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Spinal Activation of Tropomyosin Receptor Kinase-B Recovers the Impaired Endogenous Analgesia in Neuropathic Pain Rats

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BACKGROUND: Although endogenous analgesia plays an important role in controlling pain states, chronic pain patients exhibit decreased endogenous analgesia compared to healthy individuals. In rats, noxious stimulus–induced analgesia (NSIA), which is an indicator of endogenous analgesia, diminished 6 weeks after spinal nerve ligation (SNL6W). A recent study in rats with deleted noradrenergic fibers demonstrated that the noradrenergic fibers were essential to NSIA. It has also been reported that brain-derived neurotrophic factor increased spinal noradrenergic fibers. Therefore, this study examined the effect of TrkB activation, which is the receptor for brain-derived neurotrophic factor, on impaired NSIA in SNL6W rats. In addition, we also examined the effect of endogenous analgesia on acute incisional pain.

METHODS: After 5 daily intraperitoneal injections of 7,8-dihydroxyflavone (7,8-DHF, TrkB agonist, 5 mg/kg), NSIA was examined by measuring the withdrawal threshold increment in the left (contralateral to nerve ligation) hindpaw at 30 minutes after capsaicin injection (250 μg) in the forepaw. K252a (TrkB antagonist, 2 μg) was administrated intrathecally for 5 days. Idazoxan (α2 adrenoceptor antagonist, 30 μg), atropine (muscarinic antagonist, 30 μg), and propranolol (nonselective β adrenoceptor antagonist, 30 μg) were administered intrathecally for 15 minutes before capsaicin injection. Microdialysis and immunohistochemistry were performed to examine the noradrenergic plasticity in the spinal dorsal horn. A hindpaw incision was performed on the left (contralateral to nerve ligation) hindpaw. Data were analyzed by 1-way analyses of variance or 2-way repeated-measures 1-way analysis of variance followed by a Student *t* test with Bonferroni correction.

RESULTS: Five daily intraperitoneal injections of 7,8-DHF restored the attenuated NSIA in SNL6W rats (n = 7, $P = .002$; estimated treatment effect [95% CI]: 62.9 [27.0–98.7] g), with this effect blocked by 5 daily intrathecal coadministrations of K252a (n = 6, $P < .001$; −57.8 [−78.3 to −37.2] g). This effect was also inhibited by a single intrathecal administration of idazoxan (n = 8, $P < .001$; −61.6 [−92.4 to −30.9] g) and atropine (n = 8, $P = .003$; −52.6 [−73.3 to −31.9] g), but not by propranolol. Furthermore, 7,8-DHF increased the noradrenergic fiber in the spinal dorsal horn and the noradrenaline release in response to the capsaicin injection in the forepaw in SNL6W rats. In addition, repeated injections of 7,8-DHF prevented delayed recovery from incisional pain in SNL6W rats.

CONCLUSIONS: Spinal activation of TrkB may recover the attenuated endogenous analgesia by improving the adrenergic plasticity, thereby leading to prevention of pain prolongation after surgery. (Anesth Analg 2019;129:578–86)

KEY POINTS

- **Question:** Does TrkB activation have an effect on impaired endogenous analgesia and on the acute incisional pain in rats at 6 weeks after spinal nerve ligation?
- **Findings:** This study demonstrated that repeated TrkB agonist treatments led to recovery of the attenuated noxious stimulus–induced analgesia by increasing spinal noradrenergic fibers and noradrenaline release in response to the noxious stimulus, with enhancement of noxious stimulus–induced analgesia preventing the delayed pain resolution after a hindpaw incision in 6 weeks after spinal nerve ligation rats.
- **Meaning:** Spinal TrkB activation may be a new strategy for preventing chronic postoperative pain in patients with attenuated endogenous analgesia.

Endogenous analgesia is the intrinsic inhibitory system of pain that controls the noxious stimulus from the peripheral nerve in the central nervous system.[1] Recent studies have suggested that patients with chronic pain including neuropathic pain tend to exhibit decreased endogenous analgesia, which is evaluated as a conditioned

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pain modulation,[2] compared to healthy individuals.[3] In addition, the degree of endogenous analgesia correlates with the incidence of chronic postoperative pain.[4,5] It has also been reported that preoperative pain and impaired pain modulation are risk factors of chronic postoperative pain.[6] Therefore, treatments that assist in the recovery of the attenuated endogenous analgesia in chronic pain patients could potentially lead to the effective pain management and the prevention of sustained postoperative pain.

A previous study that examined spinal nerve ligation (SNL) rats reported that noxious stimulus–induced analgesia (NSIA),[7] which is an indicator of endogenous analgesia, was reduced over time after a nerve injury, with the effect almost completely diminished at 6 weeks after the SNL surgery (SNL6W).[8,9] Furthermore, there was also decrease in the spinal noradrenaline (NA) release in SNL6W rats in response to a noxious stimulus decrease.[8,9] Therefore, SNL6W rats are considered to be a model for the attenuation of endogenous analgesia, especially noradrenergic descending inhibitory system of pain.

When treating neuropathic pain, antidepressants such as amitriptyline and duloxetine or pregabalin are widely used as first-line drugs.[10] In nerve-injured rats, these analgesics activate the noradrenergic descending inhibitory system of pain and have sufficient analgesic effect for mechanical hypersensitivity.[11–14] However, the administration of amitriptyline and duloxetine, but not pregabalin, led to the recovery of the attenuated NSIA in SNL6W rats.[9,15] A recent study reported that amitriptyline and duloxetine, which act as a serotonin (5-HT)-NA reuptake inhibitor,[16] increased brain-derived neurotrophic factor (BDNF) release from astrocytes.[17] It has also been demonstrated that intrathecal administration of BDNF antibody blocked the increase of the spinal noradrenergic fibers after nerve injury.[18] However, there have been no studies that have directly investigated the effect of BDNF-TrkB signaling on endogenous analgesia.

