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Research Paper

The efficacy of duloxetine depends on spinal cholinergic plasticity in neuropathic pain model rats

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ABSTRACT

Antidepressants, such as duloxetine, are widely used to treat chronic pain, including neuropathic pain; however, their efficacy is unsatisfactory. In our previous studies, we showed that in a spinal nerve ligation (SNL) rat model, the descending noradrenergic inhibitory system, which involves in the anti-hypersensitivity mechanism of antidepressants, decrease its activity over time following peripheral nerve injury. In this study, we hypothesized that the analgesic effects of duloxetine may diminish following the attenuation of the descending noradrenergic inhibitory system. The analgesic effects of duloxetine in SNL model rats at the early (SNL2W) and chronic (SNL6W) phases following spinal nerve ligation were compared. Male Sprague-Dawley rats were randomly assigned to the SNL2W or SNL6W groups and used to evaluate the anti-allodynic effects of duloxetine using the von Frey filament test. The anti-allodynic effects of duloxetine at a dose of 10 mg/kg were lower in SNL6W rats than in SNL2W rats. Basal noradrenaline concentrations in rat spinal dorsal horns were higher in the SNL6W group than in the SNL2W group, and there was no difference in the increase in spinal noradrenaline concentrations between the 2 groups following duloxetine administration. In addition, we found that duloxetine-induced acetylcholine (ACh) release and choline acetyltransferase (ChAT) expression in the spinal dorsal horn decreased in SNL6W rats. At a dose of 30 mg/kg, duloxetine showed anti-allodynic effects even in SNL6W rats and induced ACh release in the spinal cord. Furthermore, these anti-allodynic effects were completely inhibited by intrathecal atropine (muscarinic antagonist) administration. Moreover, 5 daily intraperitoneal injections of the TrkB agonist, 7,8-dihydroxyflavone (5 mg/kg), not only restored ChAT expression, but also decreased the anti-allodynic effects of duloxetine. These findings suggest that the attenuation of the anti-allodynic effects of duloxetine at the chronic phase of SNL may be due to impaired spinal acetylcholine-mediated analgesia. In addition, the activation of BDNF-TrkB signaling may be beneficial in reversing this impairment.

Introduction

Duloxetine is a serotonin noradrenaline reuptake inhibitor (SNRI) which inhibits serotonin (5-HT) and noradrenaline (NA) reuptake and is widely used in the treatment of chronic pain, including neuropathic pain (Finnerup et al., 2015). Increase in NA concentrations in the spinal dorsal horn through reuptake inhibition is thought to be one of the analgesic mechanisms of duloxetine (Hoshino et al., 2015; Hiroki et al., 2017). Duloxetine was found to rapidly and significantly suppress mechanical hypersensitivity in an animal model of neuropathic pain (Hoshino et al., 2015; Hiroki et al., 2017). However, in human patients, number needed to treat (NNT) of duloxetine is approximately 6 (Finnerup et al., 2015). This suggests that its analgesic effects are limited. In addition, the drug administration duration required to observe analgesic effects differs between preclinical and clinical studies. Clinically, a 2-week chronic administration of duloxetine is frequently required to observe its analgesic effects in patients with neuropathic pain (Yasuda et al., 2011).

It has been shown that the anti-allodynic effects of duloxetine, as well as those of other SNRIs, on neuropathic pain significantly depend on the brainstem-spinal descending noradrenergic system (Nakajima et al., 2012; Hoshino et al., 2015). Furthermore, the efficacy of the

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noradrenergic descending system inhibition approach diminishes over time following nerve injury (Kimura et al., 2015; Matsuoka et al., 2016). These two findings suggest that using animal models of neuropathic pain during the early phase after nerve injury may not be reflective of the clinical situation in which analgesics are administered for the treatment of chronic or established neuropathic pain conditions. The primary objective of this study was to determine whether the analgesic effects of duloxetine change overtime following nerve injury using an animal model at the early (SNL2W) and chronic (SNL6W) phases.

The second aim of this study was to evaluate the role of the cholinergic nervous system in duloxetine-induced analgesia. Nerve injury has been shown to induce functional alterations in the spinal cholinergic nervous system at the early phase via a brain derived neurotrophic factor (BDNF)-mediated mechanism (Hayashida and Eisenach, 2010). Stimulation of spinal α2 adrenoceptors results in an increase in spinal acetylcholine (ACh) concentrations in nerve-injured animals (Kimura et al., 2012). The spinal cholinergic system is essential for NA-mediated analgesia at early time points following nerve injury in rodents. However, its contribution and potential alterations in the spinal cholinergic system, have not been investigated during the later stages of nerve injury. In this study, we measured changes in ACh and NA concentrations in the spinal dorsal horn following duloxetine administration. Furthermore, we compared choline acetyltransferase (ChAT) immunoreactivity in SNL2W and SNL6W rats.

Experimental procedures

The time course for each experiment is shown in Fig. 1.

Animals

This study was approved by the Animal Care and Use Committee of Gunma University Graduate School of Medicine (Maebashi, Japan. No. 16–047). Adult male Sprague-Dawley rats, weighing 200–280 g at the time of surgery, were obtained from SLC (Shizuoka, Japan). Animals were housed under a 12-h light-dark cycle, with ad libitum access to food. This study involved 147 rats, with the animals randomly assigned to each experimental group.

SNL surgery

L5 and L6 spinal nerve ligation was performed on the 141 rats, as previously described (Kim and Chung, 1992). In brief, after the rats were anesthetized with 2.0% isoflurane in oxygen, their right transverse processes were removed. Then, their right L5 and L6 spinal nerves were tightly ligated using 5–0 silk sutures, and the wounds were sutured. Two or six weeks following SNL, the rats were used for the experiments (SNL2W or SNL6W). Rats with a right-hand paw withdrawal threshold exceeding 6 g were excluded from this study. Two rats with paralysis of the right hind paw and five rats without postoperative hypersensitivity were excluded.

Intrathecal catheterization in SNL6W rats

Intrathecal catheterization was performed 5 weeks (35 days) following SNL surgery. The animals were anesthetized with 2.0% isoflurane in oxygen. After making a small puncture in the atlanto-occipital membrane of the cisterna magnum, a 7.5 cm polyethylene catheter (ReCathCO LLC, Allison Park, PA, USA) was inserted into the rats. Animals were allowed to recover for a week following surgery, after which they were habituated for the behavioral experiments. Following the completion of the behavioral tests, correct catheter placement was confirmed by intrathecal lidocaine administration (0.5 mg/10 μL) for the induction of reversible hind paw paralysis. In this study, three animals were excluded since they did not show reversible paralysis, suggesting catheter malposition. One rat was euthanized during catherization due to a possible spinal cord injury.

