

# 学位論文

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# 学 位 論 文

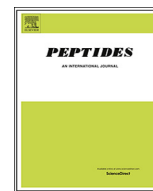
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# Microinjection of 26RFa, an endogenous ligand for the glutamine RF-amide peptide receptor (QRFP receptor), into the rostral ventromedial medulla (RVM), locus coeruleus (LC), and periaqueductal grey (PAG) produces an analgesic effect in rats

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## ABSTRACT

26RFa is an endogenous ligand for the QRFP receptor. We previously found that intracerebroventricular injection of 26RFa produces an analgesic effect in a rat formalin test. In the present study, we directly tested the hypothesis that the analgesic effects of 26RFa in the formalin test are mediated in well-recognized regions of the descending inhibitory pain pathways, such as the rostral ventromedial medulla (RVM), locus coeruleus (LC), and periaqueductal grey (PAG) in rats. Injection cannulae were stereotactically placed in the RVM, LC, or PAG through a burr hole. 26RFa (15 µg) or saline was delivered in a total volume of 0.5 µL. In a formalin test, 50 µL of 5% formalin was injected subcutaneously into the hind paw. In an antagonist study, idazoxan, an  $\alpha$ -2 antagonist, or naloxone, an opioid receptor antagonist, was administered. Microinjection of 26RFa into the RVM had no effect compared with that in saline-injected rats. Microinjection of 26RFa into the LC contralateral, but not ipsilateral, to the formalin injection site significantly decreased the number of flinching behaviors compared with that of saline-injected rats. This effect was antagonized by intrathecal injection of idazoxan. Microinjection of 26RFa into the contralateral, but not ipsilateral, PAG produced an analgesic effect, and this effect was partly antagonized by intraperitoneal naloxone. These data suggest that 26RFa microinjected into the contralateral LC induced noradrenaline release in the spinal cord and produced an analgesic effect. In the contralateral PAG, 26RFa activated the opioid system, and some analgesic effects were mediated by opioid system activation.

## 1. Introduction

26RFa, a 26-residue RFamide peptide, and its N-terminally extended form glutamine RF-amide peptide (QRFP) are endogenous ligands for GPR103, which is an orphan G protein-coupled receptor renamed the QRFP receptor [8]. The QRFP receptor has similarities with the orexin, neuropeptide FF (NPFF), and cholecystikinin receptors. The 26RFa/QRFP precursor and QRFP receptor genes are widely expressed in hypothalamic nuclei, and intracerebroventricular (ICV) injection of 26RFa stimulates food intake in mice and rats [8]. The QRFP receptor is present from fish to humans. 26RFa-expressing neurons have discrete localization in the hypothalamus. 26RFa acts as a key neuropeptide in vertebrates to control vital neuroendocrine functions [3]. 26RFa/QRFP knockout mice are markedly hypophagic, lean, and have increased anxiety-like behaviors [12].

The descending noradrenergic and serotonergic inhibitory system is involved in pain modulation [1,9,13]. The periaqueductal gray (PAG),

locus coeruleus (LC), and the dorsal raphe nucleus (DR) are key nuclei in this descending inhibitory system. In particular, activation of the LC produces noradrenaline-mediated analgesia in the spinal cord, and a serotonin/noradrenaline reuptake inhibitor, which enhances spinal noradrenergic activity, acts as an analgesic for the treatment of chronic pain [9].

We previously found that ICV injection of 26RFa produces an analgesic effect in a rat formalin test, an inflammatory pain model, and in a rat partial sciatic nerve ligation model, a neuropathic pain model [16,17]. The precise mechanisms that produce the analgesic effect of ICV injection of 26RFa are still unknown. The PAG, LC, and DR are high 26RFa binding sites in rats [2]. It is possible that ICV injection of 26RFa diffused to these nuclei to produce an analgesic effect. In the present study, we directly tested the hypothesis that an analgesic effect of 26RFa in a formalin test was mediated by the activation of well recognized regions of the descending inhibitory pain system, such as the rostral ventromedial medulla (RVM), LC, and PAG, in rats. The DR is a

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major nucleus of the RVM. We also examined whether activation of the LC or PAG by microinjected 26RfA induced noradrenaline or serotonin release in the spinal cord using microdialysis.

The effects of 26RfA were antagonized by BIBP3226, a mixed antagonist of the NPY Y1 and NPFF receptors [17]. We also examined whether the analgesic effects of 26RfA were antagonized by pretreatment of BIBP3226.

## 2. Methods

This research was performed according to a protocol approved by the Institutional Animal Care Committee of Kumamoto University. Male Sprague-Dawley rats (250–300 g; Japan SLC, Inc., Shizuoka, Japan) were housed in groups of two, maintained on a 12-hour dark-light cycle, and permitted food and water ad libitum. Animals were handled on arrival and housed for at least three days before catheter implantation. Animals were euthanized immediately after behavioral or microdialysis studies.

### 2.1. RVM, LC, and PAG cannula placement for microinjection

Implantation of the injection cannula into the RVM, LC, and PAG was performed under isoflurane anesthesia. Rats were placed in a stereotaxic apparatus, and stainless steel 26 G thin wall guide cannulae (C315 G, Plastics One, VA) were stereotactically placed at the level of RVM, LC, and PAG through a burr hole (RVM: AP: −11.0 mm, L: 0.0 mm, H: −11.0 mm from bregma; LC: AP: −10.0 mm, L: 1.4 mm, H: −8.0 mm from bregma; PAG: AP: −7.6 mm, L: 0.7 mm, H: −6.0 mm from bregma). Guide cannulae were affixed to the skull with stainless steel screws and cranioplastic cement.

