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# Nerve growth factor alters microtubule targeting agent-induced neurotransmitter release but not MTA-induced neurite retraction in sensory neurons

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## Abstract

Peripheral neuropathy is a dose-limiting side effect of anticancer treatment with the microtubule-targeted agents (MTAs), paclitaxel and epothilone B (EpoB); however, the mechanisms by which the MTAs alter neuronal function and morphology are unknown. We previously demonstrated that paclitaxel alters neuronal sensitivity, *in vitro*, in the presence of nerve growth factor (NGF). Evidence in the literature suggests that NGF may modulate the neurotoxic effects of paclitaxel. Here, we examine whether NGF modulates changes in neuronal sensitivity and morphology induced by paclitaxel and EpoB. Neuronal sensitivity was assessed using the stimulated release of calcitonin gene-related peptide (CGRP), whereas morphology of established neurites was evaluated using a high content screening system. Dorsal root ganglion cultures, maintained in the absence or presence of NGF, were treated from day 7 to day 12 in culture with paclitaxel (300 nM) or EpoB (30 nM). Following treatment, the release of CGRP was stimulated using capsaicin or high extracellular potassium. In the presence of NGF, EpoB mimicked the effects of paclitaxel: capsaicin-stimulated release was attenuated, potassium-stimulated release was slightly enhanced and the total peptide content was unchanged. In the absence of NGF, both paclitaxel and EpoB decreased capsaicin- and potassium-stimulated release and the total peptide content, suggesting that NGF may reverse MTA-induced hyposensitivity. Paclitaxel and EpoB both decreased neurite length and branching, and this attenuation was unaffected by NGF in the growth media. These differential effects of NGF on neuronal sensitivity and morphology suggest that neurite retraction is not a causative factor to alter neuronal sensitivity.

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paclitaxel; epothilone B; microtubule; chemotherapy-induced peripheral neuropathy; sensory neuron; nerve growth factor; neuropeptide; neurite outgrowth; retraction

## Introduction

The microtubule-targeted agents (MTAs), paclitaxel and epothilone B (EpoB), are effective chemotherapeutic agents, but their use is frequently limited by peripheral neuropathy (PN). The symptoms of PN include burning pain, numbness, tingling, and loss of proprioception in the hands and feet (Forsyth et al., 1997, Dougherty et al., 2004, Vahdat et al., 2012). These symptoms typically present in 30–40% of patients receiving MTA therapy, occur ~3–6 weeks after the onset of therapy, and sometimes persist after therapy is discontinued (Thompson et al., 1984, Rowinsky et al., 1991, Rowinsky et al., 1993, Postma et al., 1995, Nabholtz et al., 1996, Forsyth et al., 1997, Lee and Swain, 2006, Windebank and Grisold, 2008). There are currently no treatment options to prevent the development or reverse the symptoms of peripheral neuropathy (PN).

Nerve growth factor (NGF), a neurotrophin that serves as an important regulator of neuronal sensitivity and morphology in adult sensory neurons (Petty et al., 1994, Kimpinski et al., 1997, Svensson et al., 2003, Hoke et al., 2006, Lykissas et al., 2007), has been suggested to modulate the development of symptoms of PN induced by MTAs. In patients receiving paclitaxel, a decrease in circulating NGF levels correlates with symptoms reflecting a loss of function in a subset of sensory nerves (loss of sensitivity to vibration, loss of deep tendon reflexes, diminished sural action potential amplitude; Cavaletti et al., 2004). In animal models, systemic administration of MTAs alters nocifensive behaviors in a dose-dependent manner, with high doses enhancing mechanical sensitivity while decreasing thermal heat sensitivity (Cavaletti et al., 1997, Authier et al., 2000, Chiorazzi et al., 2009). Exogenous administration of NGF with high-dose paclitaxel prevented the loss of heat sensitivity (Apfel et al., 1991), whereas putative inhibition of NGF signaling through the trkA receptor via a nonselective tyrosine kinase inhibitor (Nakahashi et al., 2014) prevented the mechanical hypersensitivity induced by MTA administration.

In addition to changes in neuronal sensitivity, MTAs also alter sensory neuron morphology, both *in vivo* and *in vitro* (Letourneau and Ressler, 1984, Malgrange et al., 1994, Melli et al., 2006, Scuteri et al., 2006, Siau et al., 2006, Chiorazzi et al., 2009, Xiao et al., 2009, Yang et al., 2009, Ustinova et al., 2013), decreasing distal axon and neurite length, respectively. In contrast, NGF has been shown to increase the number of process-bearing neurons, as well as the total neurite length of adult DRG neurons in culture (Lindsay, 1988, Malgrange et al., 1994, Smith and Skene, 1997, Gavazzi et al., 1999, Niwa et al., 2002). Several reports have suggested that NGF can reverse MTA-induced prevention of neurite outgrowth in dissociated neurons and explant studies (Konings et al., 1994, Malgrange et al., 1994, Hayakawa et al., 1998). The question remains, however, whether NGF acts specifically to prevent MTA-induced changes in neuronal sensitivity and MTA-induced neurite retraction or

whether NGF acts strictly as a trophic agent to enhance neuropeptide content and neurite outgrowth.

To address this question, we examined neuronal function and morphology in sensory neuron cultures derived from dorsal root ganglia (DRG) from adult male rats. Our previous studies, using release of the nociceptive neuropeptide calcitonin gene-related peptide (CGRP) as a marker of sensory neuronal function (Pittman et al., 2014), demonstrated a paclitaxel-induced decrease in capsaicin-stimulated release, but a slight increase in release stimulated by high extracellular potassium (Pittman et al., 2014). These experiments were performed in the presence of added NGF in the growth media. Because of the clinical and laboratory findings with NGF, we investigated whether removing exogenous NGF from the growth media would affect the changes in neuronal sensitivity induced by paclitaxel and epothilone B (EpoB). We chose to study EpoB since a synthetic analog of EpoB, ixabepilone, induces symptoms of PN similar to those induced by paclitaxel in patients (Lee et al., 2008), and since EpoB binds within the taxane pocket of β-tubulin to stabilize microtubules; however, EpoB is structurally dissimilar to paclitaxel (Bollag et al., 1995, Giannakakou et al., 2000).

We confirmed that EpoB mimics the effects of paclitaxel on CGRP release, supporting the notion that changes in neuronal sensitivity are secondary to MTAs binding directly to microtubules and altering microtubule dynamics. We also found that maintaining cultures in the absence of NGF results in a decrease in stimulated peptide release, regardless of the stimulatory agent used to elicit release. Furthermore, MTAs decrease the total content of CGRP in the absence of NGF without altering neuronal viability. Finally, we confirmed that MTAs decrease sensory neurite length and branching, and unexpectedly found that these reductions in length and branching are not reversed by NGF.

## Methods

### Animals

All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were performed on primary cultures of sensory neurons derived from the DRG of adult male Sprague Dawley rats (150–250 g; Harlan Laboratories, Indianapolis, IN). Prior to sacrifice, animals were housed in group cages in a light controlled room with food and water available *ad libitum*.

### Isolation of primary sensory neuron cultures

DRG were harvested and cultured as described previously (Pittman et al., 2014). Dissociated DRG cells were added to plates previously coated with poly-D-lysine and laminin [~30,000 cells/well in 12-well plates for CGRP release assays and viability assays; ~30,000 cells/well in 6-well plates for biolistic transfection/neurite imaging experiments; and ~3,000 cells/well in 48-well plates for neurite length and branching experiments]. Cells were grown in F-12 growth media supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 μg/ml penicillin and streptomycin, 50 μM 5-fluoro-2-deoxyuridine, and 150 μM uridine in

the absence of added NGF or in the presence of 30 or 250 ng/ml NGF. Cells were maintained in an atmosphere of 3% $CO_2$ at 37°C, and media was changed every other day. For paclitaxel and EpoB treatments, stocks of drugs (10 mM) were prepared in 1-methyl-2-pyrrolidinone (MPL) and stored at −20°C. The stocks were further diluted in media to appropriate concentrations. Cultures were treated with paclitaxel or EpoB in media starting on day 7 in culture, and experiments were performed on day 7, before MTA exposure, or on day 12 in culture.

