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Retrograde transport of endoneurially injected botulinum toxin into mouse sciatic nerve



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**Publication Date**
2017

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**Abstract**

Recent work has shown that Botulinum toxins (BoNT) are taken up and transported in sensory nerve to cerebral spinal terminals, where they will cleave synaptic proteins (SNARES) and block hyperalgesic states with little change in motor function We sought i) to determine if such transport occurred after *endoneurial* delivery in the sciatic nerve. If such uptake occurred, we hypothesized that there would be a significant antihyperalgesic effect and SNARE cleavage at doses lower than those required after intraplantar delivery. We examined the BoNT-B serotype, which cleaves VAMP (vesicle associated membrane protein). To test this hypothesis, 1µL of saline, 0.05U, 0.1U, 0.5U, or 1.0U BoNT-B was injected into the sciatic nerves of isoflurane anesthetized C57Bl/6 male mice. Pain behavior was assessed by the counting of paw flinching initiated by unilateral intraplantar injection of formalin (20 µL/2% formalin). This study has shown that mice injected unilaterally endoneurially with 0.1U BoNT-B showed significantly reduced intraplantar formalin-induced flinching behavior as compared to saline animals. Mice that received 0.05U, 0.5U, or 1.0U BoNT-B did not have significantly different flinching behavior compared to saline animals. In previous work, intraplantar BoNT B produced comparable effects but at 10x higher doses. Ipsilateral and contralateral hind paw thermal escape latencies were not significantly different among any of the groups. Mice injected with saline, 0.5U, and 1.0U BoNT-B exhibited motor impairment of the ipsilateral hind limb. Western blotting to detect VAMP1/2 levels in the ipsilateral and contralateral DRG of saline and BoNT injected mice were not successful due to poor tissue yield. These data suggest that intrasciatic injection of BoNT may produce a potent effect upon pain behavior at doses lower than those required if given by intraplantar delivery.

**Background**

Botulinum neurotoxins (BoNT) are a class of bacterial protein toxins made of a 50 kDa light chain (LC) and 100 kDa heavy chain (HC) linked together by a disulfide bond. (1) They are among the most hazardous natural substances known. BoNT undergo uptake into nerve terminals. The relatively acidic cytosol reduces the disulfide bond, releasing LC from HC. The acidic environment also unfolds LC, activating its catalytic function. (1) The active LC cleaves the SNARE (soluble NSF attachment protein receptor) family of synaptic proteins, which are critical to vesicle fusion. Among these SNAREs are VAMP (vesicle-associated membrane protein), which will be considered in this study. Cleaving SNAREs blocks vesicle fusion, thus blocking NT (neurotransmitter) release. The effects of BoNT at the neuromuscular junction are well-investigated; BoNT prevents the release of Ach (acetylcholine), causing flaccid paralysis of the affected muscle.

Studies have also shown the BoNT reduces the spinal release of other NTs notably sP (substance P) and glutamate, indicating that BoNT affects the releasing functions of afferent neurons. (3) sP is a NT released from primary afferent nociceptors. (7) Accordingly, this effect upon sP release provides a surrogate marker for the likely affect of the BoNT on nociception.

A variety of studies have indeed shown that BoNT at the spinal level (e.g. delivered by intrathecal injection) affects the pain pathways. Thus, intrathecal injections of BoNT-B in mice reduce spinal dorsal horn sP release, as measured by the internalization of the NK1 receptor upon which sP acts, and reduce spinal activation, as measured by the incidence of neurons expressing the immediate early gene *c-fos*. (3, 4) Of particular significance, subcutaneous BoNT-B injections into mouse hindpaw (IPLT, intraplantar) reduced levels of VAMP (the target synaptic protein cleaved by BoNT-B) in the ipsilateral DRG (dorsal root ganglia). (4) Consistent with this action, IPLT BoNT-B reduces intraplantar formalin-induced flinching, capsaicin-induced plasma extravasation, NK1r internalization, and *c-fos* expression. (4) These data suggest that the IPLT BoNT delivered into the paw is taken up and trafficked centrally where it serves to cleave synaptic protein in the primary afferent and blocks release and subsequent downstream dorsal horn activation. There is also immunohistochemical evidence that intraneural, intramuscular, and sciatic nerve BoNT-A injections increased levels of truncated SNAP-25 (synaptosomal-associated protein 25, the target of BoNT-A) in lumbar spinal cord. (5) These data suggest that BoNTs in sensory axons, after uptake, may undergo retrograde transport towards the spinal cord.

