UNIVERSITY OF CALIFORNIA, SAN DIEGO

Intrathecal Botulinum Neurotoxin B: Effects on Spinal Primary Afferent Neurotransmitter Release, Inflammatory Nociception and Neuropathic Pain in the Mouse

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Polly Pu Huang

Committee in charge:

Professor Tony Yaksh, Chair
Professor Darwin Berg, Co-Chair
Professor Mauricio Montal

2010

The Thesis of Polly Pu Huang is approved and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California, San Diego

2010

TABLE OF CONTENTS

LIST OF TABLES & FIGURES

ACKNOWLEDGEMENTS

I would like to give special thanks to everyone at “Yakshville,” for their help and guidance throughout this project. I need to thank Shelle Malkmus, Joanne Steinauer, and Bethany Fitzsimmons for their assistance with surgeries, staining, and behavior studies. Without their help, my research would have taken twice as long.

Professor Imran Khan, who was a former professor at UCSD Pharmacology Department in the School of Medicine, has been an invaluable resource. My undergraduate research experience began when he offered me a laboratory position in his lab nearly 4 years ago. His intellectual and technical guidance helped optimize the models used in this series of studies and doubtlessly helped lift this project off the ground. Dr. Khan has made a tremendous contribution to my research experience and to my academic career.

I would like to give special recognition to Dr. Edgar Salazar-Grueso (deceased) of Solstice Neurosciences for his intellectual contribution to the project during preliminary stages. Solstice Neurosciences Inc. has been an invaluable materials resource, without which this research would not have gone this far.

Shelle Malkmus carried out experiments on dose tolerability of botulinum neurotoxin B (BoNT-B) in the mouse. Damon McCumber carried out experiments on BoNT-B effect on formalin-induced flinching and neuropathic pain in the rat.

Parts of this thesis were submitted as abstracts and presented in poster format at American Pain Society Conference 2009 and the Society for Neuroscience Conference 2010. Works are cited as follows, respectively:

PP Huang, IM Khan, TL Yaksh. Intrathecal Botulinum Toxin B: Spinal Substance P release and Nociception in the Mouse Formalin Pain Model. San Diego, CA: American Pain Society, 2009. The Journal of Pain 1 April 2009; 10:4, S30.

PP Huang, TL Yaksh, IM Khan. Intrathecal Botulinum Neurotoxin B: Mouse Formalin Pain Model and Spinal Nerve Ligation Neuropathic Pain Model. Program No. 457.13. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

ABSTRACT OF THE THESIS

Intrathecal Botulinum Neurotoxin B: Effect on Spinal Primary Afferent Neurotransmitter Release, Inflammatory Nociception and Neuropathic Pain in the Mouse

by

Polly Pu Huang

Master of Science in Biology

University of California, San Diego, 2010

Professor Tony Yaksh, Chair
Professor Darwin Berg, Co-Chair

Botulinum neurotoxin B (BoNT-B) mediates proteolytic cleavage of VAMP I/II (synaptobrevins I/II) in the family of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. Such cleavage of SNARE proteins inhibits vesicle-membrane

fusion at the synaptic terminal and inhibits neurotransmitter release. The aim of this study is to investigate the effects of intrathecal (IT) delivery of BoNT-B on neurotransmitter release from spinal primary afferent C-fibers and the effects on nociception in mouse. In the formalin mouse model, intraplantar (IPLT) formalin induces substance-P (SP) release from primary afferent C-fibers. SP binding to neurokinin-1 receptors (NK1-R) on dorsal horn neurons resulting in receptor internalization can be visualized using immunohistochemistry and confocal microscopy. Mice that received IT BoNT-B prior to IPLT formalin showed reduction in NK1-R internalization and spinal C-Fos protein activation. In contrast, intrathecal co-injection of BoNT-B and anti-BoNTB antibody showed prevention of these effects on neurotransmitter release. Intrathecal BoNT-B pretreatment also attenuated phase 2, and not phase 1, of formalin flinching behavior. To investigate effects of IT BoNT-B on neuropathic pain, mice received spinal nerve ligation (SNL) of the left L5 spinal nerve. SNL induced tactile allodynia 14 days post ligation upon stimulation using von Frey filaments. Mice that received IT BoNT-B showed increased paw withdrawal threshold, compared to vehicle animals, for the next 15 days. The data suggest that IT BoNT-B blocks neurotransmitter release from spinal primary afferent C-fibers in a facilitated state, attenuates formalin-induced nociceptive response, and attenuates spinal nerve injury- induced neuropathic pain, in the absence of motor impairment.

I. INTRODUCTION

Botulinum neurotoxins (BoNTs) are metalloproteases produced by *Clostridium botulinum.* They mediate proteolytic cleavage of subunits in the family of soluble N-methylaleimide-sensitive attachment protein receptor (SNARE) proteins in the synaptic terminal. There are seven BoNT serotypes (A-G). Each neurotoxin serotype specifically and non-competitively cleaves one site of a member of the SNARE protein complex. Botulinum neurotoxin serotype A (BoNT-A) cleaves SNAP-25. Botulinum neurotoxin serotype C (BoNT-C) cleaves syntaxin. Botulinum neurotoxin serotypes B, F, and G cleave VAMP/synaptobrevin protein (Schiavo et al, 1994). Such enzymatic cleavage results in inhibition of vesicle-membrane fusion and prevents the release of neurotransmitters. BoNT blockade of acetylcholine from motor neurons at the neuromuscular junction results in flaccid muscle paralysis. The muscle-relaxing effect of botulinum neurotoxin serotype A (BoNT-A) has common applications in cosmetic procedures and as a symptomatic treatment for chronic muscle spasm syndromes, such as torticollis. Botulinum neurotoxin serotype B (BoNT-B) is the only FDA approved botulinum neurotoxin for treatment of cervical dystonia. While previous studies have shown effects of BoNTs on acetylcholine release from motor neurons and effects on muscle, effects on release of other neurotransmitters from other types of neurons are less defined. While intramuscular effects and peripheral effects of BoNTs have been well-studied, this series of studies is focused on the spinal action of botulinum neurotoxin serotype B (BoNT-B).

SNARE proteins mediate neurotransmitter release

During neurotransmitter release, influx of calcium activates synapsin protein tethered at neurotransmitter vesicles, resulting in mobilization of vesicles. At the synaptic membrane,

formation of SNARE protein complex mediates vesicle-membrane fusion and neurotransmitter release via exocytosis. SNARE protein complex consists of syntaxin, synaptobrevin, and SNAP-25. Syntaxin is tethered at the plasma membrane by its C-terminus. Synaptobrevin isoforms I and II, or vesicle-associated membrane protein (VAMP I/II), are tethered at the vesicle membrane and are cleavage target of BoNT-B. SNAP-25 is anchored at the plasma membrane. Fusion of syntaxin, synaptobrevin, and SNAP-25, resulting in formation of a four-α-helix bundle is critical for vesicle fusion with the plasma membrane and release of neurotransmitters (Sutton et al, 1998). Enzymatic cleavage of SNARE proteins by BoNTs renders SNARE proteins unable to fold and form helical bundles.

BoNT translocation and action

Botulinum neurotoxin structure consists of light chain (LC) and heavy chain (HC). Light chain is the protease domain which enzymatically cleaves target SNARE protein. Heavy chain receptor domain mediates binding to membrane receptor and heavy chain translocation domain mediates translocation of toxin protein across the plasma membrane into neurons. Ganglioside GT1b, which resides on plasma membrane, has been shown to be a complementary receptor component for BoNT binding during translocation (Kozaki et al, 1998). During translocation, the heavy chain serves a conduit for light chain domain as the toxin protein is internalized into the cytoplasm, where it enzymatically cleaves its target protein (Koriazova et al, 2002; Montal, 2008). In addition to ganglioside binding, BoNT-B translocates via binding to synaptotagmin I and II, which are transiently expressed on the plasma membrane during vesicle recycling (Dong et al, 2003). However, studies to investigate translocation of BoNT-B and BoNT-F in hippocampal neurons show toxin entry independent of vesicle recycling and synaptotagmin expression

(Verderio et al, 1999; Grumelli et al, 2005). This property of BoNTs entry into neurons suggests that translocation and toxin internalization depends on combination of binding proteins and gangliosides that mediate toxin translocation in different types of neurons (Montecucco et al. 2004; Grumelli et al, 2005).

BoNT-B specifically cleaves VAMP I/II at amino acid residues Gln/76-Phe/77 (Schiavo et al, 1992). Cleavage is believed to be dependent not only on the target cleavage site, but also recognition of three-dimensional structure. Thus, amino acid residues flanking the cleavage site also plays a critical role in toxin recognition and proteolytic cleavage (Foran et al, 1994).

Based on the mechanism of binding and translocation of BoNTs, drug action and toxicity critically depends on active heavy chain domains of the toxin protein, as well as its target binding sites. Toxin activity after translocation into the cytoplasm depends on enzymatic activity of long chain protease and recognition of cleavage site. These mechanisms of BoNT-B action contribute to the specificity of BoNT-B proteolytic cleavage of VAMP I/II and effects on central and peripheral neurons.

Effects of BoNTs on neurotransmitter release

Previous studies have extensively shown effects of BoNTs on acetylcholine release from motor neurons and effects on muscle contractility. It is important, however, to note that SNARE proteins play a ubiquitous role in vesicle-membrane coupling process during neurotransmitter release in both peripheral and central nervous systems. Despite this general mechanism of neurotransmitter release, relatively little work has focused on the effects of BoNTs on neurotransmitter release in systems other than the neuromuscular junction. The issue of interest is the potential effects of BoNTs on neurotransmitter release in the central nervous

system. Several studies have demonstrated that treatment of neuronal systems and cell cultures that BoNT-A inhibits release of acetylcholine, substance P (Welch et al, 2000), glutamate (McMahon et al, 1992), and calcitonin gene-related peptide (Morris et al, 2001; Durham et al, 2003). The majority of this work has focused on *ex vivo* systems and the effects on behavior have been described in a limited fashion. The effects upon peptide release from dorsal root ganglion cells raises the interesting likelihood that BoNT effects are manifested on the release of excitatory neurotransmitters in the spinal dorsal horn from high threshold unmyelinated primary afferent neurons. As such, it suggests the speculative hypothesis that spinal action of BoNTs might produce effects on pain behavior. However, given the potent and ubiquitous effects on neurotransmitter release, spinal effects may reflect a general impairment of all spinal motor and sensory functions. In addition, while these effects of BoNT-A on neurotransmitter release have been extensively described, the effects of BoNT-B (Myobloc®) is not well-described in the literature. This thesis presents an investigation of BoNT-B on substance-P release by analysis of SP-specific neurokinin-1 receptor internalization.

Effects of BoNTs on pain

SNARE protein cleavage resulting in blockade of neurotransmitter release also has applications in blockade of sensory input. Discussion of potential role of BoNTs in pain requires a brief review of major classes of pain mechanisms mediated by primary afferents and spinal cord, which are initiated by tissue and nerve injury.

In the case of tissue injury, peptidergic sensory afferent fibers are activated and release excitatory neurotransmitters. Primary afferents terminate in the spinal dorsal horn. Such release activates local second order dorsal horn interneurons and neurons which project to higher

center. This pathway leads to supraspinally organized behavioral response to the nociceptive stimulus (e.g. escape and withdrawal response). In addition, persistent activation of sensory afferents induced by tissue injury and nerve injury lead to initiation of a facilitated state of sensory processing in the dorsal horn. Such spinal systems underlie the phenomena of hyperalgesia, in which normally innocuous stimuli leads to aversive sensations and typical behavioral response to pain (Yaksh et al, 1999).

One example of such a preclinical model to induce excitatory neurotransmitter release from primary afferents and initiate a state of facilitated spinal processing is the formalin-pain model. Intraplantar delivery of irritant chemical formalin activates primary afferent C-fibers through activation of TRPA1 channel on the afferent terminal (McNamara, 2007), resulting in an initial pronounced activation followed by persistent ongoing low level of discharge from primary afferent C-fibers. Persistent C-fiber activation by formalin, over the ensuing 60 minutes, results in biphasic activation of dorsal horn wide dynamic range (WDR) neurons (Dickenson and Sullivan, 1987) and biphasic flinching of the injected hind paw (Yaksh et al, 2001). Biphasic WDR neuron activation mirrors flinching behavior temporally and in amplitude (Puig and Sorkin, 1996). It is widely believed that phase I flinching response reflects the potent early activation of small afferents: Aδ and C-fibers. Phase 2 flinching response involves persistent small afferent input and onset of a facilitated state in the spinal dorsal horn. This persistent activation of chemically responsive C-fibers by formalin leads to long-term potentiation of excitability of second order neurons (Yaksh et al, 2001). Blockade of neurotransmitter release from primary afferents results in reduced activation and facilitation of dorsal horn interneuron, which consequently reduces central pain processing. It is important to note that phase 1 and phase 2 of formalin response reflects distinct dorsal horn processes. Thus, the spinal delivery of agents

such as opiates will reduce phase 1 and phase 2. In contrast, other agents, such as gabapentin, NK1-R antagonists, cyclooxygenase inhibitors and NMDA receptor antagonists diminish phase 2 with no effect on phase 1.

Peripheral nerve injury results in symptoms of neuropathic pain in humans and in rodents. Such nerve injury frequently leads to hypersensitivity to normally innocuous stimulus or hyperalgesia. Typically, this hypersensitivity is manifested in an exaggerated response to light tough, referred to as tactile allodynia. Several factors contribute to neuropathic pain after nerve injury. Interactions between injured and intact neurons contribute hyper-excitability of primary sensory neurons (Devor and Wall, 1990). Increased neurotransmitter release from primary afferent sensory neurons has also been shown in previous studies of nerve injury-induced neuropathic pain (Gardell et al, 2003; Coderre et al, 2005). It is important to note that neuropathic pain models and tissue injury models vary in their pharmacology, which suggests different underlying mechanisms. Blockade of neurokinin-1 receptor (NK1-R), which responds to peptidergic neurotransmitter substance-P released from primary afferent C-fibers, blocks inflammatory acute nociception. However, such blockade does not affect neuropathic pain behavior; blockade of glutamate release from primary afferents attenuates neuropathic pain behavior. Intrathecal delivery of opiods and cyclooxygenase inhibitors also have little effect on tactile allodynia, whereas agents such as NMDA receptor agonists and gabapentin are effective (Sorkin and Yaksh, 2009).

A few previous studies have examined effects of BoNTs on acute and neuropathic pain *in vivo*. Interpretation of these studies requires consideration of the pain model and route of drug delivery.

BoNTs have been described to attenuate formalin-induced licking behavior while normal grooming behavior is unaffected (Luvisetto, 2009). Plantar injection of BoNT-A has been shown to attenuate carrageenan- and formalin-induced inflammatory pain and capsicin-induced hyperalgesia (Cui et al, 2004; Luvisetto et al, 2006; Bach-Rojecky and Lackovic, 2005). Intradermal BoNT-A injection attenuates neuropathic behavior induced by infraorbital nerve constriction and inhibits neurotransmitter release from trigeminal ganglion neurons in rat (Kitamura et al, 2009). Intraplantar injection of BoNT-A in the hind paw before carageenan administration in the ipsilateral hind paw reduced hypersensitivity (Favre-Guilmard et al, 2009). BoNT-A applied to the peripheral hind paw diminished allodynia otherwise produced by nerve ligation (Park et al, 2006). Peripheral and intrathecal injection of BoNT-A also alleviated allodynia in a diabetic rat model of neuropathic pain (Bach-Rojecky et al, 2010). Such peripheral drug effects are believed to reflect local action of BoNTs on peripheral terminal excitability or central transport, where there would be block of central neurotransmitter release from primary afferents.

Based on the review on the role of BoNTs in preclinical models of pain, it is evident that topical and intrathecal treatment with BoNTs have an anti-hyperalgesic effect. Limited data on peripheral and central delivery of BoNTs also suggest that the effects may occur in the absence of toxicity and motor function impairment. An important limitation of the studies published thus far is that there has been little effort to correlate central effects with measure of neurotransmitter release and anti-nociception. The objective of this study is to investigate the effects of spinally delivered botulinum neurotoxin serotype B (BoNT-B) on primary afferent release from identified populations of nociceptive afferents and consequent changes in pain behavior.

Peripheral delivery of BoNTs has applications in cosmetic treatment for facial wrinkles and treatment of chronic muscle spasm. Peripheral injection of BoNTs provides local effects of BoNTs on nearby synaptic terminals. Intramuscular injection of BoNTs results in localized toxin penetration into motor neurons at the neuromuscular junction and prevents acetylcholine release from these neurons, resulting in flaccid muscle paralysis. Therefore, peripheral delivery of BoNTs has direct effect on peripheral synaptic terminals and on muscle function. Spinal delivery of BoNTs, in contrast, has direct effect on synaptic terminals in the central nervous system. This permits examination of toxin effects on synaptic terminals located in the CNS, such as spinal primary afferent fibers, and examination of drug effects at the spinal level in an intact animal. Consistent with the mechanisms of BoNT translocation and internalization into neuron cytosol at the synaptic terminal, spinal delivery of BoNTs shows its effect on primary sensory afferents which terminate at the spinal cord.

