Effects of Intrathecally Administered Nociceptin/Orphanin FQ in Monkeys: Behavioral and Mass Spectrometric Studies

M. C. Holden Ko, Hui Wei, James H. Woods, and Robert T. Kennedy

ABSTRACT

Nociceptin/orphanin FQ (N/OFQ) is a heptadecapeptide that is an endogenous ligand for the N/OFQ peptide (NOP) receptor. The aim of this study was to investigate the behavioral responses of N/OFQ and its major fragment N/OFQ(2–17) in monkeys following i.t. administration. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to quantify the amounts of N/OFQ and N/OFQ(2–17) in the cerebrospinal fluid at specific time points when effects of i.t. N/OFQ were sustained and disappeared. Intrathecal administration of N/OFQ dose dependently (10–100 nmol) produced long-lasting antinociception against a noxious stimulus, 50°C water, and did not elicit itch/scratching responses in monkeys. Subcutaneous pretreatment with a selective NOP receptor antagonist, (+)-J-113397 [(1-[3R,4R]-1-cyclooctymethyl-3-hydroxymethyl-4-piperidyl)-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one] (0.1 mg/kg), completely blocked i.t. N/OFQ (100 nmol)-induced antinociception. In contrast, a classic opioid receptor antagonist, naltrexone (0.01 and 1 mg/kg), failed to reverse i.t. N/OFQ-induced antinociception. MALDI-TOF-MS showed that the amount of N/OFQ(2–17) was 4-fold higher than that of N/OFQ at 1.5 h after i.t. administration of 100 nmol N/OFQ. Intrathecal N/OFQ-induced antinociception disappeared at 4.5 h, which corresponded to nearly undetectable cerebrospinal fluid levels of N/OFQ. No other metabolite of N/OFQ was detected at appreciable levels at either the 1.5- or 4.5-h time points. Although significant amounts of N/OFQ(2–17) were detected at the 1.5- and 4.5-h time points, 100 nmol N/OFQ(2–17) i.t. was inactive in changing the monkeys’ nociceptive threshold. These results provide the first functional evidence of spinal N/OFQ-induced antinociception in primates and indicate that activation of spinal NOP receptors may be a potential target for spinal analgesics.

The orphan opioid receptor has been identified in several species. It was previously called the ORL1 receptor and is now named the NOP receptor, as the fourth member within the opioid receptor family, by the International Union of Pharmacology (Mollereau et al., 1994; Foord et al., 2005). The sequence of the NOP receptor is closely related to each of the classical, well characterized μ-, δ-, and κ-opioid receptors. However, ligands that bind to these classic opioid receptors do not bind NOP receptors with high affinity (Mollereau et al., 1994). The NOP receptor is widely distributed in the central nervous system, and it may participate in a broad range of behavioral and physiological functions (Neal et al., 1999a,b). Nociceptin/orphanin FQ (N/OFQ), an endogenous peptide at the NOP receptor, inhibits cyclic AMP accumulation and Ca2+ currents and increases K+ conductance in neurons, similar to the effects of agonists at the other opioid receptors (Meunier et al., 1995; Reinscheid et al., 1995; Jennings, 2001). These in vitro studies seem to support an inhibitory function of NOP receptor activation at sensory neurons (Stanfa et al., 1996; Helyes et al., 1997; Zeilhofer and Calo, 2003). Given that the actions of N/OFQ have much in common with those of opioid peptides at the cellular level, it is important to study behavioral effects of centrally administered N/OFQ.

Although early functional studies of N/OFQ focused on supraspinal sites, subsequent studies have shown that spinal administration of N/OFQ produced antinociceptive effects in a variety of pain models in rodents (Erb et al., 1997; Zeilhofer and Calo, 2003; Obara et al., 2005). Given that i.t. administration of opioids is one of the most frequently used methods of analgesia in humans (Dougherty and Staats, 1999; Bennett et al., 2000; Rathmell et al., 2005), it is important to study further the functions of spinal NOP receptors in a primate species. Administration of μ-opioid receptor agonists by the spinal route has...
become a more popular approach to treatment of pain during the last 2 decades; however, the most common side effect of spinal morphine administration is pruritus (itch sensation), which sometimes is severe and lessens the value of spinal opioids for pain relief (Cousins and Mather, 1984; Waxler et al., 2005). Recent studies have demonstrated that the same  {\mu}-opioid receptors mediate i.t. morphine-induced antinociception and itch/scratching responses in monkeys (Ko and Naughton, 2000; Ko et al., 2004), making it difficult to separate these effects. If i.t. administration of N/OFQ produced antinociception without profound scratching in monkeys, the NOP receptor agonist may represent a novel pharmacological agent for spinal opioid analgesia in humans.

