

Bremazocine Recognizes the Difference in Four Amino Acid Residues to Discriminate Between a Nociceptin/Orphanin FQ Receptor and Opioid Receptors

Takahiro Seki, Masabumi Minami, Chiaki Kimura, Tomoya Uehara, Takayuki Nakagawa and Masamichi Satoh*

Department of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606–8501, Japan

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ABSTRACT—We investigated the molecular basis of the discrimination between nociceptin/orphanin FQ receptor (NociR) and opioid receptors (OPRs) by bremazocine, a non-type-selective opioid ligand. Construction of several chimeric receptors between NociR and κ -opioid receptor (KOPR) and mutant NociRs followed by binding experiments with [3 H]bremazocine showed that the mutation of only four amino acid residues of NociR, Ala²¹⁶, Val²⁷⁹, Gln²⁸⁰ and Val²⁸¹, to the amino acid residues located at the corresponding position of KOPR, Lys²²⁷, Ile²⁹⁰, His²⁹¹ and Ile²⁹², made it possible for the resultant mutant NociR to bind bremazocine with high affinity. Considering that these four amino acid residues are conserved among μ -, δ - and κ -OPRs, the present result suggests that bremazocine recognizes the difference in these four amino acid residues to discriminate between NociR and OPRs.

Keywords: Nociceptin/orphanin FQ receptor, Opioid receptor, Bremazocine, Chimeric receptor, Site-directed mutagenesis

A novel receptor ORL1 (opioid receptor-like 1) belonging to the opioid receptor family due to its close similarity in amino acid sequence with μ -, δ - and κ -opioid receptors (OPRs) (Fig. 1) was cloned (1, 2), but had been regarded as an orphan receptor, because no known opioid peptides or alkaloids bound to this receptor with high affinity. An endogenous peptidic agonist of ORL1 was identified by two independent groups (3, 4). Meunier et al. (3) and Reinscheid et al. (4) isolated this peptide from rat and porcine brains and termed it nociceptin and orphanin FQ, respectively. In this paper, we refer to this peptide as nociceptin and to its receptor as the nociceptin receptor (NociR).

It has been reported that intracerebroventricular (i.c.v.) administration of nociceptin induced hyperalgesia or an anti-opioid effect (3–5). On the other hand, the intrathecal (i.t.) injection of this peptide produced an antinociceptive effect at high doses (6), but allodynia or hyperalgesia at low doses (7). These findings strongly suggest that nociceptin is involved in pain transmission and/or regulation systems, but the details remain unclear. Particularly, the physiological and pathophysiological

roles of endogenous nociceptin have not been elucidated, because no antagonist of NociR has been available. Recently, a selective antagonist for NociR was produced by chemical modification of nociceptin (8), but the effects of this antagonist peptide on pain transmission or regulation have never reported up to now. Nonpeptidic agonists and antagonists specific to NociR are thought to be useful for elucidating its function, but such compounds have never been obtained.

Researchers are keenly interested in the molecular mechanism of discrimination between OPRs and NociR by opioid ligands. In this study, we tried to identify the amino acid residues that are the determinant for the discrimination between OPRs and NociR by bremazocine, a non-peptidic and non-type-selective opioid ligand. First, we constructed several chimeric receptors between κ -opioid receptor (KOPR) and NociR, since nociceptin has some analogy to dynorphin, which is thought to be an endogenous ligand of KOPR. We considered that the amino acid residues conserved among the three types of OPRs are important for the binding of bremazocine to OPRs and that, in these residues, those different in NociR are critical for the discrimination between OPRs and NociR by bremazocine. According to this concept,

* To whom correspondence should be addressed.