Based on review of spinal action of BoNTs, investigation of spinally delivered BoNT-B can shed light on an alternative method for pain management. This can also provide insight on therapeutic targets for acute nociception and neuropathic pain induced by nerve injury. Peripheral delivery of BoNTs as a potential method for clinical pain therapy has been explored in previous studies published thus far. For example, relief of chronic muscle spasms after intramuscular injection of BoNT-A or BoNT-B is believed to be the result of reduction in muscle ischemia. Activation of nociceptors by ischemic muscles is believed to be the cause of painful symptoms of chronic muscle spasm (Mense, 2004). However, little work has been done to examine spinal delivery of BoNTs for similar clinical purposes.

Hypotheses

This thesis is focused on effects of spinal botulinum neurotoxin B (BoNT-B) on primary afferent neurotransmitter release and pain in the mouse. The following hypotheses are specifically addressed:

1. Spinal delivery of BoNT-B produces a persistent block of formalin-evoked neurotransmitter substance-P (SP) release from primary afferent sensory neurons. This blockade is accompanied by a reduction in activation of non-afferent dorsal horn internerons.

   Assessment of neurotransmitter release: Intrathecal (IT) delivery of BoNT-B (Myobloc ® Solstice Neurosciences) in the lumbar region provides local delivery of BoNT-B at the location where primary afferent C-fibers from the hind paws terminate. Stimulation of the hind paws, such as chemically with intraplantar (IPLT) formalin in the hind paw or a sharp pinch of the paw, induces neurotransmitter release from spinal primary afferent C-fibers. Substance P (SP), neurotransmitter peptide released by spinal primary afferent C-fibers, specifically binds to neurokinin 1 receptors (NK1-R) in the superficial dorsal horn (laminae I and II) causing receptor internalization. Quantification NK1-R internalization is an assay for neurotransmitter release from spinal primary C-fibers (Nazarian et al, 2008). This method allows us to assess neurotransmitter release in vivo.

   Assessment of post-synaptic activation: Activation of primary sensory afferents leads to release of neurotransmitters which consequently activate second order interneurons in the spinal dorsal horn. Second order neurons project to higher centers

where sensory input is integrated as the sensation of pain, touch, heat or pressure, and produce the behavioral response to the sensory stimulus. C-Fos is an early gene expressed upon neuronal activation. C-Fos protein expression in second order neurons in superficial dorsal horn at 1-2 hours after application of painful stimulus, such as intraplantar formalin, serves as an assay for primary afferent sensory input. Central neuronal activation is proportional to primary afferent sensory input. Visualization of C-Fos protein expression after IPLT formalin serves as an additional assay for spinal BoNT-B effect on primary afferent neurotransmitter release. We hypothesize that intrathecal BoNT-B effect on formalin-induced substance-P release and NK1-R internalization is accompanied by reduction in spinal C-Fos protein expression.

2. IT BoNT-B at dose which blocks primary afferent neurotransmitter release also attenuates formalin-induced nocifensive behavioral response.

Tissue injury model of pain behavior: Intraplantar injection of formalin induces flinching behavioral response which lasts approximately 40 minutes after IPLT formalin injection. Measurements of formalin-induced flinching response using an automated motion detection device provide an assessment of BoNT-B effect on nocifensive behavior (Dubuisson and Dennis, 1977; Wheeler-Aceto et al, 1990; Yaksh et al, 2001). Formalin is a chemical stimulus which persistently activates primary afferent C-fibers, which results in 3 phases of sensory neuronal activation – early, middle, and late phase. Phasic activation of wide dynamic range (WDR) neurons located in the spinal superficial dorsal horn results in biphasic flinching behavior. Initial early phase of C-fiber activation leads to long-term potentiation (LTP) of WDR neurons, or the phenomenon termed

"wind-up." Phase II of formalin-evoked flinching response is mediated by early phase of C-fiber action and reflects the consequent 2nd phase central facilitation. Therefore, formalin-evoked flinching behavior is nocifensive behavior that is mediated by activation of primary afferent sensory fibers. BoNT-B blockade of primary afferent neurotransmitter release would also affect central facilitation of flinching behavior.

3. IT BoNT-B attenuates neuropathic hyperalgesia induced by peripheral nerve injury.

Model of neuropathic hyperalgesia: Peripheral nerve injuries result in symptoms of neuropathic pain in humans and in rodents, such as hypersensitivity to normally innocuous stimulus or hyperalgesia. Spinal nerve ligation of the L5 spinal nerve of the mouse is one of several established rodent models nerve injury-induced neuropathic pain, which mimics symptoms seen in humans (Kim and Chung, 1994; Bennett and Xie, 1988; Seltzer et al, 1990). Several factors contribute to neuropathic pain after nerve injury. Interactions between injured and intact neurons contribute hyper-excitability of primary sensory neurons (Devor and Wall, 1990). Increased neurotransmitter release from primary afferent sensory neurons has also been shown in previous studies of nerve injury-induced neuropathic pain (Gardell et al, 2003; Coderre et al, 2005). Consistent with BoNT-B effect on neurotransmitter release, we hypothesized that IT delivery of BoNT-B also has inhibitory effect on elevated neurotransmitter release induced by nerve injury and that IT BoNT-B consequently attenuates neuropathic pain behavior. The mouse spinal nerve ligation model (Kim and Chung, 1994) is used in order to assess the effect of IT BoNT-B on spinal nerve injury-induced neuropathic pain.

4. Establish tolerable doses of BoNT-B which block primary afferent neurotransmitter release and attenuates acute and neuropathic pain with no effect on motor function.

    Dose tolerability study of BoNT-B: Toxicity after intrathecal injection of BoNT-B can be assessed by observation of clinical indications and motor function. Clinical indications of toxicity include piloerection, weight decrease, motor impairment, abnormal respiration, abnormal grooming behavior, and abnormal exploratory behavior. Motor function can be assessed in terms of grip strength, gait, ambulatory ability, and motor coordination. Examination of an animal's ability to remain suspended on a wire mesh by gripping with front and hind paws provides dynamic assessment of normal motor coordination, gait, grip strength, and exploratory behavior.

5. Spinal BoNT-B effects on primary afferent neurotransmitter release, inflammatory pain and neuropathic hyperalgesia are the result of VAMP I/II specific cleavage in the mouse.

    VAMP I/II cleavage by BoNT-B: Active and mobile toxin is required to translocate into neuron cytosol and proteolytically cleave target SNARE protein. Incubation of VAMP I/II with BoNT-B is expected to result in the cleavage of VAMP I/II protein. This confirms enzymatic activity of BoNT-B. Alternatively, antibody binding to BoNT-B protein would render the toxin unable to translocate across the terminal membrane. Antibody binding also renders the enzymatic domain inactive. We hypothesize that co-injection of BoNT-B with anti-BoNT-B antibody reverses its effects on formalin-evoked neurotransmitter release. Co-injection of BoNT-B and antibody, therefore, permits assessment of BoNT-B activity *in vivo*.

Specificity of BoNT-B to mouse VAMP I/II: BoNT-B proteolytically cleaves VAMP I/II at Gln/76-Phe/77 in the mouse and human. BoNT-B resistance in the rat is due to the replacement of phenylalanine for valine at amine acid residue 77 of VAMP I protein. In order to confirm BoNT-B effects in the mouse is due to VAMP I/II specific cleavage by the toxin, resistance to BoNT-B in the rat was explored as a negative control for BoNT-B effects on formalin-evoked flinching response.

References

Back-Rojecky L, Relja M, Lackovic Z. Botulinum toxin type A in experimental neuropathic pain. *J Neural Transm*. 2005 Feb;112(2):215-9.

Bach-Rojecky L, Salkovic-Petrisic M, Lackovic Z. Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy: bilateral effect after unilateral injection. *Eur J Pharmacol*. 2010 May 10;633(1-3):10-4.

Bennett GJ and Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain.* 1988 Apr;33(1):87-107.

Coderre TJ, Kumar N, Lefebvre CD and Yu JS. Evidence that gabapentin reduces neuropathic pain by inhibiting spinal release of glutamate. *J Neurochem.* 2005 Aug;94(4):1131-9.

Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. *Pain*. 2004 Jan;107(1-2):125-33.

Devor M and Wall PD. Cross-excitation in dorsal root ganglia of nerve-injured and intact rats. *J Neurophysiol.* 1990 Dec;64(6):1733-46.

Dubuisson D and Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain.* 1977 Dec;4(2):161-74.

Durham PL, Cady R, Cady R. Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. *Headache.* 2004 Jan;44(1):35-42.

Foran P and Shone CC. Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry*. 1994 Dec 27;33(51):15365-74.

Gardell LR, Vanderah TW, Gardell SE, Wang R, Ossipov MH, Lai J and Porreca F. Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation. *J Neurosci.* 2003 Sep 10;23(23):8370-9.

Grumelli C. and Verderio C. Internalization and mechanism of action of clostridial toxins in neurons. *Neurotoxicology.* 2005 Oct;26(5):761-7.

Kim SH and Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain.* 1992 Sep;50(3):355-63.

Kitamura Y, Matsuka Y, Spigelman I, Ishihara Y, Yamamoto Y, Sonoyama W, Kuboki T and Oguma K. Botulinum toxin type A (150 kDa) decreases exaggerated neurotransmitter release from trigeminal ganglion neurons and relieves neuropathy behaviors induced by infraorbital nerve constriction. *Neuroscience*. 2009 Apr 10;159(4):1422-9.

Koriazova LK and Montal M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol.* 2003 Jan;10(1):13-8.

Kozaki S, Kamata Y and Watarai S. Ganglioside GT1b as a complementary receptor component for Clostridium botulinum neurotoxins. *Microb Pathog.* 1998 Aug;25(2):91-9.

Luvisetto S, Marinelli S, Cobianchi S, Pavone F. Antiallodynic efficacy of botulinum neurotoxin A in a model of neuropathic pain. *Neuroscience.* 2007;145: 1-4.

Luvisetto S, Marinelli S, Lucchetti F, Marchi F, Cobianchi S, Rossetto O, Montecucco C, Pavone F. Botulinum neurotoxins and formalin-induced pain: central vs. peripheral effects in mice. *Brain Res.* 2006 Apr 12;1082(1):124-31.

Luvisetto S, Marinelli S, Rossetto O, Montecucco C, Pavone F. Central injection of botulinum neurotoxins: behavioral effects in mice. *Behav Pharmacol.* 2004 May;15(3):233-40.

Luvisetto S, Rosetto O and Montecucco C. Toxicity of botulinum neurotoxins in central nervous system of mice. *Toxicon.* 2003 Mar;41(4):475-81.

McMahon HT, Foran P, Dolly JO, Verhage M, Wiegant VM, Nicholls DG. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, γ-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. *J Biol Chem.* 1992 Oct 25;267(30):21338-43.

Montal M. Translocation of botulinum neurotoxin light chain protease by the heavy chain protein-conducting channel. *Toxicon.* 2009 Oct;54(5):565-9

Montecucco C. and Schiavo G. Structure and function of tetanus and botulinum neurotoxins. *Q Rev Biophys.* 1995 Nov;28(4):423-72.

Morris JL, Jobling P, Gibbins IL. Differential inhibition by botulinum neurotoxin A of cotransmitters release from autonomic vasodilator neurons. *Am J Physiol Heart Circ Physiol.* 2001 Nov;281(5):H2124-32.

Nazarian A, Gu G, Gracias NG, Wilkinson K, Hua XY, Vasko MR, Yaksh TL. Spinal N-methyl-D-aspartate receptors and nociception-evoked release of primary afferent substance P. *Neuroscience.* 2008 Mar 3;152(1):119-27.

Schiavo G and Benfenati F. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*. 1992 Oct 29;359(6398):832-5.

Schiavo G, Malizio C, Trimble W, De Laureto PP, Milan G, Sugiyama H, Johnson EA, Montecucco C. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. *J Biol Chem.* 1994 Aug 12;269(32):20213-6.

Schiavo G. and Matteoli M. Neurotoxins affecting neuroexocytosis. *Physiol Rev.* 2000 Apr;80(2):717-66.

Schiavo G and Shone CC. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J Biol Chem.* 1993 Jun 5;268(16):11516-9.

Seltzer A, Dubner R and Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain.* 1990 Nov;43(2):205-18.

Verderio C, Coco S, Rossetto O, Montecucco C, Matteoli M. Internalization and proteolytic action of botulinum toxins in CNS neurons and astrocytes. *J Neurochem.* 1999 Jul;73(1):372-9.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U. SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron.* 2004 Feb 19;41(4):599-610.

Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia to Clostridium botulinum neurotoxins. *Toxicon.* 2000 Feb;38(2):245-58.

Wheeler-Aceto H, Porreca F, Cowan A. The rat paw formalin test: comparison noxious agents. *Pain.* 1990 Feb;40(2):229-38.

Yaksh TL and Ozaki G. An automated flinch detecting system for use in the formalin nociceptive bioassay. J Appl Physiol. 2001 Jun;90(6):2386-402.

II. METHODS

Animals

All studies undertaken in this thesis were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. Adult male C57/Bl6 mice and Sprague Dawley rats (Harlan Sprague Dawley Inc., Indianapolis, IN). Mice were housed in vivarium minimum of 2 days before use, maintained on a 12/12 hour day-night cycle, and are given food and water *ad libitum.*

Drug delivery

Mouse intrathecal injections: Animals were anesthetized using 5% isoflurane before and 3% isoflurane during the IT injection. A 30G needle was inserted into the intrathecal space at L5-L6, and a tail flick was observed as indication of correct placement of the injection. Drugs were delivered using a Hamilton syringe. Intrathecal injections of NaCl 0.9% or drug were given in volumes of 5µL for each dose given, unless otherwise stated.

Rat intrathecal injections: While anesthetized using 5% isoflurane, rats were prepared with lumbar intrathecal (IT) catheters as previously described (Yaksh TL and Rudy TA, 1976; Yaksh TL and Stevens CW, 1986). After 5-7 days recovery, rats were entered into the study to receive drug in volume of 10µL, unless otherwise stated. Each injection is followed by 10µL of saline flush.

In mouse studies, formalin was injected in the plantar surface of the hind paw using a 30G needle and Hamilton syringe. For rat studies, intraplantar formalin was delivered to the hind paw.

Drugs

Botulinum neurotoxin B (BoNT-B; Myobloc®, Solstice Neurosciences Inc., South San Francisco, CA) was provided in solution containing 5000U/mL. BoNT-B was diluted in NaCl 0.9% such that the dose to be delivered was present in a volume of 5µL for mouse studies and 10µL for rat studies, unless otherwise stated. In both rat and mouse studies, intraplantar formalin (5%, 20µL) was given in the left hind paw. For IT injections of BoNT-B titer, BoNT-B was incubated with anti-BoNT-B antibody (rabbit polyclonal; Abcam Inc., San Francisco, CA) at effective binding concentrations determined in titer assay for 1 hour at 37°C prior to IT injection.

Morbidity and motor function

Morbidity: Mice received intrathecal injection of BoNT-B at doses 0.1U-50U. Clinical observations were made for indications of toxicity: piloerection, labored breathing, normal grooming behavior, normal ambulation, and exploratory behavior. In order to assess weight loss, mice that received maximal tolerable dose of BoNT-B were weighed 7 days after intrathecal injection.

Grip strength: Five days after receiving IT NaCl 0.9% or BoNT-B 0.5U, animals were placed on top of a wire mesh mounted on a scale. The experimenter lifts the animal from the wire mesh by the tail. The grip strength is recorded as the maximal force at the point of release from the wire mesh.

Motor function: Animals were required to remain suspended underneath a wire mesh for up to 1 minute. When placed upside down under the wire mesh, animals were required to grip onto the wire grid with front and hind paws, and grip strength is required to support their

body weight. Normal exploratory movement while suspended underneath the wire mesh was also observed, as indication of exploratory behavior and motor coordination.

Formalin-induced flinching

A metal band was placed on the left hind paw of the mouse and acclimated in the behavior testing chamber at least 1 hour prior to formalin injection. Intraplantar (IPLT) injection of formalin (5%, 20µL) was administered in the marked hind paw and the animal was immediately returned to the detection device. Flinching behavior was collected using an automated system for 40 minutes following IPLT formalin injection (Yaksh TL et al, 2001).

Spinal nerve ligation (SNL)

Same spinal nerve ligation procedure was used for rats and mice. Animals were anesthetized using xylazine (10mg/kg) and ketamine (100mg/kg) cocktail and 3% isoflurane during ligation surgery. The L6 transverse process is removed to expose the L5 and L6 nerve roots. A 6-0 silk suture is tied on the L5 spinal nerve distal to the spinal cord. Animals were given 1mL lactated Ringer's solution with Carprofen subcutaneously immediately after surgery. At 5-7 days after ligation surgery, increase in tactile hypersensitivity was observed (tactile allodynia).

Tactile Allodynia Assessment

Animals were allowed to acclimate in clear plastic testing chambers on wire mesh for at least 1 hour prior to testing. To assess paw withdrawal threshold (PWT), von Frey filaments (Touch Test® Sensory Evaluators) were applied to the left hind paw which is ipsilateral to the site

of spinal nerve ligation. Using the up-down method of von Frey filament application (Chaplan et al, 1994), six readings were taken for statistical analysis of tactile threshold.

