Oxaliplatin Neurotoxicity of Sensory Transduction in Rat Proprioceptors

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Abstract

Neurotoxic effects of oxaliplatin chemotherapy, including proprioceptive impairments, are debilitating and dose-limiting. Here we sought to determine whether oxaliplatin interrupts normal proprioceptive feedback by impairing sensory transduction of muscle length and force in axons that are not damaged by dying-back neuropathy. Oxaliplatin was administered over four weeks to rats in doses that produced systemic changes, e.g. decreased platelets and stunted weight gain, but no significant abnormality in the terminal ends of primary muscle spindle sensory neurons. The absence of neuropathy enabled determination of whether oxaliplatin caused functional deficits in sensory encoding without the confounding issue of axon death. Rats were anesthetized and action potentials encoding muscle stretch and contraction were recorded intra-axonally from dorsal roots. By striking contrast with normal proprioceptors, those from oxaliplatin-treated rats typically failed to sustain firing during static muscle stretch. The ability of spindle afferents to sustain and centrally-conduct trains of action potentials in response to rapidly repeated transient stimuli, i.e. vibration, demonstrated functional competence of the parent axons. These data provide the first evidence that oxaliplatin causes persistent and selective deficits in sensory transduction that are not due to axon degeneration. Our findings raise the possibility that even those axons which do not degenerate following oxaliplatin treatment may have functional deficits that worsen outcome.

Key words: Chemotherapy, Neuropathy, Muscle spindle, Electrophysiology, and Afferent
Peripheral neuropathy is the most common dose-limiting factor of oxaliplatin chemotherapy (see reviews (Argyriou et al. 2008; Krishnan et al. 2005; Pasetto et al. 2006)). Acute neurotoxicity, predominantly sensory in nature, is seen in nearly all patients and is characterized by cold intolerance, dysthesias, and, less commonly, laryngeal spasms (Wilson et al. 2002). With continued treatment, a cumulative sensory neuropathy can develop similar to that seen in patients treated with other platinum compounds. The onset of the neuropathy is gradual and the severity worsens with an increase in cumulative dose. Chronic neuropathy is thought to primarily affect large diameter sensory fibers (for evidence and earlier citations, (Jamieson et al. 2005)) which include muscle afferents that provide the central nervous system with proprioceptive information. Consistent with this notion, patients often lose deep tendon reflexes, mediated by muscle spindle afferents, and develop proprioceptive loss seen as problems with coordinating movement, e.g. lost dexterity and sensory ataxia.

Sensory contributions to behavior and experience depend on conduction and central transmission of signals that are encoded by specialized sensory receptors. Behavior can be affected, therefore, by interrupting the flow of sensory information at any point in peripheral receptor transduction, axon conduction, and/or central synaptic transmission. With oxaliplatin treatment, axon degeneration, i.e. neuropathy, interrupts axonal conduction in sensory nerves, as suggested by the reduction of compound sensory nerve action potential amplitudes (SNAPs) and by the sensory loss that often occurs in a “stocking and glove” distribution affecting the distal limbs and progressing proximally (Argyriou et al. 2008). There are also changes in axon excitability that can disturb normal conduction of sensory signals (Krishnan et al. 2005; Lehky et al. 2004). These effects of oxaliplatin undoubtedly contribute in altering sensory feedback, although the relative contribution of impaired conduction to sensory loss is undetermined.
Substantial numbers of sensory axons maintain conduction as indicated by the partial presence of SNAPs, but their ability to properly encode sensory stimuli in the periphery is untested, and if modified could contribute significantly to sensory losses experienced by some patients in advance of neuropathy (Cascinu et al. 2002).

