DAMGO, a μ-opioid receptor selective agonist, distinguishes between μ- and δ-opioid receptors around their first extracellular loops

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Received 21 November 1994

Abstract The structural basis of μ-opioid receptor (OPR) for the specificity in its ligand binding was investigated using chimeric μ/δ-OPRs. Replacement of the region around the first extracellular loop of δ-OPR with the corresponding region of μ-OPR gave the resultant chimeric receptor the similar affinity to DAMGO compared with the native μ-OPR. The reciprocal replacement deprived the high affinity to DAMGO from μ-OPR. These results indicate that the difference(s) in the structure around the first extracellular loop is critical for DAMGO to distinguish between μ- and δ-OPRs. Furthermore, displacement studies revealed that this region is partly involved in the discrimination between μ- and δ-OPRs by other peptidic μ-selective ligands, such as dermorphin, morphiceptin and CTOP, but not by non-peptidic ligands, such as morphine and naloxone.

Key words: Opioid receptor; Chimeric receptor; Ligand binding; μ-type; DAMGO

1. Introduction

Endogenous opioid peptides and opiate drugs like morphine produce various physiological and pharmacological effects, such as analgesia, respiratory depression, euphoria and modulations of neuroendocrine, through their specific receptors. Although pharmacological studies have defined three subtypes of opioid receptors (OPRs), that is, μ-, δ- and κ-OPRs, using various subtype-specific ligands, the molecular basis for how these ligands discriminate among three subtypes of OPRs is yet unknown and of great interest.

Recently we and other groups have cloned these OPR cDNAs from the rat brain [1–3]. The amino acid sequences of the three OPRs are highly conserved in the putative transmembrane domains and the intracellular regions, and the amino acid identities in those regions are 73.1–76.3% and 62.3–74.3%, respectively. On the other hand, the sequences of the extracellular regions are considerably different (34.1–39.6% of identities), and it is likely to assume that the differences in these regions are responsible for the discrimination by the subtype-specific opioid ligands. The cloning of cDNAs for these OPRs has enable us to investigate the structural basis for subtype specificity of the OPRs in their ligand bindings using the various molecular biological techniques. The construction of chimeric OPRs is thought to be one of the very powerful approaches to the issue, as it has been demonstrated in the cases of adrenaline [4–6], acetylcholine [7], dopamine [8] and tachykinin [9,10] receptors. In the present study, to elucidate the regions of μ-OPR which are specifically recognized by μ-selective opioid ligands, we have constructed chimeric m/δ-OPRs and examined their affinities to [^{3}H]DAMGO, one of the most popular μ-selective opioid ligands, by Scatchard analyses. In addition, other μ-selective opioid ligands, that is, dermorphin, morphiceptin, CTOP, morphine and naloxone, were tested in displacement studies with [^{3}H]DAMGO and [^{3}H]DPDPE.

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Abbreviations: CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; DAMGO, [D-Ala, MePhe^4, Gly(ol)^5]enkephalin; DPDPE, [D-Pen^{2,5}]enkephalin; EL, extracellular loop; G-protein, GTP binding protein; OPR, opioid receptor; TM, transmembrane domain.

2. Materials and methods

2.1. Materials

[Tyrosyl-3,5-^{3}H(N)]DAMGO (50.5 Ci/mmol), (−)-[9-^{3}H(N)]bremazocine (29.8 Ci/mmol) and [Tyrosyl-2,6-^{3}H(N)]DPDPE (46.0 Ci/mmol) were obtained from DuPont-New England Nuclear (USA). DAMGO was purchased from Cambridge Research Biochemicals, Ltd. (UK). Morphine hydrochloride was from Takeda Chemical Industries, LTD (Japan). Naloxone hydrochloride was from Sigma Chemical Co. (USA). Dermorphin, morphiceptin and CTOP were from Peninsula Laboratories, INC. (USA).

