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INHIBITION OF AMPA RECEPTORS AND ACTIVATION OF THE GLYCINE SITE OR POLYAMINE SITE OF NMDA RECEPTORS AT THE SUPRASPINAL LEVEL INDUCES ANTINOCICEPTION

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ABSTRACT
At the supraspinal level, the role of AMPA receptors in the mechanism of pain remains to be fully clarified, even though they are found in large numbers throughout many brain regions. The results of this study revealed that intracerebroventricular administration of YM872, an AMPA receptor antagonist, exerted a dose-dependent antinociceptive effect on acute and tonic pain such as that induced by the tail flick or formalin tests without sedation or signs of abnormal behavior in rats. Moreover, the antinociceptive effect induced by intracerebroventricular administration of YM872 together with D-serine, an endogenous co-agonist for the glycine site of the NMDA receptor, or spermidine, an endogenous allosteric modulator for their polyamine site, was larger than that by application of YM872, D-serine, or spermidine alone in the formalin test. These results suggest that inhibition of AMPA receptors and/or activation of NR2B-containing NMDA receptors at the supraspinal level induces an antinociceptive effect on both acute and tonic pain.

KEYWORDS: YM872, D-serine, spermidine, AMPA receptor, NMDA receptor.

1. INTRODUCTION
[2,3-dioxo-7-(1H-imidazol-1-yl)-6-nitro-1,2,3,4-tetrahydroquinoxalin-1-yl]-acetic acid monohydrate (YM872), a potent, high selective, and water-soluble, competitive α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor antagonist, was developed as a neuroprotective drug for use in the treatment of brain ischemia.[1] Treatment with YM872 as well as MK-801, N-methyl-D-aspartate (NMDA) receptor antagonist, can reduce excitotoxicity leading to neuronal death after the middle cerebral artery occlusion.[2,3] Extensive experimental and clinical evidence suggests that AMPA and NMDA receptors in the spinal cord involved in nociception.[4] It has been suggested that at the spinal level, AMPA receptors are engaged specifically in nociceptive mediate fast excitatory transmission involving both innocuous and acute nociceptive transmission, whereas NMDA receptors particularly participate in intense and prolonged nociceptive responses.[5]

Several lines of study have demonstrated that unlike spinal level, activation of NMDA receptors at the supraspinal level induces antinociception.[6-9] Administration of D-serine, an endogenous agonist for the glycine site of the NMDA receptor, into the third cerebral ventricle led to potentiation of antinociception in the tail-flick response [9] and formalin-induced pain [10] in an opioid receptor antagonist- or benzodiazepine receptor agonist-reversible manner in rat.[11] The distribution of D-serine is similar to that of the NMDA receptor 2B subunit (NR2B) mRNA.[12-14] Spermidine is also an endogenous allosteric modulator that regulates NR2B-containing NMDA receptor via binding polyamine site.[15, 16]

In earlier studies, we demonstrated that intrathecal or intraperitoneal administration of YM872 exerted a dose-dependent antinociceptive effect on acute and tonic pain such as that induced by either the tail flick or formalin test without motor disturbance or flaccidity in rat.[5, 17] At the supraspinal level, the role of AMPA receptors in the mechanism of pain remains to be fully clarified, even though they are found in large numbers throughout many brain regions, some of which are associated with descending modulation of pain.[18] To elucidate the role of AMPA receptors at the supraspinal level in pain and how it differs from that of NMDA receptors, we

investigated the antinociceptive effect of intracerebroventricular administration of YM872 alone or in combination with D-serine or spermidine in the tail-flick and formalin tests in rat.

