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Full Length Article

The active second-generation proteasome inhibitor oprozomib reverts the oxaliplatin-induced neuropathy symptoms

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Running title: Oxaliplatin-induced neuropathy and proteasome

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Abstract

Oxaliplatin-induced neuropathy (OXAIN) is a major adverse effect of this antineoplastic drug, widely used in the treatment of colorectal cancer. Although its molecular mechanisms remain poorly understood, recent evidence suggest that maladaptive neuroplasticity and oxidative stress may participate to the development of this neuropathy. Given the role played on protein remodeling by ubiquitin-proteasome system (UPS) in response to oxidative stress and in neuropathic pain, we investigated whether oxaliplatin might cause alterations in the UPS-mediated degradation pathway, in order to identify new pharmacological tools useful in OXAIN. In a rat model of OXAIN (2.4 mg kg^{-1} i.p., daily for 10 days), a significant increase in chymotrypsin-(β5) like activity of the constitutive proteasome 26S was observed in the thalamus (TH) and somatosensory cortex (SSCx). In addition, the selective up-regulation of β5 and LMP7 (β5*i*) subunit gene expression was assessed in the SSCx. Furthermore, this study revealed that oprozomib, a selective β5 subunit proteasome inhibitor, is able to normalize the spinal prodynorphin gene expression upregulation induced by oxaliplatin, as well as to revert mechanical allodynia and thermal hyperalgesia observed in oxaliplatin-treated rats. These results underline the relevant role of UPS in the OXAIN and suggest new pharmacological targets to counteract this severe adverse effect.

This preclinical study reveals the involvement of the proteasome in the oxaliplatin-induced neuropathy and adds useful information to better understand the molecular mechanism underlying this pain condition. Moreover, although further evidence is required, these findings suggest that oprozomib could be a therapeutic option to counteract chemotherapy-induced neuropathy.

Keywords: oxaliplatin; neuropathic pain; proteasome; immunoproteasome; oprozomib.

1. Introduction

Oxaliplatin-based chemotherapy is the standard first-line treatment option for stage III of metastatic colorectal cancer treatment [1-3]. Although oxaliplatin exhibits valuable anti-cancer properties, it induces a painful neuropathy (OXAIN, oxaliplatin-induced neuropathy) as a prominent adverse effect [4-7] which represents its major dose-limiting toxicity.

Different molecular mechanisms participate in the development of OXAIN, such as P2X7R-Pannexin1 system activation [8], peroxisome alterations [9]; furthermore, the involvement of specific channels or receptors, Kv7, $5HT_{2C}$ and α7 nAChR [10-12] has been also proposed. In particular, the latter seems to play a crucial role in the pathophysiology of OXAIN being drastically decreased both in the peripheral and central nervous system of oxaliplatin-treated animals. Selective α7 nAChR agonists prevent receptor downregulation and reduce hypersensitivity. Interestingly, the pain relieving effect of α7 nAChR agonists parallels a spinal decrease of dynorphin A [13], an endogenous kappa opioid receptor agonist that strongly contributes to neuropathic pain [14, 15].

Other cellular functions have been associated with the activation and sensitization of pain signaling in sensory neurons. Indeed, we reported that during OXAIN the level of oxidative stress markers increases in the systemic circulation, in the peripheral and central nervous system [16, 17], and the activation of glial cells also occurs [18, 19]. This evidence agrees with the hypothesis that antineoplastic agents can increase pain modulators and free radical production, to whom mammalian nerves seem to be more susceptible [20, 21]. In this view, the degradation of non-functional oxidized proteins is essential for the maintenance of antioxidant defense machinery [22-24] that seems compromised in OXAIN.

The major proteolytic machinery responsible for the removal of oxidized proteins is represented by the Ubiquitin-Proteasome System (UPS) whose protein degradation process is tightly regulated through the action of three different enzymes (E1, ubiquitin activating-; E2, ubiquitin conjugating- and E3, ubiquitin ligase-enzyme). They act sequentially by adding ubiquitin molecules to the

substrate via lysine 48, thus tagging proteins for 26S proteasome degradation [25-27]. The core particle (20S - CP) of 26S constitutive proteasome consists of four heteroheptameric rings where the proteolytic subunits are located. Theβ1, β2 and β5 subunits are endowed with proteolytic activity, cutting different protein substrates with caspase-, trypsin- and chymotrypsin-like activity, respectively [28].

The contribution of UPS activity to central sensitization, hyperalgesia, and allodynia phenomena has been proposed [29]. Moreover, other studies highlighted the anti-inflammatory and anti-nociceptive effects exhibited by the proteasome inhibitor MG132 in a rat model of rheumatoid arthritis [30], further underlining UPS as a crucial molecular target to be considered in analgesic therapy [31].

Besides the constitutive proteasome ubiquitously expressed, other classes of proteasome exist. One of them is represented by the immunoproteasome which is primarily expressed in cells of hematopoietic origin or in non-hematopoietic cells exposed to inflammatory cytokines [32, 33]. In the immunoproteasome, the LMP2 (or β1*i*), MECL-1 (or β2*i*) and LMP7 (or β5*i*) subunits replace β1, β2 and β5 activities of the constitutive proteasome, respectively [34, 35]. The immunoproteasome expression can be induced by inflammatory conditions [36] and immunoproteasome inhibition can block cytokine production and attenuate arthritis progression [37].

On these bases, and aiming at identifying new pharmacological tools useful to control OXAIN, we sought to evaluate whether rats exposed to oxaliplatin exhibit alterations in the UPS-mediated degradation pathway. To address this question, we focused our attention at both spinal and supraspinal level analyzing separately the lumbar, thoracic and cervical portions of rat spinal cord and selected supraspinal regions involved in pain transmission and modulation. In particular, we analyzed the thalamus (TH) and the somatosensory cortex (SSCx), sequentially activated along the pathway conveying nociceptive information. In addition, the periaqueductal gray (PAG) matter was also investigated as a strategic hub in the descending pain control system [38, 39]. To evaluate and discern the contribution of the constitutive and immunoproteasome complexes to the cleavage activity

alterations induced by repeated exposure to oxaliplatin, we evaluated the gene expression of β5 and LMP7 (or β5*i*) proteasome subunits in the supraspinal areas.

As noted above, neuropathic pain appears to be reduced by the intrathecal administration of proteasome inhibitors [29]. At the same time, an increase of spinal dynorphin A peptide levels associated with neuropathic pain conditions, appears to be reduced by a proteasome inhibitor treatment [40]. For these reasons, we also investigated the gene expression of spinal prodynorphin in OXAIN-suffering rats as well as the behavioral and neurochemical effects of the new active second-generation proteasome inhibitor compound, oprozomib.

