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# Botulinum Toxin B in the Sensory Afferent: Transmitter release, Spinal activation and Pain Behavior

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## Abstract

We addressed the hypothesis that intraplantar Botulinum toxin B (rimabotulinumtoxin B: BoNT-B) has an early local effect upon peripheral afferent terminal releasing function and over time will be transported to the central terminals of the primary afferent. Once in the terminals it will cleave synaptic protein, block spinal afferent transmitter release and thereby prevent spinal nociceptive excitation and behavior. In mice, C57Bl/6 males, intraplantar BoNT-B (1U), given unilaterally into the hind paw had no effect upon survival or motor function but ipsilaterally decreased: i) intraplantar formalin evoked flinching; ii) intraplantar capsaicin evoked plasma extravasation in the hindpaw measured by Evans blue in the paw; iii) intraplantar formalin evoked dorsal horn SP release (NK1 receptor internalization); iv) intraplantar formalin evoked dorsal horn neuronal activation (cFos); v) ipsilateral DRG VAMP; vi) ipsilateral SP release otherwise evoked bilaterally by intrathecal capsaicin; vii) ipsilateral activation of cFos otherwise evoked bilaterally by intrathecal substance P. These results indicate that BoNT-B after unilateral intraplantar delivery is taken up by the peripheral terminal, is locally active (blocking plasma extravasation), is transported to the ipsilateral DRG to cleave VAMP and is acting presynaptically to block release from the spinal peptidergic terminal. The observations following intrathecal SP offer evidence for a possible transsynaptic effect of intraplantar BoNT. These results provide robust evidence that peripheral BoNT-B can alter peripheral and central terminal release from a nociceptor and attenuate downstream nociceptive processing via a presynaptic effect, with further evidence suggesting a possible postsynaptic effect.

## INTRODUCTION

Botulinum toxins are composed of a heavy and light chain (LC). The heavy chain is required for cell membrane receptor-mediated toxin endocytosis [13]. Once inside of the cell, LC is

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cleaved in the acidic environment of endosome and exported into the cytosol. LC is a zinc-dependent endopeptidase that targets consensus sites on the SNARE (Soluble NSF attachment protein receptor) superfamily of synaptic proteins [75], including SNAP-25 (synaptosomal-associated protein 25) or VAMP (vesicle associated membrane protein). SNARE cleavage prevents vesicle fusion and transmitter release [11]. Therapeutically, BoNTs are delivered at local sites yielding muscle relaxation by local block of acetylcholine release [31; 94]. As SNAREs mediate most vesicular release, it is not surprising that toxins such as BoNT-A and B cleaving respectively SNAP-25 or VAMP also block release of primary afferent transmitters (substance P (SP) and calcitonin gene-related peptide (CGRP)) after local application in *ex vivo* [25; 74] and *in vivo* models [38]. Peripherally delivered BoNTs have no effect upon acute pain thresholds, but exhibit a homotopic antihyperalgesic effect in rodent models of inflammation and arthritis [2; 7; 8; 20; 26; 57; 69; 71] and in models of mono- and poly-neuropathy [8-10; 56; 59; 67; 79]. These results parallel human studies where local BoNTs have no effect upon acute thresholds [12; 34; 89; 101], but reduce hyperesthesia in postherpetic neuralgia [39; 55; 104], diabetic neuropathy [108], nerve injury [30; 81; 83], residual limb pain [47] and in certain forms of migraine [22; 24].

The effect of peripheral toxins on pain processing might be ascribed to actions on peripheral nociceptors. Local release of afferent peptides (SP/CGRP) by capsaicin mediates neurogenic flare and plasma extravasation [35; 72]. BoNTs would reduce this, if toxins prevent local release, as has been reported in humans [33; 49; 98] and in animals by some [16] but not others [7; 89; 101]. Alternately, the sensory terminal could take up local BoNTs, and transport the active form centrally to block spinal terminal release. It has been considered that BoNTs in contrast to tetanus toxin are not centrally transported [88]. Current work, however, indicates that BoNTs may be taken up and undergo fast axonal transport [5; 6; 51; 69; 70; 84; 85; 90].

In the present studies we examined whether BoNT-B delivered intraplantarly (IPLT) was taken up by afferents and underwent spinopetal movement. The following hypothesized events should then transpire ipsilaterally to the BoNT-B treated paw: 1) block of local afferent transmitter release (capsaicin evoked plasma extravasation); 2) reduced DRG VAMP; 3) reduced formalin evoked dorsal horn SP release; 4) reduced flinching and activation of dorsal horn neurons following formalin; 5) reduced release of SP evoked by a central stimulus (intrathecal capsaicin); 6) delayed onset of central versus peripheral effects. Studies undertaken here to address the above issues indeed indicate that BoNT-B has a local effect and support the spinopetal transport of active BoNT-B. Unexpectedly, we also obtained evidence that there may be transsynaptic changes after intrathecal (IT) SP delivery initiated by unilateral IPLT-BoNT-B pretreatment.

# METHODS

## Animals

Adult male C57B/l6 mice, 25-30 grams (Harlan Sprague Dawley Inc., Indianapolis, IN) were housed in the vivarium a minimum of 2 days before use, maintained on a 12/12-hour day-night cycle and given free access to food and water. All studies undertaken in this study

were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

## Drug Delivery

**Mouse Intraplantar Injection—**Mice were anesthetized (2.5% Isoflurane, with 80% Oxygen and 20% room air). A 30G needle was inserted into the plantar surface of the paw subcutaneously and 30 μL of BoNT-B (Rimabotulinumtoxin B, Myobloc ®, Solstice Neurosciences), 25 μL of 1% capsaicin (Sigma-Aldrich), 20 μL of 2.5% formalin, or 30 μL of saline were injected over 10-20 sec. BoNT-B solutions were prepared from stock solutions of 5000U/mL which were then serially diluted to the final concentration in 0.9% saline. All solutions were stored at 4°C and warmed to room temperature prior to use.

**Mouse Intrathecal Injection—**Mice were anesthetized (2.5% Isoflurane, with 80% Oxygen and 20% room air). A 30G needle was inserted between the L5 and L6 vertebrae evoking a tail flick. 10 μL of vehicle, capsaicin or substance P (Sigma-Aldrich) was injected into the space over an interval of ~30 sec. Following recovery, mice were evaluated for normal motor coordination and muscle tone [38].

## Behavior

All behavioral tests were performed by investigators blinded to drug treatments.

**Formalin Flinch—**Mice received a single injection of IPLT formalin to induce flinching after BoNT-B pretreatment. BoNT-B or vehicle (saline) in volumes of 30μL was injected into the dorsum of the paw. At intervals after the BoNT-B delivery, a metal band was placed around the IPLT hind paw. The movement of the metal band can be detected by an automated device [106]. After 1 hr acclimation, mouse received an injection into the ipsilateral paw of formalin (20μL of 2.5% formalin). Flinching of the injected paw was detected and processed by the automated flinch detecting system for 1 hr after the IPLT formalin.

**Thermal Thresholds—**Mice received the intraplantar injection of BoNT-B (1U) or saline. After 4 Hours, 1, 7, and 21 days animals were examined for their ipsilateral and contralateral thermal escape latency using a Hargreaves thermal escape device [23; 62].

**Sharp probe response—**To assess the acute motor response to a sharp probe applied to the paw, mice were placed on a wire mesh grid. A 20G needle was inserted though the mesh such that the point lightly contacted the plantar surface of the ipsilateral (treated) or the contralateral (untreated) paw. This stimulus typically evokes an immediate withdrawal of the stimulated paw in a control animal [36].

**Motor Function—**To systematically assess motor function, three quantitative measures were employed [38].



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**Suspension—**When placed upside-down under the wire mesh, animals were required to grip onto the wire grid with front and hind paws. Successful completion of the test requires remaining suspended for 1 minute.

**Placing and stepping—**The dorsum of the hind paw hind paw of the animal was passed over the edge of table. This evoked a lifting and plantar placement of the paw. If successful, the response indicated that there was intact large afferent linkage and appropriate fine motor control of the examined paw.

