Upregulations of $P2X_3$ and ASIC3 involve in hyperalgesia induced by cisplatin administration in rats

（シスプラチン投与により誘発される痛覚過敏における $P2X_3$ および ASIC3 の関与）

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Abstract

The role of ion channels expressed in sensory neurons on mechanical and thermal hyperalgesia were examined in a rat model of cisplatin-induced peripheral neuropathy. Rats were injected with 3 mg/kg of cisplatin intraperitoneally once per week for 5 consecutive weeks. The von Frey test, pin-prick test and Plantar-test were performed to examine any noxious sensitivity of the skin. The Randall-Selitto test of the gastrocnemius muscle (GM) and the measurement of grip forces were performed to quantify muscle hyperalgesia. Coordination/motor was assessed by Rota-rod testing. Expressions of the ion channels TRPV1, TRPV2, $P2X_3$ and ASIC3 were examined in dorsal root ganglion (DRG) neurons and the muscle afferent neurons innervating GM. Effects were evaluated of antagonists against either $P2X_3$ or ASICs on behavioral responses. Mechanical hyperalgesia and allodynia of both skin and muscle were observed in cisplatin-treated animals. Expressions of TRPV2, $P2X_3$, and ASIC3 increased in all DRG neurons. In addition, expressions of $P2X_3$ and ASIC3 also increased in muscle afferent neurons in DRGs. Antagonists against $P2X_{3,\ 2/3}$ and ASICs showed a suppressive effect on both skin and muscle hyperalgesia induced by cisplatin administration. Upregulation of TRPV2, $P2X_3$, and ASIC3 may play important roles in the mechanical hyperalgesia induced by cisplatin. Furthermore, cisplatin treatment also induced muscle hyperalgesia in muscle afferent neurons in connection with the upregulation of $P2X_3$ and ASIC3.

Keywords: Cisplatin; TRPV1; TRPV2; $P2X_3$; ASIC3; Hyperalgesia

1. Introduction

Cisplatin is an effective antineoplastic drug used extensively in the treatment of malignancies including ovarian, bladder, and testicular cancers [27,57]. It acts by crosslinking DNA and blocking its replication in rapidly dividing cells, thereby inhibiting DNA repair mechanisms and activating apoptosis and necrosis pathways [27,55]. However, it is also associated with serious adverse effects including nephrotoxicity and peripheral neurotoxicity. Sensory neuropathy involving muscle pain with complaints of the development of painful sensory disorders is the major side effect [32], sometimes requiring the cessation of anticancer therapy.

In animal studies, mechanical allodynia and hyperalgegia were observed following repeated injections of cisplatin in rats [3]. Cisplatin-associated neuropathy is believed to stem from the preferential uptake of the drug in DRG and the loss of sensory neurons [51] affecting large myelinated sensory nerve fibers. As for an algetic mechanism caused by cisplatin, it has been suggested that a decrease in magnesium levels by cisplatin administration might enhance nociception by increasing binding between NMDA receptors and glutamate [5], although the mechanism of induced neuropathic pain at the molecular level remains largely unknown.

Recent physiological, morphological, and molecular biological studies have elucidated the molecular mechanisms whereby primary sensory neurons detect pain-producing stimuli, thus initiating the processes referred to as nociception [29]. The distribution of ion channels which lead to a functional diversity in sensory signaling in neurons associated with pain pathways is becoming better understood [56]. The transient receptor potential channel, a vanilloid subfamily member 1 (TRPV1), is gated by heat temperature (>43 °C), vanilloids (capsaicin, resineferatoxin), or non-vanilloid endogenous ligands such as protons, the endocannabinoid anandamide and lipoxygenase products [11,12,25,50] involved in several types of pain including neuropathic pain [24,43]. TRPV2, a TRPV1 homologue, is activated by a very high-threshold heat temperature (>52 °C), which has been suggested to act as a high-threshold temperature sensor in Aδ nociceptors [1,21]. $P2X_3$ receptor, one of the ligand-gated ion channels activated by extracellular adenosine triphosphate (ATP) [14], in sensory neurons plays a role in pain transmission [10]. Acid sensing ion channels (ASICs) open when the extracellular pH drops [33]. One member of the ASIC family, ASIC3, is found in large- and small-diameter primary afferents in rodents. The roles of these ion channels, however, have not yet been examined in chronic hyperalgesia induced by the administration of cisplatin.

In the present study, we adopted a rat model of cisplatin-induced peripheral neuropathy to clarify the involvement of ion channels underlying the development of painful neuropathy. In addition to cutaneous pain, we also assessed muscle pain, the mechanisms of which are poorly understood. The characterization of the molecular system that mediates hyperalgesia in this model is important in developing novel clinical tools for promoting relief from the painful symptoms of cisplatin neuropathy.

2. Materials and methods

2.1 Experimental animals

Seventy male Sprague-Dawley rats (SLC, Hamamatsu, Japan) weighing around 388 g were used in this study. They were exposed to a light-dark cycle (L: D, 12: 12-h) and kept in a temperature-controlled room (23° C) with food and water ad libitum. This study was conducted under the auspices of the local animal ethics committee in accordance with the Regulations for Animal Experiments in Nagoya University Graduate School of Medicine (permission No. 19108), the Animal Protection and Management Law of the Japanese Government (No. 105), and the guidelines of the International Association for the Study of Pain [58].

2.2. Cisplatin administration

The rats were divided into two groups comprised of a cisplatin-treated and saline-treated control group. The former group was injected with cisplatin (Sigma, St. Louis, MO, USA) according to a previously described protocol [4]. Briefly, cisplatin was diluted in normal saline (0.9% NaCl) just before administration to a final concentration between 0.08 and 0.15 mg/ml, ensuring that volumes of less than 0.5 ml would be injected into the peritoneal cavity. Cisplatin was administered intraperitoneally once a week at a dose of 3 mg/kg (cumulative dose, 15 mg/kg) for 5 weeks. Before each injection, 2 ml of sterile saline solution was given subcutaneously to prevent renal damage via

hyperhydration. Treatments were randomized within each cage of rats (cisplatin and saline). To avoid acute effects, the injections were therefore given after the behavioral tests were performed. The control group was intraperitoneally injected with the same volumes of saline (0.9% NaCl) calculated according to the animal's weight.

2.3. Assessment of general toxicity

Body weights (g) were measured before each injection and 7 days following the final injection. All rats were examined daily to detect abnormal clinical signs such as piloerection, hindlimb weakness, gait disturbance, or gastrointestinal disorders such as diarrhea.

2.4. Behavioral testing

Analyses of the behavioral experiments were conducted by experimenters blind to the experimental conditions. All behavioral tests were carried out prior to each injection of cisplatin.

2.4.1 Assessment of mechanical sensitivity of hind paws

Rats were placed in cages with an elevated metal mesh floor covered with transparent plastic boxes and allowed to acclimate to their surroundings for at least 20 min prior to the initiation of behavioral testing.

To test for the presence of mechanical allodynia, the paw withdrawal threshold value was examined with a von Frey-type filament (0.5 mm in diameter) by applying increasing force to the plantar surface of the hind paw, starting under the threshold of detection and increasing until the animal withdrew its paw (Dynamic Plantar Aesthesiometer, Ugo Basile Inc., Comerio, Italy). The force in grams when the paw was withdrawn was registered automatically. A 50-g limit was imposed as the cut-off to preclude tissue damage. Each animal was tested five times at intervals of 5 s, and the 5 measurements were averaged. The presence of mechanical hyperalgesia was determined according to previously described methods [53] by pressing the plantar surface of the hind paw with the point of a safety pin at an intensity sufficient to produce a reflex withdrawal response in normal animals but insufficient to

penetrate the skin. The duration of the pin-prick-evoked hind paw withdrawal was measured with a stopwatch. Since normal responses were of such very short duration too quick to time accurately by hand, they were therefore arbitrarily assigned a duration of 0.1 s.

2.4.2 Assessment of mechanical sensitivity of gastrocnemius muscle (GM)

Mechanical hyperalgesia of the gastrocnemius muscle (GM) was measured as a paw-withdrawal threshold in response to a mechanical stimulus across the calf muscles using a Randall-Selitto apparatus (Ugo Basile, Comerio, VA, Italy). Mechanical hyperalgesia of the skin over the GM was measured using von Frey hairs (North Coast Medical, San Jose, CA, USA). Our training sessions were carried out for at least 3 days before cisplatin injection. The animals were allowed to acclimate to their surroundings for at least 10 min before testing. They were restrained with a towel around their trunk, and the Randall-Selitto probe and von Frey hairs were applied to their calves. In the Randall-Selitto test, the force applied was increased at a constant rate of 16 g/s until the animal withdrew its paw. Measurements were performed 5 times at intervals of several seconds , and the mean value was taken as the threshold. The frequency of nociceptive responses to von Frey hairs was calculated from 5 trials. For each trial, the filament was applied at intervals of several seconds. The nociceptive threshold was defined as the minimum pressure needed to evoke nociceptive responses in at least 60% of the trials. The experimenter was blind to which group an animal belonged.

