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Research report

Loss of endogenous analgesia leads to delayed recovery from incisional pain in a rat model of chronic neuropathic pain

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HIGHLIGHTS

- Neuropathic rats with impaired endogenous analgesia show extended pain after surgery.
- Perioperative repeated amitriptyline restores noxious stimulus-induced analgesia.
- Repeated amitriptyline treatment improves delayed pain recovery after surgery.
- Enhancing endogenous analgesia is promising to avoid chronic pain after surgery.

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ABSTRACT

Background: Preoperative pain and impaired endogenous analgesia are risk factors of chronic postsurgical persistent pain (CPSP). A Chronic neuropathic pain model induced by spinal nerve ligation (SNL6W) shows impaired endogenous analgesia and delayed recovery from incisional pain. Repeated amitriptyline treatment can restore the endogenous analgesia, but its effects on delayed recovery are not clear.

Methods: A plantar incision was made on the side contralateral to the nerve ligation in SNL6W rats. Withdrawal thresholds were measured by von Frey filament test until 28 d after surgery. Amitriptyline (10 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) or vehicle was administered for 13 d perioperatively. To examine the roles of noradrenergic and cholinergic signals in the spinal dorsal horn, pharmacological antagonism, measurement of each neurotransmitter concentration, and immunohistochemistry were conducted.

Results: Recovery of the withdrawal threshold of SNL6W animals to pre-incision values required 28 d after surgery, while naive animals recovered within 14 d. Intrathecal injection of alpha2 adrenoceptor antagonist (idazoxan) or muscarinic cholinergic receptor antagonist (atropine) decreased the withdrawal threshold on POD14 and 21 in naive animals, but not in SNL6W rats. Repeated amitriptyline treatment attenuated the delayed recovery in SNL6W rats, and the effect was antagonized by muscarinic cholinergic receptor antagonist. Beside the concentration of acetylcholine and its synthetic enzyme were not altered by the treatment.

Conclusions: Noradrenergic and cholinergic analgesia, which is necessary for normal recovery, is lost in the SNL6W rats. A strategy to enhance endogenous analgesia using antidepressants, rather than simple analgesia, may help to prevent CPSP in chronic pain patients.

1. Introduction

Pain prior to surgery and severe acute postoperative pain are well recognized risk factors for chronic postsurgical pain (CPSP) (Kehlet et al., 2006; Richebé et al., 2018), but CPSP is not prevented by administering drugs which may show direct acute analgesic effects in perioperative period (Andreae and Andreae, 2012; Schmidt et al., 2013; Wong et al., 2014). Novel strategies derived from a new perspective to treat CPSP are therefore in high demand.

Impaired endogenous analgesia is also a risk factor of CPSP (Wu and

Abbreviations: CPSP, chronic postsurgical pain; SNL, spinal nerve ligation; NSIA, noxious stimulus-induced analgesia; SNL6W, spinal nerve ligation model at 6 weeks after surgery; AMI, amitriptyline; SNL6W-Veh, spinal nerve ligation model at 6 weeks-vehicle; SNL6W-AMI, spinal nerve ligation model at 6 weeks–amitriptyline; DβH, dopamine-β-hydroxylase; ChAT, choline acetyltransferase

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Raja, 2011). Previous studies reported that persistent pain attenuates endogenous analgesic systems (Potvin and Marchand, 2016; Teles et al., 2018). Patients with chronic pain exhibit prolonged postsurgical pain in areas remote to the site of the pre-existing pain (Gerbershagen et al., 2009; VanDenKerkhof et al., 2012). Impaired endogenous analgesia is detected by experimental measurements of conditioned pain modulation (Yarnitsky, 2010), and preoperative testing of conditioned pain modulation predicts the incidence of CPSP (Yarnitsky et al., 2008). Persistent pain also produces impairment in endogenous analgesia in some animal models (Ferrari et al., 2010; Hughes et al., 2013; Miranda et al., 2015). Spinal nerve ligation (SNL) leads to persistent pain, and this model also exhibits impaired endogenous analgesia at a late stage (5–6 weeks) after SNL surgery (Kimura et al., 2015; Matsuoka et al., 2016). In naive animals, subcutaneous capsaicin injection produces painful sensations and analgesia at sites remote from the injection site. This phenomenon, called noxious stimulus-induced analgesia (NSIA), is considered to reflect endogenous analgesia. NSIA is entirely lost in SNL model rats at 6 weeks after surgery (SNL6W) due to the loss of activation of the locus coeruleus to noxious stimulation. Dysfunction of the locus coeruleus leads to abnormalities in the descending noradrenergic pain inhibitory system that acts at the spinal dorsal horn. We hypothesized that SNL6W model rats exhibit persistent postsurgical pain after plantar incision due to the dysfunction of noradrenergic descending pain inhibition. We examined the time course of the paw withdrawal threshold after plantar incision on the contralateral side of the nerve ligation during a 28-d period. Repeated treatment with amitriptyline restores impaired endogenous analgesia in SNL6W animals (Matsuoka et al., 2016). To examine whether restoring endogenous analgesia prevents persistent postsurgical pain, we also examined the effects of perioperative treatment with amitriptyline and the recovery process of postsurgical hypersensitivity in SNL6W rats.

2. Results

2.1. SNL6W rats exhibited delayed recovery from postsurgical pain

The SNL6W rats used for all experiments exhibited allodynia in the right paw. A plantar incision was made on the contralateral paw and the withdrawal thresholds were measured over time. There were significant main effects of group (Naive vs SNL6W) and time (group: $F_{1, 33} = 18.06$, $P = 0.0002$, time: $F_{7, 231} = 54.07$, $P < 0.0001$, group × time: $F_{7, 231} = 6.299$, $P < 0.0001$, two-way repeated measures ANOVA). Post hoc testing revealed that SNL6W rats had a significantly lower withdrawal threshold than naive animals on POD14, 21, and 28 (POD14; $P < 0.0001$, POD21; $P < 0.0001$, POD28; $P = 0.0047$). The withdrawal threshold was not significantly different between groups on POD1, 3, 7, and 10. Furthermore, the paw withdrawal threshold of SNL6W rats returned to values statistically not significantly different from the pre-incision values by POD28, while that of naive animals returned to the pre-incision values by POD14. This finding suggested SNL6W rats exhibited delayed recovery (Fig. 2A). The estimated treatment effect (difference between groups or time) and 95% confidence interval are shown in Table 1.

