



## Nociceptin produces antinociception after spinal administration in amphibians

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### ABSTRACT

Nociceptin, also known as orphanin FQ, is a opioid-like neuropeptide that mediates its effects at the nociceptin receptor, a member of the G protein-coupled receptor superfamily. In mammals, nociceptin produces analgesia after spinal administration, however the role of nociceptin and nociceptin receptors in the modulation of noxious stimuli in non-mammalian species has not been examined. In an amphibian pain model using the acetic acid test with *Rana pipiens*, nociceptin and nociceptin<sub>1–13</sub> amide produced dose-dependent antinociception (1–100 nmol), blocked by the nociceptin antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (30 nmol), but not the opioid antagonist, naltrexone (100 nmol/g, s.c.). Conversely, the antinociceptive effects of *mu*, *delta*, and *kappa* opioid receptor agonists were not blocked by the nociceptin antagonist. Nociceptin and nociceptin<sub>1–13</sub> amide were the least potent of the opioid agonists tested. These studies demonstrate that spinal nociceptin receptors and not opioid receptors mediate the antinociceptive effect of nociceptin. Considered with previous findings, these behavioral data supports a role for nociceptin inhibition of spinal nociception in amphibians and perhaps all vertebrates.

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### 1. Introduction

Nociceptin (also called orphanin FQ) is a 17 amino acid neuropeptide found in brain extracts that was implicated in the modulation of nociceptive transmission, along with other roles (Meunier et al., 1995; Reinscheid et al., 1995). In concert with its name, nociceptin produced hyperalgesia after intracerebroventricular (i.c.v.) administration to mice (Meunier et al., 1995; Suaudeau et al., 1998; Zhu et al., 1997). Conversely, nociceptin administered by the spinal route produced analgesia (Wang et al., 1999a; Yamamoto et al., 1997). However, even this difference in the effect of nociceptin explained by route of administration was not consistent among all studies (Mogil and Pasternak, 2001; Yamamoto et al., 1999). It is now thought that the hyperalgesic effects of supraspinal nociceptin were due to reversal of stress-induced analgesia and that nociceptin administered to the spinal cord of mammals produces a classical analgesic response (Lambert, 2008; Zeilhofer and Calò, 2003).

The pronociceptin precursor protein that yields nociceptin is closely related to the family of endogenous opioid propeptides; proopiometanocortin, proenkephalin and prodynorphin. Pronociceptin has similar gene structure (e.g. conserved intron/exon boundaries, CYS residues and dibasic cleavage sites) as opioid peptide precursors, especially prodynorphin (Darland et al., 1998; Henderson and Mcknight, 1997; Meunier, 1997). Pronociceptin mRNA and nociceptin immunoreactivity is localized throughout the CNS of mammals within

neuronal pathways that function in the processing of pain (Mogil and Pasternak, 2001). Like endogenous opioid peptides, especially high densities of both nociceptin markers are found in the dorsal horn of mammalian spinal cord (Darland et al., 1998; Lai et al., 1997; Pettersson et al., 2002; Schuligoi et al., 1997).

The receptor for nociceptin (NOP) was discovered before its endogenous ligand (hence the *orphan* in its previous name and the abbreviation, ORL) by low-stringency hybridization screening using opioid receptor cDNA probes or by selective amplification of genomic DNA using degenerate primers (Meunier, 1997; Mollereau et al., 1994). The sequence of the NOP is homologous with that of classical *mu* (MOR), *delta* (DOR) and *kappa* (KOR) opioid receptor proteins. Thus the gene for NOP is the fourth member of the opioid receptor gene family. Cell lines expressing NOP receptors display a high-affinity for nociceptin binding, and binding of nociceptin to the NOP receptor stimulates the binding of GTPγS, and activates a pertussis-sensitive Gα<sub>i/o</sub> signal transduction pathway leading to inhibition of cAMP formation, a decrease in calcium conductance and an increase in potassium channel conductance (Dooley and Houghten, 2000; Fawzi et al., 1997; Hawes et al., 2000; Ikeda et al., 1997). The net effect of these intracellular sequelae is inhibition of neurotransmitter release and neuronal activity. Thus, functionally as well as structurally, the nociceptin receptor is homologous to the classic opioid receptors by acting through similar signal transduction pathways (Fawzi et al., 1997). With the exception of lofentanil and buprenorphine, most opioids do not have appreciable affinity for the nociceptin receptor (Bloms-Funke et al., 2000; Butour et al., 1997; Yamamoto et al., 2006; Zaveri et al., 2001).

