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Spinal mechanism of standard analgesics: Evaluation using mouse models of allodynia

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ABSTRACT

Spinal neurotransmission plays an important role in the perception of pain signaling. In the present study, we investigated the spinal anti-nociceptive mechanism of current standard analgesics in mouse models of tactile allodynia induced by intrathecal administration of N-methyl-D-aspartic acid (NMDA), prostaglandin E_2 (PGE_2), and bicuculline. NMDA-induced allodynia is induced by postsynaptic NMDA receptor activation, while PGE_2-induced allodynia is triggered by the enhancement of presynaptic glutamate release via EP1 receptor activation. In contrast, bicuculline induces allodynia by the blockade of γ-aminobutyric acid $(GABA)_A$ receptor-mediated inhibitory system. As the clinically available analgesics, pregabalin (α2δ-subunit calcium channel ligand), ziconotide (N-type calcium channel blocker), mexiletine (sodium channel blocker), and duloxetine (serotonin and norepinephrine reuptake inhibitors) were evaluated in these neurochemically-induced allodynia models. Pregabalin almost completely alleviated NMDA-, PGE_2-, and bicuculline-induced allodynia. Despite being classified as an agent with a similar molecular target mechanism, ziconotide could only alleviate PGE_2-induced allodynia, but not NMDA- or bicuculline-induced allodynia, as did mexiletine and duloxetine. These results taken together suggest that ziconotide, mexiletine, and duloxetine suppress spinal hyperactivity via the presynaptic site mechanism. In contrast, pregabalin could suppress via the downstream step during spinal hyperactivation such as postsynaptic NMDA activation or dysfunction of GABAergic control in addition to presynaptic mechanism. In conclusion, present findings provide implication that the spinal anti-nociceptive mechanistic site of pregabalin is different from that of ziconotide, mexiletine, and duloxetine, and pregabalin could have a broader anti-nociceptive mechanism other than N-type calcium channel blockade.

1. Introduction

Several new analgesics have been developed to date as pharmacotherapies to treat patients suffering from chronic pain. The calcium channel α2δ ligand pregabalin and the serotonin and norepinephrine reuptake inhibitors duloxetine are considered first-line medications, while the sodium channel blocker mexiletine is a second-line medication (Finnerup et al., 2005; Dworkin et al., 2007). With regard to intrathecal analgesics, the N-type calcium channel blocker ziconotide is used to treat patients with severe pain (Williams et al., 2008). All of the agents have been shown to modulate key molecules involved in the hyperactivity of somatosensory neurotransmission related to pain sensation. However, due to the complexities of the diverse interactions of neurotransmitters and receptors in both the peripheral and central nervous systems, mechanistic site of action of all analgesics in the pain pathway has not yet been fully elucidated.

The dorsal horn of the spinal cord is an important gateway in the ascending pain transmission pathway. Activation and disinhibition of neurotransmission in the dorsal horn are considered to be key mechanisms in inducing hyperactivity of the ascending pain pathway and transmitting pain signals, and a number of neurotransmitters play important roles in this part of synaptic neurotransmission. For example, glutamate is a major excitatory neurotransmitter, and the activation of N-methyl-D-aspartic acid (NMDA) receptors by glutamate on postsynaptic neurons is thought to be an essential step in excitatory neurotransmission (Meller and Gebhart, 1993). Prostaglandin E_2 (PGE_2) is another well-known potentiator of glutamatergic neurotransmission, and the activation of its receptor, EP1, facilitates glutamate release from presynaptic terminals of capsaicin-sensitive C-fibers (Minami et al., 1999). In contrast, γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter. Dysfunction of GABAergic inhibition at the spinal level has been shown to promote hyperactivity of pain signaling (Drew et al., 2004; Sivilotti and Woolf, 1994).

Mouse models of neurochemically-induced spinal activation are useful in investigating spinal anti-nociceptive mechanisms of analgesics, since evaluation of analgesics with a pure allodynic state facilitates identification of activity sites in the pain pathway. Intrathecal injection of NMDA, PGE_2, and $GABA_A$ receptor antagonist bicuculline induces spinal hyperactivity via specific receptor signaling, thereby leading to the induction of tactile allodynia, a clinically important manifestation of neuropathic pain.