### Calcitonin gene-related peptide release

Release experiments were performed as described previously (Vasko et al., 1994, Pittman et al., 2014). 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 maintained at 37°C for 3 incubations. During the first interval, cells were incubated with HEPES buffer for 20 minutes to establish resting basal release of immunoreactive CGRP (iCGRP), which we will refer to as CGRP. For the release experiments performed with neurons maintained in 0 added NGF, we increased the basal incubation time from the previously used 10 minutes (Pittman et al., 2014) to 20 minutes, in order to achieve measurable amounts of basal CGRP since neurons grown in 0 added NGF have a lower total content of CGRP compared to those grown in NGF (Park et al., 2010). During the second interval, cells were incubated with HEPES containing 30 nM capsaicin or HEPES containing 50 mM KCl (substituted for equimolar NaCl) in order to stimulate CGRP release. The third incubation was in HEPES to reestablish resting basal release. Supernatants were collected after every interval, and CGRP was measured using radioimmunoassay as previously described (Vasko et al., 1994, Pittman et al., 2014). 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.

### Viability assay

The viability of sensory neurons grown in 0 added NGF following exposure to paclitaxel was assessed by staining with propidium iodide (PI). Sensory neuronal cultures were exposed to either vehicle (.003% MPL) or 300 nM paclitaxel for 5 days. As a positive control, untreated cultures were exposed to 300 μM $H_2O_2$ for 1–2 hours at 37°C. At the end of the 1–2 hour incubation, the cultures were returned to F-12 media. Approximately 24 hours later, all cultures were rinsed with phosphate-buffered saline (PBS), rinsed with staining buffer, and then incubated with 0.5 ml of the staining solution, consisting of 6 μM PI in staining buffer, for 1 hour. Cells were visualized after the 1 hour incubation period. The dye was excited at 530 nm and the emitted light was monitored at 645 nm. Cells in three random fields were counted and scored as either viable (unstained) or non-viable (red), and the total number of neurons, classified via morphology, per treatment group was also counted. The counter was blinded to the experimental sample 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.

### Neurite length and branching via ImageXpress

Neurite length and branching were determined using the Neurite Outgrowth module software of the ImageXpress Micro XL instrument (Molecular Devices, Sunnyvale, CA). The DRG cultures were fixed with 4% paraformaldehyde for 20 minutes 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), a neuronal-specific protein (Thompson et al., 1983), 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 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 × 1.4 mm). Data are 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 $\mu m^2$) and outgrowths (maximum width: 8 μm, intensity above local background: 5000 graylevels, minimum cell growth to log as significant: 10 μm).

### Neurite outgrowth and branching via biolistic transfection and individual neuronal measurements

The Helios Gene Gun System (Bio-Rad Laboratories, Hercules, CA) was used for biolistic transfection of neurons. Optimized protocols were designed and modified from a published report (Dib-Hajj et al., 2009) and from the manufacturer's instructions (Bio-Rad Laboratories Helios Gene Gun System) to maximize transfection efficiency and preserve the health of neurons. Green fluorescent protein cDNA (10 μg) in 50 μM spermidine was coprecipitated onto 1.0 μm gold particles with $CaCl_2$ (1M). The DNA gold suspension was washed and reconstituted three times with dehydrated EtOH. After the last EtOH wash, the gold-DNA complex was reconstituted in 0.05% polyvinylpyrrolidone in ethanol. The gold suspension was used to coat the inner wall of a 24 inch piece of Tefzel tubing (Bio-Rad Laboratories). The complex rested undisturbed in the tubing for approximately 3–5 minutes before the liquid (EtOH) contents were slowly removed with a syringe at a rate of 0.5–1.0 in/sec. Next, the tubing was dried using ultrapure nitrogen and cut into cartridges for the Helios Gene Gun. This process resulted in a density of 0.75 mg of gold particles per shot and 0.75 μg of total DNA per cartridge.

Immediately before biolistic transfection, the growth media was removed from the well of a 6-well plate containing recently harvested (~24 hrs) rat DRG neurons. The pressure and velocity were chosen so as to achieve transfection in several neurons while not affecting the health of the neurons. Transfected neurons expressed EGFP, and green fluorescence was visible as early as 24 hrs post-transfection. The neurons were photographed immediately before exposure to paclitaxel on day 7 in culture, and again 2 and 5 days (day 9 and day 12

in culture, respectively) after exposure to paclitaxel. The images were analyzed with the help of the neurite outgrowth plug-in, which was compatible with the MetaMorph 7.6 software. This software measures total outgrowth of the neuron projecting from the cell body rather than the length of individual neurites. User-defined criteria, such as detection threshold and diameter of cell body, were used to quantify length of processes. The validity of the specified criteria was verified by observing traces superimposed by the software onto the original image for purposes of comparison. Data were expressed as % change in neurite extension on day 9 and on day 12 relative to that on day 7.

### Reagents

All materials unless stated otherwise were purchased from Sigma-Aldrich (St. Louis, MO). F-12 media, horse serum, antibiotics, and Alexa Fluor 488 donkey anti-rabbit antibody were purchased from Life Technologies (Carlsbad, CA). NGF was purchased from Harlan Laboratories (Indianapolis, IN). The rabbit anti-human Protein Gene Product 9.5 antibody was purchased from AbD Serotec, a Bio-Rad company (Raleigh, NC). The GFP construct, pMax-GFP was acquired from Amaxa (Lonza; Basel, Switzerland).

### 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 Tukey's or Dunnett's test, as indicated. Statistical calculations were performed with the GraphPad Prism version 6.02 statistical software (GraphPad Software, La Jolla, CA). Data are presented as mean ± SEM, and differences are considered significant if $p < 0.05$.

## Results

### The effects of epothilone B on basal and stimulated release of CGRP in the presence of added NGF

We have previously demonstrated that a five-day treatment with paclitaxel (300 nM) decreased capsaicin- but enhanced potassium- stimulated release of CGRP without altering the basal release or total content of CGRP in sensory neurons maintained in NGF (Pittman et al., 2014). To ascertain whether an alternative MTA, EpoB, will also alter transmitter release from sensory neurons, cultured sensory neurons were treated with a range of EpoB concentrations (1–30 nM) for 5 days, and then the basal and stimulated release of CGRP was measured. EpoB did not alter basal release of CGRP from the sensory neurons (white column compared to white hashed columns for each treatment group, Figure 1A). Capsaicin-evoked (30 nM) CGRP release was augmented by 5-day treatment with 1 nM EpoB; release from vehicle (0.003% MPL) and EpoB-treated cultures was 300 ±16 and 395 ±22 fmol/well/10 min, respectively (Figure 1A). The higher concentrations of EpoB (10 and 30 nM) decreased capsaicin-evoked release to 148 ± 4 and 139 ± 13 fmol/well/10 min, respectively (Figure 1A). To investigate whether the effects of EpoB were limited to modulation of neuronal sensitivity via the TRPV1 channel, release from cultures treated with EpoB (30 nM) was also stimulated by high extracellular potassium; potassium-stimulated release was significantly augmented by EpoB (Figure 1B). EpoB did not alter the total content of CGRP in the neurons: total content levels were 1347 ± 48 and 1351 ± 61 fmol/well for the vehicle

and EpoB (30 nM) treatment groups, respectively (Figure 1C). Since the EpoB treatment did not alter CGRP content, the release of CGRP normalized to the content levels reflected release values, with EpoB (30 nM) treatment decreasing capsaicin-evoked release from 22.3 ± 0.6 (vehicle) to 11.7 ± 0.4 % of the total content of CGRP and increasing potassium-evoked release from 26.1 ± 0.5 (vehicle) to 28.2 ± 0.8 % of total content (Figure 1D).