Since the original discovery of endogenous nociceptin, other peptide analogs were synthesized including the agonist, nociceptin<sub>1–13</sub> amide

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(Butour et al., 1997) and the antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (Calò et al., 2000; Pheng et al., 2000; Xu et al., 2002). Nociceptin<sub>1–13</sub> amide displaces tritiated nociceptin with the same affinity as the native peptide, nociceptin, in rat brain homogenates and after transfection of NOP receptor in CHO cell membranes (Calò et al., 2002). Like nociceptin, the agonist activity of nociceptin<sub>1–13</sub> amide was shown by stimulating the binding of GTPγS, and inhibiting cAMP formation, decreasing calcium conductance and increasing potassium channel conductance (Salvadori et al., 1999). The nociceptin antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide, also has a high affinity for the NOP receptor and the spinal analgesic effect of nociceptin<sub>1–13</sub> amide in rodent models (Butour et al., 1997) was blocked by co-administration of [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (Pheng et al., 2000; Xu et al., 2002).

To date, all of the behavioral studies of nociceptin were done using mammalian species. Given the variable results on nociceptin effects in mammals and for purposes of contributing data to a larger comparative dataset on the pharmacology of opioids and vertebrate opioid receptor proteins, the present study examined the pharmacology of nociceptin following spinal administration in amphibians. The amphibian model for assessing opioid effects is an alternative or adjunct model for pain and analgesia research (Stevens, 1992, 2004). Previous studies examined the relative antinociceptive potency of non-selective and selective opioid agonists after systemic, spinal, and supraspinal routes of administration (Mohan and Stevens, 2006; Stevens, 1996; Stevens et al., 1994, 2007b; Stevens and Rothe, 1997). Non-opioid analgesics and adrenergic agents were also tested using the amphibian model (Brenner et al., 1994; Stevens et al., 2001; Stevens and Brenner, 1996).

The present study examined the effects of nociceptin and nociceptin<sub>1–13</sub> amide following spinal administration in amphibians. For comparison, the antinociceptive effects of the MOR agonist, fentanyl (Janssen et al., 1963), the DOR agonist, [D-Pen<sub>2</sub>-D-Pen<sub>5</sub>]-enkephalin (DPDPE; (Mosberg et al., 1983) and the KOR agonist, U50488 (VonVoigtlander et al., 1983) were also tested. Finally, the receptor selectivity of nociceptin's effect was examined by the use of the non-selective opioid receptor antagonist, naltrexone, and the selective NOP receptor antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide.

## 2. Methods

All procedures were approved by the Institutional Animal Use Committee (IACUC) and adhere to the National Institutes of Health (U.S.A.) and the European Community guidelines on the use of animals for biomedical research.

### 2.1. Animals

Northern grass frogs, *Rana pipiens* (Charles Sullivan, Inc., Nashville, TN, USA) with a mean weight of 28 g were kept in groups of 48 in a flow-through, stainless steel enclosure at room temperature with running water after arrival. They were maintained with a 12-hour photoperiod (lights on 0700) and were fed live crickets twice a week. At least two days before experiments, animals were transferred to the laboratory and placed in individual plastic pans with an adequate amount of tap water. On the day of experimental study, water was adjusted to a depth such that the dorsal surface of the frog's thigh was exposed for testing. Baseline nociceptive thresholds (see below) were obtained 2 h after the water level was adjusted on the morning of the experiment and post-treatment nociceptive thresholds at 1 and 3 h after drug administration. Previous studies of analgesics administered by the intraspinal route in amphibians demonstrated that maximal effects were obtained within this time period.

