

POST-TRANSCRIPTIONAL REGULATION OF OPIOID RECEPTORS IN THE NERVOUS SYSTEM

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1. ABSTRACT

Three types of opioid receptors exist in the animals, each is encoded by a single gene, i.e., the mu opioid receptor gene, the delta opioid receptor gene, and the kappa opioid receptor gene. However, each opioid receptor gene produces multiple mRNA variants as a result of alternative promoter usages, splicing and/or polyadenylation. As such, a large reservoir of regulatory events has evolved for the control of the production of mRNA variants or differentially modified proteins from each opioid receptor gene. This review focuses on post-transcriptional events for the regulation of opioid receptor expression or activities, including alternative splicing, mRNA stability, translation, RNA polyadenylation, RNA transport, and covalent modification of the receptors. Variation at the mRNA level contributes, primarily, to the control of spatial and temporal expression of these receptors in different parts of neurons; whereas modification at the receptors is the key for controlling the duration and amplitude of signals generated from these receptors.

2. INTRODUCTION TO OPIOID RECEPTORS AND THEIR GENES

2.1. Opioid receptor as a member of G protein-coupled receptor

The initial demonstration of stereoselective binding sites for opioid ligands on brain membrane preparations suggested the presence of opioid receptors on cell membrane (1-3). Subsequently, three types of opioid receptors were found according to their selectivity toward specific ligands (4, 5) and cross-tolerance studies (6, 7). However, it was not until almost 15 years later that the actual molecular identity of opioid receptors was revealed with the cloning of the cDNA encoding the δ -opioid receptor (DOR) (8, 9). Within a short period of time, both the mu- (MOR) and the kappa- (KOR) opioid receptor cDNAs were also successfully obtained (10-14). From sequence comparison, it was concluded that all three opioid receptors belong to the superfamily of G protein-coupled receptors (GPCRs) that are a collection of membrane receptor proteins that mediate diverse signals of many hormones and neurotransmitters (15).

GPCRs consist of ~1,000 to 2,000 members comprising ~ 1% of the human genome. These GPCRs are classified by the GRAFS system into five main families: Glutamate, Rhodopsin, Adhesion, Frizzled/taste 2, and Secretin. Based on the chromosomal position of the genes and the “fingerprint” motifs within these families, GPCRs within the GRAFS family have been shown to evolve from the same ancestral gene through gene duplication and exon shuffling (16, 17). Accordingly, opioid receptors belong to the rhodopsin subfamily of these GPCRs. In regardless of which family these receptors belong to, these diverse genes products all have one feature in common, i.e., they serve as GDP/GTP exchange proteins. The existing “dogma” for GPCR signal transduction involves the transient formation of the receptor-G protein ternary complexes and subsequent breakdown of these complexes in the transmission of signals across the membrane (18-20). The activation of the GPCRs by agonists promote the dissociation of GDP and association of GTP on the G α subunits of the heterotrimeric G proteins, resulting in the dissociation of the heterotrimers into two independent signaling molecules, the GTP bound G-alpha, and the beta/gamma subunits. The endogenous GTPases in the G-alpha subunits will hydrolyze the bound GTP thus terminating the signals by the reassociation of the G-alpha and beta/gamma subunits. Due to the complexity of the G proteins heterotrimers compositions: 20 different mammalian G α subunits, 5 β and 12 γ subunits (21), the diversity in GPCR signal transduction processes can be attributed to the ability of the receptors to activate specific G protein. Reported studies suggest that opioid receptors transduce their signals by activating specific G proteins.

From the cDNAs of the MOR, DOR and KOR, these putative 7 transmembrane receptors are about 60% identical to each other, with the greatest identity found in the transmembrane domains (73-76%) and intracellular loops (86-100%). The greatest divergent areas were found in the N-terminus (9-10%), extracellular loops (14-72%) and the C-terminus (14-20%) (22). Thus, it was not surprising that these opioid receptors could regulate the same spectrum of second messenger systems. Activation of the cloned opioid receptors have resulted in the inhibition of adenylyl cyclase activity (23), in the increase in phospholipase C activity and in the transient increase in the intracellular Ca²⁺ level (24, 25), in the activation inward rectifying K⁺ channels (Kir) (26), in the inhibition of both the N-type (27) and L-type (28) Ca²⁺ channels, and in the activation of the mitogen-activated protein kinases Erk-1/2 (29, 30). There appears to be some G protein specificity in the opioid receptor functions. Studies with G α -specific antibodies suggested that G μ_2 mediates the DOR inhibition of adenylyl cyclase activity in NG108-15 cells (31) while G μ_o mediates the MOR inhibition of the adenylyl cyclase activity in SHSY5Y cells and brain membrane (32). The G μ_o proteins mediating DOR induced inhibition of Ca²⁺ channels has been demonstrated over a decade ago (33) and was later confirmed by the use of G- α_{μ_o} -specific antiserum (34). Thus, the activation of specific G proteins could mediate the opioid receptor signaling.

2.2. Scaffolding of GPCR and the opioid receptor

However, such one-dimensional signal transmission does not appear to hold true for GPCRs and the opioid receptors. In order to explain the observations that different GPCRs activate the same G proteins but different effectors, as in the case of MOR utilizing G μ_o to regulate the activities of adenylyl cyclase and Ca²⁺ channels, the concept of uneven distribution has been proposed (35-38), i.e., the compartmentation of GPCR and its effectors in specific microdomains of the plasma membrane. An excellent example of such microdomains is the caveolae, a caveolin-rich membrane structure that is shown to contain many of the proteins involved in GPCR signaling, e.g., G proteins, adenylyl cyclase, phospholipases, adenylyl cyclase, nitric oxide synthase, PKC- α , and various components of MAP Kinase cascade such as Ras, Raf and Src (39-48). Opioid receptor has been demonstrated to be distributed evenly into membrane caveolae-like microdomains (49). In these microdomains and in other compartments, opioid receptor must associate with proteins other than the α - and β /gamma-subunits of the G proteins. Hence, the signaling of the opioid receptor is not a one-dimensional event, and the proteins scaffold to the receptor could modulate the magnitude and duration of the receptor signaling processes.