**Force Pulling—**Animals were placed on top of a wire mesh mounted on a scale. The experimenter lifted the animal from the wire mesh by the tail. The grip strength was recorded as the maximal force at the point of release from the wire mesh by the hind paws.

## Plasma Extravasation

Mice received a single injection of BoNT-B into the plantar surface of the ipsilateral paw and a single injection of saline into the plantar surface of the contralateral paw. In control groups, saline was delivered into the ipsi and contralateral paws. After an interval of 4h, 1, 7, and 21 days the animals were anesthetized with isoflurane and received one injection of 1% capsaicin into the ipsilateral (BoNT-B treated) and one injection of 1% capsaicin into the contralateral (vehicle-treated) paw in the plantar surface. After 10 minutes the animals received a tail vein injection of Evans blue (50 mg/kg, Sigma-Aldrich). Following a further 10 minutes the mouse was perfused with saline and the plantar paw skin was removed and dried. The dye in the dried paws was then extracted with formamide (8 mL per 1 g dried tissue, Sigma-Aldrich). After incubation of 48 hrs, the solvent was submitted for a spectrophotometric analysis of the stain density and calibrated with a standard curve to determine the amount of dye extravasation. Dye concentrations in the ipsilateral and contralateral paws were then compared.

## Histochemistry

**Tissue Harvest—**At specified time points, mice were anesthetized and perfused with 0.9% saline followed by 4% paraformaldehyde. L4/5 DRG and L4/5 spinal cord were removed, post-fixed and cryoprotected in sucrose. Spinal cord and DRG sections were cut as free-floating or attached sections respectively. To assess marker activation tissue was harvested at 10 min for assessment of Neurokinin 1 receptor (NK1r) internalization (a marker of SP release) and 2 hrs after formalin injection for cFos (a marker of dorsal horn neuron activation).

**NK1r internalization—**To assess NK1r expression in the spinal cord dorsal horn, sections were incubated with rabbit anti-NK1r polyclonal antibody (Advanced Targeting Systems, San Diego, CA) and goat anti-rabbit secondary antibody conjugated with Alexa Fluor 488 (Invitrogen, Eugene, OR). NK1r internalization was counted using an Olympus BX-51 fluorescence microscope (Olympus Optical, Tokyo, Japan) and a 60x oil-immersion objective lens. Internalization was assessed according to the standard of previous reports [38; 48; 76]. The filter set was Omega Optical XF100 to 2 Green Bandpass Filter. Neuronal profiles that had 10 or more endosomes were considered to have internalized NK1rs. The

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total number of NK1r immunoreactive neurons in lamina I/II, with and without NK1r internalization, was counted and taken to calculate the fraction of cells showing internalization. Counting was done without knowledge of treatments. Mean counts from 3 to 5 sections per segment of L3-L5 lumbar spinal cord were used as representative counts for a given animal. Following quantification, images used for figures were subject to Photoshop processing to enhance contrast for figure presentation only.

**cFos—**Free-floating sections prepared as above, were incubated overnight at 25°C in rabbit anti-cFos polyclonal antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) and a goat anti-rabbit secondary biotinilated antibody. Sections were then subject to the ABC method and DAB was used as the chromogen. cFos-positive neurons in superficial dorsal horn (lamina I and II) were counted without knowledge of treatments. The mean counts from 4 sections per segment of L4-L5 lumbar spinal cord were used as representative counts for a given animal [38]. Following quantification, images used for figures were subject to Photoshop processing to enhance contrast for figure presentation only.

**Fluorescent double-labeling of NK1r and cFos—**Free-floating sections were incubated with rabbit anti-NK1r polyclonal antibody (1:10,000) for 16 hrs, and then with biotinylated anti-rabbit antibody (1:5,000) as well as the ABC complex. After washing, sections were incubated with Cy3-tyramide for 30-60 sec. Sections were then incubated with rabbit anti-cFos polyclonal antibody (1:1000) followed by Alexa488 conjugated anti-rabbit antibody (1:1000). There was no labeling on control sections treated with anti-NK1r polyclonal antibody (1:10,000) followed by Alexa488 conjugated anti-rabbit antibody (1:1000). Single-focal-plane images were obtained from a Leica TCS SP5 confocal system at 1024×1024 pixels. Images used for figures were subject to Photoshop processing to enhance contrast for figure presentation only.

### Western Blots

**Tissue Harvest:** Mice received unilateral intraplantar injections of BoNT-B. At 4hrs, 1, 7 and 21 days after injection, the animals were deeply anesthetized and decapitated. The vertebral column was then cut crosswise at the level of the pelvic crests and the spinal cord was ejected by the hydraulic pressure generated by a syringe filled with 0.9% saline placed at the lumbar exposure with a blunt cut 19 g needle. The spinal cords were bisected into left dorsal/ventral horns and right dorsal/ventral horns. In addition, the ipsilateral and contralateral L3- 5 DRGs were quickly dissected. Tissues were transferred into a tube of extraction buffer with protease inhibitor cocktail (P8340, 1: 100, Sigma) and phosphatase inhibitor cocktail 2 and 3 (1: 100, P5726 and P0044, Sigma). The tissue was then quickly frozen and stored at −70°C.

**VAMP:** Samples were thawed and homogenized by sonication and subject to centrifugation at which time the supernatant was collected. Samples were subject to SDS-PAGE and transferred to nitrocellulose membrane (iBlot, IB301001, Invitrogen). The membrane was incubated with the primary antibody, rabbit polyclonal anti-VAMP 1,2,3, (Synaptic Systems, Gottingen, Germany) and the anti-rabbit Horseradish Peroxidase conjugated secondary antibody (Cell Signaling Technology, Beverly, MA, USA). After washing,

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membranes were incubated per manufacturer directions with SuperSignal West Femto Chemiluminescent Substrate reagents (Pierce, Rockford, IL, USA) and the luminescent signal was exposed to film and developed for further scanning and quantification. The nitrocellulose membranes were stripped with a Re-Blot western blot recycling kit (Chemicon, Temecula, CA, USA) and reprobed with β-actin (1: 50,000; Sigma). The optical density (OD) of immunoreactive bands was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). VAMP bands were normalized relative to the corresponding β-actin band and ipsilateral tissue was compared to the corresponding contralateral tissue. The VAMP 1,2,3 antibody detects the intact VAMP protein of the three VAMP isoforms. VAMP 1 and 2 were reportedly expressed in neurons [29] and were observed in this study in the spinal cord, while only VAMP 2 was found in the DRG. VAMP 1 and 2 were both cleaved by BoNT-B in the mouse [40; 87], and therefore a decrease in either or both SNARE proteins was taken as a sign of BoNT-B activity. Following quantification, images used for figures were subject to Photoshop processing to enhance contrast for figure presentation only. One western blot was run for each time point, and figures were cropped to present non-adjacent columns next to each other for comparison.

### Statistics

Data are presented as mean ± SEM. Data in figure 6 were analyzed with paired t-test. Data in all other figures were analyzed with one-way ANOVA (Analysis of variance) followed by Bonferroni's Multiple Comparison Test. Data in supplemental figure 1 and 2 were analyzed with two-way ANOVA. All tests were performed with Prism (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was set as the criterion for statistical significance.

## RESULTS

### Dose ranging for intraplantar BoNT-B on formalin-evoked flinching and clinical appearance

Initial screening studies were undertaken to define the doses of IPLT BoNT-B required to reduce formalin-evoked flinching, and the effects upon clinical appearance at 24 hrs after delivery. As indicated in Figure 1A, IPLT BoNT at a dose of 1U/30μL resulted in a highly significant reduction in phase 2 flinching with no evident morbidity. Lower doses (0.1 to 0.5U/30μL) were without effect while the higher dose (1.5U) produced no statistically greater effect upon flinching but resulted in changes in animal appearance (ruffled fur, dehydration.) at 24hrs. Higher doses (3 to 150U/30μL) were observed to be uniformly lethal (supplemental table 1) by 24 hrs.

