AN *IN VITRO* STUDY OF THE MECHANISMS THAT UNDERLIE CHANGES IN NEURONAL SENSITIVITY AND NEURITE MORPHOLOGY FOLLOWING TREATMENT WITH MICROTUBULE TARGETING AGENTS

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DEDICATION

This work is dedicated to my best friend, Eric Herod, for opening my eyes to the need for inner activity and productive engagement in all aspects of life.

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Sherry Kathleen Pittman

AN *IN VITRO* STUDY OF THE MECHANISMS THAT UNDERLIE CHANGES IN NEURONAL SENSITIVITY AND NEURITE MORPHOLOGY FOLLOWING TREATMENT WITH MICROTUBULE TARGETING AGENTS

Microtubule targeting agents (MTAs) are chemotherapeutics commonly used in the treatment of breast, ovarian, lung, and lymphoma cancers. There are two main classes of MTAs based upon their effects on microtubule stability. The two classes are the destabilizing agents, which include the drug vincristine, and the stabilizing agents, which include paclitaxel and epothilone B. These drugs are highly effective antineoplastics, but their use is often accompanied by several side effects, one of which is peripheral neuropathy. Peripheral neuropathy can be characterized by burning pain, tingling, loss of proprioception, or numbness in the hands and feet. In some patients, the MTA-induced peripheral neuropathy is debilitating and dose-limiting; however, there are no effective prevention strategies or treatment options for peripheral neuropathy as the mechanisms mediating this side effect are unknown. The goal of this work was to investigate MTA-induced effects on neuronal activity and morphology in order to elucidate the underlying mechanisms involved in the development of MTA-induced peripheral neuropathy.

As an indicator of sensory neuronal activity, the basal and stimulated release of the putative nociceptive peptide, calcitonin gene-related peptide (CGRP), was measured from sensory neurons in culture after exposure

to the MTAs paclitaxel, epothilone B, and vincristine. Neurite length and branching were also measured in sensory neuronal cultures after treatment with these MTAs. The results described in this thesis demonstrate that MTAs alter the stimulated release of CGRP from sensory neurons in differential ways depending on the MTA agent employed, the CGRP evoking-stimulus used, the concentration of the MTA agent, the duration of exposure to the MTA agent, and the presence of NGF. It was also observed that MTA agents decrease neurite length and branching, independent of the concentration of NGF in the culture media. Thus, this thesis describes MTA-induced alterations of sensory neuronal sensitivity and neurite morphology and begins to elucidate the underlying mechanisms involved in MTA-induced alterations of sensory neurons. These findings will undoubtedly be used to help elucidate the mechanisms underlying MTA-induced peripheral neuropathy.

Jill C. Fehrenbacher, Ph.D., Chairperson

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LIST OF ABBREVIATIONS

AITC	allyl isothiocyanate
ALCAR	acetyl-*L*-carnitine
ANOVA	analysis of variance
Bcl-2	B-cell lymphoma 2
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin-gene related peptide
CRE	cAMP response element
CREB	cAMP response element-binding
DRG	dorsal root ganglia
eGFP	enhanced green fluorescent protein
EpoA	epothilone A
EpoB	epothilone B
Erk	extracellular signal–regulated kinase
iCGRP	immunoreactive CGRP
IENF	intraepidermal nerve fiber
$InsP_3R$	inositol 1,4,5-triphosphate receptor
MAPK	mitogen-activated protein kinase
MPL	1-methyl-2-pyrrolidone
MTA	microtubule targeting agent
NAC	N-acetyl-*L*-cysteine
NCS-1	neuronal-calcium sensor 1

NGF	nerve growth factor
PBN	phenyl *N*-tert-butylnitrone
PBS	phosphate buffered saline
PDL	poly-D-lysine
PGP 9.5	protein gene product 9.5
PI	propidium iodide
PI3-K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PNDC	peroxynitrite decomposition catalysts
ROS	reactive oxygen species
SEM	standard error of the mean
TEMPOL	1-oxyl-2,2,6,6-tetramethyl-4-hydroxypiperidine
TRP	Transient Receptor Potential
TRPA1	Transient Receptor Potential Ankyrin 1
TRPV1	Transient Receptor Vanilloid 1
TRPV4	Transient Receptor Potential Vanilloid 4
VDCC	Voltage-dependent calcium channel

INTRODUCTION

1) Description of Primary Afferent Sensory Neurons

Primary afferent sensory neurons transmit sensory information from the periphery to the spinal cord. These neurons are a heterogeneous population, and different classes of sensory neurons transmit peripheral information including touch, proprioception, temperature, itch, vibration, pressure, and nociception, which is the signaling of a noxious stimulus (Sternini 1997, Julius and Basbaum 2001). The cell bodies of the primary sensory neurons are located within the dorsal root ganglia (DRG), which are lateral to the vertebrae in the spinal column, and the neurons are pseudo-unipolar in that they have one axon leaving the cell body that bifurcates into two separate branches. One branch innervates the periphery, including the skin, organs, and muscle, and the other branch terminates in the spinal cord (Kandel and Schwartz, 2000). This branching system allows for primary sensory neurons to transmit peripheral sensory input to the neuronal cell body in the DRG and also onto the spinal cord for signaling to higher brain systems (Kandel and Schwartz, 2000).

This work is focused on functional and morphological effects of a class of anticancer drugs called microtubule targeting agents (MTAs) on a particular subclass of peripheral sensory neurons that process and transmit noxious peripheral sensations (Loeser and Treede 2008). Peripheral nociceptive neurons are able to detect and transmit noxious signals from chemical, mechanical, and thermal stimuli (Julius and Basbaum 2001). There are two main subdivisions of

peripheral nociceptive neurons based upon the myelination state of their axons. Unmyelinated nociceptors are termed C fibers, and lightly myelinated nociceptors are Aδ fibers. These C and Aδ nociceptive neurons are also termed small and medium diameter sensory neurons, respectively, since the diameters of their cell bodies (small: < 27 μm, medium: 27-45 μm) are smaller than the Aα and Aβ sensory neurons, or large diameter neurons (large: > 45 μm), that detect non-noxious stimuli such as proprioception, touch, vibration, and pressure (Schmalbruch 1986, Scroggs and Fox 1992, Yusaf, Goodman et al. 2001). The nociceptive neurons have a slower conduction velocity and higher thresholds to activation compared to the large diameter sensory neurons. Researchers utilize conduction velocity as a way to differentiate the subsets of sensory neurons; C fibers have a slower conduction velocity than Aδ fibers (Harper and Lawson 1985, Harper and Lawson 1985).

Small and medium diameter sensory neurons can be further subdivided into peptidergic and nonpeptidergic neurons. The peptidergic neurons express the neuropeptides substance P and calcitonin-gene related peptide (CGRP) (Lawson, Perry et al. 1993, Hiruma, Saito et al. 2000). CGRP is synthesized in the cell bodies of the sensory neurons and then trafficked to the central and peripheral terminals in large dense core vesicles (Kashihara, Sakaguchi et al. 1989). The neuropeptides are important in nociceptive signaling (Besson and Chaouch 1987) as evidenced by studies that have shown that capsaicin-induced allodynia can be alleviated by the administration of substance P and CGRP receptor antagonists in animal models (Sun, Lawand et al. 2003, Sun, Lawand et

al. 2004). Also, studies have shown that following noxious stimulation, there is a release of neuropeptides from sensory neurons into the spinal cord (Kuraishi, Hirota et al. 1985, Holzer 1988), which also suggests that the neuropeptides are involved in nociceptive signaling. In rat DRG neurons, approximately 50% of the neurons express substance P and 47% express CGRP, and there is substantial overlap of the neuropeptides in approximately 45% of the DRG neurons (Kai-Kai, Anderton et al. 1986, McCarthy and Lawson 1990, Price and Flores 2007). Approximately 49% of the CGRP-expressing DRG also express TRPV1 (Price and Flores 2007).

CGRP has several physiological functions that are important in mediating neuronal sensitization and neurogenic inflammation. After stimulation with noxious stimuli, CGRP is released from the central terminals to signal to the second order neurons which in turn signal to higher brain regions (Ryu, Gerber et al. 1988, Ryu, Gerber et al. 1988). The peripheral terminals also release CGRP. One function of CGRP in the periphery is autocrine; CGRP binds to its G-protein coupled receptor and activates the cAMP/PKA signaling cascade, augmenting neuronal sensitivity (Sun, Tu et al. 2004). CGRP can also elevate intracellular calcium levels and enhance inward sodium currents via PKA and PKC signaling within DRG neurons, thereby modulating synaptic transmission (Natura, von Banchet et al. 2005). Other functions of CGRP in the periphery include vasodilatation of blood vessels and activation and recruitment of proinflammatory immune cells (Richardson and Vasko 2002, Gracias, Cummins et al. 2011). The CGRP receptor is broadly expressed throughout the entire organism including

within sensory neuron cell bodies, the dorsal horn of the spinal cord, and the brain (van Rossum, Hanisch et al. 1997). It is also expressed in non-nervous system tissues and cells including vascular smooth muscle, kidney, heart, spleen, liver, skeletal muscle, endothelial cells, and mast cells (van Rossum, Hanisch et al. 1997).

This work uses the release of CGRP from sensory neurons in culture as an indicator of sensory neuronal activity. Dougherty and co-workers suggest that a predominant clinical effect of a commonly used MTA agent, paclitaxel, is on discrimination of sensations conducted by large diameter fibers, and his group theorizes that the function of small diameter sensory neurons is maintained (Dougherty, Cata et al. 2004); however, a role for the small diameter sensory neurons has been intimated based upon *in vivo* endpoints (Polomano, Mannes et al. 2001, Gracias, Cummins et al. 2011). As described below, low-dose paclitaxel altered nociceptive endpoints in an animal model without altering motor function (Polomano, Mannes et al. 2001). Systemic administration of paclitaxel also decreased CGRP release and subsequent capsaicin-evoked vasodilatation in the rat hindpaw, suggesting that the release of CGRP and function of nociceptive peripheral neurons is altered after treatment with paclitaxel (Gracias, Cummins et al. 2011). This work examines the effects of MTA agents on CGRP release from sensory neurons in culture as a measure of MTA-induced alterations of neuronal sensitization.

2) Neuronal Sensitization and Nerve Growth Factor

Sensitization of nociceptive neurons occurs following tissue injury by overt damage and/or infection, and sensitization is often mediated by inflammatory mediators (Basbaum, Bautista et al. 2009, Gold and Gebhart 2010). A short-lived, acute sensitization of the sensory neurons is considered to be beneficial to an organism as it can prevent further tissue damage and protect the organism during tissue repair processes, which is the underlying function of nociception (Reichling and Levine 2009). Sensitization of sensory neurons can also persist for long periods of time, which is termed chronic sensitization. This form of sensitization serves no beneficial role to the organism and is considered to be pathological in nature (Tsuda, Inoue et al. 2005, Basbaum, Bautista et al. 2009). The work in this thesis is directed at investigating the chronic sensitization of sensory neurons by MTA agents since MTAs produce chronic pain states in patients, which may be mediated by chronic sensitization of the sensory neurons. I specifically investigated the sensitization of sensory neurons of the peripheral nervous system since systemic paclitaxel administration in rats resulted in high concentrations of accumulated paclitaxel within DRG and to a slightly lesser extent within the sciatic nerve (Cavaletti, Cavalletti et al. 2000). Low concentrations were found within brain tissue and spinal cord of the central nervous system, suggesting that the peripheral nervous system is more susceptible to paclitaxel exposure (Cavaletti, Cavalletti et al. 2000).

Neurons are termed sensitized when they become more responsive to a given stimulus, in other words, a given stimulus elicits a greater response from

the neurons than prior to the sensitization event (Castellucci and Kandel 1976, Lewin, Ritter et al. 1993, Hingtgen and Vasko 1994). Sensitization of the peripheral nociceptor neurons, or peripheral sensitization, results in increased transmitter release from the peripheral and central terminals upon stimulation. This hypersensitivity of the nociceptive neurons can contribute to the development of hyperalgesia and allodynia (Basbaum 1999, Basbaum and Woolf 1999). Sensitization of sensory neurons often occurs after exposure to inflammatory mediators that augment neuronal function via transcriptional changes or post-translational modifications of receptors, ion channels, or proteins important in modulation of neuronal activity (Bhave and Gereau 2004).

One inflammatory mediator involved in neuronal sensitization during tissue injury is nerve growth factor (NGF). NGF is a trophic factor that is an important mediator of neuronal hypersensitivity and can augment nociceptive responses and pain in humans (Petty, Cornblath et al. 1994, Svensson, Cairns et al. 2003). NGF is produced and released from a variety of cells after tissue injury including macrophages, monocytes, mast cells, and Schwann cells (Lindholm, Heumann et al. 1987, Leon, Buriani et al. 1994, Lambiase, Bonini et al. 1995). Acute exposure to NGF can sensitize adult DRG neurons by several mechanisms including activation of phosphoinositide 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK), and phospholipase C (PLC) signaling cascades, likely resulting in phosphorylation of the Transient Receptor Potential Vanilloid 1 (TRPV1) channel, which is a non-selective cation channel important for the perception of noxious stimuli (Caterina, Schumacher et al. 1997, Julius and

Basbaum 2001, Zhu and Oxford 2007, Park, Fehrenbacher et al. 2010). There is some debate about the pathways mediating NGF-induced sensitization of sensory neurons as PLC inhibitors prevent NGF-induced augmentation of capsaicin-evoked CGRP release whereas MEK and PI3-K inhibitors do not, suggesting that PLC signaling mediates NGF-induced changes in neuropeptide release (Park, Fehrenbacher et al. 2010). Interestingly, there is a developmental shift in the dependency of DRG neurons on NGF for survival as well as the ability of NGF to sensitize neurons (Buchman and Davies 1993, Acosta, Fabrega et al. 2001, Zhu, Galoyan et al. 2004); NGF can sensitize TRPV1 to capsaicin and noxious heat stimuli in DRG harvested from adult rats, but it does not sensitize TRPV1 in DRG from embryonic rats (Zhu, Galoyan et al. 2004). The mechanisms underlying these developmental shifts in response to NGF within sensory neurons are unknown.

Long-term exposure to NGF alters the sensitivity of sensory neurons via several mechanisms, including increases in the expression of several channels and neuropeptides involved in neuronal sensitization, intimating NGF's involvement in peripheral sensitization. NGF regulates the expression of substance P and CGRP within peripheral sensory neurons (Lindsay, Lockett et al. 1989). In DRG cultures from adult rats, maintaining the sensory neurons in the absence of added NGF resulted in a decline of CGRP mRNA. After 5 days of continual growth in 25 ng/ml of NGF, CGRP mRNA increased 15-fold and CGRP peptide content increased 4-fold compared to control cultures (Lindsay and Harmar 1989). Similarly, growing adult DRG neurons in either 30 ng/ml or 100

ng/ml of NGF for 7 days increased CGRP content and potassium-stimulated release of CGRP from sensory neurons in culture (Park, Fehrenbacher et al. 2010). This NGF-induced increase in CGRP content was determined to be partially mediated by the Ras signaling cascade; Ras-GTP levels increased in neurons grown in 30 ng/ml or 100 ng/ml NGF without increasing total Ras protein levels, and blocking the pathway with either a lentivirus expressing a dominant negative Ras construct or with a MEK inhibitor attenuated the increase in CGRP content (Park, Fehrenbacher et al. 2010).

NGF also alters the expression of membrane channels involved in neuronal sensitivity. In adult DRG cultures, NGF increased TRPV1 mRNA levels in a trkA-dependent and concentration-dependent manner (Winston, Toma et al. 2001), and NGF increased the translocation and insertion of TRPV1 in the plasma membrane of HEK293 cells and sensory neurons in culture (Zhang, Huang et al. 2005). After inflammation, TRPV1 protein expression increased in rat DRG, and this increase was prevented with an anti-NGF antibody (Amaya, Shimosato et al. 2004). NGF has also been found to modulate sodium channel expression; in adult sensory neurons in culture and PC12 cells, NGF increased sodium channel mRNA expression by a trkA-dependent mechanism (Fjell, Cummins et al. 1999, Choi, Toledo-Aral et al. 2001). These NGF-induced changes in ion channels can contribute to sensitization of sensory neurons, leading to augmented responses to noxious stimuli.

NGF can also indirectly alter neuronal sensitivity by modulating the activity of immune cells that release inflammatory mediators and cytokines involved in

neuronal sensitization. NGF increased the number and size of mast cells in several tissue types in neonatal rats receiving NGF injections (Aloe and Levi-Montalcini 1977), and the release of proinflammatory and sensitizing substances such as histamine increased from mast cells after exposure to NGF *in vitro* (Aloe and De Simone 1989). NGF affects several other immune system cells, which potentially can augment neuronal sensitization by releasing proinflammatory cytokines and chemokines, including B- and T-lymphocytes, neutrophils, and monocytes (Gee, Boyle et al. 1983, Ehrhard, Erb et al. 1994, Torcia, Bracci-Laudiero et al. 1996, Aloe, Simone et al. 1999). NGF likely alters the sensitivity of sensory neurons directly and indirectly by altering signaling cascades, membrane ion channels, and immune cells that can all contribute to neuronal sensitization.

3) Cytoskeleton and NGF Involvement in Neurite Length

Neurons undergo complicated remodeling of axonal neurites in order to functionally adapt during development, learning, and following injury (Niwa, Hayakawa et al. 2002, Skaper 2005, Tucker and Mearow 2008). Axons undergo periods of both neurite outgrowth and retraction based upon cellular environmental demands, and the needs of the cell are communicated to neurite structural machinery via intricate signaling cascades (Spira, Oren et al. 2001, Zheng and Poo 2007). The processes involved in neurite outgrowth and retraction are delicately balanced, and microtubules, actin, motor proteins, and growth factors are all important components of neurite growth, maintenance, and

retraction (Heidemann, Joshi et al. 1985, Smith and Skene 1997, Wang, Naruse et al. 2001, Niwa, Hayakawa et al. 2002, Zheng and Poo 2007, Feng, Kang et al. 2010).

A growing body of evidence suggests that neurites are maintained by opposing forces between actin microfilament structures and microtubules (Wang, Naruse et al. 2001). These opposing forces between microfilaments and microtubules are thought to be generated by myosin motors on the microfilaments and dynein motors on the microtubules via hydrolysis of ATP (Ahmad, Hughey et al. 2000). A counterbalance of forces between the microfilaments and microtubules is important for maintaining neurite terminals and morphology (Wang, Naruse et al. 2001). Inhibition of microtubule dynamics or motor proteins disrupts the counterbalance of forces and causes neurite retraction that can be prevented by disassembly of microfilaments (Letourneau, Shattuck et al. 1987, Ahmad, Hughey et al. 2000). In an embryonic chick DRG culture model, nocodazole, a microtubule destabilizing drug, or microinjection of dynamitin, a protein that causes cessation of dynein motor activity, induced neurite retraction, suggesting that a disruption in microtubule dynamics results in neurite retraction. This neurite retraction was prevented with pre-treatment of the neurites with latrunculin, a microfilament disassembly agent, or microinjection with a dominant-negative myosin motor. Treatment with latrunculin or the dominant-negative myosin motor alone did not cause neurite retraction. This suggests that functional microfilaments and microfilament-motor proteins are necessary for neurite retraction, and the forces generated by microfilaments are

counterbalanced by microtubule forces (Ahmad, Hughey et al. 2000). Agents that disrupt the counterbalance between the cytoskeleton structures and motor protein forces can alter neurite maintenance and result in neurite retraction (Letourneau, Shattuck et al. 1987, Ahmad, Hughey et al. 2000, Wang, Naruse et al. 2001).

Coordinated efforts of microfilaments and microtubules are also involved in neurite outgrowth. During outgrowth in development and after injury, microfilaments organize into web-like structures, known as growth cones, that are responsible for elongation of the neurite and are supported by the microtubule structures (da Silva and Dotti 2002, Oertle, van der Haar et al. 2003, Erturk, Hellal et al. 2007). Destabilization of the microtubule network in DRG cultures with nocodazole caused growth cones to form into retraction bulb-like structures, thereby preventing growth cone elongation and inhibiting neurite growth (Erturk, Hellal et al. 2007). This suggests that microtubule deregulation can decrease neurite length not only by enhancing neurite retraction but also by preventing neurite elongation through the disruption of growth cones.

There are several key signaling pathways involved in the regulation of neurite length including the downstream signaling cascades initiated by NGF. NGF has been shown within numerous systems to promote DRG neurite growth (Kimpinski, Campenot et al. 1997, Hoke, Redett et al. 2006, Lykissas, Batistatou et al. 2007). Using DRG neuronal cultures derived from adult rats, Gavazzi et al. (Gavazzi, Kumar et al. 1999) showed that growing DRG neurons in 100 ng/ml NGF increased the number of process-bearing neurons and increased the total

neurite length of the neurons. Similarly Niwa et al. (Niwa, Hayakawa et al. 2002) cultured DRG explant cultures derived from embryonic, neonatal, young adult, and aged animals and found that 50 ng/ml of NGF significantly increased the neurite length from all ages of explants. In an autonomic, superior cervical ganglia explant model from adult rats, NGF promotes outgrowth of the neurites over time (Hayakawa, Sobue et al. 1994, Hayakawa, Itoh et al. 1999), and in an adult DRG culture model, NGF promotes the outgrowth of neurons in a concentration-dependent manner (Malgrange, Delree et al. 1994). NGF promotes neurite growth by initiating signaling cascades through the TrkA receptor, and both the phosphatidylinositol 3-kinase (PI3-K) and Ras/MAPK signaling cascades have been implicated in mediating NGF-induced neurite growth (Edstrom and Ekstrom 2003, Tucker, Rahimtula et al. 2008). Although still a subject of investigation, PI3-K activates Akt, a serine/threonine protein kinase necessary for neurite elongation in several model systems (Edstrom and Ekstrom 2003). Both PI3-K and Ras signaling mediate neurite growth by activating downstream effectors including the cAMP response element-binding protein (CREB) and tau, a microtubule-associated protein (Tucker and Mearow 2008).

4) Microtubule Targeting Agents

Microtubules are dynamic cellular structures that have crucial roles in many functions including cell signaling, mitosis and cell division, intracellular organelle and vesicular transport, cell shape and motility, and intracellular

organization (Burnside 1975, Wilson and Jordan 2004). Microtubules are fibrous polymers that are formed by heterodimers of two types of tubulin monomers, β and α. The β and α heterodimers form slender tube-like structures, called polymers, which undergo dynamic rearrangement. This dynamic rearrangement property of microtubules is characterized by a continuous exchange between free β and α heterodimer pools and the microtubule polymer structure. Tubulin heterodimers are continuously added to the growing, or plus end, of the polymer and removed from the shortening, or minus, end of the polymer, depending on the demands of the cell (Mandelkow and Mandelkow 1989, Mandelkow, Mandelkow et al. 1989). This dynamic rearrangement is necessary for microtubule physiology (Gelfand and Bershadsky 1991). The ends of a microtubule polymer are never stationary, and the rates of polymerization and depolymerization of the two ends are different (Horio and Hotani 1986). Because of the dynamic characteristics of microtubules and the necessity for this dynamic rearrangement in cell physiology, especially in cell division, microtubule polymers and tubulin monomers have become major targets for cancer therapeutics (Morris and Fornier 2008).

There are two main classes of MTAs, which are compounds that bind directly to the tubulin subunits to alter the dynamic rearrangement of microtubules. Compounds that bind the β-tubulin subunit and prevent polymerization of microtubules are called depolymerizing or destabilizing agents and include vincristine, vinblastine, vinorelbine, vindesine, nocodazole, and colchicine (Johnson, Armstrong et al. 1963, Sartorelli and Creasey 1969, Zhou

and Rahmani 1992). The other class of MTAs binds β-tubulin comprising the microtubule polymer and prevents the depolymerization of the microtubule polymer. These agents are called polymerizing or stabilizing agents and include paclitaxel, docetaxel, and the epothilones (Sackett and Fojo 1999, Altaha, Fojo et al. 2002). Although they have opposite effects on microtubules, both classes interfere with the dynamic rearrangement of microtubules. Alterations in microtubule dynamics is a common mechanism of blocking mitosis within dividing cells, which leads to subsequent apoptosis in cancer cells (Jordan, Wendell et al. 1996), thus MTAs have been developed and used extensively as anticancer drugs (Wilson and Jordan 2004).

The *Vinca* alkaloids are a group of microtubule destabilizing drugs that were first isolated from the leaves of the periwinkle plant, *Catharanthus roseus,* and include the naturally occurring vinblastine and vincristine, as well as synthetically made vinorelbine and vindesine (Johnson, Armstrong et al. 1963, Zhou and Rahmani 1992). The *Vinca* alkaloids are commonly used for the treatment of Hodgkin's lymphoma, non-small cell lung cancer, breast cancer, lymphomas, and head and neck cancers (Himes 1991, Moudi, Go et al. 2013). The *Vinca* alkaloids bind to two sites on the β-tubulin, and upon binding, microtubule dynamics and polymerization are interrupted (Wilson, Creswell et al. 1975). The *Vinca* alkaloids can also bind free tubulin dimers to prevent tubulin polymerization as well as the ends and outer surface of microtubules in a domain referred to as the vinca domain (Himes 1991, Dumontet and Jordan 2010). In this manner, *Vinca* alkaloids inhibit microtubule polymerization. While the *Vinca*

alkaloids are effective antineoplastic agents, their use is accompanied by common side effects including reversible myelosuppression, neutropenia, anemia, acute cardiac ischemia, gastrointestinal toxicities, and peripheral neuropathy (Moudi, Go et al. 2013).

Another commonly used class of antineoplastics are the taxanes, paclitaxel and docetaxel, which are microtubule stabilizing agents. Paclitaxel was first isolated from the bark of the yew tree, *Taxus brevifola,* in 1967 by Monroe Wall and Mansukh Wani (Wani, Taylor et al. 1971). The semi-synthetic analog, docetaxel, was derived decades later. Docetaxel is derived semisynthetically through a process using the needles of the tree. *Taxus baccata* (Ringel and Horwitz 1991, Horwitz 1992). The taxanes are used as treatments for several types of cancer including breast, ovarian, gastric, and lung cancers (Rowinsky, Gilbert et al. 1991). Taxanes alter microtubule polymerization by binding specifically to the β-tubulin subunit at one 31-amino acid binding site on the N-terminal tail (Manfredi, Parness et al. 1982, Rao, Horwitz et al. 1992, Rao, Krauss et al. 1994). This domain is separate from the vinca domain and along the lumen of the tubular microtubule polymer (Manfredi, Parness et al. 1982). It is hypothesized that upon binding to tubulin, paclitaxel induces a conformational change in the microtubule that increases the binding between tubulin subunits, making the microtubule more stable (Rao, Krauss et al. 1994). In this way, taxanes enhance microtubule polymerization as well as prevent depolymerization of the polymer (Manfredi, Parness et al. 1982) At high concentrations, paclitaxel (> 1 μM) increases microtubule polymerization, hence

the microtubule polymer mass increases, with nearly 1:1 stoichiometry of paclitaxel binding to tubulin, creating microtubule bundles within cells (Schiff and Horwitz 1980, Carboni, Farina et al. 1993), and free tubulin concentrations are decreased (Schiff, Fant et al. 1979, Horwitz 1992). Lower concentrations of paclitaxel (10-100 nM) suppress the extent and rate of microtubule shortening without altering the microtubule polymer mass. At intermediate concentrations of paclitaxel (100 nM-1 μM), microtubule dynamics are inhibited at both ends of the microtubule; however, significant changes in the microtubule polymer mass are still not observed (Derry, Wilson et al. 1995). The taxanes are effective anticancer agents, although their use is sometimes limited by side effects. Common taxane-induced side effects include neutropenia, myalgias, alopecia, cardiac rhythm abnormalities, and peripheral neuropathy (Rowinsky, Chaudhry et al. 1993, Rowinsky, Eisenhauer et al. 1993, Pronk, Stoter et al. 1995, Kuppens 2006). Neutropenia was a major dose-limiting side effect of paclitaxel; however, concurrent treatment with granulocyte-colony stimulating factor and increasing infusion time of the taxanes to 24 hours can alleviate the neutropenia side effect, leaving peripheral neuropathy as the major dose-limiting side effect of taxane therapy (Sarosy, Kohn et al. 1992, Reichman, Seidman et al. 1993, Seidman, Norton et al. 1993, Verweij, Clavel et al. 1994).

Epothilones are another class of microtubule stabilizing compounds that are primarily used in the treatment of metastatic breast cancer (Lee, Borzilleri et al. 2008). The epothilones, EpoA, EpoB and the synthetic EpoB analog, ixabepilone, are macrolides that were first isolated from the bacterium,

Sorangium cellulosum, and have been found to have similar effects on microtubules as paclitaxel, although the structures of the Epos and paclitaxel are different. EpoA and EpoB induce tubulin polymerization and enhance the stabilization of microtubule polymers similarly to paclitaxel (Bollag, McQueney et al. 1995). Both Epos are competitive inhibitors of the binding of paclitaxel to microtubules, suggesting that they bind tubulin at the same site as paclitaxel or near the same site (Bollag, McQueney et al. 1995, Kowalski, Giannakakou et al. 1997). While the exact tubulin binding site of the Epos is still unknown, molecular modeling suggests that the Epos bind tubulin within the paclitaxel-binding domain (Giannakakou, Gussio et al. 2000). The Epos were originally thought to be promising drug candidates because they are not as susceptible to cellular extrusion via the multidrug-resistant phenotype as paclitaxel. Multidrug-resistant cancer cells are capable of increasing efflux of drugs via the ATP binding cassette proteins, which results in a reduction of intracellular drug concentrations (Dumontet and Jordan 2010). Unlike paclitaxel and vincristine, the Epos are not substrates of P-glycoprotein, which is a transport protein commonly overexpressed in drug-resistant cancer (Arias, Gatmaitan et al. 1990, Dumontet and Jordan 2010). Furthermore, the Epos are more effective in reducing tumor volumes in human multidrug-resistant tumor xenograft mouse models than paclitaxel (Chou, O'Connor et al. 2001). Use of ixabepilone is accompanied by side effects including neutropenia, myalgias, alopecia, and peripheral neuropathy (Lee, Borzilleri et al. 2008).

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5) MTA-Induced Peripheral Neuropathy

MTA-induced peripheral neuropathy is a common, dose-limiting side effect of many different classes of MTA agents including *Vinca* alkaloids, taxanes, and Epos. MTA-induced peripheral neuropathy symptoms are experienced in the hands and feet of patients, termed a “glove and stocking” distribution. Peripheral neuropathy can be characterized by gain in function manifestations including burning pain, tingling, or paresthesia as well as loss of function characteristics including loss of proprioception, decreased perception of vibrations, or numbness, although peripheral neuropathy symptoms vary between patients (Lipton, Apfel et al. 1989, Forsyth, Balmaceda et al. 1997, Iniguez, Larrode et al. 1998, Miltenburg and Boogerd 2014). Sensory deficits occur more frequently than motor deficits; however, myopathy and decreased motor skills do occasionally occur in patients (Wiernik, Schwartz et al. 1987, Lipton, Apfel et al. 1989, Forsyth, Balmaceda et al. 1997, Dougherty, Cata et al. 2004). While less common, autonomic neuropathy, characterized by orthostatic hypotension and paralytic ileus, occurs in some patients receiving high-dose paclitaxel and vincristine therapy, although it is debated that autonomic neuropathy is more common in patients receiving vincristine therapy (Legha 1986, Jerian, Sarosy et al. 1993, Rowinsky, Eisenhauer et al. 1993, Miltenburg and Boogerd 2014). The peripheral neuropathy symptoms range in intensity from mild to severe, and although these symptoms resolve in some patients following cessation of treatment, they may be irreversible and can persist as chronic neuropathic pain (Connelly, Markman et al. 1996).

The development of MTA-induced peripheral neuropathy cannot currently be predicted, but there are several risk factors associated with the occurrence and intensity of MTA-induced peripheral neuropathy. The risk factors include frequency of treatment, duration of chemotherapeutic infusion, dose given per treatment cycle, the cumulative dose of the chemotherapeutic received, and co-administration with drugs that also cause peripheral neuropathy, including the platinum class of chemotherapeutics (Lee and Swain 2006). Other risk factors include diabetes mellitus, preexisting peripheral neuropathy, alcoholism, and age (Lee and Swain 2006, Windebank and Grisold 2008). There are no known treatment options which specifically prevent or reverse MTA-induced peripheral neuropathy, and most patients resort to scaling back the bolus of chemotherapeutic drug per treatment or to discontinuing administration entirely to minimize symptoms of the neuropathy (Pachman, Barton et al. 2011). Both of these interventions can interfere with optimal antineoplastic outcomes (Capri, Tarenzi et al. 1996, Pachman, Barton et al. 2011).

The mechanisms underlying the development of MTA-induced peripheral neuropathy are unknown, but there are several theories that are currently being explored. Since the peripheral neuropathy affects the hands and feet, which are innervated by the longest, distal axons of sensory neurons, and because microtubules are crucial for the transport of proteins along neuronal axons, it is theorized that the MTAs induce peripheral neuropathy by altering retrograde and anterograde transportation of proteins throughout neurons (Carlson and Ocean 2011). The cell bodies of sensory neurons in the DRG and their peripheral

nerves are not protected by the blood-brain barrier, making them more susceptible to toxicity from MTAs (Cavaletti, Cavalletti et al. 2000, van der Sandt, Gaillard et al. 2001, Kemper, Cleypool et al. 2004). While the ganglia of the autonomic system are also not protected by the blood-brain barrier, no studies have been performed comparing the relative accumulation of paclitaxel in sensory versus autonomic ganglia. It is theorized that peripheral neuropathy may be a combination effect of MTA-induced toxicity of the cell body as well as the axon, and since the neuropathy develops in the distal extremities where axons are the longest, neurite length may be an important variable in MTA-induced toxicity (Lee and Swain 2006). This is supported by the finding that paclitaxel exposure to the sensory nerve axon in a microfluidics cell culture model is essential for development of paclitaxel-induced nerve retraction, and exposure to the cell body alone did not induce retraction (Yang, Siddique et al. 2009). While exposure to the axon is necessary for paclitaxel-induced retraction, the role of accumulated paclitaxel within the cell body is currently unknown, but it may have important implications for changes in trafficking of proteins from the soma to the axons as well as transcription, and translation of proteins necessary for neuronal function (Kashihara, Sakaguchi et al. 1989, Watson and Latchman 1995, Freeland, Liu et al. 2000) Motor neuron function is also affected by MTAs; however, the cell bodies of motor neurons are protected by the blood-brain barrier within the spinal cord and the axons of motor neurons are highly myelinated, presumably decreasing their exposure to MTAs (Schmalbruch 1986, Fetcho 1987). This also suggests that exposure of the neuronal cell body to

MTAs is important in the development of peripheral neuropathy. Motor neuropathies are more common at higher doses of paclitaxel (500 mg/m^2 or higher) (Iniguez, Larrode et al. 1998). Generally, MTA-induced peripheral neuropathy is believed to be a length-dependent distal sensory neuropathy; however, to a lesser extent, motor neurons and autonomic neurons are also implicated in MTA-induced neuropathy (Carlson and Ocean 2011).

