INTRODUCTION

Neurokinin (NK) 1 and N-methyl-D-aspartate (NMDA) receptors are known to play a role in the facilitation of spinal pain transmission. In the spinal cord, NK1-positive projections mediate spinal nerve ligation (SNL)-induced neuropathic pain\(^1\) as well as facet joint injury induced nociception.\(^2\) In addition, the activation of spinal NMDA receptors was shown to be involved in chemotherapeutic\(^3\) and SNL-induced neuropathic pain.\(^4\) Substance P (SP) and NMDA act as full agonists at NK1 receptors and NMDA receptors, respectively, and the intrathecal (i.t.) injection of these agonists has been shown to induce nociceptive behavior.\(^5,6\)

Angiotensin (Ang) (1–7), an N-terminal fragment of Ang II, by the action of Ang-converting enzyme (ACE) 2. By acting on the G protein-coupled receptor MAS1, Ang (1–7) exerts a counter-regulatory effect towards the flank, biting and/or licking of the hindpaw or the tail for 5 min. Localization of MAS1 and either NK1 receptors or NMDA receptors in the lumbar superficial dorsal horn was determined by immunohistochemical observation. The nociceptive response induced by SP and NMDA was attenuated by the i.t. co-administration of Ang (1–7) (0.03–3 pmol) in a dose-dependent manner. The inhibitory effects of Ang (1–7) (3 pmol) were attenuated by A779 (100 pmol), a MAS1 antagonist. Moreover, immunohistochemical analysis showed that spinal MAS1 co-localized with NK1 receptors and NMDA receptors on cells in the dorsal horn. Taken together, the i.t. injection of Ang (1–7) attenuated the nociceptive response induced by SP and NMDA via spinal MAS1, which co-localized with NK1 and NMDA receptors. Thus, the spinal Ang (1–7)/MAS1 pathway could represent a therapeutic target to effectively attenuate spinal pain transmission caused by the activation of NK1 or NMDA receptors.

Key words angiotensin (1–7); MAS1; substance P; N-methyl-D-aspartate; spinal cord

MATERIALS AND METHODS

Animals Male ddY mice (22–24 g; Japan SLC, Japan) were used in this study. These were housed in cages with free access to food and water under controlled conditions (temperature, 22 ± 2°C; humidity, 55 ± 5%) and a 12/12 h light–dark cycle (lights on: 07:00 to 19:00). Groups of 10 mice for behavioral experiments and 3 mice for immunohistochemical experiments were used in single experiments. All experiments were performed following the approval from the Ethics Committee of Animal Experiment at Tohoku Medical and Pharmaceutical University (Approval No. A14023-cn).

Drugs and Antibodies The following drugs and reagents were used: Ang (1–7) and SP (Peptide Institute, Japan); NMDA (Nacalai Tesque, Japan); [α-Ala\(^1\)]-Ang (1–7) (A779) (Bachem, Switzerland); rabbit anti-MAS1 antibody (#AAR-013), rabbit anti-NK1 receptor antibody (#ATR-001) and rabbit anti-NMDA receptor 1 (GluN1 subunit) antibody (#AGC-001; Alomone Laboratories, Israel); Alexa Fluor 488-conjugated AffiniPure Fab fragment goat anti-rabbit immunoglobulin G (IgG) (#111-547-003; Jackson ImmunoResearch Laboratories, U.S.A.) and Alexa Fluor 568-conjugated goat anti-rabbit IgG (#A-11011; Thermo Fisher Scientific, U.S.A.); 4',6-diamidino-
2-phenylindole dihydrochloride (DAPI) (Dojindo, Japan); normal goat serum (NGS) (Invitrogen, U.S.A.).

**Intrathecal Injections**  The i.t. injections were performed in unanesthetized mice at the L5, L6 intervertebral space as previously described.1) Briefly, a volume of 5 µL was administered i.t. with a 28-gauge needle connected to a 50-µL Hamilton microsyringe, the animal being lightly restrained to ensure needle positioning. Puncture of the dura was indicated behaviorally by a slight flick of the tail. For i.t. injections, all drugs were dissolved in Ringer's solution.

**Measurement of Nociceptive Behavior**  At least 30 min before the i.t. injection, the mice were habituated to the transparent cage (22.0 × 15.0 × 12.5 cm). Immediately after the injection, the mice were placed individually in this cage and the accumulated response time of hindlimb scratching directed toward the flank, biting and/or licking of the hindpaw or the tail was measured during a 5 min period.

**Immunohistochemical Staining**  The immunohistochemical labeling for MAS1 was performed in conjunction with NK1 receptors or NMDA receptor GluN1 subunit. Since the primary antibodies used to detect these three receptors were all derived from same host species (rabbit), sequential labeling was performed using Fab fragments, instead of whole antibodies, for the first target (MAS1). Spinal cord slices were incubated with anti-Mas1 antibody (1 : 100, 4 °C, overnight) followed by an incubation with Alexa Fluor 488-conjugated Fab fragment IgG (1 : 200, 4 °C, overnight). After rinsing with PBS, the slices were incubated with anti-NK1 or -GluN1 antibody (1 : 100, 100(µmol) or vehicle alone were injected as controls. The duration of scratching, biting and licking induced by SP was determined over a 5 min period starting immediately after its administration. One-way ANOVA: F₁,₇ = 29.61, p < 0.01. Values represent the means ± standard error of the mean (S.E.M.) for 10 mice per group. **p < 0.01 compared with vehicle-injected controls, ***p < 0.01 compared with SP alone and ††p < 0.01 compared with Ang (1–7) (3 pmol) in combination with SP.