The previous BoNT work by Marino et al. employed intraplantar delivery. (4) Subcutaneous administrations of BoNT include the risk of systemic uptake and redistribution, which may cause systemic toxicity. The present study will examine the effects of delivering BoNT-B into the nerve sheath (e.g. endoneurally). We hypothesize that endoneurial BoNT will be taken up and transported. Endoneurial BoNT injection, if proven to reduce a pain response as effectively as subcutaneous injection did, would reduce the risk of systemic toxicity because its local delivery will require lower total doses. This study should provide a better understanding as how BoNT can be used to reduce hyperalgesia and pain responses.

## Methods

All protocols employed were undertaken with a protocol approved by the UCSD IACUC.

### Animals

Adult male C57BI/6 mice.

### Administration of BoNT

Mice were anesthetized with isoflurane, and the left sciatic nerve exposed. 1µL saline, 0.05U, 0.1U, 0.5U, or 1U BoNT-B was injected into the nerve with a 33G needle and Hamilton syringe. Skin and muscle were sutured, Lactate Ringer's sq given, and the mouse recovered. Injections were performed 7 days in advance of study.

### Thermal Escape

Thermal escape was performed at baseline prior to injection and 7 days after injection. Mice were placed in a chamber on the thermal stimulus device. Time from initiation of thermal stimulus to hind paw to paw withdrawal was recorded. Thermal escape investigates the effects of BoNT on the acute afferent neurons. A previous study indicated that IPLT BoNT did not affect acute afferent neuronal traffic, but this will access the effects of intrasciatic BoNT delivery.

### Clinical Scoring

This reflects motor impairment of the hind limbs. Mice were placed on a flat surface, and gait was observed. A score of 0 to 3 was assigned: 0 indicates no motor impairment; 1 indicates reduced weight-bearing abilities; 2 indicates reduced plantar-surface contact and reduced

weight-bearing abilities; and 3 indicates no weight-bearing abilities. This value was recorded at baseline and after 1, 3, and 7 days.

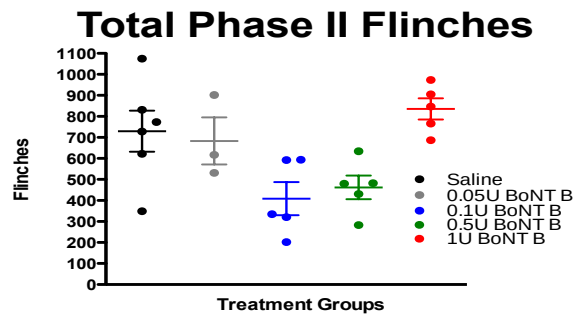
**Grip strength**
This is an additional measure of motor impairment following injection of saline and/or BoNT. Mice were placed on a wire mesh mounted on a scale. The experimenter lifted the animal by its tail, and the grip strength was recorded at its maximal value before the animal released the wire mesh. This was recorded at baseline and after 1, 3, and 7 days.

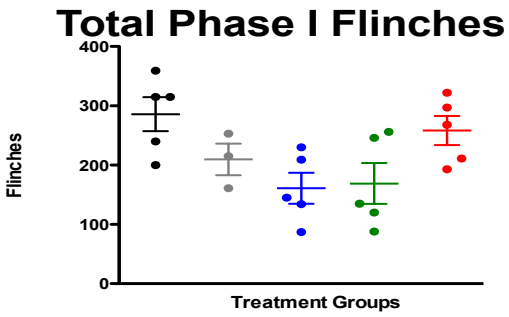
**Formalin flinch**
A metal band was placed around the ipsilateral hind paw. Mice were allowed to acclimate for 30min; then, 20uL of 2.5% formalin was be injected into the dorsal aspect of the ipsilateral hind paw. The mouse was placed in a cylindrical chamber where the movement of the metal band was detected by an automated system. Both Phase I, 0-9 minutes after injection, and Phase II flinches, 10-60 minutes after injection, were recorded. This was performed after 7 days. This experiment investigated the primary mechanism for the anti-hyperalgesic effect of BoNT: altering central sensitization. Previous studies suggest that formalin-induced flinching occurs in two phases. Phase I occurs from 0-9 minutes and is a result of direct activation of primary afferent neurons, through the TRPA1 cation channel. This is followed by Phase II, which occurs from 10-60 minutes and is a result of the sensitization of central dorsal horns. Studies have also suggested that BoNT alters the sensitization process through reducing the spinal release of sP but does not alter the activation of primary afferent neurons that is responsible for the Phase I response. It is expected that mice treated with BoNT would have a reduction in Phase II flinches but not in Phase I flinches.