### 2.2. Drugs and drug administration

Naltrexone, fentanyl, DPDPE, U50488H, and nociceptin<sub>1–13</sub> amide were obtained from the National Institute on Drug Abuse, Drug Supply

Program (with generous assistance from Mr. Kevin Gormley of the Research Technology Branch). Nociceptin was obtained from Bachem, Inc. (Torrance, CA) and the antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide, was purchased from Tocris Cookson, Inc. (Ellisville, MO). Drugs were mixed in saline to give nmol/μl solutions of the peptide or free base. Spinal administration was done using a Hamilton microsyringe and was made between the articulation of the lumbar vertebrae in a volume of 5 μl (Stevens, 1996). Saline-injected control animals were co-run with agonist and antagonist experiments, and there were no significant changes from baseline values. The nociceptin antagonist was given by concurrent spinal injection with the agonist using doses determined in pilot experiments. For opioid antagonism experiments, systemic naltrexone (100 nmol/g) or saline was administered 1 h before the spinal administration of agonists. Systemic administration was made by bolus injection into the dorsal lymph sac at a volume of 10 μl/g body weight (Stevens et al., 1994). Treatment groups consisted of six to eight animals per dose. Each animal was used only once.

### 2.3. The acetic acid test for determining nociceptive thresholds in amphibians

The acetic acid test to determine the nociceptive threshold (NT) in frogs consists of eleven concentrations of acetic acid diluted in half-log steps from glacial acetic acid. The concentrations are given a code number from 0 to 10 with the lowest code number equal to the lowest concentration of acetic acid (Pezalla, 1983). Nociceptive testing is done by placing, with a Pasteur pipette, a single drop of acid on the dorsal surface of the frog's thigh. Testing begins with the lowest concentration and proceeds with increasing concentrations until the NT is reached. The NT is defined as the code number of the lowest concentration of acid that causes the frog to vigorously wipe the treated leg. The nociceptive response of the animal is directly dependent on the pH of the acid solution applied as the noxious stimulus (Hamamoto et al., 2000). To prevent tissue damage, the acetic acid is immediately wiped off with a gentle stream of distilled water once the animal responds or after 4 s. If the animal fails to respond, testing continues on the opposite hindlimb. An animal that fails to respond to the highest concentration (#10) is assigned the cut-off of 11. Each NT determination consists of a single trial of the acetic acid solutions. The acetic acid test was done to obtain baseline NT before drug treatment and post-treatment NT at various times after treatment. Baseline NT was obtained 30 min before drug administration. The raw NT data (code number of acetic acid solution) was converted to maximum percent effect (MPE) by the following formula:

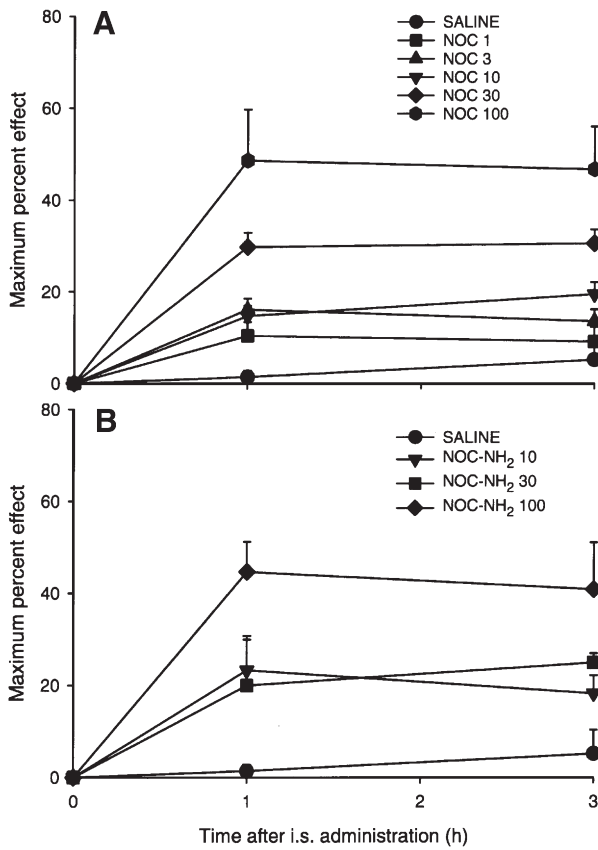
$$\text{MPE} = \frac{\text{Posttreatment NT} - \text{Baseline NT}}{\text{Cutoff value (11)} - \text{Baseline NT}} \times 100$$