In our current study, we hypothesized that activation of TrkB, which is the receptor for BDNF, might play a role in the recovery of the attenuated endogenous analgesia. To test this hypothesis, we used SNL6W rats to evaluate the effect of the TrkB agonist on the noradrenergic neuroplasticity and the impaired NSIA. Furthermore, we also examined the possibility that recovery of endogenous analgesia might lead to an improvement in the SNL-induced hypersensitivity and acute incisional pain.

METHODS

Supplemental Digital Content 1, Figure 1, http://links.lww.com/AA/C479, shows the time course for each experiment.

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-hour light-dark cycle, and fed ad libitum. This study examined 183 rats, with the animals randomly assigned to each experimental group.

SNL6W Model (6 Weeks After SNL)

L5 and L6 SNLs were performed in 126 rats, as described previously.[19] After rats were anesthetized with 2.0% isoflurane in oxygen, the right transverse process was removed, and the right L5 and L6 spinal nerves were tightly ligated using 5-0 silk suture. After closure of the wound and housing the rats for 6 weeks, the animals were used for the experiments. Three rats were excluded due to paralysis of the right hindpaw after surgery.

Intrathecal Catheterization

Intrathecal catheterization was performed in the naïve and SNL rats 10 days before the experiment. 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) was inserted. Animals were allowed to recover for 1 week after the surgery, after which they were habituated for the behavioral experiments. After completion of the behavioral tests, correct catheter placement was confirmed by administration of an intrathecal lidocaine injection (0.5 mg/10 μL) to elicit reversible hindpaw paralysis.

Behavioral Tests

Hypersensitivity. The withdrawal threshold to mechanical stimulus in the naïve and SNL6W rats was assessed using an Analgesy-meter (37215, Ugo Basile, Comerio, Italy), as described previously.[20] Before the test, all animals were trained for at least 3 days with the apparatus. Withdrawal threshold was measured 3 times in the right hindpaw, with these values then averaged, respectively. A cutoff pressure of 250 g was used to avoid any tissue injury in the animals. The person performing the behavioral tests was blinded to the group.

Noxious Stimulus–Induced Analgesia. We assessed NSIA in the naïve and SNL6W rats, as described previously.[9] The withdrawal threshold in the left (contralateral to nerve ligation) hindpaw was measured as described above. Under isoflurane anesthesia (2%), capsaicin (250 μg/50 μL) was injected into the left forepaw, with the withdrawal threshold in the left hindpaw measured at 30 minutes after the injection. The increment of the withdrawal threshold after the capsaicin injection was then calculated.

Hindpaw Incision. Mechanical hypersensitivity was determined using 8 von Frey filaments (Stoelting, Wood Dale, IL) that ranged from 0.6 to 26 g, as described previously.[21] Rats were placed in individual acrylic chambers with a plastic mesh floor and allowed to acclimate within the environment for at least 30 minutes before the test. Subsequently, filaments were then applied to the heel of the hindpaw to the bending point for 5 seconds. A brisk paw withdrawal was considered to be a positive response. The up-down method was used to determine the withdrawal threshold.[21]

Planter incision was performed on the left (contralateral to nerve ligation) hindpaw in the naïve and SNL6W rats, as described previously.[22] Animals were anesthetized with

2.0% isoflurane in oxygen. A 1-cm midline incision was made using a No. 11 surgical blade at 0.5 cm from the proximal edge of the heel. The plantaris muscle was elevated and incised longitudinally. After the incision, the wound was closed with 5-0 nylon mattress sutures. Mechanical hypersensitivity was measured before and at 1, 3, 5, 7, 14, 21, 28, and 35 days after an incision.

Microdialysis for Spinal NA and 5-HT Concentrations

Microdialysis in the spinal dorsal horn was performed in accordance with our previous study.[23] The naïve and SNL6W rats were anesthetized with 2.0% isoflurane and maintained during the measurements with 1.5% isoflurane in 100% oxygen. The rectal temperature of the animals was maintained at 37.0°C using a heating blanket. Saline was infused at a rate of 1 mL/h through a cannulated left femoral vein using an infusion pump system (Fusion 400, Chemyx, Stafford, TX). The L4–L6 spinal cord was exposed by T13-L1 laminectomy. Microdialysis probes (CX-I-8-01, Eicom Co, Kyoto, Japan) were inserted into the right spinal dorsal horn and 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 for 1 hour to stabilize the baseline. At 30-minute intervals, 2 dialysates were collected and used for the baseline values. After left forepaw capsaicin injection, NA and 5-HT concentrations were measured for 90 minutes using separate high-pressure liquid chromatography systems with electrochemical detection (HTEC-500, Eicom Co). The basal concentrations of NA and 5-HT are presented as the average of two 30-minute dialysates. The change in the NA and 5-HT concentrations after capsaicin injection is presented as a percentage compared to the baseline (100%).

Immunohistochemistry

Spinal cord tissue was collected in the naïve and SNL6W rats, as described previously.[7] After animals were anesthetized using an intraperitoneal injection of 50 mg/kg pentobarbital, the thorax was opened and 0.1 mol/L phosphate-buffered saline (PBS) containing 20% sodium nitrate followed by fixative (4% paraformaldehyde in 0.1 mol/L phosphate buffer) was perfused from the left ventricle via a peristaltic pump (20 mL/min). The L4–L6 spinal cord was removed and immersed in fixative overnight at 4°C. On the following day, the spinal cord was immersed and maintained in 30% sucrose solution until sectioning. Using a cryostat, the spinal cord was sectioned at a thickness of 16 μm. After being pretreated with 0.3% normal donkey serum (Jackson Immuno Research Laboratories, West Grove, PA), the sections were incubated with mouse monoclonal anti-dopamine beta-hydroxylase (DβH) antibody (1:500, Merck Millipore, Billerica, MA) overnight. The following day, the sections were washed with PBS and incubated with Cy2-conjugated anti-mouse IgG (1:200, Jackson Immuno Research Laboratories) for 3 hours at room temperature.

