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# Supraspinal role of protein kinase C in oxaliplatin-induced neuropathy in rat

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ABSTRACT

Oxaliplatin is a platinum-based chemotherapy drug characterized by the development of a painful peripheral neuropathy which is reproduced in rodent animal models with features observed in humans. Our focus was to explore the alterations of intracellular second messengers at supraspinal level in oxaliplatin-induced mechanical hyperalgesia. In our experiments, chronic administration of oxaliplatin to rats induced mechanical hyperalgesia which lasted for many days. When the hyperalgesic rats were submitted to paw pressure test in the presence of selective PKC inhibitor Calphostin C supraspinally administered, hyperalgesic effect could be reversed showing that PKC activity in supraspinal brain regions is needed. Concurrently, oxaliplatin chronic treatment induced a specific upregulation of γ isoforms of PKC and increased phosphorylation of γ/ε PKC isoforms within thalamus and PAG. Phosphorylation was reversed when PKC activity was inhibited by Calphostin C. Distinct PKC-activated MAPK pathways, including p38MAPK, ERK1/2 and JNK, were investigated in chronic oxaliplatin rat. A dramatic phosphorylation increase, Calphostin C sensitive, could be observed in thalamus and PAG for p38MAPK. These data show that, in oxaliplatin-induced neuropathy, enhanced mechanical nociception is strictly correlated with increased phosphorylation of specific intracellular mediators in PAG and thalamus brain regions pointing to a role of these supraspinal centers in oxaliplatin-induced neuropathic pain mechanism.



## 1. Introduction

Oxaliplatin[(*trans*-l)1,2-diaminocyclohexaneoxalatoplatinum(II)] is a platinum-based chemotherapy drug whose cytotoxicity is thought to result from inhibition of DNA synthesis. The use of the antineoplastic drug oxaliplatin for the treatment of a variety of cancers is significantly limited by the development of a painful peripheral neuropathy characterized by both an acute sensitivity to cold and lack of sensation in the hands and feet and a chronic foot/leg, hand/arm numbness [3]. It has previously been reported that oxaliplatin produces a peripheral neuropathy in rats in several well-characterized animal models with features observed in humans [5]. Thus, oxaliplatin induces acute mechanical hyperalgesia after intravenous single dose administration [11]; repeated intravenous oxaliplatin enhances mechanical hyperalgesia and allodynia, heat allodynia and cold allodynia in rodents [2] indicating sensitization in the peripheral and central nociceptive system. Although the mechanism lying beneath the acute neuronal excitability and pain induced by oxaliplatin is still to be determined, it has been ascribed, at least in part, to a specific interaction of oxaliplatin with specific isoforms of the voltage-gated sodium channels, located in the cellular membrane, increasing the excitability of sensory neurons, an effect that has been suggested to play a role in pain syndrome experienced by oncologic patients [9,10]. Although investigations of the mechanism of neuropathic pain induced by platinum-based chemotherapy drug have been a major focus of interest for the last years, neuropathic pain is a major chronic condition that presently remains difficult to treat for solid tumor chemotherapy. In the present study we tested whether alterations of intracellular second messengers at supraspinal level are revealed in oxaliplatin-induced mechanical hyperalgesia.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats weighing between 200 and 300 g (Harlan Inc., Varese, Italy) were used. During the experiment, the animals were fed with commercial cubes and drinkable water *ad libitum* and maintained in an air-conditioned housing room (23 ± 1 °C) with a 12-h light–dark cycle. All animal procedures were performed according to the European Economic Community (EEC) guidelines for animal care (DL 116/92, application of the European Communities Council Directive of 24 November 1986-86/609/EEC). Ethical policy of the University of Florence conforms with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23,

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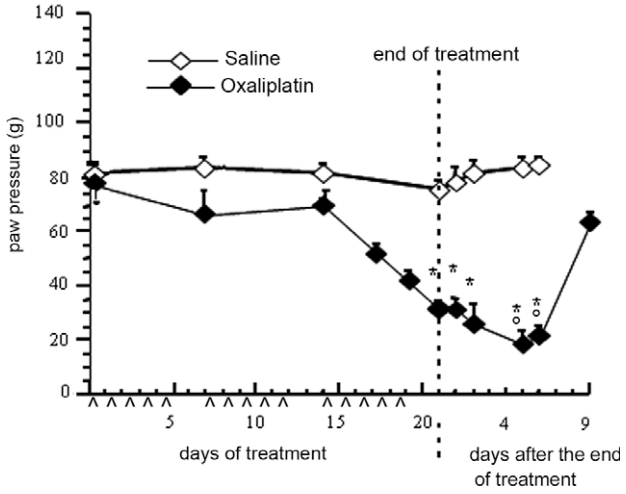


**Fig. 1.** Time-course of mechanical hyperalgesia induced by oxaliplatin treatment. Effect of administration of oxaliplatin in comparison with administration of saline on mechanical hyperalgesia. Each point is the mean ± SEM of single mechanical nociceptive threshold obtained from at least 15 animals. $^{*}p < 0.05$ significantly different from control saline. ^ indicates oxaliplatin 2.4 mg/kg i.p. administration.

revised 1996; University of Florence assurance number: A5278-01). All efforts were made to minimize animal suffering and to reduce the number of animals used.

