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# Noradrenergic inhibition of spinal hyperexcitation elicited by cutaneous cold stimuli in rats with oxaliplatin-induced allodynia: electrophysiological and behavioral assessments

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**Abstract** We investigated the spinal action of noradrenaline on cold-elicited hyperexcitation detected in dorsal horn neurons of rats with allodynia induced by an oxaliplatin (6 mg/kg, i.p.) injection. In vivo extracellular recordings from the spinal dorsal horn showed that wide dynamic range neurons responded to cutaneous acetone (10 μl) stimulation in normal rats, and cold-elicited firings in oxaliplatin-administered rats were increased with a longer duration, correlated with behavioral responses. These responses were significantly attenuated by spinal administration (50 μM) of noradrenaline or its agonists, clonidine ($\alpha_2$), phenylephrine ($\alpha_1$) and isoprenaline ($\beta$), in descending order of efficacy. Thus, the inhibitory effect of noradrenaline on spinal oxaliplatin-induced cold hyperexcitation is mediated mainly by activation of $\alpha_2$- and/or $\alpha_1$-adrenoceptors.

**Keywords** Noradrenaline · Oxaliplatin · Cold allodynia · Spinal cord · Wide dynamic range neurons · In vivo extracellular recording

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## Introduction

Oxaliplatin, a third-generation platinum derivative, is among the most effective chemotherapies for metastatic colorectal cancer when combined with fluorouracil and leucovorin [1]. The typical side effect of oxaliplatin is peripheral neuropathy, manifested as tingling of the hands, feet, and the oral or perioral regions, which is exacerbated by exposure to low temperature [2]. This oxaliplatin-induced cold hypersensitivity is the main factor that lowers the patient's quality of life and frustrates the will to undergo chemotherapy. However, effective treatment for established chemotherapy-induced peripheral neuropathy (CIPN) has yet to be found [3].

The noradrenergic endogenous analgesic system modulates noxious transmission at several sites from the peripheral nerves to the central nervous system [4]. This noradrenergic pathway is mainly from the locus coeruleus and other pontine regions, terminating axons diffusely throughout the spinal dorsal horn, and mediates strong inhibitory actions with volume transmission [5–8]. Endogenous analgesic modulation of noradrenaline was mimicked by intrathecal injection of noradrenaline and adrenoceptor agonists [9]. It was already proved that activation of the endogenous noradrenergic system alleviates inflammatory pain and nerve injury-induced neuropathic pain at the spinal cord [10–12]. In our previous study [13], bee venom acupuncture attenuated oxaliplatin-induced cold allodynia in rats, which was blocked by systemic injection of phentolamine ($\alpha$-adrenoceptor antagonist) or intrathecal injection of idazoxan ($\alpha_2$-adrenoceptor antagonist). However, there have been no electrophysiological studies demonstrating whether and how direct spinal application of noradrenaline inhibits oxaliplatin-induced cold allodynia, and its correlation with behavioral results.

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In this study, using an in vivo electrophysiological method, we examined the response properties of the spinal wide dynamic range (WDR) neurons to acetone cold stimulation on the hind paw in oxaliplatin-administered rats. Afterwards, we assessed the suppressive effect of spinal noradrenaline on the firing patterns of WDR neurons in response to acetone, and determined which type of adrenoceptor plays a major role in this inhibitory effect. Behavioral responses to cold stimulation were also assessed by the acetone test to see whether the electrophysiological results are associated with changes in pain behavior.

## Materials and methods

Young adult, male Sprague–Dawley rats (8 weeks, 180–200 g) were used for this study and they were housed in cages with food and water available ad libitum. The room was maintained with 12-h light/dark cycle and kept at $21 \pm 1$ °C. All processes involving animals were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SE)-16-020) and the National Institutes of Natural Sciences (15A-027), and were performed in accordance with the institutional guidelines for animal experiments and with the ethical guidelines of the International Association for the Study of Pain [14]. Every effort was made to reduce the number of animals in this study. At the end of the study, the animals were killed with carbon dioxide gas or by injecting an overdose of urethane.

