

## Study in vitro and in vivo of nociceptin/orphanin FQ(1–13)NH<sub>2</sub> analogues substituting N-Me-Gly for Gly<sup>2</sup> or Gly<sup>3</sup>

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### Abstract

In the present study, two analogues containing N-Me-Gly (Sarcosine, Sar) were synthesized to further investigate the structural–activity relationships of orphanin FQ/nociceptin (OFQ/NC, NC). The replacement of Gly<sup>2</sup> or Gly<sup>3</sup> with Sar increased the flexibility and decreased the hydrophobicity of the N-terminal tetrapeptide. The activity of the analogues was investigated in a series of assays in vivo and in vitro. [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> was found to (1) produce dose-dependent inhibition of the electrically induced contraction in MVD assay (pEC<sub>50</sub> = 6.14); (2) produce significant hyperalgesia effects in a dose-dependent manner when intracerebroventricularly (i.c.v.) injected in mice. The inhibitive effects of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> in MVD assay could be significantly antagonized by [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>, and partially antagonized by naloxone; the hyperalgesic effect of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> could be significantly antagonized by naloxone, and partially antagonized by [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>. On the contrary, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> showed no effects in these assays. All the findings suggest that the flexibility of the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> and between Gly<sup>2</sup> and Gly<sup>3</sup> play an important role in NC–OP<sub>4</sub> receptor interaction, and the hydrophobicity of the N-terminal tetrapeptide showed no significant effect on this interaction. The present work also helps to provide a novel method to elucidate structural and conformational requirements of the opioid peptide–receptor interaction.

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**Keywords:** Orphanin FQ/nociceptin; Sarcosine; Mouse vas deferens (MVD); Tail-flick test; Naloxone; [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>

### 1. Introduction

Nociceptin (also termed orphanin FQ, NC), a heptadecapeptide (Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln), has been identified as the endogenous ligand of the OP<sub>4</sub> [9,13]. Although structurally similar to the endogenous opioid peptide (especially dynorphin A), NC possesses different characteristics in pharmacological profiles in that it binds to the classical opioid receptors (μ, δ and κ) with very low affinity [9,13]. Since the discovery of NC, great deals of works have been done in structure–activity relationship of the peptide. Systemic structure–activity study of the activity of NC fragments determined NC(1–13)NH<sub>2</sub> to be the minimal sequence that retains full agonist activity [4], and NC(1–13)NH<sub>2</sub> has served as a template for the designing of further compounds since then. The subsequent studies on NC-related peptides

revealed that, as in case of opioid peptide, the message domain of NC coincides with the N-tetrapeptide F-G-G-F, then leaving to the highly basic C-terminal sequence NC(5–13) the function of address domain [5]. The compound [Phe<sup>1</sup>ψ(CH<sub>2</sub>–NH)Gly<sup>2</sup>]NC(1–13)NH<sub>2</sub> was synthesized in an attempt to protect the N-terminus from degradation by aminopeptidases and it was found to behave as an OP<sub>4</sub> antagonist in a variety of in vitro assays, while acting as an agonist or partial agonist in most in vivo assays [1,2]. Then the compound [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> was identified as a pure and selective antagonist of OP<sub>4</sub>, and this antagonistic properties was proved in most of in vivo and in vitro assays [12,14].

In the present study, we have focused on the N-terminal tetrapeptide of NC(1–13)NH<sub>2</sub>, which was considered to be crucial for OP<sub>4</sub> occupation and activation. In addition, the chemical requirements of this region appear to be very rigorous, as the insertion of pseudo-peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> maintains good affinity and behaves as receptor antagonist, partial agonist or agonist [1,2,8,10]. In an attempt to further study if the hydrophobicity and flexibility of the N-terminal tetrapeptide play important roles in NC–OP<sub>4</sub> receptor interaction, two analogues substituting N-Me-Gly

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(Sarcosine, Sar) for Gly<sup>2</sup> or Gly<sup>3</sup> were synthesized. These modifications would increase the flexibility of the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> or Gly<sup>2</sup> and Gly<sup>3</sup>, and decrease the hydrophobicity of this region. The analogues were tested for their activity to inhibit the electrically evoked contractions of the mouse vas deferens (MVD)—a pharmacological preparation shown to be sensitive to NC [4]; the effects of these analogue in pain modulation at supraspinal level were tested with the warm water tail-flick assay in mice.