Spinal Cord Harvest

For NK1-R internalization and spinal C-Fos expression studies, animals were sacrificed at 10 minutes and 2 hours, respectively, after IPLT formalin using intraperitenial (IP) Euthosol (200µL). Animals were intracardially perfused with 0.9% NaCl, and fixed using 4% paraformaldehyde. Laminectomy was performed to extract the spinal cord. Harvested spinal cords were incubated for 24 hours in 4% paraformaldehyde, followed by 20% sucrose and 30% sucrose. For Western blot assays for VAMP I/II cleavage, animals were anesthetized using 5% isoflurane and sacrificed by decapitation. Spinal cord was harvested by hydroextrusion. Spinal cord was placed in lysis buffer (0.5% Triton X-100, 50mM Tris, 150mM NaCl, and nanopure H_2O), with protease and phosphatase inhibitors (Sigma Aldrich Co., St. Louis, MO), and homogenized by sonication.

Immunohistochemistry

For NK1-R internalization studies, lumbar tissue samples of L2-L5 incubated in NK1-R (Advanced Targeting Systems, San Diego, CA) and NeuN (Chemicon, Billerica, MA) antibodies; followed by 2º Antibodies Fluoroscein (FITC)-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG and Rhodamine Red™-X-conjugated AffiniPure Donkey Anit-Mouse IgG (JacksonImmuno Research Laboratories, West Grove, PA).

For C-Fos expression studies, lumbar tissue samples of L2-L5 were incubated in anti-C-Fos (CalBiochem, EMD Chemicals, Gibbstown, NJ) and biotin-conjugated anti-NeuN (Chemicon,

Billerica, MA) primary antibodies. Tissue was then incubated in fluorescent conjugated 2º antibodies: Streptavidin-Alexa Fluor ® 555 conjugate and Alexa Fluor® 488 goat anti-rabbit IgG (Molecular Probes, Invitrogen Inc., Carlsbad, CA).

For BoNT-B penetration studies, 30µm lumbar tissue sections were incubated in anti-BoNTB (Abcam) and anti-NeuN (Chemicon, Billerica, MA) primary antibodies, followed by incubation in goat AlexaFluor® 555 conjugated anti-mouse and goat AlexaFluor ® 488 anti-rabbit secondary antibodies.

Dorsal horn regions of antibody-stained spinal cord tissue were scanned using confocal microscopy (Leica TCS SP5). Images were viewed on Adobe Photoshop CS2 ®.

Western Blotting

For synaptobrevin cleavage studies, spinal cord was extracted by hydroextrusion and homogenized in lysis buffer (50mM Tris, 15mM NaCl, nanopure H_2O) and protease and phosphatase inhibitor cocktails (Sigma Aldrich Co., St. Louis, MO). Tissue lysate was centrifuged at 14,500rpm at 4°C and supernatant was extracted, followed by incubation with BoNT-B at 37°C for 1 hour. Control tissue lysate and incubated tissue lysate was quantitized for protein content using BCA® Protein Assay Kit (Thermo Fischer Scientific Inc.), with Albumin Standard (Thermo Fischer Scientific Inc.). Protein samples were loaded into SDS-PAGE (NuPAGE ®,Invitrogen™, Life Technologies™, Carlsbad, CA) with DTT (Sigma Aldrich®) and LDS loading sample buffer (NuPAGE®, Invitrogen™, Life Technologies™, Carlsbad, CA). Molecular weights were compared to SeeBlue® Plus 2 Standard (Invitrogen™). SDS-PAGE gel was transferred to nitrocellulose membrane, followed by blocking with 5% non-fat milk in TBST (50mM Trizma® Pre-Set Crystals, 150mM NaCl, 0.1% Tween-20). Blot was probed using rabbit anti-synaptobrevin 1/2/3 polyclonal

(Synaptic Systems, Gottengen, Germany; diluents: 5% BSA-TBST, 10% NaN_3) and anti-rabbit-HRP conjugated secondary antibody (diluents: 5% non-fat milk in TBST).

For BoNT-B and antibody titer assay, BoNT-B was diluted in 0.9% NaCl and incubated in anti-BoNTB antibody (Abcam Inc., San Francisco, CA) in various concentrations. Samples were then run on native gel (NativePAGE®, Expedeon Inc., San Diego, CA) with sample loading buffer (Expedeon Inc.). Gel was then transferred to nitrocellulose membrane, blocked, and incubated in anti-botulinum toxin B antibody (Abcam Inc.), followed by anti-rabbit-HRP conjugated.

Development of blots was performed using SuperSignal®west Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.). For synaptobrevin cleavage studies, blots were reprobed for β-tubulin. Protein band intensity ratio was compared to β-tubulin band intensity using Image Quant software.

References

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994 Jul;53(1):55-63.

Hylden JLK and Wilcox GL. Intrathecal morphine in mice: a new technique *Eur J Pharmacol.* 1980 Oct 17;67(2-3):313-6.

Kim SH and Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain.* 1992 Sep;50(3):355-63.

Nazarian A, Gu G, Gracias NG, Wilkinson K, Hua XY, Vasko MR, Yaksh TL. Spinal N-methyl-D-aspartate receptors and nociception-evoked release of primary afferent substance P. *Neuroscience.* 2008 Mar 3;152(1):119-27.

Wheeler-Aceto H, Porreca F, Cowan A. The rat paw formalin test: comparison noxious agents. *Pain.* 1990 Feb;40(2):229-38.

Yaksh TL and Rudy TA. Chronic catheterization of the spinal subarachnoid space. *J Neurosci Methods*. 2004 Feb 15;133(1-2):65-9.

Yaksh TL and Ozaki G. An automated flinch detecting system for use in the formalin nociceptive bioassay. *J Appl Physiol*. 2001 Jun;90(6):2386-402.

III. RESULTS

Dose tolerability of BoNT-B in the mouse

Morbidity: High dose BoNT-B given intrathecally has adverse effects, such as motor impairment, respiratory difficulty, and death. Typically these effects are severe to the extent that euthanasia is required. In order to determine a maximal tolerable dose of intrathecally delivered BoNT-B in the mouse, animals received intrathecal (IT) injection of BoNT-B, were observed for clinical indications of toxicity and were tested for motor impairment and grip strength. At doses 1-50U given intrathecally, onset of motor disability was observed within 24 hours after the injection. Because of evident motor impairment and clinical indications of toxicity, animals underwent sacrifice. The maximal tolerable dose was determined to be 0.5U/5µL, with survival and normal motor function observed at 7 days after IT injection (table 1). In addition, weight loss is monitored as one clinical indication for toxicity. Animals that received BoNT-B 0.5U/5µL also did not show significant weight loss at 7 days after intrathecal injection, as compared to IT saline pre-treated animals (figure 1A).

Motor function: Mice that received IT BoNT-B 0.5U/5µL showed similar grip strength of the hind paw, as compared to IT NaCl animals at 5 days after IT injection (figure 1B). The same animals were required to hang underneath a wire mesh for up to 1 minute. Animals that received the maximal tolerable dose of BoNT-B showed normal motor coordination, grip strength and exploratory behavior on the wire mesh. The time required to remain suspended upside down underneath the wire mesh was not different for saline and BoNT-B treated groups. IT BoNT-B 0.5U was established as the maximal tolerable dose for experiments from this point forward.

Presence of cytosolic BoNT-B immunoreactivity after IT delivery

In the absence of effects on motor function at maximal tolerable dose of IT BoNT-B, BoNT-B ability to translocate into neurons was investigated. Mouse lumbar spinal cord tissue was harvested and visualized for presence of BoNT-B in the cytosol using immunohistochemistry and confocal microscopy. In the absence of IT BoNT-B, background immune-fluorescence for BoNT-B is present, but not specific co-localization of BoNT-B and NeuN immunoreactivity (figure 2A). At 2 hours after IT BoNT-B injection, high background immunofluorescence for BoNT-B was observed. However, specific co-localization of BoNT-B and NeuN was not observed at this time point (figure 2B). At 2 days after IT BoNT-B, co-localization of neuronal nuclei (NeuN) and BoNT-B immunoreactivities were observed (figure 2C-E). Appearance of BoNT-B immunoreactivity surrounding and co-localization with neuronal nuclei is indicative of internalization of BoNT-B into the cytoplasm.

BoNT-B cleavage of spinal VAMP I/II

In order to determine the cleavage of spinal VAMP I/II by active BoNT-B, spinal cord tissue lysate was incubated with BoNT-B. Western blot of control spinal cord tissue lysate shows both VAMP I (19kDa) and VAMP II (17kDa) proteins. Tissue lysate incubated with BoNT-B show significant decrease in VAMP I/II whole protein (figure 3). VAMP I/II cleavage product of smaller molecular weight was not observed, most likely due to rapid enzymatic degradation.

Characterization of NK1-R expression and internalization

In order to quantify NK1-R internalization, we first standardized characterization of NK1-R expression and internalization at high magnification (63X). In confocal images of lumbar tissue

stained for NeuN and NK1-R immunoreactivity (figure 4A-F), NK1-R expression is characterized by appearance of an outline of NK1-R immunoreactivity surrounding NeuN immunoreactivity. NK1-R internalization is characterized distortion of the NK1-R outline and appearance of endosomes indicative of NK1-R internalization. This indicates migration of NK1-R immunoreactivity as receptors undergo internalization. Confocal images of mouse lumbar dorsal horns are counted for NK1-R expression and internalization at high magnification based on these criteria.

We then characterized NK1-R expression in the superficial dorsal horn rostrocaudally along the lumbar segment. Confocal images from L1-L5 show statistically similar numbers of NeuN(+), NK1-R expressing neurons (approximately 30%)in the superficial dorsal horn (figure 4G).We next examined lumbar segments L1-L5 for maximal formalin-induced NK1-R internalization. Naïve, unstimulated mice showed minimal NK1-R internalization in the left and right dorsal horns: approximately 20-30%; this was established as constitutive NK1-R internalization in the absence of stimulation. Representative confocal images from animals that received IPLT formalin show increasing NK1-R internalization from L1 through L5. NK1-R internalization in the ipsilateral dorsal horn was maximal in L4-L5 at 10 minutes after formalin injection (figure 4H). In subsequent studies of formalin-evoked NK1-R internalization, representative confocal images from L4-L5 were counted for NK1-R expression and NK1-R internalization.

Effects of IT BoNT-B on formalin-evoked neurotransmitter release

In order to investigate the effect of spinal BoNT-B on primary afferent neurotransmitter release, the formalin model of facilitated release was employed. As reviewed in the

introduction, intraplantar (IPLT) formalin evokes sustained substance P (SP) release from spinal primary afferent C-fibers. SP specifically binds to neurokinin-1 receptors (NK1-R) in the superficial dorsal horn (laminae I and II), causing receptor internalization. Quantification of NK1-R internalization in the superficial dorsal horn was used as an assay for neurotransmitter release. Animals received IT NaCl or BoNT-B 0.1U-0.5U 2 days prior to IPLT formalin in the left hind paw. In animals that received intrathecal (IT) NaCl pretreatment, dorsal horn ipsilateral to the site of formalin injection shows marked increase in NK1-R internalization, approximately 53%, as compared to the contralateral unstimulated dorsal horn, approximately 25% internalized (figure 5A). In contrast, animals that received IT BoNT-B 0.5U pretreatment showed approximately 25% NK1-R internalization in the ipsilateral dorsal horn; this is a marked reduction in NK1-R internalization compared to ipsilateral dorsal horn of vehicle animal (figure 5B). NK1-R internalization in the contralateral dorsal horns of IT BoNT-B and NaCl pretreated animals did not show significant difference after IPLT formalin (figure 5C). In addition, at 5 days after IT pretreatment, animals that received IT BoNT-B 0.1U and 0.5U similarly showed reduction in IPLT formalin-evoked NK1-R internalization, as compared to vehicle animals (figure 6A-D). Both 2-day and 5-day pretreatment in mice showed statistically similar reduction in percentage of NK1-R internalization after IPLT formalin (figure 5C). Overall, the data suggest that IT BoNT-B pretreatment inhibits of IPLT formalin-evoked SP release from primary afferent C-fibers. Two-day and 5-day IT BoNT-B pretreatment showed comparable reduction in NK1-R internalization, which suggests a sustained effect of BoNT-B on primary afferent neurotransmitter release.

Effects of IT BoNT-B on dorsal horn C-Fos activation

Based on IT BoNT-B effects on formalin-evoked neurotransmitter release from primary afferent sensory neurons, we next investigated whether IT BoNT-B pre-treatment would result in suppression of non-afferent neuron activation after IPLT formalin. C-Fos protein expression, which is an immediate early gene expressed upon neuronal activation, is used in our study as a marker for central neuronal activation. Confocal images of spinal cord tissue showed C-Fos protein activation in the spinal dorsal horn, characterized by co-localization of C-Fos immunoreactivity and NeuN immunoreactivity (figure 7A-C). At 2 hours after IPLT injection, formalin induced elevated C-Fos protein expression in the ipsilateral dorsal horn in vehicle animals that received IT NaCl 2 days prior to formalin (figure 7D). In contrast, animals that received IT BoNT-B 0.1U and 0.5U 2-day pretreatment showed marked decrease in formalin-evoked C-Fos expression in the ipsilateral dorsal horn (figure 7E-F), as compared to vehicle animals. Confocal images of the unstimulated contralateral dorsal horn did now show statistical difference in C-Fos expression between treatment groups (figure 7G). The data suggest that IT BoNT-B pre-treatment, which blocked neurotransmitter release from primary afferent C-fibers, also reduced activation of superficial dorsal horn interneurons upon stimulation by a painful chemical stimulus.

Effects of IT BoNT-B on formalin-induced nocifensive behavior

To determine whether the effects of IT BoNT-B on primary afferents fibers at doses which block neurotransmitter release and C-Fos activation alters nocifensive behavior, the effects of IT BoNT-B on formalin-induced flinching response was examined. Mice received IT NaCl or IT BoNT-B 0.1U-0.5U pretreatment 2 days prior to formalin-evoked flinching

measurements. IPLT formalin evoked robust biphasic flinching behavior in mice that received IT NaCl pretreatment. Mice that received IT BoNT-B 0.5U 2-day pretreatment showed significant reduction in phase II, but not phase I, of formalin-evoked flinching response, as compared to vehicle animals (figure 8). The data suggest that IT BoNT-B pretreatment reduces chemically induced nocifensive behavior in the mouse.

Effects of IT BoNT-B on neuropathic pain

Spinal nerve injury induces long-term neuropathic hyperalgesia characterized by long-term hypersensitization of peripheral sensory neurons. This results in tactile allodynia or decreased paw withdrawal threshold (PWT) when stimulated by a normally innocuous stimulus. Mice that received spinal nerve ligation (SNL) of the L5 spinal nerve show significant decrease in paw withdrawal threshold in response to von Frey filaments, as compared to naïve non-ligated animals by 14-15 days after ligation surgery. Immediately following baseline withdrawal threshold measurements, single IT NaCl or BoNT-B 0.1U-0.5U was administered and withdrawal thresholds were assessed up to 15 days after IT injection. Animals that received SNL and IT NaCl maintained paw withdrawal threshold below 1g for the duration of assessment, with little change from baseline PWT. In contrast, animals that received IT BoNT-B 0.5U showed increase from baseline PWT within 6 hours after IT treatment. These animals also showed statistically significant and persistent increase in PWT from baseline for the next 15 days after IT treatment (figure 9). The data suggests long-term sustained alleviation of hyperalgesia by IT BoNT-B, as well as long-term dose-tolerability of BoNT-B in the mouse.

Prevention of BoNT-B and effect on primary afferent neurotransmitter release

Blockade of substance P release from primary afferent C-fiber by intrathecal delivery BoNT-B should required an enzymatically active and mobile neurotoxin. We hypothesized that co-injection of BoNT-B with anti-BoNTB antibody inhibits the effects on neurotransmitter release shown in previous experiments. BoNTB-antibody titer assay showed effective binding concentrations of BoNTB and antibody, as assessed on NativePAGE. BoNTB concentration equivalent to IT injection of 0.5U/5µL incubated with anti-BoNTB antibody at 1:10 dilution, and BoNTB concentration equivalent to IT BoNT-B 1U/5µL incubated with antibody at 1:5 dilution (figure 10A) resulted in loss of free toxin. Mice received IT BoNT-B 0.5U and 1U co-injected with antibody at respective titer concentrations 2 days prior to IPLT formalin. Lumbar spinal cord tissue was then analyzed for NK1-R internalization. No statistical difference was shown in NK1-R internalization between mice that received IT NaCl and animals that received IT BoNTB-antibody co-injection. In contrast, mice that received IT BoNT-B 0.5U, without antibody, showed reduced NK1-R internalization, as compare to IT NaCl pretreated animals (figure 10B). Thus, incubation with anti-BoNT-B antibody most likely renders the neurotoxin immobile and unable to be internalized across the synaptic terminal membrane. Antibody binding also most likely inhibited BoNT-B enzymatic activity on its target SNARE protein.