Oxaliplatin (Wako Pure Chemical Industries, Osaka, Japan) was dissolved in a 5% glucose solution at a concentration of 2 mg/ml and was intraperitoneally injected at a dose of 6 mg/kg [15]. The same volume of 5% glucose solution was used for the control.

Animals were habituated to the experimental circumstances for 30-60 min before the behavioral test. The animals were placed on a perforated metal sheet (37000-003, UGO Basile, Comerio, Italy), enclosed within a 20 (d) × 20 (w) × 14 (h) cm clear plastic cage (37000-006, UGO Basile). Acetone (10 μl) was applied to the ventral surface of the right hind paw by a pipette, the tip of which was connected to the paw with polyethylene tube, and the behavioral responses were then monitored for 40 s. The responses to acetone were graded according to the following 4-point scale: 0, no response; 1, quick withdrawal, flicking or stamping of the paw; 2, prolonged withdrawal or repeated flicking ($\geq 2$) of the paw; 3, repeated flicking with licking directed at the ventral side of the paw. Acetone was applied five times to the right hind paw, and cumulative scores were then generated, with the minimum score being 0 and the maximum score being 15 [16, 17]. Mechanical sensitivity was measured by the von Frey hair test. A von Frey filament (Linton Instrumentation, Norfolk, UK) with a bending force of 15 g was applied to the mid-plantar area of the right hind paw 10 times, with each application held for 5 s. The number of withdrawal responses to the von Frey filament applications were counted and then expressed as an overall percentage response [17]. Three experimenters conducted the behavioral tests and they were blinded on the types of drugs administered.

To investigate the effect of noradrenaline and its agonists in a behavioral experiment, we intrathecally administered noradrenaline, phenylephrine ($\alpha_1$-adrenoceptor agonist), clonidine ($\alpha_2$-adrenoceptor agonist) or isoprenaline ($\beta$-adrenoceptor agonist) at a concentration of 50 μM [5] with a direct lumbar puncture under isoflurane anesthesia as previously described [18]. All drugs were dissolved in PBS and the injected volume was 50 μl, which is required to spread consistently to caudal thoracic vertebrae containing the lumbar enlargement of the spinal cord [19]. Intrathecal injection of 50 μl lidocaine results in lower limb paralysis without any other collateral effects [20].

The methods for the in vivo electrophysiology were slightly modified from the previous studies [21–23]. Briefly, the rats were anesthetized with urethane (1.2-1.5 g/kg, i.p.), and the surgery was conducted on a warm plate. Thoracolumbar laminectomy was performed at the vertebral level of T13-L2 to expose the spinal segment L3–L5, and the rat was placed in a stereotaxic apparatus (ST-7, Narishige, Tokyo, Japan). The exposed part of the spinal cord was irrigated with oxygenated (95% $O_2$–5% $CO_2$ gas) Krebs solution (in mM: 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $NaH_2PO_4$, 11 glucose and 25 $NaHCO_3$) at a flow rate of 10 to 15 ml/min at $38 \pm 1$ °C. With the solution perfused, the dura membrane was removed, and the pia-arachnoid membrane was cut for making a small window to insert the tungsten electrode (impedance of 10 MΩ, FHC, ME, USA) smoothly without adjacent tissue suppression.