The goal of this study was to determine whether functional deficits in sensory encoding exist following oxaliplatin administration. To avoid the confound of recording from axons that might be in the process of degenerating, we found a dose of oxaliplatin that did not cause neurodegeneration in rats and recorded from individual sensory axons in vivo during muscle stretch to characterize sensory encoding of proprioceptive information. We demonstrate abnormal sensory transduction that persists long after removal from treatment, even in the absence of peripheral degeneration.
Methods

All procedures involving animals were approved by the Wright State University Laboratory Animals Care and Use Committee. Data were collected from 20 adult female Wistar rats (240-300g). All animals were assigned to either vehicle control (VC, n=7) or oxaliplatin-treated (OX, n=13) groups, housed individually in barrier-protected cages, and given food and water ad libitum. Animals were monitored daily for general signs of distress, and body weights and food intake were recorded twice per week. None exhibited signs of discomfort reaching preset levels for removal from the study; all were killed by intraperitoneal injection of euthasol (150 mg/kg) following terminal experiments.

Oxaliplatin (Sigma-Aldrich, St. Louis, MO) dissolved in 5% dextrose (4 mg/ml) was administered through intraperitoneal (i.p.) injections weekly for four weeks. Doses of 9-10 mg/kg were used to reach cumulative doses of 36-40 mg/kg. Five animals were used solely for immunohistochemical analysis (see below). VC rats were administered 5% dextrose using similar volumes (adjusted for weight) and dosing schedules.

At 1 week intervals preceding terminal experiments, the 15 remaining rats not used for immunohistochemical analysis were anesthetized (inhalation of isoflurane in 100% O₂) for purposes of recording sensory nerve action potentials (SNAPs) evoked electrically in the tail exactly as described by (Novak et al. 2009). SNAPs were stored on computer and analyzed for amplitude, latency and duration. Before anesthesia was discontinued, blood samples were taken from the lateral tail vein of OX rats for measurement of complete blood counts.

Terminal experiments were performed on all rats 3-5 weeks after the final treatment with oxaliplatin or vehicle alone. Anesthesia was induced and maintained by isoflurane inhalation (1-3% in 100% O₂). Either the response properties of muscle sensory nerves or the neural morphology of muscle spindle receptors were studied as detailed in our earlier studies (Haftel et
al. 2004; Haftel et al. 2005). Briefly, with rats secured in a rigid recording frame, triceps surae muscles in the left hind limb were dissected and attached through their common Achilles tendon to a servomotor which measured force and controlled muscle length. Triceps surae nerves were placed in continuity on bipolar stimulating electrodes, and other hind limb nerves were crushed, including common peroneal and posterior tibial nerves and nerves supplying hamstrings muscles. From dorsal roots L4 and L5 exposed by laminectomy and positioned on a recording platform, individual sensory axons were penetrated with sharp glass micropipettes (25-35 MΩ, 2M K-acetate) in order to record their action potentials produced in response to various stimuli.

Sensory axons were randomly sampled in dorsal roots and selected for further study when they responded with orthodromic action potentials having conduction delay < 3 ms upon electrical stimulation of the triceps surae nerves. Sensory axons were classified as either muscle-spindle or tendon-organ afferents, respectively, depending upon whether they paused or accelerated firing during the rising phase of force in isometric twitch contractions of the triceps surae muscles. Further classification and characterization of mechanotransduction by these neurons was obtained from their responses to muscle stretch in the forms of ramp-hold-release (20 mm/s ramp and release, 3 mm), triangular stretch-release, and vibration (50-250 Hz, 80 μm). Muscle spindle afferents were classified as group IA when they (a) produced high-frequency bursts of firing at the onset of ramp or triangular stretch (4 mm/s, 3 mm) and (b) fired in response to each cycle of vibrations at frequency ≥100 hz (e.g. Fig. 3; (Matthews 1972)). Those muscle spindle afferents exhibiting neither property a or b were designated group II, while those displaying mixed responses were unclassified, and were not considered further. In controls 52/153 (34%) afferents were unclassifiable vs. 25/116 (22%) in oxaliplatin treated. All stretches began at resting length (Lr) and were also performed at resting length + 1 mm (Lr+1), resting length + 2 mm (Lr+2) and resting length – 1 mm (Lr-1). Records of intra-axonal action potentials
and muscle length force were collected, digitized (20 kHz) and stored on a computer for later
analysis using Spike2 software.

