5
+ BoNT/A
+ CAP
+ BoNT/A
B
Body weight (g)
25
30
35
40
0
5
10
15
20
25
Time (days)
Control
+ CAP
+ BoNT/A
+ BoNT/A
C
Time on rotarod (s)
0
100
200
300
400
BoNT/A Injected on day 8
Control
+ CAP + BoNT/A
+ BoNT/A
0
4
8
12
16
20
24
28
32
36
Time (days)
D
Motor strength (%)
0
50
100
Control
+ BoNT/A
+ CAP + BoNT/A
**
**
0
2
3
6
Time (weeks)

Figure 2

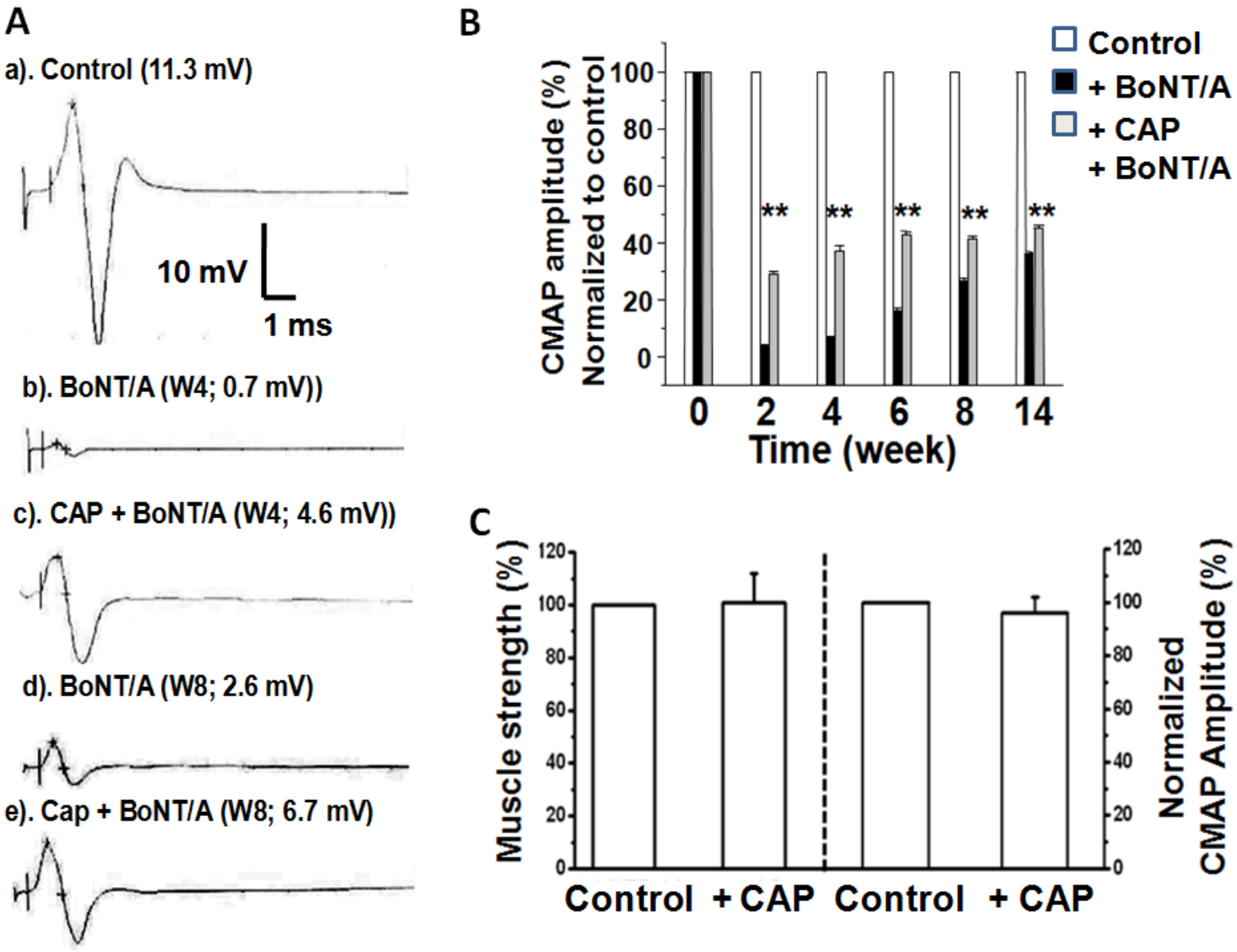

A
a). Control (11.3 mV)
10 mV
1 ms
b). BoNT/A (W4; 0.7 mV))
c). CAP + BoNT/A (W4; 4.6 mV))
d). BoNT/A (W8; 2.6 mV)
e). Cap + BoNT/A (W8; 6.7 mV)
B
Control
+ BoNT/A
+ CAP
+ BoNT/A
CMAP amplitude (%)
Normalized to control
100
80
60
40
20
0
**
0
2
4
6
8
14
Time (week)
C
Muscle strength (%)
Normalized
CMAP Amplitude (%)
120
100
80
60
40
20
0
Control
+ CAP
Control
+ CAP