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Here, we investigated the anti-nociceptive site of standard analgesics during spinal neurotransmission using mouse models of intrathecal NMDA-, PGE_2- and bicuculline-induced allodynia. We initially conducted pharmacological blocking studies using the corresponding agonist and antagonist of neurochemicals to elucidate the mechanism behind how each stimulant triggers spinal hyperactivity to produce allodynia. We then evaluated the effects of standard marketed analgesics such as pregabalin, ziconotide, mexiletine, and duloxetine in these allodynia models to address the possible difference in their anti-nociceptive mechanisms.

2. Materials and methods

2.1. Animals

Male ICR mice (SLC Japan Inc., Shizuoka, Japan) weighing 18–22 g were used. Animals were maintained with conventional laboratory chow and tap water provided *ad libitum* in a room with a 12-h light/dark cycle at a constant room temperature (23 ± 2 °C) and humidity (55 ± 10%). All animal experimental procedures were approved by the Committee for Animal Experiments of Astellas Pharma Inc. (Tokyo, Japan), and conformed to the International Guiding Principles for Biomedical Research Involving Animals (CIOMS) and the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006). All efforts were made to minimize the number of animals used and their suffering.

2.2. Experimental design

Studies on allodynia were conducted following a procedure slightly modified from the method of Minami et al. (1994a). Mice were divided into various groups, with eight animals per group. A 30-gauge stainless steel needle attached to a microsyringe was inserted between the L_5 and L_6 vertebrae of conscious mice, and a neurochemical stimulant (either NMDA, PGE_2, or bicuculline) in saline in a volume of 5 μl was injected slowly into the subarachnoid space (Hylden and Wilcox, 1980). Tactile allodynia was assessed 5, 10, 15, and 30 min after intrathecal injection of stimulant by lightly stroking the flank of the mice with a paintbrush. The allodynia response was ranked as 0 (no response), 1 (avoidance), or 2 (vigorous squeaking or strong avoidance), and the maximum cumulative score was 8 for the sum of the scores at each of the four time points.

In the first experiment, the concentrations of stimulants were increased to confirm dose-dependent induction of tactile allodynia. In the subsequent intrathecal pharmacological blocking study, stimulants were intrathecally administered simultaneously with the corresponding agonist or antagonist. In these experiments, MK-801 (1 or 3 μg) was used as an NMDA receptor antagonist, ONO-8713 (1 or 10 nmol) as an EP1 receptor antagonist, and muscimol (10 or 100 ng) as a $GABA_A$ receptor agonist. To assess the effect of analgesics, pregabalin (10, 30, and 100 mg/kg p.o.), mexiletine (10, 30, and 100 mg/kg p.o.), or duloxetine (3, 10, and 30 mg/kg p.o.) were administered 60, 60, and 240 min before the allodynic agent, respectively. Ziconotide (1 or 3 pmol) was administered simultaneously with the neurochemical stimulants via the intrathecal route. Doses of intrathecal neurochemical stimulants selected were those which produced a submaximal allodynic response in a dose escalation study (NMDA, 30 ng; bicuculline, 30 ng; PGE_2, 100 ng). In the mechanistic study on duloxetine, 5-$HT_{1B/1D}$ receptor antagonist GR127935 (10 nmol) and alpha-2 adrenergic receptor antagonist idazoxan (30 nmol) were intrathecally administered 15 min prior to the PGE_2 injection.

2.3. Drugs

NMDA, MK-801, muscimol, GR127935, and idazoxan were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ziconotide was purchased from Peptide Institute, Inc. (Osaka, Japan). Bicuculline was purchased from Sigma Aldrich Japan (Tokyo, Japan). All drugs were dissolved in saline and administered intrathecally at a volume of 5 μl. Pregabalin and duloxetine hydrochloride were synthesized by Astellas Pharma Inc. (Ibaraki, Japan), and mexiletine was purchased from Sigma Chemical Co. These compounds were dissolved in distilled water, and administered orally at a volume of 10 ml/kg. PGE_2 (Sigma Chemical Co.) was dissolved in ethanol. An aliquot of the desired solution was evaporated under nitrogen gas to remove the ethanol, and then dissolved in saline. ONO-8713 (synthesized by Astellas Pharma Inc.) was dissolved in 4% NaOH in 20% 2-hydroxypropyl-β-cyclodextrin.

