PACLITAXEL ALTERS THE FUNCTION OF THE SMALL DIAMETER SENSORY NEURONS

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DEDICATION

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ABSTRACT

Neilia Gracias

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Although paclitaxel is a commonly used anti-neoplastic agent for the treatment of solid tumors, therapy often results in a number of side effects, the most debilitating of which is peripheral neuropathy. Peripheral neuropathy is defined as a pathology of peripheral nerves, and, depending on the type of nerves damaged, the neuropathy can be classified as sensory, motor, or autonomic neuropathy. In the case of peripheral neuropathy induced by paclitaxel, the symptoms are experienced in the extremities and are sensory in nature. Patients undergoing chemotherapy with paclitaxel often report sensory disturbances such as burning, tingling, numbness, a diminished sensation to pain and temperature, loss of vibration sense, loss of proprioception, and loss of deep tendon reflexes. Electrophysiological abnormalities including decreased sensory nerve action potential amplitude and conduction confirm damage to large myelinated fibers. However, the involvement of damage to small diameter sensory neurons in the etiology of paclitaxel – induced peripheral neuropathy is still controversial. Therefore, experiments were performed to determine if paclitaxel alters the function of small diameter sensory neurons and to examine the mechanisms responsible for the change in function.

Sensory neuron mediated vasodilatation in paclitaxel – injected animals was examined as an indirect measure of calcitonin gene related peptide (CGRP) release and therefore of sensory neuron function. CGRP release was also directly measured from central terminals in the spinal cord. To examine mechanisms of paclitaxel – induced sensory neuron damage, CGRP release and neurite length was examined in paclitaxel – treated sensory neurons in culture. The results demonstrate that (1) paclitaxel decreases the ability of small diameter sensory neurons to produce an increase in blood flow in the skin; (2) paclitaxel alters the release of CGRP from the small diameter sensory neurons; (3) paclitaxel causes the neuronal processes of isolated sensory neurons to degenerate. This dissertation provides novel information showing that paclitaxel alters the function of small diameter sensory neurons and thus provides a better understanding of the mechanisms mediating the sensory disturbances characteristic of peripheral neuropathy resulting from chemotherapy with paclitaxel.

Michael R. Vasko, Ph.D, Chairperson

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AM	Adrenomedullin
ASIC	Acid sensing ion channel
CGRP	Calcitonin gene related peptide
CHO	Chinese hamster ovary
CL	Calcitonin receptor like receptor
CrEL	Cremophor EL
CsA	Cyclosporin A
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
DRR	Dorsal root reflexes
EGCG	Epigallocatechin 3-gallate
EGFP	Enhanced green fluorescent protein
EMG	Electromyography
ER	Endoplasmic reticulum
EtOH	Ethanol
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HRP	Horseradish peroxidase
iCGRP	Immunoreactive CGRP
MES	(N-morpholino) ethanesulfonic acid
MPL	1-Methyl-2-pyrrolidone
mPTP	Mitochondrial permeability transition pore
mtDNA	Mitochondrial DNA
NO	Nitric oxide
PAD	Primary afferent depolarization
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGP 9.5	Protein gene product 9.5
PVDF	Polyvinylidene fluoride
RAMP	Receptor activity-modifying *protein*
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
TBST	Tris buffered saline with 0.1% Tween-20
TPU	Tissue perfusion units
TRP	Transient receptor potential
VGCC	Voltage gated calcium channel
VGSC	Voltage gated sodium channel

INTRODUCTION

When the nerves that innervate peripheral tissues are damaged, the resulting signs and symptoms are referred to as peripheral neuropathy. Neuropathies are classified as autonomic, motor or sensory depending on the nerves affected and the symptoms experienced. Autonomic neuropathies result from a dysfunction of the sympathetic or parasympathetic nerves that regulate the function of visceral organs, vascular smooth muscle, endocrine and exocrine glands, the immune system, and soft tissues. Damage to these nerves results in impairment of the cardiovascular system, the gastrointestinal system, the urogenital system, and the thermoregulatory system, among others. The symptoms resulting from malfunction of the autonomic nerves are varied and include but are not limited to loss of bladder control, disturbances in heart rate, abnormal sweating, nausea, and vomiting. Autonomic nerve dysfunction can sometimes be life threatening, especially if cardiovascular or respiratory function is disrupted (Novak et al., 2001; Freeman, 2005, 2007). Motor neuropathies result from altered function of the somatic nerves that control voluntary movement of skeletal muscles. Consequently, the associated signs and symptoms of motor neuropathy are loss of muscle control, painful cramps, muscle atrophy, loss of dexterity, and muscle twitching or fasciculation. Sensory neuropathies result from dysfunction of the primary afferent nerves that transmit information such as vibration, temperature, pain, touch, and limb

positioning. The most commonly experienced symptoms associated with sensory neuropathy include but are not limited to burning, tingling, loss of propioception (position sense), loss of vibration sense, loss of deep tendon reflexes and sometimes pain. Peripheral neuropathy can be debilitating even in the absence of pain. Patients often report difficulty in walking resulting from impaired sense of foot position and skin lacerations resulting from kitchen accidents secondary to impaired position sense in the hands (Dougherty et al., 2004). There is currently no treatment to prevent the loss of sensation that occurs with sensory neuropathies.

Causes of peripheral neuropathy

Peripheral neuropathy may be either inherited (e.g. Charcot-Marie-Tooth disease) or acquired. Acquired neuropathy can be caused by physical injury to a nerve resulting from traumatic injury, sports-related activities, and nerve compression secondary to tumors or by slipped disks between vertebrae. Peripheral neuropathies have also been linked to systemic diseases or metabolic disorders including diabetes, deficiencies of vitamin B1, B6, and B12, kidney disorders resulting in accumulations of toxic substances that can affect nerves, and hormonal imbalances (Steiner et al., 1988; D'Amour and Butterworth, 1994; Sugimoto et al., 2000; Kucera et al., 2002; Koike et al., 2003; Koike and Sobue, 2006). Some bacterial and viral infections directly cause peripheral nerve damage. Viruses like herpes varicella-zoster, Epstein-Barr virus,

cytomegalovirus, and human immunodeficiency virus (HIV) can severely damage sensory nerves and cause sharp attacks of pain (Fujii et al., 1982; Rubin and Daube, 1999; Simpson, 2002; de Freitas, 2007; Zhou et al., 2007). Leprosy and Lyme disease, which occur after bacterial infections, are also characterized by extensive peripheral nerve damage (Ooi and Srinivasan, 2004; Mygland et al., 2006). Alternatively, bacterial and viral infections can cause indirect nerve damage by eliciting immune responses that attack nerves. Such neuropathies are described as autoimmune neuropathies (Van Brakel, 2000; Ooi and Srinivasan, 2004). Autoimmune neuropathies can be induced by antibodies against the Hu antigen on the dorsal root ganglion, GM1 antigens that are concentrated at the nodes, myelin or presynaptic calcium channels at the neuromuscular junction resulting in interference with release of acetylcholine (Ho et al., 1998).

Of greatest importance to this study is chemotherapy-induced peripheral nerve damage resulting from exposure to chemotherapeutic agents. These neuropathies can be severe and sometimes interfere with the course of therapy (Roelofs et al., 1984; Boogerd et al., 1990; Siegal and Haim, 1990; Postma et al., 1999; Peltier and Russell, 2002; Verstappen et al., 2003b; Verstappen et al., 2005; Mielke et al., 2006; Peltier and Russell, 2006; Scripture et al., 2006; Argyriou et al., 2007). The type and the severity of the neuropathy depend on the drug, the dose intensity, the cumulative dose, frequency of administration, and pre-

existing conditions like diabetes or alcoholism (Cata et al., 2006b; Windebank and Grisold, 2008). With most drug-induced neuropathies, the symptoms can persist for weeks to months after termination of therapy and in some instances the neuropathy is irreversible. Discussed below is a list of chemotherapeutic agents that are frequently associated with a high incidence of peripheral neuropathy in patients. It should be noted that although each of the following class of drugs differ in their mechanism of action, axonal degeneration is frequently observed in nerve biopsies of patients and animal models.

Anti-retroviral drugs

Of all anti-retroviral agents, the nucleoside reverse transcriptase inhibitors (NRTIs) zalcitabine, didanosine, and stavudine are frequently associated with a painful sensory neuropathy that is characterized by burning, numbness, tingling, and stiffness in the hands and the feet (Reliquet et al., 2001; Simpson, 2002). Zalcitabine, didanosine, and stavudine are phosphorylated by thymidine kinases and the phosphorylated NRTIs compete with the natural substrates for HIV reverse transcriptase to inhibit genomic DNA replication. They also decrease mitochondrial DNA (mtDNA) replication by inhibiting the enzyme γ-DNA polymerase. In virus-infected cells, there is inhibition of viral replication while in neuronal cells inhibition of mtDNA results in mitochondrial damage (Dalakas, 2001). Indeed, human biopsies of the

sciatic nerve from patients treated with zalcitabine revealed enlarged mitochondria with extensive vacuolation as well as axonal degeneration and myelin degradation (Dalakas, 2001; Dalakas et al., 2001; Reliquet et al., 2001).

Platinum containing compounds

The platins (cisplatin, carboplatin and oxaliplatin) are commonly prescribed alone or in combination with paclitaxel for the treatment of cancers of the breast, ovary, testes, lung, and bladder (Lokich and Anderson, 1998; Go and Adjei, 1999) and produce a dose dependent sensory neuropathy. The antineoplastic activity of cisplatin and carboplatin is related to their ability to form platinum-DNA adducts resulting in apoptosis of mitotic cells secondary to inhibition of DNA replication and transcription. While the neurotoxic effects of the platinum containing drugs correlate with the formation of platinum DNA adducts in dorsal root ganglion neurons (Krarup-Hansen et al., 1999; Ta et al., 2006), cisplatin has been shown to cause an increased production of reactive oxygen species (Jiang et al., 2008). Platinum-induced neuropathy is predominantly sensory and is characterized by numbness, tingling, paresthesia, reduced vibration sense, loss of proprioception, reduced deep tendon reflexes, lack of coordination, and gait disturbance (Verstappen et al., 2003a). These sensory symptoms are consistent with the observation that high concentrations of cisplatin are observed in the

dorsal root ganglion (DRG). Histological examinations of peripheral nerves and DRGs revealed degeneration of peripheral sensory nerve axons and atrophy of the dorsal root secondary to apoptosis in the DRG (Gregg et al., 1992; Krarup-Hansen et al., 1999).

In addition to crosslinking DNA, oxaliplatin interferes with axonal ion conductance and consequently alters neural excitability (Adelsberger et al., 2000; Ta et al., 2006). The alteration in neuronal excitability is a likely cause of the acute symptoms including paraesthesia, cold hypersensitivity, jaw and eye pain, and leg cramps which are observed within thirty minutes of infusion of the drug. The early symptoms completely disappear within a few days to a couple of weeks but reappear with every new course of the drug administered. In addition to acute symptoms, patients also develop also develop long lasting symptoms including paraesthesias, reduced vibration sense, loss of proprioception, reduced deep tendon reflexes, lack of coordination, and gait disturbance (Gent and Massey, 2001).

Vinca Alkaloids

The vinca alkaloids include the naturally occurring compounds vincristine and vinblastine, and the semi-synthetic compounds such as vindesine and vinorelbine. Of these drugs, vincristine, prescribed for the treatment of non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia, and other solid tumors, is most commonly associated with peripheral

neuropathy. The vinca alkaloids bind to tubulin and inhibit their polymerization into microtubules (Himes et al., 1976). Since microtubules are required for the formation of the mitotic spindle during mitosis, loss of microtubule function leads to cell cycle arrest and death. In neuronal cells, inhibition of microtubule function has been shown to inhibit fast axonal transport (Banks et al., 1971a; Dahlstrom and Heiwall, 1975; Samson et al., 1979). The most commonly observed symptoms of the neuropathy are paraesthesia, pain in fingers and toes, hyperesthesia, and weakness of the extensor muscles of the wrist and dorsiflexors of the toes (Peltier and Russell, 2002; Dougherty et al., 2007). Vincristine treatment frequently causes constipation, paralytic ileus, loss of bladder control, orthostatic hypotension and disturbed heart rate associated with autonomic neuropathy (Legha, 1986). Sural nerve biopsies obtained from vincristine-treated patients showed axonal degeneration with segmental demyelination (McLeod and Penny, 1969). Results from electrophysiological studies suggest that the axonal degeneration occurs in a distal to proximal manner (Casey et al., 1973).

Taxanes

Taxanes are a class of drugs that include paclitaxel and docetaxel. Like the vinca alkaloids, the taxanes also inhibit microtubule function, but do so by initially activating microtubule polymerization, and subsequently preventing their de-polymerization (Schiff et al., 1979; Manfredi et al.,

1982; Horwitz et al., 1986) resulting in cell cycle arrest in dividing cells. Peripheral neuropathy is more frequently encountered with paclitaxel than with docetaxel chemotherapy. Paclitaxel and docetaxel differ in their pharmacokinetics and in their pharmacodynamic profiles, which could account for dissimilarities in their clinical activity and associated toxicity (Rowinsky, 1997; Gligorov and Lotz, 2004). The experiments described in this thesis were performed to understand the effects of paclitaxel on sensory neurons, since this is the prototype drug of the taxane class.

Toxicities associated with paclitaxel treatment

Paclitaxel was originally derived from the bark of the western yew tree *Taxus brevifolia*. It is effective against carcinomas of the ovary, breast, lung, head, and neck and is often used as the first line of treatment in patients diagnosed with these kinds of cancer. In Phase I studies, administration of paclitaxel was complicated by the appearance of life-threatening type I anaphylactic reactions like urticaria, dyspnea with bronchospasm, and hypotension. The hypersensitivity reaction was attributed to the polyoxyethylated castor oil (CrEL) vehicle, which is required to maintain the lipophilic drug in solution during intravenous infusion (Wiernik et al., 1987b). Premedication with corticosteroids like dexamethasone and H1 and H2 antagonists, however, decreases the incidence of hypersensitivity reactions (Wiernik et al., 1987a; Peereboom et al., 1993).

Paclitaxel also causes neutropenia and leucopenia. These toxicities can be reversed by decreasing the infusion duration from 24 hrs to 3 hrs and co-administering granulocyte colony-stimulating factor (Sarosy et al., 1992b; Reichman et al., 1993b; Reichman et al., 1993a; Seidman et al., 1993b; Seidman et al., 1993a). However, decreasing the infusion time to 3 hours increases the frequency of peripheral neuropathy, the neurologic side effect of paclitaxel (Ohtsu et al., 1995; Mielke et al., 2005).

Peripheral neuropathy

Clinical observations

Peripheral neuropathy is a dose-limiting toxicity associated with paclitaxel and is encountered at doses greater than 200 mg/m^2 (or 6 mg/kg in an individual 72 inches tall and weighing 100 lbs) (Wiernik et al., 1987b; Cliffer et al., 1998). About 55% of patients receiving a dose of 200 mg/m^2 of paclitaxel per infusion developed neuropathy compared with only 30% of patients receiving 175 mg/m^2 (Lipton et al., 1989). Symptoms such as burning, tingling, and numbness usually appear within 24 to 72 hours, and resolve within a week after infusion. (Lipton et al., 1989; Iniguez et al., 1998). The symptoms recur and increase in severity with successive courses of infusion and eventually progress to loss of propioception, decreased thermal and vibratory threshold, and loss of deep tendon reflexes (Wiernik et al., 1987b; Lipton et al., 1989; Sarosy et al., 1992a;

Forsyth et al., 1997; Iniguez et al., 1998; Loprinzi et al., 2007). For many patients, the symptoms are most pronounced in the tips of the fingers and the toes; most experience difficulty in performing routine tasks such as writing or buttoning a shirt (Dougherty et al., 2004). Motor neuropathy is evident only at doses of 500 mg/m^2 or higher (Iniguez et al., 1998). Paclitaxel-induced peripheral neuropathy is also dependent on the frequency of administration and patients receiving weekly injections of 110 mg/m^2 as opposed to 175 mg/m^2 every three weeks developed painful symptoms in the hands and the feet. Briasoulis et al. showed that using the weekly schedule of 110 mg/m^2, the maximum cumulative dose that is tolerated by patients is 1500 mg/m^2 and the symptoms persist for 1-6 months after completion of therapy (Briasoulis et al., 2002).

Neurophysiologic studies conducted in a patient who received 34 paclitaxel courses of 275 mg/m^2 showed a decrease in the amplitude of sural and tibial nerve action potentials (Sahenk et al., 1994). Lipton et al. also observed a decrease in amplitude and conduction velocity of the sural nerve (Lipton et al., 1989).

Myalgia, or muscle pain, has also been observed in patients receiving greater than 250 mg/m^2 of paclitaxel every three weeks. Symptoms are observed during infusion, immediately after infusion, or 2 - 3 days after treatment but usually resolve within a week. Myopathies, neuromuscular diseases from myofiber dysfunction, are very rarely noted and have only been seen in patients receiving high doses of paclitaxel

(300 - 350 mg/m^2), especially when granulocyte colony stimulating factor was co-administered (Loprinzi et al., 2007). In addition, deficits in cognitive function has not been observed after treatment with the microtubule stabilizing drug, as is commonly observed with cisplatin-induced neurotoxicity. Furthermore, it is interesting that while autonomic dysfunction is frequently observed following treatment with the vinca alkaloids, the evidence that paclitaxel affects the autonomic nervous system is still inconclusive (Argyriou et al., 2005).

Although the mechanisms mediating the neuropathy are not known, the decrease in action potential amplitude or conduction velocity suggest that paclitaxel causes axonal damage. Indeed, sural nerve biopsy of a patient receiving paclitaxel showed a significant reduction in the myelinated and unmyelinated fiber density and prominent axonal atrophy (Sahenk et al., 1994). The mechanisms mediating this degeneration are not known.

Studies in animals

To study the mechanisms mediating paclitaxel-induced peripheral neuropathy, several investigators have injected paclitaxel systemically in animals and examined neurophysiologic function, sensory behaviors, and morphological changes in sensory nerves.

Investigators have observed both hypernociception and hyponociception in animals that were chronically treated with paclitaxel,

and the response observed seems dose-dependent. Animals receiving a cumulative dose of up to 10 mg/kg paclitaxel demonstrated a hypernociceptive response to mechanical stimuli (Authier et al., 2000; Dina et al., 2001; Polomano et al., 2001; Matsumoto et al., 2006; Peters et al., 2007; Nishida et al., 2008) but in one study using 25 mg/kg paclitaxel, a decreased response to noxious mechanical stimulation was observed (Cavaletti et al., 1997). The responses to thermal stimuli also varied depending on the dose of paclitaxel administered and the frequency of administration. When rats were given a cumulative dose of cumulative dose of 25 mg/kg (approximately 175 mg/m^2) or higher, they showed a decreased sensitivity (antinociception) to thermal stimuli (Apfel et al., 1991; Campana et al., 1998; Authier et al., 2000), while animals receiving a cumulative dose of 4 to 12 mg/kg had an increased sensitivity (hypernociception) to thermal stimuli (Dina et al., 2001; Polomano et al., 2001; Matsumoto et al., 2006).

Light and electron microscopy was used to examine degenerative changes in the sciatic nerve of paclitaxel-treated animals (Cavaletti et al., 1995; Authier et al., 2000; Persohn et al., 2005). In electron micrographs of sciatic nerves from animals given cumulative doses ranging from 16 to 80 mg/kg paclitaxel, aggregated microtubules were observed in sciatic nerves, and in some cases they were seen surrounding mitochondria (Cavaletti et al., 1995; Cavaletti et al., 1997; Authier et al., 2000; Persohn et al., 2005). In another study, animals receiving multiple injections of 2

mg/kg paclitaxel showed a decrease in the density of nerve fibers in the epidermis (Melli et al., 2006; Siau et al., 2006), and this decreased density has been observed in other painful sensory neuropathies (Holland et al., 1997; Polydefkis et al., 2002; Pittenger et al., 2004; Lauria et al., 2005). Other investigators observed enlarged or swollen mitochondria in electron micrographs of sciatic or saphenous nerves (Cavaletti et al., 1997; Flatters and Bennett, 2006). Overall, axonal degeneration in sciatic nerves of rats is generally associated with cumulative doses of 16 mg/kg or greater; axonal damage was not observed at cumulative doses of 8 mg/kg (Cavaletti et al., 1997; Flatters and Bennett, 2006). Decreased sensory nerve conduction velocity in rats treated with paclitaxel often correlated with axonal degeneration of the sciatic nerves (Apfel et al., 1991; Cavaletti et al., 1995; Cavaletti et al., 1997; Cliffer et al., 1998; Authier et al., 2000).

Studies on isolated sensory neurons

Few investigators have examined the effects of paclitaxel on the length of neurites in isolated sensory neurons. In some studies paclitaxel (6 to 94 nM for 48 hours) prevented outgrowth of neurites in rat DRG explants (Konings et al., 1994). In another study, paclitaxel (7 to 350 nM for 24 hours) also inhibited neurite outgrowth in isolated chick DRG neurons (Letourneau and Ressler, 1984). In contrast, other investigators demonstrated that exposure to 28 nM paclitaxel for 24 hours causes the

degeneration of already established neurites in embryonic rat DRG neurons (Melli et al., 2006; Yang et al., 2009).

Potential effects of paclitaxel on sensory neurons

Paclitaxel binds to the β subunit of tubulin and promotes the assembly of microtubules that are resistant to depolymerization by agents that promote their disassembly, namely cold temperature (4ºC) and $CaCl_2$ (Schiff et al., 1979; Kumar, 1981; Schiff and Horwitz, 1981; Manfredi et al., 1982; Horwitz et al., 1986). Thus paclitaxel treatment results in the accumulation of abnormal microtubules in the cells (Kumar, 1981; Manfredi et al., 1982). In dividing cells, this stabilization of microtubules results in cell cycle arrest in its G1 or M phase and apoptosis (Liebmann et al., 1993). Microtubule stabilization by paclitaxel has been observed in mouse fibroblast cells, synaptosomes prepared from rat forebrains, DRG neurons, and a number of cell lines (Schiff and Horwitz, 1980; Masurovsky et al., 1981; Burgoyne and Cumming, 1983).

The role of microtubule stabilization in the etiology of peripheral neuropathy is still debated. In sural nerve biopsies from patients with paclitaxel-induced neuropathy, no abnormal aggregates of microtubules in the axons and Schwann cells were observed (Wiernik et al., 1987b; Lipton et al., 1989). Similarly, morphological examination of the saphenous nerve from paclitaxel-injected animals (cumulative dose, 8 mg/kg) showed no abnormalities in microtubules despite the observation

of hypernociception in these animals (Flatters and Bennett, 2006). Microtubule accumulation was observed in rat sciatic nerve only when the cumulative dose of paclitaxel in animals exceeded 16 mg/kg (Cavaletti et al., 1997). These results support the notion that microtubule stabilization is not the mechanism that accounts for peripheral neuropathy.

Apart from stabilizing microtubules, paclitaxel has been shown to affect mitochondria. In neuroblastoma cells, paclitaxel (1 - 100 μM) causes mitochondrial swelling, induces opening of the mitochondrial permeability transition pore (mPTP) and cytochrome release (Andre et al., 2000; Andre et al., 2002). The mitochondrial permeability transition (MPT) refers to the depolarization of mitochondria that occurs as a result of oxidative stress or calcium overload in the cell. A major consequence of the MPT is uncoupling of oxidative phosphorylation resulting in decreased production of ATP and further damage to the cell (Halestrap et al., 2002). In the saphenous nerve of rats, chronic administration of paclitaxel causes mitochondrial swelling. Mitochondrial damage can cause increase in intracellular concentration of free radicals. Indeed production of reactive oxygen species has been observed in neuroblastoma cells and other non-neuronal cell types following treatment with paclitaxel (Andre et al., 2002; Wang et al., 2004; Fawcett et al., 2005; Ramanathan et al., 2005; Alexandre et al., 2006; Alexandre et al., 2007; Kim et al., 2008). While a correlation between production of reactive oxygen species and opening of the mPTP has been demonstrated in neuroblastoma cells after

exposure to paclitaxel (Andre et al., 2000), there is no evidence that the microtubule-stabilizing drug alters mitochondrial function or increases free radicals in sensory neurons and both of these events remain to be explored.

Function of microtubules in neuronal cells

Microtubules are formed by assembly of α/β tubulin heterodimers at a fast growing end, which is designated as the “plus end”, while tubulin subunits are continually lost from the opposite slow growing or the “minus end”. This continuous lengthening and shortening of microtubules defines the direction of movement and facilitates the directional transport of proteins along axons from the cell body. Accordingly, inhibition of microtubule function by drugs like colchicine, vincristine, vinblastine, and paclitaxel can potentially interfere with axonal transport. Indeed, Banks et al., observed an inhibition of transport of noradrenaline-containing vesicles by colchicine and vinblastine in an *in vitro* preparation of cat hypogastric nerve/inferior mesenteric ganglion; this inhibition was accompanied by a decrease in the number of axonal microtubules (Banks et al., 1971b; Banks et al., 1971a). A similar inhibition of acetylcholine transport in cholinergic motor axons was observed after disruption of microtubules by colchicine or vinblastine (Heiwall et al., 1976; Heiwall et al., 1978). While paclitaxel’s mechanism of microtubule inhibition is different from that of colchicine and vinblastine, paclitaxel nonetheless inhibits the transport of proteins in DRG neurons and in the sciatic nerve *in vitro* (Nakata and Yorifuji, 1999; Theiss and Meller, 2000; Shemesh and Spira, 2010).

In neurons, microtubules are especially important for the anterograde transport of mitochondria from the soma to regions of high

ATP consumption including the nodes of Ranvier, the tips of growth cones, and synaptic terminals (Morris and Hollenbeck, 1995; Gallo and Letourneau, 1999; Ligon and Steward, 2000). Mitochondrial localization at synapses is crucial for buffering the large increases in intracellular calcium that occur during depolarization. Mitochondria also provide ATP as an energy source for exocytosis and for vesicle recycling. Decreased density of mitochondria at synapses is thought to occur early in the progression of neurodegenerative diseases. Thus it seems possible that disruption of transport of mitochondria by paclitaxel could be a mechanism for drug-induced peripheral neuropathy. Indeed, disruption of microtubule function causes accumulation of mitochondria in the cell body suggesting that anterograde transport of mitochondria is inhibited (Morris and Hollenbeck, 1995; Wang et al., 2003a). Treatment of hippocampal neurons with the microtubule targeting drug vinblastine causes mitochondria to accumulate away from the plasma membrane. Consequently NMDA-evoked larger $[Ca2+]_i$ increases in vinblastine treating neurons suggesting that localization of mitochondria at the site of calcium influx Is important for sequestration for calcium (Wang et al., 2003a). One inference from these data is that inhibition of microtubules in primary afferent sensory neurons by paclitaxel inhibits transport of mitochondria to nerve endings and that this results in decreased buffering of intracellular calcium at the peripheral endings of sensory neurons.

Classification of sensory neurons

The DRGs contain the cell bodies of the primary afferent sensory neurons, and these neurons convey sensory information from the periphery to the spinal cord. The neurons are pseudounipolar: they have a single axon that bifurcates into a peripheral branch that terminates in the skin, muscle, or visceral organs, and a central branch that terminates in the spinal cord. The DRGs contain a heterogeneous population of neurons that relays a spectrum of sensory information including discriminative touch, pressure, nociception (signaling of tissue damage usually perceived as pain or itch), propioception (position in space) and temperature. As can be seen in Table 1, several criteria exist for the classification of sensory neurons.

Of primary importance to the current work are the Aδ and the C fibers that are activated by noxious thermal, mechanical, or chemical stimuli. While some of these sensory neurons are activated only by specific stimuli (only mechanical or only thermal), others respond to multiple stimuli (thermal, mechanical and chemical): these fibers are usually the C fibers and are commonly known as polymodal nociceptors (Besson, 1999). The C fibers constitute 80% of the axons terminating in the skin and together with the Aδ fibers, they are located mostly at the junction of the dermis and epidermis in close proximity with dermal blood vessels (Lumpkin and Caterina, 2007).

	Aα	Aβ	Aδ	C
Diameter of axons	15 - 20 μm	5 - 15 μm	1 - 5 μm	>1 mm
Myelination	Myelinated		Thinly myelinated	Unmyelinated
Conduction velocity of axons	72 - 120 m/s	42 - 72 m/s	12 - 36 m/s	0.5 - 1.2 m/s
Receptor type	Meissner's corpuscle, Merkel disk receptor, pacinian corpuscle, Ruffini endings		Bare endings	Bare endings
Role in perception	Muscle tension, muscle length and velocity	Light touch, Pressure, form, texture, flutter, motion, stretch, vibration	Sharp, pricking pain, burning pain, skin cooling (25ºC), hot temperature (>45ºC)	Freezing pain, burning pain, cold temperature (<5ºC), skin warming (41ºC)

Table 1. Criteria for the classification of sensory neurons.

Although it is well accepted that peripheral neuropathy induced by paclitaxel results from impaired function of myelinated fibers, the involvement of small diameter sensory neurons is ambiguous. Based on symptoms reported by patients undergoing chemotherapy with paclitaxel, Dougherty et al., postulated that myelinated fiber function is impaired, while function of the unmyelinated C fibers is preserved (Dougherty et al., 2004). Additionally, studies in animals revealed pathology of myelinated fibers in electron micrographs (Cliffer et al., 1998), while no changes in the conduction velocity of C fibers were observed following systemic administration of paclitaxel (Dina et al., 2001). In contrast, a sural nerve biopsy from a patient receiving paclitaxel showed degeneration of myelinated and unmyelinated fibers (Sahenk et al., 1994). Furthermore, in rats treated with paclitaxel (cumulative dose 80 mg/kg) degeneration of myelinated and unmyelinated fibers was observed (Authier et al., 2000). Because the hypernociception observed following systemic administration with paclitaxel suggest involvement of the small diameter peptidergic fibers (lightly myelinated Aδ and unmyelinated C fibers), it is possible that the function of these fibers is altered after systemic administration of paclitaxel.

Molecular mechanisms of sensory transduction

The transduction of sensory stimuli is mediated by molecular structures or receptors expressed on the peripheral endings of sensory neurons. The cell surface receptors that are activated by sensory stimuli include a wide repertoire of ligand gated ion channels, G-protein coupled receptors, and receptors with tyrosine kinase activity (Lumpkin and Caterina, 2007). One such family of receptors is the ion channels belonging to the transient receptor potential family (TRP). Some TRP channels like TRPM8 and TRPA1 respond to cold temperatures i.e., they are activated by cooling from normal skin temperature (about 32ºC) to below 30°C and below 20°C, respectively (McKemy, 2005; Madrid et al., 2006; Bautista et al., 2007; Caterina, 2007; Dhaka et al., 2007). Another family of channels known as the acid sensing ion channels (ASICs) are also expressed on nociceptive neurons and activated by low pH ($\leq$ 6.6) (Krishtal, 2003).

Of major importance in pain perception is the non-selective cation channel TRPV1 that is expressed on a subset of Aδ and C fibers. Activation of this channel by heat (> 43ºC) or low pH (Caterina et al., 1997) excites small diameter sensory neurons and evokes nociceptive responses. TRPV1 can also be activated by the vanilloid capsaicin, which mimics the actions of a physiological stimulus or an endogenous ligand produced during tissue injury. Capsaicin is therefore a valuable pharmacological tool for the examination of TRPV1 function. Activation of

the TRPV1 channel by capsaicin, heat or protons results in an influx of calcium and sodium ions into neuron. Sometimes the depolarization induced by divalent cations is sufficient to induce opening of voltage gated sodium channels (VGSCs). Entry of sodium through VGSCs causes the neuron to fire action potentials. Propagation of the action potentials through the axon to the central terminals in the spinal cord induces opening of the voltage gated calcium channels (VGCC) in the nerve terminals in the spinal cord. The resultant influx of calcium ions via VGCCs causes release of neurotransmitters like CGRP, substance P, and glutamate in the spinal cord.

While release of neurotransmitters in the spinal cord requires propagation of action potentials along primary afferent fibers, release of CGRP and SP from the peripheral terminals of the sensory neuron can occur in the absence of action potentials, i.e. the depolarization produced by influx of calcium and sodium ions in the periphery is sufficient to cause neurotransmitter release (Maggi, 1991). Because CGRP and SP can be released from central terminals as well as peripheral terminals, they perform dual functions as discussed below. Therefore examination of release of the neuropeptides from sensory neurons can be used as an indicator of sensory neuron function.

Dual function of CGRP: as a neurotransmitter and vasodilator

CGRP is a 37-amino acid peptide produced by alternative splicing of the calcitonin gene. It belongs to a family that also includes adrenomedullin (AM) and amylin. Two forms of CGRP exist: α-CGRP and β-CGRP. The two forms of CGRP differ from each other by 3 amino acids, although β-CGRP is expressed from a separate gene that does not produce calcitonin. Both forms of CGRP are widely distributed in the central and the peripheral nervous system and they display very similar biological activities. Of the two CGRP isoforms, α-CGRP is abundant in sensory neurons, while β-CGRP is expressed in enteric neurons and in the pituitary gland (Brain and Grant, 2004).

CGRP is synthesized in the cell bodies of sensory neurons and transported in large dense core vesicles to the nerve terminals (Kashihara et al., 1989). In the dorsal horn of the spinal cord, CGRP-like immunoreactivity is detected in the tract of Lissauer, and the presynaptic terminals in laminae I, II, and V of the spinal cord. Dorsal rhizotomy causes approximately a 95% decrease in CGRP immunostaining in the dorsal horn, suggesting that the CGRP is localized to the presynaptic terminals of the primary afferent sensory neurons (Gibson et al., 1984). In the rat, approximately 46.5% of DRG neurons stain for CGRP. These neurons are a subset of the C and Aδ type (McCarthy and Lawson, 1990).

The receptor for CGRP is a G-protein coupled receptor (GPCR) consisting of two proteins: the calcitonin receptor-like receptor (CL), which is associated with G proteins, and a receptor activity modifying protein (RAMP). RAMP is important for membrane localization of the receptor and for conferring ligand specificity (McLatchie et al., 1998; Foord and Marshall, 1999). Three RAMPS have so far been identified: RAMP1, RAMP2, and RAMP3. The association of CL with RAMP1 is the CGRP1 receptor that is activated by αCGRP (Chakravarty et al., 2000; Brain and Grant, 2004), while association of RAMP2 or RAMP3 with CL are receptors for adrenomedullin (McLatchie et al., 1998; Chakravarty et al., 2000). The binding of CGRP to the CGRP1 receptor can be blocked by $CGRP_{8-37}$ (Chiba et al., 1989). CGRP also binds to another receptor (CGRP2) and this binding is not antagonsied by $CGRP_{8-37}$ (Hay, 2007). The molecular identity of CGRP2 is not known and will not be discussed further. The CGRP1 receptor is widely expressed in the brain, in the dorsal horn of the spinal cord, and on cell bodies of sensory neurons (van Rossum et al., 1997). Outside the nervous system, CGRP1 is expressed in the heart, lung, kidney, spleen, skeletal muscle, on lymphocytes, vascular smooth muscle cells, mast cells, and endothelial cells.

CGRP release can be evoked by stimuli that activate the small diameter sensory neurons (e.g. capsaicin, low pH, bradykinin and heat). When released from the central terminals in the dorsal horn, CGRP produces a modest depolarization in second order neurons (Ryu et al.,

1988a). CGRP also has an autocrine function: in cultured DRG neurons, CGRP elevates intracellular calcium and induces inward currents through voltage-gated TTX-resistant sodium channels (Natura et al., 2005). Additionally, it enhances excitatory synaptic transmission by increasing calcium currents in DRG neurons (Ryu et al., 1988b; Ryu et al., 1988c). Furthermore, CGRP increases the spontaneous release of glutamate in spinal cord slices (Kangrga et al., 1990; Kangrga and Randic, 1990). The effects of CGRP are thought to result from the stimulation of adenylyl cyclase and production of cAMP (Hirata et al., 1988; Crossman et al., 1990; Van Valen et al., 1990; Takhshid et al., 2006).

CGRP is also released from the peripheral terminals of small diameter sensory neurons during inflammation or by application of capsaicin (Kilo et al., 1997; Kress et al., 1999; Petho et al., 2004). Local depolarization induced by capsaicin or by low pH can trigger the release of CGRP via the influx of calcium (Maggi, 1991). Notably, release by both agents is insensitive to inhibitors of voltage-sensitive Ca^{2+} channels (VSCC), lidocaine, and to high concentrations of TTX, suggesting that the influx of calcium via TRPV1 is sufficient to cause local neurotransmitter release.

