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RESEARCH PAPER

Topical application of disodium isostearyl 2-O-L-ascorbyl phosphate, an amphiphilic ascorbic acid derivative, reduces neuropathic hyperalgesia in rats

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BACKGROUND AND PURPOSE

$Ca_v3.2$ T-type calcium channels, targeted by H_2S, are involved in neuropathic hyperalgesia in rats and ascorbic acid inhibits $Ca_v3.2$ channels. Therefore, we evaluated the effects of intraplantar (i.pl.) administration of ascorbic acid or topical application of disodium isostearyl 2-O-L-ascorbyl phosphate (DI-VCP), a skin-permeable ascorbate derivative on hyperalgesia induced by NaHS, an H_2S donor, and on neuropathic hyperalgesia.

EXPERIMENTAL APPROACH

In rats mechanical hyperalgesia was evoked by i.pl. NaHS, and neuropathic hyperalgesia was induced by L5 spinal nerve cutting (L5SNC) or by repeated administration of paclitaxel, an anti-cancer drug. Dermal ascorbic acid levels were determined colorimetrically.

KEY RESULTS

The NaHS-evoked $Ca_v3.2$ channel-dependent hyperalgesia was inhibited by co-administered ascorbic acid. Topical application of DI-VCP, but not ascorbic acid, prevented the NaHS-evoked hyperalgesia, and also increased dermal ascorbic acid levels. Neuropathic hyperalgesia induced by L5SNC or paclitaxel was reversed by i.pl. NNC 55–0396, a selective T-type calcium channel blocker, ascorbic acid or DI-VCP, and by topical DI-VCP, but not by topical ascorbic acid. The effects of i.pl. ascorbic acid and topical DI-VCP in the paclitaxel-treated rats were characterized by the faster onset and greater magnitude, compared with their effects in the L5SNC rats. Dermal ascorbic acid levels in the hindpaw significantly decreased after paclitaxel treatment, but not L5SNC, which was reversed by topical DI-VCP.

CONCLUSIONS AND IMPLICATIONS

Ascorbic acid, known to inhibit $Ca_v3.2$ channels, suppressed neuropathic hyperalgesia. DI-VCP ointment for topical application may be of benefit in the treatment of neuropathic pain.

Abbreviations

CBS, cysthathionine-β-synthase; CSE, cystathionine-γ-lyase; DI-VCP, disodium isostearyl 2-O-L-ascorbyl phosphate; DRG, dorsal root ganglion; i.pl., intraplantar; L5SNC, L5 spinal nerve cutting, ODN, oligodeoxynucleotide

Introduction

The molecular mechanisms underlying neuropathic pain involve abnormal functions and/or altered expression of voltage-gated ion channels in sensory neurons and such channels include sodium (Porreca *et al.*, 1999; McGowan *et al.*, 2009), calcium (Matthews and Dickenson, 2001a,b; Boroujerdi *et al.*, 2011) and potassium channels (Cao *et al.*, 2010). Increasing evidence suggests the involvement of low-voltage-activated or transient (T)-type calcium channels (channel nomenclature follows Alexander *et al.*, 2011) in the processing of pain signals. Furthermore, the $Ca_v3.2$ isoform of T-type channels, abundantly expressed in the peripheral ending of nociceptors, plays an important role in the pathophysiology of neuropathic pain (Todorovic *et al.*, 2001; Dogrul *et al.*, 2003; Flatters and Bennett, 2004; Bourinet *et al.*, 2005; Jagodic *et al.*, 2008; Takahashi *et al.*, 2010; Okubo *et al.*, 2011; Todorovic and Jevtovic-Todorovic, 2011;). Recently, we found that hydrogen sulfide (H_2S), a gasotransmitter, formed from L-cysteine by certain enzymes including cystathionine-γ-lyase (CSE) and cysthathionine-β-synthase (CBS) (Li and Moore, 2008), activated or sensitized $Ca_v3.2$ channels in the peripheral ending of nociceptors, leading to somatic and visceral hyperalgesia in rats (Kawabata *et al.*, 2007; Maeda *et al.*, 2009; Matsunami *et al.*, 2009; 2011; Nishimura *et al.*, 2009). The CSE/H_2S/$Ca_v3.2$ system appears to play a role in the maintenance of the neuropathic hyperalgesia evoked by L5 spinal nerve cutting (L5SNC) or by repeated injections of paclitaxel, an anti-cancer drug (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). Evidence exists for the upregulation of $Ca_v3.2$ channels and/or the inhibition of neuropathic hyperalgesia by silencing expression of $Ca_v3.2$ channels in distinct neuropathic pain models (Bourinet *et al.*, 2005; Jagodic *et al.*, 2007; Messinger *et al.*, 2009; Takahashi *et al.*, 2010; Okubo *et al.*, 2011; Todorovic and Jevtovic-Todorovic, 2011), and so the identification of selective and potent inhibitors of $Ca_v3.2$ channels should help in the development of safer or more effective therapies for treatment of neuropathic pain.

Although selective and potent inhibitors of $Ca_v3.2$ channels are not currently available, there is evidence that ascorbic acid (vitamin C) inhibits $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$ channels, by initiating the metal-catalysed oxidation of a specific, metal-binding histidine residue in domain I of the channel (Nelson *et al.*, 2007). We have previously reported that ascorbic acid is capable of inhibiting the facilitation of T-type currents and concomitant neurite outgrowth by NaHS, a donor of H_2S that activates $Ca_v3.2$ channels, in NG108-15 cells (Nagasawa *et al.*, 2009; Tarui *et al.*, 2010). Systemic administration of ascorbic acid appears to inhibit formalin-evoked nociception in mice (Rosa *et al.*, 2005). Furthermore, there is clinical evidence to suggest that ascorbic acid reduces the prevalence of the complex regional pain syndrome after wrist fractures (Zollinger *et al.*, 2007) and the painful symptoms of herpetic and postherpetic neuralgia (Chen *et al.*, 2009; Schencking *et al.*, 2010; Byun and Jeon, 2011). Moreover, plasma ascorbic acid levels in patients with postherpetic neuralgia are lower than those seen in healthy individuals (Chen *et al.*, 2009; 2011). Therefore, we suggest that ascorbic acid reverses neuropathic pain, most probably by inhibiting $Ca_v3.2$ T-type calcium channels.

