

*Short Communication***Repeated Administration of Amitriptyline Reduces Oxaliplatin-Induced Mechanical Allodynia in Rats**Hikaru Sada¹, Nobuaki Egashira^{1,*}, Soichiro Ushio¹, Takehiro Kawashiri¹, Masafumi Shirahama¹, and Ryoza Oishi¹¹Department of Pharmacy, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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Abstract. Oxaliplatin is a key drug in the treatment of colorectal cancer, but it causes acute and chronic neuropathies in patients. Amitriptyline has widely been used in patients with painful neuropathy. In this study, we investigated the effect of amitriptyline on the oxaliplatin-induced neuropathy in rats. Repeated administration of amitriptyline (5 and 10 mg/kg, p.o., once a day) reduced the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia and reversed the oxaliplatin-induced increase in the expression of NR2B protein and mRNA in rat spinal cord. These results suggest that amitriptyline is useful for the treatment of oxaliplatin-induced neuropathy clinically.

Keywords: oxaliplatin, amitriptyline, peripheral neuropathy

Oxaliplatin has widely been used as a key drug in the treatment of colorectal cancer. However, it causes severe acute and chronic peripheral neuropathies. Acute neuropathy includes acral paresthesias and dysesthesia triggered or enhanced by exposure to cold, and it appears soon after administration (1). After multiple cycles of treatment, the patients develop chronic neuropathy characterized by sensory and motor dysfunction. This chronic neuropathy is a dose-limiting toxicity and a major clinical problem in oxaliplatin chemotherapy (2).

Antidepressant drugs have been recommended to be used as first-line drugs for the treatment of neuropathic pain (3, 4), and in particular, amitriptyline is demonstrated to possess an analgesic activity for neuropathic pain in many randomized controlled trials (5).

Recently, we reported that repeated administration of oxaliplatin induced cold hyperalgesia in the early phase and mechanical allodynia in the late phase in rats (6), and spinal NR2B-containing *N*-methyl-D-aspartate (NMDA) receptors are involved in the oxaliplatin-induced mechanical allodynia (7). However, the effect of amitriptyline on the oxaliplatin-induced neuropathy remains unexplored. Accordingly, we investigated the effect of amitriptyline on the oxaliplatin-induced cold hyperalgesia and mechanical allodynia in rats. Moreover, we ex-

amined the effect of amitriptyline on the oxaliplatin-induced increase in the expression of NR2B protein and mRNA in the rat spinal cord.

Male Sprague-Dawley rats (Kyudo Co., Tosu) were used. Rats were housed in groups of four to five per cage, with lights on from 7:00 AM to 7:00 PM. Animals had free access to food and water in their home cages. All experiments were approved by the Experimental Animal Care and Use Committee of Kyushu University (Fukuoka) according to the National Institutes of Health guidelines, and we followed the International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research (8).

Oxaliplatin (Elplat[®]) was obtained from Yakult Co., Ltd. (Tokyo) and was dissolved in 5% glucose solution. Amitriptyline was provided by Wako Pure Chemical Industries, Ltd. (Osaka) and was dissolved in distilled water. Oxaliplatin (4 mg/kg) or vehicle (5% glucose solution) was injected intraperitoneally (i.p.) in volumes of 1 mL/kg, twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days (from day 1). The doses of these drugs were chosen based on previous reports (6, 7, 9). Behavioral tests were performed blindly with respect to drug administration.

The cold hyperalgesia was assessed by acetone test described by Flatters and Bennett (10). The acetone test was performed before the first drug administration (on day 0) and on days 4, 7, 11, 14, 18, 21, and 28. On days

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4, 7, 11, 14, 18, and 21, test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. Fifty microliters of acetone (Wako Pure Chemical Industries, Ltd.) was sprayed onto the plantar skin of each hind paw three times with a Micro Sprayer® (Penn Century, Inc., Philadelphia, PA, USA), and the number of withdrawal response was counted for 40 s from the start of the acetone spray.

The mechanical allodynia was assessed by the von Frey test. The von Frey test was performed before the first drug administration (on day 0) and on days 7, 14, 21, 28, 35, 42, 49, and 56. On days 7, 14, and 21, test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. von Frey filaments (The Touch Test Sensory Evaluator Set; Linton Instrumentation, Norfolk, UK) ranging 1–15 g bending force were applied to the mid-plantar skin of each hind paw with each application held for 6 s. The paw withdrawal threshold was determined by a modified up-down method (11).

