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Disease-Specific Heteromerization of G-Protein-Coupled Receptors That Target Drugs of Abuse

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Abstract

Drugs of abuse such as morphine or marijuana exert their effects through the activation of G-protein-coupled receptors (GPCRs), the opioid and cannabinoid receptors, respectively. Moreover, interactions between either of these receptors have been shown to be involved in the rewarding effects of drugs of abuse. Recent advances in the field, using a variety of approaches, have demonstrated that many GPCRs, including opioid, cannabinoid, and dopamine receptors, can form associations between different receptor subtypes or with other GPCRs to form heteromeric complexes. The formation of these complexes, in turn, leads to the modulation of the properties of individual protomers. The development of tools that can selectively disrupt GPCR heteromers as well as monoclonal antibodies that can selectively block signaling by specific heteromer pairs has indicated that heteromers involving opioid, cannabinoid, or dopamine receptors may play a role in various disease states. In this review, we describe evidence for opioid, cannabinoid, and dopamine receptor heteromerization and the potential role of GPCR heteromers in pathophysiological conditions.

1. INTRODUCTION

Morphine, an alkaloid extracted from the poppy *Papaver somniferum*, and Δ^9-tetrahydrocannabinol (also known as THC or more commonly as marijuana), the main psychoactive extract from *Cannabis sativum*, are commonly abused drugs due to their mood-altering effects. Morphine is the most commonly used drug in the treatment of chronic pain, while THC is used in the alleviation of neuropathic pain and for the reduction of eye pressure in glaucoma patients.[1–5] However, long-term use of these drugs leads to a number of undesirable side effects, including the development of tolerance and addiction. In the latter context, several lines of evidence demonstrate the involvement of the dopaminergic system in the rewarding effects of drugs of abuse (reviewed in Ref. 6). Both morphine and THC exert their physiological effects via activation of $G_{\alpha i/o}$-coupled receptors, the opioid and cannabinoid receptors, respectively.[1,3–5] In an effort to develop drugs that target these receptors and exert the same physiological effects as morphine or THC but without the undesirable side effects associated with chronic drug use, several groups have investigated whether protein–protein interactions, including receptor heteromerization, could modulate the function of individual receptors. Here, we describe heteromerization as associations between different subtypes of a receptor or between two different receptors. Although heteromers involving opioid, cannabinoid, or dopamine receptors have been described, not much is known about their physiological role. In this context, studies using peptides that disrupt specific heteromers, or antibodies that selectively block heteromer signaling, suggest that GPCR heteromers may play a role in a number of pathologies.[7–11] While additional GPCR heteromers associated with other drugs of abuse have been reported, for the sake of

brevity and conciseness, in this review we have focused only on heteromers that contain either opioid or cannabinoid receptors as one of the partners. We have also included dopamine receptors since they are the primary effectors of the action of stimulants such as cocaine and amphetamines. These are described below along with the putative role of these heteromers in the development of tolerance and other disease states.

1.1. Opioid receptors

Opioid receptors are members of the rhodopsin family of GPCRs and their signal transduction involves activation of $G_{\alpha i/o}$ proteins, downstream inhibition of adenylyl cyclase activity, the opening of inwardly rectifying K^+ channels, and inhibition of Ca^{2+} channels.[1] Molecular cloning studies have identified three types of opioid receptors μ-, δ-, and κ-opioid receptors (reviewed in Ref. 2).

Opioid receptors can be activated by endogenous peptides derived from the processing of the large precursor proteins, proopiomelanocortin, proenkephalin, and prodynorphin, by prohormone convertases via the classic peptide-processing pathway[12] to generate mainly β-endorphin, Met- and Leu-enkephalin, and dynorphins.[13] It is generally thought that enkephalins exert their biological effects primarily by activating δ opioid receptors (DOR), β-endorphin by activating μ opioid receptors (MOR), and dynorphins by activating κ opioid receptors (KOR).[13,14] In addition, opioid receptors can also be activated by drugs of abuse such as morphine and heroin.[15] Agonist-mediated activation of opioid receptors leads to the modulation of many biological functions including pain perception, motivation, locomotion, hormone secretion, and reward.[1,2] Studies using genetic knockdown have further elucidated the role of each opioid receptor type. These studies have implicated MOR in the analgesic effects of morphine and in the rewarding effects resulting from other nonopioid drugs of abuse including cannabinoids,[16–20] DOR in the regulation of emotional responses including anxiety and depression,[21] and KOR in the dysphoric effects of opioids and cannabinoids as well as in stress-induced relapse.[22,23] Together, these studies reveal a broad role for opioid receptors in normal physiology and disease.

1.2. Cannabinoid receptors

Like opioid receptors, cannabinoid receptors are also members of the rhodopsin family of GPCRs and signal via the $G_{\alpha i/o}$-signaling pathway, to activate similar downstream signaling cascades.[3–5] The two main subtypes of cannabinoid receptors are CB_1R and CB_2R.[3] Recently, the orphan receptor, GPR55, has been proposed as a putative cannabinoid receptor subtype (reviewed in Ref. 24).

CB_1R and CB_2R are activated by endogenous ligands derived from membrane lipids termed “endocannabinoids” which together with the receptors constitute the “endocannabinoid system” (for review, see Refs. 25–27). The two major lipid-based endocannabinoids are anandamide and 2-arachidonoylglycerol (2-AG)[3] that are generated by phospholipases[28–32] and degraded by fatty acid amide hydrolase,[33,34] cyclooxygenases, lipooxygenases,[35,36] and monoacylglycerol lipase.[37] Recently, endogenous peptidic agonists of cannabinoid receptors have been identified.[38–42] These peptides, RVD-hemopressin and VD-hemopressin, are derived from the α-chain of hemoglobin[38] and appear to activate a signal transduction pathway distinct from that of classic CB_1R ligands.[38] However, very little information is available about the enzymatic steps involved in either the generation or degradation of RVD-hemopressin.

Studies show that the endocannabinoid system plays an important role in many physiological processes including pain perception, learning and memory, appetite control, motor coordination, lipogenesis, and modulation of immune responses (for reviews, see

Refs. 25–27,43,44). Studies with knockout mice indicate that the CB_1R subtype plays a role in the central effects of cannabinoids, including mood disorders, learning and memory, motor coordination, feeding, control of pain, and stress responses, and in the reinforcing and rewarding effects of drugs of abuse (reviewed in Refs. 45,46). Conversely, the CB_2R subtype plays a role in the peripheral effects of cannabinoids, including the development and function of the immune system, embryonic development, bone homeostasis, and pain perception, and plays an antifibrogenic role in liver diseases (reviewed in Ref. 47). These studies imply a broad role for CB_1R in central nervous system physiology and CB_2R in immune system function.

1.3. Dopamine receptors

Like opioid and cannabinoid receptors, dopamine receptors are members of the family A of GPCRs. Based on their ability to modulate cAMP levels, these receptors are classified as D1- or D2-like receptors (reviewed in Ref. 48). D1-like receptors couple to $G_{\alpha s/olf}$ proteins and are thought to be exclusively present at the presynapse (reviewed in Ref. 48). Activation of D1-like receptors leads to increases in cAMP levels, activation of cAMP-dependent protein kinases, increase in the phosphorylation levels of DARPP-32 (a molecule involved in the integration of cellular signaling responses to neurotransmitters) and ERK1/2 (the latter particularly in medium spiny neurons, MSNs), increases in L-type Ca^{2+} channel and NMDA receptor activity, inhibition of N- or P/Q-type Ca^{2+} channel and K^+ channel activity, enhancement of AMPA currents and modulation of GABA currents (reviewed in Refs. 48–50). D2-like receptors couple to $G_{\alpha i/o}$ proteins and are present both at the pre- and postsynapse (reviewed in Ref. 48). Activation of D2-like receptors decreases cAMP levels and the activity of protein kinase A, decreases the phosphorylation levels of DARPP-32, inhibits ERK1/2 phosphorylation in striatum, decreases the activity of L-, N-, and P/Q-type Ca^{2+} channels, and activates K^+ channels (reviewed in Refs. 48,49). There is some evidence suggesting that, in addition to $G_{\alpha s/olf}$ and $G_{\alpha i/o}$-mediated signaling, dopamine receptors can also signal via $G_{\alpha q}$- and G-protein-independent mechanisms (reviewed in Refs. 48–50). However, the mechanisms underlying $G_{\alpha q}$-mediated signaling are not completely understood (reviewed in Refs. 48–50). Studies show that dopamine receptors play a role in the modulation of locomotor activity, olfaction, retinal processes, cardiovascular, renal, and immune functions, memory and learning, feeding, sleep regulation, and in reward behaviors, particularly those related to drugs of abuse (reviewed in Ref. 48). Five subtypes of dopamine receptor have been identified. While D1 and D5 dopamine receptors belong to the D1-like receptor type, D2, D3, and D4 receptors belong to the D2-like receptor type (reviewed in Refs. 48–50).

As the name suggests, the endogenous agonist of dopamine receptors is dopamine. Dopamine can be synthesized from phenylalanine and tyrosine by tyrosine hydroxylase (reviewed in Ref. 50). Following synthesis in the cytosol, dopamine is taken into storage vesicles by vesicular monoamine transporters (reviewed in Ref. 50). Dopamine is released at the synapse by Ca^{2+}-mediated fusion of dopamine-containing vesicles with neuronal membranes (reviewed in Ref. 50). The synaptic activity of released dopamine is terminated by its reuptake into presynaptic nerve terminals by dopamine transporters (reviewed in Ref. 50). In the mammalian brain, four major dopaminergic pathways have been identified. These include the nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular systems (reviewed in Refs. 48–50). Interestingly, it has been shown that MSNs in the striatum and nucleus accumbens can be phenotypically distinguished depending on the expression of D1 or D2 dopamine receptors. D1 dopamine receptors (D1R) are expressed by MSNs that project into the medial globus pallidus and the substantia nigra pars reticulata, D2 dopamine receptors (D2R) are expressed by MSNs that project into the lateral globus pallidus

(reviewed in Refs. 48–50), and a small percentage of MSNs coexpress D1R and D2R (reviewed in Refs. 48–50).

In order to elucidate the physiological roles of different dopamine receptor subtypes, knockout mice have been generated. Data with these mice have been the subject of excellent reviews (see Refs. 50–53) and are not described in detail here. Briefly, studies with D1R knockout mice suggest that the latter exhibit alterations in the motivational or attentive aspects of behavior.[52] Interestingly, D1R knockout mice exhibit a decrease in the locomotor effects of cocaine.[54] Studies with D2R knockout mice suggest that they may represent an animal model for Parkinson's disease as they recapitulate the extrapyramidal symptoms of the disease.[50] Interestingly, while the release of dopamine elicited by drugs of abuse such as morphine and cocaine is higher in D2R knockout mice than in wild-type animals (reviewed in Ref. 55), the rewarding effects of these drugs are attenuated in the knockout mice (reviewed in Ref. 55). D3R knockout mice exhibit increased activities in novel and exploratory environments, in rearing behavior, and deficits in spatial working memory compared to wild-type mice (reviewed in Ref. 50). These mice also exhibited a decrease in methamphetamine-mediated locomotion[56] and behavioral sensitization to ethanol but not to amphetamines.[56,57] Moreover, the D3R knockout mice exhibit attenuation in the development of tolerance and precipitated withdrawal to chronic morphine administration.[58] Studies with D4R knockout mice show that this receptor subtype may be involved in the modulation of novelty-related exploration and avoidance behavior to unconditioned stimuli (reviewed in Ref. 50). With regard to drugs of abuse, these mice are more responsive to the locomotor effects of ethanol, cocaine, or amphetamines than wild-type mice (reviewed in Ref. 50). In addition, D4R alleles associated with novelty seeking behavior have been correlated with drug abuse, attention deficit hyperactivity disorder, and gambling (reviewed in Ref. 50). Studies show that D5R knockout animals exhibit hypertension suggesting that this dopamine receptor subtype may be involved in the regulation of blood pressure (reviewed in Ref. 50). Taken together, these studies reveal a role for dopamine receptors in normal physiology and in drug abuse.