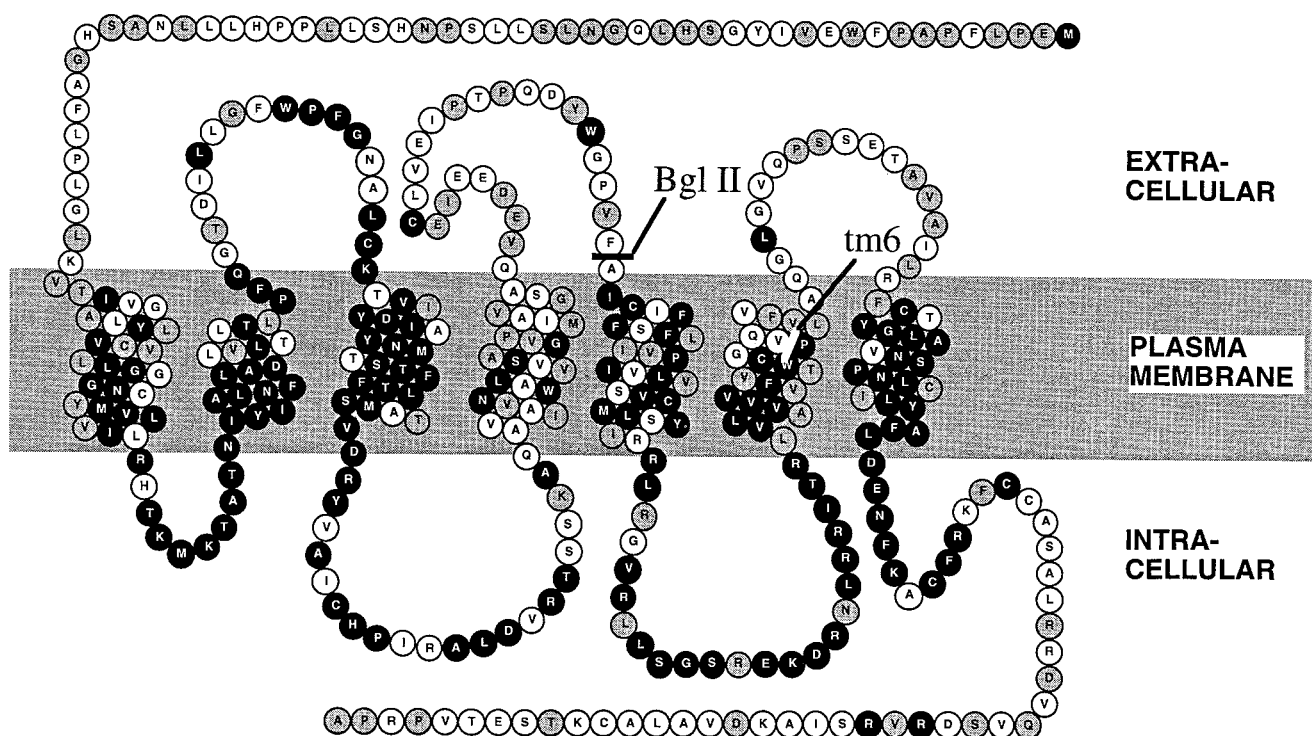


Fig. 1. Proposed membrane topology of the human NociR. Solid circles indicate the amino acid residues conserved between NociR and three types of OPRs, and gray circles indicate the residues conserved between NociR and one or two type(s) of OPR(s). Noted are the joints to construct chimeric receptors.

several mutant NociRs were constructed in the light of the results from the previous experiments with chimeric receptors. The affinities of [^3H]bremazocine for these chimeric and mutant receptors were examined.

MATERIALS AND METHODS

Materials

The rat KOPR cDNA was cloned as previously described (9). The human NociR cDNA was cloned from HL60 cells, a human myeloid leukemia cell line, by the RT-PCR method. (–)-[^3H](N)Bremazocine (29.8 Ci/mmol) and bremazocine were purchased from DuPont-New England Nuclear (Boston, MA, USA) and Research Biochemicals Inc. (Natick, MA, USA), respectively.

