Cloning and characterization of the rhesus monkey nociceptin/orphanin FQ receptor

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We succeeded in cloning the rhesus monkey nociceptin/orphanin FQ peptide (NOP) receptor. The nucleotide sequence and amino acid sequence of the rhesus monkey NOP receptor were 95.9% and 97.8%, respectively, identical to the human NOP receptor. There was no significant difference between the rhesus monkey NOP receptor and the human NOP receptor in the binding affinity of [125I]Thy14 nociceptin and the binding of [35S]guanosine 5'-O-(γ-thio)triphosphate ([35S]GTPγS) stimulated by nociceptin/orphanin FQ (N/OFQ). A selective NOP receptor antagonist, 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one (+(+)-J-113397) inhibited the [35S]GTPγS binding activated by N/OFQ using the membrane of the rhesus monkey NOP receptor. The antagonistic activity of (+)-J-113397 to the rhesus monkey NOP receptor was comparable to that to the human NOP receptor. Thus, N/OFQ acts via activation of the NOP receptor in both human and rhesus monkeys without significant species differences.

Key words: J-113397, nociceptin/orphanin FQ, NOP receptor, rhesus monkey

INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is a 17 amino acid-long peptide that has been identified as an endogenous agonist to the nociceptin/orphanin FQ peptide (NOP) receptor previously known as the opioid receptor like 1 receptor (Foord et al., 2005). The NOP receptor shows a high degree of sequence homology with the classical μ-, δ- and κ- opioid receptors (Bunzow et al., 1994; Nishi et al., 1994; Wang et al., 1994). The NOP receptor is coupled with G proteins, and its activation causes the reduction of intracellular cyclic AMP (Mollereau et al., 1994), which is one of the common intracellular second messengers and regulates cell functions (Ring et al., 2005; Calo’ et al., 2000).

The NOP receptor is widely distributed in both the central nervous system and the peripheral tissues (Anton et al., 1996; Mollereau and Mouledous, 2000). In rodents, various biological effects on nociception, locomotion, cognition and food intake are mediated via the NOP receptor (Nishi et al., 1997; Leventhal et al., 1998; Manabe et al., 1998; Calo’ et al., 2000).

An administration of N/OFQ into the tails of rhesus monkeys inhibits the nociceptive response to the thermal stimulation by capsaicin (Ko et al., 2002) and an intrathecal administration of N/OFQ inhibits the nociceptive response to the thermal stimulation by warm water (Ko et al., 2006). These anti-nociceptive effects of N/OFQ are antagonized by the subcutaneous administration of the selective NOP receptor antagonist, 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxyethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one (+(+)-J-113397) (Ko et al., 2002, 2006).

The distribution of [125I]Thy14 nociceptin binding sites has been demonstrated in brain slices prepared from cynomolgous macaques (Bridge et al., 2003). These studies predict the existence of a rhesus monkey NOP receptor. However, the functional characterization of any non-human primate NOP receptor has not yet been conducted, as the nucleotide sequence of the NOP receptor remains undetermined.

In the present study, we determined the nucleotide sequence corresponding to the rhesus monkey NOP rec-

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Abbreviations: N/OFQ; nociceptin/orphanin FQ, NOP; nociceptin/orphanin FQ peptide, +(+)-J-113397; 1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxyethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one, CHO; Chinese hamster ovary, GTPγS; guanosine 5'-O-(γ-thio)triphosphate, GDP; guanosine 5'-diphosphate, BSA; bovine serum albumin, TM; transmembrane, Kd; dissociation constant, EC50; the half maximal effective concentration, IC50; the half maximal inhibitory concentration.
ceptor. We then expressed the rhesus monkey NOP receptor in Chinese hamster ovary (CHO) cells, and compared the characteristics of this receptor to those of the human NOP receptor.

**MATERIALS AND METHODS**

**Materials** N/OFQ and (+)-J-113397 were synthesized at Banyu tsukuba research institute. [125I][Thy14]nociceptin and [35S]guanosine 5′-O-(γthio)triphosphate were purchased from GE Healthcare (Piscataway, NJ, USA). Ham’s F12 medium and Geneticin were purchased from Invitrogen (Carlsbad, CA, USA). Guanosine 5′-O-(γthio)triphosphate (GTPγS) and guanosine 5′-diphosphate (GDP) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cloning of the full-length rhesus monkey NOP receptor cDNA** Total RNA was prepared from rhesus monkey prefrontal cortex using Isogen (Nippongene, Tokyo, Japan). The first strand cDNA was synthesized with oligo (dT) primers and AMV reverse transcriptase (Invitrogen). The primers were designed to the sequence both 5′ and 3′ non-coding regions of the human NOP receptor (5′-TACCGTACAGTGATTTGCG-3′, 5′-GACAGGCACCATTGGCGA-3′). The PCR amplification was performed using Takara Ex Taq (Takarabio, Shiga, Japan) in a PCR thermal cycler MP (Takarabio) under the following conditions: 30 cycles of 94°C, 45 s; 50°C, 45 s; 72°C, 90 s. The PCR products were subcloned into the pCR3.1 plasmid using a Eukaryotic TA cloning kit (Invitrogen). The nucleotide sequences were determined by dye termination sequencing reactions on an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences have been deposited in GenBank with the accession number DD160700.