In the present study we evaluated the effects of intraplantar injection or topical application of ascorbic acid on the $Ca_v3.2$ channel-dependent hyperalgesia caused by intraplantar administration of NaHS, and on the neuropathic hyperalgesia induced by L5SNC or by repeated administration of paclitaxel in rats. In addition we examined the effect of topical application of disodium isostearyl 2-O-L-ascorbyl phosphate (DI-VCP) (Shibayama *et al.*, 2005), a skin-permeable, amphiphilic, ascorbic acid derivative, as an ointment, because ascorbic acid itself permeates skin poorly (Ebihara *et al.*, 2003). DI-VCP permeates into the skin, and is then converted into ascorbic acid following hydrolysis by phosphatases present in the tissues (Shibayama *et al.*, 2005; 2008a,b). Here we show evidence that DI-VCP ointment is a novel drug for treatment of neuropathic pain.

Methods

Animals

All animal care and experimental procedures used were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996), and adhered to the guidelines of the IASP published in Pain, 16 (1983) 109–110. The experimental work was approved by the Committee for the Care and Use of Laboratory Animals at Kinki University. Male Wistar rats (7 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). Rats were housed in a temperature- and light-controlled room (around 24°C, 12-h light/dark cycles) and had free access to food and water.

Measurement of nociceptive threshold

Nociceptive threshold was measured by the paw pressure test, using an analgesia meter (MK-300, Muromachi Kikai, Co., Tokyo, Japan). Continuously increasing pressure (30 $g \cdot s^{-1}$) was applied to the hindpaw of the rat and the weight required to elicit nociceptive responses was determined as a nociceptive threshold. A cut-off weight of 500 g was used to prevent tissue damage (Kawabata *et al.*, 2007). The baseline threshold was determined after a training session, and the threshold measured after drug administration and/or surgical operation was expressed as a percentage of the baseline threshold (% baseline). The area under the curve (AUC) of the time (min)–threshold (%) curve for the appropriate time period was also calculated by adding the areas of the trapezoid between two consecutive time points for threshold measurements.

A surgically induced neuropathic pain model in the rat

The surgically-induced neuropathic pain model was created by cutting the L5 spinal nerve (L5SNC), as described previously (Kim and Chung, 1992; Chung *et al.*, 2004; Takahashi *et al.*, 2010), with minor modifications. Under anaesthesia with sodium pentobarbital (40 $mg \cdot kg^{-1}$, i.p.; supplemented as necessary), the right L4 and L5 spinal nerves were exposed by removing the paraspinal muscles and the right L6 transverse process. The right L5 spinal nerve was ligated with a thread and completely cut at the peripheral side of the knot. For

sham operations, the animals were subjected to the same procedure except for ligation and cutting of the L5 spinal nerve.

Silencing of $Ca_v3.2$ channels in the sensory neurons

Silencing of $Ca_v3.2$ channels in the sensory neurons was achieved by repeated intrathecal administration of the antisense oligodeoxynucleotides (ODNs) for $Ca_v3.2$ channels in rats, as previously reported (Maeda *et al.*, 2009).

Chemotherapy-evoked neuropathic pain in the rat

A chemotherapy-induced neuropathy model was created by repeated administration of paclitaxel, an anti-cancer drug, as described previously (Polomano *et al.*, 2001; Okubo *et al.*, 2011). After measurements of baseline nociceptive thresholds, paclitaxel [prepared from 6 mg·mL^{-1} solution in 50% Cremophor EL® (polyethoxylated castor oil) and 50% ethanol, Taxol®, Bristol-Myers Co. Ltd, Tokyo, Japan] at a dose of 2 mg·kg^{-1} was administered (i.p.) to rats every 2 days (day 0, 2, 4 and 6), four times in all. The animals were used for experiments at least 2 weeks after the start of paclitaxel treatement, except for the time-course experiments.

Drug administration

For intraplantar (i.pl.) administration, NaHS (Kishida Chem. Co., Ltd. Osaka, Japan), a donor of H_2S, at 1 nmol per paw and NNC 55–0396 (Sigma-Aldrich, St. Louis, MO, USA), a selective T-type calcium channel blocker, at 1–10 nmol per paw, ascorbic acid (Sigma-Aldrich) at 3–30 nmol per paw and DI-VCP (HBC Science Research Center Co. Ltd, Osaka, Japan) at 3–30 nmol per paw were dissolved in saline, and administered into the plantar region of the right hindpaw in rats in a volume of 100 µL. In one set of experiments, ascorbic acid was co-administered i.pl. with NaHS. For topical application, ascorbic acid or DI-VCP ointment (80 or 240 nmol·g^{-1}) was prepared in the vehicle consisting of macrogol 400 and 4000 (1:1) (Solbase®, Dainipon Sumitomo Pharma Co., Ltd, Osaka, Japan) to which 5% 1-hexadecanol (Kishida) was added. The ointment of 250 mg containing 20, 60 or 600 nmol of ascorbic acid or DI-VCP was applied to the skin from the instep of the right hindpaw extending to the lower front surface of the crus in the rat. To prevent the rat from licking or biting the hindpaw, a Rat Elizabethan Collar (RC E1, Lomir Biomedical Inc., Quebec, Canada) was used and rats were acclimatized to the collar for 2 days before the experiments. In the experiments to test the effect on the NaHS-induced hyperalgesia, the ointment was applied to the hindpaw 90 min before i.pl. injection of NaHS.

Measurements of ascorbic acid levels in the instep skin of rat hindpaw

Ascorbic acid concentrations in the hindpaw tissue were determined in the control rats, rats in the neuropathic pain models, and also 90 min after topical application of ascorbic acid, DI-VCP or vehicle ointment to the right hindpaw in those animals. The rat was anaesthetized (i.p. urethane, 1.5 g·kg^{-1}), and the surface of the hindpaws of each rat was cleaned with KimWipes (S-200, Nippon Paper Crecia Co. Ltd, Tokyo, Japan). After transcardial perfusion with saline in a volume of 200 mL, the skin was excised from each instep of the ipsilateral (right) and contralateral (left) hindpaws, and homogenized/sonicated in 14 volumes of 5.4% metaphosphoric acid (final concentration: 5%). The amount of total ascorbic acid including ascorbic acid (reduced form) and dehydroascorbic acid (oxidized form) was determined colorimetrically by the 2,4-dinitrophenylhydrazine derivatization method, using a vitamin C assay kit (SHIMA Laboratories Co., Ltd, Tokyo, Japan). In the preliminary experiments, it was confirmed that DI-VCP itself did not react in this assay system.