## 2. Materials and methods

### 2.1. Animals

Male Kunming mice were supplied by the Animal Center of Lanzhou Medical College. All animals were cared for and experiments were carried out in accordance with the European Community guidelines for the use of experimental animals (86/609/EEC). All the protocols in this study were approved by the Ethics Committee of Lanzhou Medical College, China.

### 2.2. Drugs

The peptides used in this study: NC, [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub>, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> and [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> were synthesized by solid-phase peptide synthesis method and purified by high-performance liquid chromatography (HPLC) by our group. All the drugs were dissolved in sterilized saline. Morphine hydrochloride and naloxone were the products of Shenyang First Pharmaceutical Factory, China.

### 2.3. Mouse isolated vas deferens (MVD)

The vas deferentia were taken from male adult Kunming mice (28–30 g) which were sacrificed by cervical dislocation. Vascular and fatty tissue were removed and suspended in 10 ml organ baths containing Krebs solution oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The temperature was set at 37 °C, a resting tension of 0.3 g was applied to the mouse vas deferens. A Mg<sup>2+</sup> free Krebs solution was used in the MVD assay.

The mouse vas deferens was continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 ms duration and 0.1 Hz frequency. The electrically evoked contractions were measured isotonicly with a strain gauge transducer (Machine Equipment Corporation of GaoBeiDian, China) and recorded on a Powerlab recorder system (model 410, Australia). After an equilibration period of 60 min the contractions induced by electrical field stimulation were stable; at this time, cumulative concentration response curves to nociceptin or its analogues were performed. In certain experiments, [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> or naloxone were added to the medium 15 min before performing concentration response curves to the agonist.

### 2.4. Tail-flick assay

Male Kunming mice weighing 20–22 g were used. They had free access to food and water in an animal room which was maintained at 22 ± 1 °C with a 12-h light/12-h dark cycle. Every mouse was used only once. The effects of these analogues of NC were assessed with 48 °C warm-water tail-flick test. The i.c.v. administration was performed following the method described by Haley and McCormick [7]. Proper injection site was verified by administration and localization of methylene blue dye. To investigate whether the hyperalgesic effects of the analogue could be antagonized by [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> or naloxone, [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> or naloxone were co-injected with the analogue. The latency to the first sign of a rapid tail-flick was taken as the behavioral end point. Every mouse was first tested for baseline latency by immersing its tail in the water and recording the response time. Effects of drugs were tested with tail-flick reflex every 5 min for 30 min and then tested every 10 min for 30 min post-injection. The average tail-flick latency (TFL) of each group of mice before administration served as the control (100%). Results were calculated with the following equation: percent TFL = 100 × (test TFL/control TFL) ± S.E.M. Data have been statistically analyzed with analysis of variance followed by the LSD post hoc test; *P*-values less than 0.05 were considered to be significant. A cut-off latency of 20 s was used, the animals that did not respond within the cut-off time were removed to avoid tissue damage.

## 3. Results

### 3.1. Effects in mouse vas deferens assay

As reported previously, NC inhibits the electrically induced contractions of the mouse vas deferens (pEC<sub>50</sub> = 7.50), this response was not antagonized by the non-selective classical opioid receptor antagonist naloxone but was antagonized by selective OP<sub>4</sub> receptor antagonist [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>. This preparation was also used to evaluate the effects of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> and [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub>. [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> caused a rapid and concentration-dependent inhibition on electrically evoked contraction (pEC<sub>50</sub> = 6.14), which could be partially antagonized by non-selective classical opioid receptor antagonist naloxone and significantly antagonized by selective OP<sub>4</sub> receptor antagonist [Nphe<sup>1</sup>]N/OFQ(1–13)NH<sub>2</sub>. On the contrary, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> showed no significant inhibition effect in the MVD assay. Both [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> and [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> did not antagonize the effects of NC in MVD assay (Table 1 and Fig. 1).