2.4.3 Assessment of thermal sensitivity

Heat sensitivity of the hind paw was assessed using the Plantar-test (Ugo Basile) [6,22]. Animals were placed in cages with a glass floor covered with transparent plastic boxes and allowed to acclimate to their surroundings for a minimum of 20 min before testing in a temperature-controlled room (23 °C). A high-intensity, movable radiant heat source was placed underneath the glass and aimed at the plantar surface of the hind paw. Care was taken to initiate the test when the animal was at rest not walking, with its hind paw in contact with the glass floor of the test apparatus. The stimulus onset activated a timer that was controlled by a photocell. The hind paw withdrawal reflex interrupted the photocell's light and automatically stopped the timer. Latencies of the reflex were measured from the onset of radiant heat until hind-paw withdrawal. Each animal was tested five times at intervals of 5

min. The light intensity was adjusted at the beginning of the experiment in order to produce latencies of approximately 10 s in normal naïve animals, and kept constant thereafter.

2.4.4. Grip force assays

To evaluate muscle hyperalgesia, fore- and hindlimb grip forces were measured using a hand-made grip force analyzer similar to the one described by Kehl et al. [30]. This apparatus measured the amount of tensile force each rat exerted against a wire mesh grid (15 ×15 cm^2) attached to a force transducer (Model-RX-100, Aikoh Engineering, Nagoya, Japan). Forelimb and hindlimb grip force measurements were obtained. For measuring forelimb grip force, the apparatus was placed in front of the rat facing the wire mesh grids, to measure hindlimb grip force, it was placed behind the rat. During testing, each rat was held by its tail, gently passed over the wire mesh grids, and allowed to grasp them. The force transducer converts the grip force to a digitized signal. The duration of the force each animal applies to the mesh grid is self-dependent. Therefore, the level of force exerted is subject to evaluate factors such as muscle hyperalgesia that influence the behavioral performance of the animal. Three consecutive grip force measurements were obtained for each animal at each time point, the average of which was used to represent each animal's grip force for each time point.

2.4.5. Rota-rod performance assessment

The rota-rod (rotating rod) test [17] is widely used in rodents to assess their "minimal neurological deficit", such as impaired motor function (e.g., ataxia) and coordination [45]. To rule out the possibility of motor impairment caused by cisplatin administration affecting the results of the behavioral test, each rat's coordination/motor performance after cisplatin administration was measured using an accelerating rota-rod apparatus (model LE8500, Ugo Basile) by a method previously described [7]. Briefly, in a training session, rats were placed on the rod that was set to accelerate from 3 to 30 rpm over a 180-s period, and the performance time that each rat was able to remain on the rota-rod was recorded. Rats were subjected to three training trials at 3- to 4-h intervals on two separate days for acclimatization purposes. In the test session, rats were placed on the rota-rod and their performance times were recorded up to a cut-off time of 180 s. Each animal was tested three times at intervals of 5 min, and the 3 measurements were averaged.

2.5. Retrograde labeling of muscle sensory neurons

The GM afferent neurons in DRGs were identified via retrograde-labeling with hydroxy-stilbamidine [Fluoro-gold (FG); Fluorochrome, Englewood, CO, USA]. After the final behavioral experiment, the animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Nembutal, Abbott Laboratories, Chicago, IL, USA). A dorsal incision from the ankle to the knee was made, and the GM was exposed by opening a space between the biceps femoris muscle. Using a 100-μl Hamilton syringe with a 30-gauge needle, 4% FG in saline (50 μl) was injected into the GM. Control animals were also given FG, after which they were allowed to survive for 1 week.

2.6. Tissue preparation

One week after the injection of FG, cisplatin-treated rats (n=4) and saline-injected control rats (n=4) were each anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Nembutal, Abbot Laboratories), and transcardially perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). L5 DRGs were immediately dissected out after perfusion, and immersed in the same fixative for 4 h at 4 ºC. Post-fixed DRGs were kept in PBS containing 20% sucrose for cryoprotection. The samples were then embedded in Tissue-Tek (Sakura Finetechnical, Tokyo, Japan) and stored until cryosectioning at -20 ºC. The DRGs were serially cut on a cryostat at a thickness of 10 μm on a horizontal plane along the long axis of the ganglion. Sections were thaw-mounted on Superfrost Plus microscope slides (Matsunami, Tokyo, Japan) and dried at room temperature overnight.

2.7. Immunohistochemistry

After dilution in 0.1 M phosphate buffered saline (PBS) containing 1.5% normal goat serum and 0.3% Triton-X 100 (Sigma), DRG sections were incubated with either guinea pig polyclonal antiserum against synthetic rat ASIC3 (1:10; Neuromics Antibodies, Bloomington, MN, USA), rabbit polyclonal antiserum against synthetic rat TRPV1 (1:10; Trans Genic, Kumamoto, Japan), rabbit polyclonal

antiserum against synthetic rat TRPV2 (1:1000; Sigma), or rabbit polyclonal antiserum against synthetic rat $P2X_3$ (1:500; Neuromics). Sections for ASIC3 were reacted with reagents for 2 days at room temperature and others at 4 °C. After being rinsed with 0.1 M PBS, sections were reacted in PBS with fluorescein-isothiocyanate (FITC)-conjugated goat anti-guinea pig or -rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) at a concentration of 1:100. After being rinsed with 0.1 M PBS, the sections were cover-slipped in mounting medium (Immunon, Pittsburgh, PA, USA) and examined under a fluorescence microscope equipped with a digital camera.

Using appropriate filters, FG-labeled, FITC-labeled, and double (FG and FITC)-labeled cells were observed. To discriminate positive immunostaining from the background, the cells showing a staining intensity of 2-fold or more than that of the average background were considered positive for immunoreactivity. No specific labeling was observed in the absence of a primary antibody.

The numbers and sizes of FG-labeled and FITC-labeled cells were automatically counted and measured using a computer-assisted imaging analysis system (Scion Imaging Software, Scion Corporation, Frederick, MD, USA). The FG-labeled along with the ASIC3-, TRPV1-, TRPV2-, or $P2X_3$-immunoreactive cells were arbitrarily classified by cell diameter into 6 groups (<10 μm, 10-20 μm, 20-30 μm, 30-40 μm, 40-50 μm, >50 μm). The ratios of ASIC3-, TRPV1-, TRPV2-, and $P2X_3$-immunoreactive cells in each animal were calculated by the following formula: (total number of FITC-labeled immunoreactive cells in 4 sections of DRG/ total number of all cells in 4 sections of DRG) × 100. And the ratios of ASIC3-, TRPV1-, TRPV2-, and $P2X_3$-immunoreactive cells in muscle afferent neurons were calculated by the following formula: (total number of double (FG and FITC)-labeled cells in 4 sections of DRG / total number of FG-positive cells in 4 sections of DRG) × 100. The calculation was performed for each group of cells classified by cell size. Histological analysis was conducted blind to the treatments.

2.8. Administration of morphine or antagonists

To test whether the hind paw-withdrawal responses and grip forces qualify as valid measurements of nocifensive behavior following mechanical stimulation of the skin or muscle, the animals were tested with morphine. Morphine dissolved in saline (6 mg/kg) was injected intraperitoneally. Control animals received an equivalent volume of saline. Behavioral tests were performed 30 min after the morphine treatment.

The effects of the administration of antagonists on behavioral experiments were studied either one week after the last cisplatin injection in cisplatin-treated animals or the last saline injection in saline-treated control animals. The antagonists used were the ASICs antagonist amiloride (Sigma) dissolved in dimethyl sulfoxide (DMSO) (112.8 μmol/kg, i.p.), and $P2X_{3,\ 2/3}$ antagonist A-317491 (Sigma) dissolved in saline (100 μmol/kg, s.c.). The doses of each antagonist used in this study had proven effective in previous studies [18,35]. The effects of vehicles were also examined by administering them at the same volume as that of antagonists.

