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Intrathecal substance P (1–7) prevents morphine-evoked spontaneous pain behavior via spinal NMDA-NO cascade

Tsukasa Sakurada [a,*], Takaaki Komatsu [a], Hikari Kuwahata [a], Chizuko Watanabe [b], Toru Orito [b], Chikai Sakurada [c], Minoru Tsuzuki [c], Shinobu Sakurada [b]

[a] First Department of Pharmacology, Daiichi College of Pharmaceutical Sciences, 22-1 Tamagawa-cho, Minami-ku, Fukuoka 815-8511, Japan
[b] Department of Physiology and Anatomy, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan
[c] Department of Biochemistry, Nihon Pharmaceutical University, 10281, Komuro Ina-Machi Kitaadachi-gun, Saitama 362-0806, Japan

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ABSTRACT

Previous research has shown that injection of high-dose of morphine into the spinal lumbar intrathecal (i.t.) space of rats elicits an excitatory behavioral syndrome indicative of severe vocalization and agitation. Substance P N-terminal fragments are known to inhibit nociceptive responses when injected i.t. into animals. In this study, we investigated the effect of i.t. substance P (1–7) on both the nociceptive response and the extracellular concentrations of glutamate and nitric oxide (NO) metabolites (nitrite/nitrate) evoked by high-dose i.t. morphine (500 nmol). The induced behavioral responses were attenuated dose-dependently by i.t. pretreatment with the substance P N-terminal fragment substance P (1–7) (100–400 pmol). The inhibitory effect of substance P (1–7) was reversed significantly by pretreatment with [D-Pro^2, D-Phe^7]substance P (1–7) (20 and 40 nmol), a D-isomer and antagonist of substance P (1–7). In vivo microdialysis analysis showed a significant elevation of extracellular glutamate and NO metabolites in the spinal cord after i.t. injection of high-dose morphine (500 nmol). Pretreatment with substance P (1–7) (400 pmol) produced a significant reduction on the elevated concentrations of glutamate and NO metabolites evoked by i.t. morphine. The reduced levels of glutamate and NO metabolites were significantly reversed by the substance P (1–7) antagonist (40 nmol). The present results suggest that i.t. substance P (1–7) may attenuate the excitatory behavior (vocalization and agitation) of high-dose i.t. morphine by inhibiting the presynaptic release of glutamate, and reducing NO production in the dorsal spinal cord.

1. Introduction

Morphine is widely used as a strong opioid-analgesic compound in the clinical management of moderate to severe pain. Despite widespread use of morphine, its treatment is often accompanied with the development of tolerance to and dependence on opioid analgesics. Analgesic tolerance has led to the higher opioid requirements and increases non-analgesic side effects such as respiratory depession, urinary retention, pruritis and myoclonic seizures. Hyperalgesic responses in animals occur during a precipitated opioid withdrawal. Previous studies have demonstrated that spinal administration of morphine at far higher doses than that required for analgesia produces spontaneous pain-related behavior (nociception) and hyperalgesia, which are naloxone-insensitive in mice and rats [1–3]. This observation suggests that spontaneous nociceptive behaviors and sensory hypersensitivity evoked by high-dose intrathecal (i.t.) morphine are not an opioid-mediated event.

* *Corresponding author*. Tel.: +81 92 541 0161; fax: +81 92 553 5698.
E-mail address: tsukasa@daiichi-cps.ac.jp (T. Sakurada).
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Clinical reports have also implicated that similar changes in pain sensitivity may also occur in pain patients after spinal administration of high-dose of morphine [4–7].

Substance P has been implicated as a major neurotransmitter/neuromodulator of pain in the spinal cord [8]. The i.t. administration of substance P causes a behavioral series of spontaneous scratching, biting and licking, and a hyperalgesic response to noxious stimuli. Furthermore, antagonists to neurokinin (NK_1) receptors were reported as having antinociceptive effect in mice [9–11], which provides additional strong support for the involvement of substance P in pain transmission and modulation in the dorsal horn of spinal cord. In contrast to a pivotal role of substance P in pain transmission, a major metabolite of substance P appears to be the N-terminal heptapeptide substance P (1–7) [12–14], which is known to have the opposite effect to substance P or to bioactive C-terminal substance P fragments. A membrane-bound protease capable of degrading substance P in synaptic region releases N-terminal fragments, substance P (1–7) and (1–8) from the parent peptide [15]. It is interesting to note that substance P specific endopeptidase present in human cerebrospinal fluid (CSF) and rat spinal cord is able to release the N-terminal fragments, substance P (1–7) and (1–8), from the parent peptide [12,16]. Substance P (1–7), the predominant N-terminal metabolite of substance P in the dorsal part of spinal cord [13,14], has been found to inhibit nociceptive behaviors in several nociceptive assays when injected i.t. into mice [17–20].