Protein scaffolding could occur by the recognition of specific protein sequence motifs such as the PDZ domains. Several GPCRs have been reported to interact with various PDZ domains containing proteins via the specific sequence motifs at the carboxyl tails. The β_1 -adrenergic receptor associates with the post-synaptic density protein 95 (PSD-95) (50, 51) and membrane-associate guanylate kinase-like protein inverted-2 (MAGI-2) (51). The interaction with PSD-95 physically linked the β_1 -adrenergic receptor with the N-methyl-D-aspartate (NMDA) class of glutamate receptor channels, which activities are known to be regulated by β_1 -adrenergic stimulation in neurons (52-54). Opioid receptors do not contain specific sequence motifs that are known to participate in the protein-protein interaction. However, the receptor could be modified covalently to promote cellular proteins interactions with the receptor. One excellent example is the recruitment of β -arrestin molecules to the receptor vicinity by the phosphorylation of the receptor.

2.3. Multiple mRNA variants of opioid receptors

Besides protein modification, a number of potential regulatory events at the level of RNA were found. Immediately following the cloning of cDNA of each opioid receptor, their genes were isolated from various animal species and the genomic structures were determined (for review see ref. 55). It appears that each opioid receptor is encoded by a single gene. Comparison of their genomic structures revealed highly conserved positions of exon-intron junctions as well as the number of exons in the coding region. While pharmacologically, multiple subtypes of opioid receptors were detected in the animals, the attempt to identify splicing variants of receptors that could potentially account for the pharmacologically defined receptor subtypes was not successful. However, multiple

splicing variants of either the 5'- or the 3'-untranslated region (UTR), in particular for the MOR and the KOR, were detected in various animal species, and the protein coding region of each opioid receptor gene appeared to reside in three highly conserved exons (55). Further, the untranslated sequences of their mRNA, in either the 5'- or the 3'-ends, was found to be subjected to regulation at the level of mRNA splicing/processing and could play a role in differential transport of the mRNA in neurons, such as in the case of the KOR gene (56-58). Interestingly, the 3'-splicing variants of the MOR gene seemed to play a role in modulating mu δ opioid analgesic property (59).

Therefore, while each opioid receptor gene produces only one type of protein, or receptor, a large reservoir of regulatory events has evolved for the control of the production of opioid receptor mRNA variants or differentially modified proteins. This review focuses on these post-transcriptional events that could be involved in the regulation of opioid receptor expression or activities, including alternative splicing, mRNA stability, translation, RNA polyadenylation, RNA transport, and covalent modification of the receptors.

3. ALTERNATIVE mRNA SPLICING OF OPIOID RECEPTOR GENES AND THE REGULATION

3.1. Opioid receptor genes splicing variants

3.1.1. MOR

Extensive alternative splicing at the 5'- and 3'-ends was found for the mouse MOR gene that generated at least 14 alternatively splicing variants. Most of these splicing variants encode an identical receptor protein and if differ, only at the C-terminus of the proteins. Therefore, all these reported MOR variants are specific to mu opioids (60-62). These variable sequences appeared to be involved in modulating mu δ opioid analgesic property (59, 63). Since all these variants were identified from reverse transcription-coupled PCR rather than full length cDNA cloning, the naturally produced mRNA or protein variants remain to be substantiated. Alternative splicing of the MOR gene have also been reported for the 3'-end of the rat gene. For instance, rMOR1B utilized a novel exon 5 and differed in agonist-induced desensitization when it was expressed as a recombinant protein in cultures (64). Three alternative splicing variants for the human gene were also reported, including one that retained intron 3 and was truncated at the C-terminus (MOR1A) (65), one that was detected in SJON-SH cells (66) and one that utilized a novel down stream exon X (MOR1O) located between original exons 3 and 4 (MOR-1X) (67).

The detection of MOR splicing variants with RT-PCR has initially generated a tremendous amount of enthusiasm because it might provide a molecular explanation for the pharmacologically defined MOR subtypes. However, since no nature proteins or mRNA species for these reported MOR variants have been substantiated with a more reliable method, it remains to be confirmed whether any of these variants indeed constitute the various MOR subtypes.

3.1.2. DOR

Few studies have reported splicing variants of DOR. Only one study reported the detection of DOR splicing variants in mouse brain (68). This reported variant contains a 243 bp insertion at the splicing junction between exons 1 and 2, and generates an in-frame stop codon that would lead to the production of truncated receptor. However, the existence of this type of truncated receptor in nature also remains to be substantiated.

3.1.3. KOR

Splicing variants of KOR gene have been reported in different animals species. In mouse tissues, we have identified at least three KOR mRNA splicing variants generated from alternative splicing in the 5'-end of the transcript and from alternative promoter usage. Two functional KOR promoters are present (69). The first promoter directs the synthesis of two variants that differ in the junction of exon 1 and exon 2, generating an alternative mRNA species with a 30 bp insertion in the 5'-UTR. We have named the most predominant KOR species "variant A", or the wild type (wt). The alternatively spliced variant with the 30 bp insert was called "variant B". In addition, a second functional promoter is located within intron 1, and directs the synthesis of the third alternative mRNA species that differed also in the 5'-UTR, and was named "variant C" (56).