Image Analysis

Digital images captured by an Olympus FSX100 microscope (Olympus Co, Tokyo, Japan) using fluorescein isothiocyanate filters and a 20× objective with a resolution of 1360 × 1024 pixels were used to quantify the immunostaining. Image analysis software (Image J, National Institutes of Health, Bethesda, MD) was used to quantify the immunofluorescence changes. For quantification, a square with a fixed area (250 × 250 μm^2) covering the region of laminae II of the spinal dorsal horn was examined. The number of pixels associated with the immunoreactivity within a defined threshold was measured and averaged in each group. The same threshold value was applied to all of the images for a given antibody. The person performing the image analysis was blinded to the groups.

Drugs

7,8-Dihydroxyflavone (7,8-DHF, TrkB agonist, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was dissolved in the 0.1 mol/L PBS containing 20% dimethyl sulfoxide (DMSO). ANA-12 (TrkB antagonist, Tocris Bioscience, Bristol, UK) was dissolved in saline containing 5% DMSO and 10% polyoxyethylene (20) sorbitan monooleate (Wako Pure Chemical Industries, Ltd). 7,8-DHF (5 mg/kg/d) with or without ANA-12 (0.5 mg/kg/d) was injected intraperitoneally for 5 days from day 37 to 41 after the SNL surgery. K252a (TrkB antagonist, Wako Pure Chemical Industries, Ltd) was dissolved in saline containing 10% DMSO. K252a (2 μg/10 μL) was administrated through an intrathecal catheter at a volume of 10 μL and then flushed with saline (10 μL) just before the intraperitoneal injection of 7,8-DHF. Idazoxan hydrochloride (α2 adrenoceptor antagonist, Sigma Aldrich, St Louis, MO), propranolol (nonselective β adrenoceptor antagonist, Sigma Aldrich), and atropine (muscarinic antagonist, Sigma Aldrich) were dissolved in saline. Idazoxan (30 μg/10 μL), propranolol (30 μg/10 μL), or atropine (30 μg/10 μL) were administrated through an intrathecal catheter as described above 15 minutes before the capsaicin injection. Capsaicin (Wako Pure Chemical Industries, Ltd) was dissolved in polyoxyethylene (20), sorbitan monooleate (50%), and ethanol (50%) at a concentration of 50 μg/μL and then diluted in saline to a final concentration of 5 μg/μL. The capsaicin solution was injected at a volume of 50 μL (250 μg) in all experiments.

Statistics

Parametric data are presented as the mean ± standard deviation and nonparametric data are presented as the median and the interquartile ranges. In the microdialysis, hypersensitivity, and hindpaw incision studies, the data were analyzed by a 2-way (factors: group and time) repeated-measures analysis of variance (ANOVA). When significant differences were observed for the group × time interaction ($P < .05$), a Student t test with Bonferroni correction was performed for the group comparisons at each time point. Other data were analyzed by a 1-way ANOVA, and if significant, a Student t test with Bonferroni correction for the group comparisons was then performed. Nonparametric data were analyzed using a Kruskal-Wallis test. P values adjusted with a Bonferroni correction were reported throughout, with $P < .05$ considered statistically significant. The estimated treatment effect (difference between the groups) and 95% CI for the main outcomes are reported for all of the analyses. All statistical analyses were conducted using SigmaPlot12 (Systat Software Inc, San Jose, CA) and EZR (Saitama Medical Center, Saitama, Japan). Of the 180 rats examined, 10 rats were excluded due

to intrathecal cannula misplacement and spinal nerve injury during the laminectomy (microdialysis study). Thus, a total of 170 rats were analyzed in this study.

We performed a power analysis for the primary outcome (NSIA) to determine the appropriate sample size. In accordance with the results of our previous study, our current analysis was based on the assumption that there was a mean difference of 50 g for the withdrawal threshold in the left hindpaw and a standard deviation of 30 g in each group.[9] The power analysis indicated that the use of more than 6 rats in each group would result in the detection of significant differences with 80% power at a significant level of $\alpha = .05$. However, because we did not include a Bonferroni correction in the sample size, this may have affected the type I error rate. This lack of control of type I error is a limitation of our current study.

RESULTS

The Effect of Spinal TrkB Activation on the Impaired NSIA in SNL6W Rats

To examine the effect of TrkB activation on endogenous analgesia, we evaluated NSIA in the left (contralateral to nerve ligation) hindpaw. The Table shows the estimated treatment effect (difference between groups) with the 95% CI. There was a significant main effect on the change in the withdrawal threshold ($F_{2,27} = 11.2$, $P < .001$, 1-way ANOVA; Figure 1A), while the post hoc testing revealed that the SNL6W rats exhibited a decrease in NSIA compared to naïve rats ($P < .001$). Five daily injections of 7,8-DHF (TrkB agonist) resulted in the recovery of the impaired NSIA in the SNL6W rats ($P = .002$). This effect was inhibited by 5 daily coadministrations of ANA-12 (TrkB antagonist) ($P = .028$). To determine the site of action of the TrkB agonist, we assessed the effect of the intrathecal administration of K252a (TrkB antagonist) on the 7,8-DHF–induced recovery of NSIA. 5 daily intrathecal administrations of K252a inhibited the enhancements of NSIA that were induced by the repeated 7,8-DHF treatments in the SNL6W rats ($P < .001$, Student t test with Bonferroni correction; Figure 1B). During the 5 daily treatments, there was no difference in the body weight in any of the groups (data not shown).