Studies of peptide metabolism have indicated that N/OFQ metabolic fragments have biological activity (Terenius et al., 2000). For example, i.t. administration of N/OFQ(1–7), N/OFQ(1–9), and N/OFQ(1–13) alone had no effects, but attenuated i.t. N/OFQ-induced behavioral responses when they were coadministered i.t. with N/OFQ in mice (Sakurada et al., 2000). Although N/OFQ(2–17) has been reported as the major fragment of N/OFQ in human blood (Yu et al., 1996), it is unknown which fragment is the major biotransformation product in the cerebrospinal fluid (CSF) of primates and whether the major fragment of N/OFQ in CSF would modulate any of the behavioral responses produced by i.t. N/OFQ.

It would be interesting to compare the biotransformation products of N/OFQ in rodents and primates and to determine whether peptide metabolism generates products that are active or modulate activity (Terenius et al., 2000).

Therefore, the aim of the study was to investigate effects of i.t. N/OFQ in nonhuman primates by using behavioral and mass spectrometric assays. First, experiments were conducted to characterize the time course of antinociceptive and itch/scratching effects produced by i.t. administration of N/OFQ in monkeys. Second, a selective NOP receptor antagonist, J-113397 (Ozaki et al., 2000), and a classic opioid receptor antagonist, naltrexone, were given in conjunction with i.t. N/OFQ to determine that the effects of the peptide agonist were mediated through the NOP receptor, independent from classic opioid receptors. Third, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to identify the major fragment of N/OFQ in the CSF. In addition, synthesized 15N-labeled peptides were used in MALDI-TOF-MS analysis for medical quantification of the amounts of N/OFQ and its major fragment at specific time points following i.t. administration of N/OFQ for 2 h.

The second part of the study was to quantify the amount of N/OFQ and its major fragment in the monkey CSF using MALDI-TOF-MS. An initial experiment was conducted to collect the monkey CSF at 2- and 5-h time points following i.t. administration of 100 nmol N/OFQ. This experiment confirmed the sensitivity of MALDI-TOF-MS and identified N/OFQ(2–17) as the dominant fragment. Although other fragments such as N/OFQ(1–15) and N/OFQ(1–13) could be found at the 2-h time point, they were not detected at appreciable levels compared with N/OFQ(2–17). Further experiments were conducted using the same monkeys (n = 6) involved in the first part of the study in separate test sessions. The CSF samples were collected before 1.5 and 4.5 h after i.t. administration of 100 nmol N/OFQ. The dose of N/OFQ and the time points for CSF sampling were chosen based on the results of behavioral studies, showing that antinociception produced by 100 nmol N/OFQ i.t. was sustained at 1.5 h and disappeared completely by 4.5 h after i.t. administration. The obtained samples were cleaned by ZipTip and analyzed by MALDI-TOF-MS. The amount of N/OFQ and N/OFQ(2–17) in the CSF were quantified by using [15N6]N/OFQ and [15N6]N/OFQ(2–17) as internal standards.

The third part of the study was to determine the effects of 100 nmol N/OFQ(2–17) i.t. in the absence and presence of i.t. N/OFQ to verify whether the N/OFQ major fragment N/OFQ(2–17) could modulate N/OFQ-induced antinociception following i.t. administration. The temperature-response curve of i.t. N/OFQ(2–17) alone was determined every 0.5 h for 2 h in an additional four monkeys (n = 4). Antinociceptive effects of 100 nmol N/OFQ(2–17) i.t. with or without 100 nmol N/OFQ(2–17) (i.e., coadministration) were also compared in the same monkeys over a 2-h time course. All experiments using i.t. administration were conducted with a 10-day interinjection interval.