Construction of chimeric receptors and site-directed mutagenesis

The chimeric receptor, NKbgl, was constructed by using a restriction enzyme, Bgl II. A recognition site for Bgl II (Bgl II site) exists at the boundary region between the second extracellular loop and fifth transmembrane domain (Fig. 1) in KOPR, but not in NociR. Therefore, the Bgl II site was introduced at the corresponding position of NociR by *in vitro* site-directed mutagenesis. The

appropriate restriction enzyme fragments of KOPR and NociR cDNAs were ligated and cloned into the multiple cloning site of the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA). The chimeric receptor, NKbglNtm6, was constructed by the PCR method (10) with another joint (tm6 site) in the sixth transmembrane domain. For the site-directed mutagenesis, the coding region of NociR cDNA was subcloned into the pBluescript II (Stratagene, San Diego, CA, USA). *In vitro* site-directed mutagenesis was carried out using a TransformerTM Site-Directed Mutagenesis Kit (2nd version) (Clontech Laboratories, Inc., Palo Alto, CA, USA) as previously described (11). The fragment containing the full length coding region of each mutant NociR cDNA was subcloned into the pcDNA3 vector. The sequence of each construct was confirmed by sequencing analysis using an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer, Foster City, CA, USA).

Expression of wild type, chimeric and mutant receptors and radioligand binding assay

COS-7 cells were grown in Dulbecco's modified Eagle Medium supplemented with 10% fetal bovine serum in 5% CO_2 at 37°C. For transient expression of the wild type, chimeric and mutant receptors, each plasmid cDNA

(2–5 $\mu\text{g/ml}$) was transfected to COS-7 cells by the DEAE-dextran method. After cultivation for 65 hr, the cells were harvested and homogenized in 50 mM Tris (pH 7.4) containing 10 mM MgCl_2 and 1 mM EDTA. After centrifugation for 20 min at $30,000\times g$, the pellet was resuspended in the same buffer and used as the membrane preparation in the radioligand binding assay. Saturation binding experiments were carried out with [^3H]bremazocine. The membrane preparations were incubated with various concentrations of [^3H]bremazocine at 25°C for 1 hr. Non-specific binding was determined in the presence of 10 μM unlabeled bremazocine. The incubation was terminated by the addition of ice-cold buffer followed by rapid filtration over a Whatman GF/C glass fiber filter, which had been pretreated with 0.1% polyethyleneimine, and the radioactivity on each filter was measured by liquid scintillation counting. The K_d value of [^3H]bremazocine for each receptor was obtained by a Scatchard analysis of the data from saturation binding experiments. The results of binding assays are presented as the mean \pm S.E.M. of three to five separate experiments.

RESULTS

Determination of the regions involved in discrimination between KOPR and NociR by bremazocine

The affinities of bremazocine for KOPR, NociR and

chimeric receptors expressed in COS-7 cells were estimated by saturation binding assay using [^3H]bremazocine as a radiolabeled ligand. Although KOPR bound [^3H]bremazocine with a high affinity ($K_d=3.1\pm0.4$ nM), NociR poorly bound [^3H]bremazocine and the K_d value could not be determined (Fig. 2, Table 1). The chimeric receptor NKbgl, in which the region from the Bgl II site to the carboxyl-terminus of NociR was replaced with the corresponding region of KOPR, bound bremazocine with a high affinity ($K_d=11\pm3$ nM, Table 1) equivalent to that of the wild type KOPR. Another chimeric receptor NKbglNtm6, in which the region from the tm6 site to the carboxyl-terminus of NKbgl was further replaced with the corresponding region of NociR, did not show any high affinity for bremazocine (Table 1).