Effects of the TrkB Agonist on the Spinal NA and 5-HT Concentrations in SNL6W Rats

We examined the effect of the TrkB agonist on the noradrenergic and serotonergic descending inhibitory system of pain by measuring the catecholamine concentrations of the dialysates from the spinal dorsal horn. There was a significant main effect of group on the NA concentration ($F_{2,27} = 8.10$, $P = .002$, 1-way ANOVA; Figure 2A), with post hoc testing revealing that the basal NA concentration in the SNL6W rats was higher than that for naïve rats ($P = .003$). Furthermore, the 5 daily 7,8-DHF treatments decreased the NA concentration in SNL6W rats ($P = .008$). There was also a significant

Table. Results With Statistics

Source of Estimated Difference	Time	Estimated Treatment Effect	95% CI	*P* Value
Figure 1A: NSIA				
Naïve versus SNL6W		-70.5 g	-107.5 to −33.6 g	<.001
Vehicle versus 7,8-DHF (SNL6W)		62.9 g	27.0–98.7 g	.002
7,8-DHF versus 7,8-DHF + ANA12 (SNL6W)		−46.4 g	11.6–81.2 g	.028
Figure 1B: NSIA				
7,8-DHF versus 7,8-DHF + K252a (SNL6W)		−57.8 g	−78.3 to −37.2 g	<.001
Figure 2A: NA concentration				
Naïve versus SNL6W	Pre	0.29 pg/30 µL	0.09–0.48 pg/30 µL	.008
Vehicle versus 7,8-DHF (SNL6W)	Pre	−0.31 pg/30 µL	−0.50 to −0.13 pg/30 µL	.003
Figure 2B: NA concentration change				
Naïve versus SNL6W	30 min	−52.7%	−79.7% to −25.7%	<.001
	60 min	−53.6%	−86.5% to −20.7%	<.001
	90 min	−40.5%	−82.7% to 1.7%	.013
Vehicle versus 7,8-DHF (SNL6W)	30 min	41.5%	21.9%–61.1%	.010
	60 min	53.2%	19.0%–87.4%	<.001
	90 min	30.4%	−0.4% to 61.1 %	.076
Figure 3B: DβH immunoreactivity				
Naïve versus SNL6W		0.82%	0.28%–1.36%	.042
Vehicle versus 7,8-DHF (SNL6W)		0.85%	0.19%–1.50%	.027
Figure 4A: NSIA				
Vehicle versus idazoxan (SNL6W)		−61.6 g	−92.4 to −30.9 g	<.001
Vehicle versus atropine (SNL6W)		−52.6 g	−73.3 to −31.9 g	.002
Vehicle versus propranolol (SNL6W)		−3.0 g	−22.7 to 16.7 g	1.000
Figure 4B: NSIA				
Vehicle versus idazoxan (naïve)		−50.0 g	−70.6 to −29.5 g	<.001
Vehicle versus atropine (naïve)		13.8 g	−5.4 to 32.9 g	.430
Figure 5: Hindpaw incision				
Naïve versus SNL6W	Day 14	−7.2 g	−12.3 to −2.0 g	.003
	Day 21	−7.0 g	−12.5 to −1.6 g	.004
	Day 28	−7.3 g	−14.5 to −0.2 g	.003
Vehicle versus 7,8-DHF (SNL6W)	Day 14	9.1 g	4.7–13.4 g	<.001
	Day 21	8.6 g	0–8.6 g	<.001

Abbreviations: 5-HT, serotonin; 7,8-DHF, 7,8-dihydroxyflavone; CI, confidence interval; DβH, dopamine beta-hydroxylase; NA, noradrenaline; NSIA, noxious stimulus-induced analgesia; SNL6W, 6 wk after spinal nerve ligation.