2. MATERIALS AND METHODS

2.1. Animals and drugs

The present animal experiments were performed in strict accordance with the guidelines of Tokai University (http:// www.u-tokai.ac.jp/about/concept/guidance.html). Approval was also obtained from the Animal Investigation Committee of this institute. Male Wistar rats (Clea Japan Inc., Tokyo, Japan) weighing 180-220 g each were used. The rats were group-housed in laboratory cages and kept in a temperature-controlled room (23 ± 2ºC) under a 12-hr light/dark cycle (lights on: 07:00), with food and water freely available. D-serine was purchased from Sigma (Tokyo, Japan). Spermidine trihydrochloride was purchased from Nacalai (Kyoto, Japan). YM872 was provided free of charge by Astellas Pharma Inc. (Tokyo, Japan). Ten milligrams YM872 was dissolved in 0.97 ml sterilized water and 30 µl of 1 N sodium hydroxide to adjust the solution to a pH of 7.3-7.5. The desired concentration of YM872 was obtained by adding saline to the solution. Zero point three (0.3), 0.9, 3.0, 9.0, and 30.2 nmol YM872 are approximately equivalent to 0.1, 0.3, 1.0, 3.0, and 10.0 µg, respectively.[5] The solutions of all drugs used were prepared to the desired concentration just before use.

2.2. Intracerebroventricular administration

Infusion of drugs into the third cerebral ventricle in each rat was performed via an indwelling cannula as described previously.[9] The rats were mounted on a stereotaxic frame to allow implantation of a stainless-steel guide cannula (internal diameter of 0.35 mm) under anesthesia with pentobarbital sodium (40 mg/kg, i.p.) 5-7 days prior to the day of the experiment. The lower end of the injection cannula (30G needle, TERUMO Co., Tokyo, Japan) was aimed at the third cerebral ventricle (AP, -0.8 mm; V, +7.8 mm; L, ± 0.0 mm) according to the atlas of Paxinos and Watson,[19] through a stainless-steel guide cannula (outer diameter: 0.55 mm). The injection cannula was attached to a motor-driven, 50-µl micro-syringe by polyethylene tubing (PE-20; Clay Adams, Parsippany, NJ, USA). The drugs were infused at a volume of 10 µl for 1 min and the injection cannula left in place for 1 min before removal. The distribution of the drug solution in the cerebroventricular system was verified by infusion of 0.3% Evans blue dissolved in saline after the experiment.

2.3. Behavioral and motor function test

Behavioral tests were performed in a neutral environment by three investigators in a blinded manner. General behavior (including agitation and allodynia), sedation, catalepsy, motor function, and corneal reflex were examined.[5] Agitation was judged as spontaneous irritable movement and/or vocalization. The presence of allodynia was determined by looking for signs of agitation (escape and/or vocalization), evoked by lightly stroking the flank of the rat with a small probe. Sedation was confirmed with a righting reflex test, in which each rat was left undisturbed on its back. If the rat failed to turn itself upright within 10 sec, it was regarded as being sedated.[20] A state of catalepsy was determined using a box of 280 mm in length, 70 mm in width, and 40 mm in height. In the catalepsy test, the forepaws of the rat were placed on the upper surface of the box. If the rat remained immovable for 15 sec, it was regarded as being cataleptic. Motor function was assessed with a balance beam, which consisted of placing the rats on a round wooden beam (diameter; 25 mm).[10] The cut-off time was set at 30 sec. As rating of motor function was on an all-or-none basis, the number of animals unable to remain on the beam for the full 30 sec out of the total number receiving drugs was used to calculate the percentage loss of response. Furthermore, the rats were tested for possible side effects as evidenced by a reduction in corneal reflexes as described previously.[20]

2.4. Tail-flick test

The investigators were blind to all drug treatments carried out in these experiments. In the tail-flick test, the latency to flick the tail after contact with water warmed to a temperature of 55°C was measured at before and at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min after administration of the drugs as described previously.[9] The latency to flick the tail before drug administration was approximately 1 sec (0.8 to 1.4 sec). A cut-off time of 5 sec was used to prevent any injury to the tail. The latencies of the tail-flick responses were converted to a percentage of maximum possible effect (% MPE) for each animal at each time according to the following formula: % MPE = [(test latency – baseline latency)/(5 – baseline latency)] x 100. The area under the curve (AUC) values for the % MPE (0-120 min) were used to evaluate the analgesic effect of the drugs on each rat.