A role of nitric oxide (NO) in spinal nociceptive processing has been postulated in models of neuropathic pain [21]. NO synthase (NOS) inhibitors such as L-NAME prevent or reduce thermal hyperalgesia following chronic constriction injury. Recently, iNOS has become of significant interest in the phathophysiology of inflammatory and neuropathic pain [22–24]. NO has been shown to be involved in thermal hyperalgesia induced by endogenous and exogenous substance P in rats [25] and in substance P-induced itch-associated responses in mice [26]. In addition, it is of interest to note that an accumulation of substance P (1–7) down-regulates neuronal NOSmRNA and decreases constitutive NOS activity in the spinal cord and dorsal root ganglia [27].

The present study was to determine the effect of i.t. substance P (1–7) on the spontaneous vocalization and agitation responses evoked by high-dose i.t. morphine in rats. In addition, we measured released concentrations of glutamate and nitrite/nitrate by microdialysis in the spinal cord after high-dose i.t. morphine in the presence and absence of substance P (1–7) to determine whether the *N*-methyl-D-aspartate (NMDA)-NO cascade was influenced.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley (SD) rats were obtained from Shizuoka Laboratory (Japan). All behavioral testing occurred when the rats were between 250 and 260 g. The rats were individually housed in a colony maintained in a controlled environment (12-h light:12-h dark cycle, room temperature 23 °C, 50–60% relative humidity). The animals had unlimited access to food and water. All behavioral experiments took place during the light period between 10:00 and 17:00 h in a quiet room. The animals belonging to the various treatment groups ($n = 7$ each group) were tested in randomized order. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals [28]. Additionally, the study was approved by the Committees of Animal Care and Use of Daiichi College of Pharmaceutical Sciences and Tohoku Pharmaceutical University.

2.2. Intrathecal administration

The i.t. administration of compounds to the lumbar region of the spinal cord of rats was performed through a polyethylene catheter 10 μl in a volume. This involved inserting a length of polyethylene tubing (PE-10) following laminectomy between L3 and L4 and carefully placing the catheter tip in the subarachnoid space of L5 and L6 through a slit in the atlanta-occipital membrane [15]. The animals were allowed to recover 7 days following implantation of the catheter. The catheter was filled with sterile artificial cerebrospinal fluid (CSF), containing in (g/L) NaCl 7.4, KCl 0.19, $MgCl_2$ 0.19 and $CaCl_2$ 0.14. Drugs were administered in volumes of 10 μl followed by a flush of 15 μl of artificial CSF to ensure that each compound reached the spinal cord. Substance P (1–7) and [D-Pro^2, D-Phe^7]substance P (1–7) were administered i.t. 2 and 4 min prior to i.t. morphine (500 nmol), respectively. Rats showing motor weakness and signs of hindlimb paralysis upon recovery from anesthesia were immediately sacrificed.

2.3. Measurement of spontaneous behavior

Prior to testing, all rats were handled and habituated to an open Plexiglass chamber (34 cm × 30 cm × 17 cm) for 1 h before actual experimental sessions, which also served as an observation chamber after injection. The handling and habituation protocol were designed to limit stress-induced analgesia that has been reported to be influenced by rat strain [29]. Immediately after morphine (500 nmol) was injected i.t. using a microsyringe with a 26-gauge needle, behavioral observation was immediately started. The i.t. administration of 500 nmol morphine produced a striking behavioral syndrome consisting of spontaneous vocalization and agitation. The magnitudes of two behavioral responses were quantified; vocalization and agitation. Spontaneous vocalization was recorded by the stop-watch during each 5-min interval for a 40-min period after i.t. administration of morphine (500 nmol) in combination with artificial CSF (control), or substance P (1–7) and/or [D-Pro^2, D-Phe^7]substance P (1–7). The latency to induce the first vocalization after i.t. administration of morphine was also recorded. The second parameter studied was the appearance of spontaneous agitation. Spontaneous agitation was ranked visually every 1 min after i.t. administration of 500 nmol morphine in combination with artificial CSF (control), or substance P (1–7) and/or [D-Pro^2, D-Phe^7]substance P (1–7) as 0, no sign of excitation; 1, restlessness, scratching and biting at the flank or tail; 2, mild vocalization with restlessness, scratching and biting at the flank or tail; 3, vocalization with spontaneous running and circling; 4, vigorous vocalization with running, circling, rolling and

jumping. The score of agitation was expressed in each animal during each 5-min interval for a 40-min period after i.t. morphine (500 nmol).

2.4. Microdialysis and implantation

Concentrations of nitrite/nitrate and glutamate were determined using microdialysis. The U-shaped microdialysis cannula consisted of a 6 cm length of microdialysis fiber (200 μm inner diameter, 300 μm outer diameter, 50 kDa molecular weight cut-off; Filtral, AN69-HF). The fiber was coated with a thin layer of epoxy (Araldite, CIBA GEIGI Inc.) except for a 4 cm portion in the middle. In order to make the fiber firm enough for implantation, a Nichrome-Formvar wire (0.0026 in. inner diameter; A-M system, Inc., Everret, WA) was then passed through the fiber and the ends of the fiber were attached to a PE-10 (5.0 cm long) with cyanoacrylate. The fiber was then bent so that a U-shaped loop was formed in the middle of the uncoated portion. Under pentobarbital anesthesia (50 mg/kg, intraperitoneally) the tip of microdialysis cannula was placed in the subarachnoid space of L5 and L6. The dialysis catheters were then externalized on the back of the neck in rats.