2.9. Statistical analysis

The data were expressed as mean ± SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA) with repeated measures, two-way ANOVA with repeated measures followed by Holm-Sidak multiple comparison tests if warranted, or Student's *t*-test where appropriate. The data of von Frey testing on the calves were analyzed using non-parametric two-way repeated measures ANOVA on ranks followed by a Holm-Sidak comparison if warranted. A P value of <0.05 was considered significant. Data were analyzed using SigmaStat 3.5 statistical software (Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Assessment of general toxicity

Most animals received either cisplatin or saline injections as scheduled in the protocol. Ten percent of the rats in the cisplatin group died during the experimental period, whereas all rats in the saline control group survived the experiment.

In the cisplatin group, body weight gain was significantly reduced compared to that in the saline group from 1 week after the initial administration ($P<0.05$, −3.1%, cisplatin 376.1 ± 8.4 vs control 388.8 ± 12.1 g) to the end of the experiment, with the maximum decrease occurring 1 week after the

final administration ($P < 0.01$, −27%, 311.8 ± 10.8 vs 425.7 ± 11.7 g) (Table 1). Some of the cisplatin-treated rats showed a definite change in their behavioral activity characterized by a sick gait involving toe walking with their backs arched. However, no other behavioral changes such as reduced motor activity or motor disturbances were observed.

3.2. Behavioral testing

3.2.1. Changes in mechanical sensitivity of hind paws

Mechanical allodynia of the rat hind paws was evaluated using a dynamic plantar aesthesiometer following cisplatin injection (Fig. 1A). Mechanical allodynia developed after cisplatin injection, and a significant decrease in withdrawal thresholds compared to those of the saline group was observed in 100% of cisplatin-treated rats on days 28 and 35, with a maximum decrease on day 28 ($P < 0.01$). No change in the withdrawal threshold was observed at any time in the saline-treated group. Mechanical hyperalgesia of the hind paw was also evaluated by a pin-prick test following cisplatin injection (Fig. 1B). Significant increases in withdrawal durations compared to those in the saline group were observed on days 28 ($P < 0.01$) and 35 ($P < 0.05$) in the cisplatin group.

3.2.2. Changes in mechanical sensitivity of GM

The mechanical sensitivity of GM was measured by a Randall-Selitto apparatus applied to the GM. A significant reduction in the withdrawal threshold in the cisplatin group (100% rats) compared to the saline group was observed from day 14 to the end of the experiment, indicating the development of mechanical hyperalgesia (Fig. 1C). The maximum decrease appeared 1 week after the final injection (day 35, $P < 0.01$). With regard to the saline group, no decrease in the withdrawal threshold was observed during the course of the experiment. The mechanical withdrawal threshold on the surface skin of the GM measured by von Frey hairs showed a significant decrease in the cisplatin group compared to the saline-treated controls (Fig. 1D), which showed no change at any time.

3.2.3. Changes in thermal sensitivity

In contrast to the mechanical hyperalgesia, no significant changes in paw withdrawal latency with the plantar test were observed at any time in either the cisplatin-treated or saline-treated control rats (Fig. 1E).

3.2.4. Changes in grip force

During the first three weeks of cisplatin treatment, the grip force level did not change compared to that in the control group. In the forelimbs, signs of grip force weakness began to develop in the cisplatin group from day 21, while in the control animals no changes were observed (Fig. 2A). The maximum decrease in the cisplatin group was detected on day 28. In the hindlimbs, grip force in the cisplatin group declined significantly from day 28, showing a maximum decrease on day 35, while remaining unchanged in the control animals (Fig. 2B).

3.2.5. Changes in motor performance

In the rat rota-rod test, administrations of cisplatin had no significant effect on motor performance at any time when compared with the control rats (Fig. 2C).

3.3. Immunohistochemistry

3.3.1. Retrograde tracing and immunofluorescence

After immunohistochemistry the ion channels TRPV1, TRPV2, $P2X_3$ and ASIC3, immunopositive neurons in L5 DRG were stained a brilliant light green in color (indicated by arrows and large arrowheads in Figs. 3A, C, E and G), and could be readily distinguished from the non-positive cells. The presence of ion channels-immunoreactive GM sensory neurons were examined using retrograde-tracing of the fluorescent neuronal tracer hydroxy-stilbamidine (Fluoro-gold, FG) combined with immunofluorescence techniques. Retrogradely-labeled neurons were observed in the DRGs one week after injections of FG into the GM (denoted by arrows and small arrowheads in Figs. 3B, D, F, and H). Ion channel-immunoreactive GM sensory neurons were represented by FG-labeled cells that were also labeled with each antibody (indicated by arrows in Fig. 3).

The total number of neurons in L5 DRG was not significantly different between cisplatin-treated (282.2±14.7/section) and saline-treated control (300.9±25.5/section) animals. The frequency of FG-positive neurons in the L5 DRG neurons innervating GM showed no significant differences between cisplatin-treated (12.3±1.0％) and saline-treated control (10.7±0.8％) animals. Table 2 shows the Frequency of TRPV1, TRPV2, $P2X_3$, and ASIC3 immunoreactive neurons in all DRG neurons and retrogradely-labeled GM afferent neurons.

3.3.2 TRPV1 expression

There were no changes in the frequency of TRPV1-positive cells (Fig. 3A, arrow and large arrowhead) in any DRG neurons as well as in any group of neurons classified by cell diameter between the cisplatin-treated and saline-treated control rats (Fig. 4A, B). Using retrograde-tracing of the fluorescent neuronal tracer FG, the retrogradely-labeled GM afferent neurons were observed in the L5 DRG (Fig. 3B, arrow and small arrowhead). FG-positive cells containing TRPV1 were present (Fig. 3A arrow). The expressions of TRPV1 in the L5 DRG neurons innervating GM showed no significant differences between the cisplatin-treated and saline-treated control animals (Fig. 4B).

3.3.3 Alterations of TRPV2 expression (Fig. 5)

TRPV2-positive cells significantly increased in the small cell group of all L5 DRG neurons after cisplatin administration. Although L5 DRG cells innervating GM (FG-positive cells) expressed TRPV2, no significant differences were observed in the number of TRPV2-positive cells innervating GM compared to those in the control group.

3.3.4 Alterations of $P2X_3$ expression (Fig. 6)

$P2X_3$-positive cells significantly increased in both populations of all DRG neurons and GM afferent neurons after cisplatin administration (Fig. 6A).

3.3.5 Alterations of ASIC3 expression (Fig. 7)

ASIC3-positive cells significantly increased in the small- and medium-sized cell groups in all DRG neurons after cisplatin administration (Fig. 7A, B). The expression of ASIC3 in the L5 DRG neurons innervating GM showed significant increases in the medium-sized cell group after cisplatin administration compared to that in the control (Fig. 7A, B).

3.4. Effects of morphine and antagonists on hyperalgesia induced by cisplatin

3.4.1. Morphine treatment

At the plantar surface of the hind paw, the withdrawal threshold to mechanical stimulation and withdrawal latency to a radiant heat source rose significantly in both the control and cisplatin groups following intraperitoneal morphine (Fig. 8A, B). The mechanical threshold of GM assessed by Randall-Selitto apparatus and that of the surface skin of the GM assessed by von Frey filaments increased in the cisplatin group, but not in the control (Fig. 8C, D). As for grip force, no effect of morphine was observed in the control group, while the threshold decline in the cisplatin group was recovered (Fig. 8E). At the dose (6 mg/kg) used in this study, on the other hand, no morphine effect was observed in either the control or cisplatin group on rota-rod performance (Fig. 8F).

3.4.2. $P2X_{3,2/3}$ antagonist treatment

We examined the effect of selective $P2X_{3,2/3}$ receptor antagonist A-317491 in behavioral experiments [28]. In the cisplatin-treated animals, systemic administrations of A-317491 suppressed both cutaneous and muscle hyperalgesia as assessed by the hind paw-withdrawal threshold to mechanical stimuli or grip force without motor performance degradation (Fig. 9). A-317491 showed no effect on either cutaneous or muscle mechanical sensitivity in control animals. Vehicles of the antagonists (saline) produced no changes of mechanical sensitivity in either cisplatin-treated or control animals.

3.4.3. ASICs antagonist treatment

We also examined the effect of an ASICs antagonist amiloride. In the cisplatin-treated animals, the intraperitoneal administration of amiloride revealed an inhibition of mechanical hyperalgesia without motor performance degradation (Fig. 10), while producing no effect in control animals. The vehicle of the antagonist, DMSO, was not effective in either cisplatin-treated or saline-treated control animals.