Recently, we have identified two functional polyadenylation (PA) sites for the mouse KOR gene, each appeared to be used by all three previously defined 5'-alternatively splicing variants A, B and C (57). This would suggest another level of post-transcriptional control for the production of KOR mRNA by using alternative polyadenylation signals. The first functional PA is located immediately down stream from the termination codon, generating mature KOR mRNA of approximately 1.6 kb in size. The second functional PA is located approximately 2 kb down stream of the termination codon, generating mature KOR mRNA of approximately 3.8 kb in size. The fact that both PA signals can be utilized by transcripts initiated from either promoter 1 or promoter 2 agrees with the detection of two major groups of KOR mRNA in the size of approximately 4 kb (transcripts utilizing PA2) and 2 kb (transcripts utilizing PA1) detected on the Northern blot. As such, at least 6 types of mature KOR mRNA can be generated from the mouse KOR gene, in the combination of A type 5'-end with poly(A) 1 (A-PA1), A type 5'-end with poly(A)2 (A-PA2), B type 5'-end with poly(A) 1 (B-PA1), B type 5'-end with poly(A)2 (B-PA2), C type 5'-end with poly(A)1 (C-PA1) and C type 5'-end with poly(A)2 (C-PA2).

A KOR splicing variant with differences at the junction of exons 2 and 3 was detected in mouse T lymphocytes, which could generate an in-frame stop codon that would lead to the production of a truncated receptor (68). A potential alternative promoter in intron 1 was also reported for the rat KOR gene, that directed the synthesis of a transcript species starting from this intron. It was suggested that a KOR protein with additional 5'-sequence could be synthesized from the putatively alternative

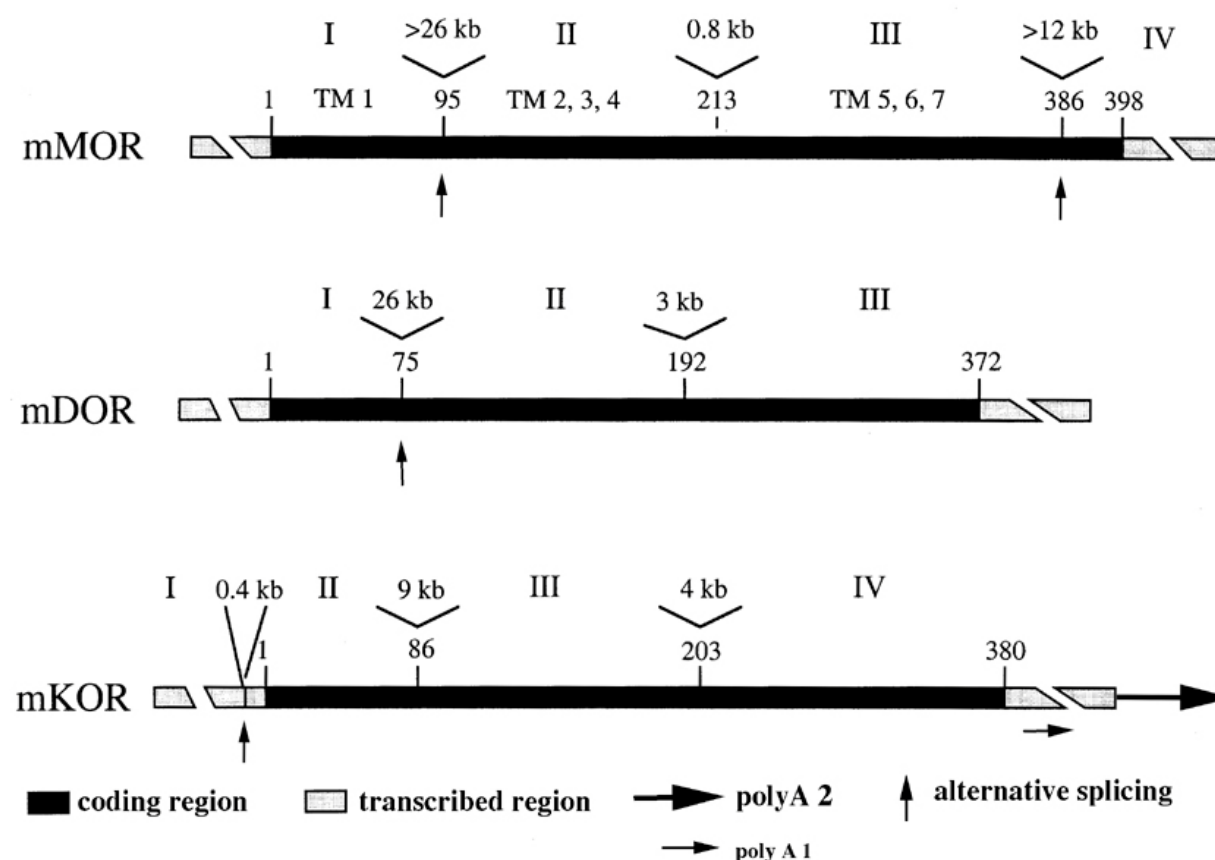


Figure 1. A summary of genomic structures, functional promoters, splicing sites and polyadenylation signals of mouse opioid receptors MOR, DOR and KOR. The major exons of each receptor were numbered I - IV with approximate sizes of introns and the amino acid residues where splicing occur denoted above the map. All three opioid receptor genes are alternatively spliced at intron 1 as indicated with an arrow under intron 1 of each receptor map. A potential alternative splicing event was also found to occur at intron 3 of MOR. Other alternative splicing events demonstrated only with RT-PCR were not indicated because of its speculative nature. Two functional polyadenylation sites were found for the KOR and indicated with horizontal arrows at the end of KOR. Filled boxes represent the coding regions of each receptor and grey boxes represent the untranslated regions. TM: transmembrane regions.

translation codon located within intron 1 (70). However, production of this type of KOR protein in nature remains to be substantiated.

Apparently, numerous mRNA variants could be produced from each opioid receptor gene, providing a rich reservoir for regulating either the amount of receptors produced or the distribution of these receptors. The three opioid receptor gene structures, their major splicing sites, and the known functional promoters and polyadenylation sites of the KOR are summarized (Figure 1).