Behavioral tests

Mechanical hypersensitivity was determined using eight von Frey filaments (Stoelting, Wood Dale, IL, USA) with masses ranging from 0.6 to 26 g, as previously described (Chaplan et al., 1994). In brief, each rat was placed in an individual acrylic chamber with a plastic mesh floor and allowed to acclimate to the environment for at least 30 min prior to testing. Subsequently, filaments were applied to the heel of the hind paw of the rats to the bending point and maintained for 6 s. A brisk paw withdrawal was considered to be a positive response. The up-down method was used to determine the withdrawal threshold (Chaplan et al., 1994). Mechanical hypersensitivity was measured before and after duloxetine administration. The individual who performed the behavioral tests was blinded to the groups.

Microdialysis for the determination of spinal NA, 5-HT, and ACh concentrations

Microdialysis on the spinal dorsal horn was performed according to a method described in our previous study (Obata et al., 2010). In brief, SNL2W and SNL6W rats were anesthetized with 2.0% isoflurane, and anesthesia was maintained during the measurements using 1.5% isoflurane in 100% oxygen. The rectal temperature of the animals was maintained at 37 °C using a heating blanket. Saline was infused into the rats at a rate of 1 mL/h through a cannulated left femoral vein using an infusion pump system (Fusion 400, Chemyx, Stafford, TX, USA). For intraperitoneal duloxetine injection, a 24-gauge intravenous catheter was inserted into the intraperitoneal space of the rats. The L4-L6 spinal cord was exposed by a T13-L1 laminectomy. Microdialysis probes (CX-I-8–01, Eicom Co., Kyoto, Japan) were inserted into the right spinal dorsal horn, which was perfused with Ringer's solution (147 mmol/L NaCl, 4 mmol/L KCl, 2.3 mmol/L $CaCl_2$) at a rate of 1 μL/min. After 60 min of constant perfusion, dialysates were collected at 30-min intervals. After the collection of two dialysates for baseline samples, duloxetine was injected intraperitoneally. Each sample was divided into two parts; one part was used for NA and 5-HT concentration measurement while the other was used for Ach concentration measurement. NA, 5-HT, and ACh concentrations were measured 240 min following duloxetine injection using separate high-pressure liquid chromatographic systems with electrochemical detection (HTEC-500, Eicom Co) (Obata et al., 2010; Kimura et al., 2015). The columns used for the measurement of NA and 5-HT concentrations and ACh concentrations were EICOMPAK SC-5ODS (3.0 × 150 mm, Eicom Co.) and EICOMPAK AC-GEL (2.0 × 150 mm, Eicom Co.) columns, respectively. The basal concentrations of NA, 5-HT, and ACh were determined as the average of their concentrations in two 30-min dialysates. Changes in the concentrations of NA, 5-HT, and ACh following duloxetine injection were determined as a percentage of the baseline concentration (100%). Seven rats were excluded due to the occurrence of spinal nerve injury during laminectomy.

Immunohistochemistry

Spinal cord tissue was collected from SNL2W and SNL6W rats, as previously described (Peters et al., 2015). In brief, after the animals were anesthetized by intraperitoneal 50 mg/kg pentobarbital injection, their thoraxes were opened, and 0.1 mol/L phosphate-buffered saline (PBS) containing 20% sodium nitrate was perfused through their left ventricles via a peristaltic pump (20 mL/min); this was followed by the perfusion of a fixative (4% paraformaldehyde in 0.1 mol/L phosphate buffer). The L4-L6 spinal cord sections of the rats were removed and immersed in a fixative agent overnight at 4 °C. On the following day, the spinal cord sections were immersed and maintained in 30% sucrose solution until sectioning. Using a cryostat, these spinal cord sections were further

