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Novel pharmaco-types and trafficking-types induced by opioid receptor heteromerization

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Abstract

Homo- and heteromerization of 7 transmembrane spanning (7TM)/G-protein coupled receptors (GPCRs) has been an important field of study. Whereas initial studies were performed in artificial cell systems, recent publications are shifting the focus to the *in vivo* relevance of heteromerization. This is especially apparent for the field of opioid receptors. Drugs have been identified that selectively target opioid heteromers of the delta opioid receptor with the kappa and the mu opioid receptors, that influence nociception and ethanol consumption, respectively. In addition, in several cases, the specific physiological response produced by the heteromer may be directly attributed to a difference in receptor trafficking properties of the heteromers compared to their homomeric counterparts. This review attempts to highlight some of the latest developments with regard to opioid receptor heteromer trafficking and pharmacology.

Introduction

Opioid receptors

Opioids, and the receptors to which they bind, have been extensively studied and are well-known for their roles in pain modulation/analgesia and reward. The peptidergic opioid receptors belong to class A of the family of 7 transmembrane spanning (7TM)/G-protein coupled receptors (GPCRs). For many years opioid receptors were subdivided into three classes: the mu opioid receptor (MOP-R), the delta opioid receptor (DOP-R) and the kappa opioid receptor (KOP-R), each of which displays at least two pharmacological “subtypes” *in vivo* [1-3]. In the early 1990's sequence homology cloning efforts resulted in the identification of a fourth “opioid” receptor, the nociceptin (orphanin FQ/ORL1) receptor NOP-R, which despite its high homology to the other opioid receptors does not bind “classic” opioid ligands with high affinity [4]. Continuing research at the molecular level has resulted in the identification of receptor splice variants of each of these receptors [5]. An additional layer of complexity is added by the ability of opioid receptors to engage in receptor-receptor interactions, forming receptor homomers and heteromers with altered pharmacological properties [6,7], first described more than a decade ago [8]. Hence, although only four opioid

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receptor genes have been identified, the pharmacological diversity in opioid response is much greater.

An important aspect of opioid pharmacology is the establishment of tolerance. Opioid tolerance can have multiple causes, but often include mechanisms involving receptor trafficking. These mechanisms, such as receptor phosphorylation/desensitization, internalization, recycling or degradation, for a large part, rely on receptor interactions with non-7TM/GPCRs, such as protein kinases and β -arrestins [9,10].

This review focuses on recent advances in understanding opioid receptor heteromerization and trafficking.

Importance of opioid receptor trafficking

Not only do the opioid receptor subtypes vary in their signaling and ligand binding properties, but importantly, they also display different trafficking properties. The most studied receptors in this respect are the MOP-Rs, which recycle after ligand activation, and the DOP-Rs, which degrade after activation [11]. However, this is not as straightforward as it seems, since the MOP-R agonist morphine does not induce receptor internalization and/or recycling and there are endogenous ligands that both do and do not differentiate between MOP-R and DOP-R [4]. Both the ability and the inability of opioid receptors to desensitize and endocytose have been proposed as mechanisms responsible for opioid tolerance [12]. Nevertheless, it is clear that desensitization alone cannot explain morphine tolerance, since active receptors still remain in the “tolerant state” and are revealed as signs of physical withdrawal upon removal of drug.

Importantly, several studies have suggested that enhancing MOP-R desensitization and endocytosis can delay tolerance [13,14]. Indeed, endogenous endorphins [14], and exogenous DAMGO [15] or methadone [16] all appear to enhance the ability of morphine to promote endocytosis of the MOP-R. One possible explanation for these findings is that morphine-occupied MOP-Rs are endocytosed because they form homomers with MOP-Rs occupied by an endocytosis-promoting ligand. Thus, these findings hint at the possible existence of MOP-R homomers [15] (Figure 1A).

Additionally, recent studies propose that DOP-Rs are located predominantly intracellularly, but can be translocated to the cell surface under several conditions (for review see [17] & Figure 2A), e.g. chronic morphine exposure, stress, inflammation or more recently, ethanol consumption [18]. It appears that both substance P [19] and the presence of MOP-Rs may play a role in the translocation of DOP-Rs to the surface [20,21]. However, the redistribution of intracellular DOP-Rs remains controversial, with recent findings arguing against this hypothesis [22].

