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# Heterodimerization of the kappa opioid receptor and neurotensin receptor 1 contributes to a novel β-arrestin-2–biased pathway

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ABSTRACT

Together with its endogenous ligands (dynorphin), the kappa opioid receptor (KOR) plays an important role in modulating various physiological and pharmacological responses, with a classical G protein–coupled pathway mediating analgesia and non-G protein–dependent pathway, especially the β-arrestin–dependent pathway, eliciting side effects of dysphoria, aversion, drug-seeking in addicts, or even relapse to addiction. Although mounting evidence has verified a functional overlap between dynorphin/KOR and neurotensin/neurotensin receptor 1 (NTSR1) systems, little is known about direct interaction between the two receptors. Here, we showed that KOR and NTSR1 form a heterodimer that functions as a novel pharmacological entity, and this heterodimer, in turn, brings about a switch in KOR-mediated signal transduction, from G protein–dependent to β-arrestin-2–dependent. This was simultaneously verified by analyzing a KOR mutant (196th residue) that lost the ability to dimerize with NTSR1. We also found that dual occupancy of the heterodimer forced the β-arrestin-2–dependent pathway back into $G_i$ protein–dependent signaling, according to KOR activation. These data provide new insights into the interaction between KOR and NTSR1, and the newly discovered role of NTSR1 acting as a switch between G protein– and β-arrestin–dependent pathways of KOR also suggests a new approach for utilizing pathologically elevated dynorphin/KOR system into full play for its analgesic effect with limited side effects.



## 1. Introduction

The kappa opioid receptor (KOR) and its specific endogenous ligands, dynorphins, are broadly expressed in the central nervous system [1,2]. Activated KOR plays important roles in the modulation of various physiological and pharmacological responses, such as analgesia, dysphoria, feeding, water dieresis, hypothermia, and immune function, and even in reducing the drug-seeking motivation in drug addicts [3,4].

In the canonical sense, activated KOR *per se* usually exerts its *bona fide* effect on the various aforementioned responses *via* classical pathways, initiated by coupling to $G_i/G_o$ proteins and subsequently followed by inhibition of adenylyl cyclase activity, blockade of calcium influx through voltage-gated $Ca^{2+}$ channels, enhanced mitogen-activated protein kinase (MAPK) phosphorylation (especially extracellular signal–regulated kinase ERK1/2), and activity of inwardly rectifying $K^+$ channel [3,5,6].

However, in the central nervous system, besides the classical G protein–coupled pathway that mediates analgesia, the non-G protein–dependent pathway, especially the β-arrestin–dependent pathway, often invokes KOR-mediated side effects [7]. Because of this, KOR ligands often elicit quite different physiological responses, at different levels, according to the particular signaling pathways. At the brain level, not only the administration of exogenous KOR agonists [8,9], but also the endogenous dynorphins, released during the exposure to stressful experiences [10], would result in a dysphoric, anxiogenic, or aversive effect, which in turn would probably enhance the risk of drug abuse or addiction [11–13]. On the other hand, at the spinal level, 6′-guanidinonaltrindole (6′-GNTI), a type of KOR agonist, may be a potent analgesic, but no such effect is observed upon its intraventricular

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administration [14]. The reasons for this phenomenon are currently unknown.

Studies of KOR-mediated ERK phosphorylation revealed that ERK activation is G protein–dependent in KOR only–transfected cell lines, while it is β-arrestin-2–dependent in cultured striatal neurons [15]. Therefore, the aforementioned inactivity of the selective KOR–DOR (delta opioid receptor) heterodimer agonist 6′-GNTI in the brain could probably be interpreted as a G protein bias [16], with a limited distribution of KOR–DOR heterodimers in the brain [14].

The striatum is a place which is closely related with compulsive or habit-like behavior formation, *e.g.*, drug abuse or addiction, and furthermore, a pronounced elevated KOR/dynorphin system is considered to be a hallmark of drug addiction [17,18]. Since the specially biased β-arrestin-2–dependent pathway is involved in KOR signal transduction in the striatum, one may anticipate that the promoted KOR/dynorphin system may significantly contribute to drug abuse and addiction *via* this pathway. Therefore, it is extremely urgent to identify the root cause for the biased β-arrestin–dependent pathway with respect to KOR activation in the striatum.

Increasing evidence has revealed the universality of existence of G protein–coupled receptor (GPCR) heterodimers and elucidated their novel structural pharmacological characteristics distinct from those of the individual proteins [19–21]. Being a major member of the GPCR-type opioid receptors, KOR is no exception [6,22]. Furthermore, it is often the case that the protein (especially GPCR) that co-forms such heterodimers also usually directly determines the subsequent signal transduction in response to particular agonists.

The above-mentioned studies strongly suggested the possibility of the existence of different forms of KOR in the brain, with functions possibly distinct from that of KOR monomers or KOR–DOR heterodimers on account of activation of the non-classical pathway. We therefore proposed the existence of heterodimers composed of KOR and different GPCRs in the brain, whose conformation may bring about a shift in KOR signaling, from classical G protein–dependent to non-G protein–dependent.