Several parameters were measured from afferent firing responses during ramp-hold-release
stretch. The first action potential in response to ramp stretch specified the thresholds
associated with muscle length and force traces. Note that there were very few instances of
firing at resting length for any of the afferents sampled here from VC or OX rats. The slope of
best-fit linear regressions on instantaneous firing rate vs. muscle length represented the change
in firing rate with muscle length. Peak firing rate was measured at the peak of ramp stretch, and
dynamic index was taken as the difference in firing rate between peak and 0.5 s into the hold
phase of stretch. The time of occurrence of the last spike during the hold phase was noted.

Finally, history dependent afferent firing was characterized by the reduced dynamic response
(RDR; see Haftel et al. 2004), which was measured as the difference in the number of spikes
fired in the first and third in a series of three successive triangular stretches, excluding the initial
burst.

From five OX rats the left medial gastrocnemius (one of the triceps surae muscles) was excised
to examine the sensory nerve supply of muscle spindles for signs neuropathy (Haftel et al.
2005). Briefly, fixed and frozen muscles were cut in 100 μm sections for immunohistochemical
labeling of muscle spindle axons using rabbit polyclonal antibody against protein gene product
9.5 (1:500, MorphoSys, Munich, Germany). Visualization of axons was achieved using
fluorescein-conjugated secondary antibodies (1:100, Jackson ImmunoResearch, West Grove,
PA). Z-axis stacks of images were obtained using a Fluoview FV 1000 confocal microscope
and a 60x objective (Olympus optical). Illustrated images are flat-plane in focus projections
obtained from z-series images using Fluoview software.
Results

Oxaliplatin toxicity without neuropathy

Rats maintained healthy appearance and behavior throughout treatment, but there were clear systemic effects of oxaliplatin. Mean body weight increased over time in VC rats, but remained relatively unchanged in OX rats (Fig. 1A). Some OX rats (4/13) developed abdominal bloating with a purulent fluid found in the abdominal cavity at necropsy, as reported in previous studies using similar intraperitoneal doses (Jamieson et al. 2005). Because results from these 4 rats appeared indistinguishable from others, the data were pooled. No animals died during the course of the experiment.

A tendency toward decreased white blood cell count was observed in OX rats (Fig. 1B) consistent with observations reported for humans (de Gramont et al. 2000) treated with oxaliplatin. The decrease achieved significance beginning week 4, just one week after completing the oxaliplatin dosage, and by week 5 began to recover.

We found no evidence of appreciable neuropathy in nerve conduction studies, similar to patients that have been treated with OX but have no reduction in SNAP amplitude. Fig. 1C illustrates that SNAP amplitude and latency (clinical measures of axon degeneration and demyelination, respectively) were not significantly different between VC and OX rats at any time point (p > 0.1). Tendencies toward larger amplitudes and shorter latencies with time in both groups may reflect maturation of peripheral nerves.

Morphological inspection of distal-most extent of sensory nerves yielded little evidence of degeneration or structural disruption. Fig. 1E illustrates that annulospiral nerve endings formed by group IA sensory axons appeared structurally normal in OX rats. In the medial gastrocnemius muscle of two untreated control animals, we observed 20 and 21 intact muscle
spindles, each exhibiting neural innervation and annulospiral endings. The same normal morphology was observed in muscles from 5 OX rats, wherein the normal number of muscle spindles were found (19, 20, 21, 21, and 21) and the great majority displayed normally appearing annulospiral endings. In rare cases, annulospiral endings were absent (3/102) or diminished in number (9/102 <5 annulospiral rings per spindle). These results demonstrate that even the most distal portion of axons encoding stretch is intact following the dose of oxaliplatin used. Thus there was no distal neuropathy in these rats that could contribute to deficits in sensory transduction.