6) Clinical Incidence of MTA-induced Peripheral Neuropathy

Peripheral neuropathy is graded using several different scales in order to categorize the severity of the neuropathy experienced. Although there is not a universal scale, many studies employ scales established by the World Health Organization, Eastern Cooperative Oncology Group, or the National Cancer Institute (Miller, Hoogstraten et al. 1981, Oken, Creech et al. 1982, Postma and Heimans 2000). Generally, a grade 1 neuropathy indicates mild neuropathy that does not interfere with daily life, grade 2 indicates a moderate neuropathy, grade 3 describes severe symptoms of neuropathy that limit activities of daily living, and grade 4 indicates life threatening neuropathy (Postma and Heimans 2000, Carlson and Ocean 2011). While these scales are mainly subjective in nature (Cavaletti and Marmiroli 2012), there are several other approaches used to measure peripheral neuropathy within patients. Commonly used approaches include quantitative sensory testing, which are techniques to assess changes in discriminative touch, sharpness, temperature, vibration, pressure and nociceptive function (Rolke, Baron et al. 2006). Clinicians also use neurophysiological

testing to assess changes in the conduction velocity of nerve fibers in patients (Cavaletti, Frigeni et al. 2007).

Incidence and severity of peripheral neuropathy from paclitaxel chemotherapy depends on treatment schedule and dose, with an increased incidence of toxicity with larger cumulative dose and more frequent dosing (Winer, Berry et al. 2004, Seidman, Berry et al. 2008). In one study, breast cancer patients received weekly treatment of a 100 mg/m^2 dose for 6 weeks followed by a 80 mg/m^2 dose of paclitaxel, which was continued until disease progression or limited by toxicity (Seidman, Berry et al. 2008). This dosing paradigm resulted in the development of grade 3 or 4 neuropathy in 9-24% of the patients (Seidman, Berry et al. 2008). In studies where higher doses of paclitaxel were administered, the incidence of peripheral neuropathy also increased; a 24-hour infusion of 250 mg/m^2 paclitaxel every 21 days caused peripheral neuropathy in 40% of the patients (Wiernik, Schwartz et al. 1987). In another study using a range of paclitaxel doses infused every 3 weeks in metastatic breast cancer patients, doses of 175 mg/m^2, 210 mg/m^2 , and 250 mg/m^2 dose dependently enhanced the incidence and severity of peripheral neuropathy (Winer, Berry et al. 2004). Pharmacokinetic studies have shown that dosing patients with 125-225 mg/m^2 of paclitaxel results in maximal plasma concentrations between 1 and 10 μM (Rowinsky, Jiroutek et al. 1999, Henningsson, Karlsson et al. 2001), with a reduction in concentration to between 10 nM and 1 μM by 20 hours.

Vincristine treatment also causes neuropathy in patients. In non-Hodgkin's lymphoma patients, a dosing paradigm of 1.5 mg/m^2 per week resulted in the development of 1-3 grade neuropathy in 35.2% of the patients (Dorchin, Masoumi Dehshiri et al. 2013). In another study of patients with non-Hodgkin's lymphoma using a liposomal encapsulation of vincristine to increase the therapeutic efficacy, a dosing paradigm of 2.0 mg/m^2 of liposomal vincristine every 2 weeks resulted in the development of grade 3 or 4 neuropathy in 31% of the patients (Sarris, Hagemeister et al. 2000).

Peripheral neuropathy is observed at a similar rate in patients receiving ixabepilone therapy. In a clinical trial including patients with metastatic breast cancer, a dosing regimen of 40 mg/m^2 every 3 weeks caused grade 3 peripheral neuropathy in 12% of the patients, whereas a dosing regimen of 20 mg/m^2 every week for 3 weeks followed by one week off caused grade 3 neuropathy in 27% of the patients, suggesting that frequency of therapy greatly affects the development of peripheral neuropathy (Fountzilas, Kotoula et al. 2013).

The onset of MTA-induced peripheral neuropathy symptoms varies between patients due to differences in the doses of drugs administered and upon the presence of risk factors described previously. Generally, burning pain and tingling symptoms are the first to appear within 24 to 72 hours after MTA-treatment, and these symptoms typically resolve within a week following the first infusion (Lipton, Apfel et al. 1989, Iniguez, Larrode et al. 1998). With succeeding MTA treatments, symptom severity increases in patients, and they show an increased incidence of loss of proprioception, decreased reflexes, and loss of

vibratory sensations (Wiernik, Schwartz et al. 1987, Lipton, Apfel et al. 1989, Sarosy, Kohn et al. 1992, Forsyth, Balmaceda et al. 1997). Motor neuropathy is less commonly experienced by patients, but decreases in fine motor skills and gait disturbances can be observed following high-dose infusions of paclitaxel (>500 mg/m^2) (Sarosy, Kohn et al. 1992, Schiller, Storer et al. 1994, Iniguez, Larrode et al. 1998). In most patients, neuropathy symptoms resolve after a few months following discontinuation of MTA therapy (Postma, Vermorken et al. 1995, Roche, Yelle et al. 2007); however, in some patients, neuropathy can persist for months and even years following cessation of treatment (Verstappen, Koeppen et al. 2005, Balayssac, Ferrier et al. 2011, Osmani, Vignes et al. 2012). There is also a “paclitaxel-associated acute pain syndrome” that occurs in up to 70% of patients receiving taxane therapy and is characterized by aching and sharp pain in the extremities as early as 24 hours after paclitaxel infusion (Loprinzi, Maddocks-Christianson et al. 2007, Saibil, Fitzgerald et al. 2010, Reeves, Dakhil et al. 2012). This syndrome has not been well characterized, but the development of the paclitaxel-associated acute pain syndrome is correlated with development of more severe paclitaxel-induced peripheral neuropathy with further treatment (Pachman, Barton et al. 2011). The development of MTA-induced neuropathy, although not fully characterized within all trials, appears to be dose and frequency dependent, and patients tend to develop sensory neuropathies before motor neuropathies.

7) Animal Models of Peripheral Neuropathy

Several animal models have been developed to examine the *in vivo* effects of paclitaxel on neuronal activity. A low-dose model, whereby paclitaxel (cumulative doses of 4, 8, or 16 mg/kg) is injected systemically, either by intravenous or intra peritoneal injection, over the course of 7 days, results in mechanical hyperalgesia and allodynia, as well as cold allodynia, without causing overt nerve damage, alterations in nerve conduction velocity, or changes in neuronal survival (Polomano, Mannes et al. 2001, Flatters and Bennett 2006, Matsumoto, Inoue et al. 2006). This dosing paradigm is similar to clinical doses of 200 mg/m^2, which is approximately 6 mg/kg in patients who have an approximate body surface area of 1.7 m^2 and weigh 55 kg (Redal-Baigorri, Rasmussen et al. 2014). A cumulative dose of 4 mg/kg of paclitaxel also results in a decrease of capsaicin-evoked bloodflow within the hindpaw of the rat, suggesting that paclitaxel alters the function of small diameter sensory neurons involved in vasodilatation of the skin (Gracias, Cummins et al. 2011). In an alternative high-dose animal model, paclitaxel (cumulative doses of 80-135 mg/kg) is injected systemically over the course of 5-9 weeks. This model produces impairments in pain-like behaviors using the tail-flick test and thermal withdrawal latencies as endpoints, decreases in coordination as measured by the rota-rod test, and decreases in nerve conduction velocity in the tail nerve accompanied by swollen, degenerated axons (Cavaletti, Cavalletti et al. 1997, Authier, Gillet et al. 2000). These data suggest that increases and decreases in neuronal sensitivity occur, dependent upon the endpoint measured and the

dosing paradigm of paclitaxel administered, which is similar to the clinical observations of MTA-induced peripheral neuropathy in patients.

The most commonly used models to examine the *in vivo* effects of vincristine use daily intravenous or systemic injections of the drug for 2 weeks (cumulative dose of 1 mg/kg). Mechanical allodynia and hyperalgesia develop as early as 5-7 days following injection, and motor performance is not altered as measured by a rota-rod test (Aley, Reichling et al. 1996, Weng, Cordella et al. 2003). Rats experienced increased spontaneous discharges in neurons of the lumbar DRG as well as increased A- and C-fiber responses to electrical stimulation (Weng, Cordella et al. 2003), suggesting a functional change in sensory neurons. Investigators do not have a clear understanding of what population of neurons is affected by paclitaxel and vincristine or the mechanisms underlying these changes in sensitivity; however, axonal damage is also minimal in low-dose vincristine animal models (Tanner, Levine et al. 1998). This suggests that MTA-induced changes in neuronal sensitivity after low-dose treatment with MTAs may be mediated by mechanisms that do not involve overt axonal damage, whereas decreases in motor function may be secondary to axonal damage following high doses of MTA drugs (Cavaletti, Cavalletti et al. 1997, Authier, Gillet et al. 2000).

Animal models for epothilones have been less extensively characterized than models for paclitaxel and vincristine (Authier, Balayssac et al. 2009). One study using female rats found that weekly intravenous administration of .25 or .5 mg/kg of EpoB for 4 weeks (cumulative dose of 1-2 mg/kg) resulted in decreased

tail nerve conduction velocities, mild sciatic nerve axonal degeneration, and a dose-related prolongation of foot withdrawal latency on a hot plate (Chiorazzi, Nicolini et al. 2009). Another study using female rats found that intravenous administration of 1.2 or 2.4 mg/kg for 4 weeks (cumulative dose of 4.8-9.6 mg/kg) of sagopilone, a synthetic epothilone similar to ixabepilone, caused a dose-dependent reduction in tail nerve conduction velocities and decreased myelination of the sciatic nerve (Chiorazzi, Hochel et al. 2012). These studies have analogous findings to the high-dose paclitaxel *in vivo* models (Cavaletti et al., 1997, Authier et al., 2000), and it would be interesting to investigate if lower doses of epothilones would result in increased nociceptive behaviors within animal models.

8) Paclitaxel- and Vincristine-induced Alterations of Neurite Length *in vitro*

In vitro model systems have also been developed in an attempt to examine the effects of MTAs on neurons in culture, and most studies have investigated the effects of MTAs on neurite length. Most *in vitro* studies use clinically relevant concentrations (10 nM- 2 μM) of MTAs as pharmacokinetic studies have shown that dosing patients with 125-225 mg/m^2 of paclitaxel results in maximal plasma concentrations between 1 and 10 μM (Rowinsky, Jiroutek et al. 1999, Henningsson, Karlsson et al. 2001), with a reduction in concentration to between 10 nM and 1 μM by 20 hours. Paclitaxel and vincristine have been found to alter neurite length in several different *in vitro* model systems. Using embryonic DRG explant cultures, treatment with paclitaxel (10 nM-10 μM) for 24-

28 hours prevented neurite outgrowth from the explants in a concentration- and time-dependent manner (Scuteri, Nicolini et al. 2006). Similarly, when paclitaxel (29.3 nM) was applied to distal axons of embryonic DRG using a compartmentalized microfluidic culture platform, paclitaxel caused degeneration of the neurites; however, paclitaxel did not alter neurite length when application was limited to the soma (Yang, Siddique et al. 2009). In an autonomic, adult rat superior cervical ganglion explant culture model, paclitaxel (100 nM- 2 μM) and vincristine (100 nM-2 μM) prevented neurite outgrowth from the explant in a concentration- and time-dependent manner (Hayakawa, Sobue et al. 1994, Hayakawa, Itoh et al. 1999). Finally, using an adult sensory neuron culture model, paclitaxel (1 μM) and vincristine (0.1 nM) were added to the cultures after a 24 hour attachment and growth period. After an additional 24 hours in culture in the presence of the MTAs, neurite length was decreased compared to vehicle-treated controls (Malgrange, Delree et al. 1994). This collective body of work suggests that paclitaxel and vincristine can decrease neurite length (Malgrange, Delree et al. 1994, Yang, Siddique et al. 2009) and prevent neurite outgrowth from axotomized explants *in vitro* (Hayakawa, Sobue et al. 1994, Hayakawa, Itoh et al. 1999, Scuteri, Nicolini et al. 2006). The mechanisms underlying the MTA-induced reduction in neurite length are unknown, and the role of these MTA-induced morphological changes in the development of neuronal sensitization is still a subject needing further investigation.

9) Putative Mechanisms for MTA-induced Changes in Neuronal Sensitivity and Morphology

a. MTA-Induced Alterations of Intracellular Trafficking in Neurons

In peripheral sensory neurons, the majority of protein synthesis occurs within the neuronal cell body, and the resultant proteins and organelles necessary for axonal functioning must be delivered to the periphery, which can be approximately 1 meter in length in some instances (Gallant 2000, Xu and Tung 2001). In order to achieve transport of necessary components throughout axons, sensory neurons have developed an intricate system of microtubules that allow for anterograde transport of proteins to the periphery and retrograde transport of components from the periphery to the cell body (Conde and Caceres 2009). The microtubules form a polar configuration within the axons, meaning that their plus ends point uniformly towards the axon tips (Conde and Caceres 2009, Shemesh and Spira 2010). Disruption of this polar configuration is thought to be involved in neuronal degenerative processes as microtubule polarity is important for proper trafficking of proteins and organelles, such as mitochondria, along the microtubules (Shemesh and Spira 2010). This transport of cell components along the polar microtubules is mediated by microtubule motor proteins including kinesins and dyneins (Goldstein and Yang 2000). Thus axonal transport is a crucially important cellular process for neuronal function and depends on several factors including proper microtubule polar configuration and functional microtubule motor proteins, and alterations of axonal transport have been implicated in peripheral neuropathy and several neurodegenerative

diseases (Shemesh and Spira 2010, Power, Srinivasan et al. 2012, Kanaan, Pigino et al. 2013).

MTAs have been shown to alter axonal microtubule function, reconfigure microtubule polar configurations within axons, and decrease organelle transport along axons. Paclitaxel (11.7 μM) has been shown to inhibit the anterograde transport of microinjected horseradish peroxidase in a reversible and time-dependent manner in cultured chick DRGs. This treatment paradigm also caused aggregation of axonal and cytoplasmic microtubules within the DRG cultures (Theiss and Meller 2000). In a cultured *Aplysia* neuronal model, application of paclitaxel (100 nM) changed microtubular polar orientations, decreased the number of dynamically polymerizing microtubules, and inhibited the transport of organelles along the axon (Shemesh and Spira 2010). Vincristine has also been shown to alter mitochondrial transport in cultured cells. Using a differentiated, neuronal-like SK-N-SH cell culture model, treatment with vincristine (100 nM) decreased the velocity of mitochondrial movement along axons (Rovini, Carre et al. 2010). Similarly, in a comparison study of paclitaxel, vincristine, and ixabepilone on vesicle motility in isolated giant squid axons, vincristine (1 μM) decreased both anterograde and retrograde transport rates of vesicles. Perfusion of paclitaxel (10 μM) decreased anterograde transport but did not alter retrograde transport, and treatment with ixabepilone (1 μM) decreased both anterograde and retrograde fast axonal transport. All three MTA drugs altered fast axonal transport in the giant squid axons without inducing detectable microtubule bundling (LaPointe, Morfini et al. 2013). Thus, MTA-

induced alterations of microtubule polar configuration, axonal transport, and organelle trafficking could all contribute to the development of MTA-induced peripheral neuropathy since disruptions in these processes have been linked to neuronal degeneration (Shemesh and Spira 2010). These disruptions could cause alterations in the trafficking of peptides, including CGRP, and organelles necessary for maintenance of sensory neurite function and morphology, like mitochondria, to the periphery. This may underlie changes in neurite length and sensitivity of sensory neurons following treatment with MTAs.

b. MTA-induced Alterations in Reactive Oxygen Species Generation and Nitroxidative Stress

Another possible mechanism that may underlie the development of MTA-induced neuronal sensitization is increased generation of reactive oxygen species (ROS). A classic ROS and reactive nitrogen species pathway can be seen in Figure 1.

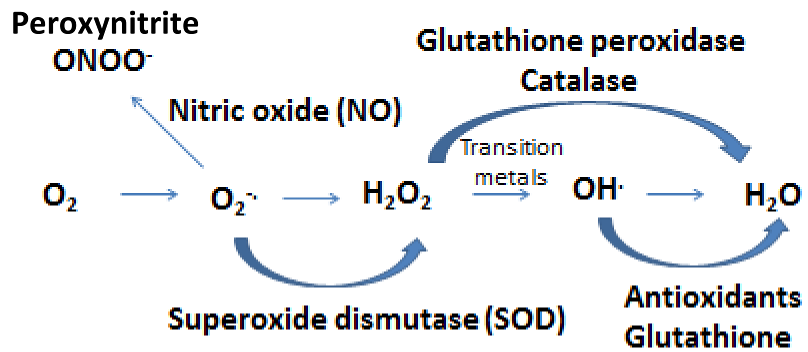


Figure 1. Reactive oxygen and nitrogen species pathway. Adapted from (Djamali 2007).

MTAs increase ROS generation in several model systems. In a human lung cancer cell model, paclitaxel treatment (0.1 μM- 5 μM) increased ROS generation in a concentration-dependent manner (Alexandre, Batteux et al. 2006). Additionally, exposing cortical neuronal cultures to paclitaxel (300 nM) increased ROS within the neurons, and this increase was reversed by treatment with the antioxidant, vitamin E (Jang, Hwang et al. 2008). Vincristine (10 μM) has also been shown to increase ROS in a concentration-dependent manner within lymphoblastoma cells. This vincristine-induced increase of ROS was partially attenuated by treatment with N-acetyl-*L*-cysteine (NAC) (20 mM), which is a compound that acts to recycle glutathione and scavenge free radicals (Tsai, Sun et al. 2007). Glutathione is an intracellular thiol compound that acts as a substrate for reduction reactions, thereby decreasing reactive species levels (Ribas, Garcia-Ruiz et al. 2014). NAC also attenuated ROS produced by paclitaxel and EpoB. In a study conducted with mouse ovarian cells and human ovarian cancer cells, pre-treatment with NAC (3 mM) attenuated both paclitaxel (199 nM)- and EpoB (27.4 nM)-induced increases of ROS production (Rogalska, Marczak et al. 2013). Although the cell types, MTA agents used, and drug concentrations vary among the above described studies, *in vitro* administration of MTAs increase ROS in several different model systems. While this may contribute to the development of MTA-induced neuronal sensitivity, there is also a current theory that MTA-induced generation of ROS may contribute to the oncolytic action of MTAs. Previous work has shown that reducing MTA-induced generation of H_2O_2 within cancer cells and mice decreased the antitumor effects

of the MTA (Alexandre, Batteux et al. 2006, Alexandre, Hu et al. 2007). This suggests that reducing MTA-induced ROS as a treatment to prevent peripheral neuropathy may be a poor strategy as it may negatively impact the antineoplastic activity of the MTAs. However, an increased production of ROS can result in a subsequent increase of reactive nitrogen species levels including peroxynitrite (Figure 1), and peroxynitrite has been implicated in the development of pain states without having known beneficial roles within systems, like superoxide's role in memory and learning (Massaad and Klann 2011, Salvemini, Little et al. 2011). Thus, targeting reactive nitrogen species may be a better strategy for preventing MTA-induced peripheral neuropathy.

The role of nitroxidative stress in paclitaxel-induced neuronal hypersensitivity and nociception has been intimated based upon studies where administration of free radical scavengers, superoxide dismutase mimetics, and peroxynitrite decomposition catalysts (PNDC) have prevented, reversed, or attenuated paclitaxel-induced nociception in *in vivo* models. In a paclitaxel animal model, systemic administration of the general free radical scavenger, phenyl *N*-tert-butylnitrone (PBN), or a superoxide dismutase mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), prevented and reversed mechanical allodynia and hyperalgesia (Kim, Zhang et al. 2010, Fidanboylu, Griffiths et al. 2011), and PBN attenuated cold allodynia (Fidanboylu, Griffiths et al. 2011). The non-specific PNDCs and superoxide dismutase mimetics, $FeTMPyP^{5+}$ and MnTE-2-PyP^{5+}, were administered by subcutaneous injections or intrathecally to rats beginning on the day of paclitaxel injections. Both

$FeTMPyP^{5+}$ and $MnTE\text{-}2\text{-}PyP^{5+}$ attenuated the development of paclitaxel-induced mechanical hyperalgesia and allodynia in a dose-dependent manner without altering baseline nociceptive behavior. Intraperitoneal or intrathecal injections of $FeTMPyP^{5+}$ and $MnTE\text{-}2\text{-}PyP^{5+}$ on day 16, after the development of paclitaxel-induced mechanical hyperalgesia and allodynia, reversed the mechanical hypersensitivity (Doyle, Chen et al. 2012). Selective PNDCs, SRI110 and SRI6 (Rausaria, Ghaffari et al. 2011, Rausaria, Kamadulski et al. 2011) were given orally to rats at the beginning of paclitaxel injections, and both compounds blocked the development of mechanical hyperalgesia and allodynia (Doyle, Chen et al. 2012), suggesting that peroxynitrite may be the reactive species responsible for the development of hypersensitivity in MTA animal models. These findings collectively show that preventing the formation of or decreasing the concentrations of non-specific ROS, superoxide, and/or peroxynitrite can prevent and reduce paclitaxel-induced mechanical hypersensitivity within animal models. This suggests that the nitroxidative stress likely plays a role in the underlying mechanisms of paclitaxel-induced changes in neuronal function.

Unexpectedly, the positive effects of decreasing ROS and reactive nitrogen species within animal models of MTA-induced peripheral neuropathy have not been translated to the clinic. Antioxidant therapy has had no effect on paclitaxel-induced peripheral neuropathy within patients (Argyriou, Chroni et al. 2006, Kottschade, Sloan et al. 2011). Additionally, treatment with glutathione, as a method to decrease reactive species, did not alleviate paclitaxel/carboplatin-induce peripheral neuropathy (Leal, Qin et al. 2014). This suggests that the

generation and role of reactive species in MTA-induced peripheral neuropathy is complicated and perhaps multifactorial, and more work in understanding the production and effects of MTA-induced nitroxidative stress is necessary.

The source and function of the reactive species generated within neurons after insult with an MTA are currently unknown. Classically, the two possible sources for reactive species are from the mitochondrial electron transport chain and membrane-bound NADPH oxidase (Herrero and Barja 2000, Turrens 2003, Chinopoulos and Adam-Vizi 2006, Doyle, Chen et al. 2012, Gao, Zhou et al. 2012), and mitochondrial dysfunction and NADPH oxidase activation have both been observed after paclitaxel exposure *in vivo* and *in vitro* within neurons (Flatters and Bennett 2006, Jang, Hwang et al. 2008, Melli, Taiana et al. 2008). Nitroxidative stress can cause a range of damage within cells including DNA damage, lipid peroxidation, and protein alterations and dysfunction (Rutkowski, Pancewicz et al. 2007, Schieber and Chandel 2014). Within neurons, nitroxidative stress can also alter neuronal sensitivity by several mechanisms including altering channel activity (Naziroglu, Cig et al. 2013, Nesuashvili, Hadley et al. 2013, Nishio, Taniguchi et al. 2013), enhancing kinase activity involved in neuronal sensitization signaling cascades (Balafanova, Bolli et al. 2002, Hongpaisan, Winters et al. 2004, Chakraborti, Das et al. 2005, Ibi, Matsuno et al. 2008), and inducing redox sensitive transcription factor activity (Bar-Shai and Reznick 2006, Yakovlev, Barani et al. 2007). The initial source and effects of MTA-generated nitroxidative stress are currently under investigation within the field.

c. MTA-induced Alterations in Mitochondrial Function

Another possible mechanism underlying MTA-induced peripheral neuropathy is via toxicity to the mitochondria, which suggests that MTAs alter and/or damage neuronal mitochondria, thereby altering energy production within the cell. This can create deficits in energy production needed for neuronal functions, ultimately causing neuronal sensitivity and dysfunction (Flatters and Bennett 2006, Xiao, Zheng et al. 2011, Janes, Doyle et al. 2013). In *in vitro* studies of non-neuronal cell cultures, paclitaxel depolarized the mitochondrial membrane potential, decreased mitochondrial calcium levels, and increased release of cytochrome *c* from the mitochondria, all indicators of mitochondrial dysfunction (Evtodienko, Teplova et al. 1996, Andre, Braguer et al. 2000, Kidd, Pilkington et al. 2002). In *in* vivo animal models, paclitaxel has been shown to increase the number of atypical and swollen mitochondria in C-fibers and myelinated axons within saphenous nerves of treated animals (Flatters and Bennett 2006). Paclitaxel also decreased mitochondrial respiration and ATP production within rat sciatic nerves, which is further support that paclitaxel impairs mitochondrial energy production within neurons (Xiao, Zheng et al. 2011, Zheng, Xiao et al. 2011). This paclitaxel-induced impairment in mitochondrial respiration was prevented with prophylactic treatment with the mitochondrial protective agent, acetyl-*L*-carnitine (ALCAR) (Zheng, Xiao et al. 2011). Furthermore, concurrent treatment of ALCAR with paclitaxel prevented and reversed paclitaxel-induced mechanical allodynia and hyperalgesia as well as prevented paclitaxel-induced increases in swollen and vacuolated mitochondria

of C-fibers (Flatters, Xiao et al. 2006, Jin, Flatters et al. 2008). This collective evidence suggests that one possible mechanism underlying MTA-induced changes in neuronal activity is mitochondrial dysfunction, which could contribute to calcium deregulation, reactive species generation, and cellular energy deficits (Xiao and Bennett 2008, Xiao, Zheng et al. 2009, Zheng, Xiao et al. 2011). Contrary to the *in vivo* ALCAR findings, the administration of ALCAR to breast cancer patients undergoing paclitaxel chemotherapy resulted in an increased incidence of peripheral neuropathy (Hershman, Unger et al. 2013). Thus, the involvement of mitochondria in the development of MTA-induced peripheral neuropathy is not fully understood, and further investigation is warranted.

d. MTA-induced Alterations in Membrane Channels

Membrane ion channels are key regulators of neuronal excitability and play a fundamental role in the detection of stimuli in nociceptive sensory neurons (Bennett and Woods 2014). Alterations in membrane channel expression and/or function may underlie changes in neuronal sensitivity following paclitaxel treatment. Spontaneous axonal activity was measured from the sural nerves of animals treated with paclitaxel or vincristine, and the spontaneous activity of myelinated and unmyelinated primary afferent fibers increased in MTA-treated animals (Xiao and Bennett 2008). The spontaneous activity of DRG from paclitaxel-treated animals has also been shown to be increased. Within the sensory neurons that exhibited an increase in spontaneous activity, the mRNA expression of several membrane channels, including potassium, sodium and

TRP channels, was altered (Zhang and Dougherty 2014), providing a possible mechanism by which paclitaxel alters excitability in the nerve fibers and DRG. Alterations of several different channel types have been investigated as possible mechanisms mediating MTA-induced changes in nociception, including several TRP channel family members and voltage-dependent calcium channels (VDCC) (Chen, Yang et al. 2011, Kawakami, Chiba et al. 2012, Materazzi, Fusi et al. 2012, Hara, Chiba et al. 2013).

MTA agents may be altering neuronal function and inducing peripheral neuropathy by altering a family of ion channels known as Transient Receptor Potential (TRP) channels, which are non-selective cation channels (Clapham, Runnels et al. 2001, Montell, Birnbaumer et al. 2002, Jara-Oseguera, Simon et al. 2008). The Transient Receptor Potential Vaniloid 1 (TRPV1) channel is an important ion channel responsible for the perception of noxious chemical stimuli including capsaicin and acidic pH as well as noxious heat and some endogenous lipids (Caterina, Schumacher et al. 1997, Tominaga, Caterina et al. 1998, Julius and Basbaum 2001, Bhave, Hu et al. 2003, Tominaga and Caterina 2004, Cortright and Szallasi 2009, Patwardhan, Scotland et al. 2009). Systemic administration of paclitaxel in the rat resulted in an increase in TRPV1 mRNA and protein expression in the DRG (Hara, Chiba et al. 2013), while in a paclitaxel mouse model, treatment with a TRPV1 antagonist, capsazepine, reversed paclitaxel-induced thermal hyperalgesia without affecting mechanical hyperalgesia (Chen, Yang et al. 2011). These data suggest that TRPV1 may mediate paclitaxel-induced changes in thermal sensitivity within animal models.

The Transient Receptor Potential Ankyrin 1 (TRPA1) channel is activated by a wide variety of stimuli, including noxious mechanical, noxious cold, and environmental chemicals (see review by Kwan, Allchorne et al. 2006). Several investigators have examined the role of TRPA1 in mediating paclitaxel-induced mechanical and cold allodynia by systemically administering a TRPA1 antagonist or by using TRPA1-deficient animals and examining the effects of these manipulations in animals treated with paclitaxel. Antagonism of the TRPA1 channel attenuated paclitaxel-induced mechanical allodynia and reversed cold allodynia, while TRPA1-deficient animals did not develop paclitaxel-induced cold allodynia (Chen, Yang et al. 2011, Materazzi, Fusi et al. 2012). Collectively, these data suggest that TRPA1 mediates mechanical allodynia and cold allodynia induced by paclitaxel.

Transient Receptor Potential Vanilloid 4 (TRPV4) is activated by a diverse range of stimuli including heat, phorbol esters, acidic pH, and hypo-osmotic stimuli (Guler, Lee et al. 2002, Watanabe, Davis et al. 2002, Gao, Wu et al. 2003, Suzuki, Mizuno et al. 2003, Suzuki, Watanabe et al. 2003). Knocking down TRPV4 protein expression within L4 and L5 DRG with TRPV4-targeting oligonucleotides reversed paclitaxel-induced mechanical hyperalgesia within a rat model (Alessandri-Haber, Dina et al. 2004), suggesting that TRPV4 may be involved in paclitaxel-induced mechanical hyperalgesia and neuropathy.

Another type of membrane channel that has been implicated in MTA-induced peripheral neuropathy are the VDCCs, which are voltage-gated ion channels that are selectively permeable to calcium (McCleskey 1999). Paclitaxel

administration in an *in vivo* animal resulted in an increase in VDCC current in DRG neurons, which was inhibited by gabapentin, an antagonist of VDCCs expressing the α2δ-1 and α2δ-2 subunits. Paclitaxel also increased α2δ-1 protein expression in DRG (Kawakami, Chiba et al. 2012). Systemic administration of ethosuximide, a putative T-type VDCC blocker, reversed paclitaxel-induced mechanical allodynia and hyperalgesia and cold allodynia as well as vincristine-induced mechanical allodynia and hyperalgesia, suggesting that T-type VDCCs may be involved in both paclitaxel- and vincristine-induced hypersensitivity in the rat (Flatters and Bennett 2004). Inhibition of VDCCs with gabapentin therapy does not translate clinically, however. Patients with established chemotherapy-induced peripheral neuropathy received either gabapentin or placebo therapy for 6 weeks, and there was no difference in peripheral neuropathy symptoms at the completion of the trial compared to placebo treatment (Rao, Michalak et al. 2007).

Alterations in the VDCC and TRP channel expression and/or function by MTAs would likely alter the intrinsic membrane properties of DRG neurons and could lead to enhanced and attenuated neuronal sensitivity, depending on the specific modifications of the channels. Membrane channels could be modified by MTAs by several possible mechanisms including transcription and localization abnormalities as well as post-translational modifications from aberrant signaling cascades.

e. MTA-induced Alterations of Intraepidermal Nerve Fibers and Neuroimmune Regulation

Despite there not being any overt axonal damage, defined by degeneration or structural abnormalities of axons within the sciatic nerve (Polomano, Mannes et al. 2001) at the level of the peripheral nerve in rats after low-dose paclitaxel or vincristine treatment, the terminals of the peripheral nerves degenerate. The number of intraepidermal nerve fibers (IENF) innervating the hindpaws of rats decreased to 24% and 44% of that in vehicle-treated animals in *in vivo* models of paclitaxel- and vincristine-induced hypersensitivity, respectively, and the decrease in IENF corresponded to the peak of hypersensitivity in the animals (Siau, Xiao et al. 2006). Whether the decreased number of innervating IENFs was a result of MTA-induced retraction or MTA-prevention of nerve fiber maintenance and growth is unknown. As described above, alterations of microtubule dynamics can lead to both neurite retraction and inhibition of neurite outgrowth by altering growth cones. The specific mechanisms underlying MTA-induced decreases in IENFs are under investigation; however, an alteration of microtubule dynamics by MTAs may be involved in these phenomena.

The involvement of the immune system in paclitaxel- and vincristine-induced decreases in IENFs has also been suggested. Paclitaxel and vincristine treatment increased epidermal Langerhans cells in the hindpaws of rats corresponding to the paclitaxel- and vincristine-induced decreases in IENFs (Siau, Xiao et al. 2006, Jin, Flatters et al. 2008). Langerhans cells are antigen-presenting immune cells of the skin (Siau, Xiao et al. 2006). In a similar study,

administration of paclitaxel increased the number of macrophages in the hindpaw (Liu, Lu et al. 2010). The paclitaxel-induced decrease of IENFs in rat hindpaws was reversed with co-treatment with minocycline, a tetracycline-derived antibiotic that is known to have immunomodulatory properties. Treatment with minocycline also prevented paclitaxel-induced mechanical hyperalgesia and allodynia (Liu, Lu et al. 2010, Boyette-Davis, Xin et al. 2011). This suggests that one possible mechanism of MTA-induced hypersensitivity may be that MTAs decrease innervating IENFs in the peripheral skin, perhaps caused by a neuroimmune interaction in the skin; however, the mechanistic link between decreased peripheral IENFs and hypersensitivity is still under investigation.

f. Vincristine and Paclitaxel Bind and Activate Neuronal-Calcium Sensor 1

While the investigations of MTA effects on neuronal function have primarily been focused on mechanisms involving alterations of microtubule dynamics, MTAs may alter neuronal function and cause peripheral neuropathy by other, microtubule-independent mechanisms. Paclitaxel binds neuronal-calcium sensor 1 (NCS-1) and B-cell lymphoma 2 (Bcl-2) (Rodi, Janes et al. 1999, Boehmerle, Splittgerber et al. 2006, Ferlini, Cicchillitti et al. 2009, Benbow, Mann et al. 2012), but a direct involvement of this binding has not been shown to mediate neurotoxicity. Dr. Barbara Ehrlich's group found that paclitaxel and vincristine bind to NCS-1, which is a high affinity calcium binding protein important in many calcium signaling events and cascades (Benbow, Mann et al.

