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Sensorimotor Analysis of Oxaliplatin Treated Rats

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SENORIMOTOR ANALYSIS OF OXALIPLATIN TREATED RATS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Krystyna Blanka Wieczerzak ENTITLED Sensorimotor analysis of Oxaliplatin treated rats BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMNTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Wieczerzak, Krystyna Blanka. Department of Neuroscience, Cell Biology, & Physiology, Wright State University, 2015. Sensorimotor Analysis of Oxaliplatin treated rats

There is currently no direct evidence that chemotherapy induced peripheral neuropathy (CIPN) necessarily explains the sensorimotor deficits seen in 90% patients treated with oxaliplatin (OX). Some patients develop sensory symptoms without CIPN. Our laboratory reported abnormal signaling from IA afferents in OX treated rats with no evidence of neuropathy. We hypothesized that in the absence of CIPN, the behavioral disability is associated with impaired sensory encoding in OX treated rats. The purpose of this study was to investigate the sensorimotor abilities of OX treated rats. The battery of behavioral tests was designed to address proprioception and sensorimotor integration. In the absence of CIPN, few OX rats revealed signs of decreased performance, as compared to controls. In conclusion, this study reproduced some of the results seen in the clinical studies and established a rat model, which can be used in further investigation of chronic sensorimotor symptoms of OX.

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I. INTRODUCTION

Antineoplastic agent oxaliplatin (OX) is one of the most effective treatments against advanced and metastatic digestive tumors such as colorectal cancer with the result that patients are among the third largest group of cancer survivors (Ganz, 2003). Furthermore, OX also exhibits promising antitumor activity effective in the treatment of number of other malignancies such as: rectal, pancreatic (Louvet, et al., 2002), gastric, ovarian (Dieras, et al., 2002; Piccart, et al., 2001), non-small-cell lung (Monnet, et al., 1998), breast cancer (Garutic, et al., 2001), and non-Hodgkin lymphoma (Chau, et al., 2001).

Oxaliplatin a third-generation organoplatinum compound was discovered in 1976 at Nagoya City University by Prof. Yoshinori Kidani (Di Francesco, et al., 2002) and was first introduced into clinical trials in 1986 by Mathe (Mathe, et al., 1986). Like other platinum compounds, OX is a DNA alkylating agent believed to form inter-, and intra-strand cross-links in DNA that prevent DNA synthesis, replication and transcription leading to cell apoptosis (Ta, et al., 2006; Barbec and Kasparkova, 2005). Contrary to its precursor cisplatin (Extra,et al., 1990), OX treatment is not associated with dose limiting symptoms such as nephrotoxicity (Fillastre and Rguenez-Viotte, 1989), ototoxicity, or myelotoxicity (Nguyen, et al., 1981). Moreover, the efficiency of cisplatin is often limited by acquired and/or intrinsic resistance, which does not occur in OX-treatment (Extra, et al., 1990).

Although OX is a leading chemotherapy for various malignancies, acute and/or chronic neurotoxic side effects are common. These acute and transient neurotoxic symptoms are observed in 85-95% of patients (Park,et al., 2009a), and are manifested during or shortly after the infusion as dysesthesias, muscle spasm, hyperalgesia, and hypersensitivity to cold (Dropcho, 2011). These symptoms are not dose dependent, and are typically resolved between treatments (Lehky, et al., 2004). Chronic symptoms include distal dysesthesias, sensory ataxia, proprioceptive deficits and functional impairment (Dropcho, 2011; Krishnan, et al., 2005). Unlike the acute symptoms, chronic neurotoxicity develops with an increase in cumulative dose, and leads to a dose reduction or even cessation of the treatment preventing up to 80% of patients from receiving a full dose, decreasing their chances of survival (Bennet, et al., 2012). Despite the claims of reversibility within 1 year (Andre, et al., 2004), studies have shown that in 79% of patients the chronic symptoms can persist even 2 years since the last dose, leading to decreased quality of life in those patients (Park, et al., 2011). Furthermore, there were reported cases of patients who experienced worsening of symptoms (a phenomenon called "coasting") after treatment was discontinued (Bennet, et al., 2012; Burakgazi, et al. 2011).

Standardized patient evaluations such as National Cancer Institute Common Toxicity Criteria (NCI-CTC) are commonly used to diagnose these chronic symptoms as chemotherapy induced peripheral neuropathy (CIPN) (National Cancer Institute:Common Toxicity Criteria version 2.0 1999). However, the clinical assessment of CIPN is very ambiguous and varies in terms of the grading scale used, and the interpretation of those scales (Postma, et al., 1998). Direct evaluation of the symptoms done by

electrophysiological examinations of patients treated with OX provide further and more direct evidence of neuropathy such as reductions in amplitude of electrically-evoked sensory nerve action potentials and in density of intra-epidermal nerve fibers (Koskinen, et al., 2011; Park, et al., 2009b).

A clinical at John's Hopkins reported several patients developing prominent symptoms such as difficulty drinking, walking, and performing dexterity tasks with no evidence of axonal loss indicating that CIPN might not be entirely responsible for OX chronic side effects (Burakgazi, et al., 2011). The clinical assessment of CIPN is strongly biased towards patients symptoms obtained from standardized evaluations, suggesting the possibility that in some patients, the chronic OX-induced symptoms of sensorimotor deficit may be in fact misdiagnosed as CIPN (Burakgazi, et al., 2011; Frigeni, et al., 2011). Furthermore, our laboratory reported evidence that chronic OX- treatment leads to corrupted peripheral signal transduction/encoding by muscle receptors in the absence of CIPN (Bullinger, et al., 2011). In an intact animal, the intra-axonal recordings from large diameter proprioceptive afferents that supply muscle spindle receptors (group IA and II afferents) and tendon organ receptors (group IB afferents) fire continuously throughout the static muscle stretch, hence they are known to be slowly adapting (Matthews, 1972). However, following a chronic OX treatment, up to 50% of proprioceptive afferents became "fast adapting" and ceased to fire in under 0.5 sec (Bullinger, et al., 2011).

The signal produced by the proprioceptive afferents provides the central nervous system (CNS) with a sensory feedback that is crucial in proper movement control, general sense of body position in space (proprioception) and body movement (kinesthesia). Hence, these afferents play an essential role in stabilizing body position and

ensuring precise trajectory of limbs (Proske, 2005; Proske and Gandevia, 2009a; Proske and Gandevia 2012). The patients that suffer from OX-induced chronic symptoms often described their deficits as “problems with balance”, “feeling unsure and unsteady”, “having difficulty walking”, “problems when driving”, “getting very clumsy”, “nearly falling over” (Bennet, et al., 2012). Although those symptoms could be elicited by dying-back neuropathy such as CIPN, other deficits in proprioceptive signal transduction have to be manifested through similar symptoms. Therefore, we hypothesized that the behavioral disability, in the absence of CIPN, is associated with impaired sensory encoding in OX treated rats.

The main purpose of this study was to reproduce the results seen in several patients from the Burakgazi study (2011), i.e. the presence of chronic sensorimotor deficits, without the evidence of CIPN, in a rat model following the OX-treatment. The secondary goal was to determine if the changes in physiological encoding of muscle proprioceptors are associated with the sensorimotor deficits upon OX treatment. Complex motor coordination and proprioceptive abilities of OX treated animals were measured by behavioral tasks known to be sensitive to sensorimotor deficits: paw placement (De Ryck, et al., 1989; Madinier, et al., 2014) and ladder rung skilled walking task (Metz and Whishaw, 2002) in the horizontal, upward and downward plane (Antonow-Schlorke, et al., 2013). Additionally, specifically for the purpose of this study we have designed a novel modification to the common balance beam test (Hicks and D'Amato, 1974; Helgren, et al., 1997) by adding four 90˚ turns. Although it did not show a prominent difference between the OX and control animals, it showed a tendency towards easier and more secure approaches to the corner, as compared with the control animals.