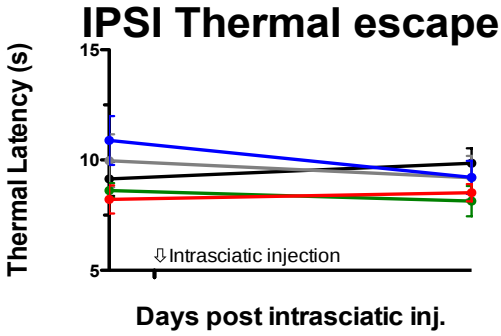
**Western Blots for VAMP**
This study would investigate and quantify the central transport of intrasciatic delivered BoNT. This data would be used in conjunction with that from the formalin flinch test to access whether any significant reduction in flinching is due to the central action of BoNT. Animals were anesthetized with isoflurane and decapitated 2 hours after the 2.5% formalin injection. The vertebral column was cut transversely at the pelvic crest. Hydraulic pressure generated by a saline-filled syringe was used to eject the spinal cord. L3-L5 DRG were dissected, and the samples were transferred into a buffer with an inhibitor cocktail. Samples were homogenized. After centrifugation, the supernatant was collected. The samples were subjected to SDS-PAGE. After transfer to a nitrocellulose membrane, the membrane was incubated with anti-VAMP 1,2,3 antibodies. These membranes were imaged and analyzed. The blots were reprobed for β-actin as a loading control.

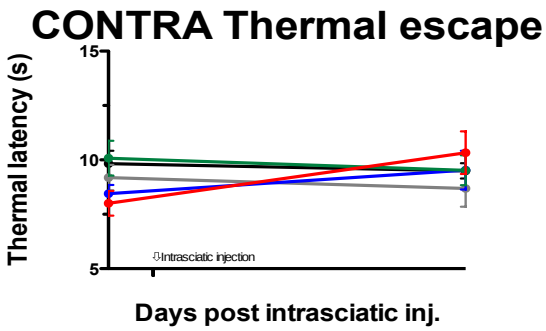
**Statistical Analysis**
Statistical analysis was performed with 1-way ANOVA, two-sample t-test, and paired t-tests.

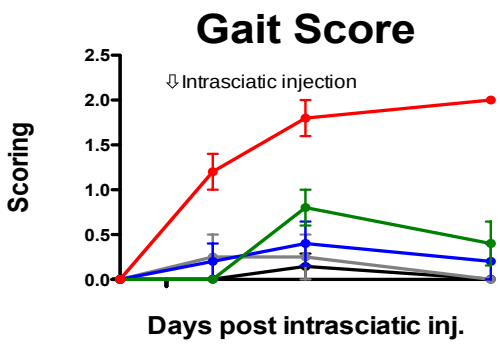
**Results**

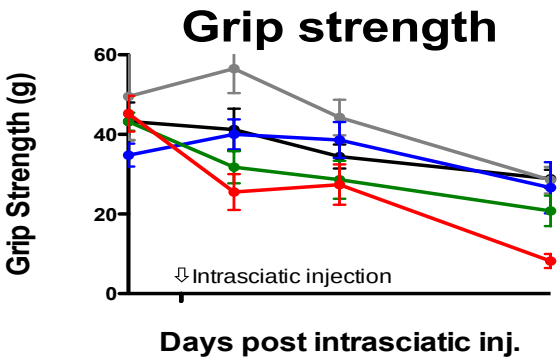



Mice injected with 0.05U BoNT had mean 683.3 phase II flinches (p-value 0.7820), 0.1U BoNT had mean 408.8 flinches (p-value 0.0351), 0.5U BoNT had mean 462.0 flinches (p-value 0.0520), and 1U BoNT had mean 835.6 flinches (p-value 0.3906). Mice injected with 0.1U BoNT had significantly fewer phase II flinches than control mice (mean 729.7), but no other treatment group had significantly different mean phase II flinches than control mice.