2.4. Statistical analysis

Data are expressed as the mean ± S.E.M. Statistical significance between groups was assessed using Wilcoxon's rank sum test or Steel's test (for multiple comparisons). Values were considered statistically significant for $P<0.05$.

3. Results

3.1. Induction of allodynia by intrathecal injections of NMDA, PGE_2, and bicuculline

Allodynia was observed within 5 min following intrathecal administration of NMDA (10, 30, and 100 ng), PGE_2 (10, 100, and 1000 ng), and bicuculline (10, 30, and 100 ng), and was consistently sustained for 30 min (Fig. 1A). All stimulants induced allodynia in a dose-dependent manner (Fig. 1B). For NMDA-induced allodynia, the percentage of maximum cumulative scores at each dose were 37.5 ± 9.4%, 81.3 ± 10.3%, and 96.9 ± 3.1%, respectively, while those for PGE_2 were 65.6 ± 12.4%, 92.2 ± 3.3%, and 95.3 ± 3.3%, and those for bicuculline were 48.4 ± 14.6%, 78.1 ± 11.0%, and 95.3 ± 4.7%, respectively. Vehicle treatment did not induce any allodynic behavior.

3.2. Intrathecal blocking studies in NMDA-, PGE_2-, and bicuculline-induced allodynia models

To elucidate the mechanism behind how each stimulant triggers spinal hyperactivity to produce allodynia, pharmacological blocking studies with corresponding agonist and antagonists were performed. Intrathecal blockers were injected simultaneously with NMDA (30 ng), PGE_2 (100 ng), or bicuculline (30 ng). As shown in Table 1, the induction of NMDA-induced allodynia was significantly alleviated by the NMDA receptor antagonist MK-801 (64% inhibition at 1 μg, 108% inhibition at 3 μg) and $GABA_A$ receptor agonist muscimol (71% inhibition at 100 ng), but not by the EP1 receptor antagonist ONO-8713. PGE_2-induced allodynia was significantly alleviated by MK-801 (88% inhibition at 3 μg), muscimol (106% inhibition at 100 ng), and ONO-8713 (98% inhibition at 10 nmol). In the bicuculline-induced model, allodynia was completely alleviated by muscimol (89% inhibition at 100 ng). In contrast, ONO-8713 showed only partial inhibition (54% inhibition at 10 nmol) while MK-801 had no effect.

3.3. Effects of analgesics on NMDA-, PGE_2-, and bicuculline-induced allodynia

In the NMDA-, PGE_2-, and bicuculline-induced allodynia models, we studied the effects of the analgesics pregabalin, ziconotide, mexiletine, and duloxetine. The effects of all analgesics on NMDA-, PGE_2-, and bicuculline-induced allodynia were almost steady over 30 min period. The temporal inhibitory profile is stable within the observation period (as a supplemental data, the time course of the effects of pregabalin and ziconotide on NMDA- and PGE_2-induced allodynia are shown).