Axon reflexes also might contribute to the release of CGRP in the periphery. The peripheral endings of the Aδ and C fibers are highly branched. Action potentials resulting from activation of peripheral endings propagate centrally, but can sometimes propagate antidromically

(backwards) from the branch point to other peripheral branches, resulting in release of neuropeptides (Figure 1).

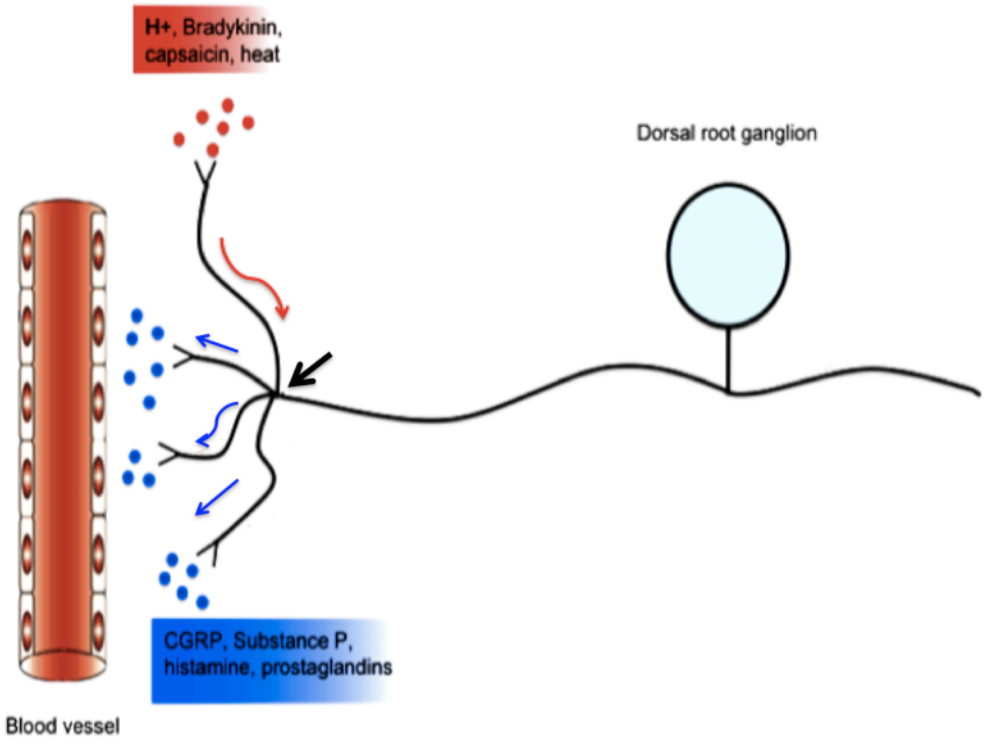

Figure 1. Proposed mechanism of axon reflex mediated vasodilatation. When stimuli such as low pH, bradykinin, capsaicin and heat activate a branch of the peripheral ending of a nociceptive neuron, the generated impulse propagates centrally to the local branch point (indicated by the black arrow). From here the impulse is reflected down the other branches to cause release CGRP on to effector organs e.g. the blood vessels.

A third potential mechanism mediating release of CGRP in the periphery involves antidromic stimulation of the Aδ and C fibers by action potentials that are triggered in the spinal cord (Lin et al., 1999; Weng and Dougherty, 2005). These action potentials are also known as dorsal root reflexes (DRRs) and they are initiated by excessive primary afferent depolarization (PAD) of the central terminals in the spinal cord. The mechanism of PAD involves the role of GABAergic interneurons. When action potentials initiated in the periphery trigger GABA release from interneurons, $GABA_A$ receptors on presynaptic terminals of sensory neurons are activated resulting in Cl^- efflux and depolarization. This depolarization then propagates to the periphery as a DRR (Willis, 1999).

Vasodilatation is an important biological effect of CGRP release from peripheral terminals of sensory neurons. When the peripheral endings of sensory neurons are activated by bioactive substances at the site of injury, the resulting release of CGRP and SP not only directly induce vasodilatation, but they also act on mast cells and other immune cells to release proinflammatory substances that contribute to inflammation i.e. redness and warmth (secondary to vasodilatation), swelling, and hypersensitivity (Richardson and Vasko, 2002). Vasodilatation results in enhanced delivery of different populations of cells of the immune system (e.g. monocytes, macrophages, T lymphocytes, and mast cells) to facilitate tissue repair and wound healing (Onuoha and Alpar, 2001; Irie et al., 2002; Li et al., 2007). CGRP-induced

vasodilatation has been demonstrated in many organs including the skin, lung, joints, heart, and kidney (Brain et al., 1985; Brain et al., 1986; Martling et al., 1988; Hughes and Brain, 1991; Brain et al., 1992; Escott and Brain, 1993; Chin et al., 1994; Escott et al., 1995). In these organs, CGRP expressing sensory neurons (Aδ and C fibers) are localized near blood vessels. Activation of the CGRP1 receptor, expressed on endothelial cells as well as vascular smooth muscle cells, (Hirata et al., 1988; Crossman et al., 1990) causes smooth muscle relaxation and subsequent vasodilatation.

CGRP produces vascular smooth muscle relaxation directly by stimulating receptors on vascular smooth muscle cells and indirectly by activating endothelial cells. In cerebral arteries from the cat and porcine coronary arteries CGRP causes relaxation in the absence of the endothelium, suggesting that CGRP acts directly on smooth muscle cells. Relaxation of these vessels correlates closely with increased intracellular cAMP in the cerebral artery of the cat and coronary arteries of the rat and pig (Edvinsson et al., 1985; Yoshimoto et al., 1998). In contrast, relaxation of the rat aorta requires an intact endothelium: the relaxation correlates with an increase in cAMP and cGMP in coronary arteries from rats and pigs, and is antagonized by nitric oxide synthase inhibitors, suggesting the involvement of nitric oxide from endothelial cells (Yoshimoto et al., 1998; Wisskirchen et al., 1999). Direct smooth muscle relaxation secondary to an increase in cAMP in these cells is thought to

result from activation of PKA, and subsequent phosphorylation of K^+ channels. Furthermore, Nelson et al. showed that arterial dilations by CGRP can be inhibited by glibenclamide, a K_{ATP} channel blocker (Nelson et al., 1990).

Methods to study peripheral neuropathy in animal models

As mentioned above, the end points currently used for the examination of paclitaxel-induced changes in peripheral nerve function in animals include nociceptive behavior, nerve biopsies, and electrodiagnostic testing. The major nociceptive behaviors that have been examined include measuring paw withdrawal in response to thermal or mechanical stimuli. A shorter withdrawal latency is interpreted as a hypernociceptive response to a stimulus and a longer latency is interpreted as anti-nociception. Electrodiagnostic studies are also often performed to assess nerve fiber integrity in animal models. There are two primary types of electrodiagnostic studies: nerve conduction studies and electromyography (EMG). Nerve conduction studies are performed to determine the shape, amplitude, latency, and velocity of action potentials conducted by a nerve and to help determine if the neuropathy is the result of axonal degeneration or demyelination. With axonal degeneration, the amplitude of the action potential is lower, while demyelination of nerves results in longer latencies and slow conduction velocities. EMG studies test spontaneous muscle fiber activity and activity after nerve stimulation. However, a limitation of electrodiagnostic studies is that they test the function of only the large, myelinated neurons and are not strong indicators of potential alterations in function of the small diameter sensory neurons (Holland et al., 1997; Griffin et al., 2001). In such cases, skin biopsies to determine intraepidermal or dermal fiber density can be

examined because the epidermis is richly innervated by small diameter fibers, namely the unmyelinated C fibers and the thinly myelinated Aδ fibers, both of which are easily distinguishable at the light microscope level after immunostaining for axonal markers (Holland et al., 1997; Periquet et al., 1999; Pittenger et al., 2004; Lauria et al., 2005; Sommer and Lauria, 2007; Tavee and Zhou, 2009). However, as described below, sensory neuron mediated cutaneous vasodilatation was used as an endpoint in the studies conducted in this thesis because it is a non-invasive method to assess small diameter sensory neuron function *in vivo*.

Measurement of vasodilatation in the study of paclitaxel-induced peripheral neuropathy

Peripheral neuropathy induced by paclitaxel encompasses symptoms that indicate dysfunction of all classes of sensory neurons. The loss of propioception, vibration sense, and deep tendon reflexes indicate that the function of the large fibers is compromised. The elevated thermal nociceptive threshold, burning, tingling, and numbness are symptoms which are characteristic of small fiber neuropathies (Tavee and Zhou, 2009). Because nerve conduction studies are not suitable for examining function of small diameter sensory neurons *in vivo*, the studies conducted for this thesis examined CGRP release indirectly - by measuring cutaneous vasodilatation. Cutaneous vasodilatation is mediated by small diameter sensory neurons that release peptides and whose peripheral

terminals are localized in the upper dermis close to blood vessels. For these studies sensory neurons were activated by capsaicin, the ligand for the TRPV1 ion channel (Caterina et al., 1997) since these channels are found on 60% of peptidergic neurons that innervate the skin (Hwang et al., 2005). Intradermal injection of capsaicin or electrical stimulation of the sciatic nerve has been used by some investigators to evoke vasodilatation in the skin (Koltzenburg et al., 1990; LeVasseur et al., 1990; Escott and Brain, 1993; Lin et al., 1999). There is precedent in the literature that diseases which result in peripheral neuropathy are associated with a decrease in vasodilatation after sensory nerve stimulation. These include diabetic neuropathy (Forst et al., 1997; Caselli et al., 2003; Kramer et al., 2004; Caselli et al., 2006), and port herpetic neuralgia in elderly patients (LeVasseur et al., 1990). Thus it seems possible that this phenomenon could occur after paclitaxel treatment. Consequently, examination of cutaneous vasodilatation is a relevant method to examine changes sensory neuron function secondary to treatment with paclitaxel.

SPECIFIC AIMS OF THE THESIS

The studies outlined in this thesis examine the effects of the cancer chemotherapeutic drug paclitaxel on small diameter sensory neurons and determine the mechanisms responsible for functional changes. Therefore, the aims of this thesis are:

1. To determine the effects of paclitaxel on release of CGRP from small diameter sensory neurons.
2. To determine if paclitaxel alters the length of neurites in isolated sensory neurons.

MATERIALS AND METHODS

Materials

Tissue culture plates were purchased from Becton-Dickinson (San Jose, CA). F-12 media, horse serum, antibiotics, and Normocin-OTM, annexin V-FITC, NuPAGE® Novex Bis-Tris Gels, NuPAGE® MES western blot running buffer, NuPAGE® sample reducing buffer, NuPAGE® MES transfer buffer, SeeBlue® Plus2 prestained standard, PVDF membranes and filter paper sandwiches were purchased from Invitrogen (Carlsbad, CA). TaqMan® Universal PCR master mix, MicroAmp® Fast 96 well plates for real time PCR, MicroAmp® optical adhesive film, probe and primer sets for relative quantification of mRNA for TRPV1, $Na_v1.9$, and the endogenous control GAPDH were purchased from Applied Biosystems (Foster City, CA). Reagents for protein quantification (bovine IgG protein and Bradford reagent), and iScript^{TM} cDNA synthesis kit, supplies for biolistic transfection of neurons, including tubing, gold, polyvinylpyrrolidone (PVP), tubing Prep Station, Helios Gene Gun, Gene Gun Barrels and Cartridge holders were purchased from Bio-Rad Laboratories (Hercules, CA). High grade dehydrated ethanol for biolistic transfection of sensory neurons was ordered from Spectrum Chemical MGF. CORP. (Gardena, CA). CGRP for radioimmunoassay and for vasodilatation experiments was purchased from Tocris Bioscience (Ellisville, MO). Rat (Tyr27)-$\alpha CGRP_{27-37}$ for radioimmunoassay was

purchased from Bachem (Torrance, CA). Radiolabeled NaI (^{125}NaI) for iodination of $CGRP_{27-37}$ and Western Lightning® *Plus*-ECL Enhanced Chemiluminescence substrate were purchased from Perkin-Elmer (Shelton, CT). Anti-goat TRPV1 antibody and blocking peptide were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HRP conjugated donkey anti-goat IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Donkey serum was purchased from Millipore (Billerica, MA). Radiographic film was purchased from RPS imaging (Michigan City, IN). PrepEase® RNA Spin Kit for extraction of RNA was purchased from USB corporation (Cleveland, OH). Trevigen CometSlides® for COMET assay were purchased from Trevigen (Gaithersburg, MD). Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Experimental animals

The Animal Care and Use Committee at Indiana University School of Medicine, Indianapolis, IN approved all procedures used in these studies. Adult male Sprague Dawley rats were purchased from Harlan (Indianapolis, IN). Whenever required, rats were housed in groups in a light controlled room (light from 6:00 to 19:00) at a constant temperature of 22^{o}C. All animals were allowed to acclimate for at least 3 days after delivery and before administering injections. Food and water were

available *ad libitum*. Animals for DRG harvests were sacrificed on the day they were delivered.

Paclitaxel administration for *in situ* experiments

To study the effects of paclitaxel on sensory neuron function *in vivo*, the injection schedule established by Polomano et al., was used (Polomano et al., 2001). This schedule was chosen because it produces a change in sensory behavior without any overt signs of toxicity. Paclitaxel was initially reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/ml, and aliquots were stored at -20°C. The drug was further diluted to 1mg/ml in phosphate buffered saline so that the final concentration of DMSO was 10%. Male Sprague Dawley rats (175 to 180 grams) were weighed prior to administering intraperitoneal injections of 1 mg/kg, 2 mg/kg, or 4 mg/kg paclitaxel every other day for a total of 4 doses. Vehicle-injected animals received intraperitoneal injections of 10% DMSO in phosphate buffered saline (PBS). Rats belonging to different treatment groups were housed in different cages. Experiments were performed one week after the last injection. As seen in Figure 2, paclitaxel-injected rats gained weight normally. On the day of the experiment, animals weighed 250 to 260 grams.

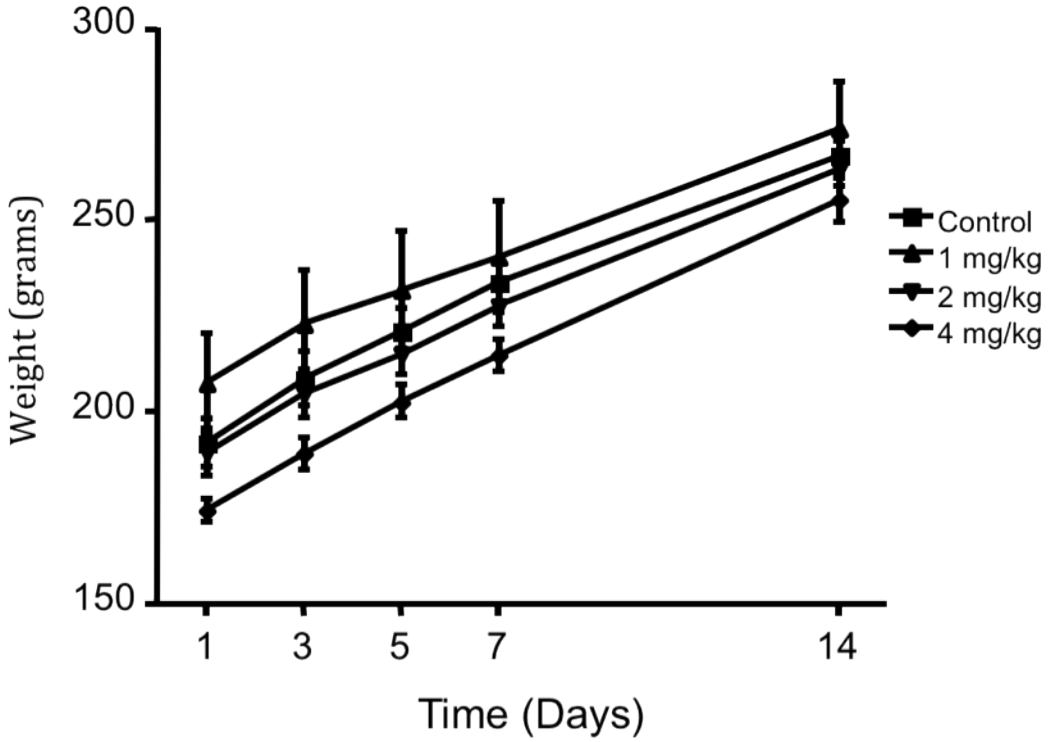

Figure 2. Paclitaxel-injected animals gain weight normally. Each data point is the mean ± SEM gram weight of 6 to 10 rats, over the times indicated.

Blood flow measurement by laser Doppler flowmetry

Cutaneous blood flow was measured by a BLF21D laser Doppler flowmeter from Transonic systems Inc. (Ithaca, NY) using a type N 11 G needle-style probe placed on the skin. With this method, vasodilatation in the dermis can be recorded non-invasively. A low intensity beam of monochromatic light travels from a laser diode in the flowmeter via a fiber optic cable through the laser probe head and illuminates the tissue being examined. The laser beam is then reflected back by the tissue components as well as by moving objects such as blood cells. The fiber optic cable guides the reflected light back to the flowmeter where it is received by a photo detector. The volume of tissue sampled is approximately 0.6 mm^3 and blood flow is recorded in terms of tissue perfusion units. Because the laser penetrates to a depth of approximately 600 μM, laser Doppler flow probe presumably detects changes in blood flow from the microvasculature in the dermis. For experimental set-up, see Figure 3.

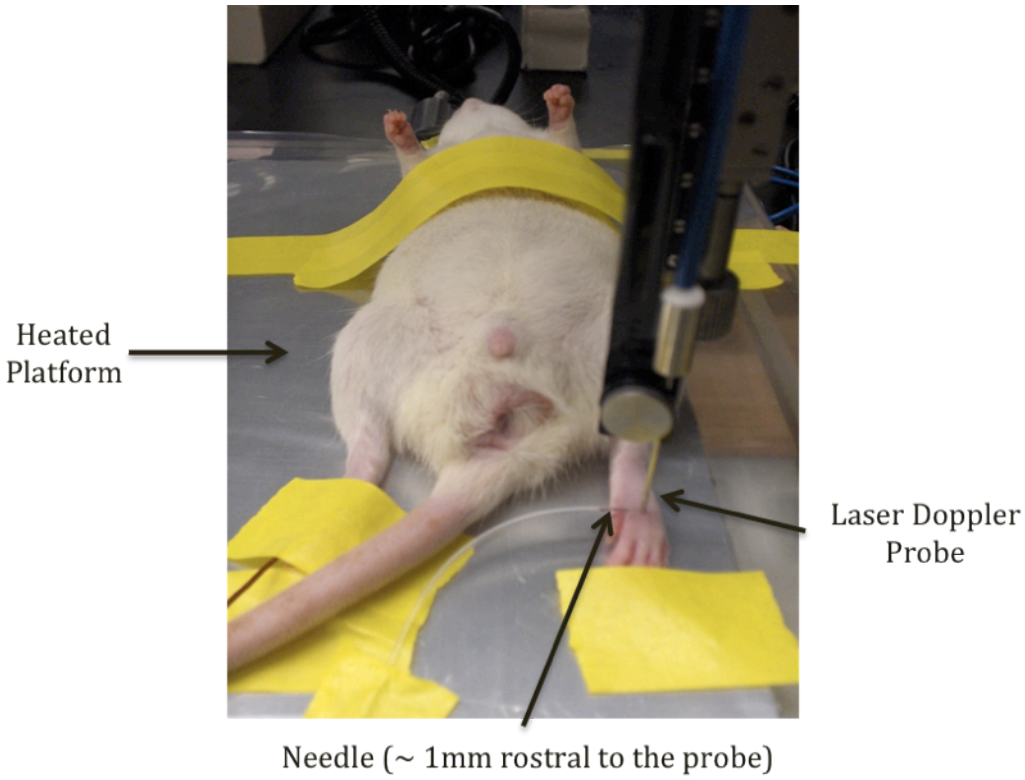

Figure 3. Experimental set-up for measurement of vasodilatation in the rat hind-paw.

All measurements were made on the hairy skin of the rat hind paw. This region of the hind paw is innervated by the L4 and L5 ganglia (Takahashi and Nakajima, 1996). Rats were placed on a heated platform to maintain body temperature at 37ºC. The laser probe was allowed to warm up for at least 10 minutes, and the heated platform was allowed to reach a stable temperature of 37ºC prior to beginning any blood flow measurements. In all experiments, vasodilatation was measured in rats weighing 250 - 260 grams. They were allowed to acclimate in their cages in the laboratory for at least 1 hour before being anesthetized with 100 mg/kg i.p. sodium thiopenthal. The hair of the dorsal surface of the paw was shaved and the rat was placed on the heated platform for 30 minutes before initiating blood flow measurements. The 30-minute period of acclimation was chosen to stabilize body temperature of the rat at 37ºC. since fluctuations in body temperature can alter basal blood flow. After the initial acclimation, basal blood flow was recorded for approximately 10 minutes until it stabilized. After stabilization of baseline blood flow, a 27G needle was inserted into the skin at an angle of 15 degrees to ensure intradermal placement of the needle. Placement of a 27G needle intradermally did not cause significant changes in baseline blood flow (Figure 4). An additional baseline blood flow in the presence of the needle was recorded for 15 minutes.

Characterization of cutaneous blood flow after intradermal injection of capsaicin

Experiments were performed initially to develop a protocol for the measurement of capsaicin-induced vasodilatation. After baseline blood flow (in the presence of the needle) stabilized, 10 μM capsaicin in 10 μl was injected intradermally. Figure 4 shows that blood flow increased from 18 ± 2 to 24 ± 1 TPU/min. This response was modest in comparison to observed effects of capsaicin in published reports (Hughes and Brain, 1991; Lin et al., 1999). This was a major concern for the planned studies because the weak response could prevent observations of changes in drug treated animals.

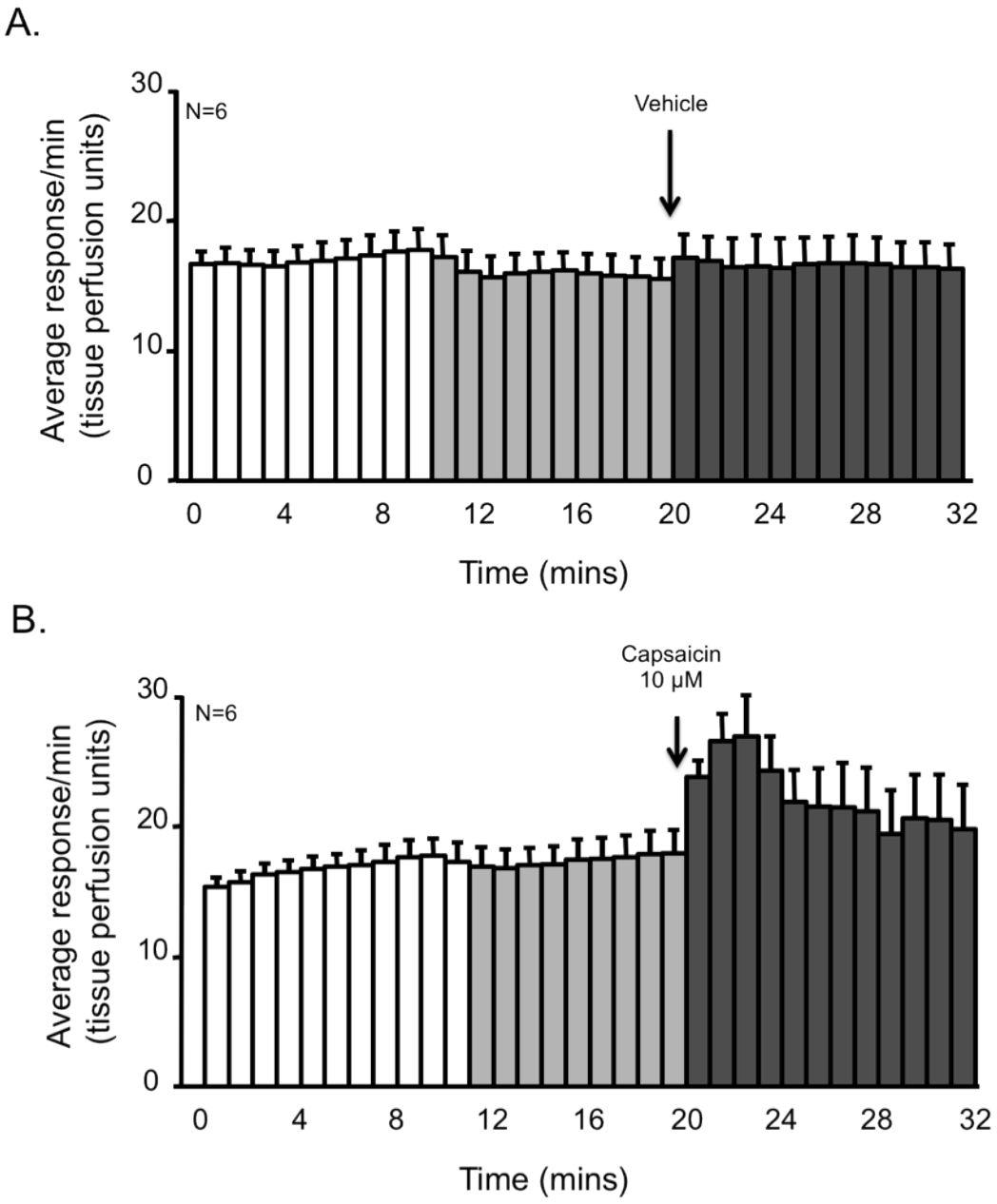

Figure 4. Intradermal injection of capsaicin increases blood flow in the skin of the rat hind paw. Each column is the mean ± SEM. The open columns and the light shaded columns represent basal blood flow before and after intradermal placement of the injection needle respectively. The dark shaded columns represent blood flow after injection of vehicle (0.01% MPL in PBS) (A) or 10 µM capsaicin (B).

There are two possible reasons for the weak response to intradermal injection of capsaicin: First, since the flow probe is able to detect changes in blood flow only within a 1 mm^2 area, the probe might be too far from the injection site. Second, the volume of injection (10 µl) might interfere with the detection of a response by the laser probe. To resolve these issues injections were made 1 mm away from the site of the probe and the injection volume was decreased to 1 µl in future experiments.

Characterization of cutaneous blood flow after electrical stimulation of the sciatic nerve

For examination of changes in blood flow in response to sciatic nerve stimulation, the animal was removed from the heated platform after stabilization of baseline. The sciatic nerve was exposed in the groove between the dorsal side of the ischeum and sacral bone. To prevent injury-induced action potentials, 1% lidocaine was applied to the trunk of the sciatic nerve with a cotton-tipped applicator. The nerve was cut proximal to the lidocaine application site and was placed into a nerve cuff fitted with a silver-stimulating electrode (Figure 5). A layer of petroleum jelly was applied over the stimulating electrode, the skin was sutured, and the animal was returned to the heated platform. Baseline blood flow following axotomy was recorded for 30 minutes to allow sufficient time for the lidocaine block to be extinguished., A stimulus amplitude of 5mA at 10 Hz for 30 seconds was used to stimulate the sciatic nerve (Koltzenburg et

al., 1990). The increase in blood flow following the stimulation was recorded for an additional 30 minutes.

To determine if the vasodilatation induced by capsaicin or sciatic nerve stimulation is mediated by CGRP, 20 μM of the CGRP receptor antagonist $CGRP_{8-37}$ was injected intradermally 5 minutes before drug injection or before electrical stimulation (Hughes and Brain, 1991; Escott and Brain, 1993). To assure that the $CGRP_{8-37}$ and capsaicin were injected at the same site, two 30 G needles were glued together. One needle was connected to a syringe containing capsaicin and the other needle to a syringe filled with the CGRP receptor antagonist.

Examination of blood flow in paclitaxel injected animals

In experiments to determine if paclitaxel altered sensory neuron function, vasodilatation in response to intradermal capsaicin or electrical stimulation was examined in paclitaxel-injected animals using the procedures detailed above. To determine if vascular smooth muscle function in paclitaxel-treated animals was intact, methacholine (100 μM) or α-CGRP (30 μM) was injected intradermally.

The data for all blood flow experiments are presented as the mean response ± SEM recorded per 3-minutes in tissue perfusion units (TPU) for each animal. To determine if there were statistically significant differences blood flow between treatment groups, I compared the mean ± SEM of the evoked response/15 minutes between treatment groups using

one-way analysis of variance (ANOVA) and Bonferroni's post hoc test. Evoked response was calculated by subtracting the baseline response for 15 minutes before the injection of capsaicin or electrical stimulation from the response for 15 minutes after stimulation.

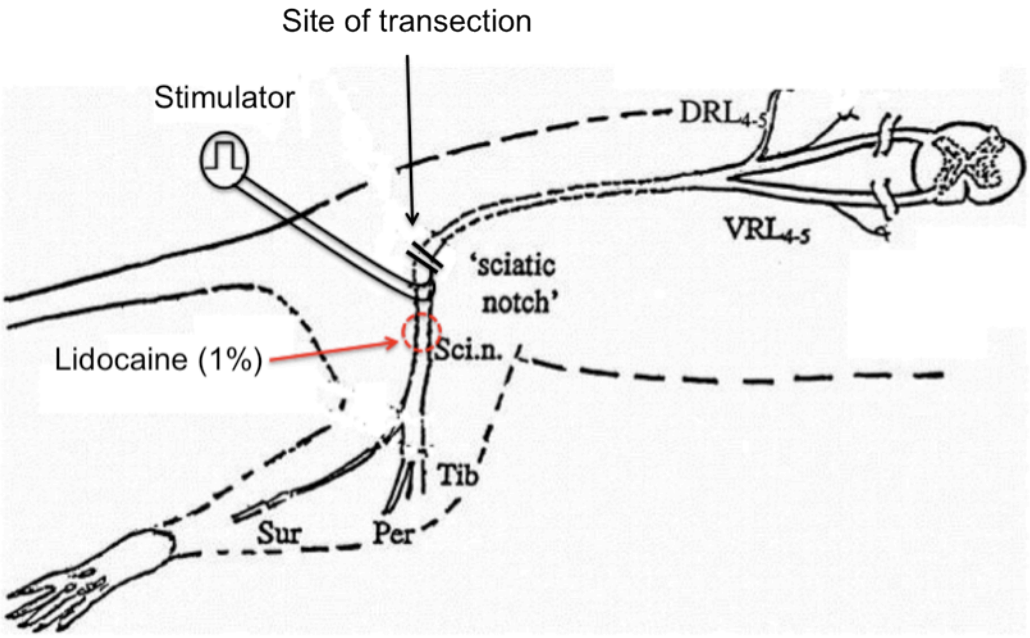

Figure 5. Schematic representation of the sciatic nerve stimulation experiment. The stimulator was placed on the trunk of the sciatic nerve (before it branched into the sural, peroneal and tibial branches). The site of transection and lidocaine application are also indicated.

Neuropeptide release from spinal cord slices

For experiments measuring release of CGRP from spinal cord slices, male Sprague Dawley rats were injected with paclitaxel or vehicle as indicated above, then sacrificed, and spinal cord slices were prepared as previously described (Chen et al., 1996). Rats were anaesthetized by carbon dioxide asphyxiation and then decapitated. The spinal column was dissected out, and the spinal cord was extruded through the rostral end of the column using PBS. The lumbar region was identified as a distinct swelling or enlargement at the caudal end, and 1 cm of this lumbar enlargement was dissected. The dorsal horn of the spinal cord was dissected and chopped, first cross-sectionally and then parasagitally into 0.3 x 0.3 mm sections. The tissue was weighed, placed into a cylindrical perfusion chamber with an internal volume of 0.5 ml, and perfused with buffer consisting of HEPES 25 mM, NaCl 135 mM, KCl 3.5 mM, MgSO4 1 mM, CaCl2 2.5 mM, dextrose 3.3 mM, bovine serum albumin 1%, and ascorbic acid 200 μM. The buffers also contained the peptidase inhibitors phe-ala (100 μM), bacitracin (20 μM), phenyl methane sulphonyl fluoride (PMSF-100 μM), and phosphoramidon (1 μM) to minimize breakdown of CGRP (Chen et al., 1996). The inhibitors were prepared from stock solutions that were stored at 1000 times the final concentration at -20ºC. A stock concentration of 10 mM capsaicin in 1-Methyl-2-pyrrolidone (MPL) was prepared and was further diluted to 500 nM with HEPES buffer. The buffers were continuously aerated with 95% 0_2 + 5% CO_2 and pH 7.4-7.5,

and maintained at 37^{o} C throughout the experiment. The tissue was perfused at a flow rate of 0.5 ml/min. The perfusate from the first 20 minutes was discarded, after which perfusate fractions were collected into test tubes containing 75 µl of 1M 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 6.7-6.9 every 3 minutes. Basal release was established by first perfusing the tissue with HEPES buffer for 18 min, after which the tissue was exposed to HEPES buffer containing either 500 nM capsaicin or 30 mM KCl substituted for equimolar NaCl for 9 minutes to evoke release of CGRP. To demonstrate a return to basal release after stimulation, the tissue was perfused with HEPES buffer for another 15 minutes. For a schematic representation of the perfusion apparatus, see Figure 6.

At the end of the experiment, the tissue was removed from the chambers and homogenized in 0.01 M HCl, then centrifuged for 20 min at 3000 x g at 4ºC in a Beckman-Coulter Allegra^{AM} 6R centrifuge (Fullerton, CA). The supernatant was diluted with HEPES buffer, and total peptide content remaining in the tissue as well as that in the perfusate fractions was measured by radioimmunoassay. The total content of iCGRP was calculated as the amount of iCGRP remaining in the tissue at the end of the experiment added to the amount in each of the 14 perfusate fractions. The CGRP release is referred to as immunoreactive CGRP (iCGRP). Release data from spinal cord slices are expressed as the mean ± SEM of the fractional release of iCGRP (% of total content). For analysis of CGRP

release from spinal cord slices, the sum of the iCGRP released in the 3 fractions before exposure to the stimulus (basal release/9 minutes) was subtracted from the sum of the iCGRP released in the 3 fractions during exposure to the stimulus (stimulated release/9 minutes) to give the evoked release of iCGRP in 9 minutes (evoked release/9 minutes). The evoked release was compared using an unpaired t test between control and paclitaxel-treated animals. Statistical significance was set at $p < 0.05$.

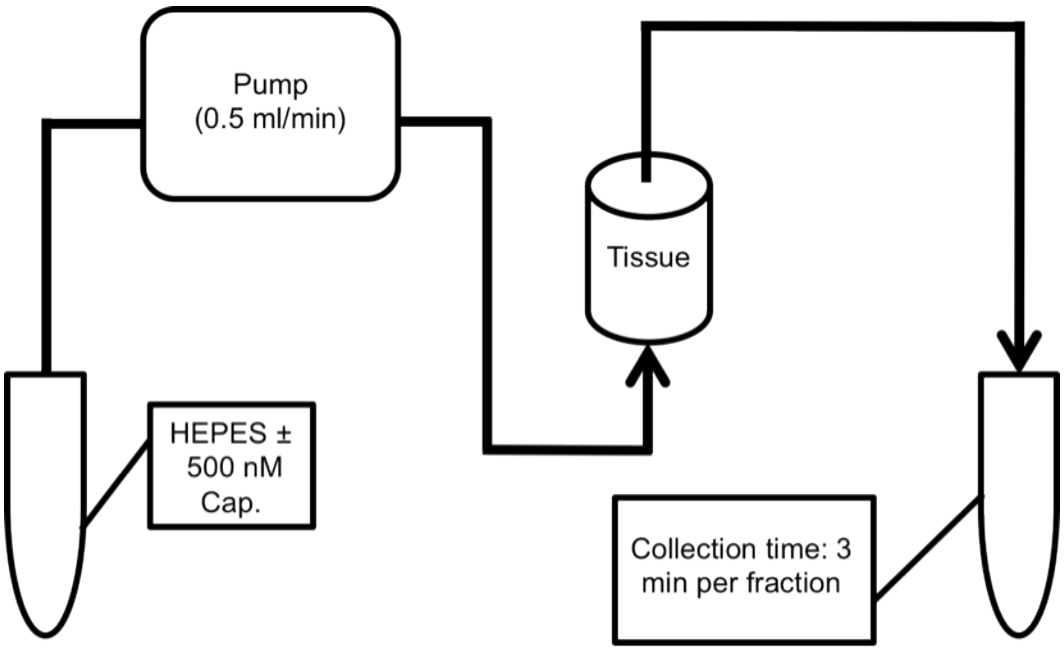

Figure 6. Schematic representation of the CGRP release assay from spinal cord slices.