2. RECEPTOR HETEROMERIZATION

In the past decade, the idea that GPCRs associate to form heteromeric complexes with novel pharmacology has emerged (for reviews, see Refs. 59–65). Initial evidence for GPCR heteromerization came from studies showing that activation of a GPCR modulated the function of another GPCR.[66–70] The possibility that GPCRs could form heteromers was supported by elegant complementation studies. These included studies using chimeras of the α_{2c}-adrenergic and m3 muscarinic receptors (α_{2c}/m3 and m3/α_{2c}) that, when expressed individually, could not bind to either radiolabeled adrenergic or muscarinic ligands but when coexpressed could.[71] Similarly, coexpression of two different mutants of the angiotensin AT1 receptor that individually could not bind to angiotensin led to the restoration of angiotensin binding upon coexpression of these mutant receptors.[72]

The first direct evidence for GPCR heteromerization came from studies with the metabotropic $GABA_B$ receptors. The expression of functional $GABA_B$ receptors at the cell surface requires the coexpression of both the $GABA_{B1}$ and $GABA_{B2}$ protein subunits.[73–76] Another example for the requirement for heteromerization for receptor function is provided by taste receptors where associations between $T1R_1$ and $T1R_2$ form a receptor that recognizes sweet tasting molecules, while those between $T1R_1$ and $T1R_3$ recognize "umami" tasting molecules.[77–79]

The availability of epitope-tagged GPCRs for immunoisolation of interacting complexes to examine whether two GPCRs are in close proximity for direct interaction has shown that a

large number of GPCRs can form heteromers (for reviews, see Refs. 59–61). Scientists in the field have applied the following criteria in order to ascertain that two GPCRs form heteromers: (i) colocalization of the two interacting receptors in the same cellular and subcellular compartment; (ii) immunoisolation of interacting receptors, using receptor-selective antibodies, from wild-type animal tissue that expresses both receptors (but not from animals lacking one of the receptors); (iii) proximity-based assays showing that the two receptors are in close proximity to interact only in cells/tissues expressing both receptors and not in cells/tissues lacking individual receptors; and (iv) unique pharmacological properties as determined using highly selective reagents targeting the heteromer.

2.1. Methods to study GPCR heteromerization

2.1.1 Techniques to study GPCR heteromerization in vitro

i. *Coimmunoprecipitation assays*: In this technique, interacting complexes in tissues are detected using antibodies selective for individual protomers. Alternatively, cells are cotransfected with the GPCRs of interest, each of which is differentially epitope tagged (e.g., with a Flag or myc epitope) and antibodies to the epitope tags used for the assay. Tissues or cells are then solubilized with a buffer-containing detergent, and antibodies to an individual receptor protomer or to the epitope tag on one of the receptors (e.g., the Flag epitope) are used to immunoprecipitate that receptor.[80,81] The immunoprecipitates are subjected to Western blot analysis and probed for the presence of the second receptor using antibodies selective for that receptor or to the epitope tag (e.g., the myc epitope) present on the receptor.[80,81] The detection of a signal on Western blots indicates that the two receptors form interacting complexes. Given the hydrophobic nature of GPCRs, appropriate controls are used in order to rule out the artifactual detection of interacting complexes. These include carrying out immunoprecipitation studies with tissue from animals lacking one receptor or with cells that express only one receptor, mixing cells that individually express each receptor prior to solubilization and immunoprecipitation, using different detergents for solubilization and/or using cross-linking reagents to cross-link cell surface proteins prior to solubilization and immunoprecipitation.[80,81] This strategy was first used to demonstrate opioid receptor heteromers in tissues[7] or in cells that coexpress both receptors.[82]

ii. *Bioluminescence resonance energy transfer assay*: In order for two receptors to form heteromers, they have to be in close proximity to interact in live cells. One of the earliest proximity-based assays used to examine GPCR heteromerization was bioluminescence resonance energy transfer (BRET) (reviewed in Ref. 83). In this assay, the C-terminus of one of the receptors is fused to *Renilla reniformis* luciferase (Rluc), while the C-terminus of the other receptor is fused to a genetic variant of green fluorescent protein (GFP) such as yellow fluorescent protein (YFP) (reviewed in Ref. 83). Cells are transfected with both receptors and then treated with the luciferase substrate, coelenterazine h. The action of luciferase on its substrate leads to the emission of light at 480 nm which can excite YFP which then emits light at 530 nm only if the two receptors are less than 100 Å apart (reviewed in Ref. 83). An advantage of this technique is that it does not require conventional light illumination, and this eliminates artifactual results due to autofluorescence (reviewed in Ref. 83). BRET assay was used to demonstrate proximity between opioid receptor types as well as between opioid and other receptors (Table 9.1). Several generations of BRET have been developed. BRET^1 uses YFP or enhanced GFP in combination with Rluc and coelenterazine h and has the advantage that the BRET signal can be detected for up to 1 h (reviewed in Ref. 83). BRET^2 uses a

GFP variant that is excited at ~400 nm and DeepBlueC as the Rluc substrate. This allows significant separation between the excitation and emission spectra of GFP. The BRET^2 signal decays rapidly; however, the use of Rluc variants, Rluc2 or Rluc8, ensures a high BRET signal that can last for hours depending on the donor and acceptor combinations chosen (reviewed in Ref. 83). BRET^3 makes use of RLuc8 and a variant of red fluorescent protein, mOrange, as donor and acceptor pairs. BRET^3 exhibits the most red-shifted emission wavelength (564 nm), and consequently yields a several-fold improvement in light output compared with other BRET assays (reviewed in Ref. 83).

iii. *Fluorescence resonance energy transfer assay*: Another technique commonly used to determine close proximity between two GPCRs is fluorescence resonance energy transfer (FRET). Here, cells are cotransfected with pairs of receptors of interest; one of the receptors is fused to a fluorescent donor and the other to a fluorescent acceptor (reviewed in Ref. 120). The most commonly used fluorescent donor is cyan fluorescent protein (CFP), while the fluorescent acceptor is YFP (reviewed in Ref. 120). Light excitation at 400 nm causes CFP to emit light at 480 nm which can then excite YFP, if it is in sufficiently close proximity, causing it to emit light at 580 nm (reviewed in Ref. 120). Given that the FRET efficiency is inversely proportional to the sixth power of the distance between the two fluorophores, a FRET signal is observed only if the donor–acceptor pair is less than 100 Å apart (reviewed in Ref. 120). An advantage of FRET is that it is amenable to confocal microscopy. Problems with FRET leading to poor signal-to-noise ratio include (i) cross talk, wherein light used to excite the donor also excites the acceptor fluorophore; (ii) bleed through, due to partial overlap between the spectra of the donor and acceptor fluorescent proteins; and (iii) photobleaching (reviewed in Ref. 120). Several modifications of the FRET technique have been developed to improve its signal-to-noise ratio including photobleaching FRET (pbFRET), photoquenching FRET, fluorescence lifetime imaging microscopy (FLIM), and time-resolved FRET. Several excellent reviews describe these techniques in detail.[120–124]

Several techniques to label GPCRs for FRET studies have been developed including (i) labeling one receptor with a FlAsH tag (a 15-amino acid polypeptide tag with a tetracysteine core (CCXXCC) that covalently binds to a fluorogenic bisarsenical fluorescein ligand whose fluorescence increases upon binding to the polypeptide tag) and fusing the other receptor with CFP, (ii) using mTurquoise instead of CFP for FLIM experiments, (iii) using Eu^{3+}- and Tb^{3+} cryptate in combination with a fluorescent protein, and (iv) using SNAP- and CLIP tags (reviewed in Ref. 124). Moreover, FRET sensors have been developed to examine ligand binding to a GPCR, receptor activation, conformational changes in receptor dimers, receptor trafficking, and receptor interaction with signaling molecules (reviewed in Ref. 124).

iv. *Protein complementation*: In recent years, protein-fragment complementation assays have been used to examine GPCR heteromerization in living cells. In this approach, cells are transfected with one GPCR fused to a fragment of luciferase and another GPCR fused to a complementary fragment (reviewed in Ref. 121). Reconstitution of active luciferase enzyme would occur only if the two receptors were in close proximity. This approach is known as bimolecular luminescence complementation (BiLC) (reviewed in Ref. 121). A combination of protein complementation with the BRET assay[125] has proven to be amenable to the development of sensors to study not only receptor heteromerization but also conformational changes following receptor activation, receptor interactions with

signaling molecules, and how the latter are modulated by ligand treatment (reviewed in Ref. 83). Moreover, recent advances in BRET technology have enabled these assays to be carried out in live animals.[126]

Another protein complementation approach uses a receptor fused with a fragment of YFP (nonfluorescent) and another receptor fused with a complementary YFP fragment (nonfluorescent). YFP fluorescence is reconstituted only when the two receptors are in close proximity. This approach is known as bimolecular fluorescence complementation (BiFC) (reviewed in Ref. 121). The advantage of BiFC over BiLC is that it can provide information about the subcellular localization of receptor interactions (reviewed in Ref. 121). Moreover, a combination of BiFC or BiLC with either BRET or FRET has been used to demonstrate the presence of GPCR trimers or tetramers.[127–130]

v. *Atomic force microscopy and crystallography*: Biophysical approaches such as atomic force microscopy (AFM) and X-ray crystallography have been useful in demonstrating the existence of GPCR dimers. AFM uses a scanning tunneling microscope to measure the movement of a cantilever beam over the sample surface.[131] Using this approach, studies showed that rhodopsin, the prototypical family A GPCR, was arranged in dimeric arrays.[132] X-Ray crystallography, a method used for determining the arrangement of atoms within a molecule, can provide information about the structural organization of a GPCR. Until very recently, the high-resolution crystal structures for only a few GPCRs were available. These included the structures for rhodopsin and extracellular domains of metabotropic glutamate receptors, which indicated that these receptors could exist as dimers or higher-order structures.[133,134] The obstacles in obtaining high-resolution structures for most GPCRs included low expression levels in endogenous tissues, difficulties in expressing the receptor at high enough levels in heterologous cells, structural instability following detergent solubilization, as well as multiple receptor conformational states. Recent approaches aimed at stabilizing a GPCR into a single conformational state, through the use of either monoclonal antibodies that stabilize the conformation of intracellular loop 3 or by replacing a portion of this loop with T4-lysozyme, together with crystallization in the presence of a receptor antagonist, have permitted the determination of high-resolution structures for a number of GPCRs including those for opioid receptors.[135–142] In this context, it is to be noted that the recent MOR and chemokine receptor, CXCR4, crystal structures reveal a dimeric receptor arrangement consistent with the idea that these receptors could exist as higher-order structures *in vivo*.[137,139]

2.1.2 Techniques to study GPCR heteromerization in vivo

i. *Ligand-aided FRET*: The presence of GPCRs in endogenous tissue can be detected by FRET assays using fluorescently labeled ligands to each receptor protomer. Since both agonists and antagonists can be used in such studies, they provide additional information on the conformational state of the receptor.[143] This approach makes use of fluorophores compatible with TR-FRET to label the ligands in order to obtain a high signal-to-noise ratio.[143] Thus, ligands are labeled with donor–acceptor pairs (typical examples of donor–acceptor pairs optimal for TR-FRET include Eu-PBBP, Lumi4-Tb, or Alex-64-d1), and the assay is then carried out in tissue patches.[143] Such an approach was used to detect the presence of oxytocin receptor dimers/oligomers in mammalian gland.[143]

ii. *Antibody-aided FRET*: The presence of GPCR heteromers in live cells can be detected by a combination of selective antibodies and FRET. For example, in live cells expressing epitope-tagged receptors, secondary antibodies labeled with either

fluorescein or rhodamine were used in pbFRET experiments to monitor the formation of SSTR1–SSTR5 and D2R–SSTR5 heteromers.[144,145] In another study, antibodies to epitope tags on the receptor were labeled with either Eu^{3+} or allophycocyanin protein and used in TR-FRET experiments to demonstrate the presence of DOR oligomers at the cell surface.[146] An extension of the latter approach could be used to examine GPCR heteromerization in endogenous tissue by labeling monoclonal antibodies that are highly selective to individual receptors in the heteromer with complementary TR-FRET donor–acceptor pairs. Such an approach was used to detect the presence of $GABA_B$ receptor tetramers on brain membranes.[147]

iii. *Proximity-based ligation assays (PLAs)*: Recently, PLAs have been used to detect the presence of GPCR heteromers in endogenous tissue.[148,149] This is an antibody-based approach where tissue sections of interest are first incubated with anti-GPCR selective antibodies.[148,149] The antibody to each protomer in the heteromeric complex under investigation is raised in a different species (rabbit or mouse). Sections are then incubated with species-specific secondary antibodies coupled to complementary oligonucleotide sequences (PLA probes).[148,149] When the antibodies to the two receptors are in close proximity (<17 nm), the PLA probes can be ligated using ligase and a ligation solution comprising of two oligonucleotides that will hybridize with the two PLA probes to form a closed circle.[148,149] The ligated closed circle is then amplified by polymerase in a rolling-circle amplification reaction in the presence of fluorescently labeled oligonucleotides.[148,149] Hybridization of the fluorescently labeled nucleotides appears as afluorescent spot that can then be visualized by fluorescence microscopy.[148,149] This assay has been used to detect the presence of CB_1R–CB_2R receptor heteromers in the pineal body and nucleus accumbens and of dopamine D2–adenosine A_{2A} receptor heteromers in the striatum.[148,149]

iv. *Heteromer-selective antibodies*: Monoclonal antibodies that selectively recognize a GPCR heteromer are useful tools that can be used to not only determine the tissue distribution of the heteromer but also its possible physiological role. A subtractive immunization strategy has been described for the development of such antibodies.[7,9,10,88] In this approach, mice are first made tolerant to nonspecific cell membrane epitopes by treatment with cyclophosphamide.[7,9,10,88] Mice are then immunized with membranes from cells expressing the heteromer of interest.[7,9,10,88] Antibodies selective for MOR–DOR, DOR–CB_1R, or DOR–AT1R heteromers have been generated using this approach.[7,9,10,88] Studies using these antibodies show that the antibodies are able to selectively block signaling by specific heteromers and that MOR–DOR heteromers are increased in brain regions involved in pain processing under conditions leading to the development of tolerance to morphine, while DOR–CB_1R heteromer levels are increased in specific brain regions during neuropathic pain, and DOR–AT1R heteromer levels are increased during ethanol-induced liver fibrosis.[7,9,10,88]