3.2. Regulatory events at the level of RNA

While all three opioid receptor genes were found to encode alternatively spliced mRNA species, only the KOR gene was examined in terms of the differential expression of, and potential regulatory events for, its mRNA variants. From studying both animal tissues and cultured cells, it was found that KOR mRNA variants were expressed at different levels in different animal tissues, as

well as in differentiating cells treated with differentiation agent retinoic acid (RA) (71). Further, these mRNA could be subjected to regulatory events that affected their steady state level of expression or cellular distribution. These include their spatial and temporal specificity, mRNA stability, mRNA splicing efficiency, polyadenylation, translation and RNA transport (Table 1). With the exception of mRNA splicing efficiency, all the other RNA-related regulatory events for the mouse KOR gene have been examined and will be discussed in the following.

3.2.1. Specificity of the expression of KOR mRNA variants

In our initial study we first reported differential expression of the three KOR mRNA variants, A, B and C in animal tissues (56). Variant A was the predominant KOR species and expressed most widely, whereas variants B and C were expressed only in the nervous tissues such as hypothalamus, brain stem, cortex and spinal cord. Further, the relative expression level of each variant changed as

Table 1. Mouse opioid receptor regulatory mechanisms involving RNA

Post-transcriptional Regulatory mechanisms	MOR	DOR	KOR
Alternative splicing	introns 1 and 3	intron 1	intron 1
RNA stability	N.A.	N.A.	8hrs vs. 12 hrs
Alternative poly (A)	N.A.	N.A.	2
Translation efficiency	N.A.	N.A.	1-3x difference
Polyadenylation	N.A.	N.A.	two sites
RNA transport	N.A.	N.A.	5'- and 3'-UTRs
Variants reported	14	2	4

animals developed. Although both variants A and B were expressed in developing embryos as early as gestation stage day 9, variant C was not detected until post-natal stages. After birth, the expression of all three KOR mRNA species was elevated, particularly in the central nervous system. Therefore, it is apparent that the three 5'-alternatively spliced KOR mRNA species are each expressed in a spatially and temporally specific manner.

In an attempt to determine whether the expression of KOR mRNA variants responded differentially to pharmacological or hormonal manipulation, the expression pattern of these KOR variants was examined in diet-manipulated animals and cultured cells. It was first found that fetuses of female animals that were rendered hypovitaminosis A expressed higher level of all three KOR mRNA variants (71). Consistently, in an embryonal carcinoma cell line P19 where KOR was constitutively expressed, RA, the most potent ingredient of vitamin A, suppressed the expression of all three KOR mRNA variants, with variant C being most sensitive to RA treatment. Further, while RA-suppression of both variants A and B could be reversed by a protein synthesis inhibitor, cycloheximide, RA suppression of variant C could only be reversed partially by the same drug in the same culture, indicating complication at the regulation of KOR variant C. The RA-mediated suppression of KOR expression was found to be mediated through a negative transcription factor, Ikaros, which was induced by RA in P19 cells (72). The result of full recovery of RA-suppression of variants A and B by cycloheximide treatment would suggest that RA-suppression was most likely at the time of transcription when induction of Ikaros by RA could be blocked by protein synthesis inhibitor. However, variant C, that was initiated from intron 1, could be regulated by RA through a combination of transcriptional and post-transcriptional events. We are in the process of identifying post-transcriptional events that specifically regulate RA suppression of KOR gene, in particular for variant C.

In a mechanical allodynia animal pain model, it was found that total KOR expression was significantly reduced in the contralateral dorsal root ganglia relative to the side of nerve injury only in animals that experienced pain (73). In contrast, variants B and C were not significantly affected, suggesting variant A to be correlated with nerve injury-induced pain in animals. This result would indicate that the feeling of pain could regulate the expression level of a specific KOR mRNA variant. The regulatory event remains to be elucidated.

3.2.2. mRNA stability

To determine the factors contributing to difference in the steady state level of the three KOR mRNA

species, we first examined the half-life of the three KOR mRNA species. In the *in vitro* culture model P19, it was found that variant A was most stable and exhibited a half-life of 12 hrs. The half-life of variant B was 8 hrs under the same condition. This was consistent with the more abundant steady state expression of variant A than B, although both variants were initiated from the same promoter (56). It was interesting that variant A differed from B only at an insertion of 30 nucleotides in the 5'-UTR. The structural basis of this difference in mRNA stability remains to be determined.

3.2.3. Regulation at protein translation

The finding that KOR variants A, B and C differ only in the 5'-UTR but not the coding region prompted us to examine the translation efficiency of each variant. Using both *in vitro* translation and *in vivo* reporter systems, it was concluded that variant A was least efficient in translation, approximately 2 folds lower than that of variants B and C. Variants B and C were similar in translation efficiency, agreeing with the fact that both variants share an identical 5'-upstream region for more than 40 nucleotides long (56).

3.2.4. Regulation at polyadenylation (PA)

As summarized earlier, the mouse KOR gene can use two functional PA sites approximately 2 kb apart (57). Further, both PA sites were functional in both neuron tissues and cultured P19 cells, but they were differentially regulated by RA treatment. The difference in RA sensitivity (suppression) was partially due to the presence of a negative regulatory sequence adjacent to PA1 and/or an enhancer adjacent to PA2. In addition, the stability of KOR mRNA using PA2 was significantly greater than that using PA1, adding a further complicated control over differential expression of KOR mRNA species via the use of alternative PA signals.

3.2.5. Regulation at RNA transport in neurons (58)

Initially, we documented an interesting phenomenon that KOR mRNA variants were differentially expressed in a variety of animal tissues. Since the majority of KOR expression resided in the nervous system, we then examined whether these mRNAs were present universally in different parts of neurons. Interestingly, in both primary neuron cultures and *in vitro* differentiated neurons derived from RA-treated P19 stem cells, the three KOR mRNA species were found to exhibit very different distribution patterns. For instance, in trigeminal nerves where axons could be easily separated from the somas, it was found that variant A was evenly distributed in both the axons and the somas, whereas variants B and C were present mostly in the soma. In *in vitro* differentiated P19 neuron cultures

where somas and neuron processes were grown on different layers and could be separated from each other, variant A was also found to be evenly distributed in both the soma and processes, but variants B and C were preferentially detected in the soma.