### The effects of paclitaxel on basal and stimulated release of CGRP in the absence of added NGF

We previously showed that paclitaxel attenuated capsaicin-stimulated release and enhanced potassium-stimulated release in the absence of any change in neuropeptide content in sensory neurons maintained in the presence of NGF (Pittman et al., 2014). To determine whether NGF contributes to the stimulus-dependent effects of paclitaxel on release, we examined release from neurons grown in the absence of added NGF. Treatment with vehicle (0.003% MPL) or paclitaxel, in the absence of added NGF, did not alter basal release of CGRP from sensory neurons (Figure 2A). However, five-day treatment with paclitaxel significantly decreased the capsaicin-evoked release of CGRP from 31 ± 3 fmol/well/10 min in vehicle-treated cultures to 10 ± 1 fmol/well/10 min in paclitaxel-treated cultures (Figure 2A). In contrast to the effects of paclitaxel in the presence of NGF, paclitaxel decreased CGRP release stimulated by potassium when the cultures were maintained in the absence of NGF. The potassium-evoked release was 50 ± 4 fmol/well/10 min from vehicle-treated cultures and 22 ± 2 fmol/well/10 min from paclitaxel-treated cultures (Figure 2B). In addition, treatment with paclitaxel in the absence of added NGF decreased the total content of CGRP within the cultures. Total CGRP content was 402 ± 13 fmol/well in vehicle-treated cultures and 213 ± 6 fmol/well in paclitaxel-treated cultures (Figure 2C). Despite the change in CGRP content, when we normalized release to the total content for each group, we still observed paclitaxel-induced decreases in release. Treatment with paclitaxel decreased the normalized CGRP release evoked by both capsaicin and potassium (Figure 2D). The normalized capsaicin-evoked release was 7.9 ± 0.5% and 4.9 ± 0.7% of total content in the absence and presence of paclitaxel treatment, respectively. Similarly, the normalized potassium-evoked release was 12.0 ± 0.4% and 10.0 ± 0.4% of total content. These paclitaxel-induced decreases in the normalized release suggest that the mechanisms by which paclitaxel alters release of CGRP include both a decrease in CGRP levels available for outflow and a decrease in the sensitivity of the neurons to a general depolarizing stimulus. Furthermore, both of these effects are reversed by the addition of NGF (Pittman et al., 2014).

### The effects of paclitaxel on neuronal viability in the absence of added NGF

We previously demonstrated that paclitaxel (300 nm or 1 μM) did not alter neuronal viability in cultures grown in media containing NGF (30 ng/ml; Pittman et al., 2014), however, others have suggested that NGF is neuroprotective against paclitaxel-induced neurotoxicity (Peterson and Crain, 1982). Therefore, we ascertained whether the survival rate of neurons grown in the absence of added NGF was compromised by paclitaxel treatment using propidium iodide (PI) viability assays. Sensory neurons were cultured in media without added NGF and treated with vehicle or paclitaxel for 5 days, after which they were stained with PI. The total number of neurons in the observation field was also counted to verify that

paclitaxel treatment did not cause neuronal death and subsequent lifting of the neurons from the culture plate, in which case the neuronal death would not be captured by PI staining. 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 neurons/field (Figure 3). Paclitaxel did not alter the percent of positive PI-staining neurons or the total number of neurons per field. Following treatment, 9 ± 2% of neurons stained positive for PI and there was a total of 21 ± 1 neurons/field (hashed columns of Figure 3). As a positive control for the 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 (Vasko et al., 2005, Pittman et al., 2014). 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 vary with $H_2O_2$ exposure (black columns of Figure 3).

### The effects of EpoB on basal and stimulated release of CGRP in the absence of added NGF

To examine whether the absence of NGF in the growth media would affect EpoB-induced changes in peptide release in a similar manner to that observed with paclitaxel, we performed release experiments following exposure to EpoB in cultures without added NGF. In these cultures, treatment with EpoB (30 nM) for 5 days decreased capsaicin-evoked CGRP release compared to vehicle treated neurons. The release values were 30 ± 2 and 12 ± 3 fmol/well/10 min from vehicle and EpoB-treated cultures, respectively (Figure 4A). Unlike cultures grown in NGF, however, EpoB decreased potassium-stimulated release from 63.5 ± 2.7 to 22.0 ± 3.4 fmol/well/10 min in vehicle and EpoB-treated cultures, respectively (Figure 4B). The total content of CGRP was decreased in neurons treated with EpoB (30 nM) from 322 ± 8 to 152 ± 5 fmol/well in vehicle- and EpoB-treated wells, respectively (Figure 4C). Since total content was decreased by treatment with 30 nM EpoB, stimulated CGRP release was normalized to the total content. EpoB treatment did not significantly alter CGRP release (% of total content released) upon stimulation with capsaicin; however, there was a decrease upon stimulation with potassium. Release stimulated by potassium represented 19 ± 1 and 13 ± 2 % of the total content, in the absence and presence of EpoB treatment, respectively.

### The effects of NGF and MTAs on neurite length and branching

Since NGF modulates the effects of paclitaxel and EpoB on CGRP release and since NGF has been suggested to be protective against paclitaxel-induced inhibition of neurite growth (Konings et al., 1994, Malgrange et al., 1994, Hayakawa et al., 1998), we developed an automated protocol to determine whether NGF protects against MTA-induced morphological changes in sensory neurons. The maintenance of sensory neurons for 12 days in the presence of 30 or 250 ng/ml of NGF slightly enhanced neurite length and branching compared to neurons grown in the absence of NGF. Representative images of cultures grown in the absence of added NGF vs. those grown in 30 ng/ml NGF are presented in Figures 5A and 5B. Total neurite lengths were 117.8 ± 7.9, 140.6 ± 2.5, and 140.7 ± 3.1 mm in the 0, 30, and 250 ng/ml NGF groups, respectively (Figure 5E). Total branching segments were 8118 ± 795, 10944 ± 356, and 11154 ± 452 segments in the 0, 30, and 250 ng/ml NGF groups, respectively (Figure 5F). Representative images for vehicle and paclitaxel-treated cultures in

the absence of added NGF and in the presence of 30 ng/ml NGF at day 12 can be seen in Figures 5A–D. In cultures grown in the absence of NGF, five days of treatment with paclitaxel (300nM) resulted in a significant decrease in neurite length from 117.8 ± 7.9 to 67.9 ± 7.0 mm and a decrease in branching from 8118 ± 795 to 3301 ± 487 segments. Maintaining cultures in either 30 or 250 ng/ml NGF did not alter the paclitaxel-induced decreases in neurite length or branching. Neurite lengths decreased to 85.9 ± 6.7 and 87.9 ± 7.0 mm (Figure 5E), whereas total branching segments decreased to 5055 ± 567 and 5245 ± 626 segments in the 30 and 250 ng/ml NGF groups (Figure 5F).

We next determined whether EpoB alters neurite length and branching in a manner similar to paclitaxel. Representative images of the effects of EpoB on neurite length and branching of neurons grown in the absence and presence of NGF are presented in Figures 6A–D. As observed previously, the vehicle-treated neurons maintained in 30 and 250 ng/ml NGF had greater total length and branching compared to the vehicle-treated neurons grown in the absence of NGF. Five-day treatment with EpoB (30 nM) reduced both neurite length and branching. In the neurons maintained in the absence of NGF, neurite length was reduced from 107.6 ± 10.3 to 66.3 ± 8.4 mm and the number of branch segments was reduced from 7525 ± 721 to 3467 ± 619. The inclusion of NGF in the growth media did not reverse the EpoB-induced decreases in neurite length or branching. Neurite lengths were reduced by EpoB from 139.0 ± 2.6 and 141.7 ± 4.1 mm to 66.4 ± 11.6 and 71.9 ± 11.4 mm in the 30 and 250 ng/ml NGF groups, respectively (Figure 6E). Similarly, the number of branching segments was decreased by EpoB from 10697 ± 321 and 11329 ± 555 segments to 3858 ± 930 and 4331 ± 936 segments in the 30 and 250 ng/ml NGF groups, respectively (Figure 6F).

We also examined the effects of paclitaxel (300nM) on neurite length using a well-established neurite length measurement technique so that we could focus on the effects of paclitaxel on individual neurons (Arantes and Andrews, 2006). We transfected cultured sensory neurons, maintained in 30 ng/ml NGF, with gold particles coated with cDNA for enhanced green fluorescent protein (EGFP) using the Helios biolistic gene gun. We then imaged individual neurons and used MetaMorph software (Molecular Devices) to measure the neurite lengths of individual neurons. The process of shooting gold particles at high velocity and pressure into sensory neurons did not affect the growth or development of neuronal processes. Neurons that were exposed to vehicle (0.01% MPL for 5 days) maintain their morphology and integrity for at least 12 days in culture (Figure 7A). As illustrated in Figure 7B, the total outgrowth on day 9 and day 12 in vehicle-treated neurons was 107.6 ± 8.0 and 95.3 ± 9.2% of the total length at day 7. In contrast, when sensory neurons were exposed to paclitaxel (300 nM), the neuronal morphology changed (Figure 7A) and the length of neurites decreased to 53.7 ± 4.8 % and 32.5 ± 4.4% of the total day 7 length on days 9 and 12, respectively (Figure 7C). Thus, as was observed with the automated imaging experiments, paclitaxel (300 nM) significantly decreased the length of neurites following a five-day exposure to the chemotherapeutic agent. Furthermore, being able to monitor the effects of paclitaxel on a single sensory neuron over time supports the notion that decreases in neurite length are secondary to active retraction or distal degeneration of the neurites (Yang et al., 2009).

