Effects of Adjuvant Analgesics on Cerebral Ischemia-Induced Mechanical Allodynia

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Received December 21, 2015; accepted January 27, 2016

Central post-stroke pain (CPSP), a potential sequela of stroke, is classified as neuropathic pain. Although we recently established a CPSP-like model in mice, the effects of adjuvant analgesics as therapeutic drugs for neuropathic pain in this model are unknown. Hence, the aim of the present study was to assess the usefulness of our model by evaluating the effects of adjuvant analgesics used for treating neuropathic pain in this mouse model of CPSP. Male ddY mice were subjected to 30 min of bilateral carotid artery occlusion (BCAO). The development of hind paw mechanical allodynia was measured after BCAO using the von Frey test. The mechanical allodynia was significantly increased on day 3 after BCAO compared with that during the pre-BCAO assessment. BCAO-induced mechanical allodynia was significantly decreased by intraperitoneal injections of imipramine (a tricyclic antidepressant), mexiletine (an antiarrhythmic), gabapentin (an antiepileptic), or a subcutaneous injection of morphine (an opioid receptor agonist) compared with that following vehicle treatment in BCAO-mice. By contrast, milnacipran (a serotonin and norepinephrine reuptake inhibitor), paroxetine (selective serotonin reuptake inhibitor), carbamazepine (antiepileptic), and indomethacin (nonsteroidal anti-inflammatory drug) did not affect the BCAO-induced mechanical allodynia. Our results show that BCAO in mice may be useful as an animal model of CPSP. In addition, BCAO-induced mechanical allodynia may be suppressed by some adjuvant analgesics used to treat neuropathic pain.

Key words central post-stroke pain; global ischemia; allodynia; neuropathic pain

Pain may occur with actual substantial or even potential histologic lesions, producing uncomfortable sensory and emotional experiences, according to the definition by the International Association for the Study of Pain. One type of pain, neuropathic pain, is caused by dysfunctional peripheral or central nerves and results in the appearance of allodynia and hypersensitivity to pain. Cerebral stroke patients may experience several types of pain, including musculoskeletal pain, headache, and neuropathic central post-stroke pain (CPSP).

Among the different types of neuropathic pain, CPSP is an especially intractable condition to treat. CPSP is associated with various symptoms, such as burning and lancinating pain, and the incidence of CPSP after cerebral stroke is reportedly 1–14%. In clinical studies, the risk factors associated with CPSP are the degree of brain infarction, sexual dimorphism, alcohol intake, depression anamnesis, statin use, dyslipidemia, diabetes, and peripheral vascular disorder. The pathogenic mechanisms for CPSP and neuropathic pain appear similar at the point of excitation of primary afferent fibers and for epi- static or central excitation or suppression pathway disorders. Recently, we established a CPSP-like animal model by experimentally inducing focal (middle cerebral artery occlusion model) or global cerebral ischemia (bilateral carotid artery occlusion model; BCAO). In our BCAO model of global ischemia, pain thresholds were significantly decreased in response to mechanical and thermal stimulation to the hind limbs compared with those of sham-operated animals. These decreased pain thresholds were associated with ischemic neuronal damage. In a previous study, we showed that the regulation of BCAO-induced CPSP may involve alterations in the signaling of astrocyte free fatty acid receptor 1, also known as GPR40, a receptor of long-chain fatty acids. Thus, this BCAO model may be useful as a mouse model of CPSP. In the clinic, CPSP is usually treated with adjuvant analgesics, such as antidepressants, antiepileptogens and narcotic analgesics, based on the treatment of neuropathic pain or surgery. However, the effect of adjuvant analgesics on the BCAO-induced pain is unclear.

In the present study, to determine the usefulness of our BCAO-induced mouse model of CPSP, we evaluated the therapeutic potential of clinically relevant neuropathic pain drugs as adjuvant analgesics in this model.

MATERIALS AND METHODS

Animals All experimental procedures were approved by the ethics committee for animals at Kobe Gakuin University (approval number: 14–21). The experiments were performed on male ddY mice (5 weeks old, 25–30 g) obtained from SLC (Shizuoka, Japan). The animals were housed at 23–24°C with a 12-h light–dark cycle (lights on at 8:00 a.m.). Food and water were available ad libitum. The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society.