4. Discussion

4.1 Cutaneous and muscle hyperalgesia in experimental model of cisplatin neuropathy

In this study, we were able to observe both mechanical allodynia and hyperalgegia after cisplatin administration. Neither motor disturbance, muscle weakness, nor reduced motor activity were observed, while diminishing body weight gains were apparent in cisplatin-treated animals. Although food and water consumption might be affected by cisplatin administration, our data confirmed the results of a previous study showing that a protocol of five weekly intraperitoneal injections of 3 mg/kg proved to be a good compromise between preserving the general clinical status of the rats and inducing of nociceptive neuropathy [4].

In the present study, cisplatin treatment induced cutaneous mechanical allodynia and hyperalgesia but no alteration in noxious thermal sensitivity. Our data of mechanical allodynia and hyperalgesia confirmed the prior study that used the same cisplatin administration-protocol [4]. On the other hand, the effects produced by cisplatin on thermal sensitivity remain controversial. Some studies have reported thermal hypoalgesia [4,9], whereas others have found no change [15,54] in the responses to thermal stimulation following treatment with cisplatin. Further studies are needed to define the alteration of thermal sensitivity in cisplatin neuropathy.

In the present study, mechanical sensitivity of the animals' calves was behaviorally assessed using the Randall-Selitto apparatus and von Frey filaments to measure the mechanical sensitivity of the GM. Scince the tip of the Randall-Selitto apparatus (ϕ 5 mm) is much larger than that of the von Frey filaments, the force applied can be transmitted deeper through the skin to measure the mechanical hyperalgesia of deep tissue such as GM. In contrast, it is unlikely that the mechanical stress applied to

the skin by von Frey filaments is transmitted deeply enough because the tip diameter of the filament is so small (< 0.5mm) [52]. In fact, Randall-Selitto thresholds of our data were about 10 times larger than von Frey filaments thresholds, supporting our assertion. In our study, cisplatin treatment reduced the mechanical withdrawal threshold of the calves as measured by both the Randall-Selitto apparatus and von Frey hairs. We interpreted this result as mechanical hyperalgesia originating from skin and/or deep tissue such as GM. We could not rule out the possibility that mechanical hyperalgesia in the overlying skin might have contributed to the decreased withdrawal thresholds assessed by the Randall-Selitto apparatus.

Muscle pain has been shown to lead to reduced grip strength through the inhibition of alpha- [31] and gamma-motor neurons [36] in agonist muscles after noxious chemical stimulation. The reduction in grip force evoked by intramuscular carrageenan was reversed by analgesic drugs, suggesting that a reduction in grip force provided a valid assessment of muscle hyperalgesia [30]. In the present study, using methods similar to those of Kehl et al. [30], we obtained a reduction in grip force after cisplatin administration. Thus both measurements used in this study, i.e, pressure hyperalgesia by the Randall-Selitto test and a reduction in grip force, indicated the presence of muscle hyperalgesia in cisplatin-treated rats.

In our cumulative-dose protocol (15 mg/kg), cisplatin treatment had no effect on motor performance in the rota-rod test. This finding is consistent with previous reports that the motor nerve conduction velocity remained electrophysiologically unaffected, while the sensory nerve conduction velocity was reduced [15]. A previous study reported that administrations of cisplatin (total dose 32 mg/kg) intraperitoneally in female Dark Agouti rats produced reductions in both rota-rod performance and gait disturbance [9]. This discrepancy with our results is most likely related to the total dosage of cisplatin we used (15 mg/kg) being less than that in the previous study, so that the effect on rota-rod performance by cisplatin might be kept to a minimum.

4.2 Involvement of ion channels in cisplatin neuropathy

There was no change in the frequency of TRPV1-positive cells in either all DRG neurons or muscle afferent neurons after cisplatin administration compared to the saline administered control animals. An increase in the TRPV1 expression level and its involvement in hyperalgesia has been reported in uninjured DRG following peripheral nerve injury [19,24]. As previously reported [2], there might be

different mechanisms underlying the pain and hyperalgesia in toxic neuropathy like those in our cisplatin-induced rats compared to those in other painful neuropathies following peripheral nerve injury. Although a protective role of TRPV1 in the development of mechanical hyperalgesia has been suggested in a study of the TRPV1 -/- mouse [8], no pronociceptive role has been reported for cisplatin-induced toxic neuropathy.

In our present study, though TRPV2-positive cells significantly increased in the small-cell group after cisplatin administration, thermal sensitivity remained unchanged. A recent study reported the upregulation of TRPV2 protein levels in DRG neurons after intraplantar injections of CFA, suggesting a role for TRPV2 in peripheral thermal sensitization during inflammation, possibly via the transduction of pain hypersensitivity to highly noxious temperatures [47]. In addition to thermal nociceptive processing, it was reported that TRPV2 might be activated by mechanical stimuli [26], which was demonstrated to mediate mechanosensation [38,41]. Because selective TRPV2 antagonists are not commercially available, so far, further mechanistic studies including TRPV2 knockout mouse might be needed to determine TRPV2 involvement in cisplatin neuropathy. In any case, it is interesting to note that TRPV2-positive cells significantly increased without any change in thermal sensitivity after cisplatin treatment.

Involvement of the $P2X_3$ subunit-containing receptors plays a central role in mediating the abnormal nociceptive responses in neuropathic pain following chronic constriction injury (CCI) [46] and spared nerve injury (SNI) [13]. In the present study, we found an increased expression of $P2X_3$ receptors in both DRG and the FG-positive-GM sensory neurons. A previous study revealed that DRG neurons innervating the gastrocnemius-soleus muscle exhibited $P2X_3$ and were sensitive to ATP [23]. It is conceivable that an up-regulation of $P2X_3$ receptors in the soma might lead to an increase in $P2X_3$ receptor expression at both the central and peripheral terminals [39]. Our study showed that systemic administration of A-317491, a non-nucleotide, $P2X_{3,2/3}$ selective antagonist [28], inhibited the mechanical allodynia, hyperalgesia, and grip-force weakness associated with cisplatin administration. Our morphological and pharmacological results indicated that the up-regulation of $P2X_3$ is involved in the mechanical allodynia and hyperalgesia observed in both skin and muscle in this model. P2X receptors were shown to play a role in the masseter muscle hyperalgesia induced by the exertion of that muscle [48]. ATP released from damaged muscle [20] might also be involved in muscle pain [23,34,44].

We found that the expression of ASIC3 receptors increased in small and medium-sized DRG neurons as well as in medium-sized FG-positive-GM sensory neurons. As a result of antagonism by amiloride, the mechanical hyperalgesia of both skin and muscle after cisplatin treatment were inhibited. Although a well-characterized ASIC antagonist, amiloride, possesses little subtype selectivity and is also actively engaged at other sodium-selective channels, our immunohistochemical and pharmacological results indicate that ASIC3 very likely participates in cutaneous and muscle hyperalgesia in this cisplatin-treatment model. ASIC channels (ASICs) belong to a superfamily of amiloride-sensitive cationic channels [16]. ASIC3, a member of the ASICs, has been shown to have a necessary function in low- and high-threshold mechanoreception as well as in acid and heat nociception [42]. A recent study reported that an increase in the ASIC3 expression level and its involvement in mechanical allodynia following L5 spinal nerve ligation induced neuropathy [40]. In the context of muscle sensation, ASIC3 has been suggested to play a critical role in the development of mechanical hyperalgesia associated with muscle inflammation, and is a mechanosensor candidate [49]. ASIC3-positive neurons also have properties thought to be as metaboreceptors of muscles, i.e, neurons that sense the metabolic state of muscles and can trigger pain when there is insufficient oxygen [37]. Our results suggest that ASIC3 is an important component in mediating mechano-nociception and in the development of mechanical hyperalgesia induced by cisplatin injection in both skin and muscle.

We explored the role of ion channels expressed in DRG neurons in the painful neuropathy associated with cisplatin administration. Upregulations of TRPV2, $P2X_3$ and ASIC3 may play important roles in the mechanical hyperalgesia induced by cisplatin. In addition to cutaneous hyperalgesia, cisplatin treatment might also induce muscle hyperalgesia associated with upregulations of $P2X_3$ and ASIC3. Interfering with these channels may prove to be a promising therapeutic target for treating painful symptoms of cisplatin neuropathy, and may further be able to ensure the continuation of anticancer therapy.

Conflict of interest

The authors declare that there are no conflicts of interest related to the study.

Acknowledgements

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Table 1
Body weights of cisplatin-treated and saline-treated control rats.
Body weights (g) were expressed as mean ± S.E.M. A significant difference with the saline group was observed from day 7 to the end of the study. Data were analyzed using a two-way repeated measurement ANOVA followed by Holm-Sidak comparisons.

