Research report

Substance P microinjected into the periaqueductal gray matter induces antinociception and is released following morphine administration

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Abstract

The aims of the present study were to investigate, in rats, the behavioral effects of substance P (SP) microinjected into the ventrolateral periaqueductal gray (PAG) and the effects of the neurokinin 1 (NK-1) receptor antagonist [D-Arg¹, D-Trp⁷, ⁹, Leu¹¹]-substance P (Spantide). The effect of morphine administration on the release of SP in the ventrolateral PAG was also investigated using microdialysis in awake rats. SP microinjected into the ventrolateral part of the PAG induced significant increases in the hindpaw withdrawal latencies (HWLs) to thermal and mechanical stimulation as an antinociceptive response. The NK-1 receptor antagonist blocked these effects but exhibited no antinociceptive effect alone. Subcutaneous administration of morphine increased basal SP-like immunoreactivity (SP-LI) release in the microdialysate obtained from the ventrolateral PAG of freely moving rats. Our results demonstrate that SP injected into the ventrolateral PAG induces an antinociceptive effect via activation of NK-1 receptors. Morphine administered systemically induces the release of SP in the ventrolateral PAG. We suggest that an increased release of SP in the PAG may contribute to opioid antinociception.

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1. Introduction

Substance P (SP) is a neuropeptide belonging to the tachykinin family and most likely acts as a neuromodulator in both nociception [30,35] and antinociception [3,5]. Noxious stimuli induce the release of SP from primary afferent terminals and activate NK-1 receptors in the dorsal horn of the spinal cord [17,29,30,37]. Efforts have been made to prove that NK-1 receptor antagonists will block nociception [15]. These expectations have not been fulfilled. In preclinical studies, although decreased responses to nociception have been found in animals sensitized by inflammation or nerve damage, NK-1 receptor antagonists have little effect on baseline nociception [27]. In clinical studies, no analgesic effects have been found for NK-1 receptor antagonists [7].

There is evidence that SP may activate the descending antinociceptive pathways [6,11,48]. SP is present in both cell bodies and terminals in the PAG [45,46], as are NK-1 receptors [2], and has a naloxone-reversible antinociceptive effect when injected into the PAG or intracerebroventricularly [20,27]. In rabbits, injections of SP antibodies into the PAG have been reported to decrease the effect of acupuncture [14].

The PAG may be the central area where the activation of the antinociception that SP seems to be involved in takes place. In the late 1960s, the antinociceptive effect of electrical stimulation in the PAG was demonstrated in rats [31,32,38]. The discovery of stimulation-produced analgesia (SPA) was followed by intense research. Today, the ventrolateral PAG is known to play a central role in endogenous antinociception [3,5,44]. The PAG projects rostrally to the medial thalamus and orbital frontal cortex [10]. The major descending neuronal outflow from the ventrolateral PAG goes via the nucleus raphe magnus down...
to the dorsal spinal cord where the inhibition of noxious stimuli will take place [3].

Morphine is generally believed to produce analgesia. The analgesic effect of morphine is thought to have both spinal and supraspinal mechanisms of action. Supraspinally, PAG is regarded to be the main target of morphine. We found in earlier studies that morphine (in 1, 3, or 10 mg/kg doses) given systemically in rats increases the tissue levels of SP in the PAG in a dose-dependent fashion [39].

In the present study, we wished to investigate the behavioral effects of SP and the NK-1 receptor antagonist microinjected into the ventrolateral PAG and the effect of systemic administration of morphine on the release of SP in the ventrolateral PAG.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were housed in cages in groups of five animals at constant room temperature (22 ± 2 °C) and exposed to a 12:12-h light/dark cycle (light onset at 0600 h). All experiments were performed in the daytime (1000–1500 h). The Ethics Committee for Animal Experiments approved all experimental protocol. Initially, the animals were randomly assigned to treatment. Food and water were provided ad libitum. Following delivery, the animals were allowed to adapt to the environment for at least 1 week before the experiments were begun. When the experiments were finished, the rats were killed by an overdose of halothane or sodium pentobarbital or were decapitated.

The microdialysis experiments were performed at Karolinska Institute in Stockholm, Sweden, and the behavioral studies at Peking University in Beijing, China.