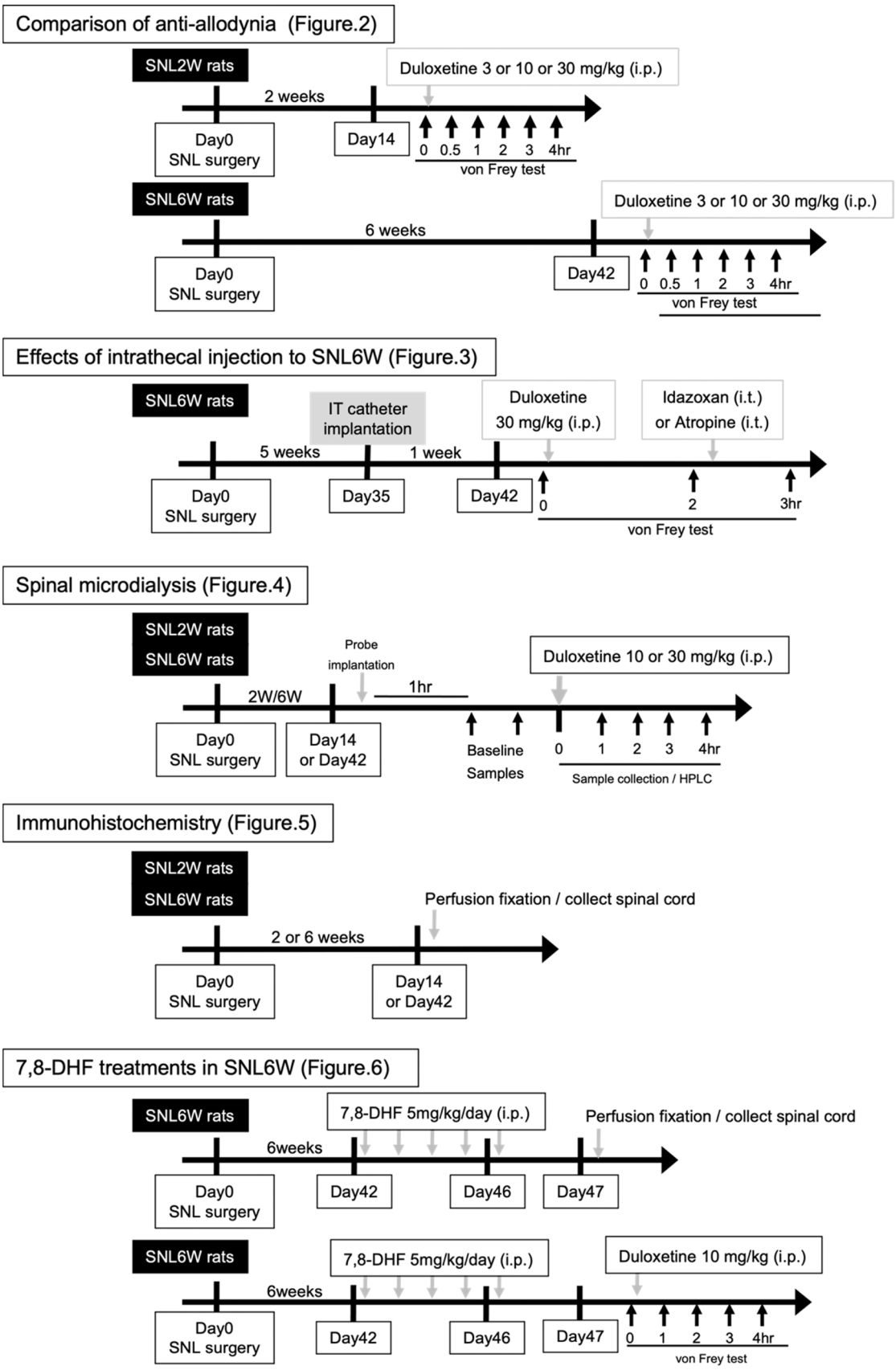

Fig. 1. Schematic representation of the study design.

sectioned at a thickness of 40 μm. After pretreatment with 3% hydrogen peroxide and 5% normal donkey serum (Nichirei Biosciences Inc., Tokyo, Japan), the sections were incubated with goat monoclonal anti-choline acetyltransferase (ChAT) antibodies (1:100, AB144P, Merck Millipore, Billerica, MA, USA) in 5% normal donkey serum overnight. The following day, the sections were washed with PBS and incubated with biotinylated anti-goat IgG and horseradish peroxidase-conjugated streptavidin processed using the SAB-PO Kit (414,011, Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer's instructions, and developed with diaminobenzidine.

Image analysis

Digital images were captured with an Olympus FSX100 microscope (Olympus Co., Tokyo, Japan) using a 10 × objective, with a resolution of 1360 × 1024 pixels, and used for the quantification of the immunostaining. An image analysis software (Image J, National Institutes of Health, Bethesda, MD, USA) was used to quantify changes in immunofluorescence. The number of pixels associated with immunoreactivity within a defined threshold was determined in a fixed square area (582 × 582 μm^2) covering the lamina II region of the spinal dorsal horn was examine and averaged for each group. The same threshold value was applied to all images for a given antibody. The individual who performed image analysis was blinded to the groups.

Drugs

Duloxetine (3, 10, 30 mg/mL/kg, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in 50% dimethylsulfoxide and saline (vehicle), and injected intraperitoneally (Ito et al., 2018, Hiroki et al., 2017, Sun et al., 2014). Idazoxan hydrochloride (α2 adrenoceptor antagonist, Sigma Aldrich, St. Louis, MO, USA) and atropine (muscarinic antagonist, Sigma Aldrich) were dissolved in saline. One hundred and sixty-five minutes following intraperitoneal duloxetine injection, 10 μL idazoxan (30 μg/10 μL) (Hayashida et al., 2008a) or atropine (30 μg/10 μL) (Hayashida et al., 2007) was administered to the rats through an intrathecal catheter, and this was followed by flushing with 10 μL of saline. 7,8-Dihydroxyflavone (7,8-DHF, TrkB agonist, Wako Pure Chemical Industries, Ltd., Osaka, Japan), dissolved in 0.1 mol/L PBS containing 20% dimethylsulfoxide. 7,8-DHF (5 mg/mL/kg/day) (Kato et al., 2019; Andero et al., 2012), was injected intraperitoneally for 5 days from week 6 (day 42) to week 7 (day 46) following SNL surgery.

Statistical analysis

Data are presented as the mean ± standard deviation. In the microdialysis and hypersensitivity studies, data were analyzed using two-way repeated-measures analyzes of variance (ANOVA), with the group (SNL2W and SNL6W) and the time following duloxetine injection considered as independent variables. When significant differences were observed for the group × time interaction, Student's t-test with Bonferroni correction was performed for group comparisons at each time point. Other data were analyzed using Student's t-test or one-way ANOVA, and if differences were significant, Student's t-test with Bonferroni correction was performed for group comparisons. P values adjusted with Bonferroni's correction were reported throughout, with $P < 0.05$ considered statistically significant. All statistical analyzes were conducted using SigmaPlot12 (Systat Software Inc., USA) and EZR (version 1.51, Saitama Medical Center, Japan). A total of 132 rats were analyzed in this study.

We performed a power analysis for the primary outcome (anti-allodynic effects) to determine the appropriate sample size. In accordance with the findings of our previous study, our current analysis was based on the assumption that a mean difference of 8 g for the withdrawal threshold in the right hind paw and a standard deviation of 5 g would be present in each group (Kato et al., 2019). The power analysis indicated that the use of more than six rats in each group would result in the detection of significant differences, with 80% power at a significance level of $\alpha = 0.05$.

Results

The same experiments were performed in SNL2W and SNL6W rats (Fig. 2, Figs. 4 and 5). Intrathecal catheterization was performed in SNL rats 35 days after SNL surgery (Fig. 3). 7,8-DHF (5 mg/mL/kg/day) or vehicle was injected for 5 days from days 42–46 following SNL surgery (Fig. 6). 7,8-DHF: 7,8-dihydroxyflavone; i.p.: intraperitoneal; i.t.: intrathecal.

Comparison of the anti-allodynic effects of duloxetine in the neuropathic pain model at the early phase (SNL2W) and the chronic phase (SNL6W)

To determine whether the anti-allodynic effects of duloxetine depend on the duration of nerve injury, we evaluated the 50% withdrawal threshold in the ipsilateral hind paws of SNL rats before and after a single administration of duloxetine 2 weeks (SNL2W) or 6 weeks (SNL6W) following surgery.

Duloxetine exhibited dose-dependent anti-allodynic effects in both SNL2W and SNL6W rats, and induced a significant increase in withdrawal threshold at doses of 10 mg/kg and 30 mg/kg as compared to the withdrawal threshold observed in the vehicle group (Fig. 2A and B). The area under the curve (time after duloxetine x withdrawal threshold) of the groups were calculated and compared using two-way ANOVA (Fig. 2C). ANOVA revealed there were significant main effect on dose ($F_{2, 24} = 29.5$, $P < 0.001$), postoperative time ($F_{1, 12} = 86.0$, $P < 0.001$), and a dose × postoperative time interaction ($F_{2, 24} = 11.0$, $P < 0.001$). Post-hoc testing revealed that the anti-allodynic effects of duloxetine (both 10 mg/kg and 30 mg/kg) were attenuated in SNL6W compared to SNL2W ($P < 0.001$ each).