MPE data was plotted for treatment groups as the time course after administration, and the maximum MPE value over that time course was pooled from individual animals at the same treatment dose for construction of dose–response curves. Pharmacological software (Pharmacological Calculation Systems, PCS v. 4.0, MicroComputer Specialists, Philadelphia, PA) was used to calculate the median effective dose (ED<sub>50</sub>) and 95% confidence interval, the slope and 95% confidence interval, and for statistical testing of the significant differences between treatment groups. Antagonist data were analyzed by a one-way ANOVA followed by the *post-hoc* Newman–Keuls test. Significant effects were considered at the  $p < 0.05$  level.

## 3. Results

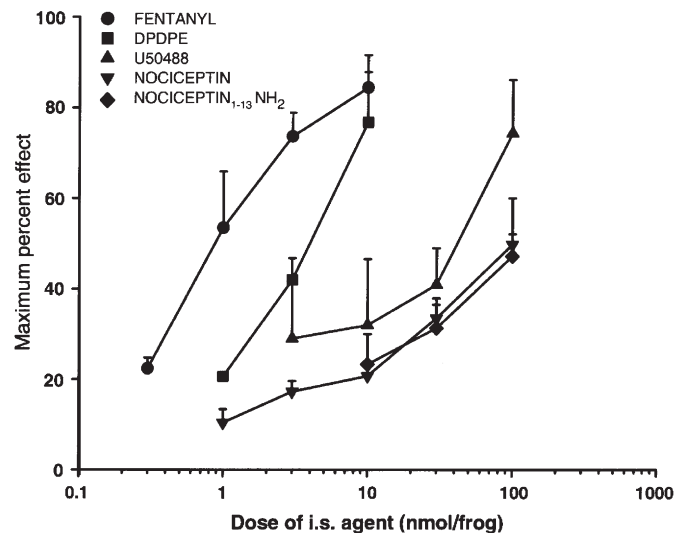
### 3.1. Time course of nociceptin and nociceptin<sub>1–13</sub> amide antinociceptive effects

Nociceptin and nociceptin<sub>1–13</sub> amide produced dose-dependent antinociceptive effects over the 3 h time course with doses from 1 to



**Fig. 1.** Time course of the antinociceptive effect of nociceptin (NOC, panel A) or nociceptin<sub>1–13</sub> amide (NOC–NH<sub>2</sub>, panel B) after spinal administration. Doses were 1–100 nmol/frog for nociceptin and 10–100 nmol/frog for nociceptin<sub>1–13</sub> amide. *N* = 6–8 animals per treatment dose and saline-injected controls.

100 nmol/frog (Fig. 1A, B). At 300 nmol/frog of nociceptin, hindlimb dysfunction was noted in 4 out of 6 animals precluding assessment of nociceptin effects at that high dose. Consistent with other analgesic agents administered to amphibians, the antinociceptive effects lasted at least 3 h, however the animals returned to normal nociceptive thresholds the following day.



**Fig. 2.** Log dose–response curves of the antinociceptive effect of nociceptin and nociceptin<sub>1–13</sub> amide, and fentanyl, DPDPE, and U50488, after spinal administration in amphibians. *N* = 6–8 animals per treatment dose.