2.2. Construction of chimeric receptors

The chimeric receptors between μ- and δ-OPRs were constructed by using the *Afl*III and *Bbs*I sites which intrinsically exist at the corresponding position of both receptor cDNAs (Fig. 1). The appropriate restriction enzyme fragments of the μ- and δ-OPRs were ligated and cloned into the *Hin*dIII–*Apa*I site of the pcDNA3 expression vector (Invitrogen, USA). The sequence of each construct was confirmed by sequencing analysis using Sequenase ver.2 DNA sequencing kit (United States Biochemical, USA). Each constructed chimeric receptor was given a name on the basis of the origins of its four extracellular domains.

2.3. Expression of native and chimeric receptors and binding assay

For transient expression of the native and chimeric OPRs, each plasmid cDNA was transfected to COS-7 cells by the DEAE-dextran method [11]. After cultivation for 65 h, the cells were harvested and homogenized in the following buffer; 50 mM Tris (pH 7.4), 10 mM $MgCl_2$ and 1 mM EDTA. After centrifugation for 20 min at 30,000 × *g*, the pellet was resuspended in the same buffer. Binding experiments for Scatchard analyses were performed with various concentrations of [^{3}H]DAMGO or [^{3}H]bremazocine. For displacement studies, various concentrations of unlabeled opioid ligands and 1 nM [^{3}H]DAMGO or 1 nM [^{3}H]DPDPE were used. Non-specific binding was determined in the presence of 10 μM unlabeled DAMGO, bremazocine or DPDPE. The incubations of membrane with opioid ligands were carried out at 25 °C for 1 h and terminated by vacuum filtration through GF/C filters (Whatman). Then the radioactivity was measured by liquid scintillation counting.