### 2.2. Drug administration

Oxaliplatin was purchased from Sequoia Research Product Ltd., USA, and administered by intraperitoneal injection (i.p.) at the dose of 2.4 mg/kg i.p. for five consecutive days every week (15 injections) according to a previously established treatment protocol [2]. Calphostin C was purchased from Calbiochem (USA) and intracerebroventricularly (i.c.v.) administered. Oxaliplatin was dissolved in 10 ml/kg saline volume; Calphostin C was dissolved in saline added to 0.5% DMSO and administered at 5 μl/rat volume.

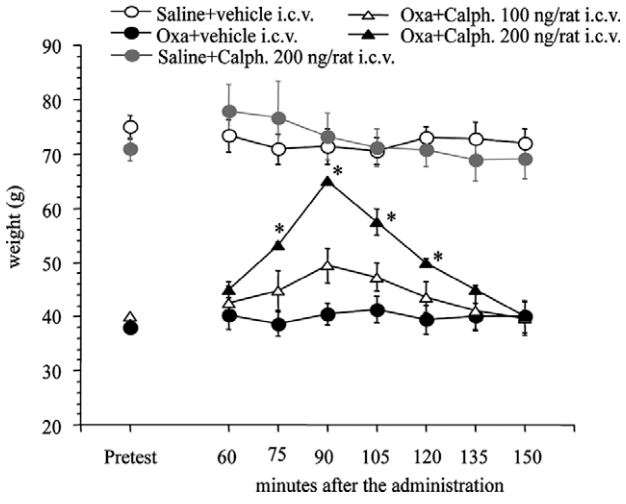


**Fig. 2.** Effect of Calphostin C on mechanical hyperalgesia induced by chronic oxaliplatin treatment. Mechanical hyperalgesia induced by oxaliplatin treatment is shown in the presence or absence of i.c.v. Calphostin C administration at different doses. Experiments were performed on day 21 after the start of oxaliplatin administration. Each point represents the mean ± SEM of at least eight rats. $^{*}p < 0.05$ significantly different from saline-treated rats. Vehicle, saline + 0.5% DMSO; Oxa, oxaliplatin; Calph., Calphostin C.

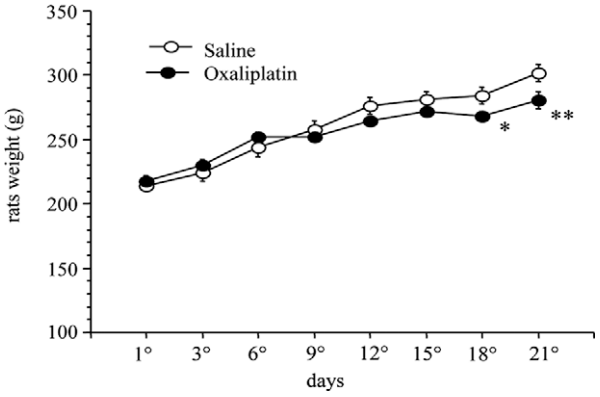


**Fig. 3.** Time-course of rat weight during repeated oxaliplatin administration. Graph describes weight increase of treated with respect to control rats. Each point represents the mean ± SEM of all treated rats. $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from saline-treated rats.

### 2.3. Intracerebroventricular injection technique

Rats, anesthetized with chloral hydrate (400 mg/kg i.p.) and located in a stereotaxic frame (Stellar, Stoelting, Wood Dale, IL, USA), were implanted with a plastic guide cannula (Metalant, Sweden) stereotaxically inserted through a skull hole drilled over the left lateral ventricle of the brain, AP = −0.8, L = −1.5 mm relative to the bregma, according to data from Paxinos and Watson [15]. The cannula was screwed into the skull hole until it reached a depth of 1 mm below the external surface of the skull and was secured to the bone with dental cement. After surgery, the rats were allowed to recover for 3 days in individual plastic cages. Saline, vehicle and drug solutions were injected in awake rats using a Hamilton 10-μl syringe that was inserted through the guide cannula to a depth of 3.8 mm below the external surface of the skull. The needle was left *in situ* for 30 s to allow the drug to diffuse.