**Table 1**  
Analysis of nociceptin and opioid antinociceptive dose–response curves after spinal administration in amphibians

Agent	ED <sub>50</sub> <sup>a</sup>	95% C.I. <sup>b</sup>	Slope	95% C.I.	R.P. <sup>c</sup>
Fentanyl	9.8	(6.7–14.1)	38.7	(23.8–53.7)	1.00
DPDPE	11.1	(5.93–20.80)	40.5	(13.7–67.2)	0.88
U50488	48.2	(20.9–111.5)	36.5	(7.0–65.8)	0.20
Nociceptin	211.3	(75.5–590.9)	18.5	(12.7–24.3)	0.04
Nociceptin <sub>1–13</sub> amide	160.5	(7.9–3252.8)	22.4	(–12.2–56.9)	0.06

<sup>a</sup> In nmol/animal, spinal administration.

<sup>b</sup> 95% confidence interval.

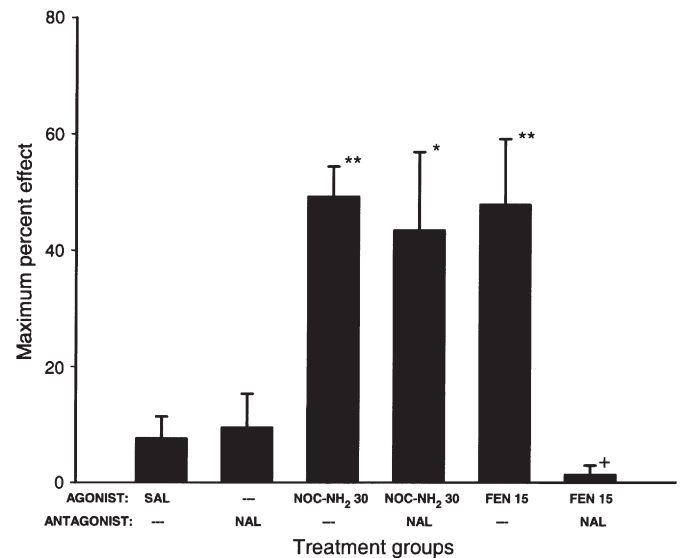
<sup>c</sup> Relative potency compared to fentanyl by the ratio of the ED<sub>50</sub> fentanyl/ED<sub>50</sub> other agent.

### 3.2. Relative antinociceptive potency of nociceptin and nociceptin<sub>1–13</sub> amide

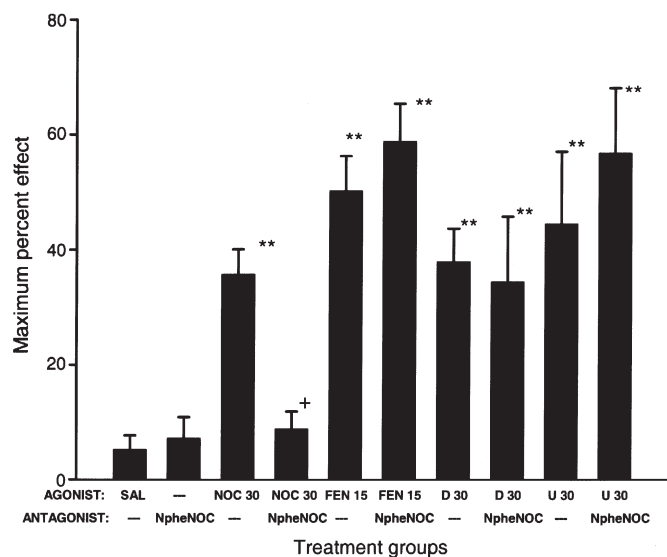
The dose–response curves of antinociceptive effect of nociceptin and nociceptin<sub>1–13</sub> amide following spinal administration in amphibians is in Fig. 2. For comparison, the dose–response curves of the selective MOR (fentanyl), DOR (DPDPE) or KOR (U50488) agonists were also obtained. The ED<sub>50</sub> values and 95% confidence intervals are given in Table 1. Nociceptin and nociceptin<sub>1–13</sub> amide had ED<sub>50</sub> values of 211.3 and 160.5 nmol/animal, respectively. The relative potency of nociceptin and nociceptin<sub>1–13</sub> amide was 0.04 and 0.06 compared to fentanyl (set at 1.00). With the exception of nociceptin<sub>1–13</sub> amide, all agents had a significant slope (*i.e.* the 95% confidence interval of the slope did not include 0) indicating dose-dependent responses. The selective MOR opioid receptor agonist, fentanyl, was the most potent agonist, followed by the DOR and KOR agonists; with nociceptin and nociceptin<sub>1–13</sub> amide the least potent.