Unlike the acute symptoms, the chronic sensorimotor deficits are not widely addressed in rat models and, furthermore there is no published study presenting chronic proprioceptive deficits in rats following the OX treatment. In the current situation, there is no apparent evidence that the dose-limiting (Bennet, et al. 2012), chronic sensorimotor deficits are caused by the CIPN. Thus, developing the rat model is crucial in order to study this problem that prevents up to 80% cancer patients from receiving a full dose decreasing their chance of survival (Park, et al. 2009b).

II. METHODS

Animals

All the procedures were carried with an approval of the Wright State University Laboratory Animal Care and Use Committee. The data were collected from 15 adult female Wistar rats weighting 240-250g at the beginning of the experiment. The animals were housed individually in barrier-protected cages in a 12 hours light-dark cycle with water and food ad libitum. 10 rats, randomly selected, were subjected to the oxaliplatin treatment (OX) and the rest were given vehicle control injections (VC). The animals were monitored daily for signs of distress and body weight loss. All animals were euthanized by overdose with 5% isofluorane after terminal experiment.

Experimental Design

The training for the ladder rung and modified balance beam test was done one week before the control data collection. The paw placement test does not require training (De Ryck, et al., 1989) thus it was not performed during the training week. The rats were trained to cross the horizontal ladder rung in two trials (Antonow-Schlorke, et al., 2013), and to cross the modified balance beam in three trials (Helgren, et al., 1997). In the following weeks all animals were subjected to OX or VC treatment (except control week 0 and week 4 due to problems with OX supply), Sensory Nerve Action Potentials (SNAPs) recording and the blood sample collection was performed on the first day of each week between 8AM-12PM. In order to eliminate the influence of time between the treatment and the test, each behavioral test was performed on different days each week

Table 1

Table 1. Experimental design, presenting the schedule of behavioral tests relatively to the treatment day. The abbreviations are as follow: OX (30)/VC- injection of the oxaliplatin to treated animals that gave the cumulative dose of 30mg/kg, or injection of vehicle control; Blood- blood sample collection from the lateral tail vein; SNAPs- recording of the Sensory Nerve Action Potentials; MB- Modified Balance Beam test, LR A- Ladder Rung test with group A (8animlas); LR B- Ladder Rung test with group B (7animals); PP-Paw Placement test.

Week	Monday	Tuesday	Wednesday	Thursday	Friday
Training	Handling	MB	LR B	LR A	
0 Control	Blood, SNAPs	LR A	MB	PP	LR B
1	Blood, SNAPs, OX (10)/VC	PP	LR B	LR A	MB
2	Blood, SNAPs, OX (20)/VC	MB	LR A	LR B	PP
3	Blood, SNAPs, OX (30)/VC	LR B	PP	MB	LR A
4	Blood, SNAPs, no OX			PP+MB	LR A+ B
5	Blood, SNAPs, OX (40)/VC	LR A	PP +MB	LR B	
6	Blood, SNAPs, OX (50)/VC	LR B	PP	LR A	MB
7	Blood, SNAPs, OX (60)/VC	LR A	MB	LR B	PP
8	Blood, SNAPs, OX (70)/VC	PP	LR A	MB	LR B
9	Blood, SNAPs	MB	LR B	PP	LR A
10	Blood, SNAPs	LR B	LR A	MB	PP
11	Blood, SNAPs	MB	PP	LR B	LR A
12	Terminal in-vivo electrophysiology				

(see Table 1) always between 9AM-12PM. Due to a large amount of trials in the ladder rung test, the animals were divided into two groups A and B with 8 and 7 rats respectively.

Treatment

The animals were anesthetized by isoflurane inhalation (2-4% in 95% O_2). The oxaliplatin dissolved in 5% dextrose was administered via IP injection (Bullinger, et al., 2011) once per week with a single dose of10mg/kg up to a cumulative dose of 70mg/kg. On the same day the control group was given a dextrose IP injection. Due to the problems with supplier the OX dose was skipped on the week 4. The blood samples (200-500µL) were collected from the lateral tail vein for the measurement of complete blood counts. Following blood collection, all rats received 5ml of Na/Cl via subcutaneous injections in order to prevent dehydration.

Sensory Nerve Action Potentials recordings

Electrophysiological symptoms of OX-induced dying-back neuropathy in patients are: axon degeneration and demyelination which are described as decreased amplitude and increased latency in compound Action Potentials (cAP), respectively (Park, et al. 2009b). Similarly, several studies using rat models also showed reduction in amplitudes in cAP in the tails of OX-treated rats (Jamieson, et al. 2005). In order to address the possibility of OX-induced CIPN in the experimental rats, the electrically evoked sensory nerve action potentials were recorded from control and experimental rat's tail weekly throughout the entire study (Novak, et al., 2009).

Figure 1

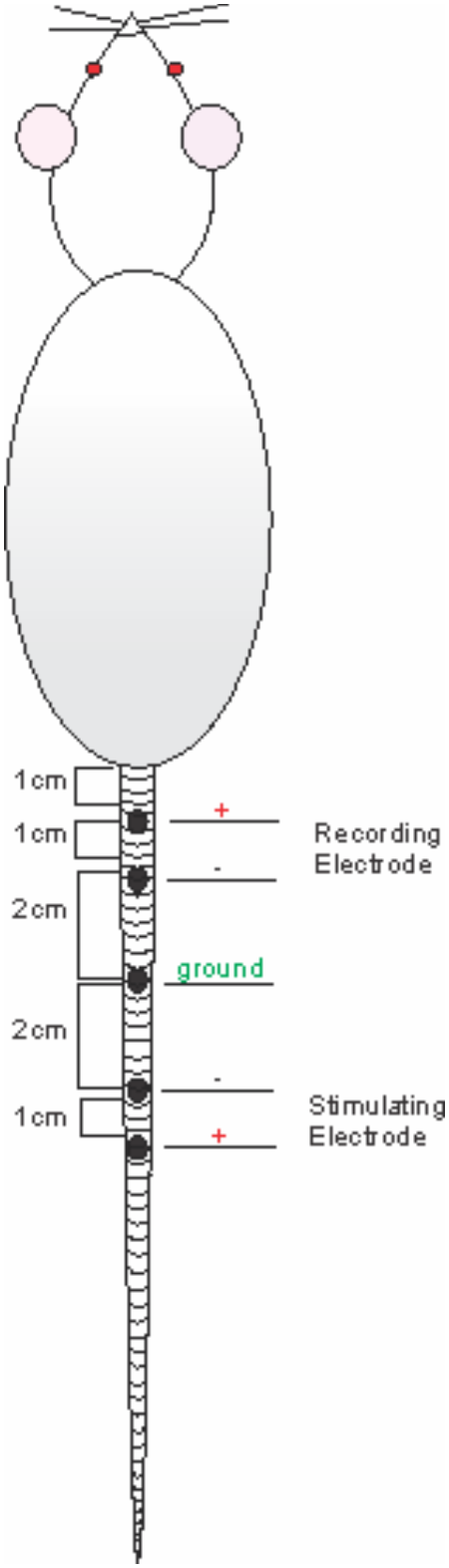

1cm
1cm
2cm
2cm
1cm
+
-
ground
-
+
Recording
Electrode
Stimulating
Electrode

Fig. 1. Sensory nerve action potential recording set up.

The animals were fully anesthetized by inhalation of isofluorane (2-3% in 100% O_2). The recording needle electrodes were placed 1cm apart starting a 1cm away from the base of the tail. The ground electrode was placed 2cm away from the recording electrode. The stimulating electrodes were placed 2cm away from the ground electrode and 1cm apart from each other (see the Fig. 1). The electrical stimulus of 10mA was applied to elicit a SNAP (Novak, et al., 2009)

Signal Encoding in Proprioceptive Afferents

The terminal experiments were performed on all rats 4-5 weeks after the final treatment with OX or VC alone. Anesthesia was induced and maintained by the inhalation of isoflurane inhalation (1-3% in 100% O_2). The animal's vitals were monitored every 15-30min and body temperature was maintained at 37˚C throughout the entire experiment. After data collection, the rats were euthanized by isoflurane overdose.