The electrode, fixed on the head of a micromanipulator (MHW-4-1, Narishige) at an angle of 45–50°, was placed into the dorsal horn of the spinal cord, and action potentials were extracellularly recorded and amplified with the bioamplifier (DAM80, WPI, USA). The data were digitized (Digidata 1440A, Axon instruments, USA) and stored in a personal computer using pCLAMP10 software (Axon instruments). Recorded action potentials were spike-sorted with Spike2 (version 6, Cambridge Electronic Design, UK) and Offline Sorter software (version 3, Plexon, Dallas, TX). Neurons were classified as WDR neurons if they elicited action potentials in response to light touch (brushing or tapping the ipsilateral hind paw) and displayed increased firing to pinching with toothed forceps by hand (11022-14, Fine Science Tools, Heidelberg, Germany), as shown

previously [24]. The force was determined as noxious that also induced withdrawal behavior in awake rats. In WDR neurons, cold stimulation (acetone drop) was applied to the receptive fields of the mechanical responses. Responses to the cold and mechanical (brushing, press and pinching to the skin for 3 s) stimuli were compared between WDR neurons in vehicle control and oxaliplatin-administered rats. For the press stimulus, the blunt tip of a camel brush with a diameter of 0.5 cm was used with a magnitude of about 20 g.

For pharmacological study, drugs were diluted in Krebs solution to a concentration of 50 μM and superfused to the surface of the spinal cord for 30 min [5, 25]. The inhibition rate was calculated as the evoked spike number after drug application of 30 min divided by that before drug application. Noradrenaline, phenylephrine, clonidine and isoprenaline were used for this study. All these drugs were purchased from Sigma (St Louis, MO, USA).

Statistical analysis was conducted with the software of Prism 5.0 (Graph Pad Software, USA). All data are presented as the mean $\pm$ SEM. $P < 0.05$ was considered significant.

## Results

All of the rats showed little response to acetone cold stimuli (10 μl) on the hind paw prior to oxaliplatin or vehicle injection. A significant cold allodynia sign was observed 3–7 days after oxaliplatin injection, as shown in previous studies [26, 27]. Figure 1 shows the behavioral responses to acetone stimuli in the vehicle control and oxaliplatin-administered rats. Total sums of the behavioral assessment scores were markedly higher in the oxaliplatin-administered group than those in the vehicle-treated group (Fig. 1a, $p = 0.0009$). The frequency of the responses to acetone out of 5 trials was also significantly higher in the oxaliplatin group than that in the vehicle group (Fig. 1b, $p = 0.0004$). Once the behavioral test was completed, in vivo extracellular recordings were made from WDR neurons in the spinal dorsal horn. Total number of recorded neurons was 34 (17 neurons from 10 control rats and 17 neurons from 11 oxaliplatin injected rats). Most WDR neurons in the control rats showed transient (2–3 s) responses (did not last more than 10 s) of firing, immediately after an administration of acetone drop on the receptive field of mechanical stimuli (see Methods section) to the ipsilateral hind paw (see upper trace in Fig. 2a). In contrast, the majority of WDR neurons in the oxaliplatin-administered rats exhibited a longer duration (more than 10 s) of firing in response to acetone cold stimuli (see lower trace in Fig. 2a). The frequency of the cold-elicited firing in oxaliplatin-administered rats was significantly
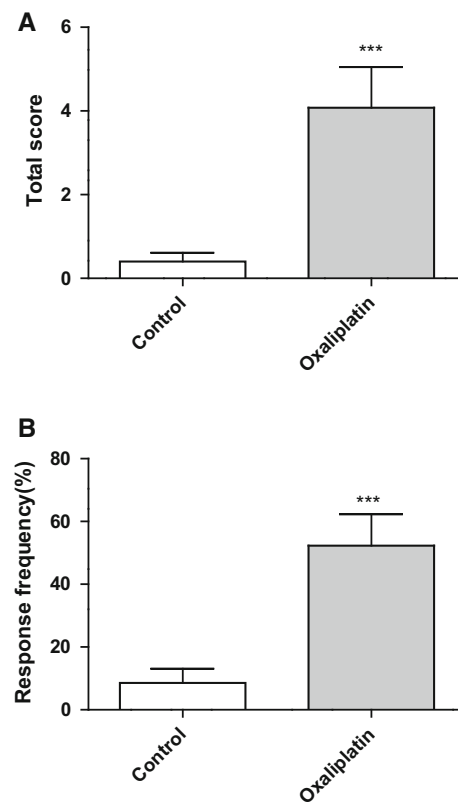