### 3.3. Effects of naltrexone on nociceptin<sub>1–13</sub> amide and fentanyl antinociception

The effect of pretreatment with the opioid antagonist, naltrexone (100 nmol/g, *s.c.*), on the antinociceptive action of fentanyl or nociceptin<sub>1–13</sub> amide is shown in Fig. 3. Saline or pretreatment with



**Fig. 3.** Naltrexone pretreatment blocks fentanyl but not nociceptin<sub>1–13</sub> amide antinociceptive effects in amphibians. Naltrexone was given by the systemic route (NAL; 100 nmol/g, *s.c.*) 1 h before spinal administration of nociceptin<sub>1–13</sub> amide (NOC–NH<sub>2</sub>; 30 nmol/frog) or fentanyl (FEN; 15 nmol/frog). Asterisks indicate significantly greater thresholds than SAL alone (\* = *p* < 0.05, \*\* = *p* < 0.01). Plus (+) indicates significantly reduced thresholds (*p* < 0.01) compared to FEN alone treatment group.



**Fig. 4.** Concurrent spinal administration of the nociceptin antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (NpheNOC, 30 nmol/frog) blocks the antinociceptive effects of nociceptin (NOC, 30 nmol/frog) but not fentanyl (FEN, 15 nmol/frog), DPDPE (D, 30 nmol/frog), or U50488 (U, 30 nmol/frog) in amphibians. Asterisks indicate significantly greater nociceptive thresholds than SAL alone (\*\*= $p < 0.01$ ). Plus (+) indicates significantly reduced thresholds ( $p < 0.05$ ) than nociceptin (NOC) alone.

naltrexone alone did not significantly affect the nociceptive threshold in amphibians. Nociceptin<sub>1–13</sub> amide (30 nmol/animal, i.s.) or fentanyl (15 nmol/animal, i.s.) alone produced significant antinociceptive effects. Pretreatment with naltrexone did not affect the antinociception produced by spinal administration of nociceptin<sub>1–13</sub> amide, but significantly blocked the antinociceptive effect of fentanyl.

#### 3.4. Effects of [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide on nociceptin and opioid antinociception

The effects of concurrent spinal administration of the nociceptin receptor antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide, with nociceptin, fentanyl, DPDPE, or U50488 is shown in Fig. 4. Spinal administration of saline or [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (30 nmol/animal) alone did not significantly alter the nociceptive threshold in amphibians. The nociceptin antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide (30 nmol/animal) blocked the antinociceptive effects of nociceptin but not fentanyl, DPDPE, or U50488.

#### 4. Discussion

The present data are the first to show that nociceptin and nociceptin<sub>1–13</sub> amide produces significant antinociception in a non-mammalian vertebrate species, the amphibian *R. pipiens*. Nociceptin administered to the spinal cord of amphibians produced a dose-dependent analgesic effect with an ED<sub>50</sub> of 211 nmol/frog. Nociceptin was a weak analgesic agent, only 0.04 times as potent as fentanyl. Nociceptin<sub>1–13</sub> amide had a similar weak analgesic potency, although the dose–response curve was too shallow to give a significant slope (Table 1). Among the selective opioid agonists tested to date in the amphibian model, the potency of nociceptin falls within the range of most KOR opioid agonists (Stevens, 1996, 2004). This is not surprising as the amino acid sequence of the nociceptin precursor, pronociceptin, is most similar to prodynorphin, and among all vertebrate opioid receptors, the NOP receptor sequence is most similar to that of the KOR protein (Stevens et al., 2007a).