SSDI 0014-5793(94)01341-1

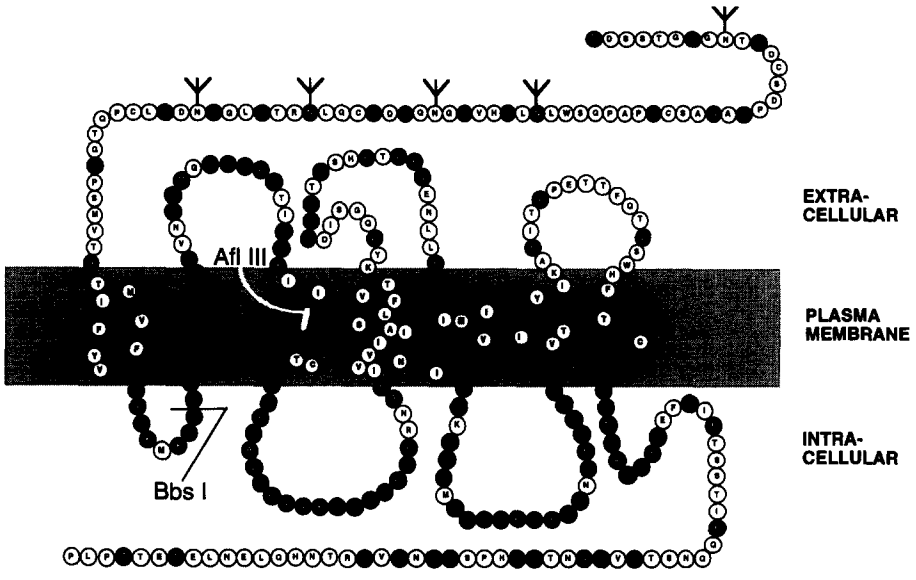
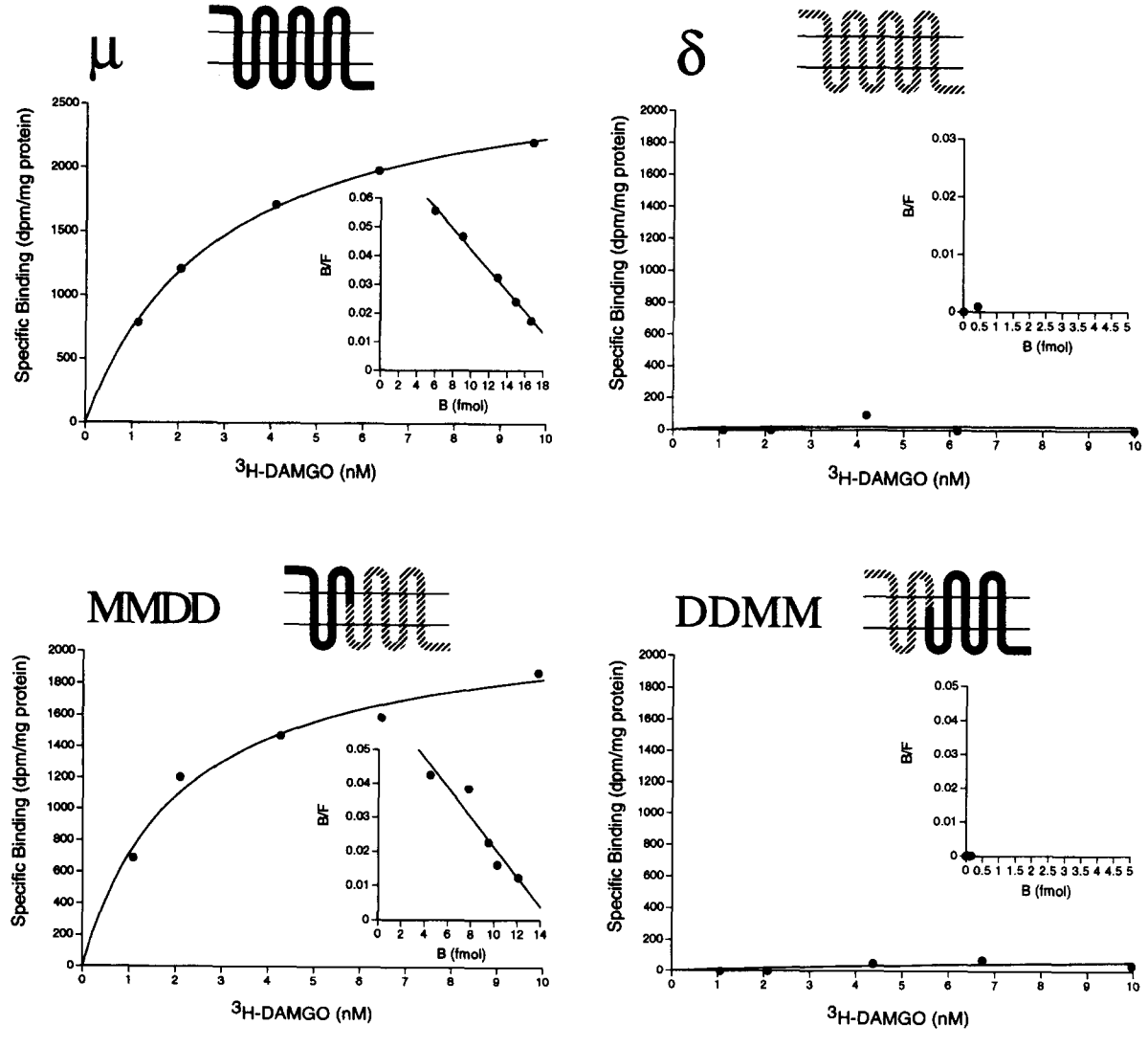


Fig. 1. Proposed model for membrane topography of the rat μ-opioid receptor. Solid circles indicate amino acid residues conserved between μ- and δ-opioid receptors. Branched structures show consensus sequences for potential N-linked glycosylation sites. Noted are the unique restriction enzyme sites used to construct chimeric receptors.