To investigate the functional changes in mRNA levels of NR2B, the L4-6 spinal cord was quickly removed on day 28. mRNA was isolated using PolyATtract® System 1000 (Promega, Corp., Madison, WI, USA). cDNA was synthesized using the PrimScript® 1st strand cDNA Synthesis Kit (Takara Bio, Inc., Otsu). The polymerase chain reaction (PCR) was carried out with Gene Taq (Nippon Gene, Co., Ltd., Tokyo). The oligonucleotide primers for NR2B were designed based on the sequences described by Lau et al. (12). The sequences of PCR primers were as follows: NR2B, 5'-TCC GTC TTT CTT ATG GG ATA TGC-3' (sense), 5'-CCT CTA GGC GGA CAG ATT AAG G-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-YGC CTG CTT CAC CAC CTT-3' (sense), 5'-TGC MTC CTG CAC CAC CAA CT-3' (antisense) (Sigma-Aldrich, St. Louis, MO, USA). Reactions were run for 40 cycles with 95°C denaturing cycle (30 s), 63°C annealing cycle (1 min), and 72°C extension cycle (30 s) for NR2B or for 30 cycles with 94°C denaturing cycle (45 s), 53°C annealing cycle (45 s), and 72°C extension cycle (1.5 min) for G3PDH. The PCR products were subjected to electrophoresis on 2% agarose gel, and the DNA was visualized by staining with ethidium bromide under ultraviolet irradiation. Then, the intensities of PCR products were semi-quantified densitometrically by Alpha Imager 2200 (Protein Simple, Santa Clara, CA, USA).

To examine the functional changes in protein levels of NR2B, the L4-6 spinal cord was quickly removed on day 28. The tissues were homogenized in a solubilization

buffer containing 20 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 0.32 M sucrose, 2 mg/ml aprotinin, and 2 mg/ml leupeptin, pH 7.4. The homogenates were subjected to 6% SDS-PAGE, and proteins were transferred electrophoretically to PVDF membranes. The membranes were blocked in Tris-buffered saline/Tween-20 (TBST) containing 5% BSA (Sigma-Aldrich) for an additional 1 h at room temperature with agitation. The membrane was incubated overnight at 4°C with rabbit polyclonal NR2B antibody (1:5000; Millipore, Corp., Billerica, MA, USA) and then incubated for 1 h with anti-rabbit IgG-horseradish peroxidase (1:5000; Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). The immunoreactivity was detected using Enhanced Chemiluminescence (Perkin Elmer, Waltham, MA, USA).

Values were expressed as the mean ± S.E.M. The values were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test to determine differences among the groups. A probability level of $P < 0.05$ was accepted as statistically significant.

In the acetone test, oxaliplatin (4 mg/kg, i.p., twice a week) significantly increased the number of withdrawal responses compared with vehicle on days 11, 14, 18, 21, and 28 (days 11, 14, and 18: $P < 0.05$, days 21 and 28: $P < 0.01$; Fig. 1A). Repeated administration of amitriptyline (5 and 10 mg/kg, p.o., once a day) did not affect the oxaliplatin-induced increase in the number of withdrawal responses.

In the von Frey test, oxaliplatin significantly reduced the withdrawal threshold compared with vehicle on days 14, 21, 28, 35, 42, 49, and 56 ($P < 0.01$, Fig. 1B). Repeated administration of amitriptyline (10 mg/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold on days 14, 21, 28, 35, 42, 49, and 56 ($P < 0.01$). In addition, repeated administration of amitriptyline (5 mg/kg) significantly inhibited the oxaliplatin-induced reduction of the withdrawal threshold (day 28: $P < 0.05$ and days 35 and 42: $P < 0.01$).

NR2B expression was examined by western blot and PCR analysis on homogenates of the spinal cord from rats. The results of western blotting and PCR showed that NR2B protein and mRNA levels in the spinal cord of oxaliplatin-treated rats significantly increased compared with that of vehicle-treated rats on day 28 ($P < 0.05$, Fig. 2). Repeated administration of amitriptyline (5 and 10 mg/kg) significantly reversed the oxaliplatin-induced increase in the NR2B mRNA levels in the spinal cord ($P < 0.05$, Fig. 2A). Similarly, repeated administration of amitriptyline (10 mg/kg) significantly reversed the oxaliplatin-induced increase in the NR2B protein levels in the spinal cord ($P < 0.01$, Fig. 2B). In addition, repeated