## Discussion

We show here for the first time that EpoB, a microtubule stabilizing agent that lacks structural homology with paclitaxel but binds to tubulin and stabilizes microtubules (Bollag et al., 1995, Buey et al., 2004), produces functional and morphological effects on sensory neurons in a manner analogous to paclitaxel. We predictably observed that a higher potency of EpoB altered neuronal release and morphology compared to paclitaxel, since EpoB binds microtubules with approximately 10 times higher affinity than paclitaxel (Kowalski et al., 1997). EpoB does not have any known microtubule-independent actions and has been used as a negative control in experiments investigating microtubule-independent effects of paclitaxel (Boehmerle et al., 2007, Zhang et al., 2010). Our results support the *notion* that MTA-induced changes in the sensitivity and growth of sensory neurons are primarily caused by the disruption of microtubules rather than elicited by off-target effects of paclitaxel, such as activation of the neuronal calcium sensor-1 (Boehmerle et al., 2006), binding to Bcl2 (Ferlini et al., 2009), or binding to the toll-like receptor 4 (TLR4) accessory protein, MD-2 (Resman et al., 2008, Zimmer et al., 2008). *The critical experiments to confirm that microtubule binding is essential for the neuronal effects of paclitaxel and EpoB, via overexpression of a mutated β-tubulin that cannot bind to paclitaxel, are complicated by the fact that β-tubulin mutations alter the stability of microtubules in the absence of any stabilizing drug* (Hari et al., 2006). Finally, we found that NGF is protective against a loss of neuronal sensitivity and decreases in CGRP content following exposure to MTAs; however, this did not correlate with a protective effect against morphological changes induced by the MTAs. These findings allow the dissociation of MTA-induced retraction from the ability of these MTAs to alter transmitter release.

We used an in vitro experimental model to investigate the effects of MTAs and NGF on neuronal sensitivity and morphology. As with any experimental model, the use of neuronal cultures to gauge MTA-induced neuropathy has limitations. Sensory neurons are dissociated from their native environment in order to create a reduced system, eliminating putative neuron to glia (Kim et al., 2015) and glia to glia (Dublin and Hanani, 2007, Zhang et al., 2009) communication via gap junctions. In addition, the sensory neuron cultures are comprised of both neurons and non-neuronal cells, thus an effect of MTAs on non-neuronal cellular function has the potential to confound the interpretations of these in vitro studies. Finally, the neurons are cultured for up to two weeks on a substrate composed of poly-D-lysine and laminin and in media which contains NGF (30ng/ml). Despite these limitations, there is strong precedence that neuronal cultures are valuable to identify the mechanisms by which inflammatory mediators and toxins alter the function of sensory neurons (Zhuang et al., 2004, Fehrenbacher et al., 2005, Patwardhan et al., 2006, Lin et al., 2011, Pittman et al., 2014). Previously, we demonstrated that treating neuronal cultures with paclitaxel for 5 days could alter the sensitivity of the neurons, emulating the effects of paclitaxel on nociceptive behavioral outcomes following systemic administration of paclitaxel (Pittman et al., 2014). We chose to continuously include the MTAs in the growth media for our in vitro experiments. We appreciate that in the clinic, paclitaxel and EpoB are administered in a cyclic manner via intravenous infusions. Despite this cyclic administration, paclitaxel accumulates within the sensory nervous tissue (Cavaletti et al., 2000, Xiao et al., 2011).

Using calculations to account for the density of peripheral nerve tissue (Beckmann et al., 1999) and the reported values of paclitaxel within the DRG of animals systemically treated with paclitaxel (Cavaletti et al., 2000, Xiao et al., 2011), we estimate that the DRG are exposed to paclitaxel levels between 400–550 nM 24 hours after the last injection of paclitaxel. Furthermore, ten days following the last injection, paclitaxel was still detectable in the DRG at concentrations of 63 ± 6 nM (Xiao et al., 2011). These data suggest that DRG serve as a reservoir for the drug, therefore sensory neurons are constantly exposed to paclitaxel as long as ten days following the last exposure, despite decreasing or non-detectable concentrations of paclitaxel in the serum in vivo. It is unclear why chemotherapeutics accumulate in peripheral nervous tissues, but enhanced vascularity of the nervous tissues by capillaries with high permeability has been implicated (Jimenez-Andrade et al., 2008).

The mechanisms by which MTAs alter neuronal sensitivity and neurite morphology are unknown. Varied hypotheses have been proposed, including: compromised transport of proteins and organelles (Shprung and Gozes, 2009, Shemesh and Spira, 2010, LaPointe et al., 2013), changes in receptor and channel localization and function (Flatters and Bennett, 2004, Matsumoto et al., 2006, Xiao et al., 2007, Nieto et al., 2008, Chen et al., 2011, Okubo et al., 2011, Kawakami et al., 2012, Hara et al., 2013, Chen et al., 2014) and/or activation of an immune response (Siau et al., 2006, Peters et al., 2007, Pevida et al., 2013, Warwick and Hanani, 2013, Zhang et al., 2013, Li et al., 2014, Li et al., 2015). Stabilization of microtubules can alter both anterograde and retrograde axonal transport of mitochondria along the microtubules (Nennesmo and Reinholt, 1988, Morris and Hollenbeck, 1995, Nakata and Yorifuji, 1999, Theiss and Meller, 2000, Shemesh and Spira, 2010, Bober et al., 2015). Alterations in mitochondrial trafficking could subsequently alter neuronal sensitivity via increased reactive oxygen and nitrogen species production (Kim et al., 2010, Fidanboylu et al., 2011, Barriere et al., 2012, Doyle et al., 2012, Janes et al., 2013), changes in calcium buffering, and local changes in energy production within the nerve terminals (Abou-Sleiman et al., 2006). Indeed, several investigators have proposed that decreased numbers of mitochondria (Ebenezer et al., 2014) and mitochondrial dysfunction are the underlying cause of peripheral neuropathy (Andre et al., 2000, Kidd et al., 2002, Flatters and Bennett, 2006, Cavaletti et al., 2007, Jin et al., 2008, Melli et al., 2008, Podratz et al., 2011, Xiao et al., 2011, Zheng et al., 2011, 2012), yet the precise mechanisms by which paclitaxel and EpoB alter mitochondrial function have not been elucidated.

There have been numerous pharmacological approaches taken to either prevent the development or reverse the symptoms of neurotoxicity in animal models of CIPN. One such approach was the exogenous administration of NGF (Apfel et al., 1991). NGF is a growth factor which is essential during development for the survival of sensory neurons, but plays a modulatory role in neuronal sensitivity and axonal growth in adult sensory neurons (Lindsay, 1988, Zhu et al., 2004). Previous studies suggest that NGF attenuates the clinical and behavioral effects of paclitaxel. A correlation between the intensity of peripheral neuropathy symptoms and treatment-induced reductions in circulating NGF levels in patients receiving paclitaxel was established in 2004 (Cavaletti et al.), following an observation that NGF administration reversed the development of thermal hypoalgesic behaviors in animals treated with high doses of paclitaxel (Apfel et al., 1991). We previously reported that, in the

presence of NGF, paclitaxel slightly enhanced the potassium-stimulated release of CGRP, decreased capsaicin-stimulated release, and had no effect on the total content of CGRP (Pittman et al., 2014). Here, we demonstrate that when NGF was excluded from the growth media, paclitaxel and EpoB decrease potassium-stimulated neuropeptide release and decrease the total content of CGRP. We confirmed that the MTAs did not decrease neuropeptide release and content via neuronal death in our adult cultures, since previous reports indicated that paclitaxel treatment decreased neuronal survival in embryonic (E16) DRG cultures maintained in low levels of NGF (5 ng/ml; Scuteri et al., 2006). There is much evidence that endogenous NGF enhances the sensitivity of sensory neurons in the presence of inflammation or nerve injury (Lewin et al., 1993, Woolf et al., 1994, Bennett et al., 1998). We previously demonstrated that NGF can regulate the sensitivity of sensory neurons *in vitro* by enhancing neurotransmitter synthesis and by increasing the expression and activity of receptors and ion channels (Park et al., 2010). Our results here establish that the MTAs compromise both CGRP expression and release and that NGF prevents both of these changes to protect the function of the neurons. Interestingly, the MTA-induced decrease in capsaicin-stimulated release is unaffected by the presence of NGF, and we hypothesize that the MTAs may have a direct effect on TRP channel localization or function to diminish TRPV1 activity. Neuroprotection by NGF, therefore, appears to prevent a loss of neuronal function within the peptidergic population of sensory neurons. This effect may underlie the NGF-induced prevention of thermal hypoalgesia in the rat since thermal sensitivity is determined by this population of sensory neurons and because NGF protects against a loss of neurotransmitters in these neurons (Apfel et al., 1991).