2. Materials and methods

2.1 Animals

Sprague Dawley rats (220-250 g; Envigo, Varese, Italy) were used. Animals were housed in the Centro Stabulazione Animali da Laboratorio (CeSal, University of Florence), four rats were housed per cage (size 26 x 41 cm^2); animals were fed with a standard laboratory diet and tap water *ad libitum* and kept at 23 ± 1 °C with a 12 h light/dark cycle (light at 7 A.M.). All animal manipulations were carried out according to the Directive 2010/63/EU of the European Parliament and of the European Union council (22 September 2010) on the protection of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Italian Ministry of Health (No. 498/2017-PR) and from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines [41]. Five animals per group were utilized in each experiment. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2 Oxaliplatin-induced neuropathic pain model and oprozomib administration

Oxaliplatin (2.4 mg kg^{-1}; Carbosynth, Compton, UK) was injected intraperitoneally (i.p.) for 5 consecutive days every week for 2 weeks for a total of 10 injections (from day 1 to day 5 and from day 8 to day 12) [42,43] with minor modification. Oxaliplatin was dissolved in a 5% glucose solution. Oprozomib (30 – 100 mg kg^{-1}), suspended in 1% carboxymethylcellulose sodium salt (CMC; Sigma-Aldrich, Milan, Italy) was acutely administered *per os* (p.o.) on day 15, at the end of oxaliplatin injection (day 15) when neuropathy was well established. Control animals received an equivalent volume of vehicles.

2.3 Assessment of mechanical hyperalgesia (Paw pressure test)

The nociceptive threshold of rats was determined by an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton et al. [44]. Briefly, a constantly increasing weight was applied to a small area of the dorsal surface of the hind paw using a blunt conical probe by a mechanical device. Mechanical weight (expressed in g) was increased until vocalization or withdrawal reflex occurred while rats were lightly restrained. An arbitrary cut-off value of 100 g was adopted.

2.4 Assessment of thermal allodynia (cold plate test)

Thermal allodynia was assessed using the cold-plate apparatus (Ugo Basile, Varese, Italy). With minimal animal-handler interaction, rats were taken from home-cages, and placed onto the cold surface maintained at a constant temperature of 4°C ± 1°C. Ambulation was restricted by a cylindrical Plexiglas chamber (diameter: 10 cm, height: 15 cm), with open top. Pain-related behavior (paw lifting

or licking) was observed, and the time (s of the first sign was recorded. The cut-off time latency was set at 30s [10].

2.5 Assessment of mechanical allodynia (Von Frey test)

Mechanical allodynia was measured with an electronic Von Frey hair unit (Ugo Basile, Varese, Italy) as described by Sakurai and colleagues [45]. Briefly, rats were placed in 20 × 20 cm plexiglas boxes equipped with a metallic screen-mesh floor, 20 cm above the bench. A habituation of 15 min was allowed before the test. The withdrawal threshold was evaluated by applying a force ranging from 0 to 50 g with a 0.2 g accuracy. The punctuate stimulus was delivered to the mid-plantar area of each posterior paw from below the meshy floor through a plastic tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum pressure required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not taken as a withdrawal response. Stimuli were applied on each posterior paw with an interval of 5 s. The measure was repeated five times and the final value was obtained by averaging the five measures.

2.6 Assessment of motor coordination (Rota-rod test)

The Rota-rod apparatus (Ugo Basile, Varese, Italy) consisted of a base platform and a rotating rod with a diameter of 6 cm and a non-slippery surface. The rod was placed at a height of 25 cm from the base. The rod, 36 cm in length, was divided into 4 equal sections by 5 disks. Thus, up to 4 rats were tested simultaneously on the apparatus, with a rod-rotating speed of 10 revolutions per minute. The integrity of motor coordination was assessed on the basis of the time the animals kept their balance on the rotating rod for a maximum of 10 minutes (600 seconds). After a maximum of 6 falls from the rod, the test was suspended and the time elapsed was recorded [46].

2.7 Tissue collection

On day 15, at the end of the last behavioural test, animals were sacrificed using a guillotine. Thalamus (TH), the somatosensory cortex (SSCx) the periaqueductal gray (PAG) and the spinal cord (dissected in lumbar, thoracic and cervical portions) were collected and immediately frozen in liquid nitrogen for further ex vivo analysis.

2.8 Proteasome activity assay

Rat tissue samples were homogenized in lysis buffer (150 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA, 2 mM ATP, 1% Triton;Sigma-Aldrich, Milan, Italy) and centrifuged at 14,000 × g at 4 °C for 15 min. Homogenates were aliquoted and kept at -80 °C. Protein concentration was determined by using Pierce ™ BCA protein assay kit (Thermo Scientific Massachusetts, USA).

The proteasome trypsin- and chymotrypsin-like activities were analyzed monitoring the cleavage of two fluorogenic substrates using 25 μg of lysate proteins. The substrates benzyloxycarbonyl-Ala-Arg-Arg-7-amino-4-methylcoumarin (Z-ARR-AMC) and succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) (both purchased from Merck Millipore, Milan, Italy) were used according to the manufacturer's instructions to measure trypsin- (β2) and chymotrypsin-like (β5) activities, respectively. The assay is based on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrates Z-ARR-AMC or LLVY-AMC. All samples were run in triplicate and added to the substrates, then incubated for 2 hours at 37 °C. The free AMC fluorescence was quantified at 380 nm excitation and 460 nm emission wavelengths using a plate reader fluorometer (GENios Tecan). According to the manufacturer's instructions, an AMC standard curve was generated for reference by preparing a dilution series of AMC standard reagent in the concentration range of 0.04 – 12.5 μM and run in triplicate. The assay was validated by analyzing proteasome positive control incubated with the inhibitor lactacystin and two independent experiments were carried out for each analyzed tissue.

Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group.

2.9 RNA isolation and qRT-PCR

Total RNA was isolated using the TRIZOL reagent (Life Technologies, USA) according to the method of Chomczynski and Sacchi [47] and its integrity was checked by 1% agarose gel electrophoresis. In brief, the amounts of RNA were determined by measuring optical densities and only RNA samples with an OD260/OD280 1.8 < ratio < 2 were used. Total RNA was reverse transcribed using GeneAmp RNA PCR kit (ThermoFisher Scientific, USA) in a final volume of 20 μl, according to manufacturer's instructions. Quantitative real-time PCR was performed on a StepOne Real-Time PCR System (Life Technologies, USA) using TaqMan Gene Expression Master Mix (ThermoFisher Scientific, USA), to analyze β5 (Rn01488742_m1, FAM-MGB) and the LMP7 (or β5*i*) (Rn00589926_m1, FAM-MGB) proteasome subunits gene expression, expressed by the constitutive and immune- proteasome complexes respectively [48] [49]. All samples were run in triplicate and were normalized to the endogenous reference gene glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) (Rn01775763_g1, VIC-MGB). Primers and probe sequences used for TaqMan gene expression were purchased from ThermoFisher Scientific, USA.

To analyze pDYN gene expression the SYBR Green PCR MasterMix was used (Life Technologies, USA). Each sample was run in triplicate and all data were normalized to GAPDH. The primers used for PCR amplification in SYBR Green PCR MasterMix were designed using Primer 3, and are here reported: Gapdh Forward 5′- AGACAGCCGCATCTTCTTGT-3′; GAPDH Reverse 5′-CTTGCCGTGGGTAGAGTCAT-3′; pDYN Forward 5′-CCTGTCCTTGTG TTCCCTGT-3′; pDYN Reverse 5′- AGAGGCAGTCAGGGTGAGAA-3′. Relative abundance of each mRNA species was

calculated by Delta–Delta Ct (ΔΔCt) method and converted to relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis [50]. Data are expressed as means ± SEM (each sample run in triplicate) and represented as fold change in mRNA levels.