2.2. Opioid receptor heteromerization

Early radiolabeled binding studies, as well as studies measuring the antinociceptive effects of selective opioid receptor ligands, suggested the presence of multiple opioid receptor subtypes.[150,151] However, to date, the cDNAs for only MOR, DOR, and KOR have been identified.[2,20] This suggested the possibility that the multiple opioid receptor subtypes detected by binding and antinociceptive studies could arise from the formation of heteromeric complexes between opioid receptor subtypes (MOR, DOR, KOR) or between the latter and other GPCRs. The availability of cDNAs for epitope-tagged receptors together

with coimmunoprecipitation and/or proximity-based assays has enabled studies showing that opioid receptors can form heteromers with a number of GPCRs (see Table 9.1). These are described below.

DOR–KOR heteromers—The first opioid receptor heteromer to be detected and characterized involved DOR and KOR.[82] Coimmunoprecipitation studies, using differentially epitope-tagged receptors, showed that DOR and KOR could form interacting complexes.[82] BRET studies showed that both receptors were in close enough proximity (<100 Å) in live cells to directly interact.[86] Analysis of the binding, signaling, and trafficking properties of DOR–KOR heteromers revealed that the latter exhibited properties distinct from that of the individual receptors. For example, the binding affinities of selective DOR and KOR ligands were reduced in the DOR–KOR heteromer; however, a combination of DOR and KOR ligands exhibited cooperativity in binding that in the case of agonists was translated into a potentiation in signaling responses.[82] This binding cooperativity was also observed with a combination of DOR and KOR antagonists.[82] Bivalent ligands consisting of DOR and KOR antagonists (naltrindole and 5′-guanidinonaltrindole (5′-GNTI), respectively) linked by spacers of variable length were used to target the DOR–KOR heteromer.[152] One of these bivalent ligands, KDN-21, exhibited a higher affinity and selectivity for DOR–KOR heteromers compared to DOR and KOR homomers.[153] Pharmacological and binding data indicate that KDN-21 binds to DOR–KOR heteromers of the DOR1–KOR2 phenotype.[152,153] Interestingly, a study identified 6′-guanidinonaltrindole (6′-GNTI) as a DOR–KOR heteromer-selective agonist that induced antinociception when administered to the spinal cord but not to the brain.[154] However, a recent study has suggested that 6′-GNTI is a KOR-biased ligand[155]; thus, it remains to be seen if 6′-GNTI represents a DOR–KOR heteromer-biased ligand. Studies that suggest a role for DOR–KOR heteromers in pain pathways are described in Section 3.

DOR–MOR heteromers—This is the most extensively studied opioid receptor heteromer due to its role in morphine-mediated antinociception and in the development of tolerance and dependence to opiates. A comprehensive body of evidence suggested the possibility of DOR–MOR heteromerization (reviewed in Ref. 156). Noteworthy are radiolabeled studies indicating that (i) there is an allosteric modulation of radiolabeled binding to DOR by MOR ligands[157,158]; (ii) intracerebroventricular potentiation of morphine-mediated antinociception by nonantinociceptive doses of DOR agonists which can be blocked by the DOR antagonists ICI174,864 and naltrindole-5′-isothiocyanate[159]; (iii) potentiation of acute morphine-mediated antinociception by DOR antagonists and inhibition of development of tolerance or reduction of already developed tolerance to morphine[160]; (iv) prevention of the development of tolerance and dependence in mice by pretreatment with DOR antagonists[161]; and (v) lack of development of tolerance to morphine in DOR knockout mice.[162]

Evidence that MOR and DOR form heteromeric complexes *in vitro* came from coimmunoprecipitation and BRET studies using differentially epitope-tagged receptors.[93–95] In addition, coimmunoprecipitation studies using receptor-selective antibodies revealed the presence of DOR–MOR heteromers in spinal cord membranes from wild-type but not DOR knockout mice.[94] More recently, antibodies that selectively recognize DOR–MOR heteromers were used to detect the presence of these heteromers in brain regions of wild-type but not MOR or DOR knockout mice.[7]

DOR–MOR heteromers exhibit distinct pharmacological properties from that of individual receptors.[93–95] Low nonsignaling doses of DOR or MOR ligands potentiate the binding and signaling of MOR or DOR receptors, respectively.[93,94,96] Moreover, the potency of highly selective synthetic MOR or DOR ligands is decreased, while the affinity for endomorphin-1

and DOR-selective agonists is increased in DOR–MOR heteromers as compared to MOR or DOR.[95,97]

Studies show that DOR–MOR heteromerization leads to changes in the signaling pathways activated by the heteromers compared to individual receptor homomers. In this context, a study showed that the DOR–MOR heteromer associates with pertussis-insensitive $G_{\alpha z}$ instead of the pertussis-sensitive $G_{\alpha i}$ and this interaction occurs in the endoplasmic reticulum.[98,99] Another study reported that activation of a protomer in the DOR–MOR heteromer leads to a switch in signaling from $G_{\alpha i}$- (as it occurs in receptor homomers) to β-arrestin-mediated signal transduction leading to changes in the spatiotemporal dynamics of ERK1/2 phosphorylation and ultimately differential activation of transcription factors.[100] It has also been reported that while the MOR-selective agonist, DAMGO, stimulates Ca^{2+} signaling in cells expressing DOR–MOR heteromers by activating phospholipase C and releasing Ca^{2+} from intracellular stores, it inhibits Ca^{2+} signaling in GH3 cells expressing only MOR by inhibiting adenylyl cyclase activity and voltage-gated Ca^{2+} channels and by activating inwardly rectifying K^{137+}. Taken together, these studies indicate that heteromerization increases the repertoire of signaling for opioid receptors.

A few studies have examined how DOR–MOR heteromerization modulates the trafficking of individual receptors. One study reported that treatment with receptor-selective agonists (DAMGO or deltorphin II but not DPDPE or DSLET) led to internalization of the DOR–MOR heteromer.[99] However, another study examining receptor turnover in cells that expressed increasing levels of DOR with constant levels of MOR reported that the two receptors endocytosed independently of each other in the context of the heteromer.[102] Finally, a recent study reported that methadone could promote the internalization of the DOR–MOR heteromer targeting it to the degradation machinery.[103] Together, these data suggest that the internalization of the DOR–MOR heteromer could be a ligand type-dependent event. Recent studies suggest a role for these heteromers in pathways leading to development of tolerance to morphine; this is discussed in Section 3.

DOR–CB_1R heteromers—Early studies examining interactions between DOR and CB_1R showed that (i) long-term exposure to a DOR agonist attenuated CB_1R-mediated stimulation of G-protein activity in N18TG2 neuroblastoma cells that coexpress DOR and CB_1R[163]; (ii) the DOR antagonist, naltrindole, blocked Δ^9-THC-mediated anxiolytic responses[105]; (iii) CB_1R levels as well as agonist-mediated G-protein activity are increased in the substantia nigra of mice lacking DOR as compared to wild-type animals[164]; and (iv) DOR activity is increased in the caudate putamen of CB_1R knockout mice compared to wild-type mice.[165]

DOR–CB_1R heteromers are detected in HEK-293 and in Neuro 2A cells coexpressing both receptors by coimmunoprecipitation and BRET assays.[88,89] DOR and CB_1R are colocalized in cortical neurons as examined by immunofluorescence studies with receptor-selective antibodies.[88] Interestingly, heteromerization leads to a differential recruitment of trafficking (adaptor) proteins; CB_1R associates with the adaptor protein AP-3 in the absence of DOR and with AP-2 in the presence of DOR.[88] This, in turn, leads to differential localization of CB_1R from intracellular vesicles, in the absence of DOR, to the cell surface in the presence of DOR.[88] In addition, lentiviral-mediated knockdown of DOR in F11 cells that endogenously coexpress CB_1R and DOR leads to a decrease in cell surface localization of CB_1R.[88] Heteromerization also alters CB_1R signaling; the potency of the CB_1R agonist, Hu-210, is decreased in cells expressing DOR–CB_1R in assays that measure G-protein activity or ERK1/2 phosphorylation, and in these cells, knockdown of DOR leads to an increase in the potency of Hu-210.[88] Moreover, DOR–CB_1R heteromerization leads to a PLC-mediated recruitment of β-arrestin 3 to the heteromeric complex, and to the activation

of novel signaling pathways that promote cell survival.[88] Recent studies suggest a role for these heteromers in pain pathways; this is discussed in Section 3.

DOR–CXCR4 heteromers—Given that both DOR and chemokine CXCR4 receptors are expressed in immune cells, coimmunoprecipitation and FRET assays were used to determine whether both receptors could form heteromers.[90] These studies showed that DOR and CXCR4 form interacting complexes.[90] Moreover, the studies showed that simultaneous activation of DOR and CXCR4 in cells coexpressing both receptors did not affect cell viability, chemokine binding, or receptor internalization but led to the formation of nonfunctional complexes.[90] This suggests that DOR–CXCR4 heteromerization provides a means to silence CXCR4 function and could be important in the regulation of immune function.

DOR–sensory neuron-specific receptor heteromers—Sensory neuron-specific receptors (SNSRs) are mainly expressed in dorsal root ganglia in the spinal cord, where they mediate pronociceptive responses.[166] Given that DOR and SNSR-4 share localization in dorsal root ganglia, can be activated by the bovine adrenal medulla peptide 22 (BAM22), and their functions are antagonistic to each other, led to studies examining whether both receptors could form heteromeric complexes. BRET assays in HEK-293 cells coexpressing differential epitope-tagged receptors indicated that both receptors were in sufficiently close proximity to directly interact in live cells.[104] In cells expressing DOR–SNSR-4 heteromers, activation of SNSR-4 alone led to $G_{\alpha q}$-mediated signaling, while activation of DOR alone resulted in $G_{\alpha i}$-mediated signaling. However, coactivation of both receptors by receptor-specific agonists led to a preferential signaling via $G_{\alpha q}$.[104] Interestingly, the endogenous ligands for SNSR are derived from the proteolytic processing of the proenkephalin precursor that also generates the antinociceptive endogenous DOR ligands, Leu- and Met-enkephalin.[167] Moreover, one of these peptides, BAM22, has been shown to bind to both DOR and SNSR-4.[167] Activation of DOR–SNSR-4 heteromers with BAM22 leads to signaling via $G_{\alpha q}$. This is the first demonstration of an endogenous peptide as a bivalent agonist of a GPCR heteromer.