2.2. SNL6W rats showed a loss of spinal noradrenergic and cholinergic analgesia, which is necessary for recovery from postsurgical pain

We performed intrathecal injections of idazoxan or atropine and compared the withdrawal threshold of the incised paw at 30 min after injection. In naive animals, intrathecal injection of idazoxan significantly decreased the withdrawal threshold on POD14 and 21, whereas it had no effect on POD28 (POD14: group; $F_{1, 19} = 11.54$, $P = 0.003$, time; $F_{1, 19} = 18.79$, P = 0.0004, group × time; $F_{1, 19} = 27.24$, P < 0.0001; POD21: group; $F_{1, 16} = 3.179$, $P = 0.0936$, time; $F_{1, 16} = 21.73$, $P = 0.0003$, group × time; $F_{1, 16} = 28.45$, $P < 0.0001$, Fig. 2B). In SNL6W rats, however, the withdrawal threshold was not changed by idazoxan at any time-point. In naive animals, intrathecal injection of atropine significantly decreased the withdrawal threshold on POD14 (group; $F_{1, 14} = 30.34$, $P < 0.0001$, time; $F_{1, 14} = 2.449$, $P = 0.1399$, group × time: $F_{1, 14} = 14.68$, $P = 0.0018$, Fig. 2C). Atropine did not affect the paw withdrawal threshold of SNL6W rats at any time-point.

2.3. NSIA restoring effect by repeated amitriptyline treatment lasts at least 1 week

Repeated administration of amitriptyline can potentiate NSIA, which is impaired in SNL6W animals (Matsuoka et al., 2016). We examined the duration of the effect. When NSIA was measured at 1 week intervals, there was significant main effect of group (SNL6W-Veh vs SNL6W-AMI) and time (group: $F_{1, 10} = 10.94$, $P = 0.0079$, time: $F_{3, 10} = 15.08$, $P < 0.0001$, group × time: $F_{3, 10} = 7.489$, $P = 0.0007$, two-way repeated measures ANOVA), and post hoc testing revealed that SNL6W-AMI rats had a significantly increased withdrawal threshold at 30 min after the capsaicin injection (Fig. 3A, $P < 0.0001$). SNL6W-Veh rats did not exhibit an increased withdrawal threshold at any time-point. There were no significant differences in the measurements performed at 2 weeks, demonstrating that the effect of amitriptyline had disappeared (Fig. 3B).

2.4. Perioperative amitriptyline improved the recovery from postsurgical pain through the cholinergic system

We examined whether the restoration of NSIA by repeated amitriptyline injections could improve the delayed recovery from postsurgical pain. To avoid the direct analgesic effect of amitriptyline, we designed a consecutive 13-d amitriptyline treatment, and measured the withdrawal threshold on POD14, the 7 d following the last amitriptyline treatment. There were significant main effects of group (SNL6W-Veh vs SNL6W-AMI) and time (group: $F_{1, 24} = 24.21$, $P < 0.0001$, time: $F_{8, 192} = 56.72$, $P < 0.0001$, group × time: $F_{8, 192} = 3.734$, $P = 0.0004$, two-way repeated measures ANOVA, Fig. 4A) and post hoc testing revealed that the withdrawal thresholds of SNL6W-AMI rats were significantly higher than that of SNL6W-Veh on POD14 and 21 (POD14; $P = 0.0156$, POD21; $P < 0.0001$). Intrathecal injection of idazoxan on POD14 had no effect on the withdrawal threshold in either group (Fig. 4B). In contrast, intrathecal atropine significantly reduced the withdrawal threshold in SNL6W-AMI rats (group: $F_{1, 24} = 0.0795$, $P = 0.7804$, time: $F_{1, 24} = 6.053$, $P = 0.0215$, group × time: $F_{1, 24} = 33.4$, $P < 0.0001$, two-way repeated measures ANOVA, Fig. 4C), but not in SNL6W-Veh rats.

2.5. Perioperative amitriptyline did not alter the spinal content of noradrenaline and acetylcholine after plantar incision in SNL6W animals.

We compared the neurotransmitter content in the left spinal dorsal horn after plantar incision on POD14, among naive, SNL6W-Veh, and SNL6W-AMI animals. For the noradrenaline content, there was a significant main effect of group ($F_{2, 18} = 14.45$, $P = 0.0002$, one-way ANOVA; Fig. 5A), and post hoc testing revealed that SNL6W animals had lower noradrenaline content than naive animals ($P = 0.0003$, $P = 0.0012$, respectively), regardless of the perioperative treatment. Noradrenaline content was not significantly different between SNL6W-AMI and SNL6W-Veh rats. For the acetylcholine content, there was a significant main effect of group ($F_{2, 19} = 4.878$, $P = 0.0195$, one-way ANOVA; Fig. 5B), and post hoc testing revealed that the SNL6W animals contained more acetylcholine than naive animals ($P = 0.0266$). Similar to noradrenaline, however, there was no significant difference in acetylcholine content between the treatment groups.