**RESULTS**

**Effect of Ang (1–7) on SP-Induced Nociceptive Behavior in Mice**  Vehicle, Ang (1–7) (0.03–3 pmol), or Ang (1–7) (3 pmol) in combination with A779 (30 or 100 pmol) were co-administered i.t. with SP (0.1 nmol). A779 (100(µmol) or vehicle alone were injected as controls. The duration of scratching, biting and licking induced by NMDA was determined over a 5 min period starting immediately after its administration. One-way ANOVA: F₁,₇ = 12.67, p < 0.01. Values represent the means ± S.E.M. for 10 mice per group. **p < 0.01 compared with vehicle-injected controls, ***p < 0.01 compared with NMDA (0.3 nmol) alone and ††p < 0.01 compared with Ang (1–7) (3 pmol) in combination with NMDA (0.3 nmol).

**Statistical Methods**  Data were expressed as means ± standard error of the mean (S.E.M.). Significant differences were analyzed by a one-way ANOVA, followed by Tukey’s multiple comparison test. All statistical analyses were performed using GraphPad Prism software, version 7.03 (GraphPad Software, U.S.A.). In all comparisons, p < 0.05 was considered statistically significant.
the basal behavioral response (Fig. 1). These results suggest that Ang (1–7) attenuates the SP-induced nociceptive behavior via spinal MAS1.

Effect of Ang (1–7) on NMDA-Induced Nociceptive Behavior NMDA is also known to induce a nociceptive behavior when it is administered i.t. Therefore, we examined the potential counter-effect of Ang (1–7) on the NMDA-induced nociceptive behavior. Indeed, the NMDA-induced behavior (0.3 nmol) was again dose-dependently suppressed by the i.t. co-administration of Ang (1–7) (0.03–3 pmol), and this inhibition was significantly reversed by the i.t. injection of A779 (100 pmol) (Fig. 2). These results suggest that Ang (1–7) attenuates the NMDA-induced nociceptive behavior via spinal MAS1.

Co-localization of MAS1 and NK1 Receptors or GluN1 Subunit in the Lumbar Superficial Dorsal Horn Immunohistochemical experiments were performed to determine whether spinal NK1 receptors or GluN1 subunit co-localized with MAS1. We found that both NK1 receptors (Fig. 3a) and GluN1 subunit (Fig. 3b) were expressed in MAS1-immunoreactive cells. Since we used primary antibodies from the same host species in each dual labeling, high concentrations of Alexa 488-conjugated Fab fragments were used as secondary antibody against the anti-MAS1 antibody. The rationale for this was to saturate and block the antigen binding sites on the primary rabbit anti-MAS1 antibody with the label thereby

![Fig. 3](image_url)

(a) Photomicrographs showing fluorescent labeling for MAS1 (green), NK1R (red), and nuclei with DAPI (blue), as well as merged images (right panels). (b) Photomicrographs showing fluorescent labeling for MAS1 (green), GluN1 (red), or nuclei with DAPI (blue), as well as merged images (right panels). (c) Photomicrographs showing fluorescent labeling for MAS1 (green), control for absence of MAS1 labeling with second secondary antibody (Ab) (red), or nuclei with DAPI (blue), as well as merged images (right panels). (d) Rate of NK1R- or GluN1-positive cells, which express MAS1, in the superficial dorsal horn (laminae I–III). (Color figure can be accessed in the online version.)
preventing the subsequent fluorophore-conjugated anti-rabbit secondary antibody from binding to these sites. To determine whether the Fab fragments could effectively block the antigen binding sites on anti-MASI antibodies, we performed a control immunohistochemical labeling in the absence of the primary anti-NK1 or GluN1 antibodies. As shown in Fig. 3c, the fluorescence produced by the Alexa 568-conjugated secondary antibody completely disappeared suggesting that the Fab fragments effectively blocked the antigen binding sites on anti-MASI antibodies. Thus, our results suggest that MAS1 and NK1 or NMDA receptors are expressed in the same cells of the superficial dorsal horn in the spinal cord (Lumbar 5–6). Further, the rate of MAS1-expressed NK1R- or GluN1-positive cells in the superficial dorsal horn (laminae I–III) was 98.8 ± 0.17 or 97.7 ± 0.36%, respectively (Fig. 3d).

DISCUSSION

In the spinal dorsal horn, the activation of NK1 receptors induced by SP leads to an increase in neuronal activity, which occurs via NMDA receptor activation. Therefore, we suggested that the SP-induced nociceptive behavior by modulating the activation of their receptors. 

In conclusion, we showed that the i.t. injection of Ang (1–7) attenuated the nociceptive behavior induced by SP and NMDA via spinal MAS1, which are co-localized with NK1 and NMDA receptors. Since spinal NK1 and NMDA receptors are involved in several types of pain, the activation of spinal Ang (1–7)/MAS1 pathway could represent an effective therapeutic approach for pain management.

Acknowledgments This study was supported in part by JSPS KAKENHI (Grant number 20K07074 to K.T., and W.N.; Grant No. 19K16376 to W.N.), NISHINOMIYA Basic Research Fund (Japan) to W.N. and Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan (Grant No. N-190701 to R.Y.).

Conflict of Interest The authors declare no conflict of interest.

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