DOR–β_2 adrenergic receptor heteromers—Interactions between DOR, a $G_{\alpha i}$-coupled receptor, and the β_2 adrenergic receptor (β_2AR), a $G_{\alpha s}$-coupled receptor, were suggested by studies showing that in the isolated isovolumic rat heart the DOR agonist, Leu-enkephalin, significantly inhibited β_2AR-mediated positive ionotropic effects and stimulation of cAMP levels, effects that were blocked by the opioid antagonist, naloxone, and by pertussis toxin treatment.[168] Moreover, in isolated rat ventricular myocytes, nanomolar concentrations of Leu-enkephalin could block β_2AR-mediated increases in sarcolemmal L-type Ca^{2+} currents, cytosolic Ca^{2+} transients, and contraction.[169] Coimmunoprecipitation and BRET assays using differentially epitope-tagged receptors demonstrated the formation of DOR–β_2AR heteromers in HEK-293 cells.[86,87] While the binding and signaling properties of individual protomers in the DOR–β_2AR heteromer were not changed by heteromerization, individual receptor trafficking was, in that DOR agonists could internalize β_2AR only in cells coexpressing both receptors.[87]

DOR–α_{2A} adrenergic receptor heteromers—A number of studies have suggested interactions between DOR and α_{2A} adrenergic receptors (α_{2A}AR). These include studies showing that (i) a selective imidazole/α_2AR agonist synergizes with the DOR agonist, deltorphin II, to block substance P elicited nociception[170]; (ii) the synergy between agonists for the two receptors was observed in α_{2C}AR and in MOR knockout mice but not in α_{2A}AR knockout mice suggesting an involvement of α_{2A}AR and of DOR in these interactions[171–173]; and (iii) cotreatment of synaptosomes isolated from the spinal cord or of

spinal slices with α_{2A}AR and DOR agonists led to potentiation of inhibition of K^+-stimulated release of neuropeptides.[84,174]

Studies using differentially epitope-tagged receptors expressed in heterologous cells together with coimmunoprecipitation and BRET assays show that α_{2A}AR and DOR are in close proximity (<100 Å apart) to form interacting complexes.[85] Not much is known about how DOR–α_{2A}AR heteromerization modulates the pharmacological, signaling, and trafficking properties of this heteromer pair. One study suggested the involvement of protein kinase C in heteromer-mediated synergy in inhibition of calcitonin gene-related peptide release from spinal cord slices,[84] while another indicated that the presence of α_{2A}AR is sufficient to potentiate DOR-mediated neurite outgrowth in Neuro 2A cells.[85] The latter study indicated that just the presence of α_{2A}AR is sufficient to modulate DOR activity; whether the converse also occurs and how ligand occupancy of both protomers in the DOR–α_{2A}AR heteromer modulates individual receptor function need to be further evaluated.

DOR–D1R heteromers—Interactions between DOR and D1R were suggested by studies showing (i) that alterations in dopaminergic neurotransmission modulate the endogenous levels of opioid peptides[175–177]; (ii) attenuation of DOR-mediated signaling by the D1R agonist, SKF 82958[178]; and (iii) enhancement of climbing behavior in mice by a combination of DOR and D1R agonists.[179]

Immunoelectron microscopy detected the presence of DOR and D1R immunoreactivity in a subset of dendrites in the dorsolateral striatum and nucleus accumbens of rats, indicating that both receptors were present in the same neuron.[91,92] Moreover, withdrawal from cocaine, a drug that blocks dopamine transporter activity and thereby alters dopamine neurotransmission by increasing and prolonging signaling by postsynaptic dopamine receptors caused a redistribution of DOR in the nucleus accumbens.[91] Although these colocalization studies indicate that DOR–D1R could form heteromers, this needs to be further evaluated by studies using epitope-tagged receptors, coimmunoprecipitation, and proximity-based assays.

MOR–KOR heteromers—Very few studies have examined heteromerization between MOR and KOR. An early study using coimmunoprecipitation of differentially epitope-tagged receptors did not detect the presence of interacting MOR–KOR complexes.[82] This does not rule out the formation of MOR–KOR heteromers if the latter are not stable under the detergent conditions used in the coimmunoprecipitation studies. Another study used BRET to show that MOR and KOR are in close proximity to interact in live cells.[114] A recent study reported the formation of MOR–KOR heteromers in spinal cord membranes of female, but not male, rats and showed that it is dependent on the stage of the estrous cycle.[115] Interestingly, this study identified dynorphin 1–17 as an MOR–KOR heteromer-selective ligand.[115]

MOR–α_{2A} adrenergic heteromers—Studies describing synergistic interactions between the antinociceptive effects of MOR and α_{2A}AR agonists[180,181] and with α_{2A}AR knockout mice that exhibit a reduction in the analgesic potency of morphine[173] suggested the possibility of MOR–α_{2A}AR heteromerization.

Differential epitope-tagged receptors together with coimmunoprecipitation, BRET, or FRET assays demonstrated the formation of MOR–α_{2A}AR heteromers.[107,108] Studies examining heteromer-mediated signaling showed that while the presence of α_{2A}AR is sufficient to enhance MOR signaling, coactivation of both receptors in the heteromeric complex leads to a significant attenuation of signaling responses.[107] Proximity-based assays (FRET, BRET) showed that, in cells coexpressing MOR–α_{2A}AR heteromers, treatment with morphine

induces conformational changes in norepinephrine occupied α_{2A}AR leading to a decrease in $G_{\alpha i}$-mediated signaling.[108]

Finally, a study examining the trafficking of MOR–α_{2A}AR heteromers showed that treatment with the agonist to one receptor protomer did not lead to cointernalization of the heteromeric partner receptor.[109] However, given that, in addition to heteromers, the cells express individual receptor homomers, gives rise to the possibility that the agonists are in fact causing internalization of the homomer rather than the targeted receptor in the heteromer. Thus further studies are required to evaluate the trafficking properties of MOR–α_{2A}AR heteromers.

MOR–CB_1R heteromers—Several studies have described functional interactions between MOR and CB_1R (reviewed in Ref. 43). These include studies showing that (i) Δ^9-THC enhances the potency of morphine in animal models[4]; (ii) conditioned place preference to Δ^9-THC is not observed in MOR knockout mice[17]; and (iii) CB_1R knockout mice exhibit a reduction in the reinforcing properties of morphine as well as in the severity of withdrawal symptoms to this opioid.[182]

Coimmunoprecipitation, BRET, and FRET assays using differentially epitope-tagged receptors detected the presence of MOR–CB_1R heteromers in heterologous expressing systems.[89,110] While the presence of unliganded CB_1R does not affect MOR-mediated signaling, ligand binding to CB_1R decreases MOR-mediated signaling.[89] Conversely, ligand binding to MOR decreases CB_1R-mediated signaling.[89] These interactions had physiological relevance as observed by inhibition of neurite outgrowth in Neuro2A cells that coexpress MOR and CB_1R following cotreatment with agonists to both receptors.[89] Electrophysiological studies with oocytes coexpressing MOR and CB_1R and where one of the receptors is fused to a chimeric G_{qi5} showed that agonists to either receptor elicited Ca^{2+}-activated Cl^- currents thus demonstrating the formation of a functional receptor heteromer.[110] However, this study did not examine the effect of coadministration of agonists to both receptors on Ca^{2+}-activated Cl^- currents.

MOR–chemokine receptor 5 heteromers—Studies have described the presence of MOR in immune cells and that receptor activation by morphine increases the expression of chemokine receptor 5 (CCR5) suggesting interactions between these two receptors.[183,184] Coimmunoprecipitation studies using differentially epitope-tagged receptors showed that MOR–CCR5 interacting complexes could be detected only in cells coexpressing both receptors.[111,112] Examination of the properties of the MOR–CCR5 heteromer showed that treatment with either the MOR agonist, DAMGO, or the CCR5 agonist, RANTES, induced chemotaxis in cells coexpressing both receptors.[111] However, pretreatment with DAMGO inhibited RANTES-induced chemotaxis, increased CCR5 phosphorylation, and decreased G-protein coupling to CCR5.[111] The converse was observed when cells were pretreated with RANTES[111] suggesting cross-desensitization of MOR and CCR5 within the heteromeric complex.

MOR–gastrin-releasing peptide receptor heteromers—Studies showing that (i) MOR agonist administration induces itching (also known as pruritus)[185–187]; (ii) scratching responses to itch-inducing stimuli including morphine administration are abrogated in mice lacking gastrin-releasing peptide receptor (GRPR)[113,188,189]; and (iii) colocalization of MOR and GRPR in the dorsal horn of the spinal cord[113] led to the examination of interactions between the MOR isoform, MOR1D (comprising exons 1–3 and 8–9 of the *Oprm* gene) and GRPR.[113]

Direct interactions between MOR1D and GRPR were suggested by coimmunoprecipitation studies carried out in heterologous cells expressing epitope-tagged receptors or with spinal cord membranes that endogenously express both receptors.[113] Examination of the signaling properties of the MOR1D–GRPR heteromer showed that morphine-induced intracellular Ca^{2+} release only in cells coexpressing both receptors.[113] Interestingly, treatment with morphine enhanced GRPR internalization, while treatment with the GRPR agonist had no effect on MOR internalization in cells coexpressing both receptors.[113]

MOR–orphanin FQ/nociceptin receptor heteromers—Interactions between the orphanin FQ/nociceptin receptor (OFQ/N) and MOR have also been described. These include studies showing that an OFQ/N receptor antagonist potentiated the analgesic effects of morphine both in naïve mice and in mice that were tolerant to this opioid.[190,191] Coimmunoprecipitation studies using differentially epitope-tagged receptors showed that MOR and OFQ/N receptors could form interacting complexes in cells coexpressing both receptors.[117,118] MOR–OFQ/N heteromers exhibited an enhanced affinity for MOR-selective ligands.[117] Interestingly, MOR–OFQ/N receptor heteromerization attenuated the potency of MOR-mediated inhibition of adenylyl cyclase activity and ERK1/2 phosphorylation.[118] Moreover, treatment with the OFQ/N receptor agonist, nociceptin, led to desensitization of both MOR- and OFQ/N receptor-mediated signaling, while treatment with the MOR agonist, DAMGO, desensitized only MOR-mediated signaling.[118] These studies suggest that MOR–OFQ/N receptor heteromerization may play a role in modulating MOR receptor function.

MOR–NK-1 receptor heteromers—Substance P exerts its effects through the activation of neurokinin-1 (NK-1) receptors. Both MOR and NK-1 receptors have been detected in dorsal horn neurons of the spinal cord as well as in brain regions implicated in mood disorders, stress responses as well as those that mediate the rewarding effects of drugs of abuse.[192,193] In addition, treatment with morphine leads to an increase in the expression of NK-1 receptors in cortical neurons leading to a greater substance P-mediated Ca^{2+} mobilization than in untreated neurons.[194] Moreover, activation of NK-1 receptors modulates neuronal morphine-mediated endocytosis and MOR desensitization.[195] These observations together with those showing that the rewarding effects of morphine are not present in NK-1 receptor knockout mice led to studies examining whether these two receptors formed heteromeric complexes.[196] Coimmunoprecipitation and BRET assays using differentially epitope-tagged receptors demonstrate the presence of MOR–NK-1 receptor heteromers in HEK-293 cells coexpressing both receptors.[116] MOR–NK-1 receptor heteromerization did not lead to changes in the binding and signaling properties of individual protomers.[116] However, treatment with the MOR-selective agonist, DAMGO, led to phosphorylation and cointernalization of NK-1 receptors, while treatment with substance P induced the phosphorylation and cointernalization of MOR.[116] Moreover, in cells coexpressing MOR and NK-1 receptors but not in cells expressing only MOR, cotreatment with substance P and DAMGO-induced cointernalization of both receptors along with β-arrestin into the same endosomal compartment leading to a delay in the recycling and resensitization kinetics of MOR.[116]

MOR–somatostatin 2A receptor heteromers—Another receptor pair investigated for the possible formation of heteromeric complexes involved MOR and somatostatin 2A receptors (sst2a) based on the fact that the latter exhibited 38% homology with the amino acid sequence of MOR.[119] Coimmunoprecipitation studies using differentially epitope-tagged receptors demonstrated the presence of interacting MOR–sst2a receptor complexes in HEK-293 cells coexpressing both receptors.[119] MOR–sst2a receptor heteromerization did not affect protomer-mediated ligand binding and signaling; however, it modulated agonist-

mediated receptor trafficking.[119] In this context, in cells coexpressing MOR and sst2a, the sst2a receptor agonist promoted the internalization of both receptors, while the MOR agonist promoted only the internalization of MOR.[119] Although it is possible that MOR–sst2a heteromers do not undergo internalization following treatment with MOR agonists (the observed internalization being that of MOR homomers), it is to be noted that MOR agonists desensitize sst2a receptor signaling and sst2a receptor agonists desensitize MOR-mediated signaling.[119]

KOR–β_2AR heteromers—Both KOR and β_2AR are present in the heart and treatment with the β_2AR agonist, isoproterenol, has been shown to increase radiolabeled U69,593 binding to KOR present in rat cardiac sarcolemma.[197,198] This suggested interactions between KOR and β_2AR. Coimmunoprecipitation studies using differentially epitope-tagged receptors expressed in HEK-293 cells indicated that KOR and β_2AR formed interacting complexes.[87] While KOR–β_2AR heteromerization did not affect the pharmacology of each protomer in the complex, it did modulate trafficking and signaling as examined by observing the phosphorylation of ERK1/2 in cells coexpressing both receptors.[87] For example, treatment with a β_2AR agonist reduced β_2AR internalization in cells coexpressing KOR–β_2AR, but could induce internalization in cells expressing only β_2AR.[87] Moreover, β_2AR-mediated increases in ERK1/2 phosphorylation are significantly attenuated in cells coexpressing KOR–β_2AR heteromers. Together, these results indicate an important physiological role for KOR–β_2AR heteromerization in modulation of β_2AR function.