Mefenamic acid (32.5 mg, Daiichi-Sankyo, Tokyo, Japan) was orally administered two times/day 0–2 days after surgery for post-operative pain control. The formalin tests were performed 7 days after cannulae implantation. All animals displayed normal feeding and drinking behaviors post-operatively. Rats showing neurological deficits after cannulae implantation were not studied.

At the completion of the experiment, 0.5  $\mu$ L of India ink was injected through the internal cannula 10 min before rats were euthanized. The brains were fixed with paraformaldehyde, and coronal tissue sections were Nissl stained to confirm the proper injection site. Only the rats whose microinjection site was located within the intended nucleus were included in the results.

### 2.2. Drugs and injection

Drugs were dissolved in saline. 26RfA (molecular weight = 2820) was purchased from the Peptide Institute Inc (Osaka, Japan). BIBP3226 (molecular weight = 474), a non-peptide mixed antagonist of the neuropeptide YY1 and neuropeptide FF receptors [7,10,14], was purchased from Sigma (St. Louis, MO). Idazoxan, an  $\alpha$ -2 adrenergic receptor antagonist, and naloxone, an opioid receptor antagonist, were also purchased from Sigma.

26RfA was administered in a total volume of 0.5  $\mu$ L over a period of 60 s using a microsyringe pump (EP-60, EICOM, Japan) and 30 G stainless steel internal cannula (C315 G, Plastics One, VA) connected via a polyethylene tube to a 10  $\mu$ L Hamilton syringe.

Fifteen micrograms of 26RfA was injected directly into the RVM, contralateral or ipsilateral LC, or contralateral or ipsilateral PAG 10 min before formalin injection into the footpad. Because of the limited solubility of 26RfA, 15  $\mu$ g was the highest dose microinjected into each nucleus. In the contralateral LC study, 5  $\mu$ g and 1.5  $\mu$ g 26RfA were also injected to determine whether 26RfA produced a dose-dependent analgesic effect. To test the mechanisms of action of 26RfA, one of the following drugs was injected 10 min before injection of 26RfA: BIBP3226 (3.6  $\mu$ g/0.5  $\mu$ L microinjection into the LC or PAG), idazoxan (60  $\mu$ g/10  $\mu$ L intrathecal [IT] in the LC and PAG study), or naloxone

(1 mg/kg intraperitoneal [IP] in the PAG study).

### 2.3. Formalin test

In the formalin test, 50  $\mu$ L of 5% formalin was injected subcutaneously into the dorsal surface of the hind paw with a 26-gauge needle under brief isoflurane anesthesia. Within 1 min after formalin injection, spontaneous flinching of the injected paw could be observed. Flinching was readily discriminated as a rapid and brief withdrawal or flexion of the injected paw. This pain-related behavior was quantified by counting the number of flinches for 1-min periods at 5-min intervals from 0 to 60 min after injection. Two phases of spontaneous flinching behavior were observed: an initial acute phase (phase 1: during the first 6 min after formalin injection) and a prolonged tonic phase (phase 2: beginning about 10 min after formalin injection). The phase 1 response is mediated by the direct stimulation of nociceptors by formalin, and the phase 2 response is mediated by the inflammatory response induced by formalin [15,18].

### 2.4. Intrathecal microdialysis and assay of the noradrenaline level in the LC study and noradrenaline and serotonin levels in the PAG study

LC is the major site of noradrenergic cell bodies in the brain. Thus, in the LC study, we focused on noradrenaline release. The PAG influences the descending pain inhibitory system through its reciprocal connection with the RVM. The RVM includes the serotonergic nucleus raphe magnus. PAG stimulation is associated with spinal noradrenaline and serotonin release [5]. Thus, in the PAG study, the levels of both noradrenaline and serotonin were measured.

Rats were anesthetized with isoflurane and placed in a stereotaxic apparatus for surgical implantation of a microdialysis probe. The intrathecal microdialysis probe (exposed tip, 10 mm, cut-off of 50 kDa; EICOM, Japan) was passed caudally 7.5 cm from the cisterna magnum to the lumbar enlargement. After surgery and anesthesia, each rat was checked for neurological deficits. Rats showing neurological deficits were excluded. After checking neurological function, rats were maintained under anesthesia using 1.0–1.5% isoflurane and dioxygen, and the probe was perfused at 2  $\mu$ L/min with artificial cerebrospinal fluid. The rat was allowed to recover from the operation for 2–3 h. After obtaining stable noradrenaline and serotonin data, formalin was injected into the rat foot pad.

Microdialysis was performed in anesthetized rats. During the experiment, the probe was perfused at 2  $\mu$ L/min with artificial cerebrospinal fluid, and dialysate samples were collected every 15 min. Three baseline fractions were collected before 26RfA or saline injection.

Fifteen minutes after pretreatment of 26RfA (15  $\mu$ g microinjection) or saline, formalin (5%, 50  $\mu$ L) was injected into the contralateral hind-paw and dialysate samples were collected during the formalin test. Samples were subsequently analyzed for noradrenaline and serotonin.

### 2.5. Noradrenaline measurement in the LC study

Noradrenaline in the dialysate samples was analyzed by reverse phase high performance liquid chromatography (HPLC) and electrochemical detection (ECD-300, EICOM, Japan). A reverse phase column (EICOMPAK, CA-50DS, 2.1  $\times$  150 mm, EICOM) was used, and the mobile phase for detection of noradrenaline was composed of 0.1 M phosphate buffer solution and 50 mg/mL EDTA $\cdot$ 2Na with methanol (95:5 v/v) in water adjusted to pH 6.0 with 400 mg/L 1-octanesulfonic acid (sodium salt). The flow rate was 0.23 mL/min (EP-300, EICOM). The column temperature was maintained at 25  $^{\circ}$ C, and the applied potential was set at + 450 mV (ATC-300, EICOM). Quantification was obtained from standard curves.