2.5. Formalin test

The investigators were blind to all drug treatments carried out in these experiments. The formalin test was performed 10 min after drug administration as described previously.[20] Fifty microliters of 5% formalin was injected subcutaneously into the dorsal surface of the right hind paw with a 27-gauge needle. Immediately after injection, the rat was placed in an open clear plastic chamber (30×30×30 cm) and its flinching or shaking paw response observed at 5-min intervals for a period of 1 hr. The number of flinches was counted for 1 min. In the formalin test, usually two phases are observed: phase 1, comprising the first 6 min after injection; and phase 2, beginning after approximately 10 min, with an interval of a few flinches between each phase. The number of flinches was used to evaluate the antinociceptive effect of the drugs on each rat. The summation of the number of flinches (SUM) was used to evaluate the analgesic effect of the drugs on each rat (phase 1, $SUM_{0\text{-}10min}$; phase 2, $SUM_{15\text{-}60min}$).

2.6. Data analysis

The results are given as the mean with standard error of the mean (SEM). The statistical analysis was conducted using Prism 6 (GraphPad Software, Inc., San Diego, CA) for a comparison across the experimental conditions. When a significant difference was observed after drug administration in a two-way (dose of drug and time) repeated-measures analysis of variance (ANOVA), the Dunnett's post-hoc test was used to define significance at each time point. When a significant difference among groups was obtained in the Kruskal-Wallis test, the Dunn's post-hoc test was used to define which group contributed to these differences. The level of statistical significance was set at $P<0.05$.

3. RESULTS

3.1. Behavioral and motor function test

Figure 1 represents the number of rats showed abnormal behavior out of 6 in each group. All the rats exhibited normal behavior (including agitation, allodynia, catalepsy, corneal reflexes, motor function and righting reflex) during the tests after intracerebroventricular administration of 3.0 nmol YM872. Apart from agitation and allodynia, all rats exhibited sedation (loss of righting reflex) and problems in motor function (including catalepsy and loss of corneal reflexes) during the test after intracerebroventricular administration of 30.2 nmol YM872. These abnormal behaviors disappeared, however, within 45 min. Intracerebroventricular administration of 9.0 or 30.2 nmol YM872 induced agitation and allodynia for 15 min.

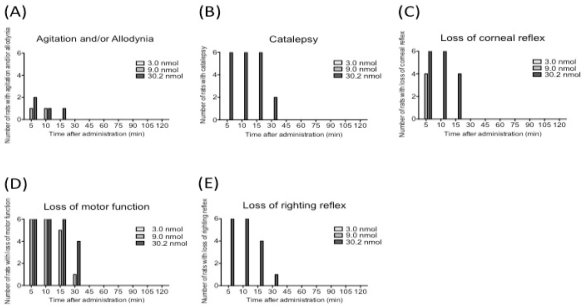

Fig.1. Results represent the number of rats out of 6 in each group. Types of behavior after intracerebroventricular administration of YM872: (A) agitation and/or allodynia; (B) catalepsy; (C) loss of corneal reflex; (D) loss of motor function; and (E) loss of righting reflex.

3.2. Tail-flick test

Figure 2 includes the results of the antinociceptive effects in the same rats used in behavioral and motor function test (3.0, 9.0 and 30.2 nmol of YM872) (Fig. 1). Intracerebroventricular administration of YM872 resulted in a significant and dose-dependent antinociceptive effect on the tail-flick response (Fig. 2). The maximum antinociceptive effect of YM872 was achieved at 45 min after administration (Fig. 2A). Because abnormal behaviors were exhibited within 45 min by administration of YM872 at a dose of 9.0 or 30.2 nmol (Fig. 1), we evaluated the $AUC_{45\text{-}120min}$ value (Fig. 2C), in addition to the $AUC_{0\text{-}120min}$ value (Fig. 2D). The ED_{50} value of % MPE at 45 min and the $AUC_{45\text{-}120min}$ were 4.622 and 8.182 nmol, respectively (Fig. 2B, 2C).