2012). Upon binding paclitaxel or vincristine, NCS-1 signaling is enhanced, and interaction between NCS-1 and the inositol 1,4,5-triphosphate receptor ($InsP_3R$) is increased (Boehmerle, Splittgerber et al. 2006, Benbow, Mann et al. 2012). Acute application of paclitaxel (937 nM) on neuroblastoma cells caused oscillations in cytosolic calcium levels that were mediated by NCS-1 and were $InsP_3R$ dependent (Boehmerle, Splittgerber et al. 2006). This suggests that MTA agents may alter neuronal calcium homeostasis in a microtubule-independent manner. Although paclitaxel and vincristine bind NCS-1, epothilone B was used as a negative control in paclitaxel/NCS-1-induced calcium oscillation experiments, suggesting that not all MTAs activate the NCS-1 signaling cascade (Zhang, Heidrich et al. 2010).

g. Nerve Growth Factor Alters MTA-induced Effects *in vitro and in vivo*

NGF may be an important variable in the development or maintenance of MTA-induced peripheral neuropathy, and *in vivo* and *in vitro* investigations suggest NGF may be protective against the effects of MTAs. Within several *in vitro* systems, NGF attenuates MTA-induced changes of different endpoints including cell survival and neurite length. In a study investigating the effects of paclitaxel and NGF on cell survival, embryonic DRG explants were exposed to paclitaxel (1 μM) for 4 days, and this caused 90% of the neurons to degenerate and die; however, when the explants were concurrently exposed to paclitaxel and NGF (300 U/ml), the DRG neurons survived comparably to control neurons (Peterson and Crain 1982). This suggests that paclitaxel may mediate changes

in neuronal function by decreasing viability of the neurons, which can be reversed with NGF treatment. A limitation to this study, however, is that embryonic neurons are dependent on NGF for survival, and the decrease in survival observed in this study may be a result of NGF deprivation as opposed to paclitaxel exposure (Buchman and Davies 1993, Acosta, Fabrega et al. 2001).

NGF can also rescue MTA-induced alterations of neurite length. As described above, paclitaxel and vincristine prevented neurite outgrowth from superior cervical ganglia explants in concentration- and time-dependent manners; however, growth of the neurites was augmented in the paclitaxel- and vincristine-treated cultures by exogenous application of 100 ng/ml NGF (Hayakawa, Sobue et al. 1994, Hayakawa, Itoh et al. 1999). Whether NGF can protect the neurites from the effects of the MTAs or whether NGF independently stimulates growth of the neurites and therefore reduces the impact of the MTAs on neurite length is unknown.

NGF also reverses MTA-induced alterations within animal models. Using a high-dosing paclitaxel schedule, mice received 6 injections of 21.6 mg/kg (129.6 mg cumulative) of paclitaxel, which resulted in thermal hypoalgesia and decreased substance P in the DRG of the mice. Concomitant injections of paclitaxel with 10 µg/ml NGF prevented the paclitaxel-induced hypoalgesia. Systemic NGF also prevented the paclitaxel-induced decrease of substance P in the murine DRG (Apfel, Lipton et al. 1991), suggesting that NGF may be capable of preventing paclitaxel-induced neurotoxicity in high-dose animal models, in which the development of paclitaxel-induced thermal hypoalgesia is apparent.

In another high-dose paclitaxel model, rats were injected with 8 mg/kg paclitaxel twice a week for 4 weeks (64 mg cumulative), and the rats developed paclitaxel-induced mechanical allodynia. NGF levels decreased in the sciatic nerves of paclitaxel-treated rats at the end of the 4 week treatment schedule compared to vehicle-treated control animals (Arrieta, Hernandez-Pedro et al. 2011). Whether the decrease in NGF levels was causally related to the development of the mechanical allodynia is unknown, but these data suggest that paclitaxel alters the baseline NGF concentrations within tissues that may be important for homeostatic cell maintenance and/or signaling cascades. Collectively these studies suggest that the MTA-induced changes in neuronal morphology and sensitivity may be attenuated by NGF treatment; however, the underlying mechanisms mediating this attenuation and/or the possible convergent signaling pathways activated by MTA and NGF are currently unknown.

h. Paclitaxel Alters NGF Levels Clinically

Circulating NGF levels have been shown to be correlated with the development of paclitaxel-induced peripheral neuropathy in the clinic. Cervical cancer patients who did not have prior peripheral neuropathy had their circulating NGF levels measured at baseline, before receiving paclitaxel and cisplatin chemotherapy, and again at the end of the trial. A significant correlation existed between patients with the greatest decrease in circulating NGF levels after treatment with paclitaxel and cisplatin and the severity of peripheral neuropathy

developed (Cavaletti, Bogliun et al. 2004). Similar observations were made in a trial of non-small-cell lung cancer patients who did not have prior peripheral neuropathy. Changes in baseline and post-treatment circulating NGF levels were determined, and there was a correlation between the greatest decrease in circulating NGF levels and development of peripheral neuropathy (Arrieta, Hernandez-Pedro et al. 2011). These correlations suggest that changes in NGF levels may be important markers in predicting the development and severity of peripheral neuropathy; however, it is unknown if NGF protects against the development of peripheral neuropathy. The role of NGF and its downstream signaling cascades in MTA-induced peripheral neuropathy are unknown, and further investigation is warranted as NGF may attenuate MTA-induced alterations.

As described throughout the introduction, there are several putative mechanisms thought to underlie the development of MTA-induced peripheral neuropathy including alterations in microtubule dynamics, intracellular trafficking, reactive species generation, membrane channel expression and/or function, mitochondrial function, and IENF innervation. It is likely that all of these changes occur within sensory neurons following MTA-treatment; however, the relative contribution of these different mechanisms is still under investigation. These described mechanisms may be linked and could propagate each other following MTA treatment; for example, MTA-induced changes in mitochondrial function may result in an increase in mitochondria-produced reactive oxygen species.

Interactions between all the proposed mechanisms need to be further investigated. This will aid in the development of effective therapies since the possible interconnected relationships of the mechanisms may be important in the development of MTA-induced peripheral neuropathy.

SPECIFIC AIMS OF THE THESIS

The studies in this thesis investigated the effects of three cancer chemotherapeutic microtubule targeting agents, paclitaxel, EpoB, and vincristine, on the function and morphology of sensory neurons in culture and the contribution of NGF to these effects. Therefore, the aims of this thesis are:

1. To determine the effects of paclitaxel, EpoB, and vincristine on the release of CGRP from sensory neurons in culture in the presence and absence of NGF.
2. To determine the effects of paclitaxel, EpoB, and vincristine on sensory neurite length and branching in culture in the presence and absence of NGF.

MATERIALS AND METHODS

1) Materials

All materials unless stated otherwise were purchased from Sigma-Aldrich (St. Louis, MO). F-12 media, horse serum, and antibiotics, were purchased from Invitrogen (Carlsbad, CA). NGF was purchased from Harlan Laboratories (Indianapolis, IN). Alexa Fluor 488 donkey anti-rabbit antibody was purchased from Life Technologies (Grand Island, NY), and the rabbit anti-human protein gene product 9.5 (PGP9.5) antibody was purchased from AbD Serotec, a Bio-Rad company (Raleigh, NC). The AITC was purchased from Fisher Scientific (Pittsburgh, PA). CGRP for radioimmunoassay was purchased from Tocris Bioscience (Ellisville, MO). Rat (Tyr27)-α$CGRP_{27-37}$ for radioimmunoassay was purchased from Bachem (Torrence, CA). Radiolabeled sodium iodine (^{125}NaI) for iodination of $CGRP_{27-37}$ was purchased from Perkin-Elmer (Shelton, CT).

2) Animals

The Animal Care and Use Committee at Indiana University School of Medicine (Indianapolis, IN) approved all procedures used in the experiments described in this body of work, and all experiments were carried out in accordance with the approved protocols. Animals were stored in group cages in light controlled rooms (light from 6:00 to 19:00), and food and water were available *ad libitum*. Animals were allowed to acclimate in the animal room upon delivery for at least 3 days before use. All experiments were performed on

primary cultures of sensory neurons derived from the DRG of adult male Sprague Dawley rats (150-200 g; Harlan Laboratories, Indianapolis, IN).

3) Isolation of Primary Sensory Neuron Cultures

Plates for cell culture were precoated starting 2 days before the harvest by exposing them to 1 ml (12-well plate) or 250 µl (48-well plate) of poly-D-lysine (PDL) (0.1 mg/ml) and storing them overnight in a 37° C, 3% CO_2 incubator. The following day, the wells were rinsed 1 time with 1 ml or 250 µl of sterile H_2O and were subsequently exposed to 1 ml or 250 µl of laminin (1 mg/ml) overnight. The laminin was removed from the wells immediately before addition of the dissociated neurons.

Adult rat DRG cultures were prepared as follows: adult rats were asphyxiated with CO_2 and then decapitated. DRG were harvested from the entire length of the vertebral column, and the DRG were digested by incubating them for 1 hr in F-12 media containing 0.125% collagenase. After the 1 hr incubation period, the DRG were centrifuged for 1 min to pellet the DRG, the media containing collagenase was removed, and the DRG pellet was resuspended in culture media, which was F-12 media supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 100 µg/ml normocin, 50 µg/ml penicillin and streptomycin, 50 µM 5-fluoro-2-deoxyuridine, and 150 µM uridine in the absence of added nerve growth factor (NGF) or in the presence of 30 or 250 ng/ml NGF. The DRG were then dissociated into a single-cell suspension by mechanically agitating the pellet with a fire-polished glass pipette. The

suspension was diluted with culture media, and approximately 30,000-40,000 cells were plated per well on the 12-well plates, and approximately 3,300-4,300 cells were plated in each well of the 48-well plates. The sensory neuron cultures were maintained in culture media in a 37° C and 3% CO_2 incubator throughout the duration of the experiments, and culture media was changed in the cultures every other day.

4) Drug Stocks and Treatments

Drug stocks of 10 mM were prepared for paclitaxel, EpoB, and vincristine in 1-methyl-2-pyrrolidinone (MPL) and stored at -20°C. These stocks were further diluted in culture media to 50 μM and then to the subsequent, appropriate concentrations immediately prior to treatment of the cultures. N-acetyl-*L*-cysteine (NAC) stocks of 200 mM were prepared in sterile H_2O and stored at 4°C for up to 1 month. The stock was further diluted to 10 mM in culture media and then to lower concentrations immediately prior to addition to culture wells. Acetyl-*L*-carnitine (ALCAR) stocks of 100 mM were prepared in sterile H_2O and stored for up to 1 month at 4°C. For experiments, ALCAR was further diluted to 100 μM in culture media. Cultures were treated with the MTA drugs or MPL vehicle control (0.003%) in media starting on day 7, 9, or 12 in culture, and media containing the drug was refreshed every other day. ALCAR and NAC, when appropriate, were added to the culture media concomitantly with paclitaxel beginning on day 7 and refreshed every other day. Experiments were performed on day 7, before drug exposure, or on day 12 in culture.

5) Calcitonin Gene-Related Peptide Release

Release experiments were performed as described previously (Hingtgen and Vasko 1994, Vasko, Campbell et al. 1994). The wells were rinsed one time with HEPES buffer containing 25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 3.3 mM D-glucose, and 0.1% bovine serum albumin, pH 7.4. The cells were then maintained in HEPES +/- a stimulatory agent at 37°C for 4, 10 min incubations. During the first and second 10 min intervals, cells were incubated with HEPES buffer to establish resting basal release of immunoreactive CGRP (iCGRP), which will be referred to as CGRP. During the third interval, cells were incubated with HEPES containing 30 nM capsaicin, 30 µM AITC, or 50 mM KCl (substituted for equimolar NaCl) in order to stimulate CGRP release. The fourth incubation was in HEPES to reestablish resting basal release. Supernatants were collected after every interval, and CGRP was measured using radioimmunoassay as described below. At the completion of the release experiment, cells were incubated for 20 min in 0.1 N HCl, and the supernatant was collected to determine total CGRP content. Total content for each well was determined by adding the CGRP released during each incubation period with the CGRP released during the HCl incubation. For the experiments using cells grown in the absence of added NGF, a slight adjustment was made for the basal release fractions; instead of collecting the fractions every 10 min and having 2 basal fractions, the cells were incubated in HEPES buffer for 20 min, and only 1 basal fraction was collected. This modification was made in order to ensure detection of the basal CGRP fractions by the radioimmunoassay.

Since total content of CGRP is lower in neurons grown in 0 added NGF, CGRP in 10 min basal fractions often falls below the level of detection of the assay.

For the acute paclitaxel experiments, neurons were grown in culture for 12 days, and the neurons were naïve at the time of the release experiment, meaning they had not been treated with an MTA drug or vehicle. At the time of the experiment, the wells were exposed to 4 successive, 10 min incubations. The first incubation was HEPES buffer alone to establish a resting basal release of CGRP. For the 3 following incubations, the cells were incubated for 10 min in 300 nM paclitaxel in HEPES buffer. The CGRP in the fractions was determined using a radioimmunoassay. The CGRP released in the last 3 fractions was compared to the CGRP released in the 1st fraction in order to determine if paclitaxel causes acute release in the absence of a known stimulatory agent.

6) CGRP Radioimmunoassay

Radioimmunoassays were performed to measure the CGRP released from the neuronal cultures. On the day of the CGRP release experiment, aliquots from the release assays were pipetted into 5 ml Sarstedt tubes, and an equal volume of CGRP antibody (100 µl), approximately 1:65,000 dilution, was added to each tube. A standard curve was made in triplicate with known CGRP concentrations ranging from 3 fmol to 300 fmol, and CGRP antibody was added to the standard curve samples. All tubes, including unknown samples and standard curve, were brought up to equal volume with HEPES buffer and incubated overnight at 4°C. The following day, ^{125}I-[0Tyr]-iCGRP$_{27\text{-}37}$ (^{125}I-

CGRP)(100 μl) containing approximately 3500 cpm was added to each tube and incubated for a second night at 4°C. Samples were also prepared to determine max binding of the assay; tubes received HEPES, CGRP antibody, and ^{125}I-CGRP. Nonspecific binding samples were made by combining HEPES and ^{125}I-CGRP only. On the third day, charcoal solution (0.1 M phosphate buffer, 50 mM NaCl, 1% bovine serum, 1% charcoal, pH 7.4) was added to each sample, and the tubes were centrifuged at 3000 X g for 20 min. The charcoal absorbed any CGRP not bound to the antibody and pelleted it to the bottom of the tube. The supernatant containing CGRP (^{125}I-CGRP or unlabeled) bound to antibody was decanted to fresh tubes, and the radioactivity was measured by gamma scintillation spectrometry. The amount of CGRP in unknown samples was then calculated based upon the standard curve and was reported as fmol of CGRP per sample.

7) Viability Assay

The viability of sensory neurons grown in 30 ng/ml or 0 added NGF following exposure to paclitaxel was assessed by staining with propidium iodide (PI). Sensory neuron cultures were exposed to vehicle (.003% MPL), 10 nM, or 300 nM paclitaxel for 5 days. As a positive control for neuronal death, vehicle-treated neurons were exposed to 300 μM H_2O_2 for 1-2 hours at 37°C. At the end of the 1-hour incubation, the neurons were returned to culture media. Approximately 24 hours later, all neurons were rinsed one time with phosphate buffered saline (PBS), rinsed once more with staining buffer, and incubated with

0.5 ml of the staining solution containing 6 μM PI for 1 hour. Cells were visualized after the 1 hour incubation period using a Nikon Eclipse Ti-S inverted microscope. The PI was excited at 530 nm and the emitted light was monitored at 645 nm. Neurons in 3 random fields were counted and scored as either viable (unstained) or non-viable (red), and the total number of neurons per treatment group was also counted. The investigator was blinded to the treatment paradigm until after assessment of the data. The data are expressed as mean ± standard error of the mean (SEM) of percent non-viable cells per total number of neurons as well as total number of neurons per field.

8) Neurite Length and Branching

Neurite length and branching were determined using the Neurite Outgrowth module software of the ImageXpress Micro XL instrument (Molecular Devices, Sunnyvale, CA). Neurons were fixed with 4% paraformaldehyde for 20 minutes on day 7 or on day 12 following MTA treatment. Fixed neuronal cultures were then stored in filtered blocking buffer (1:25 dilution of normal donkey serum in 0.3% Triton X-100 in PBS) at 4°C for 1-10 days prior to incubation with antibody. Cultures were incubated overnight with primary antibody against PGP9.5 (1:1000, rabbit), which is a neuronal-specific protein (Wilkinson, Lee et al. 1989), in filtered blocking buffer. The following day, cultures were rinsed 3 times for 10 minutes with PBS, incubated for 2 hours with Alexa Fluor 488 donkey anti-rabbit secondary antibody (1:200) in filtered blocking buffer, and

rinsed again in PBS 3 times for 10 minutes. Cultures were imaged while submerged in PBS.

Neurons were imaged with the ImageXpress Micro XL System (Molecular Devices, Sunnyvale, CA) using the FITC filter set at 10X magnification; the Alexa Fluor 488 fluorophore was excited at 482 nm and the emitted light was monitored at 536 nm. One image was taken in the center of each well, and each image captured a total area of 1.96 mm^2 (1.4 X 1.4 mm). Data is reported as average neurite length or branching segments per field. Total neurite length and branching was determined using the Neurite Outgrowth module software using established parameters for cell bodies (max width of cell body: 54 µm, intensity above local background: 30000 graylevels, minimum area: 100 $µm^2$) and outgrowths (maximum width: 8 µm, intensity above local background: 5000 graylevels, minimum cell growth to log as significant: 10 µm).

9) Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA as indicated, and post-hoc analyses were performed using the Bonferroni's, Tukey's, or Dunnett's test, as indicated. Statistical calculations were performed with the GraphPad Prism version 6.02 statistical package (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM, and differences are considered significant if $p < 0.05$.

RESULTS

1) Paclitaxel Alters Stimulated CGRP Release from Sensory Neurons in Culture

Systemic administration of paclitaxel has various effects on the sensitivity of sensory neurons, reducing the sensitivity of sensory neurons in some studies (Gracias, Cummins et al. 2011) and increasing sensitivity in others (Polomano, Mannes et al. 2001, Flatters and Bennett 2006, Matsumoto, Inoue et al. 2006). The cumulative dose of paclitaxel also affects neuronal sensitivity as lower cumulative doses (cumulative doses of 4, 8, or 16 mg/kg) result in mechanical hyperalgesia and allodynia, as well as cold allodynia in rats, whereas higher cumulative doses (cumulative doses of 80-135 mg/kg) produce impairments in pain-like behaviors and thermal hypoalgesia (Cavaletti, Cavalletti et al. 1997, Authier, Gillet et al. 2000). These data suggest that, dependent upon the endpoint measured and the dosing paradigm administered, both increases and decreases in neuronal sensitivity occur; however, there is still not a clear understanding of the mechanisms by which these changes in sensitivity develop. To determine the direct effects of long-term paclitaxel exposure on sensory neurons, sensory neurons were maintained in culture for 7 days after which cells were treated for 5 days in the presence of various concentrations of paclitaxel and stimulated with capsaicin. Capsaicin stimulates the release of neuropeptides from small diameter sensory neurons expressing the TRPV1 receptor (Holzer 1988) by increasing the permeability of the channel to calcium and inducing an

inward calcium current (Clapham et al., 2001). The basal release of CGRP and the release of CGRP stimulated with 30 nM capsaicin were measured. Five-day treatment with vehicle (.003% MPL), 10 nM, or 300 nM paclitaxel did not alter the resting basal release of CGRP (first B column of each treatment group, Figure 2A). Capsaicin stimulated the release of CGRP from a basal level of 29 ± 2 fmol/well/10min to 274 ± 23 fmol/well/10min in vehicle-treated neurons (Figure 2A). Five-day treatment with 10 nM paclitaxel enhanced the stimulated release of CGRP from the neurons. The capsaicin-evoked release was augmented to 329 ± 24 fmol/well/10min in neurons treated with 10 nM paclitaxel (C columns, Figure 2A). In contrast, treatment with 300 nM paclitaxel significantly decreased capsaicin-evoked release to 150 ± 9 fmol/well/10min. The changes in release of CGRP were not secondary to an altered content of CGRP in the neurons, as the total content of CGRP was similar in cultures treated with vehicle, 1207 ± 80 fmol/well, 10 nM paclitaxel, 1359 ± 97, or 300 nM paclitaxel,1165 ± 59, (Figure 2B).

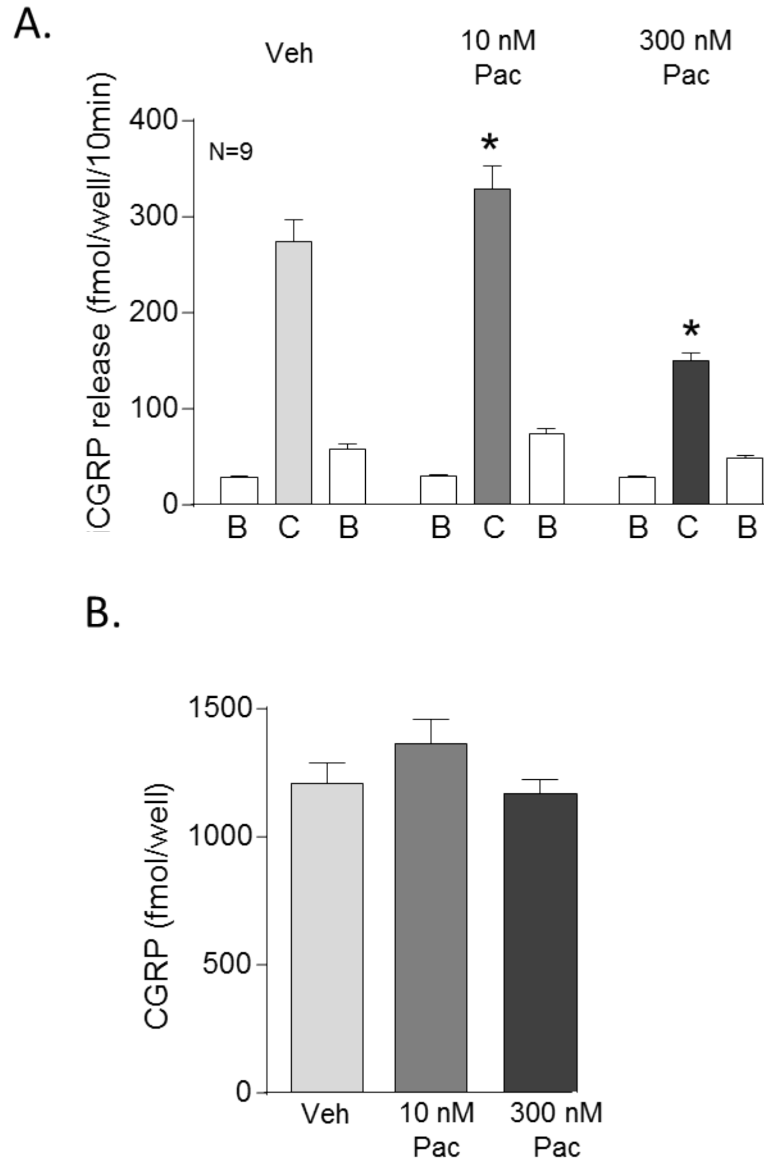


Figure 2. Paclitaxel alters capsaicin-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 10 nM or 300 nM paclitaxel for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=9) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or paclitaxel treated cultures (Pac) as indicated (N=9).

To determine if paclitaxel alters the sensitivity of the TRPA1 channel, sensory neurons were stimulated with 30 μM AITC, a TRPA1 agonist, after treatment with 10 or 300 nM paclitaxel for 5 days. AITC-evoked CGRP release was significantly greater from neurons treated with 10 nM paclitaxel as compared to vehicle-treated neurons: the stimulated release was increased from 347 ± 26 fmol/well/10min in vehicle-treated neurons to 472 ± 29 fmol/well/10min in neurons treated with 10 nM paclitaxel (A columns, Figure 3A). Treatment for 5 days with 300 nM paclitaxel decreased AITC-evoked release to 117 ± 16 fmol/well/10min (Figure 3A). Total CGRP content in the neurons was not changed by paclitaxel treatment. Content levels were 1107 ± 77, 1343 ± 93, and 895 ± 85 fmol/well in the vehicle, 10 nM, and 300 nM-treated groups, respectively (Figure 3B).

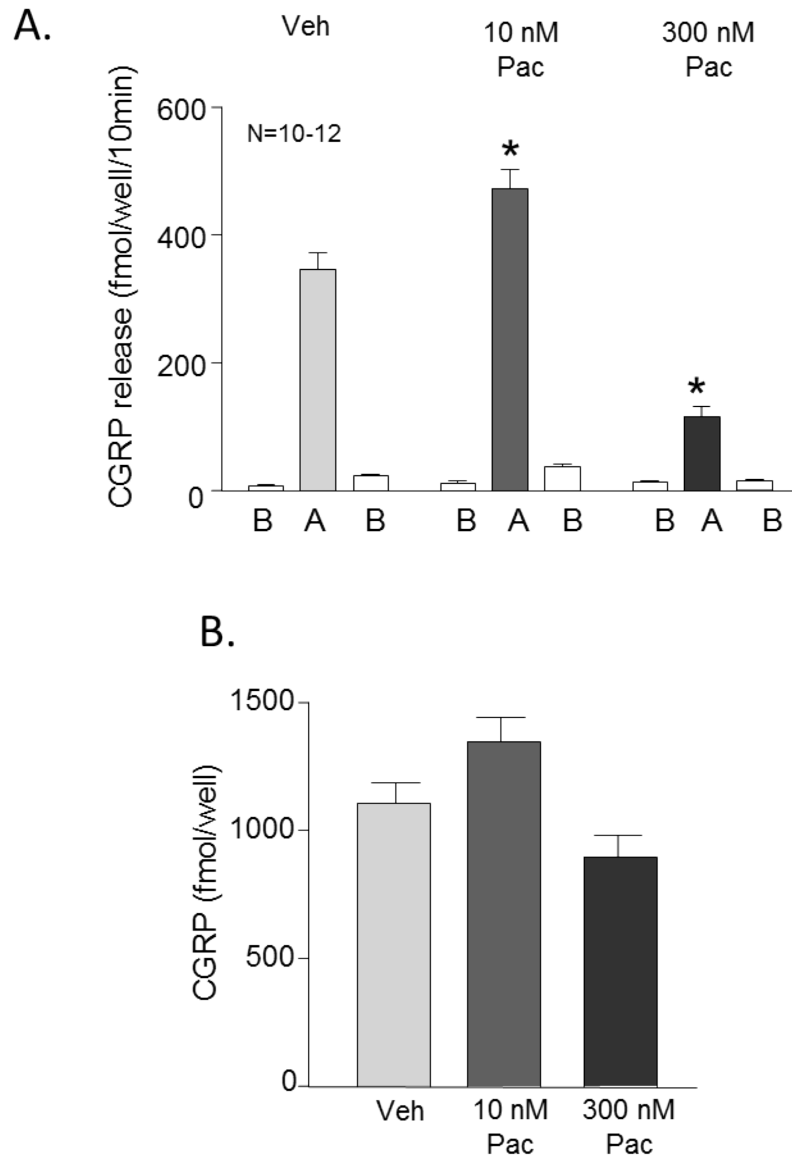
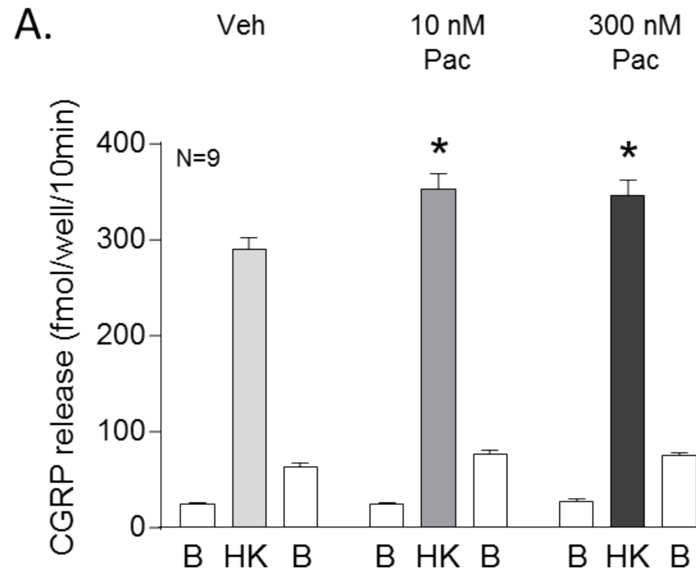


Figure 3. Paclitaxel alters AITC-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 10 nM or 300 nM paclitaxel for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 μM AITC (A), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in AITC-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=10-12) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column irepresents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or paclitaxel treated cultures (Pac) as indicated (N=10-12).

To examine whether the effects of paclitaxel were dependent on altered sensitivity of TRP channels, neuronal cultures treated with vehicle, 10 nM paclitaxel or 300 nM paclitaxel for 5 days were stimulated with a general depolarizing stimulus, 50 mM potassium chloride. In vehicle-treated cultures, high extracellular potassium increased the release of CGRP from basal levels of 25 ± 2 fmol/well/10min to 291 ± 12 fmol/well/10min (open vs shaded HK columns, Figure 4A). Exposing cultures to 10 nM paclitaxel for 5 days resulted in an increase in the potassium-stimulated release of CGRP to 353 ± 16 fmol/well/10min without altering resting basal release in a manner analogous to that observed when release was stimulated by the TRP channel agonists (Figure 4A). In contrast to the results with capsaicin- and AITC-evoked release, exposing cultures to 300 nM paclitaxel for 5 days also augmented the CGRP release evoked by potassium to 346 ± 16 fmol/well/10min (Figure 4A). Differences in potassium-evoked release were not due to an alteration of neuropeptide content, as total content for the vehicle, 10 nM, and 300 nM treated cultures was 1219 ± 78, 1277 ± 87, and 1190 ± 63 fmol/well, respectively (Figure 4B).



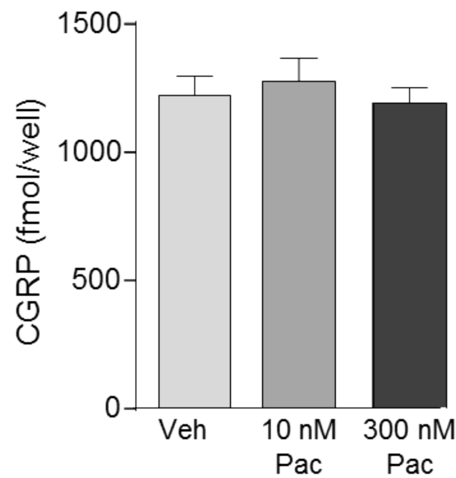


Figure 4. Paclitaxel augments potassium-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 10 nM or 300 nM paclitaxel for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 50 mM KCl (HK), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in HK-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=9) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or paclitaxel treated cultures (Pac) as indicated (N=9).

2) Paclitaxel Does Not Alter Neuronal Viability

Previous reports demonstrate that paclitaxel treatment can cause significant neuronal death in embryonic (E16) DRG cultures and that this death is mediated through necrosis (Scuteri, Nicolini et al. 2006). To determine whether paclitaxel's alteration in transmitter release could be secondary to a change in sensory neuron viability in adult DRG cultures, neuronal cultures were stained with propidium iodide (PI) to differentiate between live and dead neurons. The total number of neurons per field was also counted, based upon the cellular morphology using a phase contrast image. The total number of neurons per field was counted to ensure that the % of PI-positive neurons was not skewed by non-viable neurons lifting from the plates. In cultures treated with vehicle for 5 days, the percentage of neurons that stained positive for PI was 6 ± 1% of the total number of neurons counted, and the total number of neurons per field was 21 ± 1 neurons/field (Figure 5A and B). In cultures exposed to 10 nM paclitaxel for 5 days, the percentage of neurons that stained positive for PI was 10 ± 2%, and the total number of neurons per field was 24 ± 1 (Figure 5A and B). Five days of exposure to 300 nM paclitaxel did not significantly affect cell viability; the percentage of PI-positive staining neurons was 8 ± 2%, and the total number of neurons per field was 20 ± 1 (Figure 5A and B). As a positive control for decreases in cell viability, neuronal cultures were exposed to 300 µM H_2O_2 for 1-2 hours at 37°C, which has previously been shown to produce a significant loss in cell viability (Vasko, Guo et al. 2005). As seen in Figure 5A, H_2O_2 treatment significantly increased the percentage of PI-positive cells to 20 ± 3% of the total

number of neurons. The total number of neurons per field was not altered by treatment with H_2O_2 as there were 22 ± 1 neurons/field.

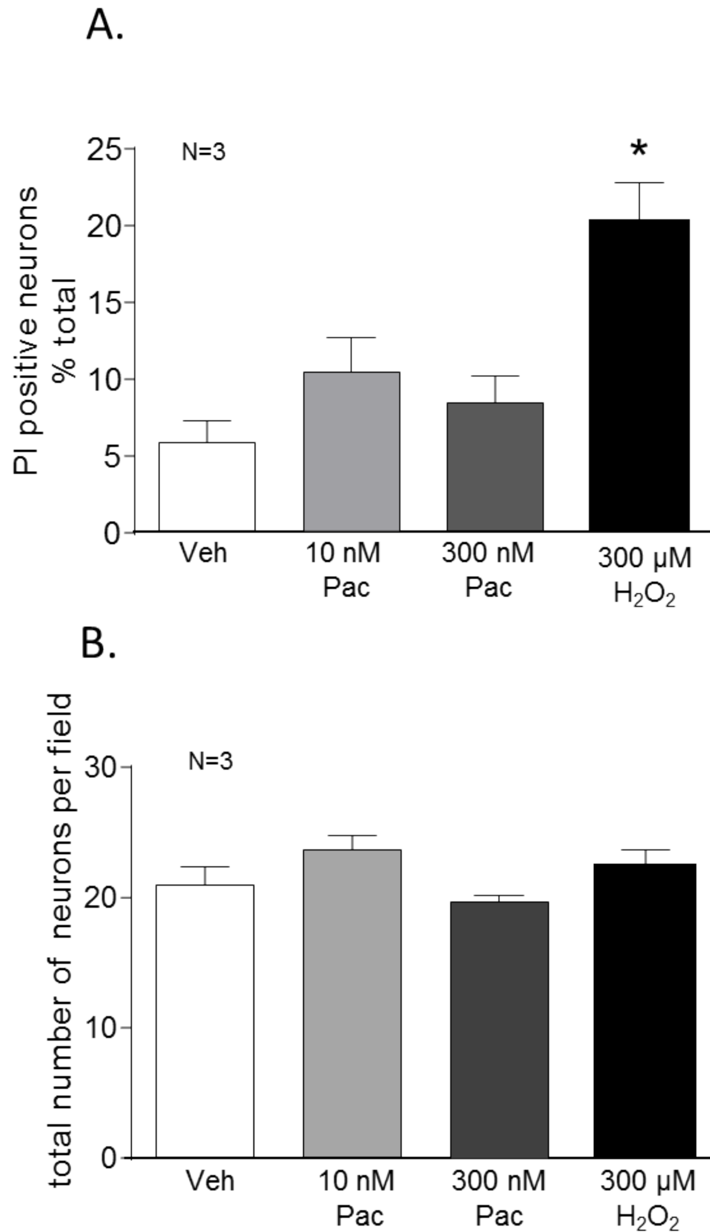


Figure 5. Paclitaxel does not decrease the survival of sensory neurons grown in 30 ng/ml NGF. Sensory neuron cultures were treated with vehicle (open columns, Veh) or with 10 nM or 300 nM paclitaxel for 5 days (Pac; gray columns) and cell viability was measured. As a positive control, vehicle-treated cultures were exposed to H_2O_2 (300 µM) for 1-2 hours 24 hours prior to analysis of cell survival (black columns). A) The number of propidium iodine (PI) positive cells were counted in a minimum of 3 fields from 3 different harvests and normalized to the total number of neurons in the field. Each column represents the mean ± SEM of % positively stained neurons. An (*) indicates a significant difference from vehicle-treated controls ($p<0.05$, N=3) using a one way-ANOVA and Tukey's post-hoc test. B) The total number of neurons in each treatment group was also counted. Each column represents the mean ± SEM of the total number of neurons counted per field (N=3).