Based on the initial dose ranging studies outlined in the preceding paragraph, we performed additional detailed evaluation of the behavioral phenotype of the 1 and 1.5U groups. At 1.5U, all animals survived through 72 hrs, but over half of the animals displayed at least moderate degrees of motor weakness as measured by failure to reach criteria on the suspension test, reduction in the force pulling test and impairment of placing and stepping (data not shown). At 1U, BoNT-B had no effect on pin prick response (supplemental table 2), motor function (all animals met the criteria on the three motor end points) and there was no statistically significant loss of body weight as compared to saline controls over the

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ensuing 21 days (supplemental Figure 1). Further, as will be noted below, there was no change in the acute nociceptive withdrawal response to thermal or pinprick stimuli, which emphasizes that normal motor function was preserved as required in these escape responses. Based on these initial observations, the IPLT dose of BoNT-B (1U/30μL) was employed as the standard dosing.

### Pain behavior – BoNT-B reduced formalin-evoked pain but had no effect on acute pain thresholds

**Formalin-evoked flinching—**Intraplantar formalin in control animals evoked a robust biphasic (phase 1 and 2) flinching of the injected hind paw. Unilateral IPLT BoNT-B (1U/30μL) delivered at 4 hours, 1, 7, or 21 days prior to receiving IPLT formalin resulted in a significant reduction in phase 2 flinching at 1 and 7 days, but not at 4 hrs or 21 days, compared to the saline treated animals (Figure 1B). No differences in phase 1 flinching were seen between saline and any of the four pretreatment groups.

**Thermal escape—**Baseline thermal escape latency in saline treated animals was 9.0 ± 1.8 sec. Thermal latencies at 4 hrs, 1, 7 or 21 days after IPLT-BoNT-B (1U/30μL) were not different from saline at any time point (supplemental figure 2).

**Sharp probe response—**Application of the 20G needle to the saline control or uninjected paw resulted in a brisk withdrawal of the stimulated paw in all animals. Application of the needle to the ipsilateral paw after IPLT BoNT-B (1U/30μL) at intervals out to 21 days revealed no change in the withdrawal response, e.g .all animals responded with a withdrawal (data not shown).

### IPLT-BoNT-B reduced neurotransmitter release from peripheral afferent terminals

To determine whether IPLT-BoNT-B blocks peripheral terminal neurotransmitter release from primary afferents, IPLT-BoNT-B was delivered ipsilaterally and IPLT saline vehicle contralaterally at 4 hours, 1, 7, or 21 days before the IPLT injection of capsaicin into each paw to evoke plasma extravasation as measured by the appearance of Evans blue in the paw skin tissue. In the saline control paw, IPLT capsaicin yielded a prominent local extravasation of the blue dye (Figure 2). In contrast, IPLT-BoNT-B induced a prominent reduction in dye extravasation compared to the saline treated paw (Figure 2). Plasma extravasation was significantly decreased at 4 hrs, 1 and 7 days, but not 21 days (Figure 2).

### IPLT-BoNT-B reduced spinal SP release and spinal activation evoked by IPLT Formalin

**SP release - NK1r internalization—**Typically in control animals, in any spinal histological section, 5 to 10 NK1r(+) cells and extensive NK1r(+) processes were found distributed across the superficial dorsal horn on one side, with the majority of the immunoreactivity being localized to the cytosolic membrane of the neuron (Figure 3A). IPLT formalin in control animals resulted in a prominent increase in the percentage of NK1r(+) cells that displayed internalization of the NK1r(+) immunoreactivity in the ipsilateral lumbar dorsal horn (Figure 3B). This evoked NK1r internalization was blocked by pretreatment of IPLT BoNT-B (1U/30μL) at 4hrs, 1 and 7 days prior to formalin, but not at

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21 days (Figure 3C). Contralateral dorsal horn labeling of NK1r was not different between any of the time points or after intraplantar saline pretreatment (data not shown).

**Spinal activation - cFos expression—**In control spinal cord, the incidence of cFos(+) cells in lumbar dorsal horn was low (typically 5 to 15/section). However, IPLT formalin resulted in a prominent increase in the number of cFos(+) neurons in the ipsilateral, but not contralateral dorsal horn (Figure 4). Unilateral IPLT BoNT-B (1U/30μL) delivered at 1 and 7 days prior to formalin significantly prevented the increase of cFos(+) cells compared to saline controls in the IPSI dorsal horn, while BoNT-B at 4 hrs or 21 days prior to formalin failed to do so. Contralateral dorsal horn labeling of cFos was not different between any of the time points and saline groups.

## IPLT-BoNT-B resulted in VAMP cleavage in the DRG but not spinal cord

In control animals, western blots revealed clearly detectable VAMP1 and VAMP2 bands in the spinal cord, while only VAMP2 was detectable in DRG homogenate (Figure 5). VAMP1 was not detected in the DRG using either immunohistochemistry (data not shown) or western blots (Figure 5). VAMP1 was not detected in either BoNT-B treated or vehicle treated DRG, which is consistent with reports that VAMP1 is largely found in motorneurons, which DRG lack, while VAMP2 is expressed widely in neuronal tissue [97]. Further mechanistic, functional, and expressional differences in VAMP isoforms were not explored. At 1 and 7 days following IPLT BoNT-B (1U/30μL) pretreatment, we observed a significant reduction in VAMP2 expression in the ipsilateral lumbar DRG homogenates. Such reduction was not observed at 4hrs or 21 days or on the contralateral side. In the spinal cord, neither VAMP1 nor VAMP2 signal was altered at any time point on the ipsilateral or contralateral side (Figure 5). These results indicate that following IPLT-BoNT-B, functionally significant quantities of an active form of BoNT-B were present in the DRG, leading to a reduction in VAMP expression in the ipsilateral side, e.g. VAMP cleavage in the ipsilateral DRG. The lack of changes in total spinal cord VAMP was considered to indicate the small component of the spinal VAMP affected by the intraplantar BoNT-B.

## Intrathecal capsaicin-evoked SP release and spinal activation was reduced by IPLT-BoNT-B

To determine whether intraplantar BoNT-B blocks the spinal release of SP evoked by a central stimulus, NK1r internalization evoked by intrathecal capsaicin was determined. IPLT BoNT-B was delivered 1 day prior to the intrathecal injection of capsaicin (10ng/10μL). Capsaicin, through an effect mediated by TRPV1 receptors on SP(+) afferent terminals, evokes a robust bilateral release of SP and subsequent internalization of dorsal horn NK1r (Figure 6). Pretreatment with unilateral IPLT BoNT-B (1U/30μL) significantly reduced the NK1r internalization evoked by intrathecal capsaicin, on the side ipsilateral but not contralateral to BoNT-B delivery, or as compared to the vehicle-treated paw (paired t-test $p<0.05$, Figure 6). These results suggest that the peripherally delivered BoNT-B reaches the ipsilateral dorsal horn afferent terminals in an active form to prevent the ipsilateral dorsal horn release of SP otherwise produced through the bilateral activation of the TRPV1(+) afferents.

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### Intrathecal Substance P activation of Second Order Spinal Neurons was blocked by IPLT-BoNT-B

The IT delivery of SP (10ng/10μL) resulted in a robust activation of dorsal horn neurons as evidenced by a bilateral increase in the incidence of cFos(+) neurons in the superficial and deeper laminae of control animals which received IPLT saline (Figure 7A and 7C). Consistent with the relative few (5-10) neurons in any section that were NK1r(+), fluorescent immunohistochemistry revealed that the majority of cFos(+) profiles are NK1r negative (Figure 7C, 7D-L). In animals that received unilateral IPLT BoNT-B and IT SP, the ipsilateral dorsal horn displayed a significant reduction of cFos as compared to the contralateral side (Figure 7B and C). NK1r(+) cells were observed to be internalized on the ipsilateral BoNT-B side, which suggests BoNT-B blocked the downstream activation of NK1r(+) cells, but not the internalization itself (supplemental Figure 3). Immunohistochemistry of DRG for NK1r(+) in naive animals could not detect NK1r immunoreactivity in the primary afferent cell bodies of the DRG, though this protocol robustly detected dorsal horn NK1r (supplemental Figure 4). This suggests a lack of NK1r protein on the primary afferent, and that the IT SP was unlikely having a presynaptic effect on afferent terminals (see further discussion below). Thus, IT SP-induced cFos activation represents a direct activation of NK1r(+) neurons and/or downstream neurons. Depending upon the extent that NK1r receptors are exclusively on the second order neurons, ipsilateral block of IT SP-evoked cFos activation would represent a transsynaptic effect of the IPLT BoNT-B.