2.5. Spinal cord microdialysis and sample collection

The dialysis experiments were performed 3–5 days after i.t. implantation of microdialysis catheters. The dialysis system was attached to a microdialysis pump under re-anesthesia with isoflurane (1.1% throughout the dialysis experiment in a 50:50 air/O_2 mixture). The dialysis tubing was connected to the syringe pump (EO-60, EICOM) and perfused with artificial CSF 30 min prior to sample collection to establish a diffusion equilibrium. Baseline concentrations were defined as the mean of two 10 min control samples taken after the initial 30 min washout period. Dialysate samples were collected at 10-min intervals for the duration of the experiment. The samples were separated for measuring nitrite/nitrate (aliquot 50 μl), and stored at −70 °C for subsequent analysis.

2.6. Measurements of glutamate and NO

The concentration of glutamate in the spinal perfusate was determined by reversed-phase high-performance liquid chromatography (HPLC) (ECD-300, EICOM) with fluorimetric detection following pre-column derivatization with o-phthaldialdehyde (OPA). Chromatography was performed on a reversed-phase C-18 column (2.1 × 150 mm, EICOM PACKED COLUMN) using a pH sodium acetate methanol gradient. The minimal detectable concentration of glutamate was 5–10 pmol/30 μl.

NO was measured as its breakdown products, nitrite (NO_2^-) and nitrate (NO_3^-). The measurement of nitrite/nitrate was made using a commercially available NO analyzer (MODEL-280NOA, Sievers, Inc.) and integrator (HO3396, Sievers, Inc.). The limit of detection of nitrite/nitrate was 1–2 pmol/30 μl.

2.7. Drugs

Drugs used and their sources were: morphine hydrochloride (Takeda, Osaka, Japan) and substance P (1–7) (Sigma Chemical Co., St. Louis, MO, USA). [D-Pro^2, D-Phe^7]substance P (1–7) was synthetized by solid phase methodology. All drugs for spinal administration were dissolved in artificial CSF. The dose, 500 nmol of morphine, was chosen since given alone it elicited a reproducible behavioral response and a significant release of glutamate and nitrite/nitrate from the spinal cord [3].

2.8. Statistics

The time-course effects of substance P (1–7) on excitatory behaviors (vocalization and agitation) evoked by high-dose i.t. morphine are presented as the mean ± S.E.M. of accumulated vocalization times and agitation score. Differences between treatment groups were evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's test. A two-way repeated measures ANOVA was used to evaluate the time-course data followed by Bonferroni's test. Differences are considered significant at a P-value of less than 0.05.

3. Results

3.1. Behavioral studies

3.1.1. Effect of substance P (1–7) on vocalization and agitation evoked by high-dose i.t. morphine

The i.t. administration of morphine at a high dose of 500 nmol into the spinal lumbar space evoked a spontaneous agitation such as vigorous vocalization accompanied by hindlimb scratching toward the flanks, and biting of hindpaw or the base of the tail, running and jumping, and occasionally myoclonic seizures. Vocalization and agitation evoked by i.t. morphine (500 nmol) peaked at 5–10 and 5–15 min, respectively. These behavioral characteristics of i.t. morphine at the high dose (500 nmol) confirms our previous reported data [3]. Fig. 1 shows the effect of i.t. administration of substance P (1–7) on the excitatory responses, vocalization and agitation, evoked by i.t. 500 nmol morphine ($n = 7$ for each group). In control group pretreated with artifical CSF, the total vocalization time for the 40-min observation after i.t. administration of 500 nmol morphine was 433.3 ± 10.4 s (Fig. 1A). The time-

Table 1 – Latency to first vocalization following intrathecal administration of high-dose morphine in combination with artificial CSF, substance P (1–7) and/or [D-Pro^2, D-Phe^7]substance P (1–7) in rats

Treatments (i.t.)	Latency (s)
CSF plus morphine	68.5 ± 5.4
SP (1–7) (100 pmol) + morphine	61.1 ± 5.7
SP (1–7) (200 pmol) + morphine	67.2 ± 6.9
SP (1–7) (400 pmol) + morphine	67.4 ± 7.3
D-SP (1–7) (10 pmol) + SP (1–7) (400 pmol) + morphine	60.3 ± 5.6
D-SP (1–7) (20 pmol) + SP (1–7) (400 pmol) + morphine	60.4 ± 5.8
D-SP (1–7) (40 pmol) + SP (1–7) (400 pmol) + morphine	60.3 ± 6.4
D-SP (1–7) (40 pmol) + morphine	61.9 ± 5.7

Substance P (1–7) and [D-Pro^2, D-Phe^7]substance P (1–7) were administered i.t. 2 and 4 min prior to i.t. morphine (500 nmol), respectively. Data are shown as the mean ± S.E.M. of seven rats. SP (1–7): substance P (1–7); D-SP (1–7): [D-Pro^2, D-Phe^7]substance P (1–7).