3) Paclitaxel Alters Capsaicin-evoked CGRP Release in a Time-dependent Manner

To investigate if paclitaxel-induced alterations in capsaicin-evoked CGRP release were dependent on the duration of paclitaxel exposure, sensory neuron cultures were treated with 10 nM or 300 nM paclitaxel for 1, 3, or 5 days or with vehicle for 5 days. The basal release of CGRP and release upon stimulation with 30 nM capsaicin was then measured. The treatment with vehicle (.003% MPL), 10 nM or 300 nM paclitaxel did not alter the resting basal release of CGRP (B columns, Figures 6A and 7A) over the course of treatment. The capsaicin-evoked CGRP release from neurons treated with 10 nM paclitaxel for 1 day did not differ from the vehicle controls; the release was 339 ± 11 and 353 ± 10 fmol/well/10min in the vehicle and 1-day-treated neurons, respectively (C columns, Figure 6A). However, the capsaicin-evoked CGRP release from the 3-day and 5-day 10 nM paclitaxel-treated wells significantly increased to 380 ± 14 and 395 ± 17 fmol/well/10min, respectively. The increase in capsaicin-evoked release of CGRP was not secondary to an altered content of CGRP in the neurons since treatments with 10 nM paclitaxel did not alter the content of the peptide in the cultures and was 1482 ± 29, 1512 ± 32, 1580 ± 39, and 1615 ± 53 fmol/well in the vehicle, 1-day, 3-day, and 5-day treated groups, respectively (Figure 6B).

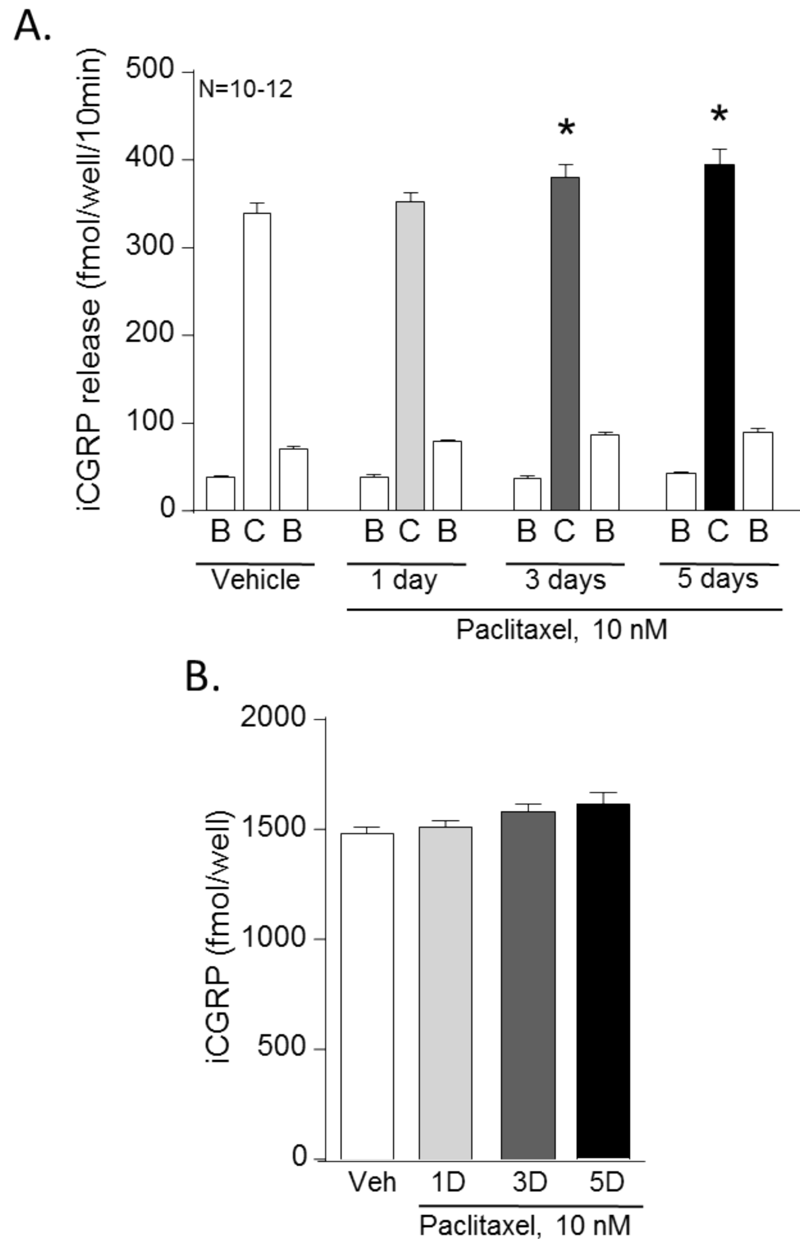
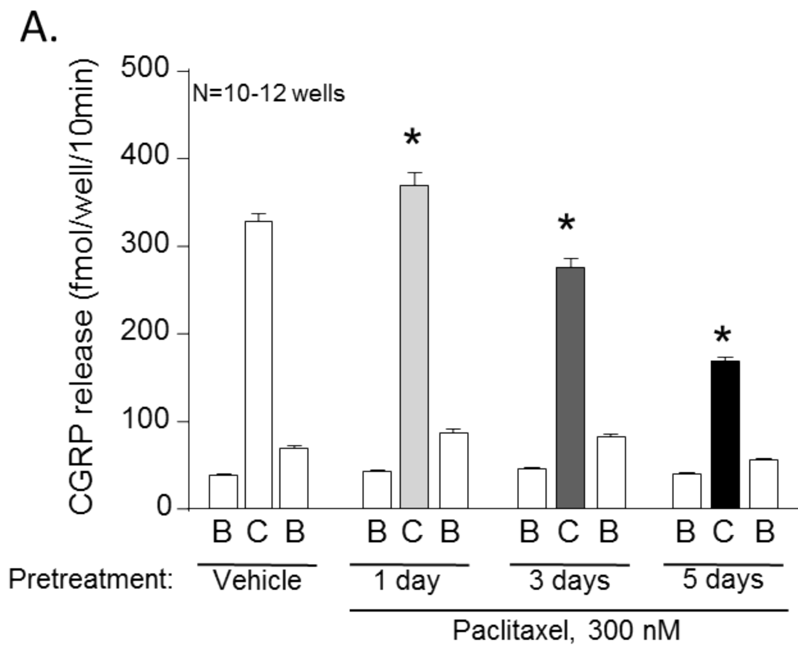
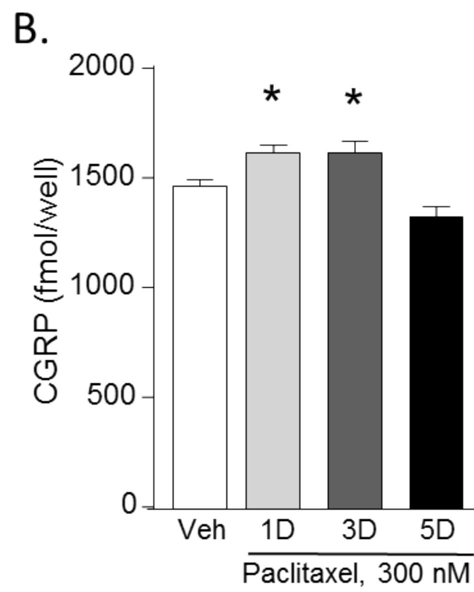


Figure 6. Paclitaxel (10 nM) increases the release of CGRP from sensory neurons in culture in a time-dependent manner. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle or 10 nM paclitaxel for 1, 3, or 5 days as indicated. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=10-12) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from cultures treated with vehicle (Veh) or paclitaxel (Pac) for the duration of time as indicated (N=10-12).

A small, but significant, augmentation in the capsaicin-evoked release of CGRP following 1 day of exposure of neuronal cultures to 300 nM paclitaxel was observed. The release of CGRP in vehicle- and paclitaxel-treated neurons was 328 ± 9 and 369 ± 15 fmol/well/10min, respectively (C columns, Figure 7A). This increase in CGRP release, however, was likely because of an increase in the content of CGRP in the cultures, since 300 nM paclitaxel increased the peptide content levels from 1470 ± 30 to 1623 ± 40 (Figure 7B). Calculating the capsaicin-stimulated release as the % of total peptide content, release from vehicle-treated neurons was 22 ± 1%, and it was 23 ± 1% from cultures treated with 300 nM paclitaxel for 1 day (Figure 7C). Following 3 or 5 days of treatment with paclitaxel (300 nM), the capsaicin-evoked release was significantly reduced to 275 ± 10 and 169 ± 5 fmol/well/10min, respectively. The total content of CGRP in the neurons following 3 and 5 days of treatment was 1626 ± 49 and 1332 ± 47 fmol/well (Figure 7B). Thus the capsaicin-stimulated release at 3 and 5 days of paclitaxel exposure represents 17 ± 1% and 13 ± 1% of the total content of CGRP, demonstrating that the reduced release was not secondary to changes in peptide levels (Figure 7C).


A.
N=10-12 wells
CGRP release (fmol/well/10min)
500
400
300
200
100
0
*
*
*
B C B
B C B
B C B
B C B
Pretreatment:
Vehicle
1 day
3 days
5 days
Paclitaxel, 300 nM


B.
CGRP (fmol/well)
2000
1500
1000
500
0
*
*
Veh
1D
3D
5D
Paclitaxel, 300 nM

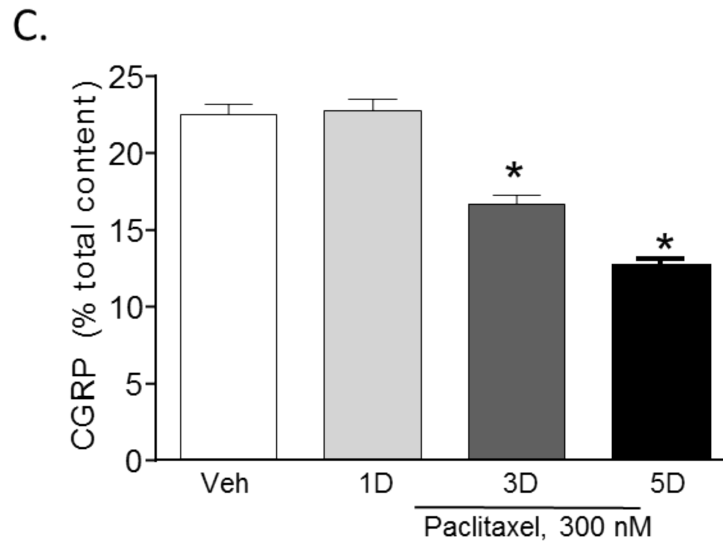


Figure 7. Paclitaxel (300 nM) increases and decreases release of CGRP from sensory neurons in culture in a time-dependent manner. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle or 300 nM paclitaxel for 1, 3, or 5 days as indicated. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=10-12) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well (N=10-12) from cultures treated with vehicle (Veh) or paclitaxel (Pac) for the duration of time as indicated. An (*) indicates a significant difference in the total content of CGRP compared to the vehicle-treated cultures ($p<0.05$, N=10-12) using a one way-ANOVA and Dunnett's post-hoc test. C) Each column represents the mean ± SEM of the percent CGRP release normalized to total content. An (*) indicates a significant difference from vehicle-treated neurons ($p<0.05$, N=10-12) using a one-way ANOVA with Dunnett's post-hoc test.

4) Epothilone B Alters Stimulated CGRP Release from Sensory Neurons in Culture

To determine if a microtubule stabilizing agent with a similar mechanism of action to paclitaxel alters CGRP release, cultured sensory neurons were treated with a range of EpoB concentrations for 5 days, and I then measured the basal and stimulated release of CGRP. The effective concentrations of EpoB on neuronal neuropeptide release were unknown within our model system; therefore, a concentration-response curve of EpoB and capsaicin-evoked CGRP release ranging from 1-300 nM of EpoB was performed. These concentrations were chosen since previous work has shown that EpoB binds microtubules with approximately 10 times higher affinity than paclitaxel, and cancer cells are 3-30-fold more sensitive to EpoB than paclitaxel (Zhang 2010, Kowalski 1997). Neurons were treated with a range of EpoB concentrations including vehicle (0.003%), 1, 3, 10, 30, 100, or 300 nM EpoB for 5 days and then stimulated with capsaicin (30 nM). EpoB did not alter basal release of CGRP from the sensory neurons (first B column of each treatment group, Figure 8A). Capsaicin-evoked (30 nM) CGRP release was augmented by 5-day treatment with 1 and 3 nM EpoB compared to vehicle and was 386 ± 17, 395 ±22, and 300 ±16 fmol/well/10 min, respectively (Figure 8A). The higher concentrations of EpoB: 10, 30, 100, and 300 nM, decreased capsaicin-evoked release to 148 ± 4, 139 ± 13, 129 ± 6, and 108 ± 10 fmol/well/10 min respectively (Figure 8A). EpoB did not alter total content of CGRP in the neurons, and total content levels were 1340 ± 52, 1480 ± 47, 1526 ± 70, 1361 ± 106, 1184 ± 89, 1155 ± 68, and 1105 ± 81 fmol/well for the

vehicle, 1, 3, 10, 30, 100, 300 nM EpoB treatment groups, respectively (Figure 8B).

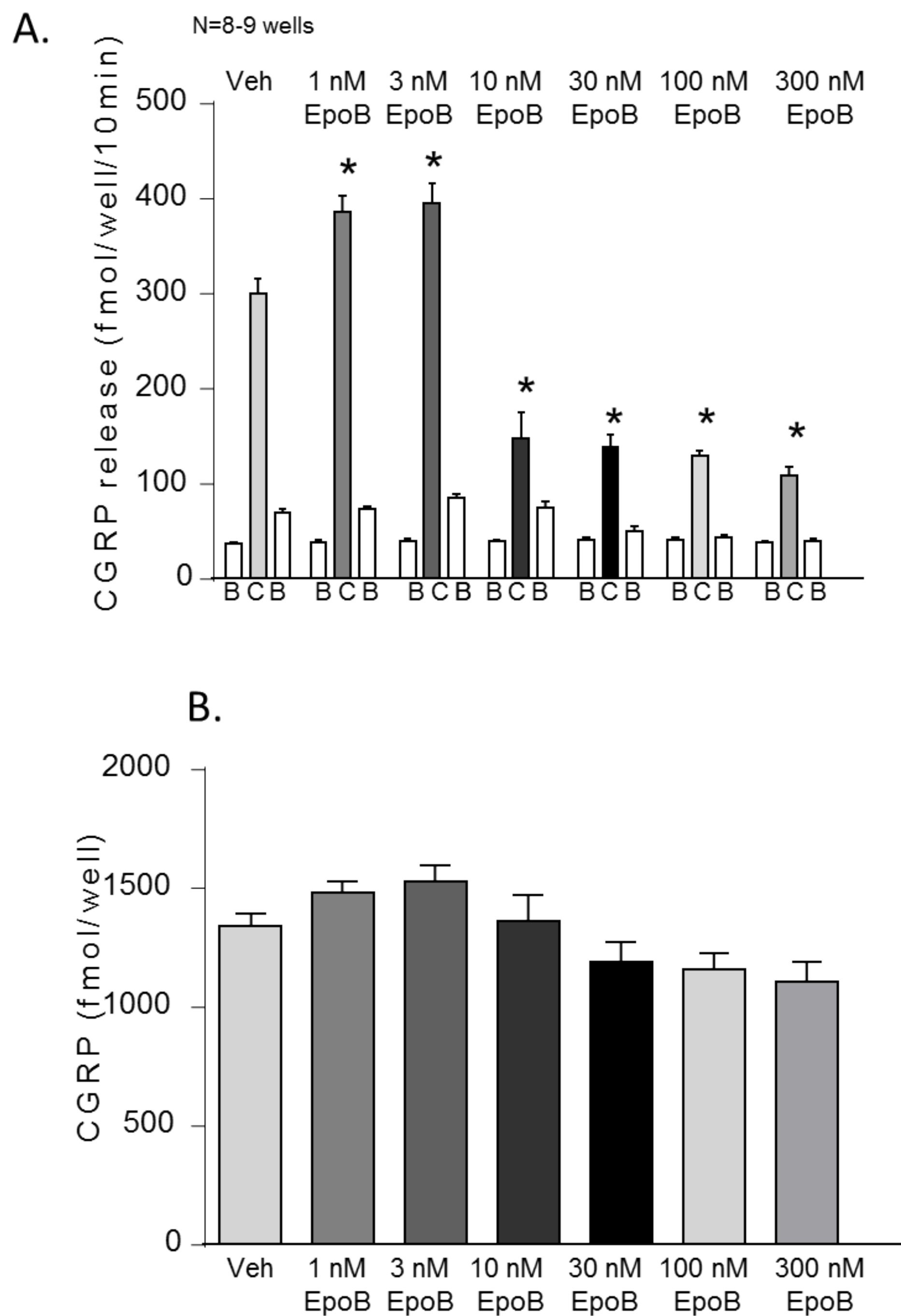


Figure 8. EpoB alters capsaicin-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 1, 3, 10, 30, 100, or 300 nM EpoB for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=8-9) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column irepresents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or EpoB treated cultures (EpoB) as indicated (N=8-9).

To determine if EpoB alters CGRP release independent of TRPV1 activity, sensory neurons were cultured in vehicle, 1 nM, or 30 nM EpoB, and CGRP release was stimulated with high extracellular potassium (50 mM). Potassium-evoked release was not changed after 5-day treatment with 1 nM EpoB compared to vehicle-treated neurons, and was 372 ± 22 and 353 ± 21 fmol/well/10 min, respectively (Figure 9A). Five-day treatment with 30 nM EpoB increased potassium-evoked CGRP release compared to vehicle and was 416 ± 21 fmol/well/10 min (Figure 9A). Treatment with EpoB did not alter total content of CGRP in the neurons and was 1353 ± 80, 1473 ± 85, 1477 ± 64 fmol/well in the vehicle, 1 nM and 30 nM EpoB treatment groups, respectively (Figure 9B).

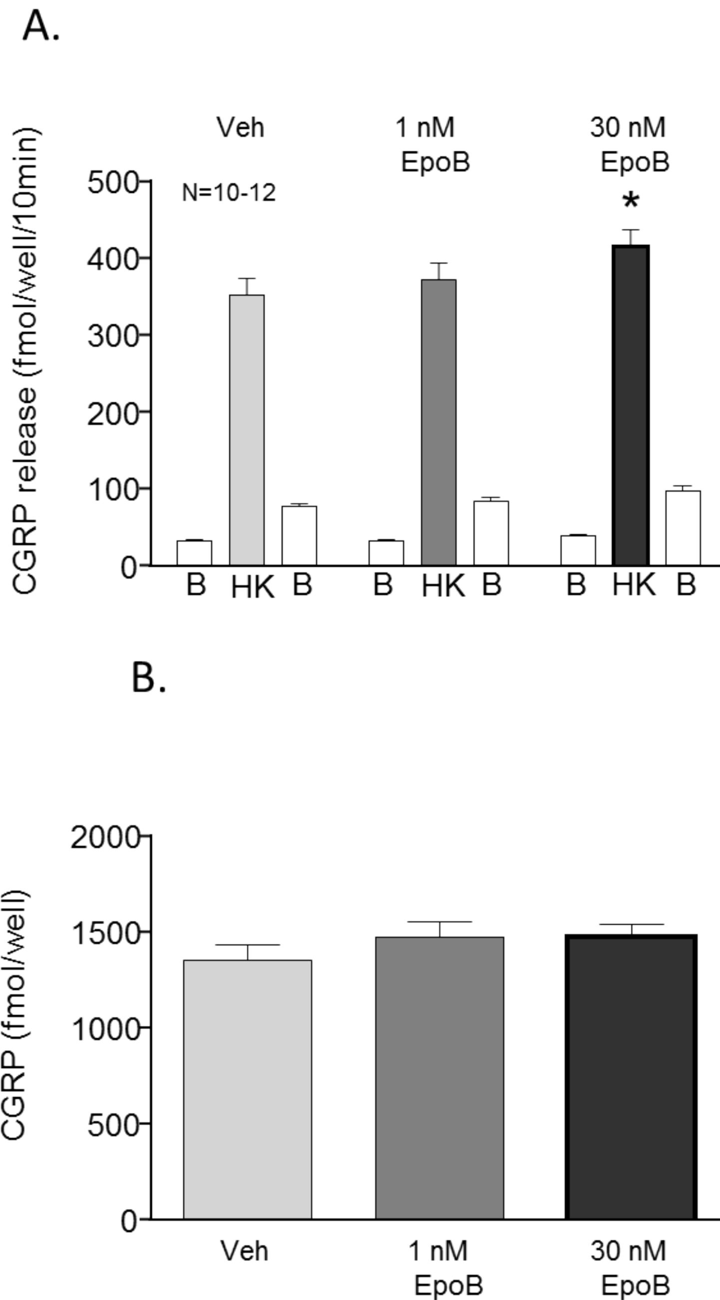


Figure 9. EpoB augments potassium-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 1 nM or 30 nM EpoB for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 50 mM KCl (HK), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in HK-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=10-12) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or EpoB treated cultures (EpoB) as indicated (N=10-12).

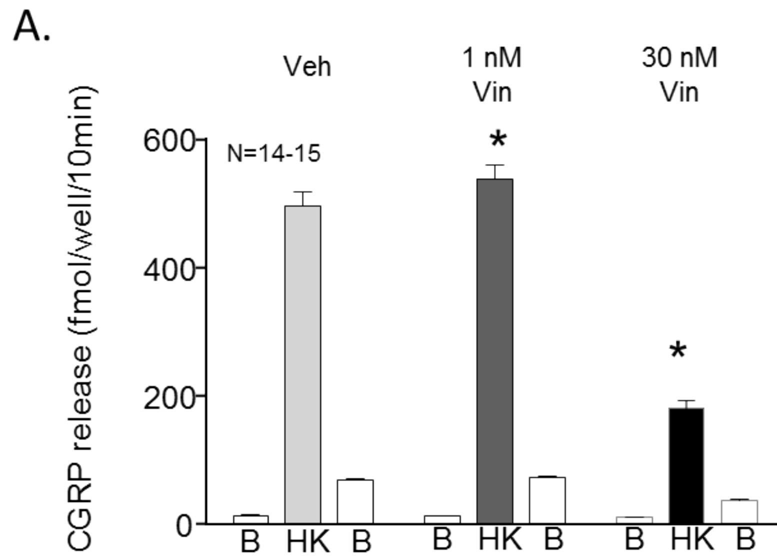
5) Vincristine Alters Potassium-evoked CGRP Release from Sensory Neurons in Culture

In order to ascertain if the alterations of stimulated CGRP release by paclitaxel and EpoB are specific to microtubule stabilization or are a result of general changes in microtubule dynamics, neurons were cultured for 5-days in the presence of vehicle, 1 nM or 30 nM vincristine, which is a microtubule destabilization agent. These concentrations of vincristine were chosen since previous unpublished work from Dr. Vasko's laboratory showed that treating neurons with 1 nM vincristine for 5-days augmented capsaicin-evoked (30 nM) release compared to a vehicle control, whereas treatment with 30 nM vincristine decreased capsaicin-evoked CGRP release. This suggests that, when using capsaicin as a stimulus, treatments with low concentrations of MTAs for 5 days increase capsaicin-evoked CGRP release, and treatments with high concentrations of MTAs decrease capsaicin-evoked CGRP release.

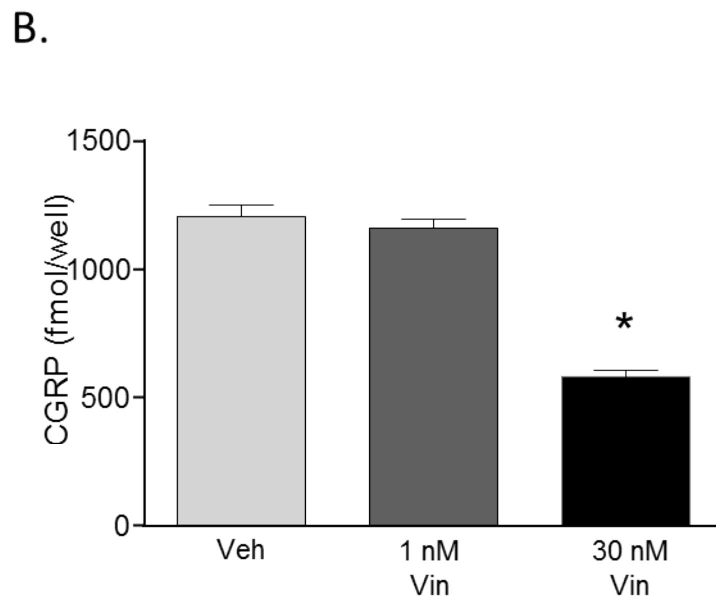
To determine if vincristine alters basal or potassium-evoked CGRP release, neurons were cultured for 5 days in vehicle, 1 nM, or 30 nM vincristine and stimulated with high extracellular potassium. Vincristine did not alter basal CGRP release from the neurons (first B column of each treatment group, Figure 10A). Treatment with 1 nM vincristine for 5 days augmented potassium-evoked CGRP release compared to vehicle and was 539 ± 21 and 498 ± 22 fmol/well/10 min, respectively, whereas treatment with 30 nM vincristine decreased potassium-evoked release to 182 ± 10 fmol/well/10 min (Figure 10A). Total content of CGRP did not change with 1 nM vincristine; however, treatment with

30 nM vincristine decreased total content. Total content was 1208 ± 44, 1165 ± 34, and 582 ± 23 fmol/well in the vehicle, 1 nM and 30 nM vincristine treatment groups, respectively (Figure 10B). In order to determine if the changes in potassium-evoked release are secondary to the changes in total content, the potassium-evoked release was normalized by total content for each group. There was no difference in normalized CGRP release between the vehicle and 1 nM treatment group at 42 ± 2% and 46 ± 2% of total content, respectively, and the normalized CGRP release for the 30 nM group was decreased to 31 ± 2% of total content (Figure 10C). The normalized CGRP release values, 31-46%, were higher than the commonly observed range of 10-15% CGRP release from sensory neurons stimulated with 50 mM potassium (Park, Fehrenbacher et al. 2010). It is suspected that the NGF concentrations in the media may have been lower than 30 ng/ml, and further experiments are being conducted to investigate this potential issue.

A.



B.



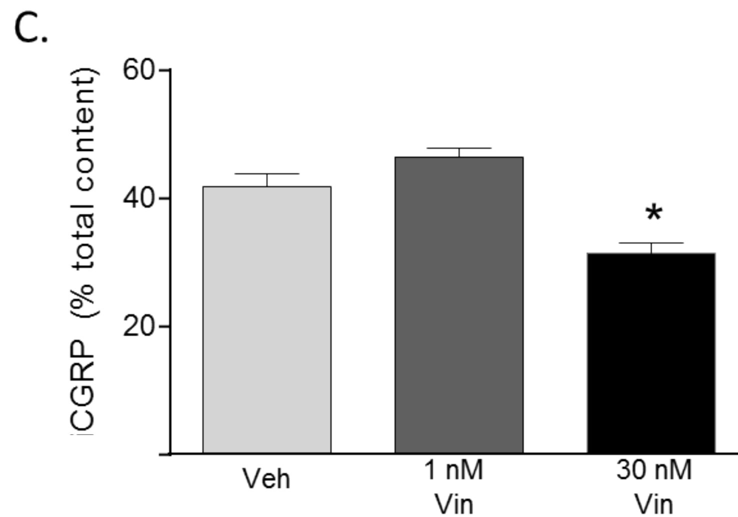


Figure 10. Vincristine alters potassium-evoked release of CGRP from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 1 nM or 30 nM vincristine for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 50 mM KCl (HK), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in HK-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=14-15) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well (N=14-15) from vehicle treated cultures (Veh) or vincristine treated cultures (Pac) as indicated. An (*) indicates a significant difference from vehicle-treated neurons ($p<0.05$, N=14-15) using a one-way ANOVA with Dunnett's post-hoc test. C) Each column represents the mean ± SEM of the percent CGRP release normalized to total content. An (*) indicates a significant difference from vehicle-treated neurons ($p<0.05$, N=14-15) using a one-way ANOVA with Dunnett's post-hoc test.

6) Acute Treatment with Paclitaxel Does Not Alter CGRP Release from Sensory Neurons in Culture

Although 5-day treatment with paclitaxel did not alter basal release of CGRP release from the neurons in culture, an acute effect of paclitaxel on the neurons may be missed with this dosing paradigm. In order to determine if paclitaxel has an acute effect on CGRP release from sensory neurons, untreated sensory neurons in culture were treated with either HEPES buffer and vehicle for 10 min or HEPES buffer and 300 nM paclitaxel for 3, 10 min incubations. CGRP release during the 1st, 2nd, and 3rd incubations with paclitaxel did not differ from the release during the vehicle incubation. Release was 48 ± 1, 50 ± 2, 42 ± 2, and 40 ± 3 fmol/well/10 min from the vehicle, 1st, 2nd, and 3rd paclitaxel incubations, respectively (Figure 11).

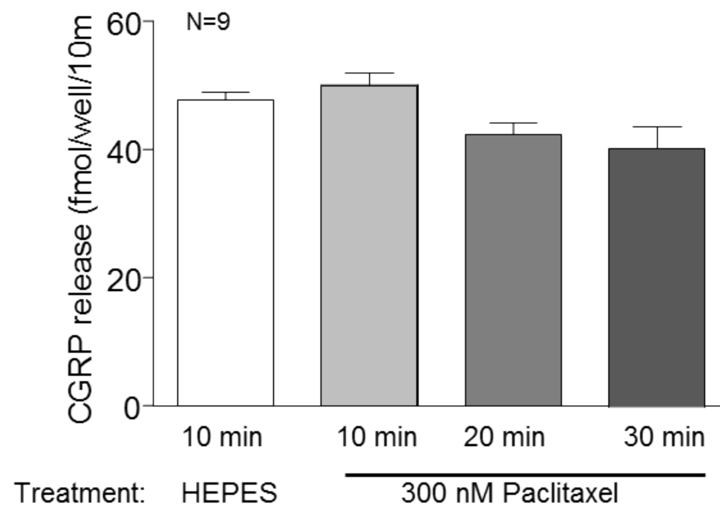
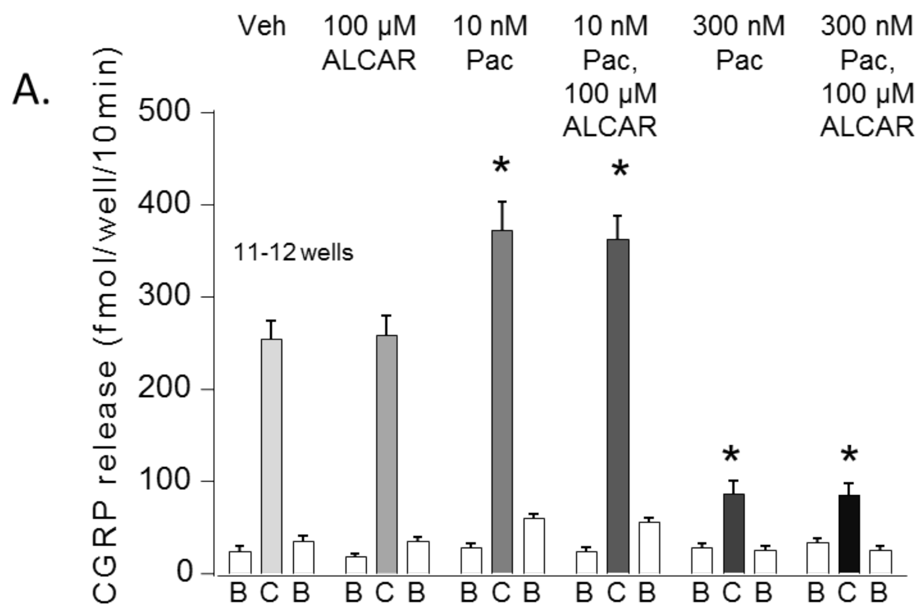


Figure 11. Acute administration of paclitaxel does not alter CGRP release from sensory neurons in culture. Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle in HEPES buffer (open bar) or 300 nM paclitaxel for 10-30 min (gray bars) (N=9).

7) <u>Acetyl-*L*-carnitine Does Not Reverse Paclitaxel-induced Alterations of CGRP Release</u>

Previous work in animal models has shown that ALCAR prevents and reverses paclitaxel-induced nociception and impairment in mitochondrial respiration within sciatic nerves by presumably protecting mitochondria (Flatters Bennett 2006, Zheng 2011). To determine if ALCAR protects against paclitaxel-induced alterations of CGRP release, neurons were cultured with either 100 μM ALCAR alone for 5 days or 100 μM ALCAR in the presence of 10 nM or 300 nM paclitaxel. This concentration of ALCAR was chosen because similar concentrations of ALCAR (100 μM, 250 μM and 1 mM) have been previously used in experiments of cultured neurons (Manfridi, Forloni et al. 1992, Kano, Kawakami et al. 1999, Abdul Muneer, Alikunju et al. 2011). In preliminary experiments, treatment with 10 mM ALCAR alone for 5 days decreased capsaicin-evoked CGRP release and 100 μM ALCAR did not alter capsaicin-evoked release. I wanted to use a concentration of ALCAR that would not alter stimulated release of CGRP alone, and I therefore used 100 μM ALCAR for the experiments described below. Five-day treatment with 100 μM ALCAR did not alter basal release of CGRP or capsaicin-stimulated (30 nM) release, and the release was 259 ± 21 fmol/well/10 min from ALCAR treated neurons and 254 ± 19 fmol/well/10 min from vehicle-treated neurons (Figure 12A). In addition, treatment with 100 μM ALCAR did not alter capsaicin-evoked release from neurons treated with low or high concentrations of paclitaxel; 5-day treatment with ALCAR + 10 nM paclitaxel or 10 nM paclitaxel alone augmented capsaicin-

evoked CGRP release to 363 ± 24 and 373 ± 30 fmol/well/10 min, respectively, and treatment with ALCAR + 300 nM paclitaxel or 300 nM paclitaxel alone decreased capsaicin-evoked release from the neurons, and the release was 84 ± 14 and 87 ± 14 fmol/well/10 min, respectively (Figure 12A). ALCAR alone or in combination with paclitaxel did not alter total content of CGRP in the neurons, and total content was 981 ± 82, 942 ± 73, 1085 ± 57, 1200 ± 79, 839 ± 70, and 826 ± 52 fmol/well from the vehicle, 100 μM ALCAR, 10 nM paclitaxel, 100 μM ALCAR + 10 nM paclitaxel, 300 nM paclitaxel, and 100 μM ALCAR + 300 nM paclitaxel treated groups, respectively (Figure 12B).