2.2. Preparation for intra-PAG injections

The animals (n = 39) were anesthetized with intraperitoneal (i.p.) sodium pentobarbital (45 mg/kg, from Apoteksbolaget, Sweden) and were mounted on a stereotaxic instrument. A stainless steel guide cannula with an outer diameter of 0.8–1.0 mm was inserted in the PAG [coordinates from the bregma: anteroposterior (AP) = 7.6 mm, lateralmedial (LM) 0.5 mm, and dorsoventral (DV) = 4.0 mm according to Paxinos and Watson [36] and measured from the surface of the brain] and fixed to the skull with dental acrylic. After surgery, the animals were returned to their cages and housed individually. The experiments began after 2 days of recovery. On the experimental days, a stainless steel needle (diameter 0.4 mm) was inserted into the guide cannula until the needle was 2 mm beyond the tip of the cannula. One microliter of solution was then infused into the PAG during 1 min. At the end of the experiments, the rats were killed with an intraperitoneal dose of sodium pentobarbitol (80 mg/kg) and the brain was dissected out and fixed in 10% formalin solution for 1 week with the guide cannula in situ. The location of the tip of the guide cannula was verified in serial, 50-μm crystal sections. Only the results from animals where the tip of the guide cannula was located within the ventrolateral PAG were used for statistical analysis.

2.3. Hindpaw withdrawal latency (HWL)

All rats were accustomed to the test conditions for 5 days before the experiment was conducted. The latencies to hindpaw withdrawal (HWL) during thermal and mechanical stimulation were measured [50]. The thermal response was assessed by the hot-plate test. The entire plantar surface of the rat’s hindpaw was placed on a hot plate maintained at 52 °C (51.8–52.4 °C). HWL was measured in seconds.

The Randall–Selitto test (Ugo Basile, Type 7200, Italy) was used to assess the HWL to mechanical stimulation, loading. A wedge-shaped pusher with a loading rate of 30 g/s—the pressure required to initiate a struggle response—was applied to the dorsal surface of the hindpaw. The HWL induced by mechanical stimulation, i.e. latency to withdrawal from the start of stimulation, was expressed in seconds. Each rat was tested with both types of stimulation. A cutoff time of 15 s was chosen in both tests to avoid tissue damage.

2.4. Injections of SP and NK-1 receptor antagonists

The solutions for the 1-μl intra-PAG injections were prepared with sterilized 0.9% saline. The concentrations of SP were 0.5, 1.0, 2.5, and 5 nmol. The concentrations of the NK-1 receptor antagonist [d-Arg1, d-Trp7, 9, Leu11]-SP (Spantide) were 1, 5, or 10 nmol. The control rats received a 1-μl injection of 0.9% saline into the PAG. The nociceptive latencies measured immediately before the intra-PAG injections served as the baseline values. The experiments were designed to allow for an internal control and were performed with both the left and the right paws of each rat.

2.4.1. Experiment 1

The animals were divided into five groups: one group for each of the SP concentrations (0.5, 1.0, 2.5, 5.0 nmol) and a control group (0.9% saline). Immediately following the baseline tests, the rats were given a PAG injection. Thereafter the HWLs were measured at 5, 10, 20, 30, and 60 min after the injections.

2.4.2. Experiment 2

The animals were divided into five groups. All groups underwent the baseline tests. Thereafter, 1 μl SP (5 nmol) was injected into the PAG in four of the groups, and the HWLs were measured. Ten minutes later the NK-1 receptor antagonist Spantide was injected at the same location in three of the groups, one group for each of the three concentrations (0.5, 2.5, and 5 nmol). The fourth experi-
mental group received a saline injection. In the fifth group, saline was injected into the PAG following the baseline tests. Ten minutes later, an injection of the NK-1 receptor antagonist (5 nmol) was made at the same location. The HWLs to the hot plate and to mechanical stimulation were measured 5, 15, 25, 35, and 65 min after the first injection.