**Construction of rhesus monkey NOP receptor expressing cells** The plasmids containing the sequence of the rhesus monkey NOP receptor were transfected into CHO cells using Transfectam reagent (Promega, Madison, WI, USA). The rhesus monkey NOP receptor expressing CHO cells were maintained in Ham’s F12 medium with 10% fetal bovine serum and 1 mg/ml Geneticin at 37°C in a humidified incubator containing 5% CO2. Clonal, Geneticin-resistant cells were obtained and identified by the [125I][Thy14]nociceptin binding.

[125I][Thy14]nociceptin binding Membrane was prepared from rhesus monkey NOP receptor expressing CHO cells as described previously (Ozaki et al., 2000; Ichikawa et al., 2001). We also prepared membrane from human NOP receptor expressing CHO cells in the same way to compare characteristics between the rhesus monkey NOP receptor and the human NOP receptor. These membranes were incubated in nociceptin binding buffer (50 mM Hepes, 10 mM NaCl, 1 mM MgCl2, 2.5 mM CaCl2, 0.1% bovine serum albumin (BSA), 0.025% bacitracin, pH7.4) containing various concentrations of [125I][Thy14]nociceptin at 37°C for 1 h. The incubation was terminated by rapid filtration through GF/C filters (PerkinElmer, Waltham, MA, USA) that were presoaked in 0.5% polyethyleneimine (Wako, Osaka, Japan). The filters were washed with the wash buffer (5 mM Hepes/Tris, 0.1% BSA (pH7.4)) three times at 4°C. The radioactivity retained on the filter was counted using a gamma counter. Nonspecific binding was defined as the amount of radioactivity under the conditions mentioned above in the presence of 1 μM N/OFQ. Specific binding was determined by subtracting nonspecific binding from total binding, which was the radioactivity without N/OFQ. Protein concentrations on these membranes were determined using a BCA protein assay kit (Sigma-Aldrich) with BSA as the standard.

[35S]GTPγS binding [35S]GTPγS binding is used to evaluate the activation of G protein coupled receptor by an agonist.Replacing GTP with the non-hydrolyzable analogue, [35S]GTPγS, allows the measurement of the exchange process in the presence of GDP. Unlike GTP, [35S]GTPγS addition results in accumulation of a stable complex between G protein and [35S]GTPγS. Therefore, if a receptor is coupled to G proteins, an agonist stimulation increases the amount of [35S]GTPγS binding (Sim et al., 1995; Traynor and Nahorski, 1995). [35S]GTPγS binding was performed as described previously (Ozaki et al., 2000; Ichikawa et al., 2001). Membranes were incubated in GTPγS binding buffer (20 mM Hepes, 100 mM NaCl, 10 mM MgCl2, 1 mM EDTA and 5 μM GDP, pH7.4) containing 200 pM [35S]GTPγS, 1.5 mg WGA PVT SPA scintillation beads (GE healthcare) and N/OFQ between 0.1 nM and 1 μM at 25°C for 2.5 h. Membrane-bound radioactivity was counted by toptocount-HTS (PerkinElmer). Nonspecific binding was defined as the amount of radioactivity under the conditions mentioned above in the presence of 10 μM GTPγS. Specific binding was determined by subtracting nonspecific binding from total binding which was the radioactivity under the conditions without GTPγS.

The antagonistic effects of (+)-J-113397 on [35S]GTPγS binding stimulated by N/OFQ were determined as follows. Membranes were incubated in GTPγS binding buffer containing 200 pM [35S]GTPγS, 1.5 mg WGA PVT SPA scintillation beads, 10 nM N/OFQ and (+)-J-113397 between 0.1 nM and 1 μM at 25°C for 2.5 h.