2.10 Statistical analysis

All experimental results were expressed as mean ± SEM and data were analyzed by t-test or by one-way ANOVA followed by Bonferroni's multiple comparison post hoc test, as appropriate. P values <0.05 were considered statistically significant. Investigators were blind to all experimental procedures. Statistical analysis was performed using GraphPad Prism software package (v7 for Windows, GraphPad Software, San Diego CA, USA, www.graphpad.com).

3. Results

3.1 Assessment of oxaliplatin-induced neuropathic signs

Two weeks of oxaliplatin repeated administrations significantly decreased the pain threshold to non-noxious (allodynia-like) and noxious (hyperalgesia-like) stimuli, evaluated on day 15 (Figure 1). The licking latency in response to a thermal non-noxious stimulus (cold plate test) decreased from 19.3 ± 0.7 s of the control group to 9.5 ± 1.1 s in oxaliplatin-treated rats (Figure1a). The withdrawal threshold to a mechanical non-noxious stimulus (von Frey test) was significantly reduced in oxaliplatin-treated group (13.1 ± 0.7 g) with respect to control (20.7 ± 1.2 g) (Figure1b). Similarly, the weight tolerated by the animals on the posterior paws (mechanical noxious stimulus, paw pressure test) decreased from 65.0 ± 0.5 g of control groups to 45.3 ± 1.0 g in oxaliplatin-treated animals (Figure 1c). Moving to the evaluation of oxaliplatin impact on motor coordination and physical endurance, the Rotarod test showed only a slight reduction of the time spent on the rotating rod with respect to the control group (Figure 1d).

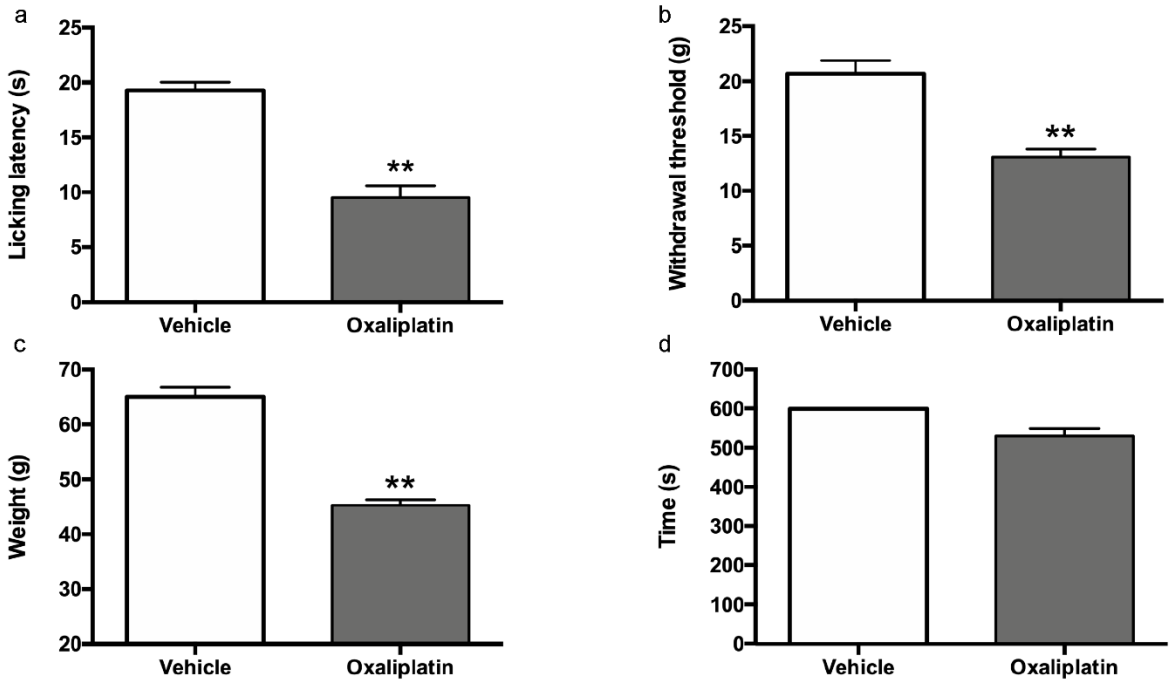

Figure 1

3.2 Proteasome activities modulation in neuropathic rats

3.2.1 Spinal cord. Intracellular trypsin- (β2) and chymotrypsin- (β5) like activities of the constitutive proteasome were assessed after oxaliplatin treatment in three different portions of spinal cord. Results indicated that oxaliplatin-treated rats did not show significant alteration at β2 subunit activity level in the three different portions of spinal cord examined (lumbar 91.44 ± 2.82 vs 100.00 ± 2.76; thoracic: 91.58 ± 3.78 vs 100.00 ± 2.41; cervical: 100.25 ± 3.49 vs 100.00 ± 1.68, n.s.) (Figure 2 a, b and c).

Similarly, the analysis of the β5 subunit activity did not show changes in the spinal cord of OXAIN-suffering rats (lumbar 95.93± 2.12 vs 100.00 ± 1.57; thoracic: 93.65 ± 6.96 vs 100.00 ± 3.28; cervical: 100.29 ± 5.75 vs 100.00 ± 5.38, n.s.) (Figure2 d, e and f).