The three KOR 5'-splicing mRNA variants were then examined to identify molecular signals that might govern their differential distribution in neurons. To address this issue, a phage RNA-binding protein motif MS2 was fused to a nuclear green fluorescent protein to serve as a tracer, and the MS2-binding RNA sequence was fused to each of the three KOR mRNA variants. Thus, the distribution of each KOR mRNA variant tagged with a MS2-binding site could be traced by following the signal of MS2-GFP which would otherwise be present only in the nuclei. When MS2-GFP bound to the MS2-KOR mRNA, it became extra-nuclear. Therefore, extra-nuclear GFP would indicate the transport of MS2-KOR mRNA that recruited the MS2-GFP, and the pattern of GFP signals would reflect the cellular distribution pattern of a particular KOR mRNA variant that has been tagged with the MS2-binding site (58). By using this approach, we have demonstrated that KOR variant A was most efficient in mobilizing GFP to P19 neuronal processes, followed by variant B and then variant C. Studies of mRNA transport to neuronal processes have been reported mostly for the lower animal species, and transport of mRNA to the axons of sensory neurons was reported only for several structural proteins. Therefore, the demonstration of KOR mRNA transport to neuronal processes including the axons of sensory neurons, mediated by the un-translated region of these KOR mRNA sequences would implicate potential regulatory mechanisms for differential transport of mature KOR mRNAs in neurons. More importantly, this regulation seemed also effective for KOR mRNA transport into the axons, a compartment thought to rely only on cargo transport of preformed protein complexes for protein supplies. It would be an important task and a huge challenge to determine the pharmacological and physiological meaning of differential distribution of KOR mRNA variants in different neuronal compartments. Currently, we are in the process of identifying signals that trigger differential transport of these KOR mRNA species in neurons.

4. COVALENT MODIFICATION OF OPIOID RECEPTORS

4.1. Phosphorylation of the receptor and its consequences

4.1.1. Demonstration of opioid receptor phosphorylation

Phosphorylation of GPCR as the trigger for cellular control of the receptor activity has been proposed by Lefkowitz and his co-workers using β_2 -adrenergic receptor as the model (74). In this model, agonist binding to the receptor results in the rapid phosphorylation of the receptor by protein kinases including the G protein-coupled receptor kinases (GRKs), thereby promoting the association of beta-arrestin. Association of beta-arrestin with the receptor uncoupled the receptor from the respective G protein that transduces the signal and thus blunted the

receptor signaling (receptor desensitization). Beta-arrestin also is involved in the agonist-induced, clathrin-coated vesicles-mediated receptor internalization. Within this endocytotic pathway, the agonist-induced receptor internalization is the initial step of receptor trafficking to other subcellular compartment such as lysosomes where receptor degradation occurs. Subsequently, there is a decrease or down-regulation of the overall cellular receptor content. Beta-Arrestin itself also serves as an adapter molecule in the β_2 -adrenergic receptor signaling such that a receptor-src kinase complex is formed through which activation of the MAP kinases Erk1/2 by the β_2 -adrenergic receptor is accomplished (75).

Phosphorylation of opioid receptor has been reported long before the cloning of the receptors. Several reports have indicated that the partially purified MOR, a 58 kDa protein, isolated from either mouse brain (76) or from the human neuroblastoma cells SK-N-SH (77), was tyrosine phosphorylated during morphine treatment. However, concrete demonstration of opioid receptor phosphorylation was first demonstrated by Pei *et al.* (78) with the DOR and by Arden *et al.* (79) with the MOR. Subsequently, agonist-induced phosphorylation of the KOR was also reported (80). Either by metabolically labeling of the ATP pools with ^{32}P or with ^{32}P -ATP, immunoprecipitation either with the monoclonal antibodies to an epitope at the N-terminus of the receptor or with the polyclonal receptor-specific antibodies revealed a rapid, agonist-dependent phosphorylation of the receptor protein. Studies with the DOR (78) or MOR (81, 82) suggested the phosphorylation of the opioid receptor is mediated via GRKs and not by the protein kinase C. Predictably, the ability of opioid ligand to induce receptor phosphorylation correlated with its efficacy (83). With the exception of morphine, agonists such as DAMGO or etorphine all were reported to induce MOR phosphorylation. Wang and co-workers reported that morphine could induce MOR phosphorylation in CHO cells, while Arden *et al.* (79) and Zhang *et al.* (84) reported morphine could not induce receptor phosphorylation in HEK293 cells. The fact that over-expression of GRK-2 in HEK293 cells resulted in the morphine-induced phosphorylation of the MOR (84) suggests that the morphine-receptor complex is a poor substrate for the GRKs. Thus, the discrepancy in the ability of morphine to induce receptor phosphorylation could be due to the differences in the level of protein kinases in the CHO and HEK293 cell lines. The difference between the morphine-receptor complex and other agonist-receptor complex was further illustrated by the ability of *in vitro* PKA catalytic subunit to phosphorylate the morphine-receptor complex but not the DAMGO-receptor complex (85). Such findings support the observations with the receptor chimeras in which the receptor domains involved in the recognition of morphine and DAMGO appeared to be different.

4.1.2. Identification of the phosphorylation sites

Initial experiments with receptor sequence deletion analyses have suggested that the carboxyl tail of the opioid receptor is the site for agonist-induced receptor phosphorylation (86, 87). Subsequent systematic mutations

of the Ser/Thr residues within the carboxyl tail sequences have identified the amino residues that are being phosphorylated. For DOR, studies from two different laboratories independently identified that Thr³⁵⁸ and Ser³⁶³ residues within the carboxyl tail motif were phosphorylated in the presence of agonist (88). Further, the agonist-induced phosphorylation was hierarchical. Phosphorylation of Ser³⁶³ must occur prior to the phosphorylation of Thr³⁵⁸ residue when the DOR was expressed in HEK293 cells (88). As for KOR, Ser³⁶⁹ of the rat receptor was identified to be the site by phosphorylated-receptor specific antibodies (89). The actual demonstration of ³²P incorporated into this amino acid residue was not reported.