Surgical preparation was performed as described previously (Bullinger, et al., 2011; Haftel, et al., 2004; Haftel, et al., 2005). The triceps surae muscles and their nerves were dissected and freed of surrounding tissue in the left hind limb, while the rest of nerves were crushed. The animal was secured in a rigid recording frame and its femur and tibia were fixed rigidly to the frame with knee and ankle joints at angles of 90˚. The muscles were attached through their common Achilles tendon to a servomotor, which measured the force and controlled muscle length. The triceps surae nerves were placed in continuity on bipolar stimulating electrodes. The dorsal roots at L4 and L5 were exposed by laminectomy and placed on the bipolar hook electrodes. Individual sensory-axons

were penetrated with sharp glass micropipettes (25-30Ω, 2M K-acetate) to record action potentials elicited by various stimuli.

Sensory axons that responded with orthodromic action potentials, and had the conduction delay of <3ms in response to electrical stimulus in triceps surae, were selected for further study. Muscle-spindle and tendon-organ afferents were identified by paused and accelerated firing during the rising phase of force in isometric twitch contractions of the triceps surae muscle, respectively (Bullinger, et al., 2011). Further, muscle- spindle afferents were classified as group IA if upon a ramp stretch (20mm/s, 3mm) or triangular stretch (4mm/s, 3mm) they responded with a high frequency initial burst and if they fired in response to each cycle of vibrations at the frequencies of ≥100Hz (Matthews, 1972). If a muscle-spindle afferents did not exhibit high frequency initial burst nor responded to vibration at the frequencies of ≥100Hz, it was classified as a group II.

The analysis of proprioceptive encoding was made by investigating the firing response of IA proprioceptive afferents to triceps surae muscles stretch in a ramp-hold-release paradigm (20mm/s ramp, 3mm). The stretches were repeated every 4 seconds. The time of occurrence of the last AP during the hold phase of stretch recorded. Records of afferent action potentials, muscle length, and muscle force were collected, digitized (20 kHz), and stored on computer for later analysis using Spike2 software (Bullinger, et al., 2011; Haftel, et al., 2004; Haftel, et al., 2005).

Behavioral Assessment of Proprioceptive Abilities

Ladder Rung and Modified Balance Beam analysis was made by an investigator blind to the treatment inspecting the video recordings frame-by-frame. The results were

analyzed and presented for the following weeks: 0 (control week), 2, 4, 8 (the last week of treatment) and 10 (two weeks after the treatment). The Paw Placement data was obtained by life-scoring, done by the blind investigator. The data for tis test was presented for each week of the study.

Ladder Rung in Horizontal, Downward and Upward dimension

The Ladder Rung test originally designed by Metz and Wishaw (Metz and Whishaw, 2002; Metz and Whishaw, 2009) has been widely used to assess skilled walking, limb placement and limb coordination in variety of models of motor dysfunctions: stroke model (Antonow-Schlorke, 2013; Riek-Burchardt, et al., 2004), spinal cord injuries (Ghosh, et al., 2009; Martinez, et al. 2010), as well in Parkinson's Disease (Metz and Whishaw, 2002; Faraji and Metz, 2007). Low scores and high error/steps ratios in those studies were concluded as a result of: "skilled motor impairment", "proprioceptive impairment" and "impairment in sensorimotor skills".

Furthermore, as described previously in Antonow-Schlorke et al. (2013), setting up the ladder rung in an 18˚ angle downward and upward inclinations provided a more sensitive analysis of motor disabilities. In their study they found that inclining the ladder rung upward required higher level of motor performance compared to horizontal walking. The upward inclination also added more load on hind limbs to move the body up, which provided an additional risk that a limb might slip from a rung if placed incorrectly. The downward inclination provided an additional challenge in spatial cognition, limb coordination, weight bearing, and balance (Antonow-Schlorke, 2013).

Table 2

Table 2. Rating scale for foot placement in the skilled ladder rung walking test

Score	Type	Characteristics
0	Total miss	The limb missed the rung and resulted in paw being below the rung plane
1	Deep slip	Deep fall after limb slipped off the rung, interrupting the gait
2	Slight slip	Limb slipped off the rung without causing a fall or interrupting the gait
3	Replacement	Limb was replaced from one rung to another before weight bearing
4	Correction	Limb aimed for one rung but was placed on another Or: Limb position on the same rung was corrected
5	Partial Placement	Limb placed on rung with either toes or heel
6	Correct Placement	Midportion of the limb placed on rung

The ladder rung apparatus was self-made according to the instructions of Metz and Wishaw (Metz and Whishaw, 2009). The test was performed in horizontal, upward and downward position with 5 trials at each (Antonow-Schlorke, 2013). The position of rungs was randomly assigned for each trial. The distance between rungs was at least 1cm and no more than 5cm (Metz and Whishaw, 2002).

Foot Fault Analysis: The qualitative evaluation of hind limb placement was performed using a 7-category scale scoring system introduced previously by Metz and Whishaw (Metz and Whishaw, 2009) and presented in the table 3. One modification was made as to the score 0. The original scale defined score 0 as deep fall after limb missed the rung. In this study, anytime the limb missed the rung and extended the limb below the level of the rungs was scored as 0 even if this miss did not result in a deep fall. All steps by hind limbs were analyzed, therefore the last step before and the first step after the stop were included in the score (Antonow-Schlorke, 2013). The data were averaged and presented as % of change, i.e. the baseline score obtained during the week 0 served as the original 100%. Therefore, the positive values indicated the improvement, while the negative values worsening.

Foot Placement Accuracy Analysis (Number of Errors): As described previously by Metz and Whishaw (Metz and Whishaw, 2002), the foot placement accuracy was defined as the number of errors per steps. Based on the scoring system described above, the error was defined as score 0, 1 and 2. The total number of errors in hind limbs was calculated with no distinction between right and left, and the mean error/step ratio was calculated for all five trails and represented as % of error.

Walking Coordination Analysis: The close investigation of the stepping cycle in the ladder rung in the control animals in the preliminary experiments revealed a following walking pattern: left front limb, right hind limb, right front limb and left hind limb. The hind limb is usually placed at exactly the same rung as the corresponding front limb. In order to investigate the inter-limb coordination, the stepping sequence along the whole distance of the ladder rung was inspected. Each time the limb was not used in the actual cycle in was considered "idle" (Antonow-Schlorke, 2013). The number of times each limb was idle was counted and then averaged for 5 trials. The result was presented as the average % of cycles in which the hind limbs were idle.

Hind Foot Placement Precision: In the preliminary study in control animals, it was observed that the animals tend to place their hind paw in exactly the same rung as the corresponding front paw. It was hypothesized that in a case of impaired proprioception; the animal would not be able to place their hind foot precisely at the same rung, since it could not correctly orient its body in space. The error was defined as any step made by a hind limb that originally was not placed on the same rung as the proceeding front limb. Therefore if the foot was placed on the incorrect rung, and then replaced to the correct one, such a step was still considered an error. A total number of errors was counted and expressed as an average of error/step ratio for both hind limbs.

Modified Balance Beam

The modification was made based on the previous observations, were OX-treated rats did not show difficulties in crossing a straight beam, however they seemed to have problems in placing the foot when they had to step from the straight beam onto a

perpendicular pathway. Therefore, the Balance Beam was modified by adding 4 90˚ turns after each 40cm long and 2cm wide straight path. The scoring system was created to distinguish the mistakes made by slip of the foot and a miss or a swipe which would indicate that the rat has difficulties orienting their foot in space.

Foot Placement Scoring. The analysis was done using a scoring system as presented in the Table 2. The analysis was made only for the hind limbs by an investigator blind to the treatment by studying frame-by-frame the recorded trials. The average of 4 trials was calculated and recorded for each rat individually. The results were presented in the form of % improvement; therefore all the values for week 0 are equalized to 0. The values in the following weeks are the % change of the original value. The positive values indicate improvement while the negative deterioration of performance.

Foot Placement Accuracy: The Foot Placement Accuracy measure was inspired by that used by Metz in the ladder rung test (Metz and Whishaw 2002). The absolute number of errors (defined as scores 0, 1, 2 and 3) was counted and divided by the total number of steps.

