Pharmacological Evidence for Involvement of Excitatory Amino Acids in Aversive Responses Induced by Intrathecal Substance P in Rats

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Rats given an intrathecal injection of substance P (0.3–10 nmol) or any of the excitatory amino acid agonists, *N*-methyl-D-aspartate (NMDA, 1–10 nmol), kainate (1 and 3 nmol) or α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, 0.3–3 nmol), showed biting or licking the hind paws, scratching with the hind paws (only after substance P) and vocalization (only after excitatory amino acid agonists). The intrathecal co-administration of the NMDA antagonist, 2-amino-5-phosphonovaleric acid (APV, 10 nmol), inhibited behavioral responses to NMDA (10 nmol) and substance P (10 nmol) but not to kainate (3 nmol). Co-administration of the non-NMDA antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 nmol), suppressed responses to kainate (3 nmol), AMPA (3 nmol) and substance P (10 nmol) but not to NMDA (10 nmol). Co-administration of the substance P antagonist, CP-96,345 (3 nmol), inhibited the behavioral responses to substance P (10 nmol), but not to NMDA (10 nmol), kainate (3 nmol) and AMPA (3 nmol). The results suggest that the aversive behavior induced by intrathecal NMDA and non-NMDA agonists is mediated by activation of the corresponding glutamate receptors, but not by NK-1 receptors, and that the behavioral action of intrathecal substance P is mediated not only by direct activation of NK-1 receptors but also indirectly by NMDA and non-NMDA receptors for glutamate.

Keywords excitatory amino acid; substance P; nociception; intrathecal injection; spinal cord

Increasing evidence suggests that excitatory amino acids (EAAs), especially glutamate (Glu), and the neuropeptide substance P (SP) are involved in the transmission of nociceptive information in the spinal dorsal horn. For example, Glu-like immunoreactivity is densely distributed in myelinated and unmyelinated primary afferent terminals in the superficial layers of the spinal dorsal horn.¹⁾ Peripheral noxious stimulation increases the release of Glu from the dorsal spinal cord.²⁾ Glu binding sites are localized in the superficial laminae of the spinal dorsal horn.^{3,4)} An intrathecal injection of an EAA antagonist produces antinociceptive effects in rodents.^{5,6)} In addition, intrathecal injections of EAA receptor agonists produce aversive behavior and hyperalgesic effects.^{7–9)} SP is present in central and peripheral terminals of primary afferent fibers and dorsal root ganglion cells^{10–13)} and noxious stimulation increases the release of immunoreactive SP within the spinal dorsal horn.^{14–18)} An intrathecal injection of anti-SP antibody^{19,20)} or SP antagonist⁵⁾ produces antinociceptive effects, while an intrathecal injection of SP elicits caudally-directed aversive behavior.^{21–23)}

Glu and SP coexist in some small primary afferent neurons²⁴⁾ and their terminals in the superficial laminae of the spinal dorsal horn,²⁵⁾ suggesting that both Glu and SP play important roles in nociceptive transmission in the spinal dorsal horn. However, complex data have been reported concerning the interaction between Glu and SP in the spinal cord. Behavioral effects following intrathecal injection of EAAs have been reported to be potentiated²⁶⁾ or to be unaffected^{7,27)} by co-administration of SP. Exogenously administered SP increases the release of Glu from the spinal cord, but Glu released by SP has been claimed not to be involved in SP-induced biting and scratching behavior.²⁷⁾ In addition, aversive behavior elicited by an intrathecal injection of *N*-methyl-D-aspartic acid (NMDA) was inhibited or unaffected by co-administration of SP ana-

logues.²⁸⁾ These findings suggest an interaction between Glu and SP in spinal nociceptive transmission, the precise mechanism of which remains obscure. Therefore, as a step towards elucidating the role and interaction of Glu and SP in nociceptive transmission in the spinal dorsal horn, we have examined the effects of selective antagonists of SP or EAA receptors on the aversive behavior induced by an intrathecal injection of SP or EAAs.

Materials and Methods

Animals Male Sprague-Dawley rats weighing 200–250 g were used in all experiments. Rats were housed three to a cage and maintained under a 12h light–dark cycle (light cycle between 8:00 and 20:00) at a room temperature of $24 \pm 1^\circ\text{C}$ with free access to rat chow and water. They were allowed to acclimatize for at least 24 h prior to the experiments and each rat was used in only one experiment. The ethical guidelines for investigations of experimental pain in conscious animals²⁹⁾ were followed.