**Materials and Methods**

**Subjects**

Ten adult intact male and female rhesus monkeys (Macaca mulatta) with body weights ranging between 6.7 and 12.5 kg were used. Six monkeys participated in the dose-response and antagonist studies of N/OFQ, and also in the mass spectrometric studies. The remaining four monkeys were used in the behavioral studies of N/OFQ’s major metabolite. The monkeys were housed individually with free access to water and were fed approximately 25 to 30 biscuits (Purina Monkey Chow;Ralston Purina, St. Louis, MO) and fresh fruit daily. No monkey had exposure to any opioid receptor agonist or antagonist for 1 month before the present study. The monkeys were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. The studies were conducted in accordance with the University Committee on the Use and Care of Animals in the University of Michigan and the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, revised 1996).

**Experimental Designs**

The first part of the study was to characterize the behavioral effects of i.t. N/OFQ over 5 h. Four male and two female monkeys (n = 6) were used in these experiments. Injections were given no more frequently than every 10 days. Behavioral responses to N/OFQ (0, 10, 30, and 100 nmol) were tested by giving different doses randomly in a single dosing procedure. In addition, behavioral responses to i.t. 100 nmol morphine were also tested for comparison. The antinociceptive effects were measured during the 25th to 40th min of each hour following i.t. administration. Subsequently, monkeys were returned to their home cages, and scratching responses were recorded during the 45th to 60th min of each hour (i.e., 15 min/session) after the antinociceptive measurement.

After establishing the dose-response curve of i.t. N/OFQ, the antagonists, naltrexone and (+)J-113397, were chosen to verify the opioid receptor mediation of the effects of i.t. N/OFQ. The smaller dose of naltrexone, 0.01 mg/kg, was used because it was selective for  {\mu}-opioid receptors, and the larger dose, 1 mg/kg, was used because it produced full blockade of  {\mu}-,  {\kappa}-, and  {\delta}-opioid receptors. This is based on naltrexone’s binding affinity in the monkey brain membranes and its in vivo antagonist potency in monkeys (Emmerson et al., 1994; Ko et al., 1998, 2006). The dose of 0.1 mg/kg (+)J-113397 was chosen because this dose was effective in blocking the NOP receptor agonist Ro 64-6198-induced antinociception in monkeys (M. H. Ko, unpublished data). Both antagonists were administered s.c. in the back (i.e., around the scapular region) 5 min before i.t. administration of N/OFQ. The antinociceptive responses were measured every 0.5 h after i.t. administration of N/OFQ for 2 h.

The second part of the study was to quantify the amount of N/OFQ and its major fragment in the monkey CSF using MALDI-TOF-MS. An initial experiment was conducted to collect the monkey CSF at 2- and 5-h time points following i.t. administration of 100 nmol N/OFQ. This experiment confirmed the sensitivity of MALDI-TOF-MS and identified N/OFQ(2–17) as the dominant fragment. Although other fragments such as N/OFQ(1–15) and N/OFQ(1–13) could be found at the 2-h time point, they were not detected at appreciable levels compared with N/OFQ(2–17). Further experiments were conducted using the same monkeys (n = 6) involved in the first part of the study in separate test sessions. The CSF samples were collected before 1.5 and 4.5 h after i.t. administration of 100 nmol N/OFQ. The dose of N/OFQ and the time points for CSF sampling were chosen based on the results of behavioral studies, showing that antinociception produced by 100 nmol N/OFQ i.t. was sustained at 1.5 h and disappeared completely by 4.5 h after i.t. administration. The obtained samples were cleaned by ZipTip and analyzed by MALDI-TOF-MS. The amount of N/OFQ and N/OFQ(2–17) in the CSF were quantified by using [15N6]N/OFQ and [15N6]N/OFQ(2–17) as internal standards.