Figure 3

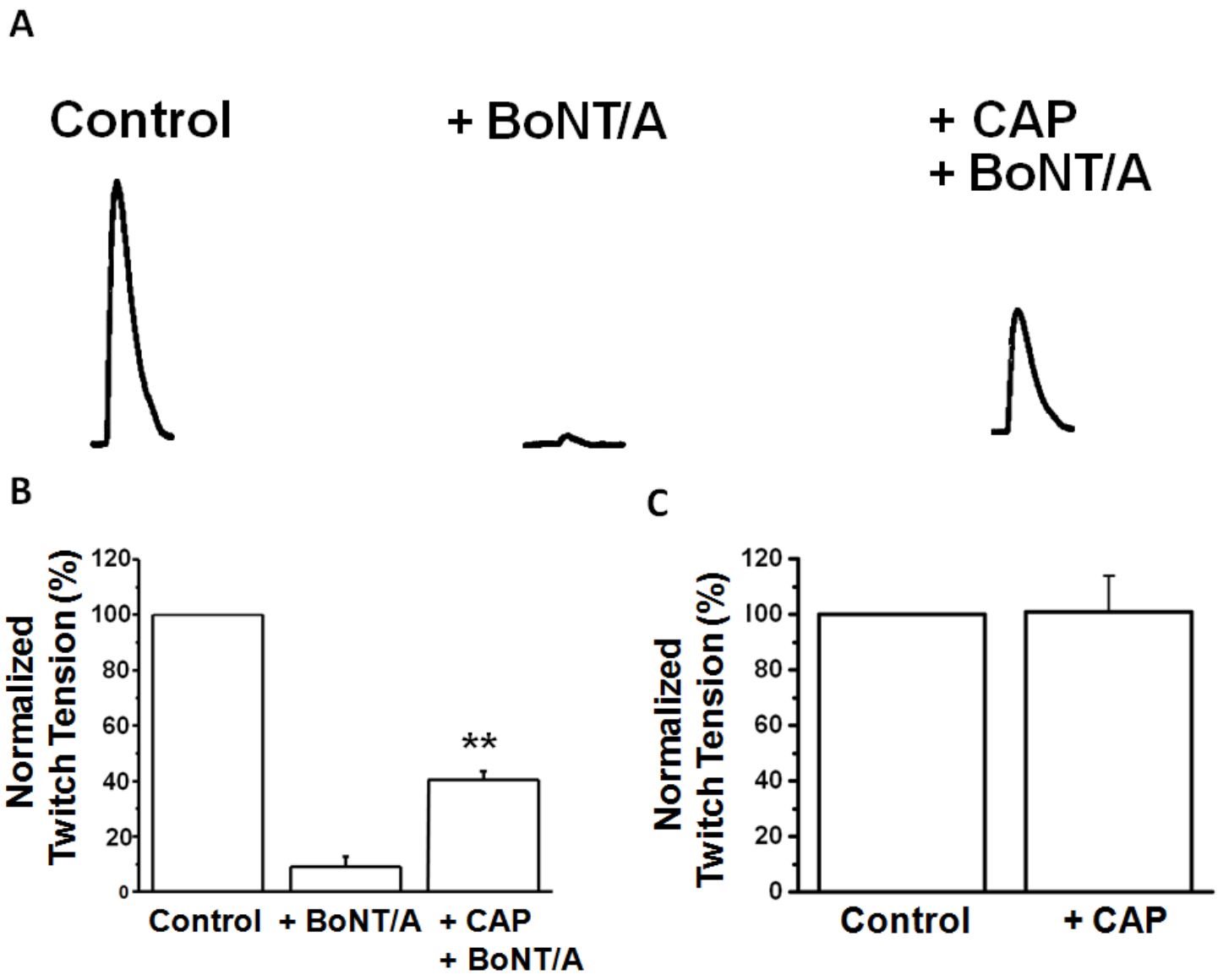

A
Control
+ BoNT/A
+ CAP
+ BoNT/A
B
Normalized Twitch Tension (%)
120
100
80
60
40
20
0
**
Control
+ BoNT/A
+ CAP