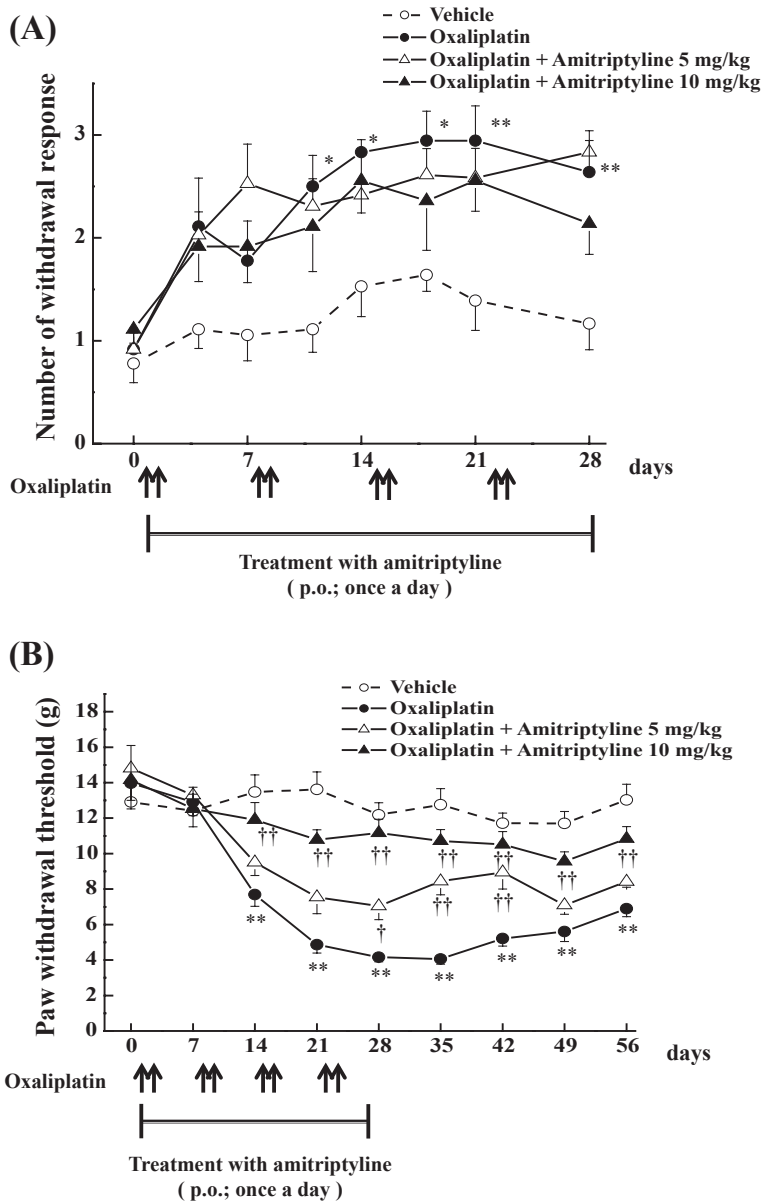


Fig. 1. Effects of amitriptyline on oxaliplatin-induced cold hyperalgesia in the acetone test (A) and mechanical allodynia in the von Frey test (B) in rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days. The acetone test was performed before the first drug administration (on day 0) and on days 4, 7, 11, 14, 18, 21, and 28. The von Frey test was performed before the first drug administration (on day 0) and on days 7, 14, 21, 28, 35, 42, 49, and 56. Values are expressed as the mean \pm S.E.M. of 6 animals (A) or 9–10 animals (B). * P < 0.05, ** P < 0.01, compared with the vehicle; † P < 0.05, †† P < 0.01, compared with oxaliplatin alone.

administration of amitriptyline (5 mg/kg) tended to reduce the oxaliplatin-induced increase in the NR2B protein levels.

In this study, repeated administration of amitriptyline reduced the oxaliplatin-induced mechanical allodynia but not cold hyperalgesia. Therefore, amitriptyline may be useful for treatment of the oxaliplatin-induced chronic peripheral neuropathy.

Furthermore, repeated administration of amitriptyline reversed the oxaliplatin-induced increase in the NR2B protein and mRNA levels in the spinal cord. Tricyclic antidepressant agents (TCAs) have been reported to have properties to bind NMDA receptors and to inhibit binding of NMDA ligand (13, 14). Amitriptyline has also

been reported to inhibit NMDA-induced pain behavior in rats (15). Recently, we reported that spinal NR2B-containing NMDA receptors are involved in the oxaliplatin-induced mechanical allodynia (7). Taken together, the reduction of amitriptyline on the expression of NR2B subunits may be involved in its inhibitory effect on the development of oxaliplatin-induced mechanical allodynia.