### 3.2. Effects in mice tail-flick test

In previous studies, NC injected supraspinally was found to produce an initial hyperalgesic response followed by

Table 1  
Apparent affinities (pEC<sub>50</sub>) and maximum effects of NC and its analogues on the electrically stimulated mouse vas deferens

Number	Abbreviated names	Agonist		Antagonist <sup>a</sup>
		pEC <sub>50</sub> (CL <sub>95%</sub> ) <sup>b</sup>	E <sub>max</sub> (%) <sup>c</sup>	
1	NC(1–17)OH	7.50(0.43)	-78.8 ± 7.3	ND <sup>d</sup>
2	[Sar <sup>2</sup> ]NC(1–13)NH <sub>2</sub>	6.14(0.42)	-60.2 ± 5.0	I
3	[Sar <sup>3</sup> ]NC(1–13)NH <sub>2</sub>	crc incom <sup>e</sup>		I

<sup>a</sup> The antagonist properties of the compounds were tested using NC(1–17)OH as agonist.

<sup>b</sup> pEC<sub>50</sub>: the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal effect. CL<sub>95%</sub>: 95% confidence limits.

<sup>c</sup> E<sub>max</sub>: the maximal effect induced by an agonist expressed as percent inhibition of electrically induced twitches.

<sup>d</sup> ND: not determined because the compound is full agonist.

<sup>e</sup> crc incom: concentration-response curves (crc) incomplete indicates that only a slight affect (<50% inhibition) was detected at the highest concentration tested (10 μM).

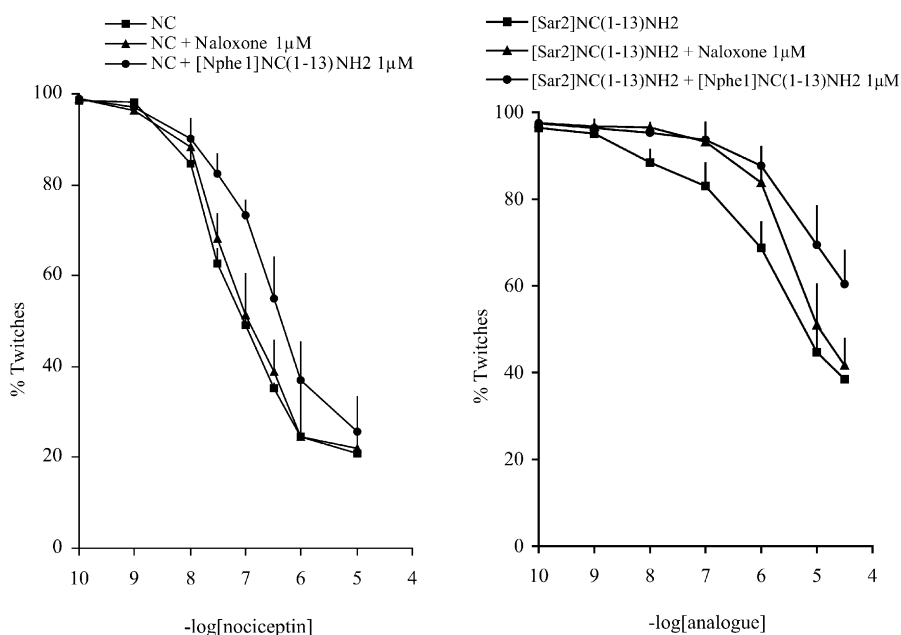


Fig. 1. Effects of naloxone (1 μM) and [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (1 μM) on concentration-response curves on nociceptin (the left panel) or [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (the right panel) in the electrically stimulated mouse vas deferens. Data are mean ± S.E.M. of at least five experiments.