### 2.4. Paw pressure test

The experiments were performed blind in a quiet room with randomization of treatment. Rats were habituated to handling by the investigator during the week before the experiments. The nociceptive threshold in rat was determined with an analgesimeter (Ugo Basile, Varese, Italy), according to the method described by Leighton

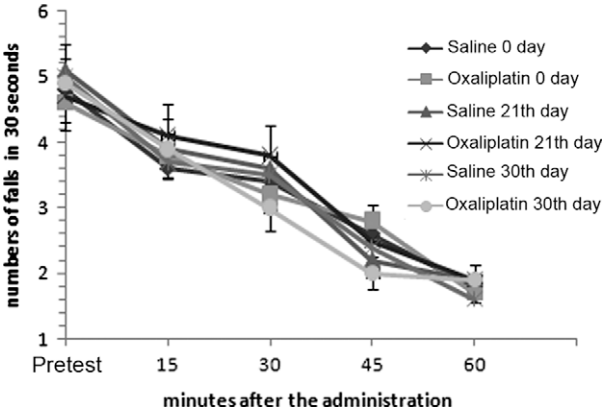


**Fig. 4.** Lack of effect of oxaliplatin treatment on rat motor coordination (rota-rod test). Graph shows that in saline and oxaliplatin-treated rat the number of falls in 30 s decreased every following session without any significant difference among the control and treated groups before starting of treatment (0 day), at the end of treatment (21st day) and 9 days after the end of treatment. Each point represents the mean ± SEM of at least 15 rats.

et al. [13]. Briefly, a constantly increasing pressure was applied to a small area of the dorsal surface of the paw using a blunt conical probe. Mechanical pressure was increased until vocalization or a withdrawal reflex occurred while the rat was lightly restrained. Vocalization or withdrawal reflex thresholds were expressed in gram. An arbitrary cut-off value of 250 g was adopted. Two series of rats were administered with oxaliplatin or saline at $t + 0$ of each treatment day. Nociceptive threshold was measured in rats 2 h before the end of days 1, 7, 14, 17, 19, 21, 22, 23, 26, 27 and 30.

### 2.5. Rota-rod test

The apparatus 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 30 cm from the base. The rod, 40 cm in length, was divided into five equal sections by six disks. The speed of the apparatus is 16 r.p.m. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 s according to Vaught et al. [21]. Those rats scoring less than 3 and more than 6 falls in the pretest were rejected. The performance time was measured before (pretest) and 15, 30, 45 and 60 min after the beginning of the test.

### 2.6. Western immunoblotting

At 21st day after oxaliplatin treatment started, the rats used for these experiments were decapitated under ethyl oxide anesthetic and the brain dissected, placed on dry ice and then stored at −80 °C. Various brain areas from control and treated rats were dissected on a cold plate. The tissue was mechanically homogenized in ice with lysis buffer containing 1 M Tris–HCl (pH 7.5), 10% sodium dodecyl sulfate (SDS), 40 mM *p*-nitrophenyl phosphate (PNFF), 57 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM sodium orthovanadate ($Na_3VO_4$), 100 mM sodium pyrophosphate (NaPP), 1.4 mg/ml aprotinin, 2 mg/ml leupeptine, 1 M NaCl, 100 mM EGTA and 50 mM EDTA. Homogenates were centrifuged at 10,000*g* for 15 min at 4 °C. The supernatant was collected and then stored at −80 °C. Protein concentration of the total fraction was quantified using Bradford's method (Protein Assay kit, Bio-Rad, Laboratories, Milan, Italy). Protein homogenates (30–100 μg) from control and oxaliplatin-treated rats were separated on 10% SDS–PAGE and transferred onto nitrocellulose membranes (110 min at 120 V) using standard procedures. Membranes were blocked in PBST 0.1% (PBS containing 0.1% Tween) containing 5% non-fat dry milk for 120 min. After washing, blots were incubated