Determination of the amino acid residue(s) in the region from the tm6 site to the carboxyl terminus critical for the high affinity binding of bremazocine

According to the concept described previously, three amino acid residues of NKbglNtm6, Val²⁷⁹-Gln²⁸⁰-Val²⁸¹, were replaced with Ile-His-Ile, which are the amino acids located at the corresponding position of KOPR, to make the mutant receptor NKbglNtm6+VQV(279–281)IHI. Different from the case of NKbglNtm6, NKbglNtm6+VQV(279–281)IHI bound bremazocine with an affinity ($K_d=29\pm3$ nM, Table 2) as high as that of NKbgl ($K_d=11\pm3$ nM, Table 1). Replacement of Val²⁷⁹-Gln²⁸⁰-Val²⁸¹

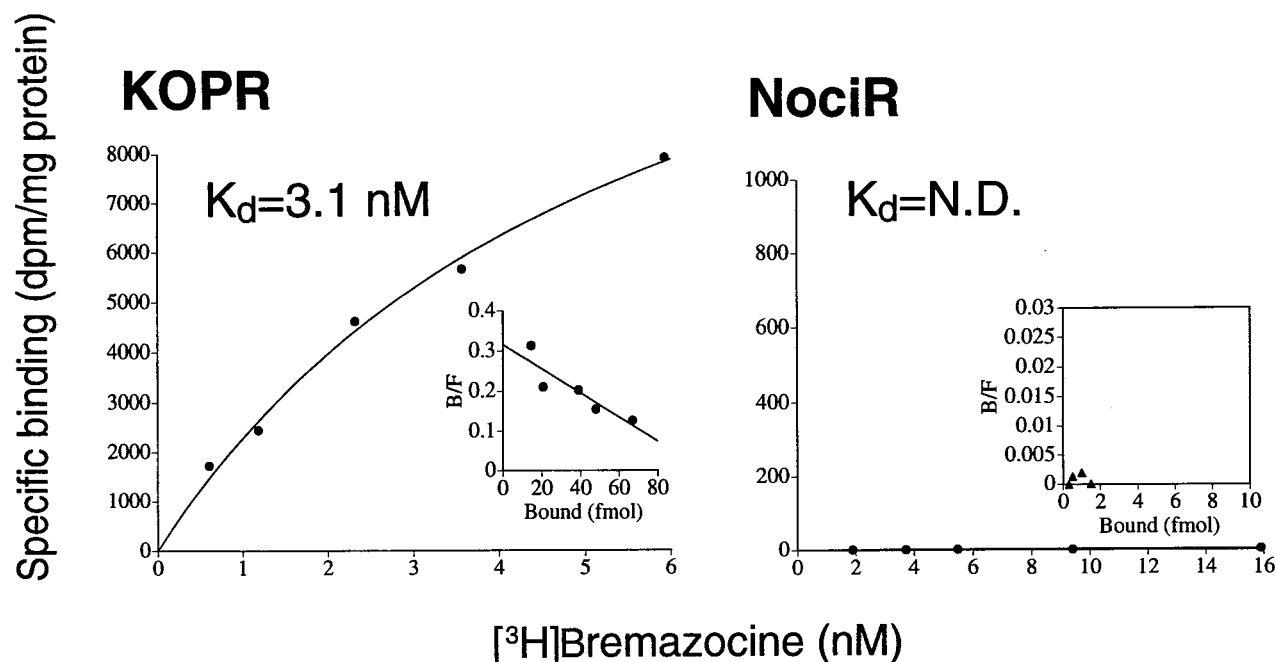


Fig. 2. Saturation binding of [^3H]bremazocine to the membrane preparations of COS-7 cells expressing KOPR (left) and NociR (right). Insets show the Scatchard analysis of [^3H]bremazocine binding.

Table 1. K_d values of [3 H]bremazocine for KOPR, NociR and KOPR/NociR chimeric receptors

Receptor	Scheme	K_d (nM)
		[3 H]Bremazocine
KOPR		3.1 ± 0.4
NociR		N.D.
NKbgl		11 ± 3
NKbglNtm6		N.D.