Animal Model of Global Cerebral Ischemia Transient global cerebral ischemia was induced by occlusion of the BCAO in mice as described previously. Briefly, mice were anesthetized with pentobarbital (60 mg/kg). The rectal temperature was maintained at 37±0.5°C with a heating blanket (FH-100, Unique Medical, Osaka, Japan) equipped with a small animal heat controller (ATC-101B, Unique Medical). The bilateral common carotid arteries were occluded for 30 min using Sugita standard aneurysm clips (Mizuho Ikakogyo Co., Ltd., Chiba, Japan).
Tokyo, Japan). Sham-operated mice were subjected to the same procedure as above without occlusion of the BCA. The final number of mice used for each drug treatment is stated in the figure legends.

**Drugs** Imipramine hydrochloride (Sigma-Aldrich, St. Louis, MO, U.S.A.), milnacipran hydrochloride (Toronto Research Chemicals, Toronto, Canada), gabapentin (Sigma-Aldrich), morphine hydrochloride (Takeda Pharmaceutical Company Limited, Osaka, Japan) and mexiletine hydrochloride (Sigma-Aldrich) were dissolved in saline. Carbamazepine (Sigma-Aldrich) was dissolved in saline with 1% Tween 20 and 2% dimethyl sulfoxide. Paroxetine hydrochloride (Toronto Research Chemicals) was dissolved in saline containing 0.75% methanol. Indomethacin (Sigma-Aldrich) was dissolved in 1% carboxymethyl cellulose sodium.

**Drug Administration** The BCAO-mice were treated with a single injection (intraperitoneal; i.p.) of imipramine (5, 20 mg/kg), milnacipran (30 mg/kg), paroxetine (40 mg/kg), gabapentin (10, 30 mg/kg), carbamazepine (20 mg/kg), mexiletine (10 or 30 mg/kg), indomethacin (10 mg/kg), or vehicle (0.1 mL/10 g body weight); morphine (1 or 10 mg/kg) was administered subcutaneously (s.c.). The doses of all drugs were selected based on the results of previous reports.4,15

**Assessment of Mechanical Allodynia** Mechanical allodynia was evaluated using von Frey filaments (Neuroscience Inc., Tokyo, Japan) as previously described.ig Mice were placed on a 5×5 mm wire mesh grid floor, covered with an opaque cup to avoid visual stimulation, and allowed to adapt for 2–3 h prior to testing. The von Frey filament was then applied to the middle of the plantar surface of the hind paw with a weight of 0.4 g. On the indicated days, withdrawal responses following hind paw stimulation were measured 10 times, and mechanical allodynia was defined as an increase in the number of withdrawal responses to the stimulation. On day 3 after BCAO, the von Frey test was performed on the BCAO mice at 10, 20, 30, and 60 min after each drug treatment.

**Statistical Analysis** All data were analyzed using two-way ANOVA followed by Tukey’s test, and are presented as the mean±standard error of the mean (S.E.M.). Differences of p<0.05 or p<0.01 were considered statistically significant.

**RESULTS**

**Effects of Antidepressant Drugs on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia** The number of escape behaviors against the stimulation induced by the von Frey filament was significantly increased on day 3 after BCAO compared with that in the sham group. The tricyclic antidepressant imipramine (20 mg/kg) significantly decreased the BCAO-induced increase in the escape behavior score in a dose-dependent manner [Fig. 1A, drug×time interaction: \( F(15, 96)=8.25, p<0.01 \), drug effect: \( F(3, 96)=93.25, p<0.01, \) time effect: \( F(5, 96)=18.92, p<0.01 \)]. However, milnacipran, a serotonin and noradrenaline reuptake inhibitor (SNRI; 30 mg/kg) and paroxetine, a selective serotonin reuptake inhibitor (SSRI; 40 mg/kg) did not alter the escape behavior score [Fig. 1B, drug×time interaction: \( F(10, 42)=2.29, p<0.05 \), drug effect: \( F(2, 42)=70.76, p<0.01, \) time effect: \( F(5, 42)=15.82, p<0.01 \), Fig. 1C, drug×time interaction: \( F(10, 42)=4.09, p<0.01, \) drug effect: \( F(2, 42)=72.57, p<0.01, \) time effect: \( F(5, 42)=15.33, p<0.01 \)].