Adult rat dorsal root ganglion culture preparation

Cell culture plates were precoated the day before the DRG harvest by exposing them to 1 ml poly-D-lysine (1mg/10 ml; 70,000 - 150,000 MW) for 1 hour, rinsing with ddH_2O and then exposing them to 1 ml laminin (1 mg/ml) at 37ºC overnight. The laminin was removed just before plating the dissociated cells (see below). Adult rat DRG cell cultures were prepared as previously described (Burkey et al., 2004). Adult male Sprague Dawley rats weighing 175 to 200 grams were euthanized by CO_2 asphyxiation. The DRGs were collected from the entire spinal column (from cervical to sacral levels), and the afferent fibers were trimmed off. Next, the ganglia were incubated in Ham's F-12 medium containing 0.125% collagenase for 1 h in 3% CO_2 at 37ºC, and then were centrifuged and resuspended in F-12 medium supplemented with 10% horse serum, 2 mM glutamine, 100 µg/ml normocin, 50 µg/ml penicillin, 50 µg/ml streptomycin, 50 µM 5-fluoro-2′-deoxyuridine, 150 µM uridine, and 30 ng/ml NGF. The DRGs were then dissociated by triturating with fire polished pasteur pipettes. An aliquot (30 µl) of the dissociated cell suspension was diluted 1:2 with trypan blue, and the number of live cells (those that exclude trypan blue) was counted using a haemocytometer. The suspension was diluted to 30,000 live cells/ml, and 1 ml of this suspension was plated in each well of a 12-well culture plate. The sensory neurons were maintained in F-12 medium containing 30 ng/ml NGF at 3% CO_2 in a 37ºC incubator. Growth medium was changed every other day.

Paclitaxel was reconstituted to 10 mM in MPL and stored at -20ºC. Subsequent dilutions of the drug was made in F-12 medium. Unless otherwise indicated paclitaxel was added to wells on day 7 in culture. All endpoints described in this thesis were examined on day 12.

Neuropeptide release from cell cultures

Release studies were performed on sensory neuron cultures as previously described (Hingtgen and Vasko, 1994). After the medium was removed, neuronal cultures were washed once with HEPES buffer consisting of 25 mM HEPES, 135 mM NaCl, 3.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 3.3 mM D-glucose, and 0.1% bovine serum albumin, pH 7.4. Following this initial wash, the neuronal cells were exposed to the 0.4 ml HEPES buffer for 10 minutes at 37ºC to establish basal release. To stimulate release of CGRP, the neurons were exposed to 0.4 ml HEPES buffer containing 30 nM capsaicin at 37ºC for 10 minutes. This concentration of capsaicin was chosen because it lies on the linear region of the capsaicin concentration vs. iCGRP release curve. Basal release was re-established by incubating the cells with 0.4 ml HEPES buffer for 10 minutes at 37ºC. After completion of the release protocol, the cells were lysed by adding 0.4 ml of 0.1M hydrochloric acid to the wells and total content of the peptide was measured from this lysate.

After each 10-min incubation, the buffer was removed and the amount of iCGRP was measured by radioimmunoassay. An aliquot (50 -

300 µl) of the incubation buffer and 20 µl of the cell lysate was assayed for iCGRP. Release of iCGRP was normalized to the total content of CGRP. The total content of iCGRP was defined as the sum of CGRP released during each of the 3 incubations and the amount in the cell lysate. Basal and capsaicin-stimulated release is expressed as the percent of total content of iCGRP/10 minutes. Total content of CGRP is reported as fmol/well.

Iodindation of CGRP for radioimmunoassay

The resins used for purification of freshly iodinated peptide, P-4 and AG 1-X8 (chloride form) were swollen 24 hours in advance in 1 M acetic acid plus 0.1% BSA and 200 mM sodium acetate buffer pH 5.0, respectively. The P-4 resin was packed in a plastic column to a height of 15 cm and AG 1-X8 resins were packed in Pasteur pipettes to a height of 5 cm. The columns and washed once with 1 M acetic acid plus 0.1% BSA and 200 mM sodium acetate buffers respectively. Rat (Tyr27)-αCGRP$_{27-37}$ was reconstituted in ddH_2O to a concentration of 1mg/ml. The iodination reaction mixture consisted of 90 µl potassium phosphate buffer (250 MM), 10 µl diluted (Tyr27)-αCGRP$_{27-37}$, 20 µl chloramine-T solution (4.4 mM), and 10 µl ^{125}I-Na. The reaction was stopped after 40 seconds by adding 40 µl sodium metabisulfite (26 mM). The reaction mixture was added to the AG 1-X8 column. After the volume of liquid disappeared into the column, 500 µl sodium acetate buffer was added to the column to elute the iodinated peptide. This was repeated until a total of 4 individual

fractions were collected. A 10 µl aliquot of each of the 4 fractions were counted on the gamma counter. The fraction with the highest amount of radioactivity was passed through the P-4 column for further purification. The iodinated peptide was eluted off the P-4 column with 1 M acetic acid plus 0.1% BSA and fractions were collected at 5 minute intervals for a total of 14 fractions. The amount of radioactivity in 10 µl of each fraction was determined. The three fractions with the highest amount of radioactivity were tested for non-specific binding. For this, a small aliquot of each fraction was diluted to 10,000 counts/100 µl of 200 mM Tris buffer and 100 µl added to 400 µl 200 mM Tris buffer in triplicate. To these samples, 0.5 ml of 1% Norite charcoal solution in 0.1 M phosphate buffer also containing 50 mM NaCl and 1 % bovine serum (pH 7.4) was added. The mixture was centrifuged at 3000 x *g* for 10 minutes in a Beckman-Coulter Allegra^{AM} 6R centrifuge (Fullerton, CA). The charcoal binds to free, unbound radiolabeled CGRP samples. This supernatant was decanted to a new tube, and the radioactivity in the supernatant was measured by gamma scintillation spectrometry on a 2470 WIZARD^2 automatic gamma counter from Perkin Elmer (Shelton, CT). The fractions with the lowest nonspecific binding (less than 1%) were diluted to 1,000,000 cpm/100 µl, aliquoted, and stored at -20ºC.

Radioimmunoassay of iCGRP

Immunoreactive CGRP (iCGRP) was assayed as previously described (Chen et al., 1996). CGRP for standard curves was reconstituted in 50 mM Tris HCl pH 4.0 and further diluted in CGRP assay buffer pH 7.4 containing 0.2 M Tris base, 0.1% BSA, and 0.06% Dextran (T70). A standard curve with known amounts of CGRP (0-250 fmol) was prepared in duplicate for each assay. To each standard and sample being assayed, 25 µl each of ^{125}I-[0Tyr]-iCGRP$_{27-37}$ containing approximately 2000 cpm and a 1:65,000 dilution of CGRP antibody (a generous gift from M. Iadorola, NIH) was added. The samples were incubated for 16-20 h at 4ºC to allow the binding between the antibody to the CGRP and CGRP$_{27-37}$ to reach equilibrium. Peptides bound to antibody were separated from unbound peptides by adding 0.5 ml of 1% Norite charcoal solution in 0.1 M phosphate buffer that also contained 50 mM NaCl, and 1 % bovine serum (pH 7.4). The peptides were then centrifuged at 3000 x *g* for 10 minutes. Unbound peptide is trapped by the charcoal, while the supernatant contains antibody bound to peptide (radiolabeled or unlabeled). The supernatant was decanted to a new tube, and the radioactivity was measured by gamma scintillation spectrometry. The amount of iCGRP in perfusate samples was quantified by comparing the radioactivity in samples to those values in a standard curve using a 4 point non-linear least squares regression analysis. Using this method, the minimal detectable amount of iCGRP was 10 fmoles (95% confidence interval).

Western blot and densitometry

For immunoblotting of proteins from sensory neurons in culture, cells were scraped in cold PBS using a cell scraper and centrifuged at 14,000 x *g* at 4°C for 10 minutes in an Eppendorf Centrifuge 5415R (Hauppauge, NY). The pelleted cells were resuspended in modified RIPA lysis buffer containing 50 mM Trizma base, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM Na_3VO4, and 25 mM NaF. The cells were sonicated on ice for 10 seconds using a 559 Sonic Dismembrator from Fisher Scientific (Pittsburg, PA). The lysate was centrifuged at 14000 x *g* at 4ºC, and the pellet containing membrane fragments was discarded. The supernatant consisting of cytoplasmic and membrane proteins was assayed for protein content by the Bradford assay. This assay is a dye-binding assay in which a differential color change of a dye (Coomassie® Brilliant Blue G-250) occurs in response to varying concentrations of protein. The absorbance maximum for the dye is 465 nm, which shifts to 595 nm after it binds to protein.

To determine protein concentration in each lysate, a standard curve was prepared with 0-500 µg/ml bovine I_gG protein; 10 µl of each standard or diluted lysate was added into separate microtiter wells of a 96-well plate. 200 µl of the diluted dye reagent (1ml dye + 4 ml ddH_2O) was added to each well. The dye was allowed to react with the protein at room temperature for 5 minutes. The absorbance at 560 nm of the samples was

determined using a Tecan SpectraFluor Plus spectrophotometer with Magellan 5.03 software (Crailsheim, Germany). Linear regression analysis of the graph of absorbance vs. concentration of standard was used to calculate the concentration of protein in the cell lysates.

Approximately 20 µg of the protein was loaded on precast bis-Tris polyacrylamide gels and run at 200 mV for 30 minutes. The proteins on the gel were transferred to a polyvinylidene fluoride (PVDF) membrane 30 mV for 1 hr. The membrane with transferred proteins was washed once with ddH_2O and blocked for 1 hour with 5% donkey serum in Tris buffered saline containing 0.1% tween 20 (TBST). The membrane was incubated overnight at 4ºC with a 1:500 dilution of the TRPV1 antibody in 5% donkey serum. The blot was washed 3 times with TBST, each time for 10 minutes. Next, the blot was incubated with a 1:10000 dilution of a goat anti-donkey antibody coupled to horseradish peroxidase (HRP) for 1 hour at room temperature. After washing off the secondary antibody 3 times with TBST for 10 minutes per wash, the blot was incubated with Western Lightning® chemiluminescent substrate solution, exposed to light sensitive radiographic film, and developed.

Before immunoblotting for actin, the membrane was stripped off all bound antibodies, washed with TBST and blocked with 5% donkey serum for 1 hour at room temperature. Anti-actin antibody (1:5000) was incubated with the blot for 1 hour. Excess antibody was washed off with TBST for 30 minutes (10 minutes x 3 times). HRP conjugated secondary

antibody in 5% donkey serum (anti-mouse) was allowed to incubate with the blot for 1 hour at room temperature. The secondary antibody was washed off the blot by rinsing with TBST 3 times for 10 minutes each. The blot was then exposed to chemiluminescent substrate and developed as mentioned above.

Optimization of the Western blot to examine TRPV1 immunoreactivity

Proteins were extracted as outlined above. To confirm antibody specificity for TRPV1, a lysate of DRG cells in culture was analyzed by western blot. using neutralized TRPV1 antibody. For these experiments, 20 µgs of sensory neuron lysate, lysates of Chinese hamster ovary (CHO) cell over-expressing TRPV1 (positive control), and CHO cell lysate not expressing TRPV1 (negative control) were loaded in duplicates on two gels and separated by electrophoresis as described above. The gels were transferred to a PVDF membrane and blocked with 5% donkey serum in TBST at room temperature for 1 hour. Meanwhile, a 1:500 dilution of the anti-TRPV1 antibody in 5% donkey serum was incubated with the blocking peptide (1 part antibody + 4 parts blocking peptide) for 1 hour on a rocking platform to neutralize the antibody. One of the two blots was incubated with the neutralized antibody, while the other was blotted with non-neutralized anti-TRPV1 antibody. Both membranes were allowed to incubate overnight at 4ºC, washed, and then incubated with the secondary antibody as before. The blots were exposed to chemiluminescent

substrate and developed. The results are shown in Figure 7. The membrane incubated with anti-TRPV1 antibody revealed a band migrating at ≈98 kD in the sensory neuron lysate and TRPV1-expressing CHO cell lysate but not in the non-TRPV1 expressing CHO cell lysate (Figure 7, left panel). Furthermore, the antibody did not recognize any bands in the lane containing the negative control (non-TRPV1 expressing CHO cell lysate). No immunoreactivity for TRPV1 was detected in the membrane blotted with neutralized antibody (Figure 7, right panel). These data confirm that the band migrating at 98 kD is immunoreactive for TRPV1.

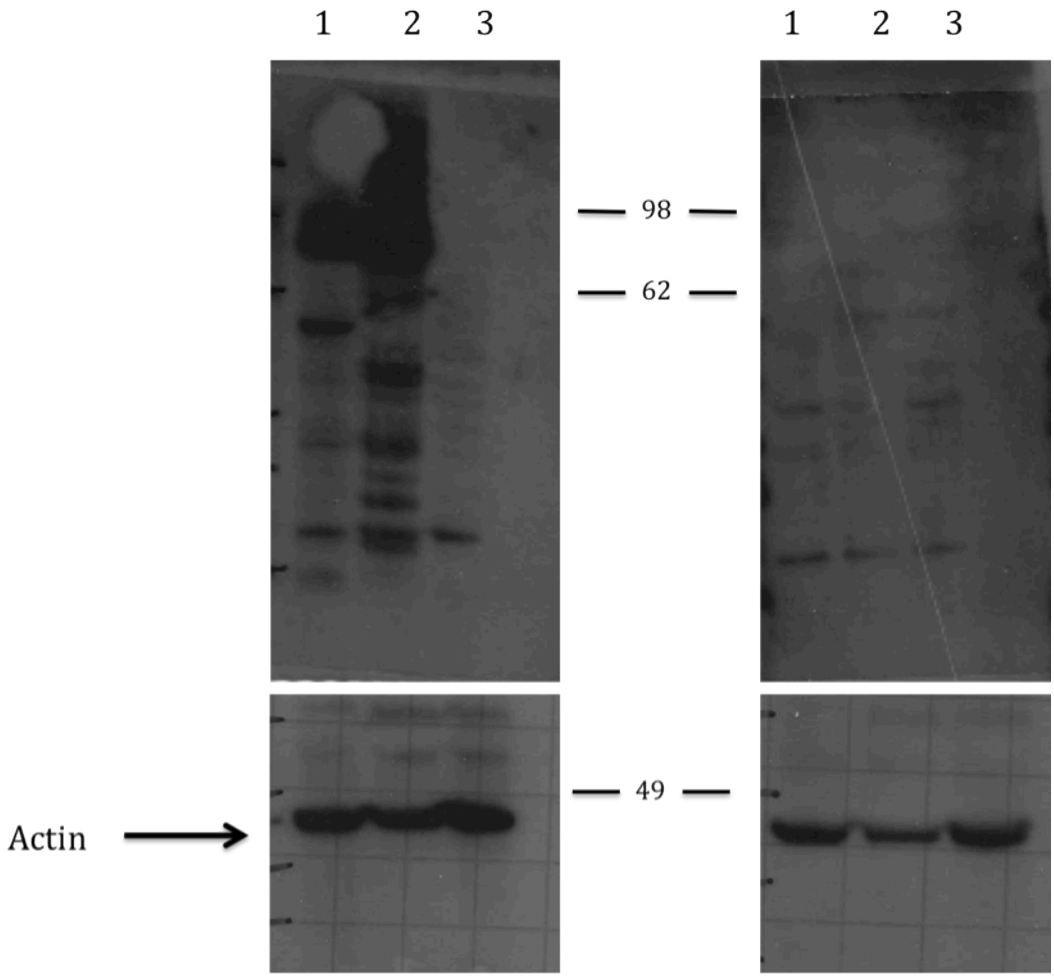

Figure 7. Identification of the immunoreactive TRPV1 band. The left panel is the PVDF membrane that was blotted with anti-TRPV1 antibody. The right panel represents the membrane that was blotted with neurtralized anti-TRPV1 antibody. Lanes 1, 2 and 3 were loaded with lysates from sensory neurons, CHO cells over-expressing TRPV1 (positive control), and non-TRPV1 expressing CHO cells (negative control).

Real Time polymerase chain reaction

Extraction of RNA

Total RNA was extracted from sensory neuronal cultures using the PrepEase® RNA Spin Kit according to the instructions supplied. Total RNA was quantified using the Nanodrop ND1000 spectrophotometer from Thermo Fisher Scientific (Wilmington, DE); 1 OD unit at 260 nm = 40 µg/ml of RNA. The RNA yield was considered pure if the 260/280 nm absorbance ratio was of 1.9-2.2 units.

Reverse transcription of RNA to cDNA

cDNA was synthesized from 1 µg of RNA using a Bio-Rad Laboratories' iScript cDNA Synthesis Kit. The reaction mixture consisted of 1 µg RNA, 4 µl of the iScript reaction mix, and 1 µl of iScript reverse transcriptase and nuclease free water so that the total volume of each reaction was 20 µl. Using a MultiGene® thermocycler from Labnet International, INC (Edison, NJ), the reverse transcriptase reaction was performed for 5 minutes at 25°C, for 30 minutes at 42°C, then for 5 minutes at 85ºC.

Validation of the $2^{-\Delta\Delta C}_T$ method for relative quantification

Quantification of mRNA for specific genes was performed by the $2^{-\Delta\Delta C}_T$ method. For this method to be valid, the amplification efficiency of the primer-probe sets for the target sequence (TRPV1 or $Na_v1.9$) must be identical to that of the endogenous control (Glyceraldehyde 3-phosphate dehydrogenase - GAPDH). The efficiencies can be determined by looking at how ΔC_T varies with template dilution (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). The C_T (threshold cycle) for the target gene is subtracted from the C_T for GAPDH: this value is referred to as the ΔC_T value. If the slope of ΔC_T (absolute value) versus cDNA dilution is close to zero, it implies that the efficiencies of the target and housekeeping genes are very similar, and the $2^{-\Delta\Delta C}_T$ method can be used for relative quantification.

A standard curve was prepared using 0.01 to 100 ngs of cDNA in 10 fold increments. Each dilution of cDNA was amplified in 2 sets of triplicates: one set contained 1 µl/well of the probe-primer mix for the target gene, and the other set contained 1 µl/well of the probe and primer mix for GAPDH. Each well also contained 10 µl of the TaqMan master mix. The real time PCR reaction was performed in a StepOne Plus® 7500 Fast Real-Time PCR system from Applied Biosystems (Foster City, CA) using universal amplication parameters. Figures 8 and 9 show the absolute value of the slope of ΔC_T vs concentration of cDNA as less than

0.1 and close to 0. Therefore the $2^{-\Delta\Delta C}_T$ can be used for relative quantification of TRPV1 or Na_v 1.9.

Relative quantification of TRPV1 or Na_v1.9 from paclitaxel-treated sensory neurons in culture

For quantification of mRNA (TRPV1 or Na_v1.9) from paclitaxel-treated cells, RNA isolation and cDNA synthesis were performed as already described. For the relative quantification reaction, 30 ngs of cDNA per well was used as the template. Each sample was amplified in 2 sets of triplicates; one set for target gene amplication and the other for GAPDH. The rest of the procedure was identical to the one above. The mean ± SEM of the expression of mRNA for TRPV1 or Na_v1.9 expressed as % of untreated control was compared by one-way ANOVA and Bonferroni's post test.

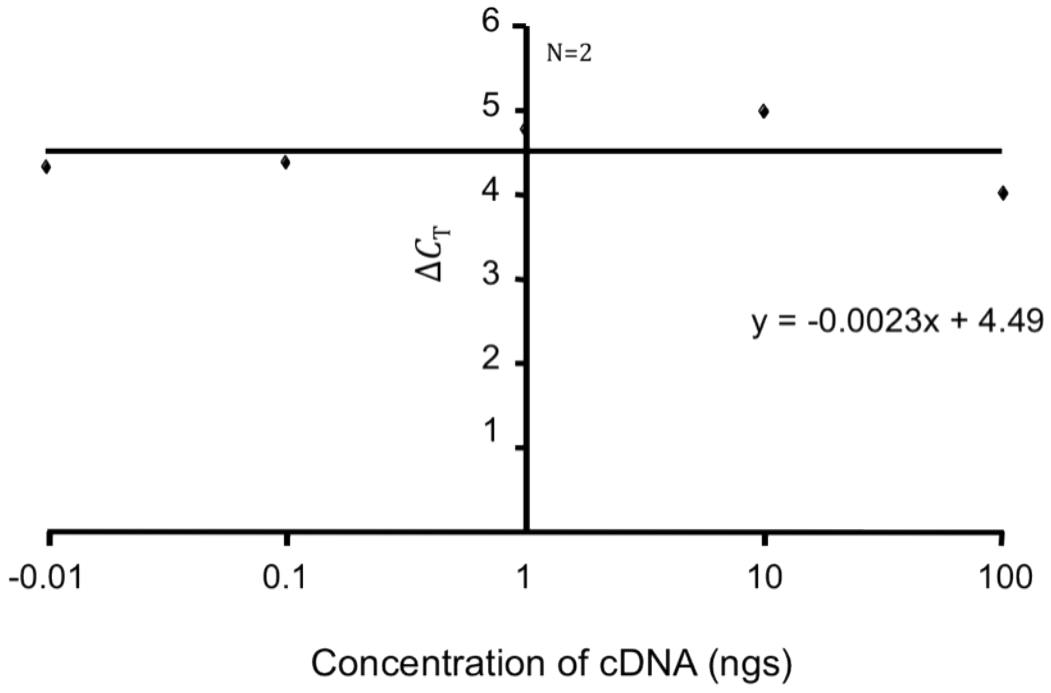

Figure 8. Validation of the $2^{-\Delta\Delta C}{}_T$ method for relative quantification of TRPV1. Different cDNA dilutions synthesized from total RNA from isolated sensory neurons were amplified. The efficiency of amplification of TRPV1 and GAPDH was examined using real-time PCR and TaqMan detection. The ΔC_T ($C_{T,TRPV1}$ - $C_{T,GAPDH}$) was calculated for each cDNA concentration. The data were fit using least-squares linear regression analysis.

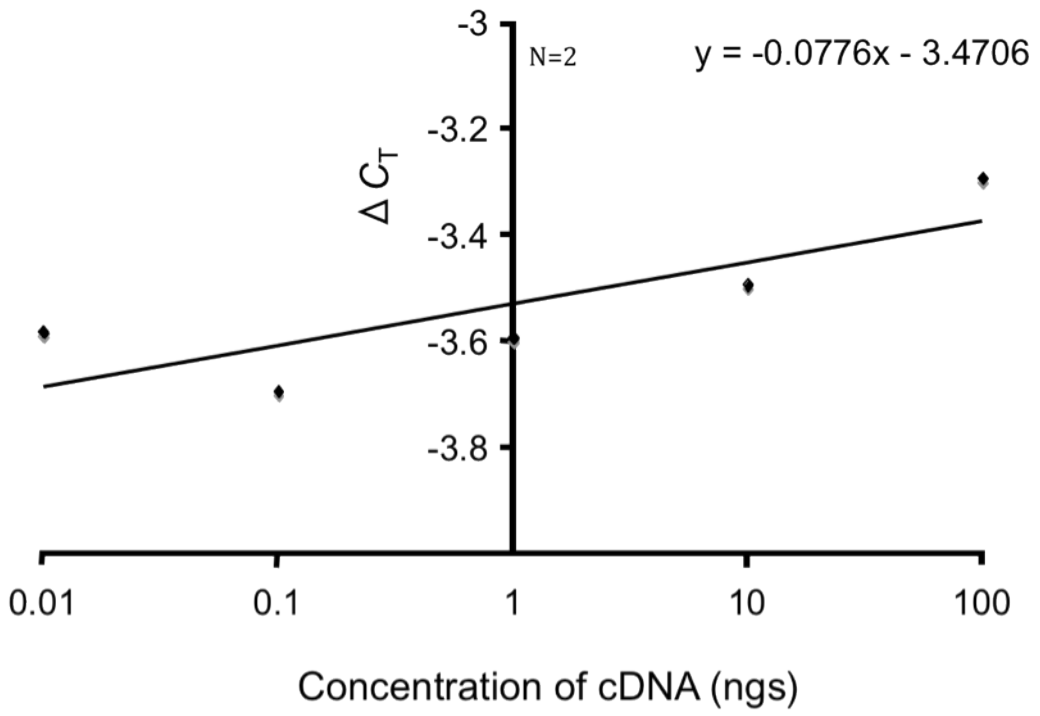

Figure 9. Validation of the $2^{-\Delta\Delta C_T}$ method for relative quantification of $Na_v1.9$. Different cDNA dilutions synthesized from total RNA from isolated sensory neurons were amplified. The efficiency of amplification of $Na_v1.9$ and GAPDH was examined using real-time PCR and TaqMan detection. The ΔC_T ($C_{T,Nav1.9}$ - $C_{T,GAPDH}$) was calculated for each cDNA concentration. The data were fit using least-squares linear regression analysis.

Propidium iodide (PI) and annexin V double labeling

Viability of sensory neurons following exposure to paclitaxel was assessed by double staining with PI and annexin V. Sensory neuronal cultures were exposed to either 300 nM or 1 μM paclitaxel for 2 or 5 days. As a positive control, untreated neurons were exposed to 300 μM H_2O_2 for 1 hour at 37ºC (Vasko et al., 2005). At the end of the 1-hour incubation the neurons were returned to F12 medium. Approximately 24 hours later, all neurons were incubated with 0.4 ml of the staining solution containing 25 μl annexin V - FITC in 10 ml HEPES buffer and 6 μM PI. After washing off the staining solution, cells were visualized. The excitation of PI/annexin V was 530/485 nm and the emitted light was monitored at 645/530 nm. PI is a DNA intercalating agent that is only able to penetrate the membrane of dead cells. Annexin V has high affinity for phosphatidylserine (PS), which is located in the inner (cytoplasmic) leaflet of the cell membrane. Following induction of apoptosis, PS is translocated to the outer (cell surface) leaflet of the cell membrane, where it is now easily accessible to annexin V in the staining solution. After staining with PI and annexin V nuclei appear red while cells that are in the initial stages of apoptosis display a green colored ring around the cells. Dead cells more diffused staining with annexin V. Neurons in five random fields were counted and scored as either viable (unstained) or non-viable (red or green). The data were expressed as mean ± SEM % of total number of neurons counted.

Biolistic transfection of neurons for assessment of neurite length

The biolistic transfection method utilized the Helios Gene Gun System from Bio-Rad Laboratories (Hercules, CA, U.S.A). Optimized protocols were designed and modified from a recently published report (Dib-Hajj et al., 2009) and from the manufacturer's instructions (Bio-Rad Laboratories Helios Gene Gun System) to increase efficiency and preserve the health of neurons. Briefly, a 24 inch piece of tubing was cut to fit the saddle of the Tubing Prep Station, and ultrapure grade nitrogen was allowed to flow (0.3-0.4 LPM) through the plastic tubing for approximately 20 minutes to purge ambient oxygen and completely dry the interior of the tubing. A 15 mg (1:1 ratio of mg gold to µg cDNA) aliquot of gold microcarriers (1 µm size) was weighed. Fresh aliquots were made of PVP and spermidine in fresh, high grade (dehydrated) ethanol (EtOH) from stock solutions of 20 mg/mL and 1 M, respectively, and were stored at -20ºC. The final concentrations of PVP and spermidine aliquots used for the experiment were 0.05 mg/ml and 0.05 M respectively.

For transfection of rat DRG neurons, 10 µg of cDNA for enhanced green fluorescent protein (EGFP) in a volume of 10 µl was used. Spermidine (0.05 M), $CaCl_2$ (1M) and cDNA were used at a 1:1:1 ratio. First, spermidine was added to the gold microcarriers and the complex was vigorously mixed by vortex and sonicated to break up large particles. cDNA was then added to this suspension and mixed. Next, 1M CaCl2 was added drop-wise to the slurry of gold particles on the vortex. The complex

was allowed to sit for 10 min before centrifuging to remove the volume. The gold-DNA pellet was then washed and reconstituted three times with dehydrated EtOH. After the last EtOH wash, the gold-DNA complex was reconstituted using the entire volume of the PVP aliquot and transferred to a 15 mL conical tube. The contents of the conical tube were drawn into the nitrogen-purged tubing using a syringe and were carefully placed back into the tubing prep station saddle. 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, after which the automatic rotation of the plastic tubing was started. Next, the nitrogen was turned back on at a flow of 0.3-0.4 LPM for approximately 20 min. Cartridges were cut and placed into the Helios Gene Gun cartridge holder and contents were shot using the helium pressure within the gene gun onto 6-well plates containing recently harvested (~24 hrs) rat DRG neurons. The pressure and velocity was chosen so as to achieve transfection in only a few 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. Shown in Figure 10 is a neuron that stably expresses EGFP.

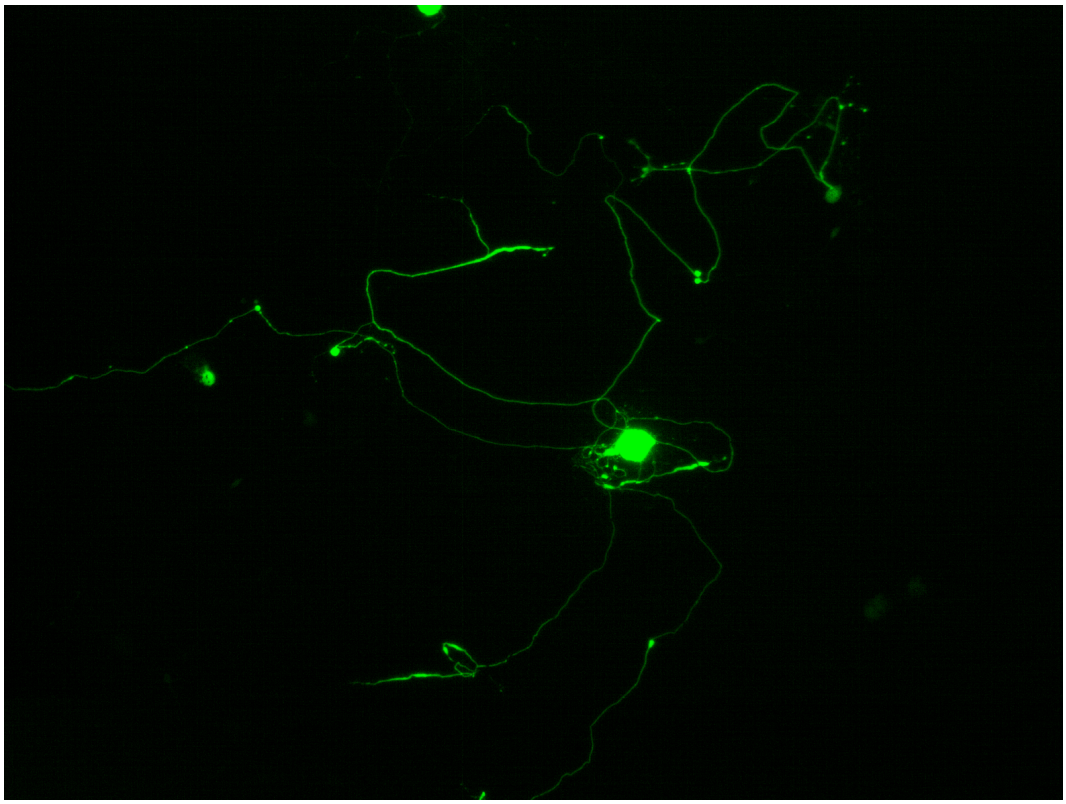

Figure 10. Sensory neuron expressing EGFP 48 hours after transfection using the gene gun method.

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 10, 100 or 300 nM 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 can be verified by observing traces superimposed by the software onto the original image for purposes of comparison. Data were expressed as % change in the length of neurites on day 9 and on day 12 relative to that on day 7.

Data Analysis

All data were analyzed using Prism 4.0 by GraphPad. Unless otherwise indicated, statistical significance was established using one-way ANOVA with Bonferroni's post test, $p < 0.05$.

RESULTS

Capsaicin increases blood flow in a concentration dependent manner

There are several reports that activation of sensory neurons causes release of CGRP in the periphery resulting in vasodilatation (Brain et al., 1985; Brain et al., 1986; Kilo et al., 1997). Consequently, examination of cutaneous vasodilatation after activation of small diameter sensory neurons can be used as an indirect method to measure the release of CGRP from peripheral nerve endings of these neurons. We performed an initial series of experiments to establish a model for capsaicin-induced neurogenic vasodilatation in a manner analogous to previous studies (Buckley et al., 1990; Hughes and Brain, 1991; Escott and Brain, 1993). We initially established that intradermal injection of capsaicin could increase cutaneous blood flow (Figure 4B).

To find a concentration of capsaicin that lies on the linear region of the concentration vs. vasodilatation curve, I recorded changes in blood flow in response to 1 µl of different concentrations of capsaicin. The capsaicin-evoked response was plotted against the concentration of capsaicin to generate the dose response curve. The evoked response was calculated by subtracting the basal blood flow recorded in the presence of the needle for 15 minutes from the blood flow recorded for fifteen minutes after injection of capsaicin. The evoked response at different concentrations of capsaicin is listed in Table 2.

Concentration of capsaicin	Average evoked response/15 minutes (tissue perfusion units)
0	9.9 ± 3.3
300 nM	91.4 ± 14.4
1 μM	123.4 ± 13.7
3 μM	166.5 ± 6.8
10 μM	175.5 ± 25.1
30 μM	253.3 ± 21.2
100 μM	295.8 ± 20.8
300 μM	321.7 ± 20.9
1 mM	281.8 ± 35.6

Table 2. Evoked response/15 minutes at different concentrations of capsaicin.

Figure 11 illustrates that capsaicin induces vasodilatation in a concentration-dependent manner. The EC50 (12 μM) was calculated using a 4-point non-linear least squares regression analysis. Since 10 μM lies on the linear region, all future experiments on capsaicin-evoked cutaneous vasodilatation were performed with this concentration.

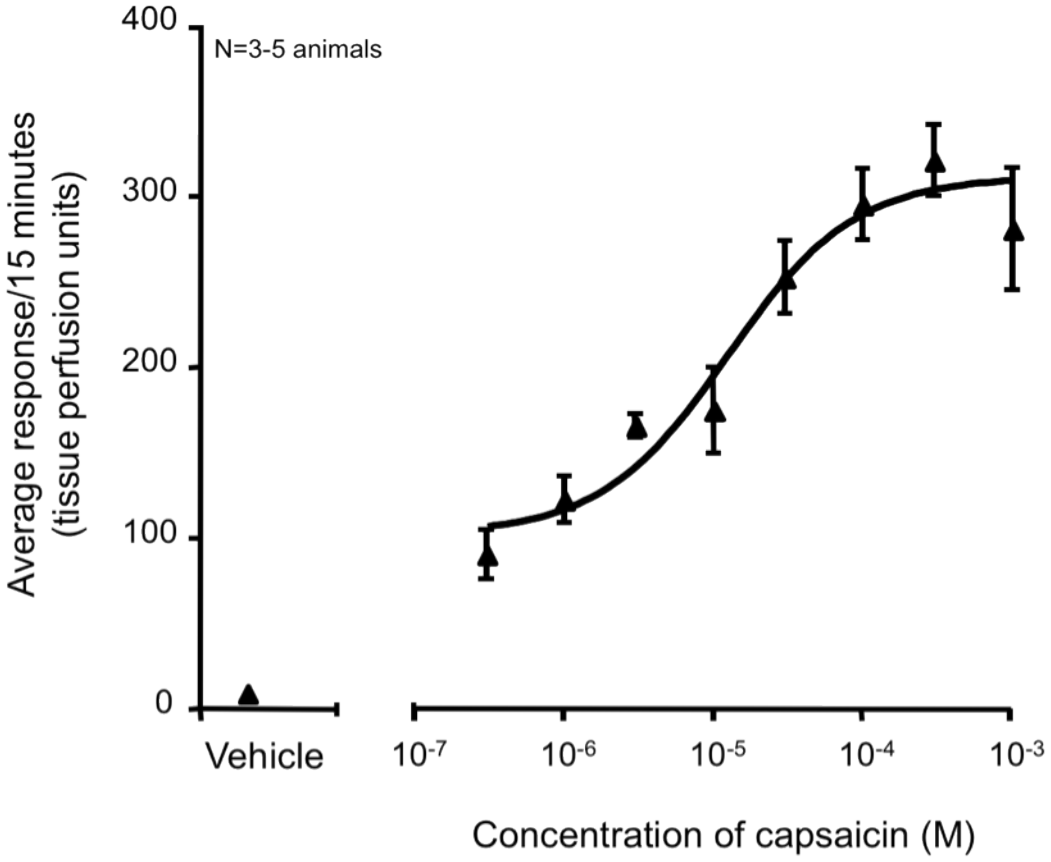

Figure 11. Intradermal injection of capsaicin increases cutaneous blood flow in dose dependent manner. Each point is the mean ± SEM of evoked blood flow in TPU/15 min for vehicle (0.01% MPL in PBS) or various concentrations of capsaicin injected intradermally in the hind paws of rats. The data were fit using 4 point non-linear least squares regression analysis. The EC_{50} was 12 μM.