K_d values of [3 H]bremazocine were determined by Scatchard analyses of the data from saturation binding experiments. Values are the mean \pm S.E. of three to five experiments. N.D. means impossible to determine K_d values due to the very low affinity for [3 H]bremazocine. Black and hatched parts in the schemes of the receptors indicate the receptor regions derived from NociR and KOPR, respectively.

of NociR with Ile-His-Ile did not give any high affinity for bremazocine to the resultant mutant receptor NVQV-(279–281)IHI, and the K_d value was not determined (Table 2).

Determination of the amino acid residue(s) in the region from the Bgl II site to the tm6 site critical for the high affinity binding of bremazocine

In addition to the mutation of Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ to Ile-His-Ile, Ala²¹⁶ at the boundary region between the second extracellular loop and the fifth transmembrane domain of NociR was replaced with Lys, which is the amino acid found at the corresponding position of KOPR. The resultant mutant receptor NA216K+VQV-(279–281)IHI showed a high affinity for bremazocine ($K_d = 31 \pm 14$ nM, Table 2), equivalent to that of NKbglNtm6+VQV(279–281)IHI ($K_d = 29 \pm 3$ nM, Table 2). Mutation of only Ala²¹⁶ of NociR to Lys did not give any high affinity for bremazocine to the resultant mutant receptor NA216K, and the K_d value was not determined (Table 2).

Elucidation of the contribution of Ile²⁹⁰, Ile²⁹² and Ile³¹⁶ of KOPR to the bremazocine binding

To elucidate the contribution of Ile²⁹⁰ and Ile²⁹² of

Table 2. K_d values of [3 H]bremazocine for the mutant receptors

Receptor	Scheme	K_d (nM)
		[3 H]Bremazocine
NKbglNtm6 +VQV(279–281)IHI		29 ± 3
NVQV(279–281)IHI		N.D.
NA216K +VQV(279–281)IHI		31 ± 14
NA216K		N.D.
NA216K +Q280H		N.D.
NA216K +VQV(279–281)IHI +T305I		23 ± 5

K_d values of [3 H]bremazocine were determined by Scatchard analyses of the data from saturation binding experiments. Values are the mean \pm S.E. of three to five experiments. N.D. means impossible to determine K_d values due to the very low affinity for [3 H]bremazocine. Black and hatched parts in the schemes of the receptors indicate the receptor regions derived from NociR and KOPR, respectively. White circles indicate the amino acid residues which were mutated in this study.

KOPR to the bremazocine binding, a mutant receptor NA216K+Q280H was constructed. This mutant receptor did not bind bremazocine with a high affinity and the K_d value was not determined (Table 2). In addition to the mutation of Ala²¹⁶ and Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ to Lys and Ile-His-Ile, respectively, Thr³⁰⁵ of NociR was mutated to Ile to construct another mutant receptor NA216K+VQV-(279–281)IHI+T305I, but this additional mutation did not give any definite increase in the affinity for bremazocine ($K_d = 23 \pm 5$ nM, Table 2) compared with that of NA216K+VQV(279–281)IHI.

DISCUSSION

In this study, first, two chimeric receptors were constructed to determine the region(s) critical for the discrimination between KOPR and NociR by bremazocine, a non-peptidic and non-type-selective opioid ligand. The chimeric receptor NKbgl, in which the region from the Bgl II site to the carboxyl-terminus of NociR was replaced

with the corresponding region of KOPR, bound bremazocine with a high affinity ($K_d = 11 \pm 3$ nM, Table 1) equivalent to that of the wild type KOPR. This result indicates that all of the amino acid residues critical for the binding of bremazocine exist in the region from the Bgl II site to the carboxyl-terminus. Another chimeric receptor NKbglNtm6, in which the region from the tm6 site to the carboxyl-terminus of NKbgl was further replaced with the corresponding region of NociR, did not show any high affinity for bremazocine (Table 1), suggesting that at least one region critical for bremazocine binding exists between the tm6 site and the carboxyl-terminus.