## DISCUSSION

As summarized in Table 1, unilateral IPLT BoNT-B has a local early effect upon ipsilateral capsaicin evoked plasma extravasation, indicating a local uptake and effect upon afferent terminals. Delayed cleavage of VAMP in ipsilateral DRG and block of afferent evoked SP release, emphasizes a central homolateral spinal redistribution of BoNT-B. These effects are relevant to spinal nociceptive processing, as with the same time course the IPLT-BoNT-B reduced phase 2 flinching and neuronal cFos expression produced by intraplantar formalin. The ipsilateral decreases in IT SP evoked cFos suggests that a component of these changes in processing may be mediated by a transsynaptic action of transported toxin.

### Afferent terminal effects of BoNT-B

TRPV1 channels are present on the central and peripheral terminals of peptidergic afferents [17; 43]. Capsaicin thus mobilizes SNAREs, leading to terminal release of peptidergic afferent transmitters (SP/CGRP) [78; 80].

### Peripheral terminals

In the periphery, release of SP and CGRP by capsaicin leads to protein extravasation [4; 32; 58]. IPLT-BoNT-B resulted in an early reduction in ipsilateral but not contralateral plasma extravasation, as reported with BoNT-A in humans [33; 49; 98] and in rodent [16], suggesting a local block of afferent peptide release.

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### Spinal terminals

SP is expressed in terminals of small TRPV1(+) C fibers, which distribute into the superficial dorsal horn [95]. Release of this peptide activates NK1r on dorsal horn neurons. These receptors then undergo internalization. Extensive work supports correlation between SP release and NK1r internalization: i) *Ex vivo*, there is covariance between SP concentration and NK1r internalization [68]; ii) *In vivo*, dorsal horn NK1r internalization is prevented by depletion of primary afferent SP [48]; iii) NK1r internalization evoked by afferent input, but not by direct activation with IT SP, is prevented by IT opiates [48], and calcium channel blockers [92]. Correspondingly, IPLT-BoNT-B reduced NK1r internalization following IPLT-formalin, but internalization evoked by IT SP was unaltered by IPLT-BoNT-B (supplementary Figure 3), suggesting that IPLT-BoNT-B blocked release, not internalization.

An important concern is whether IPLT-BoNT-B effects on IPLT-formalin evoked spinal SP release resulted from block of local formalin evoked afferent activation. Four lines of evidence indicate that IPLT-BoNT-B affects spinal release from central afferent terminals, indicating spinopetal movement of the toxin.

- **i.** Direct spinal terminal activation: IT-capsaicin, through spinal TRPV1 receptors, leads to spinal SP release [42; 82; 105] and bilateral increases in dorsal horn cells showing NK1r internalization. After unilateral IPLT-BoNT-B, NK1r internalization in ipsilateral but not contralateral spinal cord was reduced. It might be argued that BoNT-B altered TRPV1 activation. We are aware that after IPLT BoNT-A, nerve injury evoked increases in TRPV1 receptor expression are prevented, but TRPV1 expression always remained above control levels [103]. So, absent a direct effect on TRPV1 activation, block of IT-capsaicin evoked release by IPLT BoNT-B reflect effects upon spinal terminal SP release.
- **ii.** DRG VAMP: Western blotting shows that DRGs express VAMP2 and that IPLT-BoNT-B reduced VAMP in ipsilateral, but not contralateral DRG. Changes in spinal VAMP were not detected. We believe this is because VAMP losses in the primary afferent are small compared to total VAMP found in spinal non-afferent cells and processes.
- **iii.** Time course/ipsilaterality: Local effects of IPLT-BoNT-B on extravasation showed an early onset (by 4 hrs). Effects upon DRG VAMP and formalin evoked cFos were not noted until after a 24 hr delay. Were a systemic redistribution relevant to these effects, we would anticipate that all effects would have shown a similar time of onset and IPLT effects would not show consistent homolaterality.
- **iv.** Systemic toxicity. Systemic uptake and redistribution would have shown not only weight and strength defects in mice at the doses injected, but systemic uptake at 1U (the IP LD50) would have caused morbidity in mice as defined by mouse bioassays, which determine concentrations based on the toxin's lethality in mice dosed with IP BoNT. We observed no morbidity with IPLT dosing of 1U/30uL.

These results support the hypothesis that IPLT-BoNT-B was taken up locally, moved to ipsilateral DRGs, cleaving target protein, ipsilaterally blocking SP release and cFos

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activation. Such transport has been observed with BoNT-A in brain [5; 84] and from the periphery to spinal cord, with effects prevented by axon transport blockade [69; 70].

## Possible transsynaptic actions

IT SP bilaterally activates neurons in superficial and deep dorsal horn (cFos) [93], and results in an agitation/hyperalgesia mediated by spinal NK1r [41; 63]. This cFos expression occurs in both NK1r(+) neurons, of which there are few, and NK1r(−) neurons, of which there are many. Thus, NK1r(+) cells would be activated bilaterally by IT SP and they in turn, through release of an excitatory transmitter, would activate downstream NK1r(−) neurons. Unexpectedly, we observed that unilateral IPLT-BoNT-B resulted in a reliable reduction in cFos activation ipsilateral to the IPLT-BoNT-B delivery. An important question is whether this increased incidence of cFos(+) neurons after IT SP reflects an effect by IT SP on primary afferent terminals or on second order NK1r(+) neurons? In the former case, IPLT-BoNT-B might act on the primary afferent spinal terminal, while in the latter case IPLT-BoNT-B effects might represent a transsynaptic action (e.g. movement from the afferent to a second order neuron). It remains under debate whether DRG neurons possess functional NK1r. Rodent DRG cells can express NK1r mRNA [3; 53; 102], and SP can evoke depolarizing currents in DRG cells [53; 109]. Conversely, immunohistochemical evidence for NK1r on sensory neurons and their terminals is limited (supplemental data) [14; 54; 64; 77]. More importantly, NK1r KO and inhibitor studies show that loss of NK1r increased SP release from primary afferents, which indicates a negative feedback autoreceptor. This suggests that IT SP would serve, if anything, to reduce evoked release of SP [52; 61] as well as glutamate and aspartate from primary afferent terminals [44-46]. Thus, while a stimulatory effect of IT SP on the primary afferent terminal cannot be excluded, the present results, in which IT SP evoked cFos activation is unilaterally blocked by IPLT-BoNT-B, suggest a transsynaptic event. The results are in accord with a growing body of literature, which reports BoNT-A transcytosis in neurons [1; 5; 50; 65; 84; 86; 96] and glia [60; 67]. Such transsynaptic effects are important in understanding how peripherally delivered BoNTs can alter phenomena such as migraine, which arises from meningeal afferents, and secondary hyperalgesia [33; 49]. We also note that it has been suggested that BoNT-B may activate endogenous opioid systems [28; 100].

## Time course

IPLT-BoNT-B resulted in a block of plasma extravasation by 4 hrs. DRG VAMP cleavage and suppression of flinching occurred by 24 hrs, with each effect having a similar duration of action (greater than 7 less than 21 days). This ordering is consistent with a central movement of the IPLT-BoNT-B and duration of action reported *in vivo* [6].

## Motor function

IPLT BoNT-B at the concentration/dose employed had no effect upon hind paw placing and stepping, nor the withdrawal response evoked by a thermal stimulus or pinprick. These observations reveals the continued functionality of large low threshold (Aß) afferents which initiates withdrawal involving hip flexors, gastrocnemius and soleus musculature [21; 27; 91]. Thermal and pinprick escape reflects activity in small high threshold myelinated and

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unmyelinated afferents [15; 99]. Thus, these results suggest that at the dose/concentration employed IPLT-BoNT-B had no effect upon either motor function or upon acute afferent traffic.