Fig. 1 Increased behavioral response to cold stimulation caused by an oxaliplatin injection. Behavioral assessment was performed with 4-grade scoring for each behavioral response from applying 10 μl of acetone on the rat's hind paw as innocuous cold stimulation: *0* no response, *1* brisk withdrawal or flick of the paw, *2* repeated flicking of the paw, *3* repeated flicking of the hindpaw and licking of the paw. Scores acquired from five trials of cold stimulation on the rat's hind paw were summed (**a**). $N = 15$ for each group. Response frequency was acquired from the five trials (**b**). $N = 15$ for each group. ***$p < 0.001$, by Mann–Whitney test (**a**) and unpaired *t*-test (**b**)

higher than that in the vehicle treated rats (Fig. 2b). These results indicate that the spinal WDR neurons are more sensitive to ipsilateral peripheral cold stimuli following oxaliplatin administration as represented in the marked increase of firing rate and duration, which is highly correlated with increased behavioral responses to cold stimuli.

For other stimuli (i.e., mechanical stimuli), such as light brush, moderate press and noxious pinch, a higher frequency of neuronal firing was also observed in the oxaliplatin group as compared to the vehicle control group (Supplementary Fig. 1A–C). Paw withdrawal responses to von Frey hair (15 g) stimuli were also significantly increased after oxaliplatin administration, indicating the development of mechanical hypersensitivity (Supplementary Fig. 1D–E). However, total response duration of spinal WDR neurons to press or pinch stimulation was not significantly augmented by oxaliplatin, although it was markedly increased in response to cold acetone stimulation (Supplementary Fig. 2A). In addition, 5 of 17 recorded