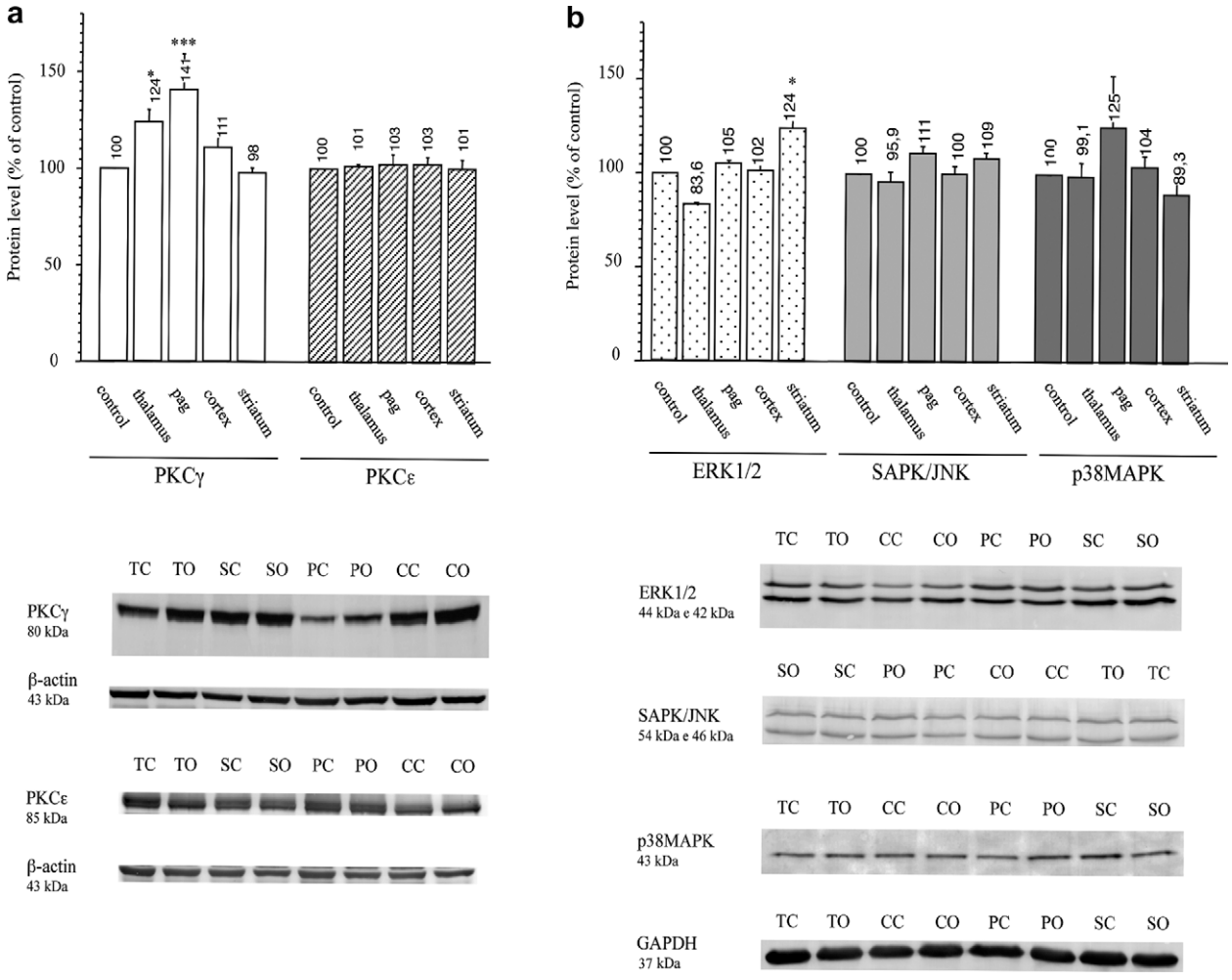


**Fig. 5.** Western blot assay of PKC and MAPK protein level in different brain regions. Effect of chronic oxaliplatin treatment on PKCγ (80 kDa) and PKCε (85 kDa) protein level in different brain regions in comparison with saline treatment are reported in (a). Effect of chronic oxaliplatin treatment on ERK1/2 (42–44 kDa), SAPK/JNK (46–54 kDa) and p38MAPK (43 kDa) level is reported in (b). Each bar represents the mean ± SEM of density level obtained from three independent experiments. Each single experiment is achieved from five pooled animals; density level is expressed for each considered brain region as percent of the control saline normalized to β-actin (43 kDa) (a) and GAPDH (37 kDa) (b). Homogenates from each single experiment were submitted at least three times to Western blotting. $^{*}p < 0.05$, $^{***}p < 0.001$ significantly different from control. A representative Western blot of a single experiment is visible at the bottom. TC, thalamus control; TO, thalamus oxaliplatin; CC, pre-frontal cortex control; CO, pre-frontal cortex oxaliplatin; PC, PAG control; PO, PAG oxaliplatin; SC, striatum control and SO, striatum oxaliplatin.

overnight at 4 °C with specific antibodies against PKCγ phosphorylated on Thr514 (p-PKCγ, 1:1000 dilution) (Biosource, Camarillo, CA, USA); PCKγ (1:1000), PCKε phosphorylated on Ser729 (p-PCKε, 1:750), PKCε (1:800) (Santa Cruz Biotechnology Inc., CA, USA); p38MAPK phosphorylated on Thr180/Tyr182 (p-p38MAPK 1:250), p38MAPK (1:500), ERK1/2 phosphorylated on Thr202/Tyr204 (p-ERK1/2 1:500), ERK1/2 (1:500), SAPK/JNK phosphorylated on Thr183/Tyr185 (p-SAPK/JNK 1:750) and SAPK/JNK (1:750) (Cell Signaling Technology). After being washed with PBS containing 0.1% Tween, the nitrocellulose membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antiserum (1:2000–1:5000) (Santa Cruz Biotechnology Inc., CA, USA) and left for 1–2 h at room temperature. Blots were then extensively washed according to the manufacturer's instruction and developed using enhanced chemiluminescence detection system (Millipore, Milan, Italy) or with colorimetric method (Opti-4CN substrate Kit Bio-Rad, USA). Densitometric analysis of scanned images was performed on a Macintosh iMac computer. Measurements in control samples were assigned a relative value of 100% β-Actin and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) were used as loading controls, respectively, for PKC and MAPK.