### Nociceptive processing

IPLT-formalin though TRPA1 receptors [73] results in biphasic flinching of the injected paw [106] and an increase in cFos(+) neurons in ipsilateral dorsal horn [19]. IPLT-BoNT-B had no effect upon phase 1, but reduced phase 2 flinching. Phase 1 represents the acute afferent barrage generated by the formalin, while phase 2 is dependent upon initiation of a central sensitization and a low level of ongoing traffic through these small afferents [106]. The lack of effect upon phase 1 flinching is consistent with the observation that at the intraplantar doses used (BoNT-B, 1U/30μL) there was no effect upon either acute thermal escape or upon the response to the acute application of a sharp probe. This profile recapitulates the results of other studies with IPLT-BoNT-A in preclinical and human models [59; 66]. The lack of effect upon acute nociception, even after IT delivery [38], argues that the BoNT's effect reflects more than a simple block of small afferent terminal release. Intrathecal SP (NK1r) and glutamate (NMDA receptor) antagonists have minimal effects upon acute nociception, but reduce facilitated states [18; 37; 107]. Whether this apparent BoNT selectivity is due to a differential effect upon populations of afferents or an incomplete block of transmitter release in all afferents and/or an effect upon post- synaptic systems is not known.

In summary, these results, in conjunction with evolving literature, are consistent with the human observations, both clinical and experimental, that in addition to pronounced effects of local BoNT on muscle tone, there are changes in nociceptive processing that reflect a central afferent movement of the BoNT to modify the functionality of the first order synapse and perhaps the second order neuron.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**Summary**

Peripheral botulinum toxin-B attenuates peripheral and central terminal neurotransmitter release and nociceptive processing via a presynaptic effect with a possible postsynaptic component.

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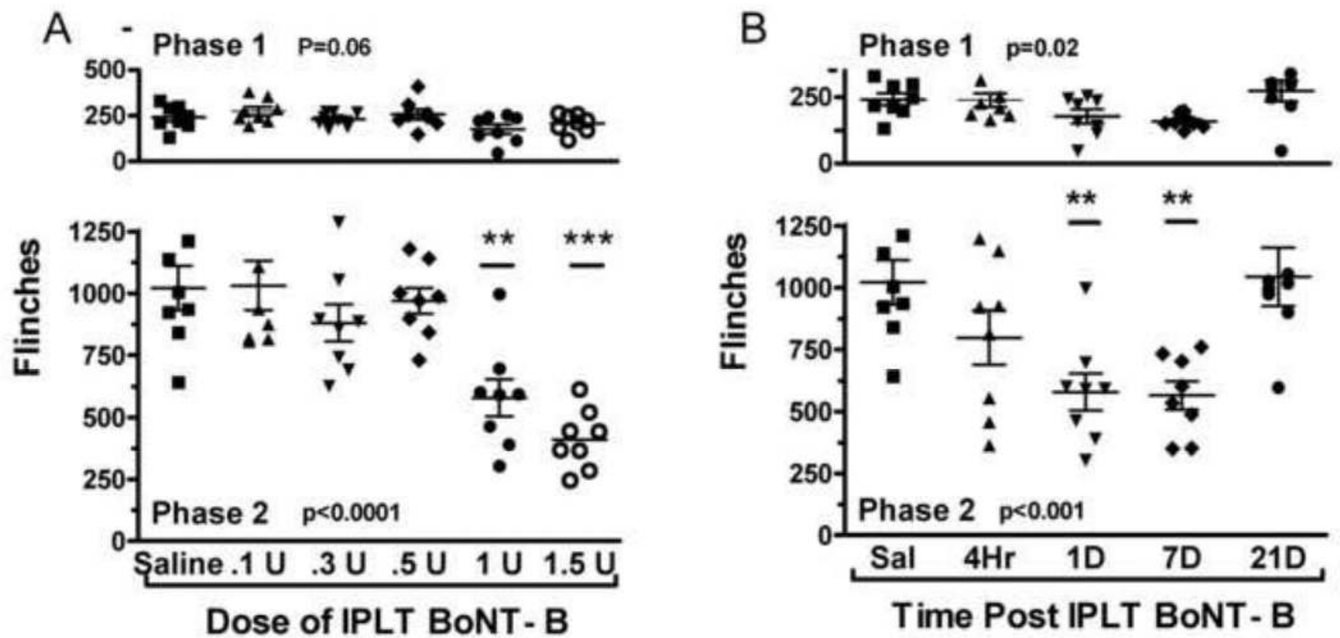


**Figure 1.**

Intraplantar (IPLT) botulinum toxin B (BoNT-B) inhibits formalin-evoked flinching behavior. Plots indicate mean ± SEM for cumulative flinches observed during Phase 1 (0-10min, top) and Phase 2 (11-60min, bottom) following unilateral formalin injection into the hindpaw. As indicated, unilateral IPLT formalin evoked a biphasic flinching response. 1A) Mice were pretreated for 24hrs with 0.1, 0.3, 0.5, 1 or 1.5U of IPLT BoNT-B or saline before formalin. IPLT BoNT-B at 1 and 1.5U produced a significant reduction in phase 2 flinching. 1B) Mice received IPLT saline or IPLT BoNT-B (1U) 4 hr, 1d, 7d, or 21 d before formalin (saline data are pooled). Pretreatment with IPLT BoNT-B (1U) for 1d and 7d (but not 4h or 21 days) resulted in a significant reduction in phase 2 flinching. No differences in phase 1 flinching were detected between groups in 1A or 1B. Saline and 1U-1d data are the same in the two graphs. **p<0.01 and ***P<0.001 vs. saline; one-way ANOVA. N=7-8 animals per group.

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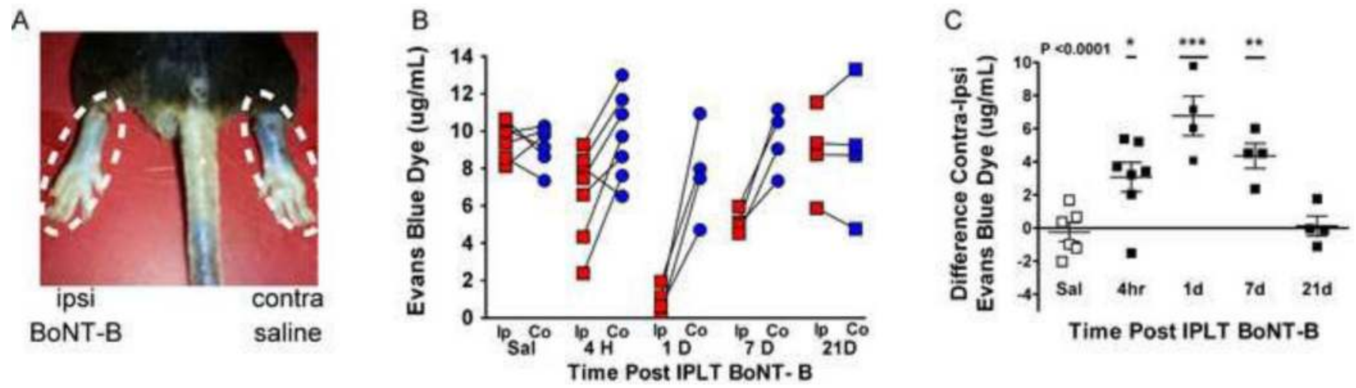


**Figure 2.**

IPLT-BoNT-B inhibits capsaicin-induced plasma extravasasion in the injected paw. Mice were pretreated with IPLT BoNT-B (1U) and saline (contralateral paw) for 4hr, 1, 7 or 21d and plasma extravasation was measured (Evans blue in the paw tissue) 10 min after bilateral IPLT injection of capsaicin. A) Paw skin color on the saline side (right) is darker than the BoNT-B side (left). B) Plot (mean ± SEM) depicts dye concentration in formamide extract of ipsi- and contralateral paw tissue. C) Plot depicts differences in dye concentration between the contra- and ipsilateral paw. A significant difference was detected between the saline group and the 4hr, 1 and 7d but not the 21d groups. *p<0.05; **p<0.01; ***p<0.001 vs. saline; one-way ANOVA. N=4-6 animals per group.