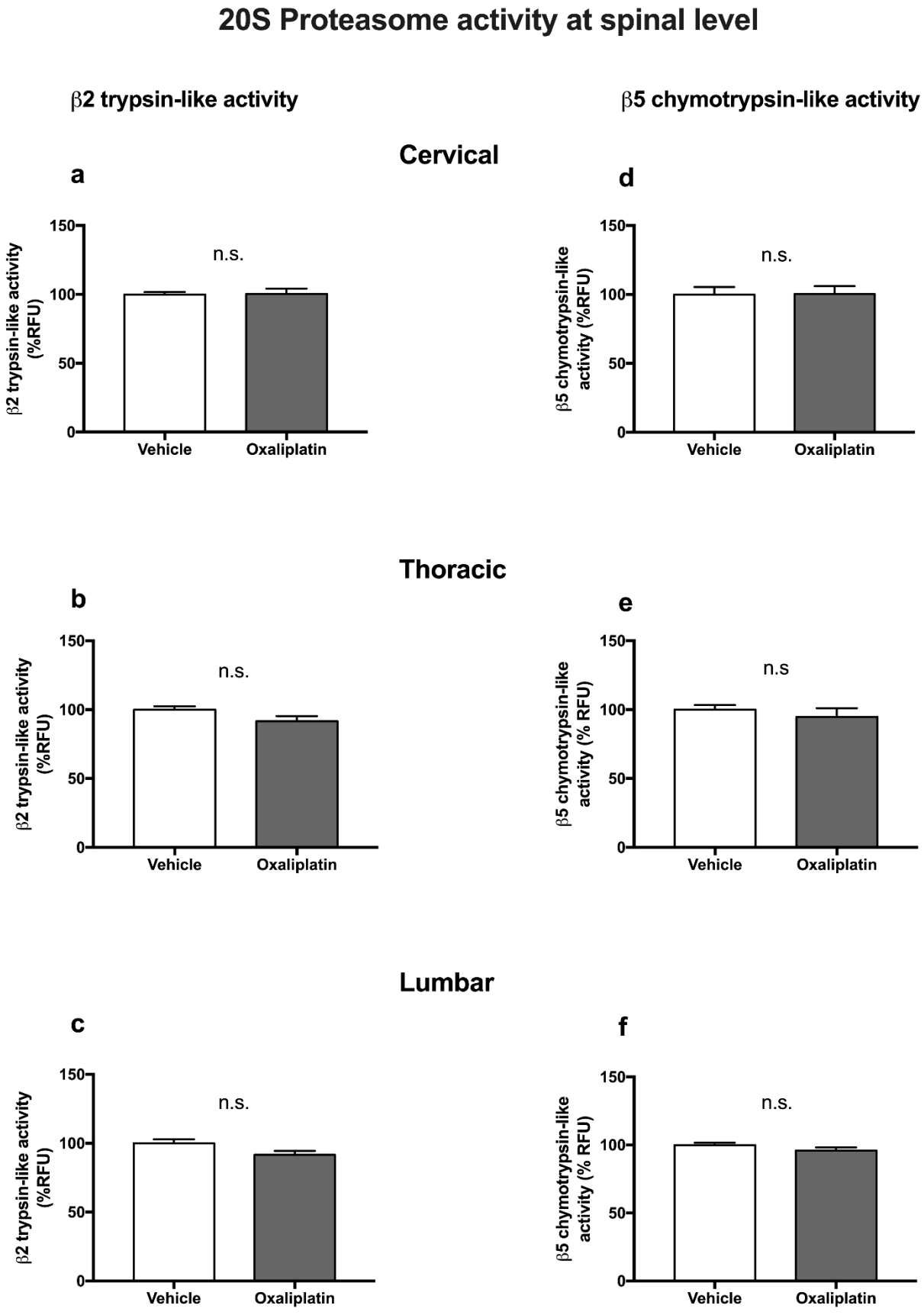
20S Proteasome activity at spinal level
β2 trypsin-like activity
β5 chymotrypsin-like activity
Cervical
a
d
β2 trypsin-like activity (%RFU)
β5 chymotrypsin-like activity (%RFU)
150
100
50
0
n.s.
Vehicle
Oxaliplatin
Thoracic
b
e
β2 trypsin-like activity (%RFU)
β5 chymotrypsin-like activity (% RFU)
n.s.
n.s
Vehicle
Oxaliplatin
Lumbar
c
f
β2 trypsin-like activity (%RFU)
β5 chymotrypsin-like activity (% RFU)
n.s.
n.s.
Vehicle
Oxaliplatin

Figure 2

3.2.2 Supraspinal areas. In the TH and in the SSCx, neuropathic rats exhibited no alteration of β2 subunit cleavage activity (TH: 105.78 ± 5.68 vs 100.00 ± 8.78; SSCx: 106.13 ± 3.76 vs 100.00 ± 2.81, n.s.) (Figure 3 a and b). In contrast, the analysis of the β5 subunit showed a significant increase of the chymotrypsin-like activity in both TH and SSCx areas (TH: 115.10 ± 3.75 vs 100.00 ± 4.48, $p < 0.05$; SSCx: 137.61 ± 5.73 vs 100.00 ± 2.76, $p < 0.001$) (Figure3 d and e). The analysis of β2 and β5 subunit cleavage activities showed no alteration in the PAG of oxaliplatin-treated rats compared to controls (β2 activity: 108.27 ± 5.39 vs 100.00 ± 4.85; β5 activity: 93.93 ± 9.73 vs 100.00 ± 8.01, n.s.) (Figure 3c and f).

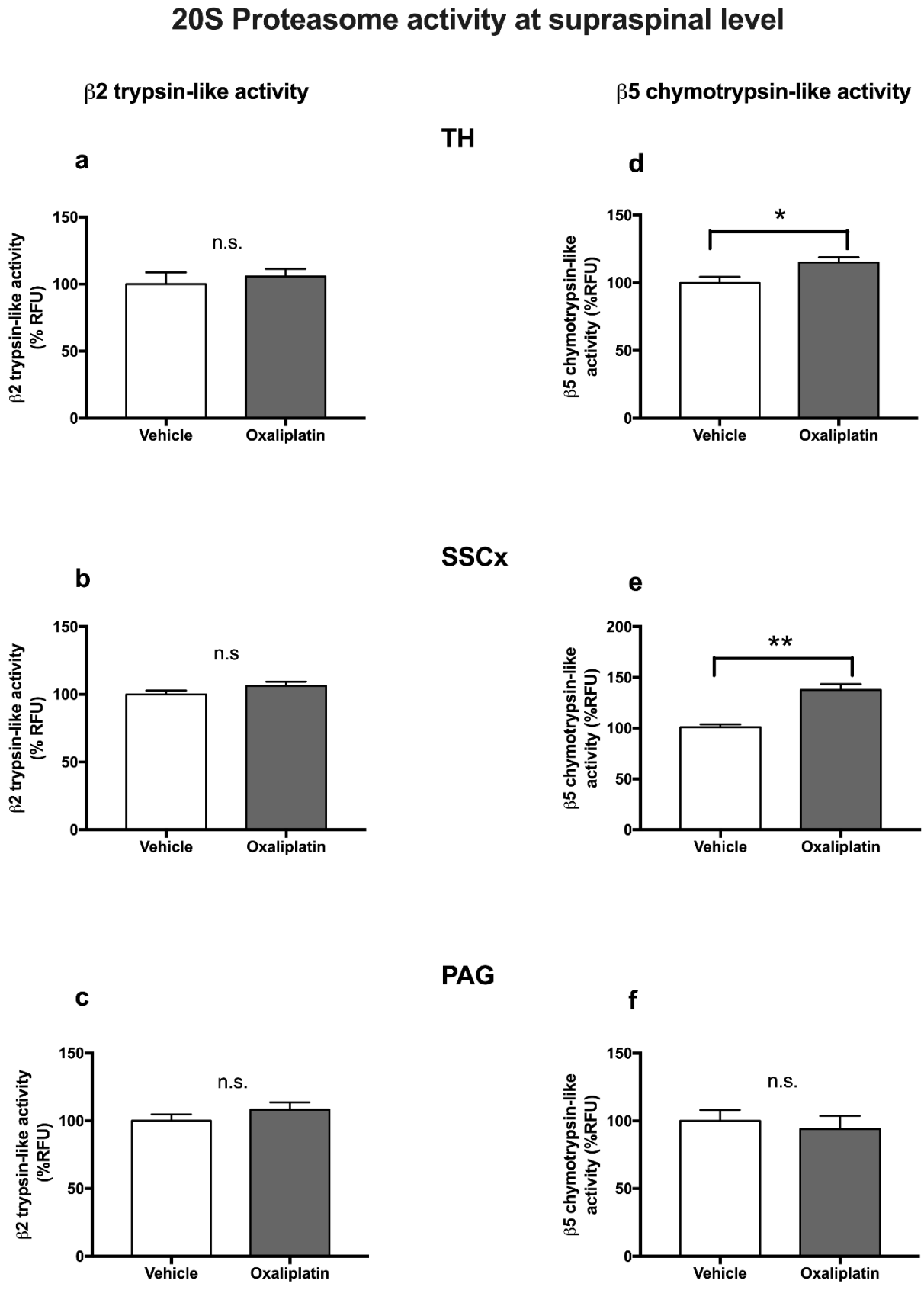
20S Proteasome activity at supraspinal level
β2 trypsin-like activity
β5 chymotrypsin-like activity
TH
a
d
β2 trypsin-like activity (% RFU)
β5 chymotrypsin-like activity (%RFU)
n.s.
*
Vehicle
Oxaliplatin
SSCx
b
e
n.s
**
PAG
c
f