**Effect of Antiepileptogenic Drugs on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia** On day 3 after BCAO, the increase in the number of escape behaviors was completely blocked by the administration of the calcium channel \( \alpha_{2}\beta \)-ligand gabapentin (30 mg/kg) in a dose-dependent manner [Fig. 2A, drug×time interaction: \( F(15, 90)=8.82, p<0.01, \) drug effect: \( F(3, 90)=111.19, p<0.01, \) time effect: \( F(5, 90)=42.05, p<0.01 \)] but not by the sodium channel inhibitor carbamazepine (20 mg/kg) [Fig. 2B, drug×time interaction: \( F(10, 24)=5.54, p<0.01, \) drug effect: \( F(2, 24)=387.40, p<0.01, \) time effect: \( F(5, 24)=26.15, p<0.01 \)].

**Effect of an Antiarrhythmic Drug on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia** The BCAO-induced mechanical allodynia was dose-dependently and significantly suppressed (30 mg/kg) by treatment with the sodium channel blocker mexiletine (10 or 30 mg/kg) [Fig. 3, drug×time interaction: \( F(15, 72)=13.81, p<0.01, \) drug effect: \( F(3, 72)=57.16, p<0.01, \) time effect: \( F(5, 72)=40.14, p<0.01 \)].

**Effect of a Narcotic Analgesic on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia** The strong opioid analgesic morphine (1 or 10 mg/kg) dose-dependently and significantly decreased the BCAO-induced mechanical allodynia [Fig. 4, drug×time interaction: \( F(15, 72)=11.57, p<0.01, \) drug effect: \( F(3, 72)=46.61, p<0.01, \) time effect: \( F(5, 72)=25.41, p<0.01 \)].

**Effect of a Nonsteroidal Anti-inflammatory Drug (NSAID) on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia** The NSAID indomethacin (10 mg/kg) had no effect on the BCAO-induced mechanical allodynia [Fig. 5, drug×time interaction: \( F(10, 48)=4.51, p<0.01, \) drug effect: \( F(2, 48)=83.43, p<0.01, \) time effect: \( F(5, 48)=21.47, p<0.01 \)].

**DISCUSSION**

CPSP can be defined as one of the central neuropathic pain conditions occurring after cerebral stroke in which the affected areas are those body parts corresponding to cerebrovascular lesions of the somatosensory system.16–18 However, no standard treatment exists for CPSP. One reason for this lack of an effective standard treatment is that no animal model had been available to determine the mechanisms mediating the development of CPSP. Recently, we and other researchers established CPSP-like models using brain infarction (BCAO model) or thalamic hemorrhage.19,20 We used BCAO in mice to model global cerebral ischemia, with mice showing a resulting CPSP-like behavior. Here, we assessed the usefulness of this mouse model by evaluating the effects of adjuvant analgesics, including antidepressants, antiepileptogenics, an antiarrhythmic, a narcotic analgesic, and an NSAID, on the BCAO-induced mechanical allodynia.

The tricyclic antidepressants, SSRIs and SNRIs are widely used for the management of neuropathic pain.21,22 In the present study, the tricyclic antidepressant imipramine markedly decreased the BCAO-induced mechanical allodynia, whereas the SNRI milnacipran and the SSRI paroxetine had no effect. There are some studies examining the suppressive effects of tricyclic antidepressants on the development of pain behaviors, including neuropathic pain. It is well known that imipramine inhibits neuropathic pain by blocking noradrenaline and serotonin reuptake.4,12,23 Noradrenaline and serotonin are highly
involved in the activity of the descending pain inhibitory pathways.\textsuperscript{24–26)} By contrast, pro-inflammatory and anti-inflammatory cytokines, such as interleukin-1, play crucial roles in the establishment and maintenance of neuropathic pain.\textsuperscript{27)} Tricyclic antidepressants reportedly inhibited the release of interleukin-1 in mixed glial and microglial cell cultures from rats.\textsuperscript{28)} Additionally, gamma-aminobutyric acid (GABA) receptor mechanism(s) may modulate imipramine-induced antinociception.\textsuperscript{29)} The GABA signaling system has been proposed as a target of possible therapies used to promote recovery from

