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Abstract

Opioid receptors are the sites of action for morphine and most other clinically used opioid drugs. Abundant evidence now demonstrates that different opioid receptor types can physically associate to form heteromers. Owing to their constituent monomers' involvement in analgesia, mu/delta opioid receptor (M/DOR) heteromers have been a particular focus of attention. Understandings of the physiological relevance and indisputable proof of M/DOR formation in vivo are still evolving. This aspect of the field has been slow to progress in large part by the limitations of most available experimental models; recently however, promising progress is being made. As a result, the long-repeated promise of opioid receptor heteromers as selective therapeutic targets is now being realized.

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1 Introduction

There is increasing convincing evidence that G-protein coupled receptors can form functional complexes as dimers or heteromers and that such complexes extend to the family of opioid receptors. Existent opioid receptor heteromers were first described by Jordan and Devi (1999) where functional and physical interaction was demonstrated between kappa (KOR) and delta (DOR) opioid receptors. Although, the concept of an opioid receptor complex was proposed earlier following the observation of noncompetitive binding interactions between mu opioid receptor (MOR) and DOR ligands (Rothman et al. 1992). Subsequently, many studies have provided further evidence for the existence of opioid receptor heteromers using various experimental approaches including co-immunoprecipitation, immunocytochemistry with novel heteromer antibodies, bioluminescence and Foerster resonance energy transfer, and electrophysiology. The proximity and interaction assay research supporting the existence of opioid heteromers has previously been thoroughly reviewed elsewhere (Costantino et al. 2012; Stockton and Devi 2012). However, there has been much debate of the physiological significance of such complexes and, initially, whether they truly existed in vivo. A preponderance of early evidence for their existence relied upon the use of heterologous expression systems in immortalized cell lines. These tools provide unparalleled experimental control. They permit the generation of precise conditions with maximum favorability for the detection of opioid receptor heteromers and a wealth of approaches to intricately dissect their functionality. A great deal of information about the mu–delta opioid receptor (M/DOR) heteromer has been gained using these models. Until recently, the deficit lay in the uncertain physiological relevance of those precisely engineered conditions. That is, opioid receptors are typically expressed in limited quantities in neuronal tissue with each opioid receptor type under tight and differential translational and trafficking control, notwithstanding the lack of clear subcellular co-localization. Meanwhile, these models often expressed these receptors in very large quantities (e.g., using CMV promoters) in HEK293 or CHO cells lacking the same control mechanisms. While these contrived models were very useful in providing information about the potential for MOR–DOR interactions and how M/DOR as a distinct receptor species behaved, the degree to which these interactions and behaviors occur in normal, physiological systems was a matter of some debate. This review will focus on recent in vivo and ex vivo research demonstrating the cellular localization, function, and unique signalling of MOR and DOR (M/DOR) heteromers. Three criteria have been recently proposed for demonstrating heteromers in native tissue: (1) physical proximity via direct interaction or allosteric interaction, (2) unique pharmacology of the heteromer from the individual receptor type, and (3) disruption of the heteromer leads to loss of the heteromer-specific

properties (Gomes et al. 2016). To date, only a few heteromer complexes fit all three of these criteria, including the M/DOR heteromer (Gomes et al. 2016).

2 Historical Physiological Interactions

Indications for MOR–DOR interactions first arose from seemingly paradoxical findings of two related, but distinct research avenues: (1) DOR analgesia as a therapeutic target, and (2) the mechanisms of tolerance to MOR-mediated analgesia.

Delta Opioid Receptor Analgesia There has been interest in developing DOR agonists as novel therapeutics for treating pain (in particular, selective small molecule drugs). The DOR system is upregulated in various models of chronic pain and selective ligands show encouraging analgesic profiles in reducing pain hypersensitivities associated with tissue and nerve injury (Cahill et al. 2007). The attractiveness of this target emanated from studies showing that activation of DOR had: (1) minimal or no rewarding properties that may trigger addiction liability, (2) no life-threatening effects of respiratory depression, and (3) did not alter nociceptive responses in the absence of injury or pathology (Gendron et al. 2016; Spahn and Stein 2017).

Mu Opioid Receptor Analgesic Tolerance Perplexingly, many studies also support the development of DOR antagonists as novel analgesic therapeutics based on research demonstrating that this same receptor limits MOR function, possibly through the formation of MOR–DOR complexes.

A concerted effort has been made to understand the mechanisms of opioid analgesic tolerance because that tolerance limits effectiveness of pain treatment. This analgesic tolerance also jeopardizes compliance, because tolerance to other pharmacological effects such as constipation does not develop at the same rate. Opioid tolerant states have also been reported in certain chronic pain states, even though subjects may be "opioid-naïve." For example, neuropathic pain, defined as pain caused by damage or dysfunction of the nervous system, is a challenge to treat as it is often refractory to many pharmacotherapies, including opioid analgesics (Gilron et al. 2006).

Understandably, most studies have focused on understanding functional changes in the MOR as the majority of opioid analgesics target this receptor type. The first inclination that DOR restricted MOR activity was with pharmacological in vivo studies. Morphine analgesic tolerance was attenuated by co-administration of naltrindole, a DOR antagonist (Abdelhamid et al. 1991; Fundytus et al. 1995), and similar effects were evident by reducing expression of DORs by antisense knockdown (Kest et al. 1996), or constitutive DOR knockout mice (Chefer and Shippenberg 2009; Zhu et al. 1999) or disruption of cyclin-dependent kinase 5, which is required for phosphorylation of Thr-161 and trafficking of DOR to plasma membranes (Xie et al. 2009).