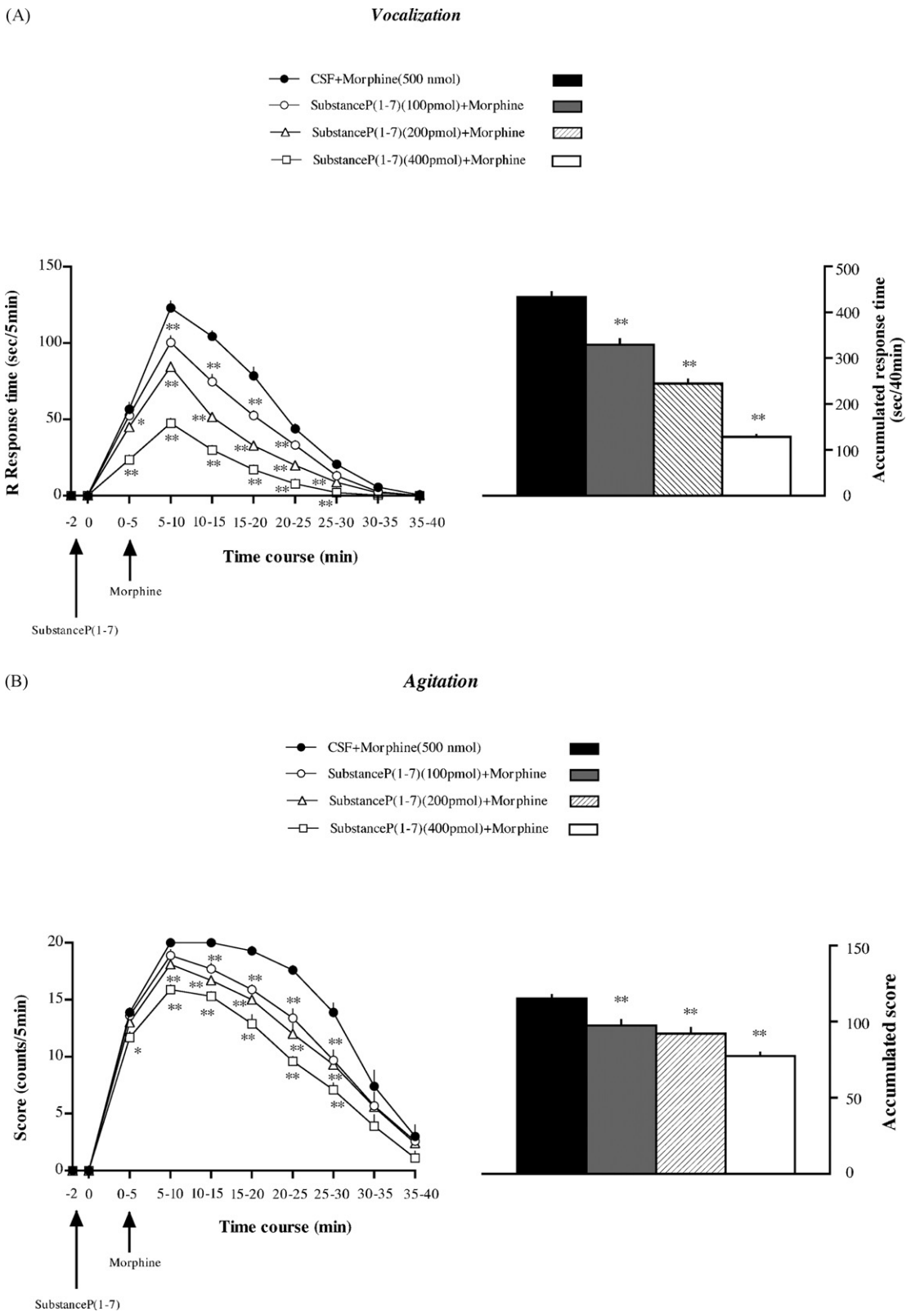

Fig. 1 – Effect of substance P (1–7) on vocalization (A) and agitation (B) evoked by intrathecal (i.t.) administration of morphine (500 nmol) in rats. Substance P (1–7) was administered i.t. 2 min prior to i.t. morphine. (A) Duration of vocalization time was observed during each 5-min interval for the 40-min period (left panel). Each bar represents the total vocalization time for the 40 min period (right panel). (B) Agitation was scored every 1 min for the 40-min period. Each point represents the accumulated score of agitation during each 5-min interval (left panel). Each bar represents the total score of agitation for the 40-min period (right panel). Data are shown as the mean ± S.E.M. of seven rats. $^{**}P < 0.01$; $^{*}P < 0.05$ when compared to CSF plus morphine (500 nmol).