If that was the case, which protein might constitute a KOR partner in the brain, especially in the striatum? Intriguingly, the neurotensin (NT)/neurotensin receptor 1 (NTSR1) system has been widely acknowledged for its multiple roles in the central nervous system [23]. Although NT or most of its derivatives should be directly applied to the central nervous system to exert their physiological effects [24,25], the unique role of NTSR1 activation in pain modulation, *e.g.*, spinal antinociceptive effect in neuropathic pain [26], central thermal antinociception [27], and antipsychotic effect [28,29], attracted our attention because the above effects significantly overlap with that of opioid receptors, especially KOR. Therefore, NTSR1 might be a promising interacting partner for KOR in the brain, especially in the striatum. Interestingly, based on laser confocal microscopy experiments verifying the co-localization of KOR and NTSR1, preliminary experiments using *in situ* proximity ligation assays (PLA) have already revealed heterodimerization of KOR and NTSR1 in cultured striatal neurons, providing a solid starting point for our prediction. Many studies have verified the importance of transmembrane domain (TM) 4/5 of GPCRs in the formation of homo- or heterodimers [30,31], while TM4 plays a critical role in the activation of opioid receptors, and the mutation of this TM of KOR can even subvert classical antagonists into agonistic ones [32]. To identify the relevant residue that would be relatively crucial for the KOR/NTSR1 interaction, site-directed mutagenesis was here used to generate mammalian KOR expression plasmids, mutating leucine to alanine within or in the proximity of TM4 (residues 184 or 196). Thus, since KOR/NTSR1 heterodimers were found to be exist in the primary cultured striatal neurons in our preliminary experiments, the current study aimed to obtain further validation of the KOR/NTSR1 heterodimerization, to verify the key residue involved in the formation of KOR–NTSR1 heterodimers, and furthermore, to reveal the possible alteration of KOR signal transduction by the KOR/NTSR1 interaction.

## 2. Materials and methods

### 2.1. Primary cultures of striatal neurons

Primary cultures of striatal neurons were prepared as described previously, with minor modifications [7]. Primary striatal neurons were isolated from newborn Sprague-Dawley rats of both sexes. After cutting into very small pieces with surgical scissors, tissue fragments were suspended in 0.125% Trypsin-EDTA and incubated at 37 °C for 15 min. The reaction was stopped by the addition of MEM containing 10% fetal bovine serum (FBS). After trituration with flame-polished glass Pasteur pipettes, the cell suspension was filtered through 200-mesh filter screen to discard incompletely fragmented tissues, followed by centrifuged at 800 $\times g$ for 3 min. The pellet was resuspended in Neurobasal-A Medium (Gibco, Carlsbad, CA, USA) with 0.5 mM GlutaMax and 100 U/mL penicillin/streptomycin, and the dissociated neurons were plated on poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA)–coated glass cover slips or in poly-D-lysine–coated 6-well plates at a density of (5–10) $\times 10^5$ cells/cm$^2$. B-27 (2%; Gibco) was added to the culture medium from day 2 onward, to prevent proliferation of non-neuronal cells. Cells were maintained in a humidified atmosphere at 37 °C with 5% $CO_2$ until use after 7 d.

### 2.2. Construction of recombinant plasmids

Plasmids pcDNA3.1(+)-NTSR1, pcDNA3.1(+)-KOR, and pcDNA 3.1(+)-HA–NTSR1 were obtained from Missouri S&T cDNA Resource Center (Rolla, MO, USA). KOR mutants with point leucine to alanine mutations were constructed by overlapping PCR using high fidelity *Pfu* polymerase, as previously described [33], using the following mutagenic primers: forward, 5′-ATC TGC ATC TGG GCG CTG TCG TCA TCT GTT G-3′, and reverse, 5′-C AAC AGA TGA CGA CAG CGC CCA GAT GCA GAT-3′, for the L184A mutant; and forward, 5′-TCT GCA ATA GTC GCT GGA GGC ACC AAA GTC-3′, and reverse, 5′-GAC TTT GGT GCC TCC AGC GAC TAT TGC AGA-3′, for the L196A mutant. Plasmid pcDNA3.1(+)-KOR was used as a template. Using purified PCR-amplified overlapping segments of the L184A mutant or the L196A mutant as templates, full-length cDNA of $KOR_{184}$ or $KOR_{196}$ was amplified using T7 forward primer (5′-TAA TAC GAC TCA CTA TAG GGA GAC-3′) and reverse primer (5′-CCGCTCGAGTAC TGG TTT ATT CAT CCC ATC GAT G-3′), and then confirmed by restriction digestion and subcloned into expression vector pcDNA3.1(+). Both $KOR_{184}$ and $KOR_{196}$ sequences were subsequently verified by sequence analysis. NTSR1–Rluc, NTSR1–eCFP, $KOR_{WT}$–Venus (Venus-tagged wild-type KOR), $KOR_{184}$–Venus, $KOR_{196}$–Venus, $G\alpha_{i2}$–Rluc, β-arrestin-1–Rluc, and β-arrestin-2–Rluc were constructed as previously described [34,35], encoding C-terminal *Renilla* luciferase (Rluc), enhanced cyan fluorescent protein (eCFP), or mutant yellow fluorescent protein (Venus), as indicated, except for Rluc–$G\alpha_{i2}$ where Rluc was inserted between residues 91 and 92. To generate Myc-tagged KORs, sequences encoding the Myc epitope tag (EQKLISEEDL) were PCR-inserted at the N-terminus of KORs. All reconstructed plasmids were verified by commercial DNA sequencing.

### 2.3. Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 10% heat inactivated FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate. The cultures were maintained in a humidified incubator at 37 °C with 5% $CO_2$. One day prior to transfection, confluent cell cultures were washed with phosphate-buffered saline (PBS) and subcultured using 0.05% trypsin/0.53 mM EDTA. Confluent cells (50–70%) were selected for transient transfection the following day. Transient transfection [34,36] was carried out using Lipofectamine 2000 reagent