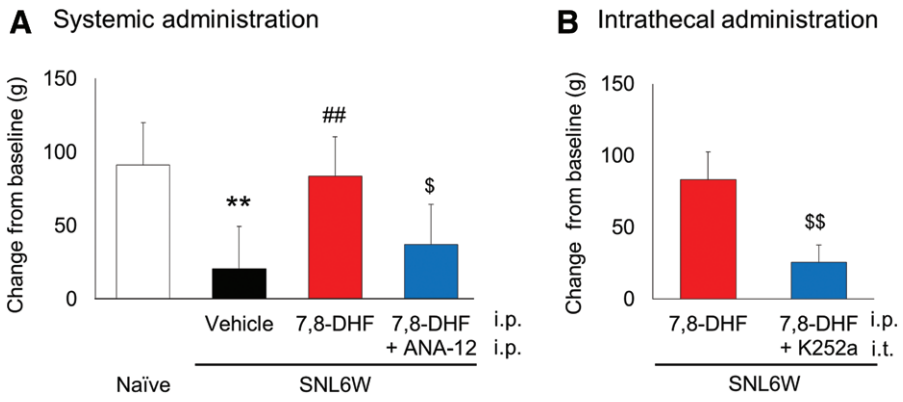

Figure 1. The effect of spinal TrkB activation on the impaired noxious stimulus-induced analgesia (NSIA) in 6 wk after spinal nerve ligation (SNL6W) rats. NSIA in naïve and SNL6W rats was evaluated by measuring the withdrawal threshold increment in the left (contralateral to nerve ligation) hindpaw at 30 min after a forepaw injection of capsaicin (250 µg/50 µL). After 5 daily intraperitoneal (i.p.) injections of 7,8-dihydroxyflavone (7,8-DHF; 5 mg/kg) with i.p. injection of ANA-12 (0.5 mg/kg) (A) or with intrathecal (i.t.) administration of K252a (2 µg/10 µL) (B), NSIA was measured. Data are presented as the mean ± standard deviation for the 8 naïve rats, the 7 SNL6W + vehicle rats, the 7 SNL6W + 7,8-DHF rats (A), the 6 SNL6W + 7,8-DHF rats, and the 6 SNL6W + 7,8-DHF + K252a rats (B). $^{**}P < .01$ versus naïve, $\#\#P < .01$ versus SNL6W + vehicle, $\$P < .05$, $\$\$P < .01$ versus SNL6W + 7,8-DHF (1-way analysis of variance with Bonferroni multiple comparisons (A) or Student *t* test (B). ANA indicates N-[2-[[(Hexahydro-2-oxo-1H-azepin-3-yl)amino]carbonyl]phenyl]benzo[b]thiophene-2-carboxamide.

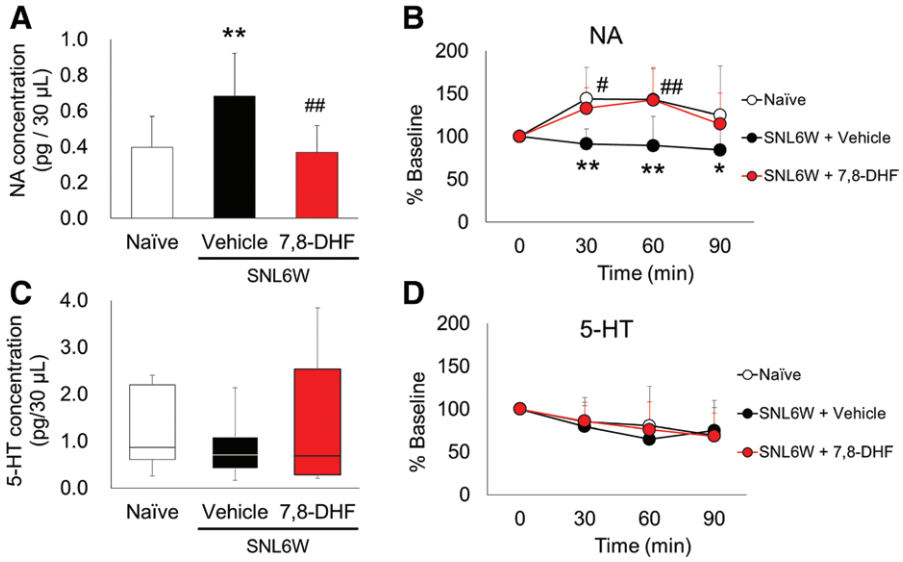

Figure 2. Effects of the TrkB agonist on the spinal noradrenaline (NA) and serotonin (5-HT) concentrations in 6 wk after spinal nerve ligation (SNL6W) rats. Basal concentrations of NA (A) and 5-HT (C) in the microdialysates from the right lumber spinal dorsal horn are presented as the average of the two 30-min samples. The change in the NA (B) and 5-HT (D) concentrations in response to the forepaw capsaicin injection (250 µg/50 µL) are presented over time as a percentage compared to baseline. Data are presented as the mean ± standard deviation or median and the interquartile ranges (C) for the 10 rats in each group (A, B), or for the 10 naïve rats, the 10 SNL6W + vehicle rats, and the 9 SNL6W + 7,8-dihydroxyflavone (7,8-DHF) rats (C, D). $^{*}P < .05$, $^{**}P < .01$ versus naïve, $\#P < .05$, $\#\#P < .01$ versus SNL6W + vehicle (1-way analysis of variance [A] or Kruskal-Wallis [C] with Bonferroni multiple comparisons or 2-way repeated-measures ANOVA with Bonferroni multiple comparisons [B, D]).

main effect on the NA release in response to the capsaicin injection (group: $F_{2,27} = 10.5$, $P < .001$, time: $F_{3,80} = 5.80$, $P = .001$, group × time: $F_{6,80} = 2.61$, $P = .023$, 2-way repeated-measures ANOVA; Figure 2B). Post hoc testing using a Student *t* test with Bonferroni correction revealed that the NA release was significantly decreased in the SNL6W rats compared to that observed for the naïve rats at 30, 60, and 90 minutes after the capsaicin injection (30 minutes; $P < .001$, 60 minutes; $P < .001$, 90 minutes; $P = .013$). Repeated 7,8-DHF treatments restored the NA release in the SNL6W rats, with the change in the NA concentration significantly higher than that observed for vehicle treatments at 30 and 60 minutes (30 minutes; $P = .010$, 60 minutes; $P < .001$, 90 minutes; $P = .076$).

In contrast, there were no differences in any of the groups for the 5-HT concentration in the spinal dorsal horn ($P = .566$, Kruskal-Wallis; Figure 2C) or for the 5-HT release in response to the capsaicin injection (group: $F_{2,26} = 0.136$, $P = .873$, time: $F_{3,77} = 11.9$, $P < .001$, group × time: $F_{6,77} = 0.646$, $P = .693$, 2-way repeated-measures ANOVA; Figure 2D).

TrkB Agonist–Induced Spinal Noradrenergic Plasticity

Figure 3A depicts representative immunoblotting images of DβH in the spinal dorsal horn from naïve and SNL6W rats. There was a significant main effect of group on the DβH-immunoreactivity ($F_{2,20} = 15.1$, $P < .001$, 1-way ANOVA;

Figure 3B), with post hoc test revealing that there was a higher reactivity for spinal noradrenergic fibers in the SNL6W versus for naïve rats (P = .042). In addition, repeated 7,8-DHF treatments increased the expression to a greater extent compared to the vehicle treatments in the SNL6W rats (P = .027).