**Fig. 2** Oxaliplatin increases the neuronal response to cold stimulation in the spinal dorsal horn. **a** Representative extracellular recording raw traces of wide dynamic range (WDR) neuron's responses to cold stimulation (a drop of 10 μl acetone to the hind paw) in naïve (*top panel*) and oxaliplatin-administered (*bottom panel*) rats. Sorted two neuron's action potentials (*insets*) and their timing (as *longitudinal bars*) are shown below each trace. **b** High frequency and persistent response of spinal neuronal firing elicited by cold stimulation in oxaliplatin-administered rats. $N = 17$ for each group. \*\*$p < 0.01$, \*\*\*$p < 0.001$, by unpaired *t*-test
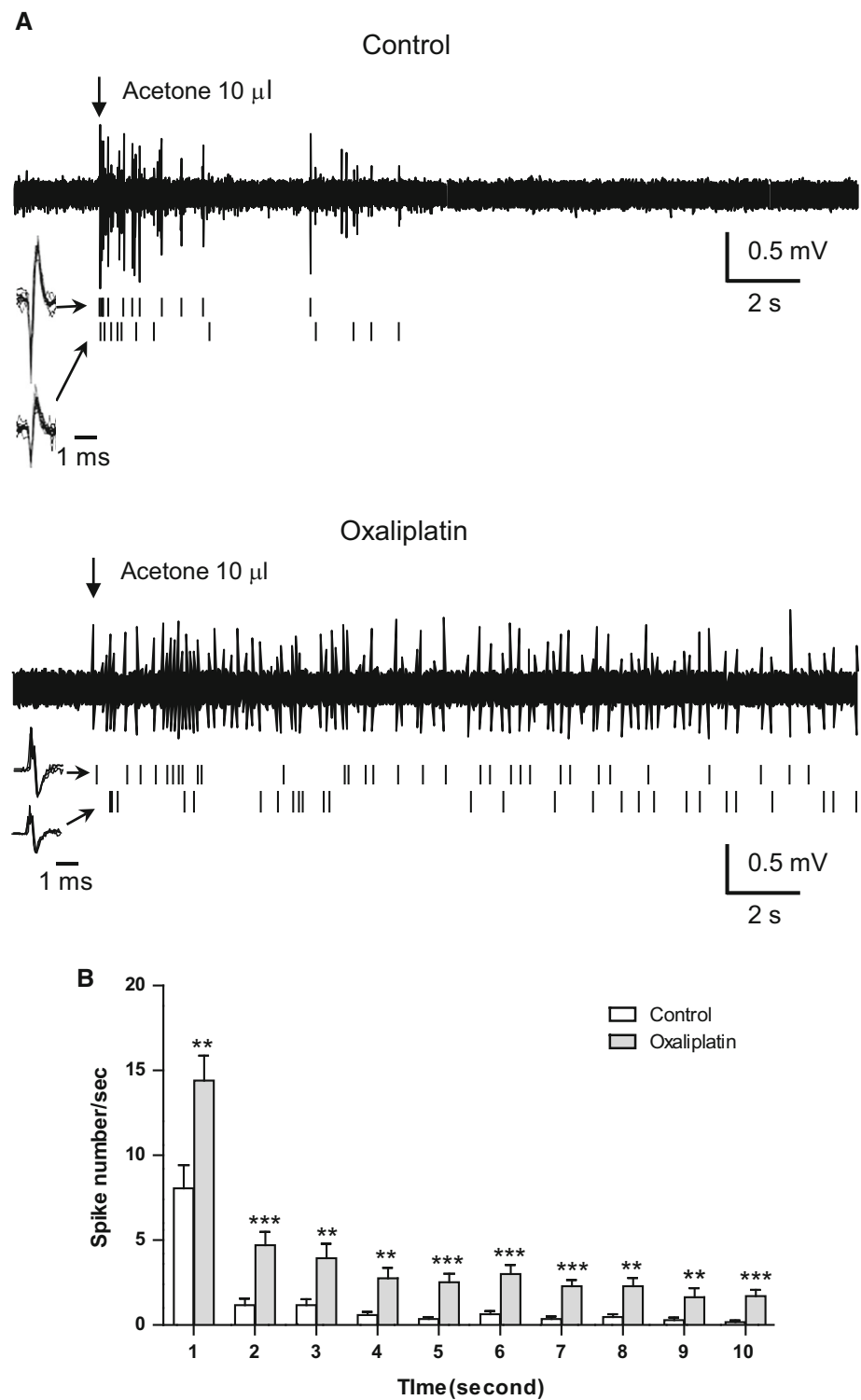


cells (29.4%) in the control group and 7 of 21 cells (33.3%) in the oxaliplatin group exhibited afterdischarge activity to pinch, resulting in no significant difference in spike frequency after the end of stimulation between the two groups (Supplementary Fig. 2B). Following press stimulation, afterdischarges were observed only in 2 of 21 recorded

cells (9.5%) in the oxaliplatin group, but not observed in control rats. This leads to only very slight differences in spike frequency after the end of press between the two groups (Supplementary Fig. 2C). Regarding these results for afterdischarges, only a small number of animals (1 of 7 rats in control group and 2 of 8 rats in oxaliplatin group)

exhibited prolonged pain behaviors (i.e., shaking and licking) following von Frey hair stimuli.

Noradrenaline (50 μM) is well known to have an inhibitory action on nociceptive transmission in the spinal cord [6, 28]. We evaluated whether noradrenaline could inhibit the longer duration of cold-elicited responses in WDR neurons of oxaliplatin-administered rats. As shown in Fig. 3a, noradrenaline applied to the surface of the spinal cord inhibited cold-elicited action potentials in oxaliplatin-administered rats. The cold-elicited responses were also suppressed by clonidine (50 μM, see lower trace in Fig. 3a) and phenylephrine (50 μM, trace is not shown). The spinal application of noradrenaline produced the most potent inhibition rate (87.2%). Clonidine (75.9%) and phenylephrine (68.8%) also showed a strong, but slightly lower, suppressive effect, whereas isoprenaline (50 μM) exhibited