A.
Veh
100 µM ALCAR
10 nM Pac
10 nM Pac, 100 µM ALCAR
300 nM Pac
300 nM Pac, 100 µM ALCAR
CGRP release (fmol/well/10min)
500
400
300
200
100
0
11-12 wells
*
*
*
*
B C B
B C B
B C B
B C B
B C B
B C B

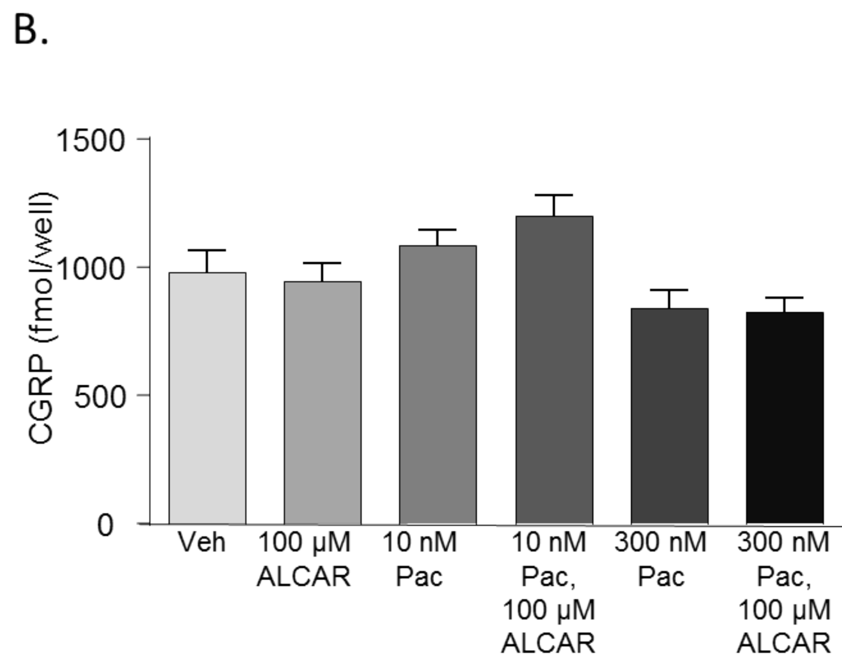
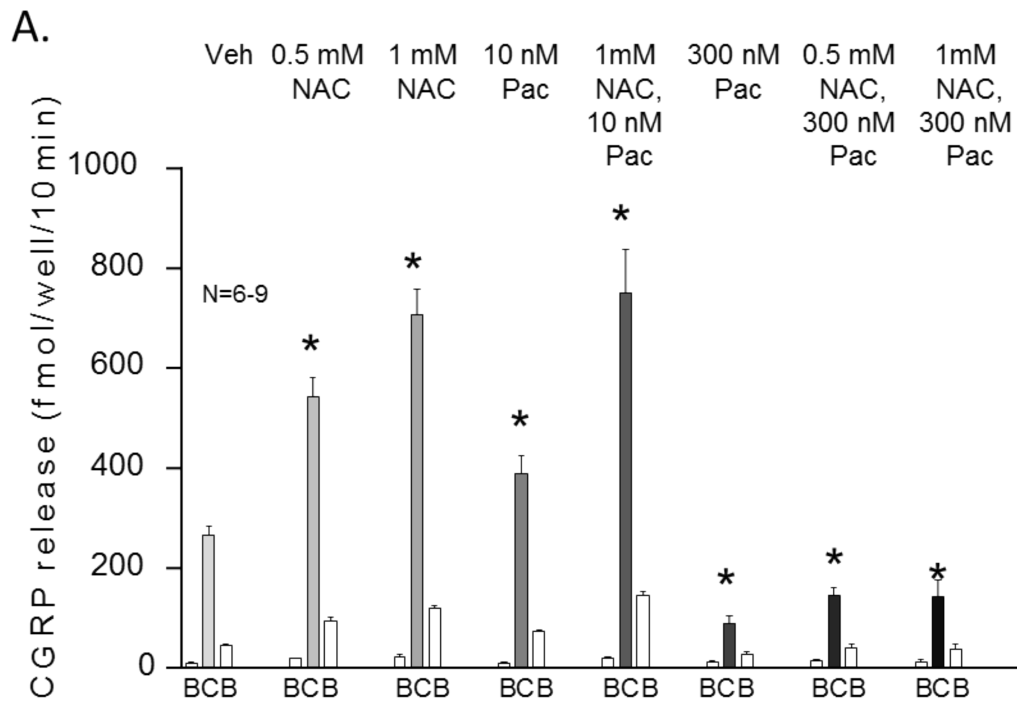

B.
CGRP (fmol/well)
1500
1000
500
0
Veh
100 µM ALCAR
10 nM Pac
10 nM Pac, 100 µM ALCAR
300 nM Pac
300 nM Pac, 100 µM ALCAR

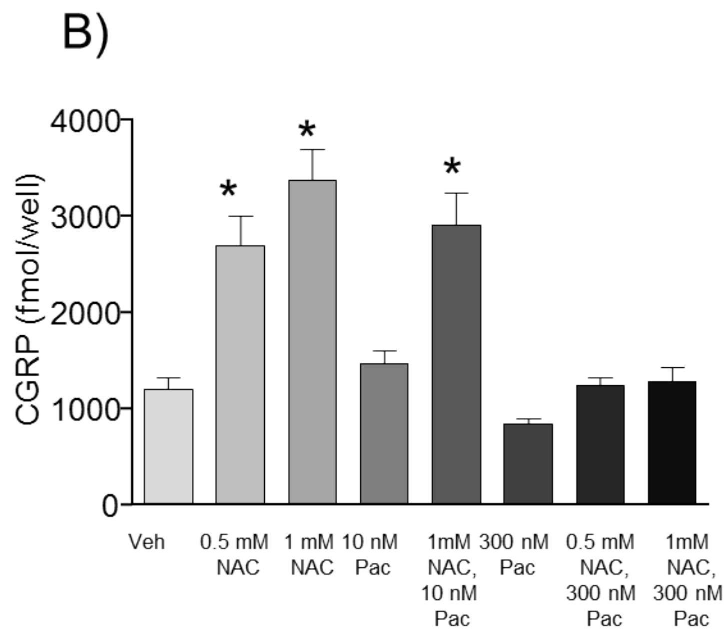
Figure 12. Acetyl-*L*-carnitine does not alter paclitaxel-induced changes in capsaicin-stimulated CGRP release from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 10 nM or 300 nM paclitaxel ± 100 μM ALCAR for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=11-12) using a two-way ANOVA with Bonferroni's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well (N=11-12) from vehicle treated cultures (Veh) or paclitaxel treated cultures (Pac) ± ALCAR as indicated.

8) N-acetyl-*L*-cysteine Does Not Reverse Paclitaxel-induced Alterations of CGRP Release

Administration of NAC in *in vitro* models has been shown to reverse ROS produced by exposure to paclitaxel, vincristine, and EpoB (Tsai Chao 2007, Rogalska Jozwiak 2013). Since administration of free radical scavengers has prevented and reversed paclitaxel-induced nociceptive behaviors *in vivo (*Kim Abdi 2010, Fidanboylu 2011)*,* I wanted to investigate if using a free radical scavenger and glutathione recycler, such as NAC, would alter paclitaxel-induced changes on sensory neuronal activity. To determine if NAC reverses paclitaxel-induced changes of stimulated CGRP release from sensory neurons, neurons were cultured in media containing 0.5 mM or 1 mM NAC for 5 days alone or in the presence of 10 nM or 300 nM paclitaxel. These concentrations of NAC were used because a similar concentration (2 mM) has been used in another study using sensory neuron cultures (Naziroglu, Cig et al. 2013). NAC did not alter basal CGRP release (first B column of each treatment group, Figure 13A). Both concentrations of NAC increased capsaicin-evoked CGRP release compared to vehicle-treated neurons, and release was 268 ± 18, 544 ± 37, 708 ± 53 fmol/well/10 min from the vehicle, 0.5 mM, and 1 mM NAC treatment groups, respectively (Figure 13A). Five-day treatment with 10 nM paclitaxel or 1 mM NAC + 10 nM paclitaxel caused an increase in capsaicin-evoked CGRP release to 392 ± 35 and 752 ± 87 fmol/well/10 min, respectively (Figure 13A). The 300 nM paclitaxel, 0.5 mM NAC + 300 nM paclitaxel, and the 1 mM NAC + 300 nM paclitaxel treatments resulted in a decrease in capsaicin-evoked CGRP release

compared to vehicle and were 91 ± 15, 146 ± 17, 144 ± 33 fmol/well/10 min, respectively (Figure 13A). Total content of CGRP increased in the 0.5 mM NAC, 1 mM NAC, and 1 mM NAC + 10 nM paclitaxel groups; however, total content did not change compared to vehicle in the 10 nM paclitaxel, 300 nM paclitaxel, 0.5 mM NAC + 300 nM paclitaxel, and 1 mM NAC + 300 nM paclitaxel groups. Total content in the neurons was 1209 ± 117, 2702 ± 312, 3378 ± 322, 1476 ± 129, 2909 ± 335, 848 ± 57, 1253 ± 64, and 1281 ± 148 fmol/well in the vehicle, 0.5 mM NAC, 1 mM NAC, 10 nM paclitaxel, 1 mM NAC + 10 nM paclitaxel, 300 nM paclitaxel, 0.5 mM NAC + 300 nM paclitaxel, and 1 mM NAC + 300 nM paclitaxel groups, respectively (Figure 13B). To determine if the increases in capsaicin-evoked release were secondary to an increase in total content, the released CGRP was normalized to the total content for each group and was 22 ± 1, 21 ± 2, 22 ± 1, 27 ± 1, 28 ± 1, 10 ± 1, 11 ± 1, 11 ± 2% total content, respectively, in the vehicle, 0.5 mM NAC, 1 mM NAC, 10 nM paclitaxel, 1 mM NAC + 10 nM paclitaxel, 300 nM paclitaxel, 0.5 mM NAC + 300 nM paclitaxel, and 1 mM NAC + 300 nM paclitaxel groups (Figure 13C). Normalized stimulated release increased compared to vehicle in the 10 nM paclitaxel and 1 mM NAC + 10 nM paclitaxel groups, and normalized stimulated release decreased compared to vehicle in the 300 nM paclitaxel, 0.5 mM NAC + 300 nM paclitaxel, and 1 mM NAC + 300 nM paclitaxel groups (Figure 13C).


A.
Veh
0.5 mM NAC
1 mM NAC
10 nM Pac
1mM NAC, 10 nM Pac
300 nM Pac
0.5 mM NAC, 300 nM Pac
1mM NAC, 300 nM Pac
CGRP release (fmol/well/10 min)
1000
800
600
400
200
0
N=6-9
*
BCB


B)
CGRP (fmol/well)
4000
3000
2000
1000
0
*
Veh
0.5 mM NAC
1 mM NAC
10 nM Pac
1mM NAC, 10 nM Pac
300 nM Pac
0.5 mM NAC, 300 nM Pac
1mM NAC, 300 nM Pac

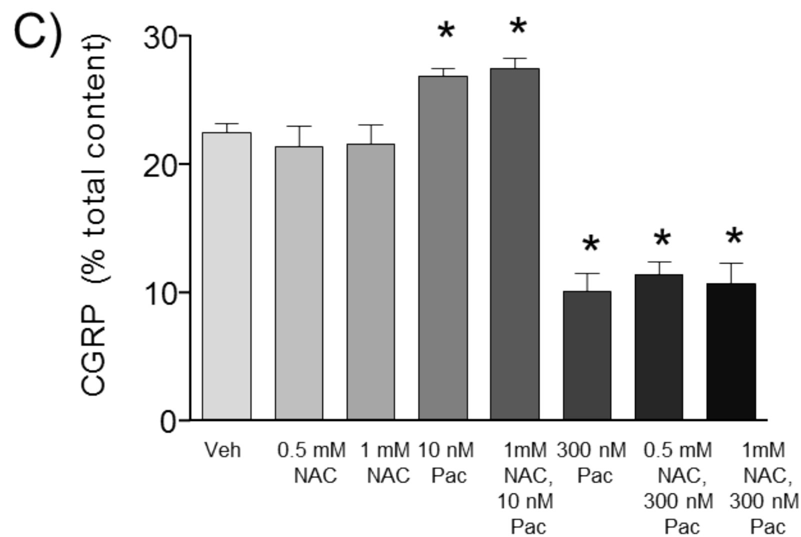
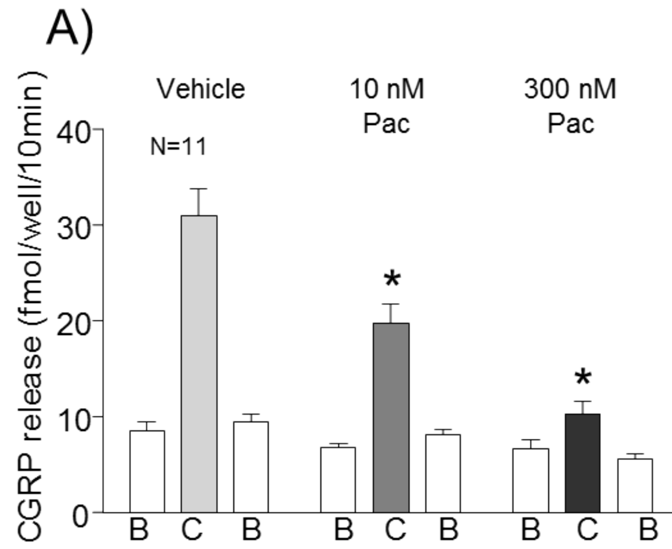


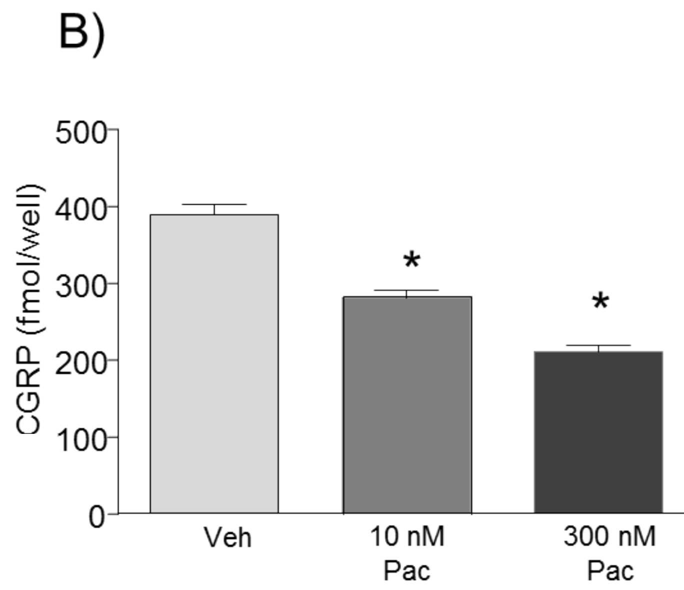
Figure 13. N-acetyl-*L*-cysteine does not alter paclitaxel-induced changes in capsaicin-stimulated CGRP release from sensory neurons in culture. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with 10 nM or 300 nM paclitaxel ± 0.5 or 1 mM NAC for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated neurons ($p<0.05$, N=6-9) using a two-way ANOVA with Tukey's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle treated cultures (Veh) or paclitaxel treated cultures (Pac) ± NAC as indicated. An (*) indicates a significant different from the vehicle-treated neurons using a one-way ANOVA ($p<0.05$, N=6-9) with Dunnett's post-hoc test. C) Each column represents the mean ± SEM of the percent CGRP release normalized to total content. An (*) indicates a significant difference from vehicle-treated neurons ($p<0.05$, N=6-9) using a one-way ANOVA with Dunnett's post-hoc test.

9) Paclitaxel Alters Stimulated CGRP Release and Decreases Total CGRP Content in the Absence of NGF

All experiments previously described in this work were conducted with cultures that had been maintained in media containing 30 ng/ml NGF. Because NGF alters neuronal sensitivity (Zhu, Galoyan et al. 2004, Zhang, Huang et al. 2005, Zhu and Oxford 2007) and other groups have found that NGF can alter paclitaxel-induced changes in cells (Peterson and Crain 1982, Apfel, Lipton et al. 1991), we asked if paclitaxel alters neuronal activity in the absence of added NGF. To determine if NGF alters the effects of paclitaxel on neuronal activity, I cultured sensory neurons in media with 0 added NGF and treated them with a low (10 nM) or high (300 nM) concentration of paclitaxel for 5 days. I then measured the basal release of CGRP as well as the release of CGRP following stimulation with capsaicin (30 nM). Five-day treatment with paclitaxel in the absence of added NGF did not alter basal release of CGRP from sensory neurons treated with vehicle (0.003% MPL), 10 nM paclitaxel, or 300 nM paclitaxel (first B column of each treatment group, Figure 14A). Five-day treatment of both 10 nM and 300 nM paclitaxel significantly decreased capsaicin-evoked release of CGRP compared to vehicle and was 20 ± 2, 10 ± 1, and 31 ± 3 fmol/well/10 min, respectively (Figure 14A). The total content of CGRP within the neurons also decreased following paclitaxel treatment and was 390 ± 13 fmol/well in vehicle cultures, 281 ± 9 fmol/well in 10 nM paclitaxel cultures, and 212 ± 7 fmol/well in 300 nM paclitaxel cultures (Figure 14B). In order to determine if the changes in capsaicin-evoked release were secondary to a

decrease in total content, I determined the capsaicin-evoked release normalized to the total content for each group; there was no difference in normalized CGRP release following treatment with 10 nM paclitaxel compared to vehicle treatment, 7 ± 2% and 8 ± 2% of total content, respectively. Treatment with 300 nM paclitaxel decreased normalized CGRP release and was 5 ± 2% of total content (Figure 14C).


A)
Vehicle
10 nM
Pac
300 nM
Pac
N=11
CGRP release (fmol/well/10min)
40
30
20
10
0
*
*
B C B B C B B C B


B)
CGRP (fmol/well)
500
400
300
200
100
0
*
*
Veh
10 nM
Pac
300 nM
Pac

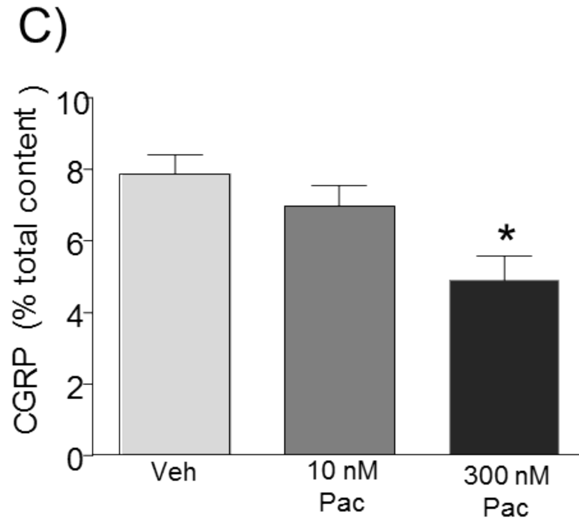
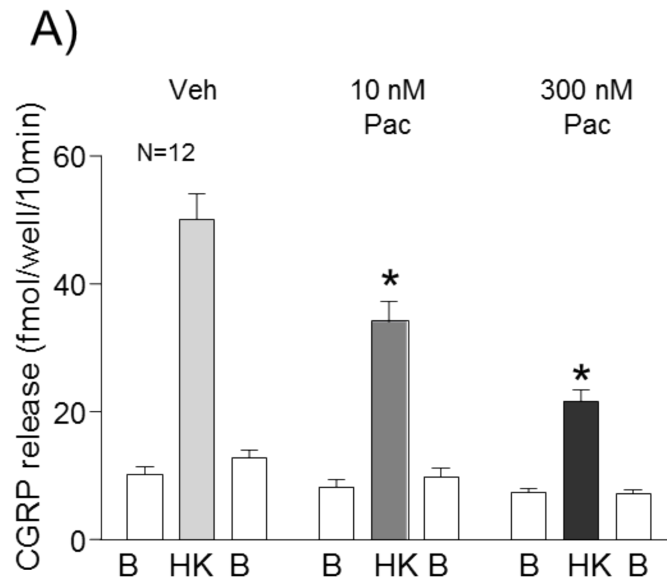
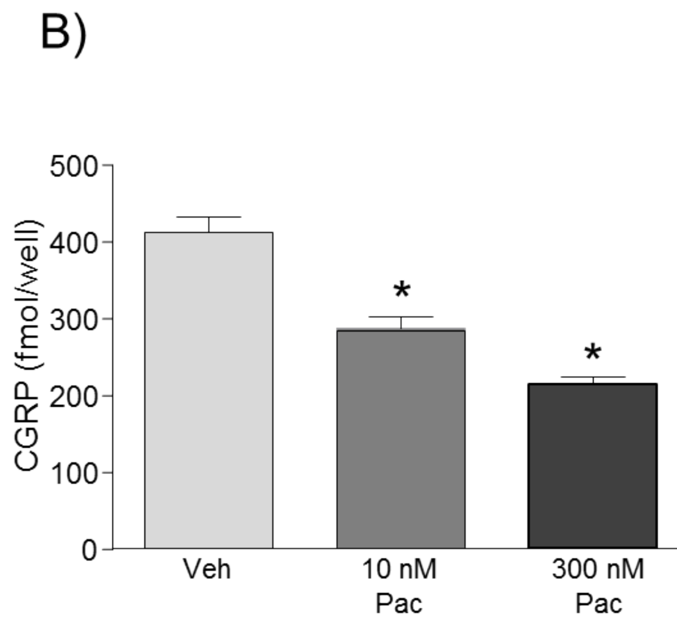


Figure 14. Paclitaxel attenuates capsaicin-evoked release of CGRP from sensory neurons grown in the absence of NGF. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle, 10 nM, or 300 nM paclitaxel for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated cultures ($p<0.05$, N=12) using a two-way ANOVA with Tukey's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle-treated cultures (Veh) or paclitaxel-treated cultures (Pac) as indicated. An (*) indicates a significant difference in total CGRP content compared to the vehicle-treated cultures ($p<0.05$, N=12) using a one-way ANOVA with Dunnett's post-hoc test. C) Each column represents the normalized mean ± SEM of the capsaicin-evoked CGRP divided by the total CGRP content for each group. An (*) indicates a significant difference in normalized CGRP compared to vehicle-treated cultures ($p<0.05$, N=12) using a one-way ANOVA with Dunnett's post-hoc test.

In order to bypass possible effects of NGF on TRP channel activity (Zhang, Huang et al. 2005, Zhu and Oxford 2007), I examined the effects of 0 added NGF on potassium-stimulated CGRP release after treatment with paclitaxel. I stimulated the release of CGRP from paclitaxel-treated cultures using a general depolarizing stimulus, high extracellular potassium chloride (50 mM), from neurons grown in 0 added NGF. The profile of potassium-evoked release from the cultures treated with vehicle, 10 nm, or 300 nM paclitaxel was similar to when I stimulated with capsaicin; both concentrations of paclitaxel decreased CGRP release compared to vehicle. The potassium-stimulated release was 50 ± 4 fmol/well/10 min from vehicle cultures, 34 ± 3 fmol/well/10 min from 10 nM paclitaxel cultures, and 22 ± 2 fmol/well/10 min from 300 nM paclitaxel cultures (Figure 15A). Total content decreased in the cultures treated with paclitaxel and was 412 ± 21, 287 ± 16, and 215 ± 10 fmol/well from the vehicle, 10 nM, and 300 nM paclitaxel treated cultures, respectively (Figure 15B). When potassium-stimulated release was normalized to the total content for each group, there was no difference in normalized CGRP release from cultures treated with 10 nM paclitaxel or vehicle and was 12 ± 1% and 12 ± 1% of total content, respectively (Figure 15C). Normalized CGRP release decreased in cultures treated with 300 nM paclitaxel, and was 10 ± 1% of total content (Figure 15C).


A)
Veh
10 nM
Pac
300 nM
Pac
N=12
CGRP release (fmol/well/10min)
60
40
20
0
*
*
B
HK
B
B
HK
B
B
HK
B


B)
CGRP (fmol/well)
500
400
300
200
100
0
*
*
Veh
10 nM
Pac
300 nM
Pac

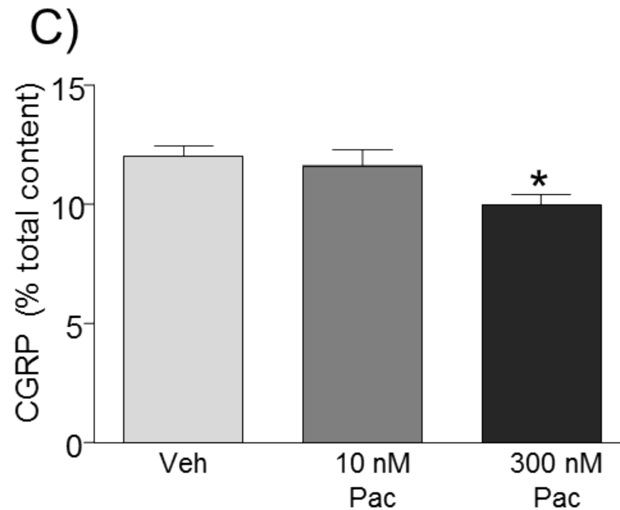


Figure 15. Paclitaxel attenuates potassium-evoked release of CGRP from sensory neurons grown in the absence of NGF. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle, 10 nM, or 300 nM paclitaxel for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 50 mM potassium (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in potassium-evoked release compared to release from the vehicle-treated cultures ($p<0.05$, N=12) using a two-way ANOVA with Tukey's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle-treated cultures (Veh) or paclitaxel-treated cultures (Pac) as indicated. An (*) indicates a significant difference in total CGRP content compared to the vehicle-treated cultures ($p<0.05$, N=12) using a one-way ANOVA with Dunnett's post-hoc test. C) Each column represents the normalized mean ± SEM of the potassium-evoked CGRP divided by the total CGRP content for each group. An (*) indicates a significant difference in normalized CGRP compared to vehicle-treated cultures ($p<0.05$, N=12) using a one-way ANOVA with Dunnett's post-hoc test.

10) Paclitaxel Does Not Alter Neuronal Viability in the Absence of NGF

Previously I have shown that 5-day treatment with 10 nM or 300 nM paclitaxel does not alter neuronal viability when neurons are grown in media containing 30 ng/ml of NGF. To ascertain whether the decrease in total CGRP content level in neurons grown in 0 added NGF was due to neuronal death, I performed PI viability assays. Neurons were cultured in media with 0 added NGF, treated with vehicle, 10 nM, or 300 nM paclitaxel for 5 days, and stained with PI. In cultures treated with vehicle, the percentage of neurons that stained positive for PI was 7 ± 2% and the total number of neurons per field was 22 ± 2 (Figure 16A and B). Paclitaxel did not alter the percent of positive PI-staining neurons in the cultures, and 8 ± 1 and 9 ± 2% of the neurons stained positive for PI in the 10 nM and 300 nM paclitaxel groups, respectively (Figure 16A and B). Paclitaxel also did not alter the total number of neurons per field as there were 24 ± 2 and 21 ± 1 neurons/field in the 10 nM and 300 nM paclitaxel groups, respectively (Figure 16A and B). As a positive control for increasing PI-staining (induction of neuronal death), neurons were treated with 300 µM H_2O_2 for 1-2 hours at 37°C in media, and PI-staining was performed approximately 24 hours after the removal of the H_2O_2. This has previously been shown to decrease neuronal viability (Gracias published thesis, Indiana University). The percentage of positively stained neurons increased to 21 ± 2% after H_2O_2 exposure; however, the total number of neurons per field did not change and was 24 ± 1 neurons/field (Figure 16A and B).

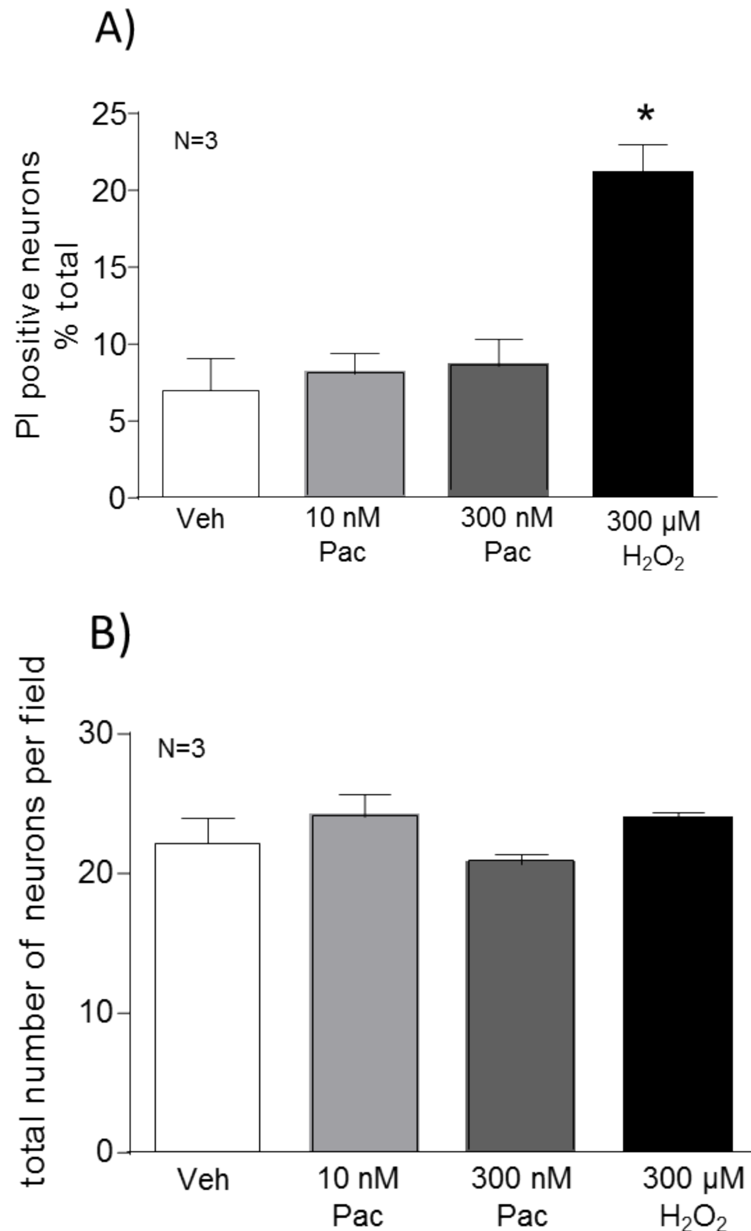
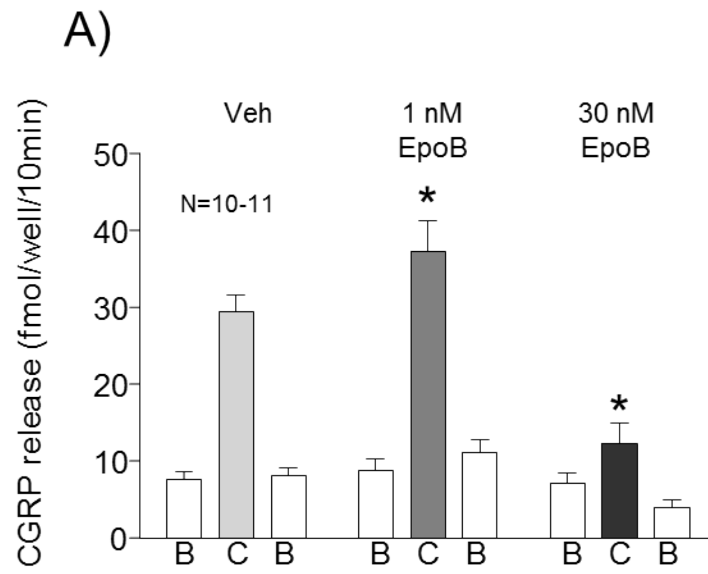


Figure 16. Paclitaxel does not decrease the survival of sensory neurons grown in the absence of NGF. Sensory neuronal cultures were treated with vehicle (open columns, Veh) or with 10 nM or 300 nM paclitaxel for 5 days (Pac; gray columns) and cell viability was measured. As a positive control, vehicle-treated cultures were exposed to H_2O_2 (300 µM) for 1-2 hours 24 hours prior to analysis of cell survival (black columns). A) The number of propidium iodine (PI) positive cells were counted in a minimum of 3 fields from 3 different harvests and normalized to the total number of neurons in the field. Each column represents the mean ± SEM of % positively stained neurons. An (*) indicates a significant difference from vehicle-treated controls ($p<0.05$, N=3) using a one way-ANOVA and Tukey's post-hoc test. B) The total number of neurons in each treatment group was also counted. Each column represents the mean ± SEM of the total number of neurons counted per field.

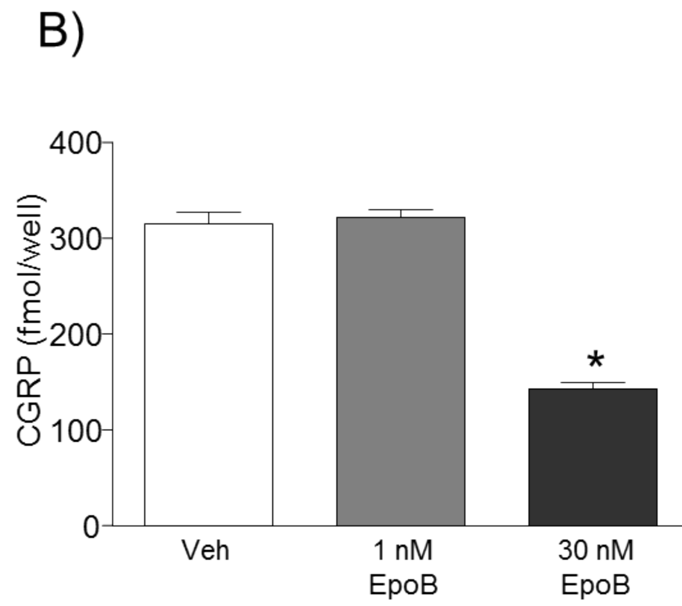
11) Epothilone B Alters Stimulated CGRP Release and Decreases Total CGRP Content in the Absence of NGF

Paclitaxel alters CGRP release differently in the presence and absence of NGF, suggesting that the presence of NGF alters the effects of MTAs on sensory neuron function or that the differential effects of paclitaxel on CGRP release, depending on the presence of NGF, is caused by NGF interacting with one of paclitaxel's microtubule-independent actions. In order to determine if NGF alters CGRP release induced by another microtubule stabilizer, neurons were cultured in media with 0 added NGF and treated with 1 nM or 30 nM EpoB for 5 days. Basal and capsaicin-evoked release were then measured. In cultures grown in 0 added NGF, 1 nM EpoB increased capsaicin-evoked CGRP release compared to vehicle treated neurons, and the release was 30 ± 2 and 37 ± 4 fmol/well/10 min from vehicle and 1 nM EpoB cultures, respectively (Figure 17A). Five-day treatment with 30 nM EpoB decreased release to 12 ± 3 fmol/well/10 min (Figure 17A). Total content of CGRP was not changed after treatment with 1 nM EpoB but was decreased in neurons treated with 30 nM EpoB. Total content of CGRP was 316 ± 12, 321 ± 8, 144 ± 18 fmol/well the vehicle, 1 nM, and 30 nM EpoB groups, respectively (Figure 17B). Since total content was altered with 30 nM EpoB, stimulated CGRP release was normalized by total content. There was no difference in the % release of total content between the groups and was 9 ± 1, 12 ± 1, and 8 ± 2 % of total CGRP in the vehicle, 1 nM, and 30 nM EpoB groups, respectively (Figure 17C).