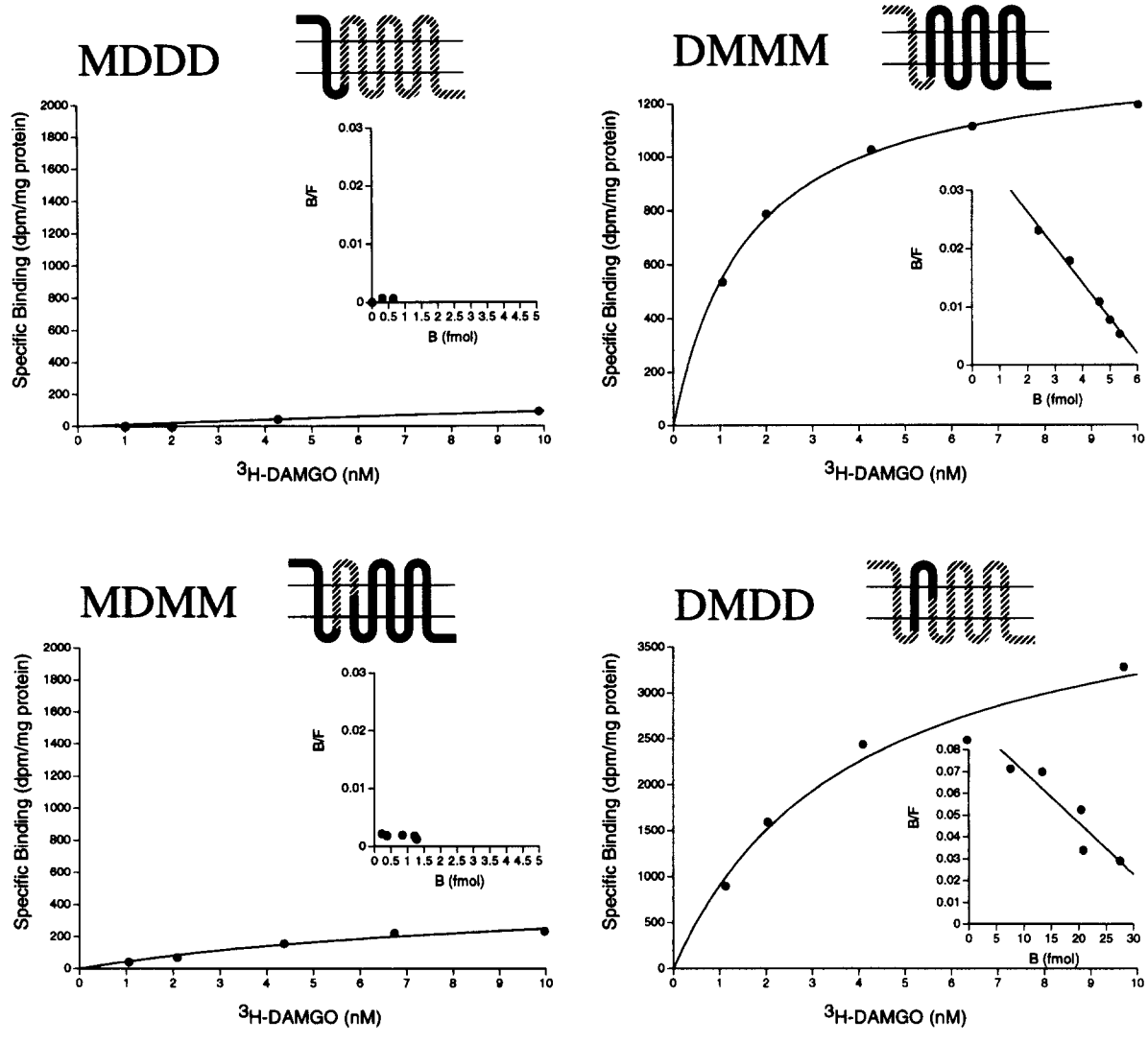


Fig. 3. Saturation binding of [^{3}H]DAMGO to the membrane of COS-7 cells which expressed chimeric MDDD, DMMM, MDMM or DMDD receptor. Insets show the Scatchard analyses of [^{3}H]DAMGO binding.

3. Results and discussion

Firstly, to determine which the first or the latter half of μ-OPR is important to its high affinity to DAMGO, we constructed two chimeric receptors using restriction enzyme *Afl*III. In native μ-OPR, the K_d value for DAMGO was 3.46 ± 0.84 nM (n = 3) (Fig. 2). The chimeric receptor MMDD, in which the carboxy-terminal half of the μ-OPR was replaced with the corresponding region of δ-OPR, exhibited an equivalent affinity (K_d = 2.13 ± 0.40 nM; n = 3) to DAMGO compared with the native μ-OPR, while the reciprocal chimeric receptor DDMM poorly bound [^{3}H]DAMGO and the K_d value could not be determined as in the case of the native δ-OPR. These results suggest that the first halves of μ- and δ-OPRs are critical for DAMGO to distinguish between these receptors.