Corner Analysis: Based on the preliminary data, three different approaches to corners were defined: Precise- animal moved close to the edge and reposition its body to be in the same plane as the next path before proceeding. It was indicated by stepping with an inner feet 1cm away or closer from the edge; Cut- animal step over the corner without getting close to the edge; Hop- animal jump over the corner so the both hind limbs were not touching the beam. Additionally, the ratio of errors occurring during a step, or a hop needed to cross the turn was calculated and expressed as the error/turn ratio.

Figure 2

Fig.2. Modified Balance Beam Apparatus from a top-view. It consists of five 40cm long and 2cm wide straight paths, interrupted by four 90˚angle turns. The area of 1cm from the corner was marked, in order to distinguish the precise approach to turning

Table 3

Table 3. Rating scale for foot placement in the modified balance beam test

Score	Type	Characteristics
0	Fall	Rat fell off the beam after the limb missed or slipped of the beam
1	Major Slip	Rat lost balance after the limb slipped of the beam
2	Miss/Swipe	Limb missed the beam. Each time is counted
3	Minor Slip	Limb slipped off the beam, without causing the disturbance to the gait
4	Correction	Limb is placed on the beam and replaced before it is weight bearing
5	Partial Placement	Majority of the foot is placed on the side of the beam
6	Correct Placement	Majority of the foot is placed on the beam

Paw Placement

Paw Placement is one of the most commonly used tests for proprioception in rodent models. It is used in variety of motor dysfunction, and sensorimotor deficit models such as: stroke models, cortical ablation, Parkinson's disease and spinal cord injuries (De Ryck, et al., 1992). The low scores in this test are concluded to be due to impair proprioception.

Animals were hold by the body trunk and placed on the table top with all 4 paws. Next, the rat was moved over to the edge so that the tested limb lost contact with the surface (De Ryck, et al., 1989). The trial was reported as correct if the rat reacted immediately and placed its foot on the surface. Missed placement or delayed response was considered as incorrect. Each animal was subjected to 5 trials testing both hind limbs. The number of errors for all 5 trials were summed and presented as % of errors.

Differentiating between symptomatic and asymptomatic OX-treated rats

The OX patients develop the symptoms at different cumulative dose and with different intensity. Furthermore, some do not develop any deficits in sensorimotor function (Park, et al. 2009a). In order to distinguish the symptomatic rats from asymptomatic, in all of the behavioral tests the data from control animals was averaged and represented as mean± 0.95 Confidence Interval, while the data from the OX rats was presented as a scatterplot with individual points for each animal. This confidence interval of a mean control value served as a standard range. The animal was considered symptomatic if it showed a consistent deterioration compering to its own value from the control week, consistently performed worse than the standard range, and persisted to perform below the standard range till the week 10. The animals were considered

"somehow symptomatic" if they performed worse than the standard range in some weeks, however scored within the range on week 8 and/or 10.

III. RESULTS

Assessment of General Toxicity

Rats showed no sign of distress following the treatment and they maintained healthy appearance and appetite throughout the entire study. The body weight gain in OX animals was smaller comparing to VC animals, which is consistent with the systematic effects of OX in rats (Bullinger, et al., 2011; Cavaletti, et al., 2001) and in humans. The increase in weight in OX animals was more rapid between week 3 and 5, which correlates with the date of the missed dose (week 4), further suggesting OX toxic effect (Fig. 3a).

Sensory Nerve Action Potentials recordings

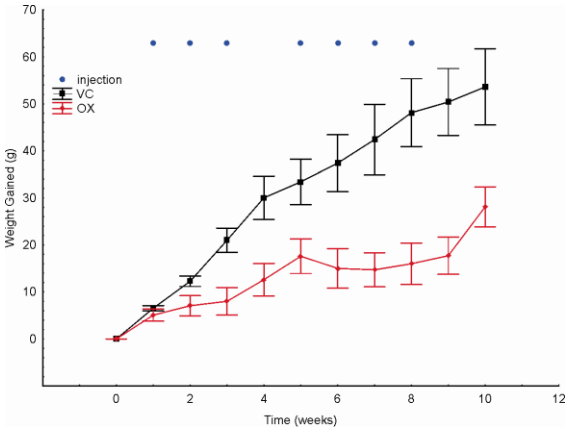
The longitudinal SNAPs recordings showed no evidence of CIPN. Similar to previous observations from our lab (Bullinger, et al., 2011) and from clinical studies (Burakgazi, et al., 2011), there were no signs indicating neither axon degeneration nor demyelination, i.e. there was no significant reduction in SNAPs amplitude or increase in latency at any point during or after the treatment (Fig.3b and c respectively). Furthermore, in both OX and VC animals, there was tendency overtime towards larger amplitudes and shorter latencies, which is a result of maturation of peripheral nerves.

Oxaliplatin Influence on the Sensory Encoding

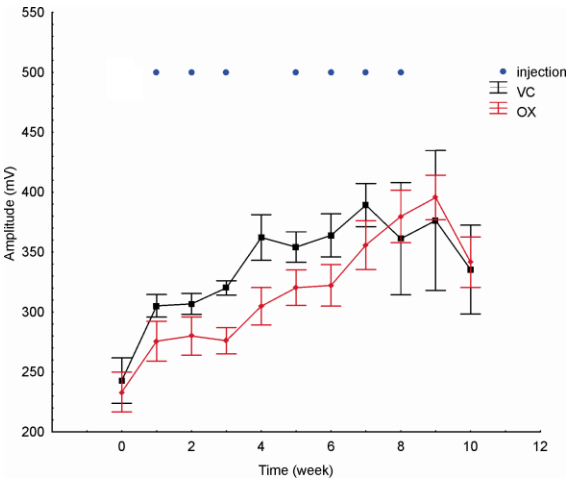
The IA afferents in OX rats exhibited an apparent failure to sustain the firing during a static muscle stretch during the hold phase of the ramp-hold-release paradigm (20mm/s, 3mm). The example of a control slowly adapting and OX rapidly adapting IA afferent is shown in the figure 4A and B respectively. On average, in the control IA

Figure 3

A


injection
VC
OX
Weight Gained (g)
Time (weeks)

B


injection
VC
OX
Amplitude (mV)
Time (week)

C

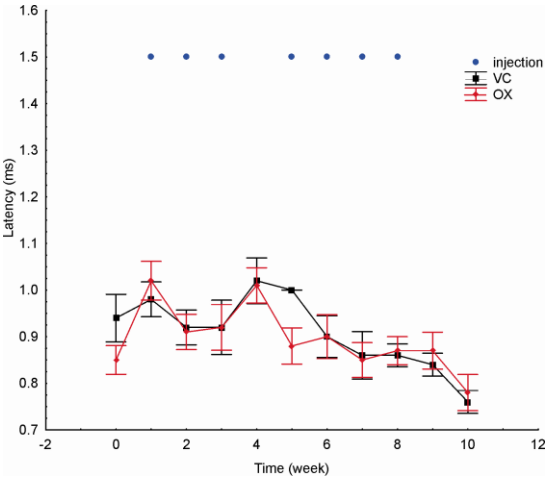

injection
VC
OX
Latency (ms)
Time (week)

Fig.3. Chronic oxaliplatin toxicity without sensory neuropathy. (A) Plot of control (VC) and oxaliplatin treated (OX) rats' average body weight gained; (B) the amplitudes of Sensory Nerve Action Potentials; (C) the latencies of the SNAPs, all plotted versus time expressed in weeks, starting with the control week 0