KOR–apelin receptor heteromers—The apelin receptor (APJ) binds to the apelin peptide and plays a role in the modulation of several functions including cardiovascular functions, neuroprotection, pain perception, and regulation of food and water intake.[199,200] Given that both KOR and APJ exhibit a similar distribution and function in the cardiovascular and nervous system, studies examined the possible heteromerization between these two receptors.[106] Coimmunoprecipitation, colocalization, and BRET assays show that KOR and APJ can form interacting complexes in HEK-293 cells.[106] KOR–APJ heteromerization leads to potentiation of ERK1/2 phosphorylation following treatment with either the APJ or KOR agonist compared to what is observed in cells expressing only homomers for either receptor.[106] Moreover, KOR–APJ heteromerization leads to a decrease in the activity of protein kinase A, an increase in the activity of protein kinase C, and an increase in cell proliferation.[106]

2.3. Cannabinoid receptor heteromerization

Compared to opioid receptors, fewer studies have examined heteromerization of cannabinoid receptors. While heteromerization between opioid receptor subtypes and CB_1R has been described in Section 2.1, in this section we describe cannabinoid receptor heteromers that do not involve opioid receptors (see Table 9.2).

CB_1R–adenosine 2A receptor heteromers—Several studies suggested interactions between CB_1R and adenosine 2A receptors ($A_{2A}R$). These include studies showing that (i) activation of $A_{2A}R$ blocks, while inhibition of $A_{2A}R$ promotes, CB_1R-mediated effects on evoked glutamate release[210]; (ii) the rewarding and aversive effects of Δ^9-THC were significantly reduced in $A_{2A}R$ knockout mice[211]; (iii) $A_{2A}R$ antagonist treatment blocks the synergy between MOR and CB_1R for CRE-mediated gene transcription[212]; (iv) locomotor behavior induced by administration of $A_{2A}R$ antagonists is attenuated by CB_1R antagonists or in CB_1R knockout mice[213]; and (v) low doses of $A_{2A}R$ antagonists attenuated while higher doses potentiated the self-administration of Δ^9-THC and anandamide.[214]

The formation of interacting CB_1R–$A_{2A}R$ complexes in rat striatum was detected by coimmunoprecipitation studies using receptor-selective antibodies.[201] BRET using differentially epitope receptors demonstrated that CB_1R and $A_{2A}R$ are in close proximity to interact in HEK-293 cells.[201] Studies carried out in SH-SY5Y neuroblastoma cells, that endogenously express CB_1R and $A_{2A}R$, show that CB_1R activation inhibits forskolin-mediated increases in cAMP levels, and that the latter is blocked by an $A_{2A}R$ antagonist.[201] Moreover, $A_{2A}R$-mediated increases in cAMP levels are blocked by a CB_1R antagonist.[201]

CB_1R–AT1 angiotensin receptor heteromers—Early studies demonstrating an upregulation of CB_1R in liver cells that have resident AT1 angiotensin receptors (AT1R) prompted the examination of interactions between these two receptors.[215–218] The heteromerization of CB_1R and AT1R was demonstrated by coimmunoprecipitation, colocalization, and BRET assays using differentially epitope-tagged receptors.[10] Examination of the signaling properties of the CB_1R–AT1R heteromer shows that, in cells coexpressing both receptors, the AT1R agonist induces a rapid, robust dose-dependent increase in ERK1/2 phosphorylation that is drastically reduced either by RNAi-mediated knockdown of CB_1R or by inhibiting the activity of diacylglycerol lipase, the enzyme responsible for the synthesis of the endocannabinoid 2-AG.[10] In addition, CB_1R agonists potentiate while antagonists block AT1R-mediated increases in ERK1/2 phosphorylation.[10] Interestingly, an antibody that selectively recognizes the CB_1R–AT1R heteromer blocks AT1R-mediated ERK signaling in cells coexpressing both receptors.[10] Moreover, in cells expressing the CB_1R–AT1R heteromer, CB_1R-mediated ERK1/2 phosphorylation is only observed following activation of AT1R by a very low dose of agonist that by itself does not induce ERK1/2 phosphorylation.[10]

Examination of receptor G-protein coupling shows that, in cells expressing the CB_1R–AT1R heteromer, AT1R-mediated ERK1/2 phosphorylation occurs via $G_{\alpha i}$ instead of $G_{\alpha q}$. Moreover, AT1R-mediated intracellular Ca^{2+} mobilization occurs via $G_{\alpha q}$ but requires the presence of CB_1R since it is significantly attenuated by RNAi-mediated knockdown of CB_1R.[10] Interestingly, activation of CB_1R did not increase intracellular Ca^{2+} mobilization, although the CB_1R agonist could potentiate AT1R-mediated effects.[10] Taken together, these studies show that CB_1R–AT1R heteromerization provides a mechanism for modulation of AT1R function by CB_1R that could be of clinical relevance in pathologies involving these receptors (see Section 3).

CB_1R–β_2AR heteromers—Based on coexpression of CB_1R and β_2AR in several tissues and on inhibition of noradrenergic neurotransmission by CB_1R a study examined whether both receptors formed interacting complexes.[202] BRET assays using differentially epitope-tagged receptors showed that both receptors were in close proximity to form complexes in live cells.[202] The BRET signal was increased in the presence of a CB_1R antagonist suggesting that the latter either caused a change in receptor conformation (bringing in close proximity the acceptor and donor pairs) or caused an increase in the surface levels of this heteromer.[202] Heteromerization between CB_1R and β_2AR was shown to lead to alterations in the signaling properties of individual receptors in that it led to an increase in CB_1R-mediated ERK1/2 phosphorylation and a decrease in CREB phosphorylation compared to cells expressing only CB_1R.[202] Moreover, β_2AR-mediated ERK1/2 phosphorylation was attenuated by an inverse agonist but not by a neutral agonist to CB_1R.[202] CB_1R–β_2AR heteromerization modulated the trafficking properties of individual receptors in that treatment with a β_2AR receptor agonist induced CB_1R internalization in cells expressing both receptors.[202]

CB_1R–CB_2R heteromers—A recent report has described heteromerization between CB_1R and CB_2R *in vivo*.[148] The authors used BRET assays with differentially epitope-

tagged receptors in HEK-293 cells to show that CB_1R and CB_2R are in close proximity in live cells.[148] They also carried out a proximity ligation assay in SH-SY5Y neuroblastoma cells (endogenously express CB_1R) transfected with epitope-tagged CB_2R, in pinealocytes obtained from rat pineal glands, and in rat nucleus accumbens slices to show that both receptors are in close proximity (<17 nm) to form interacting complexes.[148] The authors examined the signaling properties of CB_1R–CB_2R heteromers in SH-SY5Y cells transfected with CB_2R and reported that a CB_2R agonist induces Akt/PKB phosphorylation only in cells that coexpressed both receptors, and that this is significantly attenuated by cotreatment with the CB_1R agonist.[148] Interestingly, this translated into a physiological response given that CB_2R-mediated neurite outgrowth in cells expressing CB_1R–CB_2R heteromers was blocked by the CB_1R agonist.[148] These results suggest that the interactions between CB_1R and CB_2R are antagonistic in nature.

CB_1R–dopamine D2 receptor heteromers—A number of studies have reported interactions between CB_1R and D2R. These include studies showing that (i) the D2R agonist, quinpirole, mediated motor behavior is stimulated by the CB_1R antagonist, SR141716A[203]; (ii) G-protein coupling to CB_1R is modulated by D2R in cells coexpressing both receptors[204,205]; and (iii) in primary striatal neuronal cultures treatment either with the CB_1R agonist, Hu-210, or with the D2R agonist, quinpirole, leads to inhibition of forskolin-stimulated cAMP levels,[204] whereas coadministration of Hu-210 and quinpirole resulted in increases in cAMP levels mediated via CB_1R stimulation of $G_{\alpha s}$.[204] Interestingly, while the presence of D2R is required for CB_1R coupling to $G_{\alpha s}$, persistent activation of D2R switches the coupling from $G_{\alpha s}$ to $G_{\alpha i}$.[205] The formation of CB_1R–D2R heteromeric complexes was demonstrated by coimmunoprecipitation studies using differentially epitope-tagged receptors that show that cotreatment with subsaturating concentrations of agonists to both receptors causes an increase in the levels of CB_1R–D2R heteromers.[206]

CB_1R–orexin-1 receptor heteromers—Coimmunoprecipitation studies along with homogenous TR-FRET assays using SNAP- and CLIP-tagged substrates were used to show that CB_1R and orexin-1 receptors (OX1R) form multimeric complexes.[207] Examination of the trafficking properties of this heteromer showed that the OX1R agonist was more efficient at promoting heteromer internalization compared to OX1R internalization.[207] In heterologous cells coexpressing CB_1R and OX1R, a $G_{q/11}$-coupled receptor, treatment with the OX1R agonist led to a potent and long-lasting increase in ERK1/2 phosphorylation compared to cells expressing only OX1R, and that this increase was blocked by a CB_1R antagonist or by pertussis toxin pretreatment.[208] Moreover, coadministration of CB_1R and OX1R agonists did not result in additive effects suggesting that just the presence of CB_1R was sufficient to modulate OX1R signaling.[208] Given the involvement of CB_1R and OX1R in feeding, targeting this heteromer pair could lead to the identification of novel therapeutics for the treatment of obesity.

CB_1R–D2R–$A_{2A}R$ heteromers—Above, we described studies showing heteromerization between CB_1R and D2R and between CB_1R and $A_{2A}R$. In addition, D2R–$A_{2A}R$ heteromerization has been reported.[219] This led to the hypothesis that the three receptors could associate to form an oligomeric complex. This was tested with the sequential use of BRET and FRET assays.[209] BiFC assay was combined with BRET to show that the three receptors were in close proximity to interact.[129] Although this finding is exciting, further studies are needed to determine whether these heterotrimers are present endogenously, and if so, what is their tissue distribution and physiological role.

2.4. Dopamine receptor heteromerization

Several studies have examined heteromerization between dopamine receptor subtypes and between the latter and other GPCRs. Since heteromerization between dopamine receptors and opioid receptor subtypes or CB_1R have been described in Sections 2.2 and 2.3, we describe dopamine receptor heteromers that do not involve opioid or cannabinoid receptors in this section (see Table 9.3).