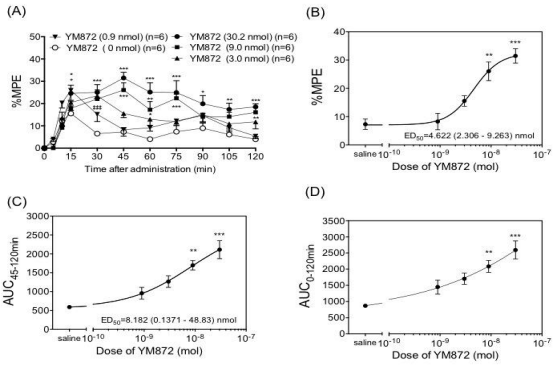

Fig. 2. Antinociceptive effect of intracerebroventricular administration of YM872 on tail-flick response. Data represent mean and SEM. (A) Time course of change in % MPE after administration of YM872 (0.9 nmol, n=6; 3.0 nmol, n=6; 9.0 nmol, n=6; 30.2 nmol, n=6) or saline (0 nmol, n=6). Significantly different from values in group receiving saline by Dunnett's post-hoc test following two-way repeated measures ANOVA ($P<0.0001$); *$P<0.05$, **$P<0.01$ and ***$P<0.001$. (B) %MPE value at 45 min after YM872 administration. Significantly different from values in group receiving saline by Dunn's post-hoc test following Kruskal-Wallis test ($P<0.0001$); **$P<0.01$ and ***$P<0.001$. (C) $AUC_{45\text{-}120min}$ for values of % MPE. Significantly different from values in group receiving saline by Dunn's post-hoc test following Kruskal-Wallis test ($P=0.0003$); **$P<0.01$ and ***$P<0.001$. (D) $AUC_{45\text{-}120min}$ for values of % MPE. Significantly different from values in group receiving saline by Dunn's post-hoc test following Kruskal-Wallis test ($P<0.0001$); **$P<0.01$ and ***$P<0.001$.

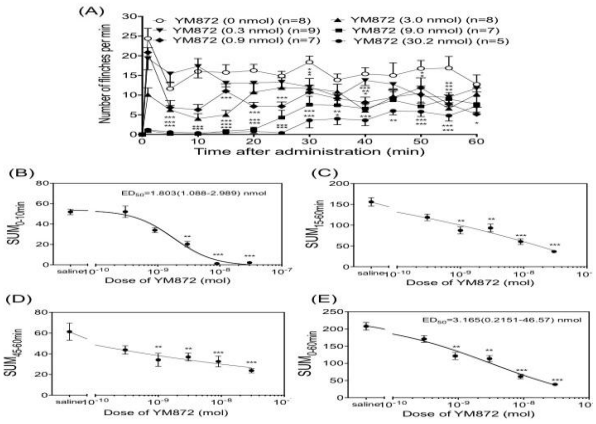

Fig.3. Antinociceptive effect of intracerebroventricular administration of YM872 on formalin-induced flinching response. Data represent mean and SEM. (A) Time course of change in

number of flinches per min after formalin injection under pretreatment with YM872 (0.3 nmol, n=9; 0.9 nmol, n=7; 3.0 nmol, n=8; 9.0 nmol, n=7; 30.2 nmol, n=5) or saline (0 nmol, n=8). Significantly different from values in group receiving saline by Dunnett's post-hoc test following two-way repeated measures ANOVA (*P*<0.0001); **P*<0.05, ***P*<0.01 and ****P*<0.001. Panel (B), (C), and (D) show number of flinches in phase 1, 2, $SUM_{45\text{-}60min}$, and all phases of formalin test, respectively. Significantly different from values in group receiving saline by Dunn's post-hoc test following Kruskal-Wallis test (phase 1, *P*<0.0001; phase 2, *P*<0.0001; $SUM_{45\text{-}60min}$, P<0.0001; all phases, *P*<0.0001); ***P*<0.01 and ****P*<0.001.