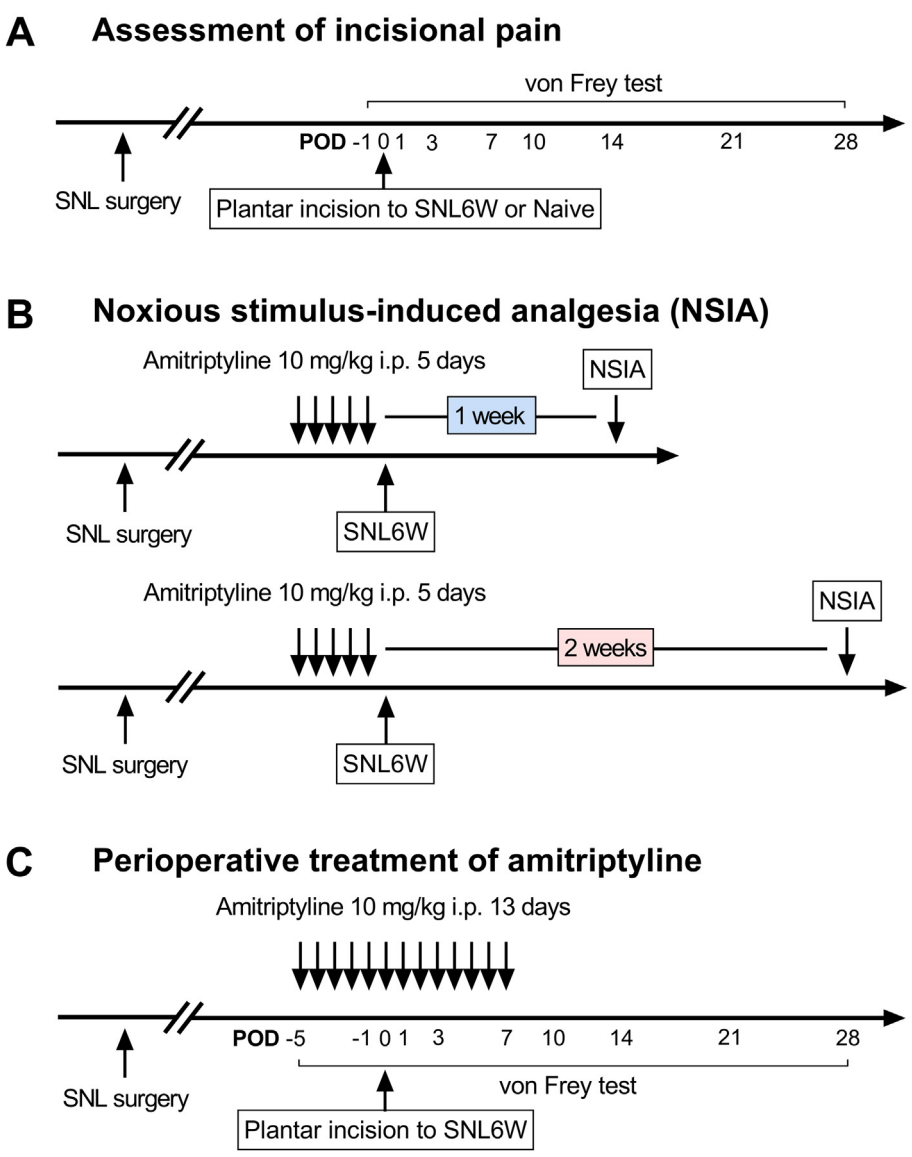

Fig. 1. Schematic representation of the study. (A) Time course of behavioral tests to assess incisional pain in naive and SNL6W rats. Numbers under filled triangles indicate each time-point after the plantar incision and von Frey measurement. (B) Time course of 5 daily intraperitoneal injections of amitriptyline and subsequent NSIA measurement in SNL6W rats. (C) Time course of 13 daily intraperitoneal injections of amitriptyline or vehicle and assessment of incisional pain in SNL6W rats. NSIA = noxious stimulus-induced analgesia; i.p. = intraperitoneal.

2.6. Perioperative amitriptyline did not change the spinal density of noradrenergic and cholinergic fibers after plantar incision in SNL6W animals

We assessed the density of axon fibers of spinal noradrenergic and cholinergic neurons after plantar incision on POD14. DβH and ChAT-IR fibers in the left spinal dorsal horn were measured and analyzed (Fig. 6A). For DβH-IR, there was a significant main effect of group ($F_{2, 32} = 3.44$, $P = 0.0044$, one-way ANOVA; Fig. 6B) and post hoc testing revealed that SNL6W-AMI rats had a lower density of DβH-IR fibers than naive animals. There was no significant difference between the SNL6W-Veh and SNL6W-AMI rats.

For ChAT-IR, there was a significant main effect of group ($F_{2, 33} = 31.38$, $P < 0.0001$, one-way ANOVA; Fig. 6C), and post hoc testing revealed that SNL6W-AMI and SNL6W-Veh rats had a higher density of DβH-IR fibers than naive animals ($P < 0.0001$). There was no significant difference in ChAT-IR between the two groups of SNL6W animals.

3. Discussion

In the present study, we found that SNL6W rats that show impaired NSIA required a longer period to recover to the pre-incision level after paw incision compared to naive animals (28 vs 14 d). The descending noradrenergic and muscarinic cholinergic systems play important roles in the recovery from postsurgical pain in naive animals. These two analgesic mechanisms are lost in SNL6W rats. 13 daily injections of amitriptyline significantly improved the delayed recovery from postsurgical pain in the rats. These findings suggest that the delayed recovery from postsurgical pain can be at least partially improved by restoring endogenous analgesia.

3.1. Endogenous analgesia and recovery from pain after surgery

CPSP is often difficult to treat by currently available analgesic drugs, and thus preventative strategies are required. Severe acute postoperative pain is a well-recognized risk factor for CPSP (Kehlet et al., 2006; Wu and Raja, 2011). It seems logical that well-designed

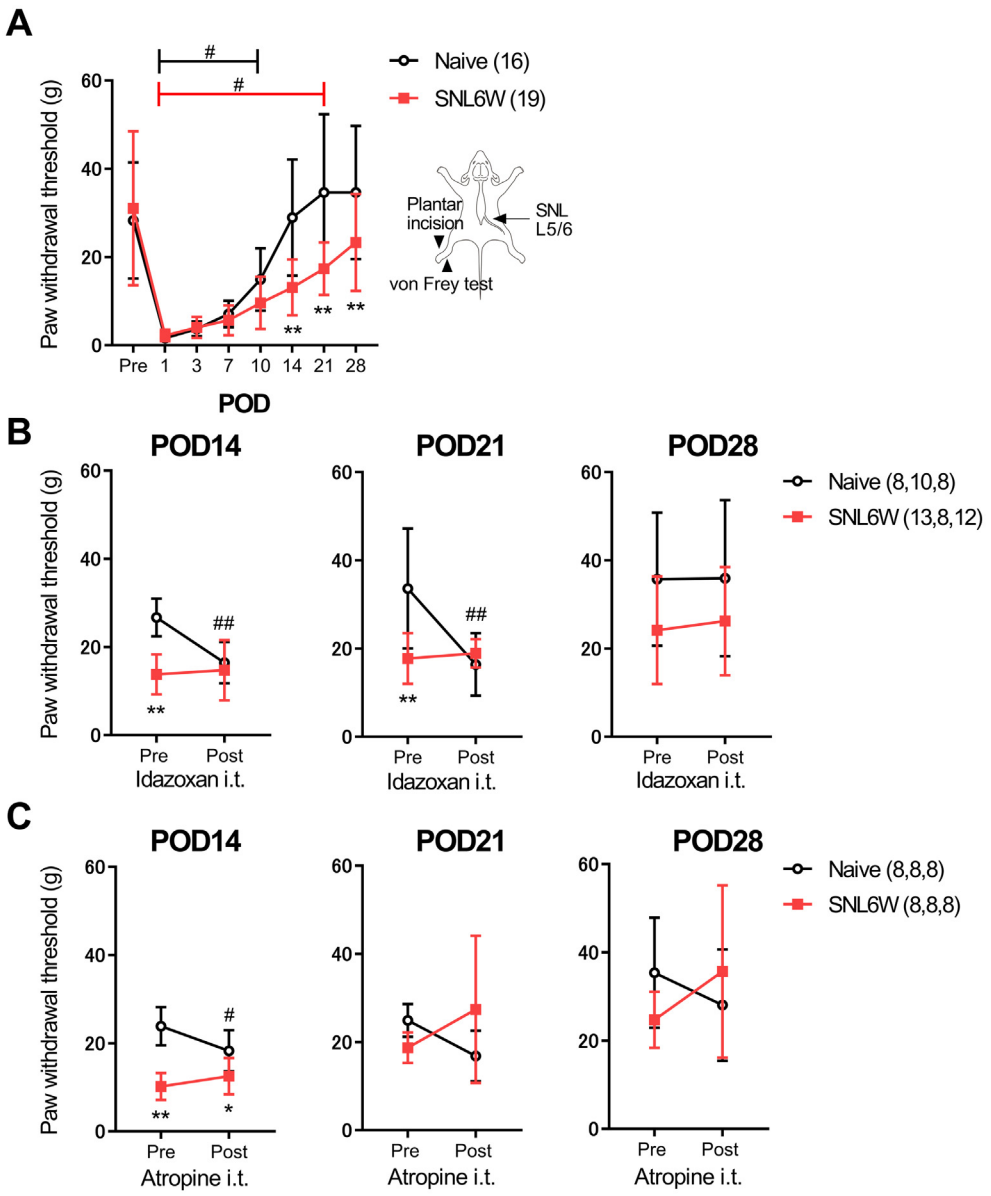

Fig. 2. SNL6W animals exhibited delayed recovery from incisional pain compared with naive animals. The withdrawal threshold around the incision in the left hind paw (contralateral to nerve ligation) was measured until POD28. The withdrawal threshold returned to the pre-incision value on POD14 in naive animals, but not until POD28 in SNL6W rats (A). Intrathecal injection of idazoxan (30 μg/10 μl) in naive animals reduced the withdrawal threshold on POD14 and 21, but did not affect the threshold in SNL6W rats (B). Intrathecal injection of atropine (30 μg/10 μl) also reduced the threshold in naive rats on POD14 (C). Intrathecal atropine did not affect the withdrawal threshold of SNL6W rats at any time-point. Data are expressed as mean ± SD, analyzed by two-way repeated measures ANOVA with Bonferroni's adjustment for multiple comparisons. $^{**}P < 0.01$, $^{*}P < 0.05$ vs naive, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs pre. ANOVA = analysis of variance; POD = postoperative day; i.t. = intrathecal.