Contribution of spinal noradrenergic and cholinergic signals to anti-allodynic effects of duloxetine in SNL6W rats

To investigate the contributions of spinal noradrenergic and cholinergic signaling on the anti-allodynic effects of duloxetine at the chronic phase of nerve injury, we evaluated the withdrawal threshold following a single administration of duloxetine (30 mg/kg) in SNL6W rats, with or without the intrathecal administration of the α2 adrenoceptor antagonist, idazoxan, or the muscarinic receptor antagonist, atropine.

Anti-allodynic effects of duloxetine diminished following the intrathecal administration of idazoxan ($P = 0.004$) or atropine ($P = 0.005$) (drug × time: $F_{4, 30} = 4.75$, $P = 0.004$, two-way ANOVA; Fig. 3). These results indicated that duloxetine exerted anti-allodynic effects in SNL6W rats through both spinal noradrenergic and cholinergic signaling.

Effects of neuropathic pain duration on NA, 5-HT, and Ach concentrations in the spinal dorsal horn

To determine the effects of the duration of neuropathic pain on duloxetine-induced reuptake inhibition and ACh concentration in the spinal dorsal horn, we measured duloxetine (10 mg/kg)-induced alterations in NA, 5-HT, and ACh concentrations in SNL2W and SNL6W rats through microdialysis. Surprisingly, there were no differences in the increase in NA ($F_{4, 64} = 1.301$, $P = 0.279$, two-way ANOVA; Fig. 4A) and 5-HT ($F_{1, 12} = 0.6633$, $P = 0.4299$, two-way ANOVA) concentrations between the SNL2W and SNL6W rats. Baseline NA concentrations in the microdialysates were higher in SNL6W rats than in SNL2W rats (2.5 ± 0.78 pg/30 μL vs 1.4 ± 0.43 pg/30 μL, $P = 0.007$).

On the other hand, duloxetine increased spinal ACh release in SNL2W, but not in SNL6W (neuropathic pain duration: $F_{1, 16} = 11.2$;

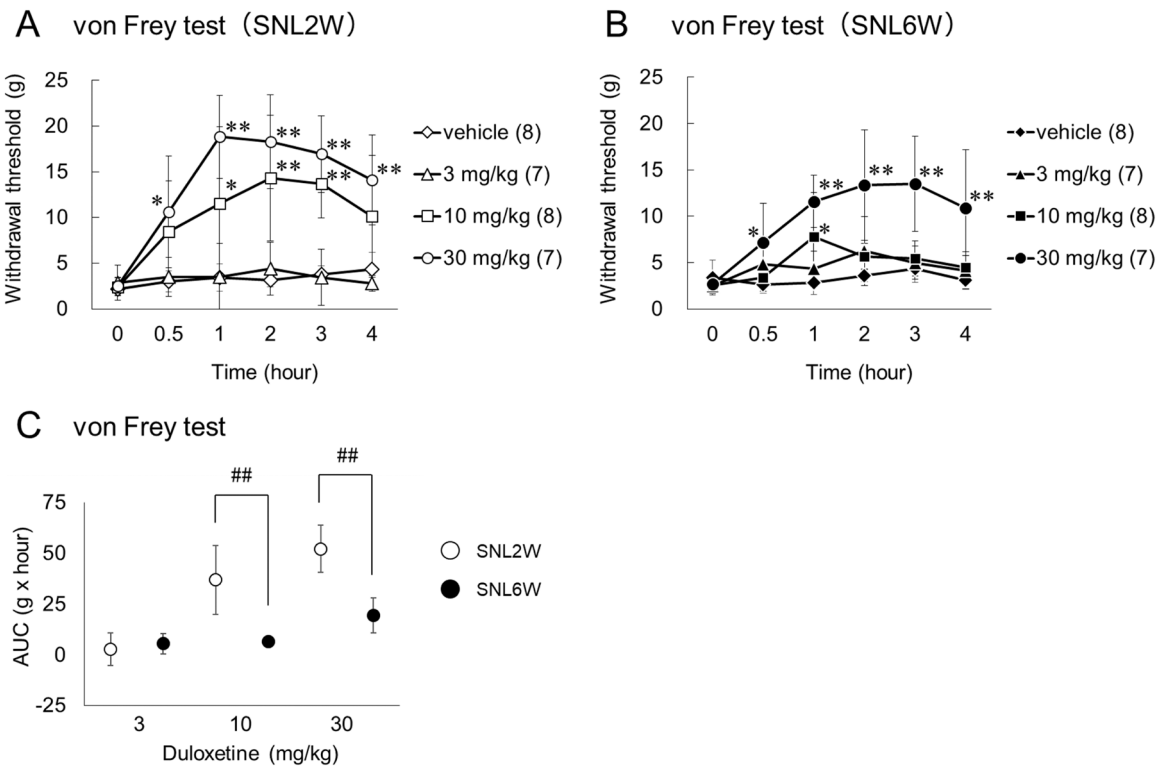

Fig. 2. Comparison of the anti-allodynic effects of duloxetine between SNL2W and SNL6W rats. Withdrawal threshold in the ipsilateral hind paw was measured before treatment (baseline, 0 h), and 0.5, 1, 2, 3, and 4 h following intraperitoneal duloxetine injection (3, 10 and 30 mg/mL/kg) in SNL2W(A) and SNL6W rats (B). The area under the curve (AUC: g × h) of the withdrawal threshold curve at 4 h following duloxetine injection was calculated for each dose in SNL2W and SNL6W rats (C). Data are expressed as the mean ± standard deviation of the values obained for 8 (vehicle and duloxetine 10 mg/kg) or 7 (duloxetine 3 mg/kg and duloxetine 30 mg/kg) rats. *P < 0.05, * *P < 0.01 vs vehicle. (A and B) and ##P < 0.01 vs SNL2W (C) two-way repeated measures ANOVA followed by Student t test with Bonferroni's correction.