Days after initial administration	**Pre**	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**
cisplatin (*n*=12)	379.9±7.6	376.1±8.4 *	364.6±9.5 **	356.9±11.1 **	336.9±7.8 **	311.8±10.8 **
control (*n*=13)	369.3±12.5	388.8±12.1	401.4±11.1	411.5±12.5	419.1±11.5	425.7±11.7

* P < 0.05, ** P < 0.01.

Table 2
Frequency of TRPV1, TRPV2, $P2X_3$, and ASIC3 immunoreactive neurons in all dorsal root ganglion neurons and retrogradely labeled GM afferent neurons.
Frequencies of ion channels immunoreactive neurons were compared between cisplatin-treated and saline-treated control rats. Four sections from each L5 DRG were sampled from each rat. Statistical analysis was performed using Student's t-test.

		All DRG neurons				***GM afferent neurons***			
	Treatment	Immunoreactive cells/section	Total cells /section	%		Immunoreactive cells/section	Total cells /section	%	
TRPV1	cisplatin	48.8± 6.3	245.5±28.9	19.9±0.9		6.5±2.1	35.3±8.0	18.9±4.64	
	control	54.5±6.9	275.8±46.2	20.3±1.8		6.3±0.3	30.3±2.7	21.1±1.8	
TRPV2	cisplatin	42.3±6.3	320.3±35.3	13.3±1.3	*	10.5±1.9	42.5±8.3	25.4±2.4	
	control	24.0±4.1	267.3±51.7	9.2±0.6		5.5±0.7	29.5±3.1	19.1±2.6	
$P2X_3$	cisplatin	85.5±5.2	291.8±23.9	30.0±3.2	*	10.5±1.7	29.0±4.5	36.5±2.2	*
	control	67.8±17.8	325.5±79.0	20.4±0.8		7.8±1.9	29.5±6.3	25.0±3.1	
ASIC3	cisplatin	144.0±13.8	271.3±25.4	53.7±5.3	**	23.5±5.1	36.3±7.2	63.9±2.7	**
	control	88.3±14.0	335.0±28.6	26.4±3.4		10.0±2.0	29.5±4.3	33.8±5.7	

* P < 0.05, ** P < 0.01.

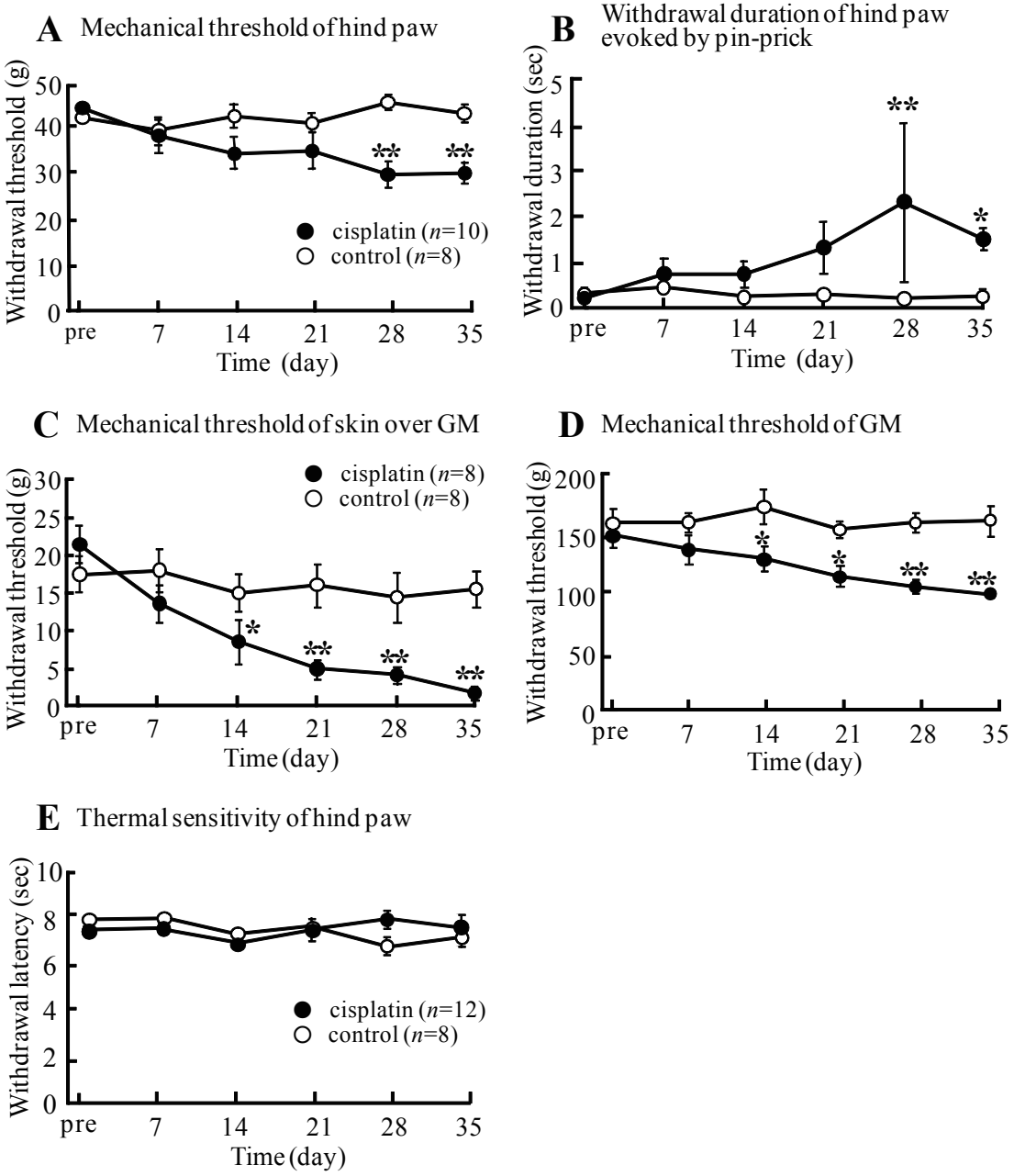

Fig. 1. Alteration of mechanical (A, B, C and D) and thermal (E) sensitivity of the hind paw in cisplatin-treated (●cisplatin) and saline-treated (○control) rats. Each group received a single intraperitoneal injection per week for 5 weeks. A: Time course of paw withdrawal thresholds measured by Dynamic plantar aesthesiometer. A significant decrease in mechanical threshold ($P<0.05$) was observed in cisplatin-treated group compared to the saline group. B: Time course of paw withdrawal duration measured by Pin-prick test. The cisplatin treated group developed significant mechanical hyperalgesia compared to the saline group. C: Withdrawal thresholds to von Frey filaments applied to the calves. Significant decrease in mechanical threshold compared to the saline group was observed from day 14 to day 35. D: Withdrawal threshold was determined by applying incremental pressure to the gastrocnemius muscle by Randall-Selitto apparatus. Cisplatin-treated group displayed lower withdrawal thresholds compared to the saline group. E. Paw withdrawal latency to noxious heat was measured by applying radiant heat to the plantar surface of the hind paw. No change was observed in both cisplatin-treated and saline-treated rats in the response latency during the experimental periods. * $P < 0.05$, ** $P < 0.01$.