Resistance to BoNT-B in the rat

In order to confirm BoNT-B effects on primary afferent sensory C-fiber neurotransmitter release and nociception in the mouse are indeed the result of BoNT-B specific cleavage of VAMP I/II, BoNT-B resistance in the rat was used in this study as a negative control for VAMP I/II cleavage. In the mouse and human, BoNT-B cleaves VAMP I protein at amino acid residues

Gln/76-Phe/77, which results in inhibition of neurotransmitter release. However, rat resistance to BoNT-B is most likely due to the replacement of amino acid residue Val for Gln in VAMP I protein at the cleavage site. In a separate dose tolerability study, IT BoNT-B 100U did not produce motor impairment or toxicity in the rat (data not shown). This dosage is equivalent to concentration administered in intramuscular injections in humans. Single IT BoNT-B, NaCl or placebo was administered 15 minutes or 3 days prior to IPLT formalin. Animals that received IT NaCl or placebo showed typical biphasic flinching behavior measured using an automated motion detection device up to 40 minutes. Rats that received IT BoNT-B in doses of 50U-100U 15-minute and 3-day pretreatments did not show difference in IPLT formalin-evoked flinching behavior when compared to rats that received IT placebo or IT NaCl (figure 11A-B). IPLT BoNTB pretreatment in the hind paw also did not show effect on IPLT formalin-evoked flinching behavior, as compared to vehicle animals (figure 9C). Furthermore, IT BoNT-B did not reduce SNL-induced tactile allodynia in the rat (figure 12), as compared to allodynic rats that received IT NaCl or IT placebo. The data in the rat suggest that BoNT-B specific and active enzymatic cleavage of VAMP I/II is required to produce effects of BoNT-B on neurotransmitter release, nociception, and SNL-induced hyperalgesia in the mouse.

Acknowledgements

Shelle Malkmus carried out experiments on dose tolerability of botulinum neurotoxin B (BoNT-B) in the mouse. Damon McCumber carried out experiments on BoNT-B effect on formalin-induced flinching and neuropathic pain in the rat.

Parts of this chapter were submitted as abstracts and presented as a poster at American Pain Society Conference 2009 and the Society for Neurocience Conference 2010. Works are cited as follows, respectively:

PP Huang, TL Yaksh, IM Khan. Intrathecal Botulinum Neurotoxin B: Mouse Formalin Pain Model and Spinal Nerve Ligation Neuropathic Pain Model. Program No. 457.13. 2009 Neuroscience Meeting Planner. Chicago, IL: Society for Neuroscience, 2009. Online.

PP Huang, IM Khan, TL Yaksh. Intrathecal Botulinum Toxin B: Spinal Substance P release and Nociception in the Mouse Formalin Pain Model. San Diego, CA: American Pain Society, 2009. The Journal of Pain 1 April 2009; 10:4, S30.

Table 1. BoNT-B dose tolerability in the mouse.
Table shows summary of clinical observations following intrathecal injection of BoNT-B. Maximal tolerable dose was determined to be BoNT-B 0.5U/ 5µL. * Clinical observation was made at time of sacrifice.

BoNT-B Doses	Route	Survival (days)	N	Clinical Obs*
50U/10uL	IT	1	3	moribund
5U/5uL	IT	1	2	moribund
1U/5uL	IT	1	2	motor impairment
0.5U/5uL	IT	>2	2	normal
0.1U/5uL	IT	>2	2	normal
0.5U/5uL	IT	>7	4	normal
0.1U/5uL	IT	>7	4	normal

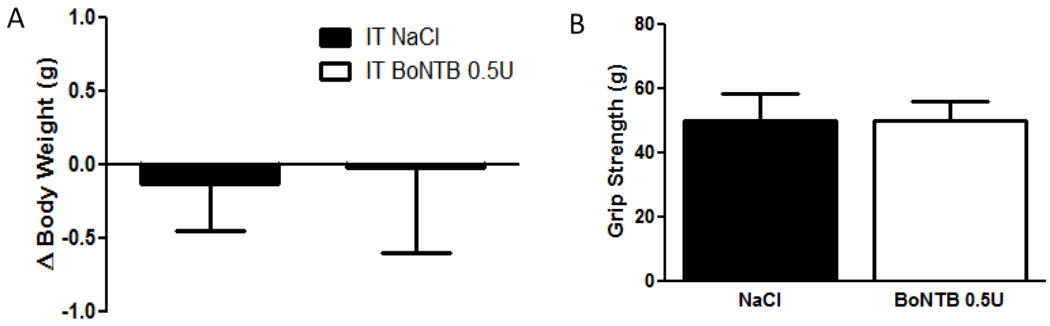


Figure 1. BoNT-B effect on motor function and toxicity in the mouse
A) Grip strength of mice that received IT NaCl (vehicle, n=5) or IT BoNTB 0.5U (n=5) 5 days prior to measurement on top of a wire mesh mounted on a weigh scale. Grip strength was recorded as the maximum force at the point of release from the wire mesh. B) Histogram shows change in body weight of mice that received IT NaCl (vehicle, n=4) and IT BoNTB 0.5U (n=6) measured 7 days after IT injection.

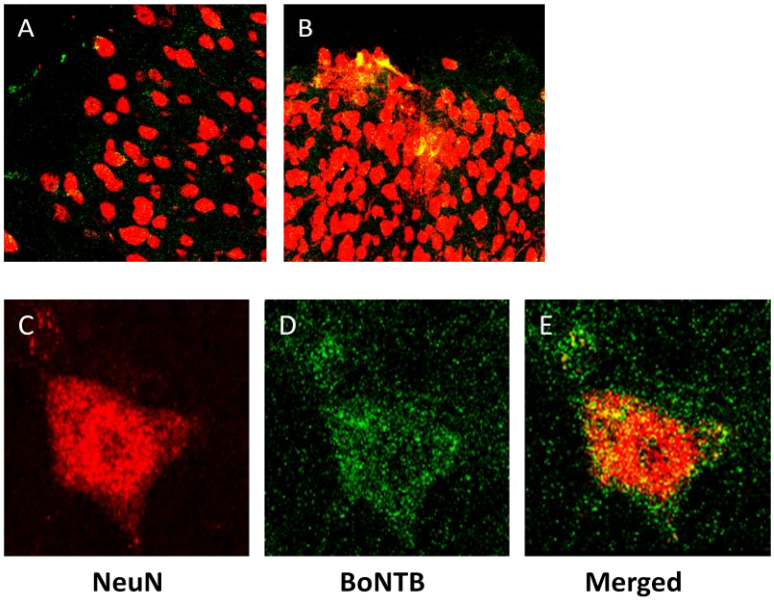


Figure 2. Cytosolic BoNT-B

Representative confocal images spinal cord section immune-stained for BoNT-B (green) and NeuN (red). A) Spinal cord section of a mouse that received IT NaCl 2 days prior to sacrifice and tissue harvest. B) Shows BoNT-B immunoreactivity throughout the spinal cord section from a mouse that received IT BoNT-B 0.5U two hours prior to tissue harvest. C-E) Confocal image showing BoNT-B in cytoplasm of interneurons in superficial dorsal horn. Tissue harvested 2 days after IT BoNT-B 0.5U injection. Image shows specific co-localization (merged, yellow) of NeuN (red) and BoNT-B (green) immunoreactivity.

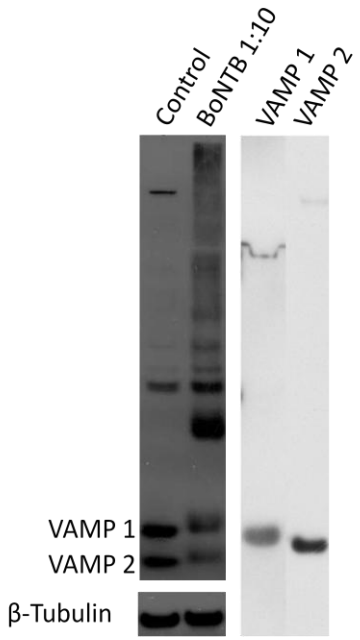


Figure 3. BoNT-B cleavage of spinal VAMP I/II

Immunoblots show VAMP I/II protein of control lumbar spinal cord tissue lysate and tissue lysate incubated with BoNT-B (1:10 concentration). VAMP I and II bands are confirmed by VAMP I (19kDa) and VAMP II (17kDa) synthetic peptides. Band intensity for control and BoNTB treated tissue VAMP I and II is compared to β-Tubulin.

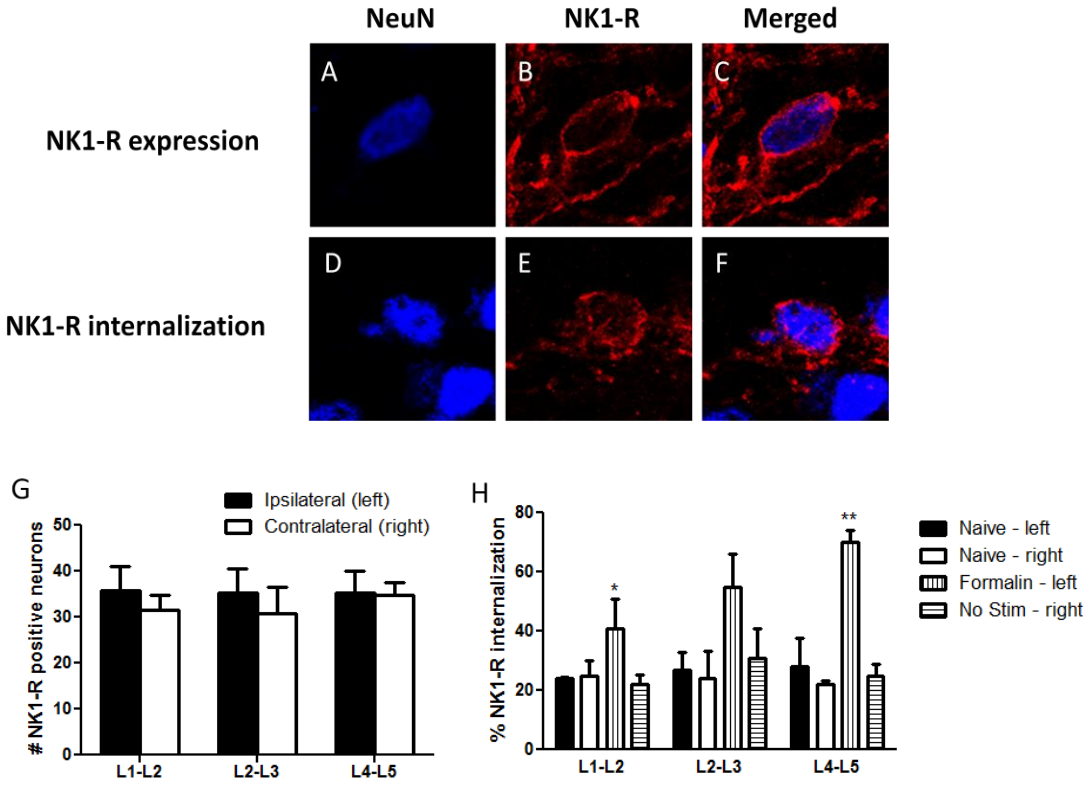


Figure 4. Characterization of NK1-R expression and internalization in the spinal dorsal horn. A-C) Examples of neuron in the dorsal horn showing expression of neurokinin-1 receptors. NK1-R expression is characterized by co-localization of NeuN and NK1-R immunoreactivity. Merged image shows outlined of NK1-R on the membrane. D-F) Neuron showing NK1-R internalization, characterized by co-localization of NeuN and NK1-R, as well as distortion of membrane outline marked by NK1-R immunoreactivity and formation of endosomes indicative of receptor internalization. G) Graph showing number of neurons showing NK1-R expression in spinal dorsal horn along the lumbar segment. NK1-R expression is shown to be consistent along the lumbar spinal cord. H) Graph shows NK1-R internalization along the lumbar segment at 10 minutes after IPLT formalin in the left hind paw (all n=3). (*and ** $P<0.05$)

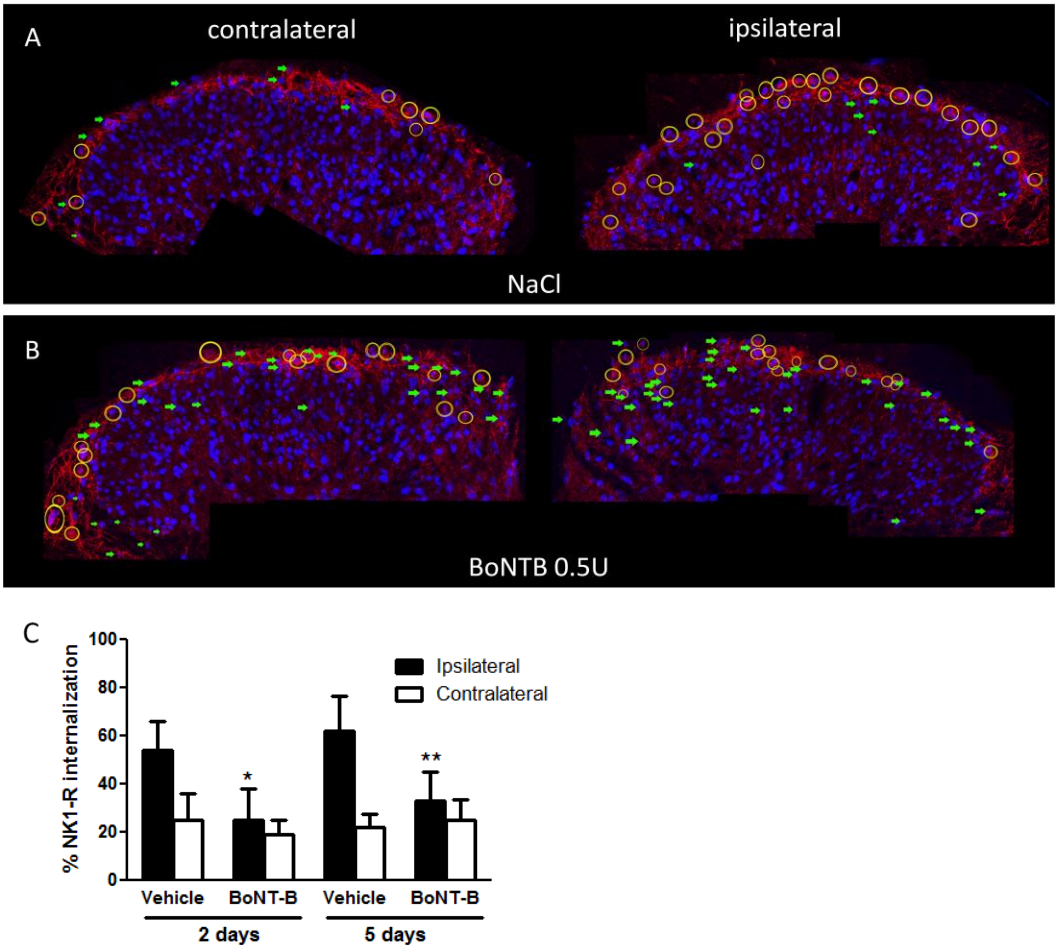
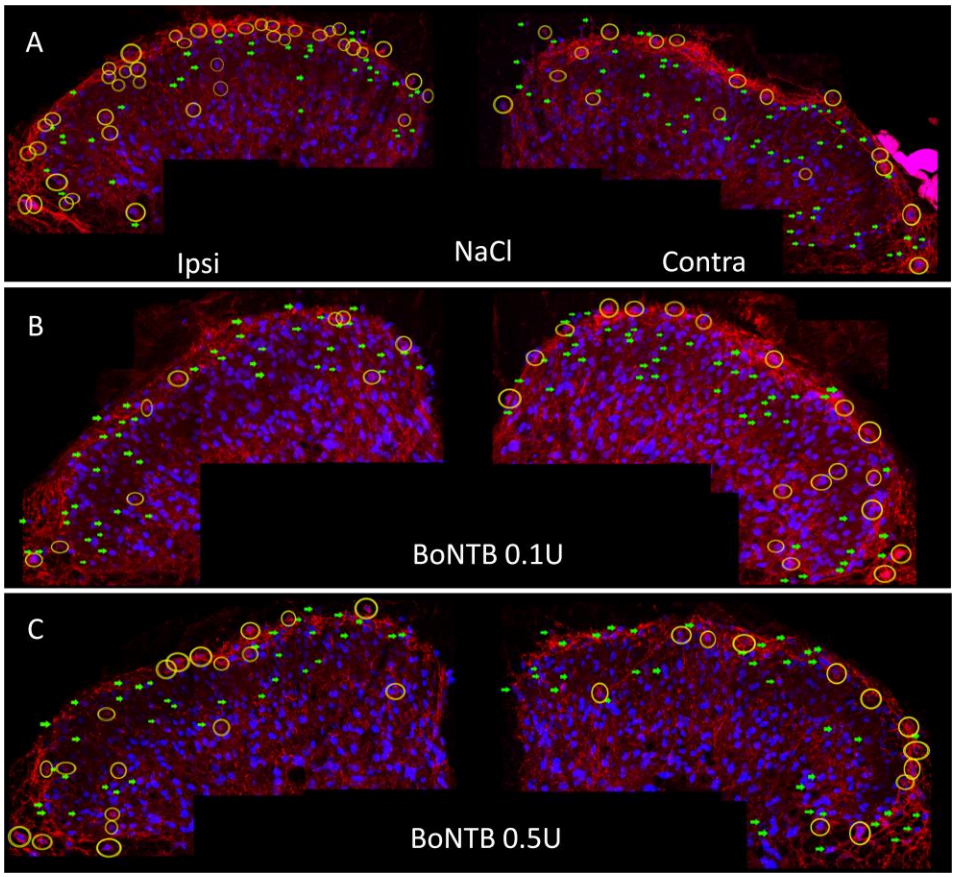
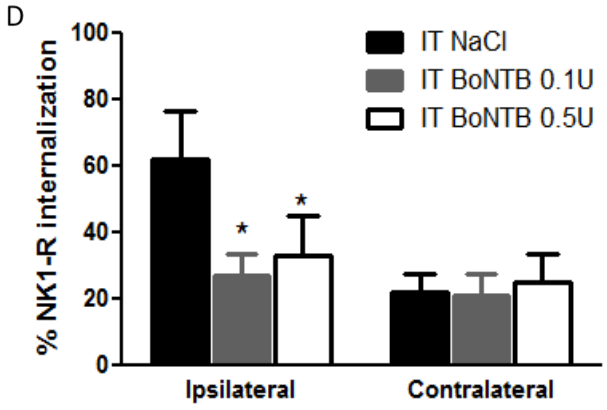


Figure 5. IT BoNT-B on neurotransmitter release from spinal primary afferent C-fibers.
A-B) Figure shows representative confocal images of dorsal horns of 2-day IT BoNTB pretreated and vehicle animals. Both the ipsilateral and contralateral dorsal horns are counted for NK1-R expression (green arrows), and for NK1-R internalization (yellow circles). At 10 mins after IPLT formalin IT NaCl pretreated animal shows significant increase in NK1-R internalization in the dorsal horn ipsilateral to the site of formalin injection. In contrast, in the IT BoNTB pre-treated animal, ipsilateral formalin-induced NK1-R internalization is significantly reduced, as compared to the vehicle animal. There was no significant difference in contralateral NK1-R internalization between the BoNTB pretreated and vehicle animals. C) Percentage of NK1-R expressing neurons in the dorsal horn that show internalization after IPLT formalin. Ipsilateral dorsal horn shows marked increase in NK1-R internalization in the vehicle animals (n=5) after IPLT formalin. Animals that received IT BoNTB 0.5U pretreatment 2 days (n=5) and 5 days (n=4) prior to formalin showed marked reduction in NK1-R internalization after IPLT formalin in the hind paw. Both vehicle and BoNTB pre-treated animals showed similar NK1-R internalization in the contralateral dorsal horn (* and ** $P \leq 0.05$).