D1R–D2R heteromers—Interactions between D1R and D2R were suggested by the colocalization of D1R and D2R in MSNs in the striatum (reviewed in Refs. 48–50). Further evidence for direct interaction was suggested by the findings that (i) there is a requirement of D1R activation for D2R-mediated locomotor activity[241]; (ii) enhancement of the acute effects of cocaine requires activation of D1R and of D2R[242]; and (iii) potentiation in the release of arachidonic acid is observed upon coactivation of D1R and D2R in cells expressing both receptors.[243]

Evidence for direct interactions between D1R and D2R came from coimmunoprecipitation and confocal FRET studies carried out with rat striatal membranes, primary rat striatal neuronal cultures or with cells coexpressing epitope-tagged receptors.[220–224] In addition, protein complementation studies where coexpression of D1R fused to a fragment of luciferase and of D2R fused to a complementary luciferase fragment resulted in the reconstitution of active luciferase supported D1R–D2R heteromerization.[125] Signaling assays showed that while activation of D1R led to $G_{\alpha s/olf}$-mediated signaling and of D2R to $G_{\alpha i}$-mediated signaling, coactivation of both receptors led to increases in intracellular Ca^{2+} levels.[220,221] Since the increase in intracellular Ca^{2+} levels is not observed in cells that express only D1R or D2R this suggested that the D1R–D2R heteromer can couple to $G_{\alpha q}$ to induce Ca^{2+}-mediated signaling.[220,221] Interestingly, occupancy of D1R in cells expressing D1R–D2R heteromers results in rapid desensitization of heteromer-mediated Ca^{2+} signaling that is regulated by both the catalytic and RGS domains of GRK2.[223]

The D1R agonist, SKF83959, has been proposed as a D1R–D2R heteromer-selective ligand based on the finding that (i) it did not increase adenylyl cyclase activity in cells coexpressing D1R–D2R or expressing only D1R; (ii) it increased intracellular Ca^{2+} release in cells coexpressing D1R–D2R, and this effect was potentiated by coadministration of quinpirole; and (iii) it increased [^{35}S]GTPγS binding to $G_{\alpha q}$ and to $G_{\alpha i}$ in cells coexpressing D1R–D2R.[221] Finally, D1R–D2R-mediated signaling by SKF83959 in primary striatal neurons via $G_{\alpha q}$ was found to lead to activation of both cytosolic and nuclear Ca^{2+}/calmodulin-dependent kinase IIα resulting in increased expression of BDNF and ultimately rapid neuronal maturation and differentiation.[222] Moreover, treatment with SKF83959 increased grooming behavior in rats and decreased the phosphorylation of the GluR1 subunit of AMPA receptors via Ca^{2+}/calmodulin-dependent kinase IIα in the nucleus accumbens.[224]

D1R–D3R heteromers—Interactions between D1R and D3R were suggested by studies showing that (i) D1R agonists could induce the expression of mRNA and increase D3R levels, and that this could be blocked by a D1R antagonist[244,245]; (ii) the coactivation of D1R and D3R caused a potentiation in the expression of substance P mRNA[246,247]; (iii) D3R knockout mice exhibit a decrease in D1R-mediated expression of the immediate early gene, c-fos[248]; and (iv) cross talk between D1R and D3R may play a role in rearing habituation and reward behavior to low doses of cocaine.[249]

Evidence for direct interactions came from coimmunoprecipitation studies showing that D1R and D3R form interacting complexes in cells coexpressing both receptors and in rat striatal membranes.[225] BRET assays showed that the two receptors were in close proximity in living cells.[225] Treatment with dopamine increased the BRET signal, and this effect could

be blocked by expression of a dominant negative mutant of dynamin leading to the suggestion that the increased signal could be due to internalized D1R–D3R heteromers.[225] Binding studies with a radiolabeled D1R antagonist showed that dopamine binds with higher affinity to D1R in the presence of D3R.[225] This is translated into more potent dopamine-mediated stimulation of adenylyl cyclase activity in cells coexpressing D1R and D3R than in cells expressing only D1R.[225] Examination of the trafficking properties of D1R–D3R heteromer showed that treatment with either a D1R or a D3R agonist did not induce heteromer internalization; however, cotreatment with agonists to both receptors induced the internalization of the D1R–D3R heteromer.[225]

D2R–D3R heteromers—Functional complementation studies suggested that D2R and D3R could form heterodimers.[227] For example, expression of D2R with a mutant D3R receptor (that cannot signal) restores the ability of D3R agonists to inhibit adenylyl cyclase activity.[227] Coimmunoprecipitation assays with differentially epitope-tagged receptors showed that D2R and D3R could form interacting complexes.[227] Signaling assays show that preferential D3R versus D2R agonists (pramipexole, ropinirole, or S32504) were more potent at inhibiting the activity of a chimeric adenylyl cyclase (does not respond to D3R activation) in cells coexpressing D2R and D3R compared to cells expressing only D2R (reviewed in Ref. 228). In contrast, preferential D2R agonists such as pergolide showed a marginal increase in potency in cells coexpressing the D2R–D3R heteromer while no changes were observed for the full D2R/D3R agonist, quinpirole (reviewed in Ref. 228). Interestingly, partial D2R/D3R agonists with antipsychotic activity did not inhibit adenylyl cyclase activity in cells coexpressing both receptors and also could block quinpirole-mediated inhibition of cAMP levels (reviewed in Ref. 228). This suggests that in cells expressing D2R–D3R heteromers partial D2R/D3R agonists can function as antagonists (reviewed in Ref. 228).

D1R–adenosine A_{1A}R heteromers—Interactions between D1R and A_{1A}R receptors were suggested by studies showing that (i) A_{1A}R activation leads to a decrease in D1R-mediated locomotion and oral dyskinesia in animals,[250] while A_{1A}R antagonists enhance D1R-mediated locomotion[251]; (ii) radiolabeled D1R antagonist affinity is decreased by A_{1A}R agonist[252]; (iii) A_{1A}R agonists modulate D1R-mediated electroencephalographic arousal[11]; and (iv) in cells coexpressing A_{1A}R and D1R, A_{1A}R antagonists cause an increase in both basal- and dopamine-mediated increases in cAMP levels while an A_{1A}R agonist blocks dopamine-mediated effects.[253]

Coimmunoprecipitation studies using receptor-selective antibodies detected the presence of D1R–A_{1A}R interacting complexes in cells coexpressing both receptors.[226] In fibroblast cells that coexpress D1R and A_{1A}R, pretreatment with agonists to both receptors decreases D1R-mediated increases in cAMP levels, while pretreatment with either receptor agonist does not. Additional studies using proximity-based assays to confirm the formation of D1R–A_{1A}R heteromers *in vivo* as well as studies to evaluate whether the D1R–A_{1A}R heteromer exhibits novel properties compared to individual receptor homomers need to be carried out.

D2R–adenosine A_{2A}R heteromers—Interactions between D2R and A_{2A}R are suggested by studies showing that (i) D2R and A_{2A}R are colocalized in GABAergic striatopallidal neurons[254]; (ii) selective A_{2A}R agonists decrease D2R binding and signaling[229,255,256]; (iii) Blockade or knockdown of D2R impairs locomotive behavior, and this effect can be attenuated by A_{2A}R antagonists[257,258]; and (iv) antagonists of D2R produce catalepsy and this is blocked by A_{2A}R antagonists.[259] The formation of interacting D2R–A_{2A}R complexes was demonstrated by coimmunoprecipitation studies and by proximity-based assays such as BRET and FRET.[219,230–232] Investigation of the pharmacological properties of the D2R–A_{2A}R in cells coexpressing both receptors showed

that D2R agonists caused a decrease in the affinity for radiolabeled $A_{2A}R$ agonist binding.[229] Moreover, signaling assays showed that D2R agonists decreased the efficacy and potency of $A_{2A}R$ agonists in cells coexpressing both receptors.[229] In addition, studies showed that treatment with either $A_{2A}R$ or the D2R agonists causes internalization of the D2R–$A_{2A}R$ heteromer.[219,260]

Bivalent ligands comprising of a D2R agonist joined by spacers of different lengths (26–118 atoms) to an $A_{2A}R$ antagonist have been used to show that in striatal membranes coexpressing both receptors, these ligands cause a greater displacement of radiolabeled ligand binding to either D2R or $A_{2A}R$ than the corresponding monovalent ligands.[261]

D2R–ghrelin receptor heteromers—A recent study examined D2R and ghrelin receptor (GHSR1a) interactions because of their robust colocalization to a subset of hypothalamic neurons.[233] Time-resolved FRET carried out in heterologous cells and with hypothalamic membrane preparations demonstrated that the two receptors are in close proximity to form heteromers.[233] In cells coexpressing D2R and GHSR1a, activation of D2R led to increases in intracellular Ca^{2+} release and not to $G_{\alpha i}$-mediated signaling and this could be blocked by a D2R or a GHSR1a antagonist.[233] Moreover, cotreatment with agonists to both receptors led to additive effects on intracellular Ca^{2+} release.[233] In addition, cross-desensitization of Ca^{2+} release is observed in cells coexpressing both receptors in that a 30-min pretreatment with agonist to one receptor attenuated intracellular Ca^{2+} release mediated via the other receptor.[233] Studies with wild-type and ghrelin receptor knockout mice showed that administration of a D2R agonist caused a reduction in food intake in wild-type but not in knockout mice.[233] This reduction in food intake can be blocked by a GHSR1a antagonist.[233] These results indicate that D2R–GHSR1a heteromerization could play an important role in the modulation of food intake.

D2R–neurotensin receptor heteromers—Interactions between dopamine and neurotensin receptors were demonstrated by studies showing that (i) type 1 neurotensin receptors (NTS1) are expressed in substantia nigra and ventral tegmental area dopaminergic neurons[262]; (ii) NTS1 has excitatory effects on dopaminergic neurons present in the midbrain[262]; (iii) neurotensin causes a modest decrease in the affinity of D2R for receptor agonists[262]; (iv) D2R autoreceptor function is decreased by NTS1 activation via a protein kinase C- and Ca^{2+}-dependent mechanism[262]; and (v) activation of D2R attenuates NTS1-mediated Ca^{2+} mobilization.[262]

A combination of immunoprecipitation of the long isoform of D2R ($D2R_L$) with fluorescent spectroscopy for detection of the presence of C-terminally epitope tagged (with enhanced CFP) NTS1 in the immunoprecipitate indicated that the two receptors formed interacting complexes only in cells coexpressing both receptors.[234] Radiolabeled binding experiments with membrane preparations from cells coexpressing $D2R_L$ and NTS1 showed that the NTS1 agonist decreased the affinity of radiolabeled agonist binding to $D2R_L$.[234] However, dopamine ligands (agonist or antagonist) had no effect on radiolabeled agonist binding to NTS1.[234] Signaling assays showed that the potency of $D2R_L$-mediated signaling is increased in the presence of neurotensin.[234] Internalization assays showed that in cells coexpressing $D2R_L$ and NTS1 treatment with NTS1 agonist increased $D2R_L$ internalization via a PKC-dependent mechanism.[235]

D2R–oxytocin receptor heteromers—Studies showing (i) the involvement of striatal oxytocin receptors (OTR) in pair bond formation in monogamous prairie female voles and in regulating social and emotional behaviors in animals (rodents, sheep, humans)[263–267]; (ii) the involvement of ascending dopaminergic neurons in social attachment behavior[1,2]; (iii) that a D2R antagonist can block partner preference[268,269]; and (iv) administration of a D2R

agonist into the nucleus accumbens can induce partner preference and this is blocked by an OTR antagonist[268,269] suggest interactions between D2R and OTR.

BRET assays in cells coexpressing both receptors showed that D2R and OTR are in close proximity in live cells.[236] *In situ* proximity ligation assays detected the presence of D2R–OTR heteromers in the neuropil of nucleus accumbens and in the dorsal striatum of rats.[236] Binding assays with nucleus accumbens membrane preparations showed that a low, but not high, concentration of the OTR agonist increase the maximal number of binding sites of a radiolabeled D2R antagonist and this is blocked by an OTR antagonist.[236] Signaling assays with nucleus accumbens membrane preparations showed that the OTR agonist increases the efficacy and potency of D2R-mediated G-protein activity.[236] These studies indicate that D2R–OTR heteromers may play a role in modulating social and emotional behavior.

D2R–somatostatin receptor heteromers—Interactions between dopamine and somatostatin receptors were suggested by studies showing that (i) a decrease of somatostatin levels attenuated dopamine receptor-mediated stereotypy as well as locomotor responses to amphetamine[270]; (ii) dopamine enhances somatostatin receptor-mediated inhibition of adenylyl cyclase activity[271,272]; and that (iii) a dopamine receptor agonist attenuates the chronic effects of a long-acting somatostatin analogue.[273] This led investigators to examine heteromerization between D2R and somatostatin receptor subtypes.