**Oxaliplatin modifies sensory transduction**

Muscle proprioceptors in OX rats exhibited a conspicuous failure to sustain firing during static muscle stretch. Fig. 2 illustrates this failure, i.e. rapid adaptation, for one spindle afferent from an OX rat (Fig. 2B) in comparison with the sustained, slowly adapting firing from a VC rat (Fig. 2A). Spindle afferents are normally characterized as slowly adapting sensory neurons (Matthews 1972), as were 93% (50/54) of spindle afferents which fired continuously through most of the hold phase of muscle stretch (≥ 0.9 sec) for VC rats, in contrast with only 56% (34/61) that sustained firing in OX rats (Fig. 2 C,D). On average, the last action potential occurred about 300 ms earlier than normal for all spindle and tendon-organ afferents (see Table 1).

Rapid adaptation of spindle afferent firing recorded in the dorsal roots might have been explained by impaired function of either excitability of the parent axon, which has been reported to change with oxaliplatin treatment (Park et al. 2009) or mechanotransduction by sensory receptors. Possible problems with action potential production were ruled out by demonstrating that axons fired continuously and at normal rates when muscle vibration was superimposed on the hold phase of stretch (Fig. 3). Of the 27 muscle spindle afferents with rapidly adapting
responses, all 27 were capable of resuming initiation and conduction of action potentials when vibration was added to the muscle stretch (Fig. 3B). Thus, all spindle afferents maintained the capacity to generate and conduct action potentials in OX rats, but many lost the ability to transduce selected features of mechanical stimuli, namely static muscle stretch.

Apart from selective impairment of sustained firing, the sensory code generated by proprioceptors in OX rats was indistinguishable from normal. Spindle afferent encoding of the dynamic features of muscle stretch was similar in VC and OX rats (Table 1). Firing rate increased with muscle length during the ramp portion of muscle stretch (e.g. Figs. 2B and 3A), with a relationship (slope pps/mm) that was unchanged by oxaliplatin. The absolute firing rates achieved at the peak of the ramp were not significantly different, although nominally lower firing rates for group IA afferents (18 pps) in OX rats was noteworthy since it could contribute to reduced deep tendon reflexes. Several other response properties associated with the ramp portion of stretch were unchanged. Included were the length threshold for firing, and the high frequency burst of firing (initial burst) observed in spindle group IA afferents which was quantified here for cases when it occurred at resting muscle length (Lr). The tendency for firing to decrease progressively in the ramp phases of successive triangular stretches (reduced dynamic response, RDR; (Haftel et al. 2004)) did not differ significantly in OX and VC rats.

Sensory axon hyperexcitability has been observed in acute oxaliplatin toxicity experienced by patients. We tested for expression of hyperexcitability in chronic OX rats and found no abnormality. No spontaneous firing was observed, nor was variance in action potential intervals during ramp stretch greater in OX than in VC rats. Thus the functional deficit reported here is distinct from that observed in acute oxaliplatin toxicity.
The data sample included tendon organ afferents, which respond most readily to active muscle contraction but also respond to stretch of a passive muscle. As for group IA spindle afferents, tendon-organ afferents were less responsive (lower average firing rate) during the hold phase of muscle stretch in OX compared to VC rats (Table 1). By contrast with spindle afferents, however, the responses of tendon-organ afferents during the ramp phase of stretch (Table 1) were modified in OX rats.
Results presented here provide the first evidence that oxaliplatin treatment in rats causes persistent deficits in sensory transduction, the process whereby sensory neurons transduce features of physical stimuli, e.g. duration of muscle stretch, into trains of action potentials. The deficits cannot be accounted for by axon degeneration, at least for group IA afferents for which there was no apparent evidence for degeneration of annulospiral endings at the oxaliplatin dose used. Studies in patients will be necessary to determine whether persistent deficits in sensory transduction contribute to sensory loss after treatment with oxaliplatin.