Figure 6. Long-term effects of IT BoNTB on formalin-evoked NK1-R internalization
A-C) Figure shows representative confocal images of dorsal horns of IT BoNTB pretreated and vehicle animals. Mice received IT injections 5 days prior to IPLT formalin. Both the ipsilateral and contralateral dorsal horns are counted for NK1-R expression (green arrows), and for NK1-R internalization (yellow circles). At 10 mins after IPLT formalin IT NaCl pretreated animal shows significant increase in NK1-R internalization in the dorsal horn ipsilateral to the site of formalin injection. In contrast, in the IT BoNTB 0.1U (n=4) and BoNTB 0.5U (n=4) pre-treated animal, ipsilateral formalin-induced NK1-R internalization is significantly reduced, as compared to the vehicle animals (n=4). There was no significant difference in contralateral NK1-R internalization between the BoNTB pretreated and vehicle animals. D) Quantitation of NK1-R internalization in IT BoNTB 0.1U – 0.5U pre-treated and vehicle animals after IPLT formalin. Mice received IT BoNTB or NaCl 5 days before formalin. (* and ** $P \leq 0.05$)


A
Ipsi
NaCl
Contra
B
BoNTB 0.1U
C
BoNTB 0.5U


D
% NK1-R internalization
100
80
60
40
20
0
IT NaCl
IT BoNTB 0.1U
IT BoNTB 0.5U
*
*
Ipsilateral
Contralateral

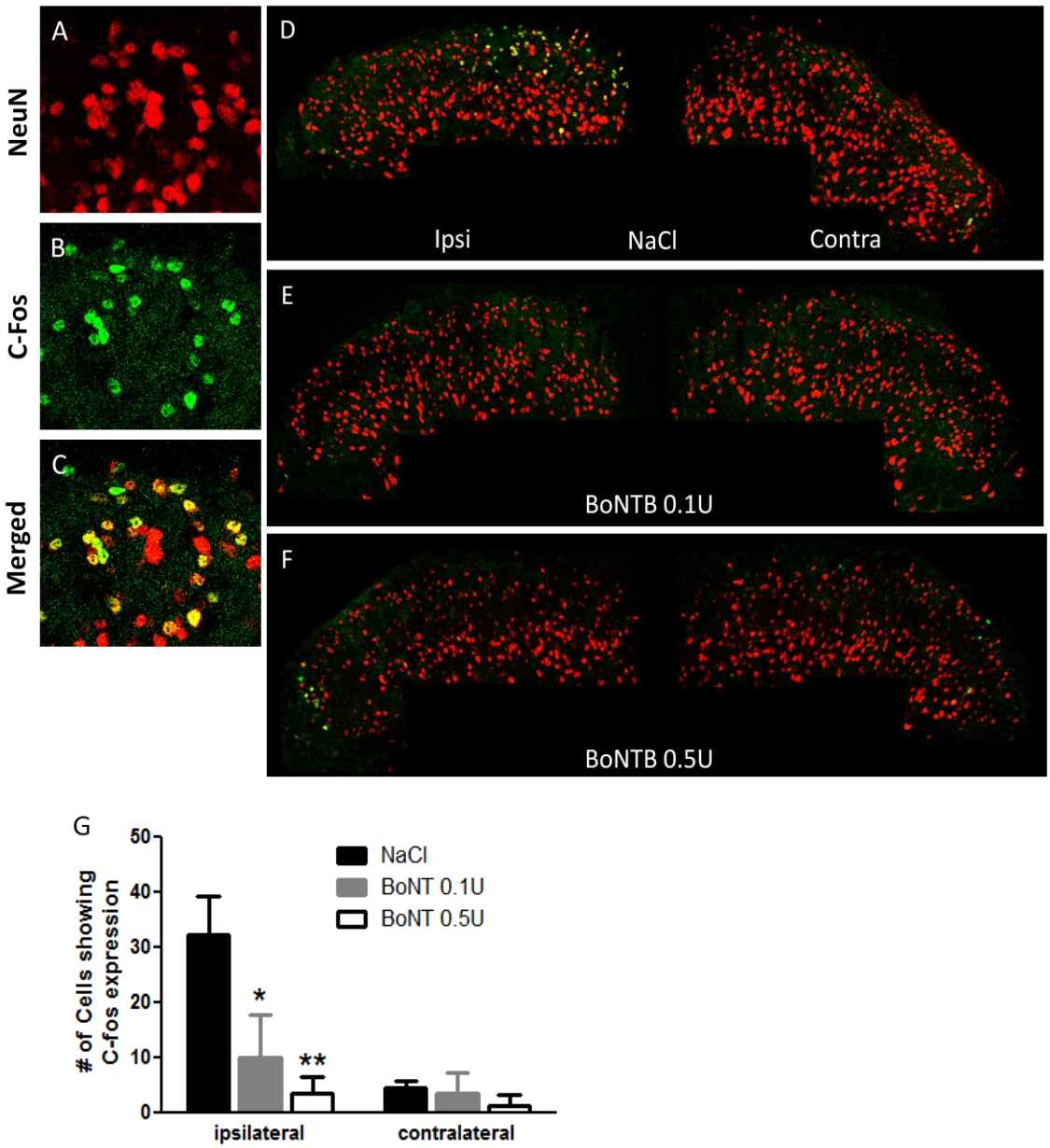


Figure 7. IT BoNT-B reduces IPLT formalin-evoked C-Fos activation.
A-C) Co-localization of NeuN and C-Fos immunoreactivity indicating C-Fos expression at 2 hrs after IPLT formalin. D-F) Confocal images representative of C-Fos expression after IPLT formalin. In animals that received IT NaCl 2 days prior to formalin, IPLT formalin induced marked increase of C-Fos expression in ipsilateral dorsal horn interneurons. In contrast, mice that received IT 2-day pretreatment of either BoNTB 0.1U (n=4) or 0.5U (n=5) showed significant reduction in C-Fos protein expression, as compared to IT NaCl pretreated animals (n=5). G) Quantification of C-Fos protein expression in the superficial dorsal horn at 2 hours after IPLT formalin. (* and **P<0.01)

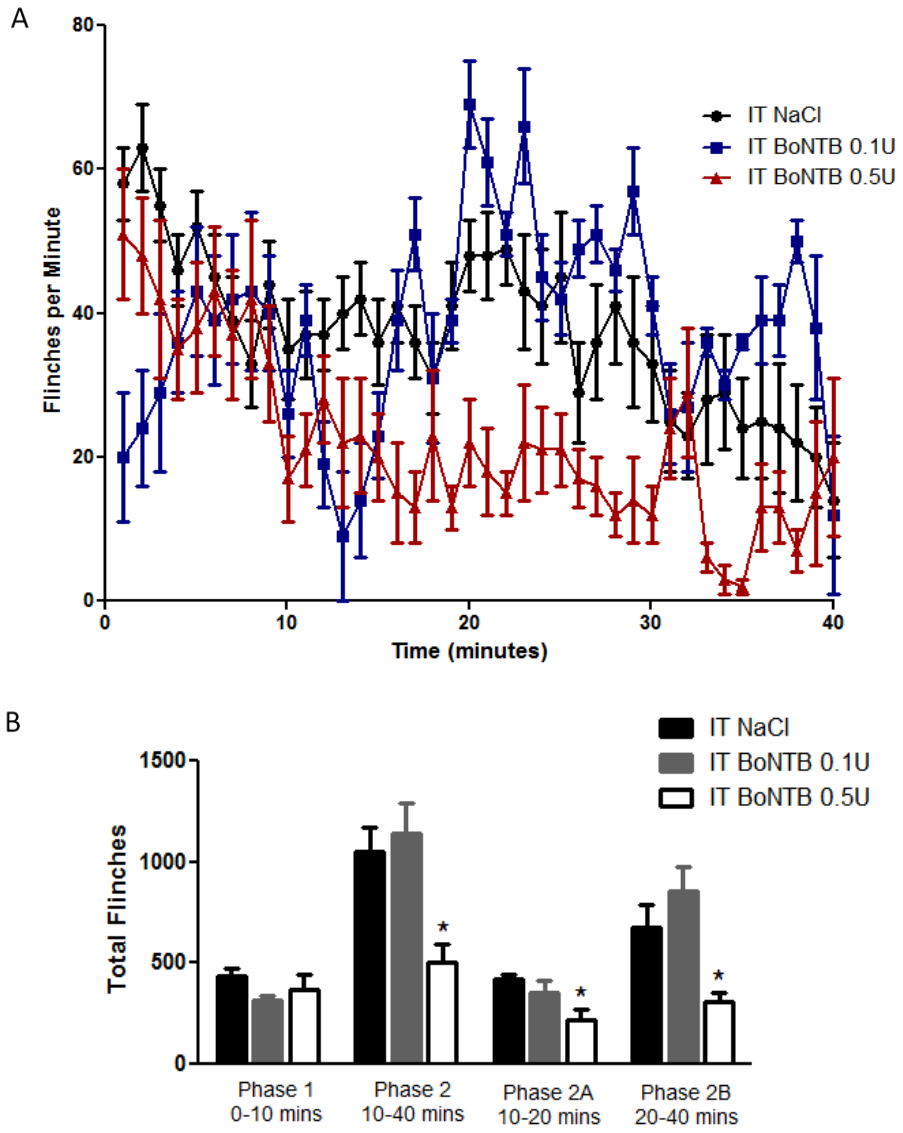


Figure 8. Effects of BoNT-B on formalin-evoked flinching behavior in the mouse
Mice received IT BoNTB 0.1U (n=8) or 0.5U (n=11) and IT NaCl (n=12) 2 days prior to IPLT formalin injection. A) Time course of formalin-evoked flinching behavior measured up to 40 mins after formalin. In IT NaCl pre-treated animal (black), IPLT formalin evoked biphasic flinching behavior. In contrast, IT BoNTB 0.5U pretreated animal showed marked decrease in phase II (10-40mins), but not phase I (0-10mins), flinching behavior. B) Cumulative number of flinches compared between phases. IT BoNTB 0.5U pretreated animals showed significant decrease in phase II of formalin-induced flinching behavior (*P<0.05).

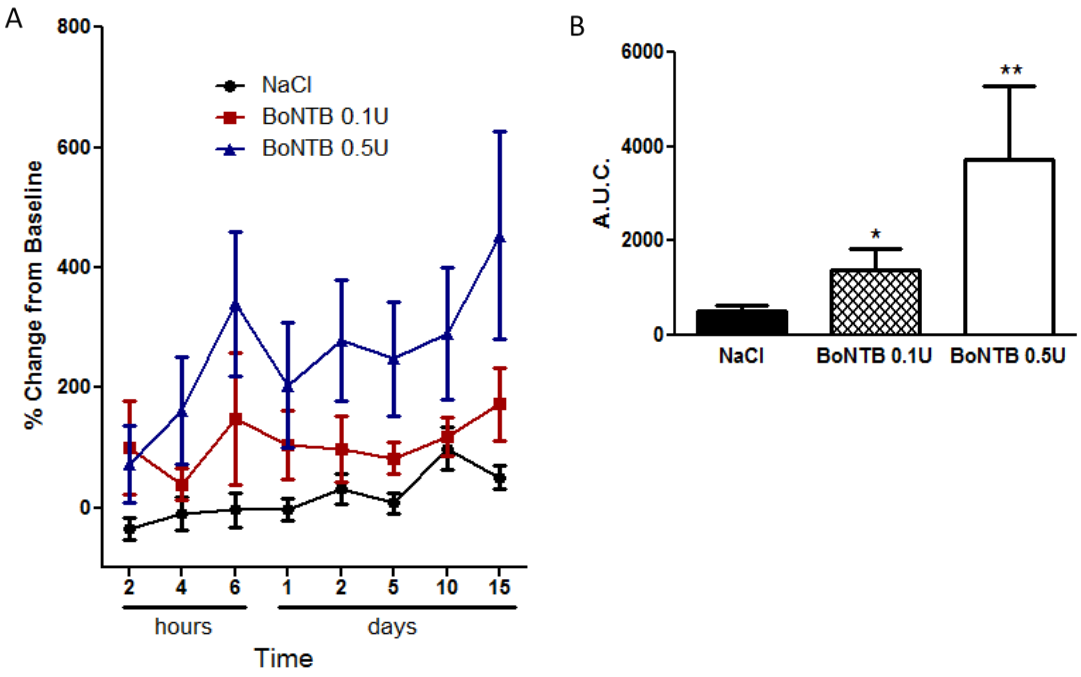


Figure 9. Effects of IT BoNTB on neuropathic hyperalgesia
SNL-induced tactile allodynia after IT BoNT-B. Paw withdrawal threshold (PWT) was measured using von Frey filaments at 14-15 days after SNL surgery and continued up to 15 days after IT injection. A) Time course of tactile allodynia up to 15 days after IT treatment, expressed as % change from baseline. At 14-15 days after SNL surgery, baseline PWT was measured: naïve (n=8), 1.35±0.53; NaCl, 0.69±0.32; BoNTB 0.1U, 0.58±0.32; BoNTB 0.5U, 0.45±0.24. Mice received IT NaCl (n=8) or BoNTB 0.1U-0.5U (n=6) immediately after baseline PWT was measured at day 14-15 days after SNL (t=0). B) Area under curve of % change from baseline PWT over 15 days after IT treatment (* and ** $P<0.05$).