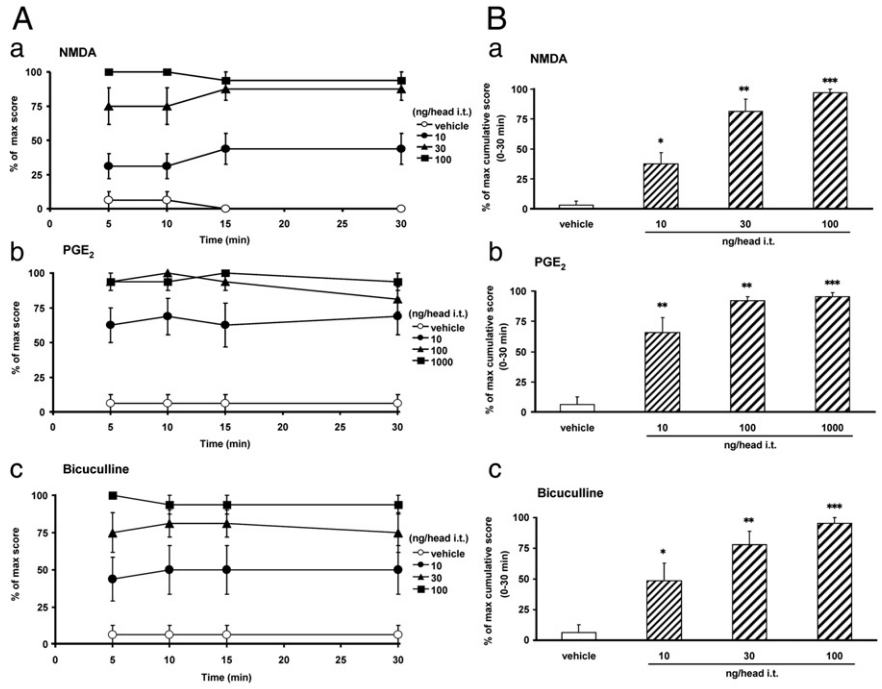

Fig. 1. Tactile allodynia in mice after intrathecal (i.t.) injection of a) NMDA, b) PGE_2, and c) bicuculline. Time course of allodynia induction from 5 to 30 min after i.t. administration (A), and dose-dependencies in the cumulative score of allodynia within 30 min of i.t. stimulation (B). Values (mean ± S.E.M. for 8 animals) are expressed as percentage of the maximum cumulative score. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ by Steel's test compared with the vehicle treatment group.

We first examined calcium channel blockers. Oral administration of pregabalin effectively alleviated NMDA-, PGE_2-, and bicuculline-induced allodynia (Fig. 2A–C, left panel). Further, significant improvements were observed at 30 and 100 mg/kg against both NMDA- (87% and 98% inhibition, respectively) and PGE_2-induced allodynia (88% and 100% inhibition, respectively) as well as at 100 mg/kg against bicuculline-induced allodynia (83% inhibition). Ziconotide also significantly alleviated PGE_2-induced allodynia at a dose of 3 pmol when administered intrathecally (77% inhibition) but did not affect NMDA- or bicuculline-induced allodynia (Fig. 2A–C, right panel).

Following examination of calcium channel blockers, we then expanded the study to determine the anti-allodynic effects of analgesics from other mechanistic classes, namely mexiletine and duloxetine. Oral treatment with the sodium channel blocker mexiletine significantly alleviated PGE_2-induced allodynia at 30 and 100 mg/kg (79% and 75% inhibition, respectively), but did not affect NMDA- or bicuculline-induced allodynia up to 100 mg/kg (Fig. 3A–C, left panel).

Similarly, the serotonin and norepinephrine reuptake inhibitors duloxetine significantly alleviated PGE_2-induced allodynia at 10 and 30 mg/kg (65% and 74% inhibition, respectively), but also failed to block NMDA- or bicuculline-induced allodynia up to 100 mg/kg (Fig. 3A–C, right panel). We further studied the effects of antagonists of 5-$HT_{1B/1D}$ receptor (GR127935) and alpha-2 adrenergic receptor (idazoxan) on the anti-nociceptive effect of duloxetine in the PGE_2-induced allodynia model. The analgesic effect of duloxetine at 30 mg/kg on PGE_2-induced allodynia was significantly blocked by combination of intrathecal injection of GR127935 at 10 nmol and idazoxan at 30 nmol (Fig. 4).

4. Discussion

In the present study, we conducted a pharmacological blocking study to clarify the mechanistic basis of NMDA-, PGE_2-, and bicuculline-induced allodynia models in triggering spinal hyperactivity. We then evaluated the effects of standard clinically available analgesics in these

Table 1
Mechanistic basis of NMDA-, PGE_2-, and bicuculline-induced allodynia models.

	MK-801			ONO-8713			Muscimol		
NMDA	vehicle	9.4 ± 4.6		Vehicle	6.3 ± 6.3		Vehicle	6.3 ± 4.1	
	0 μg	87.5 ± 6.7		0 nmol	92.2 ± 4.0		0 ng	84.4 ± 6.6	
	1 μg	37.5 ± 11.1 [b]	(64%)	1 nmol	73.4 ± 11.7	(22%)	10 ng	76.6 ± 6.9	(13%)
	3 μg	3.1 ± 2.0 [c]	(108%)	10 nmol	78.1 ± 10.0	(16%)	100 ng	29.7 ± 10.0 [b]	(71%)
PGE_2	vehicle	12.5 ± 6.7		Vehicle	9.4 ± 6.6		Vehicle	12.5 ± 8.2	
	0 μg	79.7 ± 8.8		0 nmol	82.8 ± 8.5		0 ng	85.9 ± 7.3	
	1 μg	68.8 ± 8.8	(16%)	1 nmol	59.4 ± 12.4	(32%)	10 ng	73.4 ± 12.4	(17%)
	3 μg	20.3 ± 10.5 [b]	(88%)	10 nmol	10.9 ± 6.4 [b]	(98%)	100 ng	7.8 ± 6.2 [b]	(106%)
Bicuculline	vehicle	9.4 ± 6.6		Vehicle	6.3 ± 4.1		Vehicle	6.3 ± 4.1	
	0 μg	84.4 ± 8.1		0 nmol	87.5 ± 7.1		0 ng	89.1 ± 6.4	
	1 μg	79.7 ± 10.0	(6%)	1 nmol	68.8 ± 4.1	(23%)	10 ng	73.4 ± 9.3	(19%)
	3 μg	73.4 ± 8.3	(15%)	10 nmol	43.8 ± 12.0 [a]	(54%)	100 ng	15.6 ± 8.4 [b]	(89%)