Involvement of Spinal α2, β Adrenergic and Cholinergic Signaling in the TrkB Agonist–Induced Recovery of NSIA

This part of the study examined the involvement of spinal α2 and β adrenergic, and the cholinergic signaling on the TrkB-induced recovery of the impaired NSIA. After 5 daily injections of 7,8-DHF, we intrathecally administered idazoxan, propranolol, or atropine at 15 minutes before the capsaicin injection. There was a significant effect of group on ($F_{3,29}$ = 11.8, P < .001, 1-way ANOVA; Figure 4A), with post hoc testing revealing that idazoxan and atropine blocked the 7,8-DHF–induced enhancement of NSIA in SNL6W rats (idazoxan; P < .001, atropine; P = .003). However, propranolol did not affect the enhancement (P = 1.000). In contrast, idazoxan, but not atropine, blocked NSIA in the naïve rats ($F_{2,20}$ = 25.5, P < .001, 1-way ANOVA, idazoxan; P < .001, atropine; P = .430; Figure 4B).

Effect of Repeated Administrations of TrkB Agonist on the Hypersensitivity After Nerve Injury

To examine the effect of the recovery of the impaired endogenous analgesia on the neuropathic pain, we evaluated the

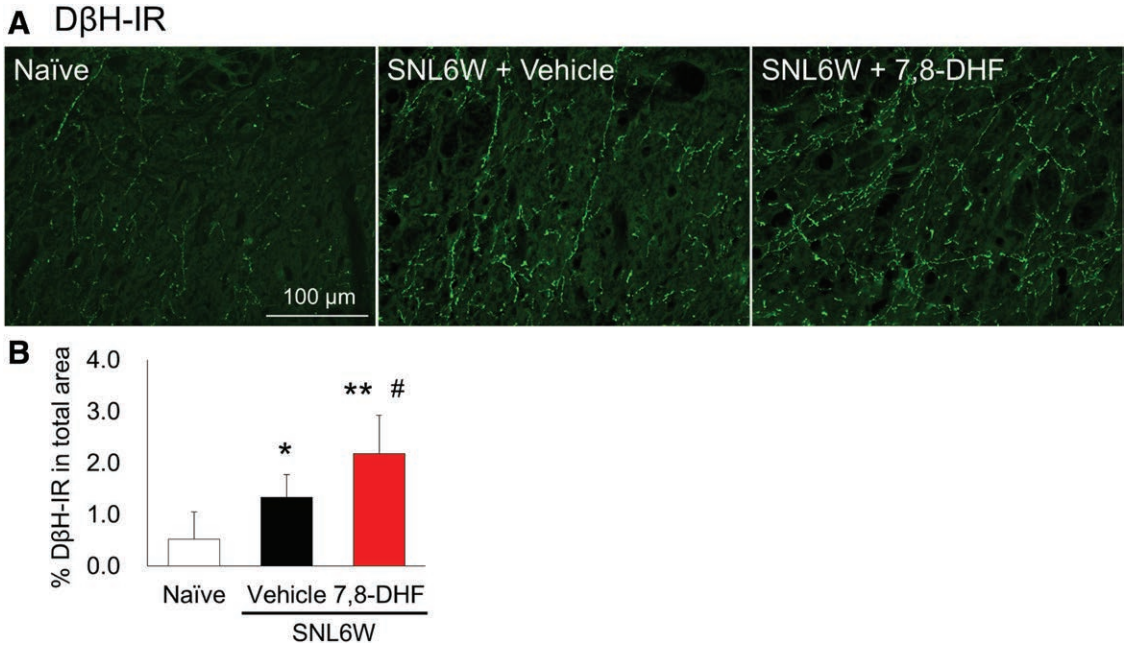

Figure 3. TrkB agonist–induced spinal noradrenergic plasticity. Photomicrographs and quantification of DβH-IR in the lumber spinal dorsal horn collected from the naïve rats and 6 wk after spinal nerve ligation (SNL6W) rats after 5 daily injections of vehicle or 7,8-dihydroxyflavone (7,8-DHF; 5 mg/kg). Scale bar (white line) = 100 μm. Data are presented as the mean ± standard deviation for the 7 naïve rats and the SNL6W groups (8 rats in each group). *P < .05, **P < .01 versus naïve, # P < .05 versus SNL6W + vehicle (1-way analysis of variance with Bonferroni multiple comparisons). DβH-IR indicates dopamine β hydroxylase-immunoreactivity.