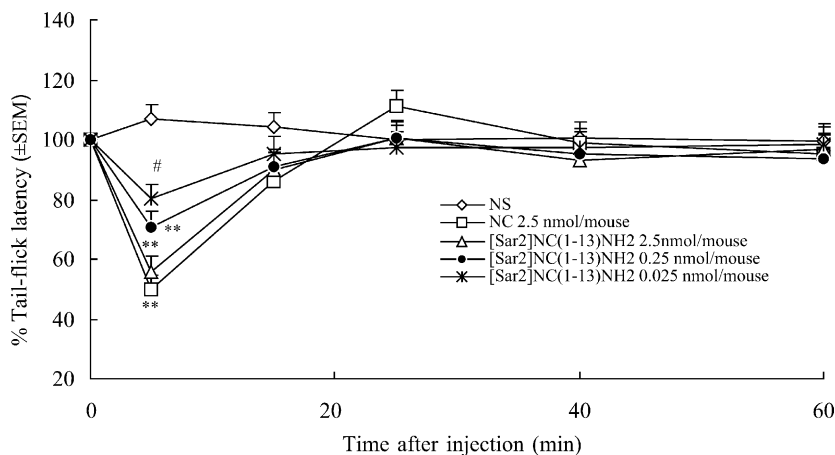


Fig. 2. Effects of i.c.v. administration of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> in tail-flick test in mice. [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> produced a hyperalgesia effect. Each time point is presented as percent tail-flick latency ± S.E.M. (*n* = 15). \*\**P* < 0.01, #*P* < 0.05, compared with saline group.

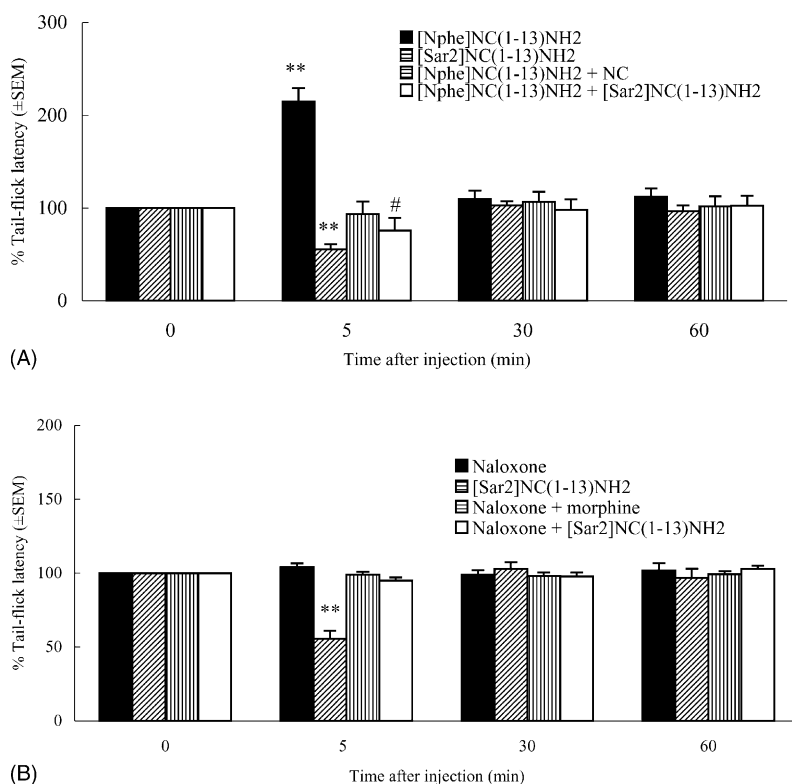


Fig. 3. Effects of i.c.v. coinjection of [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (A) or naloxone (B) on hyperalgesic effects of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub>. Mice were i.c.v. injected with [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (2.5 nmol/mouse) alone or together with [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5 nmol/mouse) or NC (2.5 nmol/mouse) (A). Naloxone (2.5 nmol/mouse) was i.c.v. injected alone or together with [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5 nmol/mouse) or morphine (2.5 nmol/mouse) (B). Assessment of tail-flick latency was made at 5, 30, 60 min. Data were expressed as tail-flick latency  $\pm$  S.E.M. ( $n = 10$ ). \*\* $P < 0.01$ , # $P < 0.05$ , compared with saline group (data of saline not shown).