NMDA (30 ng), PGE_2 (100 ng), or bicuculline (30 ng) was injected simultaneously (i.t.) with MK-801 (NMDA receptor antagonist), ONO-8713 (EP1 receptor antagonist), or muscimol ($GABA_A$ receptor agonist) respectively. The values (mean ± S.E.M. for 8 animals) are expressed as percentage of the maximum cumulative score (% of inhibition). $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ by Steel's test compared with the vehicle treatment group.

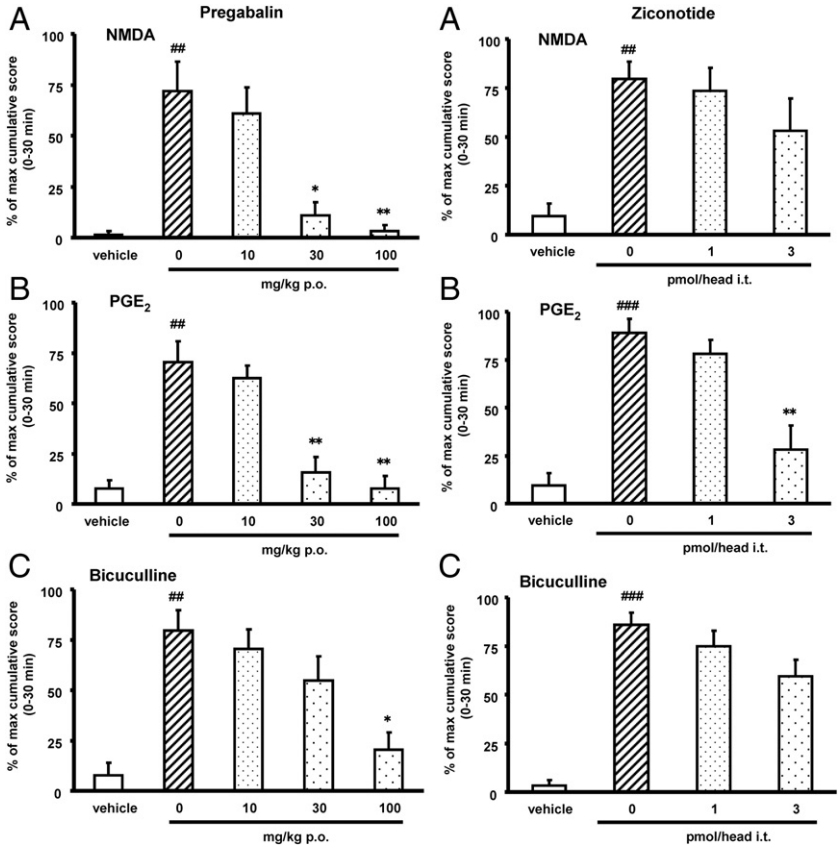

Fig. 2. Effects of calcium channel blockers on A) NMDA (30 ng)-, B) PGE_2 (100 ng)-, and C) bicuculline (30 ng)-induced allodynia in mice (pregabalin; left panel, ziconotide; right panel). Values (mean ± S.E.M. for 8 animals) are expressed as percentage of the maximum cumulative score. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ by Wilcoxon's rank sum test compared with the vehicle treatment group. $^{*}P<0.05$, $^{**}P<0.01$ by Steel's test compared with the vehicle treatment group.