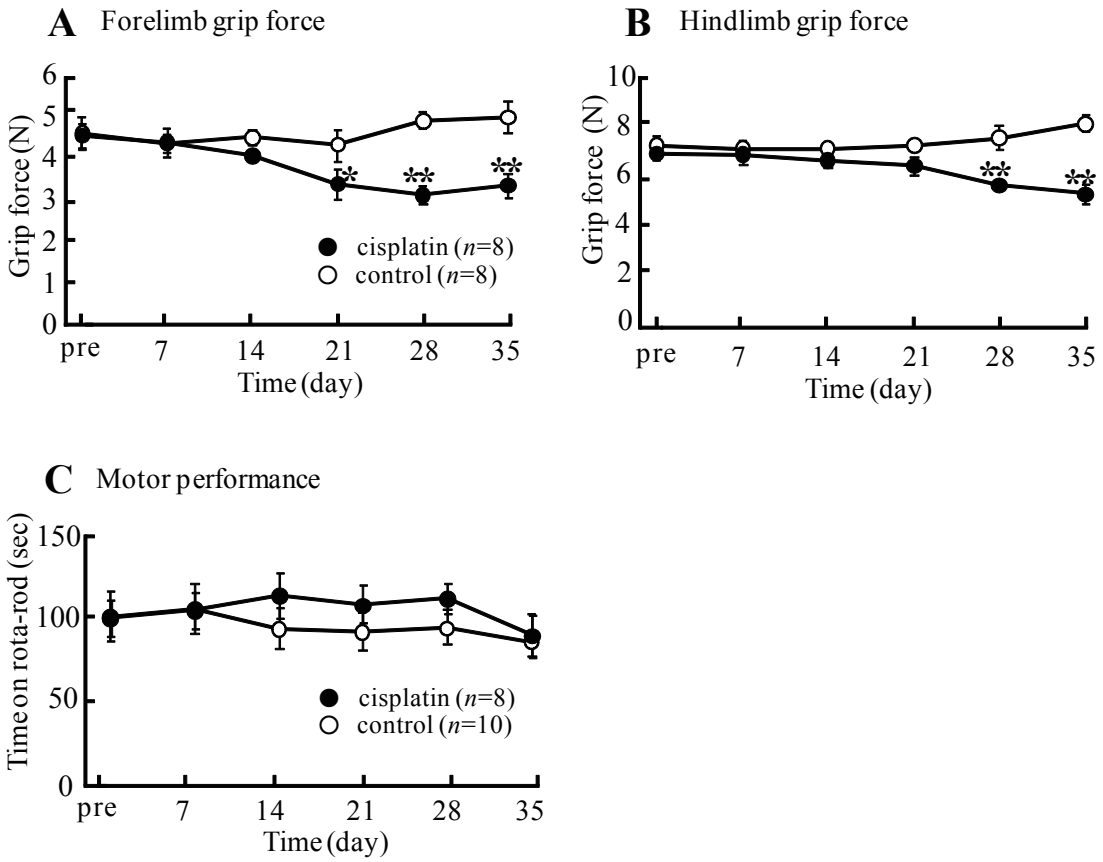

Fig. 2. A, B. Alteration of grip force in cisplatin treated (●cisplatin) and saline treated (○control) rats. Each group received one intraperitoneal injection per week for 5 weeks. A: Forelimb grip force. B: Hindlimb grip force. Significant decreases of both fore- and hindlimb grip forces were observed in the cisplatin-treated group compared to the saline-treated group. * $P < 0.05$, ** $P < 0.01$. C. Motor performance of rats treated with either cisplatin (●cisplatin) or saline (○control) was evaluated using the rota-rod test. No motor deficits were observed in either cisplatin-treated or saline-treated rats.

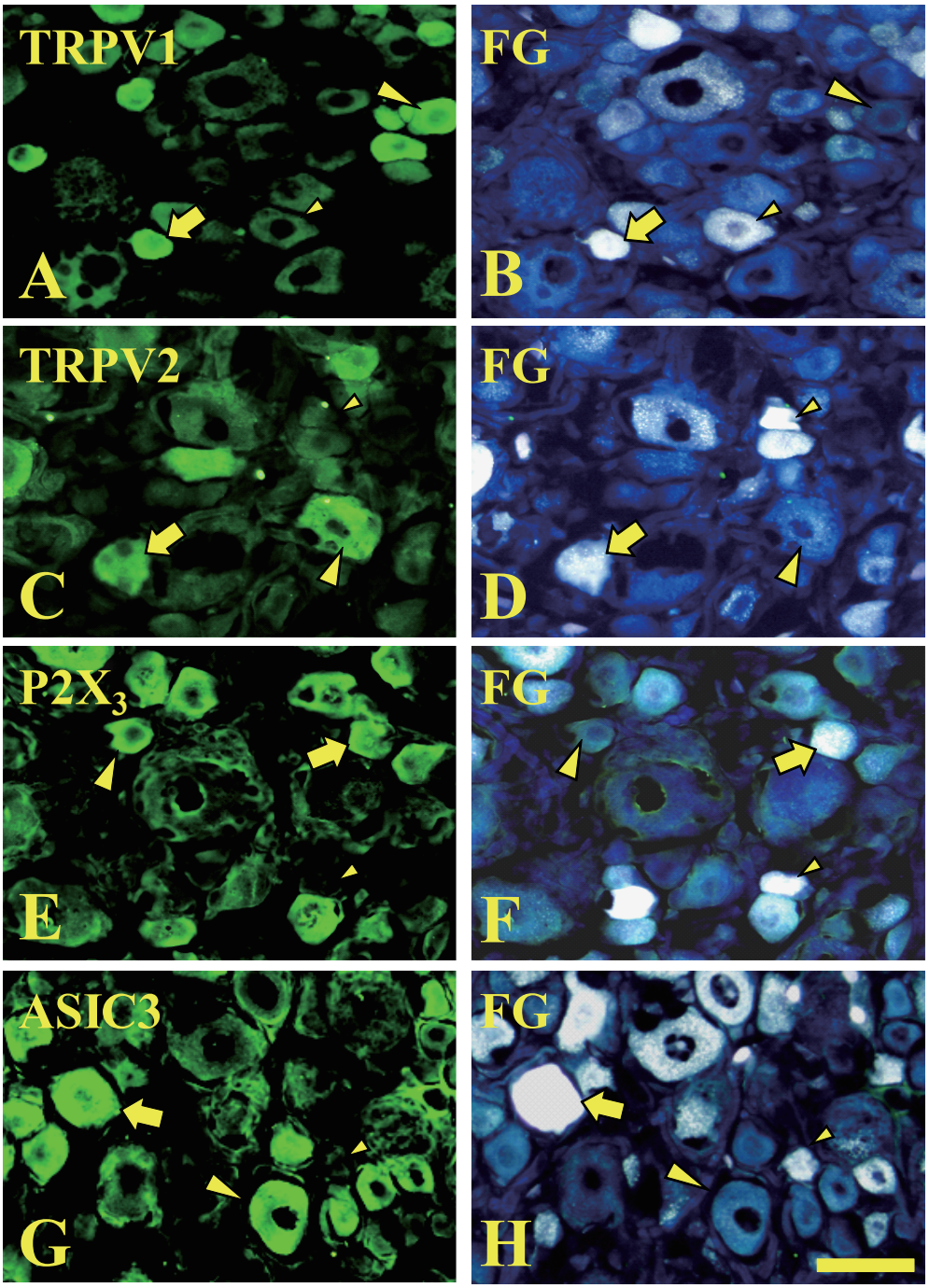

Fig. 3. Immunofluorescent labeling of L5 dorsal root ganglia (DRGs) neurons in cisplatin-treated animals. The 4 sets of micrographs (A, B; C, D; E, F and G, H) show representative DRGs sections that have been labeled with antibodies to TRPV1 (A), TRPV2 (C), $P2X_3$ (E), and ASIC3 (G), and retrogradely labeled with fluorogold (FG) applied to the gastrocnemius muscle (B, D, F, H). Large arrows indicate FG labeled cells that were also labeled with each antibody, while examples of FG-labeled cells that were not labeled by each antibody are indicated by small arrowheads. Large arrowheads indicate cells labeled with each antibody, but not with FG. Size scale bar, 50 μm.

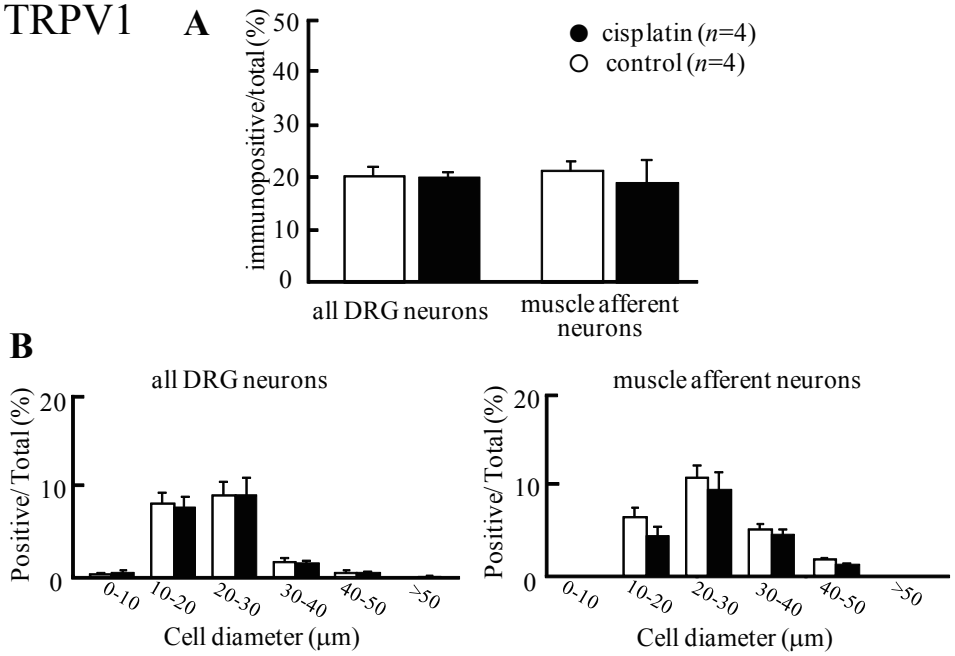

Fig. 4. Proportion of TRPV1 immunopositive neurons in L5 DRG neurons and muscle afferent L5 DRG neurons in cisplatin-treated and control animals (n=4). Ratios of TRPV1-positive cells in all DRG neurons and muscle afferent neurons were shown in A. Ratios of immunopositive cells in group of cells classified by cell size were shown in B. There was no change in the proportion of TRPV1-positive cells in DRG neurons.