Materials The drugs injected intrathecally were NMDA (Cambridge Research Biochemicals, Ltd., Cambridge, England), kainic acid (Nacalai Tesque, Inc., Kyoto, Japan), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (Sigma Chemical Co., St. Louis, U.S.A.) and SP (Peptide Institute, Inc., Minoh, Japan). To evaluate the role of receptor subtypes in the aversive behavior elicited by EAA agonists or SP in the rat, three receptor antagonists were injected intrathecally in this study. The antagonists used were 2-amino-5-phosphonovaleric acid (APV, a competitive antagonist of the NMDA receptor³⁰⁾; Cambridge Research Biochemicals, Ltd., Cambridge, England), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a competitive non-NMDA receptor antagonist³¹⁾; Tocris Neuramin, Buckhurst Hill, England) and $(\pm)$ -CP-96,345 (2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine, a selective antagonist of the NK-1 receptor³²⁾; a kind gift from Central Research Division, Pfizer Inc., Groton, U.S.A.). CNQX was dissolved in 50% dimethyl sulfoxide (DMSO; special grade, Nacalai Tesque) in physiological saline and the other drugs were dissolved in physiological saline.

Intrathecal Injection All drugs were injected intrathecally, in a volume of 10 μl , according to the method of Satoh *et al.*^{33,34)} In brief, the rats were anesthetized with ether and the skin over the L₂–L₆ vertebrae was dissected. The next day, a stainless steel needle (25-gauge) connected to a microinjector was inserted into the subarachnoid space through the foramen intervertebrale, between L₃ and L₄. Ten microliters of physiological saline or 50% DMSO in saline were also injected intrathecally

alone to obtain control data. When an agonist and an antagonist were intrathecally injected in single animals, they were simultaneously administered in one injection of 10 μ l.

Behavioral Experiments The SP- and EAA-elicited behavior was observed using an acrylic resin cage (22 $\times$ 22 $\times$ 24 cm) as an observation chamber in which the rats were allowed to adapt for at least 15 min before the experiment. Immediately after the intrathecal injection, the rats were placed individually in the transparent observation cage and the duration of aversive behavior (biting or licking the hind paws and scratching with the hind paws towards the thorax) was recorded at 4-min intervals for 20 min. Concurrently animal behavior was recorded using an 8-mm videocamera (TR-55, Sony, Tokyo, Japan), which allowed re-examination of the elicited responses and gross behavior.

Statistical Analysis The results are expressed as means $\pm$ S.E.M. Statistical analysis was performed using the Mann-Whitney *U*-test; *p* < 0.05 was considered significant.

Results

Behavioral Responses to Intrathecal SP (Fig. 1) When injected intrathecally, SP (0.3–10 nmol/rat) elicited dose-dependent aversive behavior (biting or licking the hind paws and scratching with the hind paws towards the thorax), which lasted for about 20 min at a dose of 10 nmol/rat. Vocalization was not observed after any of the intrathecal doses of SP tested. SP was used at a dose of 10 nmol/rat in the following experiments.

Behavioral Responses to Intrathecal NMDA (Fig. 2) An intrathecal injection of NMDA (1.0–10 nmol/rat) produced dose-dependent aversive behavior (biting or licking the hind paws), which peaked within 4 min and lasted for about 8 min. At a dose of 10 nmol/rat, vocalization was evoked in all rats tested, but not measured as an aversive response. At 0.3 nmol/rat no behavioral effects were produced. A dose of 10 nmol/rat of NMDA was used in the following experiments. Injection of vehicle (physiological saline) in the same volume did not elicit any aversive behavior and appeared to have no effect on gross behavior.

Behavioral Responses to Intrathecal Kainate (KA) (Fig. 3) An intrathecal injection of KA (1.0 and 3.0 nmol/rat) produced dose-dependent aversive behavior (biting or licking the hind paws), which lasted for about 20 min, while no behavioral effects were observed at a dose of 0.3 nmol/rat. KA, at a dose of 3.0 nmol/rat, produced vocalization in all

rats tested, but was not measured as an aversive response. Intrathecal KA, at a dose of 6.0 nmol/rat, produced seizures, which began immediately after injection and lasted for at least 20 min. Hence a dose of 3.0 nmol/rat of KA was adopted in the following experiments.

Behavioral Responses to Intrathecal AMPA (Fig. 4) An intrathecal injection of AMPA (0.3–3.0 nmol/rat) produced dose-dependent aversive behavior (biting or licking the hind paws), which peaked within 4 min and disappeared by 12 min. Vocalization was observed in 4 of 6 rats after 3.0 nmol/rat of AMPA. AMPA, at a dose of 10 nmol/rat but not lower, caused hind limb paralysis, which appeared immediately after injection and subsided by 8–12 min. Therefore, in the following experiments, AMPA was used at a dose of 3.0 nmol/rat.

In the next series of experiments, the durations of aversive responses for the first 4 min after intrathecal injection of the above agonists are used as indexes for evaluating the effects of various antagonists.