analgesia in mice [15,16]. Our findings confirmed these earlier results. The i.c.v. administration of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5, 0.25, 0.025 nmol/mouse) was found to produce a significant decrease in reaction time to remove off the tail from the heat stimulus; i.c.v. injection of saline did not alter the tail-flick latency significantly. This dose-dependent hyperalgesia of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> was evoked and reached a maximum at about 5 min and then declined until cessation at about 25 min after injection, but showed no significant delayed analgesia effect (Fig. 2.). [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (2.5 nmol/mouse) injected i.c.v. alone produced significant analgesia, which reached the maximum at 5 min and lasted for about 30 min. When i.c.v. co-injected, [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> (2.5 nmol/mouse) partially antagonized hyperalgesia induced by [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5, 0.25 nmol/mouse) (Fig. 3A). Naloxone (2.5 nmol/mouse) injected i.c.v. alone showed no significant effect. Co-injection of naloxone could significantly antagonized the hyperalgesia induced by [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5, 0.25 nmol/mouse) (Fig. 3B). However, i.c.v. injection of [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> showed no significant effect on the latency in the tail-flick test. Co-injection of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> or [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> did not antagonize the effects of i.c.v. injection of NC in mice tail-flick assay.

#### 4. Discussion

In earlier reports, it has been demonstrated that NC(1–13)NH<sub>2</sub> was the shortest fragment that retained the full bioactivities of NC [4,5]. Taking NC(1–13)NH<sub>2</sub> as a template, a series of analogues were designed and synthesized to study the structure–activity relationship of the N-terminal tetrapeptide of NC [2,3,6,14]. In an attempt to protect NC(1–13)NH<sub>2</sub> from degradation by aminopeptidases, the pseudopeptide [Phe<sup>1</sup>ψ(CH<sub>2</sub>–NH)Gly<sup>2</sup>]NC(1–13)NH<sub>2</sub> ([F/G]NC(1–13)NH<sub>2</sub>) was designed [1,2]. Studies showed that this pseudopeptide maintains good affinity and shows a variety of activities including antagonist [2,6], partial agonist [10] and agonist [8,11] activities at OP<sub>4</sub> receptor, depending on the tissue preparation. As to the mechanism of the change in chemical terms, the replacement of CO with CH<sub>2</sub> in this pseudopeptide eliminates the possibility of forming hydrogen bonds; at the same time it increases the flexibility of the N-terminal part of the molecule by transforming the amide into an amine function. These work showed that the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> of NC play important roles in occupying and activating to the OP<sub>4</sub> receptor.

In the present study, in an attempt to study further the structure–activity relationships of the N-terminal tetrapeptide of NC, two analogues substituting sarcosine for Gly<sup>2</sup> or Gly<sup>3</sup> were prepared. Based on principles of chemistry, replacing Gly<sup>2</sup> or Gly<sup>3</sup> with Sar could eliminate the ability to form hydrogen bond and increase flexibility of the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> or Gly<sup>2</sup> and Gly<sup>3</sup>. Preserving CONH, the flexibility between Phe<sup>1</sup> and Gly<sup>2</sup> or Gly<sup>2</sup> and Gly<sup>3</sup> was higher than that in the native peptide but lower than in [F/G]NC(1–13)NH<sub>2</sub>. As it was reported, the hydrophilicity of Sar was higher than Gly. Therefore, the insertion of Sar will produce a decrease of the hydrophobicity of the N-terminal tetrapeptide at the same time.

These analogues were tested for its ability to inhibit electrically evoked contraction (twitch response) of the mouse vas deferens (MVD), a pharmacological preparation shown to be sensitive to NC [4]. The analogue substituting Sar<sup>2</sup> for Gly<sup>2</sup>, [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub>, was found to produce a dose-dependent inhibition of the electrically evoked contraction of the mouse vas deferens, and this effect could be slightly antagonized by pre-incubation of naloxone, but significantly inhibited by pre-incubation of [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>. Furthermore, the analogue showed no significant influence on NC-induced inhibitions on electrically evoked contractions of MVD. However, the other analogue, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub>, showed no significant effects on the electrically evoked contractions or the NC-induced inhibitions in the MVD assay. These results suggested that the increasing of the flexibility of the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> in the NC(1–13)NH<sub>2</sub> sequence reduced the binding activity to OP<sub>4</sub> receptor, while increased the ability to activate classical opioid receptors. The increasing of the flexibility of the peptide bond between Gly<sup>2</sup> and Gly<sup>3</sup> caused an entire losing of the binding activity of NC(1–13)NH<sub>2</sub> on opioid receptors. Comparing [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> to [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub>, there is no significant difference in hydrophobicity of the N-terminal tetrapeptide. Compared to NC(1–13)NH<sub>2</sub>, [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> shows an increased hydrophobicity of the N-terminal tetrapeptide. Therefore, it seems that the change of the hydrophobicity of the N-terminal tetrapeptide of NC(1–13)NH<sub>2</sub> showed no significant effect on the binding activity of NC(1–13)NH<sub>2</sub> in MVD assay.