It is still unclear whether NGF directly interferes with the ability of the MTAs to attenuate neuronal sensitivity and neuropeptide content or whether NGF acts as a general trophic factor to enhance neuronal sensitivity and content. In addition to its trophic effects, NGF positively modulates axonal transport and accumulation to enhance the number of mitochondria in axons of sensory neurons (Gallo and Letourneau, 1998, Chada and Hollenbeck, 2004). NGF also regulates the membrane potential of mitochondria (Verburg and Hollenbeck, 2008), thus the growth factor enhances the function of mitochondria to enhance ATP generation, and possibly overcome the deleterious effects of MTAs. Moreover, NGF may counteract an MTA-induced generation of ROS/RNS by the upregulation of catalase, glutathione and glutathione peroxidase in sensory neurons (Podratz and Windebank, 2005). Further research is necessary to identify whether these mechanisms contribute to the protective effects of NGF on release and neuropeptide content.

Intraepidermal nerve fiber (IENF) loss is a hallmark symptom of CIPN in patients (Boyette-Davis et al., 2013, Ebenezer et al., 2014) and has been demonstrated in animal models of MTA-induced neuropathy (Siau et al., 2006, Chiorazzi et al., 2009, Boyette-Davis et al., 2011). MTA-induced decreases in neurite length have also been modeled in sensory neurons *in vitro* (Letourneau and Ressler, 1984, Bamburg et al., 1986, Horie et al., 1987, Malgrange et al., 1994, Melli et al., 2006, Scuteri et al., 2006, Yang et al., 2009, Ustinova et al., 2013). Our findings demonstrate that NGF, even at a supraphysiological concentration (250 ng/ml), does not prevent or attenuate the effects of MTAs to decrease neurite length and branching in mature adult sensory neurons. These findings contrast with previous reports, which demonstrate an NGF-dependent reversal of the effects of MTAs when examining neurite

outgrowth from embryonic DRG explants, adult superior cervical ganglion explants, or adult dissociated DRG cultures (Konings et al., 1994, Malgrange et al., 1994, Hayakawa et al., 1998). Importantly, we investigated the effects of the MTAs and NGF to alter retraction or degeneration from mature neurites of neurons derived from adult animals, whereas other studies have focused on neurite outgrowth from the soma of recently axotomized neurons. We focused on retraction as the clinical literature describes an MTA-induced dieback or retraction of distal axons which innervate the skin of patients (Boyette-Davis et al., 2013). The signaling cascades which mediate retraction differ from those which mediate outgrowth or branching (see reviews by Goldberg, 2003, Luo and O'Leary, 2005). Furthermore, the effects of NGF on axonal retraction are unclear, whereas a positive effect of NGF on axonal extension and branching is well-established (Gallo and Letourneau, 1998, Spillane et al., 2013, Wong et al., 2015), thus NGF could promote outgrowth in the presence of MTAs, but not alter MTA-induced retraction. Although our findings suggest that NGF would not prevent retraction in patients receiving MTAs, NGF may help to resolve the symptoms of CIPN following the discontinuation of MTA therapy by enhancing the regeneration of distal axons.

It has been hypothesized that retraction or degeneration of sensory neurons is a causative factor to alter the sensitivity of the neurons to noxious and innocuous stimuli in patients receiving paclitaxel chemotherapy (Siau et al., 2006, Boyette-Davis et al., 2011, Boyette-Davis et al., 2013). Our findings that NGF can protect against some of the changes of neuronal sensitivity induced by MTAs without reversing the morphological changes, suggest that changes in morphology do not predict changes in sensitivity and *in vivo* evidence of a dissociation between IENF loss and behavioral sensitivity has recently been reported (Yilmaz and Gold, 2015). Whether there is a relationship between axonal retraction and changes in neuronal sensitivity or *vice versa* still needs to be examined in detail; however, this *in vitro* model system in which neuronal function and neurite length and branching can be investigated concomitantly will facilitate studies to answer this question and help to elucidate the causative mechanisms underlying the development of PN.

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## Abbreviations

| | |
|---|---|
| **CGRP** | Calcitonin gene-related peptide |
| **CIPN** | Chemotherapy-induced neuropathy |
| **DRG** | Dorsal root ganglia |
| **EpoB** | Epothilone B |
| **MTA** | Microtubule-targeted agent |
| **NGF** | Nerve growth factor |

**Pac** Paclitaxel

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## Highlights

- MTAs alter the sensitivity of sensory neurons in an NGF-dependent manner
- MTAs decrease neurite length and branching in an NGF-independent manner
- These data suggest that retraction does not cause changes in neuronal sensitivity