Whole-cell patch-clamp recordings

Whole-cell patch-clamp recordings were performed as described previously (Kawabata *et al.*, 2007). The NG108-15 cells were seeded in culture dishes (35 mm in diameter) (1×10^4 cells/dish), and cultured for one day in the culture medium containing 1% fetal calf serum (FCS). The cells were washed with an extracellular solution containing (in mM): 97 N-methyl-D-glucamine, 10 $BaCl_2$, 10 HEPES, 40 tetraethylammonium chloride and 5.6 glucose, adjusted to pH 7.4. Ba^{2+} currents were recorded from the cells at room temperature using a whole-cell patch-clamp amplifier. A patch pipette was filled with an intracellular solution containing (in mM): 140 CsCl, 4 $MgCl_2$, 5 EGTA and 10 HEPES, adjusted to pH 7.2. The resistance of patch electrodes ranged from 3 to 7 MΩ. Series-resistance was compensated by 80%. The cell membrane voltage was held at –80 mV, and whole-cell Ba^{2+} currents were elicited by step pulses of 200 ms duration from –120 mV to +40 mV with increments of 10 mV. T-type currents were measured as the difference between currents at a peak and 150 ms after the beginning of a test pulse at –20 mV. Data were acquired and digitized with a Digidata interface (Digidata 1440, Axon Instruments, Foster City, CA, USA) and analysed by a personal computer using pClamp10.2 software (Axon Instruments). NaHS at 1.5 mM was added to the NG108-15 cells after recording of the control T-currents in a cell, and the effects of NaHS on T-currents were determined 2 min after their application in the same cell. Ascorbic acid at 0.1–1 µM was added to the cells 10 s before the addition of NaHS.

Statistics

Data are represented as mean ± SEM. Student's *t* test for unpaired or paired data and Tukey's test were used for analysing statistical significance of differences between two groups and among three or more groups respectively. Differences among experimental groups were considered significant when $P < 0.05$.

Results

Effects of i.pl. administration or topical application of ascorbic acid or DI-VCP on hyperalgesia induced by i.pl. NaHS in rats and on the ascorbic acid levels in rat skin

Using NG108-15 cells that abundantly express $Ca_v3.2$ T-type calcium channels (Nagasawa *et al.*, 2009; Tarui *et al.*, 2010),

we first confirmed that ascorbic acid in a range of 0.1–1 μM inhibited the facilitation of T-type currents induced by 1.5 mM NaHS in a concentration-dependent manner (Figure 1A), thus confirming our previous study (Tarui *et al.*, 2010). Ascorbic acid at the same concentration range had no effect on T-type currents in the absence of NaHS (data not shown). Next, we determined whether ascorbic acid inhibits the $Ca_v3.2$ channel-mediated hyperalgesia caused by H_2S, which we reported previously (Kawabata *et al.*, 2007; Maeda *et al.*, 2009). Local , i.pl., injection of NaHS, a donor of H_2S, at 1 nmol per paw produced prompt and short-lasting mechanical hyperalgesia (see Figure 1D), as reported previously (Kawabata *et al.*, 2007; Maeda *et al.*, 2009). We then confirmed that the hyperalgesia caused by i.pl. NaHS was significantly reduced by the silencing of $Ca_v3.2$ channels in the primary afferents under the present experimental conditions; the relative nociceptive threshold (%) 15–20 min after i.pl. administration of vehicle and NaHS was 110.5 ± 6.5 and 77.3 ± 3.1, respectively, in the rats treated with the mismatch ODN, and 107.9 ± 5.1 and 99.4 ± 3.7 respectively, in the rats treated with the antisense ODN for $Ca_v3.2$ channels (n = 7–8). The i.pl. NaHS-induced hyperalgesia was inhibited by co-administered ascorbic acid at 10 nmol per paw (Figure 1B), but not by ascorbic acid ointment at 60 nmol per paw applied topically 90 min before i.pl. NaHS (Figure 1C). In contrast, DI-VCP, the amphiphilic ascorbic acid derivative, as an ointment, when applied topically in the same dose and manner, significantly suppressed the NaHS-induced mechanical hyperalgesia (Figure 1D, E). Dermal ascorbic acid levels in the instep of the ipsilateral hindpaw significantly increased 90 min after topical application of DI-VCP ointment at 60 nmol per paw, but not after treatment with ascorbic acid ointment at the same dose or vehicle (Figure 1F).