Figure 3

3.3 Gene expression analysis of β5 and LMP7 (or β5*i*) proteasome subunits

Since the chymotrypsin-like activity assay does not allow to discriminate between the constitutive and immunoproteasome activation and based on the proteasome activity alterations detected in the TH and SSCx, we further evaluated the gene expression analysis of β5 and LMP7 proteasome subunits in the supraspinal areas.

Oxaliplatin treatment induced a significant gene expression increase of β5 and LMP7 proteasome subunits in the SSCx (β5 mRNA levels: 1.78 ± 0.08 vs 1.00 ± 0.08, $p < 0.001$; LMP7 (or β5*i*) mRNA levels: 1.32 ± 0.09 vs 1.00 ± 0.06, $p < 0.05$) (Figure 4c and d), which is more pronounced for the constitutive β5 subunit. On the contrary, gene expression analysis conducted in the TH did not show significant alterations in the gene codifying for both β5 and LMP7 proteasome subunits (β5 mRNA levels: 1.07 ± 0.04 vs 1.00 ± 0.07; LMP7 (or β5*i*) mRNA levels: 1.03 ± 0.06 vs 1.00 ± 0.15, n.s.) (Figure 4a and b).

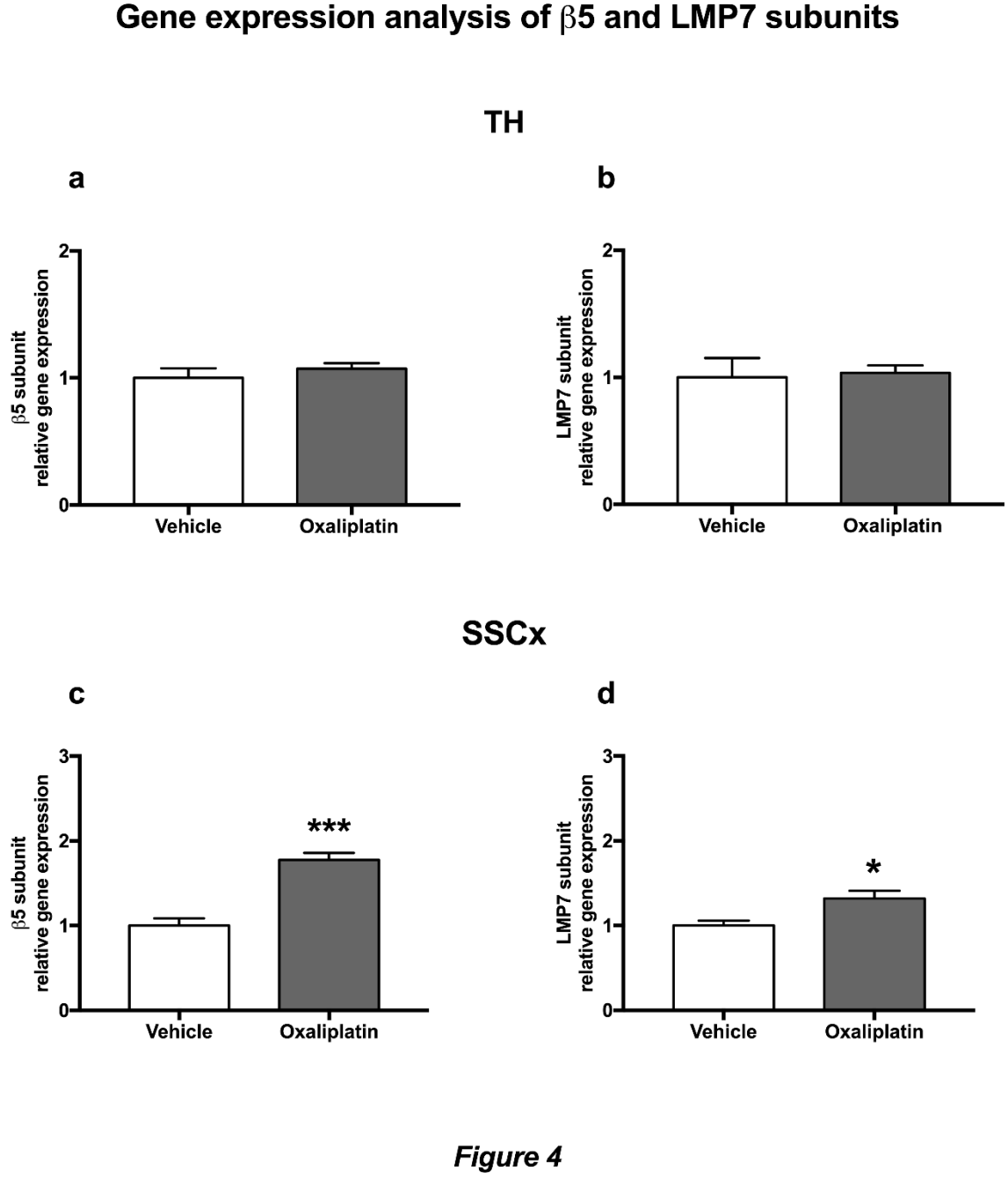

Figure 4

3.4 Acute oprozomib abolishes oxaliplatin-induced increase of spinal prodynorphin gene expression

Results indicated that oxaliplatin-treated rats exhibit significant alterations in spinal pDYN gene expression. In fact, in all the three different portions of spinal cord examined, a significant increase in pDYN mRNA levels was ascertained (cervical: 1.44 ± 0.08 vs 0.96 ± 0.14, $p < 0.05$; thoracic: 2.42

± 0.19 vs 1.01 ± 0.09, $p < 0.001$; lumbar 1.69 ± 0.12 vs 1.01 ± 0.09, $p < 0.001$; in oxaliplatin- or vehicle-treated groups, respectively) (Figure 5a, b and c).

Differently, pDYN mRNA levels measured in a separate group of oxaliplatin-exposed rats that were acutely treated with 30 mg kg^{-1} i.p. of the proteasome inhibitor oprozomib on day 15 were significantly reduced compared with oxaliplatin-exposed animals in all spinal cord segments (cervical: 0.53 ± 0.12 vs 1.44 ± 0.08, $p < 0.001$; thoracic: 0.48 ± 0.14 vs 2.42 ± 0.18, $p < 0.001$; lumbar 0,60 ± 0.07 vs 1.69 ± 0.12, $p < 0.001$; in oxaliplatin-oprozomib or oxaliplatin-vehicle treated groups, respectively) (Figure 5a, b and c).