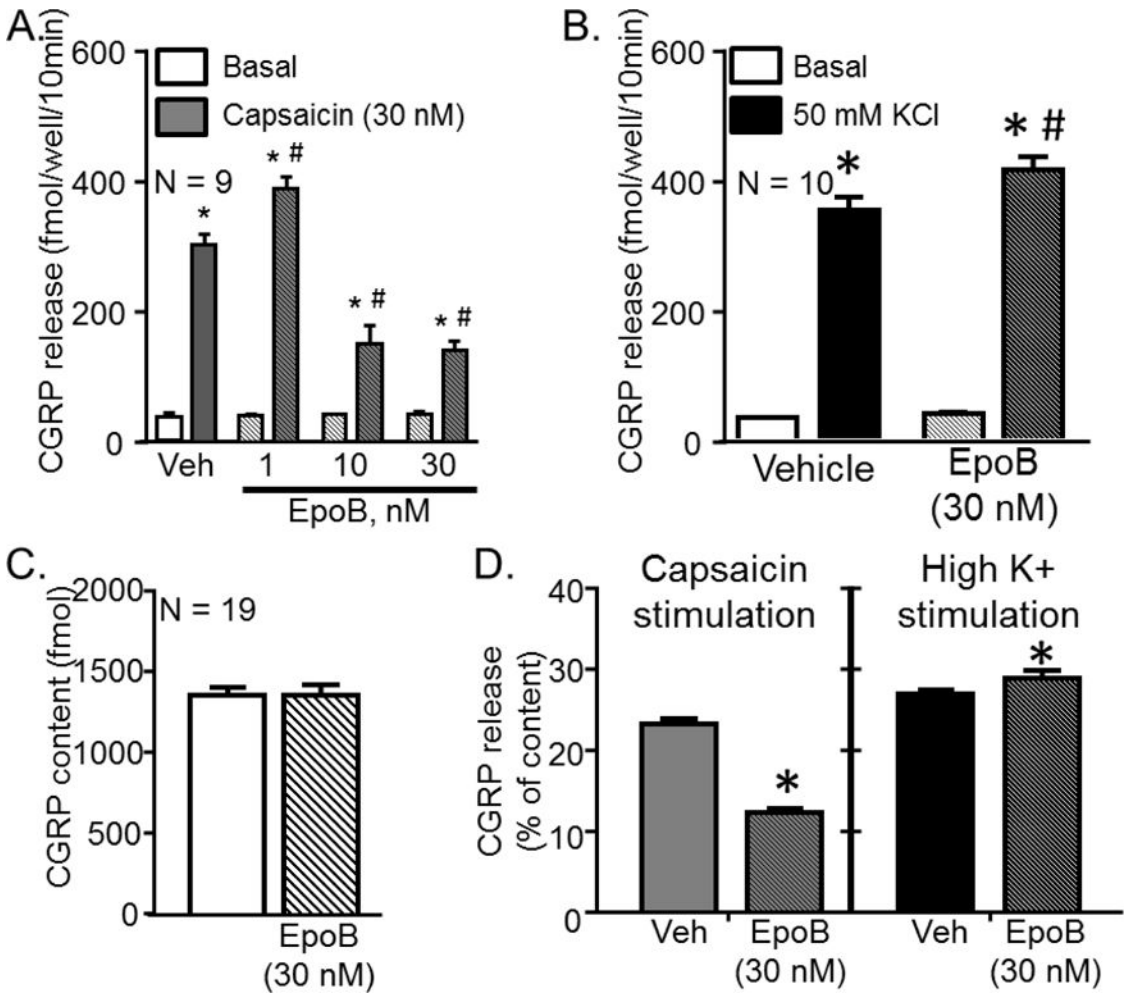


**Figure 1.**

Epothilone B alters capsaicin- and potassium-stimulated release of CGRP without altering the total content of CGRP in sensory neurons grown in NGF (30 ng/ml). A and B. Each column represents the mean ± SEM of CGRP released (fmol/well/10 min) from wells treated with vehicle or epothilone B (EpoB; 1 nM – 30 nM) for 5 days. The solid columns of each group represent basal and capsaicin-stimulated (A) or potassium-stimulated (B) release in the absence of EpoB treatment, whereas the hashed columns represent release following EpoB treatment. An (*) indicates a significant difference in stimulated release compared to baseline release, whereas a (#) indicates a significant difference in the stimulated release from the vehicle-treated cultures ($p<0.05$, N=9–10) using a two-way ANOVA with Tukey's post-hoc test. C. Each column represents the mean ± SEM of total CGRP content (fmol/well) from vehicle-treated cultures (Veh) or EpoB-treated cultures as indicated. D. Each column represents the normalized mean ± SEM of the stimulated release of 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=9–10) using a t-test.

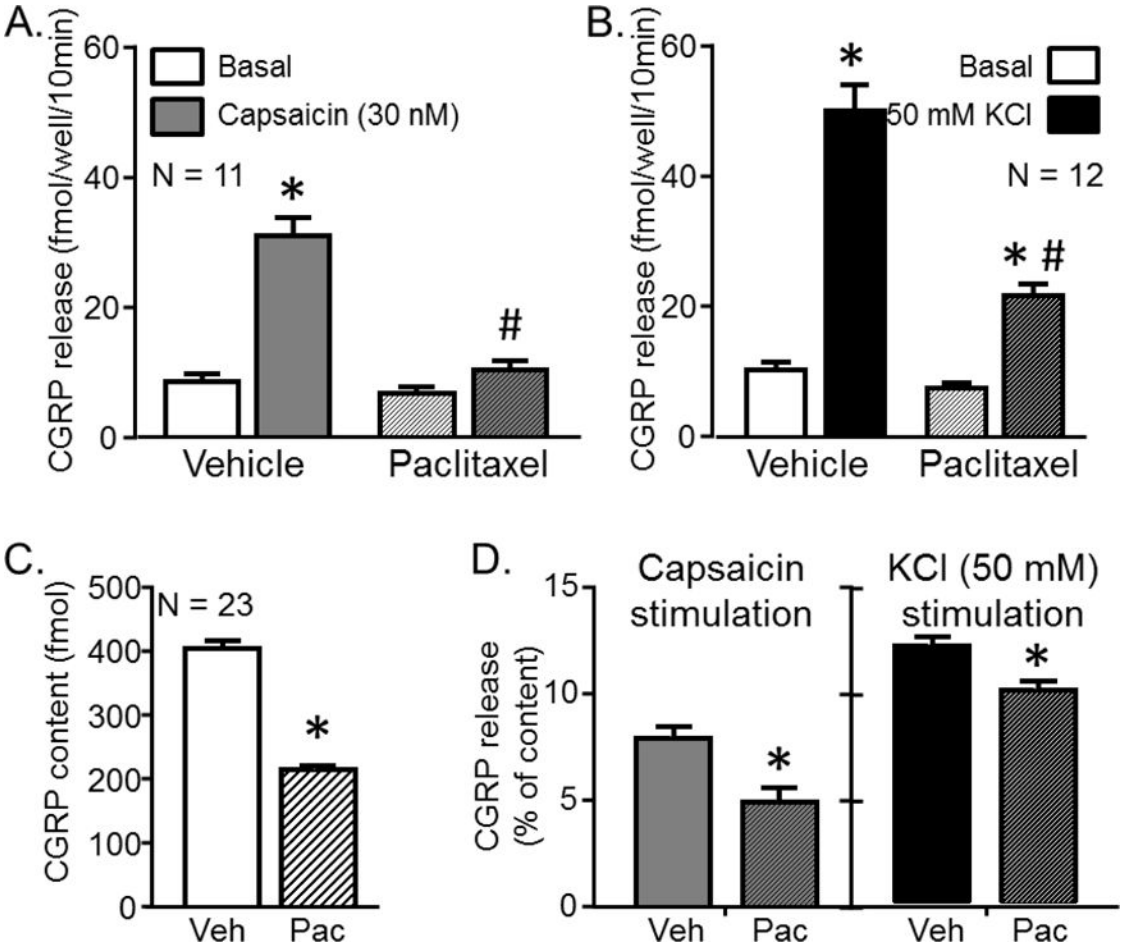


**Figure 2.**

Paclitaxel attenuates the stimulated release and total content of CGRP in sensory neurons grown in the absence of NGF. A and B. Each column represents the mean ± SEM of CGRP released (fmol/well/10 min) from wells treated with vehicle or paclitaxel (300 nM) for 5 days. The solid columns of each group represent basal and stimulated release in the absence of paclitaxel treatment, whereas the hashed columns represent release following paclitaxel treatment. An (*) indicates a significant difference in capsaicin-stimulated (A) or potassium-stimulated (B) release compared to baseline release, whereas a (#) indicates a significant difference in the stimulated release from the vehicle-treated cultures ($p<0.05$, N=11–12) using a two-way ANOVA with Tukey's post-hoc test. C. Each column represents the mean ± SEM of total CGRP content (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=23) using a t-test. D. Each column represents the normalized mean ± SEM of the stimulated release of 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=11–12) using a t-test.

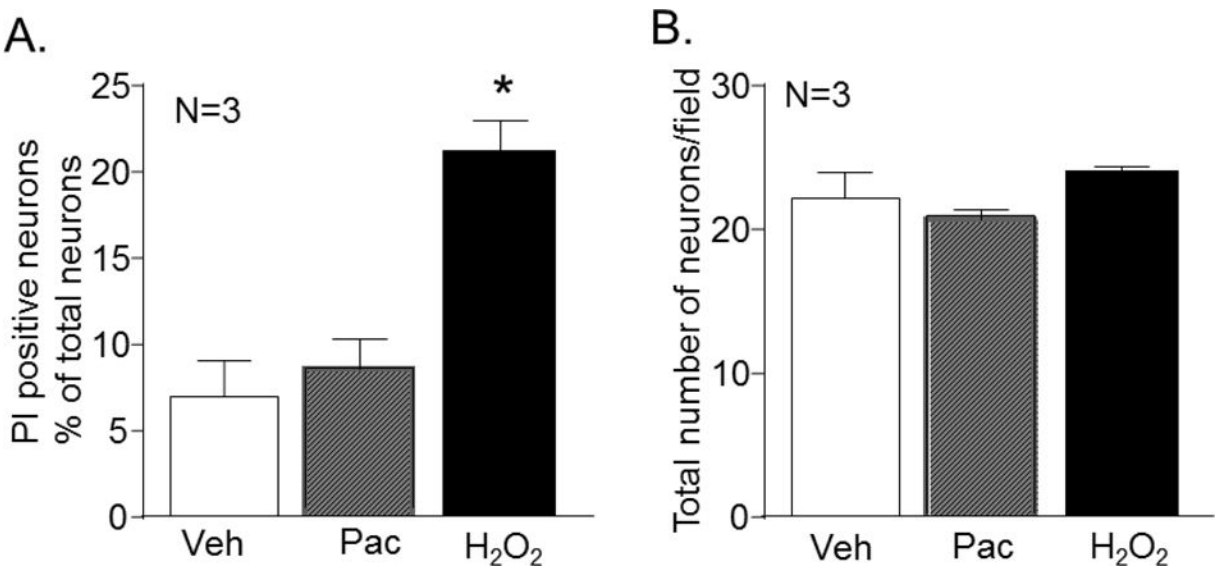


**Figure 3.**

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 paclitaxel for 5 days (Pac; hashed 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.