Fig. 3 Inhibitory effects of spinal administration of noradrenaline and its agonists on the increased firing of wide dynamic neurons in response to cold stimulation in oxaliplatin-injected rats. a Representative raw traces of WDR neuron's activities evoked by cold stimulation (a drop of 10 μl acetone to the hind paw) before and after spinal application of noradrenaline (top panel) or α$_2$ agonist, clonidine (bottom panel). b Inhibition rate of cold stimulation-evoked activities of wide dynamic range neurons by noradrenaline (50 μM, n = 11) or its agonists phenylephrine (α$_1$ agonist, 50 μM, n = 4), clonidine (α$_2$ agonist, 50 μM, n = 9), and isoprenaline (β agonist, 50 μM, n = 6). ***$p < 0.001$, by 1-way ANOVA. c Behavioral assessment with five cold stimulations after intrathecal drug administration shows that noradrenaline, α$_2$, and α$_1$ agonists generate analgesic effects on cold allodynia in accordance with electrophysiological data. **$p < 0.01$, ***$p < 0.001$, by 2-way ANOVA
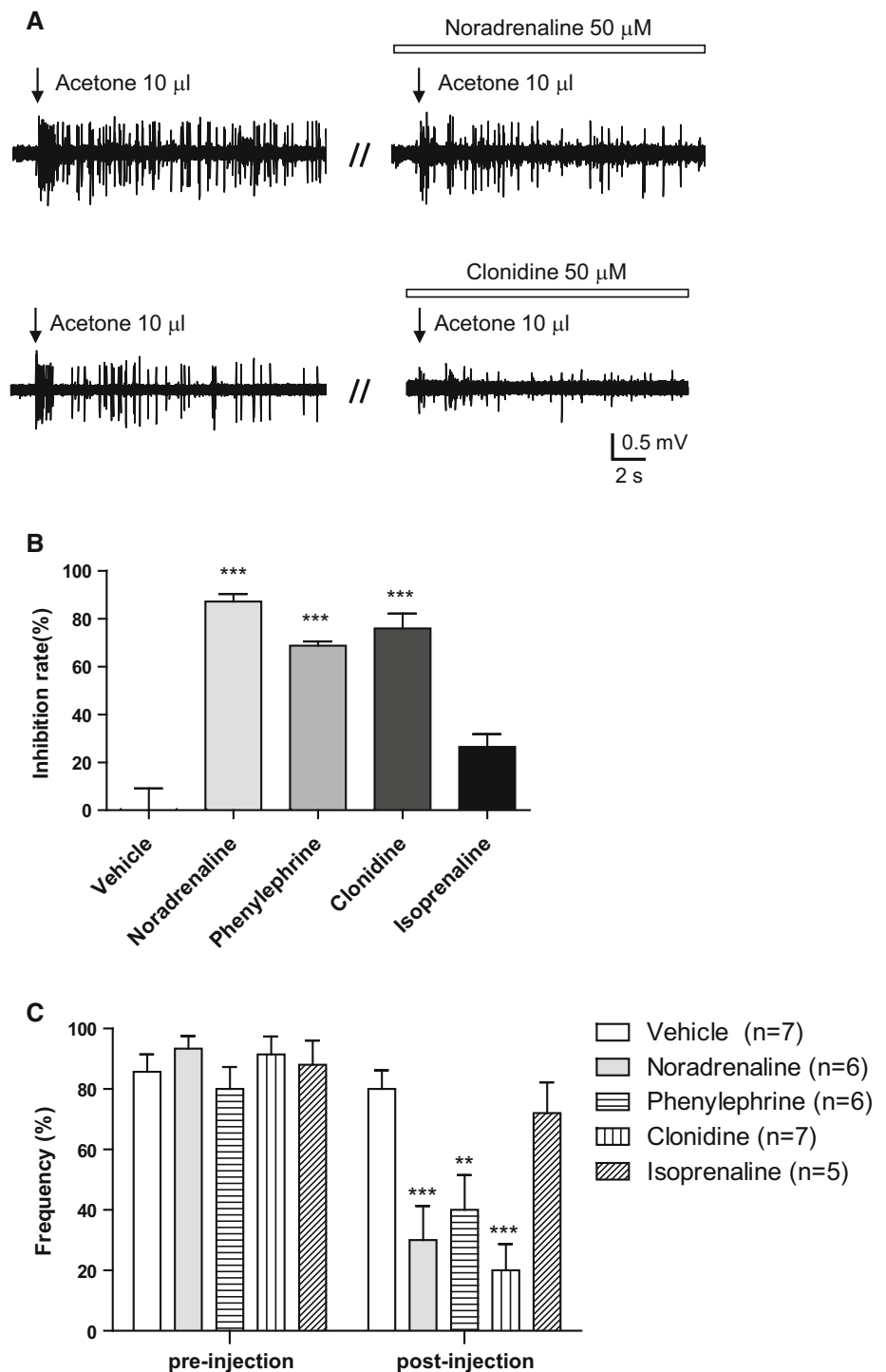
just a very low effect (26.5%) (Fig. 3b). Being consistent with these electrophysiological data, behavioral results with intrathecal injection of noradrenaline and its agonists showed that $\alpha_2$ and $\alpha_1$ agonists, but not $\beta$ agonist, induced significant analgesic effects (Fig. 3c).