The third part of the study was to determine the effects of 100 nmol N/OFQ(2–17) i.t. in the absence and presence of i.t. N/OFQ to verify whether the N/OFQ major fragment N/OFQ(2–17) could modulate N/OFQ-induced antinociception following i.t. administration. The temperature-response curve of i.t. N/OFQ(2–17) alone was determined every 0.5 h for 2 h in an additional four monkeys (n = 4). Antinociceptive effects of 100 nmol N/OFQ(2–17) i.t. with or without 100 nmol N/OFQ(2–17) (i.e., coadministration) were also compared in the same monkeys over a 2-h time course. All experiments using i.t. administration were conducted with a 10-day interinjection interval.
Procedures

Behavioral Assays. Antinociception. The warm water (50°C) tail-withdrawal assay was used to evaluate thermal antinociceptive effects of the test compound (Ko et al., 1998). In brief, monkeys were seated in primate restraint chairs, and the lower part of their shaved tails (approximately 15 cm) were immersed in a thermal flask containing water maintained at 42, 46, 50, or 54°C. Tail-withdrawal latencies were measured using a computerized timer by an experimenter who was blinded to experimental conditions. In each test session, monkeys were tested once with four temperatures given in a random order, and only the stimulus 50°C water was tested twice to confirm the full antinociceptive effect. If the monkeys did not remove their tails within 20 s (cut-off), the flask was removed, and a maximum time of 20 s was recorded. Test sessions began with control determinations at each temperature. Subsequent tail-withdrawal latencies were determined at multiple time points after i.t. administration.

Scratching responses. Scratching responses, inferred as an itch sensation (Ko and Naughton, 2000), were recorded on videotapes when monkeys were in their home cages. Each recording session was conducted for 15 min/test session. A scratch was defined as one short-duration (<1 s) episode of scraping contact of the forepaw or hind paw on the skin surface of other body parts. Scratches usually occurred repetitively at the same location. Scratching responses were scored by trained individuals who were blinded to experimental conditions.

Spinal drug delivery and sampling. Monkeys were positioned in primate restraint chairs and anesthetized by i.v. propofol (2.5–4.0 mg/kg for bolus infusion and 0.3–0.4 mg/kg/min for continuous infusion; AstraZeneca, Wilmington, DE). The lower back of the trunk was shaved and prepared steriley with Betadine. A spinal needle (22-gauge × 1.5; Becton Dickinson, Franklin Lakes, NJ) was inserted into the subarachnoid space between the L4 and L5 lumbar vertebrae. Needle position was confirmed by a free flow of clear CSF. A 1-ml solution of the test compound was infused slowly through the spinal needle within 30 s. This procedure was also used to collect CSF samples at specific time points for mass spectrometric analysis. Monkeys normally recovered from anesthesia within 5 to 10 min following termination of propofol infusion.

Data analysis. Mean values (mean ± S.E.M.) were calculated from individual values for all behavioral endpoints. Comparisons were made for the same monkeys across all test sessions in the same experiment. All data obtained from a single dosing procedure were analyzed by two-way analysis of variance followed by the Newman-Keuls test for multiple (post hoc) comparisons. The criterion for significance was set at p < 0.01.

Mass Spectrometry. Mass spectra were acquired with a MALDI-TOF Spec 2E mass spectrometer (Micromass, Milford, MA) equipped with a delayed extraction source and 337-nm pulsed (4 ns) nitrogen TOF Spec 2E mass spectrometer (Micromass, Milford, MA) equipped onto a MALDI target plate. Over 200 shots were summed to generate the spectra for quantification. The spectra were smoothed, and baseline was subtracted and then centered to give a value for the peak area for each monoisotopic peak. The relative intensity of each analyte to its synthetic internal standard was measured as the summed peak area of the first three monoisotopic peaks of the analyte divided by the summed peak area of the first three monoisotopic peaks of the internal standard. With this ratio, the analyte concentration in the sample was calculated from a standard curve. The construction of the standard curves is described in the following.

Five solutions were prepared containing 5.4 μM [15N6]N/OFQ and 0.54 to 10.0 μM N/OFQ. Each solution was mixed with equal volume of α-cyano-4-hydroxycinnamic acid. Six spots were deposited for each solution via dried-droplet method, analyzed by MALDI-TOF-MS, and mass spectra were acquired and processed as described above. A standard curve was constructed by plotting the relative intensity of N/OFQ to [15N6]N/OFQ as a function of the relative concentration of N/OFQ to [15N6]N/OFQ. The same procedures were taken to construct the standard curve for N/OFQ(2–17) except that six solutions were prepared that contained 2 μM [15N6]N/OFQ(2–17) and 0.06 to 3.0 μM N/OFQ(2–17).