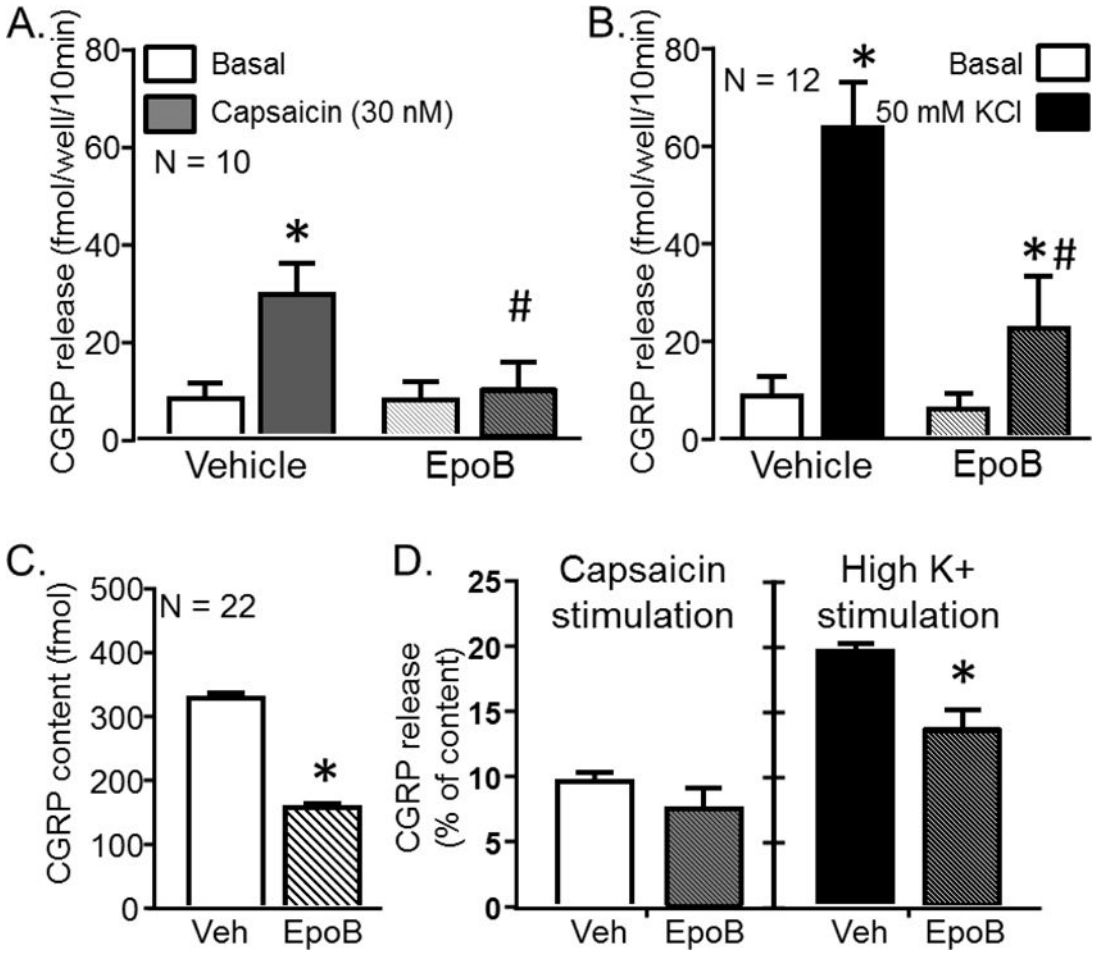


**Figure 4.**

Epothilone B attenuates the stimulated release and total content of CGRP in sensory neurons grown in the absence of NGF. A and B. Each column represents the mean ± SEM of CGRP released (fmol/well/10 min) from wells treated with vehicle or epothilone B (EpoB; 30 nM) for 5 days. The solid columns of each group represent basal and stimulated release in the absence of EpoB treatment, whereas the hashed columns represent release following EpoB treatment. An (*) indicates a significant difference in capsaicin-stimulated (A) or potassium-stimulated (B) release compared to baseline release, whereas a (#) indicates a significant difference in the stimulated release from the vehicle-treated cultures ($p<0.05$, N=11–12) using a two-way ANOVA with Tukey's post-hoc test. C. Each column represents the mean ± SEM of total CGRP content in fmol/well from vehicle-treated cultures (Veh) or EpoB-treated cultures as indicated. An (*) indicates a significant difference in total CGRP content compared to the vehicle-treated cultures ($p<0.05$, N=23) using a t-test. D. Each column represents the normalized mean ± SEM of the stimulated release of 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=11–12) using a t-test.

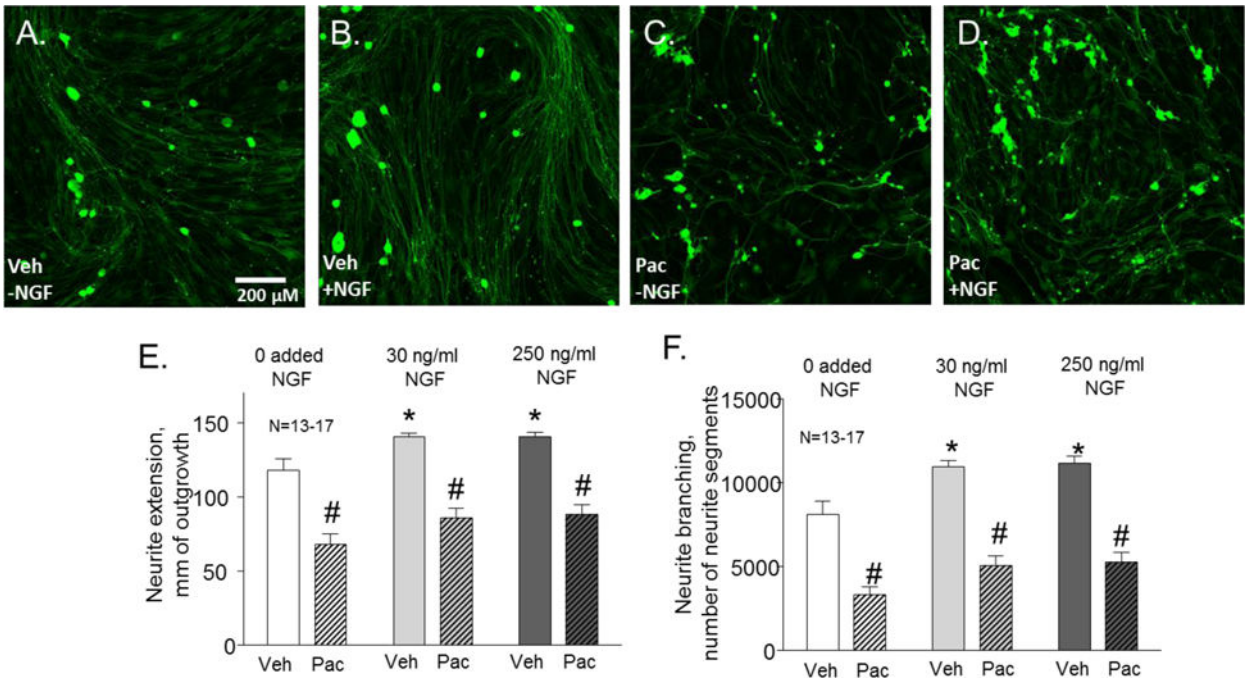


**Figure 5.**

Paclitaxel and NGF 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 or 300 nM of paclitaxel on day 7 and fixed on day 12. A–D) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in the absence or presence of NGF (30 ng/ml for 12 days in cultures) and paclitaxel (300 nM from day 7 to day 12 in culture) and fixed on day 12. Each column represents the mean ± SEM of total neurite length (E) or total segments (F). An (*) indicates a significant difference in neurite length or branching 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=13–17) using a two-way ANOVA and Tukey's post-hoc test.