To demonstrate that the capsaicin-induced vasodilatation is mediated by CGRP, I determined whether the CGRP1 receptor antagonist $CGRP_{8-37}$ could attenuate the effects of capsaicin. For these experiments, two 30 gauge needles were glued together, each needle was connected via tubing to two different syringes; one syringe was filled with capsaicin (10 μM) and the other with $CGRP_{8-37}$ (20 μM). This way $CGRP_{8-37}$ and capsaicin can be injected at the same site. After the initial baseline was recorded, the needle was inserted intradermally and a second baseline was recorded for fifteen minutes. As can be seen in Figures 11 and 12, insertion of the needle did not significantly alter blood flow. When 10 μM capsaicin was injected intradermally, blood flow increased from 11.7 ± 1.9 to 25.4 ± 3.2 TPU/ 3 minutes. However, when the CGRP receptor antagonist was injected 5 minutes before capsaicin, there was no change in blood flow. The response recorded before injection of $CGRP_{8-37}$ was 16.6 ± 1.9 and after injection of capsaicin was 14.0 ± 20 TPU/3 minutes (Figure 12B). Additionally, intradermal injection of $CGRP_{8-37}$ or vehicle (0.1 % MPL in PBS) alone did not alter baseline blood flow. Blood flow recorded before injection of the antagonist was 13.3 ± 4.6 and after injection was 12.9 ± 2.2 TPU/3 minutes (Figure 13A). Blood flow before and after injection of vehicle was 14.0 ± 1.3 and 14.3 ± 1.3 TPU/ 3 minutes (Figure 13B).

As discussed in the methods, the mean ± SEM of the evoked response over a 15-minute period was compared. Figure 14 A shows that

blood flow over 15 minutes increased from 53 ± 7 TPU/15 min to 121 ± 15 TPU/15 min after intradermal injection of capsaicin. In the presence of $CGRP_{8-37}$ and capsaicin, blood flow was 74 ± 9 TPU/15 min before capsaicin and 70 ± 11 TPU/15 min after injection of capsaicin, suggesting that $CGRP_{8-37}$ blocked the vasodilatation by capsaicin. The capsaicin-evoked blood flow was 68 ± 11 TPU/15 minutes in the absence of the $CGRP_{8-37}$, whereas in the presence of $CGRP_{8-37}$ the evoked response was -4 ± 5 TPU/15 minutes (Figure 14B).

$CGRP_{8-37}$ at the concentration used in our study (20 μM) did not by itself alter blood flow. The basal blood flow before and after injection of the antagonist was 63 ± 21 and 58 ± 10 TPU/ 15 min respectively (Figure 14C) and the evoked response was -4 ± 13 TPU/15 min (Figure 14D). This suggests that there is no tonic control of blood flow by CGRP in our preparation. Furthermore injection of the vehicle did not alter basal blood flow. The blood flow recorded before and after intradermal injection of vehicle was 68 ± 6 TPU/15 min and 70 ± 10 TPU/15 min respectively (Figure 14C), and the evoked response was 2 ±11 TPU/15 minutes (Figure 14D). Together, these data demonstrate that the increase in blood flow caused by capsaicin is mediated by CGRP.

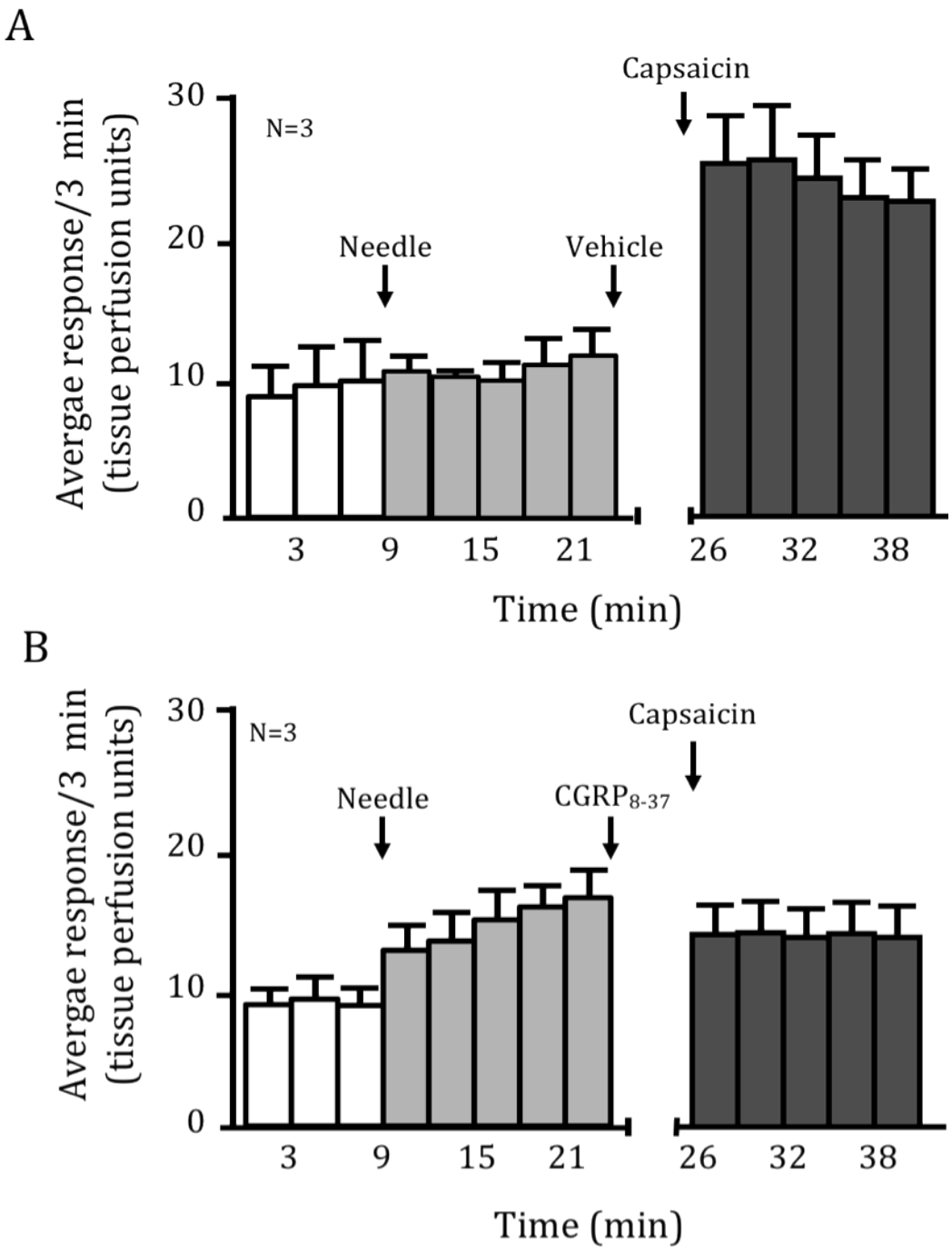

Figure 12. The CGRP receptor antagonist, $CGRP_{8-37}$, blocks capsaicin-induced cutaneous vasodilatation. Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after intradermal insertion of the needle, respectively. The dark columns represent blood flow after injection of vehicle (0.01% MPL in PBS) then 10 μM capsaicin (A) or 20 μM $CGRP_{8-37}$ then 10 μM capsaicin (B).

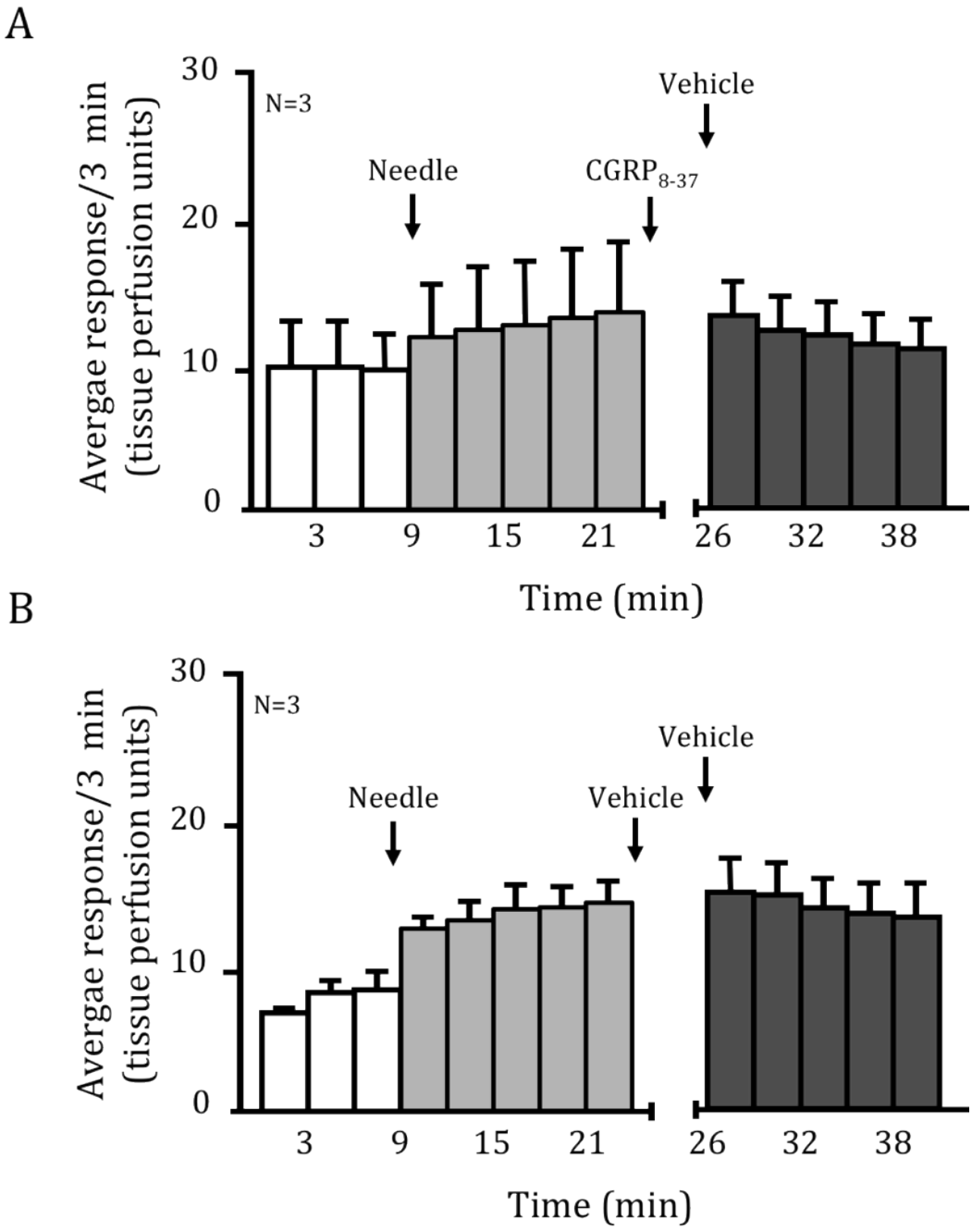

Figure 13. The CGRP receptor antagonist, $CGRP_{8-37}$ or the vehicle (0.01% MPL in PBS) do not alter blood flow. E, Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after intradermal insertion of the needle respectively. The dark columns represent blood flow after injection of $CGRP_{8-37}$ before vehicle (0.01% MPL in PBS) (A) or vehicle before a second injection of vehicle (B).

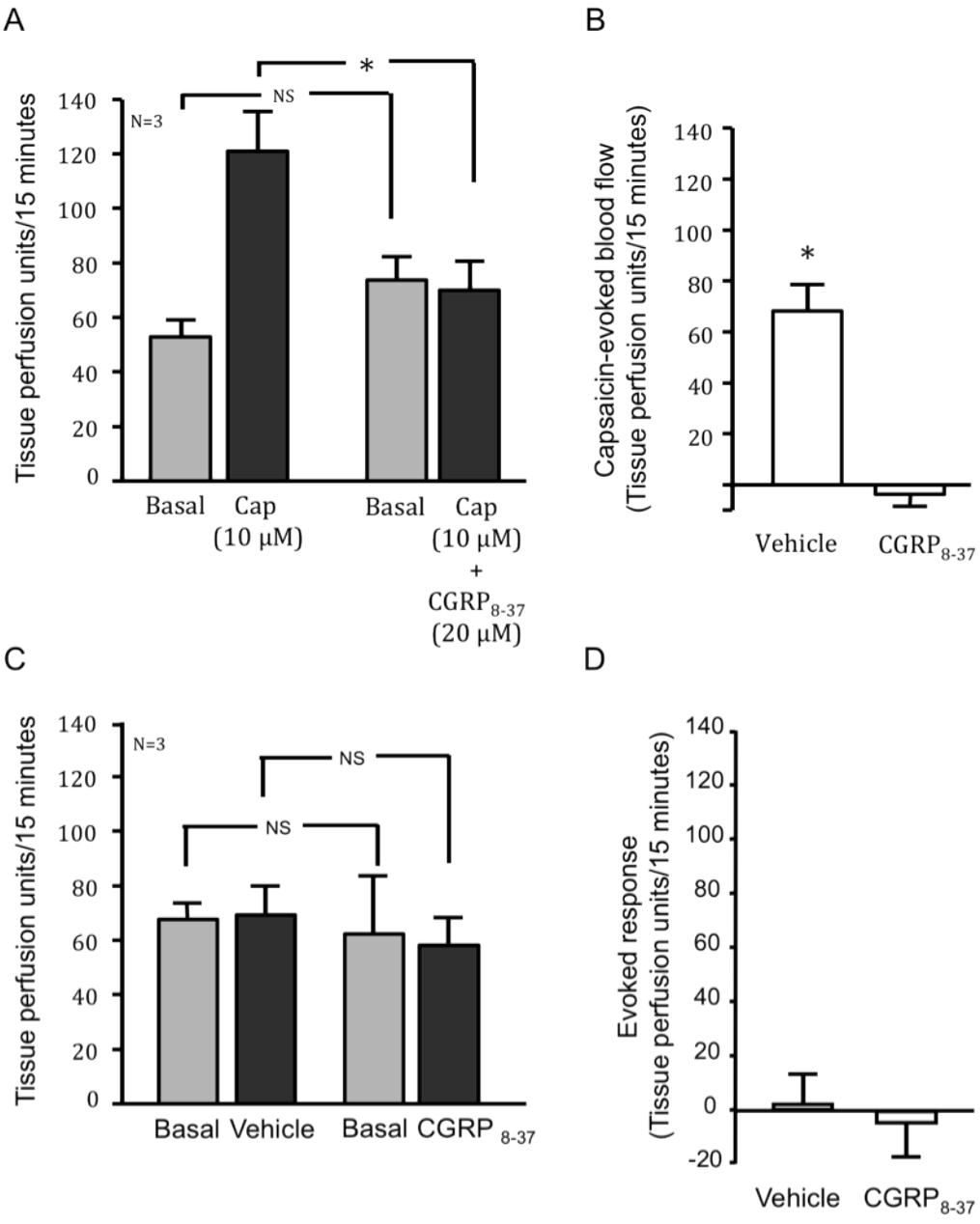

Figure 14. The capsaicin-evoked response in presence of $CGRP_{8-37}$ is significantly different from that recorded in presence of the vehicle. Each column is the mean ± SEM. Lightly shaded represent TPU recorded for 15 minutes following placement of the needle. Dark columns represent TPU recorded for 15 minutes following injection of capsaicin ± $CGRP_{8-37}$ (A) or vehicle ± $CGRP_{8-37}$ (C). Capsaicin-evoked response is statistically higher in absence of $CGRP_{8-37}$ (B). Capsaicin-evoked response in presence of vehicle was not different from that in presence of $CGRP_{8-37}$ (D). An asterisk indicates statistical significance by one-way ANOVA with Bonferroni's post tests, $p < 0.05$.

Electrical stimulation mediated cutaneous vasodilatation is mediated by CGRP

It has been demonstrated by Luo et al., that human umbilical vascular endothelial cells in culture synthesize and release CGRP after exposure to capsaicin (Luo et al., 2008). Therefore it is possible that the cutaneous vasodilatation mediated by capsaicin is secondary to release of CGRP from endothelial cells. To demonstrate vasodilatation after direct activation of sensory neurons, the sciatic nerve was stimulated using an electrical stimulus. The sciatic nerve arises from the lumbar ganglia L4, L5, and L6, and it innervates the hind paw of the rat (Takahashi et al., 2003). Koltzenburg et al., showed that electrical stimulation of the sciatic nerve using a stimulus intensity of 5 mA at 10 Hz for 30 seconds activates not only the large low threshold sensory neurons but also the high threshold and slowly-conducting unmyelinated C fibers and the thinly-myelinated Aδ fibers including those containing CGRP (Koltzenburg et al., 1990). Therefore this stimulus intensity was used to activate sensory neurons and induce vasodilatation in the skin.

After recording basal blood flow for 10 minutes, the trunk of the sciatic nerve was transected at the mid-thigh level (Figure 5). In the initial experiments, I observed a significant increase in basal blood flow after transection, which was likely due to action potential discharges initiated by the injury (data not shown). Therefore, 1% lidocaine was applied distal to the stimulation site immediately prior to cutting the sciatic nerve. After

placing the stimulator on the sciatic nerve, the animal was sutured and allowed to recover for 30 minutes before application of the electrical stimulus.

As can be seen from Figure 15A, electrical stimulation of the sciatic nerve significantly increased blood flow from 20 ± 3 to 64 ± 1 TPU/3 minutes. The response over 15 min increased from a basal value of 103 ± 15 TPU/15 minutes to 329 ± 8 TPU/15 minutes (Figure 16A). The increase in blood flow was maintained for at least 30 minutes after the 30-second stimulation. The average evoked response (obtained by subtracting the basal response from the response after stimulation) was 226 ± 19 TPU/15 minutes (Figure 16B).

To determine if the increase in blood flow by electrical stimulation was mediated by CGRP released from sensory neurons, $CGRP_{8-37}$ was injected into the paw prior to electrical stimulation of the sciatic nerve. In the presence of 20 μM $CGRP_{8-37}$, electrical stimulation did not result in a significant increase in blood flow over time. Basal blood flow was 23 ± 2 TPU/3 minutes, while stimulated blood flow in the presence of $CGRP_{8-37}$ was 24 ± 2 TPU/3 minutes (Figure 15B).

Figure 16A shows that basal blood flow before electrical stimulation was 112 ± 8 TPU/15 minutes, and after stimulation in the presence of $CGRP_{8-37}$ was 126 ± 11 TPU/15 minutes. The evoked response in the presence of $CGRP_{8-37}$ was 14 ± 10 TPU/15 minutes, which was

significantly lower than that recorded in the absence of the $CGRP_{8-37}$ (226 ± 19 TPU/15 minutes, Figure 16B).

Together, these data suggest that the increase in blood flow by electrical stimulation of the sciatic nerve is mediated by CGRP released from the peripheral endings of sensory neurons.

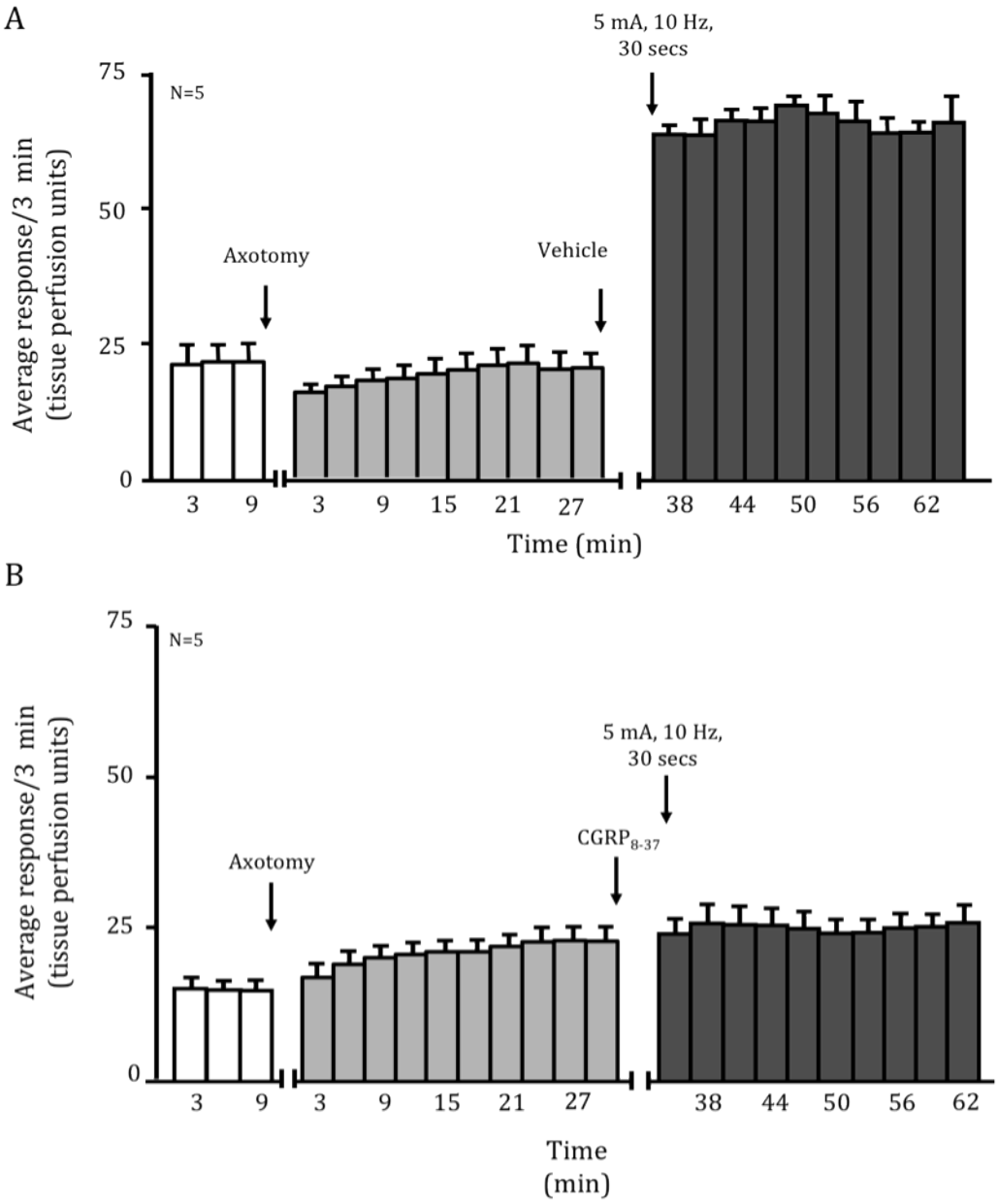

Figure 15. $CGRP_{8-37}$ blocks electrical stimulation-evoked cutaneous vasodilatation. Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after axotomy respectively. Dark columns represent blood flow changes after electrical stimulation preceded either by intradermal vehicle (A) or by intradermal $CGRP_{8-37}$ (B).

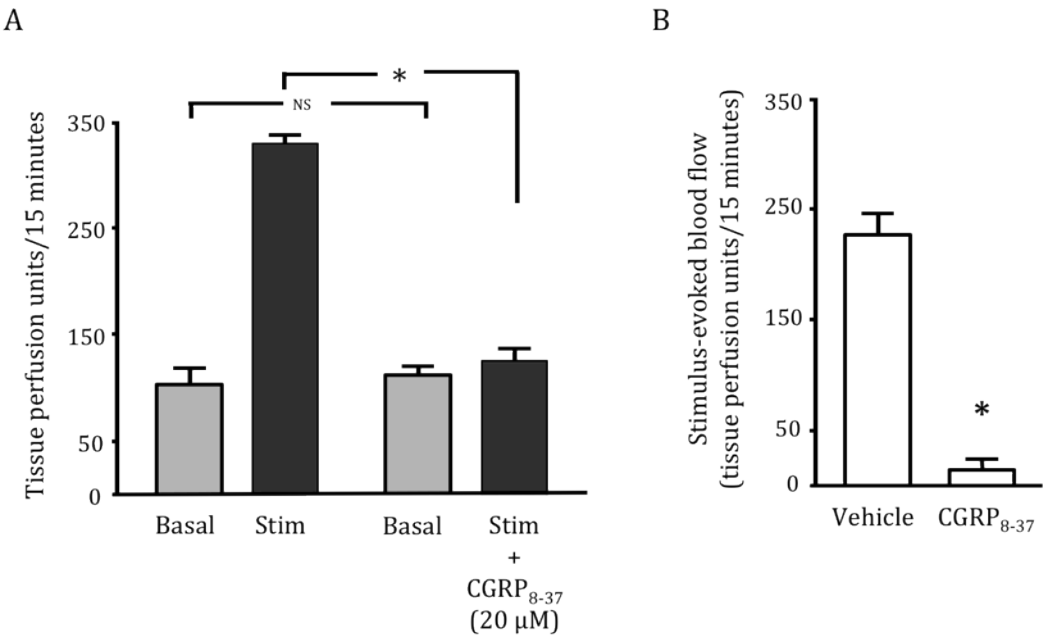

Figure 16. The electrical stimulation-evoked cutaneous vasodilatation is dependent on CGRP. Each column is the mean ± SEM. Lightly shaded represent TPU recorded for 15 minutes following axotomy while dark columns represent TPU recorded after electrical stimulation ± $CGRP_{8-37}$ (A). Responses evoked by electrical stimulation are decreased when $CGRP_{8-37}$ is injected before stimulation (B). An asterisk indicates statistical significance by one-way ANOVA with Bonferroni's post tests, $p < 0.05$.

Chronic administration of paclitaxel reduces sensory neuron mediated cutaneous vasodilatation

To determine if paclitaxel alters the release of CGRP from sensory neurons, we measured the ability of capsaicin or electrical stimulation to vasodilate after chronic administration of the microtubule-stabilizing drug. As demonstrated above, cutaneous vasodilatation induced by activation of sensory neurons is mediated by CGRP, hence as an indirect measure of CGRP release we measured changes in blood flow after stimulation of peripheral endings of sensory neurons. Male Sprague Dawley rats were injected with 1 mg/kg paclitaxel every other day for a total of 4 doses, and vasodilatation was examined 2 weeks after the first injection.

In vehicle injected animals, injection of 10 μM capsaicin in 1 μl increased blood flow from 18 ± 1 to 56 ± 3 TPU/ 3 minutes (Figure 17A). The blood flow over 15 minutes increased from 88 ± 4 to 277 ± 19 TPU/15 minutes (Figure 18A), which represents an evoked release of 190 ±19 TPU/ 15 minutes (Figure 18B). In contrast, in rats injected with 1 mg/kg paclitaxel every other day for a week, and studied one week after the last injection, 10 μM capsaicin increased blood flow from 17 ± 1 to 31 ± 4 TPU/ 3 minutes (Figure 17B). The increase over 15 minutes was 158 ± 16 TPU from 82 ± 6 TPU/15 minutes (Figure 17A). The evoked blood flow was 76 ± 17 TPU/15 minutes, which was significantly lower than that in vehicle treated rats (Figure 17B).

Paclitaxel also decreased vasodilatation induced by electrical stimulation of the sciatic nerve. Stimulation of the sciatic nerve in vehicle-treated animals produced more than a 3-fold increase in blood flow over baseline; blood flow increased from 26 ± 5 to 85 ± 5 TPU/3 minutes in (Figure 19A). Over a period of 15 minutes, the blood flow increased from 128 ± 21 to 426 ± 24 TPU/15 min (Figure 20A), and the evoked response was 298 ± 30 TPU/15 minutes. In animals that were injected with paclitaxel, the increase in blood flow after electrical stimulation was 30 ± 4 to 56 ± 5 TPU/ 3 minutes (Figure 19B). Over 15 minutes, the blood flow increased from 145 ± 19 to 296 ± 28 TPU (Figure 20A), which represents an evoked response of 151 ± 13 TPU/15 minutes (Figure 20B).

In summary, paclitaxel decreased cutaneous vasodilatation mediated by activation of sensory neurons. Because cutaneous vasodilatation is mediated by CGRP, these data suggest that paclitaxel decreases the release of CGRP from small diameter sensory neurons.

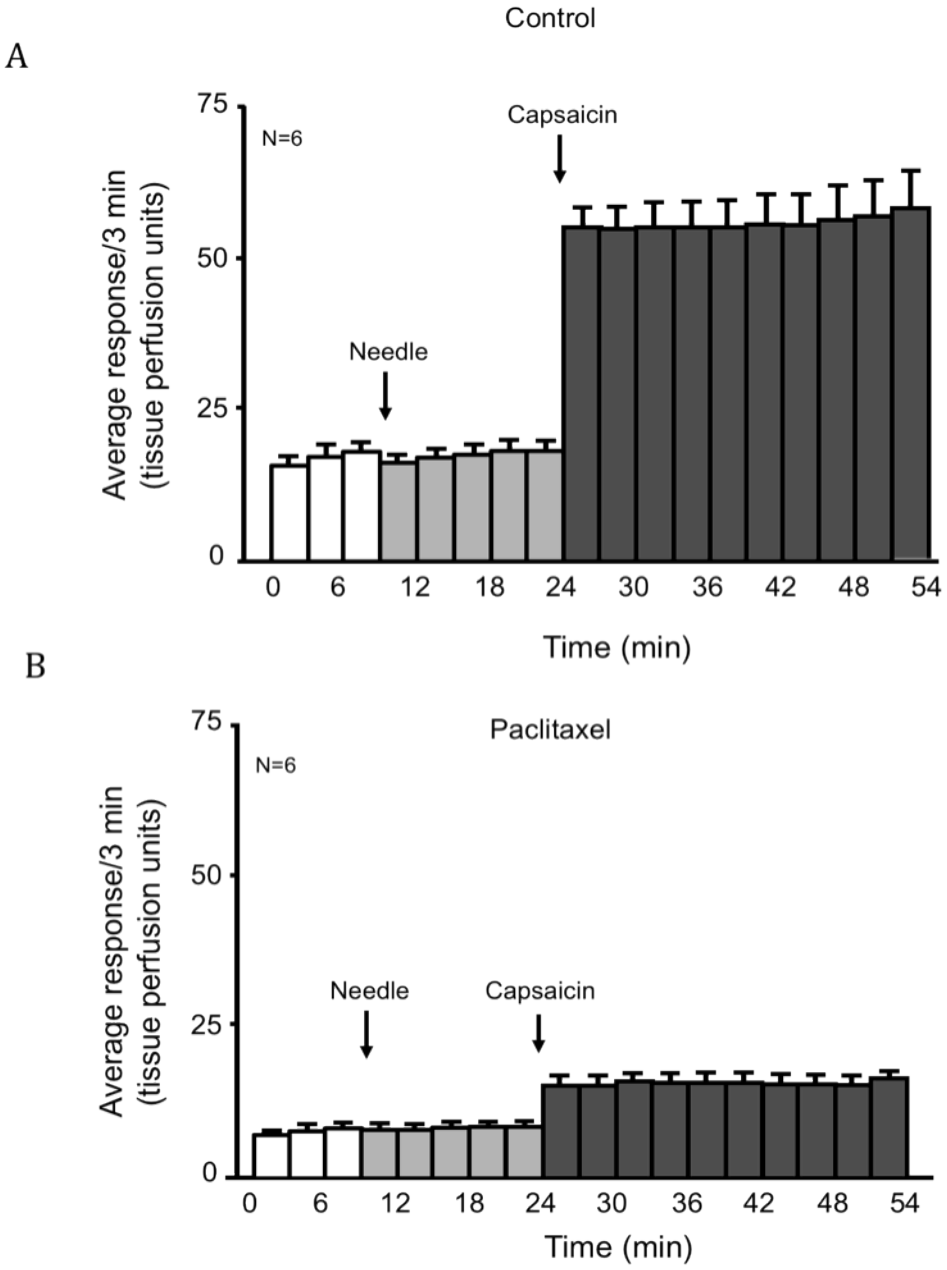

Figure 17. Chronic administration of paclitaxel decreases capsaicin-induced cutaneous vasodilatation in the rat hind paw. Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after intradermal insertion of the injection needle, respectively. The dark columns represent blood flow after injection of 10 μM capsaicin in rats chronically administered with vehicle (A) or 1 mg/kg paclitaxel (B).

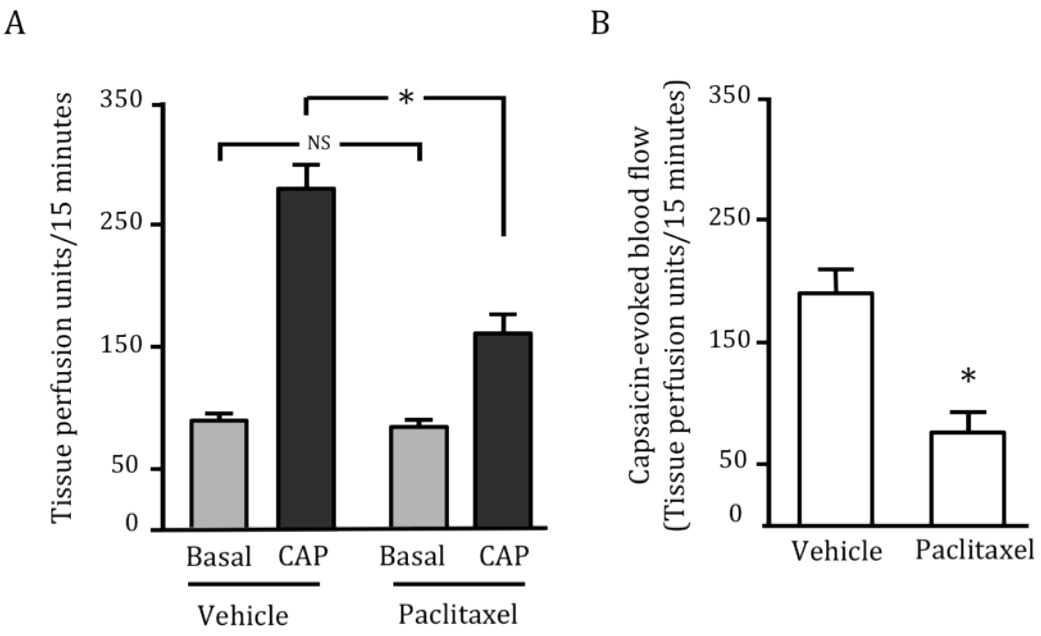

Figure 18. The capsaicin-evoked vasodilatation is significantly attenuated in paclitaxel-injected animals. Each column is the mean ± SEM. A: Lightly shaded represent TPU recorded for 15 minutes following placement of the needle. Dark columns represent TPU recorded for 15 minutes following injection of capsaicin in vehicle or paclitaxel-treated animals B: Capsaicin-evoked blood flow over 15 minutes in vehicle or paclitaxel-injected animals. In A and B, an asterisk indicates statistical significance between the paclitaxel-treated group and the vehicle-injected group by one-way ANOVA with Bonferroni's post tests, $p < 0.05$.