## 2.6. Noradrenaline and serotonin measurement in the PAG study

Noradrenaline and serotonin in the microdialysis samples were also analyzed by reverse phase HPLC and electrochemical detection (ECD-300). A reverse phase column (EICOMPAK CAX, 2.0 x 200 mm, EICOM) was used, and the mobile phase for detection of noradrenaline and serotonin was composed of 0.1 M ammonium acetate buffer solution and 50 mg/mL EDTA-2Na, 0.05 mol/L sodium sulfate with methanol (7:3, v/v) in water adjusted to pH 6.0. The flow rate was 0.25 mL/min (EP-300). The column temperature was maintained at 35 °C, and the applied potential was set at + 450 mV (ATC-300). Quantification was obtained from standard curves.

## 2.7. Statistical analysis

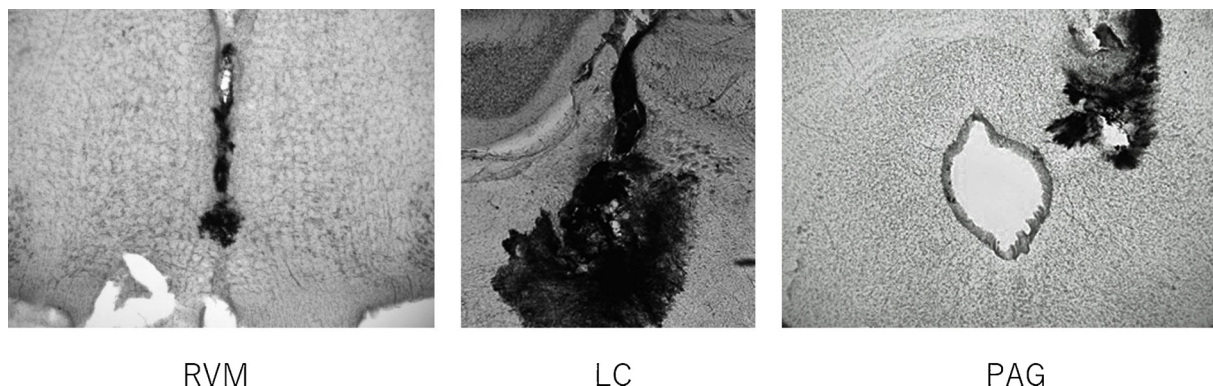
The time-response data are presented as the mean flinches  $\pm$  SEM per min for the periods between 1–2 min and 5–6 min after formalin treatment and then for 1-min periods at 5-min intervals up to 60 min. The data from phase 1 (0–6 min) and phase 2 (10–60 min) observations were considered separately. In each case, the cumulative instances of formalin-evoked flinches during phase 1 and phase 2 were calculated for each rat. The effect of 26RFa injected into the RVM, LC, and PAG was compared with that of saline using a Student's *t*-test. In the contralateral LC study, dose-dependency was analyzed by one-way analysis of variance (ANOVA). For the antagonist study, ANOVA was used. For multiple comparisons, a Tukey test was used. The effect of 26RFa was also compared with that of saline, 26RFa + BIBP3226, 26RFa + idazoxan, or 26RFa + naloxone at all measurement times by ANOVA with a Dunnett-test.

Microdialysis data are expressed as the percentage of basal values (calculated as the mean of three samples before injection). The basal concentrations of lumbar enlargement noradrenaline and serotonin in the dialysates, uncorrected for recovery, were  $6.5 \pm 2.4$  nM and  $0.71 \pm 0.54$   $\mu$ M, respectively. The values were expressed as a percentage of baseline for each rat, and the mean and standard error were determined for each treatment group. All data were given as mean  $\pm$  SEM and not corrected for 'recovery' of the dialysis procedure. Two-factor ANOVA was used to examine the possibility of significant differences between groups. For multiple comparison, the Holm-Sidak method was used.

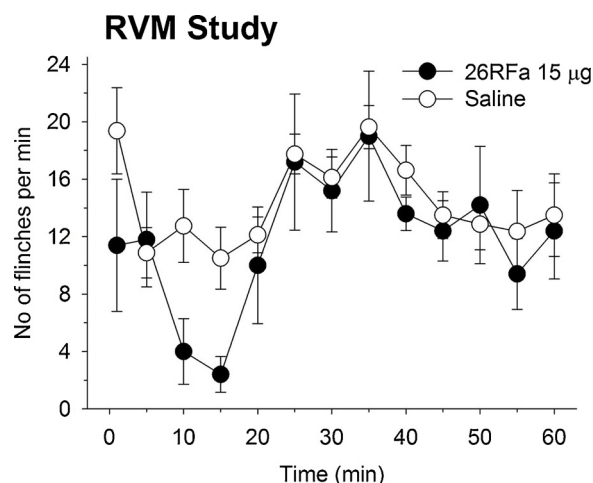
Critical values of  $p < 0.05$  were considered significant.

## 3. Results

In saline-treated control rats, a subcutaneous injection of formalin resulted in a highly reliable biphasic display of flinching of the injected paw, and this behavior was comparable to that previously reported [18].



**Fig. 1.** Injection site in the RVM, LC, and PAG. The spread of 0.5 mL of India ink injected through internal cannula. Nissl-stained sections were used to confirm the injection site. RVM, LC, and PAG were located within the regions stained by India ink.