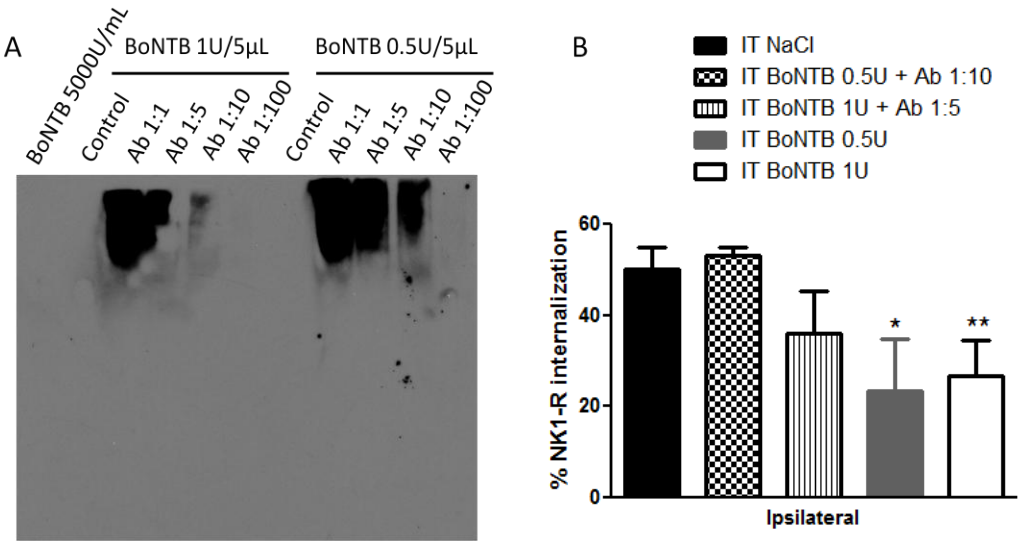
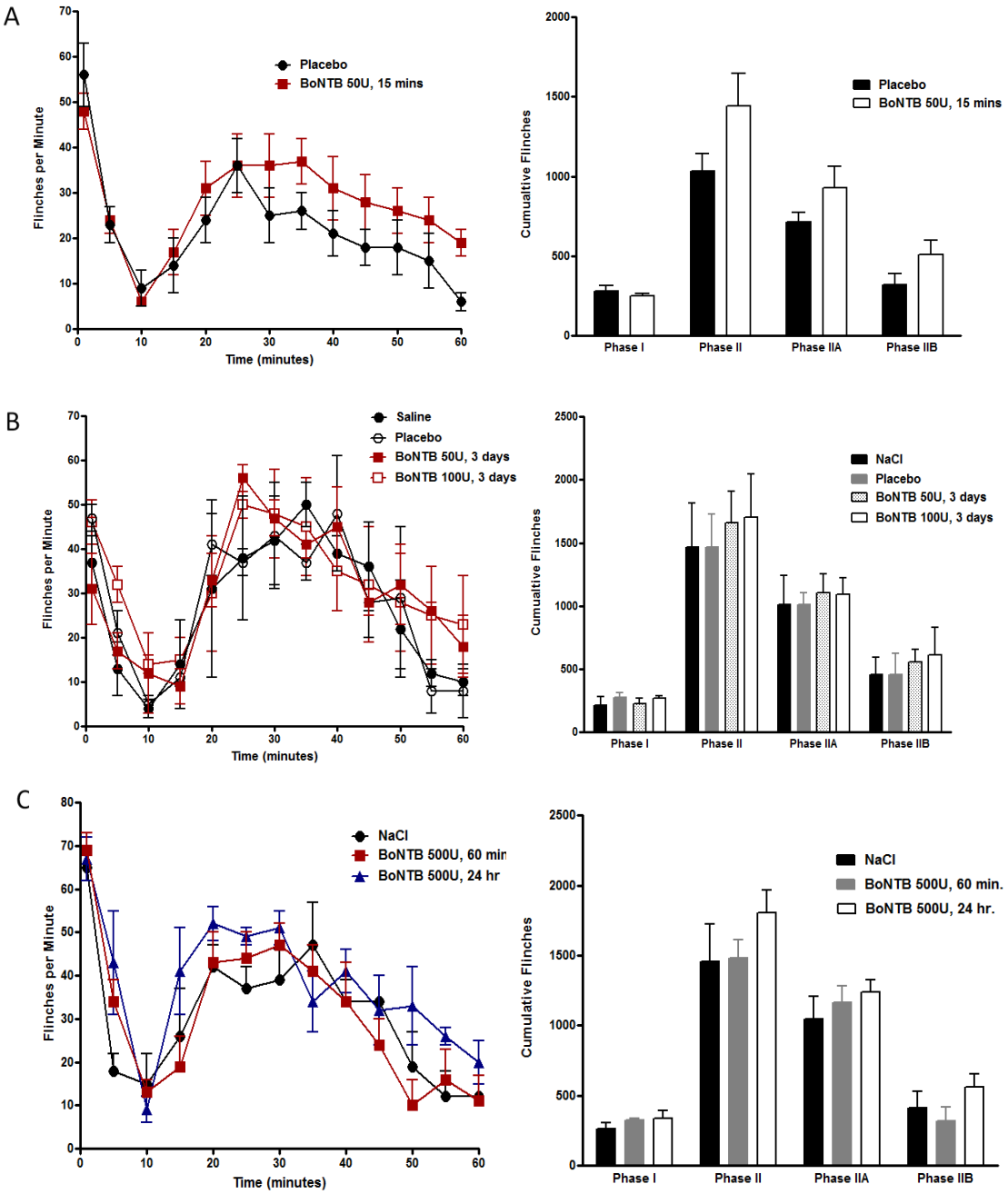


Figure 10. Prevention of BoNT-B effects on primary afferent neurotransmitter release
Effective binding concentrations of BoNT-B and antibody were determined by titer assay on NativePAGE. Co-injection of BoNT-B and anti-BoNTB antibody was administered in mice 2 days prior to IPLT formalin, and spinal cord tissue was harvested at 10 minutes after formalin for analysis of NK1-R internalization. A) Immunoblot of BoNTB-antibody titer assay. BoNT-B was incubated with anti-BoNTB antibody (Ab) at dilutions 1:1, 1:5 and 1:10. The effective binding concentrations were determined to be BoNTB 0.5U/5µL incubated with anti-BoNTB antibody at 1:10 dilution, and BoNTB 1U/5µL incubated with antibody at 1:5 dilution. B) Histogram of NK1-R internalization in the ipsilateral dorsal horn in animals that received IT BoNTB-antibody titer (n=3), BoNTB 0.5U-1U (n=3), and IT NaCl (n=4). *$P \leq 0.05$.

Figure 11. Resistance to BoNT-B in the rat
Figures A, B and C show time course (left panel) of observed flinching behavior and histogram of phasic cumulative flinches (right panel) comparing phase I (0-10 mins), phase II (10-40 mins), phase IIA (10-20 mins) and phase IIB (20-40 mins). A) Rats were pretreated with IT BoNT-B (50U, 10µL; n=7) 15 minutes prior to IPLT formalin in the hind paw, as compared to placebo (n=6) pretreated animals. B) Rats received IT BoNTB (50U, 10µL; 100U, 20µL; n=3) 3 days prior to IPLT formalin, as compared to placebo (10µL; n=3) and NaCl (10µL; n=3) pretreated animals. C) Animals received IPLT BoNT-B (500U, 100µL) in the hind paw 24 minutes (n=4) or 60 hrs (n=4) prior to IPLT formalin in the pretreated hind paw, as compared to NaCl (n=4) pretreated animals. D) Spinal nerve ligation of the L5 spinal nerve was performed in rats 7-10 days prior to IT injection. On day 0, rats received IT BoNT-B (100U,20µL; n=4), saline (n=2), or placebo (n=2). Paw withdrawal threshold in response to von Frey filaments was measured up to each day for 3 days following IT injections.


A
Flinches per Minute
Time (minutes)
Placebo
BoNTB 50U, 15 mins
Cumualtive Flinches
Phase I
Phase II
Phase IIA
Phase IIB
B
Saline
Placebo
BoNTB 50U, 3 days
BoNTB 100U, 3 days
NaCl
C
NaCl
BoNTB 500U, 60 min
BoNTB 500U, 24 hr
BoNTB 500U, 60 min.
BoNTB 500U, 24 hr.
Cumulative Flinches

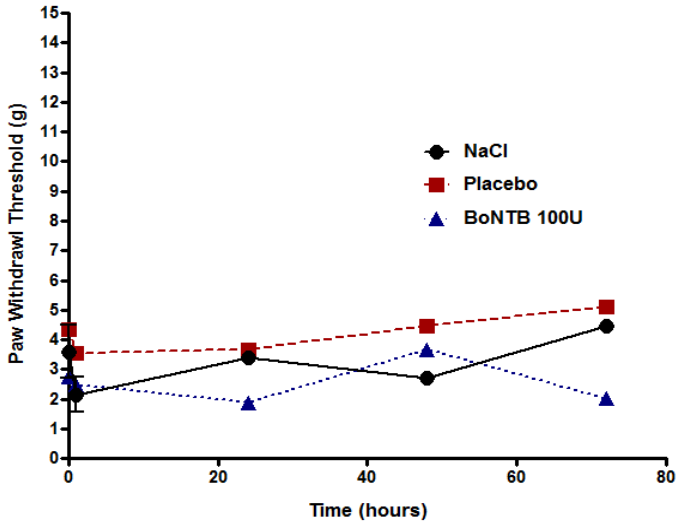


Figure 12. Absence of BoNT-B effect on neuropathic pain in the rat

Spinal nerve ligation of the L5 spinal nerve was performed in rats 7-10 days prior to IT injection. On day 0, rats received IT BoNT-B (100U,20µL; n=4), saline (n=2), or placebo (n=2). Paw withdrawal threshold in response to von Frey filaments was measured up to each day for 3 days following IT injections.

IV. DISCUSSION

Botulinum neurotoxins, such as BoNT-A (Botox ®) and BoNT-B (Myobloc®), are used in cosmetic and therapeutic applications for their enzymatic cleavage of SNARE proteins which results in blockade of neurotransmitter release from motor neurons and consequently flaccid muscle paralysis. As reviewed in the introduction, this cleavage prevents formation of SNARE protein complex which mediates vesicle-membrane fusion at the synaptic terminal during neurotransmitter release. The role of SNARE proteins in mediating depolarization-evoked and calcium-mediated vesicular release is believed to be ubiquitous throughout central and peripheral nervous systems. It is therefore a reasonable hypothesis that botulinum neurotoxins have effects on primary afferent sensory neurons which mediate peripheral sensory input. Blockade of neurotransmitter release from primary afferent sensory neurons using botulinum neurotoxins may be a potential therapeutic approach to pain management. Although the mechanisms and action on muscle function have been extensively investigated in many previous studies, there is relatively little investigation on BoNT effects on nociceptive pathways and neurotransmitter release *in vivo*.

The first aim of this series of studies was to examine the effects of spinally delivered BoNT-B on the release of neurotransmitters from primary afferent nociceptors. Such small afferents contain and release a variety of neurotransmitters, including substance P (SP) and glutamate. While glutamate is widely distributed in many classes of neurons, SP is present mainly in polymodal C-fibers. SP release is considered to be representative of small afferent excitation. In addition, SP binds to neurokinin-1 receptors (NK1-R) which are located on spinal neurons that project supraspinally. Based on many several lines of evidence, NK1-R-expressing neurons play a critical role in spinal nociceptive processing (Todd, 2002). An additional issue

related to the use of SP as a marker for small afferent release is that NK1-R displays internalization when activated by its ligand. Internalization permits *in situ* assessment of SP release and thus terminal activation (Mantyh, 2002). Systematic analysis of NK1-R internalization in the dorsal horn in conjunction of the intrathecal delivery of BoNT-B permits direct *in vivo* assessment of BoNT-B effects on neurotransmitter release from primary afferents.

The second principal aim was to determine whether blockade of neurotransmitter release by spinally delivered BoNT-B at the spinal level is associated with the behavioral response to a noxious stimulus. Small unmyelinated primary afferents, such as C-fibers, are activated by nociceptive stimuli and contribute to pain processing. Treatment of blocking terminal release from these peptidergic polymodal nociceptors can significantly attenuate pain transmission. Previous studies have shown that intrathecal delivery of mu-opioid agonists block neurotransmitter release from small primary afferents, which results in attenuation of painful sensation (Yaksh et al, 1980; Kondo et al, 2005). Accordingly, we speculated that intrathecal delivery of BoNT-B also yields a potent anti-nociceptive effect.

In pursuit of these over-arching themes, this thesis has shown that intrathecal delivery of BoNT-B produces an effective blockade of neurotransmitter release from primary afferent C-fibers *in vivo*. At doses that block substance-P release, IT BoNT-B produced a reduction of acute inflammatory nociceptive behavior and reduction of spinal nerve injury-induced neuropathic pain. While high doses indeed caused paralysis, the effects on SP release and pain behavior occurred at doses which were not accompanied by motor impairment. The following section will consider issues pertinent to the interpretation of these results.

IT BoNT-B effects on motor function

High doses of BoNTs indeed cause general muscle paralysis and death by respiratory and heart failure. Maximal tolerable dose, or LD50, for intracerebroventricular delivery of BoNT-A and BoNT-B has been previously established to be 3.75-15 pg of toxin per mouse of average body weight. Normal grooming behavior and ambulation were observed at these doses (Luvisetto et al, 2006). The maximal tolerable intrathecal dose reported in this thesis is 5pg per mouse of average body weight. The maximal tolerable dose established in this thesis is within range of tolerable toxin concentration previously described for central delivery of BoNT-B. The absence of intrathecal BoNT-B effect on motor function at doses that block neurotransmitter release could be due to various factors: i) spinal localization of BoNT-B effects, ii) penetration of the toxin into deep laminae, and iii) failure of retrograde transport of toxin to the peripheral neuromuscular junction.

Spinal localization of BoNT-B effects

Drug delivery to the spinal cord was accomplished by percutaneous intrathecal puncture. The model is widely used for spinal drug delivery in the mouse (Hylden and Wilcox, 1980). The certainty of the spinal injection is typically confirmed by the minor twitch of the tail, which is observed upon penetration into the intrathecal space. Though this likely reflects contact with a nerve root in the cauda equine, this does not have evident effect upon motor function in the absence of drug. There are three issues to consider regarding drug disposition after spinal delivery: i) the rostrocaudal distribution, ii) penetration into parenchyma, and iii) penetration of BoNT-B into the cell.

Rostrocaudal distribution of the injectate following intrathecal delivery largely depends on volume of the injectate. Five microliters used in this series of studies is the typical volume used in the mouse. Intrathecal delivery of a drug is relatively restricted to the location of injection. Only 3% of intrathecal injectate is found in the brain 10 minutes after intrathecal injection (Hylden and Wilcox, 1980). Therefore, intrathecal delivery of drug is biased toward higher concentrations than concentrations in tolerated in the brain. In a separate study, the intrathecal injection of a blue dye produced distribution up through the mid-thoracic level, which clearly covers spinal segments where primary afferents from the hind paws terminate at the spinal cord.

While the effects of interest in this series of studies reflect the effect of IT BoNT-B on SP release, it is important to appreciate that this target is the spinal terminals of SP-containing primary afferents. Synaptic terminals of primary afferents in the mouse lie in the superficial dorsal horn (laminae I and II), about 25µm from the surface. Motor neurons lie more deeply in the ventral horn. Penetration of the parenchyma after IT delivery depends on concentration gradient, lipid partition coefficient for small molecules, and molecular weight, with large molecules showing a diffusion rate inversely proportional to molecular weight. BoNT-B is a 150kDa protein. Studies of drug diffusion after intrathecal injection have shown that large molecules diffuse poorly into the parenchyma (Nicholson, 1999). Accordingly, superficial localization of BoNT-B allows relatively restricted distribution of the toxin to the site of injection, which accounts for the potent effect on sensory function and absence of effect on motor function.

Translocation of BoNTs, as reviewed in the introduction, has been well studied (Grumelli et al, 2005; Fischer and Montal, 2007; Fischer et al, 2008; Dong et al, 2007; Foran et al, 1994).

Binding and translocation of BoNT-B into the cell requires specific recognition of membrane ganglioside and synaptotagmin (Kohda et al, 2007), as well as formation of a channel by the translocating domain (Montal, 2009). Retrograde axonal transport and transcytosis of BoNT-A after intrahippocampal injection has been previously described (Antonucci et al, 2008). In the present work, immunohistochemistry and confocal microscopy was used to confirm BoNT-B penetration into neurons after IT BoNT-B injection at maximal tolerable dose. At 2 days after IT injection, BoNT-B immunoreactivity is clearly distributed in the superficial dorsal horn and is co-localized with NeuN immunoreacitivity. These observations reflect movement of BoNT-B into cell body, but not specifically into the terminal. Such terminal characterization was precluded by resolution of the confocal imaging. We believe that movement occurs directly into dorsal horn terminals, as it does into the peripheral terminal of the motor end plate where it acts to prevent local acetylcholine release. The absence of effect on motor function at low doses of BoNT-B is probably due to low penetration into deep laminae and had relatively little effect on motor neurons.

In addition, intrathecal space is protected from the general vasculature. Lymphatic and vascular transport of BoNT-A has been shown as the primary mechanism of toxin spread after intramuscular injection in cats (Wiegand et al, 1976). Same mechanisms of transport have been proposed in a monkey hand model of toxin spread after intramuscular injection (Arezzo et al, 2002). Compared to peripheral injection of BoNTs, intrathecal injection of BoNTs relatively restricts distribution of the toxin outside of the general vasculature and thus minimizes vascular transport of the toxin into peripheral terminals.

Substance P-containing primary afferents

Substance-P-containing primary afferents are small, unmyelinated C-fibers. They are activated by high intensity thermal, mechanical, and chemical stimuli, hence the designation as polymodal nociceptors. Anatomically, these afferents project largely into the superficial dorsal horn (laminae I and II), or marginal layer and substantia gelatinosa. Here, they make synaptic contact with NK1-R-expressing neurons, many of which are large neurons (marginal neurons) that project supraspinally and are considered to be part of nociceptive pathways.