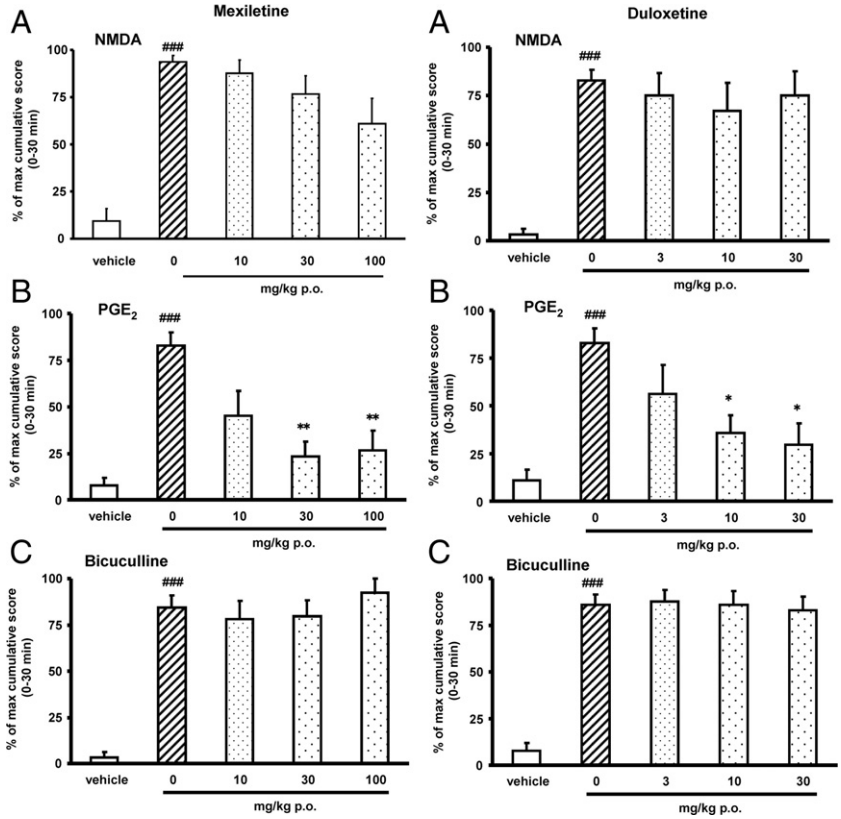

Fig. 3. Effects of mexiletine (left panel) and duloxetine (right panel) in a mouse model of A) NMDA (30 ng)-, B) PGE_2 (100 ng)-, and C) bicuculline (30 ng)-induced allodynia. Values (mean ± S.E.M. for 8 animals) are expressed as percentage of the maximum cumulative score. $^{\#\#\#}P<0.001$ by Wilcoxon's rank sum test compared with the vehicle treatment group. $^{*}P<0.05$, $^{**}P<0.01$ by Steel's test compared with the vehicle treatment group.

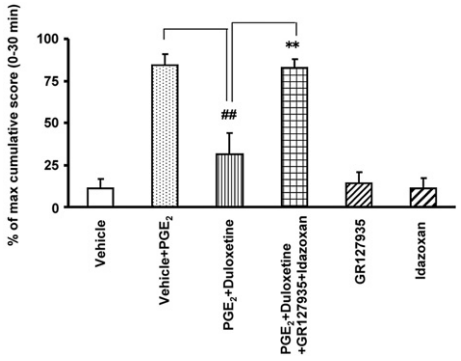

Fig. 4. Effect of GR127935 and idazoxan on anti-nociceptive effect of duloxetine in the PGE_2-induced allodynia model. Duloxetine (30 mg/kg p.o.) was administered 240 min before PGE_2 (100 ng), and 5-$HT_{1B/1D}$ receptor antagonist, GR127935 (10 nmol) and alpha-2 adrenergic receptor antagonist, idazoxan (30 nmol) were intrathecally administered 15 min before PGE_2.Values (mean ± S.E.M. for 8 animals) are expressed as percentage of the maximum cumulative score. $^{\#\#}P<0.01$ and $^{**}P<0.01$ by Wilcoxon's rank sum test compared with the vehicle treatment group.

allodynia models to investigate their anti-nociceptive mechanisms. Overall, we found these models useful in elucidating sites of action of analgesics in pain signaling pathways in spinal cord.