The most common etiology of sensory deficits in neuropathy is axon degeneration. In oxaliplatin neuropathy however, some patients have sensory deficits in the absence of electrophysiologic evidence for degeneration, e.g. no detectable abnormality in sensory nerve action potentials (Cascinu et al. 2002). One theoretical concern when one does not find evidence of axon degeneration in the setting of sensory deficits is that axons were not examined distally enough. We examined the distal most extent of sensory terminal axon morphology by examining annulospiral endings. No signs of significant degeneration were observed for group IA axons in direct visualization of muscle spindle annulospiral endings. In addition, oxaliplatin treatment had no detectable effect on several response properties of muscle proprioceptors, which exhibited their normal brief conduction delay and were classifiable by various measures of sensory modality as normal spindle and tendon-organ afferents. These changes in response properties would not be expressed if terminal axons had disconnected from receptors (Johnson and Munson 1991). Thus we ruled out axon degeneration as a contributor to deficits observed during muscle stretch in the rats studied. Despite the absence of axon degeneration and the retention of some normal properties, muscle proprioceptors exhibited distinct signs of abnormality in selected functions. Muscle proprioceptors were in some, but not all respects less sensitive to muscle stretch and were notably less able to sustain firing throughout the stimulus
as they are normally. These abnormalities would alter muscle length and force feedback from
spindle and tendon-organ afferents, respectively, potentially leading to dysfunction at multiple
sites in the sensory motor system.

Oxaliplatin neuropathy is already known to include functional effects distinct from degeneration.
Functional deficits (dysesthesias and paresthesias) occur within hours of oxaliplatin
administration, presumably reflecting the direct action of oxaliplatin on ion channels in sensory
axons (Gamelin et al. 2002; Grolleau et al. 2001; Wilson et al. 2002). The best known acute
cellular effects of oxaliplatin on neurons are its actions on voltage-gated Na channels.
Oxaliplatin modifications of Na current include slowing of inactivation kinetics (Adelsberger et al.
2000; Wu et al. 2009), reduction in current amplitude (Benoit et al. 2006; Grolleau et al. 2001;
Wu et al. 2009) and a hyperpolarized shift in the voltage dependence of inactivation (Benoit et
al. 2006). Effects on sodium channels are suggested to alter axon excitability in patients treated
with oxaliplatin (Kiernan and Krishnan 2006; Webster et al. 2005). We considered the
possibility that these direct effects of oxaliplatin might have produced results presented here
because oxaliplatin can take many weeks to clear the blood (Levi et al. 2000). However, the
sensory deficits we found are not consistent with acute effects of oxaliplatin on axon excitability.
Sensory axons expressed normal excitability, giving no evidence of spontaneous firing or firing
that was more variable than normal in response to muscle stretch. If anything, the cessation of
firing during stretch suggests reduced axon excitability. This cannot be the case, however,
since vibration superimposed on stretch completely restored normal action potential generation
and propagation. Thus, sensory axons appeared to have normal excitability as they were as
competent as normal in propagating action potentials from peripheral receptors to dorsal roots
entering the spinal cord.
Failure of muscle proprioceptors to maintain firing during static muscle stretch provides insight into the underlying mechanism. The receptor potential produced in muscle spindle proprioceptors during sustained stretch is produced by persistent inward Na current (Simon et al. 2010). The ability of vibration to restore sensory transduction demonstrates that transient potentials underlying sensory transduction function normally. Cessation of firing during static stretch may be caused, therefore, by an effect of oxaliplatin on a mechanically-gated Na channel involved in generating persistent inward current. Reduction of persistent Na current underlying the receptor potential in proprioceptors would reduce their sensitivity and lead to negative symptoms, including loss of coordinated movement.

Earlier studies demonstrated two mechanisms contributing to sensory deficits in oxaliplatin neuropathy. One is an acute effect on sensory axon excitability causing dysaesthesias, and the other is axon degeneration that leads to chronic sensory deficits. Our results from studies in rats suggest another possible mechanism, by which altered sensory transduction could impair sensory feedback. Proprioceptive deficits occurring with oxaliplatin therapy might be intensified if sensory encoding is truncated in those axons which do not degenerate. Further study will be necessary to determine whether the deficits in sensory transduction identified in rats occur in patients treated with oxaliplatin.