Fig. 1. Effects of Antidepressant Drugs on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia

The development of mechanical allodynia was analyzed using the von Frey test. The filament used was 0.4 g, and during each test trial, the right hind paw was stimulated 10 times for 6 s each. “Pre” indicates the measurement obtained before BCAO. On day 3 after BCAO, the antidepressant drugs (A) imipramine (5 or 20 mg/kg), (B) milnacipran (30 mg/kg), or (C) paroxetine (40 mg/kg) were administered (i.p.) prior to the von Frey filament test. Tests were performed 10, 20, 30, and 60 min after drug administration. Results are presented as the mean±S.E.M. °°*p<0.01, *p<0.05: compared with vehicle (veh)-sham group; ##p<0.01, #p<0.05: compared with veh-BCAO group. Tukey’s multiple comparisons test. (A) each groups: n=5. (B) veh-sham: n=3; veh-BCAO: n=4; milnacipran-BCAO, 30 mg/kg: n=3. (C) veh-sham: n=3; veh-BCAO: n=4; paroxetine-BCAO, 40 mg/kg: n=3.
Fig. 2. Effects of Antiepileptogenic Drugs on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia

The development of mechanical allodynia was analyzed using the von Frey test. The filament used was 0.4 g, and during each test trial, the right hind paw was stimulated 10 times for 6 s each. "Pre" indicates measurement before BCAO. On day 3 after BCAO, the antiepileptogenic drugs (A) gabapentin (10, 30 mg/kg) and (B) carbamazepine (20 mg/kg) were administered (i.p.) prior to the von Frey filament test. Tests were performed at 10, 20, 30, and 60 min after drug administration. Results are presented as the mean ± S.E.M. ** p < 0.01: compared with vehicle (veh-)sham group, ## p < 0.01: compared with vehicle veh-BCAO group, Tukey’s multiple comparisons test. (A) each groups: n=5. (B) each groups: n=3.

Fig. 3. Effect of an Antiarrhythmic Drug on the Mechanical Allodynia on Day 3 after Global Cerebral Ischemia

The development of mechanical allodynia was analyzed using the von Frey test. The filament used was 0.4 g, and during each test trial, the right hind paw was stimulated 10 times for 6 s each. "Pre" indicates measurement before BCAO. On day 3 after BCAO, the antiarrhythmic drug mexiletine (10, 30 mg/kg, i.p.) was administered before the von Frey filament test. Tests were performed at 10, 20, 30, and 60 min after mexiletine administration. Results are presented as the mean ± S.E.M. ** p < 0.01: compared with vehicle (veh-)sham group, * p < 0.05: compared with veh-BCAO group, Tukey’s multiple comparisons test. Each groups: n=5.
neuropathic pain.\(^\text{30,31}\) In some clinically, it is confirmed that the tricyclic antidepressants are effective for CPSP patients.\(^\text{9,32}\) Thus, the suppressive effect of imipramine on the CPSP may be mediated by the various mechanisms described above. The SNRI milnacipran inhibited neuropathic pain by blocking noradrenaline and serotonin reuptake.\(^\text{14,33}\) Sciatic nerve ligation in mice induces pain behavior and is used as a model of neuropathic pain. The pain behavior in these mice was not affected by a single injection of milnacipran s.c. but was significantly suppressed by repeated milnacipran injections.\(^\text{34}\) Additionally, it is reported that SSRIs and SNRIs were useful for the control of CPSP in clinical study.\(^\text{10,35}\) Especially, the SSRI paroxetine suppressed pain behavior in patients with CPSP following 3 months of treatment.\(^\text{35}\) Repeated injections but not a single injection of paroxetine s.c. also significantly suppressed the pain behavior observed in sciatic nerve-ligated mice.\(^\text{34}\) These results suggest that repeated milnacipran and paroxetine administration may be necessary to show an effect against the pain behaviors observed in our BCAO mouse model of CPSP.