Effects of CP-96,345 on Behavioral Responses Elicited by

Fig. 2. The Aversive Responses to Various Doses (0.3–10 nmol/rat) of NMDA Injected Intrathecally in Rats

▲, 0.3; ■, 1.0; ▼, 3.0; ●, 10.0; ○, vehicle. Agent was injected at time 0. The duration of biting and licking elicited by NMDA was measured for 4-min periods. Each point represents the mean and S.E.M. from six rats. *a)* *p* < 0.05 when compared with vehicle.

Fig. 1. The Aversive Responses to Various Doses (0.3–10 nmol/rat) of SP Injected Intrathecally in Rats

▲, 0.3; ■, 1.0; ▼, 3.0; ●, 10.0; ○, vehicle. Agent was injected at time 0. The duration of biting, licking and scratching elicited by SP was measured for 4-min periods. Each point represents the mean and S.E.M. from six rats. *a)* *p* < 0.05 when compared with vehicle.

Fig. 3. The Aversive Responses to Various Doses (0.3–3.0 nmol/rat) of KA Injected Intrathecally in Rats

▲, 0.3; ■, 1.0; ▼, 3.0; ○, vehicle. Agent was injected at time 0. The duration of biting and licking elicited by KA was measured for 4-min periods. Each point represents the mean and S.E.M. from six rats. *a)* *p* < 0.05 when compared with vehicle.

Fig. 4. The Aversive Responses to Various Doses (0.3—3.0 nmol/rat) of AMPA Injected Intrathecally in Rats

▲, 0.3; ■, 1.0; ▼, 3.0; ○, vehicle. Agent was injected at time 0. The duration of biting and licking elicited by AMPA was measured for 4-min periods. Each point represents the mean and S.E.M. from six rats. *a*) $p < 0.05$ when compared with vehicle.

Fig. 5. The Antagonist Effect of Intrathecal CP-96,345 on the Aversive Behavior Elicited by Intrathecal Injection of SP and EAA Agonists (NMDA, KA and AMPA) in Rats

Various doses (0.3—10 nmol/rat) of CP-96,345 were co-administered with either SP (10 nmol/rat), NMDA (10 nmol/rat), KA (3.0 nmol/rat) or AMPA (3.0 nmol/rat) in a 10 μ l volume. The duration of biting, licking and scratching was measured for the first 4-min of each period. The doses of CP-96,345 are indicated under each bar. The number of experiments in each group is shown in parentheses. The vertical line indicates S.E.M. *a*) $p < 0.05$ when compared with agonist alone.

Fig. 6. The Antagonist Effect of Intrathecal APV on the Aversive Behavior Elicited by Intrathecal Injection of EAA Agonists (NMDA and KA) and SP in Rats

Various doses (0.3—10 nmol/rat) of APV were co-administered with either NMDA (10 nmol/rat), KA (3.0 nmol/rat) or SP (10 nmol/rat) in a 10 μ l volume. The duration of biting, licking and scratching was measured for the first 4-min of each period. The doses of APV are indicated under each bar. The number of experiments in each group is shown in parentheses. The vertical line indicates S.E.M. *a*) $p < 0.05$ when compared with agonist alone.

SP, NMDA, KA and AMPA (Fig. 5) The intrathecal co-administration of CP-96,345, at doses of 0.3—3.0 nmol/rat, inhibited in a dose-dependent manner the aversive behavior elicited by SP (10 nmol/rat), although the inhibitory effect of a higher dose of 10 nmol/rat was no larger than that of 3.0 nmol/rat. A significant 71.1% inhibition was produced by CP-96,345 at a dose of 3.0 nmol/rat. The co-administration of CP-96,345 (3.0 nmol/rat) failed to affect the aversive behavior elicited by NMDA (10 nmol/rat), KA (3.0 nmol/rat) or AMPA (3.0 nmol/rat). An intrathecal injection of CP-96,345 (3.0 nmol/rat) alone had no apparent effect on motor function and gross behavior.