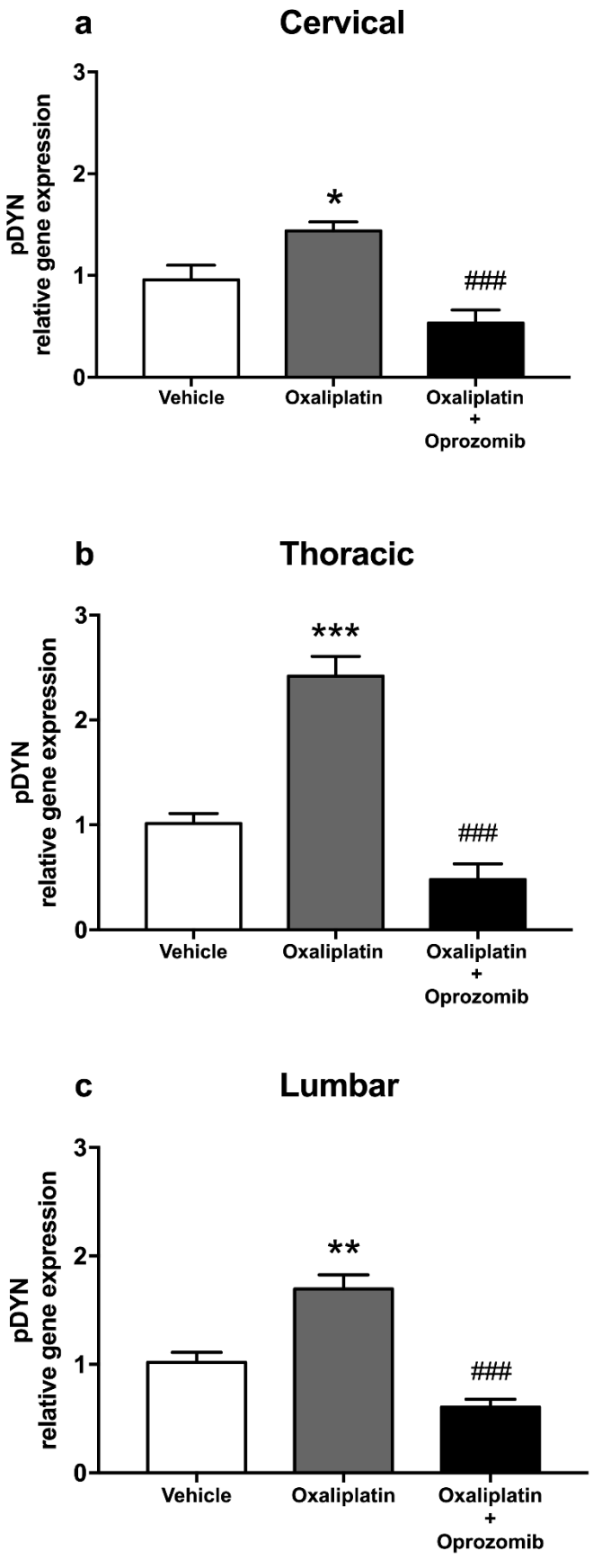
a
Cervical
pDYN
relative gene expression
3
2
1
0
*
###
Vehicle
Oxaliplatin
Oxaliplatin
+
Oprozomib
b
Thoracic
pDYN
relative gene expression
3
2
1
0
***
###
Vehicle
Oxaliplatin
Oxaliplatin
+
Oprozomib
c
Lumbar
pDYN
relative gene expression
3
2
1
0
**
###
Vehicle
Oxaliplatin
Oxaliplatin
+
Oprozomib

Figure 5

3.5 Acute oprozomib administration reverts oxaliplatin-induced hyperalgesia

Oprozomib was tested on day 15 of oxaliplatin protocol. After a single p.o. administration, oprozomib exerted an anti-hypersensitivity effect in a dose-dependent manner as depicted in Figure 6. In particular, oprozomib 100 mg kg^{-1}, completely counteracted the thermal allodynia 30 min after treatment, lasting up to 60 min. The lower dose (30 mg kg^{-1}) significantly increased the licking latency time between 30 min and 60 min (Cold plate test, Figure 6a). Comparable results were obtained with the von Frey test, the response to a non-noxious mechanical stimulus was significantly enhanced by oprozomib administration in a dose dependent-manner (Figure 6b). The higher dose was effective from15 min after administration up to 60 min while the dose of 30 mg kg^{-1} was active from 30 min to 60 min. The anti-hyperalgesic effect of oprozomib against oxaliplatin neuropathy was evaluated by the Paw pressure test where the compound was able to revert the hypersensitivity to a noxious mechanical stimulus in a dose dependent-manner (Figure 6c).

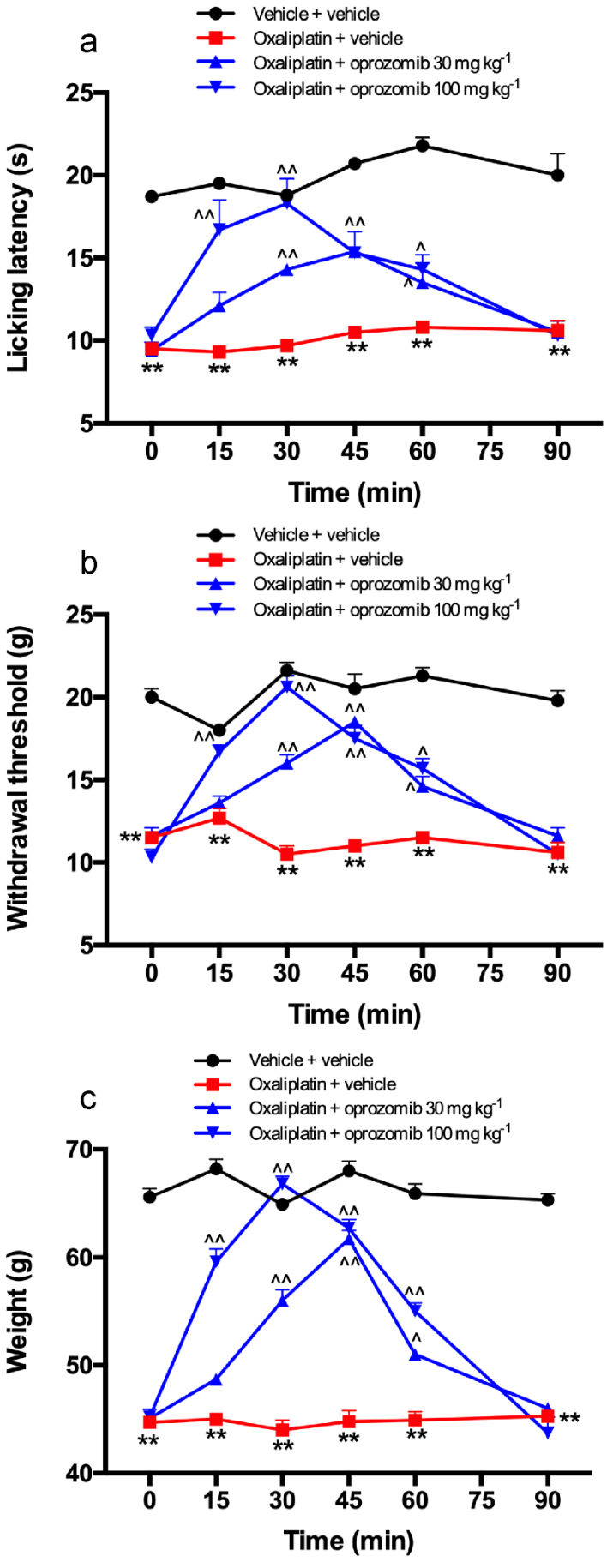
a
Vehicle + vehicle
Oxaliplatin + vehicle
Oxaliplatin + oprozomib 30 mg kg-1
Oxaliplatin + oprozomib 100 mg kg-1
Licking latency (s)
25
20
15
10
5
0
15
30
45
60
75
90
Time (min)
b
Vehicle + vehicle
Oxaliplatin + vehicle
Oxaliplatin + oprozomib 30 mg kg-1
Oxaliplatin + oprozomib 100 mg kg-1
Withdrawal threshold (g)
25
20
15
10
5
0
15
30
45
60
75
90
Time (min)
c
Vehicle + vehicle
Oxaliplatin + vehicle
Oxaliplatin + oprozomib 30 mg kg-1
Oxaliplatin + oprozomib 100 mg kg-1
Weight (g)
70
60
50
40
0
15
30
45
60
75
90
Time (min)