Previous reports have shown that gabapentin, a calcium channel\(^{\alpha2-\delta}\) ligand, inhibits the hyperalgesia by suppressing the upregulation of N-type calcium channels.\(^\text{36–39}\) Furthermore, gabapentin (10 and 30 mg/kg, i.p.) dose-dependently decreased cold hypersensitivity, with a statistically significant difference observed at 30 mg/kg.\(^\text{14}\) In clinical study, it was found that gabapentin is considered as a first line therapy or as add-on therapy for suppressing the pain behavior in CPSP patients.\(^\text{40}\) Thus, gabapentin may suppress BCAO-induced mechanical allodynia through calcium channels. By contrast, carbamazepine, another antiepileptogenic drug, did not affect the BCAO-induced mechanical allodynia in the present study. In general, carbamazepine is currently the first choice for treatment of trigeminal neuralgia, a type of neuropathic pain, because it has been shown to reduce pain symptoms in approximately 70% of the cases.\(^\text{41,42}\) However, the effectiveness of carbamazepine against other neuropathic pain is still controversial.\(^\text{43}\) In addition, one previous report showed that
carbamazepine had little effect on CPSP in a patient, suggesting that carbamazepine cannot be used for treatment of CPSP.

The mechanism of action for mexiletine, an antiarrhythmic agent, is to block voltage-gated sodium channels. Mexiletine is also a well-known adjuvant analgesic. In previous reports, mexiletine significantly attenuated hyperalgesia in formalin-treated rats and suppressed cold hypersensitivity. It is also clinically used to treat painful diabetic neuropathy. Moreover, it was proved that mexiletine is effectiveness and safety in the regulation of thalamic pain. In the present study, mexiletine dramatically suppressed the BCAO-induced mechanical allodynia. Therefore, these results suggest that mexiletine may be a beneficial adjuvant analgesic against treatment of CPSP. By contrast, carbamazepine which was voltage-gated sodium channel blocker same as mexiletine did not affect the BCAO-induced mechanical allodynia in the present study. It is well known that carbamazepine has used as first choice against treatment of trigeminal neuralgia, one of neuropathic pain. However, for other neuropathic pain condition including CPSP, the effect is much week. On the other hands, mexiletine has activating action of descending pain control system and suppressive action of substance P, one of the pain transmitter, release other than effect of voltage-gated sodium channel blocker. That is, it is possible that the mexiletine may act compositively some pain regulation systems.

Opioid analgesics are widely used for the treatment of moderate to severe pain during the perioperative period. However, opioids remain controversial as a choice for chronic neuropathic pain. Studies in animals and humans suggest that chronic neuropathic pain may respond poorly to opioid therapy, although placebo-controlled studies have demonstrated analgesia. A double-blind dose–response clinical study demonstrated that the reduction in neuropathic pain achieved with higher doses of the potent -opioid agonist levorphanol (through the use of a higher strength formulation) was significantly greater than that achieved with lower doses. In the present study, morphine markedly suppressed the BCAO-induced mechanical allodynia, suggesting that it may be useful in treatment of CPSP.

NSAIDs, comprising both non-selective and COX-2-selective inhibitors, are a standard and effective treatment for inflammatory pain. However, NSAIDs are reported to be ineffective against neuropathic pain, including CPSP in basic or clinical study. Indomethacin, an NSAID, had no effect on the paw withdrawal threshold throughout the assessment period. Additionally, another NSAID, diclofenac, had no effect on oxaliplatin-induced cold hypersensitivity. In the present study, we also found that administration of the NSAID indomethacin had no effect on the BCAO-induced mechanical allodynia, suggesting that NSAIDs are not useful in CPSP treatment.

Our present study suggests that BCAO in mice may provide a useful animal model of CPSP and that some adjuvant analgesics may be clinically relevant in the treatment of CPSP. In addition, this model may be useful for screening drugs for potential therapeutic use in patients with CPSP.

Acknowledgments This study was supported by Grants-in-Aid and by special coordination funds from Grants-in-Aid for Scientific Research (C) (25462458) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of Interest The authors declare no conflict of interest.

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