**Fig. 2.** The effects of 26RFa microinjected into the RVM ( $n = 5$ ) in the rat formalin test. 26RFa did not attenuate phase 1 ( $p = 0.257$ ) or phase 2 ( $p = 0.189$ ) flinching behaviors compared with that in saline-treated rats ( $n = 8$ ).

Ordinate: number of flinches per min; abscissa: time after drug administration (min).

Injection sites in the RVM, LC, and PAG are shown in Fig. 1. India ink (0.5  $\mu$ L) injected through the internal cannula spread to the RVM, LC, and PAG.

### 3.1. Behavioral study

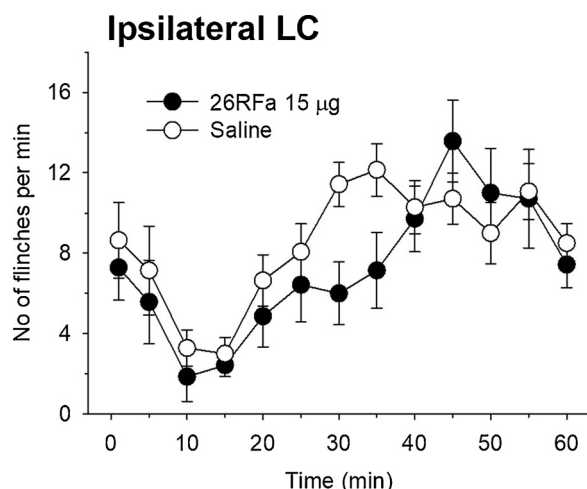
#### 3.1.1. Effects of 26RFa injected into the RVM (Fig. 2)

Microinjection of 15  $\mu$ g 26RFa into the RVM ( $n = 5$ ) produced no significant effect on either the phase 1 ( $23.2 \pm 4.4$ ;  $p = 0.257$ ) or phase 2 ( $130 \pm 21.6$ ;  $p = 0.189$ ) response compared with that in saline-injected rats ( $n = 8$ ; phase 1:  $30.3 \pm 3.8$ ; phase 2:  $158 \pm 8.6$ ).

#### 3.1.2. Effects of 26RFa injected into the LC (Figs. 3–5)

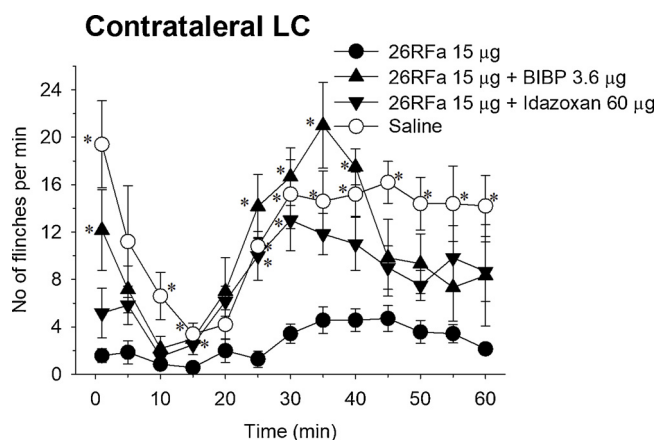
Microinjection of 15  $\mu$ g 26RFa into the LC ipsilateral to the formalin-injected side ( $n = 7$ ) produced no significant effect on either the phase 1 ( $12.9 \pm 3.4$ ;  $p = 0.568$ ) or phase 2 ( $81.1 \pm 8.7$ ;  $p = 0.305$ ) response compared with that in saline-injected rats ( $n = 14$ ; phase 1:  $15.8 \pm 3.1$ ; phase 2:  $94.1 \pm 7.5$ ).

Microinjection of 15  $\mu$ g 26RFa into the LC contralateral to the formalin-injected side ( $n = 7$ ) significantly reduced phase 1 ( $3.4 \pm 1.0$ ;  $p < 0.001$ ) and phase 2 ( $31.1 \pm 4.1$ ;  $p < 0.001$ ) flinching behavior compared with that in saline-treated rats ( $n = 5$ ; phase 1:  $30.6 \pm 4.0$ ; phase 2:  $129 \pm 8.3$ ). A significant analgesic effect of 26RFa in phase 2 was observed between 25 and 60 min after formalin injection. In both



**Fig. 3.** The effects of 26RFa microinjected into the ipsilateral LC ( $n = 7$ ) in the rat formalin test. 26RFa did not attenuate phase 1 ( $p = 0.568$ ) or phase 2 ( $p = 0.305$ ) flinching behaviors compared with that in saline-treated rats ( $n = 14$ ).

Ordinate: number of flinches per min; abscissa: time after drug administration (min).



**Fig. 4.** The effects of 26RFa microinjected into the contralateral LC ( $n = 7$ ) in the rat formalin test. The effects of BIBP3226 ( $n = 6$ ) or idazoxan ( $n = 6$ ) on the analgesic effect of 26RFa were examined. 26RFa attenuated both phase 1 ( $p < 0.001$ ) and phase 2 ( $p < 0.001$ ) flinching behaviors compared with that in saline-treated rats ( $n = 5$ ). Pretreatment of BIBP3226 antagonized the effect of 26RFa on both phase 1 ( $p = 0.012$ ) and phase 2 responses ( $p < 0.001$ ). Pretreatment of intrathecal idazoxan antagonized the effect of 26RFa on the phase 2 response ( $p < 0.001$ ) but not the phase 1 response ( $p = 0.161$ ).

\*:  $p < 0.05$  as compared with 26RFa-treated rats at each time point.