Figure 6

4. Discussion

Previous research provided robust evidence revealing the involvement of several factors associated with the development and severity of OXAIN [51], such as the overexpression of organic cation transporter-2 in neurons [52], the involvement of specific receptor systems [8, 12, 53] and also the dysfunction of different voltage-gated ion channels [54, 55].

Among this plethora of mechanisms associated with oxaliplatin-induced neurotoxicity, it is worth to mention the contribution of UPS to the development and maintenance of OXAIN and, in this regard, the present study was aimed at exploring whether the proteasome degradation machinery was involved in this neuropathic condition. Our major finding is that repeated exposure to oxaliplatin evokes alterations of the 26S proteasome degradation machinery which are strictly dependent on the pain transmission – related region. Indeed, the analysis of β2 and β5 enzymatic activities indicates that the repeated oxaliplatin exposure promotes a significant increase of intracellular chymotrypsin-(β5) like activity at supraspinal level only, in particular in the TH and SSCx, without changes in the three different portions of the spinal cord. On the other hand, the activity changes observed in the SSCx are accompained by alterations in gene expression, whereas in the TH only enzymatic activity is increased without gene expression activation.

These results are consistent with evidence demonstrating that antineoplastic agents may increase the pro-inflammatory mediator signaling [20, 21] likely through the activation of 26S proteasome complex [56]. In this view, the nuclear factor κ enhancer binding protein (NF-κB) transcription factor family activation requires the ubiquitination of inhibitory proteins of κB family (IκB) [57, 58]. The 26S proteasome proteolytic activity allows IκB post-translational modification and its degradation and the subsequent NF-κB translocation to the nucleus, where it regulates the expression of several genes including inflammatory mediators such as cytokines and chemokines [59-61]. In other words, it is likely that repeated oxaliplatin administration promotes the increase of proteasome activity inducing the NF-κB transcription factor family activation as a consequence [62, 63]. This hypothesis is supported by recent evidence from Wang and colleagues showing that different inhibition strategies

of NF-κB p65 activation, such as siRNA targeting NF-κB p65, decreases thermal hyperalgesia induced by the antineoplastic agents [62]. The involvement of NF-κB in a variety of pain conditions such as rheumatoid arthritis, migraine, and nerve injury has been thoroughly established [64-66]. In this regard, previous studies indicate the contribution of NF-κB activation in patients with acute spinal cord injury [67] and also in rat model of intervertebral disk herniation [68]. Moreover, sciatic nerve injury also promote supraspinal neuro-inflammation accompanied by high expression of NF-κB in rat brain [69].

In this frame, the inhibition of NF-κB activation through the use of proteasome inhibitor MG132 has been indicated as useful strategy to counteract the NF-κB induction of inflammatory pathways [70, 71], and to modulate inflammatory pain in a rheumatoid arthritis rat model [30, 72]. Therefore, the increase of proteasome activity reported in our experimental model corroborates the possibility that proteasome inhibitors could be effective in the treatment of painful states, including the peripheral neuropathy induced by oxaliplatin. Indeed, proteasome inhibitor administration seems to attenuate, prevent and revert pain behavioral signs in different neuropathic pain models [29, 40].

The considerable increase of proteasome activity in the TH and SSCx and the selective up-regulation of β5 and LMP7 (or β5*i*) genes in the SSCx also indicates a peculiar activation of this supraspinal region which is mainly involved in the discriminative aspect of pain and sensory coding [73].

The protein degradation mediated by UPS is a crucial aspect in the synaptic plasticity regulation and, particularly, in the long-term synaptic modifications typical for chronic abnormal pain [31]. The main involvement of β5 isoforms, belonging to the constitutive and immune proteasomes respectively, suggests a relevant role played by these specific subunits in the oxidative stress processes induced by prolonged exposure to oxaliplatin. In this regard, β5 subunit represents the most important active site at the 20S-CP [74, 75], and it also participates in the increased response to oxidative stress carrying out a cytoprotective action [76, 77]. Indeed, it has been demonstrated that the overexpression of the β5 subunit is able to increase the proteasome activity and to promote cell survival following H_2O_2 exposure [76, 77]. Therefore, it is reasonable to speculate that proteasome activity increase and the

selective β5 and LMP7 (or β5*i*) gene up regulation at supraspinal level could represent an adaptive response to the prolonged oxidative stress condition as that evoked by oxaliplatin also in the central nervous system [16,17]. This hypothesis is supported by the efficacy of antioxidant compounds in reducing the proteasome activation [78]. The same was reported by using the natural antioxidant melatonin [79].

Parallel to the increase of proteasome activity, we also observed a significant increase of pDYN gene expression in all three portions of the spinal cord. This observation confirms the involvement of the dynorphinergic system in the development of OXAIN as expected, given its role in several chronic pain conditions [15, 80-82]. In this regard, Malan and colleagues associated the increase of dynorphin peptide level at spinal level, from a spatial and temporal point of view, to the appearance of mechanical allodynia [83].

An interesting hypothesis suggests that the increase of DYN release induced by oxaliplatin treatment may lead to its interaction with NMDA receptors [82] determining the presence of allodynia signs through the production of radical species and pro-inflammatory cytokines, which in turn contribute to the development of spinal neuronal damage [82, 84]. Indeed, it is known the DYN ability to modulate nociception through its action on immune function [85]. In particular, the increase of dynorphin levels could be associated with the activation of a signaling cascade which, starting from the NMDA receptor, activates the transcription factor NF-κB and increase the expression of pro-inflammatory cytokines, nitric oxide and also DYN itself, as a consequence [84]. Therefore, the reduction of pDYN mRNA levels observed after oprozomib treatment could be associated to the ability of this second-generation proteasome inhibitor compound to inhibit NF-κB activation.