2.5. Microdialysis experiments

For microdialysis in freely moving rats (n = 26), the animals were anesthetized with a 1.5% halothane:air mixture. The skull was fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A 1.5-mm hole was drilled in the skull, the dura was exposed, and a guide cannula (external diameter 1.0 mm) was implanted in the ventrolateral part of the PAG. The tip of the guide cannula rested at a depth of 4.6 mm from the surface of the dura (coordinates from the bregma: AP = −7.3 mm and LM +2.5 mm according to the Paxinos atlas [30]; angle to the sagittal plane: 22°). The cannula was fixed to the skull with dental cement. The animals were then allowed to recover, one to a cage. Two days after the operation, a microdialysis probe (CMA/12, with an external membrane diameter of 0.5 mm and a membrane length of 2 mm and made of polycarbonate with a molecular cutoff of 20,000 Da) was inserted into the guide with the dialyzing part of the probe descending into the ventrolateral PAG to a depth of approximately 6.6 mm. The animal was placed in a freely moving animal system (CMA Microdialysis, Stockholm, Sweden) and the probe was perfused (7 μl/min) with a Krebs–Ringer solution of the following composition: 138 mM NaCl, 11 mM NaHCO3, 5 mM KCl, 1 mM NaH2PO4, 1 mM CaCl2, 1 mM MgCl2, and 5.9 mM glucose (pH 7.4). To minimize the binding of SP to the plastic surfaces and to reduce peptidase activity, bovine serum albumin (BSA, 0.2%) and bacitracin (0.03%) were included in the perfusion fluid. Sample collection was started 1 h after insertion of the probe, and a total of 16 samples (one every 15 min and 105 μl each) were collected in each experiment. Stimulation with potassium (100 mM in the perfusion fluid) was performed during two 15-min periods (i.e., during collection of sample no. 5 and 12). The samples were immediately frozen and stored at −20 °C until they were assayed for SP-LI.

2.6. Injections of morphine

In one series of experiments, morphine (3 mg/kg) was injected subcutaneously (s.c.) during the resting period between two potassium (100 mM) stimulations as described above. Another group of animals received saline (2 ml/kg, s.c.) during the resting period, and a third group was only stimulated twice with potassium. The two last groups served as controls. After the experiments, the animals were killed by an overdose of sodium pentobarbital, i.p., or halothane (by air). The brain was dissected out and frozen for later verification of the probe position by slicing the brain into 30- to 40-μm sections in a freeze microtome followed by a microscopic examination of the probe tract.

2.7. Methodological studies and considerations in the microdialysis technique

The recovery of neuropeptides is low compared to that of monoamines or amino acids. The main reason is the high molecular weight of the neuropeptides. However, this method can be useful provided that the radioimmunoassay (RIA) for SP detection is sufficiently sensitive. In this study, we used a flow rate of 7 μl/min. The rate can be considered high compared to the flow rate used in measuring monoamines. A high flow rate was chosen in light of the results from an in vitro study by Lindefors et al. [25] where the absolute recovery (that is, the amount of peptide sampled per time unit) for SP increased with an increase in the flow rate. Recovery was determined to within 4–6%. In another in vitro study undertaken in our group, the absolute recovery of SP was not found to be markedly affected by flow rates in the range of 3–7 μl/min. Further, in this study the SP-LI release was stimulated with different concentrations of potassium (50, 100, or 150 mM) in the perfusion fluid during a 15-min period. To test the calcium dependence of the basal and the potassium-stimulated SP release, calcium was omitted and the magnesium concentration was increased to 12 mM in the perfusion medium beginning with sample no. 9 (see Results).

2.8. Radioimmunoassay

The amount of SP in the microdialysis dialysate was measured using a modified RIA with increased sensitivity [8,25]. To avoid any loss of SP by its adsorption to plastic surfaces during pipetting, the assays were carried out directly in the test tubes used to collect the perfusate fractions during microdialysis. The perfusate fractions (105 μl) and standard concentrations of synthetic SP (Peninsula, CA, USA) diluted in Krebs–Ringer solution (105 μl) were preincubated for 24 h at 4 °C with a specific C-terminal directed antiserum, SP2 [9]. The antiserum, which shows negligible (<0.01%) cross-reactivity with the other mammalian tachykinins neurokinin A and neurokinin B, was diluted (1:30,000) in 25 μl 0.1 mM barbital buffer containing 0.2% BSA. The tracer, 125I-[TYR8]-SP, was then added (1000 cpm per tube) in 25 μl 0.02 M barbital buffer in Krebs–Ringer solution, and all samples were further incubated for 48 h. Separate samples without the addition of synthetic SP and several without antiserum were included in all assays to measure maximal and nonspecific binding, respectively. After incubation, antibody-bound and unbound radioligand were separated by the addition of 250 μl of a sheep anti-rabbit antibody-coated sepharose suspension (Pharmacia decanting suspension, Kabi-Pharmacia, Stockholm, Sweden). After incubation for 30 min, the samples were centrifuged at 2000 × g.
for 10 min and the supernatant discarded by aspiration. The pellets containing the bound fraction were analyzed in a gamma counter. The assay was sensitive to 1.8 fmol/ml (0.18 fmol/sample) of SP or less.