### 2.7. Statistical analysis

All experimental results are given as mean + SEM. Analysis of variance ANOVA, followed by Fisher's post hoc comparison, was used to verify significance between two means. Data were analyzed with the StatView software for the Macintosh (1992). *p* values of less than 0.05 were considered significant.

## 3. Results

### 3.1. Effect of oxaliplatin on mechanical nociceptive threshold

The mean baseline paw-withdrawal threshold of oxaliplatin-treated rats was not significantly different from that of control rats until 14th day after treatment started (Fig. 1). After that, paw-withdrawal threshold gradually decreased to lower mechanical nociceptive threshold (i.e., hyperalgesia) which lasted for 1 week after the end of treatment (Fig. 1). Baseline paw-withdrawal threshold was restored at 9th day after completion of oxaliplatin administration. When the oxaliplatin-treated rats were i.c.v. administered with protein kinase C (PKC)-specific inhibitor Calphostin C at different doses and then submitted to

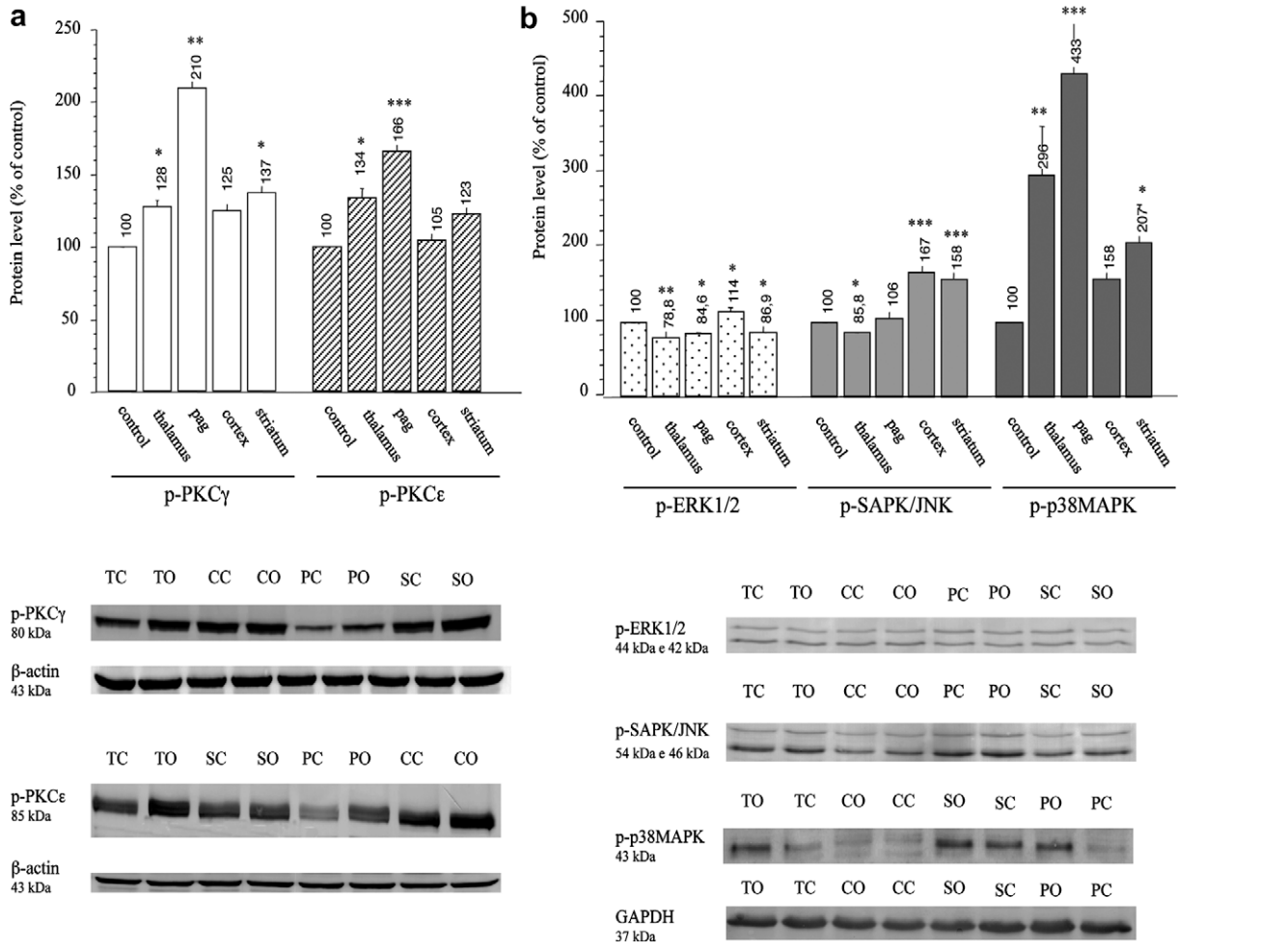


**Fig. 6.** Western blot assay of phosphorylated PKC and MAPK level in different brain regions. Effect of chronic oxaliplatin treatment on p-PKCγ and p-PKCε level in different brain regions in comparison with saline treatment is reported in (a). Effect of chronic oxaliplatin treatment on p-ERK1/2, p-SAPK/JNK and p-p38MAPK phosphorylation level is reported in (b). Each bar represents the mean ± SEM of density level obtained from three independent experiments. Each single experiment is obtained from five pooled animals. Density analysis was performed as previously. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from control. A representative Western blot of a single experiment is visible at the bottom. TC, thalamus control; TO, thalamus oxaliplatin; CC, pre-frontal cortex control; CO, pre-frontal cortex oxaliplatin; PC, PAG control; PO, PAG oxaliplatin; SC, striatum control and SO, striatum oxaliplatin.

paw-withdrawal test, the hyperalgesic effect was reversed at the higher Calphostin C dose. Calphostin C, when administered alone, did not induce any significant difference with respect to basal value (Fig. 2).