## Discussion

The acute neurotoxicity observed after oxaliplatin injection occurs in nearly all patients [29]. The current recommendations for the management of the acute and cumulative neurotoxicity from oxaliplatin include education about exposure to cold, dose modification, "stop and go", and use of neuromodulatory agents [29]. However, no certain consensus has been established on the treatment of the neurotoxicity that causes cold allodynia.

In a mouse model of chronic oxaliplatin-induced cold allodynia using a long-term repeated injection protocol, increased frequency of neuronal firing in response to cold stimulation was reported [30]. In addition to this increase in spike frequency, we also found increased duration of firing evoked by cold stimulation; even though the stimulation has ceased, afterdischarge was observable for more than 10 s in oxaliplatin-injected rats. Prolonged afterdischarges of WDR neurons have been identified as an important element in describing central sensitization in pain conditions, in which intrinsic changes of the membrane properties were seen as a consequence of nerve injury [31]. Interestingly, mechanical stimuli (i.e., press and pinch) did not significantly increase the duration of neuronal firing in contrast to the case of cold stimulation (Supplementary Fig. 2A). These results correspond well with clinical reports that the characteristic and major side effect of oxaliplatin administration is cold hypersensitivity [32, 33].

We also confirmed that a single injection of oxaliplatin induces significant mechanical hypersensitivity and increases firing frequency of spinal WDR neurons in response to mechanical stimuli (Supplementary Fig. 1). Thus, oxaliplatin-induced acute mechanical hypersensitivity should not be ignored. However, little differences in afterdischarge activity following pinch or press stimulation were observed between the vehicle control and oxaliplatin groups (Supplementary Fig. 2). In addition, only a minority of animals in the control group (14.3%) and in the oxaliplatin group (25.0%) exhibited prolonged pain behaviors following von Frey hair mechanical stimuli.

The sensation of pain is known to be modified by endogenous pain inhibitory systems [4, 7]. Several endogenous analgesic systems originate from the separated supraspinal nuclei and project down to the spinal cord widely, thereby controlling sensory inputs within the spinal deep dorsal horn [34]. Noradrenaline, especially, is one of the important endogenous analgesic systems at the spinal level. In the spinal cord of naïve rats, we revealed that noradrenaline induces inhibition of pain transmission [5, 35]. Previous studies also demonstrated that noradrenaline is the major contributor in the spinal inhibition of nociception with mechanical allodynia and thermal hyperalgesia caused by peripheral neuropathy [36, 37]. However, it remains to be elucidated whether noradrenaline suppresses the evoked neuronal activities in the spinal cord in a rat model of oxaliplatin-induced neuropathic cold allodynia. In this study, we therefore evaluated the inhibitory effects of noradrenaline and its agonists on the response of the spinal WDR neurons to peripheral cold stimuli in oxaliplatin-injected rats.