2.9. Calculations and statistical analysis

In the microdialysis experiments, the criteria for including an individual experiment in the calculations were (1) a correct position of the dialyzing part of the probe in the ventrolateral PAG; (2) a basal SP level, prior to the first stimulation, not exceeding 15 fmol/ml; and (3) an increase of more than 50% in the SP release that was evoked in response to the first potassium stimulation. The basal level of SP in the dialysate was defined as the concentration of SP during the collection period (15 min) immediately preceding the first stimulation. The evoked SP release was defined as the cumulative increase in SP release over the basal level during three collection periods from the start of the potassium stimulation. The results are presented as the mean ± standard error of the method (S.E.M.). The Kruskal–Wallis test and the Friedman test, followed by pairwise comparisons, were used for comparing unrelated and related samples, respectively. An ANOVA was used to define a statistical difference between the groups.

Data from the behavioral tests after SP microinjections were presented as the mean ± S.E.M. The withdrawal latencies of the left paw are presented (no significant difference was seen between the right and the left paw, data not shown). A two-way analysis of variance (ANOVA) was used and as a post hoc test, the Newman–Keuls test was used for statistical analysis. Differences were considered significant when \( P<0.05 \).

3. Results

3.1. Effects of SP microinjected into the PAG

Rats received intra-PAG injections of 1 µl 0.5 nmol \((n=6)\), 1 nmol \((n=8)\), 2.5 nmol \((n=8)\), or 5 nmol of SP \((n=8)\), or 1 µl of 0.9% saline as a control \((n=9)\). The HWL to thermal stimulation was tested before and 5, 10, 20, 30, and 60 min after the administration of SP.

In the hot-plate tests, significant increases in HWL to thermal stimulation were seen in the groups receiving 1 nmol \((P<0.05)\), 2.5 nmol \((P<0.01)\), and 5 nmol of SP \((P<0.001)\)—but not in the group receiving 0.5 nmol \((P=0.54)\)—compared to the control group. The results are shown in Fig. 1a.

In the Randall–Selitto tests, significant increases in HWL to mechanical stimulation were seen in the groups receiving 1 nmol \((P=0.05)\), 2.5 nmol \((P<0.01)\), and 5 nmol of SP \((P<0.001)\)—but not in the group receiving 0.5 nmol \((P=0.22)\)—compared to the controls. The results are shown in Fig. 1b.

3.2. Effects of an NK-1 receptor antagonist (Spantide) microinjected into the PAG

Rats received intra-PAG injections of 1 µl 5 nmol of SP, followed 10 min later by Spantide in concentrations of 0.5 nmol \((n=7)\), 2.5 nmol \((n=8)\), or 5 nmol \((n=8)\), or 1 µl 0.9% saline as a control \((n=8)\). In the hot-plate tests, the HWL to thermal stimulation was tested before and 5, 15, 25, 35, and 65 min after the injection of SP. Compared to the group receiving the combination of SP and saline, the groups receiving 2.5 \((P<0.001)\) or 5 nmol of Spantide \((P<0.001)\)—but not the group receiving 0.5 nmol \((P=0.15)\)—experienced significant decreases in HWL to thermal stimulation at 15 and 25 min. The results are shown in Fig. 2a. In the Randall–Selitto tests, the HWL to mechanical stimulation decreased significantly 15 and 25 min after the administration of 2.5 \((P<0.001)\) or 5 nmol of Spantide \((P<0.001)\), but not after 0.5 nmol \((P=0.22)\). The results are shown in Fig. 2b.
The control group of rats that received saline followed 10 min later by Spantide (n = 8) experienced no differences in HWL, neither in the hot-plate test nor in the Randall–Selitto test. The NK-1 receptor antagonist Spantide alone induced no effect at all.

3.3. Effects of morphine on the SP-LI release in the PAG

In awake animals, the basal SP-LI concentration was above the sensitive limit of the assay, reaching an average of 5.33 ± 0.8 fmol/ml (n = 26, Fig. 3) in all experiments. A recovery was estimated to be 2–5%.

An acute dose of morphine (3 mg/kg, s.c.), given between the two potassium stimulations (i.e., beginning with the collection of sample no. 9), induced a significant increase in the basal release of SP-LI (P<0.05, P<0.01, P<0.001) compared to the controls (sample no. 4, Fig. 3c).

A general statistical difference between the groups was also confirmed (P<0.05, Fig. 4).

The increased release, which lasted until the end of the experiments, also differed from the release seen in experiments with untreated animals (controls) and with animals treated with saline. This effect was demonstrated in rats responding to a local depolarizing stimulus of 100 mM potassium administered via the dialysis probe, with a several-fold increase in the SP-LI level. In a previous study, the local depolarization stimulus used was 50 mM potassium, and no significant differences could be shown in the basal SP-LI release after morphine treatment [40].