Mice injected with 0.05U BoNT (mean 209.67 flinches), 0.5U BoNT (169.00), 1.0U BoNT (258.20), and saline (285.80) did not have significantly different number of Phase I flinches (p-value 0.0546). Mice injected with 0.1U BoNT had significantly few Phase I flinches (mean 161.0, p-value 0.0122).

The thermal escape latency among treatment and control groups did not differ significantly in the ipsilateral paw (p-value 0.2249) or contralateral paw (p-value 0.1236) before the injection. It also did not different significantly among the groups 7 days after the injection (ipsilateral p-value 0.4377, contralateral p-value 0.6696). The pre-injection and post-injection thermal latencies also

did not differ significantly across any group (saline ipsilateral p-value 0.5491, 0.05U p-value 0.7123, 0.1U p-value 0.2263, 0.5U p-value 0.4659, 1.0U p-value 0.6943).

The gait scores for control mice and mice injected with 0.05U, 0.1U, 0.5U BoNT were not significantly different at days 1 (p-value 0.5006), 3 (p-value 0.1363), and 7 (p-value 0.2221). Mice injected with 1U BoNT had significantly increased gait scores at days 1 (mean 1.20, p-value 0.001), 3 (mean 1.80, p-value <0.0001) and 7 (mean 2.00) relative to the control group.

Mean grip strength did not differ significantly among control and treatment groups before the injection. It did not differ significantly among the control, 0.05U, 0.1U, and 0.5U BoNT groups at post-injection day 7. Post-injection day 7 grip strengths were significantly decreased from pre-injection strengths in saline (28.86g vs 43.29g, p-value 0.0039), 0.5U (20.80g vs 43.20g, p-value 0.0032), and 1.0U BoNT groups (8.20g vs 45.20g, p-value 0.0024). It did not significantly differ in 0.05U (28.50g vs 49.50g, p-value 0.1219) and 0.1U BoNT (26.60g vs 34.80g, p-value 0.3216) groups. Mean grip strength of mice with 1.0U BoNT at post-injection day 7 (8.20g) was significantly reduced compared to saline mice (mean 28.60g, p-value <0.0001).

Western Blots for VAMP in the L3-L5 DRG were unsuccessful due to poor protein yield from the tissue isolation.

**Discussion**

In regards to Phase II flinching, only the group injected with 0.1U BoNT had statistically significant reduction in flinching compared to control mice. Mice injected with 0.5U BoNT had fewer, but statistically insignificant, Phase II flinches compared to mice injected with saline. Previous studies showed that IPLT delivery of BoNT produced reduced Phase II flinches. The results of this study are somewhat consistent with the previous results. This suggests that delivering BoNT directly into the mouse sciatic nerve can produce anti-hyperalgesic effects at lower doses than IPLT delivery. Interestingly, however, these effects were limited to just the 0.1U BoNT dosage, and the 0.5U BoNT dosage did not achieve such effects. This may be explained by errors in technique during the administration of the BoNT.

Initially, this study used methylene blue as an indicator to ensure proper delivery of saline or BoNT into the sciatic nerve. However, the BoNT was ineffective even with diluted concentrations of methylene blue, likely due to the photooxidation of BoNT in the presence of methylene blue and light (8). From that point, no dye was used in the injections. Due to the clear color of the saline and BoNT solutions, it was not entirely certain if each injection delivered the full amount of saline or BoNT into the sciatic nerve of the animal. It is possible that some of the solutions did not enter the nerve and leaked into the surrounding tissue. Given the results of previous studies (4), it was expected that direct injection of BoNT into the sciatic nerve would produce similar responses but with lower doses compared to IPLT administration as a higher proportion of the administered BoNT would be transported centrally. This result was somewhat achieved, but it is uncertain why the 0.5U BoNT dosage did not have similar effects. It is possible that some mice in that group did not receive the full volume of the solution.