Opioid receptor heteromerization

7TM/GPCRs were initially thought to function solely as monomeric entities. However, a vast amount of data has been amassed over the last two decades suggesting that 7TM/GPCRs can interact with themselves or each other to form receptor homo- and heteromers [6]. The opioid receptors were some of the first heteromers that were comprehensively studied using functional (changes in pharmacology) as well as a biochemical (immunoprecipitation, cross linking) and biophysical (FRET, BRET) methods [23]. The DOP-R can form heteromers with both the MOP-R [24-27] as well as the KOP-R [27,28]. The MOP-R can also heteromerize with the NOP-R [29]. The existence of MOP/KOP-R heteromers is uncertain; Wang et al showed that all opioid receptors have a similar affinity to form receptor homo- and heteromers [27]. However, other studies found no such interactions [28].

It is possible that opioid heteromers could represent some of the opioid receptor subtypes that have been pharmacologically defined, but not attributed to splice variants, such as DOP-R1 (with a preference for DPDPE and BNTX) and DOP-R2 (with a preference for deltorphin II and naltriben) [3]. For example, there is some evidence that the DOP-R1 could be a DOP/KOP-R heteromer [30,31], while the DOP-R2 could be a DOP/MOP-R heteromer [32]. However, Gomes *et al.* found that the DOP/MOP-R heteromer showed enhanced affinity for some DOP-R1 (BNTX) but also some DOP-R2 (deltorphin II) ligands but not others (DPDPE and naltriben) [33]. Intriguingly, some behavioural effects of DOP-R1, but not DOP-R2 ligands are affected by disruption of MOP-R, again suggesting that the DOP-R1 is a DOP/MOP-R heteromer [34,35].

Similar to the DOP-R, two MOP-R subtypes exist: MOP-R1, which is inhibited by naloxanazine, and MOP-R2 which is not [1]. Three KOP-R subtypes have thus far been identified pharmacologically: KOP-R1 binds arylacetamides and KOP-R2 does not [2], while KOP-R3 [36] is insensitive to the KOP-R ligand U50,488. Some of these subtypes may actually represent the same receptors e.g. MOP-R2 may be DOP-R2 [37], whereas DOP-R1 and KOP-R2 may be the same DOP/KOP-R heteromer [30]. Additionally, opioid receptors may engage in the formation of heteromers with receptors outside the opioid receptor family. MOP-R has been described to form heteromers with many receptors including NK1 [38], CCR5 [39] and the α 2A-adrenergic receptors [40], whereas both DOP-R and KOP-R can form receptor heteromers with the β 2- adrenergic receptor [41]. Importantly, these heteromers typically exhibit unique pharmacological properties, showing differences in ligand affinities, signalling and receptor trafficking.

It is difficult to distinguish whether the need for two opioid receptors to produce a biological effect is due to heteromerization or convergence in a circuit. Importantly, in at least one case the MOP-R1 and DOP-R1 have been shown to co-immunoprecipitate in CNS tissue [25] and antibodies that selectively recognize the DOP/MOP-R heteromer *in vitro* recognize a receptor complex *in vivo* as well [42]. Furthermore, in at least one case, a ligand has been identified that selectively activates a heteromer *in vitro* and produces a biological effect *in vivo* [43]. Thus, while there is still some debate as to the *in vivo* role of DOP/MOP-R or DOP/KOP-R heteromers, there is mounting evidence that these targets do exist, at least in some tissues, *in vivo*.

Opioid receptor heteromer trafficking

There is still much ongoing debate on the ontogeny and fate of receptor heteromers. It is yet to be verified whether receptor homo- and heteromers are formed i) in the endoplasmatic reticulum and Golgi apparatus during receptor synthesis and maturation or ii) while expressed on the cell surface. As evidence of this debate, early studies suggested that the heteromerization of DOP/MOP-R may only occur at the cell surface [26], while other reports have shown that DOP/MOP-R heteromers are formed in the ER [44]. In addition, a group of receptor chaperone proteins, called receptor transporting proteins (RTPs) and receptor expression enhancing proteins (REEPs) have recently been identified, which may assist in the transport of heteromers to the cell surface [42,45] (2A).

Following their endocytosis, the MOP-R and DOP-R appear to be sorted to different compartments. Specifically, while the MOP-R is recycled to the plasma membrane [46,47], the DOP-R is targeted for degradation [11]. Thus, from a post-endocytic trafficking viewpoint, the DOP/MOP-R heteromer could display a unique trafficking profile, although there is some question as to whether the DOP/MOP-R internalizes as receptor heteromer *in vitro* [26]. In addition, heteromerization of the KOP-R and DOP-R also appears to affect trafficking. For example, while DOP-Rs are rapidly endocytosed in response to activation by etorphine, KOP-

Rs are not, and when the two receptors are co-expressed KOP-Rs prevent the endocytosis of DOP-Rs [48].