Effects of APV on Behavioral Responses Elicited by NMDA, KA and SP (Fig. 6) Although the intrathecal co-administration of APV at doses of 0.3 and 1.0 nmol/rat did not suppress the aversive behavior elicited by NMDA (10 nmol/rat), higher doses of APV inhibited in a dose-dependent manner the NMDA action by 32.5 and 94.2% at doses of 3.0 and 10 nmol/rat, respectively. The

Fig. 7. The Antagonist Effect of Intrathecal CNQX on the Aversive Behavior Elicited by Intrathecal Injection of EAA Agonists (KA, AMPA and NMDA) and SP in Rats

Various doses (0.3–10 nmol/rat) of CNQX were co-administered with either KA (3.0 nmol/rat), AMPA (3.0 nmol/rat), NMDA (10 nmol/rat) or SP (10 nmol/rat) in a 10 μ l volume. The duration of biting, licking and scratching was measured for the first 4-min of each period. The doses of CNQX are indicated under each bar. The number of experiments in each group is shown in parentheses. The vertical line indicates S.E.M. a) $p < 0.05$ when compared with agonist alone.

co-administration of APV (10 nmol/rat) had no significant effect on the KA (3.0 nmol/rat)-elicited aversive behavior. However, the aversive responses induced by SP (10 nmol/rat) were significantly inhibited by the co-administration of APV (10 nmol/rat), reaching 18.4% of the control value (SP alone). An intrathecal injection of APV (10 nmol/rat) alone had no apparent effect on motor function and gross behavior.

Effects of CNQX on Behavioral Responses Elicited by KA, AMPA, NMDA and SP (Fig. 7) When co-administered intrathecally, CNQX at doses of 0.3–1.0 nmol/rat suppressed in a dose-dependent manner the aversive behavior elicited by KA (3.0 nmol/rat); a significant 76.7% suppression was observed at 10 nmol/rat. CNQX (10 nmol/rat) also produced significant inhibition 73.5 and 66.2% of the behavioral effects of AMPA (3.0 nmol/rat) and SP (10 nmol/rat), respectively, but had no significant effect on the aversive behavior induced by NMDA (10 nmol/rat). CNQX (10 nmol/rat) alone and the vehicle (50% DMSO in saline) had no apparent effect on motor function and gross behavior.

Discussion

In the present experiments, the SP antagonist CP-96,345 at an intrathecal dose of 3 nmol/rat significantly suppressed the behavioral effect of intrathecal SP, without affecting the action of NMDA, KA and AMPA. CP-96,345 has been shown to interact with Ca^{2+} channels,³⁵ which raises the possibility that such an action, rather than the blocking of the NK-1 receptors, is responsible for the inhibition of SP-induced behavior. However, CP-96,345 at 3 nmol/rat produced neither inhibition of the EAA-induced behavior nor suppression of motor function. It has been reported that CP-96,345 does not produce motor weakness even at high intrathecal doses up to about 350 nmol (200 μ g).³⁶ Therefore, the inhibition by CP-96,345 of SP-induced behavior may be due to blockade of the NK-1 receptors.

Aversive behavior induced by intrathecal SP was suppressed by either APV, an NMDA antagonist, or CNQX, a non-NMDA antagonist. It has been demonstrated

that CNQX, at higher concentrations, inhibited the responses to NMDA as well as KA in the dissociated dorsal horn neurons of the rat.³⁷ Since in the present experiments, CNQX at an intrathecal dose of 10 nmol/rat inhibited behavioral responses to KA and AMPA but not to NMDA, this antagonist at least at the dose used may not block NMDA receptors. Therefore, activation of both NMDA and non-NMDA receptors may be involved in the behavioral effects of intrathecal SP. Taking account of findings that exogenous SP increases the release of Glu and aspartate from the rat spinal cord,^{27,38} our results suggest that the aversive behavior produced by an intrathecal injection of SP is partly due to the release of these EAAs induced by this neuropeptide. As SP has been demonstrated to potentiate the excitatory responses to NMDA, KA or AMPA in the dissociated spinal dorsal horn neurons of the rat³⁹ and those of monkey spinothalamic neurons to NMDA,⁴⁰ such a post-synaptic effect of SP may also be involved in the behavioral action of intrathecal SP. In contrast to our present results, experiments using mice have shown that the co-administration of phencyclidine or APV inhibited the behavioral responses to intrathecal NMDA, but not to intrathecal SP.⁷ This discrepancy may be partly due to a species difference.

The behavioral effects of NMDA and non-NMDA agonists were suppressed by corresponding antagonists, but not by CP-96,345. This failure of CP-96,345 to inhibit the action of NMDA is consistent with experiments in mice,²⁸ in which [D-Phe⁷,D-His⁹]SP(6–11), an NK-1 selective SP analogue, did not inhibit aversive behavior induced by intrathecal NMDA. The results suggest that SP and NK-1 receptors are not involved in the aversive behavior induced by activation of NMDA and non-NMDA receptors in the spinal cord.

In summary, the present results suggest that the aversive behavior induced by intrathecal SP is mediated not only by the activation of NK-1 receptors but also by the activation of NMDA and non-NMDA receptors, probably through SP-induced release of glutamate from the spinal cord. The aversive behavior induced by intrathecal injection of

NMDA and non-NMDA agonists may be mediated by the activation of the corresponding glutamate receptors, but not by NK-1 receptors.

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