The reports concerning the roles of NC in pain modulation showed that i.c.v. injection of NC-induced hyperalgesic effects, i.e. it reduced the tail flick [13] or hot plate response latency in mice [9]. Some subsequent studies revealed that i.c.v. NC was able to elicit both a rapid hyperalgesia and a delayed analgesia in mice in the tail-flick assay [15,16]. Our results of NC are consistent with these reports. In the present study, we found i.c.v. administration of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> (2.5, 0.25, 0.025 nmol/mouse) produced potent hyperalgesic effects in the tail-flick test, but it did not induce delayed analgesia. On the contrary, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> showed no significant effect on the latency in the tail-flick

test. [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> was first reported by Rizzi et al. [14] as an antagonist of OP<sub>4</sub> in mouse colon assay. [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> injected i.c.v. alone was found to induce an antinociceptive effect which was attributed to block of OP<sub>4</sub>; when [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> was given together with NC, it was found to reverse the hyperalgesia of NC in mice [3]. The selective and competitive antagonistic effect of [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> on OP<sub>4</sub> has been demonstrated in a variety of preparations both in vitro and in vivo [3,14]. Naloxone was widely accepted as an antagonist of classical opioid receptor. In this study, [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> and naloxone were used as tools to investigate the mechanisms of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> in pain modulation. We found that the hyperalgesia effect of [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> could be partially antagonized by [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub>, and could be significantly inhibited by co-injection of naloxone. It has been reported that [Nphe<sup>1</sup>]NC(1–13)NH<sub>2</sub> could fully antagonize the hyperalgesia of NC, while naloxone showed no effect on it. It implied that the replacement of Gly<sup>2</sup> by Sar gives a compound that acts on OP<sub>4</sub> as well as on the traditional opioid receptors. And these results suggest that [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> produced a similar effects as NC in mice tail-flick test, and maybe it elicits this effect via a different mechanism from NC and may produce some effects via activating classical opioid receptors; [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> entirely lost the activating activity to the OP<sub>4</sub> receptor and the classical opioid receptors. It seems to prove that the flexibility of the peptide bond between Phe<sup>1</sup> and Gly<sup>2</sup> or Gly<sup>2</sup> and Gly<sup>3</sup>, especially between Gly<sup>2</sup> and Gly<sup>3</sup>, play a important role in activating activity of NC to OP<sub>4</sub> receptor. Any slight change of the flexibility of these two peptide bond will cause a significant loss of bioactivities of NC in pain modulation at supraspinal level. As in the case of MVD assay, [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> shows no significant difference in hydrophobicity of the N-terminal peptide compared to [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub>, but shows an increased hydrophobicity of the region compared to the native peptide. However, [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> entirely losing of the effects of NC(1–13)NH<sub>2</sub> in pain modulation at supraspinal level while [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> shows similar effect as NC(1–13)NH<sub>2</sub> in pain modulation. It seems to suggest that slight change of the hydrophobicity of the N-terminal tetrapeptide of NC showed no significant effect on pain modulation effect of NC(1–13)NH<sub>2</sub> in tail-flick test.

Collectively, all the results obtained with [Sar<sup>2</sup>]NC(1–13)NH<sub>2</sub> and [Sar<sup>3</sup>]NC(1–13)NH<sub>2</sub> suggested that the hydrophobicity of the N-terminal tetrapeptide showed no significant effect on the binding and activating activities of NC to OP<sub>4</sub> receptor, but the flexibility of this region seems to play very important roles in the NC–OP<sub>4</sub> receptor interaction. The present work also helps to provide a novel and simplified method to perform structure–activity study, which could investigate the roles of the hydrophobicity and flexibility of defined fragment in the native peptide at the same time through a simple replacement.

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