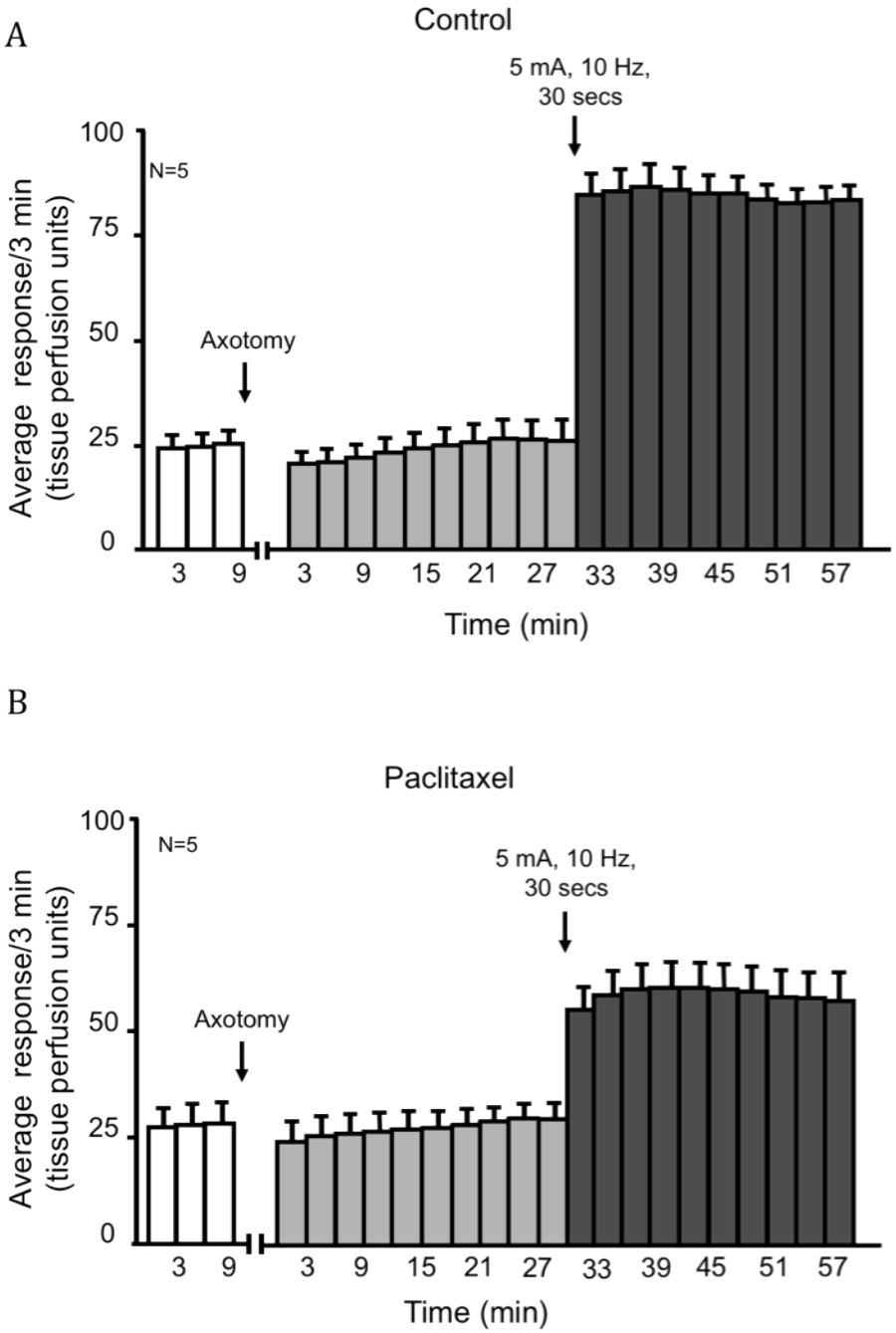

Figure 19. Chronic administration of paclitaxel decreases electrical stimulation-induced cutaneous vasodilatation in the rat hind paw. Each column represents the mean ± SEM. The open and light shaded columns represent the basal blood flow before and after axotomy respectively. The dark columns represent blood flow after electrical stimulation of the sciatic nerve in rats that were administered vehicle (A) or paclitaxel (B).

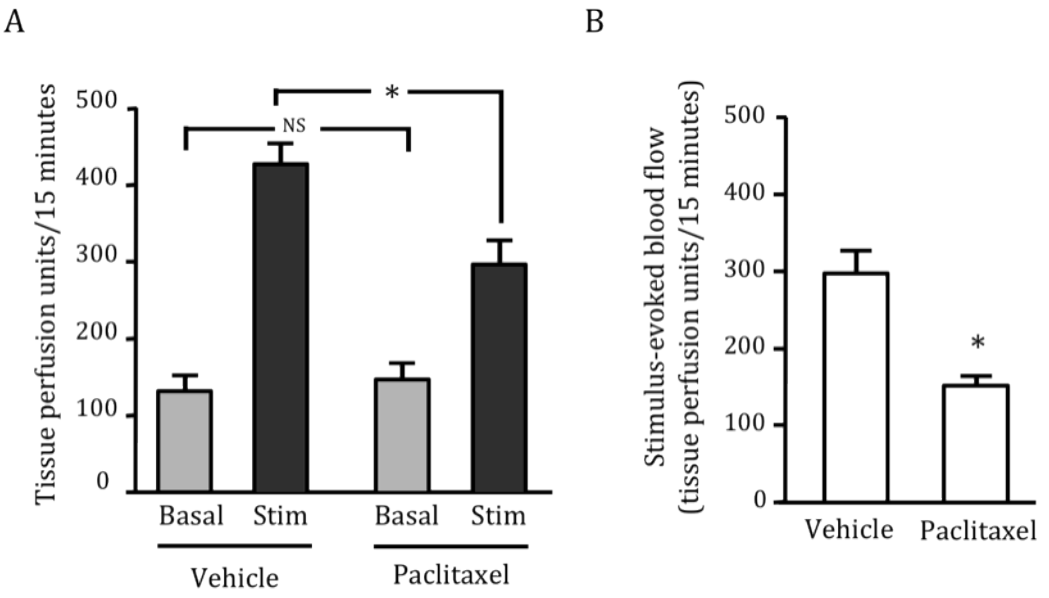

Figure 20. The electrical stimulation-evoked vasodilatation is significantly attenuated in paclitaxel-injected animals. Each column is the mean ± SEM. A: Lightly shaded represent TPU recorded for 15 minutes before stimulation. Dark columns represent TPU recorded for 15 minutes following electrical stimulation. B: Electrical stimulation-evoked changes in blood flow over 15 minutes in vehicle or paclitaxel-injected animals. In A and B, an asterisk indicates statistical significance between the paclitaxel-treated group and the vehicle-injected group by one-way ANOVA with Bonferroni's post tests, $p < 0.05$.

Chronic administration of paclitaxel does not affect vasodilatation-induced by intradermal injection of CGRP or methacholine

While it is possible that the decreased vasodilatation results from decrease in sensory neuron function, altered expression or function of the CGRP1 receptor on blood vessels could also contribute to the attenuation in sensory neuron mediated increase in skin blood flow. Therefore vasodilatation in response to intradermal injection of CGRP was examined in paclitaxel-treated rats.

In vehicle treated animals, blood flow increased from 20 ± 2 to 60 ± 2 TPU/3 minutes (Figure 21A). Over 15 minutes the blood flow increased from 97 ± 11 to 316 ± 14 TPU (Figure 22A), the evoked response was 219 ± 8 TPU/ 15 minutes (Figure 22B). In paclitaxel-injected animals, the blood flow increased from 20 ± 3 to 59 ± 1 TPU/3 minutes (Figure 21B). Over 15 minutes, the blood flow increased from 96 ± 15 to 302 ± 8 TPU (Figure 22A), which represents an evoked response of 206 ± 15 TPU/15 minutes (Figure 22B). Paclitaxel did not alter the ability of blood vessels to dilate in response to intradermal CGRP, suggesting that the expression or function of the CGRP receptor was not altered in paclitaxel injected animals.

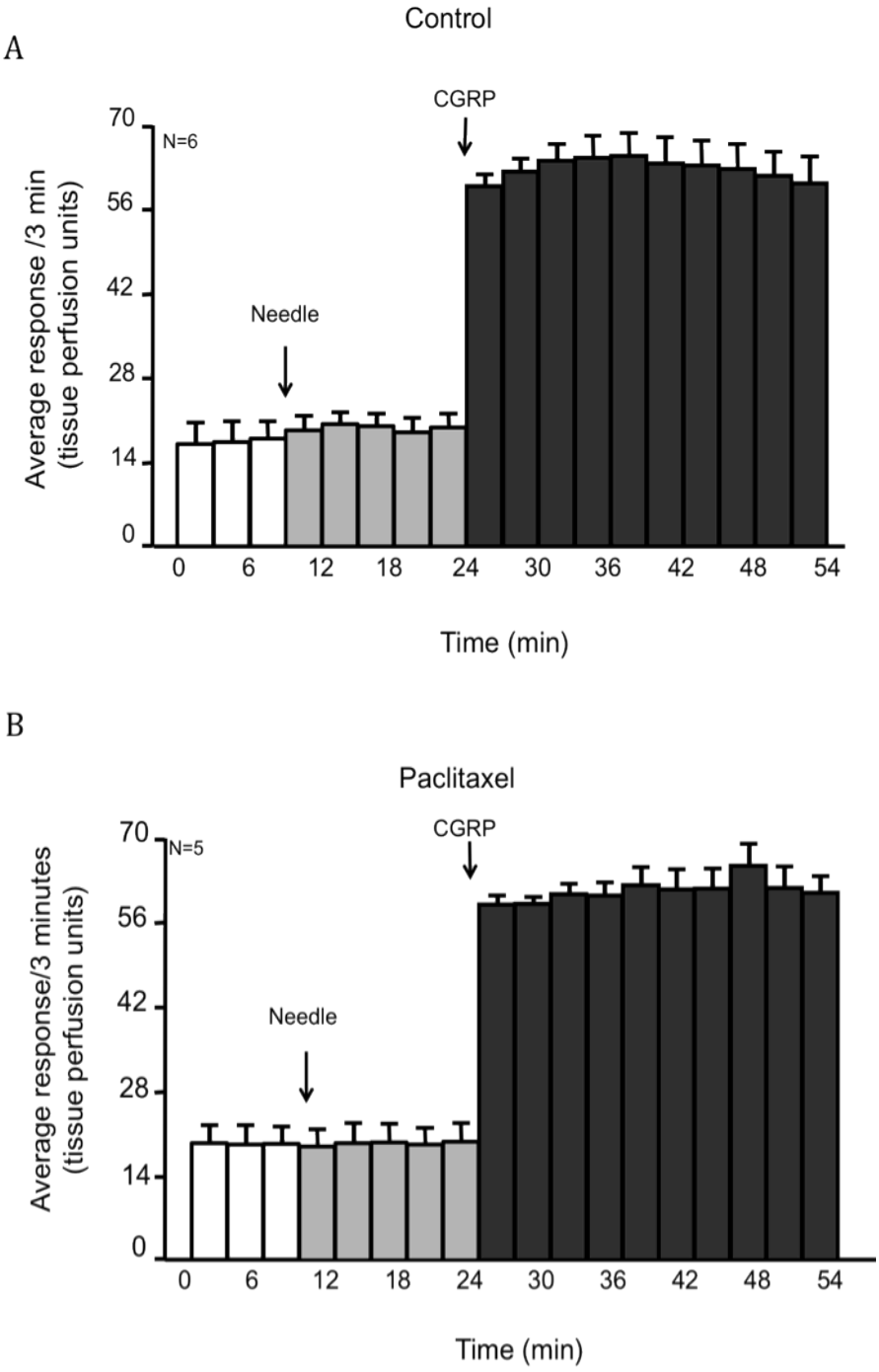

Figure 21. Chronic administration of paclitaxel does not alter CGRP-induced cutaneous vasodilatation. Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after intradermal placement of the needle, respectively. The shaded columns represent blood flow after injection of 30 μM CGRP in either vehicle (A) or paclitaxel-injected animals (B).

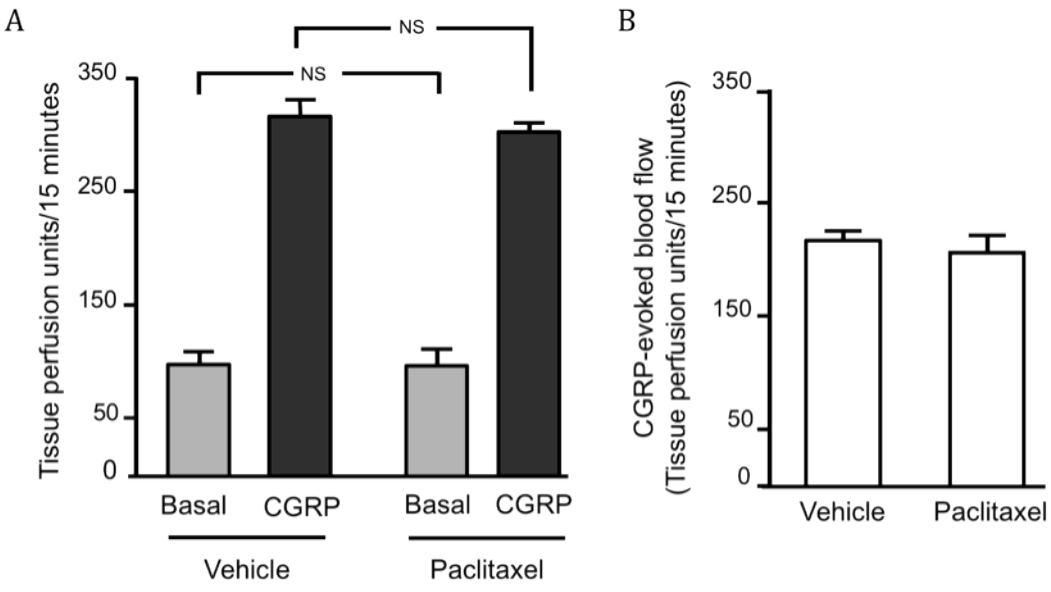

Figure 22. Vasodilatation evoked by intradermal CGRP is not altered in paclitaxel-injected animals. Each column represents the mean ± SEM. A: Lightly shaded columns represent TPU recorded for 15 minutes after intradermal placement of the needle. Dark columns represent TPU recorded for 15 minutes following injection of CGRP. B: CGRP-evoked changes in blood flow over 15 minutes in vehicle or paclitaxel-injected animals.

To determine whether paclitaxel interferes with smooth muscle relaxation, vasodilatation was examined in response to methacholine, the muscarinic receptor agonist. Methacholine induces vasodilatation in a nitric oxide-dependent manner (Kimura et al., 2007). When injected intradermally in vehicle-injected animals, 100 μM methacholine caused a significant increase in blood flow from 20 ± 2 to 53 ± 3 TPU/3 minutes (Figure 23A). Over a period of 15 minutes, methacholine increased blood flow from 97 ± 8 to 276 ± 11 TPU (Figure 24A), the evoked response in this case was 179 ± 15 TPU/15 minutes (Figure 24B). In paclitaxel injected animals, intradermal injection of 100 μM methacholine caused an increase in blood flow over 3 minutes from 18 ± 2 to 56 ± 5 TPU (Figure 23B). Over 15 minutes, the blood flow increased from 88 ± 8 to 290 ± 23 TPU (Figure 24A). The evoked response was 201 ± 23 TPU/15 minutes (Figure 24B). These data demonstrate that paclitaxel does not inhibit the ability of the vascular smooth muscle to relax and produce vasodilatation.

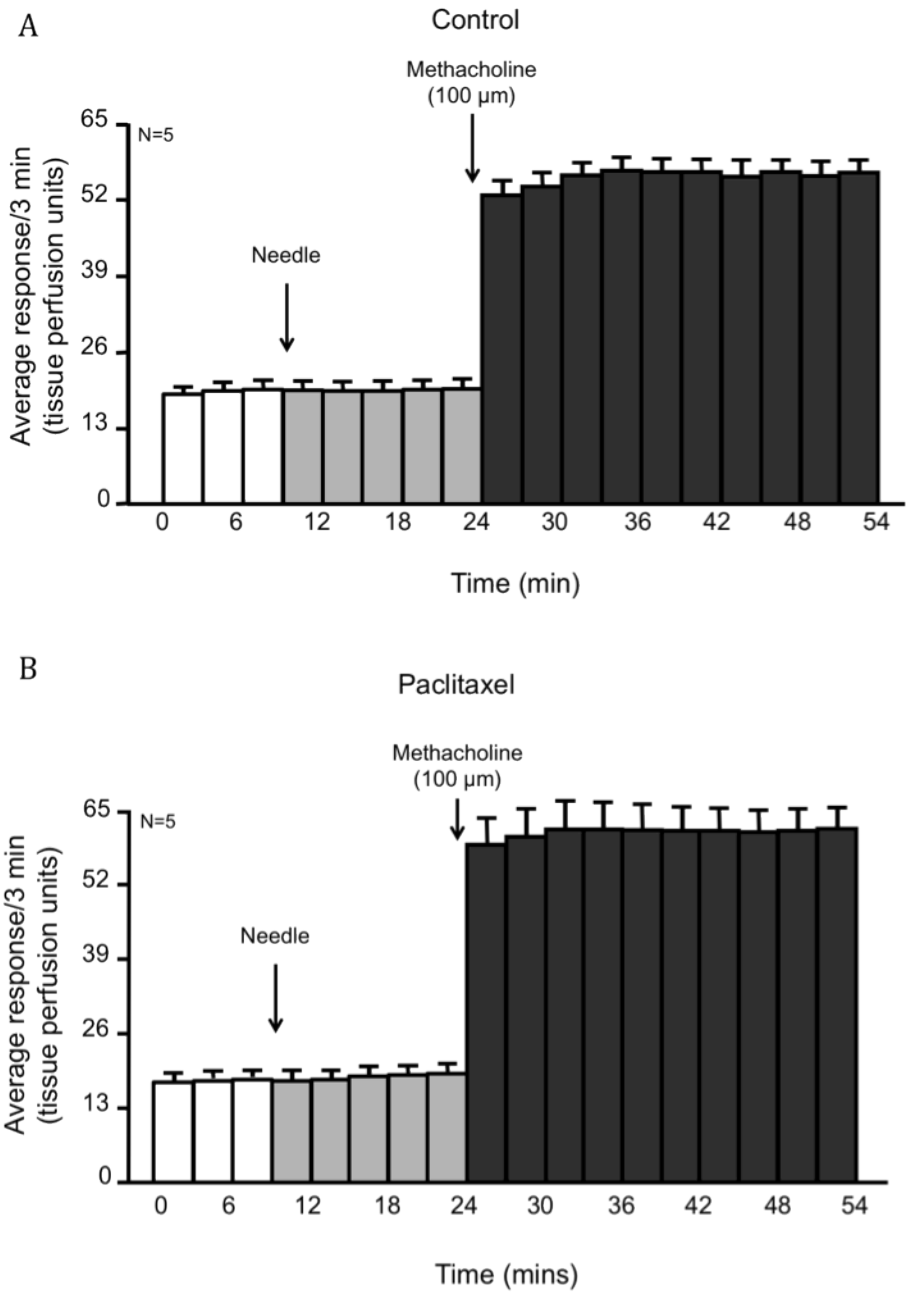

Figure 23. Chronic administration of paclitaxel does not alter methacholine-induced cutaneous vasodilatation. Each column represents the mean ± SEM. The open columns and the light columns represent basal blood flow before and after intradermal placement of the injection needle, respectively. The dark columns represent blood flow after injection of 100 µM methacholine in either vehicle injected animals (A) or paclitaxel injected animals (B).

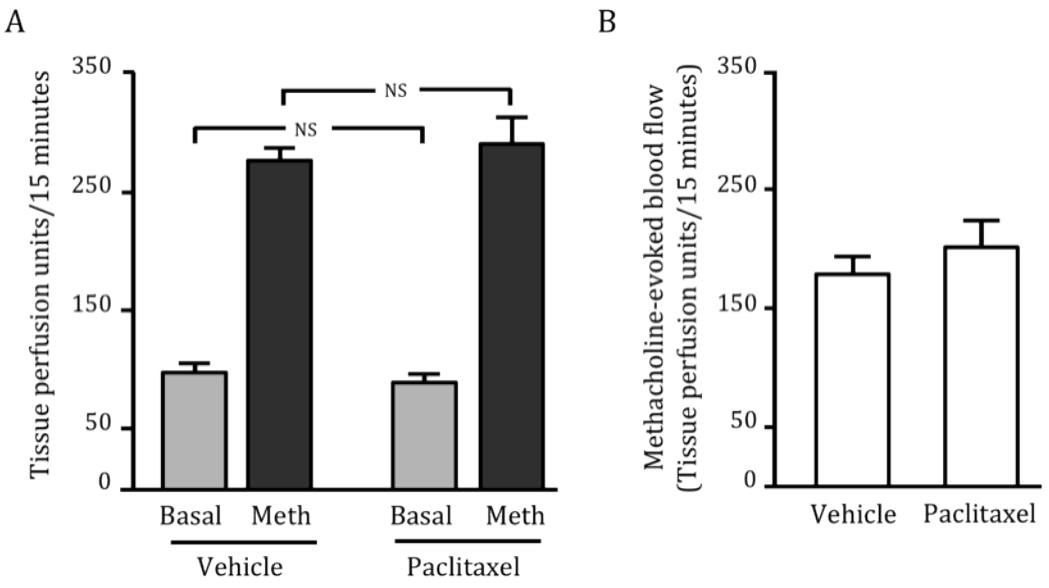

Figure 24. Vasodilatation evoked by intradermal methacholine is not altered in paclitaxel-injected animals. Each column represents the mean ± SEM A: Lightly shaded columns represent TPU recorded for 15 minutes after placement of needle. Dark columns represent TPU recorded for 15 minutes after injection of methacholine B: Methacholine-evoked changes in blood flow over 15 minutes in vehicle or paclitaxel-injected animals.

Chronic administration of paclitaxel does not alter the release of iCGRP from dorsal spinal cord

Microtubules participate in the transport of organelles and vesicles from the cell body to the nerve ending since inhibition of microtubule function has been shown to interfere with axonal transport (Banks et al., 1971b; Banks et al., 1971a; Mayor et al., 1972; Smith et al., 1975; Heiwall et al., 1978; Nakata and Yorifuji, 1999; Theiss and Meller, 2000). Thus, it is possible that paclitaxel decreases the content of CGRP and consequently the release of the neuropeptide at all nerve endings of the sensory neurons. To examine this, CGRP content and release was directly quantified from the central terminals of small diameter sensory neurons in spinal cord slices from paclitaxel-injected rats.

Slices were prepared from the dorsal lumbar region of the spinal cord because this region is enriched with sensory nerve endings that innervate the hind paws of the rat. As can be seen in Figure 25A and B, treating rats with paclitaxel did not alter basal release of iCGRP compared to rats treated with vehicle. Before stimulation, the amount of CGRP released in slices from vehicle injected animals was 0.77 ± 0.1 % total content/9 minutes while that from paclitaxel injected animals was 0.67 ± 0.07 % content/9 minutes (Figure 26A). After stimulation with 500 nM capsaicin CGRP release increased to 4.5 ± 0.5 % content/9 minutes and 3.6 ± 0.5 % content/9 minutes in vehicle or paclitaxel injected animals respectively (Figure 26A) giving an evoked release of 3.8 ± 0.5 %

content/9 minutes and 2.9 ± 0.5 % content/9 minutes in the vehicle-injected group and the paclitaxel-injected group respectively (Figure 26B). The total content of iCGRP in the tissue from vehicle-injected animals was 505.9 ± 33.8 and that from paclitaxel-treated animals was 496.8 ± 30.6 fmol/mg of tissue (Figure 26C).

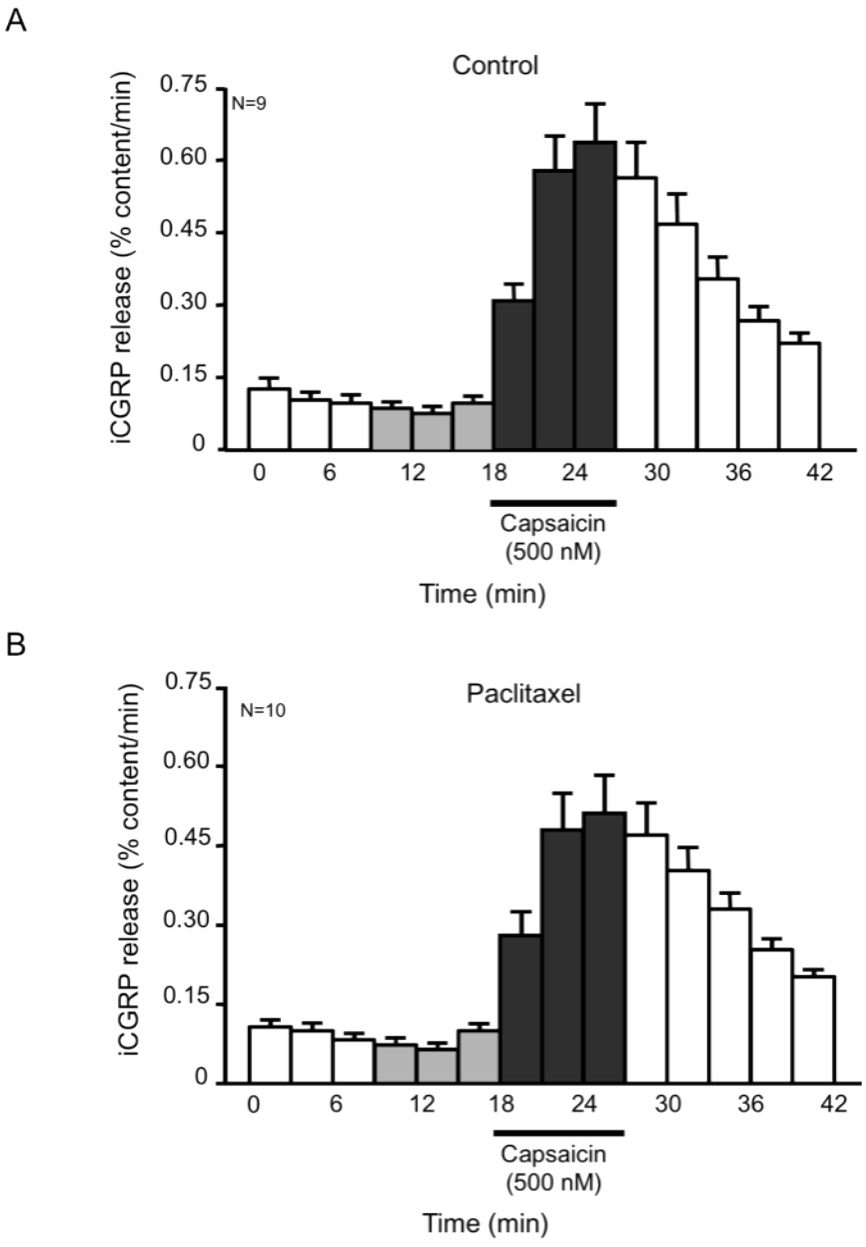

Figure 25. Chronic administration of 1 mg/kg paclitaxel does not alter capsaicin-evoked iCGRP release from rat spinal cord slices. Each column represents the mean ± SEM. The horizontal bar indicates the time when the tissues were exposed to capsaicin. iCGRP release in each 3-minute perfusion sample is expressed as percent of total peptide content per minute. A: iCGRP release in slices taken from vehicle-treated rats, B: iCGRP release in slices taken from paclitaxel-injected rats.

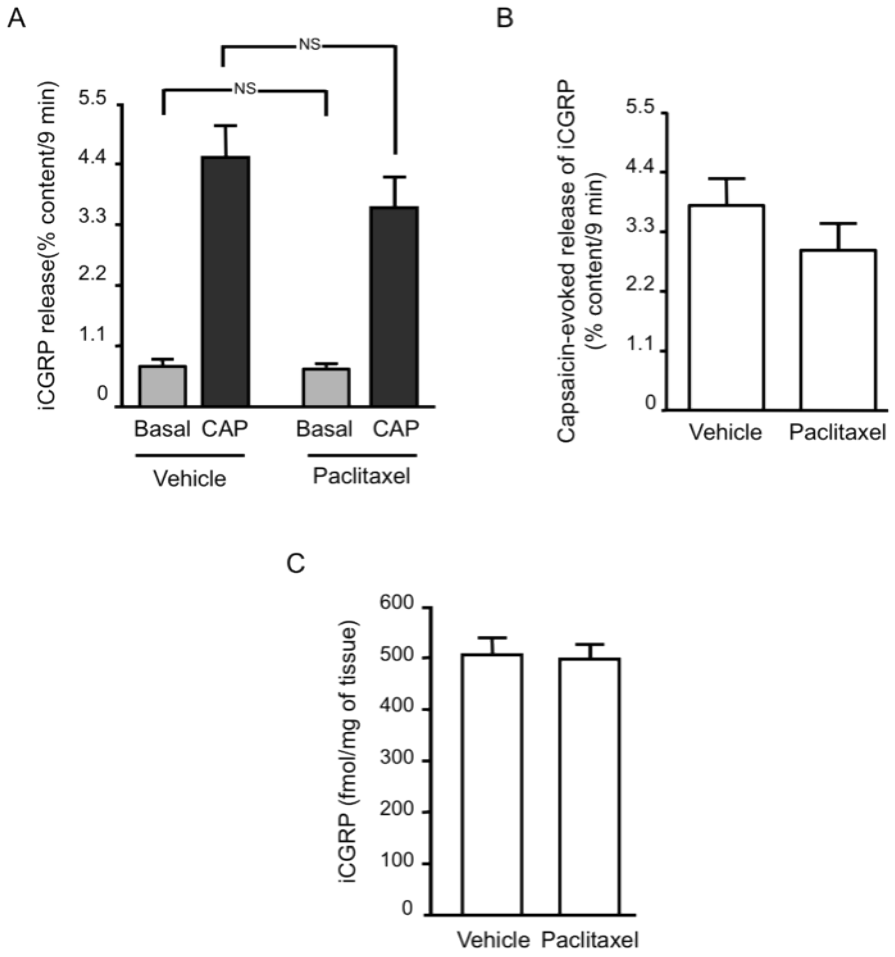

Figure 26. Capsaicin-evoked release of iCGRP is not altered in paclitaxel-treated animals. Each column is the mean ± SEM. A: Basal release is the sum of the 3 collections prior to capsaicin, whereas capsaicin-stimulated release is the sum of the three collections in the presence of capsaicin B: Evoked release of iCGRP in vehicle or paclitaxel-treated rats. C: The total content of iCGRP in spinal cord slices from vehicle- or paclitaxel injected animals.

The effects of paclitaxel on release of CGRP was examined after stimulation with a general depolarizing stimulus; 30 mM KCl. As in the previous series of studies, chronic administration with paclitaxel did not alter basal release of iCGRP (Figure 27A and B). Before stimulation, release of iCGRP from vehicle-injected animals was 0.80 ± 0.14 while that from paclitaxel-injected animals was 0.81 ± 0.07 % total content/9 min (Figure 28A). The amount of iCGRP release after stimulation of the slices with 30 mM KCl increased to 1.93 ± 0.23 and 2.10 ± 0.34 % total content/10 min in vehicle-injected and paclitaxel-injected animals respectively. The evoked release of iCGRP from sensory neurons was 1.13 ± 0.31 and 1.29 ± 0.36 % total content/9 minutes (Figure 28B). The total amount of iCGRP in vehicle-injected animals was 642.66 ± 36.37 while that in paclitaxel-injected animals was 565.95 ± 51.56 fmol/mg tissue (Figure 28C). These data indicate that paclitaxel does not alter the ability of sensory neurons to depolarize nor does it inhibit the mechanisms of neurotransmitter release.

When the evoked release in both of the above described experiments were compared by the unpaired t-test, there were no statistically significant differences between the vehicle-injected animals and the paclitaxel-injected animals. Furthermore, the total content of iCGRP was not different between the 2 groups of animals. This suggests that paclitaxel at 1 mg/kg does not alter the transport of CGRP from the cell body to the central terminals of the primary afferent sensory neuron.

Furthermore, the dosing regimen of paclitaxel used does not alter the mechanism mediating CGRP release.

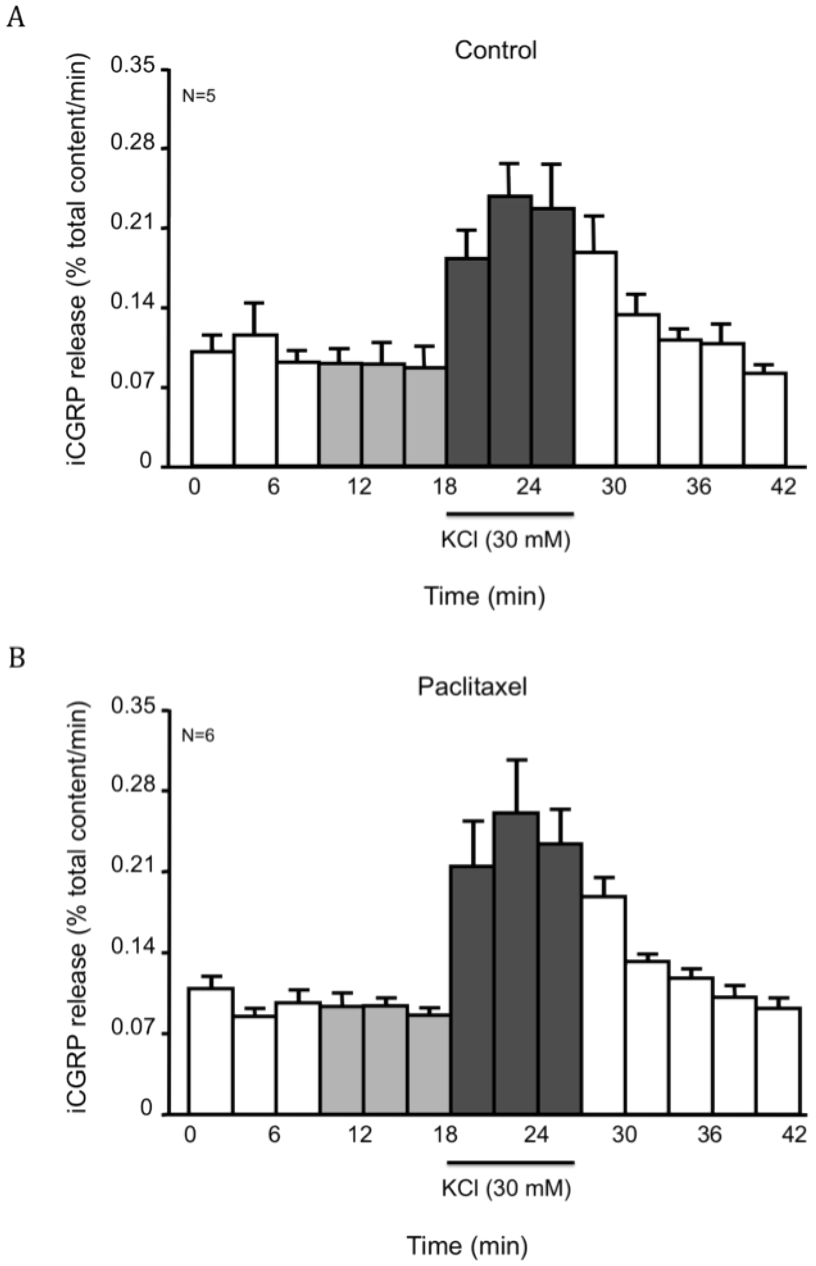

Figure 27. Chronic administration of 1 mg/kg paclitaxel does not alter iCGRP release stimulated by 30 mM KCl from rat spinal cord slices. Each column represents the mean ± SEM. The horizontal bar indicates the time when the tissues were exposed to 30 mM KCl. iCGRP release in each 3-minute perfusion sample is expressed as percent of total peptide content per minute. A: iCGRP release in slices taken from vehicle-treated rats, B: iCGRP release in slices taken from paclitaxel-injected rats.