However, for the MOR, the identity of the amino acid residues being phosphorylated in the presence of agonist has been controversial. Pak *et al.* reported the mutation of Thr³⁹⁴ to Ala within the MOR sequence resulted in the blunting of the agonist-induced receptor desensitization (90). Subsequently Deng *et al.* (91) reported that Thr³⁹⁴ was indeed phosphorylated in the presence of agonist by GRKs. However, using similar mutational analyses approach, El-Kouhen *et al.* reported that Thr³⁹⁴ was not phosphorylated when the mutant receptor was expressed in HEK293 cells. Instead, two residues within the carboxyl tail motif of the MOR, Thr³⁷⁰ and Ser³⁷⁵ were phosphorylated in the presence of agonist (92). Although the amino sequences between the carboxyl motifs of MOR and DOR are dissimilar, there appears to be a consensus motif recognized by protein kinases involved in the phosphorylation of the opioid receptor. For both MOR and DOR expressed in HEK293 cells, there is a Pro residue immediately upstream from the Ser residue being phosphorylated. The phosphorylated Thr residue is 5 amino acid residues upstream from the Ser residue, or ~ 2 alpha-helical turns. Thus, the consensus agonist-induced phosphorylation motif for the opioid receptor is defined by the sequence T-X-X-X-P-S, where X is any amino acids. Thr³⁹⁴ located at the carboxyl terminal of the MOR lies outside of such consensus motif.

4.1.3. Identities of protein kinases involved in receptor phosphorylation

The protein kinases participate in the agonist induced receptor phosphorylation most likely are members of GRKs. Expression of the dominant negative mutant of GRK or over expression of GRK5 resulted in the attenuation or potentiation of agonist-dependent phosphorylation of DOR (78). Though over expression of GRK2 in HEK293 could potentiate etorphine or morphine induced phosphorylation of the MOR (84), the same over expression of GRK-2 had minimal effect on the DAMGO induced receptor phosphorylation (82). Using purified GRKs and *in vitro* phosphorylation assays, GRK2 could phosphorylate the Ser³⁷⁵ but not the Thr³⁷⁰ residue of the MOR (unpublished observations). Further, the receptor carboxyl tail domain could not be phosphorylated by the purified GRK5. Hence, there is a possibility that other protein kinases are involved in the phosphorylation of the receptor. The candidates are Ca²⁺/calmodulin-dependent protein kinase II, PKA and the Erk-1/2. There are some indirect evidences to support the involvement of PKA and

Erk1/2 kinases, such as the ability of the MEK inhibitor: PD98059, or the PI3K inhibitors: wortmannin or LY294002 to block the chronic DAMGO effect (93) or the ability of chronic morphine treatment to blunt the *in vitro* morphine-induced receptor phosphorylation by PKA (85). However, the direct phosphorylation of the receptor by these protein kinases has not been demonstrated *in vivo*. At best, the MEK inhibitors PD98059 or U0126 could attenuate the agonist-induced MOR phosphorylation (94). Since the MOR is a poor *in vitro* substrate for Erk2 as reported in the same study, it is unlikely that these kinases directly phosphorylate the receptor themselves. On the other hand, though the agonist-dependent phosphorylation of the MOR and DOR is not mediated by the phorbol ester stimulated PKC (78, 81, 82) basal phosphorylation of the MOR appears to involve the CaM kinase as indicated by CaM kinase inhibitor studies (95). Koch *et al.* (96) reported by mutating Ser²⁶¹ and Ser²⁶⁶ of the MOR to Ala, two putative consensus sites for CaM kinase II, the increase in rate of receptor desensitization when the CaM kinase II is over expressed can be blocked either in HEK293 cells or in *Xenopus* oocytes. The same authors reported a blunting of the agonist-induced receptor phosphorylation by the mutation of these putative CaM kinase II sites. However, whether these two sites are being phosphorylated by PKC is debatable. Other reports do not support such a conclusion. In both truncation and cyanogens bromide cleavage studies, the agonist-induced phosphorylation sites have been located at the carboxyl tail domain of the receptor (91, 92). Further, Ser³⁴⁴ within the carboxyl tail motif of the DOR has been identified to be the residue being phosphorylated by PKC in the presence of agonist (97).

4.1.4. The sequels of receptor phosphorylation

The consequences for opioid receptor phosphorylation have not been resolved completely. According to the existing model, the receptor phosphorylation will lead to the β -arrestin recruitment and the blunting of the receptor signaling processes. There appears to be a causal relationship between opioid receptor phosphorylation and desensitization. Desensitization of the DOR was reported to correlate with the phosphorylation of the receptor protein in the SK-N-BE cells (98). Over expression of GRK or its dominant negative mutant could modulate the DOR desensitization (78). Mutation of the last 4 Thr and Ser residues at the C-terminus of the DOR to Ala would block the GRK and arrestin mediated desensitization (99). A direct correlation between MOR phosphorylation and desensitization was established by Zhang *et al.* (81) while over expression of beta-arrestin resulted in the rapid morphine-induced receptor desensitization and internalization (100). The *in vivo* tolerance development to morphine antinociceptive response was blunted in the beta-arrestin2 knockout mice (101, 102). This is reflected in the lack of agonist-induced receptor desensitization in the fibroblasts isolated from these animals (101). Recent data with the GRK3 knockout mice suggested that tolerance development to the kappa-opioid agonist was attenuated in these animals (103). Thus, these data supported the model that agonist-induced phosphorylation of the opioid receptor resulted in the

formation of the beta-arrestin-receptor scaffolds and the blunting of the signals.