Direct interactions between D2R and somatostatin receptor 2 subtype (SSTR2) was examined by coimmunoprecipitation and pbFRET assays that demonstrated that these two receptors formed complexes in heterologous cells coexpressing both receptors as well as in primary striatal neuronal cultures.[237] Binding studies showed that in cells coexpressing D2R and SSTR2 a combination of agonists to both receptors displaced radiolabeled antagonist binding to D2R more efficiently than the D2R agonist alone.[237] Interestingly, in these cells, the SSTR2 agonist could dose-dependently displace radiolabeled antagonist binding to D2R.[237] Also, the total number of binding sites for the radiolabeled ligand binding to SSTR2 was increased in the presence of dopamine.[237] Examination of signaling properties of the D2R–SSTR2 heteromer showed that D2R-mediated inhibition of cAMP levels was lower for some agonists (dopamine) and not others (quinpirole) than that observed in cells that expressed only D2R.[237] Moreover, the presence of the SSTR2 agonist caused an increase in the potency of quinpirole and in both the potency and efficacy of dopamine in inhibiting cAMP levels.[237] Examination of the trafficking properties of the D2R–SSTR2 heteromer showed that treatment with D2R agonist enhanced agonist-mediated internalization of SSTR2.[237]

Heteromerization between D2R and SSTR5 was suggested by the functional rescue of a C-tail deletion mutant of human SSTR5.[144] Only when coexpressed with D2R does agonist activation of the mutant SSTR5 lead to inhibition in forskolin-mediated increases in cAMP levels.[144] pbFRET supported the formation of D2R–SSTR5 heteromers.[144] Interestingly, treatment with agonists to either receptor or cotreatment with agonists to both receptors led to an increase in FRET efficiency, while a decrease in the latter was observed with D2R antagonist.[144] This would suggest that either agonist treatment leads to an increase in the level of D2R–SSTR5 heteromers or, alternatively, it changes receptor conformation leading to a decrease in the distance between the fluorescent probes.[144] The pharmacological properties of the D2R–SSTR5 heteromer were found to be distinct from those of individual receptor homomers in that the heteromers exhibited a greater affinity for the binding of each protomer agonist.[144] Moreover, occupancy of one of the receptors in the D2R–SSTR5 heteromer led to an increase in the binding affinity and signaling by the agonist to the second receptor.[144]

D2R–serotonin receptor heteromers—Studies showing that typical antipsychotic drugs used to treat schizophrenia are D2R antagonists and that some symptoms of schizophrenia are similar to those observed with hallucinogenic serotonin receptor agonists led to the examination of heteromerization between D2R and serotonin 5-HT_{2A} receptors (5-HT_{2A}R).[274,275]

Coimmunoprecipitation studies show that D2R and 5-HT_{2A}R form interacting complexes.[238] Examination of the pharmacological properties of the D2R–5-HT_{2A}R heteromer in cells or in striatal membranes coexpressing both receptors show that a D2R agonist increased the binding affinity of a 5-HT_{2A}R agonist, and this is blocked by a D2R antagonist.[238] The presence of D2R had no effect on serotonin-mediated inositol phosphate production, but it increased the potency of a hallucinogenic 5-HT_{2A}R agonist.[238] Interestingly, D2R activation had no effect on serotonin-mediated inositol phosphate production, but decreased the potency of the hallucinogenic 5-HT_{2A}R agonist.[238] Behavioral studies with 5-HT_{2A}R knockout mice show that the antipsychotic effects of the D2R antagonist, haloperidol, require the presence of 5-HT_{2A}R.[238]

D4R–α adrenergic receptor heteromers—Heteromer formation between D4R and α adrenergic receptors (αAR) was examined based on studies showing that the D4R gene is the most prominently expressed dopamine receptor gene in the rat pineal gland, and that its mRNA expression is circadian and controlled by adrenergic signaling.[276]

Coimmunoprecipitation and BRET studies with differentially epitope-tagged receptors show that D4R and α_{1B}AR form interacting complexes.[239] Pharmacological characterization of D4R–adrenergic receptor heteromers shows that the D4R agonist decreases the ability of an α_{1B}AR agonist to displace radiolabeled antagonist binding to α_{1B}AR.[239] Signaling assays in cells expressing the D4R–α_{1B}AR heteromer or in pineal gland preparations show that pretreatment or cotreatment with a selective D4R agonist leads to an inhibition of α_{1B}AR-mediated phosphorylation of ERK1/2 and of Akt/PKB.[239] Interestingly, a D4R receptor antagonist could also block α_{1B}AR-mediated signaling and the converse also occurred in that adrenergic antagonists could block D4R-mediated signaling.[239] Given that D4R expression is circadian, and that α_{1B}AR plays a role in modulating melatonin synthesis and release from the pineal gland, the formation of D4R–α_{1B}AR heteromers provides a means for regulating melatonin production and activity.[239]

D4R–β_1 adrenergic receptor heteromers—Studies examining D4R–αAR heteromerization also examined whether D4R and β_1 adrenergic receptors (β_1AR) formed heteromers. Coimmunoprecipitation and BRET studies with differentially epitope-tagged receptors show that D4R and β_1AR form interacting complexes.[239] Pharmacological characterization of D4R–β_1AR heteromers shows that the D4R agonist decreases the ability of a β_1AR agonist to displace radiolabeled antagonist binding to β_1AR.[239] Signaling assays in cells expressing either the D4R–β_1AR heteromer or in pineal gland preparations show that pretreatment or cotreatment with a selective D4R agonist leads to an inhibition of β_1AR-mediated phosphorylation of ERK1/2 and of Akt/PKB.[239] Interestingly, a D4R receptor antagonist could also block β_1AR-mediated signaling and the converse also occurred in that adrenergic antagonists could block D4R-mediated signaling.[239] These studies suggest that like the D4R–α_{1B}AR heteromer the D4R–β_1AR heteromer could also play a role in regulating melatonin production and activity.[239]

D4R–β_2 adrenergic receptor heteromers—Based on studies showing (i) colocalization of D2R-like receptors with β-AR in rat primary cortical neurons and in prefrontal cortex[277] and that (ii) D2R-like receptor activation inhibited β-AR-mediated increases in cAMP levels,[277] heteromerization between D4R and β_2-AR was examined.

Coimmunoprecipitation, BRET, and binding assays with β_2-AR immunoprecipitates, obtained from detergent solubilized mouse brain extracts using alprenolol coupled to Sepharose beads, show that D4R and β_2-AR form interacting complexes and are in close proximity in cells or tissues coexpressing both receptors.[240] However, further studies are needed to evaluate whether the D4R–β_2-AR heteromer exhibits novel properties compared to individual receptor homomers.

D2R–metabotropic glutamate receptor 5–adenosine$_{2A}$ receptor heteromers— The possibility of formation of heteromers involving D2R, metabotropic glutamate receptor 5 (mGluR5), and adenosine$_{2A}$ receptors ($A_{2A}R$) was suggested by studies showing (i) similar localization of these receptors in striatopallidal neurons[278–280]; (ii) that a mGluR5 agonist inhibited D2R-mediated contralateral turning behavior, and this was potentiated by an $A_{2A}R$ agonist and attenuated by an $A_{2A}R$ antagonist[281]; and (iii) that a mGluR5 agonist reduced the high affinity state for the D2R agonist, and this was potentiated by an $A_{2A}R$ agonist.[281]

Coimmunoprecipitation studies carried out with cells coexpressing D2R, $A_{2A}R$, and mGluR5, and also with rat striatal membranes, show that these receptors form interacting complexes.[127] BRET assays with cells expressing a constant ratio of $A_{2A}R$ tagged with luciferase and D2R tagged with YFP show that increasing amounts of mGluR5 can disrupt the BRET signal.[127] Similarly, increasing amounts of $A_{2A}R$ could disrupt the BRET signal between D2R tagged with luciferase and mGluR5 tagged with YFP.[127] This suggests that the three receptors are in close proximity to interact.[127] The formation of D2R–$A_{2A}R$–mGluR5 was confirmed by a combination of BRET and BiFC assays.[127] For this, cells were transfected with $A_{2A}R$ tagged with luciferase, mGluR5 fused with an N-terminal fragment of YFP and D2R fused with a C-terminal fragment of YFP.[127] A BRET signal was observed only when all three receptors were expressed in a cell suggesting that these receptors are in close proximity to form heterotrimers.[127] Further studies are needed in order to elucidate whether the D2R–$A_{2A}R$–mGluR5 heterotrimer exhibits novel properties compared to individual receptor homomers or heterodimers.

3. HETEROMERS IN DISEASE

Although it has become increasingly evident that GPCRs can form heterodimers,[61,63,64,282] very littleinformation is available aboutthe physiological role of GPCR heteromers. Pharmacological and coimmunoprecipitation studies using receptor-selective antibodies suggest that some receptor heteromers may play a role in the disease state. However, care has to be taken in the interpretation of such data given that such studies do not rule out a contribution of receptor homomers to the disease state. The recent development of antibodies that selectively recognize receptor heteromers, but not receptor homomers, and of TAT-based peptides that can disrupt heteromer formation, has provided us with tools to study the role of GPCR heteromers in the disease state.[7–10] In the following sections, we describe a few cases where a role for GPCR heteromers involving opioid, cannabinoid, and dopamine receptors in pathology has been implicated.

Analgesia and addiction

Antibodies that selectively recognize the MOR–DOR heteromer detect increased heteromer levels in brain regions involved in pain processing following chronic morphine administration under a paradigm that leads to the development of tolerance.[7] Taken with the fact that MOR–DOR heteromers signal via the β-arrestin-mediated signaling cascade[100] and that β-arrestin or DOR knockout mice do not develop tolerance to morphine[162,283] would suggest that MOR–DOR heteromers play a role in the development of tolerance to this opioid. This is supported by a study showing that the administration of a MOR

transmembrane 1 TAT peptide fusion protein, which selectively disrupts the MOR–DOR heteromer, resulted in an enhanced analgesic response to morphine with a concomitant decrease in tolerance to the drug.[8]

Studies have also examined whether the DOR–MOR heteromer could be a target for the development of therapeutics that are as efficacious as morphine but lack the associated adverse side effects. In this context, a study showed that low doses of the DOR antagonists, TIPPψ or naltrindole, could potentiate intrathecal morphine-mediated analgesia.[94,160] Moreover, DOR antagonist administration to animals exhibiting tolerance to morphine led to a restoration of the analgesic response and potency of morphine.[160] Based on similar observations, bivalent ligands targeting the DOR–MOR heteromer were developed.[284] These bivalent ligands comprised a MOR agonist pharmacophore, oxymorphone, joined to a DOR antagonist pharmacophore, naltrindole, by spacers of variable lengths (16–21 atoms).[284] The bivalent compounds exhibited antinociceptive activity.[284] Moreover, compounds with spacer lengths between 19 and 21 atoms did not exhibit antinociceptive tolerance and physical dependence and were less rewarding than morphine.[284,285]

An involvement of DOR–KOR heteromers in thermal allodynia was suggested by studies demonstrating the presence of these heteromers in peripheral sensory neurons.[286] Moreover, the antiallodynic effects of the KOR agonist, 6′-GNTI, to prostaglandin E2-induced thermal allodynia were blocked by either DOR or KOR antagonists.[286] This, together with the finding that the antiallodynic effects of the DOR agonist, DPDPE, were potentiated by a DOR–KOR heteromer-selective antibody supports a role for the DOR–KOR heteromer in modulating thermal allodynia.[286]

A recent study has implicated the CB_1R–DOR heteromer in neuropathic pain.[9] This study found that levels of CB_1R and DOR are increased in distinct brain regions following peripheral nerve lesion.[9] An antibody selective for the CB_1R–DOR heteromer detected increased heteromer levels in the cortex, hypothalamus, and striatum of lesioned animals.[9] In addition, CB_1R–DOR heteromerization led to a decrease in DOR activity in the cortex of lesioned animals[9] and this decrease could be attenuated by low, nonsignaling doses of CB_1R ligands.[9] Thus, the CB_1R–DOR heteromer is a novel target for the treatment of neuropathic pain.

Liver fibrosis

Antibodies that selectively recognize the CB_1R–AT1R heteromer detect a significant upregulation of this heteromer in hepatic stellate cells of rats chronically treated with ethanol.[10] This leads to increased AT1R-mediated signaling that can be blocked by a CB_1R antagonist or by inhibiting the synthesis of the endocannabinoid, 2-AG.[10] In addition, the AT1R-mediated increase in profibrogenic markers in hepatic stellate cells of rats chronically treated with ethanol was completely blocked by a CB_1R antagonist.[10] These results suggest that the profibrogenic activity of AT1R in ethanol-induced liver fibrosis requires CB_1R activity. Thus, the CB_1R–AT1R heteromer represents a novel therapeutic target for the treatment of this disease.

Psychiatric disorders

An involvement of dopamine receptor subtype heteromers in major depression has been suggested by studies showing an increase in D1R–D2R interacting complexes in the striatum of postmortem individuals that suffered from major depression.[287] Interestingly, bilateral infusion into the rat prefrontal cortex of a TAT peptide that disrupts D1R–D2R heteromers results in a decrease in the levels of D1R–D2R heteromers and leads to

antidepressant-like effects in the forced swim test.[287] This suggests an involvement of D1–D2 receptor heteromers in the pathology associated with major depression.