Figure 4

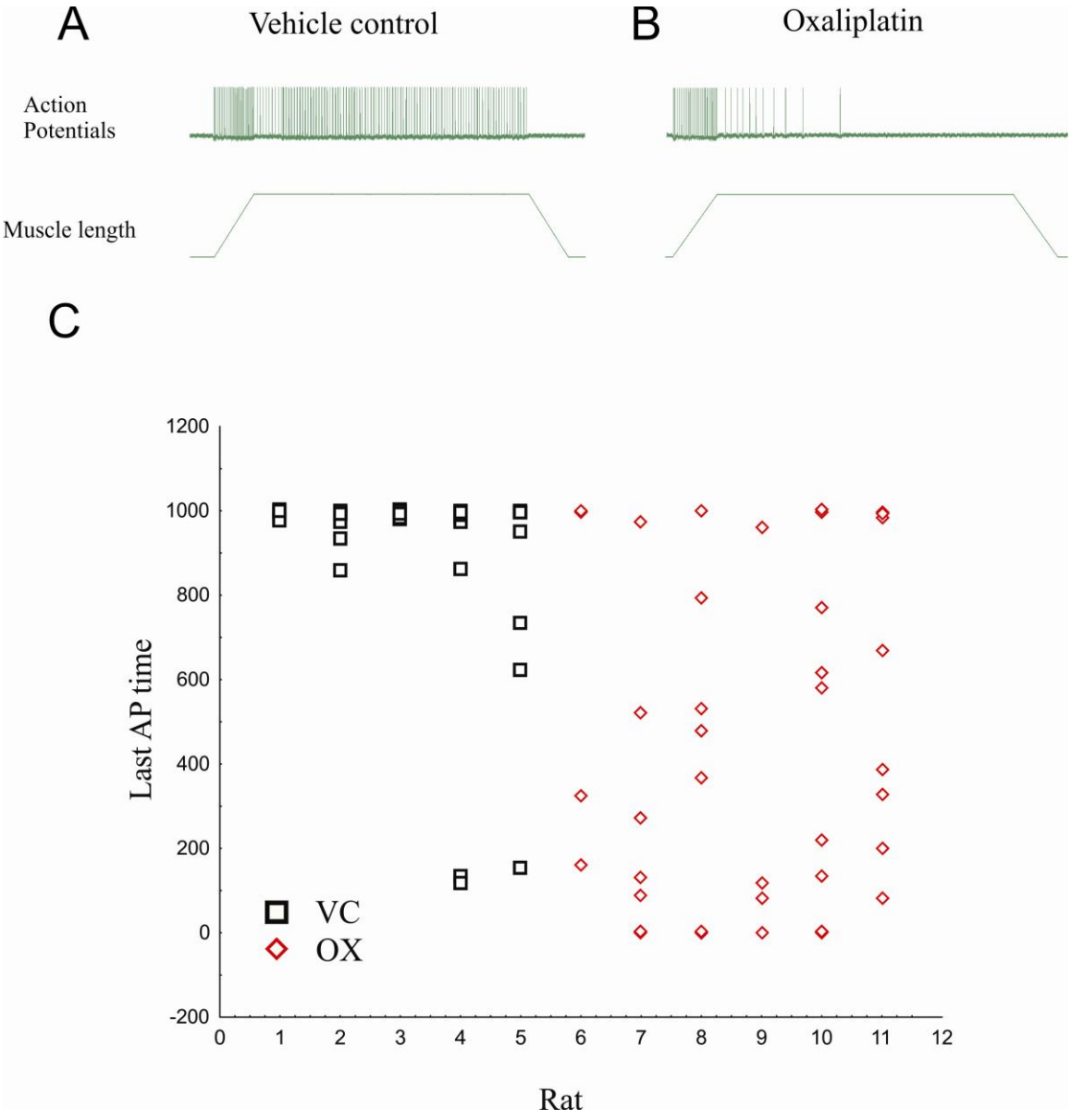

A
Vehicle control
B
Oxaliplatin
Action Potentials
Muscle length
C
Last AP time
Rat
VC
OX
1200
1000
800
600
400
200
0
-200
0
1
2
3
4
5
6
7
8
9
10
11
12

Fig.4. Chronic treatment with oxaliplatin modified the sensory coding in group IA muscle-spindle afferents. A and B represent the intra-axonal records of action potentials (APs) recorded from IA afferents in response to ramp-hold-release muscle stretch from control (A) and OX-treated (B) animals. C represents the time of occurrence of the last AP during the static hold phase (1s) of the ramp-hold-release paradigm in all IA afferents sampled per rat. The numbers on the x-axis indicates individual control (1-5) and OX-treated (6-11) rats.

afferents the last action potential during the hold phase occurred at 935.19±201.79ms. In the OX IA afferents the last action potential occurred on average at 504.3±404.21ms. 84.1% (37/44) of control and 26.1% (12/46) of OX IA afferents sustained firing through the most of the hold phase of muscle stretch (≥900ms).

Behavioral Assessment of Proprioceptive Abilities

Ladder Rung in Horizontal, Downward and Upward plane

Foot Fault Score Analysis. The control data for this analysis is presented as a mean score with 0.95 confidence interval (CI), while the results from OX-treated animals are shown as a scatterplot, with each individual point corresponding to one rat. The OX animals that showed consistently a worse performance than standard range are identified on the plot by an ID-number (see Fig. 5A)

Horizontal. There were three OX animals that felt out of the established standard range at each of the analyzed weeks: OX 3, OX 9 and OX 10. The OX7 had the % change in score below the standard on week 2, 4 and 8. OX 3 and OX 9 never reached the score obtained in the week 0.

Upward. The rats OX3, OX7 and OX 9 were considered symptomatic. OX3 and OX9 exhibited % score change below the standard range on the week 4, 8 and 10. On week 2 they both also exhibited decrease in the Foot Fault score as compare to the week 0. OX7 performed worse than on week 0 and then the standard range on week 8and 10.

Downward. The control animals showed a tendency towards improvement of the score. OX 9 performed worse than the established standard on the week 4, 8 and 10. No

other animal show a consistency in decreased performance as compared to the standard range.

Foot Placement Accuracy Analysis (number of errors). The results of this analysis are shown in a figure 5B. In the horizontal plane the rat OX 9, showed a strong tendency to increase the amount of errors during the course of treatment, and exhibit higher % of errors than the standard range on weeks 4, 8 and 10. The animal OX10 showed a tendency to increase the number of errors between weeks 0 and 4, and exhibited higher % of errors on week 2 and 4. However on the week 10 the % errors made by this rat felt among the standard range.

Upward. The animal OX 9 showed a tendency to increase the % of errors between week 0 and 8. However, only in the week 4 and 8 the % of errors was bigger than the standard range. Furthermore OX 9 exhibited a decrease in % of error on week 10 and was. On week 4, OX3 showed the increase in %error comparing to the control week, however it was at border of the standard range. In the week 8 and 10, OX3 increased the % of error even further, to a point it was outside of the standard range. OX10 animal showed a tendency to decrease the number of errors from week 0 to week 4. On week 8 and 10, the % of error made by OX10 was higher than the standard range, as well as higher than the % of error made by this animal on the week 0.

Downward. The animal OX9 showed a small decrease in the % of errors between week 0 and 2. However in the following weeks, this rat showed a great increase in % of errors, which was also much higher than the standard range. The OX10 animal exhibited

a higher than the standard range % of error on the week 4 and 10. On week 8 the % of errors made by this animal was within the range and lower than the original value.

Hind Foot Placement Precision

The results of this analysis are shown in a figure 5C.

Horizontal. The rat OX9 showed a higher % of errors in hind limb precision than the standard range on each analyzed week after the treatment. Furthermore the OX9 showed the tendency to increase the % of errors over the time of the study. The OX 10 animal showed initially a deterioration of performance from week 0 to week 2 leading to a higher than standard range % of errors in the hind limb precision. From week 2 until week 8, OX 10 had a tendency to improve its performance as a result on the week 8 it fell into the standard range. On the week 10 the % error for OX 10 was, again outside of the range.

Upward. The OX 9 rat showed a greater than the standard range % of errors in the hind limb precision on the week 4, 8 and 10. Furthermore, these values were also greater than the % of error in the control week.

Downward. The OX 9 was the only rat that showed a consistent tendency and a higher % of errors in hind limb precision in weeks: 4, 8, and 10. Additionally the values in week 8 and 10 were also higher than the original value from the control week.

Hind Limb Idleness

The results of this analysis are shown in figure 5D.