+ BoNT/A
C
Normalized Twitch Tension (%)
120
100
80
60
40
20
0
Control
+ CAP

Figure 4

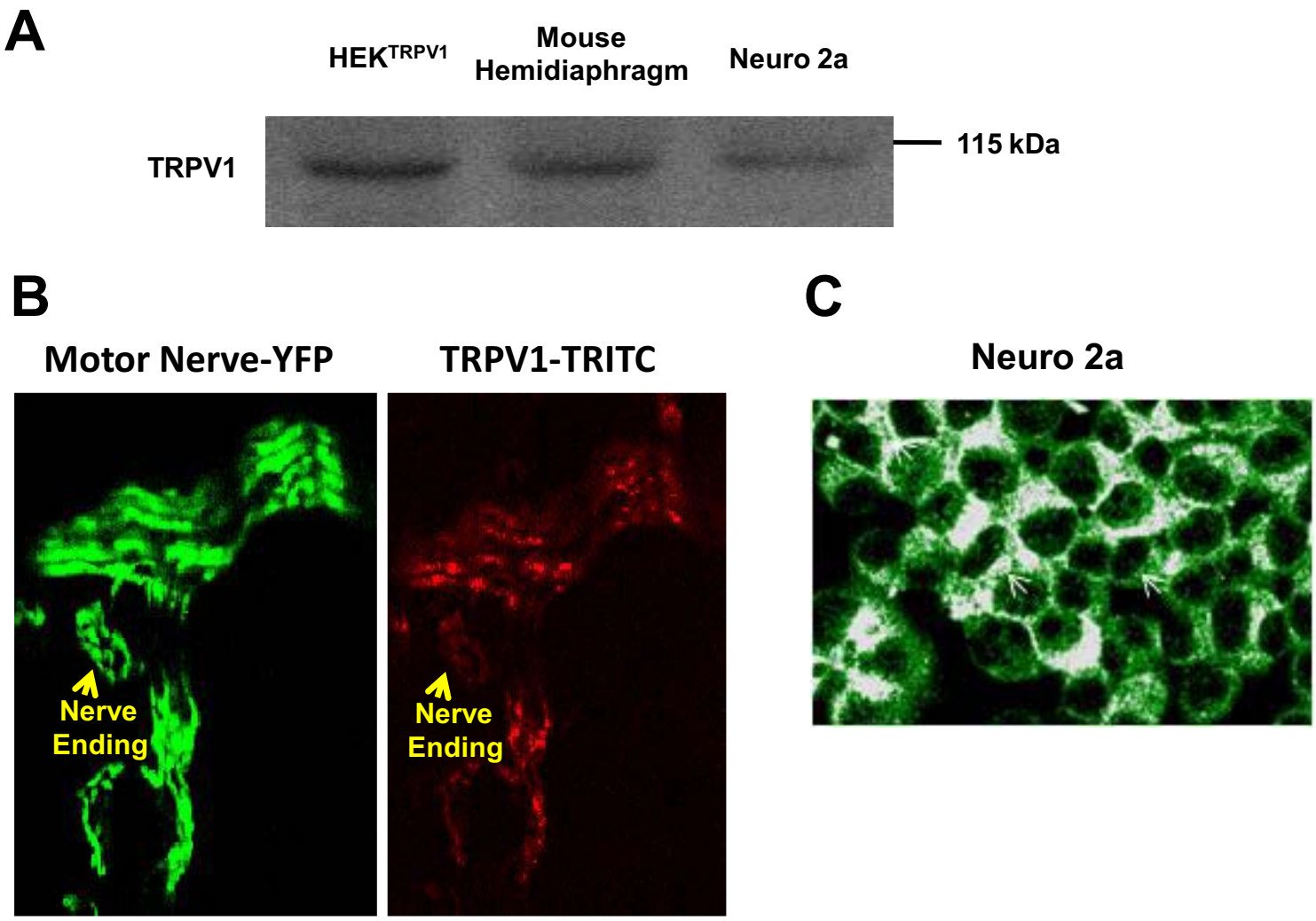
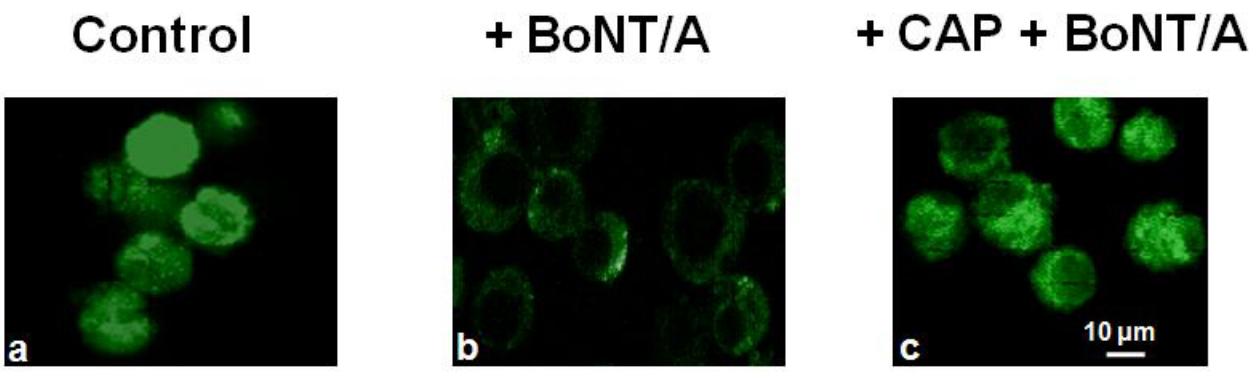