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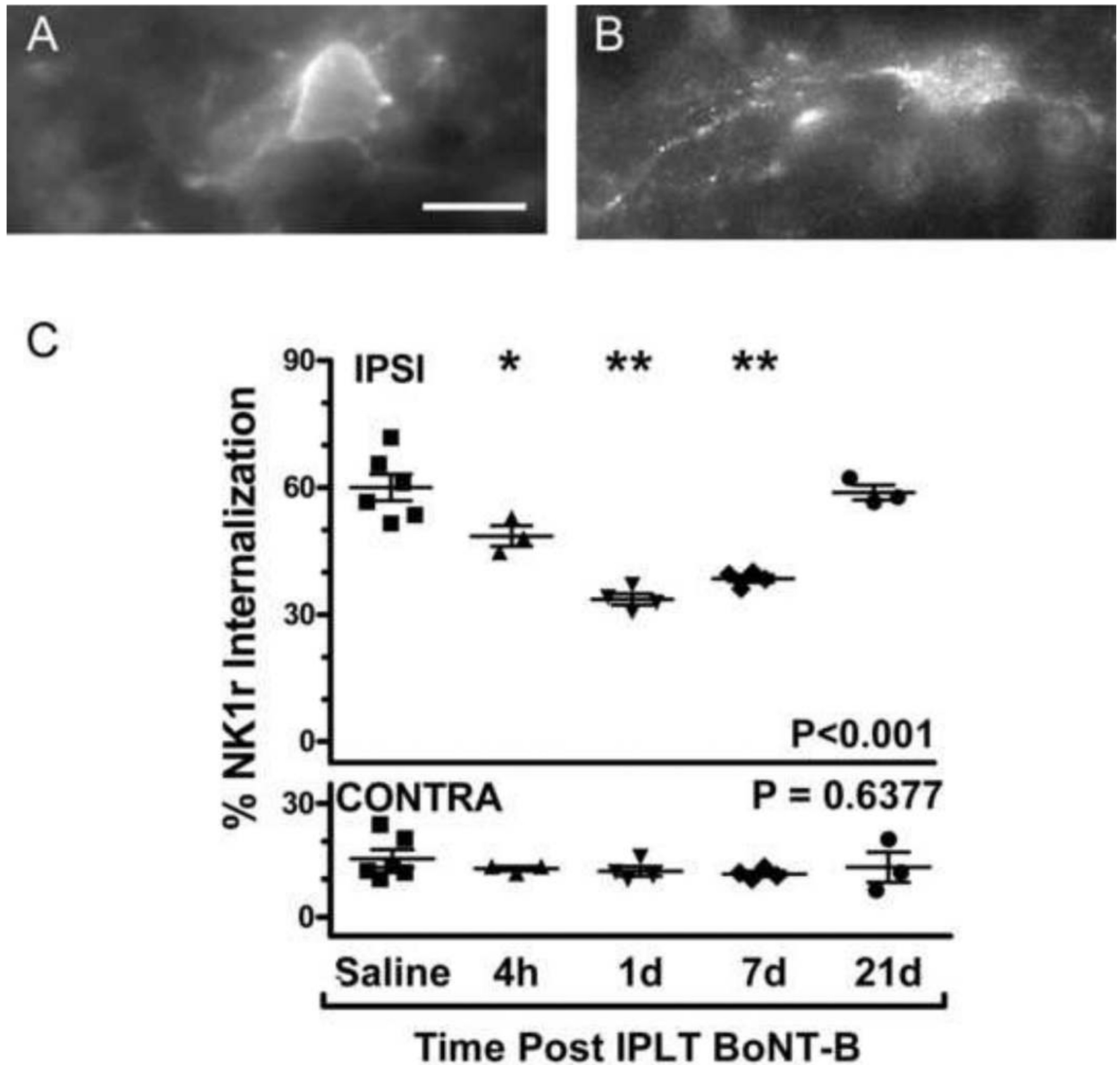


**Figure 3.**

Intraplantar (IPLT) botulinum toxin B (BoNT-B) inhibits IPLT formalin-evoked NK1 receptor (NK1r) internalization in the spinal dorsal horn. A and B) Representative immunohistochemistry images showing A) a neuron with uninternalized NK1r and B) a neuron with NK1r internalization in the ipsilalateral dorsal horn after IPLT formalin in a control animal. C) Plots indicates percentage of NK1r internalization in the ipsilateral and contralateral superficial layer of the lumbar spinal dorsal horn. IPLT formalin evokes an increase in the incidence of NK1r Internalization in the ipsilateral, but not contralateral dorsal horn in the L4, L5 and L6 spinal segments. Pretreatment with IPLT BoNT-B (1U) for 4 hr, 1 and 7d but not 21d prevented Nk1r internalization. *p<0.05; ** p<0.01 vs. saline;

one-way ANOVA. N=3-6 animals per group with 9-12 spinal cord sections counted per animal. Scale bar, 15μm.

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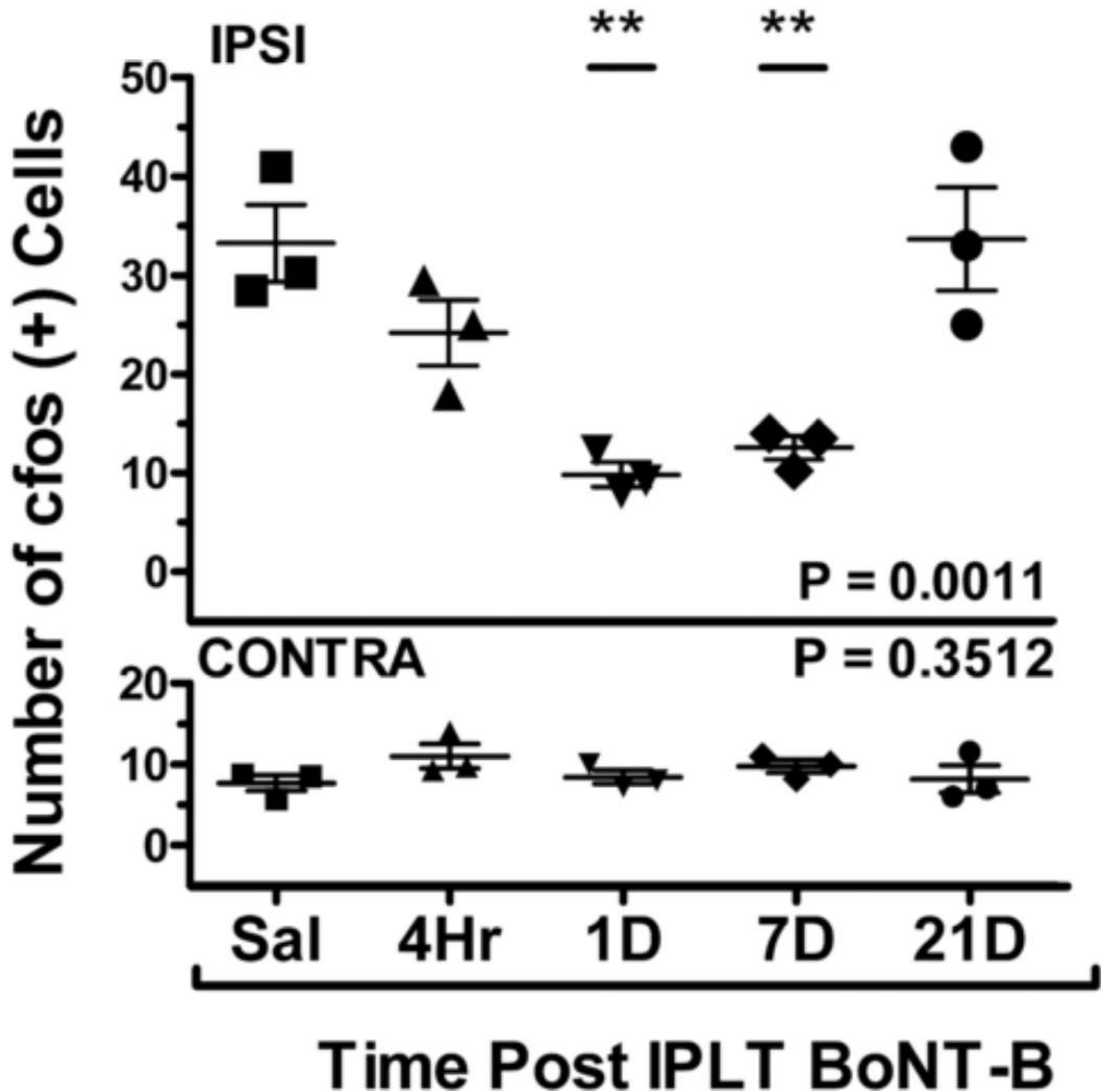


**Figure 4.**

Intraplantar (IPLT) botulinum toxin B (BoNT-B) inhibits IPLT formalin-induced cFos expression in the spinal dorsal horn. The plot presents mean ± SEM of number of cFos (+) neurons in the spinal dorsal horn in the L4, L5 and L6 segments. IPLT formalin evokes a unilateral increase in cFos(+) neurons in the ipsi- but not contralateral dorsal horn. This formalin-induced cFos increase was blocked by pretreatment with IPLT BoNT-B (1U) for 1 and 7d but not 4hr and 21d. ** $p<0.01$ vs. saline; one-way ANOVA. N=3 animals per group with 9-12 spinal cord sections counted per animal.