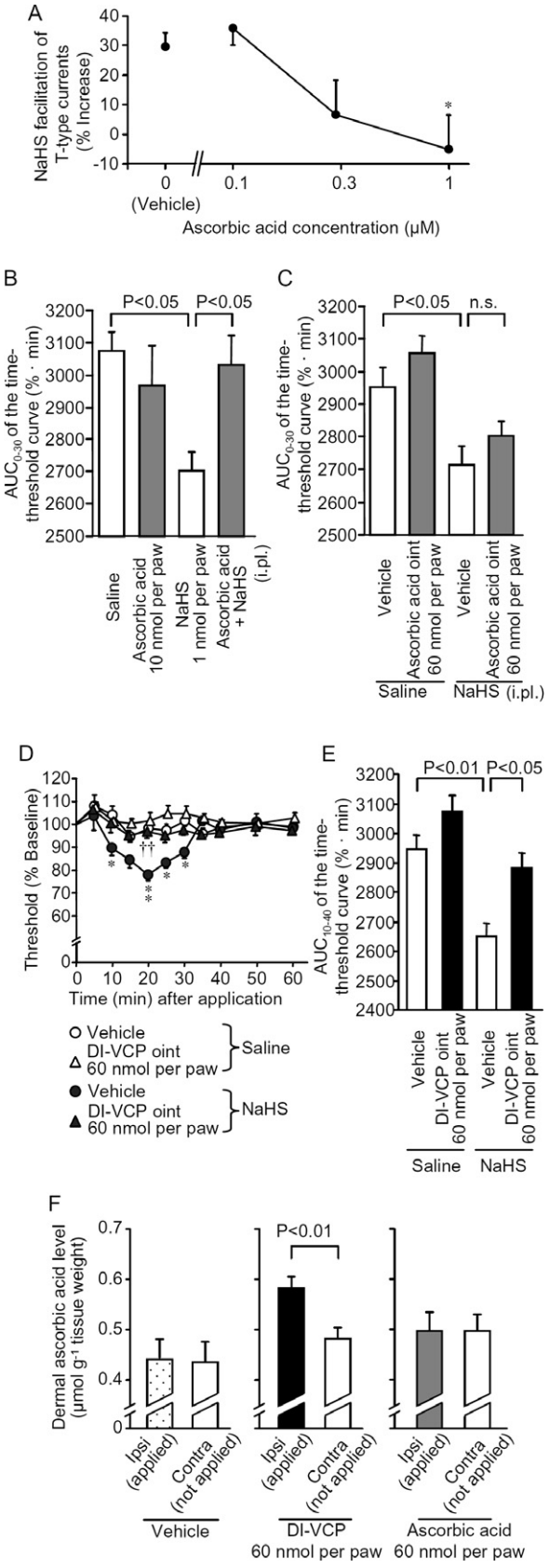

Figure 1

Effects of ascorbic acid or DI-VCP on T-type currents in NG108-15 cells and on the hyperalgesia induced by NaHS and the dermal ascorbic acid levels in rats. (A) Effect of ascorbic acid on T-type currents facilitated by NaHS in NG108-15 cells. T-type currents were determined before and 2 min after application of NaHS at 1.5 mM, in the presence or absence of ascorbic acid at 0.1–1 μM. Data show the means ± SEM from 6–13 different cells. *P < 0.05 significantly different from vehicle. (B) and (C) Effects of i.pl. administration (B) or topical application (C) of ascorbic acid on the NaHS-induced hyperalgesia. The rats received i.pl. (B) NaHS (1 nmol per paw) and ascorbic acid (10 nmol per paw, or (C) i.pl. NaHS, 90 min after topical application of ascorbic acid ointment (oint; 60 nmol per paw). The results are presented as the AUC of the time–threshold curve for early 30 min. (D) and (E) Effects of topical application of DI-VCP ointment on the NaHS-induced hyperalgesia. DI-VCP (60 nmol per paw) was applied topically 90 min before i.pl. NaHS (1 nmol per paw). The results are presented as the time–threshold curve (D) and the AUC for 10–40 min after i.pl. administration (E). *P < 0.05, **P < 0.01 significantly different from vehicle plus saline. ††P < 0.01 significantly different from vehicle plus NaHS. (F) Dermal ascorbic acid levels in the instep of ipsilateral (Ipsi) and contralateral (Contra) hindpaws 90 min after topical application of DI-VCP or ascorbic acid ointment (60 nmol per paw) to the right hindpaw. Data show the means ± SEM from 7–8 (B), 6–8 (C), 6–8 (D, E) and 4–5 (F) rats.

Effects of i.pl. administration of NNC 55–0396, a selective T-type calcium channel blocker, and of ascorbic acid or DI-VCP on the neuropathic hyperalgesia induced by L5SNC or by repeated treatment with paclitaxel in rats

The mechanical nociceptive threshold in the ipsilateral hindpaw gradually decreased after L5SNC or repeated administration of paclitaxel, reaching a plateau within 2 weeks (Figure 2A, B), as reported previously (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). Like mibefradil (Takahashi *et al.*, 2010), NNC 55–0396, a selective T-type calcium channel blocker, administered i.pl. at 1–10 nmol per paw, dramatically reversed neuropathic hyperalgesia in rats with L5SNC in a dose-dependent manner (Figure 2C). Similarly, the dose-dependent anti-hyperalgesic effect of i.pl. NNC 55–0396 in the same dose range was confirmed in rats treated with paclitaxel (Figure 2D), in agreement with our previous study (Okubo *et al.*, 2011). Local, i.pl., administration of ascorbic acid or DI-VCP at 3–30 nmol per paw significantly reversed neuropathic hyperalgesia caused by L5SNC (Figure 3A, B). The anti-hyperalgesic effects of ascorbic acid and DI-VCP gradually developed and peaked or plateaued around 100 min after i.pl. injection (Figure 3A). The anti-hyperalgesic effect of DI-VCP lasted for 300 min (5 h) or more and disappeared within 24 h, whilst the effect of ascorbic acid disappeared about 240 min after i.pl. administration (Figure 3A). Sequential administration of ascorbic acid and NNC 55–0396 at 10 nmol per paw, their maximal dose, also significantly inhibited the hyperalgesia in L5SNC rats (Figure 3C), and the magnitude of the inhibitory effect of the sequential administration was 73.4% (Figure 3C), which was not greatly different from that of the effects of a single administration of ascorbic acid and NNC 55–0396 (56.9 and 68.8% respectively) (Figures 2C, 3B). In contrast, paclitaxel-induced neuropathic hyperalgesia was rapidly and completely reversed by i.pl. administration of ascorbic acid (Figure 3D, E). The rapid onset of the anti-hyperalgesic effect of i.pl. ascorbic acid in paclitaxel-induced neuropathic pain rats (Figure 3D) was in contrast to its slowly developing effect in the L5SNC rats (see Figure 3A).