Secondly, for further clarification of the region responsible for selective binding of DAMGO, we constructed four chimeric receptors using the restriction enzyme *Bbs*I in addition to *Afl*III. The chimeric receptor DMMM, in which the N-terminal and first transmembrane domain (TM-1) of μ-OPR were replaced with the corresponding region of δ-OPR, exhibited an equivalent affinity to DAMGO (K_d = 3.16 ± 0.91 nM; n = 3) compared with the native μ-OPR (Fig. 3). Conversely, MDDD did not display any specific binding to [^{3}H]DAMGO. On the other hand, the chimeric receptor DMDD, in which the segment containing the first extracellular loop (EL-1), TM-2 and a part of TM-3 of δ-OPR was substituted for the correspondence of μ-OPR, exhibited high affinity to [^{3}H]DAMGO (K_d = 5.24 ± 0.86 nM; n = 3), while the reciprocal chimera MDMM did not exhibit the high affinity binding to

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Fig. 2. Saturation binding of [^{3}H]DAMGO to the membrane of COS-7 cells which expressed native μ- or δ-OPR or chimeric MMDD or DDMM receptor. Insets show the Scatchard analyses of [^{3}H]DAMGO binding.

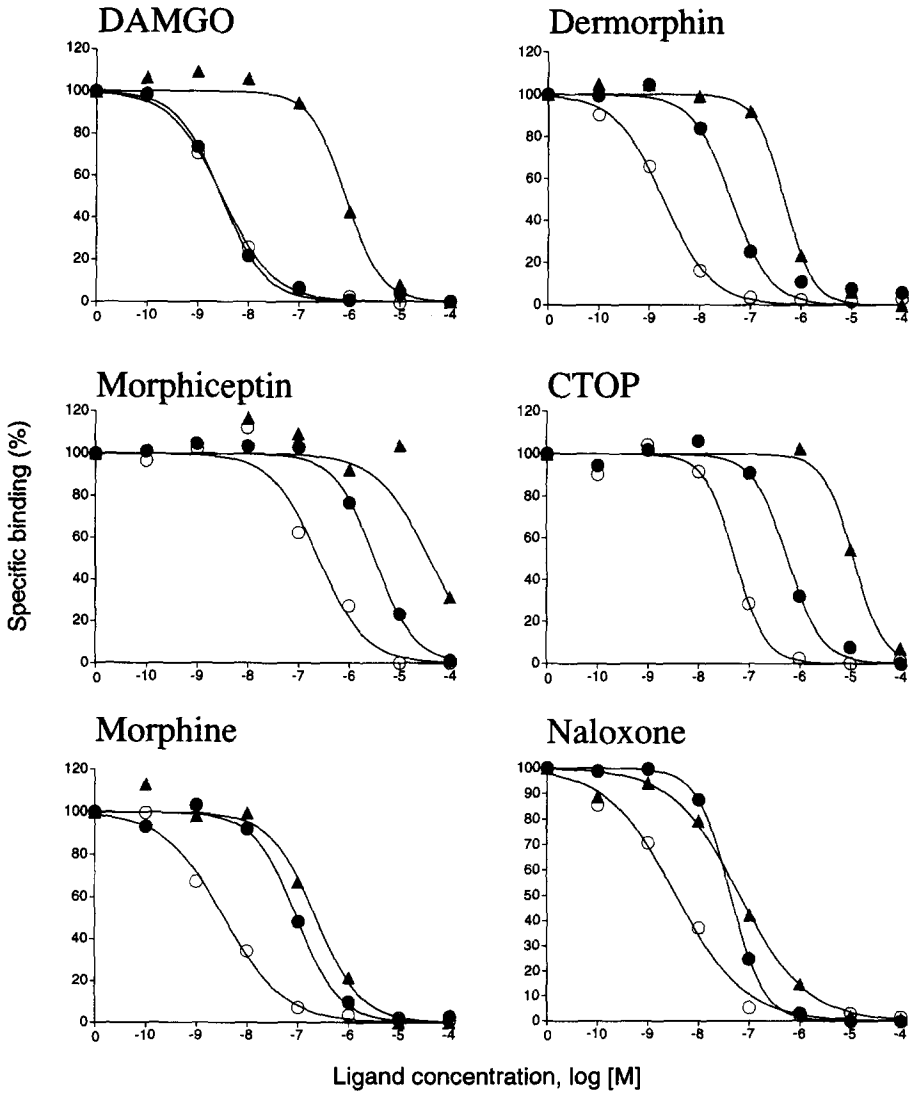

Fig. 4. Displacements of the specific binding of [^{3}H]DAMGO to the membrane of COS-7 cells expressing μ- (○) or DMDD (●) receptor and of [^{3}H]DPDPE to the membrane expressing δ- (▲) receptor with unlabeled μ-selective opioid ligands.

[^{3}H]DAMGO. Because all of the four chimeric receptors were verified to bind [^{3}H]bremazocine with high affinity (data not shown), the lack of binding of [^{3}H]DAMGO to chimeric receptors MDDD and MDMM was not due to low expression of these chimeric receptors. These results indicate that the region around the EL-1 which containing the EL-1, TM-2 and a part of TM-3 is critical for DAMGO to distinguish between μ- and δ-OPRs. Although much more differences in the amino acid sequences between μ- and δ-OPRs are found in the amino-terminal, EL-2 and EL-3 than EL-1, these regions are not likely to be concerned in the discrimination between these two receptors by DAMGO. This assumption is partly supported by the report which demonstrated that the removal of the amino-terminal of μ-OPR did not change the affinity to DAMGO [12].