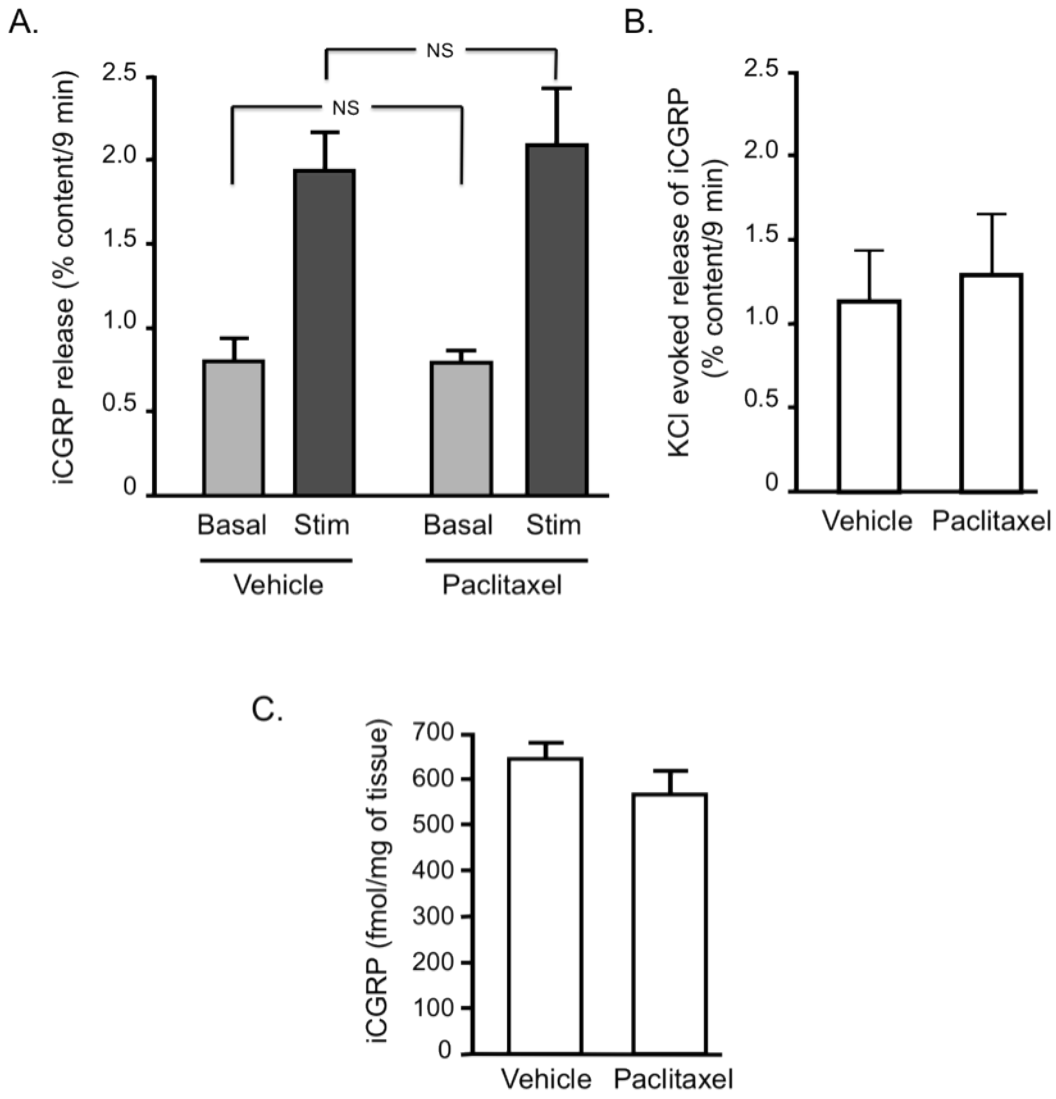

Figure 28. Potassium-evoked release of iCGRP is not altered by chronic administration of paclitaxel. Each column is the mean ± SEM. A: Basal release is the sum of the 3 collections prior to 30 mM KCl, whereas KCl-stimulated release is the sum of the three collections in the presence of 30 mM KCl B: Evoked release of iCGRP (KCl-stimulated release minus basal release presented in Figure A) in vehicle- or paclitaxel-treated rats, as indicated. C: The total content of iCGRP in the spinal cord slices from vehicle or paclitaxel injected animals.

It is known that paclitaxel does not readily cross the blood brain barrier (Lesser et al., 1995; Cavaletti et al., 2000; Fellner et al., 2002), which might explain the lack of systemic effects of the drug on CGRP release. To determine if higher doses of paclitaxel might alter release of CGRP from spinal cord slices, animals were injected with 2 mg/kg paclitaxel on 4 consecutive days.

The basal release of iCGRP in animals injected with 2mg/kg paclitaxel is not significantly different from that in vehicle-injected animals (Figure 29A and B). In vehicle-injected animals, iCGRP release increased from 1.13 ± 0.12 to 4.98 ± 0.57 % content/3 minutes after stimulation with capsaicin. The basal release in paclitaxel-treated animals was 0.94 ± 0.14 % content/9 minutes while capsaicin-stimulated release was 3.28 ± 0.51 % total content/9 minutes (Figure 30A). The evoked release of iCGRP was 3.85 ± 0.62 and 2.34 ± 0.55 % total content/9 minutes in vehicle-injected and paclitaxel-injected animals respectively (Figure 30B). Furthermore, 2 mg/kg paclitaxel did not alter the total content of iCGRP in the spinal cord: the total amount of iCGRP in vehicle-injected animals was 503.7 ± 44.82 and that in paclitaxel-injected animals was 497.7 ± 46.31 fmol/mg respectively (Figure 30C).

In summary, paclitaxel at the doses administered did not alter the release of iCGRP from sensory nerve terminals in spinal cord slices irrespective of the stimulus used. Since these doses do alter release in the

periphery, the data suggest that paclitaxel alters the peripheral nerve endings of sensory neurons while the central terminals remain unaffected.

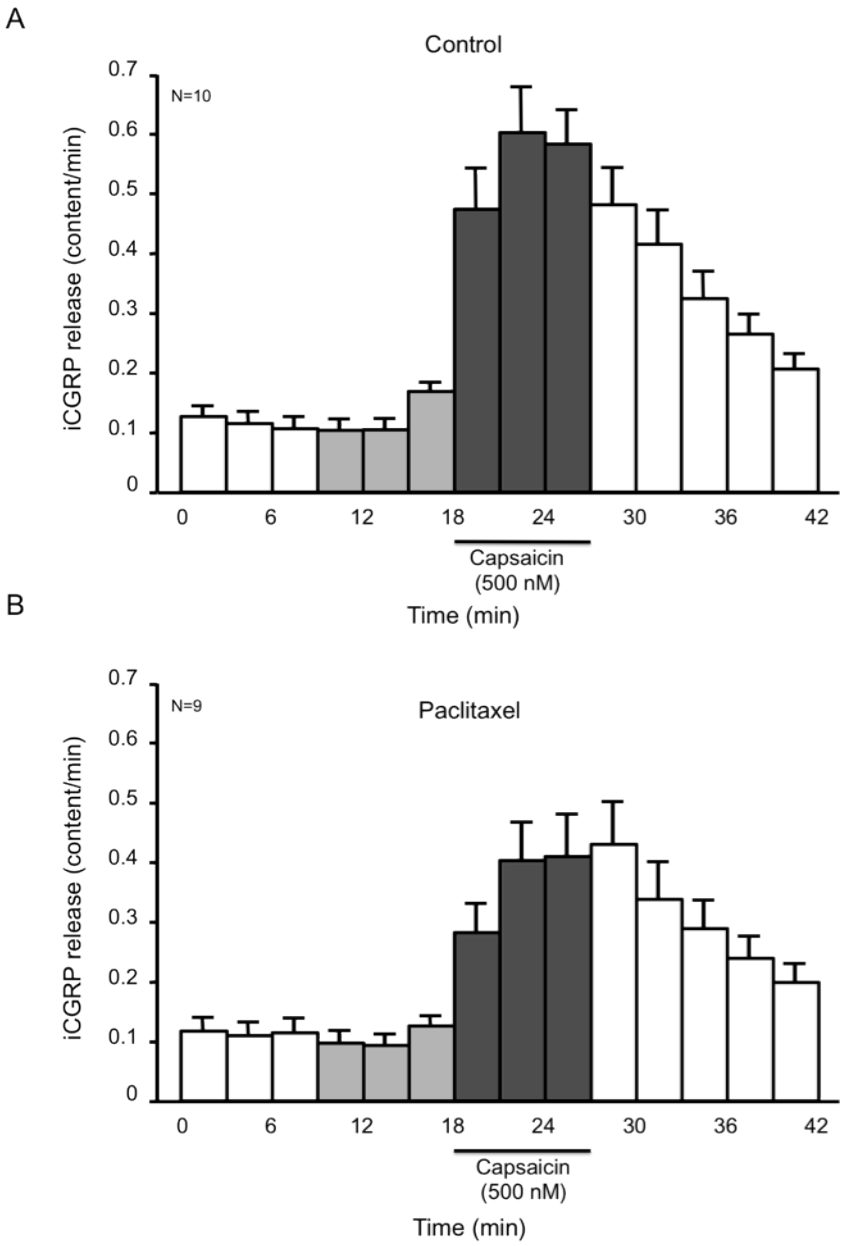

Figure 29. Chronic administration of 2 mg/kg paclitaxel does not alter capsaicin-evoked iCGRP release from rat spinal cord slices. Each column represents the mean ± SEM. The horizontal bar indicates the time when the tissues were exposed to capsaicin. iCGRP release in each 3-minute perfusion sample is expressed as percent of total peptide content per minute. A: iCGRP release in slices taken from vehicle-treated rats.
B: iCGRP release in slices taken from paclitaxel-injected rats.

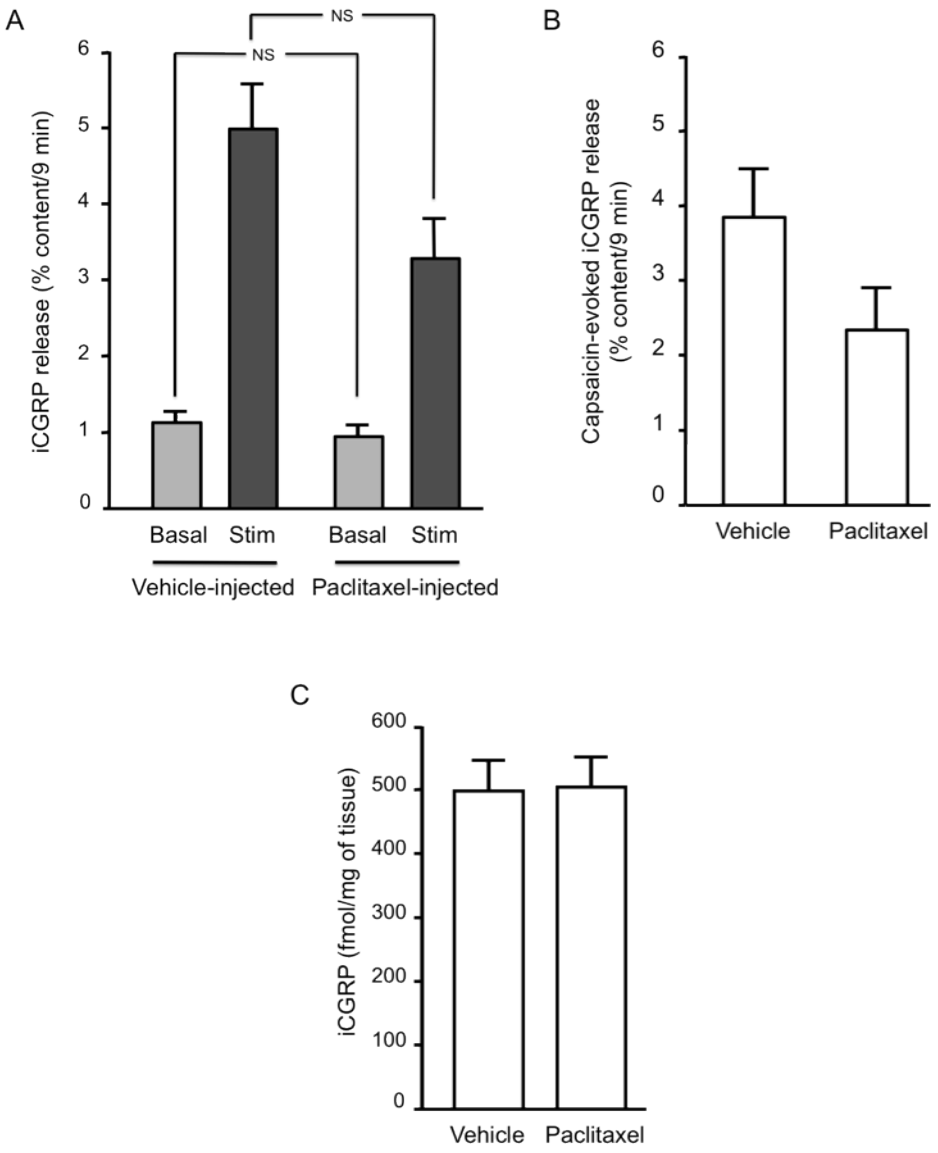

Figure 30. Release of iCGRP induced by capsaicin is not statistically significant between slices from vehicle-injected or 2 mg/kg paclitaxel-injected animals. Each column represents the mean ± SEM. A: Basal release is the sum of the 3 collections prior to capsaicin, whereas capsaicin-stimulated release is the sum of the three collections in the presence of capsaicin B: Evoked release of iCGRP in vehicle or paclitaxel-treated rats. C: The total content of iCGRP in the spinal cord slices from vehicle or paclitaxel injected animals.

Exposure of sensory neurons in culture to 300 nM or 1 μM paclitaxel decreases capsaicin-evoked iCGRP release

Since paclitaxel appears to alter the release of CGRP from the peripheral terminals of sensory neurons, this phenomenon was further examined in isolated sensory neurons so that the mechanisms mediating this change in function can be further examined.

Dorsal root ganglia were harvested from male Sprague Dawley rats, were dissociated, maintained in culture and exposed to various concentrations of paclitaxel for 5 days. As can be seen in Table 3, basal release of iCGRP and total content of iCGRP was not altered by any concentration of paclitaxel that were tested. In contrast paclitaxel did alter the capsaicin-evoked release of iCGRP from sensory neurons in a concentration dependent manner (Figure 31A). The amount of iCGRP released from untreated neurons was 19.91 ± 1.71 % content/10 minutes. Exposure of sensory neurons to 10 or 30 nM paclitaxel significantly increased capsaicin-evoked release of iCGRP to 25.63 ± 0.9 and 24.06 ± 0.8 % content/10 min respectively. In contrast, exposure to 100 nM, 300 nM or 1 μM paclitaxel significantly decreased capsaicin-evoked release to 13.32 ±1.16, 8.16 ± 0.79 and 4.82 ± 0.85 % content/10 minutes respectively. The altered release of iCGRP was not secondary to an altered content of the peptide (see Table 2 and Figure 31B). Some cultures were also exposed to the vehicle for paclitaxel (0.01% MPL) for 5 days to determine if the vehicle itself altered basal or capsaicin-evoked

release of iCGRP. Capsaicin-stimulated release from vehicle treated neurons was 20.64 ± 1.43 % total content/10 minutes. These values were not significantly different from those in untreated neurons.

It is possible that paclitaxel decreases the ability of capsaicin to stimulate TRPV1. Therefore, I examined if release of iCGRP by 100 nM capsaicin was altered by 1 μM paclitaxel. In untreated neurons release of iCGRP increased from a basal value of 0.92 ± 0.12 to 39.90 ± 1.21 % total content/10 minutes after stimulation by 100 nM capsaicin. In neurons exposed to 300 nM or 1 μM paclitaxel for 5 days the release of iCGRP increased from 1.59 ± 0.35 to 22.53 ± 1.05 % total content/10 minutes and from 1.62 ± 0.37 to 18.51 ± 1.66 % total content/10 minutes respectively (Figure 32A). Overall, iCGRP release evoked by 100 nM capsaicin in paclitaxel-treated neurons was still significantly lower than release from untreated neurons.

Treatment	Basal release (% content/10 min)	Total Content (fmol/well)
Control	1.16 ± 0.2	2030.06 ± 148.37
Vehicle (0.01% MPL)	0.99 ± 0.21	2096.23 ± 232.74
10 nM	0.93 ± 0.05	1596 ± 136.80
30 nM	1.05 ± 0.07	1934 ± 173.90
100 nM	1.49 ± 0.16	1439 ± 112.90
300 nM	1.65 ± 0.19	1899.37 ± 156.67
1 μM	1.60 ± 0.32	1935.35 ± 350.01

Table 3. Basal release and total content of iCGRP from untreated, vehicle and paclitaxel-treated sensory neurons in culture.

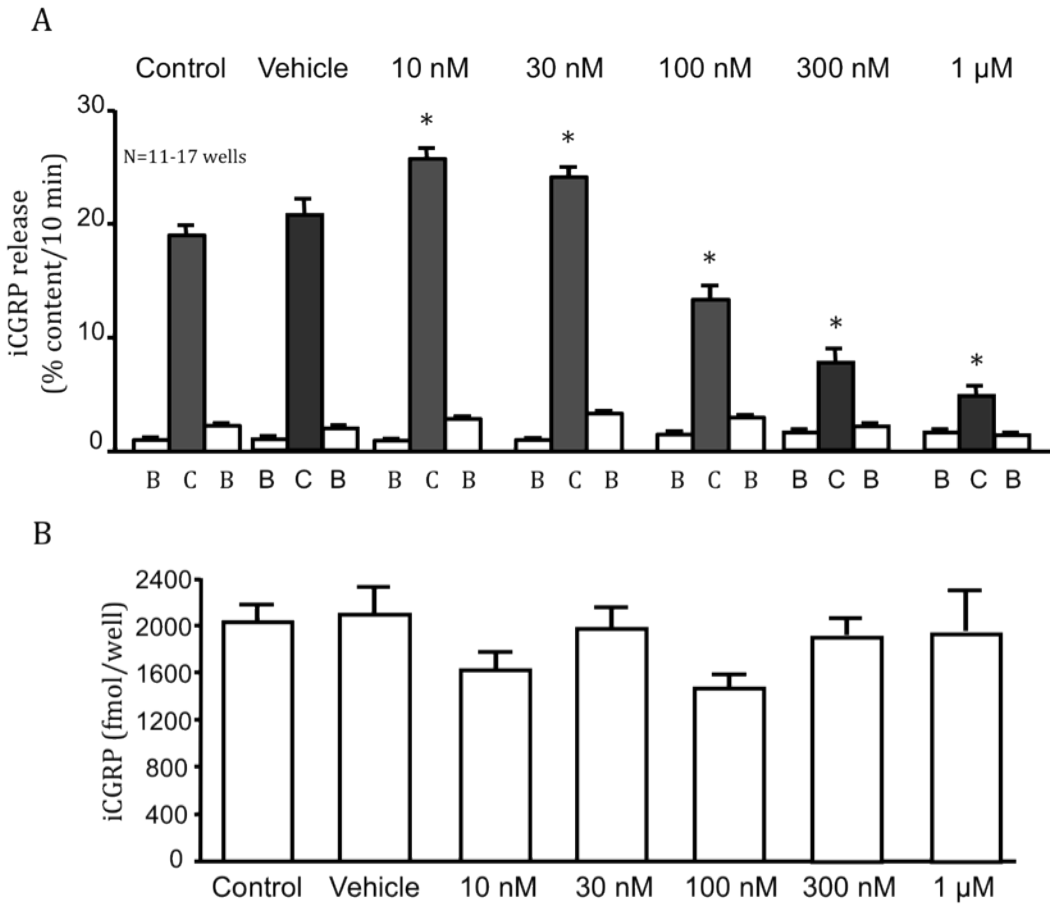

Figure 31. Paclitaxel alters release of iCGRP from isolated sensory neurons in response to 30 nM capsaicin. Columns are the mean ± SEM of iCGRP released as % total content/10 min. A: Open columns represent basal release of iCGRP during a 10 minute incubation with buffer. Solid columns represent release of iCGRP stimulated by 10 minute incubation with 30 nM capsaicin. B: Total content of iCGRP in sensory neurons. Asterisks indicate statistically significant differences in capsaicin-evoked iCGRP release between paclitaxel-treated and untreated neurons by one-way ANOVA with Bonferroni's post test, $p < 0.05$

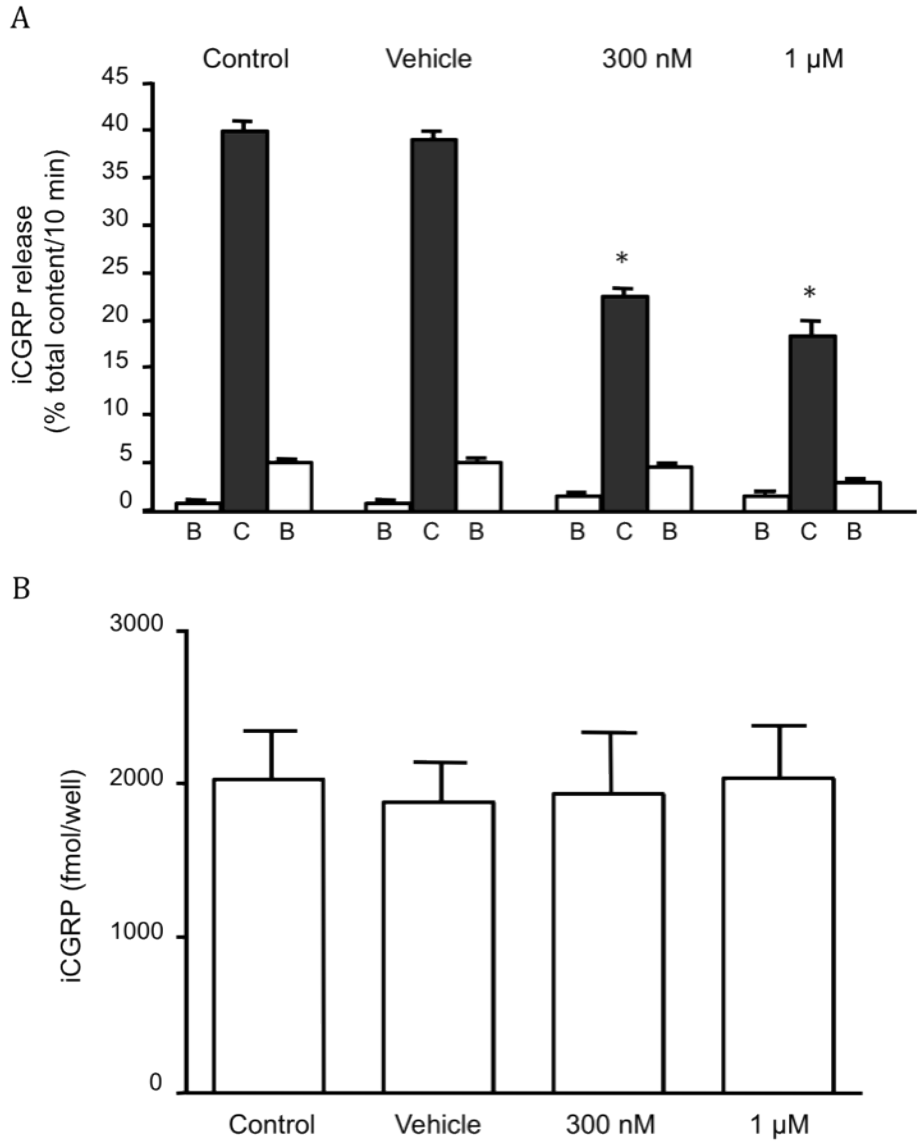

Figure 32. Paclitaxel (300 nM or 1 μM for 5 days) decreases iCGRP release from isolated sensory neurons evoked by 100 nM capsaicin. Columns are the mean ± SEM of iCGRP released as % total content/10 min. A: Open columns represent basal release of iCGRP during a 10 minute incubation with buffer. Solid columns represent release of iCGRP stimulated by 100 nM capsaicin. B: Total content of iCGRP in sensory neurons treated with paclitaxel at the concentrations indicated. An asterisk indicates statistical significance between paclitaxel-treated and untreated neurons by one-way ANOVA with Bonferroni's post test, $p < 0.05$.

To determine if paclitaxel alters the viability of sensory neurons, neuronal cultures that were exposed to paclitaxel were stained with PI and Annexin V. PI is a DNA intercalating agent that is impermeant in living cells because of it size but is able to penetrate the membrane of dead cells. After staining with PI, it is possible to discriminate between live and dead cells, but not between apoptotic and necrotic cell death. Therefore simultaneous labeling with annexin V was performed. Annexin V has a high affinity for phosphotidylserine (PS), which is located in the inner (cytoplasmic) leaflet of the cell membrane. Following induction of apoptosis, PS is translocated to the outer (cell surface) leaflet of the cell membrane, where it is now easily accessible to annexin V in the staining solution. Cells that are in the initial stages of apoptosis display a green colored ring around the cells, while dead cells show more diffused staining.

As shown in Figure 33A and B, the number of untreated sensory neurons that stained positive for annexin V or PI was 8.3 ± 1.9 % and 8.0 ± 2.3 % of the total number of neurons counted. When neurons were exposed to 300 nM paclitaxel for 5 days, the number of annexin V positive neurons was 10.7 ± 2.3 % while those that stained positive for PI was 10.3 ± 3 % of total number of neurons counted. Paclitaxel at 1 μM for 5 days caused 4 ± 2 % and 9 ± 3 % of the total number of neurons to stain positive for annexin V and PI respectively. When neurons were exposed to

300 μM H_2O_2 as the positive control, the annexin V and PI positive cells were 63.7 ± 14% and 69.3 ± 18.6 % respectively.

From these data it can be seen that paclitaxel at 300 nM or 1 μM for 5 days, did not decrease the survival of sensory neurons, thus the decrease in release of CGRP is not likely the result of cell death.

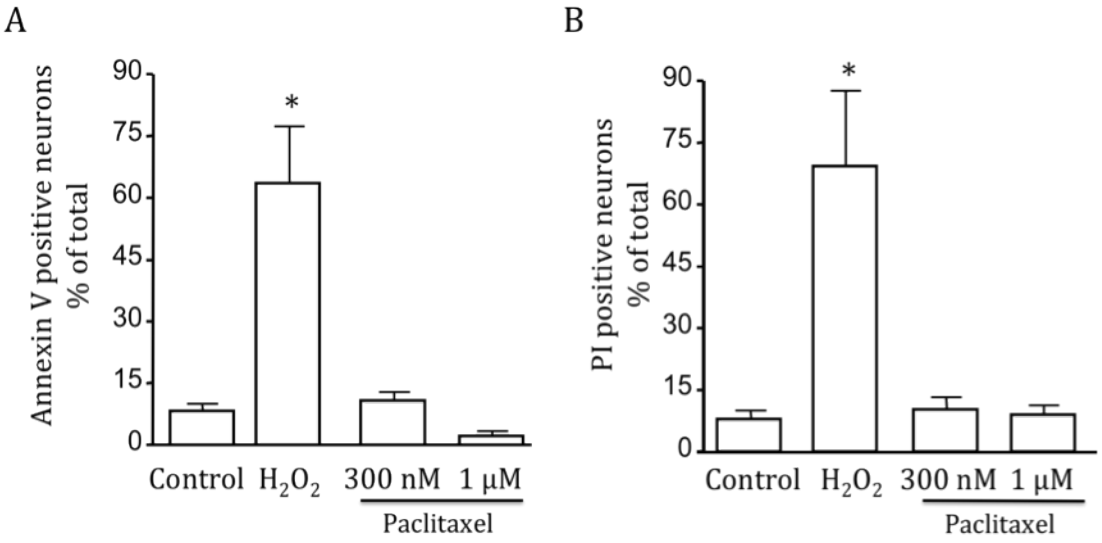

Figure 33. Paclitaxel at 300 nM or 1 µM for 5 days does not decrease the survival of sensory neurons in culture. Each column is the mean ± SEM. The number of annexin V positive (A) or PI positive (B) neurons were counted in a minimum of 5 fields from 3 different harvests and was normalized to the total number of neurons in the field. An asterisk indicates significant difference from untreated controls by one way - ANOVA and Bonferroni's post test, $p < 0.05$.

Exposure of sensory neurons in culture to 300 or 1 μM paclitaxel does not decrease KCl evoked release of iCGRP

A possible explanation for the diminished release of capsaicin-evoked release of CGRP by higher concentrations paclitaxel is that the microtubule-stabilizing drug directly alters the release mechanism for CGRP. Therefore a general depolarizing stimulus, namely 30 mM KCl was used to evoke release of CGRP from sensory neurons that were exposed to paclitaxel to determine if the anticancer drug alters release by a depolarizing stimulus.

Figure 34A shows that exposing untreated or vehicle-treated sensory neurons to 30 mM KCl increased iCGRP release from a basal value of 1.32 ± 0.16 to 14.19 ± 1.35 and from 1.44 ± 0.21 to 15.02 ± 1.07 % content/10 minutes respectively. Basal release in neurons exposed to 300 nM or 1 μM paclitaxel was 1.37 ± 0.12 and 1.69 ± 0.16 % content/ 10 minutes respectively. After incubation with 30 mM KCl for 10 minutes, iCGRP release increased to 16.4 ± 2.64 and 16.58 ± 1.44 % content/ 10 minutes in neurons exposed to either 300 nM or 1000 nM paclitaxel respectively. The stimulated release in paclitaxel- treated neurons (300 nM or 1 μM) was not significantly different from untreated controls. Additionally, the total content of iCGRP was not different across the treatment groups (Figure 34B). Content of the peptide was 2052 ± 144.7, 1955 ± 206.4, 1853 ± 234.4 and 1822 ± 211.5 fmol/well in untreated neurons, vehicle treated, and 300 nM or 1 μM paclitaxel-treated neurons.

These data clearly demonstrate that paclitaxel does not alter potassium-evoked release of iCGRP from sensory neurons suggesting that the drug does not affect the exocytosis process.

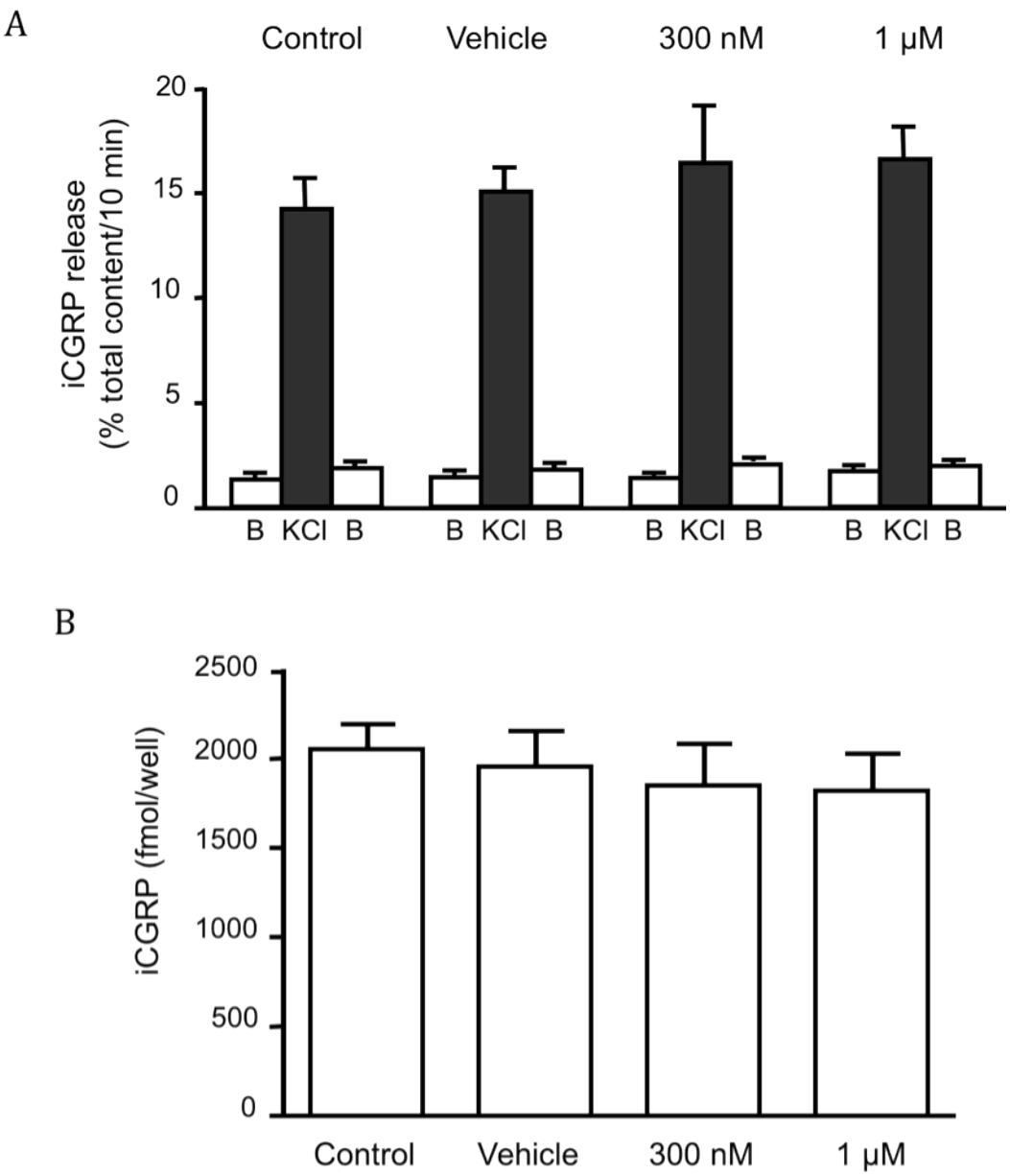

Figure 34. Paclitaxel (300 nM or 1 μM for 5 days) does not alter iCGRP release evoked by high extracellular potassium from isolated sensory neurons. Columns are the mean ± SEM of iCGRP released as % total content/10 min. A: Open columns represent basal release of iCGRP during a 10 minute incubation with buffer. Solid columns represent release of iCGRP stimulated by 30 mM KCl for 10 minutes. B: Total content of iCGRP in sensory neurons.

Expression of TRPV1 after exposure to sensory neurons to paclitaxel

One mechanism that could account for the ability of paclitaxel to attenuate capsaicin-evoked release of iCGRP could be a change in the TRPV1 receptor. Consequently, I examined the expression of TRPV1 by western blot and real-time PCR to quantify mRNA.

Representative blots demonstrating expression of TRPV1 after exposure to 300 nM or 1 μM paclitaxel at different times are shown in Figure 35A. The summary data from these blots are shown in Figure 36A. The immunoreactivity for TRPV1 after exposure to 300 nM paclitaxel for 1 day was 67.42 ± 18.52 % of control and this decrease was not significantly different from that in untreated control. However, the decrease in immunoreactivity to 36 ± 12.12 %, 30.61 ± 5.79 % and 5.56 ± 3.6 % of control after 1, 2, 3 and 5 days respectively was significantly lower than that in untreated neurons.

Exposure to 1 μM paclitaxel for 1 day also did not significantly decrease immunoreactivity of TRPV1 (70. 01 ± 24.87 % of control). Exposure for 2, 3 and 5 days decreased immunoreactivity to 38 ± 7.02 %, 21.55 ± 5.7 % and 2.18 ± 1.70 % of control respectively (Figure 36B).

As a positive control I also examined immunoreactivity of a syntaxin, a membrane-associated SNARE protein that is involved in the exocytosis of neurotransmitters. Interestingly, as shown in Figure 35B and 36C, 300 nM or 1 μM paclitaxel for 5 days did not alter the expression of syntaxin (113.5 ± 2.05 % and 122.9 ± 3.47 % of control respectively).

These data clearly suggest that paclitaxel decreases the expression of the protein for TRPV1 in a manner that is dependent on the concentration and the period of exposure. Since exposure to 300 nM or 1 μM paclitaxel for 5 days did not alter expression of syntaxin, I did not examine syntaxin expression at earlier time points.