perioperative pain control should provide benefits to reduce the incidence of CPSP by reducing central sensitization. Therefore, peripheral nerve block and epidural analgesia seem to be a logical strategy to prevent CPSP. Some studies showed that these procedures are effective for reducing the incidence of CPSP (Lavand'homme et al., 2005; Salengros et al., 2010), but the results are inconclusive (Andreae and Andreae, 2012). Thus, not only simple perioperative analgesia, but also novel concepts are required to prevent CPSP.

Pain prior to surgery and impaired endogenous analgesia are risk factors of CPSP (Kehlet et al., 2006; Wu and Raja, 2011), as are the concomitant symptoms of chronic pain (Lewis et al., 2012). A previous report suggested that testing for endogenous analgesia prior to surgery can predict the incidence of CPSP (Yarnitsky et al., 2008). The coincidence of chronic pain and impaired endogenous analgesia is reported in patients with various types of chronic pain (Lewis et al., 2012). Recently, we reported that SNL6W rats exhibit persistent allodynia and impaired endogenous analgesia, as measured by NSIA, due to dysfunction of the noradrenergic descending pain inhibition system (Kimura et al., 2015). We also reported that repeated treatment with amitriptyline, but not pregabalin, ameliorates the impairment (Matsuoka et al., 2016). On the basis of these previous findings, we hypothesized that restoring endogenous analgesia with amitriptyline could prevent the prolongation of postsurgical pain and would thus be a novel approach for preventing CPSP.

In the present study, SNL6W rats had a lower withdrawal threshold than naive animals after paw incision made in the contralateral side of the nerve ligation on POD14 to 28. Recovery of the withdrawal threshold to the pre-incision level required 28 d, i.e., twice as long as naive animals, which is considered to be delayed recovery. Furthermore, while intrathecal idazoxan reduced the pain threshold of naive animals on POD14 and 21, it had no effect on SNL6W rats, suggesting that the descending noradrenergic inhibitory system is normally required to maintain the withdrawal threshold, and this system is lost in SNL6W rats. Conversely, the hypersensitivity on POD1-7 was not different between groups. Tissue damage and the subsequent local inflammation lead to nociceptor activation and peripheral sensitization (Pogatzki-Zahn et al., 2007; Pogatzki et al., 2002). The descending noradrenergic inhibitory system can reduce central sensitization, but it might not be effective for suppressing local inflammation and peripheral sensitization. Delayed recovery was caused by differences in the rate of pain resolution and was not related to the initial pain intensity after surgery. Clinical findings suggest the importance of the acute pain

Table 1
Overview of the statistical analysis. Estimates of treatment effects and 95% confidence interval were summarized.

Source	Estimated Treatment Effects (g)	95% CI	P value
Fig. 2A: Incisional pain			
Naive vs SNL6W			
POD14	15.8	6.9 to 24.8	< 0.0001
POD21	17.3	8.3 to 26.3	< 0.0001
POD28	11.3	2.3 to 20.3	0.0047
Within naive			
Pre vs POD1	26.7	17.8 to 35.6	< 0.0001
Pre vs POD14	−0.6	−9.5 to 8.2	> 0.9999
Within SNL6W			
Pre vs POD1	28.8	20.7 to 37.0	< 0.0001
Pre vs POD28	7.7	−0.4 to 15.9	0.0745
Fig. 3A: NSIA, 1 week			
Within SNL6W-Veh			
0 vs 30	−10.7	−44.0 to 22.7	> 0.9999
Within SNL6W-AMI			
0 vs 30	−90.7	−124.0 to −57.3	< 0.0001
Fig. 3B: NSIA, 2 weeks			
Within SNL6W-Veh			
0 vs 30	−5.0	−41.7 to 31.7	> 0.9999
Within SNL6W-AMI			
0 vs 30	−16.7	−53.4 to 20.0	0.7751
Fig. 4A: Perioperative AMI			
SNL6W-Veh vs -AMI			
POD14	−10.6	−19.9 to −1.2	0.0156
POD21	−20.5	−29.8 to −11.1	< 0.0001

SNL6W = spinal nerve ligation model at 6 weeks after surgery; POD = post operative day; AMI = amitriptyline; Veh = vehicle; NSIA = noxious stimulus-induced analgesia.

trajectory as it assumes a distinctive pattern in patients who eventually develop CPSP (Lavand'homme et al., 2014). The acute pain trajectory analysis allows us to predict the long-term characteristics of post-surgical pain (Chapman et al., 2011). It is possible that the delay observed in the SNL6W animals may not fully reflect clinical conditions of CPSP in human patients. Further investigations are necessary on the validity of SNL6W as an animal model of CPSP.

Intrathecal atropine also reduced the withdrawal threshold on POD14 in naive animals. Cholinergic signaling has analgesic effects for postsurgical pain (Klamt et al., 1997; Prado and Segalla, 2004). Although the contribution may be temporary, a strategy to augment the cholinergic system might be effective for accelerating recovery. The loss of the two analgesic mechanisms may result in the delayed recovery observed in the SNL6W rats. In SNL rats at an early stage after nerve ligation (1–4 weeks), noradrenaline stimulates acetylcholine release in the spinal dorsal horn, and produce analgesia (Kimura et al., 2012). In SNL6W rats, painful stimulation does not activate the locus coeruleus (Kimura et al., 2015). This may result in the reduction of stimulation-responsive release of noradrenaline in the spinal dorsal horn (Kato et al., 2018). Therefore, it can be speculated that the loss of noradrenergic activity may lead to a decrease in cholinergic analgesia.

3.2. *Effects of amitriptyline on NSIA and the delayed recovery from postsurgical pain*

Our previous study reported that 5 daily treatments with amitriptyline restores NSIA in SNL6W rats (Matsuoka et al., 2016). The present study demonstrated that the effect of amitriptyline on NSIA lasts for 7 d after terminating treatment. According to these results, we performed 13 daily perioperative injections with amitriptyline, and measured the withdrawal threshold until POD28, which is 21 d after the last amitriptyline treatment on POD7. SNL6W rats treated with amitriptyline had a higher threshold than vehicle-treated SNL6W rats on POD14 and 21. The withdrawal threshold of the SNL6W-AMI rats recovered to the pre-incision level by POD14, and the duration was not different from that in the naive animals. The effect of amitriptyline on NSIA might be maintained at this time-point, and therefore it is possible that the difference between SNL6W-AMI and SNL6W-Veh rats is because of NSIA restoration induced by the repeated amitriptyline treatment.