Figure 5

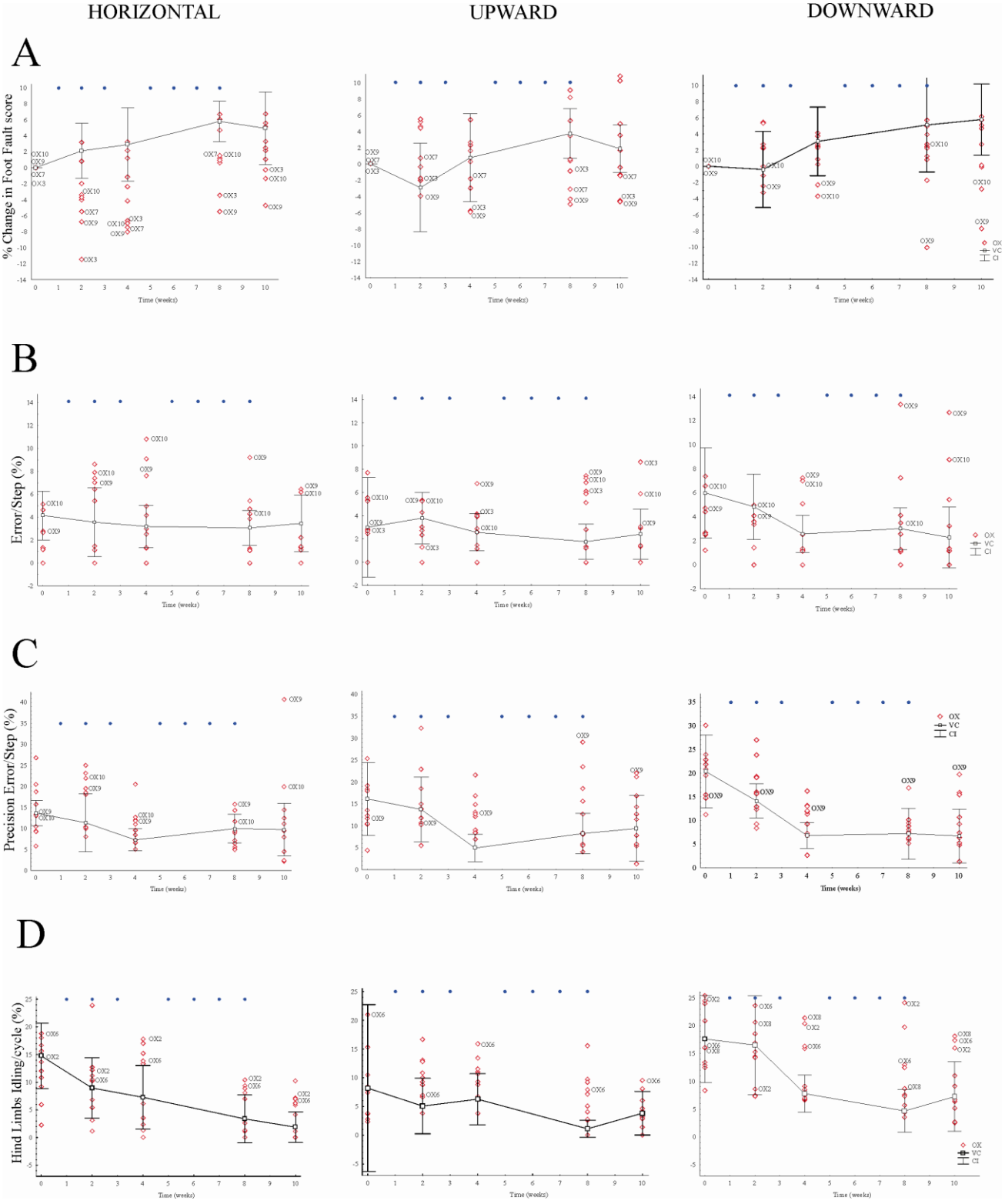

HORIZONTAL
UPWARD
DOWNWARD
A
% Change in Foot Fault score
B
Error/Step (%)
C
Precision Error/Step (%)
D
Hind Limbs Idling/cycle (%)
Time (weeks)
OX
VC
CI

Fig.5. Ladder Rung test results. The control data is represented as a mean value for all five control rats ±0.95 confidence interval. The OX-animal data is presented as a scatterplot, where each point represents one animal. Only the animals that showed consistent variation from the control mean±0.95 CI were identified on the plot. (A) The average scores from the week 0 was presented as 0 and the average % change in scores in following weeks was plotted against time (weeks) for horizontal, upward and downward planes respectively. The positive values indicate improvement whereas negative, deterioration as compared to control week 0. (B) The errors per steps ratio, presented as %of errors, in horizontal, upward and downward plane. Higher values in in this analysis indicate a greater amount of errors per step. (C) The hind limb precision placement represented as the % of hind limb steps when the rat did not placed the hind foot at the same rung as corresponding front paw in horizontal, upward and downward planes. The lower values in this test represent a better precision. (D) The hind limb idling in horizontal, upward and downward plane. The number of times the hind limbs were idle is presented in this plot as the ratio of idling occurrence per a step cycle. The higher values indicate more idling.

Horizontal. The OX 2 and OX 6 exhibited the tendency to decrease the % of idling, but revealed higher than the standard range ratio of idling/cycles consistently starting on week 4.

Upward. The animal OX6 showed a tendency to lower the number of hind limb idleness throughout the study. At the same time it exhibited a higher than the standard range ration of idling/cycles on week 4, 8 and 10.

Downward. The rats OX 2, OX 6 and OX 8 exhibited a higher ratio of idling consistently starting on week 4, but lower than their original values from the week 0.

Modified Balance Beam

The results of this test are presented in the figure 6.

Foot Placement Scoring. There were no OX animals that consistently performed worse than the established standard range. Additionally, the score in all OX animals for the week 8 and 10 were improved comparing to the week 0, i.e. the % change in score for all OX animals was above 0. The OX 1 animal performed worse than the standard range in week 2, but in the following weeks the % of change was within the standard range and higher than the control week. The OX5 rat showed a lower % of change in score than the standard range only in week 2. Although within the range, the % of change in week 10 was negative, indicating deterioration comparing to the week 0.