The control group of rats that received saline followed 10 min later by Spantide (n = 8) experienced no differences in HWL, neither in the hot-plate test nor in the Randall–Selitto test. The NK-1 receptor antagonist Spantide alone induced no effect at all.

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There are no certain explanations for the difference between using 50 or 100 mM potassium, but one hypothesis is that the higher the concentration of potassium needed to deplete SP from the terminals, the higher the turnover of SP. The concentration–response curve obtained for the potassium-induced SP release showed that 50, 100, and 150 mM potassium in the perfusion fluid increased the SP-LI release in the PAG by approximately 200%, 300%, and 400%, respectively. Both the basal and the potassium-evoked SP-LI release were significantly reduced by the omission of calcium and the higher concentration of magnesium in the perfusion fluid of sample no. 9 and above throughout the rest of the experiment. The basal release decreased by more than 50% (from 7.7 ± 0.3 in sample no. 4 [n = 5] to 3.5 ± 0.1 fmol/ml in sample no. 10 [n = 5]) and remained at low levels throughout the experiment (P < 0.01 or P < 0.05) (No figure shown).

4. Discussion

In the present study, SP microinjected into the ventrolateral PAG resulted in antinociception in the behavioral studies. This effect was blocked by the administration of the NK-1 receptor antagonist. The NK-1 antagonist itself had no perceivable antinociceptive effect at all. Antinociceptive effects of SP have been observed in animal tests [16,20]. It has been suggested that the antinociceptive effect is linked to the N-terminal region of the SP molecule. The N-terminal portion of SP is known to inhibit biting and scratching behavior elicited by the C-terminal portion of SP [24]. Whether the N-terminal segment of SP is a μ agonist or not has been the topic of discussions, but researchers have been able to block the antinociceptive effect of the N-terminal segment of SP using naloxone [19,23]. It is also possible that the N-terminal SP fragments may induce antinociception by downregulating nitric oxide synthesis in the nociceptive pathways [21].

That there is a close interaction between opioids and SP is supported by studies showing that SP stimulates the release of endogenous opioids [12,18,47]. It has also been reported that μ receptors co-localize with NK-1 receptors on the same neuron. One suggestion is that opiates modulate SP-induced nociceptive responses directly at postsynaptic sites, instead of inhibiting the release of SP from primary afferents [1].

The lack of antinociceptive effect of the NK-1 receptor antagonist in the behavioral tests was unexpected. Other researchers, in preclinical data, have found that the NK-1 receptor antagonist has an antinociceptive effect, not as strong as that of morphine, but comparable to that of non-steroidal anti-inflammatory drugs (NSAID) [27,42]. In humans, NK-1 receptor antagonists have failed to produce potent antinociceptive effects; instead, convincing proof that these antagonists have an anxiolytic effect has appeared [15,22,41–43]. It is possible that the lack of antinociceptive effect of the NK-1 receptor antagonist seen in the present study is dependent on the choice of antagonist. We used the antagonist [d-Arg1, d-Trp7,9, Leu11]-SP (Spantide) in our study. Non-peptide antagonists of the NK-1 receptor might produce different responses. Another more important suggestion to consider is the influences of stress on the results we have obtained. All kinds of injuries, in animals or in humans, will produce not only pain but also stress [26]. More studies on this subject are needed.

In the present study, we also found that the basal SP-LI release in the PAG increases after a single dose of morphine (3 mg/kg). Not only does SP induce antinociception but morphine also stimulates the release of endogenous SP. It is possible that the effect of morphine on the SP neurons in the PAG could be caused by a reduction in the tonic inhibition of the SP release, which in turn may increase the descending inhibitory control of nociceptive input. These results are in accordance with those of other studies [4,13,33,49]. In addition to morphine-produced antinociception, in vitro studies have indicated that SP induces antinociception via activation of met-enkephalin release in PAG slices [11,34] and that SP injected into the PAG produces naloxone-reversed antinociception [28].

In conclusion, SP microinjected into the PAG increases limb withdrawal latencies in rats in acute nociceptive tests, an effect that is blocked by an NK-1 antagonist. A single dose of morphine increases SP release in the PAG in awake, freely moving rats. These findings are in accordance with the idea of the role of SP in the PAG as a neuromodulator which activates the descending pain inhibitory pathways that mediate opioid analgesia in rats.

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