Substance P is contained in large dense core vesicles in the dorsal horn. Extensive *in vivo* and *in vitro* work has shown that SP is released by depolarization of dorsal horn terminals. This release is dependent on activation of voltage-gated calcium channels, which is a mechanism that plays a ubiquitous role in neuron action in the peripheral and central nervous systems. Intrathecal treatment with blockers of N-type calcium channel has been shown to depress SP release (Smith et al, 2002). Additionally, SP-containing afferents also contain and release other neurotransmitters, notably the excitatory amino acid glutamate. Terminal depolarization, accordingly, leads to release of both neurotransmitters, each resulting in post-synaptic depolarization with a distinct temporal signature. Glutamate results in rapid onset and short-lasting depolarization. SP results in slow, but persistent depolarization. Accordingly, blockade of release from these primary afferents is also likely to block release of both neurotransmitters.

Validity of NK1-R internalization as assay for SP release

Neurokinin-1 receptor is a G-protein coupled receptor. Like others in this class of receptors, when occupied by an agonist, the receptor displaces coupling to β-arrestin and

internalizes. This internalization is, therefore, taken as an index of extracellular concentration of SP, based on four lines of evidence.

1. There are three sources of SP in the dorsal horn: primary afferents, bulbospinal projections and intrinsic interneurons to a lesser extent. Previous works have described depletion of SP in bulbospinal pathway has no effect on NK1-R internalization in the superficial dorsal horn. Treatment of the animal with depleting doses of capsaicin prevents NK1-R internalization evoked by noxious stimulus (Kondo et al, 2005).
2. Intrathecal morphine, which reduces extracellular spinal SP through pre-synaptic action, reduces the fraction of spinal neurons that show NK1-R internalization after stimulation with a noxious stimulus (Yaksh et al, 1980). Conversely, intrathecal capsaicin, which is known to evoke SP release through activation of TRPV1 receptors, increases spinal NK1-R internalization (Jhamandas et al, 1984).
3. *In vitro* studies using spinal cord slices has also demonstrated covariance between local SP concentration and increase in spinal NK1-R internalization (Mantyh et al, 1995).
4. Finally, this thesis has shown that while NK1-R expression was shown to be consistent along L1-L5 lumbar segment, NK1-R internalization in the dorsal horn ipsilateral to the site of IPLT formalin injection was shown to be maximal at L4-L5. This is consistent with the location of terminals from sensory input originating from the hind paw. There is no statistical difference in NK1-R internalization between contralateral dorsal horn and unstimulated animals. These results established the minimal and constitutive magnitude of NK1-R internalization. Based on these observations, spinal NK1-R internalization is a robust reflection of SP release from spinal primary afferents.

Confirmation of enzymatic mechanism of BoNT-B on neurotransmitter release and behavior

As reviewed in the introduction, inhibition of neurotransmitter release is believed to be the result of enzymatic cleavage of VAMP I/II of SNARE protein complex. This cleavage renders synaptic proteins unable to form protein complex which plays a critical role in vesicle-membrane fusion during neurotransmitter release. A number of experiments were performed to confirm that this is indeed the mechanism underlying effects of IT BoNT-B on neurotransmitter release and pain behavior.

1. Western blot of VAMP I/II protein in spinal cord tissue lysate incubated with BoNT-B showed decrease in VAMP I/II. This ascertains an enzymatically active neurotoxin.
2. Co-injection of BoNT-B with anti-BoNTB antibody showed prevention of BoNT-B effects on primary afferent SP release. Incubation of antibody renders the neurotoxin-antibody complex too large to translocate across the membrane into the cytoplasm of neurons. Antibody binding possibly inactivated protease domain of BoNT-B, which rendered the toxin enzymatically inactive. Additionally, this prevention of BoNT-B effect suggests likely persistency of antibody binding. Results from this study follows several lines of evidence on the potent effect of BoNT-neutralizing antibodies which have been proposed as potential vaccination (Aoki et al, 2010; Xu et al, 2009; Zhou et al, 2009).
3. Finally, rat resistance to BoNT-B was examined as a negative control for BoNT-B effects in the mouse. BoNT-B proteolytically cleaves VAMP I and II at amino acid residues Gln/76-Phe/77 in mouse and human. In the rat VAMP I protein, Gln/76 is replace by valine residue, which prevents BoNT-B cleavage. Indeed, BoNT-B effects on nociceptive behavior seen in the mouse was absent in the rat. These results jointly suggest that

specific VAMP I/II cleavage is required for effect on formalin-evoked neurotransmitter release and nocifensive response.

Specificity of IT BoNT-B effects on spinal SP release

The present studies have demonstrated that IT BoNT-B pretreatment reduces SP release. Downstream effects include decrease in NK1-R internalization, reduction of formalin-evoked C-Fos protein expression, decrease in nocifensive behavioral response, and attenuation in neuropathic hyperalgesia. These effects are likely due to not reflect unique effect on substance P release. As noted in the introduction, nocifensive behavioral response to a noxious stimulus is mediated by activation of peptidergic and non-peptidergic primary afferent sensory fibers. Post-synaptic excitation, or activation of interneurons that project supraspinally, is mediated by glutamate action on AMPA and NMDA receptors. Previous works have shown that BoNTs block glutamate release from motor neurons and hippocampal neurons (McMahon et al, 2007); these effects are also mediated by cleavage of SNARE proteins (Verderio et al, 2007). Neuropathic hyperalgesia is mediated by hyper-excitation of sensory neurons and increase of neurotransmitter release, such as glutamate release (Coderre et al, 2005). Accordingly, the effects of BoNT-B on inflammatory nocifensive behavior and neuropathic pain are not uniquely the result of blockade of SP release from primary afferent C-fibers.

Mechanisms of intrathecal BoNT-B effects on nociception

Intrathecal injection of maximal tolerable dose of BoNT-B reduced phase II, but not phase I, of IPLT formalin-induced flinching behavior. Subcutaneous BoNT-A blockade of formalin-induced inflammatory pain has been previously described (Cui et al, 2003).

Intracerebroventricular BoNT-B has been shown to abolish interphase inhibitory response and phase II of formalin-induced pain (Luvisetto et al, 2006). Second phase of formalin-induced pain response is mediated by sustained primary afferent activation of dorsal horn neurons (Pitcher and Henry, 1996). Formalin induces sustained activation of primary afferent fibers. This produces phasic activation that mirrors behavioral response to intraplantar formalin. Robust early phase activation of C-fibers leads to long-term potentiation, or second phase facilitation of C-fibers. This is a phenomenon termed “wind up”. Sustained activation of wide dynamic range (WDR) neurons, which make up the majority of neurons in superficial laminae of the dorsal horn, produces second phase of formalin response behavior. Sustained, tonic substance P release from primary afferent C-fibers contributes to phase 2 formalin behavioral response (Kantner et al, 1986). Blockade of NK1-R receptors (Yamamoto and Yaksh, 1991; Henry et al, 1999) and NMDA receptors in WDR neurons has also shown to block second phase of formalin response (Chaplan et al, 1997; Hanai, 1998). These are consistent with our observation that IT BoNT-B blocks second phase of formalin-induced nocifensive response.

However, the question arises as to why blockade of neurotransmitter release affected phase 2, not phase 1, of formalin response. Upon IPLT formalin injection, WDR neurons are activated by primary afferent C-fibers, Aβ fibers, and Aδ fibers. Like C-fibers, Aβ- and Aδ-fibers also primary afferents that activate dorsal horn neurons and mediate behavioral response to painful stimulus. Unlike C-fibers, Aβ- and Aδ-fibers are non-peptidergic primary afferents and are active during phase 1 of formalin response (Puig and Sorkin, 1996). A-type primary afferent fibers are myelinated and fast fibers. C-fibers are unmyelinated and slow fibers. Phase 2 facilitation is mediated by C-fibers which respond to the chemical stimulus formalin. This leads to the interesting observation that IT BoNT-B pretreatment blocks C-fiber phase-2 facilitation

upon IPLT formalin in the hind paw in the mouse. Based on the absence of BoNT-B effect on phase 1 of formalin response, IT BoNT-B effect on thermal hyperalgesia is worthy of further investigation.

An important additional observation that emphasizes that IT BoNT-B altered dorsal horn excitation is presented by the observation that formalin-evoked C-Fos protein activation in dorsal horn neurons was almost completely abolished in animals that received IT BoNT-B pretreatment at doses which altered formalin response. C-Fos is a protooncogene which appears within approximately 1-2 hours following neuronal activation. Extensive work with this immediate early gene has shown its utility in demonstrating activation of dorsal horn neurons after nociceptive stimulation (Coggeshall, 2005). The observation that IT BoNT-B prevented this activation in the absence of effect on general motor function reflects upon the surprisingly functional specificity of this toxin treatment.

Spinal nerve injury induced by ligation of spinal nerve leads to long-term hypersensitization such that normally innocuous stimuli, such as light touch, leads to escape behavior suggesting a pain state. Mechanisms of this hypersensitivity have many complex contributing factors. However, it is important to note that tactile allodynia is mediated by interaction of primary afferents after nerve injury to activate a nociceptive pathway (Devor and Wall, 1990). Spinal opiates which act on small primary afferents do not strongly attenuate tactile allodynia (Yaksh et al 1995). Interestingly, spinal delivery of NK1-R antagonists have been reported to attenuate tactile allodynia. This effect may reflect a phenotypic change after nerve injury, in which myelinated afferents begin to synthesize and release substance P(Pitcher and Henry, 2004). Increase of neurotransmitter release from primary afferents after peripheral nerve injury has also been shown in previous studies, and proposed as a contributing factor to

development of neuropathic pain (Gardell et al, 2003; Coderre et al, 2005). The present observation that IT BoNT-B attenuates tactile allodynia suggests BoNT-B action through blockade of glutamate and other neurotransmitter release from primary afferents.

While the above comments reflect on the spinal action of BoNT-B, there has been an enduring clinical interest in the possibility that peripherally delivered BoNTs may also have an anti-nociceptive effect. Intramuscular injection of BoNTs has been described to attenuate painful sensation (Colhado et al, 2009; Pickett, 2010). Excessive muscular contraction compresses small blood vessels, which causes tissue to become ischemic. Muscle tissue ischemia results in increased release of bradykinins and excitation of nociceptors (Mense, 2004). Intramuscular BoNT blockade of acetylcholine from motor neurons inhibits muscle contraction, which attenuates ischemia and painful sensation. The present study follows the same principles of BoNT-B inhibition of neurotransmitter release from primary afferent C-fibers. However, intrathecal BoNT-B provides direct and local blockade of neurotransmitter release at the spinal level, at doses that did not affect motor function. Therefore, BoNT-B blockade of neuropathic hyperalgesia and inflammatory nociception is not likely due to inhibition of muscle ischemia.

An alternate possibility is that peripherally delivered BoNTs may be taken up and into neurons and retrogradely transported to central terminals where BoNTs can access the CNS (Antonucci et al, 2008). Peripheral and intrathecal delivery of BoNT-A have been shown to block allodynia in diabetic model of neuropathy. The block occurred at 5 days after peripheral delivery and 2 days after spinal delivery. Not only suggests retrograde transport of BoNT-A from the periphery to CNS, but also the difference in time of onset which reflects central transport of peripherally delivered BoNT (Bach-Rojecky et al, 2010). Further investigation into this retrograde transport mechanism is required.

Conclusions and clinical relevance of findings

This series of studies showed that IT BoNT-B effectively blocked neurotransmitter release from primary afferent C-fibers in a facilitated state, reduced inflammatory nociception and neuropathic hyperalgesia in the mouse, in the absence of toxicity and effect on normal motor function. This blockade is evidently due to BoNT-B enzymatic cleavage of VAMP I/II of the SNARE protein complex.

Data presented in this thesis provide additional insight on BoNT effects on mechanisms of spinal nociceptive processing, as well as new directions for future research. NK1-R internalization model of assessment of neurotransmitter release has been employed in several previous studies in the rat, though characterization in the mouse has thus far remained relatively undefined. The detailed characterization of formalin-evoked and substance P-mediated NK1-R internalization in the spinal dorsal horn provides additional validation for the use of receptor internalization as an assay for neurotransmitter release in the mouse. Effective prevention of IT BoNT-B effects on neurotransmitter release by incubation with antibody shows potency of antibody binding and confirms validity of current proposals to employ neutralizing antibodies as vaccination against BoNTs. Furthermore, the long-term effect of IT BoNT-B on nerve injury-induced neuropathic pain suggests a persistent effect of BoNT-B on VAMP I/II protein. Whether this persistency is due to persistency of BoNT-B action inside neurons, or due to turn-over rate of synaptic proteins after cleavage, and other factors contributing to persistency of BoNT-B effects deserve further investigation.

Based on data presented in this series of studies on spinal action of BoNT-B, spinal action of other BoNTs, which is relatively undefined in the literature, deserves future investigation. A study of retrograde transport mechanisms of spinally delivered versus

peripherally delivered BoNTs would reveal additional insight on BoNTs as a potential therapeutic agent for pain management.

Present studies also provide an initial assessment of therapeutic potential for intrathecal BoNT-B on inflammatory nociception induced by tissue injury and on management of neuropathic pain induced by nerve injury. Importantly, the observed effects of BoNT-B were not accompanied by motor function impairment in the mouse. Nevertheless, considerable work related to safety and potential side effects of spinally delivered BoNTs are required prior to considering this delivery method as a therapeutic alternative.

References

Adler M, Nicholson JD and Hackley BE. Efficacy of a novel metalloprotease inhibitor on botulinum neurotoxin B activity. *FEBS Lett.* 1998 Jun 16;429(3):234-8.

Adler M, Nicholson JD, Starks DF, Kane CT, Cornille F and Hackley BE. Evaluation of phosphoramidon and three synthetic phosphonates for inhibition of botulinum neurotoxin B catalytic activity. *J Appl Toxicol*. 1999 Dec;19 Suppl 1:S5-S11

Allan AL, Cortright DN and McCarson KE. Formalin- or adjuvant-induced peripheral inflammation increases neurokinin-1 receptor gene expression in the mouse. *Brain Res.* 2003 Jan 24;961(1):147-52.

Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. Long-distance retrograde effects of botulinum neurotoxin A. *J Neurosci.* 2008 Apr 2;28(14):3689-96.

Aoki K, Smith LA, Atassi M. Mode of action of botulinum neurotoxins: current vaccination strategies and molecular immune recognition*. Crit Rev Immunol.* 2010;30(2):167-87.

Arezzo JC, Litwak MS, Caputo FA, Meyer KE and Shopp GM. "Spread of paralysis to nearby and distant noninjected muscles in a monkey hand model: comparison of BoNT-B and BoNT-A". *Scientific and Therapeutic Aspects of Botulinum Toxins.* Lippincott Williams & Wilkins, Philadelphia: 123-133, 2002.

Bach-Rojecky L and Lackovic Z. Antinociceptive effect of botulinum toxin type A in rat model of carageenan and capsaicin induced pain. *Croat Med J.* 2005 Apr;46(2):201-8.

Back-Rojecky L, Relja M, Lackovic Z. Botulinum toxin type A in experimental neuropathic pain. *J Neural Transm*. 2005 Feb;112(2):215-9.

Bach-Rojecky L, Salkovic-Petrisic M, Lackovic Z. Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy: bilateral effect after unilateral injection. *Eur J Pharmacol*. 2010 May 10;633(1-3):10-4.

Baldwin MR and Barbieri JT. Association of botulinum neurotoxins with synaptic vesicle protein complexes. *Toxicon*. 2009 Oct;54(5):570-4..

Bennett GJ and Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain.* 1988 Apr;33(1):87-107.

Caleo M and Schivo G. Central effects of tetanus and botulinum neurotoxins. *Toxicon.* 2009 Oct;54(5):593-9.

Chaddock JA and Purkiss JR. Retargeted Clostridial endopeptidases: inhibition of nociceptive neurotransmitter release *in vitro*, and antinociceptive activity in *in vivo* models of pain. *Mov Disord.* 2004 Mar;19 Suppl 8:S42-7.

Chai Q, Arndt JW, Dong M, Tepp WH, Johnson EA, Chapman ER, Stevens RC. Structural basis of cell surface receptor recognition by botulinum neurotoxin B. *Nature.* 2006 Dec 21;444(7122):1096-100.

Chaplan SR, Malmberg AB, Yaksh TL. Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. *J Pharmacol Exp Ther.* 1997 Feb;280(2):829-38.

Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994 Jul;53(1):55-63.

Chen S, Hall C, and Barbieri JT. Substrate recognition of VAMP-2 by botulinum neurotoxin B and tetanus neurotoxin. *J Biol Chem.* 2008 Jul 25;283(30):21153-9.

Coderre TJ, Kumar N, Lefebvre CD and Yu JS. Evidence that gabapentin reduces neuropathic pain by inhibiting spinal release of glutamate. *J Neurochem.* 2005 Aug;94(4):1131-9.

Coggeshell RE. Fos, nociception and the dorsal horn. Prog Neurobiol. 2005 Dec;77(5):299-352.

Colhado OC, Boeing M, Ortega LB. Botulinum toxin in pain treatment. *Revista Brasileira de Anestesiologia.*2009; 59(3): 366-381.

Cui M, Khanijou S, Rubino J, Aoki KR. Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. *Pain*. 2004 Jan;107(1-2):125-33.

Deinhardt K and Schiavo G. Endocytosis and retrograde axonal traffic in motor neurons. *Biochem Soc Symp*. 2005;(72):139-50.

Devor M and Wall PD. Cross-excitation in dorsal root ganglia of nerve-injured and intact rats. *J Neurophysiol.* 1990 Dec;64(6):1733-46.