Drugs
[15N6]N/OFQ, [15N6]N/OFQ(2–17), and N/OFQ(2–17) were synthesized from the Protein Structure Facility at the University of Michigan (Ann Arbor, MI). N/OFQ, naltraxone HCl, morphine sulfate, and (+)-J-113397, provided by National Institute on Drug Abuse (Bethesda, MD), were dissolved in sterile water. Doses are presented in the compound forms listed above. For systemic administration, naltraxone and (+)-J-113397 were administered at a volume of 0.1 ml/kg.

Results

Figure 1 compares the behavioral effects of i.t. administration of N/OFQ with morphine in monkeys (n = 6). Intrathecal N/OFQ produced antinociception in 50°C water that was both dose [F(3,15) = 17.2; p < 0.01] and time [F(4,20) = 16.6; p < 0.01] dependent. Post hoc comparisons indicated that both 30 and 100 nmol of i.t. N/OFQ produced significant antinociception at the 0.5- and 1.5-h time points (p < 0.01). In particular, the antinociceptive effects of 100 nmol N/OFQ i.t. lasted for 2.5 h (p < 0.01), and i.t. N/OFQ did not elicit significant scratching responses at any dose or time point. In contrast, i.t. 100 nmol morphine produced both antinociception and scratching for 5 h (p < 0.01). Administration of N/OFQ at these doses did not cause any observable side effects, such as sedation and motor impairment (data not shown).

Figure 2 illustrates the effects of opioid receptor antagonists on antinociception produced by 100 nmol N/OFQ i.t. in monkeys (n = 6). There were significant differences in the blockade of i.t. N/OFQ-induced antinociception by various antagonists [F(3,15) = 143.6; p < 0.01]. Post hoc comparisons indicated that pretreatment with a NOP receptor antagonist [0.1 mg/kg (+)-J-113397] significantly blocked i.t. N/OFQ-induced antinociception (p < 0.01). However, both doses of
naltrexone (0.01 and 1 mg/kg) failed to attenuate i.t. N/OFQ-induced antinociception. Doses of s.c. naltrexone and (−)-J-113397 alone did not change the monkeys’ thermal nociceptive threshold (data not shown).

Figure 3 presents typical MALDI-TOF-MS spectra of CSF sampled from a single monkey. MALDI-TOF-MS identified two dominant peaks, N/OFQ and its fragment N/OFQ(2–17) (i.e., at m/z of 1810 and 1663), in the MS spectrum of CSF sampled 2 h after i.t. administration of 100 nmol N/OFQ (Fig. 3, top). Although N/OFQ(1–15) and N/OFQ(1–13) was detected at the 2-h time point, neither fragment was detected at appreciable levels compared with N/OFQ(2–17). More interesting, the fragment N/OFQ(2–17) still existed and was the only apparent peak that was identified in the MS spectrum of CSF sampled at 5 h after i.t. N/OFQ (Fig. 3, bottom).

Figure 4A shows the calibration curves for N/OFQ and N/OFQ(2–17). The absolute quantification of N/OFQ and N/OFQ(2–17) was performed using synthesized [15N6]N/OFQ and [15N6]N/OFQ(2–17) as internal standards. The chemical equivalence of the stable isotopically labeled internal standard and the target molecule eliminated the discrimination between the analyte and standard in purification and sample preparation steps (Gobom et al., 2000). Therefore, the quantification procedure of N/OFQ and N/OFQ(2–17) was based on the ratio of the relative intensity of analyte and the internal standard. The relative standard deviation of each spot on the curve was less than 10% (n = 6) and high linearity (i.e., R² = 0.995–0.999) was obtained. Thus, quantification of N/OFQ and N/OFQ(2–17) could be performed based on the equations derived from the two standard calibration curves.