However, there are reports that do not support phosphorylation of the receptor as the critical step for receptor desensitization, and hence the recruitment of beta-arrestin molecules. The time course of receptor phosphorylation was rapid. It did not correlate with the desensitization of the receptor, which was slow (82). Overexpression of the GRK and arrestin did not increase this slow desensitization process (82). Deletion of the last 31 amino acids of DOR resulted in the abolition of both GRK- or PKC-mediated agonist-dependent phosphorylation of the receptor, but did not block the agonist-induced receptor desensitization (104, 105). The complete mutation of all Ser/Thr residues within the 3^d intracellular loop and the C-terminus of MOR did not prevent the DAMGO-induced receptor desensitization (106). Further, overexpression of beta-arrestin 1 resulted in the blunting of the DOR and KOR but not MOR activity (107), while interaction of beta-arrestin with these receptors could be established (108). Though prolonged morphine treatment could elicit loss of response (109), morphine normally does not induce receptor phosphorylation. These data and others suggest that the beta-arrestin recruitment by opioid receptor does not require the phosphorylation of the receptor. This is not too surprising since agonist-induced phosphorylation of other GPCRs results in the increase in the receptor affinities for the beta-arrestin. Indeed, the hyper-phosphorylation of the receptor in the presence of agonist by over expressing GRK2 resulted in the ability of morphine to induce rapid MOR internalization (84), a beta-arrestin-dependent process that normally does not occur in the presence of morphine.

The ability of opioid receptor to recruit beta-arrestin without being phosphorylated was illustrated best by the receptor truncation studies. By removing the putative agonist-induced phosphorylation sites via the deleting the carboxyl tail sequence of DOR after Ser³⁴⁴ or the MOR after Ser³⁶³ residues, both Whistler *et al.* (110) and Qiu *et al.* (111) could demonstrate agonist-induced receptor endocytosis. Furthermore, by monitoring the beta-arrestin association with the receptor using the arrestin-GFP fusion protein, it is possible to demonstrate the translocation of the beta-arrestin-GFP fusion protein from the cytosol to plasma membrane in the presence of opioid agonist still occurs with the truncation of the MOR carboxyl tail sequence (111) or the mutation of the Ser/Thr residues involved in agonist-induced phosphorylation of the DOR (unpublished observations). Similar observations were reported by monitoring the DOR carboxyl tail interaction with beta-arrestin using BIACORE (112). Thus, the formation of the beta-arrestin-receptor complex does not require phosphorylation of the opioid receptor.

Beta-Arrestin also serves as a scaffolding molecule in the beta₂-adrenergic receptor signaling. A receptor-src kinase complex is formed via the beta-arrestin scaffold, through which activation of the MAP kinases Erk1/2 or the Jun kinases by the beta₂-adrenergic receptor is accomplished (113). Such receptor activation of the

kinases can be blocked by the beta-arrestin dominant negative mutant. The activated MAP kinase can phosphorylate the beta-arrestin and GRK. The phosphorylated forms of these proteins would not interact with the receptors, and thus blunting the desensitization signals (114). Similar beta-arrestin-opioid receptor scaffolding was proposed by Coscia and his co-workers in the receptor activation of Erk1/2. Using dominant negative beta-arrestin mutant, they reported the blunting of the opioid receptor activation of Erk1/2 (115). However, such observation could not be repeated by several laboratories. As a matter of fact, others reported that in regardless of the opioid receptor types, the activation of the Erk1/2 by opioid agonist can be separated from the agonist-induced receptor internalization process (116, 117). The use of dominant negative beta-arrestin mutant did not block the agonist activation of Erk1/2. These MAP kinases could be activated by morphine while the same agonist could not induce internalization of the MOR (118). Thus, probably, the beta-arrestin recruited to the opioid receptor vicinity after receptor phosphorylation does not serve as a scaffolding protein in the agonist-mediated activation of Erk1/2.

4.2. Receptor ubiquitination and its consequences

In addition to phosphorylation, opioid receptor has been reported to be ubiquitinated in the presence of agonist. Ubiquitin molecule, a 76 amino acid polypeptide, is expressed in all eukaryotic cells. The conjugation of this polypeptide to the target proteins by the multi-enzyme cascade involving the E1s, E2s and E3s enzymes has long been known to direct the degradation of cytosolic and nuclear proteins by proteasomes (see review in Ref. 119). Normally, this involves the addition of multi-ubiquitin chains, i.e., the carboxy-termini glycine of ubiquitin is linked to the Lys⁴⁸ of the preceding ubiquitin, to the epsilon-amino group of the lysine residue of the target protein. However, there are accumulating evidences to suggest a role of monoubiquitination in the endocytosis of plasma membrane proteins and their trafficking to the lysosomes (120, 121). In *Saccharomyces cerevisiae*, many of the plasma membrane proteins require ubiquitination in their cytoplasmic domains for their internalizations (122). Similarly, for GPCRs, there is accumulating evidence to suggest receptor ubiquitination participates in the agonist-induced receptor internalization. For GHRs (123, 124), CXCR4 receptor (125) and beta₂-adrenergic receptor (126), inclusion of proteasome inhibitors during chronic agonist treatment could prevent the down-regulation of these receptors. The monoubiquitination process appears to participate in the endosomal sorting of the receptor, preventing the recycling of the proteins and shuttling the molecules to the multivesicular bodies of the late endosomes and degradation in the lysosome. In addition to directing the lysosomal trafficking, ubiquitination of *trans*-acting endocytic protein(s) could also affect the agonist-induced receptor internalization. In the case of beta₂-adrenergic receptor, the ubiquitination of beta-arrestin, which also serves as the adaptor molecule for the E3 ligase, is essential for the endocytosis of the receptor (126). In addition, the ubiquitinated beta-arrestin molecules could alter the dissociation of the receptor with the arrestin

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molecules, and the subsequent cellular trafficking of the receptor (127). The ubiquitination of proteins such as beta-arrestin could result in the formation of multimers with other ubiquitinated proteins such as the receptor in the assembling of the complex that is needed for the budding of endocytic vesicles (120). Such complexes could represent a dynamic control of the cellular trafficking of the receptor.