In contrast to our expectation, intrathecal idazoxan did not reduce the withdrawal threshold of SNL6W-AMI rats on POD14, whereas intrathecal atropine did. Unfortunately, neither our neurotransmitter concentration measurements nor our immunohistochemistry revealed any effects of amitriptyline. Another possibility is that the amitriptyline

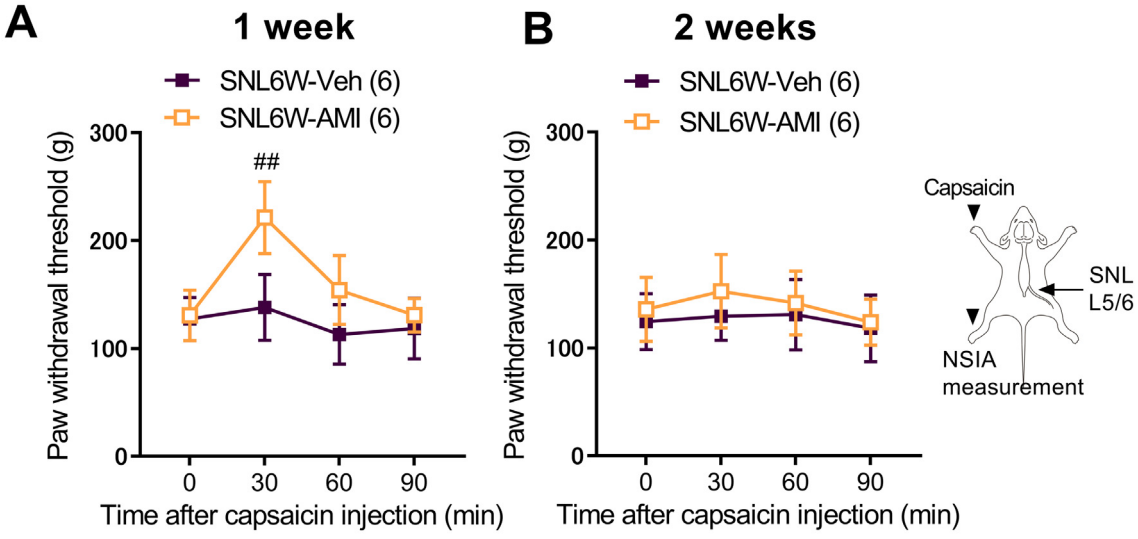

Fig. 3. Five daily injections of amitriptyline restored noxious stimulus-induced analgesia (NSIA) in SNL6W rats over a 1-week period. SNL rats were treated with 5 daily intraperitoneal injections of amitriptyline (AMI, 10 mg·kg^{-1}·d^{-1}) or vehicle just before SNL surgery, and NSIA was determined at 1 or 2 weeks. The noxious withdrawal threshold after subcutaneous injection of capsaicin (250 μg/50 μl) was measured in the left hind paw (contralateral to nerve ligation). Amitriptyline reactivated NSIA in SNL6W rats even at 1 week after treatment (A). At 2 weeks after treatment, NSIA was not observed (B). Data are expressed as mean ± SD, analyzed by two-way repeated measures ANOVA with Bonferroni's adjustment for multiple comparisons. $^{\#\#}P < 0.01$ vs time 0. NSIA = noxious stimulus-induced analgesia; i.p. = intraperitoneal; AMI = amitriptyline; ANOVA = analysis of variance.