Moreover, this latest finding agrees with previous studies showing the ability of proteasome inhibitors to down-regulate spinal DYN release [40].

The present, novel evidence of DYN involvement in the pathophysiology of OXAIN agrees with the parallelism existing between the α7 nAChR-mediated neuropathic pain relief and DYN decrease. In

a model of spinal nerve ligation, the activation of α7 nAChR alleviated neuropathic pain behaviors and reduced DYN levels in the ipsilateral spinal cord [13]. In OXAIN, α7 nAChR expression is strongly downregulated in the nervous system, selective agonists are able to relieve pain by a glia-mediated mechanism and restore receptor levels [19]. A relationship between α7 nAChR downregulation and DYN increase may be hypothesized in OXAIN mechanisms, strongly strengthening the role of treatments able to normalize DYN. Furthermore, previous studies demonstrated a temporal change in the inflammatory response that accompanies OXAIN. In this view, it has been demonstrated that oxaliplatin treatment induces a marked increase of microglial density in the dorsal horns of the lumbar spinal cord after 7 days of treatment which decreases to control following 14 and 21 days. On the contrary, spinal astrocytes engagement is long lasting, suggesting a pivotal role of this cell population in the development of chronic pain as a part of central sensitization machinery [18].

Our data show a rapid DYN up-regulation at spinal level and a proteasome activity increase at supraspinal level that could be related to the central sensitization. Nevertheless, the lack of proteasome alteration at spinal level represents an unexpected result which may suggest that this defense complex may be subjected to a temporal activation according to the area or cell population like microglia cells.

In addition, our data show that although oxaliplatin induces a significant increase of β5 subunit belonging to the constitutive proteasome, it is also able to promote the LMP7 (or β5i) subunit gene expression up-regulation. In this regard, some evidence demonstrated a prominent role of immunoproteasome in the adaptive immune response implicated in several biological and pathological processes. In fact, the immunoproteasome is known to be crucial in the degradation of damaged proteins generated by oxidative stress [86] and in inflammatory processes [87]. Therefore, given the role played by oxidative stress in peripheral neuropathy induced by oxaliplatin [16], we tested the ability of an immune proteasome inhibitor to reduce OXAIN signs. In our study, a single oprozomib administration was able to counteract hyperalgesia and allodynia induced by oxaliplatin

repeated injections that mimicks, in an animal model, the clinical adverse effects of this antineoplastic drug [88]. The results obtained on the efficacy of oprozomib could help to achieve the effect of oxaliplatin therapy reducing its toxicity. In particular, the use of immunoprotesome inhibitors has proved to be a potential useful approach especially in inflammatory and immune-related conditions. Indeed, it seems that the use of selective inhibitors for immuno subunits may result in a reduction of pro-inflammatory cytokines production [37, 87].

Moreover, our data suggest for the first time that oprozomib is able to revert the increased prodynorphin gene expression occurring in OXAIN, thus indicating one of the possible molecular mechanisms underlying this pathological condition and identifying dynorphin as a possible target of immunoproteasome inhibitors, for new therapeutic approaches. We are also aware of the difficulty to directly associate oprozomib effects upon OXAIN to the UPS inhibitory action of the drug. This fact may represent a limit of our study and warrants further investigation.

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Disclosures

Authors declare that they have no conflict of interest.

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Legends to Figures

Figure 1. Behavioral measurements. Pain: non-noxious stimuli. (a) The response to a thermal stimulus was evaluated by the Cold plate test measuring the latency (s) to pain-related behaviors (licking or lifting of the paw); (b) The von Frey test was used to measure the pain threshold as a response to a mechanical stimulus. Pain: noxious stimulus: (c) the paw pressure test was used to measure the sensitivity to a mechanical stimulus. Motor coordination. (d) The integrity of the animals' motor coordination was assessed using the Rota rod apparatus measuring the time spent to keep their balance. Animals were daily treated with 2.4 mg kg^{-1} oxaliplatin intraperitoneally (i.p.), over two weeks for a total of 10 injections. Behavioral evaluations were performed on day 15 when neuropathy was well established. Control animals were treated with vehicle. Each value represents the mean ± SEM of five rats per group. Statistical analysis is one-way ANOVA followed by Bonferroni's post-hoc comparison test. **p<0.01 vs vehicle group.

Figure 2. β2 trypsin-like activity (left panels: a,b and c) and β5 chymotrypsin-like activity (right panels: d,e and f) of 20S Proteasome in the cervical, thoracic and lumbar portion of rat spinal cord after vehicle or oxaliplatin (2.4 mg kg^{-1}, i.p., daily for 10 days) treatment. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group (n.s. p>0.05 vs vehicle, *t*-test,).

Figure 3. β2 trypsin-like activity (left panels: a,b and c) and β5 chymotrypsin-like activity (right panels: d,e and f) of 20S Proteasome in the TH, SSCx and PAG of rat after vehicle or oxaliplatin (2.4 mg kg^{-1}, i.p., daily for 10 days) treatment. Data are expressed as percentage of relative fluorescence (RFU) and are reported as mean ± SEM of five rats per group (n.s. p>0.05; *p < 0.05; **p < 0.01 vs vehicle; *t*-test).

Figure 4. Relative gene expression of β5 subunit of constitutive proteasome (left panels: a,c) and LMP7(β5i) subunit of immunoproteasome (right panels: b,d) in the TH and SSCx of rat after Vehicle or Oxaliplatin (2.4 $mgkg^{-1}$i.p., daily for 10 days) treatment. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of five rats per group (n.s. $p>0.05$; *$p < 0.05$; ***$p < 0.001$ vs Vehicle; analyzed by *t*-test).

Figure 5. Relative gene expression of pDYN in the a) cervical, b) thoracic and c) lumbar portion of rat spinal cord. Data represent 2^{-DDCt} values calculated by DDCt method and are expressed as mean ± SEM of five rats per group (*$p < 0.05$; ***$p < 0.001$ vs Vehicle; $^{###}p < 0.001$ vs Oxaliplatin-treated group; analyzed by One-way ANOVA followed by Bonferroni multiple comparisons test).

Figure 6. Behavioral measurements. Pain: non-noxious stimuli. (a) The response to a thermal stimulus was evaluated by the cold plate test measuring the latency (s) to pain-related behaviors (licking or lifting of the paw); (b) The von Frey test was used to measure the pain threshold as a response to a mechanical stimulus. Pain: noxious stimulus: (c) the paw pressure test was used to measure the sensitivity to a mechanical stimulus. Animals were daily treated with oxaliplatin 2.4 mg kg^{-1} intraperitoneally (i.p.), oprozomib (30 – 100 mg kg^{-1}, p.o.) administration and behavioral evaluations were performed on day 15, before and 15, 30, 45, 60 and 90 min after oprozomib administration. Control animals were treated with vehicle. Each value represents the mean ± SEM of five rats per group. Statistical analysis is one-way ANOVA followed by Bonferroni's post-hoc comparison. **$p <0.01$ vs Vehicle + vehicle group; ^$p <0.05$ and ^^$p <0.01$ vs Oxaliplatin + vehicle group