Figure 4B compares mass spectra of CSF samples, spiked with internal standard, obtained 1.5 and 4.5 h after i.t. ad-
administration of 100 nmol N/OFQ in a single monkey. The signal intensity of N/OFQ was higher than its internal standard (21.6 μM spiked [15N6]N/OFQ) at 1.5 h (Fig. 4B, 1) and was much lower than the internal standard at 4.5 h, even though a lower concentration of [15N6]N/OFQ (8.6 μM) was used at this time (Fig. 4B, 3). In contrast, the signal intensity of N/OFQ(2–17) was much higher than its internal standard (30.0 μM spiked [15N6]N/OFQ(2–17)) at 1.5 h (Fig. 4B, 2), and it was still detected by a signal higher than the internal standard (8.0 μM spiked [15N6]N/OFQ(2–17)) at 4.5 h (Fig. 4B, 4). These data demonstrate that strong signals can be detected without interference for both the peptide and the internal standard.

Figure 5 shows the quantified amount of N/OFQ and N/OFQ(2–17) collected before (i.e., control samples) and 1.5 and 4.5 h after 100 nmol N/OFQ i.t. in monkeys (n = 6). The amounts of N/OFQ and N/OFQ(2–17) in the control samples were below the detection limit of the method; therefore, they were reported as 0 pmol (n = 6). The average amount of N/OFQ at 1.5 h (204 pmol) was significantly higher than those obtained from the control samples and 4.5-h samples (1.7 pmol) (p < 0.01), and there was no difference between the control and 4.5-h samples. Likewise, the averaged amount of N/OFQ(2–17) at 1.5 h (803 pmol) was significantly higher than those obtained from the control samples and 4.5-h samples (29.5 pmol) (p < 0.01). Although the amount of N/OFQ(2–17) had decreased dramatically at 4.5 h, it was still detectable, and it was significantly higher than the control sample (p < 0.05). More interesting, the amount of N/OFQ(2–17) was significantly higher than N/OFQ at both time points (i.e., 1.5 and 4.5 h) (p < 0.01).

Figure 6 illustrates behavioral effects of i.t. administration of N/OFQ(2–17) in monkeys (n = 4). Intrathecal 100 nmol N/OFQ(2–17) alone did not alter the thermal nociceptive threshold at 30 min after i.t. administration (Fig. 6, top). Measurement of tail-withdrawal responses continued for 2 h and confirmed that i.t. N/OFQ(2–17) did not produce either antinociception or hyperalgesia through the entire test session. In addition, i.t. 100 nmol N/OFQ(2–17) did not change antinociception produced by 100 nmol N/OFQ i.t. (Fig. 6, bottom). Combinations of 100 nmol N/OFQ and 100 nmol N/OFQ(2–17) i.t. produced similar antinociception against 50°C water as did 100 nmol N/OFQ i.t. alone.

Discussion
The behavioral study showed that i.t. administration of N/OFQ dose dependently produced antinociception that was blocked by a NOP receptor antagonist, (+)-J-113397, in monkeys. Although some rodent studies have characterized the spinal actions of N/OFQ and reported different behavioral effects, most researchers have concluded that the dominant spinal action of N/OFQ is inhibitory and results in analgesia or antihyperalgesia (Mogil and Pasternak, 2001; Zeilhofer and Calo, 2003). The present study is the first to document the inhibitory action of spinal N/OFQ in a primate species. Antinociceptive effects of i.t. N/OFQ lasted 2.5 h in monkeys. In contrast, most rodent studies showed that the duration of antinociception induced by i.t. N/OFQ was between 30 and 60 min (Erb et al., 1997; Obara et al., 2005). Future studies can further investigate whether N/OFQ is more resistant to biotransformation in the primate CSF. Full antinociceptive effects could also be observed following i.t. administration of selective μ-opioid, but not δ- and κ-opioid,
agonists in monkeys under the same procedure (Ko et al., 2003). These findings suggest that activation of spinal NOP receptors produced comparable antinociception as spinal μ-opioid agonists in monkeys evaluated in this acute pain model. It is important to investigate further whether both spinal N/OFQ and μ-opioid agonists produce the same degree of antinociception in monkeys under experimental models using different pain modalities.