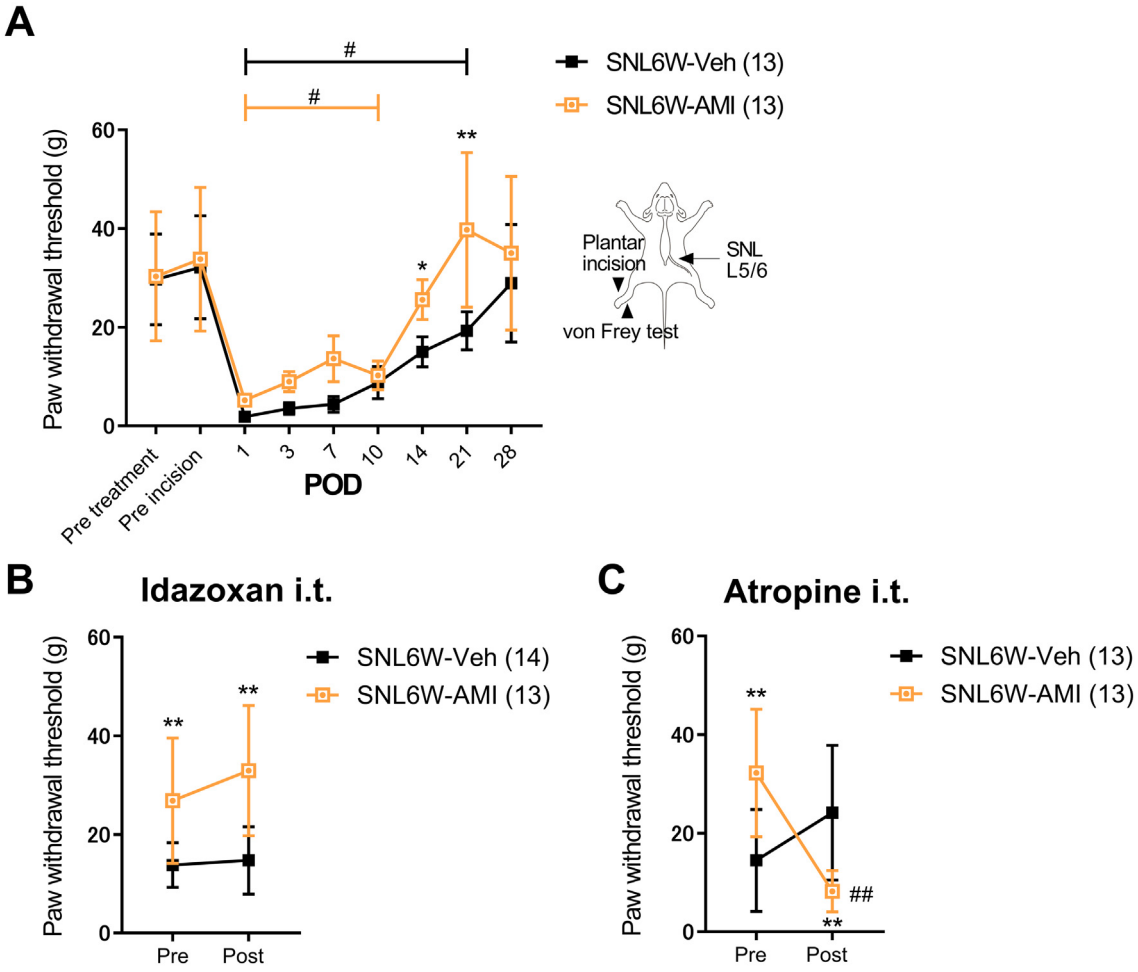

Fig. 4. Perioperative repeated amitriptyline treatment improved delayed recovery from incisional pain in SNL6W rats by activating the cholinergic system. Mechanical hypersensitivity after plantar incision was compared between the groups treated with 13 daily intraperitoneal injections of amitriptyline (SNL6W-AMI) or vehicle (SNL6W-Veh). SNL6W-AMI had a higher withdrawal threshold on POD14 and POD21 compared with the vehicle treated group (A). On POD14, intrathecal injection of idazoxan (30 μg/10 μl) did not reverse the restoring effect of amitriptyline on POD14 (B), whereas intrathecal atropine (30 μg/10 μl) reversed the restoring effect of amitriptyline (C). Data are expressed as mean ± SD, analyzed by two-way repeated measures ANOVA with Bonferroni's adjustment for multiple comparisons. $^{**}P < 0.01$, $^{*}P < 0.05$ vs SNL6W-Veh, $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs pre, POD = postoperative day; i.t. = intrathecal; AMI = amitriptyline; ANOVA = analysis of variance.

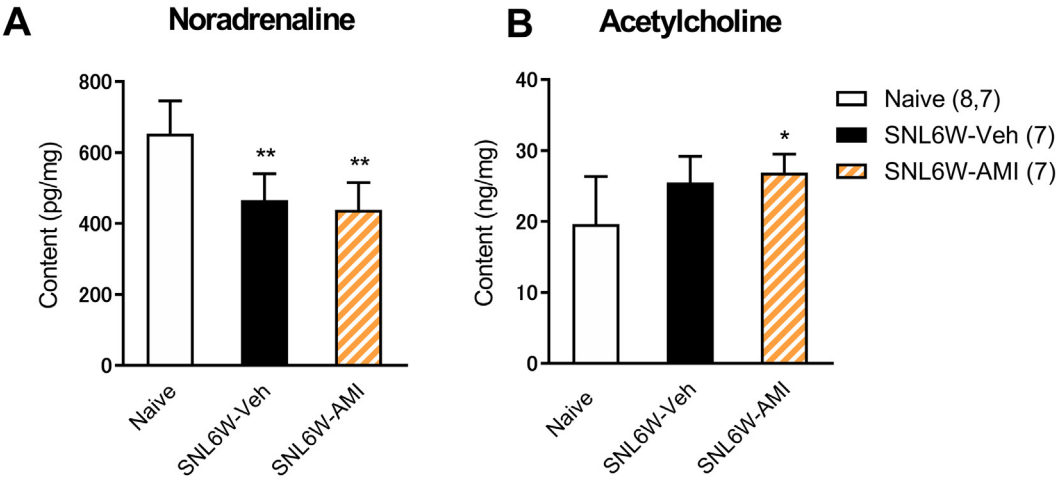

Fig. 5. Perioperative repeated amitriptyline treatment did not change the spinal noradrenaline and acetylcholine content in SNL6W rats. The left dorsal horn of the lumbar spinal cord in naive and SNL6W rats receiving 13 daily intraperitoneal injections of amitriptyline (SNL-AMI) or vehicle (SNL6W-Veh) was collected on POD14, and the noradrenaline (A) and acetylcholine (B) content was measured. The content of the two neurotransmitters did not significantly differ between the SNL6W-AMI and SNL6W-Veh rats. Data are expressed as mean ± SD, analyzed by a one-way ANOVA with Bonferroni's adjustment for multiple comparisons. $^{**}P < 0.01$, $^{*}P < 0.05$ vs naive. AMI = amitriptyline; ANOVA = analysis of variance.