A)



B)



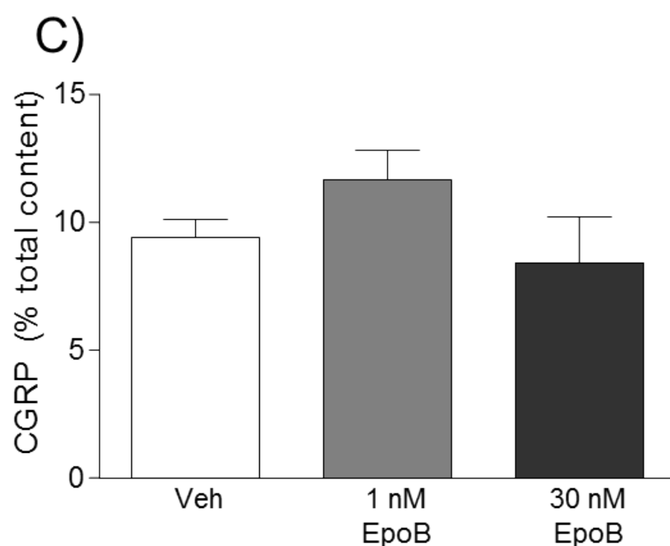
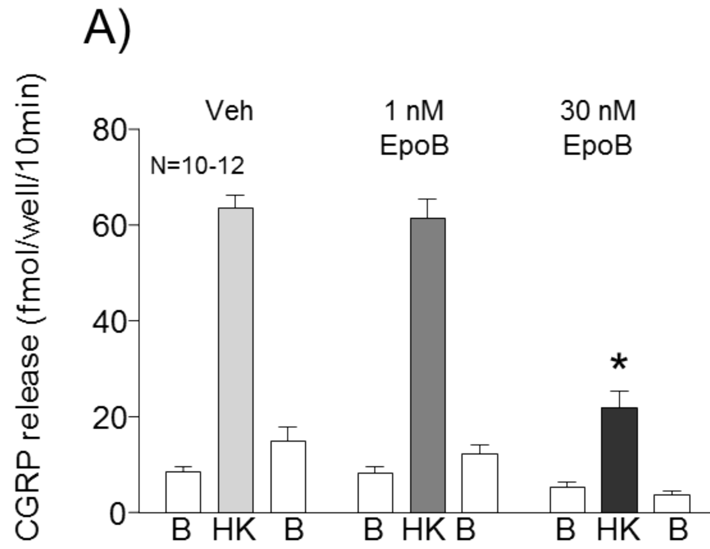
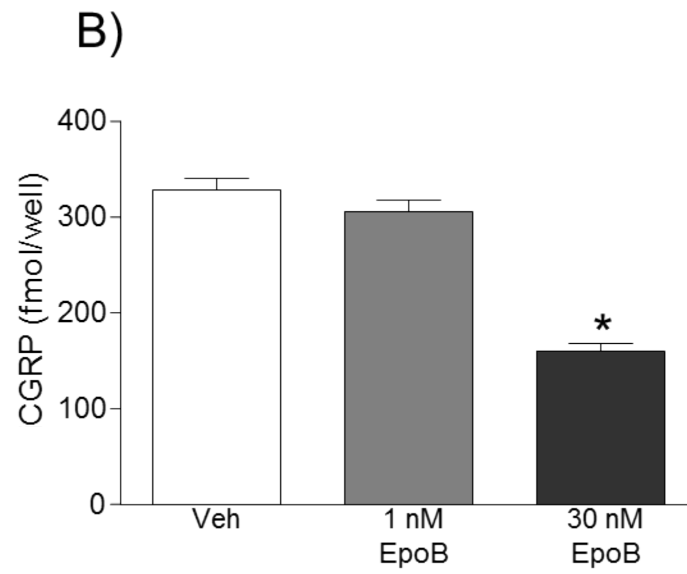


Figure 17. EpoB alters capsaicin-evoked release of CGRP from sensory neurons grown in the absence of NGF. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle, 1 nM, or 30 nM EpoB for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 30 nM capsaicin (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in capsaicin-evoked release compared to release from the vehicle-treated cultures ($p<0.05$, N=10-11) using a two-way ANOVA with Tukey's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle-treated cultures (Veh) or EpoBl-treated cultures (EpoB) as indicated. An (*) indicates a significant difference in total CGRP content compared to the vehicle-treated cultures ($p<0.05$, N=10-11) using a one-way ANOVA with Dunnett's post=hoc test. C) Each column represents the normalized mean ± SEM of the capsaicin-evoked CGRP divided by the total CGRP content for each group (N=10-11).

Potassium-evoked CGRP release was also measured from neurons grown in 0 added NGF and treated with 1 nM or 30 nM EpoB for 5 days. Treatment with 1 nM EpoB did not alter potassium-evoked CGRP release from the neurons and was 63 ± 3 and 61 ± 4 fmol/well/10 min from the vehicle and 1 nM EpoB groups, respectively (Figure 18A). Treatment with 30 nM EpoB decreased potassium-evoked release to 22 ± 3 fmol/well/10 min (Figure 18A). Total content was altered similarly to the capsaicin groups; there was no change in content between the vehicle and 1 nM EpoB groups, and 30 nM EpoB decreased total content of the neurons. Total content was 328 ± 11, 305 ± 13, and 160 ± 8 fmol/well, respectively (Figure 18B). Stimulated CGRP release was normalized to total content for each group, and normalized release was not different between the vehicle and 1 nM EpoB groups and was 19 ± 1 and 20 ± 1% of total content, respectively; however, 30 nM EpoB decreased normalized CGRP release to 13 ± 2% of total content (Figure 18C).


A)
CGRP release (fmol/well/10min)
80
60
40
20
0
Veh
1 nM
EpoB
30 nM
EpoB
N=10-12
*
B
HK
B
B
HK
B
B
HK
B


B)
CGRP (fmol/well)
400
300
200
100
0
*
Veh
1 nM
EpoB
30 nM
EpoB

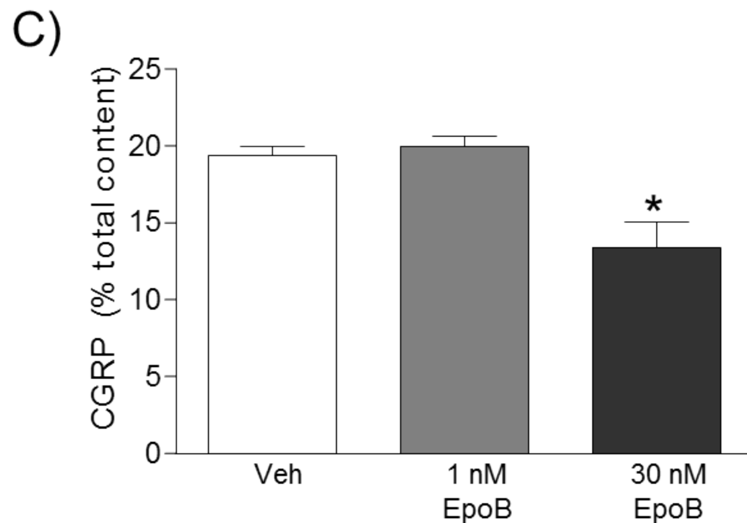
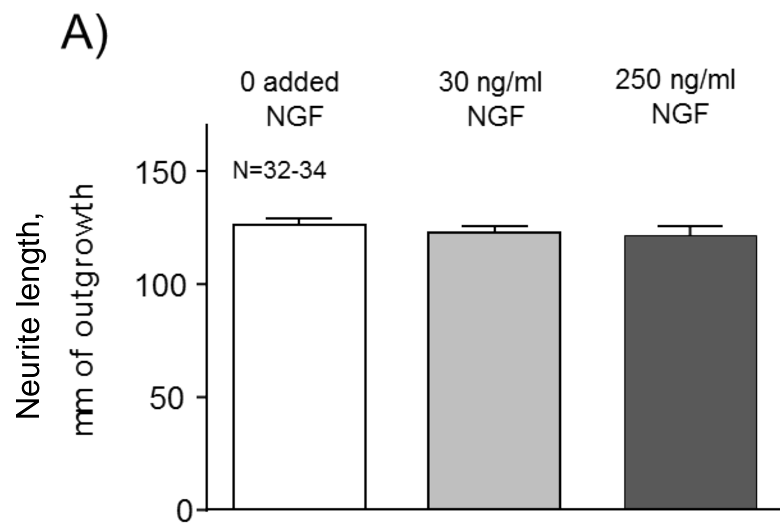


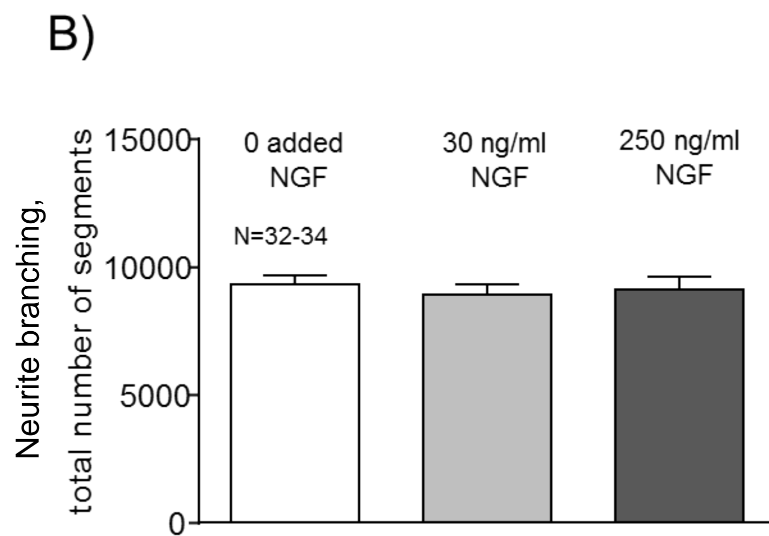
Figure 18. EpoB attenuates potassium-evoked release of CGRP from sensory neurons grown in the absence of NGF. A) Each column represents the mean ± SEM of CGRP released in fmol/well/10 min from wells treated with vehicle, 1 nM, or 30 nM EpoB for 5 days. The first open column of each group represents basal release (B), the shaded column represents release in the presence of 50 mM potassium (C), and the second open column of each group represents the recovery of basal release following stimulation (B). An (*) indicates a significant difference in potassium-evoked release compared to release from the vehicle-treated cultures ($p<0.05$, N=10-12) using a two-way ANOVA with Tukey's post-hoc test. B) Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle-treated cultures (Veh) or EpoB-treated cultures (EpoB) as indicated. An (*) indicates a significant difference in total CGRP content compared to the vehicle-treated cultures ($p<0.05$, N=10-12) using a one-way ANOVA with Dunnett's post-hoc test. C) Each column represents the normalized mean ± SEM of the potassium-evoked CGRP divided by the total CGRP content for each group. An (*) indicates a significant difference in normalized CGRP compared to vehicle-treated cultures ($p<0.05$, N=10-12) using a one-way ANOVA with Dunnett's post-hoc test.

12) NGF and Paclitaxel Alter Neurite Length and Branching of Sensory Neurons in Culture

Since NGF alters the effects of paclitaxel on CGRP release and NGF has been suggested to be protective against paclitaxel-induced inhibition of neurite growth, I investigated: 1) if NGF alone alters neurite length and branching within our cultures; 2) if paclitaxel alters neuronal length and branching in established neuronal cultures; and 3) if NGF protects against paclitaxel-induced changes in neurite length and branching. Sensory neurons were grown in the presence of 0 added NGF, 30 ng/ml NGF, or 250 ng/ml NGF for 7 days or 12 days. For the 12-day treatment groups, neurons were maintained in culture for 7 days and then treated with 300 nM paclitaxel for an additional 5 days. After 7 days in culture, there was no difference in the total length of neurites between the different NGF groups; total lengths in the cultures were 126.0 ± 3.1, 122.3 ± 3.0, and 121.4 ± 4.1 mm in the 0 added, 30 ng/ml, and 250 ng/ml NGF cultures, respectively (Figure 19A). Total branching also did not change in the groups. Total branching segments in the 0 added, 30 ng/ml, and 250 ng/ml NGF cultures were 9289 ± 388, 8916 ± 400, and 9102 ± 533 total segments, respectively (Figure 19B). Representative images for each day 7 treatment group can be seen in Figure 19C.



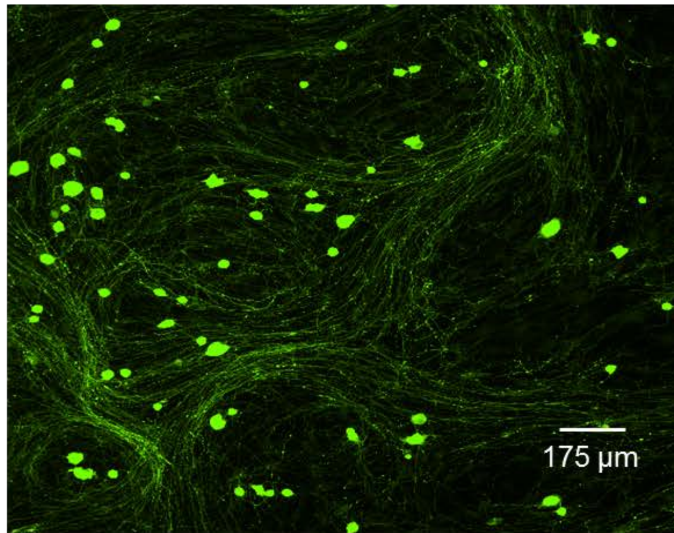
A)
0 added NGF
30 ng/ml NGF
250 ng/ml NGF
N=32-34
Neurite length, mm of outgrowth
150
100
50
0



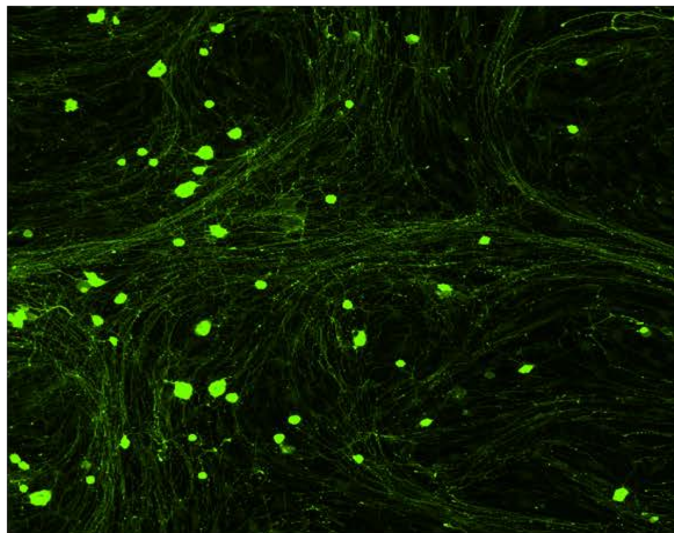
B)
0 added NGF
30 ng/ml NGF
250 ng/ml NGF
N=32-34
Neurite branching, total number of segments
15000
10000
5000
0

C)

0 NGF



30 ng/ml NGF



250 ng/ml NGF

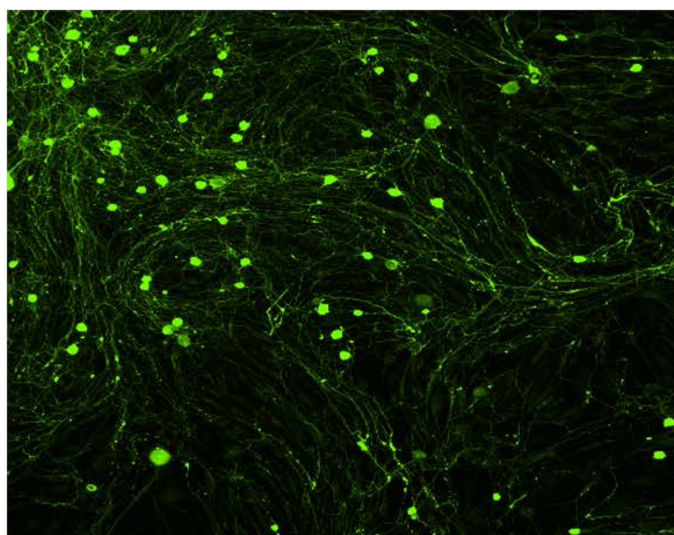
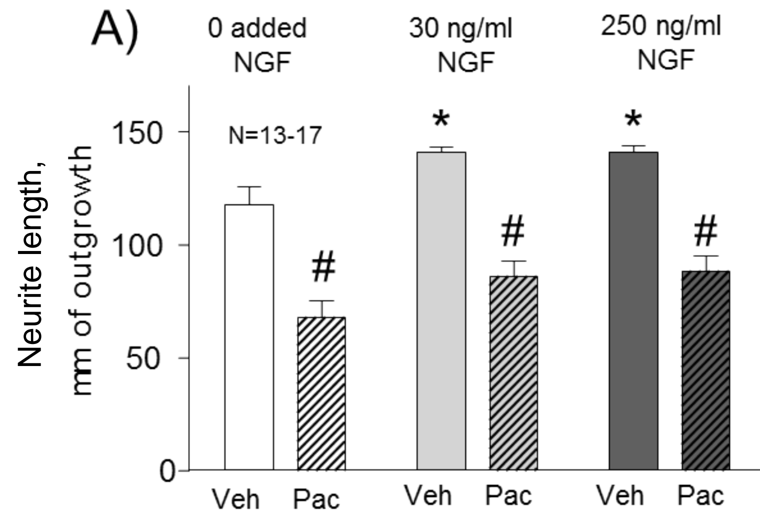
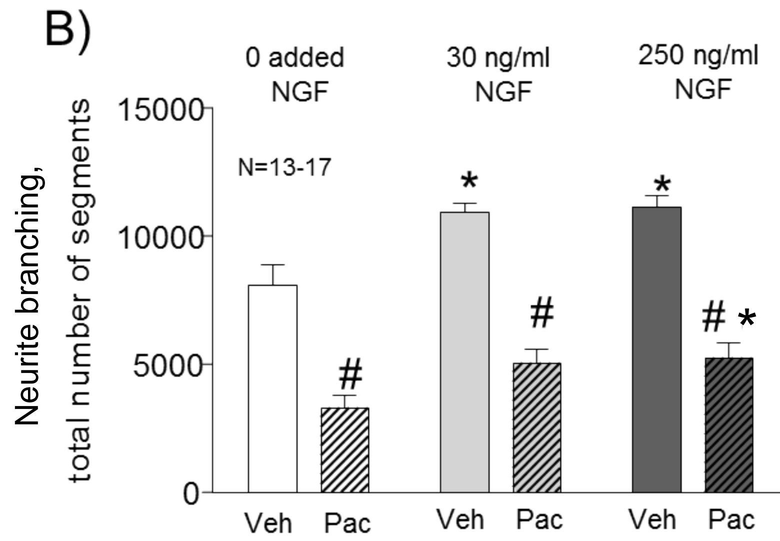


Figure 19. NGF does not alter neurite length or branching of sensory neurons after 7 days in culture. Neurite length and branching were determined in sensory neurons maintained in culture media with 0 added NGF, 30 ng/ml NGF, or 250 ng/ml NGF and fixed on day 7. A) There were no differences in neurite length between the NGF groups. Each column represents the mean ± SEM of the total neurite outgrowth per well (N=32-34). B) There were no differences in neurite branching between the NGF groups. Each column represents the mean ± SEM of the total number of branch points per well (N=32-34). C) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in increasing concentrations of NGF and fixed on day 7.

After 12 days in culture, the 5-day vehicle treated neurons maintained in 30 ng/ml NGF and 250 ng/ml NGF had greater total length and branching compared to the vehicle treated neurons in 0 added NGF. Total lengths were 140.6 ± 2.5, 140.7 ± 3.1, and 117.8 ± 7.9 mm in the 30 ng/ml, 250 ng/ml, and 0 added NGF groups, respectively (Figure 20A). Total branching segments were 10944 ± 356, 11154 ± 452, and 8118 ± 795 total segments in the 30 ng/ml, 250 ng/ml, and 0 added NGF groups, respectively (Figure 20B). Five-day treatment with 300 nM paclitaxel decreased total length and branching in all 3 NGF groups compared to the corresponding vehicle-treated neurons, and lengths were reduced to 67.9 ± 7.0, 85.9 ± 6.7, and 87.9 ± 7.0 mm in the 0 added, 30 ng/ml, and 250 ng/ml NGF groups, respectively (Figure 20A). Total branching segments also decreased to 3301 ± 487, 5055 ± 567, and 5245 ± 626 total segments in the 0 added, 30 ng/ml, and 250 ng/ml NGF groups, respectively (Figure 20B). There was no difference between the NGF groups after paclitaxel treatment except between the total branching of the 0 added NGF and 250 ng/ml NGF cultures (Figure 20B). Representative images for each day 12 treatment group can be seen in Figure 20C.


A)
0 added
NGF
30 ng/ml
NGF
250 ng/ml
NGF
N=13-17
Neurite length,
mm of outgrowth
150
100
50
0
*
*
#
#
#
Veh
Pac
Veh
Pac
Veh
Pac


B)
0 added
NGF
30 ng/ml
NGF
250 ng/ml
NGF
N=13-17
Neurite branching,
total number of segments
15000
10000
5000
0
*
*
#
#
*
Veh
Pac
Veh
Pac
Veh
Pac

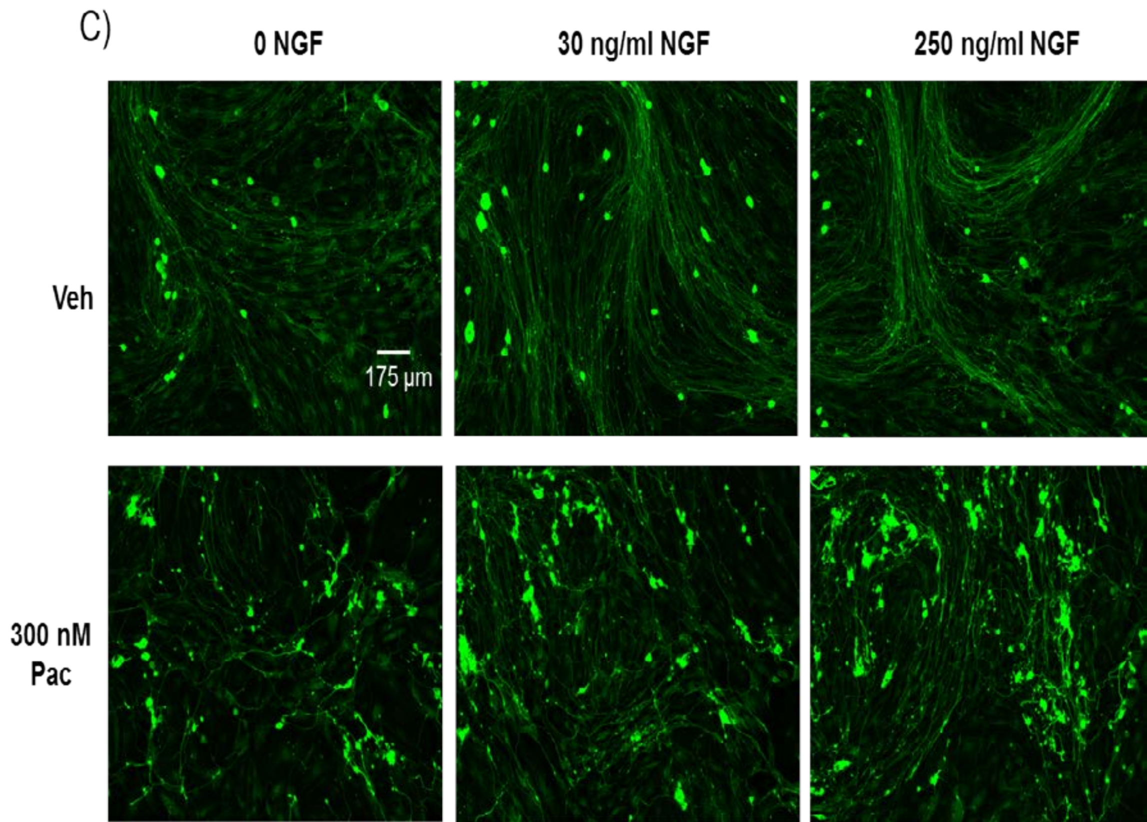
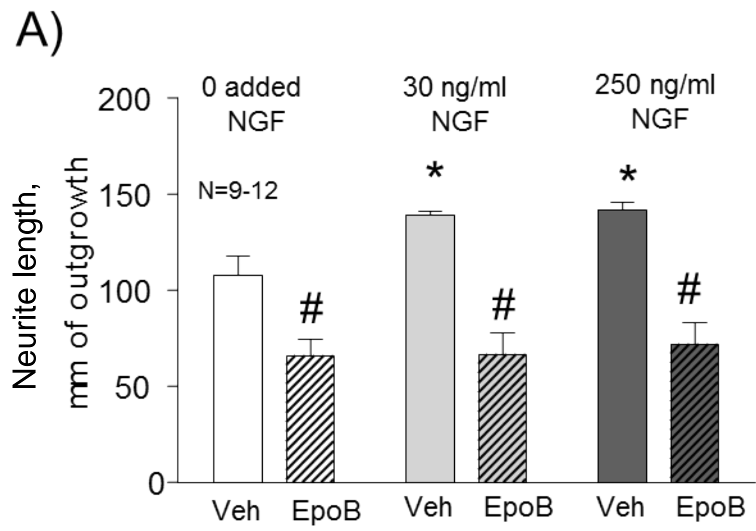


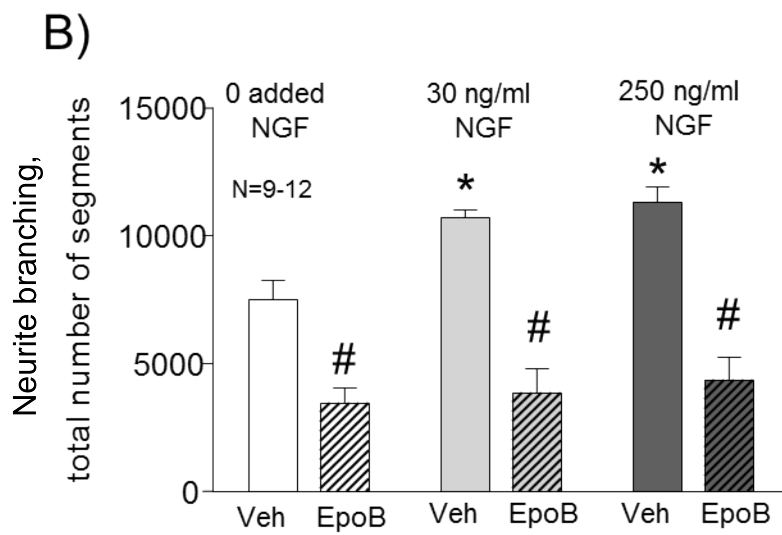
Figure 20. NGF and paclitaxel alter neurite length and branching of sensory neurons after 12 days in culture. Sensory neurons were grown in 0 added NGF, 30 ng/ml NGF, or 250 ng/ml NGF and treated with vehicle (open bars) or 300 nM of paclitaxel (hashed bars) on day 7 and fixed on day 12. A) Each column represents the mean ± SEM of total neurite length. An (*) indicates a significant difference in neurite length from the 0 added NGF vehicle treatment ($p<0.05$, N= 13-17) using a two-way ANOVA and Tukey's post-hoc test. B) Each column represents the mean ± SEM of total segments. An (*) indicates a significant difference in neurite branching from the 12 day, 0 added NGF vehicle treatment, a (#) indicates a significant difference in neurite branching from the corresponding 12 day NGF vehicle treatment, and a (&) indicates a significant difference from the 0 NGF 300 nM paclitaxel treatment ($p<0.05$, N= 13-17) using a two-way ANOVA and Tukey's post-hoc test . C) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in increasing concentrations of NGF and fixed on day 12.

13) Epothilone B Alters Neurite Length and Branching of Sensory Neurons in Culture

Paclitaxel alters neurite length and branching, but the underlying mechanisms for paclitaxel-induced alterations of neurite morphology are unknown. In order to determine if paclitaxel's effect on neurite length and branching is microtubule dependent, neurons treated with a different microtubule stabilizing drug, EpoB, were measured for neurite length and branching changes after 5 days. These experiments were performed in 0 added NGF, 30 ng/ml NGF, and 250 ng/ml NGF in order to determine if EpoB-induced alterations in neurite length and branching can be reversed by NGF. Similarly to what was observed in the paclitaxel experiments, neurite length and branching increased in the 30 ng/ml and 250 ng/ml NGF treatment groups compared to the 0 added NGF group. The neurite lengths were 139.0 ± 2.6, 141.7 ± 4.1, and 107.6 ± 10.3 mm in the 30 ng/ml, 250 ng/ml, and 0 added NGF groups, respectively (Figure 21A). Total branching segments were 10697 ± 321, 11329 ± 555, and 7525 ± 721 total segments in the 30 ng/ml, 250 ng/ml, and 0 added NGF groups, respectively (Figure 21B). Treatment with 30 nM EpoB for 5 days decreased neurite length and branching compared to the corresponding vehicle control group. Neurite lengths decreased to 66.3 ± 8.4, 66.4 ± 11.6, and 71.8 ± 11.4 mm and branching segments decreased to 3467 ± 619, 3858 ± 930, and 4331 ± 936 total segments in the 30 nM EpoB groups for the 0 added, 30 ng/ml, and 250 ng/ml NGF conditions, respectively (Figure 21A and B). NGF did not reverse the

EpoB-induced decreases in neurite length and branching. Representative images for each day 12 treatment group can be seen in Figure 21C.


A)
0 added NGF
30 ng/ml NGF
250 ng/ml NGF
N=9-12
Neurite length, mm of outgrowth
200
150
100
50
0
*
*
#
#
#
Veh
EpoB
Veh
EpoB
Veh
EpoB


B)
0 added NGF
30 ng/ml NGF
250 ng/ml NGF
N=9-12
Neurite branching, total number of segments
15000
10000
5000
0
*
*
#
#
#
Veh
EpoB
Veh
EpoB
Veh
EpoB

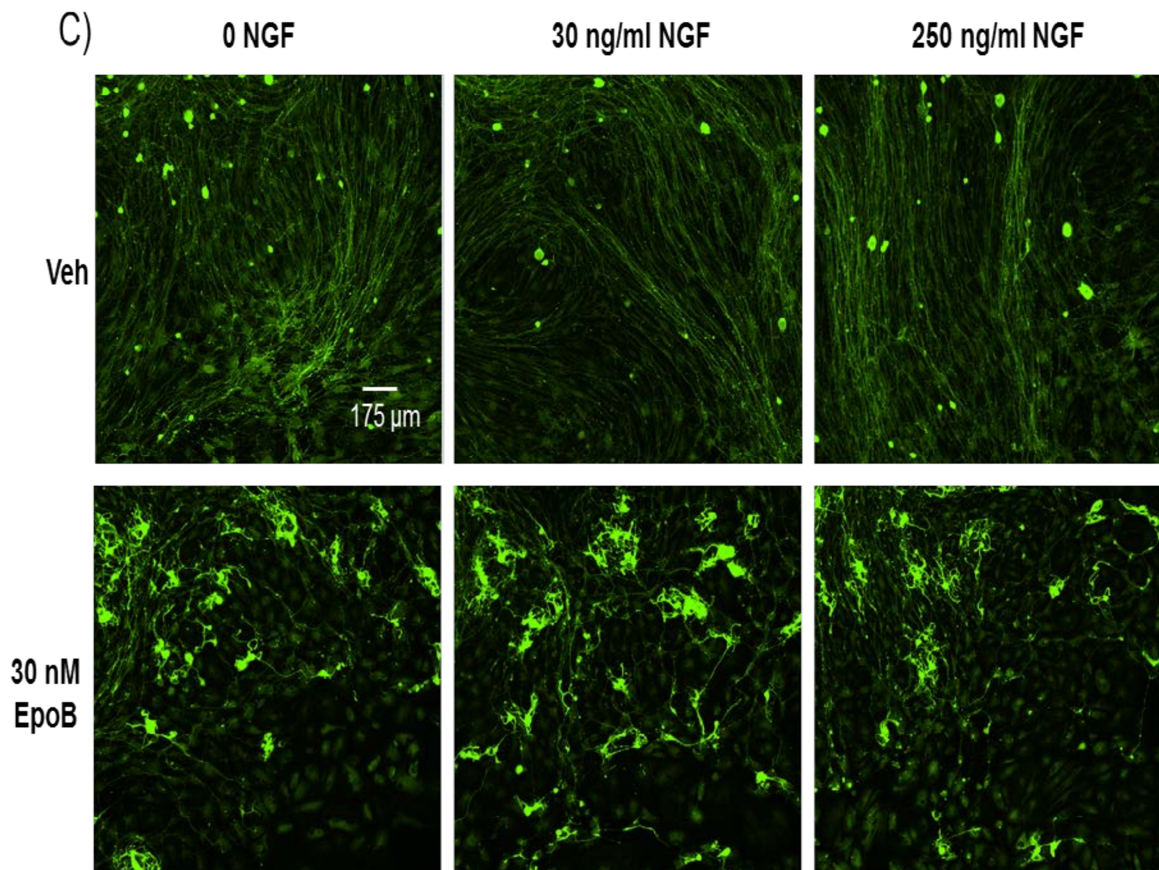


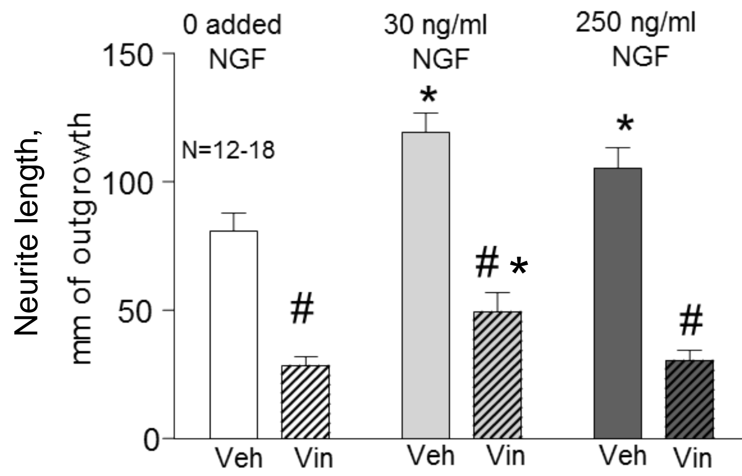
Figure 21. NGF and EpoB alter neurite length and branching of sensory neurons after 12 days in culture. Sensory neurons were grown in 0 added NGF, 30 ng/ml NGF, or 250 ng/ml NGF and treated with vehicle (open bars) or 30 nM of EpoB (hashed bars) on day 7 and fixed on day 12. A) Each column represents the mean ± SEM of total neurite length. An (*) indicates a significant difference in neurite length from the 12 day, 0 added NGF vehicle treatment, and a (#) indicates a significant difference in neurite length from the corresponding 12 day NGF vehicle treatment ($p<0.05$, N= 9-12) using a two-way ANOVA and Tukey's post-hoc test. B) Each column represents the mean ± SEM of total segments. An (*) indicates a significant difference in neurite branching from the 12 day, 0 added NGF vehicle treatment, and a (#) indicates a significant difference in neurite branching from the corresponding 12 day NGF vehicle treatment ($p<0.05$, N= 9-12) using a two-way ANOVA and Tukey's post-hoc test. C) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in increasing concentrations of NGF and fixed on day 12.