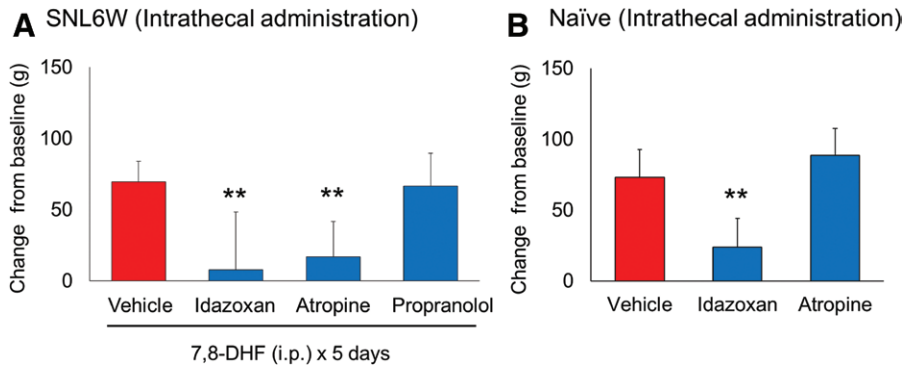

Figure 4. Involvement of spinal α2, β adrenergic and cholinergic signaling in the TrkB agonist–induced recovery of noxious stimulus-induced analgesia (NSIA). After 5 daily intraperitoneal injections of 7,8-dihydroxyflavone (7,8-DHF; 5 mg/kg), NSIA was measured in rats 6 wk after spinal nerve ligation (SNL6W) (A) and naïve (B) rats. Idazoxan (30 μg/10 μL), atropine (30 μg/10 μL), or propranolol (30 μg/10 μL) was administrated intrathecally 15 min before the forepaw capsaicin injection (250 μg/50 μL). Data are presented as the mean ± standard deviation for the 9 vehicle rats and the 8 idazoxan, atropine, and propranolol rats (A), and for the 8 vehicle and atropine rats and the 7 idazoxan rats (B). **P < .01 versus vehicle (1-way analysis of variance with Bonferroni multiple comparisons). i.p. indicates intraperitoneal.

withdrawal threshold in the right (ipsilateral to nerve ligation) hindpaw during repeated treatments with the TrkB agonist in SNL6W rats. However, the 5 daily intraperitoneal injections of 7,8-DHF did not affect the SNL-induced mechanical hypersensitivity (estimated treatment effect [95% CI]: Pre; −0.6 [−13.9 to 12.7] g, day 5; −11.9 [−27.3 to 3.6] g, group: $F_{1,14} = 0.232$, $P = .638$, time: $F_{6,84} = 30.9$, $P < .001$, group × time: $F_{6,84} = 0.986$, $P = .440$, 2-way-repeated-measures ANOVA; Supplemental Digital Content 2, Figure 2, http://links.lww.com/AA/C480).

Effect of TrkB Agonist on the Pain Prolongation After a Hindpaw Incision

To examine the effect of the endogenous analgesia on acute pain, we evaluated the recovery from hypersensitivity after a hindpaw incision. Because the withdrawal threshold in the heel of the left (contralateral to nerve ligation) hindpaw was not different in any of the groups, we evaluated the hypersensitivity in the left hindpaw over time after a hindpaw incision.

There was a significant main effect of treatment on the hypersensitivity (group: $F_{2,17} = 4.70$, $P = .024$, time: $F_{8,136} = 59.7$, $P < .001$, group × time: $F_{16,136} = 3.34$, $P < .001$, 2-way repeated-measures ANOVA; Figure 5). Post hoc testing using a Student *t* test with Bonferroni correction revealed that there was delayed recovery from the hypersensitivity after a hindpaw incision in the SNL6W rats, with the withdrawal threshold significantly decreased in SNL6W rats as compared to that for the naïve rats at day 14, 21, and 28 after a hindpaw incision (day 14, $P = .003$; day 21, $P = .004$; day 28, $P = .003$). Repeated treatments of 7,8-DHF improved the delayed recovery in the SNL6W rats, with a significantly increased withdrawal threshold observed at days 14 and 21 after a hindpaw incision (day 14, $P < .001$; day 21, $P < .001$).

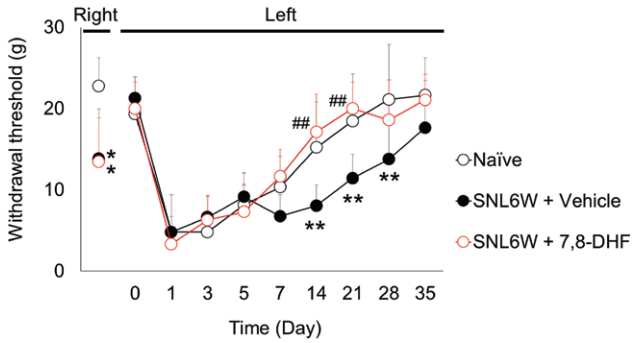

Figure 5. Effect of TrkB agonist on the pain prolongation after a hindpaw incision. Time course of the hypersensitivity after a hindpaw incision in the naïve rats and the rats 6 wk after spinal nerve ligation (SNL6W) treated with vehicle or 7,8-dihydroxyflavone (7,8-DHF) (5 mg/kg) for 5 days. Withdrawal threshold in the left (contralateral to nerve ligation) hindpaw around the incision sites was measured by von Frey filaments. Data are presented as the mean ± standard deviation for the 6 naïve rats and the SNL6W groups (7 rats in each group). *$P < .05$, **$P < .01$ versus naïve, ##$P < .01$ versus SNL6W + vehicle (2-way repeated-measures ANOVA with Bonferroni multiple comparisons).

DISCUSSION

The present study demonstrated that 5 daily administrations of 7,8-DHF (TrkB agonist) restored the impaired NSIA in SNL6W rats. In addition, 7,8-DHF–induced enhancement of NSIA was blocked not only by the systemic coadministration of ANA-12 (TrkB antagonist) but also by intrathecal K252a (TrkB antagonist). Repeated treatments of 7,8-DHF increased the noradrenergic fibers in the spinal dorsal horn and recovered the diminished NA release in response to a noxious stimulus in SNL6W rats. These results demonstrate that the spinal activation of TrkB recovered the attenuated noradrenergic descending inhibitory system of pain in SNL6W rats.