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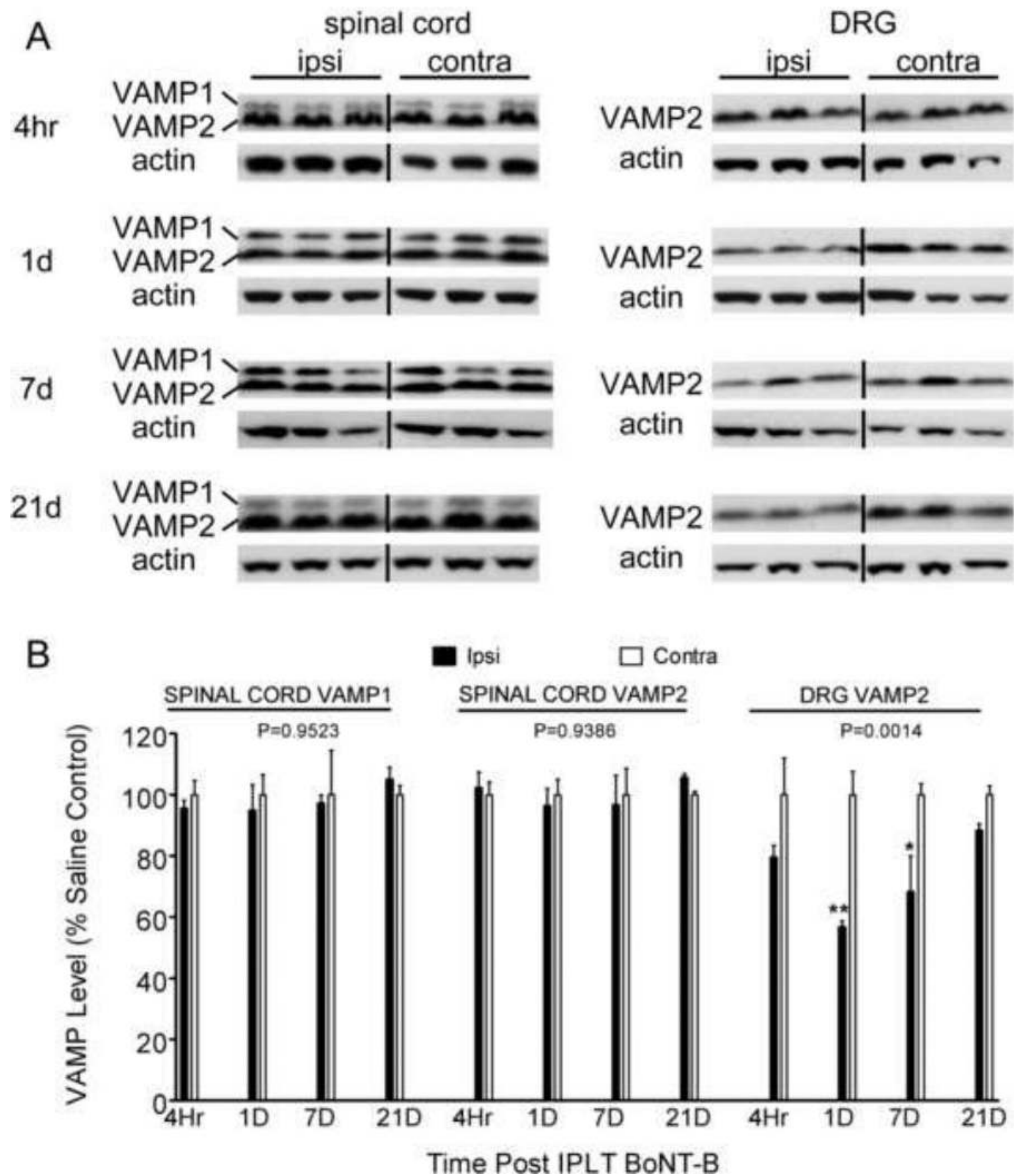


**Figure 5.**

Intraplantar (IPLT) botulinum toxin B (BoNT-B) reduces vesicle associated membrane protein (VAMP) expression in the ipsilateral DRG. A) Western blots showing VAMP and -actin (loading control) in the DRG and spinal cord ipsi- and contralateral to IPLT injection of BoNT-B (1U). Mice were treated with IPLT BoNT-B for 4h, 1, 7 or 21 days prior to tissue harvest. B) Histogram depicts the relative levels of VAMP expression quantified by densitometric measurement. Saline control tissues (set as 100%, not shown) were used for normalization. A significant decrease in VAMP expression in the ipsilateral DRG was

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observed after 1d and 7d BoNT-B pretreatment. Values are mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ vs. the contralateral side; one-way ANOVA. N=3-4 animals per group.

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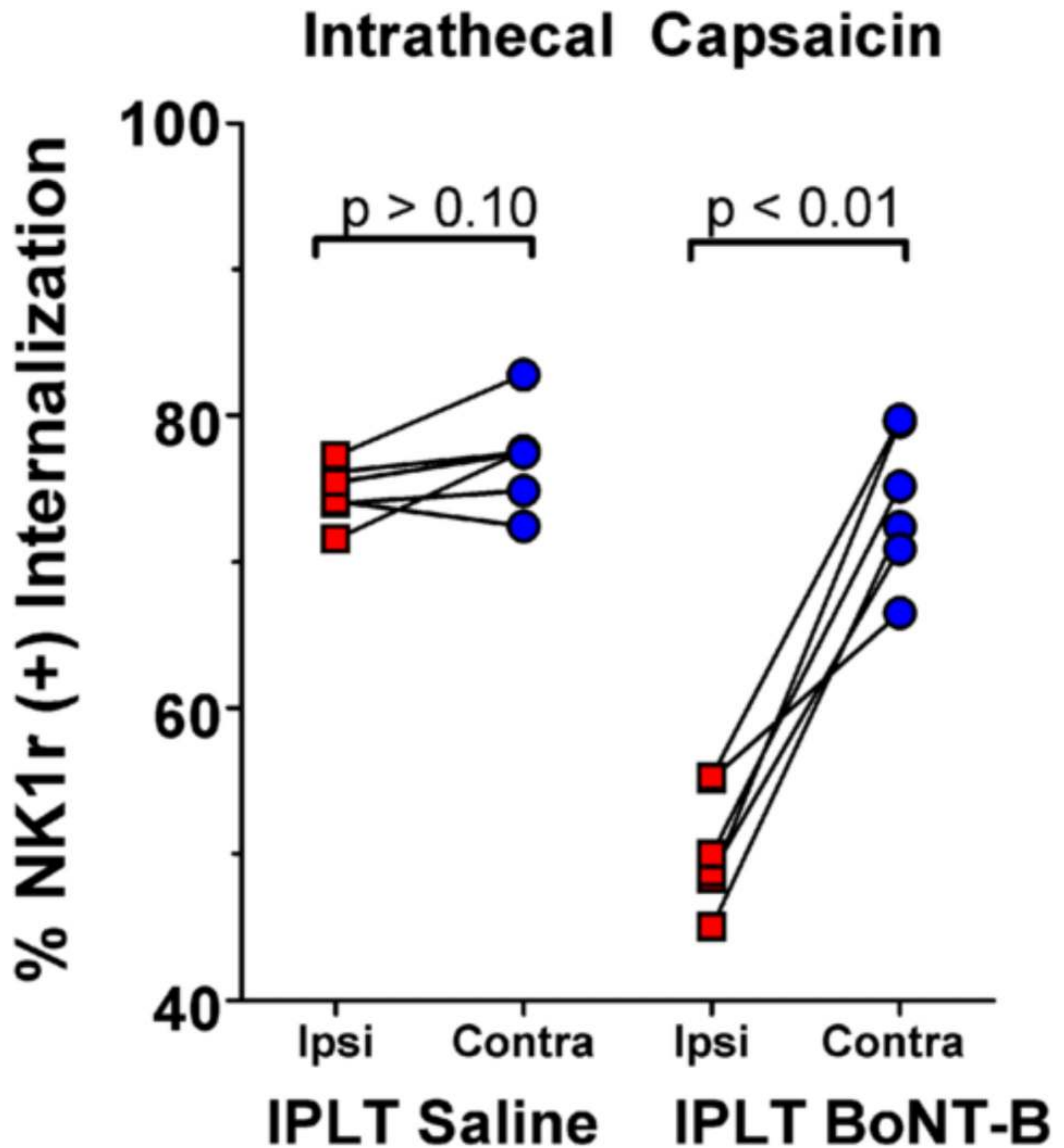