Next, several mutant receptors were constructed to elucidate the amino acid residue(s) critical for the discrimination between KOPR and NociR by bremazocine. Since bremazocine binds all of the μ -, δ - and κ -OPRs with high affinity, the amino acid residues conserved among these three types of OPRs are considered to be important for bremazocine binding. Furthermore, it is also considered that the residues different between KOPR and NociR are critical for the discrimination between these two receptors. In addition, it is assumed that the amino acid residues critical for the ligand binding are located in the extracellular or transmembrane domains, but not in the intracellular domain. The amino acid residues that meet these three conditions and are located in the region from the Bgl II site to the carboxyl-terminus of NociR are shown by open circles in Fig. 3. Some of these were mutated by site-directed mutagenesis.

In the region from the tm6 site to the carboxyl terminus, there are four amino acid residues that meet the three conditions (Fig. 3). Three residues, Val²⁷⁹-Gln²⁸⁰-Val²⁸¹, in the sixth transmembrane domain of NKbglNtm6 were changed to Ile-His-Ile, which are amino acids located at the corresponding positions of OPRs. Different from the case of NKbglNtm6, the resultant mutant receptor NKbglNtm6+VQV(279–281)IHI bound bremazocine with a high affinity ($K_d = 29 \pm 3$ nM, Table 2) similar to that of NKbgl ($K_d = 11 \pm 3$ nM, Table 1), suggesting that in the region from the tm6 site to the carboxyl terminus, the difference between Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ of NociR and Ile²⁹⁰-His²⁹¹-Ile²⁹² of KOPR is critical for the discrimination between these receptors by bremazocine. Replacement of Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ of NociR with Ile-His-Ile did not give any high affinity for bremazocine to the resultant mutant receptor NVQV(279–281)IHI (Table 2), suggesting that another amino acid residue(s) involved in the bremazocine binding exists in a region other than that from the tm6 site to the carboxyl terminus.

The amino acid residue(s) critical for the discrimination between KOPR and NociR by bremazocine was elucidated in the region between the Bgl II site and the tm6 site. There are six amino acid residues that meet the

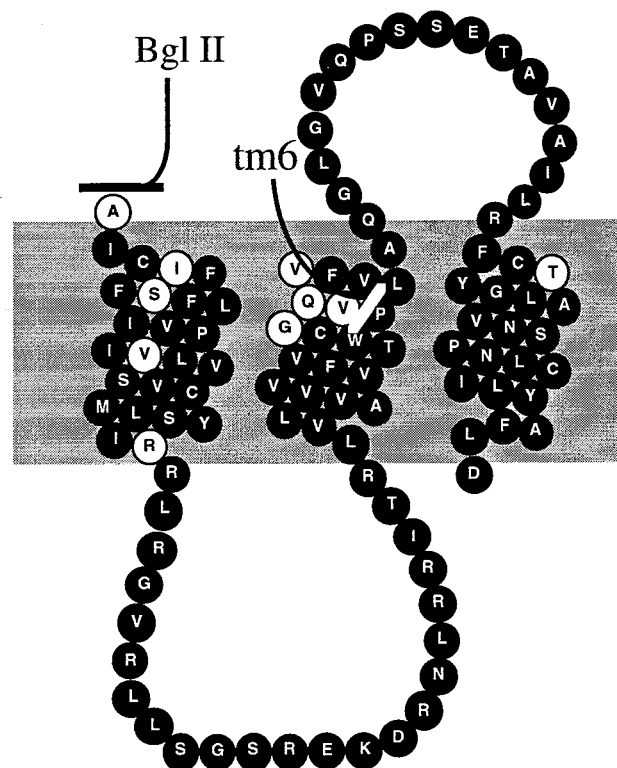


Fig. 3. The amino acid sequence from the fifth to the seventh transmembrane domains of human NociR. Open circles indicate the amino acid residues that are different between NociR and OPRs while conserved among the three types of OPRs.