Figure 6

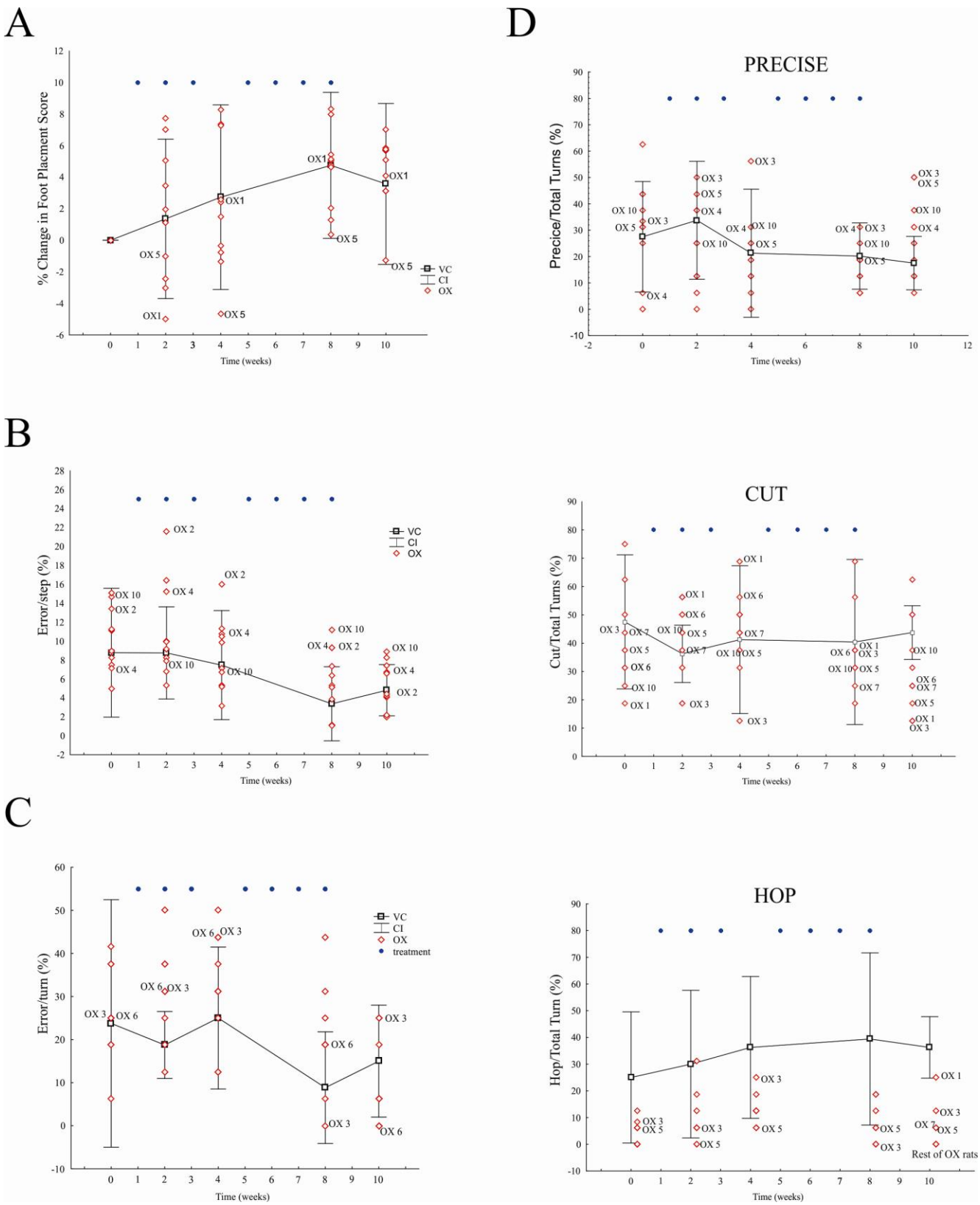

A
% Change in Foot Placment Score
Time (weeks)
VC
CI
OX
B
Error/step (%)
Time (weeks)
C
Error/turn (%)
Time (weeks)
treatment
D
PRECISE
Precice/Total Turns (%)
Time (weeks)
CUT
Cut/Total Turns (%)
Time (weeks)
HOP
Hop/Total Turn (%)
Time (weeks)
Rest of OX rats

Fig.6. Modified Balance Beam test results. The control data is represented as a mean value for all five control rats ±0.95 confidence interval. The OX-animal data is presented as a scatterplot, where each point represents an individual rat. Only the animals that showed consistent variation from the control mean±0.95 CI were identified on the plot. (A) The % change in foot placement score. The positive values indicate improvement whereas negative, deterioration as compared to control week 0. (B) Errors to steps ratio occurring during crossing the modified beam, presented as the % of error. Higher values indicate more mistakes made by rats during crossing the beam. (C) The error/turn ratio. The errors committed during stepping, or hopping over the 90 ˚corner was calculated and expressed as a percentage of turns with an error. The higher values indicate more mistakes done during this action. (D) Corner approach analysis. The ratio of three different approaches (precise, cut and hop), was calculated and presented on separate plots in order to visualize the change, and compare it between the control and OX-treated rats.

Foot Placement Accuracy. The animal OX2 showed a higher % of error than the standard range in weeks: 2, 4, and 8. Additionally, it also showed an increase in % of errors in week 2 and 4 comparing to the week 0.The animal OX4 showed a higher than the standard range % of errors on week 2 and 8. In week 4 and 10 this animal did not show a higher than the control range % and furthermore, the % of errors in week 10 was smaller than in the control week.

Corner Analysis. There was no OX animal that showed a consistent increase in error during stepping and/or hopping over the corner. The OX3 and OX6 animal were the only ones that showed a greater %of errors in two weeks (see figure 6C). The animal OX3 approached the turn in the precise manner more often than the standard range in week 4 and 10. The rats: OX4, OX5, OX9 and OX10 exhibited this type of turn more often than the control range in the week 10. Furthermore all of these animals increased the carful way of turning, comparing to the week 0. On the week 10 the rats: OX1, OX3, OX5, OX6 and OX7 exhibit lower % of the cut approach to the turn than the standard range. Additionally all of them performed less "cutting" the corner than on the week 0. The animal OX3 showed a tendency to decrease the amount of the "Cut corners". All of the OX animals performed less hops than the control range. The OX5 animal performed less hops than the standard range in every analyzed week. The OX 3 animal also performed less hops on the week 8. The mean control data with 0.95CI serving as a standard range, along with the scatterplot for the OX animals is shown in the figure 6D.

Paw Placement

The animal OX2 showed consistently a higher than the standard range, % of trails with an error starting with the week 4 till the end of the study. Additionally, in those weeks (except week 3) the % of error was also higher than the original value. The animal OX4 showed an out-of- range high % error in week 1, 5 and 6, and all of them were higher than in the control week. The mean control data along with 0.95CI was plotted on the same graph as the OX scatterplot data, and presented in the figure 7.

Differentiating between symptomatic and asymptomatic OX-treated rats

Based on the standard range the rat OX9 was diagnosed as symptomatic in 8 out of 15 tests, and somehow symptomatic in 1 of them; OX 6 was considered symptomatic in 4 tests and somehow symptomatic in 1; OX10 was symptomatic in 4 tests, and somehow symptomatic in 2; OX2 and OX3 were both symptomatic in 3 tests; OX7 was symptomatic in 1 test and also somehow symptomatic in 1; OX8 was symptomatic only in one test. Additionally the approach to the corner revealed a consistent lower % of hops in OX5 rat starting week 2. OX3 have shown a lower % of hops in week 8 and 10. All the OX rats had a lower than the standard range % of hops in week 10. The summary of this analysis is presented in the table 4.