Dong M, Richards DA, Goodnough MC, Tepp WH, Johnson EA, Chapman ER. Synaptotagmin I and II mediate entry of botulinum neurotoxin B into cells. *J Cell Biol.*2003 162: 1293-1303.

Dong M, Tepp WH, Liu H, Johnson EA, Chapman ER. Mechanism of botulinum neurotoxin B and G entry into hippocampal neurons. *J Cell Biol.* 2007 Dec 31;179(7):1511-22.

Dubuisson D and Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain.* 1977 Dec;4(2):161-74.

Duggan MJ, Quinn CP, Chaddock JA, Purkiss JR, Alexander FCG. Inhibition of release of neurotransmitters from rat dorsal root ganglia by a novel conjugate of a Clostridium botulinum toxin A endopeptidase fragment and Erythrina cristagalli lectin. *J Biol Chem*. 2002 Sep 20;277(38):34846-52.

Durham PL, Cady R, Cady R. Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. *Headache.* 2004 Jan;44(1):35-42.

Favre-Guilmard C, Auguet M, Chabrier P. Different antinociceptive effects of botulinum toxin type A in inflammatory and peripheral polyneuropathic rat models. *Eur J Pharmacol.* 2009 Sep 1;617(1-3):48-53.

Fasshauer D, Sutton RB, Brunger AT, Jahn R. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc Natl Acad Sci, USA.* 1998 Dec 22;95(26):15781-6.

Fischer A and Montal M. Cruicial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. *J Biol Chem.* 2007 Oct 5;282(40):29604-11.

Fischer A and Montal M. Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. *Proc Natl Acad Sci U S A.* 2007 Jun 19;104(25):10447-52.

Fischer A, Mushrush DJ, Lacy DB, Montal M. Botulinum neurotoxin devoid of receptor binding domain translocates acdtive protease. *PLoS Pathog.* 2008 Dec;4(12):e1000245.

Foran P and Shone CC. Differences in the protease activities of tetanus and botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments. *Biochemistry*. 1994 Dec 27;33(51):15365-74.

Galli T and Haucke V. Cycling of synaptic vesicles: How far? How fast! *Sci STKE.* 2001 Jun 26;2001(88):re1.

Gardell LR, Vanderah TW, Gardell SE, Wang R, Ossipov MH, Lai J and Porreca F. Enhanced evoked excitatory transmitter release in experimental neuropathy requires descending facilitation. *J Neurosci.* 2003 Sep 10;23(23):8370-9.

Gazarani P, Staahl C, Drewes AM, Arendt-Nielsen L. The effects of botulinum toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. *Pain*. 2006 Jun;122(3):315-25.

Green GM and Dickenson A. GABA-receptor control of amplitude and duration of neuronal responses to formalin in rat spinal cord. *Eur J Pain.* 1997;1(2):95-104.

Grumelli C. and Verderio C. Internalization and mechanism of action of clostridial toxins in neurons. *Neurotoxicology.* 2005 Oct;26(5):761-7.

Guan Y and Raja SN. Wide-dynamic-range neurons are heterogeneous in windup responsiveness to changes in stimulus intensity and isoflurane anesthesia level in mice. *J Neurosci Res.* 2010 Aug 1;88(10):2272-83.

Henry JL, YashpalK, Pitcher GM, Chabot J, Coderre TJ. Evidence for tonic activation of NK-1 receptors during second phase of the formalin test in the rat. *J Neurosci.* 1999 Aug 1;19(15):6588-98.

Hanai F. C-fiber responses of wide dynamic range neurons in the spinal dorsal horn. *Clin Orthop Relat Res.* 1998 Apr;(349):256-67.

Hylden JLK and Wilcox GL. Intrathecal morphine in mice: a new technique *Eur J Pharmacol.* 1980 Oct 17;67(2-3):313-6.

Jhamandas K, Yaksh TL, Harty G, Szolcsanyi J, Go VLW. Action of intrathecal capsaicin and its structural analogues on the content and release of spinal substance P: selectivity of action and relationship to analgesia. *Brain Res.* 1984 Jul 23;306(1-2):215-25..

Jin R, Rummel A, Binz T, Brunger AT. Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. *Nature.* 2006 Dec 21;444(7122):1092-5

Kaneko M and Hammond D. Role of spinal γ-amnobutric $acid_A$ receptors in formalin-induced nociception in the rat. *J Pharmacol Exp Ther.* 1997 Aug;282(2):928-38.

Kim SH and Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain.* 1992 Sep;50(3):355-63.

Kitamura Y, Matsuka Y, Spigelman I, Ishihara Y, Yamamoto Y, Sonoyama W, Kuboki T and Oguma K. Botulinum toxin type A (150 kDa) decreases exaggerated neurotransmitter release from trigeminal ganglion neurons and relieves neuropathy behaviors induced by infraorbital nerve constriction. *Neuroscience*. 2009 Apr 10;159(4):1422-9.

Kohda T, Ihara H, Seto Y, Tsutsuki H, Mukamoto M, Kozaki S. Differential contribution of the residues in C-terminal half of the heavy chain of botulinum neurotoxin type B to its binding to the ganglioside GT1b and the synaptotagmin 2/GT1b complex. *Microb Pathog.* 2007 Feb-Mar;42(2-3):72-9.

Kondo I, Marvizon JC, Song B, Salgado F, Codeluppi S, Hua XY, Yaksh TL. Inhibition by spinal mu- and delta-opioid agonists of afferent-evoked substance P release. *J Neurosci.* 2005 Apr 6;25(14):3651-60.

Koriazova LK and Montal M. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat Struct Biol.* 2003 Jan;10(1):13-8.

Kozaki S, Kamata Y and Watarai S. Ganglioside GT1b as a complementary receptor component for Clostridium botulinum neurotoxins. *Microb Pathog.* 1998 Aug;25(2):91-9.

LaBuda CJ and Little PJ. Pharmacological evaluation of the selective spinal nerve ligation model of neuropathic pain in the rat. *J Neurosci Methods.* 2005 Jun 15;144(2):175-81.

Larson M. Ionotropic glutamate receptors in spinal nociceptive processing. *Mol Neurobiol.* 2009 Dec;40(3):260-88.

Lew MF, Adornato BT and Duane DD. Botulinum toxin type B: a double-blind, placebo-controlled safety and efficacy study in cervical dystonia. *Neurology.* 1997 Sep;49(3):701-7.

Luvisetto S, Marinelli S, Cobianchi S, Pavone F. Antiallodynic efficacy of botulinum neurotoxin A in a model of neuropathic pain. *Neuroscience.* 2007;145: 1-4.

Luvisetto S, Marinelli S, Lucchetti F, Marchi F, Cobianchi S, Rossetto O, Montecucco C, Pavone F. Botulinum neurotoxins and formalin-induced pain: central vs. peripheral effects in mice. *Brain Res.* 2006 Apr 12;1082(1):124-31.

Luvisetto S, Marinelli S, Rossetto O, Montecucco C, Pavone F. Central injection of botulinum neurotoxins: behavioral effects in mice. *Behav Pharmacol.* 2004 May;15(3):233-40.

Luvisetto S, Rosetto O and Montecucco C. Toxicity of botulinum neurotoxins in central nervous system of mice. *Toxicon.* 2003 Mar;41(4):475-81.

Mantyh PW. Neurobiology of substance P and the NK1 receptor. *J Clin Psychiatry.* 2002;63 Suppl 11:6-10.

Mantyh PW, Allen CJ, Ghilardi JR, Rogers SD, Mantyh CR, Liu H, Basbaum AI, Vigna SR, Maggio JE. Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. *Proc Natl Acad Sci, USA*. 1995 Mar 28;92(7):2622-6.

Marvizon JC, Wang X, Matsuka Y, Neubert JK, Spigelman I. Relationship between capsaicin-evoked substance P release and neurokinin 1 receptor internalization in the rat spinal cord. Neuroscience. 2003;118(2):535-45.

McMahon HT, Foran P, Dolly JO, Verhage M, Wiegant VM, Nicholls DG. Tetanus toxin and botulinum toxins type A and B inhibit glutamate, γ-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. *J Biol Chem.* 1992 Oct 25;267(30):21338-43.

McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, Hayward NJ, Chong JA, Julius D, Moran MM, Fanger CM. TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci, USA*. 2007 Aug 14;104(33):13525-30..

Mense S. Neurobiological basis for the use of botulinum toxin in pain therapy. *J Neurol.* 2004 Feb;251 Suppl 1:I1-7.

Montal M. Translocation of botulinum neurotoxin light chain protease by the heavy chain protein-conducting channel. *Toxicon.* 2009 Oct;54(5):565-9

Montecucco C. and Schiavo G. Structure and function of tetanus and botulinum neurotoxins. *Q Rev Biophys.* 1995 Nov;28(4):423-72.

Morris JL, Jobling P, Gibbins IL. Differential inhibition by botulinum neurotoxin A of cotransmitters release from autonomic vasodilator neurons. *Am J Physiol Heart Circ Physiol.* 2001 Nov;281(5):H2124-32.

Nagy GG, Watanabe M, Fukaya M, Todd AJ. Synaptic distribution of the NR1, NR2A and NR2B subunits of the N-methyl-d-aspartate receptor in the rat lumbar spinal cord revealed with an antigen-unmasking technique. *Eur J Neurosci.* 2004 Dec;20(12):3301-12.

Nazarian A, Gu G, Gracias NG, Wilkinson K, Hua XY, Vasko MR, Yaksh TL. Spinal N-methyl-D-aspartate receptors and nociception-evoked release of primary afferent substance P. *Neuroscience.* 2008 Mar 3;152(1):119-27.

Nicholson C. "Spinal Drug Delivery." *Structure of extracellular space and physiochemical properties of molecules governing drug movement in brain and spinal cord*. Yaksh TL, editor. Elsevier 1999.

Park JH, Lee Y, Lee J, Park C, Moon DE. The effects of botulinum toxin A on mechanical and cold allodynia in rat model of neuropathic pain. *Can J Anaesth.* 2006 May;53(5):470-7.

Pellett S and Tepp WH. A neuronal cell-based botulinum neurotoxin assay for highly sensitive and specific detection of neutralizing serum antibodies. *FEBS Lett.* 2007 Oct 16;581(25):4803-8.

Pickett A. Re-engineering clostridial neurotoxins for the treatment of chronic pain: current status and future prospects. *BioDrugs.* 2010 Jun;24(3):173-82.

Pitcher GM and Henry JL. Nociceptive response to innocuous mechanical stimulation is mediate via myelinated afferents and NK-1 receptor activation in a rat model of neuropathic pain. *Experimental Neurology.* 2004 186(2): 173-179.

Pitcher GM and Henry JL. Second phase of formalin-induced excitation of spinal dorsal horn neurons in spinalized rats is reversed by sciatic nerve block*. Eur J Neurosci.* 2002 May;15(9):1509-15.

Puig S and Sorkin LS. Formalin-evoked activity in identified primary afferent fibers: systemic injection of lidocaine suppresses phase-2 activity*. Pain.* 1996 Feb;64(2):345-55.

Rossetto O, Rigoni M and Monetcucco C. Different mechanisms of blockade of neuroexocytosis by presynaptic neurotoxins. *Toxicology Letters.*2004; 149: 91-101.

Schiavo G and Benfenati F. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*. 1992 Oct 29;359(6398):832-5.

Schiavo G, Malizio C, Trimble W, De Laureto PP, Milan G, Sugiyama H, Johnson EA, Montecucco C. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. *J Biol Chem.* 1994 Aug 12;269(32):20213-6.

Schiavo G. and Matteoli M. Neurotoxins affecting neuroexocytosis. *Physiol Rev.* 2000 Apr;80(2):717-66.

Schiavo G and Shone CC. Botulinum neurotoxin serotype F is a zinc endopeptidase specific for VAMP/synaptobrevin. *J Biol Chem.* 1993 Jun 5;268(16):11516-9.

Seltzer A, Dubner R and Shir Y. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain.* 1990 Nov;43(2):205-18.

Shi X and Curran JE. The biological activity of ubiquinated BoNT/B light chain *in vitro* and in human SHSY-5Y neuronal cells. *J Cell Biochem.* 2009 Oct 15;108(3):660-7.

Shone CC and Roberts AK. Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neurotoxin. *Eur J Biochem.* 1994 Oct 1;225(1):263-70.

Shone CC and Quinn CP. Proteolytic cleavage of synthetic fragments of vesicle-associated membrane protein, isoform-2 by botulinum type B neurotoxin. *Eur J Biochem.* 1993 Nov 1;217(3):965-71.

Smith MT, Cabot PJ, Ross FB, Robertson AD, Lewis RJ. The novel N-type calcium channel blocker, AM336, produces potent dose-dependent antinociception after intrathecal dosing in rats and inhibits substance P release in rat spinal cord slices. *Pain.* 2002 Mar;96(1-2):119-27.

Sorkin LS and Yaksh TL. Behavioral models of pain states evoked by physical injury to the peripheral nerve. *Neurotherapeutics.* 2009 Oct;6(4):609-19.

Sutton RB, fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Angstrom resolution. *Nature*. 1998; 395: 347-353.

Todd AJ. Anatomy of primary afferents and projection neurons in the rat spinal dorsal horn with particular emphasis on substance P and the neurokinin 1 receptor. *Exp Physiol.* 2002 Mar;87(2):245-9.

Todd AJ, Polgar E, Watt C, Bailey ME, Watanabe M. Neurokinin 1 receptor-expressing projection neurons in laminae III and IV of the rat spinal cord have synaptic AMPA receptors that contain GluR2, GluR3 and GluR4 subunits. *Eur J Neurosci.* 2009 Feb;29(4):718-26.

Ungar D and Hughson FM. SNARE protein structure and function. *Annual Review: Cell Developmental Biology.* 2003; 19: 493-517.

Verderio C, Coco S, Rossetto O, Montecucco C, Matteoli M. Internalization and proteolytic action of botulinum toxins in CNS neurons and astrocytes. *J Neurochem.* 1999 Jul;73(1):372-9.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U. SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron.* 2004 Feb 19;41(4):599-610.

Vrljic M, Strop P, Ernst JA, Sutton B, Chu S, Brunger A. Molecular mechanism of the synaptotagmin-SNARE interaction in Ca^{2+}-triggered vesicle fusion. *Nat Struct Mol Biol.* 2010 Mar;17(3):325-31.

Welch MJ, Purkiss JR, Foster KA. Sensitivity of embryonic rat dorsal root ganglia to Clostridium botulinum neurotoxins. *Toxicon.* 2000 Feb;38(2):245-58.

Wheeler-Aceto H, Porreca F, Cowan A. The rat paw formalin test: comparison noxious agents. *Pain.* 1990 Feb;40(2):229-38.

Wiegand H, Erdmann G, Wellhoner HH. ^{125}I-labelled botulinum A neurotoxin: pharmacokinetics in cats after intramuscular injection. Naunyn Schmiedebergs Arch Pharmacol. 1976;292(2):161-5.

Wu SX, Wang W, Li H, Wang YY, Feng YP, Li YQ. The synaptic connectivity that underlies the noxious transmission and modulation within superficial dorsal horn of the spinal cord. *Prog Neurobiol*. 2010 May;91(1):38-54.

Xu Q, Pichichero ME, Simpson LL, Elias M, Smith LA, Zeng M. An adenoviral vector-based mucosal vaccine is effective in protection against botulism. *Gene Ther.* 2009 Mar;16(3):367-75.

Yaksh TL, Hua XY, Kalcheva I, Nozaki-Taguchi N, Marsala M. The spinal biology in humans and animals of pain states generated by persistent small afferent input. *Proc Natl Acad Sci, USA*. 1999 Jul 6;96(14):7680-6.

Yaksh TL, Jessell TM, Gamse R, Mudge AW, Leeman SE. Intrathecal morphine inhibits substance P release from mammalian spinal cord in vivo. *Nature.* 1980 Jul 10;286(5769):155-7.

Yaksh TL, Pogrel JW, Lee YW, Chaplan SR. Reversal of nerve ligation-induced allodynia by spinal alpha-2 adrenoceptor agonists*. J Pharmacol Exp Ther.* 1995 Jan;272(1):207-14.

Yaksh TL and Rudy TA. Analgesia mediated by a direct spinal action of narcotics. *Science.* 1976 Jun 25;192(4246):1357-8.

Yaksh TL and Rudy TA. Chronic catheterization of the spinal subarachnoid space. *J Neurosci Methods*. 2004 Feb 15;133(1-2):65-9.

Yaksh TL and Ozaki G. An automated flinch detecting system for use in the formalin nociceptive bioassay. *J Appl Physiol*. 2001 Jun;90(6):2386-402.

Yamamoto T and Yaksh TL. Stereospecific effects of non-peptidic NK1 selective antagonist, CP-96,345: antinociception in the absence of motor dysfunction. *Life Science.* 1991; 49(26): 1955-1963.

Zhou H, Zhou B, Pellett S, Johnson EA, Janda KD. Selection and characterization of human monoclonal neutralizing antibody for Clostridium Botulinum neurotoxin serotype B. *Bioorg Med Chem Lett*. 2009 Feb 1;19(3):662-4.