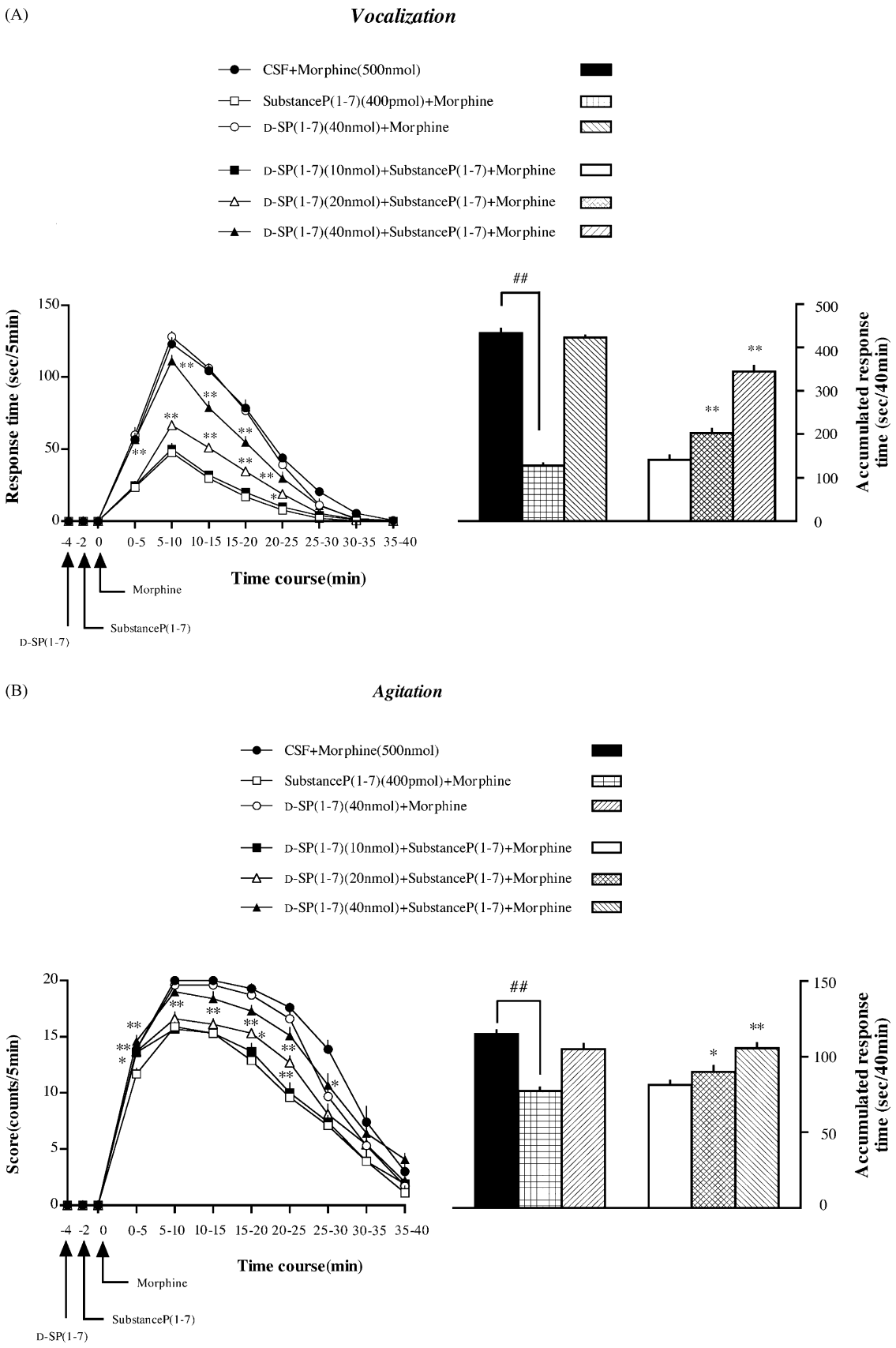

Fig. 2 – Pretreatment effect of [D-Pro^2, D-Phe^7]substance P (1–7) on the substance P (1–7)-induced inhibition in behavioral responses to intrathecal (i.t.) morphine (500 nmol). [D-Pro^2, D-Phe^7]substance P (1–7) and substance P (1–7) were administered i.t. 4 and 2 min prior to i.t. morphine, respectively. (A) Duration of vocalization time was observed during each

course analysis indicated that morphine-evoked vocalization was inhibited by i.t. pretreatment with substance P (1–7) at doses of 100, 200 and 400 pmol (Fig. 1A. $P < 0.001$, two-way repeated measures ANOVA). The appearance of the first vocalization response was unchanged in rats pretreated with substance P (1–7) at doses employed in the experiment (Table 1). In artifical CSF-pretreated group, the total agitation score for the 40-min observation after i.t. administration of 500 nmol morphine was 115.0 ± 2.4 (Fig. 1B). Pretreatment with substance P (1–7) (100–400 pmol) caused a small but significant reduction of morphine-evoked agitation in a dose-dependent manner (Fig. 1B, $P < 0.001$, two-way repeated measures ANOVA).

3.1.2. *Effect of [D-Pro^2, D-Phe^7]substance P (1–7) on substance P (1–7)-induced inhibition in behavioral responses to high-dose i.t. morphine*

The time courses for the vocalization and agitation responses are shown in Fig. 2A and B, and demonstrate that the inhibitory effect of substance P (1–7) on these behavioral responses evoked by high-dose i.t. morphine was reversed by i.t. pretreatment with the substance P (1–7) antagonist [D-Pro^2, D-Phe^7]substance P (1–7) (20 and 40 nmol) 4 min before morphine administration (400 nmol, i.t.) ($P < 0.001$, two-way repeated measures ANOVA). A lower dose of 10 nmol [D-Pro^2, D-Phe^7]substance P (1–7) failed to block the inhibitory effect of substance P (1–7) on morphine-induced vocalization and agitation. [D-Pro^2, D-Phe^7]substance P (1–7) alone at a maximum dose of 40 nmol used in the experiment had no significant effect on morphine-induced vocalization and agitation.