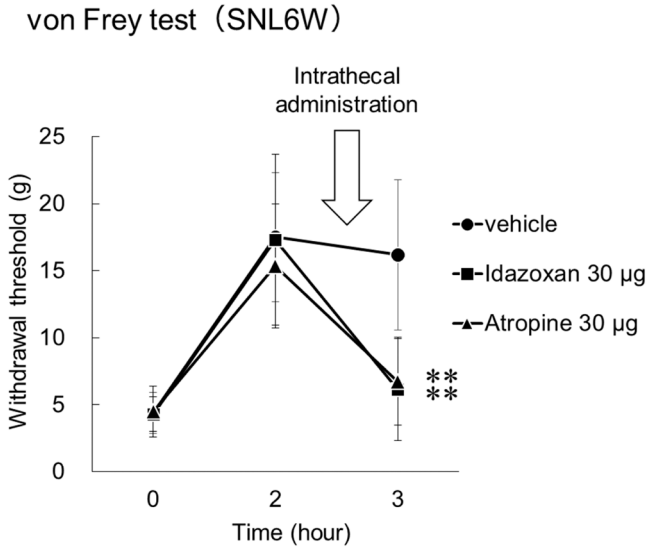

Fig. 3. Intrathecal idazoxan or atropine injection reversed duloxetine (30 mg/kg)-induced analgesia in SNL6W rats. Withdrawal threshold in the ipsilateral hind paw was measured before treatment (baseline, 0 h), and 120 and 180 min following intraperitoneal duloxetine injection (30 mg/mL/kg) in SNL6W rats. Idazoxan (30 μg/10 μL) or atropine (30 μg/10 μL) was intrathecally administrated 165 min after intraperitoneal duloxetine injection. At a dose of 30 mg/kg, duloxetine alone induced an increase in withdrawal threshold in SNL6W rats, and idazoxan or atropine reversed this effect. Data are presented as the mean ± standard deviation of the values obtained for 6 rats. * *P < 0.01 vs vehicle two-way repeated measures ANOVA followed by Student t test with Bonferroni's correction.

P = 0.004; time: $F_{4, 64} = 2.67$; P = 0.040; neuropathic pain duration × time: $F_{4, 64} = 4.40$; P = 0.003, two-way repeated measures ANOVA; Fig. 4B). Post-hoc testing revealed that ACh release was decreased in SNL6W rats than in SNL2W rats between 1 and 3 h following duloxetine injection (1 h; P = 0.002, 2 h; P < 0.001, 3 h; P = 0.042). In addition, there was no difference in the duloxetine-induced increase in spinal NA concentration in SNL6W rats at doses of 10 mg/kg and 30 mg/kg (dose: $F_{1, 17} = 5.22$; P = 0.035; time: $F_{4, 68} = 22.1$; P < 0.001, dose × time: $F_{4, 68} = 1.65$, P = 0.172, two-way repeated measures ANOVA; Fig. 4C). On the other hand, duloxetine at a dose of 30 mg/kg increased spinal ACh release in SNL6W (dose: $F_{1, 4} = 12.0$, P = 0.003, time: $F_{4, 60} = 3.36$, P = 0.015, dose × time: $F_{4, 60} = 3.53$, P = 0.012, two-way repeated measures ANOVA; Fig. 4D).

Alterations in the cholinergic nervous system in the spinal dorsal horns of SNL6W rats

ChAT immunoreactivity in the right spinal dorsal horn was shown in Fig. 5A. SNL6W rats showed decreased ChAT immunoreactivity than naïve rats (1.76 ± 1.4% vs 8.44 ± 3.4%, P = 0.0017) and SNL2W rats (1.76 ± 1.4% vs 6.43 ± 2.8%, P = 0.0248) (Fig. 5B, $F_{2, 20} = 15.1$, P < 0.001, one-way ANOVA). In addition, there was a reduction in ChAT immunoreactivity in the left spinal dorsal horn in SNL6W rats compared to naïve and SNL2W rats (1.34 ± 1.1% vs 10.75 ± 5.4%, P = 0.0011 and 1.34 ± 1.1% vs 10.76 ± 2.9%, P = 0.0011, respectively, Fig. 5B).

Baseline ACh concentrations in the microdialysates are shown in Fig. 5C. Contrary to immunostaining findings, spinal dorsal horn ACh concentrations were higher in SNL6W rats than in SNL2W rats (12.9 ± 9.2 nmol/L vs 6.33 ± 5.8 nmol/L, P = 0.014).