Ordinate: number of flinches per min; abscissa: time after drug administration (min).

phase 1 and phase 2, the dose-dependency was observed at a dose between 1.5  $\mu\text{g}$  and 15  $\mu\text{g}$  ( $p < 0.001$ ). Fifteen micrograms of 26RFa reduced the phase 2 response significantly more than 5  $\mu\text{g}$  26RFa ( $p = 0.002$ ).

The effects of 26RFa injected into the contralateral LC were completely antagonized by BIBP3226 microinjected into the contralateral LC ( $n = 6$ ; phase 1:  $19.3 \pm 5.0$ ,  $p = 0.012$ ; phase 2:  $116 \pm 17.8$ ,  $p < 0.001$ ). There was no significant difference between saline-treated rats and 26RFa + BIBP3226-treated rats (phase 1:  $p = 0.110$ , phase 2:  $p = 0.729$ ). IT injection of the  $\alpha_2$  adrenergic receptor antagonist idazoxan antagonized the analgesic effect of 26RFa injected into the contralateral LC in the phase 2 response but not in the phase 1 response ( $n = 6$ ; phase 1:  $p = 0.161$ , phase 2:  $p < 0.001$ ). There was a significant difference between saline-treated rats and 26RFa + idazoxan-

treated rats (phase 1:  $p = 0.001$ , phase 2:  $p = 0.03$ ).

### 3.1.3. Effects of 26RFa injected into the PAG (Figs. 6 and 7)

Microinjection of 15  $\mu\text{g}$  26RFa into the PAG ipsilateral to the formalin-injected side ( $n = 5$ ) produced no significant effect on the phase 1 ( $22.4 \pm 6.0$ ;  $p = 0.559$ ) and phase 2 ( $108.8 \pm 11.2$ ;  $p = 0.106$ ) response compared with that in saline-injected rats ( $n = 6$ ; phase 1:  $17.7 \pm 5.1$ ; phase 2:  $82.2 \pm 9.8$ ).

Microinjection of 15  $\mu\text{g}$  26RFa into the PAG contralateral to the formalin-injected side ( $n = 5$ ) significantly reduced phase 1 ( $6.4 \pm 1.9$ ;  $p = 0.038$ ) and phase 2 ( $29.6 \pm 6.4$ ;  $p = 0.0035$ ) flinching behavior compared with that in saline-treated rats ( $n = 5$ ; phase 1:  $19.4 \pm 4.9$ ; phase 2:  $104.2 \pm 17.1$ ). A significant analgesic effect of 26RFa in phase 2 was observed at 15, 30, 40, and 50 min after formalin injection.

The effects of 26RFa injected into the contralateral PAG on the phase 2 response, but not the phase 1 response, were antagonized by BIBP3226 microinjected into the contralateral PAG ( $n = 4$ ; phase 1:  $5.3 \pm 1.4$ ,  $p = 0.970$ ; phase 2:  $107 \pm 18.6$ ,  $p = 0.009$ ). There was a significant difference in phase 1 ( $p = 0.035$ ), but not phase 2 ( $p = 0.989$ ), between saline-treated rats and 26RFa + BIBP3226-treated rats. Intraperitoneal (IP) injection of the opioid receptor antagonist naloxone reversed the analgesic effects of 26RFa injected into the contralateral PAG on the phase 1 response, but not the phase 2 response ( $n = 6$ ; phase 1:  $24.0 \pm 5.1$ ,  $p = 0.034$ ; phase 2:  $62.8 \pm 23.3$ ,  $p = 0.416$ ). There was no significant difference between saline-treated rats and 26RFa + naloxone-treated rats (phase 1:  $p = 0.740$ , phase 2:  $p = 0.269$ ). Pretreatment of IT idazoxan did affect the analgesic effect of 26RFa microinjected into the contralateral PAG ( $n = 5$ , phase 1:  $2.2 \pm 1.2$ ,  $p = 0.10$ ; phase 2:  $17.4 \pm 3.9$ ,  $p = 0.14$ ).

## 3.2. Microdialysis study

### 3.2.1. LC Study (Fig. 8)

Microinjection of 15  $\mu\text{g}$  26RFa into the contralateral LC ( $n = 4$ ) significantly increased the release of noradrenaline from the spinal cord compared with that of saline ( $n = 4$ ) ( $p = 0.028$  by 2 way ANOVA).