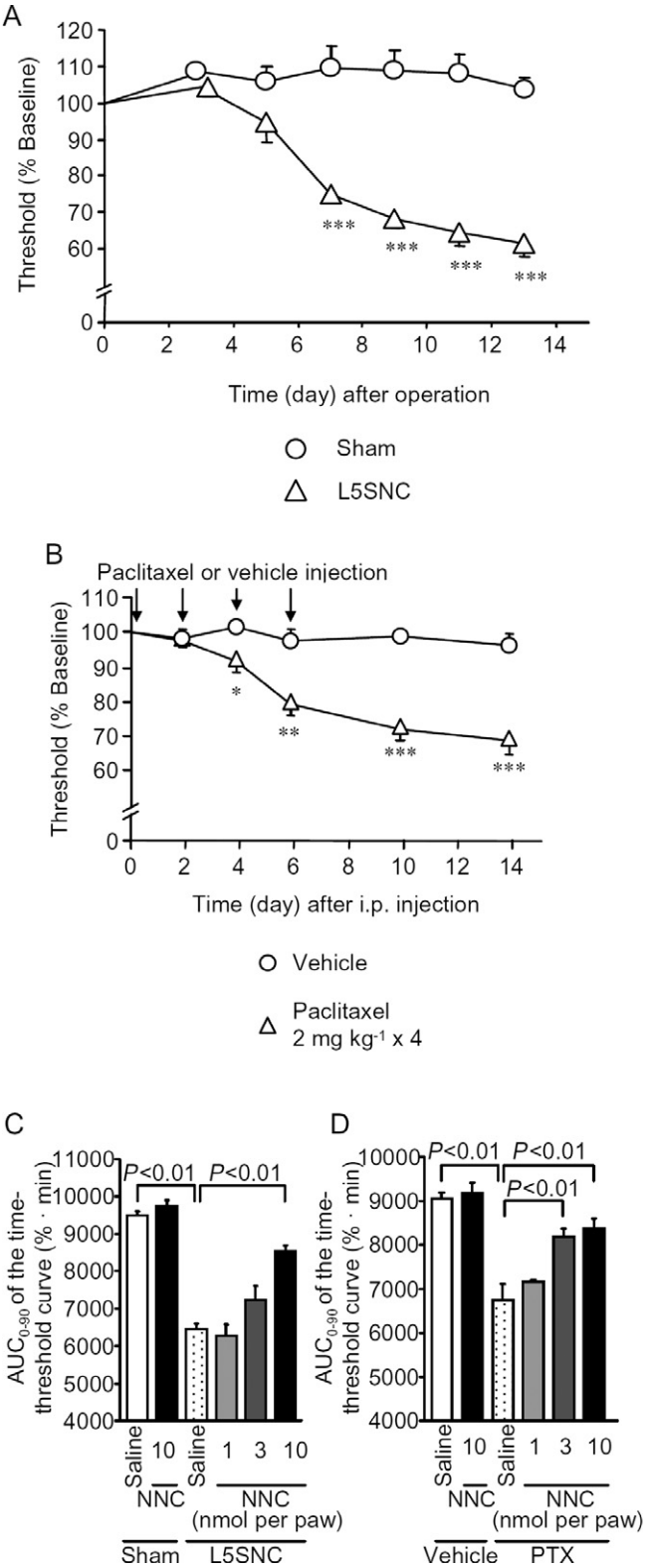

Figure 2

Reversal of neuropathic hyperalgesia by NNC 55–0396, a selective T-type calcium channel blocker, in rats with L5SNC or treated with paclitaxel. (A) and (B) The time-courses of the development of mechanical hyperalgesia after L5SNC (A) or repeated administration of paclitaxel (2 mg·kg^{-1}; B). *P < 0.05, **P < 0.01, ***P < 0.001 significantly different from sham or vehicle. (C) and (D) Anti-hyperalgesic effects of i.pl. administration of NNC 55–0396 (1–10 nmol per paw) in rats with L5SNC (C) or treated with paclitaxel (PTX; D). The results are presented as the AUC of the time-threshold curve for the first 90 min after i.pl. administration of NNC 55–0396. Data show the means ± SEM from 5–6 (A), 9–10 (B), 4–5 (C) and 5–7 (D) rats.

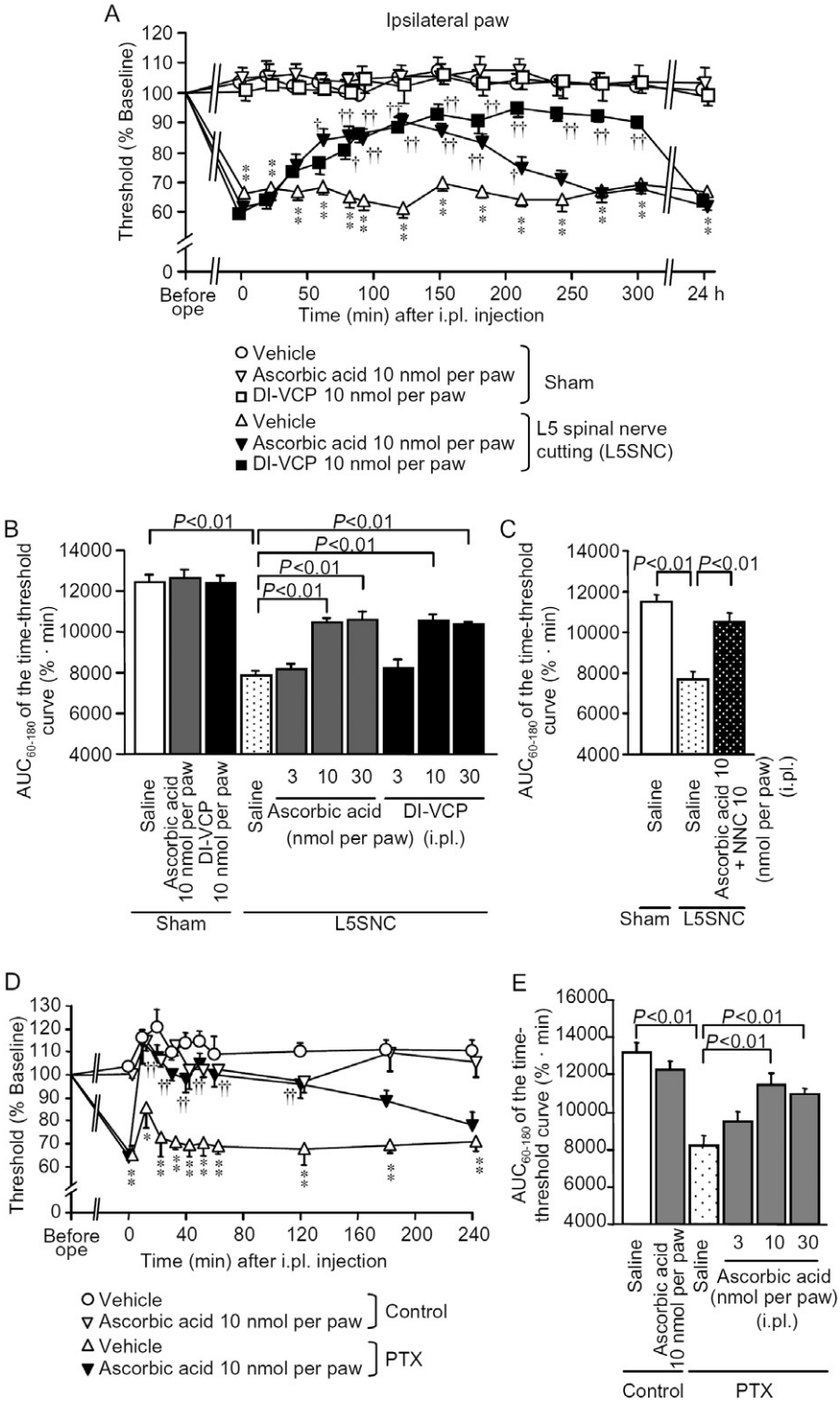

Figure 3

Anti-hyperalgesic activity of i.pl. administration of ascorbic acid or DI-VCP in the rats subjected to L5SNC or treated with paclitaxel. Ascorbic acid (3–30 nmol per paw; A, B, D, E), DI-VCP (3–30 nmol per paw; A, B), or ascorbic acid (10 nmol per paw) in combination with NNC 55–0396 (NNC; 10 nmol per paw; C) was administered i.pl. to the rats with neuropathy induced by L5SNC (A, B, C) or by paclitaxel (PTX; D, E). The results are presented as the time-threshold curves (A, D) and the AUC for 60–180 min (B, C, E) after i.pl. administration. (C) Effect of sequential administration of ascorbic acid and NNC in L5SNC rats. Ascorbic acid (10 nmol per paw) and NNC (10 nmol per paw) were administered i.pl. consecutively. The results are presented as the AUC for 60–180 min. $^{**}P < 0.01$ significantly different from vehicle in the sham rat. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ significantly different from vehicle in the rats with the neuropathy induced by L5SNC or paclitaxel. Data show the means ± SEM from 5–8 (A, B), 4–5 (C) and 5–7 (D, E) rats.