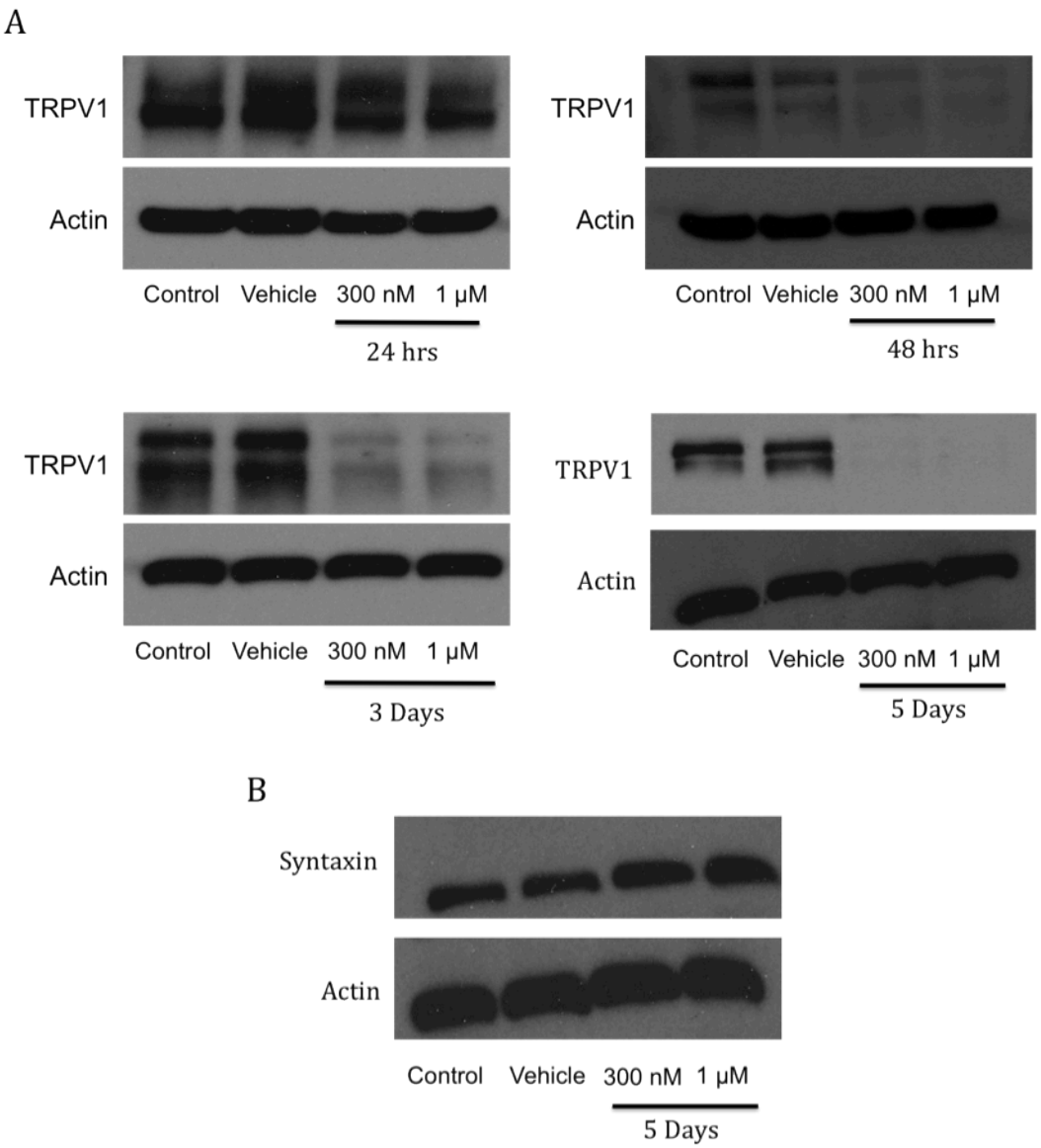

Figure 35. Exposure of sensory neurons to 300 nM or 1 μM paclitaxel decreases expression of TRPV1 protein but not of syntaxin. A: Representative blots demonstrating immunoreactivity for TRPV1 after exposure to paclitaxel at the concentrations and times indicated B: Representative blots demonstrating immunoreactivity for syntaxin.

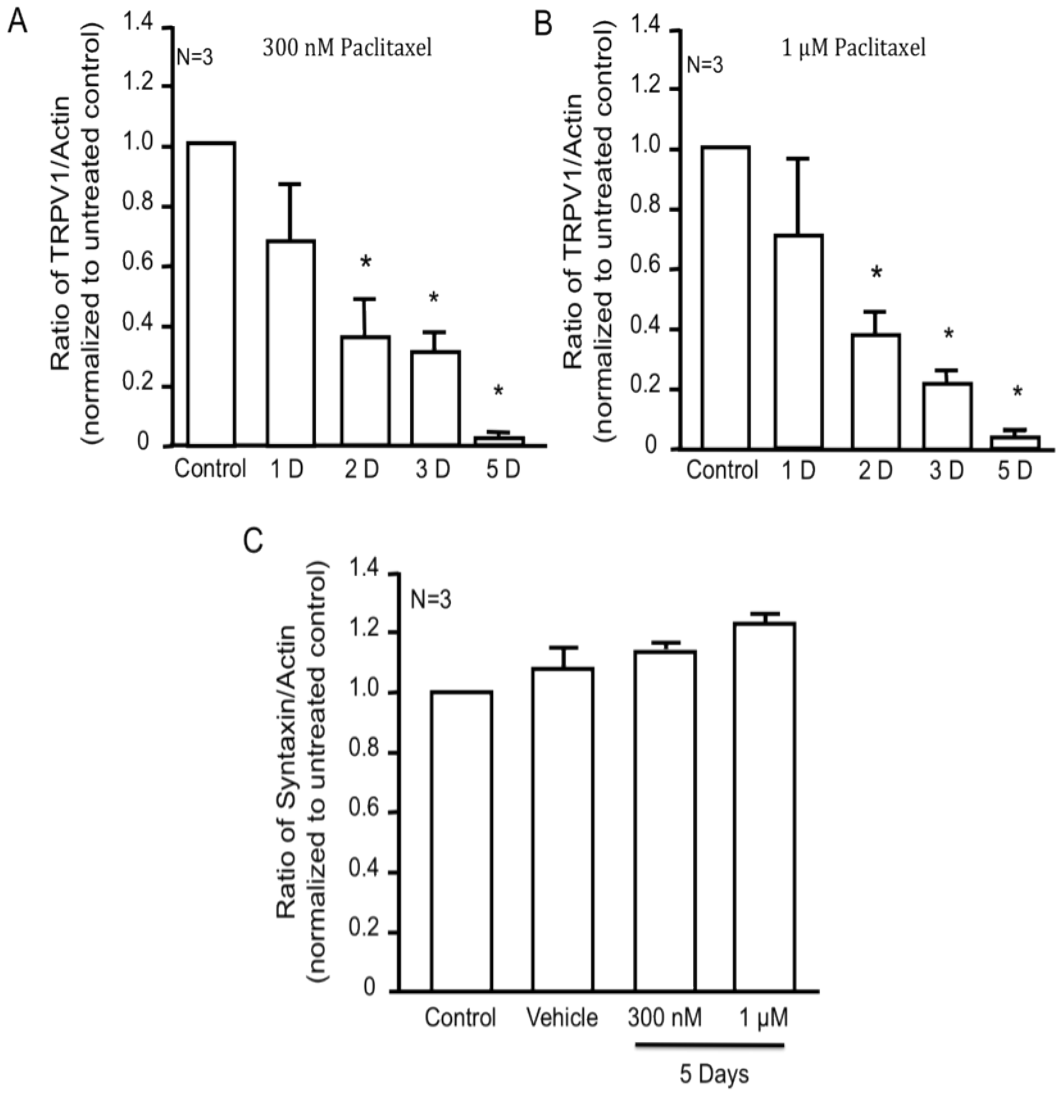

Figure 36. Densitometry of immunoreactive TRPV1 and syntaxin bands. A: Densitometric analysis of signal for TRPV1 after exposure to 300 nM paclitaxel for the duration indicated. Each column is the mean ± SEM of the ratio of TRPV1/actin normalized to that of untreated control. B: Densitometric analysis of signal for TRPV1 after exposure to 1 µM paclitaxel for the duration indicated. Each column is the mean ± SEM of the ratio of syntaxin/actin normalized to that of untreated control. C: Densitometric analysis of signal for syntaxin after exposure to 300 nM or 1 µM paclitaxel for 5 days. An asterisk indicates statistical significance determined by one-way ANOVA with Bonferroni's post test to determine differences in TRPV1 immunoreactivity between paclitaxel-treated and untreated neurons ($p < 0.05$).

Exposure of isolated sensory neurons to 300 nM or 1 μM paclitaxel decreases mRNA of TRPV1 and $Na_V1.9$

The decrease in TRPV1 immunoreactivity could result from increased degradation of the protein, or to decreased transcription resulting in decreased translation or both. To ascertain whether paclitaxel can decrease TRPV1 message, real-time PCR experiments were performed and relative quantification was achieved by the comparative C_T method, also known as the $2^{-\Delta\Delta C_T}$ method.

Exposing sensory neurons to vehicle (0.01% MPL) for 5 days did not alter the mRNA for TRPV1 in the cultures (Figure 37A). Exposure to 300 nM paclitaxel for 1, 2 or 3 days significantly decreased mRNA to 57.52 ± 7.7 %, 25.96 ± 3.32 %, 13.45 ± 2.18 % of control respectively (Figure 37B). The decrease in mRNA by 1 μM paclitaxel was 53.13 ± 6.89 %, 18.83 ± 1.6 %, 11.41 ± 2.37 % after exposure for 1, 2 and 3 days respectively (Figure 37C).

I also examined TRPV1 mRNA expression in sensory neurons exposed to 10 nM, 30 nM and 100 nM paclitaxel for 5 days. In neurons exposed to 10 nM of the drug the mRNA for TRPV1 was not different from untreated control (93.18 ± 8.53 % of control, Figure 37D). However, exposure to 30 nM and 100 nM for 5 days significantly decreased TRPV1 mRNA to 66.23 ± 5.28 and 36.6 ± 4.07 % of control respectively (Figure 37D).

I next determined if paclitaxel alters the expression of mRNA for the voltage-gated sodium channel $Na_v1.9$ as a control. Exposure of sensory neuron cultures to 0.01% MPL for 5 days did not alter the mRNA for $Na_v1.9$ - 77.23 ± 5.6 % of control (Figure 38A). Exposure of sensory neurons to 300 nM paclitaxel for 1, 2 or 3 days significantly decreased $Na_v1.9$ mRNA to 57.52 ± 7.7 %, 25.96 ± 3.32 %, 13.45 ± 2.18 % of control respectively. The $Na_v1.9$ mRNA after 1 μM paclitaxel for 1, 2 and 3 days decreased to 37.89 ± 6.67 %, 13.58 ± 3.38 %, 9.83 ± 1.28 % of control respectively (Figure 38B and C). Exposure to 10 nM of the anti-cancer drug for 5 days did not alter expression of $Na_v1.9$ mRNA (95.04 ± 3.34 % of control, Figure 38D). However exposure to 30 nM, 100 nM, 300 nM or 1 μM paclitaxel for 5 days significantly decreased $Na_v1.9$ mRNA to 58.19 ± 3.29, 28.63 ± 2.99, 11.73 ± 1.94, and 4.73 ± 0.72 % of control respectively (Figure 38B, C and D).

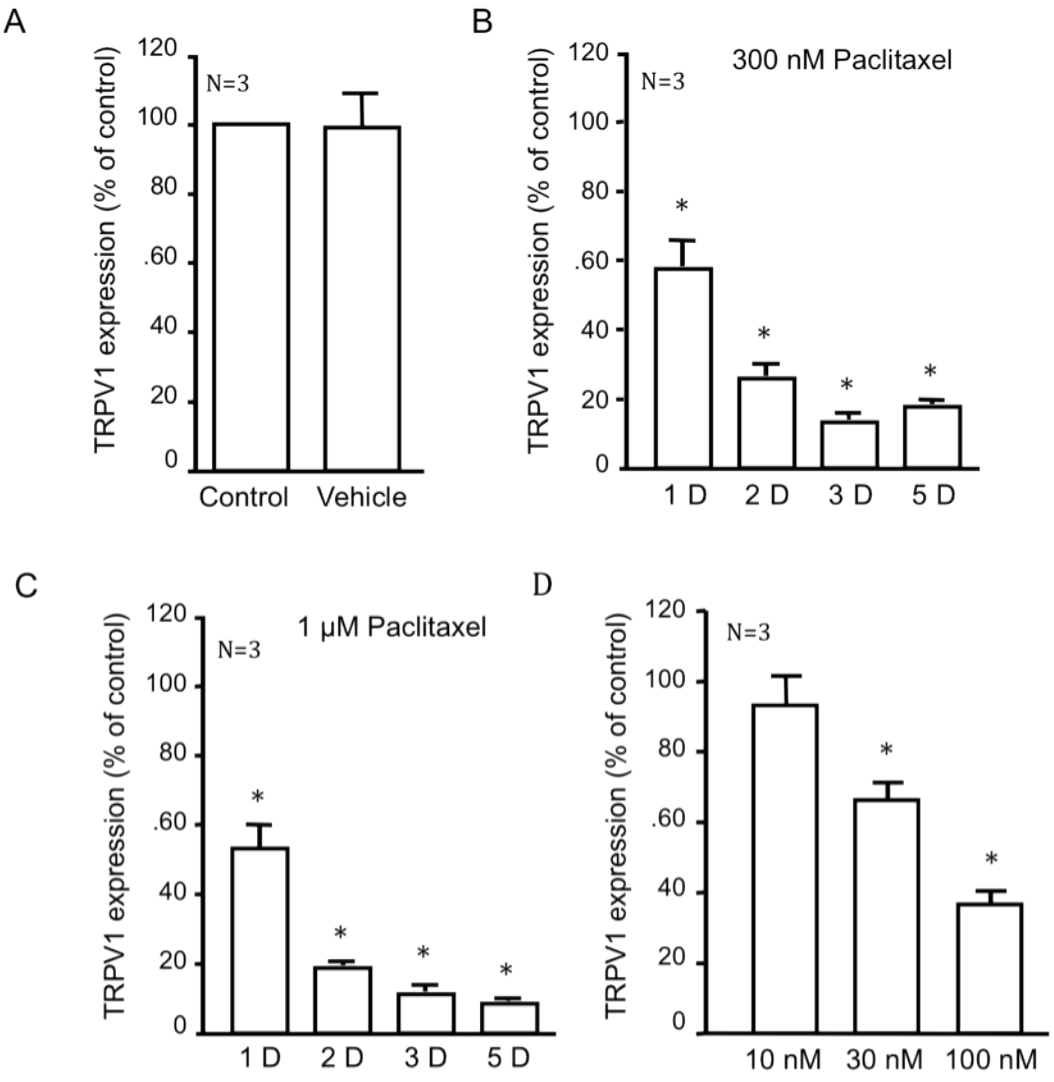

Figure 37. Paclitaxel alters TRPV1 mRNA in isolated sensory neurons. Total RNA was extracted from untreated, vehicle (0.01% MPL) or paclitaxel-treated neurons at the concentrations and durations indicated and reverse transcribed to cDNA. Relative quantification was achieved by the $2^{-\Delta\Delta C}{}_{T}$ method. Each column is the mean ± SEM of the fold- change in mRNA expression relative to untreated control. A: Fold change in TRPV1 mRNA in vehicle treated or untreated neurons. B: Fold change in TRPV1 mRNA in neurons exposed to 300 nM for the times indicated. C: Fold change in TRPV1 mRNA in neurons exposed to 1 μM paclitaxel for the times indicated. D: Fold change in TRPV1 exposed to 10 nM, 30 nM and 100 nM paclitaxel for 5 days. Asterisks indicate statistically significant differences between paclitaxel-treated neurons and untreated controls determined by one-way ANOVA and Bonferroni's post test, $p < 0.05$.

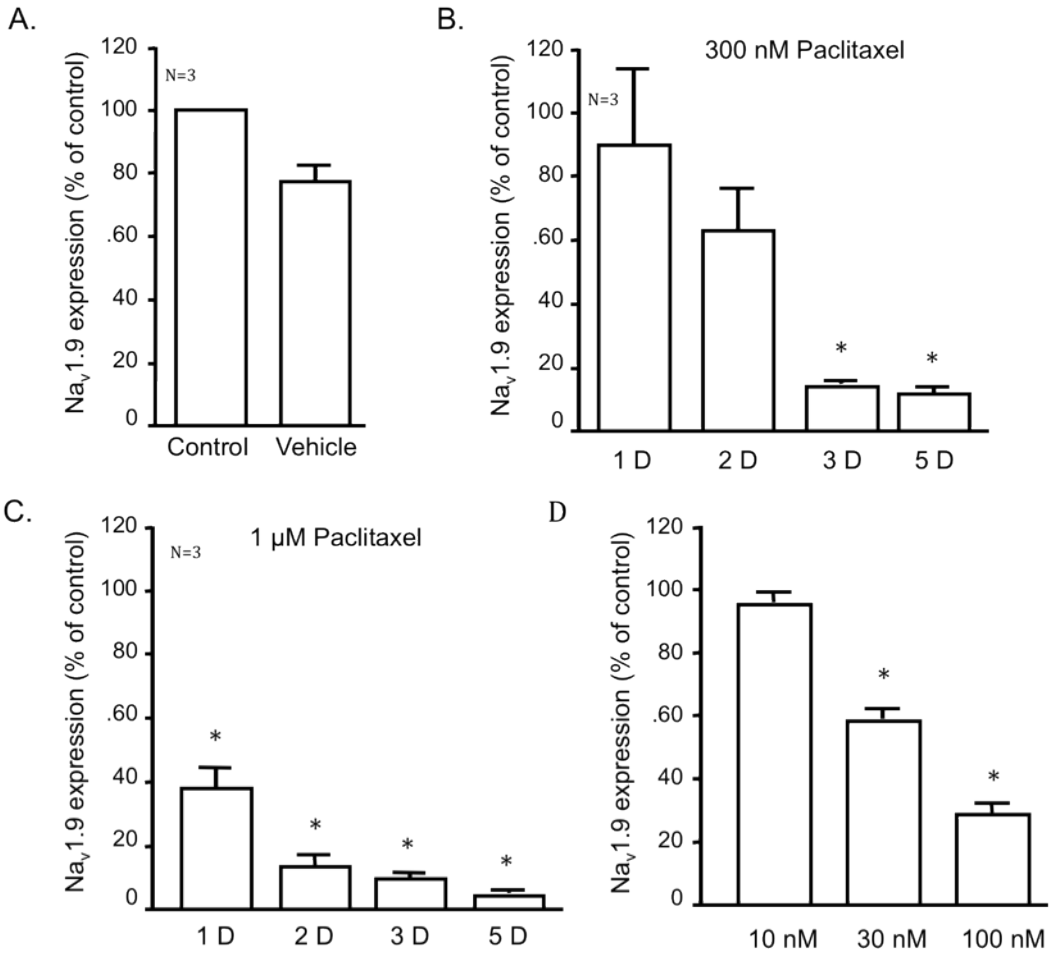

Figure 38. Paclitaxel alters $Na_v1.9$ mRNA in isolated sensory neurons. Total RNA was extracted from untreated, vehicle (0.01% MPL) or paclitaxel-treated neurons at the concentrations and durations indicated and reverse transcribed to cDNA. Relative quantification was achieved by the $2^{-\Delta\Delta C}{}_T$ method. Each column is the mean ± SEM of the fold- change in mRNA expression relative to untreated control. A: Fold change in $Na_v1.9$ mRNA in vehicle treated or untreated neurons. B: Fold change in $Na_v1.9$ mRNA in neurons exposed to 300 nM for the times indicated. C: Fold change in $Na_v1.9$ mRNA in neurons exposed to 1 μM paclitaxel for the times indicated. D: Fold change in $Na_v1.9$ exposed to 10 nM, 30 nM and 100 nM paclitaxel for 5 days. Asterisks indicate statistically significant differences between paclitaxel-treated neurons and untreated controls determined by one-way ANOVA and Bonferroni's post test, $p < 0.05$.

Chronic administration of 2 mg/kg or 4 mg/kg but not 1 mg/kg paclitaxel decreases mRNA for TRPV1

Because paclitaxel altered the expression of TRPV1 *in vitro*, experiments were performed to determine if expression was altered *in vivo*. As previously described male Sprague Dawley rats were injected with either 1 mg/kg, 2 mg/kg or 4 mg/kg paclitaxel every other day for a total of 4 doses. Two weeks after the 1st injection, the L4, L5, and L6 lumbar DRGs were removed and total RNA was extracted for real time PCR experiments. The lumbar ganglia were chosen because these DRGs innervate the paw where the measurements of blood flow were made. As shown in Figure 39, 1 mg/kg paclitaxel did not alter TRPV1 mRNA. When expressed as percent of control, TRPV1 mRNA in paclitaxel-injected animals was 100.4 ± 7.9 %. When the dose of paclitaxel was increased to 2 mg/kg or 4 mg/kg, mRNA levels were decreased to 61.09 ± 2.56 % and 54.72 ± 7.24 % of control respectively.

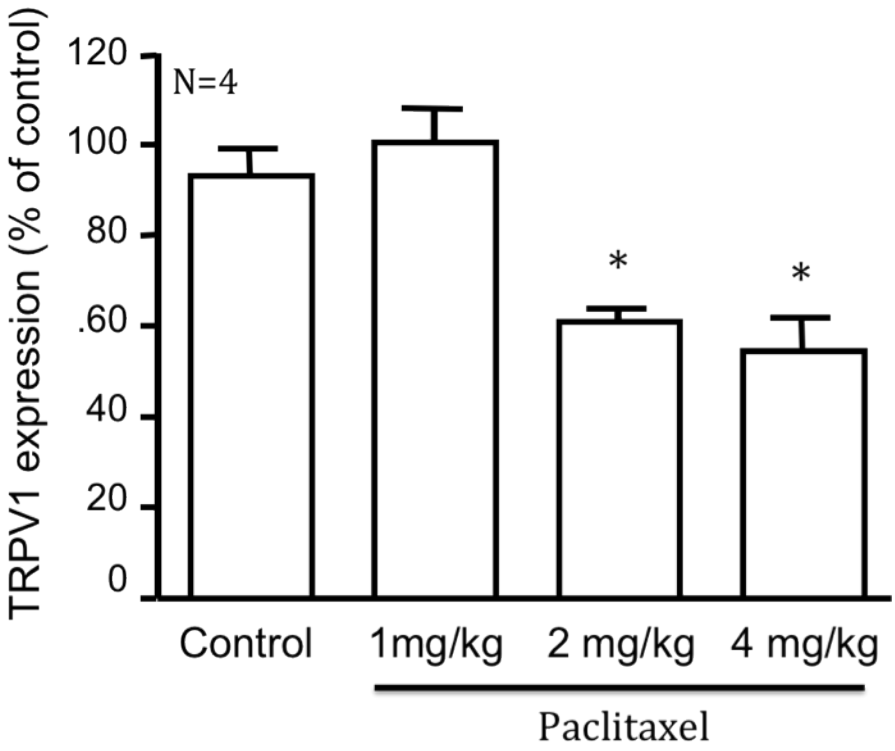

Figure 39. Chronic administration of 2mg/kg or 4 mg/kg but not 1 mg/kg paclitaxel in rats decreases TRPV1 mRNA in the dorsal horn of the spinal cord. Columns are the mean ± SEM of fold changes in TRPV1 relative to vehicle-injected control. An asterisk indicates statistically significant differences in TRPV1 mRNA from paclitaxel-injected or vehicle-injected animals by one-way ANOVA and Bonferroni's post test, $p < 0.05$.

Paclitaxel decreases the length of neurites in isolated sensory neurons

It is possible that distal degeneration of the peripheral process of DRGs contributes to the decreased vasodilatation after activation of sensory neurons. Indeed axonal degeneration has been observed in sural nerve biopsies from patients and it has already been demonstrated that paclitaxel alters the length of neurites in sensory neurons in culture (Yang et al., 2009). To determine if paclitaxel causes neurite degeneration, I established an experimental model to examine length of neurites in isolated sensory neurons following exposure to the anti-cancer drug. As outlined in the methods section isolated sensory neurons were shot with gold particles that were previously coated with the cDNA for EGFP. 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 up to day 12 in culture. (Figure 40).

Images of untreated sensory neurons were acquired on day 7 in culture. Some cultures were exposed to 10 nM, 100 nM or 300 nM paclitaxel immediately after acquisition of images. Additional images of neurons were obtained on day 9 (2 days after addition of paclitaxel), and finally on day 12 (after 5 days of exposure to paclitaxel). Representative images of neurons exposed to 300 nM paclitaxel show that the length of neurites decreased over time following addition of the drug (Figure 41).

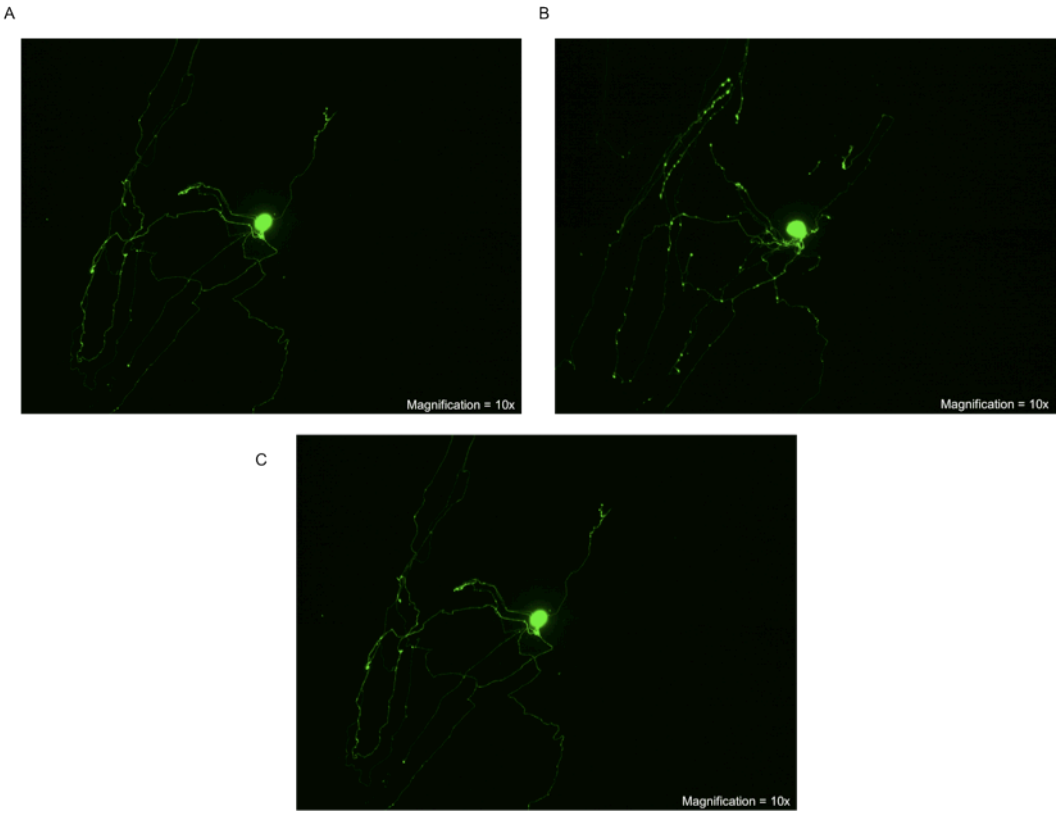

Figure 40. Sensory neurons exposed to vehicle (0.01% MPL) for 5 days do not undergo changes in neurite length. A: Sensory neuron expressing EGFP on day 7 in culture, 0.01% MPL was added immediately after image acquisition. B: The same neuron on day 9 (2 days after addition of vehicle). C: Neuron on day 12 (5 days after addition of vehicle).

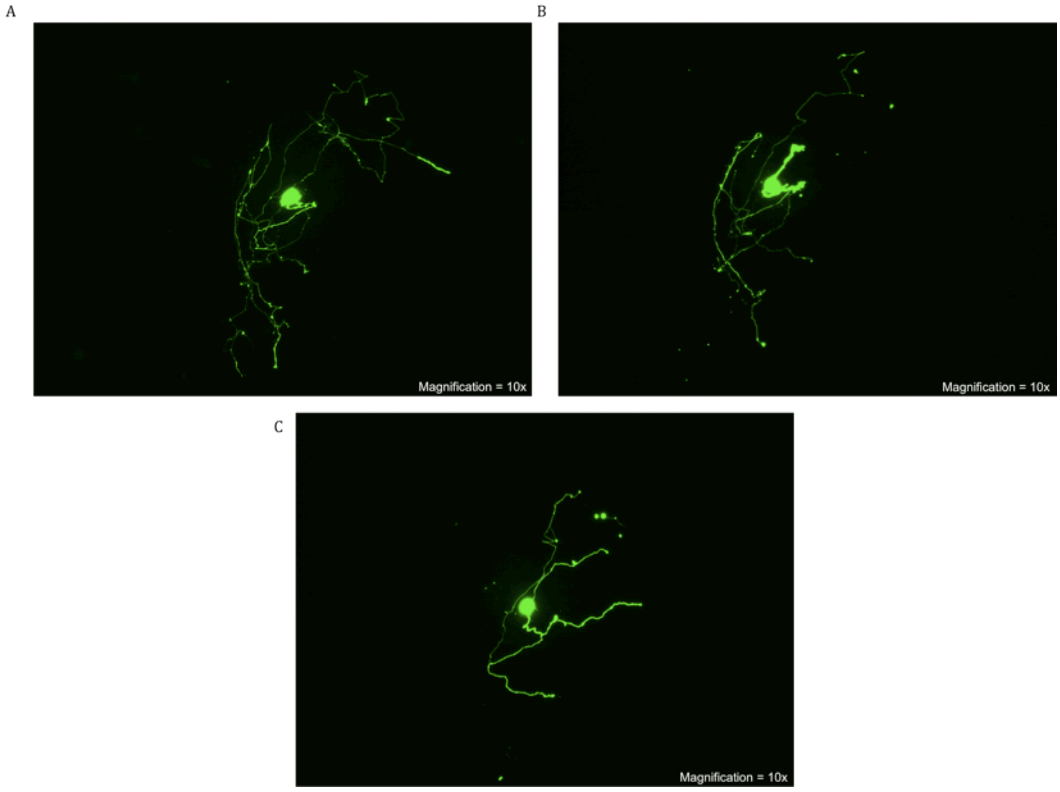

Figure 41. Paclitaxel at 300 nM decreases the length of neurites in isolated sensory neurons. A: Sensory neuron expressing EGFP on day 7 in culture. Paclitaxel was added immediately after image acquisition. B: The same neuron on day 9 (2 days after addition of paclitaxel). C: Neuron on day 12 (5 day after addition of paclitaxel).

The neurite outgrowth software was used for the calculation of total outgrowth - the cumulative length of all neurites protruding from a single cell body. The outgrowth after exposure to vehicle or paclitaxel (on day 9 and day 12) is normalized to that before addition of vehicle or paclitaxel (day 7) and is expressed as % of total outgrowth.

As illustrated in Figure 42A, the total outgrowth on day 9 and day 12 after exposure to 0.01% MPL is 107.6 ± 8 and 95.3 ± 9.24 % of the total length respectively, which is not significantly different from the length on day 7. This suggests that the vehicle did not alter the length of neurites. When neurons were exposed to 10 nM paclitaxel, the length of neurites on day 9 was 117.1 ± 16.12 % of total length, and on day 12 was 104.9 ± 17.74 % of total length (Figure 42B). Since these values were not significantly different from length on day 7, it can be concluded that 10 nM paclitaxel does not alter the length of neurites.

The length of neurites after exposure to 100 nM paclitaxel decreased to 77.1 ± 5.5% and 73.7 ± 6.4 % of the total length on day 9 and day 12 respectively. (Figure 42C). In neurons exposed to 300 nM paclitaxel, the length of neurites decreased to 53.7 ± 4.8 % on day 9 and to 32.5 ± 4.4% on day 12 (Figure 42D). The length of neurites after exposure to 100 or 300 nM paclitaxel was significantly different from total length. Overall, 100 and 300 nM but not 10 nM paclitaxel decreased the length of neurites.

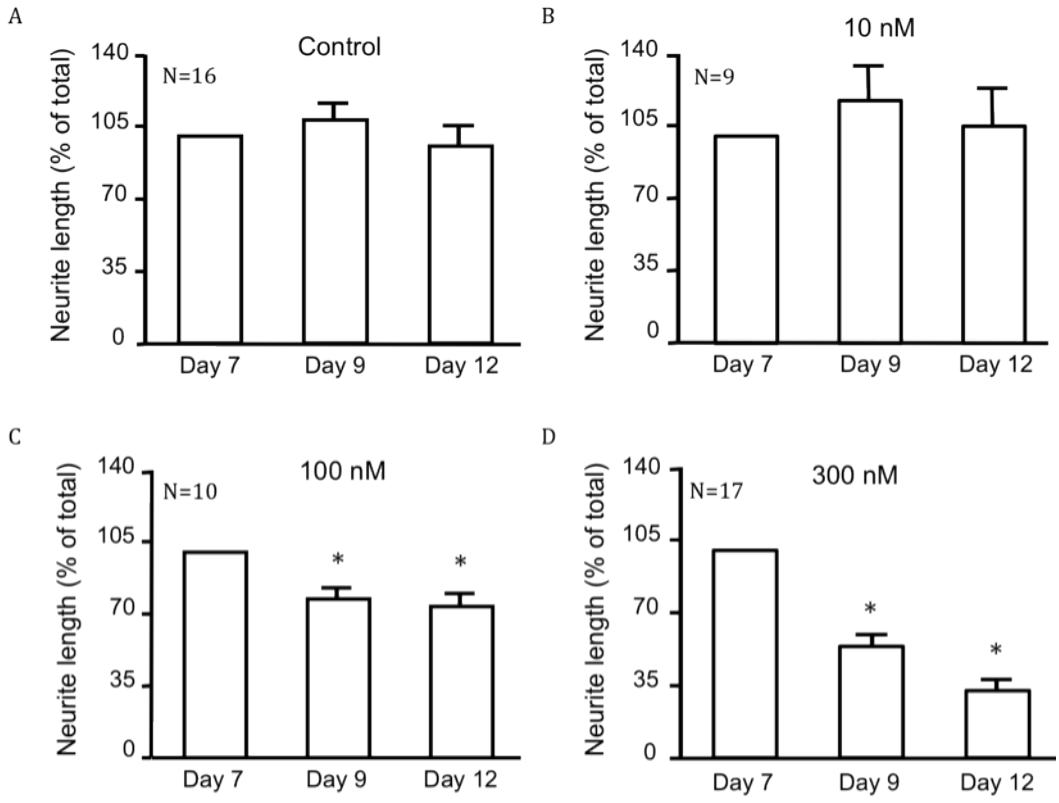

Figure 42. Exposure of sensory neurons in culture to 100 or 300 nM but not 10 nM paclitaxel decreases the length of neurites. The cDNA for EGFP was introduced into sensory neurons by a gene gun on day 2 in culture. Neurons stably expressed EGFP on day 7 in culture. Columns are mean ± SEM. The length of neurites was reported an % change relative to that on day 7 in neurons treated for 5 days with vehicle (0.01% MPL) (A), 10 nM paclitaxel (B), 100 nM paclitaxel (C) or 300 nM paclitaxel (D). An asterisk indicates statistically significant differences in neurite length relative to day 7. Significance was tested by one-way ANOVA and Bonferroni's post test. $p < 0.05$

DISCUSSION

Paclitaxel-induced peripheral neuropathy is largely a sensory neuropathy since its major actions are directly or indirectly on sensory neurons. The symptoms associated with the neuropathy are experienced in the hands and the feet, and occur at doses greater than 200 mg/m^2 (Lipton et al., 1989; Mielke et al., 2005), and no treatments are currently available to prevent or reverse the neurotoxicity because the mechanisms mediating the toxicity are still not known. Therefore this study was performed to examine the mechanisms mediating the paclitaxel-induced change in function of sensory neurons. The experiments conducted in this study provide evidence that paclitaxel alters the function of peripheral endings of small diameter sensory neurons *in vivo* which was demonstrated by a reduction in the ability of the peptidergic sensory neurons to increase peripheral blood flow. However, the microtubule-stabilizing drug does not alter the release of CGRP from the central terminals of the peptidergic neurons. Experiments performed in isolated sensory neurons demonstrated that paclitaxel directly alters the release of the neuropeptide from sensory neurons, and alters the expression of the capsaicin receptor TRPV1 and the voltage-gated sodium channel $Na_v1.9$. Lastly, paclitaxel causes degeneration of neurites in isolated sensory neurons.