3.3. Formalin test

Because abnormal behaviors were exhibited within 45 min by administration of YM872 at a dose of 9.0 or 30.2 nmol (Fig. 1), we evaluated the $SUM_{45\text{-}60min}$ value (Fig. 3D), in addition to phase 1 and 2 (Fig. 3B, 3C). YM872 significantly and dose-dependently reduced the number of flinches in phase 1, 2, $SUM_{45\text{-}60min}$ and all

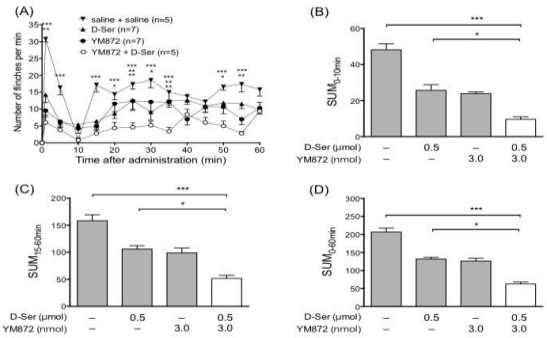

Fig. 4. Antinociceptive effect of intracerebroventricular administration of YM872 (3.0 nmol) in combination with D-serine (0.5 μmol) on formalin-induced flinching response. Data represent mean and SEM. (A) Time course of change in number of flinches per min in YM872+D-serine-, YM872-, D-serine-, and saline-treated rats after formalin injection. Significantly different from values in group receiving YM872+D-serine by Dunnett's post-hoc test following two-way repeated measures ANOVA (*P*<0.0001); **P*<0.05, ***P*<0.01 and ****P*<0.001. Panel (B), (C), and (D) show number of flinches in phase 1, 2, and all phases of formalin test, respectively. Significantly different from values in group receiving YM872+D-serine by Dunn's post-hoc test following Kruskal-Wallis test (phase 1, *P*=0.0005; phase 2, *P*=0.0004; all phases, *P*=0.0004); **P*<0.05 and ****P*<0.001.

phases ($SUM_{0\text{-}60min}$) of the formalin test (Fig. 3). The ED_{50} of phase 1 and all phases were 1.803 and 3.165 nmol, respectively (Fig. 3B, 3E). The antinociceptive effect induced by intracerebroventricular administration of YM872 (3.0 nmol, approximately equivalent to ED_{50} of $SUM_{0\text{-}60min}$, 3.165 nmol) in combination with D-serine (0.5 μmol, approximately equivalent to ED_{50} of $SUM_{0\text{-}60min}$, 0.5064 μmol)[10] was larger than that of YM872 or D-serine alone in phase 1, phase 2, and all phases in the formalin test (Fig. 4).