14) Vincristine Alters Neurite Length and Branching of Sensory Neurons in Culture

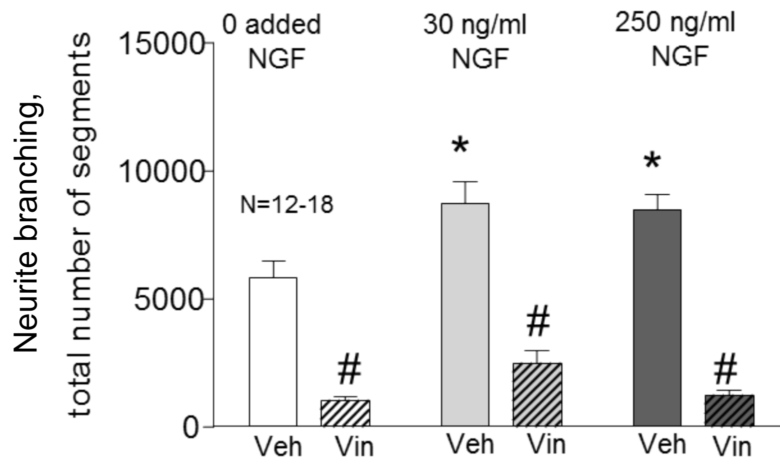
Both microtubule stabilizing agents, paclitaxel and EpoB, decreased neurite length and branching. It is unknown whether this phenomenon is due to the microtubule stabilization mechanism or is a result of a change in microtubule dynamics. In order to determine if inhibition of microtubule dynamics is underlying the decrease in neurite length and branching caused by paclitaxel and EpoB, I treated neurons for 5 days with 30 nM vincristine, a microtubule destabilization agent, and measured neurite length and branching in 0 added NGF, 30 ng/ml, and 250 ng/ml NGF. Similarly to what was observed above, growth in 30 ng/ml and 250 ng/ml NGF increased neurite length and branching compared to the 0 added NGF groups. Neurite lengths were 119.5 ± 7.2, 105.6 ± 7.9, and 81.1 ± 7.1 mm, and branching segments were 8728 ± 813, 8455 ± 609, and 5810 ± 6444 total segments in the 30 ng/ml, 250 ng/ml, and 0 added NGF vehicle treated groups, respectively (Figure 22A and B). Treatment for 5 days with 30 nM vincristine decreased neurite lengths and branching segments in all NGF groups to 28.6 ± 3.6, 49.6 ± 7.2, and 30.2 ± 39.0 mm and 1021 ± 167, 2488 ± 458, and 1241 ± 204 total segments in the 0 added, 30 ng/ml, and 250 ng/ml NGF groups, respectively (Figure 22A and B). NGF did not protect against a decrease in neurite length or branching except for the neurite length of the 30 ng/ml NGF group; neurite length was higher in the 30 ng/ml NGF group compared to the 0 added NGF group when treated with vincristine (Figure 22A).

Representative images for each day 12 treatment group can be seen in Figure 22C.

A)



B)



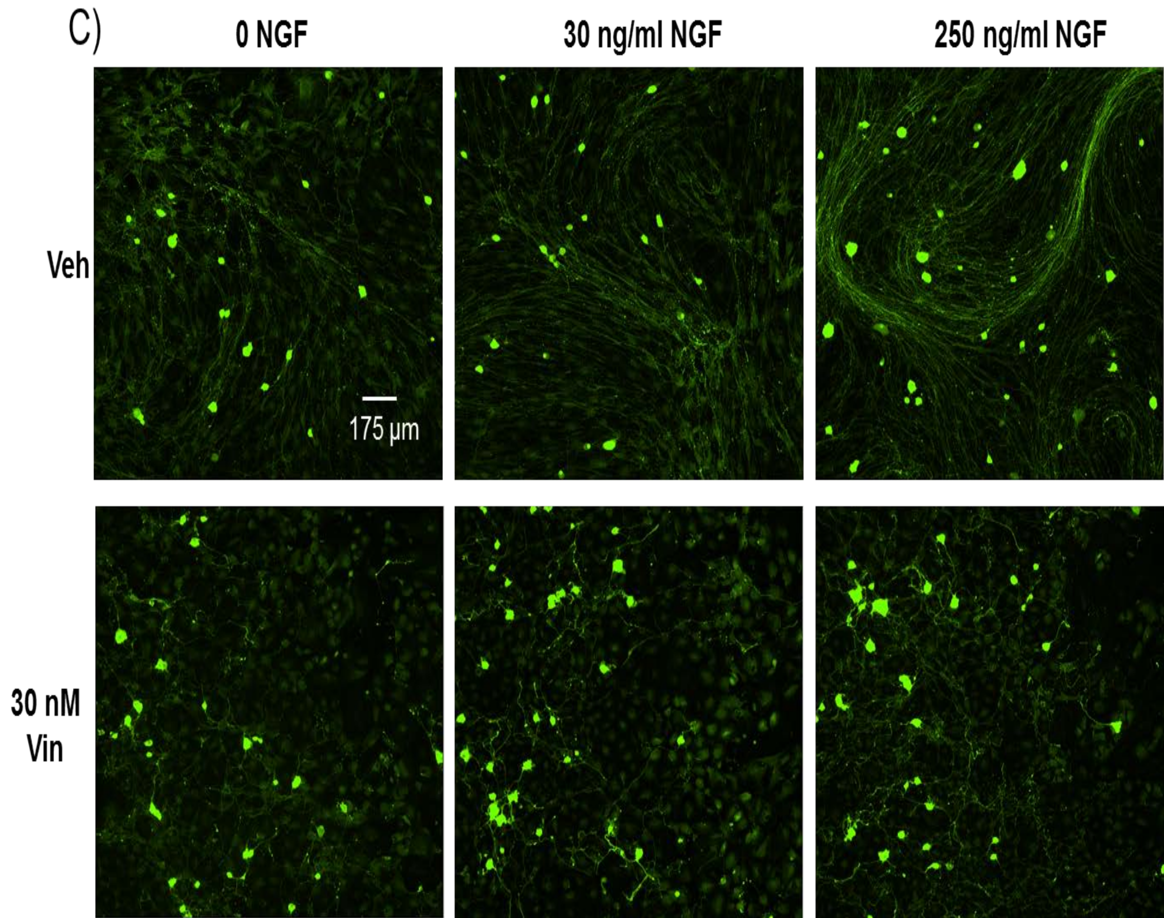


Figure 22. NGF and vincristine alter neurite length and branching of sensory neurons after 12 days in culture. Sensory neurons were grown in 0 added NGF, 30 ng/ml NGF, or 250 ng/ml NGF and treated with vehicle (open bars) or 30 nM of vincristine (hashed bars) on day 7 and fixed on day 12. A) Each column represents the mean ± SEM of total neurite length. An (*) indicates a significant difference in neurite length from the 0 added NGF vehicle treatment ($p<0.05$, N= 12-18) using a two-way ANOVA and Tukey's post-hoc test. B) Each column represents the mean ± SEM of total segments. An (*) indicates a significant difference in neurite branching from the 12 day, 0 added NGF vehicle treatment, and a (#) indicates a significant difference in neurite branching from the corresponding 12 day NGF vehicle treatment ($p<0.05$, N= 12-18) using a two-way ANOVA and Tukey's post-hoc test. C) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in increasing concentrations of NGF and fixed on day 12.

DISCUSSION

Currently there are no recommendations for preventing MTA-induced peripheral neuropathy and only limited recommendations for treating MTA-induced peripheral neuropathy in the clinic (Hershman, Lacchetti et al. 2014). One of the reasons for this paucity of treatment options for MTA-induced peripheral neuropathy is that the mechanisms underlying the development of the neuropathy have not been carefully and systematically investigated in neuronal systems. The goal for this thesis was to develop and characterize *in vitro* sensory neuron models of MTA-induced neurotoxicity in order to answer two basic questions: 1) do MTAs alter sensory neuronal function and/or morphology, and 2) what are the cellular mechanisms mediating these MTA-induced changes?

The work presented in this thesis demonstrates that MTAs alter the stimulated release of CGRP from sensory neurons as well as the length and branching of the neurite processes. This work has also begun to elucidate the underlying mechanisms involved in these MTA-induced alterations. We observed differential effects on CGRP release dependent on MTA agent, CGRP evoking stimulus, duration of exposure to the MTA agent, and presence of added NGF, and we now have the infrastructure in place to start to construct a preliminary understanding of the cellular machinery and signaling cascades changed in sensory neurons by MTAs. We have also attempted to prevent, although unsuccessfully, paclitaxel-induced changes in CGRP release with agents targeting specific, previously hypothesized mechanisms; however, the

interpretation of these studies is limited by a lack of understanding of the physiological effects of the agents on sensory neurons. For example, agents that prevent the development of paclitaxel-induced hypersensitivity in animals, like ALCAR, do not prevent paclitaxel-induced changes of neuronal activity within our culture model; however our interpretation of these experiments is limited as we do not know if the concentrations of ALCAR within our experiments were sufficient to increase mitochondrial energy production. Finally, we have established an *in vitro* method to investigate sensory neurite length and branching, and we have shown that the MTAs decreased neurite length and branching in established cultures, independent of the surrounding NGF environment. My thesis work has utilized two *in vitro,* sensory neuronal assays that can be implemented in future mechanistic studies. This comprehension of MTA-induced alterations of sensory neurons will undoubtedly be used as a tool to help elucidate the mechanisms underlying MTA-induced peripheral neuropathy.

The nociceptive behavioral changes observed in MTA-induced peripheral neuropathy animal models mimic the side effects in cancer patients treated with MTAs, and therefore they have been useful in the initial work involved in understanding the mechanisms of MTA-induced peripheral neuropathy. For example, initial findings that ALCAR, a mitochondrial protective agent, prevented the development of paclitaxel-induced mechanical hyperalgesia and allodynia within animal models (Flatters, Xiao et al. 2006, Jin, Flatters et al. 2008) have caused investigators to question the involvement of mitochondrial dysfunction in

peripheral neuropathy. Also, the MTA-induced decrease in IENFs of rat hindpaw skin suggested that the MTA-induced hypersensitivity could be secondary to a loss of skin nerve innervation, and this hypothesis has since guided investigations in patients where a similar paclitaxel-induced loss of IENF has been observed (Boyette-Davis, Cata et al. 2013). While the animal models have been helpful in developing hypotheses about the physiological alterations that may underlie MTA-induced peripheral neuropathy, the animal model findings have not translated clinically. Both gabapentin and ALCAR therapy decreased nociceptive hypersensitization within the animal models; however, gabapentin had no effect on reducing MTA-induced peripheral neuropathy within patients (Rao, Michalak et al. 2007), and ALCAR administration increased the incidence of paclitaxel-induced peripheral neuropathy in cancer patients (Hershman, Unger et al. 2013). These findings suggest that the etiologies underlying MTA-induced peripheral neuropathy are complex and likely multifactorial, and the complexity of the condition cannot be fully explored in an animal model. Findings may differ between animal models and human patients due to several reasons including differences in the pharmacokinetics of the drugs in the two species. The *in vitro* models developed in this body of work provide a reductionist approach in which the effects of the MTAs responsible for altering neuronal function and sensitivity can be thoroughly characterized on a cellular level, independent of pharmacokinetic variables like differences in drug metabolism or distribution. In this way, the different animal model-generated hypotheses can be carefully explored with a reductionist and causative methodology, and the underlying

mechanisms of MTA-induced changes, specifically within sensory neurons, can be elucidated.

1) Paclitaxel Alters Stimulus-evoked Release of CGRP from Sensory Neurons

Paclitaxel impairs the function of several subpopulations of sensory nerves including large, medium, and it has been suggested, to a lesser extent, small diameter neurons (Dougherty, Cata et al. 2004). We chose to limit our investigation to the effects of paclitaxel, and other MTAs, on the activity of CGRP-expressing sensory neurons since the MTAs alter nociceptive endpoints in animal models (Aley, Reichling et al. 1996, Polomano, Mannes et al. 2001, Weng, Cordella et al. 2003) and produce pain symptoms in patients (Forsyth, Balmaceda et al. 1997, Carlson and Ocean 2011). Our collaborators have also previously shown that capsaicin-induced bloodflow is altered in rats treated with paclitaxel, suggesting a functional change in the release of CGRP from nociceptive neurons in the periphery (Gracias, Cummins et al. 2011). CGRP is expressed and released by the nociceptive C and A δ fibers (McCarthy and Lawson 1990, Lawson, McCarthy et al. 1996, Lawson, Crepps et al. 2002), and therefore, we can use the basal and stimulated release of CGRP as a direct marker of nociceptive sensory nerve activity within our cultures.

There are several limitations to our model system. Although we are using CGRP release as an endpoint to measure activity of nociceptive small and medium diameter sensory neurons, not all small and medium diameter sensory

neurons express CGRP (Cavanaugh, Chesler et al. 2011). This suggests that there is a subset of small and medium diameter sensory neurons of which we cannot determine changes in activity. Also, peripheral neuropathy is characterized by symptoms likely involving large diameter sensory neurons including loss of proprioception, touch, and vibration (Sarosy, Kohn et al. 1992, Forsyth, Balmaceda et al. 1997). Our assay cannot address changes in activity of the large diameter sensory neurons as they do not express CGRP (Lawson, Perry et al. 1993). In order to investigate changes of activity in all sensory neurons in culture, we could employ a glutamate release assay so not to limit our studies to only CGRP-expressing neurons (Lever, Bradbury et al. 2001, Bardoni, Torsney et al. 2004, Seal, Wang et al. 2009). We could also use calcium imaging or electrophysiology methods in order to observe MTA-induced changes of the entire population of neurons in our system. Another limitation to our model is that maintaining our cultures in media containing NGF enriches them with TRPV1- and trkA-expressing sensory neurons (Khodorova, Richter et al. 2009, Park, Fehrenbacher et al. 2010). Alternatively, we could grow the neurons in other growth factors including GDNF and NT-3/BDNF to enrich for TRPV1-negative small diameter sensory neurons and myelinated sensory neurons, respectively (Khodorova, Richter et al. 2009, Park, Fehrenbacher et al. 2010).

The experiments investigating MTA-induced alterations of CGRP release within this thesis were performed with cultures maintained for 12 days. This treatment paradigm was chosen because we wanted to examine the effects of MTAs on fully regenerated neurons (Gracias 2011), so not to confound our

findings with other variables such as neurite outgrowth; for example, MTAs may affect sensory neurons that are in a process of regeneration differently than neurons that have reached their maximum length. It is suggested that competence for axonal growth is decreased in mature DRG neurons, and there is a switch in signaling pathways and gene regulation between embryonic neurons undergoing the process of elongation versus mature neurons undergoing maintenance and reorganization of terminal arbors (Hall and Sanes 1993, Smith and Skene 1997, Liu and Snider 2001). After nerve axotomy, the profile of gene transcription transitions from an arborizing profile to an elongation profile to allow for axonal growth and regeneration within mature DRG (Smith and Skene 1997). Another consideration is that MTAs are administered to patients who have mature peripheral nerves likely expressing arborizing signaling pathway and gene transcription profiles at the time of treatment, and modeling the effects of MTAs on fully extended neurons in culture more closely mimics the clinical setting.

The majority of release experiments were conducted after 5 days treatment with MTAs in an attempt to model clinically-relevant, long-term administration with the chemotherapeutic agents. The clinical onset of peripheral neuropathy symptoms is typically 3-6 weeks following the first dose of paclitaxel (Forsyth, Balmaceda et al. 1997), and we chose 5 days exposure to paclitaxel to best model long-term exposure of MTAs. There are previous studies in which a direct excitatory effect of paclitaxel on neuropeptide release has been observed, and paclitaxel and vinorelbine induce release of substance P from DRG cultures

in time- and concentration-dependent manners (Miyano, Tang et al. 2009, Materazzi, Fusi et al. 2012). Furthermore, some patients receiving paclitaxel treatment feel immediate or acute pain with paclitaxel infusions (Loprinzi, Maddocks-Christianson et al. 2007, Reeves, Dakhil et al. 2012). We therefore decided to also examine the acute effects of paclitaxel, 10-30 min following paclitaxel exposure, on CGRP release as well as a time course of paclitaxel exposure, including 1, 3, and 5 days following treatment with paclitaxel. With the findings of the time course experiments, we hoped to gain a better understanding of the paclitaxel-induced changes in neurons over time.

Exposure to 10 nM paclitaxel for 5 days increased capsaicin- and AITC-evoked release from the sensory neuron cultures while 300 nM paclitaxel for 5 days decreased capsaicin- and AITC-evoked release (see Figure 23). In contrast, both concentrations of paclitaxel augmented potassium-evoked release. This suggests that paclitaxel causes an overall sensitization or gain in function in the neurons that is TRP channel-independent, and it suggests that a high concentration of paclitaxel alters TRP channel expression and/or function. Had potassium-evoked CGRP release not been augmented by 300 nM paclitaxel, it might suggest that the decrease in capsaicin- and AITC-evoked release was due to a decrease in neuronal viability or dysfunctional release machinery. A decrease in neuronal viability is unlikely as 300 nM paclitaxel did not change total CGRP content in the neurons and because this high concentration of paclitaxel augmented potassium-evoked release. This was confirmed as there was no difference in neuronal viability after exposure to paclitaxel, as evidenced by the

	+ NGF			- NGF	
Concentration of MTA:	Cap (30 nM)	AITC (30 µM)	HK (50 mM)	Cap (30 nM)	HK (50 mM)
Pac: 10 nM	↑	↑	↑	↓	↓
300 nM	↓	↓	↑	↓	↓
EpoB: 1 nM	↑		n.c.	↑	n.c.
30 nM	↓		↑	↓	↓
Vin: 1 nM			↑		
30 nM			↓		

Figure 23. Changes in evoked CGRP release after treatment with MTAs. N.c.= no change in release compared to control. An up arrow indicates an augmentation of CGRP release, and a down arrow indicates an attenuation of CGRP release compared to control.

	+ NGF	- NGF
	Total Content	Total Content
Concentration of MTA:		
Pac: 10 nM	**n.c.**	↓
300 nM	**n.c.**	↓
EpoB: 1 nM	**n.c.**	**n.c.**
30 nM	**n.c.**	↓
Vin: 1 nM	**n.c.**	
30 nM	↓	

Figure 24. Changes in total CGRP content after treatment with MTAs. N.c.= no change in total CGRP content compared to control. A down arrow indicates a decrease in total CGRP content compared to control.

PI assay and total neuron cell counts (Figure 5). Augmentation of potassium-evoked release after 300 nM paclitaxel also argues against a possible microtubule-induced defect in the synthesis and/or release of CGRP as a potential mechanism by which paclitaxel induced a decrease in capsaicin- and AITC-evoked release.

Since a decrease in neuronal viability and an alteration in CGRP release machinery are ruled out as possible explanations for the paclitaxel-induced decrease in capsaicin- and AITC-evoked release, paclitaxel likely alters TRP channel expression and/or function. Previous unpublished work found that paclitaxel decreased TRPV1 protein and mRNA expression in neuronal cultures (Gracias 2011). In preliminary work not described in this manuscript, we tried to reproduce these findings, but we observed that paclitaxel exposure caused a slight increase or no change in TRPV1 protein expression, although our findings were not predictable or reproducible. We first presumed that the protein isolation techniques used in Gracias' work and ours must be different, resulting in differential isolation of membrane-bound versus cytoplasmic proteins. The laboratory therefore attempted to carefully isolate and perform Western blotting for TRPV1 with membrane and cytoplasmic fractions, and we attempted biotinylation experiments with TRPV1 with variable results. Our findings were not reproducible, and further investigation involving *in vitro* paclitaxel-induced changes in TRPV1 protein expression are warranted. These results suggest that there is an unknown variable within our cultures that affects TRPV1 immunoreactivity and/or expression. Interestingly, Hara et al. found that

systemic administration of paclitaxel in the rat increased TRPV1 mRNA and protein expression in the DRG (Hara, Chiba et al. 2013), suggesting that the observed reduction in capsaicin-stimulated release is not due to a decrease in TRPV1 protein expression. Currently there is not an effective antibody available to ask whether paclitaxel exposure changes TRPA1 protein expression within sensory neurons. We contemplated using mRNA levels to determine whether the expression of TRPA1 and/or TRPV1 was altered by paclitaxel, but mRNA expression does not always strongly correlate with or predict protein expression (Chen, Gharib et al. 2002, Greenbaum, Colangelo et al. 2003, Guo, Xiao et al. 2008), and we were interested in paclitaxel-induced changes of the channel proteins, which confer function.

It is likely that the decrease in capsaicin- and AITC-evoked release after exposure to high concentrations of paclitaxel is caused by a change in activity of TRP channels. The mechanisms underlying this change in activity are unknown, but paclitaxel may be causing a desensitization of the TRP channels. TRPV1 is desensitized by a calcium-dependent mechanism involving dephosphorylation of the channel by calcineurin (Mohapatra and Nau 2005). TRPV1 can also be internalized upon desensitization and degraded by lysosomes in order to mediate long-term TRPV1 desensitization (Sanz-Salvador, Andres-Borderia et al. 2012), which would support unpublished findings from Gracias' work (Gracias 2011). TRPA1 is also desensitized by calcium-dependent mechanisms; TRPA1 can be desensitized following calcium-dependent depletion of phosphatidylinositol 4,5-bisphosphate (PIP_2), which has been shown to regulate the activity of several

TRP channels (Clapham 2003, Akopian, Ruparel et al. 2007). Calcium-dependent desensitization mechanisms may be candidates for a decrease in TRPV1 and TRPA1 function after exposure to paclitaxel; acute application of paclitaxel, on the scale of seconds to 15 minutes, has been shown to increase intracellular calcium levels within neuroblastoma cells and DRG (Boehmerle, Splittgerber et al. 2006, Miyano, Tang et al. 2009). The effects of long-term paclitaxel exposure on intracellular calcium levels within DRG are unknown, and these questions are currently being investigated within our laboratory. Intracellular calcium levels would likely be altered if paclitaxel modifies mitochondrial function (Flatters and Bennett 2006, Zheng, Xiao et al. 2011, Xiao, Zheng et al. 2012); however, it is still unclear whether paclitaxel-induced changes in mitochondria precede or follow neuronal sensitivity. Experiments could be performed in the future to investigate if the reduction in TRP channel function is caused by calcium-dependent desensitization. Since calcineurin has been found to mediate calcium-dependent TRPV1 desensitization (Docherty, Yeats et al. 1996, Mohapatra and Nau 2005), calcineurin could be inhibited with cyclosporin A to see if paclitaxel-induced decreases in TRP activity are reversed. Alternatively, agents that are known to phosphorylate and reverse calcium-dependent TRPV1 desensitization, including forskolin, could be used (Mohapatra and Nau 2005). Another possibility would be to use a PLC inhibitor to investigate the role of calcium-dependent PLC-mediated PIP_2 depletion, as PIP_2 has been shown to regulate the activity of several TRP channels (Akopian, Ruparel et al. 2007, Rohacs 2009). With these proposed experiments, it may be difficult to

differentiate between changes in release caused by sensitization of the TRP channels versus inhibition of desensitization of the channels. Investigating the effects of paclitaxel on TRP channel current in a heterologous expression system, where point mutations can systematically be made to the calcineurin and PKA consensus sites, may be necessary to fully understand this possible mechanism. Our laboratory has also performed preliminary calcium imaging experiments that suggest that exposure to paclitaxel (1 μM) for 24 hours may increase basal intracellular calcium levels within sensory neurons (Darby, personal communication, March 2014), suggesting that changes in intracellular calcium levels occur in sensory neurons following exposure to paclitaxel. The exact mechanisms mediating the reduced function of the TRPV1 and TRPA1 channels after paclitaxel exposure are unknown, but future studies should be directed towards investigating the possibility of calcium-dependent desensitization mechanisms in mediating the reduced capsaicin- and AITC-evoked CGRP release from the neurons in culture.

One possibility for paclitaxel and EpoB alterations of TRPV1 activity could be that tubulin interactions with TRPV1 may be important and/or necessary for proper TRPV1 activity. It has been shown that several membrane proteins, including membrane channels, interact with microtubule complexes and with membrane tubulin (Goswami 2012). It has also been shown that TRPV1 specifically binds tubulin dimers at its C-terminal and N-terminal cytoplasmic tail regions (Goswami, Dreger et al. 2004, Lainez, Valente et al. 2010), and the tubulin binding sequence of TRPV1 is conserved among several other TRP

channels (Goswami, Kuhn et al. 2010, Sardar, Kumar et al. 2012). The exact role for tubulin binding to TRPV1 is still under investigation, but it has been suggested that TRPV1 insertion at the membrane is aided by the microtubule cytoskeleton (Storti, Bizzarri et al. 2012); however, the exact form of tubulin, whether it be microtubule filaments, free tubulin, or tubulin dimers, that interacts with TRPV1 is also unclear at this point. It has also been suggested that the TRPV1 and tubulin interaction regulates the ability of the TRPV1 channel to be sensitized (Goswami, Kuhn et al. 2011). The binding of TRPV1 and tubulin blocks a serine residue on the TRPV1 C-terminal tail, which is a site that is phosphorylated by PKCε and involved in TRPV1 sensitization (Mandadi, Tominaga et al. 2006, Goswami, Kuhn et al. 2011). This suggests that the sensitivity of TRPV1 is directly linked to its binding with tubulin, and the effects of tubulin on TRPV1 activity need to be considered when investigating possible TRPV1 desensitization mechanisms by MTAs, as discussed above. Although still a subject of speculation, further studies should be performed to determine if an alteration of tubulin and/or microtubule dynamics with TRPV1 channels could be the mechanism underlying paclitaxel- and EpoB-induced alterations of capsaicin- and perhaps AITC-evoked release. Although there is no evidence that tubulin binds TRPA1, tubulin has been found to physically interact with TRPV4 (Goswami, Kuhn et al. 2010), and the tubulin binding sequence is conserved among several TRP channels including TRPV4, TRPC5, and TRPM8 (Sardar, Kumar et al. 2012). Tubulin may also interact with TRPA1, and a disruption of tubulin and/or microtubule dynamics may mediate alterations in TRPA1 channel

function, which should also be investigated in future work. Since TRP channel activity is tightly regulated and can be affected by calcium signaling and tubulin interactions, it is likely that a combination of altered calcium signaling and aberrant tubulin/TRP interactions causes desensitization of the TRP channels following exposure to MTA agents. The specific mechanisms mediating a possible MTA-induced desensitization of TRP channels needs to be carefully explored in future investigations.

The mechanisms for paclitaxel-induced increases in CGRP release are also unknown, but they are TRP-channel independent. Similarly as described above, possible paclitaxel-mediated alterations in calcium homeostasis, whether it be through mitochondria dysfunction, NCS-1 activation, increases in VDCC subunit expression and current (Kawakami, Chiba et al. 2012), and/or other possible unknown mechanisms, could all contribute to enhanced neuronal sensitivity and activity. Although speculative, the concomitant increase in potassium-evoked release and decrease in capsaicin- and AITC-evoked release from the same culture of CGRP-containing neurons could underlie the simultaneous gain and loss of function experienced in patients receiving MTA therapy. This suggests that patients may experience a perceived gain or loss of function depending on the stimulating agent acting on their peripheral sensory neurons.

Because some patients experience acute pain from paclitaxel therapy whereas others develop peripheral neuropathy over time, we decided to investigate the effects of 10 nM and 300 nM paclitaxel on capsaicin-evoked

release over a time course of 1, 3, and 5 days. We were also interested in investigating the time course of the paclitaxel-induced decrease in neuropeptide release we observed with TRP channel activation. The low concentration of paclitaxel did not increase CGRP release until day 3 of exposure, whereas the high concentration of paclitaxel increased capsaicin-evoked release after 1 day exposure but decreased release after 3 and 5 days. This suggests that the effects of paclitaxel on CGRP release are a function of concentration and time. A low concentration for a long time or a high concentration for a short period augmented release, while a high concentration over a longer period of time, decreased capsaicin-evoked release. This suggests that the paclitaxel-induced decrease of TRPV1 channel activity, and likely that of TRPA1, is dependent on a high magnitude of paclitaxel exposure, which supports the findings that high cumulative doses of paclitaxel *in vivo* produced impairments in pain-like behaviors and thermal hypoalgesia (Cavaletti, Cavalletti et al. 1997, Authier, Gillet et al. 2000).

Previous groups have found that paclitaxel acutely stimulated the release of CGRP from esophagus tissue and the release of substance P from cultured DRG neurons (Miyano, Tang et al. 2009, Materazzi, Fusi et al. 2012). We did not observe an acute paclitaxel-induced increase in CGRP release. One difference between our work and the previous work from other labs is that the concentrations of paclitaxel used in the esophagus experiments and substance P release experiments, 30-50 μM and 10 μM respectively, were higher than the 300 nM used in our acute release experiments. The magnitude of paclitaxel

exposure in our conditions was, therefore, not as great, and perhaps not sufficient for evoking CGRP release acutely. Future experiments could be conducted to test if higher concentrations of paclitaxel cause acute release of CGRP from sensory neurons without other stimuli; however, we did not pursue this as we are not sure that higher concentrations of paclitaxel are physiologically relevant. Although it is difficult to equate the concentrations of paclitaxel used in isolated cells with concentrations achieved in tissues in patients, pharmacokinetic studies show that dosing patients with 125-225 mg/m^2 of paclitaxel results in maximal plasma concentrations between 1 and 10 μM (Rowinsky, Jiroutek et al. 1999, Henningsson, Karlsson et al. 2001), with a reduction in concentration to between 0.01 μM and 1 μM by 20 hours. We therefore did not attempt to test higher concentrations of paclitaxel on acute CGRP release.

Together, this work suggests that paclitaxel alters the stimulus-evoked release of CGRP in a concentration- and time-dependent manner, depending on the magnitude of paclitaxel exposure. Paclitaxel decreases TRP channel activity, and paclitaxel increases neuronal sensitivity, independent of TRP channel function. Our findings support a role for sensory neurons in paclitaxel-induced neurotoxicity. Furthermore, the described *in vitro* model will facilitate future studies to identify the signaling pathways by which paclitaxel alters neuronal sensitivity.

2) Epothilone B and Vincristine Alter Stimulus-evoked Release of CGRP from Sensory Neurons

In order to determine if the paclitaxel-induced alterations of CGRP release are microtubule dependent, we treated neuronal cultures with EpoB, which is a compound with a similar microtubule-stabilizing effect as paclitaxel. EpoB is a competitive antagonist of paclitaxel binding to tubulin, and EpoB binds tubulin within the same paclitaxel binding domain (Bollag, McQueney et al. 1995, Kowalski, Giannakakou et al. 1997, Giannakakou, Gussio et al. 2000). While EpoB has a similar function as paclitaxel in stabilizing microtubules, it does not have any known microtubule-independent actions, and EpoB has been used as a negative control in experiments investigating microtubule-independent effects of paclitaxel (Zhang, Heidrich et al. 2010). The synthetic analog of EpoB, ixabepilone, also creates a profile similar to paclitaxel of peripheral neuropathy in patients, suggesting that microtubule stabilization may underlie paclitaxel- and ixabepilone-induced peripheral neuropathy (Lee, Borzilleri et al. 2008). We hypothesized that if the alterations of CGRP release are somehow downstream of microtubule stabilization, EpoB treatment would have a similar effect on stimulated CGRP release from sensory neurons as paclitaxel. These results would rule out other possible microtubule-independent mechanisms of paclitaxel in altering neuronal peptide release, assuming that paclitaxel and EpoB do not have analogous, unknown microtubule-independent actions within sensory neurons.

Previous work has shown that EpoB binds microtubules with approximately 10 times higher affinity than paclitaxel, and cancer cells are 3-30-fold more sensitive to EpoB than paclitaxel (Kowalski, Giannakakou et al. 1997, Zhang, Heidrich et al. 2010). Since the effective concentrations of EpoB within our model system were unknown, we performed a concentration-response curve of EpoB and capsaicin-evoked CGRP release ranging from 1-300 nM of EpoB. We observed a similar capsaicin-evoked release profile as with paclitaxel, and EpoB was approximately 10-fold more potent at altering capsaicin-evoked CGRP release. Similar to paclitaxel, treating sensory neurons for 5 days with a low concentration of EpoB, 1 or 3 nM, increased capsaicin-evoked CGRP release whereas treatment with a high concentration, 10, 30, 100, or 300 nM, decreased capsaicin-evoked release, suggesting that EpoB alters TRPV1 channel function. Also, 1 nM of EpoB did not change potassium-evoked CGRP release, and 30 nM EpoB augmented potassium-evoked release, suggesting that EpoB sensitizes sensory neurons in a TRP-independent manner. Collectively, these data suggest that the paclitaxel- and EpoB-induced alterations of CGRP release are due to stabilization of microtubules, although the precise mechanisms mediating these changes in neuronal activity are unknown.

EpoB and paclitaxel may be altering TRV1 channel activity by decreasing tubulin interactions with TRPV1 as described above. Another scenario that might explain how paclitaxel and EpoB, which have completely different structures but have the same microtubule-stabilizing mechanism of action, could alter stimulated CGRP release from neurons may involve alterations in mitochondrial

function. As discussed above, several groups propose that mitochondrial dysfunction is an underlying cause of neuronal alterations and peripheral neuropathy; however, the mechanisms by which paclitaxel and EpoB putatively induce mitochondrial dysfunction are currently unknown. An alteration in mitochondrial trafficking and function might produce several changes that could alter neuronal activity such as increased ROS production, altered calcium buffering and subsequent calcium homeostasis, and a change in cellular energy production (Abou-Sleiman, Muqit et al. 2006).

One possibility for microtubule-stabilizing-induced alterations of mitochondria could involve a change in tubulin binding to voltage-dependent anion channels (VDACs) of mitochondria, which are important regulators of the transport of anions and other key metabolic metabolites across the outer mitochondrial membrane (Blachly-Dyson and Forte 2001). It has been shown that tubulin is present within isolated mitochondrial membranes and that the tubulin interacts with the membrane by binding to VDACs (Carre, Andre et al. 2002). Other investigators have shown that tubulin reversibly blocks the conductance of VDACs by binding and blocking the VDAC pore with the C-terminal tail (Rostovtseva, Sheldon et al. 2008). Microtubule stabilizing agents like paclitaxel and EpoB may alter mitochondrial function by disrupting the balance of free tubulin within the cell or by sequestering VDAC-bound tubulin, subsequently un-blocking the VDAC pore and altering the regulation of transport of anions and metabolites across the outer mitochondrial membrane. Opening of VDACs can lead to mitochondrial swelling, mitochondrial calcium efflux, and

release of proteins from the mitochondria including cytochrome c and procaspases (Abou-Sleiman, Muqit et al. 2006, Rostovtseva and Bezrukov 2008), all of which can result in mitochondrial dysfunction and possible alterations in neuronal sensitivity. Future studies can investigate if paclitaxel and EpoB alter tubulin binding to VDACs of mitochondria and mitochondrial function.