A
HEK^TRPV1
Mouse Hemidiaphragm
Neuro 2a
TRPV1
115 kDa
B
Motor Nerve-YFP
TRPV1-TRITC
Nerve Ending
Nerve Ending
C
Neuro 2a

Figure 5

A


Control
+ BoNT/A
+ CAP + BoNT/A
a
b
c
10 µm

B

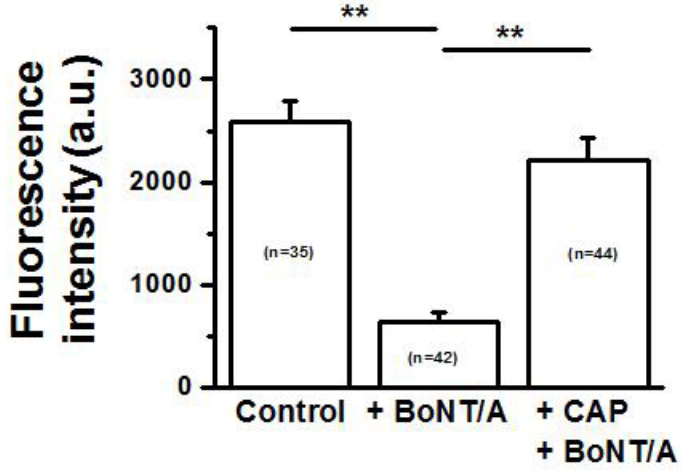

**
**
Fluorescence intensity (a.u.)