Heteromers between the β 2-AR and either the DOP-R or the KOP-R also display altered trafficking properties compared to receptor homomers/monomers. For example, when the β 2-AR and DOP-R are co-expressed, activation of either receptor leads to co-internalization of the other receptor. On the other hand, the β 2-AR/KOP-R heteromer does not endocytose when activated by agonists for either protomer [41] (Figure 2B). Similarly, when the MOP-R and NK1 receptor [38] are co-expressed, the receptors co-internalize with activation by agonists selective for either protomer. Heteromerization between MOP-R and NK1-R could explain why there is a loss of morphine place preference in NK1 $-/-$ mice [49] and why chronic morphine treatment alters substance P-induced internalization of the NK1-R [50]. On the other hand, MOP-R heteromerization with CCR5 only produces cross desensitization but not cross internalization [39].

A recent finding suggests that MOP-Rs are located in lipid rafts on the cell surface, but when activated by specific ligands (etorphine, but not morphine) move out of these rafts [51]. Mechanisms like this could play a role in the formation or breakup of heteromers and shed more light on receptor heteromer ontogeny (Figure 2C).

Targeting opioid heteromers

One method of targeting homomers and heteromers is the co-administration of two drugs that can interact with the two binding pockets of the homomer/heteromer (Figure 1 A). For instance, it was suggested that the DOP-R antagonist TIPP ψ enhances morphine analgesia *in vivo*, by acting on the DOP/MOP-R heteromer [25]. In addition, several efforts have been made to design single ligands that bridge opioid heteromers and, thus, potentially bring together individual receptors into heteromeric complexes and/or stabilize an existing heteromer. The early work in this area targeted homomers and was motivated by a desire to develop both tools to distinguish between different types of opioid receptors and more potent ligands. These ligands often did exhibit enhanced potency (presumably due to their higher affinity) [52]. Recently, these efforts have been expanded to probe the existence of heteromers, receptor allosteric coupling, and potential novel antinociceptive sites [53-55]. For example, the μ -delta agonist-antagonist (MDAN) series of ligands consists of a MOP-R agonist (oxymorphone) and a DOP-R antagonist (naltrindole) moiety linked by a spacer of variable length. The underlying hypothesis behind these ligands is the finding that DOP-R antagonists (naltrindole) attenuate acute morphine tolerance and dependence [56]. Some of the ligands from the MDAN series show activities *in vivo* consistent with a unique activity at a heteromer, i.e. reduced tolerance and naloxone precipitated withdrawal signs. This is exemplified by the finding that the novel properties of one molecule in this series, MDAN-19, can not be recapitulated by adding the two pharmacophores separately, and that its novel properties are dependent on the spacer length (Figure 1B). The proposed mechanism for these bivalent ligands is the ability of the DOP-R antagonist to negatively modulate the MOP-R receptor [54]. In addition, the bivalent ligand KDN-21 is a DOP/KOP-R bivalent antagonist that blocks antinociception produced by both the DOP-R1 agonist DPDPE and the KOP-R2 agonist brexazocine in the spinal cord [30]. Several bivalent ligands have been synthesized that have dual affinity for MOP-R and KOP-R [57,58] as well as “tri-functional” ligands harbouring DOP-R antagonistic, MOP-R agonistic and KOP-R partial agonistic properties [59]. Some of these ligands have recently been tested *in vivo* [60]. However, while these ligands were potent analgesics, activity did not differ from the monovalent parent compounds, and therefore are not uniquely targeting KOP/MOP-R heteromers. In short, since all bivalent ligands described to date retain activity at homomers/monomers, it has been difficult to draw conclusions as to the existence or functional importance of heteromers using these tools.

However, there is at least one case in which a ligand has been described that shows novel activity at a opioid heteromer distinct from that on either homomer, and is an analgesic *in vivo*. 6'-GNTI is a small molecule with high affinity for DOP-R and KOP-R. However, while this ligand is an antagonist at DOP-R and a weak partial agonist at KOP-R, it is a potent agonist on DOP/KOP-R heteromers and produces antinociception when administered intrathecally [43], consistent with the localization of DOP/KOP-R heteromers in the spinal cord but not in the brain [43] (Figure 1C).