Topical application of DI-VCP, but not ascorbic acid, reverses the neuropathic hyperalgesia induced by L5SNC or by treatment with paclitaxel in rats

As expected, topical application of ascorbic acid ointment at 60 nmol per paw did not alter the decreased nociceptive threshold in the rat subjected to L5SNC (Figure 4A, C). In contrast, topical application of DI-VCP ointment at the same dose gradually elevated the nociceptive threshold in the L5SNC rat, an effect reaching a plateau in approximately 200 min and lasting for 600 min (10 h) or more, followed by the recovery of the hyperalgesia within 24 h (Figure 4A). The anti-hyperalgesic effect of DI-VCP ointment was significant and dose-dependent in a dose range of 20–600 nmol per paw (Figure 4A, B). In rats with paclitaxel-induced neuropathic hyperalgesia, topical application of DI-VCP, but not ascorbic acid, ointment at 60 nmol per paw completely reversed the decreased nociceptive threshold, an effect reaching a plateau in about 90 min and lasting for 240 min (4 h) or more, followed by the complete recovery of the hyperalgesia 24 h after the application (Figure 5). The inhibitory effect of DI-VCP ointment on the paclitaxel-induced neuropathic hyperalgesia was characterized by its rapid onset (Figure 5A), compared with its anti-hyperalgesic effect in the L5SNC-induced neuropathic pain rats (see Figure 4A).

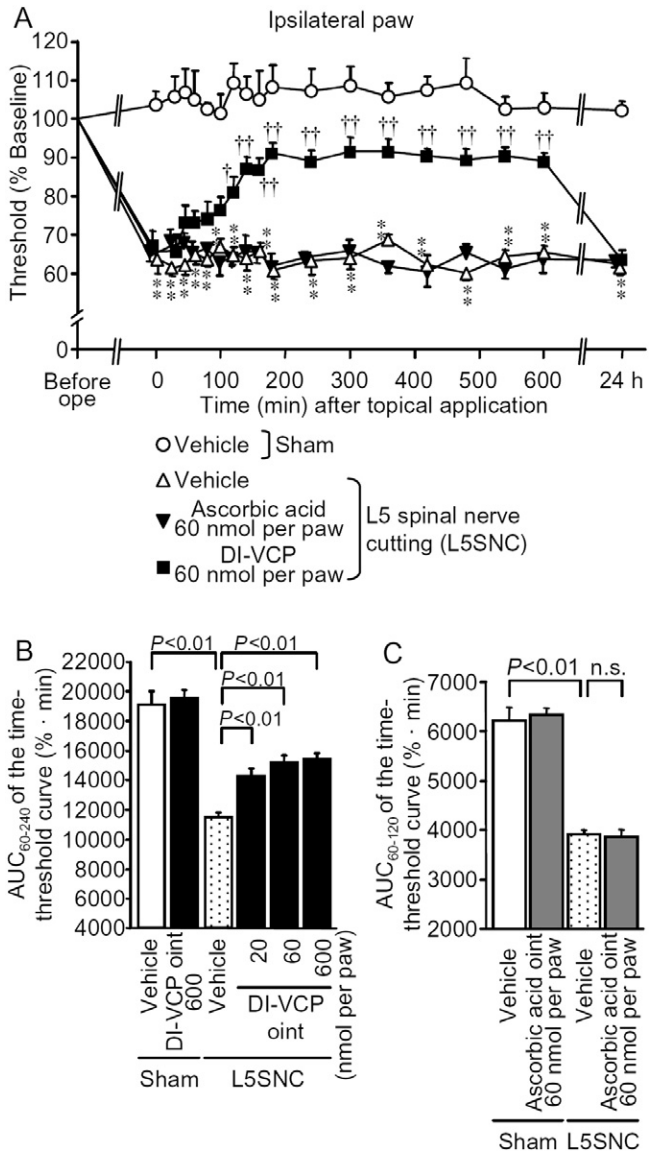

Figure 4

Anti-hyperalgesic activity of topical application of DI-VCP ointment in the rats subjected to L5SNC. DI-VCP (20, 60 or 600 nmol per paw) and ascorbic acid (60 nmol per paw) as ointment (oint) were applied topically to the ipsilateral hindpaw in the rat with L5SNC-induced neuropathy. The results are presented as the time-threshold curves (A) and the AUC for 60–240 (B) or 60–120 (D) min after i.pl. administration. $^{**}P < 0.01$ significantly different from vehicle in the rat subjected to sham operation. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ significantly different from vehicle in the L5SNC rats. Data show the means ± SEM from 4-7 rats.

Decreased dermal ascorbic acid levels in the instep of the hindpaw in the rats with neuropathy induced by paclitaxel, but not by L5SNC

Given the clinical evidence for decreased plasma ascorbic acid levels in patients with neuropathic pain (Chen *et al.*, 2009; 2011), we determined ascorbic acid levels in the instep skin of the 'hyperalgesic' hindpaw(s) in rats with the neuropathy induced by L5SNC or by paclitaxel. Surprisingly, dermal ascorbic acid levels in the instep of the bilateral hindpaws significantly decreased in the rats with paclitaxel-induced neuropathic pain (Figure 6B), while those with L5SNC-induced neuropathic pain did not exhibit significant alterations in the instep dermal ascorbic acid levels in each of ipsilateral and contralateral hindpaws (Figure 6A). In rats with paclitaxel-induced neuropathic pain, topical application of DI-VCP ointment to the right hindpaw significantly reversed the decreased ascorbic acid levels in the instep skin of the ipsilateral hindpaw 90 min after application (Figure 6C), although DI-VCP ointment was also capable of increasing the dermal ascorbic acid levels in naïve rats (see Figure 1E).