3000
2000
1000
0
(n=35)
(n=42)
(n=44)
Control
+ BoNT/A
+ CAP + BoNT/A

Figure 6

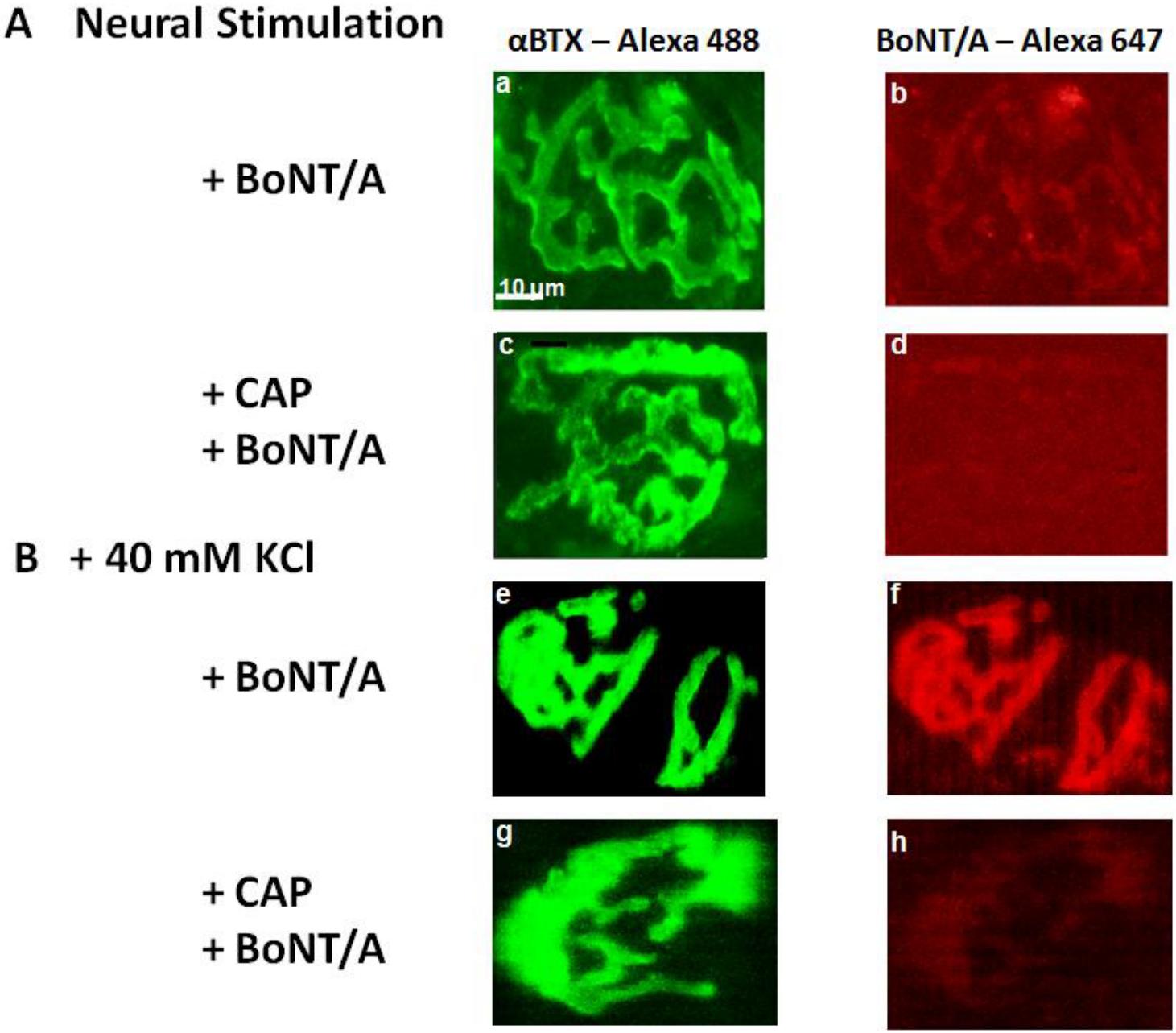

A Neural Stimulation
αBTX – Alexa 488
BoNT/A – Alexa 647
+ BoNT/A
a
b
10 µm
+ CAP
+ BoNT/A
c
d
B + 40 mM KCl
+ BoNT/A
e
f
+ CAP
+ BoNT/A
g
h