Major noradrenaline receptors are divided into $\alpha_1$, $\alpha_2$, and $\beta$ adrenoceptors [7]. $\alpha$ adrenoceptors are further classified into subtypes $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$, and $\beta$ adrenoceptors into subtypes $\beta_1$, $\beta_2$, and $\beta_3$ [38]. Subtypes of $\alpha_1$ and $\alpha_2$ adrenoceptors, mainly $\alpha_{1A}$ and $\alpha_{2A}$, are reported to be expressed throughout the spinal cord, whereas $\beta$ adrenoceptors are few in the spinal cord [39, 40]. In several behavioral studies, spinal $\alpha_2$ agonist has been reported to be responsible for distinct analgesic effects in normal and neuropathic pain animals [7, 41–44]. Also it is known that $\alpha_1$ agonist mediates the elevation of nociceptive threshold in normal animals, whereas $\beta$ agonist does not [41, 45]. In superficial lamina, we showed that $\alpha_2$ agonist operates on primary afferent terminals of afferent fibers and dorsal horn neurons in the spinal cord, therefore inducing suppressive effects on pain transmission [5, 7, 35]. In addition, $\alpha_1$ agonist acts on presynaptic terminals of inhibitory interneurons to facilitate miniature GABA or glycine release on substantia gelatinosa neurons, and also excites inhibitory neurons in deeper lamina to enhance spontaneous large amplitudes of GABA and glycinergic responses in substantia gelatinosa neurons [46]. In contrast, no clear electrophysiological and behavioral evidence supporting the inhibitory action of $\beta$ adrenoceptor in the spinal dorsal horn has been reported [7, 41, 47]. Our data in this study are largely in agreement with the above results from previous studies, although the location of the recorded neurons might be different. We clearly show that noradrenaline suppressed the augmented neuronal firings evoked by peripheral cold stimuli in the oxaliplatin-induced cold allodynia model. Furthermore, $\alpha_2$ and $\alpha_1$ agonists, but not $\beta$ agonist, mimicked such inhibitory effects. We also demonstrate that direct spinal administration of noradrenaline inhibited the exacerbated behavioral response to non-noxious peripheral cold stimuli, which was reproduced by $\alpha_2$ and $\alpha_1$ agonists. These highly correlated electrophysiological and behavioral results suggest that spinal noradrenaline produces analgesic effects on oxaliplatin-induced cold allodynia, which is mediated by

activation of $\alpha_2$ and $\alpha_1$ adrenoceptors, but not by that of $\beta$ adrenoceptors.

In conclusion, our study demonstrates a significant increase in frequency and duration of the spinal WDR neuronal firings in response to peripheral cold stimulation in oxaliplatin-administered rats. Moreover, we suggest that the inhibitory effect of spinal noradrenaline on oxaliplatin-induced cold allodynia is mediated by activation of $\alpha_2$ and $\alpha_1$ adrenoceptors, but not by $\beta$ adrenoceptor. Although there have been no reports showing the suppressive effects of spinal application of noradrenaline or its agonists on chemotherapy-induced mechanical hypersensitivity, several articles demonstrated that systemic injection of $\alpha_2$ agonists had beneficial effects on oxaliplatin- and vincristine-induced mechanical allodynia [48–50]. Further electrophysiological and behavioral studies are needed to determine whether the therapeutic use of the spinal noradrenergic system could be applicable not only to oxaliplatin-induced cold allodynia, but also to mechanical hypersensitivity and other CIPN.

**Compliance with ethical standards**

**Conflicts of interest** The authors declare no conflict of interest.

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