Discussion

The results shown in this study demonstrate that i.pl. administration of ascorbic acid, known to inhibit $Ca_v3.2$, but not $Ca_v3.1$ or $Ca_v3.3$ channels (Nelson *et al.*, 2007), blocked the transient hyperalgesia induced by the H_2S donor, NaHS, and reversed the persistent neuropathic hyperalgesia induced by L5SNC and by paclitaxel. These findings are consistent with our previous studies which suggested a role of the CSE/H_2S/$Ca_v3.2$ channel pathway in those neuropathic pain models (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). The long-lasting anti-hyperalgesic activity of topical application of DI-VCP, a skin-permeable ascorbic acid derivative, as an ointment suggests that this compound could be of therapeutic value in the treatment of clinical neuropathic pain. The faster onset and greater magnitude of the anti-hyperalgesic effects of i.pl. ascorbic acid and topically applied DI-VCP ointment in the rats treated with paclitaxel, as compared with their effects in

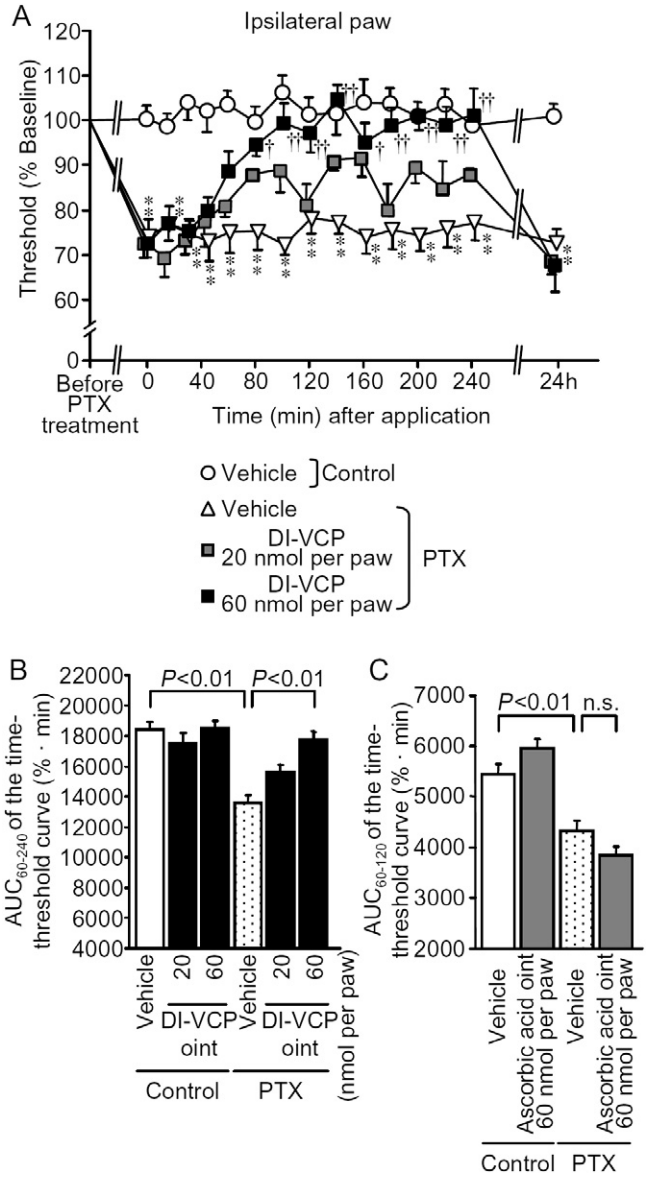

Figure 5

Anti-hyperalgesic activity of topical application of DI-VCP ointment in the rats treated with paclitaxel (PTX). DI-VCP (20 or 60 nmol per paw) and ascorbic acid (60 nmol per paw) as ointment (oint) were applied topically to the right hindpaw in the rats with paclitaxel-induced neuropathy. The results are presented as the time-threshold curves (A) and the AUC for 60–240 (B) or 60–120 (D) min after i.pl. administration. $^{**}P < 0.01$ significantly different from vehicle in the rat subjected to sham operation. $\dagger P < 0.05$, $\dagger\dagger P < 0.01$ significantly different from vehicle in the paclitaxel-treated rats. Data show the means ± SEM from 5-7 rats.

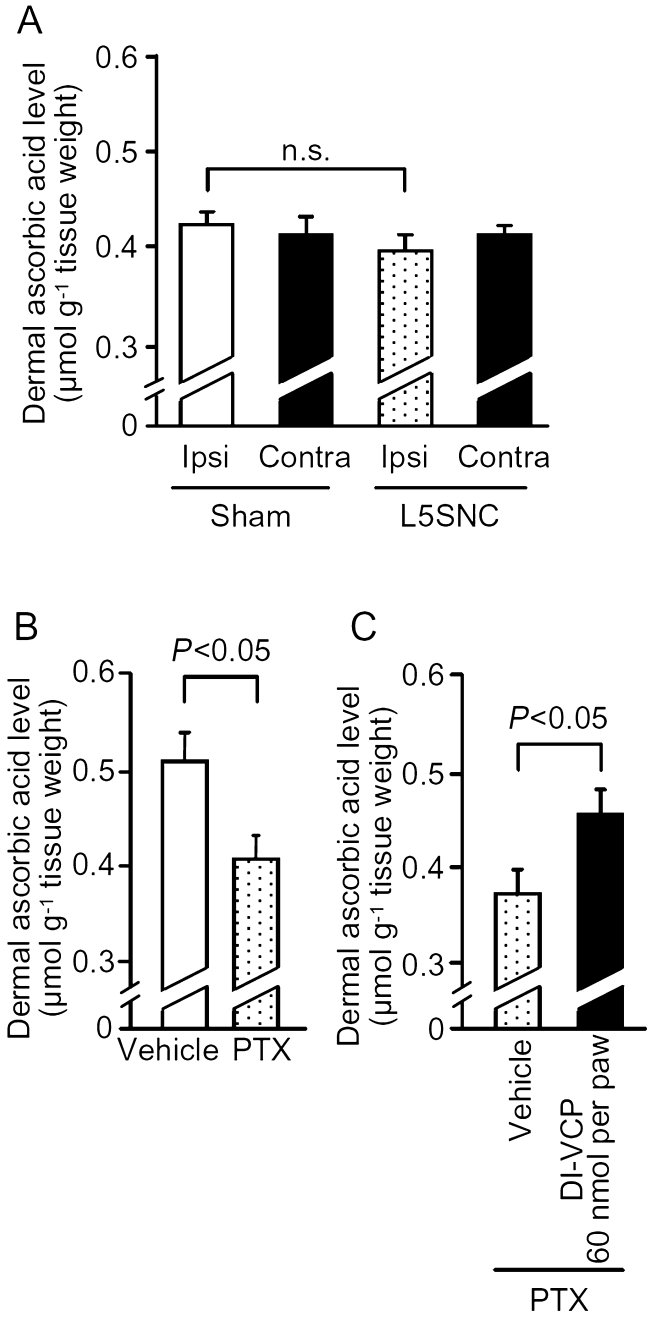

Figure 6

Dermal ascorbic acid levels in the rats with neuropathic pain. Ascorbic acid levels were determined in the instep skin of ipsilateral (Ipsi) and contralateral (Contra) hindpaws excised from the rats with neuropathy induced by L5SNC (A) or by paclitaxel (PTX; B, C). (C) In the paclitaxel-treated animals, the dermal ascorbic acid levels in the ipsilateral hindpaw were measured 90 min after topical application of DI-VCP at 60 nmol per paw to the right hindpaw. Data show the means ± SEM from 4-5 (A), 7-9 (B) and 6 (C) rats.