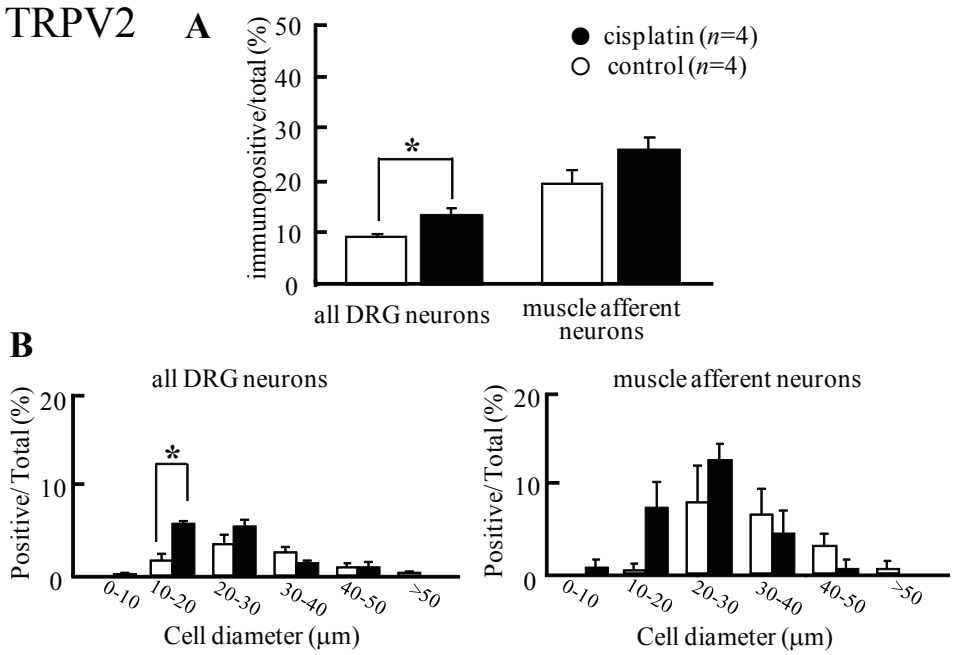

Fig. 5. Proportion of TRPV2 immunopositive neurons in L5 DRG neurons and muscle afferent L5 DRG neurons in cisplatin-treated and control animals (n=4). Ratios of TRPV2-positive cells in all DRG neurons and muscle afferent neurons were shown in A. Ratios of immunopositive cells in group of cells classified by cell size were shown in B. Proportion of TRPV2-positive cells increased in DRG neurons. * $P < 0.05$.

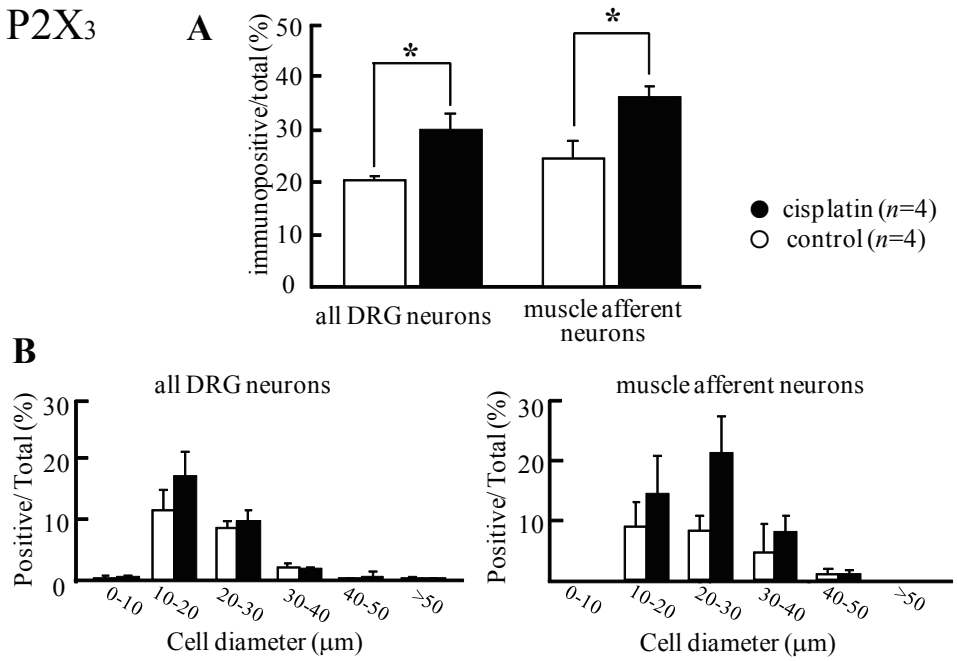

Fig. 6. Proportion of $P2X_3$ immunopositive neurons in L5 DRG neurons and muscle afferent L5 DRG neurons in cisplatin-treated and control animals (n=4). Ratios of $P2X_3$-positive cells in of all DRG neurons and muscle afferent neurons were shown in A. Ratios of immunopositive cells in group of cells classified by cell size were shown in B. Proportion of $P2X_3$-positive cells increased in all DRG neurons and muscle afferent neurons.* $P < 0.05$.

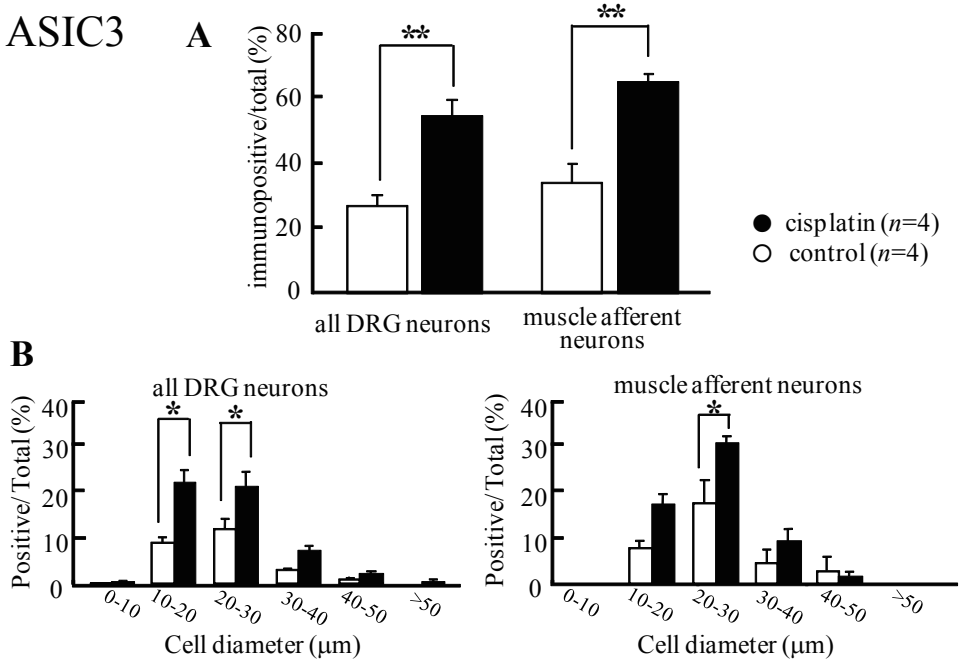

Fig. 7. Proportion of ASIC3 immunopositive neurons in L5 DRG neurons and muscle afferent L5 DRG neurons in cisplatin-treated and control animals (n=4). Ratios of ASIC3-positive cells in all DRG neurons and muscle afferent neurons were shown in A. Ratios of immunopositive cells in group of cells classified by cell size were shown in B. Proportion of ASIC3-positive cells increased in small and medium sized groups in all DRG neurons and muscle afferent neurons . * $P < 0.05$, ** $P < 0.01$.