Involvement of D1R–D2R heteromers in schizophrenia was suggested by studies showing that clozapine, a drug used to treat this condition, binds to both D1R and D2R but that the high affinity binding to D1R decreases in the presence of D2R.[288,289] Moreover, FRET assays showed that clozapine appears to modulate the formation of D1R–D2R heteromers in that high concentrations of the drug increase the FRET signal between D1R and D2R while low concentrations attenuate this signal.[288,289] Further support for the involvement D1R–D2R heteromers in schizophrenia came from studies showing that acute treatment with a heteromer-selective agonist increased the striatal expression of brain-derived neurotrophic factor, a molecule that has been linked to schizophrenia (for review, see Ref. 290).

An involvement of D2R–5-HT_{2A}R heteromers in schizophrenia is suggested by studies showing that coexpression of D2R in cells increases the signaling potency of a hallucinogenic 5-HT_{2A}R agonist, but activation of D2R decreases it.[238] Moreover, the antipsychotic effects of the D2R antagonist, haloperidol, are decreased in 5-HT_{2A}R knockout mice[238] suggesting that the D2R–5-HT_{2A}R heteromer is a target of this drug.

An involvement of CB_1R–D2R–A_{2A}R heteromers in schizophrenia has been suggested based on studies showing upregulation of CB_1R, D2R, and A_{2A}R in the postmortem brains of schizophrenics.[291–293] Moreover, in animal models of schizophrenia, treatment with antipsychotics decreases CB_1R levels in the nucleus accumbens.[294] These reports together with studies describing CB_1R–D2R and CB_1R–A_{2A}R interactions (described in Section 2) and studies showing that these three receptors can form heteromers in live cells suggest that the CB_1R–D2R–A_{2A}R heteromer could be a novel therapeutic target in the treatment of schizophrenia.

Parkinson’s disease

The involvement of D2R–A_{2A}R heteromers in Parkinson’s disease is suggested by studies showing that the antagonistic D2R–A_{2A}R interactions were increased in the striatal membranes from rat models of Parkinson’s disease (reviewed in Ref. 295). In addition, studies indicate that A_{2A}R antagonists have anti-Parkinsonian effects (reviewed in Ref. 295). For example, A_{2A}R antagonist administration could attenuate dopamine-mediated neuronal degeneration in animal models of Parkinson’s disease (reviewed in Ref. 295). Given that D2R–A_{2A}R heteromers are constitutively formed, it is possible that A_{2A}R antagonists targeting the heteromer could increase D2R-mediated signaling leading, in turn, to an attenuation of Parkinson’s disease symptoms (reviewed in Ref. 295).

The heterotrimer involving D2R–mGluR5–A_{2A}R could also be a novel therapeutic target in the treatment of Parkinson’s disease (reviewed in Ref. 295). This is supported by studies showing that mGluR5 antagonist-mediated induction of motor activity requires the presence of both D2R and A_{2A}R and that a combination of mGluR5 and A_{2A}R antagonists could potentiate locomotor behavior in reserpinized mice (reviewed in Ref. 295).

Another heterotrimer that could be a novel target for the treatment of Parkinson’s disease involves CB_1R–D2R–A_{2A}R (reviewed in Ref. 295). Low doses of CB_1R and A_{2A}R antagonists could synergize to potentiate D2R-mediated anti-Parkinsonian effects during the early stages of Parkinson’s disease when a substantial release of dopamine still occurs (reviewed in Ref. 295).

4. CONCLUSIONS

Recent evidence indicates that some GPCR heteromers including those associated with drugs of abuse are upregulated under pathophysiological conditions. However, the physiological role of these heteromers in pathology is not clearly understood due to the lack of tools to distinguish between heteromer and homomer-mediated effects. For this, heteromer-specific ligands, agonists and/or antagonists, are needed to elucidate the role of GPCR heteromers in normal and pathophysiological conditions. Although heteromer-specific ligands for a few GPCR heteromers have been reported they are mostly receptor agonists and need to be extensively evaluated for their heteromer selectivity in both *in vitro* and *in vivo* assays. Moreover, the availability of heteromer-selective antagonists would help in the evaluation of heteromer levels in the normal and diseased states as well as in studies examining heteromer pharmacology. The recent development of antibodies that selectively recognize a GPCR heteromer in endogenous tissue, and of TAT peptides that can specifically disrupt the heteromer of interest, could help to elucidate the role of GPCR heteromers in pathological conditions.[7–10] This is clearly shown in the case of MOR–DOR heteromers where heteromer-selective antibodies demonstrate that these heteromers are increased in brain areas involved in pain perception following chronic treatment with morphine under a paradigm that leads to the development of tolerance.[7] In addition, studies with TAT peptides that selectively disrupt the MOR–DOR heteromer suggest a role for the heteromer to keep morphine-mediated signaling via MOR in the desensitized state.[8]

Studies demonstrating that GPCR heteromers are upregulated during a disease state suggest that these heteromers could be novel targets for the development of therapeutics to treat these diseases. If heteromers are disease-specific and/or exhibit unique tissue specificity, they would serve as ideal drugs for the development of therapeutics with potentially lesser side effects than the ones that are currently available.

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Table 9.1

Opioid receptor heteromers

		Changes in heteromer properties			
Heteromer pair	**Detection techniques**	**Binding**	**Signaling**	**Trafficking**	**References**
DOR–α_{2A}AR	Co-IP, BRET	Not reported	Synergy in inhibition of neuropeptide release; ↑ DOR-mediated neurite outgrowth	Not reported	84,85
DOR–β_2AR	Co-IP, BRET	Not changed	Not changed	β_2AR internalized by DOR agonists	86,87
DOR–CB_1R	Co-IP, BRET	Not reported	↓ CB_1R-mediated signaling	Change in CB_1R localization	88,89
DOR–CXCR4	Co-IP, FRET	Not changed	Nonfunctional	Not changed	90
DOR–D1R	Immuno-EM	Not reported	↓ DOR signaling by D1R agonists	DOR redistribution by cocaine withdrawal	91,92
DOR–KOR	Co-IP, BRET	↓ Affinity for selective DOR or KOR ligands; binding cooperativity with DOR+KOR ligands	↑ With a combination of DOR +KOR agonists	Altered DOR trafficking	82,86
DOR–MOR	Co-IP, BRET	↓ Affinity for receptor-selective agonists; allosteric modulation of MOR binding by DOR ligands and vice versa	Switch from $G_{\alpha i}$ to $G_{\alpha z}$; heteromer coupling to β-arrestin; potentiation of MOR binding by DOR ligands and vice versa	MOR and DOR endocytosed independently of each other; some DOR agonists induce heteromer internalization	7,93–103
DOR–SNSR	BRET	Not reported	Preferential $G_{\alpha q}$ signaling	Not reported	104
KOR–β_2AR	Co-IP	Not changed	↓ β_2AR signaling	Altered	105
KOR–APJ	Co-IP, BRET	Not reported	↑ By agonists to either receptor	Not reported	106
MOR–α_{2A}AR	Co-IP, BRET, FRET	Not reported	α_{2A}R ↑ MOR signaling; α_{2A}R ligands ↓ MOR signaling	Agonist to one receptor does not cause heteromer internalization	107–109
MOR–CB_1R	Co-IP, BRET, FRET	Not reported	CB_1R ↓ MOR signaling; MOR ↓ CB_1R signaling	Not reported	89,110
MOR–CCR5	Co-IP	Not reported	MOR agonist ↓ CCR5 signaling; CCR5 agonist ↓ MOR signaling	Not reported	111,112
MOR–GRPR	Co-IP	Not reported	MOR-mediated Ca^{2+} signaling only in cells expressing the heteromer	MOR-mediated GRPR internalization	113
MOR–KOR	Co-IP, BRET	↓ Affinity for MOR-selective ligands	Not reported	Not reported	82,114,115
MOR–NK-1	Co-IP, BRET	Not changed	Not changed	MOR internalization by NK-1 agonist and vice versa	116
MOR–OFQ/N	Co-IP	↑ Affinity for MOR-selective ligands	↓ Potency of MOR ligands	Not reported	117,118
MOR–SSTR2	Co-IP	Not changed	Not changed	↑ Heteromer internalization by sSTR2 agonist	119

Table 9.2

Cannabinoid receptor heteromers[a]

		Changes in heteromer properties			
Heteromer pair	**Detection techniques**	**Binding**	**Signaling**	**Trafficking**	**References**
CB_1R–$A_{2A}R$	Co-IP, BRET	Not reported	CB_1R antagonist ↓ $A_{2A}R$ signaling; $A_{2A}R$ antagonist ↓ CB_1R signaling	Not reported	201
CB_1R–β_2AR	BRET	Not reported	Alteration in signaling of individual protomers	CB_1R internalization by β_2AR agonist	202
CB_1R–AT1R	Co-IP, BRET	Not reported	AT1R signals via $G_{\alpha i}$ instead of $G_{\alpha q}$; CB_1R agonists ↑ AT1R signaling; CB_1R antagonists ↓ AT1R signaling	Not reported	10
CB_1R–CB_2R	BRET, proximity ligation assay	Not reported	Antagonistic interactions	Not reported	148
CB_1R–D2R	Co-IP	Not reported	CB_1R signals via $G_{\alpha s}$ in the presence of D2R agonist	Not reported	203–206
CB_1R–OX1R	Co-IP, TR-FRET	Not reported	CB_1R ↑ OX1R signaling	OX1R agonist ↑ heteromer internalization	207,208
CB_1R–D2R–$A_{2A}R$	BRET+FRET; bimolecular fluorescence complementation +BRET	Not reported	Not reported	Not reported	129,209

[a]This table does not include opioid–cannabinoid receptor heteromers. These are described in Table 9.1.

Table 9.3

Dopamine receptor heteromers[a]

		Changes in heteromer properties			
Heteromer pair	**Detection techniques**	**Binding**	**Signaling**	**Trafficking**	**References**
D1R–D2R	Co-IP, FRET, protein complementation assay	Not changed	$G_{\alpha q}$-mediated signaling by the heteromer	Not reported	125,220–224
D1R–D3R	Co-IP, BRET	↑ Dopamine binding	↑ Dopamine signaling	Heteromer internalized only by agonists to both receptors	225
D1R–A_{1A}R	Co-IP	Not reported	↓ D1R signaling by combination of agonists to both receptors	Not reported	226
D2R–D3R	Co-IP, functional complementation	Not reported	↑ Potency with preferential protomer agonists	Not reported	227,228
D2R–A_{2A}R	Co-IP, BRET, FRET	Not reported	D2R ↓ efficacy and potency of A_{2A}R agonists	Heteromer internalization by agonist to either protomer	219,229–232
D2R–GSHR1a	TR-FRET	Not reported	D2R signals via $G_{\alpha q}$ and not $G_{\alpha i}$	Not reported	233
D2R–NTS1	IP+fluorescence spectroscopy	↓ Affinity for D2R agonist in the presence of NTS1 agonist	↑ Potency of D2R in the presence of NTS1 agonist	NTS1 agonist ↑ D2R internalization	234,235
D2R–OTR	BRET, proximity ligation assays	↑ D2R antagonist binding by low doses of OTR agonist	↑ Efficacy and potency of D2R signaling by OTR agonist	Not reported	236
D2R–SSTR2	Co-IP, pbFRET	Modulation of binding properties of individual protomers	SSTR2 agonist ↑ D2R signaling	D2R agonist ↑ SSTR2 internalization	237
D2R–SSTR5	Functional complementation, pbFRET	↑ Affinity for each protomer agonist	Agonist to one protomer ↑ signaling by agonist to second protomer	Not reported	144
D2R–5-HT_{2A}R	Co-IP	D2R agonist ↑ affinity of 5-HT_{2A}R agonist	D2R agonist ↑ potency of hallucinogenic 5-HT_{2A}R agonist	Not reported	238
D2R–mGluR5–A_{2A}R	Co-IP, BRET+bimolecular fluorescence complementation	Not reported	Not reported	Not reported	127
D4R–α_{1B}AR	Co-IP, BRET	D4R agonist ↓ binding displacement by α_{1B}AR agonist	D4R agonist ↓ α_{1B}AR signaling	Not reported	239
D4R–β_1AR	Co-IP, BRET	D4R agonist ↓ binding displacement by β_1AR agonist	D4R agonist ↓ β_1AR signaling	Not reported	239
D4R–β_2AR	Co-IP, BRET	Not reported	Not reported	Not reported	240

[a]This table does not include heteromers involving opioid or cannabinoid receptors. These are described in Tables 9.1 and 9.2.