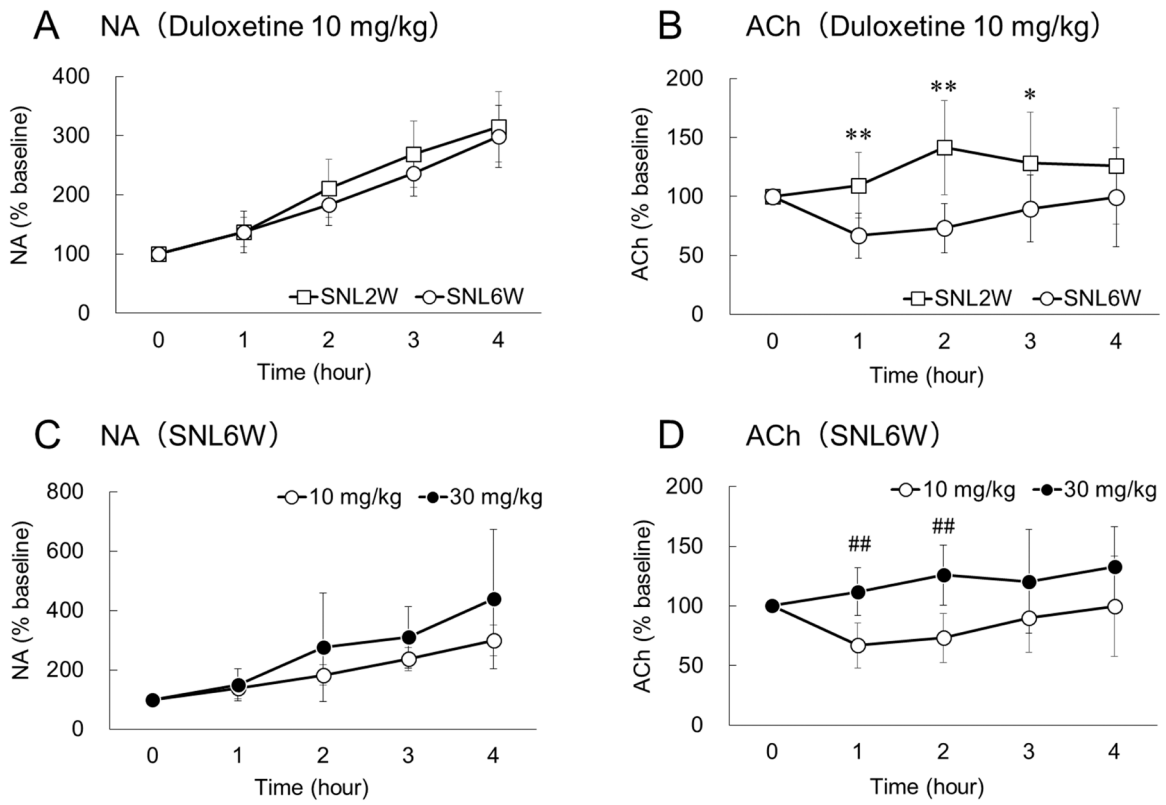

Fig. 4. Neuropathic pain duration and duloxetine-induced NA and ACh release in the spinal dorsal horn. The concentrations of NA and ACh in microdialysates obtained from the right lumber spinal dorsal horns of SNL2W and SNL6W rats were determined. Changes in NA (A) and ACh (B) concentrations following intraperitoneal duloxetine injection (10 mg/mL/kg) over time are presented as a percentage and are compared to the value obtained before injection (0 h). C and D show NA and ACh concentration changes in SNL6W rats following intraperitoneal duloxetine injection at doses of 10 mg/kg and 30 mg/kg. Data are presented as the mean ± standard deviation of the values obtained for 9 rats (A, B); 9 rats at a dose of 10 mg/kg and 10 rats at a dose of 30 mg/kg (C); 9 rats at a dose of 10 mg/kg and 8 rats at a dose of 30 mg/kg (D). *P < 0.05, * *P < 0.01 vs SNL2W (B) and ##P < 0.01vs 10 mg/kg (D) two-way repeated measures ANOVA followed by Student t test with Bonferroni's correction. NA: noradrenaline; ACh: acetylcholine.

Effects of repeated treatment with a TrkB agonist on ChAT-IR and duloxetine-induced-analgesia in SNL6W rats

After five daily intraperitoneal 7,8-DHF injections, we evaluated spinal ChAT immunoreactivity (Fig. 6A and B) and the anti-allodynic effects of duloxetine at a dose of 10 mg/kg (Fig. 6C). In SNL6W rats, 7,8-DHF reversed the decrease in ChAT expression in the right and left spinal dorsal horns (10.4 ± 7.0% vs 1.85 ± 1.7%, P = 0.012 and 10.7 ± 6.3% vs 1.83 ± 2.7%, P = 0.010, respectively, Student's t-test). In addition, repeated treatment with 7,8-DHF improved the impaired anti-allodynic effects of duloxetine in SNL6W rats, with a significant increase in withdrawal threshold observed between 1 and 4 h following intraperitoneal duloxetine injection as compared to treatment with repeated vehicle injections (drug: $F_{1,\ 14} = 37.7$, P < 0.001, time: $F_{4,\ 56} = 35.0$, P < 0.001, drug × time: $F_{4,\ 56} = 11.3$, P < 0.001, two-way repeated measures ANOVA; Fig. 6C).

Discussion

In this study, we found that the acute anti-allodynic effects of duloxetine in rats were lower during the late stage (SNL6W) of nerve injury than the early stage (SNL2W). In SNL6W rats, ChAT immunoreactivity and duloxetine-induced spinal ACh release were reduced. Repeated administration of a TrkB agonist restored ChAT immunoreactivity and the anti-allodynic effects of duloxetine in SNL6W rats.

Possible mechanisms underlying attenuation of duloxetine analgesia

In this study, we showed that a higher duloxetine dose is required to relieve mechanical allodynia in SNL6W rats than in SNL2W rats. To determine the mechanisms underlying this attenuation, first, we evaluated differences in spinal NA concentrations following duloxetine administration between SNL2W and SNL6W rats. Surprisingly, the duloxetine-induced increase in spinal NA content was similar between the two phases, suggesting that the duration of neuropathic pain did not affect the efficacy of duloxetine to inhibit NA reuptake. However, we observed a decrease in spinal ACh release following duloxetine administration in SNL6W rats. Duloxetine at a dose of 30 mg/kg increased spinal ACh, and the anti-allodynic effects were blocked by intrathecal atropine injection. Therefore, we speculate that the decrease in the anti-allodynic effects of duloxetine in SNL6W rats may result from a dysfunction cholinergic nervous system in the spinal dorsal horn.

The findings of previous studies in rat models of nerve injury suggest that the analgesic effects of duloxetine significantly depend on the brainstem-spinal descending noradrenergic system (Hoshino et al., 2015; Hiroki et al., 2017). In addition, the activity of the noradrenergic descending inhibitory system in SNL6W rats was found to gradually attenuate overtime following nerve injury and to subsequently disappear (Kimura et al., 2015; Matsuoka et al., 2016). Our study suggests that not only noradrenergic descending inhibitory system, but also spinal cholinergic system changes anatomically and functionally overtime following peripheral nerve injury.

Alterations in duloxetine mediated ACh release overtime following SNL surgery

In this study, we revealed that duloxetine increased spinal NA and ACh release in both SNL2W and SNL6W rats. Previous reports suggested