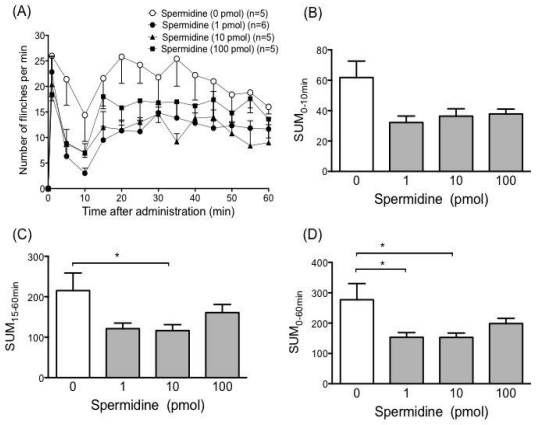

Fig. 5. Antinociceptive effect of intracerebroventricular administration of spermidine on formalin-induced flinching response. Data represent mean and SEM. (A) Time course of change in number of flinches per min after formalin injection under pretreatment with spermidine (1 pmol, n=6; 10 pmol, n=5; 100 pmol, n=5) or saline (0 pmol, n=5). There was no significant difference between the groups (two-way repeated-measures ANOVA; *P*=0.8588). Panel (B), (C), and (D) show number of flinches in phase 1, 2, and all phases of formalin test, respectively. Significantly different from values in group receiving saline by Dunn's post-hoc test following Kruskal-Wallis test (phase 1, *P*=0.1095; phase 2, *P*=0.0191; all phases, *P*=0.0362); **P*<0.05.

Spermidine (1 and10 pmol) significantly reduced the number of flinches in phase 2 and all phases of the formalin test (Fig. 5). The antinociceptive effect induced by intracerebroventricular administration of YM872 (3 nmol) in combination with spermidine (1 pmol) was larger than that of YM872 or spermidine alone in phase 1, phase 2 and all phases in the formalin test (Fig. 6).

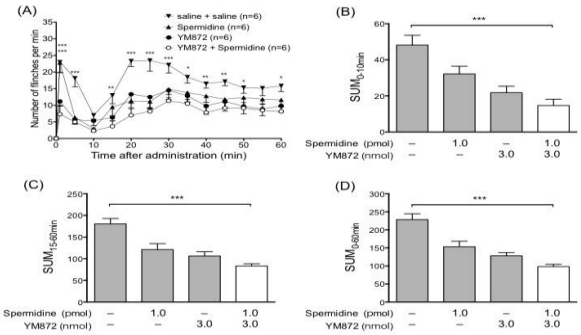

Fig. 6. Antinociceptive effect of intracerebroventricular administration of YM872 (3.0 nmol) in combination with spermidine (1.0 pmol) on formalin-induced flinching response. Data represent mean and SEM. (A) Time course of change in number of flinches per min in YM872+spermidine-

, YM872-, spermidine-, and saline-treated rats after formalin injection. Significantly different from values in group receiving YM872+spermidine by Dunnett's post-hoc test following two-way repeated measures ANOVA (*P*=0.0028); **P*<0.05, ***P*<0.01 and ****P*<0.001. Panel (B), (C), and (D) show number of flinches in phase 1, 2, and all phases of formalin test, respectively. Significantly different from values in group receiving YM872+spermidine by Dunn's post-hoc test following Kruskal-Wallis test (phase 1, *P*=0.0090; phase 2, *P*=0.0015; all phases, *P*=0.0008); ***P*<0.01.

4. DISCUSSION

The results of the present study demonstrated that intracerebroventricular administration of YM872 produced a dose-dependent antinociceptive effect on thermal- or formalin-induced acute and tonic pain in rat. The effective dose (0.9 – 3 nmol) induced no behavioral side effects. This suggests that inhibition of AMPA receptors at the supraspinal level leads to potentiation of the antinociceptive effect on acute and tonic pain without toxicity under physiological conditions. This finding is consistent with the previous studies showing that intrathecal or intraperitoneal administration of YM872 exerted a dose-dependent antinociceptive effect on the tail flick or formalin tests without signs of abnormal behavior in rat.[5, 17]