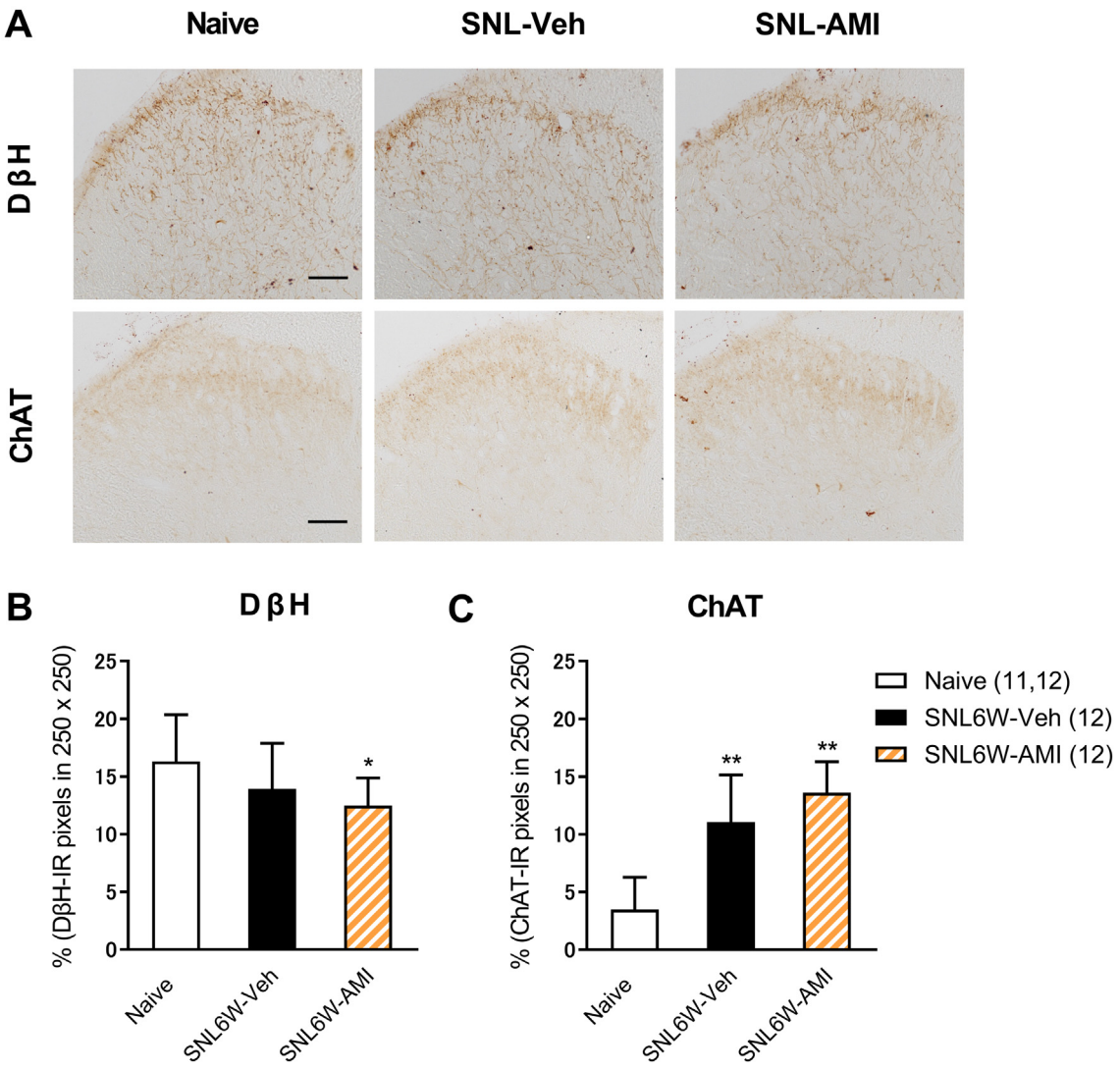

Fig. 6. Perioperative repeated amitriptyline treatment did not change the density of noradrenergic and cholinergic fibers in the spinal dorsal horn of SNL6W rats. The micrographs of DβH-IR and ChAT-IR in the left dorsal horn of the lumbar spinal cord (L4-6) on POD14 are shown in Fig. 6A. DβH-IR and ChAT-IR did not significantly differ between SNL6W rats treated with repeated amitriptyline (SNL6W-AMI) and SNL6W rats treated with vehicle (SNL6W-Veh) (B, C). Scale bar = 100 μm, Data are expressed as mean ± SD, analyzed using one-way ANOVA with Bonferroni's adjustment for multiple comparisons. $^{**}P < 0.01$, $^{*}P < 0.05$ vs naive. AMI = amitriptyline; DβH-IR = dopamine beta-hydroxylase-immunoreactive; ChAT-IR = choline acetyltransferase-immunoreactive; ANOVA = analysis of variance.

changes the interaction between noradrenergic descending neurons and cholinergic interneurons. Brain-derived neurotrophic factor (BDNF) levels increase after SNL surgery and promote G protein switching (inhibitory to excitatory), which results in acetylcholine release stimulated by noradrenergic signals (Hayashida and Eisenach, 2010). Amitriptyline also increases BDNF in the central nervous system (Arsenault and Sawynok, 2009; Hisaoka-Nakashima et al., 2016). Our recent findings suggested that the effect of amitriptyline to restore NSIA is eliminated by TrkB receptor antagonism (Suto et al., 2019). Therefore, amitriptyline may promote G protein switching in the spinal dorsal horn of SNL6W rats to increase the analgesic efficacy of acetylcholine. We currently have no direct results to explain the change in α2 adrenoceptor-coupled G proteins. Furthermore, because amitriptyline has diverse pharmacologic actions, we should have used a drug whose pharmacologic actions are simpler, such as duloxetine, which can enhance endogenous analgesia (Ito et al., 2018). A recent clinical study tested the effect of duloxetine in humans who underwent total knee arthroplasty with central sensitization prior to surgery, similar to the SNL6W rats (Koh et al., 2019). The findings demonstrated duloxetine efficacy at a later period rather than immediately after surgery, and the mechanism to restore endogenous analgesia may be the same as in our study.

3.3. Clinical implications

A strategy to enhance endogenous analgesia using antidepressants, rather than simple analgesia, may be beneficial for avoiding persistent pain after surgery in chronic pain patients, because various types of chronic pain can impair endogenous analgesia.

4. Experimental procedures

4.1. Animals

All animal procedures and protocols were approved by the Animal Care and Use Committee of the Gunma University School of Medicine (Maebashi, Japan 16–046). Only male Sprague-Dawley rats (180 g, SLC, Shizuoka, Japan) were used in all experiments. Animals were housed in

cages under a 12-h dark/light cycle, with food and water available *ad libitum*. A total of 306 rats were randomly assigned, and each experiment was performed in separate groups of rats. Fifty rats that exhibited no allodynia and three exhibiting paralysis of the right hind paw after SNL surgery were excluded from the study. Therefore, data from a total of 253 rats were analyzed.

4.2. Spinal nerve ligation (SNL6W model)

Unilateral SNL surgery was performed as described by Kim and Chung (Kim and Chung, 1992). Briefly, following induction of anesthesia with 2% isoflurane, the right L6 transverse process was removed. The right L5 and L6 spinal nerves were tightly ligated with 5–0 silk suture and the wound was closed. SNL animals were housed until 6 weeks after surgery. The mechanical withdrawal threshold of the right paw was assessed with von Frey filaments (Stoelting, Wood Dale, IL, USA) using the up-down step method (Chaplan et al., 1994). Animals exhibiting allodynia with the cut-off threshold of 6 g were used in the subsequent experiments.

4.3. Plantar incision

We used a plantar incision for the postsurgical pain model as proposed by Brennan (2011). Planter incision was performed in the left hind paw (contralateral to SNL). After inducing anesthesia with 2% isoflurane, a 1-cm incision was made starting 0.5 cm from the heel edge. The plantar muscle was lifted and stretched, then incised longitudinally. The skin was closed with 5–0 nylon suture, which was removed on postoperative day (POD) 3 after assessing the hypersensitivity as described below.

4.4. Assessment of postsurgical pain

To compare the recovery from postsurgical pain between naive and SNL6W animals, mechanical hypersensitivity after the incision was measured over time. The paw withdrawal threshold was assessed using von Frey filaments pre-incision, and at POD1, 3, 7, 10, 14, 21, and 28 (Fig. 1A).

4.5. Intrathecal injections

To evaluate the roles of spinal noradrenergic and cholinergic signaling in the recovery from postsurgical pain, we performed intrathecal injections of idazoxan hydrochloride (α2 adrenoceptor antagonist, Millipore Sigma, St. Louis, MO, USA) or atropine (muscarinic acetylcholine receptor antagonist, Millipore Sigma) on POD14, 21, and 28. Under anesthesia with 2% isoflurane, idazoxan (30 μg/10 μl) or atropine (30 μg/10 μl) was injected into the L5/6 intervertebral space using a 30-gauge needle.

4.6. Repeated amitriptyline treatment and noxious stimulus-induced analgesia

Repeated administration of amitriptyline restores impaired NSIA in SNL6W rats (Matsuoka et al., 2016). NSIA is a validated method for evaluating endogenous analgesia in animals (Peters et al., 2015). While the NSIA paradigm is still under discussion, the dose of the conditioned stimulus used in the present study is well-verified to produce analgesia (Gear et al., 1999; Peters et al., 2015; Matsuoka et al., 2016). We examined the duration of NSIA reversed by repeated amitriptyline treatment. Five daily intraperitoneal injections (10 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$) of amitriptyline were administered to SNL animals just before 6 weeks after SNL surgery, and NSIA was measured at 1 or 2 weeks (Fig. 1B). The withdrawal threshold in the left hind paw was measured using a Randall-Selitto analgesiometer (Ugo Basile, Comerio, Italy) (RANDALL and SELITTO, 1957). The cutoff threshold was set to 250 g to avoid tissue injury. The animals were trained for 3 d with this apparatus before we measured the baseline values. Following induction of anesthesia with 2% isoflurane, 250 μg capsaicin (Millipore Sigma) in a 50-μl volume was subcutaneously injected into the right forepaw. Withdrawal thresholds in the left hind paw (contralateral to nerve ligation) were measured at 30, 60, and 90 min after capsaicin injection. These experiments were performed without plantar incision.