To test whether this region is involved in the selective binding of other μ-selective opioid ligands, we carried out the displacement study using μ- and δ-OPRs and chimeric receptor DMDD (Fig. 4). [^{3}H]DAMGO was used to label μ- and DMDD receptors and [^{3}H]DPDPE to label δ-OPR. K_d values of [^{3}H]DAMGO to μ- and DMDD receptors and of [^{3}H] DPDPE to δ-OPR were almost equivalent (3.46 ± 0.84 nM at μ-OPR, 5.25 ± 0.86 nM at DMDD receptor and 3.13 ± 0.32 nM at δ-OPR). Unlabeled DAMGO displaced the binding of the tritiated ligand to μ- and DMDD receptors in the same degree, while the potency of unlabeled DAMGO to inhibit the specific binding of the tritiated ligand to δ-OPR was about 100 times lower than to μ- and DMDD receptors. Peptidic agonists, dermorphin and morphiceptin, and a peptidic antagonist, CTOP, showed the moderate affinity to DMDD chimeric receptor, that is, the affinity was higher than to δ-OPR but lower than to μ-OPR. A non-peptidic agonist, morphine, and a non-peptidic antagonist, naloxone, bound to μ-OPR with much higher affinity than to δ-OPR and to DMDD chimeric receptor, and the differences in the affinities of these non-peptidic ligands between δ-OPR and DMDD receptor were much smaller than those in the cases of the peptidic ligands. These results suggest that the region around the EL-1 is very critical just for DAMGO to distinguish between μ- and δ-OPRs, while this

region is partly involved in the subtype-specific binding of other peptidic μ-selective ligands, but not in the discrimination between μ- and δ-OPRs by non-peptidic ligands. These differences in the recognition sites for subtype-specific binding between DAMGO and other μ-selective ligands are not so surprising, because it has been reported that the different ligands recognize the different sites of the same receptor in the cases of many receptors belonged to the G-protein coupled receptor family, such as β-adrenergic, neurokinin-1 and δ-opioid receptors [5,13,14].

In this study, using chimeric m/δ-OPRs, we demonstrated that the region around the EL-1 plays an important role in the discrimination between μ- and δ-OPRs by DAMGO. There are only seven different amino acid residues between μ- and δ-OPRs in the amino acid sequence of this region (Fig. 1), and it is likely that one or a few of these amino acid residues are responsible for the subtype selectivity of DAMGO. To determine which amino acid residue(s) in this region plays a key role in the discrimination by DAMGO between μ- and δ-OPRs, site-directed mutagenesis experiments are now proceeding in our laboratory.

Acknowledgments: The authors are grateful to Dr. Fukuda, K. for providing the rat δ-opioid receptor cDNA. This study was partly supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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