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Table 1. Sensory encoding by muscle proprioceptors

<table>
<thead>
<tr>
<th></th>
<th>Muscle Spindle Group I A</th>
<th>Muscle Spindle Group II</th>
<th>Tendon Organ Group IB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VC</td>
<td>OX</td>
<td>VC</td>
</tr>
<tr>
<td>Axonal conduction delay</td>
<td></td>
<td></td>
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<tr>
<td>(ms)</td>
<td>1.6 ± 0.8 n=37</td>
<td>1.7 ± 0.6 n=52</td>
<td>1.8 ± 0.3 n=17</td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.6 n=52</td>
<td>1.8 ± 0.3 n=17</td>
<td>2.0 ± 0.6 n=9</td>
</tr>
</tbody>
</table>

Ramp:
- **length threshold (mm)**
  - VC: 0.2 ± 0.1 n=37
  - OX: 0.3 ± 0.4 n=52
- **force threshold (g)**
  - VC: 0.7 ± 0.5 n=17
  - OX: 1.2 ± 1.0 n=9
- **initial burst (pps)**
  - VC: 344 ± 102 n=12
  - OX: 373 ± 101 n=27
- **slope (pps/mm)**
  - VC: 32 ± 14 n=37
  - OX: 38 ± 14 n=50
- **peak firing rate (pps)**
  - VC: 167 ± 52 n=37
  - OX: 149 ± 47 n=52

Hold:
- **average firing rate (pps)**
  - VC: 57 ± 30 n=37
  - OX: 34 ± 21* n=52
- **last spike time (ms)**
  - VC: 920 ± 217 n=37
  - OX: 631 ± 375* n=52
- **slope (pps/sec)**
  - VC: -32 ± 22 n=35
  - OX: -43 ± 25 n=45

Miscellaneous:
- **Dynamic Index**
  - VC: 115 ± 39 n=34
  - OX: 113 ± 43 n=31
- **RDR (spike number)**
  - VC: 14 ± 7 n=36
  - OX: 10 ± 6 n=52

Values are mean ± SD for data from n afferents pooled within groups; nested ANOVA and Tukey’s honestly significant difference (HSD) post hoc tests were used to test for the significance of group differences (*p<0.05). VC = vehicle control, OX = oxaliplatin.
Figure Legends

**Figure 1.** Chronic oxaliplatin toxicity without sensory neuropathy. Plots of rat body weight (A), white blood cell count (B) and SNAP properties (C), all versus time in weeks from the beginning of oxaliplatin treatment (baseline data taken immediately before treatment) until the time that the first rats were taken for terminal experiment. *p<0.05 ANOVA. (D) Records of SNAPs from individual rats at week 5 representing similarity between groups; difference between vertical dashed lines measures latency from stimulus to SNAP onset. Annulospiral endings were indistinguishable in OX and VC rats (E).

**Figure 2.** Chronic oxaliplatin modified sensory transduction. Intra-axonal records of action potentials recorded from group IA muscle-spindle afferents in response to ramp-hold-release muscle stretch from VC (A) and OX rats (B). Time of occurrence of the last action potential (AP) during 1 sec muscle stretch hold phase for all spindle afferents sampled per rat (n=54 afferents in VC rats (C); n=61 in OX rats)(D).

**Figure 3.** Chronic oxaliplatin selectively affects sensory transduction. Records from group IA afferent in an OX rat show that failure to fire during hold phase of muscle stretch (A) was overcome by superimposing vibration (not to scale) (B). Time of occurrence of last action potential (AP) during hold phase of stretch alone (o) extended to end of hold phase when vibration was superimposed (Δ) for 27/27 spindle afferents tested (C).