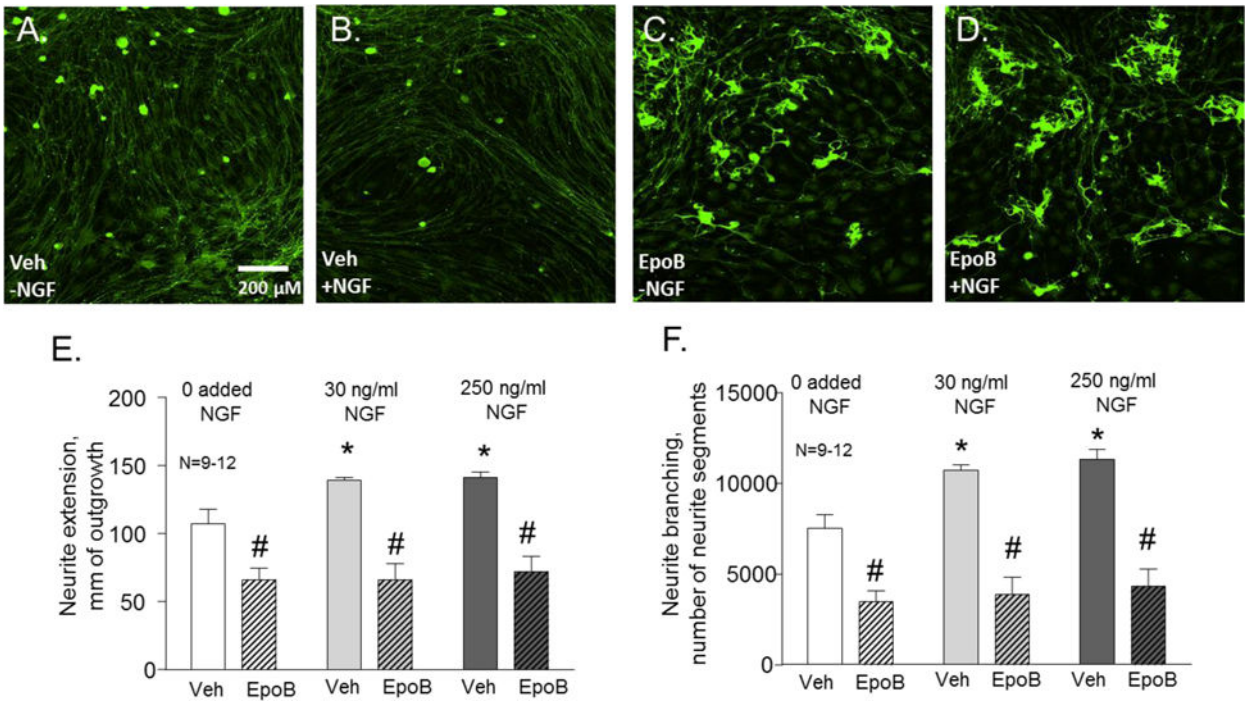


**Figure 6.**

EpoB and NGF 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 or 30 nM of EpoB on day 7 and fixed on day 12. A–D) Representative images of PGP9.5 immunoreactivity (green fluorescence) in cultures grown in the absence or presence of NGF (30 ng/ml for 12 days in cultures) and EpoB (30 nM from day 7 to day 12 in culture) and fixed on day 12. Each column represents the mean ± SEM of total neurite length (E) or total segments (F). An (*) indicates a significant difference in neurite length or branching 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.

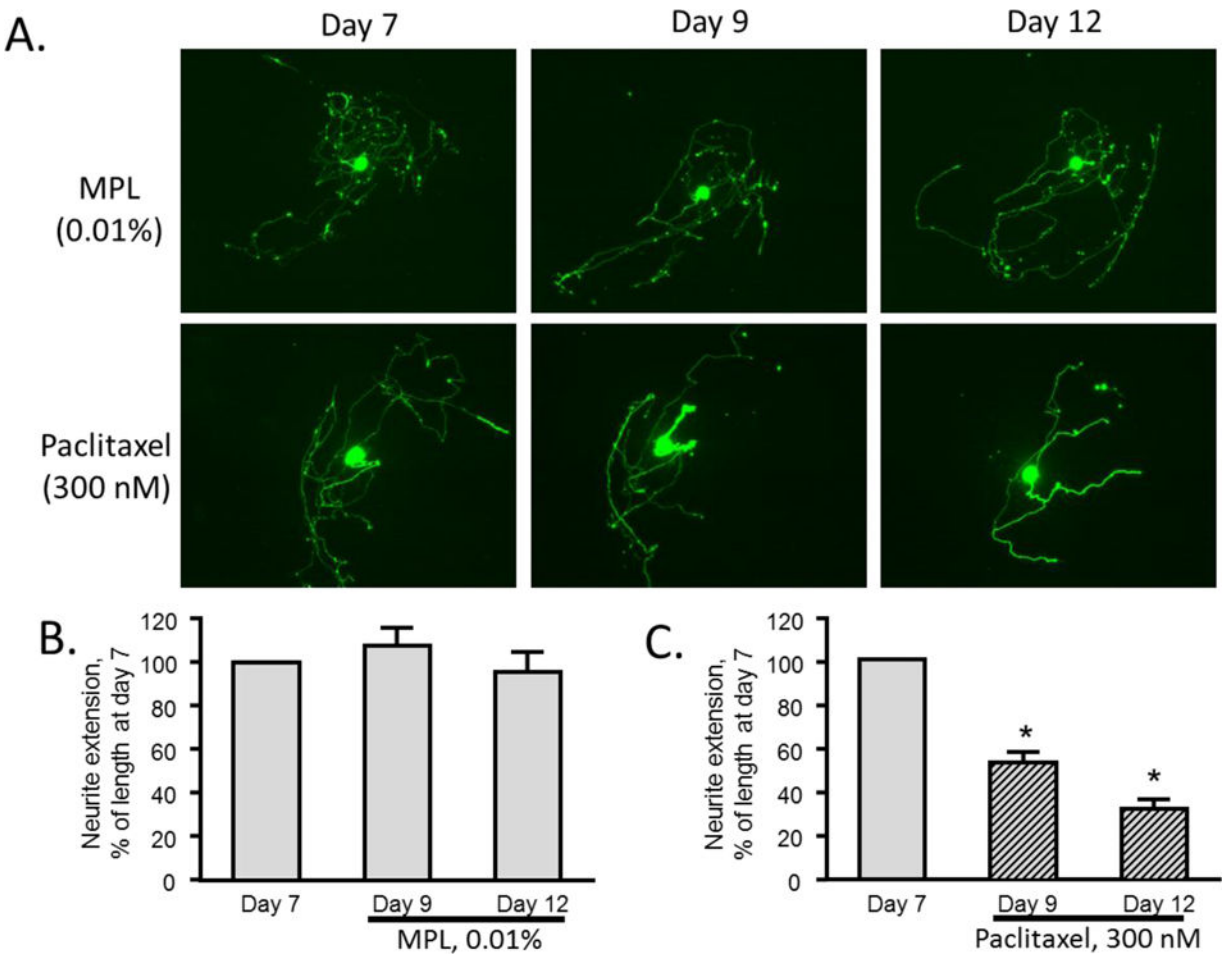


**Figure 7.**

Exposure of sensory neurons in culture to 300 nM paclitaxel decreases the length of neurites, as measured in individual neurons. Sensory neurons were grown in 30 ng/ml NGF, transfected with cDNA for EGFP via gene gun on day 2 in culture, and treated with vehicle or 300 nM paclitaxel from day 7 to day 12. A. Representative images of EGFP immunofluorescence (green) in cultures grown in vehicle (top row) or paclitaxel (bottom row) from day 7 to day 12. B and C. Quantification of neurite extension. Each column represents the mean ± SEM of the length of neurites, reported as % change relative to that on day 7 in neurons treated for 5 days with vehicle (B) or paclitaxel (C). An (*) indicates a significant difference in neurite extension from day 7 ($p<0.05$, N=16) using a one way-ANOVA and Tukey's post-hoc test.