In this study, we did not evaluate the acute effect of amitriptyline on pain behaviors. Furthermore, its inhibitory effect on the development of oxaliplatin-induced mechanical allodynia persisted up to 56 days after the end of amitriptyline administration. These results suggest that the preventive effect of amitriptyline is not a tran-

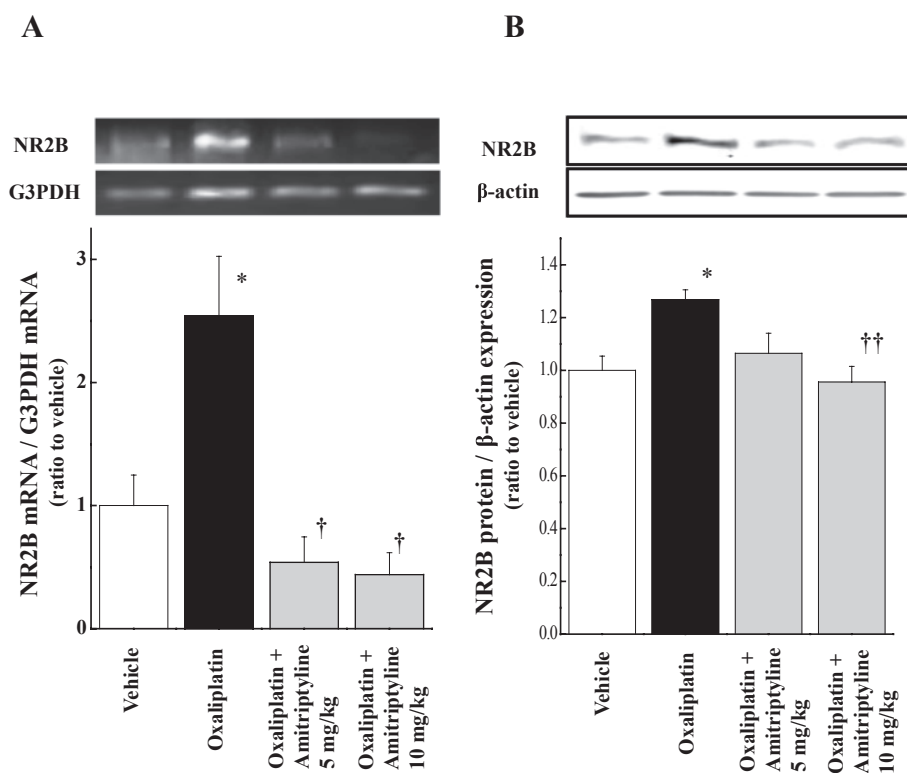


Fig. 2. Effects of amitriptyline on NR2B mRNA (A) and protein (B) levels in the spinal cord in oxaliplatin-treated rats. Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks (days 1, 2, 8, 9, 15, 16, 22, and 23). Amitriptyline (5 and 10 mg/kg) was administered p.o. once a day for 27 days. The L4-L6 spinal cord was harvested on day 28. Values are expressed as the mean \pm S.E.M. of 5–7 animals (A) or 9–10 animals (B). * $P < 0.05$, compared with the vehicle; † $P < 0.05$, †† $P < 0.01$, compared with oxaliplatin alone.

sient analgesic effect via activation of the descending pain inhibitory system related to monoamine reuptake inhibition and likely due to inhibition of the expression of NR2B subunits. In addition, chronic administration of imipramine for 16 days has been reported to reduce the expression of NMDA-receptor subunit mRNA in mouse brain (16). Therefore, imipramine has the potential to prevent the oxaliplatin-induced mechanical allodynia.

In the present study, we observed that the oxaliplatin-induced mechanical allodynia was gradually recovered on days 42–56 after the end of oxaliplatin administration. The present result is consistent with the previous one (11). Clinically, the reversibility of sensory neurotoxicity is observed in patients treated with oxaliplatin (17).

In conclusion, the present results suggest, for the first time, that repeated administration of amitriptyline reduces the oxaliplatin-induced mechanical allodynia, at least in part, by inhibiting the expression of NR2B subunits.

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