Figure 7

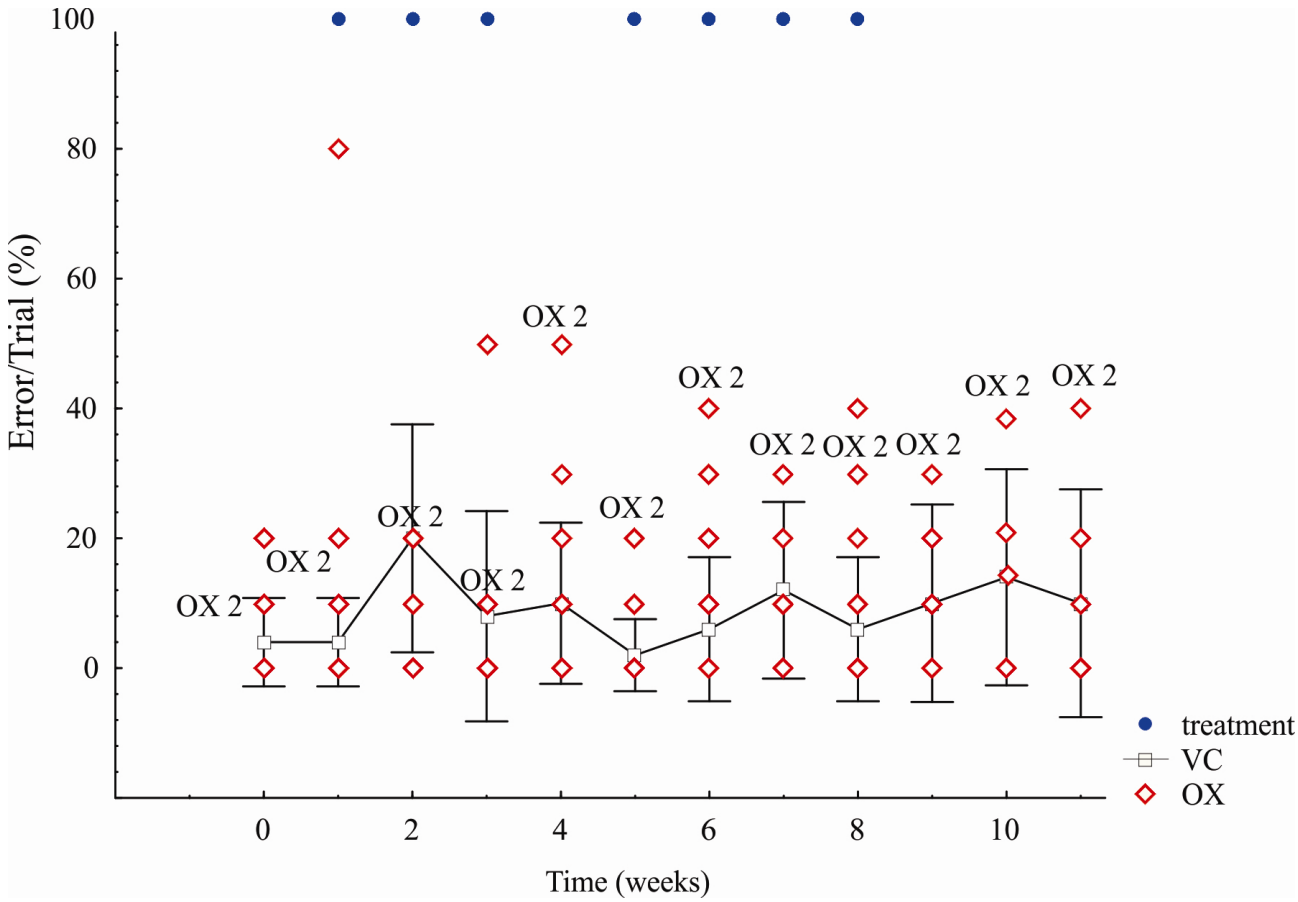

100
80
60
40
20
0
Error/Trial (%)
0
2
4
6
8
10
Time (weeks)
OX 2
treatment
VC
OX

Fig.7. Paw Placement test results. The control data is represented as a mean value for all five control rats ±0.95 confidence interval. The OX-animal data is presented as a scatterplot, where each point represents an individual rat. Only the animals that showed consistent variation from the control mean±0.95 CI were identified on the plot. The % of error in this plot indicates the number of times the rat failed to immediately place its paw back on the surface and/or failed to reach the surface resulting in swipe.

Table 4

Table 4. Symptomatic and "Somehow" Symptomatic Animals as revealed in this study

Test	Consistently Symptomatic Animals	Somehow Symptomatic Animals
H LR change in score	OX3, OX9, OX10	OX7
U LR change in score	OX3, OX7, OX9	
D LR change in score	OX9	OX10
H LR errors/steps	OX9	OX 10
U LR errors/steps	OX3, OX10	OX9
D LR errors/steps	OX9	OX10
H LR Hind Limb precision	OX9	OX10
U LR Hind Limb precision	OX9	
D LR Hind Limn precision	OX9	
H LR Hind Limb idling	OX2, OX6	
U LR Hind Limb idling	OX6	
D LR Hind Limb idling	OX2, OX6, OX8	
Paw Placement	OX2	

IV. DISCUSSION

It has been nearly 30 years since the oxaliplatin was first introduced to clinical trials in 1986 (Mathe, et al., 1986). The reports of its neurotoxic chronic and acute effects followed soon after. However, despite such a long time of research, the animal model that would address the chronic, dose-limiting, sensorimotor symptoms has not been yet established. This study provides the first step towards developing such a model. Furthermore, we were able to reproduce the results seen in clinical studies, where "several subjects reported symptoms that interfered with drinking, walking or performing dexterity task" with no apparent evidence of CIPN (Burakgazi, et al., 2011). In this study none of the OX treated animals manifested signs of CIPN; however, they exhibited an apparent failure to sustain the firing during a static muscle stretch. Although the presented data did not show prominent sensorimotor deficits in all OX-rats, several treated animals exhibited consistent difficulties performing tasks that are known to address proprioceptive abilities, sensorimotor integration and movement coordination.

For each of the tests, the rats were considered to show sensorimotor deficits, i.e. they were symptomatic if they showed a tendency to perform worse than the standard range (control mean value±0.95 confidence interval) that persisted till the week 10. Using this system, 7 out of 10 animals were identified as symptomatic at least in one of the tests. The animal that was diagnosed as symptomatic most often was OX9 and showed decreased performance in 8 out 16 tests (Table 4).

The Ladder Rung Task is a common sensorimotor test designed to detect proprioceptive and sensorimotor impairments (Metz and Whishaw, 2002). It has been widely used to investigate skilled walking, limb placement and limb coordination in variety of sensorimotor deficits models such as: Parkinson's Disease (Faraji and Metz, 2007) stroke model (Riek-Burchardt, et al., 2004), and spinal cord injuries (Martinez, et al., 2010; Ghosh, et al., 2009). Furthermore, the rungs from the ladder were positioned irregularly, and changed from trial to trial, therefore the animals were not capable of anticipating the rung location and learning a specific gait pattern, as a result, each step required adjustment in paw placement and stride length. The animals with a corrupted proprioceptive feedback that prevents them from precise control of trajectory of their limbs (Prochazka and Ellaway, 2012; Proske and Gandevia 2009a) would have to experience lower scores, higher number of errors, decreased hind limb precision and increased idling compare to the intact animals. The OX animals diagnosed as symptomatic performed worse than the control range and often below their original scores, indicating that there is a correlation between the OX-treatment and low level of performance. Furthermore, the animal OX9, when diagnosed as symptomatic, always performed bellow its original scores showing not only lack of improvement but worsening of the performance, which strongly suggests a proprioceptive deficit in this animal.

Additionally, The Modified Balance Beam Test revealed the tendency of OX animals to use a safer approach during turning. All the OX animals were in the lower values of the standard range in the terms of the amount of hops. During the precise turn, the animals stepped as close to the edge of the corner as possible, as a result they

repositioned their bodies, before they continued on the next straight path. This strategy provided them with lower probability of error. In contrast, the correct hop or cut required steeping from a straight path to a perpendicular path. In the presence of proprioceptive deficits such an action requires proper positioning of limbs in the space. Although this data is hard interpret, due to the wide range in control animals, it seems like OX animals tend to choose more secure ways of approaching the corners.

The incidence of sensorimotor impairment in OX treated patients is 90% (Bennet et al., 2009). However the dose at which this deficit starts to manifests, as well as the symptoms are variable between patients (Park et al., 2011). Therefore, the inconsistency that we observed in behavioral tests corresponds to the inconsistency seen in patients. Furthermore, the dose-limiting sensorimotor deficits develop in patients with the cumulative OX dose ≥540-850mg/m^2 (Gamelin, et al., 2002; Andre, et al., 1999). In rats this dose range corresponds to approximately 90-141.6mg/kg (Freireich, et al., 1966). Therefore, the reason why we have not observed more prominent sensory symptoms could be that the cumulative dose was not sufficient enough to elicit functional deficits. In order to create a better animal model to address the OX-induced chronic sensorimotor deficits, it is necessary to establish a dosing regimen that would correspond to the one given in humans. It has been previously shown that not only the amount of a single and/or cumulative dose but also the schedule between the injections has a great influence on the OX-induced toxicity (Cavaletti, et al. 2001).

The reason why the other rats did not show prominent sensorimotor deficit, despite showing the evidence of impair proprioceptive signaling, could be that we did not use the appropriate behavioral tests to address this deficit more clearly. The IA afferents

in OX-treated animals show cessation of the firing during the static phase of the muscle stretch. The dynamic phase is intact. The tests that we have used were mostly dynamic and required rats to walk, which would give us a false negative results about sensorimotor impairment. Therefore, for further investigation of the proprioceptive deficits in OX-treated rats, it is crucial to carefully choose and/or design the behavioral tests to ensure that they target the impaired proprioception during the static phase.

Another factor that could have a negative impact on our study was the sample size, especially the control sample size. We only used 5 control rats that we have averaged and created a standard range based on the mean value±0.95 confidence interval. The small sample size, created a situation like the one in Modified Beam that the confidence interval stretched over the values from 5% to 70%. A bigger sample would allow us to exclude the outliers, which possibly could create narrower control range.

In conclusion, the data presented in this study showed evidence of sensorimotor deficits in one animal, and suggested such impairment in six, without the sign of CIPN. Considering the variability of symptoms seen in patients, the lack of consistency in rats is expected in a good model. Therefore, although this study did not show direct correlation between the symptoms and corrupted proprioceptive encoding, it succeeded in creating an adequate rodent model for studying the sensorimotor deficits following OX treatment.

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