A number of investigators have examined the effects of various dosing regimens of paclitaxel in animal models on different endpoints

including sensory nerve conduction (Apfel et al., 1991; Cavaletti et al., 1995; Cavaletti et al., 1997; Cliffer et al., 1998; Authier et al., 2000; Persohn et al., 2005; Matsumoto et al., 2006; Jamieson et al., 2007; Nishida et al., 2008), response to thermal and mechanical stimuli (Apfel et al., 1991; Cavaletti et al., 1995; Cavaletti et al., 1997; Campana et al., 1998; Authier et al., 2000; Dina et al., 2001; Polomano et al., 2001; Persohn et al., 2005; Matsumoto et al., 2006; Peters et al., 2007; Nishida et al., 2008), and morphological changes in sensory nerves (Cavaletti et al., 1995; Schmidt et al., 1995; Cavaletti et al., 1997; Campana et al., 1998; Authier et al., 2000; Polomano et al., 2001; Persohn et al., 2005). In most of these studies, the effects of paclitaxel on the above mentioned endpoints are dependent on the total amount of paclitaxel administered. For example, rats administered greater than 16 mg/kg paclitaxel showed decreased sensory nerve conduction velocity, a decrease in sensitivity to thermal stimuli, and altered morphology of sensory nerves (Sahenk et al., 1994). On the other hand, when lower doses of paclitaxel (10 mg/kg or less) are administered, the drug produces an increased sensitivity to thermal and mechanical stimuli analogous to models of neuropathic pain syndromes. These dose dependent changes in sensitivity of sensory neurons have been observed in patients as well. Furthermore, the hypernociception observed in animal models was not accompanied by visible signs of nerve damage (Dina et al., 2001; Polomano et al., 2001; Weng et al., 2005).

Although the animal models do exhibit symptoms experienced by patients (altered sensitivity to thermal and mechanical stimuli and altered sensory nerve conduction), a limitation of most of these models including the one used in this study is that the effects of paclitaxel are examined in the absence of tumors. Therefore the contribution of cells of the immune system to changes in sensory neuron function cannot be examined in these animal models. Another limitation is that the severity of the symptoms of peripheral neuropathy in patients increases when other anticancer drugs e.g. cisplatin is administered in combination with paclitaxel, and when there are preexisting conditions including diabetes, alcoholism or other metabolic disorders (vitamin deficiencies); animal models for the examination of peripheral neuropathy do not account for these variables.

Effects of paclitaxel on sensory neuron function *in vivo*

The injection regimen in the experiments conducted in this study (1 mg/kg every other day for a total of 4 doses) was chosen because the dose is low enough that produces a change in sensory neuron function in the absence of any overt signs of toxicity (Polomano et al., 2001). My work also differs from others in the choice of vehicle used. Cremophor EL (CrEL), which is a polyethoxylated castor oil and ethanol in a ratio of 1:1 is the vehicle currently used when paclitaxel is administered to patients and by many investigators injecting the drug in animals. However CrEL itself is

associated with hypersensitivity reactions and other toxicities (Gelderblom et al., 2001; Ten Tije et al., 2003). Therefore, to eliminate effects produced by this vehicle, I used 10% DMSO as the vehicle. Moreover, Polomano et al., showed that 10% DMSO does not produce a change in basal nociceptive behavior or any signs of toxicity (Polomano et al., 2001).

In a number of studies using animal models the paclitaxel is given intravenously because this route is most frequently used in the clinic. In contrast, I chose the i.p route of administration because it enables injections of larger volumes of the drug and it eliminates the need for canulation of blood vessels for the administration of the drug. Secondly, Soma et al., showed that when injected intraperitoneally, paclitaxel persists in extra-peritoneal tissues longer than when injected intravenously (Soma et al., 2009) and for this reason intraperitoneal administration of paclitaxel has been proposed to be more beneficial in the treatment of ovarian cancers (Markman et al., 1994; Francis et al., 1995; Ishigami et al., 2009; Ishigami et al., 2010).

Measurement of cutaneous vasodilatation to examine release of CGRP from peripheral endings of senory neurons *in vivo*

The various symptoms associated with paclitaxel-induced peripheral neuropathy could result from an altered function of both myelinated and unmyelinated sensory fibers. A loss of vibration sense, loss of propioception and loss of deep tendon reflexes suggest a

dysfunction of the large myelinated fibers (Aβ). The decrease in thermal nociceptive threshold, burning pain, and paraesthesia experienced by patients with paclitaxel-induced peripheral neuropathy parallel observations made in patients with other small fiber neuropathies (Tavee and Zhou, 2009), suggesting that paclitaxel causes abnormalities in the function of the small diameter fibers (Aδ and C). Furthermore, a sural nerve biopsy from a patient receiving paclitaxel and one animal study showed degeneration of myelinated as well as unmyelinated fibers after administration of multiple injections of paclitaxel (Sahenk et al., 1994; Authier et al., 2000).

In contrast to the above observations of decreased nociceptive threshold after paclitaxel administration, Dougherty et al., observed that patients undergoing chemotherapy with paclitaxel demonstrated increased touch thresholds suggesting a loss of Aβ fiber function, while their ability to detect thermal stimuli were the same as in control subjects, suggesting that the neurons that convey thermal stimuli (Aδ and C) are unaffected by the drug treatment (Dougherty et al., 2004). In animal studies using an intravenous dosing regimen of paclitaxel (1 mg/kg per day for 10 days), no changes were observed in the thermal threshold or the conduction velocity of C fibers suggesting that paclitaxel did not alter excitability of small diameter sensory fibers (Dina et al., 2001). Whether the function of the small diameter fibers is altered by paclitaxel is still uncertain because studies examining nerve conduction and behavior do not reflect function of

the small diameter nerve fibers. Electrodiagnostic tests are able to distinguish between the large diameter neuropathies while small fiber function often appears normal in these studies (Mendell and Sahenk, 2003; Tavee and Zhou, 2009). Therefore, the studies conducted in this thesis were designed to specifically examine if paclitaxel alters release of CGRP from the peripheral and central terminals of the small sensory fibers *in vivo* and *in vitro*. Release of CGRP from the peripheral endings of sensory neurons was measured by examining vasodilatation after activation of the peripheral terminals of sensory neurons. It is well established that a subset of small diameter sensory neurons release CGRP from the peripheral endings in response to a stimulus (Franco-Cereceda et al., 1987; Rydh et al., 1992; Kilo et al., 1997; Averbeck and Reeh, 2001) and this release results in an increase in blood flow in peripheral targets (Brain et al., 1986; Merhi et al., 1998; Grant et al., 2002). Vasodilatation induced by sensory neuron activation is an important component of neurogenic inflammation and is produced at the site of injury after activation of sensory neurons by thermal, mechanical or thermal stimuli (Koltzenburg and McMahon, 1986; Holzer, 1998). Thus, as an indirect measure of CGRP release, cutaneous vasodilatation using laser Doppler flowmetry was examined. To implement the method, experiments were performed to characterize vasodilatation after activation of sensory neurons. In the initial experiments, injection of capsaicin produced only a modest increase in blood flow compared to that observed

by others (Hughes and Brain, 1991; Lin et al., 1999). Several variables could account for the weak detection of the response. The volume of tissue sampled by the laser probe is approximately 0.6 mm^3. Therefore the placement of the probe relative to the site of injection is important for detection of changes in signal. Accordingly, injecting drugs 1 mm away from the site of the probe enables detection of signal. Initial experiments were performed by injecting 10 μM capsaicin into the subcutaneous layer of the skin. Since the laser penetrates to a depth of approximately 600 μm, the laser probe will not detect blood flow increases induced by injection of capsaicin into deeper layers of the skin; only those changes occurring in the superficial layers (dermis) can be detected. Therefore, in subsequent experiments the needle was inserted at a 15-degree angle to ensure intradermal injection of drug. The volume of injection can also influence detection of blood flow by the laser probe. Larger volumes (≥ 10 μl) could interfere with the path of the laser; therefore I chose to use 1 μl in subsequent experiments. Injecting 1 μl capsaicin in the superficial layer of the skin allowed detection of almost 200% increase in blood flow in response to injection of 10 μM capsaicin, thus this protocol was used in all future experiments of cutaneous vasodilatation.

For experiments to examine vasodilatation in response to electrical stimulation, a modification of the protocol described by Koltzenburg et al., was used (Koltzenburg et al., 1990). The sciatic nerve was axotomized before stimulation of the sciatic nerve in order to eliminate dorsal root

reflexes that arise in the spinal cord. Although the data was not shown, in initial experiments, axotomy produced an increase in basal blood flow possibly resulting from injury induced firing of action potentials. Consequently, application of 1% lidocaine to the area distal to the site of cutting the sciatic nerve was used to prevent action potential conduction. This treatment eliminated the increase in basal blood flow following axotomy (data not shown). The sciatic nerve was then stimulated at 5 mA at 10 Hz for 30s because this stimulus activates the high threshold Aδ and C fibers along with the low threshold Aβ to produce maximum vasodilatation (Koltzenburg et al., 1990). The vasodilatation produced by electrical stimulation of the sciatic nerve is similar to that observed by Escott et al., after stimulation of the saphenous nerve (Escott and Brain, 1993).

To determine if vasodilatation caused by activation of sensory neurons is mediated by CGRP, I examined if the CGRP receptor antagonist ($CGRP_{8-37}$) would block the increase in blood flow induced by electrical stimulation of the sciatic nerve or by intradermal injection of capsaicin. $CGRP_{8-37}$ displaces the specific binding of human αCGRP in rat liver membranes and inhibits the production of cAMP by human CGRP in a dose dependent manner (Chiba et al., 1989). Furthermore, Dennis et al., demonstrated that $CGRP_{8-37}$ is a more potent antagonist in the right guinea pig atrium and the guinea pig ileum but a weaker antagonist in the rat vas deferens suggesting that $CGRP_{8-37}$ discriminates between different

types of the CGRP receptor (Dennis et al., 1990). This antagonist also inhibits the increase in blood flow induced by CGRP (Hughes and Brain, 1991).

To examine the effects of $CGRP_{8-37}$ I chose to use 30 μM in part because this concentration does not alter basal blood flow (Hughes and Brain, 1991). Furthermore, this concentration inhibited the increase in blood flow induced by 100 μM capsaicin injected intradermally in the rat hind paw (Hughes and Brain, 1991). Using 30 μM $CGRP_{8-37}$ in my preparation completely inhibited increase in blood flow induced by capsaicin or by electrical stimulation confirming previous work that the activation of sensory neurons induces vasodilatation in a CGRP dependent manner, (Hughes and Brain, 1991; Escott et al., 1995).

One significant finding of my work is that paclitaxel significantly decreases vasodilatation induced by activation of sensory neurons. The decreased vasodilatation cannot be attributed to a decrease in expression of TRPV1 in the skin as has been observed in skin biopsies of patients with diabetic neuropathy (Facer et al., 2007), because electrical stimulation mediated vasodilatation was similarly attenuated by paclitaxel. Many other factors could contribute to decreased vasodilatation induced by paclitaxel. It is possible that the drug alters the function of blood vessels to cause a decrease in sensory neuron mediated vasodilatation because the paclitaxel has been shown to alter the contractility of aortic smooth muscle cells (Sauro et al., 1995), block proliferation and motility of

endothelial cells *in vitro* (Belotti et al., 1996) and prevent formation of new blood vessels (Wang et al., 2003b; Kirchmair et al., 2007; Lainer-Carr and Brahn, 2007). It is also possible that paclitaxel decreases the expression or function of the CGRP receptor on vascular smooth muscle cells. Therefore, to determine if the decreased vasodilatation induced by paclitaxel is secondary to a post-synaptic action, the effects of paclitaxel on vasodilatation induced by intradermal injection of CGRP and the muscarinic receptor agonist, methacholine (Kimura et al., 2007) was examined. Chronic administration of paclitaxel did not alter the increase in blood flow induced by either methacholine or CGRP. These data suggest that paclitaxel does not decrease the expression of the CGRP receptor, or interfere with vascular smooth muscle relaxation and support the notion that paclitaxel decreases cutaneous vasodilatation by inhibiting release of CGRP from sensory nerve endings.

It is possible that the decreased release of CGRP from peripheral endings in paclitaxel-injected animals is secondary to decreased axonal transport of CGRP containing vesicles. There is morphological evidence that microtubules associate with synaptic vesicles and functional evidence that microtubules participate in fast and slow axonal transport (Smith, 1971; Smith et al., 1975; Bird, 1976; Theiss and Meller, 2000). Since CGRP is synthesized in the cell body and transported to the sensory nerve endings by microtubules (Kashihara et al., 1989), it is possible that disruption of microtubules by paclitaxel will interfere with the transport of

synaptic vesicles containing CGRP. Indeed, ultrastructural studies showed that paclitaxel exposure causes an accumulation of vesicular structures in spinal cord and DRG co-cultures reflecting an inhibition of axonal transport (Masurovsky et al., 1983). Although I did not measure axonal transport directly, I measured CGRP release from central terminals of sensory neurons as an indirect index of paclitaxel's effects on transport of CGRP or on the release mechanism.

Paclitaxel could attenuate CGRP release from sensory endings of small diameter fibers by inhibiting the neuropeptide release mechanism. There is evidence that microtubules directly participate in the release of hormones from secretory cells (Khar et al., 1979). Inhibition of microtubules by paclitaxel interferes with stimulated release of noradrenalin from chromaffin cells in culture (Thuret-Carnahan et al., 1985) and with glucose-stimulated insulin release from isolated islets of Langerhans (Howell et al., 1982). Furthermore, disruption of microtubules by colchicine interferes with the release of neurotrophin-3 from axon terminals (Wang et al., 2002).

It is possible to measure CGRP release in the skin (Kilo et al., 1997; Kessler et al., 1999; Petho et al., 2004) and from whole DRGs (Eberhardt et al., 2008). However, in this study, CGRP release from whole DRGs was not examined because the ganglia contain the cell bodies of primary afferent neurons where CGRP is synthesized; consequently the contribution of microtubule inhibition to decreased release of CGRP

cannot be examined. Direct release of CGRP from the skin was not examined because CGRP is synthesized and released by endothelial cells (Ye et al., 2007; Luo et al., 2008), therefore interpretation of the site of release of CGRP in the periphery is difficult. For these reasons, CGRP release from central terminals in spinal cord slices was examined.

The total content of CGRP in the spinal cord of paclitaxel-injected animals was not different from vehicle-injected animals. In addition, neither 1 nor 2 mg/kg paclitaxel administered every other day for a week, altered the KCl or capsaicin-stimulated release of CGRP in spinal cord. These data suggest that paclitaxel at these doses does not affect the expression nor the transport of CGRP from the cell body to the central terminals in the spinal cord.

The lack of effect of paclitaxel at central terminals may be due to an inability of the drug to readily cross the blood brain barrier to enter the central nervous system and affect the central endings of the primary sensory neurons. Indeed, Cavaletti et al., showed that 5 mg/kg paclitaxel injected on day 1, 2, 3, 9 and 10 resulted in the lowest concentrations (11 ng/g tissue) of the drug in the spinal cord while the concentrations in the sciatic nerve and the dorsal root ganglia were 5-fold and 30-fold higher respectively (Cavaletti et al., 2000). Therefore, it is likely that the injection regimen used in my study does not result in a significant accumulation of paclitaxel in the spinal cord. Since sensory neurons have their cell bodies in the dorsal root ganglia and a single axon extending from the periphery

to the dorsal spinal cord, it is reasonable to expect that any accumulation of the drug in the cell body could affect the entire neuron. If the drug was affecting the cell bodies, one would expect release to be altered at both central and peripheral endings of the neurons. Since I did not observe a change in release at the central terminals, it seems likely that paclitaxel alters release of CGRP at the peripheral endings of sensory neurons not through it's actions at the cell bodies.

Paclitaxel alters capsaicin-evoked release of CGRP from sensory neurons in culture

To directly assess the effects of paclitaxel on transmitter release from sensory neurons and to explore mechanisms that contribute to any effects of the drug, sensory neurons in culture as were used as an experimental model. In these experiments, the effects of paclitaxel on release of CGRP from sensory neurons after exposure to a general depolarizing stimulus (30 mM KCl) or to capsaicin were examined.

Exposure of sensory neurons to 300 nM or 1 μM paclitaxel for 5 days did not inhibit KCl evoked release of CGRP. However, depending on the concentration of paclitaxel used, a biphasic effect of the drug on capsaicin-evoked release of CGRP was observed: 10 or 30 nM paclitaxel for 5 days increased release of CGRP while 100 nM to 1 μM paclitaxel decreased capsaicin-evoked release of CGRP. The altered release was not secondary to a change in the total content of the peptide. These data

clearly demonstrate that paclitaxel does not interfere with general depolarization of sensory neurons or with the neurotransmitter release mechanism; it specifically alters (increases or decreases) the ability of capsaicin to activate sensory neurons.

Paclitaxel alters gene expression

Since paclitaxel decreased release of CGRP only when capsaicin was used as the evoking stimulus, it is possible that the anti-cancer drug alters the expression of the TRPV1 receptor. Indeed paclitaxel at 300 nM to 1 μM for 2 to 5 days significantly decreased TRPV1 immunoreactivity in isolated sensory neurons. The decreased immunoreactivity was secondary to decreased transcription of TRPV1, because mRNA for TRPV1 was decreased in paclitaxel-treated neurons. The decrease in mRNA occurred at concentrations as low as 30 nM paclitaxel.

Paclitaxel at 2 mg/kg or 4 mg/kg but not 1 mg/kg decreased the mRNA for TRPV1 in DRGs. It must be noted that 2 mg/kg paclitaxel did not alter the capsaicin-evoked release of CGRP in spinal cord slices even though the mRNA for TRPV1 is decreased. It is possible that when CGRP release assays in spinal cord slices were performed (1 week after the last injection of paclitaxel), a significant turnover of the TRPV1 receptor had not yet occurred. Therefore, even if a small decrease in the immunoreactivity of TRPV1 were to be detected, it is possible that residual

vanilloid receptors at central terminals is sufficient to cause significant increases in Ca^{2+} influx that is required for neurotransmitter release.

I also examined if the mRNA expression of $Na_v1.9$ is altered in sensory neurons because this ion channel is co-expressed with TRPV1 in sensory neurons (Amaya et al., 2006), and its expression has been shown to decrease in injured peripheral nerve fibers in neuropathic pain models (Decosterd et al., 2002). In isolated sensory neurons exposed to paclitaxel (30 nM to 1 μM), I observed a decrease in the mRNA for the voltage-gated sodium channel $Na_v1.9$.

From these data, it can be concluded that the decrease in release of CGRP in neurons exposed to 100 nM to 1 μM paclitaxel results from decreased expression of TRPV1. However, it must be noted that although 30 nM paclitaxel for 5 days decreased expression of TRPV1, this concentration nonetheless increased capsaicin-evoked release of CGRP. Therefore the effects of paclitaxel at lower concentrations do not seem to involve expression of TRPV1 since 10 and 30 nM of the drug actually sensitize sensory neurons. Paclitaxel actually increases the formation of microtubules that are resistant to depolymerization, resulting in depletion of tubulin monomers (Letourneau and Ressler, 1984; Roytta et al., 1984; Thuret-Carnahan et al., 1985). Thus, it is possible that at lower concentrations of the drug all processes that rely on microtubule function are enhanced or stimulated, which might explain the increased release of CGRP from sensory neurons exposed to the microtubule-inhibiting drug.

Paclitaxel decreases the length of neurites

In studies of other painful neuropathies involving small diameter sensory neurons, a decrease in epidermal innervation has been observed in skin biopsies (Kennedy and Wendelschafer-Crabb, 1996; Holland et al., 1997; Kennedy and Wendelschafer-Crabb, 1999; Periquet et al., 1999; Polydefkis et al., 2001; Polydefkis et al., 2002; Sommer and Lauria, 2007). Furthermore, Bickel et al. showed that in patients with small fiber neuropathies, the size of the flare correlates with the nerve fiber count in the vicinity of the dermal blood vessels (Bickel et al., 2009). A similar decrease in nerve fiber density has been observed in rats injected with 2 mg/kg paclitaxel every other day for a total of four doses (Siau et al., 2006). Therefore it is possible that by causing the peripheral endings to degenerate paclitaxel decreases innervation of dermal blood vessels, resulting in decreased cutaneous vasodilatation in response to capsaicin or electrical stimulation.

Letourneau et al., and Yang et al., independently showed that exposure to 700 nM or 30 nM paclitaxel respectively decreased the length of already formed neurites of embryonic DRG neurons *in vitro* (Letourneau and Ressler, 1984; Yang et al., 2009). Therefore, inhibition of neurite degeneration or treatments that promote regeneration are strategies that can be used to prevent or reverse peripheral nerve damage by paclitaxel. Indeed Melli et al., showed that recombinant human erythropoietin

prevented paclitaxel-induced distal degeneration *in vitro* and loss of sensory nerve fibers from the epidermal layer *in vivo* (Melli et al., 2006).

In order to examine mechanisms contributing to neurite degeneration, I established a model to examine the effects of paclitaxel on neurites in isolated sensory neurons. Individual cells were injected with gold particles that were previously coated with the cDNA for EGFP according to the method described by Dib-Hajj et al., (Dib-Hajj et al., 2009). In this way I could visualize individual neurons expressing EGFP and measure the length of their neurites after addition of drug. The velocity at which particles were injected into individual neurons was previously optimized so that the use of gold particles did not result in either death or degeneration of neurons. The neurons expressing EGFP were allowed to grow in culture for 7 days before exposure to the anti-cancer drug. Paclitaxel (100 to 300 nM) causes decrease in the length of the neurites. This decrease cannot be attributed to the stress induced by injecting gold particles because it is possible to record whole cell currents and action potentials about 48 hours after transfection by this method (Dib-Hajj et al., 2009).

While many investigators examined the effects of the microtubule-stabilizing drug on initiation of neurite outgrowth (Konings et al., 1994; Hayakawa et al., 1998; Hayakawa et al., 1999; Scuteri et al., 2006), very few studies examine the effects of paclitaxel on length of already established neurites (Letourneau and Ressler, 1984; Melli et al., 2006;

Yang et al., 2009). Yang et al., observed an approximately 25% decrease in axonal length after a 24 hour exposure to 30 nM paclitaxel. In my study a 25% decrease was observed when sensory neurons were exposed to a higher dose (100 μM paclitaxel) for a longer time (5 days). Such a discrepancy can be explained by the fact that my work examines the effects of paclitaxel on adult DRG neurons; whereas all other studies in the literature were performed on embryonic neurons. Yang and coworkers made an interesting observation that application of paclitaxel only to axons in a compartmentalized culture system resulted in their degeneration while addition of the drug to the compartment containing cell bodies did not cause shortening of neurites. This observation suggests that inhibition of axonal microtubules resulting in decreased delivery of nutrients and other molecules required for axonal growth and maintenance contributes to the degeneration of neurites (Yang et al., 2009). Furthermore, there is evidence that paclitaxel affects mitochondrial function (Andre et al., 2002; Kidd et al., 2002; Flatters and Bennett, 2006; Jin et al., 2008). Mitochondria are highly concentrated at synapses and at the tip of growth cones where they are important for providing energy for neurotransmitter release and sequestration of calcium at synaptic sites. Therefore, it is possible that paclitaxel-induced mitochondrial damage leads to axonal degeneration due to disruption of calcium homeostasis or lack of energy production required for axon growth and maintenance.

Relevance of *in vivo* and *in vitro* observations

There is an apparent inconsistency between the paclitaxel-induced hypersensitivity demonstrated by Polomano et al., and Weng et al., (Polomano et al., 2001; Weng et al., 2005) and my data demonstrating a decrease in sensory neuron mediated blood flow. Hypernociception is often attributed to hyperexcitability of sensory neurons, while decreased blood flow after activation of sensory neurons suggests a loss of function of sensory neurons. The question thus arises: how could diminished release of neurotransmitter from sensory neurons result in hypernociception? It is possible that changes in nociceptive behavior observed by others is a result of changes in function of neurons in the central nervous system as a result of central sensitization (Polomano et al., 2001; Weng et al., 2005). Central sensitization is the increased activation of synapses in the dorsal spinal cord due to repeated stimulation of the primary afferent sensory neurons. The possibility that paclitaxel administration results in central sensitization is supported by the observation by Weng et al., that the dosing regimen I used (1mg/kg, 4 times a week) decreases glutamate transporters in the dorsal spinal cord (Weng et al., 2005). Furthermore, the wide dynamic range (WDR) neurons in the spinal cord from paclitaxel-treated animals have an increase in spontaneous activity, an increase in after-discharges in response to mechanical and thermal stimuli and an increase in wind-up in response to cutaneous stimuli (Cata et al., 2006a).

Increased presynaptic and/or postsynaptic excitability could contribute to the increased firing of neurons in the central nervous system (Woolf, 2007; Seybold, 2009; Stein et al., 2009). Therefore, an increase in capsaicin-evoked release of CGRP in the spinal cord of paclitaxel-injected animals might account for the paclitaxel-induced increase in spontaneous activity of WDR neurons. However, paclitaxel (1 or 2 mg/kg) did not alter capsaicin-evoked release of CGRP from central terminals of primary afferent sensory neurons. A possible explanation is that in my studies release from spinal cord slices was measured one week after the last injection of paclitaxel. Therefore it is possible that an increase in transmitter release leading to central sensitization might occur earlier in the dosing schedule. Thus, it is important in future studies to examine release of CGRP in the spinal cord of paclitaxel injected animals at earlier time points to determine if its release is enhanced during the initial stages of the dosing regimen. Furthermore, I only examined the function of a subset of nociceptive neurons (only the peptidergic neurons) in paclitaxel injected animals. It is known that paclitaxel affects all types of sensory neurons, therefore it is possible that the drug has differential effects on non-peptidergic sensory neurons. Further experiments are necessary to examine if paclitaxel alters the release of glutamate in spinal cord perfusate from paclitaxel-injected animals.

Another explanation for increased sensitivity in the skin of paclitaxel-injected animals is that attenuated vasodilatation results in

peripheral ischemia resulting in high proton concentrations in the periphery. In ischemic conditions, pH levels as low as 5.4 has been measured (Steen et al., 1992; Steen and Reeh, 1993) and Issberner et al., showed that in experimental subjects, decreased intradermal pH directly correlates with increased pain perception (Issberner et al., 1996). The link between low pH and pain has been made by the discovery of molecular transducers expressed on sensory neurons, namely TRPV1 (Fischer et al., 2003; Caterina, 2007) and those belonging to the ASIC family, which respond to high extracellular proton concentration (Krishtal and Pidoplichko, 1981a; Krishtal, 2003). Protons have the ability to not only sensitize sensory neurons to non-noxious stimuli via their actions on TRPV1 and ASICs, but they can also directly activate these channels to transduce pain stimuli. Therefore, the sensitization of nociceptors as a result of peripheral ischemia might help explain another symptom of neuropathy - the paradoxical burning pain that patients experience when their skin is exposed to cold temperatures (Dougherty et al., 2004). In healthy individuals, exposure to cold temperatures results in vasoconstriction and a consequent decrease in skin blood flow. This vasoconstriction is balanced by reflex vasodilatory mechanisms in which sensory neurons have been proposed to play an important role (Bunker et al., 1990; Terenghi et al., 1991). Therefore, It is possible that in pathological situations where sensory neuron mediated vasodilatation is absent, vasoconstriction induced by cold temperatures is prolonged,

therefore leading to ischemia and subsequent activation of proton sensitive channels on nociceptive neurons to evoke sensations like burning and tingling (Krishtal and Pidoplichko, 1981a, b; Steen et al., 1992).

Burning pain is also associated with many other small fiber painful neuropathies such as diabetic neuropathy, human immunodeficiency virus-associated sensory neuropathies, neuropathies secondary to anti-retroviral drugs, and post-herpetic neuralgia (Tavee and Zhou, 2009). Decreased sensory neurons mediated cutaneous vasodilatation been observed in patients with diabetic neuropathy (Forst et al., 1997; Pitei et al., 1997; Hamdy et al., 2001; Caselli et al., 2003; Kramer et al., 2004; Caselli et al., 2006; Khalil et al., 2007; Quattrini et al., 2007). More importantly, a direct link between decreased cutaneous vasodilatation and pain can be seen in the study by LeVasseur et al; they observed decreased capsaicin-evoked vasodilatation at skin sites where pain was experienced by elderly patients with post herpetic neuralgia, while normal (non-painful) skin sites demonstrated normal responses to capsaicin injection (LeVasseur et al., 1990). Furthermore, decreased CGRP mediated vasodilatation has been postulated to be a contributing factor to the pathology of Raynaud's phenomenon, a condition which is characterized by chronic digital pain following exposure to cold temperatures (Brain et al., 1990). Therefore my observation of decreased cutaneous vasodilatation in paclitaxel-injected animals is in agreement

with similar observations made in other neuropathies that are characterized by burning pain.

Neurogenic control of skin blood flow is important for temperature regulation of the skin (Roosterman et al., 2006); in patients with diabetic and hereditary autonomic neuropathy, a dysregulation of skin temperature has been observed (Kitaoka et al., 1989; Polo et al., 2000). Therefore, it is possible that decrease in cutaneous vasodilatation might explain the coldness in the extremities experienced by patients receiving chemotherapy with paclitaxel as is observed in patients with complex regional pain syndrome and Raynaud's phenomenon (Bunker et al., 1990; Dougherty et al., 2004). Clearly, examination of blood flow is not only an important endpoint for the study of sensory neurons in this study, but changes in vasodilatation occurring secondary to peripheral neuropathy could account for at least some of the sensory abnormalities experienced by patients.

My observation that paclitaxel decreased the length of neurites of sensory neurons coupled with the finding by Siau et al., that intraepidermal nerve fiber density is decreased in paclitaxel injected animals could explain the decreased vasodilatation observed in paclitaxel injected animals (Siau et al., 2006). Bickel et al., observed a direct correlation between density of nerve endings in the skin and electrical stimulation-induced vasodilatation in patients with neuropathy (Bickel et al., 2009). A possible explanation for this correlation is that retraction of

nerve endings could decrease sensory innervation of dermal blood vessels and remove CGRP from the nerve muscle synapse, thus reducing the effectiveness of CGRP release from free nerve endings in producing neurogenic vasodilatation. Further studies are therefore warranted to examine the mechanisms that mediate neurite retraction so that effective strategies to preserve the integrity of sensory neurons may be devised.

Another interesting and novel contribution of this study was the observation that paclitaxel's effects on CGRP release is biphasic, i.e. increased CGRP release at exposure to 10 and 30 nM paclitaxel and decreased release occurring at 100 nM to 1 μM paclitaxel. The increased release observed with lower concentrations of paclitaxel is consistent with the hypersensitivity to noxious stimuli observed in animals that receive multiple injections of lower doses (e.g.1 mg/kg) paclitaxel. The decrease in CGRP release observed at higher concentrations of paclitaxel is in agreement with the loss of sensitivity to thermal and mechanical stimuli that is observed at multiple injections of higher concentrations of paclitaxel (e.g. 16 mg/kg) in animal models. Furthermore, many patients experience pain following the first cycle of infusion of paclitaxel (250 mg/m^2), which often subsides within a week (Wiernik et al., 1987a; Lipton et al., 1989; Sahenk et al., 1994). Loprinzi et al., speculated that this acute pain sensation experienced in the initial stages of chemotherapy is a result of sensitization of nociceptive neurons (Loprinzi et al., 2007). With successive cycles of administration of paclitaxel, the symptoms are further

exaggerated and increased in duration. Gradually the symptoms evolve to loss of sensation evidenced by increased thermal and vibratory thresholds (Lipton et al., 1989). Therefore, my observations on effects of paclitaxel on capsaicin-evoked CGRP release from isolated sensory neurons correlates well with behavioral studies in animals and symptoms experienced by patients undergoing chemotherapy with the drug.

CONCLUSIONS AND FUTURE DIRECTIONS

The experiments conducted as part of this thesis has provided a better understanding of the mechanisms that mediate the sensory disturbances after treatment with paclitaxel than what is currently known. The current study demonstrates that administration of paclitaxel causes a decrease in the release of CGRP from peripheral terminals of sensory nerves, and from sensory neurons in culture. Furthermore, in isolated sensory neurons paclitaxel causes degeneration of neurites, which might be a mechanism to account for the decrease in peripheral blood flow observed in animals and the burning pain and coldness in extremities experienced by patients. Therefore, whether the symptoms of peripheral neuropathy resolve or persist depends on the extent of damage to sensory neurons. It is important to identify the factors that mediate long-term changes in sensory neuron function.

One possible mechanism by which paclitaxel produces neurotoxic effects is via mitochondrial damage. In neuroblastoma cells, paclitaxel (1 - 100 μM) causes mitochondrial swelling, induces opening of the mitochondrial permeability transition pore (mPTP) and cytochrome release (Andre et al., 2000; Andre et al., 2002) and mitochondrial swelling in the saphenous nerve of rats chronically treated with paclitaxel (Flatters and Bennett, 2006). By damaging mitochondria paclitaxel can cause altered calcium homeostasis (Siau and Bennett, 2006) and increased production of free radicals (Andre et al., 2002; Wang et al., 2004; Fawcett

et al., 2005; Ramanathan et al., 2005; Alexandre et al., 2006; Alexandre et al., 2007; Kim et al., 2008). It must be noted that free radicals at moderate concentrations are harmless and are eliminated by cellular anti-oxidants. When free radicals accumulate to high concentrations, they can damage major cell constituents by oxidizing proteins and targeting them for proteolysis, oxidizing DNA and in doing so altering gene expression, and inducing lipid peroxidation resulting in increased membrane permeability and cell death (Sevanian and Hochstein, 1985; Richter, 1987). Therefore, experiments are warranted to examine mitochondrial function and measurement of free radicals after exposure of isolated sensory neurons to paclitaxel. Furthermore, the role of anti-oxidants in prevention of neurite degeneration must also be examined.

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CURRICULUM VITAE

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EDUCATION

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PUBLICATIONS

1. Gracias NG, Cummins TR, Kelley MR, Basile DP, Iqbal T, Vasko MR. Paclitaxel reduces cutaneous vasodilatation induced by activation of sensory neurons Neurotoxicology. 2011 Jan;32(1):140-9..

2. Shrestha S, Gracias NG, Mujenda F, Khodorova A, Vasko MR, Strichartz GR. Local antinociception induced by endothelin-1 in the hairy skin of the rat's back. J Pain. 2009 Jul;10(7):702-14

3. Sun YG, Gracias NG, Drobish JK, Vasko MR, Gereau RW, Chen ZF. The c-kit signaling pathway is involved in the development of persistent pain. Pain. 2009 Jul;144(1-2):178-86.

4. Nazarian A, Gu G, Gracias NG, Wilkinson K, Hua XY, Vasko MR, Yaksh TL. Spinal N-methyl-D-aspartate receptors and nociception-

evoked release of primary afferent substance P. Neuroscience. 2008 Mar 3;152(1):119-27.

ABSTRACTS AND PRESENTATIONS

1. H. Meng, C.Guo, N. Gracias, N. Liu, X.M. Xu, M. R. Kelley, M. R. Vasko; Systemic injection of cisplatin impairs cognitive functions in rats. Society for Neuroscience National Meeting, San Diego, CA 2010. Poster Presentation.

2. N. Gracias, C. Guo, M. Kelley, M. R. Vasko. The antimitotic-anticancer drugs, paclitaxel and vincristine decrease capsaicin-evoked release of calcitonin gene-related peptide and TRPV1 expression in sensory neurons in culture. Society for Neuroscience National Meeting, Chicago, IL, 2009. Poster Presentation.

3. N. Gracias, X. Pu, J. E. Klaunig, M. Kelley, M. R. Vasko. Paclitaxel treatment alters release of iCGRP and consequently contributes to decreased cutaneous blood flow. Society for Neuroscience National Meeting, Washington, DC, 2008. Poster Presentation.

4. S. Shrestha, N. Gracias, F. Mujenda, A. Khodorova, M. Vasko, G. R. Strichartz. Subcutaneous endothelin-1 produces local analgesia in hairy skin through local vasoconstriction. Society for Neuroscience National Meeting, Washington, DC, 2008. Poster Presentation.

5. N. Gracias, C. Guo, M. Kelley, M. R. Vasko. Paclitaxel-induced augmentation of capsaicin-evoked release of CGRP from sensory neurons is attenuated by antioxidants. Society for Neuroscience National Meeting, San Diego CA, 2007. Poster Presentation.

SCHOLARSHIPS AND AWARDS

1. Indiana University School of Medicine Education Enhancement Grant Award, 2008.

2. Indiana University School of Medicine Translational Research Fellowship Award, January 2008 – August 2008.

3. Indiana University School of Medicine Graduate Student Organization Travel Award, 2007.

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