### 3.2. Effect of oxaliplatin on rat weight

Before initiation of oxaliplatin administration, the control and oxaliplatin-treated rats weighted, respectively, 214.4 ± 3.3 and 218.0 ± 4.1 g. Animals in treated and control group gained weight comparable throughout the first 2 weeks of the study. At the third week, the weight increase was significantly lower in oxaliplatin-treated rats (Fig. 3).

### 3.3. Rota-rod

The endurance time on the rotating rod, evaluated immediately before and 15, 30, 45 and 60 min at the end of the 3-week treatment (21st day) showed a lack of any impairment in the motor coordination of control and treated animals (Fig. 4). The same test performed before starting and 9 days after the end of treatment did not show any significant difference in motor coordination between treated and control groups.

### 3.4. PKC protein and phosphorylation level in chronic oxaliplatin-administered rat

Western analysis of PKC protein level was performed in thalamus, periaqueductal gray (PAG) matter, cortex and striatum samples from 21-day treated and control rats. There was a higher level of γ isoform of PKC in thalamus and PAG whereas ε level was not significantly different from control (Fig. 5a). A significant increase with respect to control was detected in thalamus and PAG for both γ and ε isoforms when phosphorylated, respectively, on Thr514 and Ser729 (Fig. 6a). Phosphorylated PKCγ level was significantly higher with respect to control in striatum (Fig. 6a). In the presence of PKC inhibitor Calphostin C, phosphorylation levels were completely or partially reversed to control value, respectively, in thalamus and PAG for PKCγ; phosphorylation levels returned to basal PKCε value (Fig. 7). PKC phosphorylation level did not show any significant difference from control in the sample from spinal cord for both γ and ε isoforms (Fig. 8).

### 3.5. p38MAPK protein and phosphorylation level in chronic oxaliplatin-administered rat

p38MAPK protein level was not significantly different from control in the considered brain regions (Fig. 5b). Phosphorylated