**Data analysis** Sequence analysis was performed using Dnasis (version 3, Hitachisoftware, Tokyo, Japan). Data analysis of both [125I][Thy14]nociceptin binding and [35S]GTPγS binding was performed using Prism (version
Cloning of rhesus monkey NOP receptor

Fig. 1. Nucleotide sequence alignment between rhesus monkey NOP receptor and human NOP receptor. The dot (.) indicates the identical nucleotide.
RESULTS

Cloning of the rhesus monkey NOP receptor  
The nucleotide sequence of rhesus monkey NOP receptor was determined by sequencing the cDNA, that was synthesized from RNA of rhesus monkey prefrontal cortex. The nucleotide sequence of the rhesus monkey NOP receptor was 95.9% identical to that of the human NOP receptor (Fig. 1). The amino acid sequence of the rhesus monkey NOP receptor was 97.8% identical to that of the human NOP receptor (Fig. 2). There were seven amino acid differences between the rhesus monkey NOP receptor and the human NOP receptor. These amino acids were located at the extracellular N-terminal segment, the extracellular segment II, the extracellular segment III, the transmembrane (TM) segment V, the TM segment VI and the intracellular C-terminal segment.

Characterization of the rhesus monkey NOP receptor  
Specific binding of \[^{[25I]}\text{Thy}^{14}\]nociceptin was observed with the membranes prepared from the rhesus monkey NOP receptor expressing CHO cells. The dissociation constant (K_d) of N/OFQ were 27 ± 4 pM and 28 ± 2 pM for the rhesus monkey NOP receptor and the human NOP receptor, respectively (Fig. 3).

N/OFQ increased \[^{[35S]}\text{GTP}\gamma\text{S}\] binding with the membrane prepared from rhesus monkey NOP receptor expressing CHO cells. The half maximal effective concentration (EC_{50}) of N/OFQ were 2.0 ± 0.3 nM and 1.4 ± 0.3 nM for the rhesus monkey NOP receptor and the human NOP receptor, respectively (Fig. 4).

A selective NOP receptor antagonist, (+)-J-113397, inhibited \[^{[35S]}\text{GTP}\gamma\text{S}\] binding activated by 10 nM N/OFQ with the membrane from rhesus monkey NOP receptor expressing CHO cells. The half maximal inhibitory concentration (IC_{50}) of (+)-J-113397 were 0.72 ± 0.08 nM and 0.79 ± 0.17 nM for the rhesus monkey NOP receptor and

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Fig. 2. Amino acid sequence alignment between rhesus monkey NOP receptor and human NOP receptor. The dot (.) indicates the identical amino acids. The dashed lines below the human NOP receptor amino acid sequence indicate the transmembrane segments, numbered I–VII.
Cloning of rhesus monkey NOP receptor

There was no significant difference between the rhesus monkey NOP receptor and the human NOP receptor.

DISCUSSION

The present study is the first to report both the nucleotide sequence and the amino acid sequence of the rhesus monkey NOP receptor. The amino acid sequence of the rhesus monkey NOP receptor showed 97.8% homology with the human NOP receptor; only seven amino acids differed between the rhesus monkey NOP receptor and the human NOP receptor and these were scattered throughout the NOP receptor sequences.

Site-directed mutagenesis studies indicated that amino acids in TM III, V and VI in human NOP receptor were

### Fig. 3
Saturation binding (A) and Scatchard plot (B) of \[^{[125]}\text{I}^{\text{Tyr}}\text{nociceptin}\] binding with the membrane prepared from rhesus monkey NOP receptor expressing CHO cells. Saturation binding (C) and Scatchard plot (D) of \[^{[125]}\text{I}^{\text{Tyr}}\text{nociceptin}\] binding with the membrane prepared from human NOP receptor expressing CHO cells. Membranes were incubated in nociceptin binding buffer containing \[^{[125]}\text{I}^{\text{Tyr}}\text{nociceptin}\] as indicated. Values are specific binding counts of a representative experiment. Experiments were repeated three times independently.

### Fig. 4
[^{35}\text{S}]\text{GTP} \gamma \text{S} binding assay with the membrane prepared from rhesus monkey NOP receptor expressing CHO cells (closed circles) and human NOP receptor expressing CHO cells (open circles). These membranes were incubated in GTP\gamma S binding buffer containing 200 pM \[^{35}\text{S}]\text{GTP} \gamma \text{S} and N/OFQ as indicated. Values were shown as the percentage of the control value. Values are mean ± S.E.M. from three independent experiments.