three conditions in this region (Fig. 3). Among them, we focused on Ala²¹⁶, which is located in the boundary region between the second extracellular loop and the fifth transmembrane domain, because we previously showed that amino acid residues located at the boundary region between the extracellular loops and transmembrane domains were important for the discrimination between μ -OPR and δ - or κ -OPR by DAMGO, a μ -selective ligand (11, 12). Ala²¹⁶ of NociR was mutated to Lys in addition to the mutation of Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ to Ile-His-Ile. The resultant mutant receptor NA216K+VQV(279–281)IHI showed a high affinity for bremazocine ($K_d = 31 \pm 14$ nM, Table 2), equivalent to that of NKbglNtm6+VQV(279–281)IHI ($K_d = 29 \pm 3$ nM, Table 2). The mutant receptor NA216K, in which only Ala²¹⁶ of NociR was mutated to Lys, poorly bound bremazocine. These results indicate that, in the region between the Bgl II site and the tm6 site, the difference between Ala²¹⁶ of NociR and Lys²²⁷ of KOPR is critical for the discrimination between these receptors by bremazocine, but it is not sufficient for high affinity binding of bremazocine by itself.

The present results show that the difference between Ala²¹⁶, Val²⁷⁹, Gln²⁸⁰ and Val²⁸¹ of NociR and Lys²²⁷,

Ile²⁹⁰, His²⁹¹ and Ile²⁹² of KOPR is critical for the discrimination between these receptors by bremazocine. Considering that these four amino acid residues are conserved among μ -, δ - and κ -OPRs, it is thought that bremazocine recognizes the difference in these four amino acid residues to discriminate between NociR and three types of OPRs. This result is partly consistent with the report by Meng et al. (13). They showed that the replacement of Ala²¹³ or Val²⁷⁶-Gln²⁷⁷-Val²⁷⁸ of rat NociR with Lys or Ile-His-Ile, respectively, increased the affinity of dynorphin A. Furthermore, they reported the additional replacement of Thr³⁰² of rat NociR with Ile besides the replacement of Val²⁷⁶-Gln²⁷⁷-Val²⁷⁸ with Ile-His-Ile gave an additional increase in affinity for dynorphin A, although we showed the additional replacement of Thr³⁰⁵ of NociR with Ile besides the replacement of Ala²¹⁶ and Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ with Lys and Ile-His-Ile, respectively, did not additionally increase the affinity for bremazocine. Mollereau et al. revealed that replacement of only Gln²⁸⁰ with His increased the affinities for several nonpeptidic opioid ligands such as lofentanil and etorphine (14). In this context, we constructed the mutant receptor NA216K+Q280H to examine whether the replacement of only Gln²⁸⁰ out of Val²⁷⁹-Gln²⁸⁰-Val²⁸¹ with His is sufficient to produce the high affinity for bremazocine. Bremazocine did not bind to NA216K+Q280H with any high affinity (Table 2), suggesting some contribution of Ile²⁹⁰ and Ile²⁹² of KOPR to the bremazocine binding.

The nociceptin-NociR system is considered to play important roles in pain transmission/regulation, but its detailed function remains to be elucidated. Furthermore, since NociR is also abundantly expressed in the hippocampus and hypothalamus (1, 2), it is possible that nociceptin is involved in processes of learning and memory or regulations of feeding and hormonal secretion. However, these possibilities have not been proved yet. To investigate these possibilities and to fully elucidate the physiological and pathological roles of the nociceptin-NociR system in pain transmission/regulation, non-peptidic agonists and antagonists specific to NociR are considered indispensable. In the present study, we showed that replacement of Ala²¹⁶, Val²⁷⁹, Gln²⁸⁰ and Val²⁸¹ in NociR with Lys, Ile, His and Ile, respectively, made it possible for the resultant mutant receptor to bind bremazocine with high affinity. These findings will give us a clue to develop a novel non-peptide agonist or antagonist specific for the wild type NociR by modification of the bremazocine molecule.