3.2. *Microdialysis study*

Baseline levels of NO breakdown products, nitrite/nitrate, and glutamate were detectable in all rats treated under isoflulane through the microdialysis study. The means of baseline samples collected before i.t. administration was used for determination of nitrite/nitrate and glutamate in the dorsal spinal cord extracellular fluid. Fig. 3 shows the effect of substance P (1–7) on the elevated concentrations of extracellular nitrite/nitrate and glutamate evoked by 500 nmol morphine ($n = 7$ for each group). The high-dose of morphine after artificial CSF evoked a significant elevation of nitrite/nitrate release in the time intervals of 10–20 and 20–30 min post-i.t. administration (Fig. 3A). Pretreatment with substance P (1–7) at a dose of 400 pmol, 2 min prior to i.t. administration of morphine (500 nmol), attenuated an elevated concentration of nitrite/nitrate in samples collected in the time interval of 10–20 min following i.t. administration of morphine (Fig. 3A, $P < 0.05$, two-way repeated measures ANOVA). The effect of substance P (1–7) on the release of nitrite/nitrate was not antagonized significantly by pretreatment with the substance P (1–7) antagonist [D-Pro^2, D-Phe^7]substance P (1–7) (40 nmol).

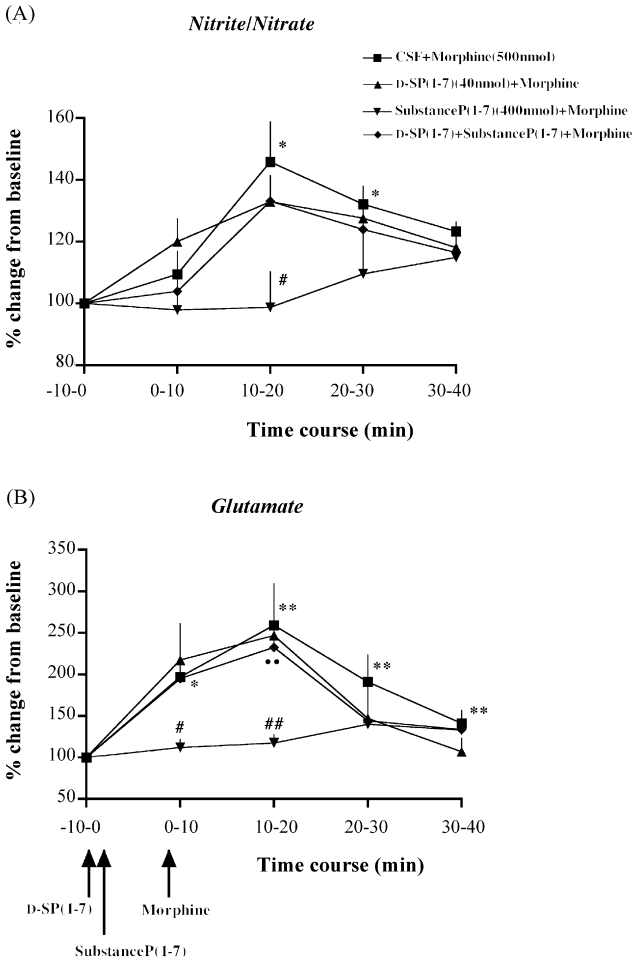

Fig. 3 – Effects of substance P (1–7) and its antagonist [D-Pro^2, D-Phe^7]substance P (1–7) on the increased spinal release of nitrite/nitrate (A) and glutamate (B) evoked by intrathecal (i.t.) administration of morphine (500 nmol). Substance P (1–7) and [D-Pro^2, D-Phe^7]substance P (1–7) were administered i.t. 2 and 4 min prior to i.t. morphine, respectively. Concentrations of nitrite/nitrate and glutamate in spinal microdialysate are expressed as the mean ± S.E.M. percentage change from baseline. Each value on the graph represents the mean ± S.E.M. for seven rats. $^{**}P < 0.01$, $^{*}P < 0.05$ when compared to the basal level. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ when compared to CFS plus morphine. $^{\bullet\bullet}P < 0.01$ when compared to substance P (1–7) plus morphine. D-SP (1–7); [D-Pro^2, D-Phe^7]substance P (1–7).

[D-Pro^2, D-Phe^7]substance P (1–7) (40 nmol) alone had no effect on morphine (500 nmol)-evoked release of nitrite/nitrate.