Recent studies have demonstrated that activation of spinal μ-opioid receptors mediated both antinociception and itch/scratching concurrently in monkeys (Ko and Naughton, 2000; Ko et al., 2004). For instance, i.t. 32 to 100 μg of morphine produced antinociception against a noxious stimulus, 50°C water, and it also produced profound scratching responses in monkeys (Ko and Naughton, 2000). This observation parallels closely the behavioral effects of spinal morphine in humans (Bailey et al., 1993; Palmer et al., 1999). Accordingly, monkey subjects can be used to validate whether other opioid analgesics possess an itch-eliciting property (Ko et al., 2004). The present study clearly showed that i.t. N/OFQ produced antinociception in the absence of scratching responses. These findings together support the notion that the spinal NOP receptor is an interesting target for research and development of spinal opioid analgesics (Zeilhofer and Calo, 2003). In particular, activation of spinal NOP receptors produced analgesia devoid of pruritus, the most common side effect associated with spinal μ-opioid analgesics in humans (Cousins and Mather, 1984; Waxler et al., 2005).

Antagonist studies verified that i.t. N/OFQ-induced antinociception in monkeys was mainly mediated by the NOP receptors and was independent from classic opioid receptors. The doses of naltrexone used in this study were sufficient to block the effects of μ-, δ-, and κ-opioid agonists based on its binding affinity in the monkey brain membranes and its in vivo antagonist potency in monkeys (Emmerson et al., 1994; Ko et al., 1998, 2006). Lack of effects of naltrexone against i.t. N/OFQ-induced antinociception suggested that spinal N/OFQ and μ-opioid agonists produced antinociceptive effects independently by activating distinct receptors. Interestingly, spinal administration of J-113397 increased pain-relevant behaviors in the rat formalin
will be important to investigate whether NOP and at peptide linkage Phe1-Gly2 seems to be the predominant antinociceptive fragment of N/OFQ in the monkey CSF. The cleavage applied in this study also identified N/OFQ(2–17) as a dominant fragment of N/OFQ in human blood (Yu et al., 1996). MALDI-TOF-MS allowed the sensitive and specific detection of all major biotransformation products of N/OFQ.

Spinal N/OFQ inhibits excitatory glutamatergic neurotransmission without affecting GABA or glycine receptor-mediated synaptic responses, which may account for the fact that its antinociceptive action had a pattern similar to that for classic opioids (Luo et al., 2002; Zeilhofer and Calo, 2003). Although there is no anatomical study of the NOP receptor in the CNS of monkeys, studies have shown that messenger RNA of N/OFQ and the NOP receptor are highly expressed in the rat spinal cord (Neal et al., 1999a,b). What is not clear is how activation of spinal NOP receptors produces analgesia without pruritus. This phenomenon may be partially explained by an immunostaining study illustrating that the NOP and µ-opioid receptors were expressed predominantly on different fiber systems in the rat spinal cord (Monteillet-Agius et al., 1998). Given that a neural pathway for central µ-opioid-evoked itch has not been identified in any species, it may have different cellular localizations on specific sensory neurons in the primate spinal cord. N/OFQ(2–17) was identified as the major fragment of N/OFQ in human blood (Yu et al., 1996). N/OFQ(2–17) was approximately 4-fold greater than that of N/OFQ(1–11), N/OFQ(1–9), and N/OFQ(1–7) were the constituted half of the original concentration of i.t. N/OFQ. The prolonged presence of N/OFQ in the monkey CSF seemed to correspond with the duration of i.t. N/OFQ-induced antinociception because the antinociception had subsided at 4.5 h with a nearly undetectable level of N/OFQ. Although other fragments such as N/OFQ(1–15) and N/OFQ(1–13) were detected by MALDI-TOF-MS, they were much lower than that of N/OFQ(2–17) in the monkey CSF sampled at 1.5 h. In contrast, N/OFQ(1–13), N/OFQ(1–11), N/OFQ(1–9), and N/OFQ(1–7) were the together with 100 nmol N/OFQ, did not change i.t. N/OFQ-induced antinociception. These results indicated that N/OFQ(2–17) played a minimal role in changing the nociceptive threshold and are in line with other studies indicating N/OFQ(2–17) to be inactive in other biological systems (Champion and Kadowitz, 1997; Kapusta et al., 1999).