In response to the forepaw capsaicin injection, naïve rats in the present study exhibited an increase in the spinal NA, but not the 5-HT release. In contrast, SNL6W rats exhibited an increase in the basal NA concentration and noradrenergic fibers in the spinal dorsal horn compared to that for naïve rats, and a decrease in the NA release in response to the capsaicin injection. These results indicate that the descending noradrenergic pathway is constantly excited and thus cannot be activated in response to a noxious stimulus in SN6W rats. Repeated 7,8-DHF treatments further increased spinal noradrenergic fibers and restored the decreased NA release in response to the capsaicin injection. This improvement of the noradrenergic plasticity in the spinal cord led to the recovery of NSIA in SNL6W rats.

Other studies have demonstrated that nerve injury increased the spinal interaction of the α2 adrenergic and cholinergic signaling. For example, dexmedetomidine, which is an α2 adrenoceptor agonist, increased acetylcholine release in the spinal cord in SNL, but not naïve rats,[24] due to a G protein shift from the inhibitory Gi/o protein to the excitatory Gs protein after nerve injury.[25] Furthermore, the antihypersensitivity effects of intrathecal clonidine, which is an α2 adrenoceptor agonist, was shown to be inhibited by intrathecal administration of atropine only in the nerve-injured rats.[26] In addition, it has also been reported that the systemic administrations of adrenaline exhibit an analgesic effect via the β2 adrenergic receptor in the inflammatory pain model.[27] Therefore, based on these previous findings, we decided to examine whether these signals participate in the TrkB agonist–induced recovery of the attenuated endogenous analgesia. Our current study showed that intrathecal idazoxan blocked NSIA in both naïve and 7,8-DHF–treated SNL6W rats. However, intrathecal atropine blocked NSIA only in the latter. Thus, this result suggests that both the spinal α2 adrenergic signaling and the cholinergic signaling might be involved in the TrkB agonist–induced recovery of endogenous analgesia in SNL6W rats. In contrast, there is little involvement of the β adrenergic signaling in this recovery.

Previous studies have reported SNL surgery increases the spinal BDNF concentration, and that BDNF-TrkB signaling contributes to the onset of neuropathic pain.[28,29] Another study reported finding increases in the spinal BDNF immediately after SNL surgery, and which was maintained at a high level for 2 weeks.[30] However, even though the spinal BDNF concentration declined to presurgery levels at 4 weeks after SNL surgery, the associated hypersensitivity was shown to remain.[30] Based on these previous reports, it appears that the activation of spinal BDNF-TrkB signaling may have different roles at different periods after the SNL surgery. In addition, our current results suggest that TrkB signaling does not exhibit either a pronociceptive effect or an antihypersensitivity effect, at least in the SNL6W rats.

Interestingly, the 7,8-DHF–induced recovery of the impaired NSIA did not have any influence on the SNL-induced hypersensitivity in the SNL6W rats. Although the TrkB agonist can restore the responsive activation findings, our current findings that the TrkB agonist itself does not activate the noradrenergic pain inhibitory system. Furthermore, we preliminarily confirmed that a single administration of 7,8-DHF did not affect the spinal NA concentration in SNL6W rats (data not shown). Similarly, it has also been reported that knockdown of the glutamate transporter-1, which increases the activity of the LC neurons and the descending noradrenergic tone, reduced the gabapentin-induced activation of the noradrenergic descending inhibition, even though it did not affect the hypersensitivity in the SNL rats.[26] Therefore, repeated administrations of the TrkB agonist is able to cause changes that ultimately lead to a more efficient functioning of the noradrenergic descending inhibitory system of pain in response to the noxious stimulus.

In humans, there is a correlative relationship between endogenous analgesia and sustained pain after surgery, with patients who exhibit weak endogenous analgesia tending to have an increased incidence of chronic postoperative pain.[4,5] In addition, a recent study demonstrated that the rats with deleted noradrenergic fibers exhibited a slow resolution of the hyperalgesia after an incision.[31] These studies indicate that the efficient endogenous analgesia that occurs via the noradrenergic descending inhibitory system of pain plays an important role in preventing sustained pain after surgery. In our current study, SNL6W rats with a decreased NSIA exhibited a slower recovery from the hypersensitivity after a hindpaw incision compared to naïve rats. Furthermore, and 7,8-DHF–induced enhancement of the attenuated NSIA led to preventing the delayed recovery. Based on these results, confirmation of the degree of the endogenous analgesia might make it possible to predict the risk of chronic postoperative pain. Moreover, enhancement of endogenous analgesia by the activation of TrkB could potentially be used within a clinical practice to improve the delayed recovery from postoperative pain.

However, the fact that repeated 7,8-DHF treatments improved the delayed recovery from hypersensitivity after a hindpaw incision even though there is no recovery of the SNL-induced hypersensitivity appears to be contradictory. We speculate that the difference of pain quality between the acute incisional pain and the neuropathic pain contribute to these results, although the specific details of these mechanisms remain unknown at this time. Additional studies that evaluate the effect of the long-term or multidose administration of 7,8-DHF on the SNL-induced hypersensitivity will need to be performed to definitively determine whether the recovery of NSIA leads to pain relief in SNL6W rats. In addition, because we did not include a Bonferroni correction in the sample size, this may have affected the type I error rate. This lack of control of type I error is one of the limitations of our current study.

In summary, repeated injections of 7,8-DHF restored impaired endogenous analgesia in SNL6W rats by increasing the spinal noradrenergic fibers and recovering NA release in responses to a noxious stimulus. In addition, 7,8-DHF also improved the delayed recovery from the hypersensitivity that occurs after a hindpaw incision in SNL6W rats. These results suggest that spinal activation of TrkB might be effective in recovering the attenuated endogenous analgesia in chronic pain patients. Furthermore, the agents that increase the spinal BDNF, such as antidepressants, could be a promising treatment for chronic postoperative pain in patients with impaired endogenous analgesia.

DISCLOSURES

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