Thus, the regulation of opioid receptor trafficking by agonist could involve the ubiquitination of the receptor. Ubiquitination of the opioid receptor has been reported. Petaja-Repo *et al.* reported that >50% of the newly synthesized DOR in HEK293S cells were retained within the endoplasmic reticulum, and these receptors, probably misfolded, were deglycosylated and ubiquitinated for proteasome degradation (128, 129). The same authors demonstrated subsequently that these receptors could be rescued with lipophilic opioid ligands that serve as chaperone for the receptor trafficking to the plasma membrane (130). Chaturvedi *et al.* (131) reported that agonist-induced MOR and DOR down-regulation was not affected by inhibitors of lysosomal proteolytic enzymes, but was attenuated by the inhibitors of proteasome inhibitors. Though these results are in disagreement with the confocal microscopy studies indicating the colocalization of the opioid receptor with lysosomal markers (132, 133), the ability of proteasome inhibitors to affect down-regulation of the receptor suggests ubiquitination of the opioid receptor might be involved in directing the receptor trafficking. However, by mutating all the 8 cytosolic facing Lys residues in DOR, Tanowitz and von Zastrow reported the agonist-induced receptor endocytosis rate and degradation rate were not altered in HEK293 cells (134). Such studies suggested that ubiquitination of the opioid receptors was not required in the endocytosis and sorting of the receptors. Whether ubiquitination of opioid receptor participates in cellular regulation of the opioid receptor trafficking needs to be investigated further.

4.3. Receptor glycosylation and its consequences

In addition to phosphorylation and ubiquitination, other covalent modification of the receptor might participate in the scaffolding of opioid receptor with other cellular proteins. One such covalent modification of the receptor is glycosylation of the receptor molecule. Opioid receptors have been identified from the start as a glycoprotein. Though the cloned sequences predicted molecular masses of these receptors to be 39 to 41 KDa, the observed M.W. of these opioid receptors as determined by SDS-PAGE analyses have varied from 55 KDa for δ -opioid receptor to 70 KDa for the MOR. This could be attributed to the differences in the number of Asn residues at the N-terminal extracellular sequence as putative glycosylation sites, with DOR having 2, MOR having 5, and KOR having 3 such residues. The significance of the glycosylation of these Asn residues is not clear. The extracellular transport of the opioid receptor does not appear to require glycosylation, as demonstrated by the glycosylation inhibitor studies and mutational analyses (135, 136). In particular, a N-terminus truncated MOR exhibited normal cell surface expression with no detectable differences in

ligands' affinities and selectivities (137, 138). Only in an isolated report on the polymorphism of the human MOR, the A118G single nucleotide polymorphism had resulted in a N40D mutation that led to an increase in beta-endorphin binding affinity and potency as well as an increased activation of G protein coupled-potassium channels in AV-12 cells and *Xenopus* oocytes (139). However, a later study by Befort *et al.* (140) did not report any change in both the function and the affinity of beta-endorphin for this N40D mutation when this receptor was expressed in cos1 cells. Hence, whether glycosylation of the opioid receptor could modulate the function of the receptor remains debatable.

5. PERSPECTIVE

While each opioid receptor is encoded by a single gene, multiple mRNA variants can be transcribed from each gene as a result of alternative splicing and promoter usage. However, variation in these mRNA sequences occurs, primarily, in the 5'- or the 3'-UTR and only one type of receptor molecule is produced from each gene. Attempt to identify receptor "subtypes" produced from different mRNA variants of each gene has been largely unsuccessful. Therefore, the molecular identity of pharmacologically defined opioid receptor subtypes remains to be determined. Recently, the discovery of differential distribution of mRNA variants, such as that of MOR and KOR, in animal tissues and cell cultures, and the demonstration of heterodimeric receptors in transfected cells provide ample opportunities for the examination of opioid receptor subtypes in the future.

Opioid receptor expression can be regulated by multiple mechanisms, including transcriptional and post-transcriptional events. Post-transcriptional regulation occurs at the level of mRNA or protein, such as alternative splicing, alternative polyadenylation, varied mRNA stability, different translation efficiency, mRNA transport, and complicated covalent modification of receptor molecules. While these mRNA variants were detected in both cultured cells and freshly isolated animal tissues, most of these regulatory mechanisms were demonstrated using cultured cells. Thus it remains to be established whether these regulatory events occur in the context of animal physiology. Further, a pharmacological relevance of these regulatory processes, particularly in the whole animals, awaits further investigation.

Opioid receptors, similar to other GPCRs, are being covalently modified resulting in alteration in receptor function. In the past, covalent modification of GPCR such as receptor phosphorylation has been considered to be the cellular mechanism in the termination of receptor signals. However, it is clear that covalent modification of GPCRs could lead the recruitment of other cellular proteins. The scaffolding of signaling molecules and receptor compartmentation are being considered to be the norm for the signaling of the GPCRs. The signaling of the GPCRs will depend on the identities of the molecules present within the complex. Opioid receptor is not an exception. The probable compartmentation and scaffolding of the receptor is best illustrated by the uneven distribution of the

receptor in caveolae (49). The probable receptor ubiquitinylation might not influence the intracellular receptor trafficking as demonstrated by the Lys mutation studies by Tanowitz and von Zastrow (134). However, protein ubiquitination is known to enhance protein-protein interaction. The ubiquitin on opioid receptor could serve as recognition site for proteins that by their presence in the receptor vicinity could alter the opioid receptor function. Phosphorylation of the receptor is known to recruit β -arrestin molecules. Although thus far the presence of β -arrestin only blunted the opioid receptor signals, this molecule has been shown with other GPCRs to be a scaffolding molecule in their signaling. Whether β -arrestin would have similar activity in the opioid receptor remains to be demonstrated. Nevertheless, the unequivocal covalent modification of the opioid receptor could function as the key for generating a dynamic receptor complex that both the duration and amplitude of the signals would depend on the proteins present within the scaffold.

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Abbreviations: GPCR, G-protein coupled receptor; MOR, mu opioid receptor; DOR, delta opioid receptor; KOR, kappa opioid receptor

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