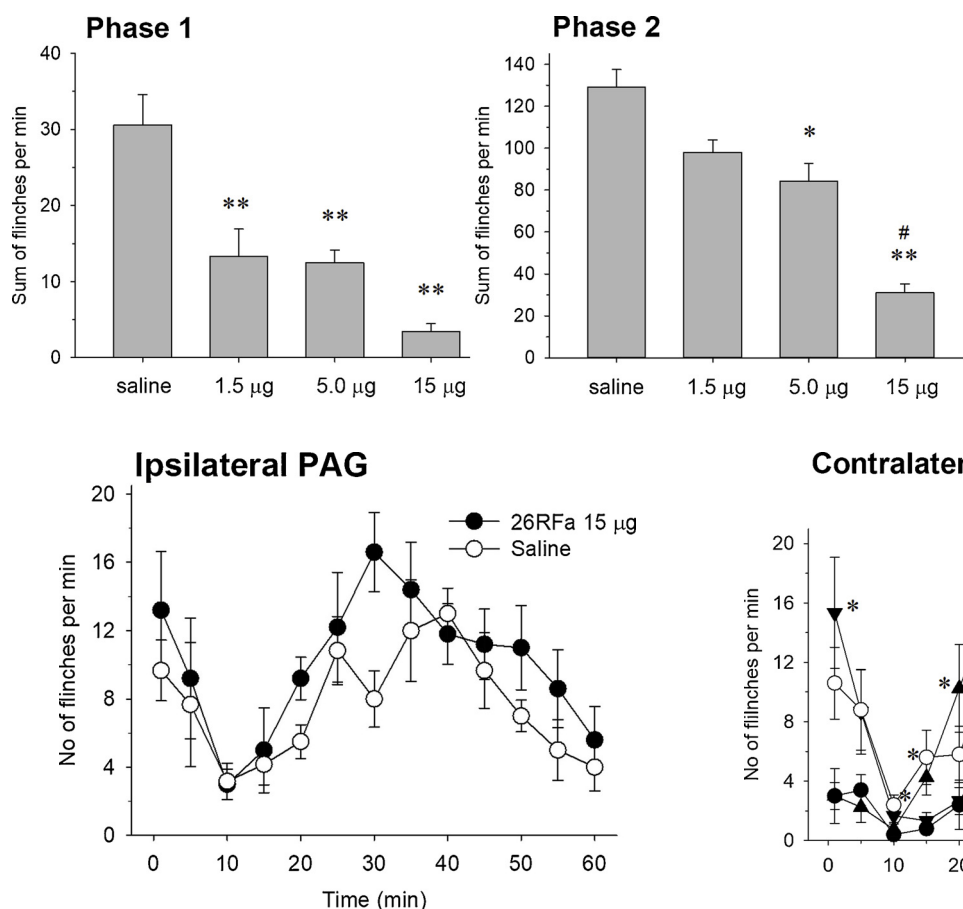
### 3.2.2. PAG study (Fig. 9)

Microinjection of 15  $\mu\text{g}$  26RFa into the contralateral PAG ( $n = 5$ ) had no effect on the release of serotonin from the spinal cord compared with that of saline ( $n = 5$ ) ( $p = 0.828$  by 2 way ANOVA). Microinjection of 15  $\mu\text{g}$  26RFa into the contralateral PAG ( $n = 5$ ) significantly decreased the release of noradrenaline from the spinal cord compared with that of saline ( $n = 5$ ) ( $p = 0.038$  by 2 way ANOVA).

## 4. Discussion

Microinjection of 26RFa into the LC or PAG contralateral, but not ipsilateral, to the formalin-injected side produced an analgesic effect in the rat formalin test. We previously reported that paw formalin injection induced glutamate release in the contralateral, but not the ipsilateral, LC [11]. There are no data about whether paw formalin injection activates the contralateral, but not the ipsilateral, PAG. According to our data, it is possible that paw formalin injection activated only the contralateral, but not the ipsilateral, LC and PAG. We previously found that spinal nociceptive neurons were innervated by both ipsilateral and contralateral LC neurons using retrograde neuron tracing [11]. This suggests that contralateral LC activated by 26RFa inhibits nociceptive input at the spinal cord.

In the present study, 15  $\mu\text{g}$  of 26RFa was administered into the RVM, LC, and PAG. In mice, ICV injection of 1  $\mu\text{g}$  26RFa provokes a significant increase in food intake [4] and stimulates locomotor activity [19]. These data suggest that microinjection of 15  $\mu\text{g}$  of 26RFa into a particular nucleus is considerably high. In a dose-response study in the contralateral LC, a dose-dependent analgesic effect of 26RFa was



**Fig. 6.** The effects of 26RFa microinjected into the ipsilateral PAG ( $n = 5$ ) in the rat formalin test. 26RFa did not attenuate phase 1 ( $p = 0.559$ ) or phase 2 ( $p = 0.106$ ) flinching behaviors compared with that in saline-treated rats ( $n = 6$ ).

Ordinate: number of flinches per min; abscissa: time after drug administration (min).

observed at doses between 1.5 and 15 µg. In particular, 15 µg of 26RFa had a more significant analgesic effect than 5.0 µg of 26RFa. Thus, we administered 15 µg of 26RFa into the RVM, LC, and PAG.

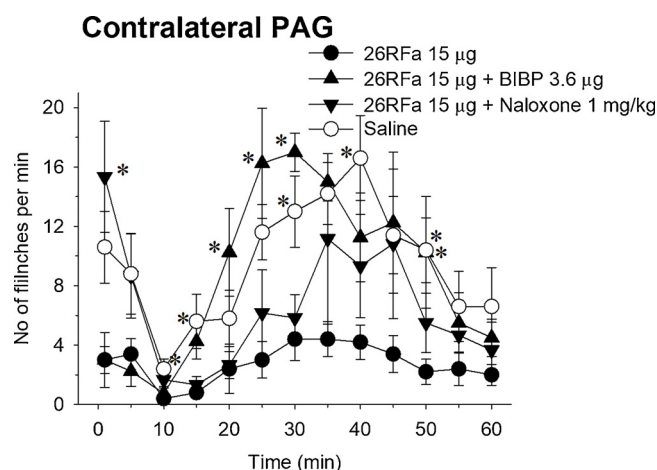
Elhabazi et al [6] reported that, in mice, ICV injection of 3 nmol (8 µg) 26RFa decreased the latency time to withdraw tail from a 48 °C water bath via NPFF receptor activation, which suggests that ICV injection of 26RFa induces heat hyperalgesia. On the other hand, we previously reported that ICV 26RFa produces an analgesic effect in a rat formalin test in a dose-dependent manner at doses between 0.3 and 30 µg [17]. In this study, microinjection of 15 µg of 26RFa into the contralateral LC and PAG produced an analgesic effect in the rat formalin test. The sensitivity of 26RFa may be species-dependent, and 26RFa may have opposite effects in rats and mice. 26RFa injected into the RVM had no effect on formalin-induced flinching behaviors. The dorsal raphe nucleus is a key area in the RVM, a high binding site for 26RFa, and has elevated QRFP receptor mRNA expression [2]. Although the RVM exerts a bidirectional pain modulatory effect, both inhibiting and facilitating pain, binding of 26RFa to the dorsal raphe nucleus was not involved in the descending pain modulatory system.