**Figure 6.**

Intraplantar (IPLT) botulinum toxin B (BoNT-B) inhibits intrathecal capsaicin-evoked NK1 receptor (NK1r) internalization in the spinal dorsal horn. Plot presents the percentage of NK1r(+) neurons displaying NK1r internalization in the superficial dorsal horn after intrathecal delivery of capsaicin (10μg/10μL). Mice were pretreated with unilateral IPLT saline or BoNT-B (1U/30μL) 1 day in advance of the intrathecal capsaicin. Both groups received IT capsaicin. In saline control mice, IT capsaicin induced bi-lateral NK1r

internalization. IPLT BoNT-B pretreatment blocks NK1r internalization on the ipsi- but not contralateral side. $p<0.01$, ipsi vs. contra-lateral; paired t-test.

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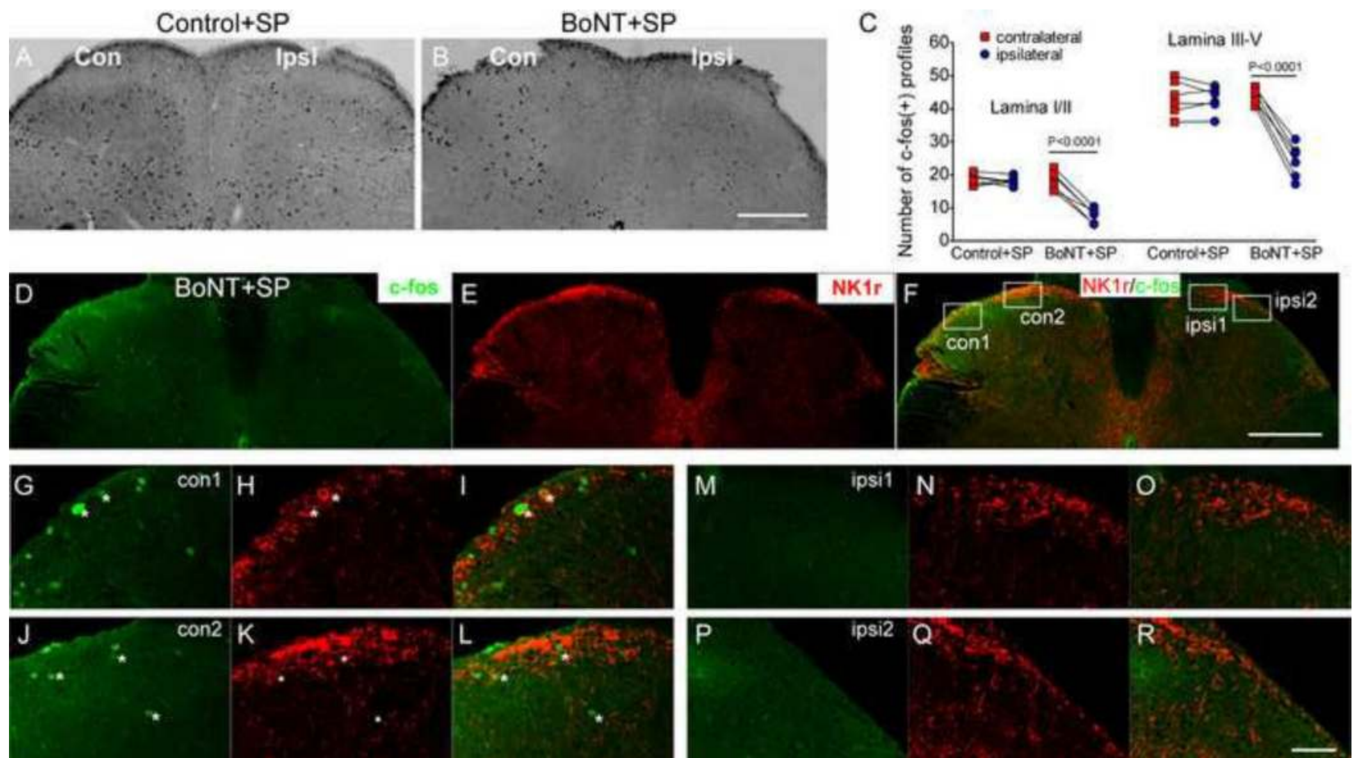


**Figure 7.**

Representative Immunolabeling of cFos and NK1r in the lumbar L4 spinal cord dorsal horn of animals which received unilateral hindpaw intraplantar (IPLT) BoNT-B or saline control, then IT substance P after 48 hrs. A) In control animals received IPLT saline, IT SP induced a bilateral increase of cFos(+) profiles in the dorsal horn. B) IT SP resulted in an increased incidence of cFos(+) neurons in the dorsal horn contralateral but not ipsilateral to the IPLT BoNT. C) Scatter plot shows the numerical differences of cFos profiles between the contralateral and ipsilateral dorsal horn. There was a significant difference between the BoNT-B group and the control group. ****P<0.0001 vs. the contralateral side; one-way ANOVA. D, E and F) Double labeling of D) c-fos and E) NK1r in the spinal cord of a mouse received IPLT BoNT-B and IT SP. G-R) Higher magnification images of the two areas in contralateral (con1 and con2) and ipsilateral (ipsi1 and ipsi2) dorsal horns shown in panel F) outlined by the boxes. In con1 and con2, a combined total of 26 c-fos profiles were counted. * denotes 5 neurons (19%) that are c-fos (+) and NK1r (+). On the ipsilateral side, there was a much lower number of c-fos(+) profiles and no c-fos(+)/NK1r(+) profiles were noted. D-R) Images from a single focal plane were shown. Scale bars, A, B, D-F, 250μm; G-R, 50μm.

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**Table 1**

Effects of intraplantar BoNT-B on the ipsilateral side.

| Experiment | | BoNT-B pretreatment time | | | |
|---|---|---|---|---|---|
| | | **4hr** | **1d** | **7d** | **21d** |
| VAMP degradation | DRG[b] | - | ** | * | - |
| | Spinal cord[b] | - | - | - | - |
| Peripheral release (plasma extravasation)[a] | | * | *** | ** | - |
| formalin | NK1r internalization[a] | * | ** | ** | - |
| | cFos[a] | - | ** | ** | - |
| | Phase2 flinching[a] | - | ** | ** | - |
| IT capsaicin – NK1r internalization[b] | | | ** | | |
| IT SP – cFos (2d BoNT-B pretreatment)[b] | | | **** | | |

"-", P>0.05

compared to

"/", experiment not performed at the indicated time point.

[*] P<0.05

[**] P<0.01

[***] P<0.001

[****] P<0.0001

[a] saline or the

[b] contralateral side.