Control mice and mice injected with 0.05U, 0.5U, and 1.0U BoNT did not have significantly different mean number of Phase I flinches; however, mice with 0.1U BoNT had significantly fewer mean Phase I flinches compared to control mice. This result was unexpected, as previous models found that IPLT BoNT did not affect the activation of primary afferent neurons that are responsible for the Phase I response to formalin. It is possible that result is specific to IPLT BoNT, and that BoNT directly administered to the sciatic nerve also interfered with the primary

afferent neuronal traffic. This would subsequently reduce the number of Phase I flinches; however, this would not explain why the 0.5U BoNT did not also have a significant reduction in Phase I flinches. It is unlikely that motor impairment is responsible for this reduction in flinches as mice with 0.1U BoNT did not exhibit any significant motor impairment. Intrasciatic delivery of BoNT may inhibit the release of neurotransmitters, namely glutamate, from primary afferent neurons. If this were true, thermal escape latencies would be expected to be significantly increased in this group as thermal stimuli are transmitted through similar primary afferent nociceptors; however, this was not the case as latencies did not differ significantly among groups. Mice injected with 0.1U BoNT also did not exhibit significant motor impairment that would suggest a more nonspecific effect of intrasciatic BoNT compared to IPLT BoNT. This reduction in Phase I flinching in this group, without effects in thermal latency and motor function, suggests that intrasciatic BoNT specifically inhibits the release of glutamate in TRPA1-expressing primary afferent neurons, for unknown reasons.

Both ipsilateral and contralateral thermal escape latencies were unaffected in mice before and after injection with BoNT. This is consistent with results in previous studies, suggesting that BoNT has no effect on acute afferent neurons, locally or systemically.

Gait was not affected in mice injected with 0.05U, 0.1U, and 0.5U BoNT. Gait was only affected at 1.0U BoNT. This indicates that the surgery and lower doses of BoNT did not affect the animal's ability to bear weight and walk; however, grip strength provides another, albeit imperfect, indication of the hind limb strength. The grip strength tests provided inconsistent results regarding the effects of the surgery on the mice. Control animals had a significant decrease in hind limb strength at 7 days after surgery, suggesting that the surgery and injection were detrimental to the motor abilities of the mice. Mice injected with 0.05U and 0.1U BoNT, however, did not have a significant decrease in strength after the injection. It is possible that the lower doses of BoNT provided anti-hyperalgesic effects that helped them recover strength more quickly. Mice with 0.5U BoNT also had significantly reduced grip strength at post-injection day 7 compared to pre-injection, but not significantly different compared to saline mice at day 7. This suggests that the reduction in 0.5U BoNT mice is due to the surgery and injection and not from the BoNT, but it is also uncertain why this group of mice experienced significantly reduced motor strength from the procedure while mice with lower doses did not. Mice with 1.0U BoNT also experienced significant reductions in motor strength after the injection, and their strength was also significantly decreased compared to control mice at day 7. This suggests that the additional loss of motor strength in this group is likely due to the direct paralysis caused by the high dose of BoNT.

Overall, this study could have benefited most from refined technique, perhaps in the use of a different dye that did not interfere with BoNT to ensure proper delivery of the BoNT or saline into the sciatic nerve of the animals, as well as a larger sample size. Improved technique may have also allowed for greater tissue yield in the DRG isolation. The VAMP WB would have greatly aided in the quantification in the effects of intrasciatic delivered BoNT at the DRG. Unfortunately, due to the issues detailed above, this could not be done during this study period.

**Conclusion**

This study suggests that intrasciatic delivery of BoNT may produce anti-hyperalgesic effects at lower doses compared to IPLT delivery. The optimal dose is likely 0.1U to 0.5U BoNT. The data also suggest that intrasciatic BoNT may also effects on primary afferent neurons, which were not seen in IPLT BoNT. Further studies, such as successfully acquiring the VAMP WB would provide further quantification of the delivery and central transport of the BoNT. Furthermore,

refinement in technique would be required to avoid or reduce the level of detriment to the motor strength of the animals.

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