#### 4.1. Antagonist study

A high dose of 26RFa (15 µg) was used, and it is possible that 26RFa may produce an analgesic effect by non-specific activity. LC BIBP3226 completely antagonized the analgesic effect of 26RFa microinjected into the contralateral LC on the phase 1 and phase 2 response. This

**Fig. 5.** Dose-response analysis in the contralateral LC study. 26RFa microinjected into the contralateral LC decreased the sum of flinches per min in both phase 1 and phase 2 in a dose-dependent manner.

\*  $p < 0.05$  as compared with saline-treated rats. \*\*  $p < 0.001$  as compared with saline-treated rats. #  $p < 0.005$  as compared with 5.0 µg 26RFa-treated rats.



**Fig. 7.** The effects of 26RFa microinjected into the contralateral PAG ( $n = 5$ ) in the rat formalin test. The effects of BIBP3226 ( $n = 4$ ) or naloxone ( $n = 6$ ) on the analgesic effect of 26RFa were examined. 26RFa attenuated both phase 1 ( $p = 0.038$ ) and phase 2 ( $p = 0.0035$ ) flinching behaviors compared with that in saline-treated rats ( $n = 5$ ). Pretreatment of BIBP3226 antagonized the effect of 26RFa on the phase 2 response ( $p = 0.009$ ) but not the phase 1 response ( $p = 0.970$ ). Pretreatment of intraperitoneal naloxone antagonized the effect of 26RFa on the phase 1 response ( $p = 0.034$ ) but not the phase 2 response ( $p = 0.416$ ).

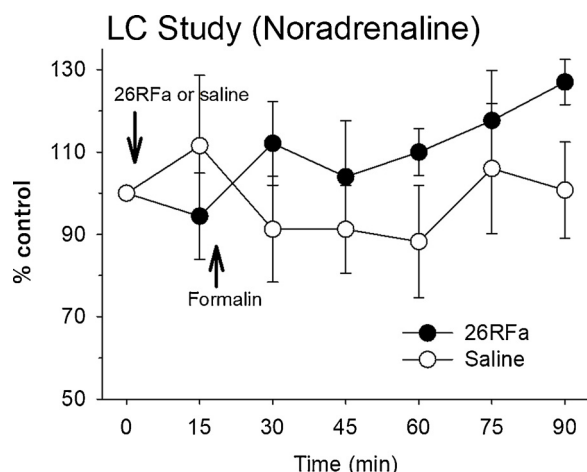
\*:  $p < 0.05$  as compared with 26RFa-treated rats at each time point.

Ordinate: number of flinches per min; abscissa: time after drug administration (min).

suggests that the analgesic effect of 26RFa microinjected into the contralateral LC was mediated by the activation of BIBP3226-sensitive receptors such as NPY Y1 or NPFF. There is no commercially available QRFP receptor antagonist. Moreover, there is no data whether BIBP3226 interferes with the QRFP receptor. Thus, we were not able to determine whether 26RFa injected into the contralateral LC produced an analgesic effect by activation of the QRFP receptor, NPY Y1 receptor, or NPFF receptor.

We previously reported that ICV injection of 26RFa decreases the number of both phase 1 and phase 2 flinching behaviors, and the analgesic effect of 26RFa injected into the ICV on the phase 1 response, but not the phase 2 response, was antagonized by BIBP3226 [17]. The antagonistic effects of BIBP3226 injected into the ICV during the rat formalin test were not comparable to that of BIBP3226 injected into the contralateral PAG or LC. This suggests that the contralateral PAG and LC were not the sole targets of the analgesic effect of 26RFa injected into the ICV, and that the contralateral PAG and LC may interact with each other. Thus, there may be other sites for the analgesic actions of 26RFa close to the third ventricle.

The analgesic effect of 26RFa injected into the contralateral LC on the phase 2 response, but not the phase 1 response, was antagonized by

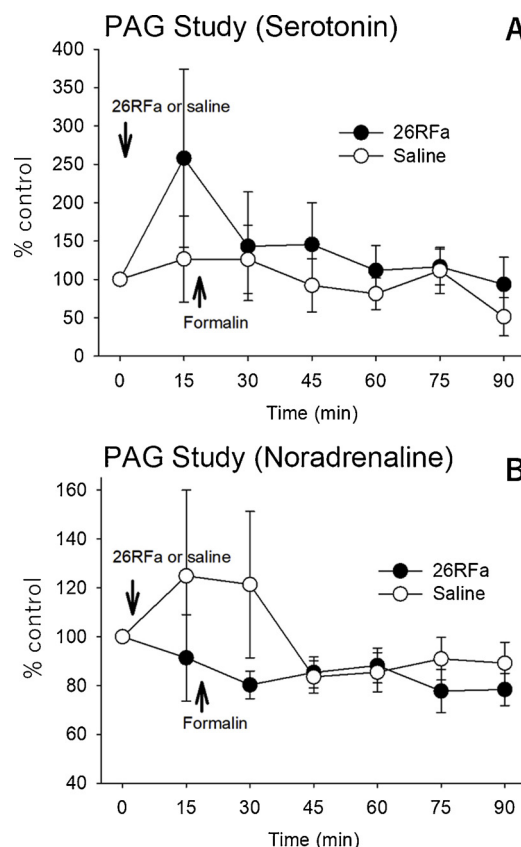


**Fig. 8.** Microdialysis sampling of noradrenaline release in the spinal cord after 26RFa was injected into the contralateral LC. Noradrenaline levels were expressed as a percentage of three baseline samples for each animal (% control). Mean value of “% control” during 15–75 min after formalin injection in 26RFa ( $n = 4$ ) treated rats was significantly larger than that in saline treated rats ( $n = 4$ ) ( $p = 0.028$ ). This indicated that 26RFa significantly increased noradrenaline release compared with that in saline-treated rats.

