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Research Paper

# Neurotoxic mechanisms of paclitaxel are local to the distal axon and independent of transport defects

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ABSTRACT

Chemotherapy-induced peripheral neuropathy (CIPN) is a dose-limiting side effect of paclitaxel and other chemotherapeutic agents. Paclitaxel binds and stabilizes microtubules, but the cellular mechanisms that underlie paclitaxel's neurotoxic effects are not well understood. We therefore used primary cultures of adult murine dorsal root ganglion neurons, the cell type affected in patients, to examine leading hypotheses to explain paclitaxel neurotoxicity. We address the role of microtubule hyperstabilization and its downstream effects. Paclitaxel administered at 10–50 nM for 1–3 days induced retraction bulbs at the tips of axons and arrested axon growth without triggering axon fragmentation or cell death. By correlating the toxic effects and microtubule stabilizing activity of structurally different microtubule stabilizing compounds, we confirmed that microtubule hyperstabilization, rather than an off-target effect, is the likely primary cause of paclitaxel neurotoxicity. We examined potential downstream consequences of microtubule hyperstabilization and found that changes in levels of tubulin posttranslational modifications, although present after paclitaxel exposure, are not implicated in the paclitaxel neurotoxicity we observed in the cultures. Additionally, defects in axonal transport were not implicated as an early, causative mechanism of paclitaxel's toxic effects on dorsal root ganglion neurons. By using microfluidic chambers to selectively treat different parts of the axon with paclitaxel, we found that the distal axon was primarily vulnerable to paclitaxel, indicating that paclitaxel acts directly on the distal axon to induce degenerative effects. Together, our findings point to local effects of microtubule hyperstabilization on the distal-most portion of the axon as an early mediator of paclitaxel neurotoxicity. Because sensory neurons have a unique and ongoing requirement for distal growth in order to reinnervate the epidermis as it turns over, we propose that the ability of paclitaxel to arrest their growth accounts for the selective vulnerability of sensory neurons to paclitaxel neurotoxicity.



## 1. Introduction

Paclitaxel is a widely used chemotherapeutic that frequently causes peripheral neuropathy. The neuropathy, which can be dose-limiting, primarily affects sensory neurons and leads to a distal axonopathy; patients with paclitaxel-induced peripheral neuropathy experience loss of sensation and ongoing pain in the hands and feet and demonstrate degeneration of the nerve fibers that innervate the skin (Boyette-Davis et al., 2013). Paclitaxel binds to the interior of microtubules and stabilizes them, interfering with the normal cycling of microtubule depolymerization and repolymerization. In dividing cells such as cancer cells, this disrupts normal spindle dynamics, interferes with mitosis, and ultimately leads to cell death (Weaver, 2014). Neurons, which do not divide, are nevertheless susceptible to paclitaxel, and the mechanisms underlying paclitaxel neurotoxicity remain incompletely elucidated (reviewed in Gornstein and Schwarz, 2014). Developing interventions for paclitaxel-induced peripheral neuropathy will require understanding the mechanisms by which paclitaxel compromises peripheral axons.

Axons are rich in microtubules, which provide structural support and serve as tracks for axonal transport throughout the life of the neuron. While neurons have a larger population of stable microtubules than non-neuronal cells, their microtubules are not static. Given paclitaxel's action in dividing cells, it has been presumed that increased microtubule stabilization contributes to paclitaxel neurotoxicity. However, alternative binding targets leading to effects on the ER and mitochondria have been proposed that may be relevant (Boehmerle et al., 2006; Ferlini et al., 2009; Rodi et al., 1999). Determining whether microtubule stabilization is the primary cause of paclitaxel neurotoxicity is a first step in defining the mechanistic pathways leading to neuronal damage.

If microtubule stabilization is the primary cause of paclitaxel neurotoxicity, the cellular mechanisms that link microtubule

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hyperstabilization and axon degeneration are unknown. One known consequence of increased microtubule stabilization is a change in the levels of tubulin posttranslational modifications, namely increased acetylation, polyglutamylation and detyrosination (Hammond et al., 2010; Mansfield and Gordon-Weeks, 1991; Garnham and Roll-Mecak, 2012). Tubulin post-translational modifications can alter the binding of microtubule-associated proteins and motors, and alterations in these modifications have been linked to axon degeneration and regeneration. For example, tyrosinated tubulin is necessary for the binding of microtubule plus-end interacting proteins that are required for transport initiation at the distal axon (Moughamian et al., 2013; Peris et al., 2006). Tubulin hyperglutamylation has been linked to Purkinje cell degeneration and increases the activity of the microtubule severing protein, spastin (Lacroix et al., 2010; Rogowski et al., 2010). Additionally, microtubule deacetylation is required for axon regeneration after injury (Cho and Cavalli, 2012), and kinesin-1 based transport is affected by acetylation and detyrosination (Cai et al., 2009; Dompierre et al., 2007; Dunn et al., 2008; Konishi and Setou, 2009; Maas et al., 2009). Thus it is plausible that the alterations in tubulin post-translational modifications observed in neurons upon paclitaxel treatment contribute to paclitaxel neurotoxicity, a hypothesis which has not yet been tested.

A prevalent hypothesis for how microtubule stabilization could ultimately lead to axon degeneration is through the disruption of axonal transport. Previous studies have observed paclitaxel-induced defects in axonal transport. These studies, however, mostly employed cell types other than the clinically affected mammalian sensory neuron (Das et al., 2014; LaPointe et al., 2013; Shemesh and Spira, 2010), or used paclitaxel concentrations higher than the peak plasma concentrations typically reached in patients (Nakata and Yorifuji, 1999; Theiss and Meller, 2000). The relevance of potential transport defects as an early, causative event in paclitaxel neurotoxicity is uncertain.