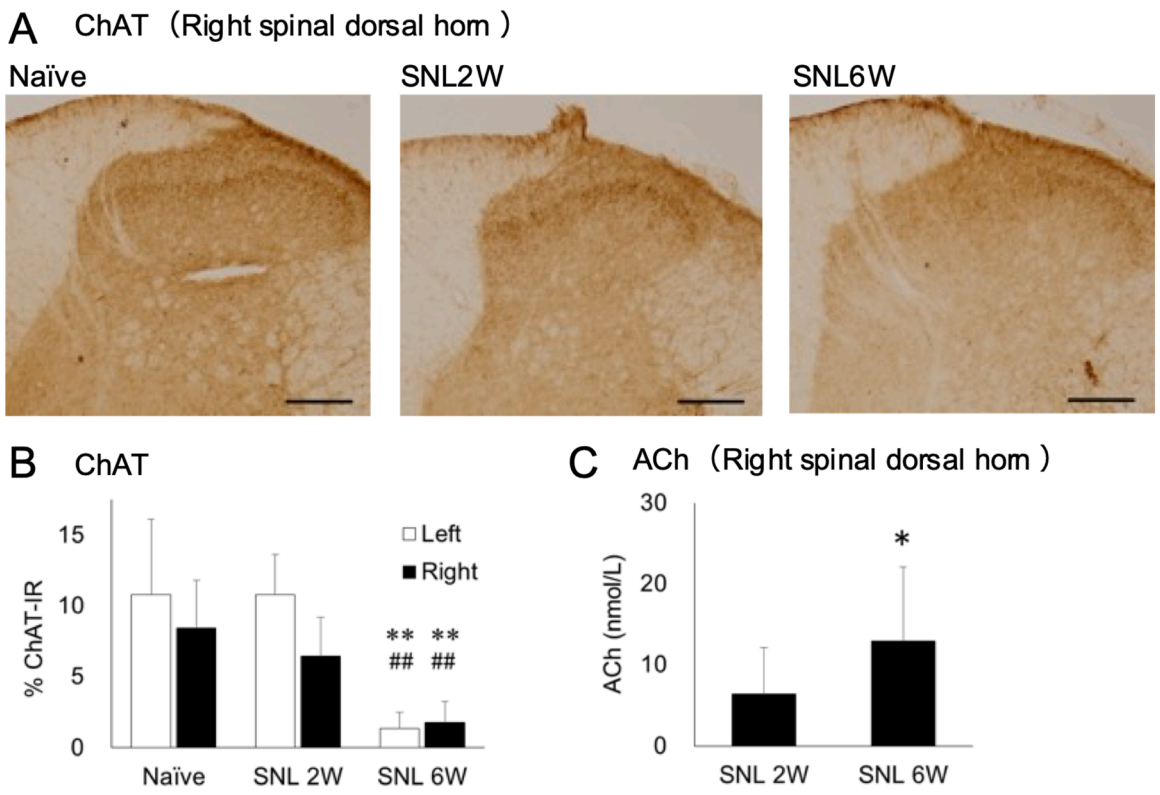

Fig. 5. ChAT immunoreactivity in the spinal dorsal horn. Representative spinal ChAT immunohistochemistry images for naïve, SNL2W, and SNL6W rats (A). Scale bar = 200 μm. ChAT-IR quantification (B). Data are presented as the mean ± standard deviation of the values obtained for 6 rats. * *P < 0.01 vs naïve and #P < 0.05, ##P < 0.01 vs SNL2W one-way ANOVA followed by Student t test with Bonferroni's correction. ChAT-IR: choline acetyltransferase-immunoreactivity in the spinal dorsal horn of each rat.Baseline ACh concentrations in microdialysates collected from the spinal dorsal horns of SNL2W and SNL6W rats (C). Data are presented as the mean ± standard deviation of the values obtained for 9 rats. #P < 0.05 vs SNL2W Mann-Whitney U test.

that activation of α2 adrenoceptors on spinal cholinergic interneurons results in an increase in spinal ACh concentrations during the early phase following SNL (equivalent to SNL2W) (Hayashida and Eisenach, 2010, Kimura et al., 2012). It is probable that duloxetine increases spinal ACh concentrations by this mechanism. In addition, our microdialysis analysis revelated that a higher duloxetine dose was required to observe an increase in ACh concentration in SNL6W rats. we also observed a decrease in ChAT immunoreactivity in the bilateral spinal dorsal horns of SNL6W rats. Furthermore, as opposed to the findings of the immunohistochemical analysis, basal ACh concentrations, as determined by the microdialysis experiments, were higher in SNL6W rats than in SNL2W rats.

Based on these findings, several possible mechanisms may be involved in the attenuation of ACh release following duloxetine administration. First, reduced spinal ACh synthesis may be a possible explanation for the reduced ACh release; however, the fact that basal ACh concentrations were found to be higher in SNL6W rats than in SNL2W rats suggests that this is unlikely. Second, SNL6W rats are less likely to respond to NA; baseline spinal NA concentrations are higher in SNL6W rats than in SNL2W rats. Therefore, the downregulation of α2 adrenoceptors expressed in cholinergic interneurons may simultaneously reduce ACh release following the increase in NA concentrations. Although this mechanism is most acceptable, there are no direct results to support this hypothesis and further investigations are required. The third possible mechanism is the autoinhibition of ACh release via presynaptic muscarinic receptors (Muramatsu et al., 2019); The high basal ACh concentrations observed in SNL6W rats may inhibit ACh release. Serotonin (5-HT) is also involved in the regulation of spinal ACh release (Kommalage and Höglund, 2005); however, there were no significant differences in 5-HT concentrations between SNL2W and SNL6W rats.

Repeated treatment with 7,8-DHF restored ChAT immunoreactivity and duloxetine analgesia in SNL6W rats

Repeated treatments with a TrkB agonist (7,8-DHF) restored spinal ChAT immunoreactivity and enhanced anti-allodynic effect of duloxetine in SNL6W rats. It is reported that in rat models of nerve injury at early phase endogenous TrkB signals increase ChAT and promote cholinergic analgesia (Hayashida and Eisenach, 2011), however, our findings suggest reduction of endogenous TrkB signals at chronic phase, and extrinsic TrkB agonist can promote similar effects on ChAT.

A recent preclinical study in mice demonstrated that following nerve injury, cholinergic tone might increase, and that downstream cholinergic signaling is altered under neuropathic conditions (Dhanasobhon et al., 2021). In addition, the cholinesterase inhibitor, donepezil, induces analgesia in human patients (Basnet et al., 2014). Our findings suggest duloxetine may produce analgesia by acetylcholine mediated mechanism. Therefore, activation of cholinergic signals may be beneficial for patients less responsive to duloxetine.

In this study, we showed that the analgesic effects of a single duloxetine administration following nerve injury attenuate overtime. In contrast, we showed that repeated duloxetine administration exerts anti-hypersensitivity effects in SNL6W rats (Ito et al., 2018). Duloxetine increases BDNF production in the central nervous system (Hisaoka-Nakashima et al., 2016; Xu et al., 2016), and this may lead to increased ChAT expression, thereby enhancing their anti-allodynic effects. These findings may provide an explanation as to why duloxetine needs to be repeatedly administered in order to exert adequate analgesic effects in clinical practice.