Morphine, injected i.t. into artificial CSF-pretreated control rats, produced a remarkable increase in glutamate concentration during the first 30 min when compared to baseline levels (Fig. 3B). Two-way repeated measures ANOVA revealed that

5-min interval for the 40-min period (left panel). Each bar represents the total vocalization time for the 40 min period (right panel). (B) Agitation was scored every 1 min for the 40-min period. Each point represents the accumulated score of agitation during each 5-min interval (left panel). Each bar represents the total score of agitation for the 40-min period (right panel). Data are shown as the mean ± S.E.M. of seven rats. $^{\#}P < 0.05$ when compared to CSF plus morphine. $^{**}P < 0.01$; $^{*}P < 0.05$ when compared to substance P (1–7) plus morphine. D-SP (1–7): [D-Pro^2, D-Phe^7]substance P (1–7).

the i.t. administration of substance P (1–7) (400 pmol) prior to i.t. morphine (500 nmol) reduced the elevated concentration of glutamate evoked by i.t. morphine in the fractions collected during the first 10 min and 10–20 min (Fig. 3B, $P < 0.01$, two-way repeated measures ANOVA). This clear-cut action of substance P (1–7) was reversed by pretreatment with the substance P (1–7) antagonist [D-Pro^2, D-Phe^7]substance P (1–7) (40 nmol) (Fig. 3B, $P < 0.05$, two-way repeated measures ANOVA). The significantly elevated concentration of glutamate evoked by i.t. morphine was not altered by pretreatment with [D-Pro^2, D-Phe^7]substance P (1–7) (40 nmol) alone.

4. Discussion

The present study demonstrates that i.t. administration of the N-terminal fragment of substance P, substance P (1–7), inhibited spontaneous vocalization and agitation after high-dose i.t. morphine in rats. Substance P (1–7), injected i.t. at pmol doses, attenuated behavioral signs of vocalization response rather than agitation seen after high-dose i.t. morphine. The significantly elevated concentrations of glutamate and nitrite/nitrate evoked by high-dose i.t. morphine were inhibited by i.t. pretreatment with substance P (1–7). This definite effect of substance P (1–7) in behavioral and microdialysis experiments was reversed significantly by its antagonist [D-Pro^2, D-Phe^7]substance P (1–7). It is thus likely that an important mechanism for spinal action of substance P (1–7) is through presynaptic inhibition in increased releases of glutamate and nitrite/nitrate evoked by high-dose i.t. morphine in primary afferent fibers of the spinal cord level.

Animals studies have documented spontaneous or precipitated withdrawal hyperalgesia after acute and chronic exposure [30–35]. Hyperalgesia could be not only produced by stopping opioid exposure, but also developed during its continuous administration [30,34]. In addition, single i.t. administration of morphine at a high-dose concentration into opioid naive animals leads to hyperalgesia in response to noxious stimuli [36]. Several lines of evidence indicate that single i.t. administration of high-dose morphine elicits a spontaneous excitatory behavior such as vocalization and agitation accompanied by scratching, biting and licking, and touch-evoked allodynia in rats and mice [1–3,37,38]. These enhanced responses have been observed in pain patients with opioid therapy. Indeed, it has been shown in some clinical studies that spontaneous or precipitated withdrawal hyperalgesia occurs in pain patients after short- and long-term opioid administration [39–42]. It should be also noted in clinical reports that patients receiving a high-dose of opioid rather than a low opioid dose during surgery suffered from increased postoperative pain despite a greater opioid consumption [43,44].

The mechanism underlyling abnormal pain evoked by high-dose i.t. morphine has yet to be elucidated. In this study, we demonstrate that a marked inhibitory effect on morphine-associated vocalization is obtained by i.t. administration of substance P (1–7). This result fits in well with results from our previous study in mice, indicating that pain-related behavior (scratching, biting and licking) evoked by high-dose i.t. morphine could be preventable by i.t. co-administration of substance P (1–7) at a pmol amount order [2]. In prior binding studies utilizing brain and spinal cord in mice and rats, [D-Pro^2, D-Phe^7]substance P (1–7) has been shown to inhibit [^{3}H]substance P (1–7) binding [45,46]. Behavioral experiments have also demonstrated that the double D-amino-substituted analogue of substance P (1–7) prevents the antinociceptive effects of i.t. injected substance P (1–7) and capsaicin [47,48]. Consistent with these results, we found that pretreatment with the substance P (1–7) antagonist [D-Pro^2, D-Phe^7]substance P (1–7) prevented the inhibitory effect of substance P (1–7) on morphine-induced vocalization and agitation. These results suggest an interaction with N-terminally directed substance P binding sites in the spinal cord, possibly a specific binding site for substance P (1–7).

NMDA receptors are believed to play a pivotal role of the nociceptive processing in the spinal cord, and in the development of opioid-associated hyperalgesia or allodynia. NMDA receptor antagonists block pain transmission in dorsal horn spinal neurons [49,50], and pain-related behavior in animal pain model [51,52]. It is conceivable that activation of the NMDA receptors at the level of the spinal cord is critical for the expression of pain-related behavior in mice [53] and vocalization in rats [3]. In fact, we have previously demonstrated that i.t. co-administration of the competitive antagonists, D-APV and CPP, and the non-competitive antagonist MK-801, both inhibited the morphine-evoked behavioral responses in mice [53]. Similar to the experiment in mice, morphine-evoked behavioral excitation (vocalization and agitation) could be inhibited by pretreatment with the NMDA receptor antagonists in rats [3]. These NMDA receptor antagonists have also been found to inhibit an increase of glutamate concentration in the extracellular level after i.t. administration of high-dose morphine.