Ordinate: the percentage of three baseline samples (% control); abscissa: time after drug administration (min).

IT idazoxan, an  $\alpha$ -2 adrenergic receptor antagonist. This suggested that 26RFa injected into the contralateral LC induced the release of noradrenaline in the spinal cord, which produced an analgesic effect in the phase 2 response, but not the phase 1 response. In the microdialysis study, we found that 26RFa injected into the contralateral LC induced noradrenaline release from the spinal cord 15–75 min after formalin injection. Therefore, a significant noradrenaline release only occurred during the phase 2 period and not during the phase 1 period, and intrathecal idazoxan antagonized only the phase 2 response. The analgesic effect of 26RFa injected into the contralateral LC was antagonized by BIBP3226. Thus, during phase 2, 26RFa activated BIBP3226-sensitive receptors in the LC and induced noradrenaline release in the spinal cord. 26RFa also activated BIBP3226-sensitive receptors during phase 1, but it is still unknown how 26RFa injected into the contralateral LC produces an analgesic effect during phase 1.

In the PAG study, BIBP3226 antagonized the analgesic effect of contralateral 26RFa in the phase 2 response, but not the phase 1 response. IP injection of naloxone antagonized the analgesic effect of contralateral 26RFa on the phase 1 response, but not the phase 2 response. These data suggested that, during phase 1, 26RFa injected into the contralateral PAG activated the opioid system and that this opioid system activation was not mediated by the activation of BIBP3226-sensitive receptors in the PAG. There are no commercially available QRFP receptor antagonists, and we were not able to determine whether 26RFa injected into the contralateral PAG activated the QRFP receptor. It is possible that QRFP receptor activation at the contralateral PAG activated the opioid system during phase 1. BIBP3226 antagonized the contralateral 26RFa-mediated analgesic effect during phase 2, which suggests that 26RFa injected into the contralateral PAG activated BIBP3226-sensitive receptors and produced an analgesic effect. The mRNA density of the QRFP receptor in the PAG is low [2], which suggests that the effects of 26RFa are not mediated through QRFP receptors in the PAG and the opioid system, but rather through BIBP-sensitive receptors to produce an analgesic effect during phase 2. We also found that the analgesic effect of 26RFa microinjected into the contralateral PAG was not antagonized by IT idazoxan. Microdialysis data indicated that 26RFa injected into the contralateral PAG reduced spinal noradrenaline release and did not affect spinal serotonin levels. As described above, 26RFa microinjected into contralateral LC



**Fig. 9.** Microdialysis sampling for serotonin (A) and noradrenaline (B) release in the spinal cord after 26RFa was injected into the contralateral PAG. Noradrenaline and serotonin levels were expressed as a percentage of three baseline samples for each animal (% control). (A) 26RFa ( $n = 5$ ) had no effect on serotonin release compared with that in saline-treated rats ( $n = 5$ ) ( $p = 0.828$ ). (B) Mean value of “% control” during 15–75 min after formalin injection in 26RFa ( $n = 5$ ) treated rats was significantly less than that in saline treated rats ( $n = 5$ ) ( $p = 0.038$ ) and this indicated that 26RFa significantly attenuated noradrenaline release compared with that in saline-treated rats. Ordinate: percentage of three baseline samples (% control); abscissa: time after drug administration (min).

increased spinal noradrenaline release and the analgesic effect of 26RFa was antagonized by intrathecal idazoxan. These data suggested that spinal noradrenaline at over the base-line level produced an analgesic effect and that spinal noradrenaline at less than the base-line level had no effect on the spinal nociceptive transmission. 26RFa microinjected into the contralateral PAG activated BIBP3226-sensitive receptors and produced an analgesic effect not by the noradrenaline/serotonin-mediated descending inhibitory pain system but by another system. We do not know why opposite effects occurred at the LC and the PAG.

The analgesic magnitude and duration of 26RFa injected into the contralateral LC was higher and longer, respectively, than that of 26RFa injected into the contralateral PAG. Moreover, the ability of antagonists to block the analgesic effect differed between the LC and PAG. Both the LC and PAG have a high density of 26RFa binding sites [2]. The expression of QRFP receptor mRNA is high in the LC and low in the PAG [2], which suggests that the systems used in the LC are different from those in the PAG.

The mechanism of 26RFa to produce an analgesic effect during phase 1 was sometimes different from that during phase 2. The phase 1 response is mediated by the direct stimulation of nociceptors by formalin, and the phase 2 response is mediated by the inflammatory response induced by formalin [15,18]. The phase 2 response is attenuated by spinal NMDA receptor antagonists, which suggests that the phase 2 response is mediated by NMDA receptors [18]. It is possible that

different characteristics between phase 1 and phase 2 may have some impact on the effects of 26RFa in phase 1 and phase 2.

## 5. Conclusions

26RFa injected into the contralateral LC and contralateral PAG, but not the RVM, ipsilateral LC, and ipsilateral PAG, produced an analgesic effect in a rat formalin test. The analgesic effects of 26RFa were mediated through either BIBP3226-sensitive receptors or the QRFP receptor in the rat formalin test.

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