Figure 7

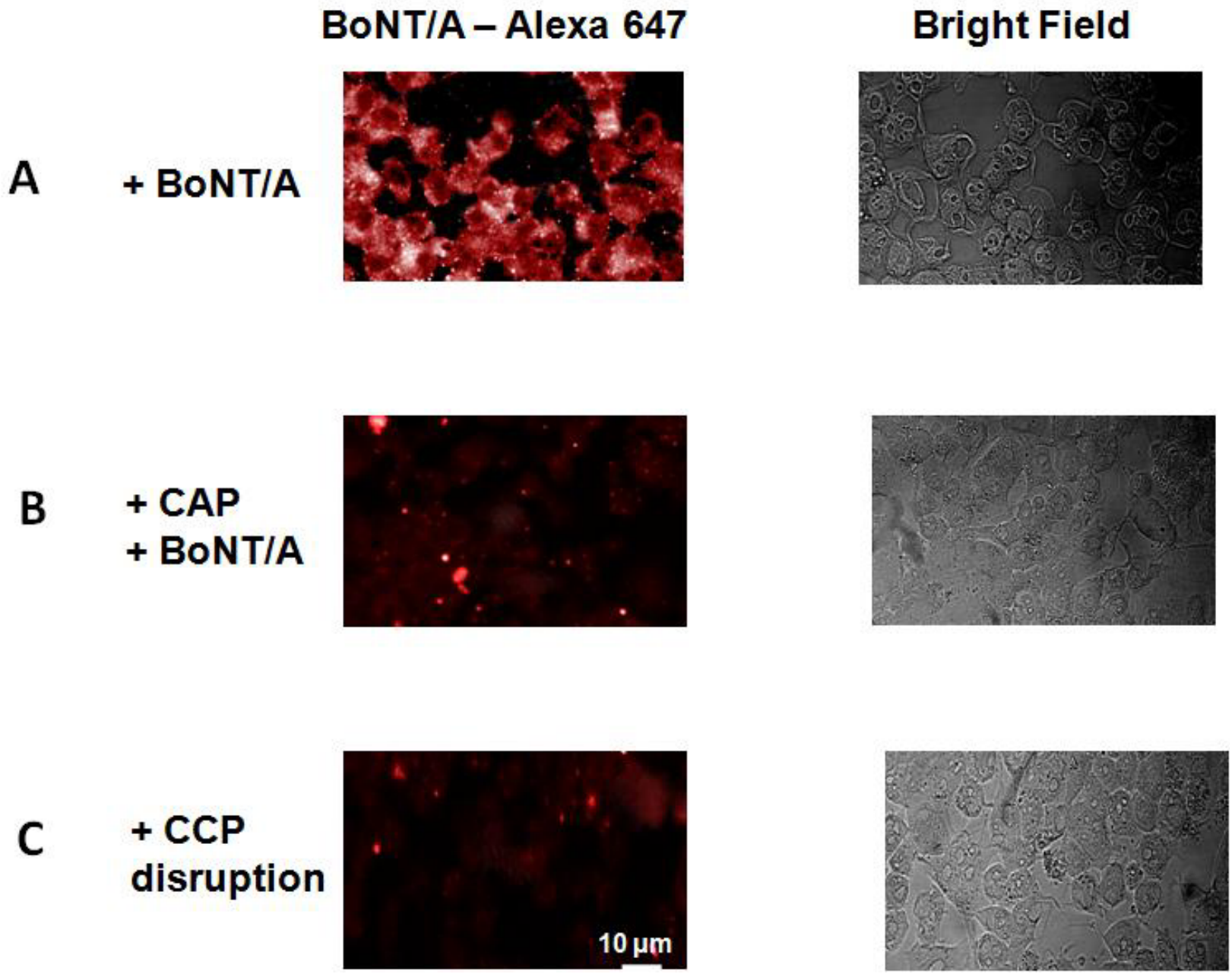

BoNT/A – Alexa 647
Bright Field
A
+ BoNT/A
B
+ CAP
+ BoNT/A
C
+ CCP
disruption
10 µm