L5SNC rats, may be associated with our findings that dermal ascorbic acid levels in the instep of the hindpaw significantly decreased after paclitaxel, but not L5SNC treatment.

Although ascorbic acid is capable of inhibiting $Ca_v3.2$ channels (Nelson *et al.*, 2007), it is still debatable whether the effects of i.pl. ascorbic acid and topical application of DI-VCP ointment on the neuropathic hyperalgesia result from inhibition of $Ca_v3.2$ channels. Nonetheless, our data emphasize that local supplementation of ascorbic acid by topical application of DI-VCP may help in clinical treatment of neuropathic pain. This possibility is supported by clinical evidence

for the therapeutic value of systemic administration of ascorbic acid in patients with persistent pain including complex regional pain syndrome after wrist fractures (Zollinger *et al.*, 2007) and herpetic or postherpetic neuralgia (Chen *et al.*, 2009; Schencking *et al.*, 2010; Byun and Jeon, 2011). The analgesic property of systemic administration of ascorbic acid has also been demonstrated in animal studies using the formalin-induced nociception test (Rosa *et al.*, 2005).

We have reported that CSE inhibitors, T-type calcium channel blockers and the silencing of $Ca_v3.2$ channels in the dorsal root ganglion (DRG) neurons reverse the neuropathic hyperalgesia induced by L5SNC or by paclitaxel administration, suggesting a role of the CSE/H_2S/$Ca_v3.2$ channel pathway in the maintenance of neuropathic pain (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). Interestingly, expression of $Ca_v3.2$ channels in DRG neurons is dramatically upregulated in the rat subjected to L5SNC (Takahashi *et al.*, 2010). In contrast, H_2S content in the hindpaw tissue significantly increased in rats with paclitaxel-induced neuropathy, although expression levels of $Ca_v3.2$ channels or CSE in the DRG or hindpaw tissues levels remain constant after paclitaxel treatment (Okubo *et al.*, 2011). This increased H_2S levels in the hindpaw might be correlated with the decreased dermal ascorbic acid levels after paclitaxel treatment, as shown in the present study. Thus, apart from the L5SNC rats, it is likely that the impaired balance between H_2S and ascorbic acid, the endogenous activator and inhibitor of $Ca_v3.2$ channels, respectively, might be associated with the pathophysiology of paclitaxel-induced neuropathy. This concept is consistent with clinical evidence that plasma ascorbic acid levels in patients with postherpetic neuralgia are lower than those in healthy volunteers (Chen *et al.*, 2009; 2011).

In various vertebrates, tissue ascorbic acid levels in the liver, muscle and brain are 0.8–1, 0.4 and 2–10 mM, respectively, whilst plasma ascorbic acid levels are 0.04–0.06 mM (Rice, 2000; Harrison and May, 2009). Thus, the extracellular levels of ascorbic acid are considered to be much lower than the intracellular levels (Rice, 2000; Harrison and May, 2009). The dermal ascorbic acid levels in the present study ranged from 0.4–0.5 μmol g^{-1} tissue which was roughly equivalent to 0.4–0.5 mM (see Figures 1F, 6). The magnitude of change in dermal ascorbic acid levels caused by topical application of DI-VCP ointment or by repeated administration of paclitaxel ranged from 20% to 30% (see Figures 1F, 6B). The increase or decrease rate reflects the changes in the sum of extracellular and intracellular ascorbic acid levels. Considering the much lower extracellular levels of ascorbic acid than the intracellular levels in a resting state, the increase and decrease rates in extracellular ascorbic acid levels after topical application of DI-VCP and treatment with paclitaxel, respectively, could be much greater than 20–30%. Our findings that neither ascorbic acid nor DI-VCP, when administered i.pl., affected the baseline threshold in the sham-operated animals or vehicle-treated animals (see Figure 3), are consistent with the results that T-type calcium channel blockers suppressed neuropathic hyperalgesia in rats, but did not affect the baseline threshold in the control animals (see Figure 2C, D), as reported previously (Takahashi *et al.*, 2010; Okubo *et al.*, 2011). Therefore, it is likely that T-type calcium channels do not play a role in the processing of nociceptive signals under normal physiological conditions, but contribute to the persistent hyperalgesia in pathological conditions. In this context, we suggest that ascorbic acid might function to suppress $Ca_v3.2$ T-type calcium channels in physiological conditions, which might be the reason why T-type calcium channel blockers did not affect the baseline nociceptive threshold, and that the decreased levels of endogenous ascorbic acid in the tissue might lead to cancelling of the suppression of $Ca_v3.2$ channel functions in the rats with paclitaxel-induced hyperalgesia. In rats subjected to L5SNC, $Ca_v3.2$ channels in nociceptors were greatly upregulated (Takahashi *et al.*, 2010), which might suggest why ascorbic acid enhanced nociceptive thresholds in L5SNC rats, but not in sham-operated rats.

Another benefit of DI-VCP is its long-lasting effect after the i.pl. administration and topical application (see Figures 3A, 4A, 5A). Once DI-VCP penetrates into the skin, it is thought to be gradually converted into ascorbic acid by phosphatase (Shibayama *et al.*, 2005; 2008a,b). In cultured human fibroblasts, DI-VCP exerts long-term facilitating effects on proliferation and collagen synthesis through its sustained conversion into ascorbic acid (Shibayama *et al.*, 2008b). This observation might indicate a long-lasting anti-hyperalgesic effect of DI-VCP ointment *in vivo*. Our study strongly suggests that topical application of DI-VCP ointment would be useful for treatment of various types of human neuropathic pain including chemotherapy-induced neuropathy and herpetic/postherpetic neuralgia that has already been reported to be reduced by systemic administration of ascorbic acid (Chen *et al.*, 2009; Schencking *et al.*, 2010; Byun and Jeon, 2011). We also suggest that DI-VCP ointment is utilised in the treatment of diabetes-evoked neuropathy that involves functional upregulation of T-type calcium channels (Jagodic *et al.*, 2007; Latham *et al.*, 2009; Messinger *et al.*, 2009).

In conclusion, ascorbic acid, known to selectively inhibit the $Ca_v3.2$ isoform of T-type calcium channels (Nelson *et al.*, 2007), also suppressed the peripheral sensitization of nociceptors involved in the pathogenesis of neuropathic pain. DI-VCP ointment for topical application is considered a promising drug for the treatment of various types of neuropathic pain.

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Conflicts of interest

None.

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