### Fig. 5
Concentration-response curves of (+)-J-113397 to \[^{35}\text{S}]\text{GTP} \gamma \text{S} binding stimulated by N/OFQ with the membranes from rhesus monkey NOP receptor expressing CHO cells (open circles) and those from human NOP receptor expressing CHO cells (closed circles). Values are shown as the percentage of the control value in the absent of (+)-J-113397. Values were mean ± S.E.M. from six independent experiments.
contributed to interaction between NOP receptor and N/OFQ. Mouledous et al. (2000) reported that aromatic residues of Tyr131, Phe220, Phe224 and Trp276 in human NOP receptor were contributed to interaction between NOP receptor and N/OFQ. Akuzawa et al. (2007) reported Thr138 and Gln280 contributed to interaction between NOP receptor and N/OFQ.

In the present study, three amino acid substitutions were found in TM V and VI, but not TM III of rhesus monkey NOP receptor compared to human NOP receptor. Val285 (Ile in human NOP receptor) and Ile230 (Val in human NOP receptor) were in TM V, and Val285 (Ala in human NOP receptor) was in TM VI. The rest of amino acid substitutions, Ser241, Ala250, Gly253 and Glu342 were out of the TM segment of rhesus monkey NOP receptor compared to human NOP receptor. Substituted amino acids in TM V and VI were not considered to be critical amino acids for interaction between NOP receptor and N/OFQ based on studies with human NOP receptor (Mouledous et al., 2000; Akuzawa et al., 2007). The rest of substituted amino acids were scattered in N-terminal, third extracellular segment and C-terminal segment.

In order to examine whether these substitution of amino acids altered functions of monkey NOP receptor from those of human NOP receptor, we examined effects of agonist (N/OFQ) and antagonist ((+)-J-113397) on both monkey NOP receptor and human NOP receptor.

The $K_d$ value of N/OFQ for the rhesus monkey NOP receptor was comparable to that for the human NOP receptor. Thus, the seven amino acid changes in the rhesus monkey NOP receptor did not affect the binding affinity of N/OFQ.

The EC50 value of N/OFQ in the [35S]GTPyS binding with the membrane from rhesus monkey NOP receptor expressing CHO cells was comparable to that with the membrane from human NOP receptor expressing CHO cells. N/OFQ reduced the intracellular cAMP concentration caused by forskolin in rhesus monkey NOP receptor expressing CHO cells (Data not shown).

One of the seven amino acids, the valine residue at position 285 in the rhesus monkey NOP receptor, is neighboring to the glutamine residue at position 286 in the human NOP receptor, which is critical to reduce cAMP accumulation by N/OFQ, as determined in the previous report mentioned above (Mouledous et al., 2000). However, the present results indicate that the Val285 residue as well as the other six amino acid differences in the rhesus monkey NOP receptor do not affect the [35S]GTPyS binding as well as the changes in intracellular cyclic AMP concentration stimulated by N/OFQ.

To date, no study has shown that site-directed mutagenesis in the human NOP receptor affects the antagonistic activity of (+)-J-113397. We investigated the antagonistic activity of (+)-J-113397 with the membrane from rhesus monkey NOP receptor expressing CHO cells, and compared it with that for the membrane from human NOP receptor expressing CHO cells.

The IC50 value of (+)-J-113397 for the rhesus monkey NOP receptor was comparable to that for the human NOP receptor. Thus, the seven amino acid differences between the rhesus monkey NOP receptor and the human NOP receptor do not affect the inhibition of (+)-J-113397 to the [35S]GTPyS binding stimulated by N/OFQ.

The nucleotide sequence of N/OFQ in monkey is predicted by a computational analysis (GeneBank accession number: XM_001110309), but not obtained a gene corresponding to monkey N/OFQ. Amino acid sequence of N/OFQ from the predicted monkey nucleotide sequence is the same as that of human N/OFQ. Therefore, amino acid sequence of N/OFQ as well as that of NOP receptor are highly conserved between human and monkey.

The NOP receptor played various biological roles on nociception, locomotion, cognition and food intake in rodents (Nishi et al., 1997; Leventhal et al., 1998; Manabe et al., 1998; Calo’ et al., 2000). However, reports on the functional roles of the NOP receptor in non-human primates are limited (Ko et al., 2002, 2006). It is reasonable to use selective antagonists in non-human primates to understand the biological roles of the NOP receptor. The present results demonstrate that (+)-J-113397 inhibits the activation of the rhesus monkey NOP receptor by N/OFQ. Thus, (+)-J-113397 is a useful tool to determine the roles of N/OFQ and NOP receptor in locomotion, cognition and food intake in non-human primates.

In conclusion, the rhesus monkey NOP receptor exhibits a high degree of sequence homology with the human NOP receptor, with only seven amino acid differences between the two. These differences do not influence the binding affinity of N/OFQ, the binding of [35S]GTPyS stimulated by N/OFQ, or the antagonistic activity of (+)-J-113397.

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Cloning of rhesus monkey NOP receptor