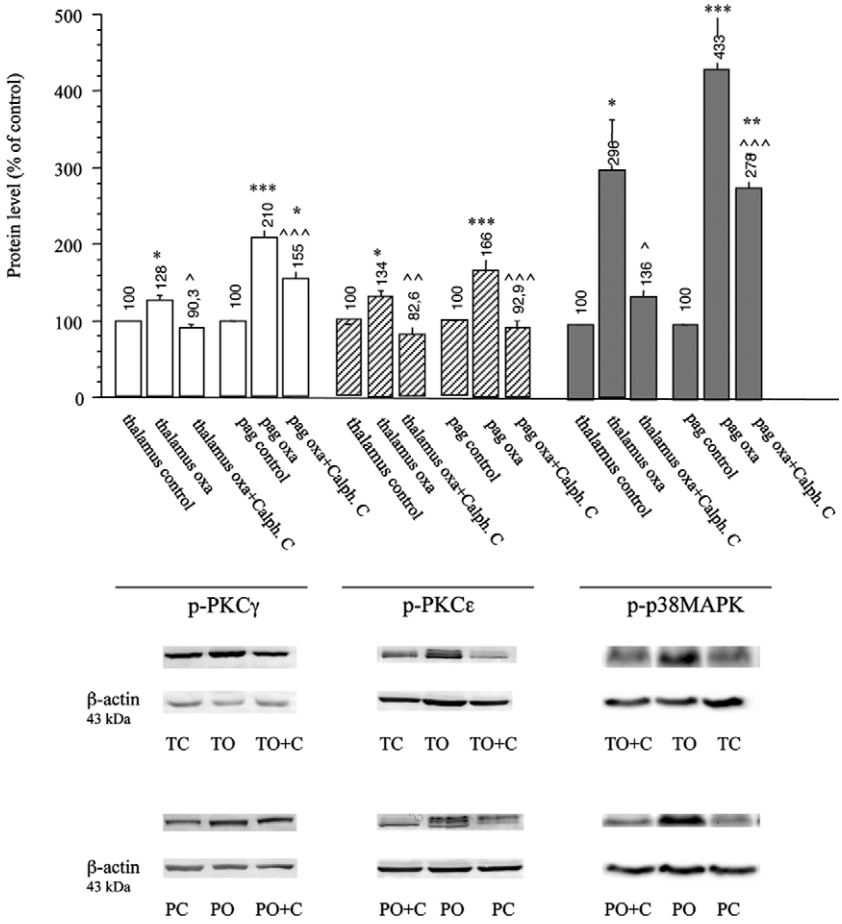


**Fig. 7.** Effect of Calphostin C on PKCγ, PKCε and p38MAPK phosphorylation level. Density analysis of PKCγ, PKCε and p38MAPK phosphorylated form level in PAG and thalamus after saline/control, oxaliplatin treatment in the presence or absence of Calphostin C (200 ng/rat i.c.v.) is represented in the upper figure. Each bar represents the mean ± SEM of at least five animals. Brain areas have been dissected at the time of the maximum effect (90 min after the administration of Calphostin C). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from saline/control and $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ significantly different from oxaliplatin. TC, thalamus control; TO, thalamus oxaliplatin; TC + C, thalamus oxaliplatin + Calphostin C.

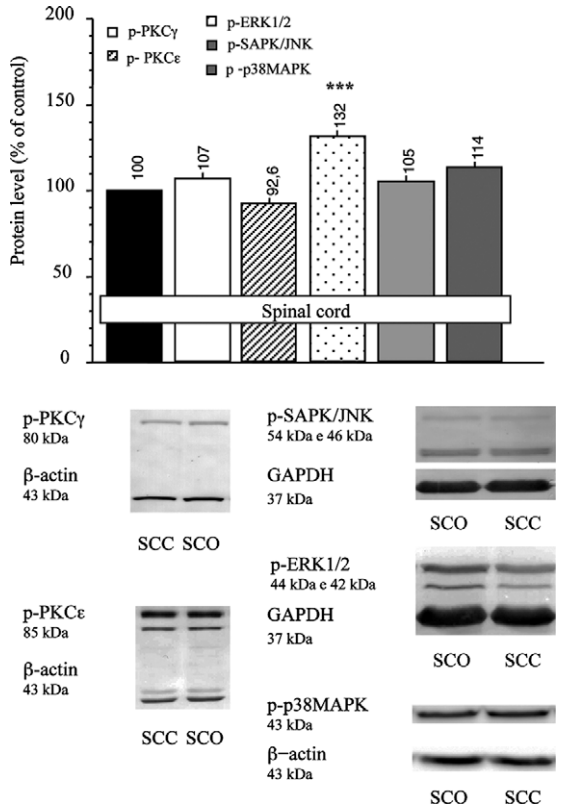


**Fig. 8.** Phosphorylation level of PKC and MAPK protein levels in the spinal cord. Effect of oxaliplatin treatment on p-PKCγ, p-PKCε, p-ERK1/2, p-SAPK/JNK and p-p38MAPK level in comparison with saline treatment. Results are expressed as the mean ± SEM of five different animals. Density level is expressed for each protein as percent of the control saline normalized to β-actin and GAPDH. $^{***}p < 0.001$ significantly different from saline/control. SCC, spinal cord control; SCO, spinal cord oxaliplatin.

p38MAPK level dramatically increased in PAG and thalamus (Fig. 6b); a significant increase was detected in striatal area (Fig. 6b). Calphostin C administration completely and partially reversed these values to control, respectively, in thalamus and PAG samples (Fig. 7). No significant change in phosphorylation could be detected in spinal cord (Fig. 8).

### *3.6. ERK1/2 and SAPK/JNK protein and phosphorylation level in chronic oxaliplatin-administered rat*

ERK1/2 and SAPK/JNK protein levels were not significantly different from control in the considered brain regions except in striatum where a mild significant increase could be detected for ERK1/2 (Fig. 5b). Phosphorylated ERK1/2 level significantly increased in cortex and decreased in striatum, thalamus and PAG (Fig. 6b). Phosphorylated SAPK/JNK level highly significantly increased in striatum and cortex whereas decreased in thalamus (Fig. 6b). Phosphorylation increase was revealed in spinal cord for ERK1/2 (Fig. 8).