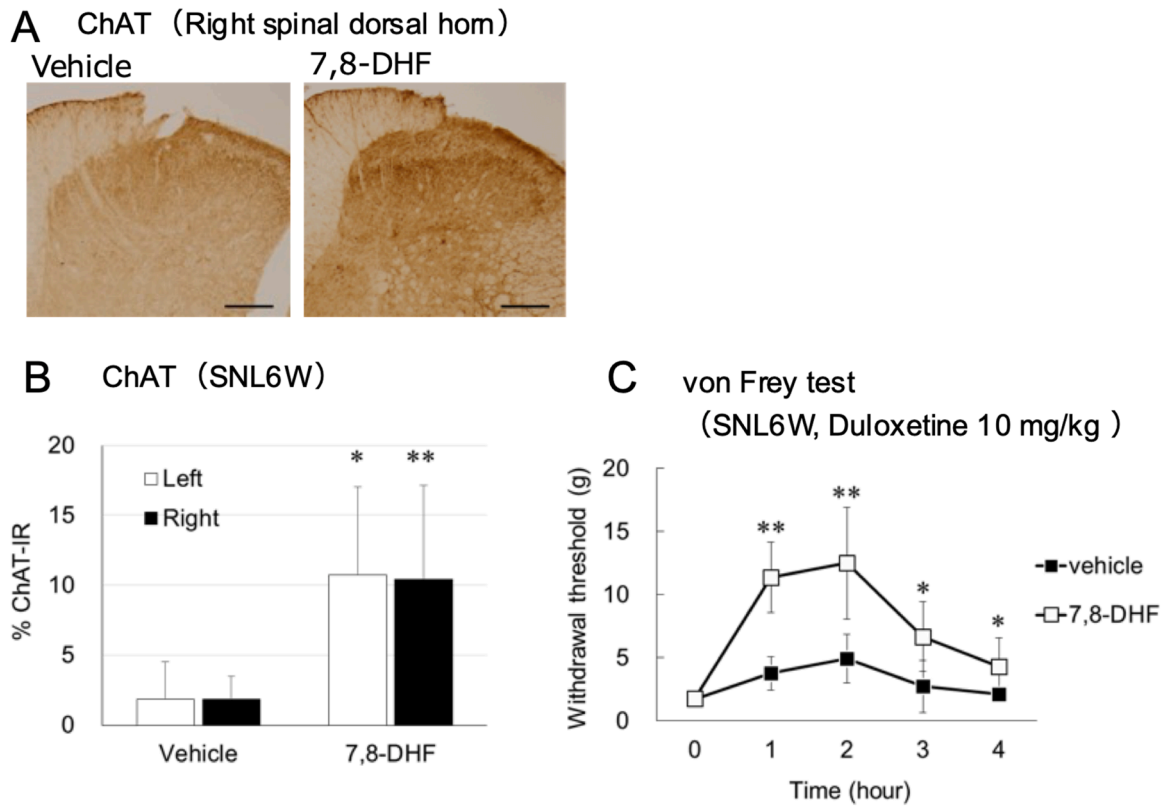

Fig. 6. Repeated treatment with a TrkB agonist enhanced duloxetine-induced analgesia in SNL6W rats by increasing ChAT-IR in the spinal dorsal horn. After five daily intraperitoneal injections of the vehicle or 7,8-DHF (5 mg/kg/day), ChAT-IR in the lumber spinal dorsal horns of SNL6W rats was quantified. Representative images are shown in A. Scale bar = 200 μm. Data are presented as the mean ± standard deviation of the values obtained for 6 rats (B). *P < 0.05, * *P < 0.01 vs vehicle Student t test in each hind paw. Withdrawal threshold in the ipsilat-eral hind paw was measured before treatment (baseline, 0 h), and 1, 2, 3, and 4 h following intraperitoneal duloxetine injection (10 mg/kg) in SNL6W rats, after the injection of five daily 7,8-DHF doses. Data are presented as the mean ± standard deviation of the values obtained for 8 rats (C). *P < 0.05, * *P < 0.01 vs vehicle two-way repeated measures ANOVA followed by Student t test with Bonferroni's correction. ChAT-IR: choline acetyltransferase-immunoreactivity.

Limitations

First, there are no results showing spinal NA and ACh concentration after vehicle administration. Our previous study reported that vehicle administration did not alter spinal NA concentration in naïve rats (Hoshino et. al. 2015). However, further experiments are needed to reveal how duloxetine affects spinal NA and ACh concentrations in SNL rats.

Second, there are some limitations in our 7,8-DHF experiments. It is reported that repeated 7,8-DHF treatment increases the spinal noradrenergic fiber density (Kato et al., 2019) and normalizes the simulation-induced NA release by normalizing the regulation of locus coeruleus (Suto et al., 2019). In line with this, treatment with trkB can enhance the duloxetine-induced increase in NA levels in the spinal dorsal horn, thereby enhancing NA-mediated anti-allodynic effects. NA mediated ACh release may also be enhanced. In addition, it is speculated that BDNF-TrkB signaling induces α2 adrenoceptor coupled G protein switching from the inhibitory Gi/o protein to the excitatory Gs protein in the cholinergic neurons following nerve injury (Hayashida and Eisenach, 2010), In this study, we did not confirm whether 7,8-DHF facilitated NA mediated ACh release in SNL6W rats. We hope that further studies will be conducted in the future to clarify these points.

Conclusions

In conclusion, we demonstrated that a single duloxetine administration induces attenuated anti-allodynic effects in animals at the chronic phase of nerve injury (SNL6W) by decreasing spinal ChAT immunoreactivity. Restoring acetylcholine-mediated analgesia by enhancing TrkB-BDNF signaling may be beneficial for patients experiencing chronic neuropathic pain.

Author contribution and attestation

Daiki Kato – **Contribution**: The author helped in designing and conducting the study, analyzed the data, and prepared the manuscript; **Attestation**: Daiki Kato attests to having approved the final manuscript and reviewed the original study data and data analysis. Daiki Kato attests to the integrity of the original data and the analysis. **Takashi Suto** – **Contribution**: The author helped in designing and conducting the study, analyzed the data, and prepared the manuscript; **Attestation**: Takashi Suto attests to having approved the final manuscript and reviewed the original study data and data analysis. Takashi Suto attests to the integrity of the original data and the analysis. **Hideaki Obata** – **Contribution**: The author helped design the study and prepare the manuscript; **Attestation**: Hideaki Obata attests to having approved the final manuscript. Hideaki Obata attests to the integrity of the original data and the analysis. **Shigeru Saito** – **Contribution**: The author helped design the study and prepare the manuscript; **Attestation**: Shigeru Saito attests to having approved the final manuscript. Shigeru Saito attests to the integrity of the original data and the analysis. Shigeru Saito is the archival author.

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CRediT authorship contribution statement

Daiki Kato: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Takashi Suto:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing – review & editing, Visualization. **Hideaki Obata:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing, Supervision. **Shigeru Saito:** Conceptualization, Writing – review & editing, Supervision.

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