Using cultured adult mammalian dorsal root ganglion (DRG) neurons, we examined the role of microtubule stabilization and its downstream consequences in paclitaxel neurotoxicity. Our results suggest that increased microtubule stabilization, rather than an off-target effect, is likely to be responsible for paclitaxel neurotoxicity. However, our results do not point to an early causative role of changes in tubulin post-translational modifications or axonal transport defects in paclitaxel's toxic effect on axons, but indicate instead a direct vulnerability to paclitaxel of the distal-most portion of the axon. Inhibiting growth at the distal axon may account for the sensory neuropathy.

## 2. Results

### 2.1. Modeling paclitaxel neurotoxicity in cultured adult DRG neurons

In order to interrogate mechanisms of paclitaxel neurotoxicity, we established a model of paclitaxel-induced degeneration in cultured adult mammalian DRG neurons, the neuron type relevant to the clinical problem of paclitaxel-induced peripheral neuropathy. The use of cultures necessarily restricted the study to cell autonomous aspects of the neuropathology although other cell types are also likely to contribute (Lisse et al., 2016; Zhang et al., 2016). For probing mechanisms of paclitaxel neurotoxicity, we thought it important to use paclitaxel concentrations that resulted in a slow time course of degeneration in order to untangle whether potential mechanisms were a likely primary cause rather than indirect consequence of degeneration. To this end, we used dissociated cultures of DRGs from 8 to 10 week old mice and exposed them to nanomolar concentrations of paclitaxel that led to degenerative effects over the course of days. Treatment of these sensory neuron cultures with 10–50 nM paclitaxel led within three days to a dose-dependent increase in bulbous swellings mostly at the axon tips, an appearance consistent with retraction bulbs (Fig. 1A–B). Axon area relative to untreated cultures decreased with a similar time course (Fig. 1C). After one day in 10 nM paclitaxel, there were scattered bulbous axon swellings, which were more frequent upon exposure to 25 or 50 nM paclitaxel. At each concentration, their frequency was further increased relative to control after three days of paclitaxel exposure (Fig. 1B). After three days in 25 nM or 50 nM paclitaxel, axon area per field was also decreased, axon width was increased, and axons appeared abnormally wavy. Nonetheless, the number of cell bodies per imaging field did not change compared to control, indicating that in these treatment conditions, paclitaxel was toxic to axons but did not cause cell death (Fig. 1D). Because low nanomolar concentrations of paclitaxel led to a slow time course of quantifiable degenerative effects, we used this concentration range to probe paclitaxel neurotoxicity mechanisms. Peak plasma concentrations after common dosing regimens in patients are in the range of 228 nM–4.3 uM (Huizing et al., 1993), but the concentrations experienced by the neurons are not known. The range of 10–50 nM therefore seemed appropriate for dissociated DRG neurons in culture that likely have better access and less transient exposure to the drug than neurons in vivo.

We characterized further the retraction bulb-like swellings that formed after paclitaxel exposure. Unlike the growth cones of control axons, the retraction bulbs did not have actin extending beyond the microtubules (Fig. 1E). Microtubule polymerization was disordered in the retraction bulbs as evidenced by swirling EB3-GFP comets (Movies S1, S2). Additionally, while the level of acetylated tubulin overall increased after paclitaxel treatment, indicative of stable microtubules, tubulin in retraction bulbs was predominantly deacetylated (Fig. 1F). Furthermore, retraction bulbs had accumulations of mitochondria (Fig. 1G). These observations are consistent with descriptions of CNS retraction bulbs, which are characterized by disorganized microtubules (Erturk et al., 2007).

We wondered whether neuronal types differed in their response to paclitaxel in vitro and therefore whether neuronal type may be an important consideration when selecting a model for mechanistic studies of paclitaxel neurotoxicity. We found that different neuron types had different sensitivities and morphological responses to paclitaxel. In particular, cultured hippocampal neurons were more sensitive than DRG neurons to paclitaxel when both were exposed to 50 nM paclitaxel for three days. Microtubules were fragmented in hippocampal neurons and their axons were no longer intact (Fig. 1H). This fragmentation was not observed in DRG cultures, even after three days in 1 μM paclitaxel (data not shown). Prior to fragmentation, hippocampal neurons did have some axonal swellings that might be akin to the retraction bulbs of DRG neurons. After one day of 50 nM paclitaxel treatment, bulbous structures were observed in hippocampal neurons near the soma at the tips of very short neurites (data not shown); this response to paclitaxel has been previously reported at higher concentrations in younger embryonic cortical neuron cultures (Chuckowree and Vickers, 2003). Previous work on younger hippocampal cultures has shown that microtubule stabilization causes neurons to form multiple axons when far lower doses of paclitaxel (3 nM) are applied (Witte et al., 2008). Adult DRG axons are thicker, more robust, and more tubulin-rich than embryonic hippocampal neurons and thus the different responses of the neurons to 50 nM paclitaxel may reflect either age-dependent (Kole et al., 2013) or cell-type specific differences. The axonal effects seen in adult DRG neurons after paclitaxel treatment better resemble the “dying back” axon retraction characteristic of paclitaxel-induced peripheral neurotoxicity (Boyette-Davis et al., 2013) and thus may be preferable to hippocampal neurons for mechanistic studies.

### 2.2. The neurotoxicity of epothilone B and paclitaxel correlates with their microtubule stabilization

As a first step in defining mechanistic pathways that lead to paclitaxel neurotoxicity, we tested the hypothesis that increased microtubule stability, rather than off-target effects, is the primary cause of paclitaxel neurotoxicity. Although a change in microtubule dynamics after paclitaxel exposure has been a dominant hypothesis to explain paclitaxel neurotoxicity, alternative paclitaxel binding targets have been