Therapeutic potential of opioid heteromers

The availability of ligands with selective or novel activity at receptor heteromers would not only enable the study of heteromer localization *in vivo*, but their dynamics as well. In addition, if opioid receptor heteromers are expressed in a tissue-selective manner, they could be exploited to prevent the side effects of opiates, such as respiratory depression, constipation and dependence that arise as a consequence of systemic use of these drugs. In addition, if the expression of select heteromers is altered during the development of morphine tolerance, they could represent unexplored and selective targets for reversing, preventing or modulating tolerance and dependence. Furthermore, receptor heteromers could be expressed in a gender-specific manner, and thereby explain some of the gender specific effects of opioids, in particular KOP-R opioids [61].

By way of example, 6'-GNTI was an effective analgesic only when administered in the spinal cord but not when injected intracerebroventricularly [43]. Thus, a drug like 6'-GNTI could show reduced side effects such as respiratory depression if the target of 6'-GNTI (the DOP/KOP-R heteromer) is selectively expressed in the spinal cord. In another example, TAN-67, a DOP-R1 selective agonist, reduces ethanol consumption in mice. The activity of TAN-67 is dependent on the presence of DOP-R as well as MOP-R (Figure 1B). These results suggest that DOP-R1 is a DOP/MOP-R heteromer that can be selectively targeted with an agonist to reduce ethanol consumption [34], without the side effects of dysphoria produced by opioid receptor antagonists.

In addition, drug "cocktails" that target either homomer or heteromers could be therapeutically valuable. For example, an opioid cocktail consisting of morphine and either methadone or DAMGO promotes morphine-induced endocytosis and thus takes advantage of the homomeric nature of the MOP-R, leading to reduced tolerance and dependence [15,16], Figure 1A. Another example is a cocktail of morphine and serotonin, which drives the endocytosis of the MOP-R/5-HT_{2A} heteromer, suggesting that serotonin might be expected to reduce the development of tolerance and dependence to morphine also by altering receptor trafficking [62].

Conclusions

Although evidence in support of the existence of opioid receptor homo- and heteromers has and continues to accumulate, their functional relevance remains ambiguous [63]. In most studies to date, receptor heteromers have either been recombinantly co-expressed in a cell line or they have been studied in cell lines that endogenously express receptors at physiological levels. However, utilizing these systems for the detection of novel activities at heteromers is challenging at best, since in all cases both homomers and heteromers are expressed. Although some recent effort has been undertaken in cloning obligatory receptor heteromers to circumvent this problem [64], only drugs with novel activity at heteromers will truly enable our ability to delineate the existence and therapeutic relevance of opioid receptor heteromers. Attempts to model receptor heteromers have shown promise [65,66]. Hopefully, these endeavours will decrease the serendipity factor in designing receptor heteromer selective drugs. Indeed, the potential of receptor heteromers as novel pharmacological targets, possibly with select tissue distribution and thus reduced side effects, warrants the large effort that continues in this area.

Research on opioid receptors has been conducted for more than a century. The discovery of opioid receptor homo- and heteromers has opened up a new way of investigating how opioids and opioid receptors function, as well as how receptor trafficking affects heteromerization and vice versa. A better understanding of these processes may lead to the development of analgesic drugs that produce less tolerance, and potentially reduced side effects.

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Figure 1. Targeting receptor homo- and heteromers

A. Targeting the MOP-R homomer using a “cocktail” consisting of morphine (circle) and DAMGO or Methadone (star) to promote internalization and prevent tolerance. **B.** Targeting the DOP/MOP-R heteromer using an agonist (e.g. TAN-67, diamond) to decrease ethanol consumption, or using a bivalent ligand, consisting of a MOP-R agonist and a DOP-R antagonist (e.g. MDAN-19), to decrease morphine tolerance. **C.** Targeting the DOP/KOP-R heteromer using 6'-GNTI (triangle), which produces potent analgesia through the heteromer when administered i.t.

Figure 2. Opioid receptor heteromerization affects trafficking

A. A population of DOP-R is located intracellularly. MOP-R and/or receptor transporter proteins (RTP4) may assist in the translocation of DOP-R from large dense core vesicles (LDCV) or the Golgi/endoplasmatic reticulum (ER) to the cell surface. **B.** Heteromers of the β_2 -AR/DOP-R and the β_2 -AR/KOP-R have altered endocytic properties compared to the respective receptor homomers: β_2 -AR activation by isoproterenol (cross) induces DOP-R internalization, whereas KOP-R prevents β_2 -AR internalization. **C.** Differential trafficking of activated MOP-Rs out of lipid rafts. MOP-Rs are located in lipid rafts on the cell surface. Activation of MOP-R with etorphine (diamond) but not morphine (circle) causes MOP-Rs to move away from the lipid rafts, which could potentially affect homomer formation.