In earlier studies, injection of AMPA into the rostral ventromedial medulla (RVM) induced dose-dependent antinociception [21], while that of 6,7-dinitro-quinoxaline-2,3-dione (DNQX), an AMPA/kainate receptor antagonist, enhanced a hyperalgesic response.[22] Injection of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA/kainate receptor antagonist, into the RVM reduced morphine-induced analgesia, although not significantly.[23] Moreover, injection of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX), a more selective AMPA receptor antagonist than CNQX and DNQX [24], into the RVM did not induce an antinociceptive effect.[25] One reason for this inconsistency with the present results may lie in differences in the selectivity of these antagonists for the AMPA receptor. Although CNQX, DNQX, and NBQX are competitive antagonists at AMPA receptors, all three compounds block kainate receptors. CNQX and DNQX also act as antagonists at the glycine binding site on NMDA receptors.[26, 27] Taken together with the present results, this suggests that injection of CNQX, DNQX or NBQX into the RVM may act on pain through different types of receptor. Another possibility is that difference in injection site may affect the action of the drug. In the present study, administration of YM872 into the third cerebral ventricle significantly induced antinociception in temperature- or formalin-induced acute and tonic pain. These results are in good agreement with those of an earlier study showing that administration of DNQX into the nucleus cuneiformis (CnF), a reticular nucleus of the midbrain, induced antinociception.[28] The midbrain periaqueductal gray (PAG) matter is continuous with the periventricular gray matter surrounding the third cerebral ventricle in brain.[29] The CnF, which is located just ventrolateral to the PAG, plays an important role in sensory/motor integration relevant to pain transmission.[30] The PAG sends descending fibers to the RVM on the floor of the medulla oblongata.[31] Administration of AMPA[21], CNQX[23], DNQX[22] or NBQX[25] into rat RVM leads to results inconsistent with those of the present and previous studies.[28] This suggests that the effect of AMPA receptors on descending modulation of pain differs among brain regions.

Interestingly, the results of our present and previous studies[9-11] indicate that AMPA and NMDA receptors exert opposing effects on antinociception with drug administration into the third cerebral ventricle. Several lines of evidences support this. First, administration of NMDA receptor antagonist (MK-801 or AP7) into the CnF attenuated the antinociceptive effects of morphine, whereas that of DNQX showed only a partial antinociceptive effect and potentiated the analgesic effect of morphine.[28] Second, injection of NMDA into the PAG induced antinociception in the tail-flick test.[7] Third, the CnF and PAG operate concordantly to control ventral medullary pain pathways.[32] The CnF is located just ventrolateral to the PAG surrounding the third cerebral ventricle in rat brain.[19,29] Taken together with the present results, these suggest that administration of YM872 and D-serine into the third cerebral ventricle results in coordinated action between the CnF and PAG. Further microinjection study using YM872 or D-serine is needed, however, to clarify this hypothesis.

In earlier studies[9,10], we demonstrated that the potentiation of the antinociception produced by D-serine was attenuated by the intracerebroventricular administration of L-701,324, a high selective antagonist for the glycine site of the NMDA receptors.[33] Therefore, on the basis of the above findings, the antinociceptive effect of intracerebroventricular administration of D-serine was found to be mainly involved in the glycine site on NMDA receptors. Although many studies concerning the functional role of D-serine have focused on the regulation of NMDA receptor activation, it has recently been found that D-serine can also act as antagonists on AMPA receptors.[34-37] These findings, together those of the present study, suggest that intracerebroventricular administration of D-serine might induce an antinociceptive effect, in part by acting as an AMPA receptor antagonist.

Spermine or spermidine at low and high dose was shown to act as a subunit-selective positive and negative allosteric modulator of NR2B, respectively.[15,38] Intrathecal administration of spermine at low dose (0.1-10,000 fmol) and high dose (2.5-10 nmol) induced nociceptive and antinociceptive effects in mice, respectively.[15,38] In the present study,

intracerebroventricular administration of spermidine at low dose (1 and 10 pmol), but not 100 pmol, produced antinociceptive effects in the rat formalin test. This is agreed with the results of intracerebroventricular administration of D-serine, a co-agonist of glycine site of NMDA receptor.[9,10] The distribution of D-serine is similar to that of NR2B mRNA.[12-14] These findings, together with those of earlier studies, suggest that activation of NR2B-containing NMDA receptors exerts an antinociceptive effect at the supraspinal levels.

5. CONCLUSION

The results of the present study, together with those of earlier studies, demonstrate that inhibition of AMPA receptors and/or activation of NR2B-containing NMDA receptors at the supraspinal level exerts an antinociceptive effect on both acute and tonic pain.

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