Comparison of the antinociceptive potency of spinally administered nociceptin or nociceptin<sub>1–13</sub> amide in amphibians to similar

studies in rodents is problematic due to the surprising lack of published ED<sub>50</sub> values. Additionally, previous studies did not directly compare the relative analgesic potency of nociceptin with selective opioid agonists. However, in mammalian studies where spinal nociceptin produced an analgesic effect, the highest dose tested did not produce as potent an analgesic effect as typically observed following morphine or other opioid agonists (Candeletti et al., 2000; Corradini et al., 2001; King et al., 1997; Ko et al., 2006; Nazzaro et al., 2006; Wang et al., 1999a,b). The low potency nature of nociceptin and nociceptin<sub>1–13</sub> amide has led to the search for more potent NOP agonists (Gunduz et al., 2006).

The antinociceptive effects of spinal nociceptin in amphibians were blocked by the concurrent administration of the NOP antagonist, [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide. This is in concert with the results from mammalian studies whereby the analgesic effects of nociceptin after spinal administration were blocked by co-administration of [Nphe<sup>1</sup>]-nociceptin<sub>1–13</sub> amide in normal (Lu et al., 2001; Xu et al., 2002) and neuropathic rodents (Corradini et al., 2001; Obara et al., 2005). The non-selective MOR, DOR, and KOR opioid antagonist, naltrexone, did not block the antinociceptive effect of nociceptin<sub>1–13</sub> amide after spinal administration in amphibians in the present study. Comparison of these results with the mammalian literature is less convergent as some studies report naloxone or naltrexone blockade of nociceptin analgesic effects whereas others report no effect of opioid antagonists on nociceptin effects (Xu et al., 2000). More recently, two papers strengthened the case for the spinal antinociceptive effects of nociceptin by anatomically and functionally linking nociceptin action to the inhibition of substance P release from primary afferent fibers (Inoue et al., 2003; Mika et al., 2003).

The putative target of nociceptin action is the NOP receptor in amphibian spinal cord. Although numerous studies showed that NOP receptors were expressed in mammalian brain and spinal cord (see above), there is only a single report of [<sup>3</sup>H]-nociceptin<sub>1–13</sub> amide binding in amphibians using brain tissue homogenates from the European water frog, *Rana esculenta* (Benyhe et al., 1999). These results showed that the nociceptin analog bound to a single, high-affinity site with an affinity of 0.55 nM and a density of 180 fmol/mg protein. More recently, the amphibian NOP receptor was cloned from two different species, and was shown to be expressed in amphibian brain and spinal cord (Stevens et al., 2007a; Walthers et al., 2005). These results, along with the present pharmacological data using NOP and opioid receptor antagonists, strongly suggest that the antinociceptive effects of nociceptin and its agonist analogues are mediated by NOP receptors in amphibian spinal cord.

The relatively weak antinociceptive effect of spinal nociceptin compared to opioid agonists appears to be conserved in amphibians and mammals. With regard to differences in the vertebrate opioid receptors, comparative bioinformatics of all vertebrate opioid-like sequences suggests that the NOP receptor is the most ancestral of the four members of the opioid receptor family, with the *mu* opioid receptor (MOR) being the most derived (Stevens et al., 2007a). In this sense, the vertebrate nociceptin system may represent a primitive endogenous analgesic system later surpassed by the endogenous opioid system with its more potent opioid receptors. There is some support for this notion given that the early-evolved sturgeon fish, *Acipenser transmontanus*, referred to as a 'living fossil', expresses a hybrid opioid- and nociceptin-like pronociceptin protein (Danielson et al., 2001).

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