REFERENCES

- 1 Mollereau C, Parmentier M, Mailleux P, Butour JL, Moisand C, Chalon P, Caput D, Vassart G and Meunier JC: ORL1, a novel member of the opioid receptor family. *FEBS Lett* **341**, 33–38 (1994)
- 2 Fukuda K, Kato S, Mori K, Nishi M, Takeshima H, Iwabe N, Miyata T, Houtani T and Sugimoto T: cDNA cloning and regional distribution of a novel member of the opioid receptor family. *FEBS Lett* **343**, 42–46 (1994)
- 3 Meunier JC, Mollereau C, Toll L, Suaudeau C, Moisand C, Alvinerie P, Butour JL, Guillemot JC, Ferrara P, Monsarrat B, Mazarguil H, Vassart G, Parmentier M and Costentin J: Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **377**, 532–535 (1995)
- 4 Reinscheid RK, Nothacker HP, Boursan A, Ardati A, Henningsen RA, Bunzow JR, Grandy DK, Langen H, Monsma FJ Jr and Civelli O: Orphanin FQ: a neuropeptide that activates an opioid like G protein-coupled receptor. *Science* **270**, 792–794 (1995)
- 5 Mogil JS, Grisel JE, Reinscheid RK, Civelli O, Belknap JK and Grandy DK: Orphanin FQ is a functional anti-opioid peptide. *Neuroscience* **75**, 333–337 (1996)
- 6 Xu XJ, Hao JX and Wiesenfeld-Hallin Z: Nociceptin or antinociceptin: potent spinal antinociceptive effect of orphanin FQ/nociceptin in the rat. *Neuroreport* **7**, 2092–2094 (1996)
- 7 Okuda-Ashitaka E, Tachibana S, Houtani T, Minami T, Masu Y, Nishi M, Takeshima H, Sugimoto T and Ito S: Identification and characterization of an endogenous ligand for opioid receptor homologue ROR-C: its involvement in allodynic response to innocuous stimulus. *Mol Brain Res* **43**, 96–104 (1996)
- 8 Guerrini R, Calo G, Rizzi A, Bigoni R, Bianchi C, Salvadori S and Regoli D: A new selective antagonist of the nociceptin receptor. *Br J Pharmacol* **123**, 163–165 (1998)
- 9 Minami M, Toya T, Katao Y, Maekawa K, Nakamura S, Onogi T, Kaneko S and Satoh M: Cloning and expression of a cDNA for the rat κ -opioid receptor. *FEBS Lett* **329**, 291–295 (1993)
- 10 Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR: Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. *Gene* **77**, 61–68 (1989)
- 11 Minami M, Nakagawa T, Seki T, Onogi T, Aoki Y, Katao Y, Katsumata S and Satoh M: A single residue, Lys108, of the δ -opioid receptor prevents the μ -opioid-selective ligand [D -Ala², N -MePhe⁴, Gly-ol⁵]enkephalin from binding to the δ -opioid receptor. *Mol Pharmacol* **50**, 1413–1422 (1996)
- 12 Seki T, Minami M, Nakagawa T, Ienaga Y, Morisada A and Satoh M: DAMGO recognize four residues in the third extracellular loop to discriminate between μ - and κ -opioid receptors. *Eur J Pharmacol* **350**, 301–310 (1998)
- 13 Meng F, Taylor LP, Hoversten MT, Ueda Y, Ardati A, Reinscheid RK, Monsma FJ, Watson SJ, Civelli O and Akil H: Moving from the orphanin FQ receptor to an opioid receptor using four point mutations. *J Biol Chem* **271**, 32016–32020 (1996)
- 14 Mollereau C, Moisand C, Butour JL, Parmentier M and Meunier JC: Replacement of Gln²⁸⁰ by His in TM6 of the human ORL1 receptor increases affinity but reduces intrinsic activity of opioids. *FEBS Lett* **395**, 17–21 (1996)