## 4. Discussion

In our experiments, chronic administration of oxaliplatin to rats induced mechanical hyperalgesia as expected [12]. Oxaliplatin-treated rats showed no relapse in general status; weight time course of oxaliplatin-treated rats paralleled controls until 15th day after starting of the treatment even if weight increase was lower in treated animals at the time point 18–21 days. Since an altered motor coordination could lead to a misinterpretation of the results obtained, a rota-rod test was therefore performed and any alteration of the motor activity induced by chronic oxaliplatin administration was excluded. The neurotoxicity induced by oxaliplatin in humans and rodents is paired with a long time course painful peripheral hyperalgesia which often disappears within a few days to several weeks reappearing with repeat cycles of chemotherapy [11]. In our experiments, administration of oxaliplatin to rats five times a week for 2 weeks did not induce any significant change in paw pressure threshold. During the third week of treatment, paw-withdrawal threshold gradually decreased reaching values corresponding to the hyperalgesic state which lasted for many days after the end of treatment before returning to basal value. A significant difference in the intensity of hyperalgesia could not be found between the earlier and the later times after last oxaliplatin administration. We tested whether intracellular second messenger contributes to oxaliplatin-induced mechanical hyperalgesia in rats which were in a hyperalgesic state and were no more submitted to oxaliplatin administration from two days. When hyperalgesic rats were submitted to paw pressure in the presence of selective PKC inhibitor Calphostin C supraspinally administered, hyperalgesic effect could be reversed at the higher dose. This finding suggests that PKC activity in brain regions must be required to maintain the mechanical hyperalgesic state. Western blots, probed with specific antibodies, demonstrated that oxaliplatin chronic treatment induced a region-specific upregulation of γ isoforms of PKC within thalamus and PAG. Both these brain sites are implicated in nociceptive transmission, with the thalamus being the main termination site for spinothalamic pathway and the brainstem containing important descending modulatory structures such as PAG [8]. Western blots were also probed with polyclonal antibodies that specifically detect phosphorylation of Thr514 and Ser729, respectively, on γ and ε PKC isoforms. A dramatic phosphorylation increase for both isoforms could be observed in PAG from chronic oxaliplatin-treated rat. A significant increase was also observed in thalamus for both isoforms and in striatum only for PKCγ. Both γ and ε isoforms of PKC have been previously shown to be implicated in hyperalgesia [1,7]. A number of studies over the last several years indicate that brainstem descending pathways linking the PAG, the rostral ventromedial medulla (RVM) and the spinal cord constitute a major mechanism in the modulation of pain transmission [8]. In addition to descending inhibition, recent studies indicate that persistent pain after tissue or nerve injury is linked to an enhanced activation of descending modulatory influences from the brainstem or forebrain. Particularly, an increased net descending facilitatory drive leads to an amplification and spread of the pain [16,17,19,20]. When phosphorylation was investigated in rats chronically treated with oxaliplatin in the presence of PKC inhibitor Calphostin C supraspinally administered at the highest dose, PKCγ/ε phosphorylation was partially reversed in PAG and completely in thalamus suggesting that these isoforms of PKC are implicated in hyperalgesic effects induced by oxaliplatin administration. Activation of the PKC pathway can lead to phosphorylation of several intracellular targets recruiting distinct MAPK pathways, including p38MAPK, ERK1/2 and JNK. These MAPK pathways play a role as regulators of nociceptive sensitivity in various models of inflammatory pain and hyperalgesia to mechanical and thermal stimuli [14]. In our experiments, Western blot protein level detection of ERK, SAPK/JNK and p38MAPK was unmodified with respect to control in thalamus, PAG and cortex from chronic oxaliplatin rats. Phosphorylation level of these molecules decreased in thalamus for ERK and SAPK/JNK and in PAG and striatum for ERK whereas increased consistently in cortex and striatum for SAPK/JNK and mildly for ERK in spinal cord as previously shown in other kinds of neuropathic pain [4,6,18]. A dramatic increase in p38MAPK phosphorylated form could be observed in thalamus and PAG. These data show for the first time that alterations in protein and phosphorylation level of intracellular

signaling molecules at multiple supraspinal brain sites are induced by chronic oxaliplatin administration to rats. When PKC was inhibited at supraspinal sites by the administration of Calphostin C to rats previously chronically administered with oxaliplatin, a partial whereas significant reversion of phosphorylation in p38MAPK was obtained in PAG and thalamus whereas phosphorylation did not decline in other assayed signaling molecules. These data show that, in oxaliplatin-induced neuropathy, enhanced mechanical nociception is strictly correlated with increased phosphorylation of specific intracellular mediators in PAG and thalamus brain regions pointing to a role of these supraspinal centers in oxaliplatin-induced neuropathic pain mechanism through PKC/p38MAPK molecular signaling. Pharmacological modulation of these intracellular mediators may open the door for novel analgesic effective in oxaliplatin-induced neuropathic pain.

## Acknowledgment

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