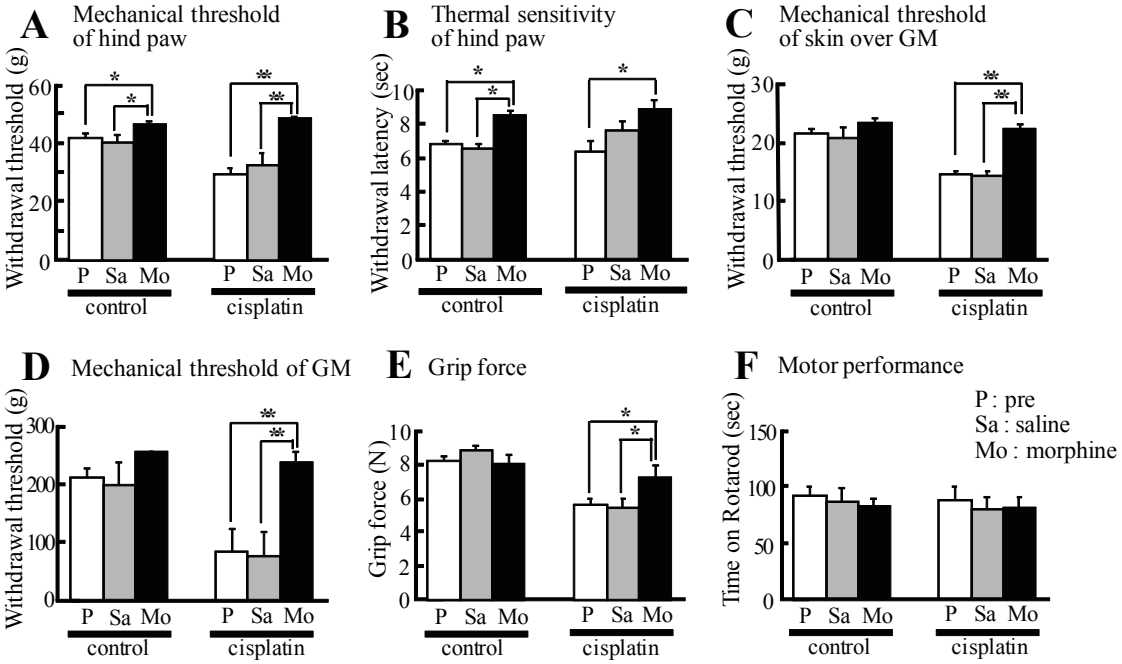

Fig. 8. The effects of morphine on nocifensive behavior induced by cisplatin administration. Behavioral tests in both skin and muscle were performed either one week after the last cisplatin injection or saline injection. Effect of morphine was tested 30 min after intraperitoneal injection (6 mg/kg). Data were analyzed using one-way ANOVA with repeated measurements followed by Holm–Sidak comparisons. * $P < 0.05$, ** $P < 0.01$.

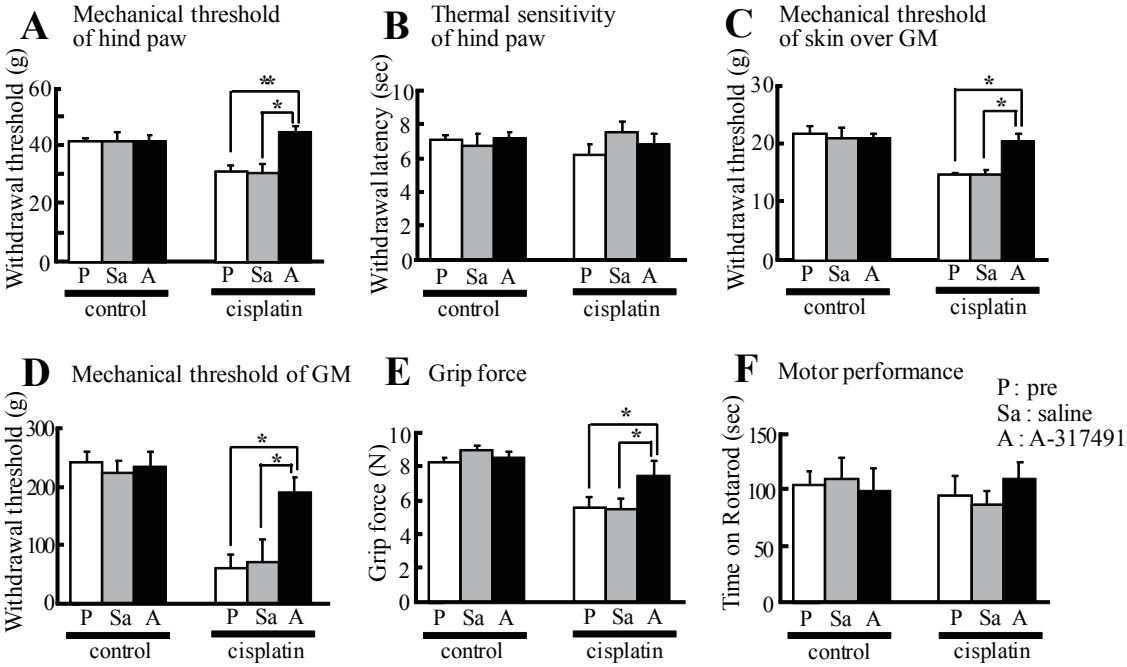

Fig. 9. The effects of selective $P2X_{3,2/3}$ receptor antagonist A-317491 on nocifensive behavior induced by cisplatin administration. Behavioral tests in both skin and muscle were performed either one week after the last cisplatin injection or saline injection. Effects of antagonists were tested 30 min after subcutaneous injection of A-317491. Data were analyzed using one-way ANOVA with repeated measurements followed by Holm–Sidak comparisons. * $P < 0.05$, ** $P < 0.01$.

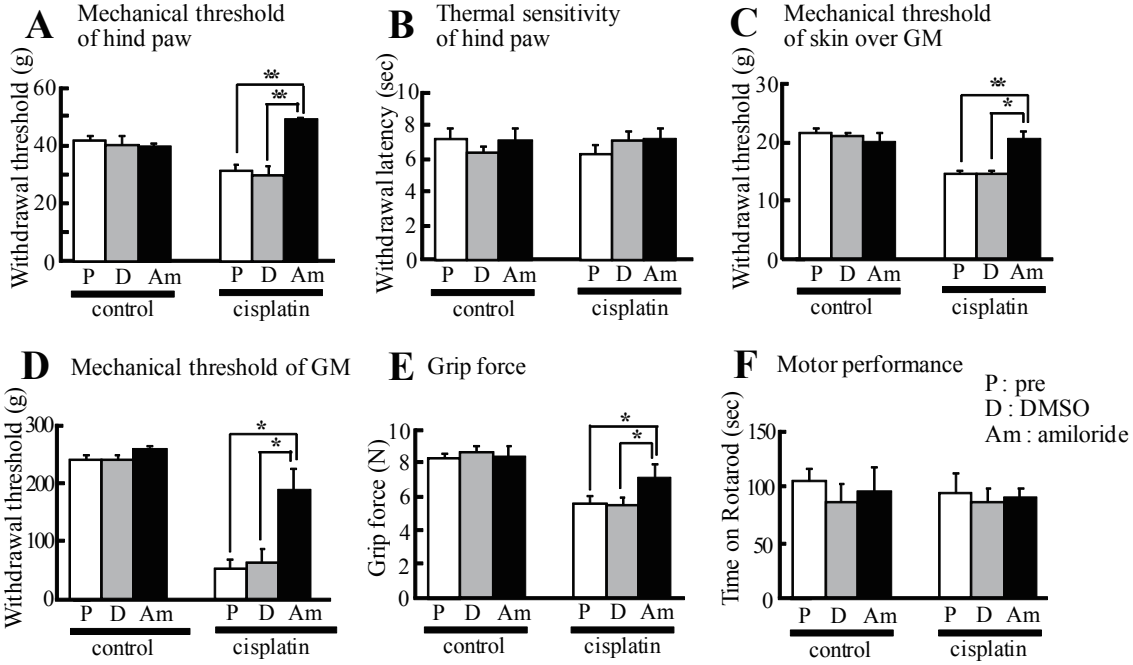

Fig. 10. The effects of ASICs antagonist amiloride on nocifensive behavior induced by cisplatin administration. Behavioral tests in both skin and muscle were performed either one week after the last cisplatin injection or saline injection. Effects of antagonists were tested 30 min after subcutaneous injection. Data were analyzed using one-way ANOVA with repeated measurements followed by Holm–Sidak comparisons. * $P < 0.05$, ** $P < 0.01$.