Mitochondrial dysfunction has been suggested to underlie other types of peripheral neuropathies. In diabetic patients, hyperglycemia can alter the electron transport chain of the mitochondria, resulting in increased generation of superoxide species (Sandireddy, Yerra et al. 2014). This is thought to cause neurotoxicity via ROS stress, resulting in diabetic peripheral neuropathy (Sandireddy, Yerra et al. 2014). It is suggested that platinum compounds, like cisplatin, may induce peripheral neuropathy by creating mitochondrial DNA damage, which subsequently leads to defective electron transport chain components and an increased production of ROS (Melli, Taiana et al. 2008). Mitochondrial dysfunction is likely a common characteristic underlying the development of peripheral neuropathy resulting from several chemotherapeutics and diabetes; however, the mechanisms underlying the mitochondrial dysfunction are likely diverse. These mechanisms may include alterations in tubulin interactions, mitochondrial DNA damage, and alterations of the electron transport chain (Jaggi and Singh 2012, Sui, Xu et al. 2013). Subsequently, drug- and disease-induced mitochondrial dysfunction can result in aberrant ROS generation, defective calcium buffering, reduced energy production, and

increased apoptotic signaling, which can all cause neurotoxicity (Sui, Xu et al. 2013).

In order to determine if the effects of paclitaxel and EpoB on CGRP release are due to their common mechanism of stabilization of microtubules or caused by a general change in microtubule dynamics, we asked if vincristine, which alters microtubule dynamics by depolymerizing microtubules, alters stimulated CGRP release from sensory neurons. I treated neuronal cultures for 5 days with low and high concentrations of vincristine, 1 and 30 nM, respectively, and then stimulated the neurons with potassium. Unexpectedly, treatment with 30 nM vincristine decreased potassium-evoked CGRP release from the neurons and decreased the total content of CGRP. The decrease in stimulated release was not dependent on total content as the normalized CGRP release was also attenuated compared to the vehicle-treated control neurons. Treatment with 1 nM vincristine augmented potassium-evoked release. This suggests that the gain in function observed with high concentrations of paclitaxel and EpoB may be specific to microtubule stabilization. It can also be argued that a high concentration of a microtubule depolymerization agent, like vincristine, may be more toxic to neurons than a microtubule stabilizing agent since it caused a decrease in total CGRP content. Although the mechanisms mediating these differences are unknown, it suggests that disruption of microtubule dynamics, whether by stabilization or destabilization, alters neuronal sensitivity, and stabilizing agents and destabilizing agents differ in how they alter stimulated CGRP release.

One important question that should be answered in the future is if vincristine altered neuronal viability in our cultures; this might account for the decrease in CGRP content in the cultures after exposure to vincristine, and it may be another difference between high concentration exposure of microtubule stabilizing drugs versus microtubule depolymerizing agents. Our laboratory is working to perform concentration-response curves of vincristine and capsaicin- and potassium-evoked CGRP release in order to determine if vincristine has a similar biphasic effect on capsaicin-evoked release and an augmentation of potassium-evoked release at lower concentrations of vincristine.

3) Acetyl-*L*-carnitine and N-acetyl-*L*-cysteine Do Not Prevent or Attenuate Paclitaxel-induced Alterations of Stimulated CGRP Release

Using our *in vitro* model, I tested the established hypothesis that paclitaxel alters neuronal sensitivity via decreased mitochondrial energy production. This hypothesis was previously postulated since ALCAR prevents and reverses paclitaxel-induced changes in mechanical allodynia and hyperalgesia *in vivo* and prevents paclitaxel-induced mitochondrial swelling within C-fibers in rats, presumably by alleviating mitochondrial damage (Flatters and Bennett 2006, Flatters, Xiao et al. 2006, Jin, Flatters et al. 2008). The exact mechanism of how ALCAR protects mitochondria is not fully known, but it is suggested that ALCAR assists in shuttling fatty acids into the mitochondria that are important for ATP production, thus aiding in cellular energy production (Hagen, Wehr et al. 1998). An ALCAR interaction with the VDAC pore has not been described in the

literature, which is a suggested hypothesis for MTA-induced mitochondrial dysfunction described above. Treatment with ALCAR for 5 days had no effect on total CGRP content or on capsaicin-evoked CGRP release in the absence or presence of paclitaxel, suggesting that ALCAR does not protect the neurons in culture from paclitaxel. One caveat to these experiments is that a positive control for mitochondria energy production was not included, thus the possibility exists that 100 μM ALCAR was not an effective concentration to protect against possible paclitaxel-induced decreases of mitochondrial function within our system. Future experiments can be performed to determine if ALCAR aided cellular energy production within the cultured sensory neurons as presumed. Interestingly, ALCAR administration prevented and reversed paclitaxel-induced hypersensitivity within animal models (Flatters, Xiao et al. 2006, Jin, Flatters et al. 2008), whereas concurrent administration of ALCAR with paclitaxel chemotherapy caused an increased incidence of peripheral neuropathy within patients (Hershman, Unger et al. 2013). This clinical finding, in combination with our *in vitro* data, suggests that ALCAR has either no effect or a detrimental effect on paclitaxel-induced changes of neuronal sensitivity, and ALCAR may be preventing paclitaxel-induced mechanical allodynia and hyperalgesia within animal models by an unknown mechanism.

These findings do not convincingly eliminate mitochondrial dysfunction or aberrant trafficking as possible mechanisms underlying paclitaxel-induced changes of neuronal sensitivity. If ALCAR aids mitochondria by increasing the transport of fatty acids into the mitochondria, ALCAR may have no effect on

possible paclitaxel-induced mitotoxicity. As discussed above, paclitaxel may alter the gating of VDAC pores, which ALCAR administration may not affect. Also, ALCAR likely does not attenuate aberrant mitochondrial trafficking in axons of neurons after paclitaxel exposure (Shemesh and Spira 2010). Future experiments should be designed to carefully investigate the effects of chronic exposure to paclitaxel on mitochondrial membrane potential, calcium levels, and gating properties within sensory neurons before further conclusions about paclitaxel-induced mitotoxicity and alterations of neuronal sensitivity can be made. It is likely that chronic exposure to paclitaxel will alter the mitochondrial membrane potential as paclitaxel (293 nM) has been shown to decrease mitochondrial membrane potential in cultured DRG after 3 hr exposure (Melli, Taiana et al. 2008).

Using our *in vitro* model, we also tested whether paclitaxel-induced alterations of stimulated CGRP release could be reversed with treatment of the free radical scavenger, NAC. The MTAs have been shown to increase ROS within different cell model systems including DRG (Barriere, Rieusset et al. 2012), and NAC can partially attenuate MTA-induced increases in ROS (Tsai, Sun et al. 2007). Accordingly, we asked whether NAC could reverse or attenuate paclitaxel-induced changes in capsaicin-evoked CGRP release, which would suggest that the changes in capsaicin-evoked release and alterations of TRPV1 function may be mediated by ROS generation. The results from the NAC and paclitaxel combination experiments are inconclusive; we were unable to find a concentration of NAC (0.5 and 1 mM) that did not alter total CGRP content in the

neurons or capsaicin-evoked release in the absence of paclitaxel. Lower concentrations of NAC were not attempted since 0.5 mM and 1 mM did not reverse paclitaxel-induced alterations of stimulated CGRP release. This suggests that neuronal CGRP content is tightly regulated, and a shift in the balance of the redox environment, or reduced and oxidized states of the cell, may be responsible for altering neuronal CGRP content and release. Interestingly, the NAC-induced increase in CGRP content was prevented by combination treatment with 300 nM paclitaxel. Perhaps CGRP content is regulated by the redox environment, and an increase in CGRP content may be produced by a NAC-induced decrease in ROS species, which may be balanced if paclitaxel increased ROS. This idea is purely speculative at this point, and in the future, ROS and/or redox status of transcription factors associated with CGRP expression could be determined after NAC treatment with and without paclitaxel in order to begin investigating this observation.

A limitation of the NAC experiments is that NAC acts as an antioxidant by scavenging free radicals such as the hydroxyl radical and hydrogen peroxide (Aruoma, Halliwell et al. 1989) as well as by recycling glutathione pools within cells, which mainly act to decrease hydroxyl radical and hydrogen peroxide levels (Kerksick and Willoughby 2005, Kalyanaraman 2013). Attempting to reverse paclitaxel-induced effects on neurons with an agent that decreases only a few, specific ROS may miss a possible paclitaxel/reactive nitroxidative effect. For example, if paclitaxel alters neuronal sensitivity by increasing the superoxide radical and downstream reactive nitrogen species like peroxynitrite, NAC would

not inhibit this since it mainly decreases hydrogen peroxide and hydroxyl radical levels (Aruoma, Halliwell et al. 1989). Thus NAC could potentially have no impact on the nitroxidative damage caused by paclitaxel. In order to further investigate if paclitaxel alters CGRP release via nitroxidative damage, it is important to conduct carefully designed experiments that systematically target specific types of nitroxidative species, one at a time. It may also be useful to measure possible paclitaxel-induced increases in the production of nitroxidative species within neurons. There are different techniques that can determine changes in specific reactive nitroxidative species or the amount of nitrotyrosine in proteins, and employing these techniques may assist in determining if paclitaxel alters reactive nitroxidative species, the specific species that are changed by paclitaxel, and the sources of the reactive species (Doyle, Chen et al. 2012). Clinically, antioxidants have not been effective at relieving peripheral neuropathy (Argyriou, Chroni et al. 2006, Kottschade, Sloan et al. 2011, Leal, Qin et al. 2014); however, many of the antioxidant therapies attempted might fail given the same argument described above, and they may not be acting to reverse the reactive species actually involved in the development of peripheral neuropathy. By using our *in vitro* model system, future investigations can determine if specific reactive nitroxidative species may be important targets for paclitaxel-induced alterations of neuronal sensitivity.

4) Paclitaxel Alters Stimulus-evoked Release of CGRP and Content from Sensory Neurons in the Absence of Added NGF

The work discussed was all performed using sensory neuronal cultures maintained in 30 ng/ml NGF throughout the time in culture. This concentration of NGF has been chosen by our group for the maintenance of sensory neurons because it has been previously shown to optimize conditions for the CGRP release assay by enhancing the expression of CGRP without compromising the ability of the neurons to be sensitized by inflammatory mediators (Park, Fehrenbacher et al. 2010). In an independent set of preliminary experiments not described in this work, we observed that removal of NGF from the culture media changed the paclitaxel effects on stimulated CGRP release. Because of this observation and the findings by other groups that NGF can alter paclitaxel-induced changes in cells, we asked if paclitaxel alters neuronal activity in the absence of added NGF. Sensory neurons were cultured and treated with low and high concentrations of paclitaxel for 5 days as performed previously; however, the neurons were maintained for the 12 days in media with 0 added NGF. We then performed capsaicin- and potassium-evoked CGRP release experiments.

In the absence of added NGF, the capsaicin- and potassium-evoked release profiles from the neurons were similar. The evoked release of CGRP decreased in a concentration-dependent manner; however, paclitaxel also decreased total CGRP content in a concentration dependent manner. In order to determine if the decrease in evoked release was secondary to a decrease in total

CGRP content, we determined the normalized evoked release from the treatment groups. The low concentration of paclitaxel, 10 nM, did not alter normalized capsaicin- or potassium-evoked release whereas the higher concentration, 300 nM, decreased both normalized capsaicin- and potassium-evoked release. The three main differences in CGRP release after treatment with paclitaxel in the absence of added NGF are: 1) there was no gain in function or augmentation of evoked CGRP release using either capsaicin or potassium as stimuli, 2) there was a decrease in potassium-evoked release after a high magnitude exposure to paclitaxel, and 3) paclitaxel decreased CGRP content in a concentration-dependent manner.

These findings suggest that the mechanisms underlying paclitaxel's gain in function effect are NGF dependent or dependent on CGRP total content. The mechanisms are currently unknown, but future investigation can be directed at identifying how NGF augments paclitaxel-induced CGRP release. Perhaps the presence of NGF in the media supports the development of a channel milieu at the membrane of the neuron that is necessary for a paclitaxel-induced gain in function. NGF is known to increase the expression and membrane insertion of channels involved in neuronal sensitivity like TRPV1 and sodium channels (Fjell, Cummins et al. 1999, Fjell, Cummins et al. 1999, Zhang, Huang et al. 2005). It is possible that exposure to NGF increases the membrane expression of channels that may be responsible for mediating the paclitaxel-induced gain in function. NGF is known to sensitize sensory neurons by several other mechanisms including phosphorylation of TRPV1 channels through protein kinase C (PKC)

and extracellular signal-regulated kinase (Erk) signaling cascades (Nicol and Vasko 2007) as well as by altering purinergic P2X receptors and acid sensing ion channels (Ramer, Bradbury et al. 2001, Mamet, Lazdunski et al. 2003). The differential effects of paclitaxel on stimulated CGRP in the absence of added NGF could be a result of differences in overall NGF sensitization of the neurons.

Another possibility for an NGF-dependent gain in function of release could be that NGF is altering the location and distribution of organelles affected by or functionally changed by paclitaxel. In accordance with the hypothesis that mitochondrial dysfunction is important for mediating paclitaxel-induced changes in neuronal sensitivity, NGF has been shown to mediate axonal transport of mitochondria and accumulation of mitochondria in terminals and axons of sensory neurons at locations of focal stimulation with NGF, which is dependent on actin microfilaments and PI3-K signaling cascades (Chada and Hollenbeck 2003, Chada and Hollenbeck 2004). In a simple scenario, perhaps NGF is responsible for increasing the number of mitochondria within terminals and axons of sensory neurons in culture prior to treatment with paclitaxel. Paclitaxel exposure may then halt any further mitochondrial trafficking to the terminals and axons of sensory neurons; however, the neurons exposed to NGF may have a greater number of accumulated mitochondria within their terminals and axons. In this way, application of paclitaxel to NGF-primed neurons, neurons with higher numbers of terminal and axonal mitochondria, may create a greater sensitizing effect compared to neurons that were not NGF-primed. This could be investigated with future experiments designed to investigate if NGF and/or

paclitaxel alter mitochondria trafficking in our system or if NGF alters paclitaxel-induced effects on mitochondria function.

When we observed that paclitaxel decreased CGRP content in a concentration-dependent manner in the absence of added NGF, our first idea was to test if neuronal viability was altered by paclitaxel, or in other words, we questioned if NGF protected the neurons from paclitaxel-induced cell death. Although we did not observe a change in viability of sensory neurons grown in 30 ng/ml NGF, previous reports demonstrated that paclitaxel treatment caused significant neuronal death in embryonic (E16) DRG cultures, mediated by necrosis (Scuteri, Nicolini et al. 2006), suggesting that paclitaxel may be toxic to sensory neurons depending on age and perhaps NGF signaling. We found that paclitaxel did not decrease neuronal viability or the total number of neurons in the absence of added NGF, suggesting that the concentration-dependent decrease in CGRP content was not secondary to neuronal death and is perhaps due to changes in CGRP expression.

CGRP transcriptional regulation has been shown to be mediated by several different pathways. Both cAMP and NGF signaling cascades can induce CGRP transcription by mediating phosphorylation of the cAMP response element-binding protein (CREB), which then binds the cAMP response element (CRE) within the promoter of the CGRP gene, thereby inducing transcription of the gene (Watson and Latchman 1995, Freeland, Liu et al. 2000). While phosphorylation of CREB is sufficient for mediating cAMP-induced CGRP transcription, CREB phosphorylation is necessary but not sufficient for mediating

NGF-induced CGRP transcription; activation of the MAPK cascade is also necessary for mediating NGF-induced CGRP transcription (Freeland, Liu et al. 2000, Durham and Russo 2003). The mechanisms mediating the NGF-dependent maintenance of neuronal CGRP content in the presence of paclitaxel are unknown. One possibility is that paclitaxel negatively alters transcriptional control of CGRP perhaps by interrupting signaling cascades involved in phosphorylation of CREB, which can be overridden when NGF is present. Paclitaxel could decrease phosphorylation of CREB by several different mechanisms including decreasing kinase activity or increasing phosphatase activity. One possible way that paclitaxel could be altering kinase or phosphatase activity is by increasing ROS production, which may alter redox balance within the neurons. There is substantial evidence that ROS are important mediators of gene expression by altering kinase, phosphatase, and transcription factor activity, including CREB (Sen and Packer 1996, Adler, Yin et al. 1999, Korbecki, Baranowska-Bosiacka et al. 2013). This is also suggested by our findings that NAC independently increased CGRP content within neurons in culture, which was prevented by the presence of high concentrations of paclitaxel. CGRP expression may be tightly regulated under redox-sensitive signaling pathways altered by paclitaxel and NGF. The effects of NGF and paclitaxel on CGRP transcription could be investigated by using viruses containing the CGRP promoter and a reporter construct, like eGFP (Durham, Dong et al. 2004). In this way, the activity of the CGRP promoter could be measured after treatment with paclitaxel and NGF within our system. ROS have

also been shown to decrease CREB protein and mRNA levels within hippocampal neurons and cardiomyocytes (Pugazhenthi, Nesterova et al. 2003, Ozgen, Guo et al. 2009). Although a matter of speculation, perhaps paclitaxel is causing ROS-dependent decreases in CREB protein expression. This decrease in CREB protein and subsequent decrease in CGRP transcription and protein expression may be masked by a NGF-mediated phosphorylation of remaining pools of CREB. This could be further studied by investigating if paclitaxel decreases CREB protein levels and/or alters CREB protein phosphorylation via ROS production.

Another possibility that may account for the MTA-induced decrease in total CGRP content within the neurons in the absence of NGF could be that the retrograde trafficking of the trkA receptor is decreased. Upon binding NGF, trkA is phosphorylated, internalized, and retrogradely trafficked to the nucleus in order to activate transcription factors that regulate downstream gene expression, including gene expression of CGRP (Ehlers, Kaplan et al. 1995). Inhibiting microtubule dynamics may attenuate retrograde trafficking of trkA and subsequent expression of CGRP, and this may be overridden when NGF is present in the media. The exact mechanisms mediating NGF-dependent gain in function of sensory neurons and CGRP maintenance in the presence of paclitaxel are unknown, but they are likely multifactorial and may involve receptor and channel localization, organelle transport and function, redox transcriptional regulation, and retrograde trafficking of trkA.

In vivo studies have found that NGF can reverse thermal hypoalgesia and the paclitaxel-induced decrease in substance P in the DRG (Apfel, Lipton et al. 1991). Although these *in vivo* findings are not directly comparable to our *in vitro* work, we also observed that NGF prevented paclitaxel-induced decreases in CGRP content. This suggests that NGF is important for maintaining neuropeptide concentrations within the DRG after exposure to paclitaxel. Within the clinic, a greater paclitaxel-induced decrease in circulating NGF levels is correlated with the severity of peripheral neuropathy and can predict the development of peripheral neuropathy in patients (Cavaletti, Bogliun et al. 2004, Arrieta, Hernandez-Pedro et al. 2011). These findings are slightly contradicted by our results showing that paclitaxel-induced increases in CGRP release, which can be loosely extrapolated to neuronal sensitivity in patients and to gain of function side effects like pain, do not occur in the absence of added NGF. However, the peripheral neuropathy is not characterized in the studies described above; an increase in severity of neuropathy could mean an increase in gain of function symptoms and/or an increase in loss of function symptoms (Griffith, Couture et al. 2014). In the case of a possible enhancement of loss of function symptoms when NGF decreases in patients, our findings may agree: we observed a paclitaxel-induced decrease in CGRP release and total CGRP content in the absence of added NGF. Collectively, our work suggests that NGF alters paclitaxel-induced effects on CGRP release in culture, and NGF is protective against paclitaxel-induced decreases of CGRP expression. Future experiments can be conducted *in vivo* to determine if NGF can reverse

paclitaxel-induced mechanical hypersensitivity in a low-dose animal model. Furthermore, it will be important for future investigators to consider the NGF environment when investigating paclitaxel-induced alterations in all settings, clinical, *in vivo,* and *in vitro*.

5) Epothilone B Alters Stimulus-evoked Release of CGRP from Sensory Neurons in the Absence of Added NGF

NGF signaling could potentially alter microtubule-independent as well as microtubule-dependent mechanisms of paclitaxel within neurons. In order to determine if NGF altered a microtubule-dependent effect of paclitaxel, I performed stimulated-release experiments after neurons were maintained in 0 added NGF and treated with EpoB for 5 days. In the presence of NGF, paclitaxel and EpoB have virtually the same effect on capsaicin- and potassium-stimulated CGRP release. In the absence of added NGF, the two microtubule stabilizing agents affected release slightly differently, suggesting that the compounds have slightly different, unknown mechanisms mediating neuronal sensitivity. Also, the development of peripheral neuropathy may be highly dependent on the circulating NGF levels within individuals (Cavaletti, Bogliun et al. 2004, Arrieta, Hernandez-Pedro et al. 2011). In the absence of NGF, EpoB increased (1 nM) and decreased (30 nM) capsaicin-evoked release, similarly to when NGF was present. When normalized to total content, there was no capsaicin-induced increase of CGRP release in the absence of NGF, and CGRP content decreased only after exposure to high concentrations of EpoB. There was no change in

potassium-evoked CGRP release following treatment with 1 nM EpoB; however, stimulated release decreased after treatment with 30 nM EpoB. Similarly to the paclitaxel experiments, there was no EpoB-induced increase in potassium-stimulated CGRP release, suggesting that NGF is necessary for MTA-induced neuronal sensitization.

Paclitaxel binds and activates NCS-1, and NCS-1 and NGF are known to converge on similar signaling cascades, including the PI3-K signaling cascade (Weiss, Hui et al. 2010). Potentially, the differential effects of paclitaxel and EpoB in the absence of NGF could be due to paclitaxel-specific and microtubule-independent functions. For example, there may be more crosstalk and/or interactions between paclitaxel-enhanced NCS-1 signaling and NGF signaling cascades within neurons following paclitaxel exposure compared to EpoB. Further, paclitaxel is known to have several microtubule-independent effects within cells, whereas there are no known other functions of EpoB. This may be due to a paucity of investigation on the molecular functions of EpoB, and EpoB could also have microtubule-independent functions which are currently unknown. Different effects of paclitaxel and EpoB on key pathways mediating neuronal sensitization are likely the causes of the different stimulated-release profiles in the absence of added NGF, and growing the neurons in NGF likely masks these signaling differences. Finally, paclitaxel has been implicated in altering nitroxidative species *in vivo* while EpoB has not been investigated in this context (Doyle, Chen et al. 2012). The different effects of EpoB and paclitaxel could result from a different effect on nitroxidative signaling and damage and/or redox-

sensitive signaling, thus the effects of EpoB on reactive species generation and signaling should be further investigated.

6) NGF Increases Neurite Length and Branching but Does Not Protect Against MTA-induced Decreases in Neurite Length and Branching

In order to investigate the effects of MTA agents on neurite length and branching of sensory neurons, I implemented an *in vitro* system where sensory neurons were cultured for 7 or 12 days, fixed on the final day in culture, labeled with neuron-specific antibodies tagged with a fluorophore, and then imaged with the high-throughput imaging system, ImageXpress Micro XL instrument. This system allowed for us to collect neurite length and branching data from entire fields with several neurons per field and from numerous wells and plates to increase our statistical power. While developing this method, I first attempted to infect the cultures with eGFP-expressing lentiviruses in order to fluorescently visualize the neurons. I moved away from this technique because the lentiviruses used were under the control of a CMV promoter, making the expression of the eGFP non-specific. Because the supporting cells were also expressing eGFP, I was unable to effectively discriminate between extensions from supporting cells and the actual neuronal processes. In order to avoid confounding our neurite length and branching data with complications from viral infection, we decided to move to a method of culture fixation and labeling with antibodies. In this way, I collected neurite data from cultures at fixed time points. This is a powerful method in that we can collect data from hundreds of neurons

within a relatively short amount of time, and we can use neuron-specific antibodies for labeling, therefore excluding any signal that may arise from the supporting cells within our cultures. One limitation to this method is that we cannot follow neurite length and branching from the same neurons over time.

Our method is also novel since we are investigating the effect of MTAs on neurite length and branching on established neurons in culture. Previous work has mostly focused on the effects of MTAs on the initiation of neurite outgrowth, or in other words, the regrowth of neurites after neurite axotomy (Hayakawa, Sobue et al. 1994, Hayakawa, Itoh et al. 1999, Scuteri, Nicolini et al. 2006). There are few studies that have investigated the effects of MTAs on neurons that were already established in culture, and the ones that have used established cultures have used embryonic-derived neurons (Letourneau, Shattuck et al. 1987, Yang, Siddique et al. 2009). Our studies are some of the first to describe the effects of MTAs on adult, fully established DRGs in culture. Dr. Gracias in Dr. Vasko's laboratory performed preliminary experiments using sensory neurons maintained in culture for 12 days. She found that paclitaxel (100 and 300 nM) decreased total neurite length of individual neurons (Gracias published thesis, Indiana University). Gracias' work and the work in this thesis more closely model the clinical exposure of patients to MTAs since their peripheral neurons are established upon commencement of MTA therapy.

NGF has been previously shown to promote neurite length in DRG cultures and explant systems (Gavazzi, Kumar et al. 1999, Niwa, Hayakawa et al. 2002). We confirmed this finding within our model system. I found that

neurite length and branching was greater in cultures grown in 30 ng/ml or 250 ng/ml NGF compared to 0 added NGF groups after 12 days in culture. Interestingly, when neurite length and branching was measured after only 7 days in culture, there were no differences in length or branching between neurons grown in 0, 30, or 250 ng/ml NGF. This suggests that NGF is most important for promoting neurite length and branching days 7-12 in culture, after the neurons have fully established themselves, or it suggests that NGF is necessary to maintain neurite length and branching between days 7 and 12 in culture; however, the former option is more likely since neurite length and branching did not decrease in 0 added NGF groups between the 7 day and 12 day time points.

Exposure to high concentrations of paclitaxel, EpoB, or vincristine over long periods of time decreased neurite length and branching of the sensory neurons, which is similar to previous work performed in embryonic DRG systems and in other neuronal systems (Hayakawa et al., 1994, Hayakawa et al., 1999, Yang et al., 2009); however, NGF did not attenuate or prevent this MTA-induced effect. This is contrary to the work from Hayakawa et al. that found that exogenous application of NGF to superior ganglia explants attenuated the effects of paclitaxel on prevention of neurite outgrowth (Hayakawa et al., 1994, Hayakawa et al., 1999). One possibility for this difference is that NGF has different effects on autonomic neurons compared to sensory neurons, and a second explanation could be that NGF may attenuate MTA-induced prevention of neurite outgrowth but have no effect on preventing MTA-induced neurite retraction.

All of the MTA agents, regardless of microtubule interaction, reduced neurite length and branching, independent of the NGF environment, which suggests that the decrease in neurite length and branching is due to an alteration in microtubule dynamics, as opposed to microtubule-independent effects of the MTAs. This is supported by the growing body of evidence that has shown that microtubules are crucial factors in the maintenance of neurites; neurite length is maintained by opposing forces between actin microfilaments in the tip of the neurite and microtubules. Disrupting this counterbalance of forces by altering microtubule dynamics can result in neurite retraction (Letourneau, Shattuck et al. 1987, Ahmad, Hughey et al. 2000, Wang, Naruse et al. 2001). The MTA-induced decreases in neurite length and branching in our system are likely a result of a disruption of microtubule dynamics, which would subsequently cause an alteration of the opposing structural forces within neurites. Another possibility that may underlie the MTA-induced decreases in neurite length and branching is that the disruption in microtubule function may alter mitochondrial function, as discussed above, and this could be important for maintenance of neurite length and branching (Morris and Hollenbeck 1993, Ruthel and Hollenbeck 2003). A disruption in mitochondrial-produced energy and calcium buffering could be important for the maintenance of neurites, and damaging mitochondria with MTAs may be involved in MTA-induced decreases in neurite length and branching.

Since all three of the MTAs had a similar effect on neurite length and branching, this helps to rule out microtubule-independent mechanisms that could

be connected to neurite length alterations. For example, NCS-1 is an important calcium regulator that has been shown to regulate neurite length via regulation of the TRPC5 channels (Hui, McHugh et al. 2006). EpoB has no known interaction with NCS-1 (Zhang, Heidrich et al. 2010) and decreases neurite length and branching similarly to vincristine and paclitaxel, which suggests that the MTA-induced deceases in neurite length and branching are not secondary to NCS-1 and TRPC5 alterations. MTAs are likely altering neurite length and branching within the established cultures by disrupting microtubule dynamics, however, whether the neurite alterations are due to the destruction of the fine balance of microfilament and microtubule interactions or via a different mechanism, like altering mitochondria function, is still a subject for future studies.

Our *in vitro* findings mirror the effects of MTA agents on IENF *in vivo* and clinically. Vincristine and paclitaxel decreased IENF in the rat hindpaw as well as in skin biopsies taken from patients receiving paclitaxel or vincristine therapy (Siau, Xiao et al. 2006, Boyette-Davis, Cata et al. 2013). This suggests that our model is effective at modeling the actions of MTA agents on sensory neurites. It is currently unknown if peripheral neuropathy develops because neurites retract or if neurites retract independent of changes in neuronal sensitivity; however, the data in this thesis suggests that changes in neuronal sensitivity may be independent of decreased neurite length since paclitaxel, EpoB, and vincristine decrease neurite length in an analogous manner but have different effects on CGRP release. Although the peak of hypersensitivity in MTA animal models corresponds to decreases of IENF within the hindpaws of the animals (Siau, Xiao

et al. 2006), the hypersensitivity may not be casually related to decreases of IENF. The mechanistic link between these MTA-induced symptoms needs to be further investigated, and our *in vitro* model can be used as a tool for future studies to investigate if neuronal sensitivity and or desensitization is secondary to MTA-induced decreases in neurite length.

CONCLUSIONS AND FUTURE DIRECTIONS

This work establishes that MTAs augment and attenuate stimulated CGRP release from peptidergic, sensory neurons *in vitro*, which is dependent on the magnitude of MTA exposure, the CGRP-evoking stimulus, the MTA treatment duration, and the surrounding NGF environment. MTAs also decrease neurite length and branching, independent of the surrounding NGF environment. These findings are analogous to MTA-induced changes in animal models and within patients receiving MTA therapy as they intimate gain and loss of function of nociceptor fibers as well as decreases in IENF. This suggests that our *in vitro* system is an effective model to investigate the molecular effects of MTAs on sensory neuron activity and morphology, and both sensitizing and desensitizing mechanisms may contribute to the clinical symptoms of peripheral neuropathy, dependent on the dosing and on the experimental endpoints measured. Our model is accompanied by some limitations that can be addressed with future work investigating glutamate release, intracellular calcium levels, and excitability of the entire population of sensory neurons in culture.

Future work should be directed at investigating the signaling pathways contributing to the changes in CGRP release induced by MTA agents. This includes investigating possible pathways involved in MTA-induced decreases in TRP channel function, which may include desensitization mechanisms of the TRP channels and/or aberrant TRP and tubulin interactions. Signaling pathways that are involved in MTA-induced gain in function should also be investigated and

may include tubulin-mediated mitochondrial dysfunction and/or alterations in membrane channels including VDCC channels.

Another possible avenue for future work is the investigation of ROS and reactive nitrogen species generation within neurons after MTA exposure. Aberrant nitroxidative signaling could have huge implications for neuronal activity as nitroxidative species have been implicated in the development of pain states of several different etiologies (Salvemini, Little et al. 2011), and ROS and reactive nitrogen species can alter signaling cascades and transcription factor activity (Bar-Shai and Reznick 2006, Bar-Shai and Reznick 2006, Yakovlev and Mikkelsen 2006). MTA-induced changes in the generation of ROS and reactive nitrogen species, the source of ROS production, and the effects of altered nitroxidative stress and signaling within neurons are all subjects needing further investigation.

Future work should also be conducted to elucidate how NGF alters MTA-induced changes in neuronal activity. Investigating changes in NGF-mediated mitochondria localization, CGRP expression, and signaling pathways involved in neuronal sensitization in the presence of MTAs may be helpful in understanding how NGF may facilitate protection from paclitaxel clinically.

Finally, the mechanisms underlying MTA-induced decreases in neurite length and branching and whether this is involved in changes in neuronal sensitivity are important topics for future investigation. It is likely that neurite length decreases due to microtubule alterations following treatment with MTAs, and future work can be directed at understanding whether neurite length is

affected by other possible MTA-induced alterations in calcium homeostasis and/or mitochondrial function.

The model systems developed in this thesis can be used to further identify the underlying mechanisms resulting in MTA-induced alterations of CGRP release and neurite morphology. These findings will also direct future work aimed at identifying and substantiating putative inhibitors of MTA-induced peripheral neuropathy. Further studies will test different hypothesized signaling pathways to determine whether they contribute to the illustrated alterations in neuropeptide release and neurite length. The long-term goal for this work is that it will be used to further understand the underlying mechanisms of MTA-induced changes in neuronal sensitivity and morphology in order to develop therapies to specifically prevent the development of MTA-induced peripheral neuropathy within patients.

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Publications:

1. Pittman SK, Gracias N, Vasko MR, Fehrenbacher J. 2014. Paclitaxel alters the evoked release of calcitonin gene-related peptide from rat sensory neurons in culture. Experimental Neurology. 253: 146-153.

2. Schmutzler BS, Roy S, Pittman SK, Meadows RM, Hingtgen CM. 2011. Ret-dependent and Ret-independent mechanisms of Gfl-induced sensitization. Molecular Pain. 7:22.

3. Finnegan MC, Pittman S, Delorenzo ME. 2008. Lethal and Sublethal Toxicity of the Antifoulant Compound Irgarol 1051 to the Mud Snail *Ilyanassa obsoleta*. Archives of Environmental Contamination and Toxicology. 56: 85–95.

4. Aguirre K, Pittman S, Ward C. 2007. Production of Inflammatory Cytokines by Microglia after Incubation with CD8+ T Cells from *Cryptococcus neoformans*-Infected Mouse Brain. Journal of Behavioral and Neuroscience Research. 5: 1-7

Presentations:

1. Pittman S, Meng H, Schneider BP, Fehrenbacher JC. “The effect of low-dose paclitaxel treatment on the expression of RWDD3 and sumoylation of proteins in lumbar dorsal root ganglia.” Society for Neuroscience Conference. New Orleans, LA. October 2012.

2. Phillips S, Hingtgen C. “Sexual dimorphic and neurofibromin-dependent alterations in the adenylyl cyclase/cAMP cascade.” Children’s Tumor Foundation Conference. New Orleans, LA. June 2012.

3. Phillips S, Hingtgen C. "Regulation of the Adenylyl Cyclase (AC)/cAMP Cascade by Neurofibromin." Society for Neuroscience Conference. Washington, D.C. November 2011.

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6. Pittman S, Aguirre K. "Investigation of CD8+ T cell cytotoxicity within the central nervous system when infected with *Cryptococcus neoformans.*" National Conference of Undergraduate Research (NCUR). Asheville, NC. April 2006.

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Honors:
-2006. President's Award recognizing 4.0 GPA. Coastal Carolina University, SC.
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