Intrathecal administration of NMDA (10, 30, 100 ng), PGE_2 (10, 100, 1000 ng), and bicuculline (10, 30, 100 ng) all dose-dependently induced tactile allodynia in mice. Our intrathecal blocking study using NMDA receptor antagonist, EP1 receptor antagonist, and $GABA_A$ receptor agonist elucidated the possible mechanism behind how each model triggers spinal hyperactivity. NMDA-induced allodynia has been reported to be induced by postsynaptic NMDA receptor activation in the dorsal horn (Minami et al., 2001). In the present study, both the NMDA receptor antagonist MK-801 and $GABA_A$ receptor agonist muscimol, but not the EP1 receptor antagonist ONO-8713, almost completely prevented NMDA-induced allodynia. This finding implies that the postsynaptic $GABA_A$ receptor controls NMDA-mediated spinal activation.

In contrast, PGE_2 has been reported to be a spinal allodynic agent that activates presynaptic EP1 receptor-mediated glutamate release from C-fibers interacting with low-threshold mechanoreceptors in Aβ-fibers (Minami et al., 1994b, 1999; Cervero and Laird, 1996). In line with the findings, we found that both the NMDA receptor antagonist MK-801 and EP1 receptor antagonist ONO-8713 almost completely prevented PGE_2-induced allodynia. The activation of the EP1 receptor therefore could enhance presynaptic glutamate release, leading to postsynaptic activation via NMDA receptor activation. These results are consistent with the inability of ONO-8713 to inhibit NMDA-induced allodynia. Furthermore, given our observation that muscimol completely prevented PGE_2-induced allodynia, we confirmed that the $GABA_A$ receptor controls this excitatory pathway as well.

Along with the activation of the excitatory system, the inactivation of inhibitory circuits is a key mechanism in pain signaling. Bicuculline-induced allodynia is a surrogate model reflecting spinal hyperactivity induced by the blockade of the $GABA_A$ receptor-mediated inhibitory system in the spinal cord. In the present study, bicuculline-induced allodynia was prevented almost completely by muscimol and partially by ONO-8713, whereas MK-801 had no effect with regard to prevention. These results are consistent with those of previous reports showing that bicuculline-induced allodynia in rats was prevented by the EP1 receptor antagonist SC-51322 and the cyclooxygenase-2-selective inhibitor NS-398, but not by the NMDA receptor antagonists d-2-amino-5-phosphopentanoic acid, ketamine, or 7-chloro-kynurenic acid (Onaka et al., 1996; Zhang et al., 2001; Copeland et al., 1994). Taken together, these findings indicate that suppression of the $GABA_A$ receptor likely induces spinal hyperactivity via activation of non-NMDA system. Furthermore, activation of the EP1 receptor by PGE_2 in the spinal cord may also be involved in this pathway, at least in part. Our demonstration here of pain pathway triggering mechanisms in mouse models of allodynia confirms previously reported observations in rats.

To clarify the mechanisms of several analgesics that have been used clinically to treat chronic pain, we evaluated the effects of these analgesics on our three allodynia models. In the first experiment, the anti-nociceptive effects of calcium channel blockers were determined. Pregabalin binds to the α2δ-subunit of the N- and P/Q type calcium channels and modulates channel function (Charles et al., 2007; Mark et al., 2006). Ziconotide binds to the N-type calcium channel pore and blocks the influx of calcium ions (Jason et al., 2008). In the present study, pregabalin dose-dependently prevented induction of allodynia by NMDA and PGE_2. Those findings are closely similar to those of another report involving gabapentin, a compound structurally related to pregabalin (Thrasivoulos et al., 2008), which found that gabapentin prevented NMDA- and PGE_2 analogue sulprostone-induced allodynia (Gil et al., 2008). In the present study, we demonstrated for the first time that pregabalin is also able to dose-dependently suppress bicuculline-induced allodynia. The effects of pregabalin on NMDA- and bicuculline-induced allodynia could be explained by findings by others that pregabalin activated the GABA-mediated inhibitory system by increasing the rate of functional GABA transport and increasing the rate of GABA release (Whitworth and Quick, 2001), and by our result in the blocking study that $GABA_A$ receptor agonist muscimol inhibited NMDA- and bicuculline-induced allodynia.