4.7. Perioperative amitriptyline treatment and postsurgical pain

To examine whether perioperative amitriptyline modulates the course of recovery, we administered 13 daily (5 d before through 7 d after incision) intraperitoneal injections of amitriptyline (SNL6W-AMI, 10 $mg{\cdot}kg^{-1}{\cdot}d^{-1}$, LKT Laboratories, St. Paul, MN, USA) (Fig. 1C). The control group was injected with vehicle (SNL6W-Veh, 1 $ml{\cdot}kg^{-1}{\cdot}d^{-1}$). The withdrawal threshold was measured using von Frey filaments at pretreatment, pre-incision, and at POD1, 3, 7, 10, 14, 21, and 28. Measurement was performed just before the amitriptyline treatment.

4.8. Spinal noradrenaline and acetylcholine content

We examined the relationship between spinal neurotransmitter content in the dorsal horn (ipsilateral to incision) and recovery of mechanical hypersensitivity after the incision. The spinal cords were collected on POD14.

For noradrenaline measurements, the animals were decapitated under deep anesthesia and the spinal cord was quickly harvested. The left portion of the lumbar enlargement was immediately dissected into 4-mm lengths of dorsal horn and weighed. The samples were homogenized in 1000 μl of homogenization solution (0.2 M perchloric acid containing 0.1 mM EDTA-2Na, 10 ng/ml isoproterenol as internal standard) and centrifuged at 20,000g at 0 ˚c for 15 min. The supernatants were filtered through a centrifugal filter with a 0.45-μm pore (PALL, Puerto Rico) at 2400g at 0 ˚C for 15 min, and adjusted to pH 3.5 with 1 M sodium acetate. Samples (10 μl) were injected into an HTEC-500 high-performance liquid chromatography (HPLC)-electrochemical detection system (Eicom Co.). The chromatographic conditions were as follows: The mobile phase comprised 0.1 M phosphate buffer (pH 6.0) containing 5 mg/L EDTA-2Na, 175 mg/L sodium 1-octanesulfonate acid, and 17% methanol. The column was an EICOMPAK SC-5ODS column (3.0 × 15 mm, Eicom Co).

For acetylcholine measurements, decapitation and sample collection was performed the same as for noradrenaline. The samples were homogenized in 1000 μl of homogenization solution (0.1 M perchloric acid containing 0.1 mM EDTA-2Na, 10^{-6} M ethylhomocholine as internal standard), and centrifuged and filtered as for noradrenaline, and then adjusted to pH 5 with 1 M potassium bicarbonate. Samples (10 μl) were injected into the HPLC. The chromatographic conditions were as follows: The mobile phase comprised 50 mM potassium bicarbonate containing 50 mg/L EDTA-2Na, 400 mg/L sodium 1-octanesulfonate acid. The column used was an EICOMPAK AC-GEL (2.0 × 150 mm, Eicom Co) and AC-ENZYM3 (3.0 × 4.0 mm, Eicom Co).

4.9. Immunohistochemistry

We examined the relationship between spinal density of noradrenergic and cholinergic axon fibers in neurons and the recovery of mechanical hypersensitivity after incision by immunostaining for dopamine-β-hydroxylase (DβH) and choline acetyltransferase (ChAT). Three groups of the animals (naive, SNL6W-AMI, and SNL6W-Veh) were assessed. Spinal cords were collected on POD14. After inducing deep anesthesia with an intraperitoneal injection of pentobarbital (100 mg/kg), the rats were intracardially perfused with cold 0.01 M phosphate buffer (PB) containing 1% sodium nitrite and then with 4% paraformaldehyde in 0.1 M PB. The spinal cord was harvested by rapid expulsion and postfixed for 4 h. After cryoprotection with 30% sucrose

in 0.1 M PB for 5 d at 4 ˚C, a 4-mm length of spinal cord at the L4-L6 level was dissected and sectioned on cryostat at 40 μm-thickness. We used an SAB-PO kit (Nichirei, Japan) for immunostaining. After pre-treating the sections with 0.3% hydrogen peroxide and 1.5% normal rabbit serum, the sections were incubated at 4 ˚C for 24 h with a mouse monoclonal anti-DβH antibody (1:1000, MAB308, Chemicon International Inc, Temecula, CA, USA) or a goat anti-ChAT antibody (1:500, AB144P, Millipore Sigma) in 1.5% normal rabbit serum and then incubated with biotinylated anti-mouse or anti-goat IgG for 1 h and peroxidase labeled-streptavidin for 30 min at room temperature. The color reaction was induced with 0.5 mg/ml 3-3′-diaminobenzidine substrate and 0.01% hydrogen peroxide in 0.05 M PB-saline. For quantification of DβH and ChAT immunoreactivity (IR), four to five sections were randomly selected from each animal. Digital images of the left spinal dorsal horn were captured with a Nikon ECLIPSE Ni-E microscope (Nikon, Japan) and a 10x objective with a resolution of 1608 × 1608 pixels. Image analysis software (Image J, National Institutes of Health, Bethesda, MD, USA) was used to quantify a square with a fixed area (250 × 250 pixels) covering the region of laminae I to V in the left spinal dorsal horn. The number of pixels associated with DβH- or ChAT-IR within a constant threshold was averaged in each group. The person performing the image analysis was blinded to the groups.

4.10. Statistics

All data are presented as the mean ± standard deviation (SD) and analyzed by two-way repeated-measures analyses of variance (ANOVA) for time-course data or one-way ANOVA for data from the three groups. When significant differences were observed ($P < 0.05$), a Student's *t* test with Bonferroni's correction for multiple comparisons was performed for between-group comparisons or comparisons at each time-point. The estimated treatment effect (difference between groups or time) and 95% confidence interval for the main outcome measures are reported for all of the analyses (Table 1). All statistical analyses were conducted using GraphPad Prism 7 (GraphPad Software Inc, San Diego, CA, USA). The primary aim of this study was to evaluate whether SNL6W rats exhibit delayed recovery of mechanical hypersensitivity after plantar incision compared with naive animals. According to our previous study (Kato et al., 2018), we determined the appropriate sample size, with the assumption of a mean difference of 10 g in the paw withdrawal threshold on POD14 and a SD of 8 g in each group. The power analysis indicated that 12 animals per group would be required to detect a significant difference with 80% power at a significance level of $\alpha = 0.05$.

CRediT authorship contribution statement

Jo Ohta: Conceptualization, Investigation, Formal analysis, Writing - original draft, Visualization, Funding acquisition. **Takashi Suto:** Methodology, Investigation, Formal analysis, Writing - review & editing, Funding acquisition, Project administration. **Daiki Kato:** Investigation, Formal analysis. **Tadanao Hiroki:** Writing - review & editing. **Hideaki Obata:** Conceptualization, Writing - review & editing, Supervision. **Shigeru Saito:** Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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