Figure 8

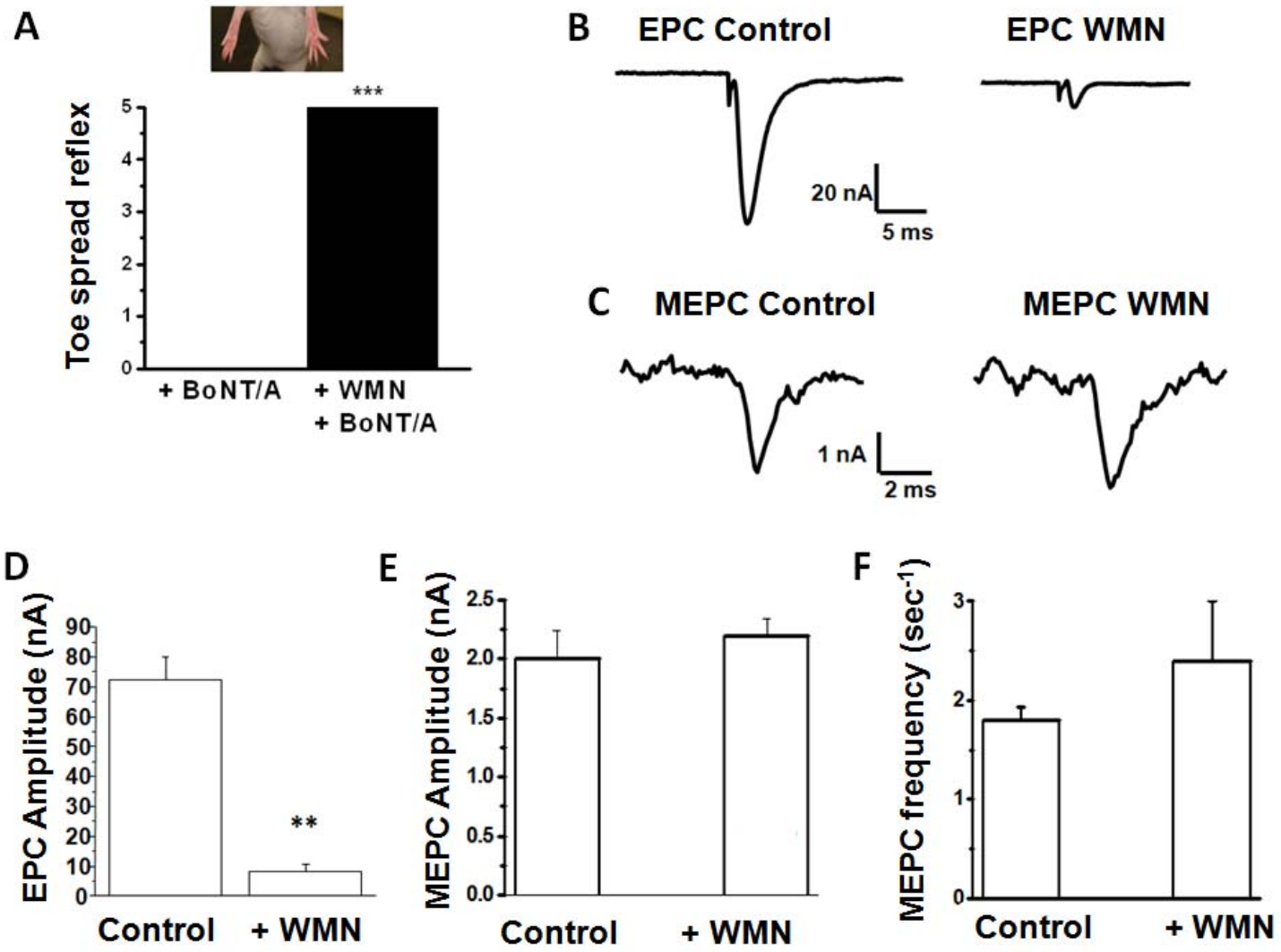
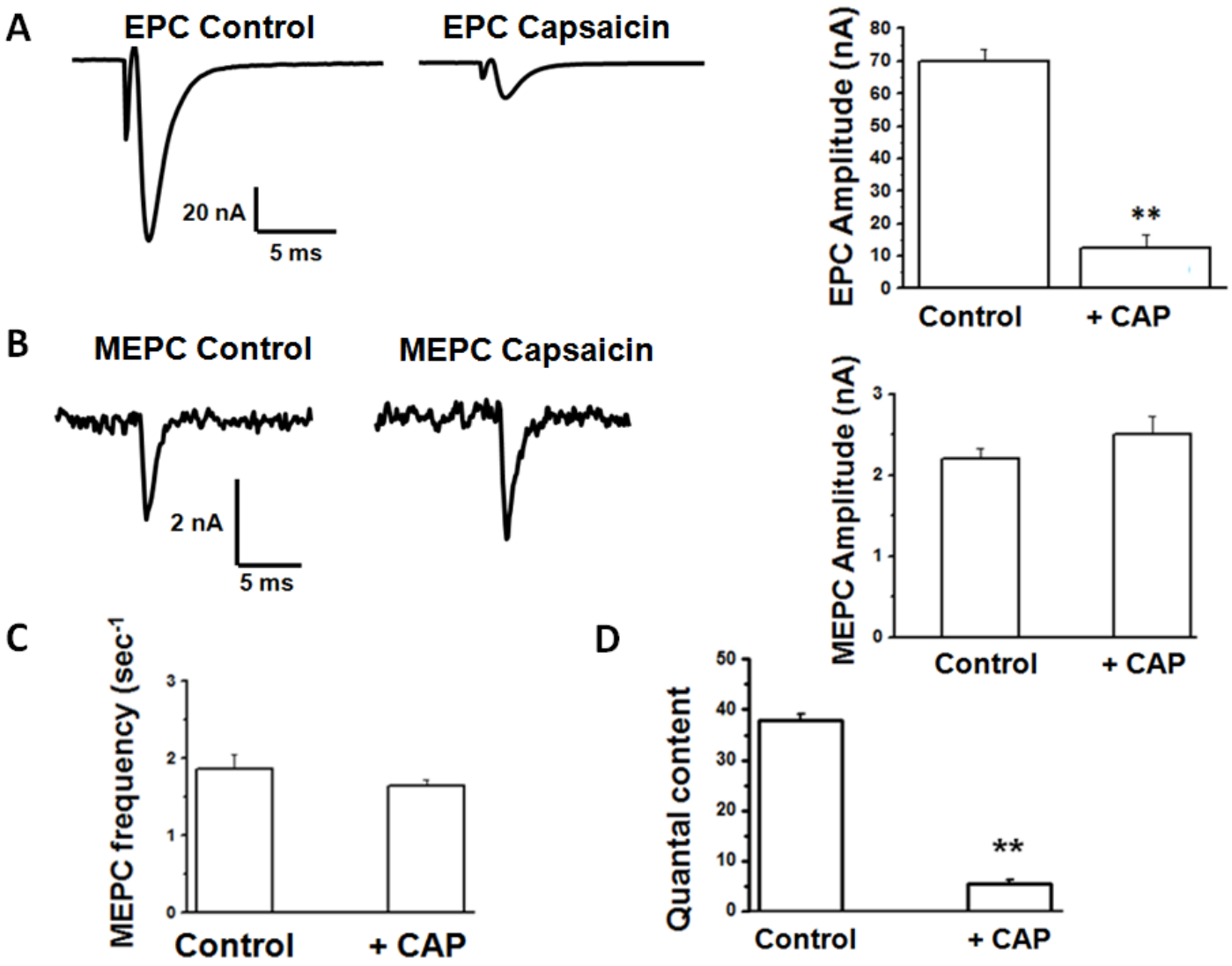

A
Toe spread reflex
***
+ BoNT/A
+ WMN
+ BoNT/A
B
EPC Control
EPC WMN
20 nA
5 ms
C
MEPC Control
MEPC WMN
1 nA
2 ms
D
EPC Amplitude (nA)
**
Control
+ WMN
E
MEPC Amplitude (nA)
Control
+ WMN
F
MEPC frequency (sec-1)
Control
+ WMN

Figure 9


A
EPC Control
EPC Capsaicin
20 nA
5 ms
EPC Amplitude (nA)
80
70
60
50
40
30
20
10
0
**
Control
+ CAP
B
MEPC Control
MEPC Capsaicin
2 nA
5 ms
MEPC Amplitude (nA)
3
2
1
0
Control
+ CAP
C
MEPC frequency (sec-1)
3
2
1
0
Control
+ CAP
D
Quantal content
50
40
30
20
10
0
**
Control
+ CAP