MALDI-TOP-MS allowed the sensitive and specific detection and quantification of all major biotransformation products of neuropeptides (Gobom et al., 2000; Wei et al., 2004). At the 1.5-h time point, the concentration of N/OFQ (i.e., 200 pmol/20 µl) in sampled CSF was approximately 1/10 of the original concentration (i.e., 100 nmol in 1 ml) delivered i.t., and the concentration of N/OFQ(2–17) was approximately 800 pmol/20 µl. These results suggested that the total amount of N/OFQ and N/OFQ(2–17) detected at the 1.5-h time point together constituted half of the original concentration of i.t. N/OFQ. The prolonged presence of N/OFQ in the monkey CSF seemed to correspond with the duration of i.t. N/OFQ-induced antinociception because the antinociception had subsided at 4.5 h with a nearly undetectable level of N/OFQ. Although other fragments such as N/OFQ(1–15) and N/OFQ(1–13) were detected by MALDI-TOF-MS, they were much lower than that of N/OFQ(2–17) in the monkey CSF sampled at 1.5 h. In contrast, N/OFQ(1–13), N/OFQ(1–11), N/OFQ(1–9), and N/OFQ(1–7) were the

**Results.**

**Fig. 5.** Comparison of quantified amounts of N/OFQ and N/OFQ(2–17) collected before and 1.5 and 4.5 h after 100 nmol N/OFQ i.t. in monkeys. Ordinate, amount (picomoles) quantified from a 20-µl sample. Each value represents mean ± S.E.M. (n = 6). CTRL represents samples obtained before i.t. administration of N/OFQ. The asterisks represent a significant difference between corresponding samples as shown herein (*, p < 0.01). For other details, see Results.

**Fig. 6.** Behavioral effects of i.t. administration of N/OFQ(2–17) in monkeys. Upper, temperature-response curves measured at 0.5 h after i.t. administration. The temperature-response curves remained unchanged at 1, 1.5, and 2 h after i.t. administration and therefore not shown for the sake of clarity. Lower, antinociception produced by i.t. N/OFQ with or without additional N/OFQ(2–17). Ordinate, latency to withdraw the tail in 50°C water. Each value represents mean ± S.E.M. (n = 4). Symbols represent different dosing conditions for the same monkeys. For other details, see Materials and Methods.
main fragments of N/OFQ identified in the rat brain and spinal cord tissues (Terenius et al., 2000). Although some fragments were active in changing the nociceptive threshold, most of the behavioral responses were mild (i.e., 2- decrease in response) and transient (i.e., 10-20 min duration) (Sakurada et al., 1999; Suder et al., 1999; Terenius et al., 2000). Future studies using spinally catheterized monkeys can further address the question of whether N/OFQ and its other fragments produce any significant behavioral responses during the first 60 min following i.t. administration.

Taken together, this study demonstrated that i.t. N/OFQ produced antinociception, which was mediated mainly by the NOP receptor, not the classic opioid receptors, in monkeys. It is the first study to provide functional evidence of spinal N/OFQ effects in a primate species, indicating that spinal NOP receptor activation may produce analgesia without pruritus in humans. MALDI-TOP-MS permits a sensitive and specific measurement for determining the role of major fragments of neuropeptides. This study only focused on the behavioral effects of spinal N/OFQ and its major fragment by using nonpeptidic NOP agonists and to explore further the potential interaction between the NOP and classic opioid receptors in primates. Given that activation of either peripheral or spinal NOP receptors can produce antinociception in both rodents and primates under different pain modalities (Ko et al., 2002; Obara et al., 2005), newly developed compounds targeting NOP receptors may produce desired analgesia without side effects (Zeilhofer and Calo, 2003).

Acknowledgments
We thank John Busenkab, Tristan Edwards, and Wayne Yang for excellent technical assistance. Isotope-labeled peptides were synthesized by Protein Structure Facility at the University of Michigan, which is supported in part from the Michigan Diabetes Research and Training Center funded by Grant NIH5P60DK20572 from the National Institute of Diabetes and Digestive and Kidney Diseases.

References