In contrast to pregabalin, ziconotide specifically prevented PGE_2-induced allodynia, indicating that these two agents exert different anti-nociceptive actions against spinal hyperactivity despite being classified as agents with a similar molecular target mechanism. Activation of the calcium channel is well known to play a major role in the augmentation of presynaptic glutamate release, and its blockade leads to the prevention of postsynaptic hyperactivity (Dooley et al., 2007), leading to the reasonable conclusion that both pregabalin and ziconotide would subsequently prevent PGE_2-induced allodynia. Pregabalin would therefore suppress not only presynaptic activation by calcium channel blockade but also postsynaptic activation by NMDA and blockade of $GABA_A$ receptor-mediated inhibitory control. The latter actions could be elicited via an N-type calcium channel independent mechanism.

In addition to pregabalin and ziconotide, mexiletine and duloxetine are also used as analgesics to treat chronic pain. Findings from the present study showed that mexiletine, a sodium channel blocker, significantly prevented PGE_2-induced but not NMDA- or bicuculline-induced allodynia, indicating that the agent preferentially suppresses hyperactivity triggered by presynaptic activation but not that by postsynaptic excitatory NMDA signaling or the removal of a $GABA_A$ receptor-mediated control. Mexiletine has been reported to suppress spinal hyperactivity initiated by Aδ- and C-fibers (Victoria et al., 1998). Consistent with the previous reports, data from the present study showed that mexiletine almost completely prevented PGE_2-induced allodynia, which is predominantly triggered by the facilitation of nociceptive neurotransmission involving C-fibers.

Duloxetine was shown to have anti-nociceptive profile similar to that of mexiletine in the present study. Duloxetine is a dual serotonin and norepinephrine reuptake inhibitors which potentiates descending pain inhibitory control by increasing synaptic concentrations of both serotonin and norepinephrine in the spinal cord (Thor et al., 2007). Our findings suggest that elevated monoamines in the dorsal horn of the spinal cord act predominantly on the terminal of primary afferent fibers to suppress presynaptic neurotransmitter release, and any potential postsynaptic mechanism would therefore contribute relatively little to its analgesic mechanism. We also studied the effects of 5-$HT_{1B/1D}$ receptor antagonist (GR127935) and alpha-2 adrenergic receptor antagonist (idazoxan) on anti-nociceptive effect of duloxetine in the PGE_2-induced allodynia model, as activation of 5-$HT_{1B/1D}$ receptor and alpha-2 adrenergic receptor at terminals of primary afferents in the

spinal cord is considered to produce analgesic effects (Millan, 2002; Li and Eisenach, 2001). The analgesic effect of duloxetine on PGE_2-induced allodynia was significantly blocked by combination of intrathecal injection of GR127935 and idazoxan. The result supports the possibility of presynaptic mechanism of duloxetine. These results taken together suggest that ziconotide, duloxetine, and mexiletine are most likely to exhibit their anti-nociceptive effect against the presynaptic target mechanism of spinal hyperactivity.

We have previously reported the effects of these agents on locomotor activity to assess potential of sedation in mice (Kiso et al., 2008). In the study, duloxetine did not affect locomotor activity up to 100 mg/kg p.o., on the other hands, pregabalin at 30 and 100 mg/kg, and mexiletine at 100 mg/kg significantly decreased locomotor activity. These results suggest that both pregabalin and mexiletine have sedative effects. Our present study demonstrated that sedative dose of mexiletine at 100 mg/kg had no effects on NMDA- and bicuculline-induced allodynia at all. These results suggest that the allodynic behaviors are unlikely to be influenced by the sedative effect of drugs in our model. Thus, the effect of pregabalin on NMDA- and bicuculline-induced allodynia is likely due to anti-nociception.

In conclusion, the present study employed models of three kinds of spinal activation induced by intrathecal administration of neurochemicals, namely PGE_2-induced presynaptic activation, NMDA-induced postsynaptic activation, and bicuculline-induced removal of $GABA_A$ receptor-mediated inhibitory control. Our results suggest that standard analgesics have different sites of action when modifying spinal hyperactivity processes. Ziconotide, mexiletine and duloxetine appear to suppress spinal hyperactivity triggered by presynaptic mechanism, but not by postsynaptic and GABAergic mechanism. In contrast, pregabalin appears to work on hyperactivity induced by presynaptic and postsynaptic activations and GABAergic dysfunction, possibly with a broader site of action potentially involving target molecules other than N-type calcium channels. Our findings could provide novel insight into the analgesic mechanism of these drugs and also implicate that the models employed in present study are useful in elucidating sites of action of analgesics.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2010.02.025.

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