The present study has revealed that i.t. administration of substance P (1–7), a major metabolite of substance P, produced a dose-dependent reduction on the vocalization and agitation responses evoked by high-dose i.t. morphine. Regarding the degree of substance P (1–7) effectiveness, it should be noted that the inhibitory percentages of substance P (1–7) (400 pmol) on morphine-evoked vocalization and agitation were approximately 71 and 33%, respectively. These results agree with previous observations in the same model using NMDA receptor antagonists [3]. It is conceivable that substance P (1–7) could prevent preferentially the nociceptive vocalization evoked by morphine rather than the agitation including not only nociceptive behaviors (scratching or biting) but also spontaneous motor components (running or circling). Overall, these data seem to suggest that vocalization may be more important than agitation in expressing morphine-evoked nociception. In the present study, however, i.t. substance P (1–7) failed to prolong the first vocalization after i.t. administration of 500 nmol morphine. This contrasts with the result of NMDA receptor antagonists, in which the appearance of the first vocalization response was prolonged significantly by pretreatment with NMDA receptor antagonists [3]. The difference may be due to the different site of action between substance P (1–7) and NMDA receptor antagonists. It has been shown that substance P (1–7) decreases the release of glutamate at the primary afferent terminals, which may involve presynaptic inhibition of glutamate release [54]. This

may be an important mechanism for the inhibitory effect of substance P (1–7) in response to high-dose i.t. morphine. It should be noted in the present study that a relatively modest concentration of substance P (1–7) could inhibit glutamate release evoked by i.t. morphine. Presynaptic NMDA glutamate receptors are located on substance P-containing primary afferents to facilitate nociception through the release of glutamate and substance P [55,56]. On the other hand, there is functional evidence that activation of some of presynaptic NMDA receptors inhibits glutamate release from primary sensory neurons in the rat spinal dorsal horn [57]. These observations led us to speculate that noxious stimulation evokes the release of substance P, which is degraded enzymatically into substance P (1–7), and substance P (1–7) may act presynaptically to reduce the release of glutamate and substance P. It is plausible that there may be a presynaptic negative autoregulation by substance P (1–7) in primary afferent terminal. This notion is further supported by the microdialysis experiment in the present study that morphine-evoked increase of glutamate was inhibited 0–10 min after i.t. administration of substance P (1–7). This contrasts to the previous results that NMDA receptor antagonists, CPP and MK-801, could not prevent the increased release of glutamate during the first 10 min, but a significant effect of these compounds was observed 10 min afterwards. The time difference in appearance of first vocalization response seen after i.t. administration of substance P (1–7) and NMDA receptor antagonist may be explained by presynaptic and postsynaptic inhibition of glutamate. We cannot rule out the involvement of postsynaptic action following i.t. administration of substance P (1–7). Scratching, licking and biting elicited by i.t. substance P have been interpreted as a direct activation on the postsynaptic site in the spinal cord neurons. The NK_1 receptor agonist-induced behavior could be reduced by i.t. co-administration of substance P (1–7) [17–19], suggesting the possible postsynaptic inhibition of substance P (1–7). Taken together, the evidence suggests that substance P (1–7) inhibits synaptic transmission in the dorsal horn spinal cord neurons reducing transmitter release presynaptically and acting substance P (1–7) binding sites postsynaptically.

NO is believed to contribute to synaptic transmission between primary afferent neurones and dorsal horn cells in spinal nociceptive processing. The release of NO could be required for facilitated synaptic transmission in the spinal cord, since inhibitors of NO synthase diminish nociceptive behavior in variety of pain models [23,24,58–60], whereas spinally delivered NO-donor at high doses causes hyperalgesia [61]. Similar to these previous findings, we have reported a significant elevation in nitrite/nitrate concentration following the spinal administration of morphine at a high-dose of 500 nmol [3]. There is now considerable evidence for roles of NMDA receptor activity and NO production in the nociceptive processing. It is widely held concept that the activation of the postsynaptic NMDA receptors leads to a calcium influx that triggers the production of NO, which diffuses intracellularly to stimulate further glutamate release [62]. Thus, a role of NO in the nociception evoked by high-dose i.t. morphine would not be unexpected. Inhibiting the release of glutamate by i.t. administration of substance P (1–7) has therefore produced a similar effect to that of NMDA receptor antagonism, and this, in turn, could be expected to attenuate NO production in neurons with NMDA receptors.

In conclusion, our work demonstrates the effectiveness of i.t. substance P (1–7) in attenuating vocalization and agitation responses in rat model of spinally mediated morphine-induced pain. Substance P (1–7) also led to inhibit the elevated concentration of extracellular glutamate and NO metabolites evoked by high-dose i.t. morphine. The effect of substance P (1–7) in the behavioral and microdialysis experiments was reversible completely by the substance P (1–7) antagonist [D-Pro^2, D-Phe^7]substance P (1–7). The present results suggest that spinal glutamate release and NO metabolites might play a critical role in spinal excitatory synaptic neurotransmission. This study also has implications for clinical use of substance P (1–7) to provide effective treatment for morphine-evoked pain or hyperalgesia.

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