This research was surprising given the previous reports showing analgesic synergy between DOR and MOR agonists in both naïve and morphine tolerant mice (Porreca et al. 1987). Interestingly, even ultralow dose DOR antagonists suppressed morphine-induced analgesic tolerance (Abul-Husn et al. 2007). This suggested that the effect might not be driven solely by the absence/presence or blockade/activation of DOR, but perhaps an interaction between the two receptors. In this case, DOR ligands would act allosterically; this would explain the matching effects of DOR agonism and DOR antagonism.

Evidence that opioid receptors, like other GPCRs, could form heteromeric complexes was subsequently demonstrated by multiple research groups. The existence of MOR and DOR (M/DOR) heteromers in native tissue was first identified by Devi and colleagues using novel antibodies for the heteromer created by immunization subtraction methods (Gupta et al. 2010). Importantly, this study demonstrated that M/DOR heteromers abundance was augmented in animals following chronic morphine treatment. The existence of M/DOR heteromers was confirmed using an innovative approach to insert a TAT domain peptide into the membrane in the correct orientation where it could interrupt the formation of the M/DOR complex. TAT fusion-interfering peptide corresponding to the second intracellular loop of the DOR (Tat-DOR-2L) reduced cell surface expression of DOR and disrupted the formation of M/DOR heteromers (Xie et al. 2009) as well as reduced the development of morphine tolerance in a model of inflammatory pain (Chen et al. 2012). Further, systemic administration of MOR^{TM1}-TAT, which corresponds to the first transmembrane domain of the MOR, but not MOR^{TM3}-TAT, disrupted the formation of the M/DOR heteromer, and consequently increased morphine antinociception and attenuated the development of morphine analgesic tolerance (He et al. 2011).

One caveat to consider for experiments evaluating morphine tolerance is the influence of memory with repeated testing. This is an experimental confound which manifests as a gradual reduction in pain threshold with repeated testing. It is perhaps not surprising that rodents may learn that they will be removed from an environment associated with a noxious stimulus with repeated testing. The use of appropriate saline-injected controls may not capture changes in baseline nociceptive threshold due to a floor effect in that most tests have calibrated instrumentation to produce short latencies because they are predicting analgesic effects following morphine administration. Together, this change in pain thresholds and this methodological limitation may overstate analgesic tolerance. There are reports in the literature of this behavioral sensitization/tolerance. Behavioral tolerance was reported after exposing rats to a nonfunctional hot plate that involved habituation to the novel distractive stimuli (Bardo and Hughes 1979). Another study examined the differential effects of weekly compared to daily exposure of a rat to the hotplate test. In this study, a sensitization phenomenon was evident, where nociceptive thresholds decreased with weekly testing (Espejo and Mir 1994). More relevant was the finding that morphine can facilitate memory, which was proposed to contribute to associative learning in antinociceptive tolerance to morphine. Thus, repeated administration of morphine in the same or different environments or when animals were moved to a different context showed that morphine antinociceptive tolerance was significantly

reversed by the change in context (Nakama-Kitamura and Doe 2003). These findings indicate that morphine develops associative and nonassociative antinociceptive tolerance, indicating that antinociceptive tolerance to morphine has contextual specificity. This is relevant to the conclusion that DOR contributes to morphine tolerance because DOR is necessary for hippocampal learning. DOR knockout mice or administration of the DOR antagonist naltrindole impaired hippocampal-dependent novel object recognition learning, demonstrating that DOR activity modulates learning and memory performance (Le Merrer et al. 2013). DOR antagonism (pharmacological or functional) may inhibit morphine-induced effects on memory.

3 Physical Evidence

Many immunohistochemical studies identified MOR and DOR co-localization. These studies have come under intense criticism due to purported lack of DOR antibody specificity, where immunolabeling remained present in constitutive knockout mice (Gendron et al. 2016). The generation of an MOR-mcherry knockin mouse (Erbs et al. 2015; Gardon et al. 2014) allowed for breeding with the DOR-eGFP (Scherrer et al. 2006) to create a double knockin mouse. This mouse was used for extensive mapping of MOR and DOR throughout the peripheral and central nervous systems (Erbs et al. 2015), where receptors could be visualized with subcellular resolution. MOR and DOR were often co-expressed with high density in many brain regions and also identified to be co-localized within large dorsal root ganglia neurons (Erbs et al. 2015), in contrast to previous findings using immunohistochemical techniques in DOR-eGFP mice (Scherrer et al. 2009). Conversely, this same mouse [DOR-eGFP] was used to show MOR and DOR co-localization within enteric neurons of the myenteric plexus (Poole et al. 2011), which may account for the ability of DOR to inhibit gastrointestinal secretion and motility. Other electrophysiological (Egan and North 1981) and pharmacologic (Fox-Threlkeld et al. 1994) studies support DOR/MOR co-expression by enteric neurons.

Although the existence of GPCR heteromers was proposed almost two decades ago, there remains some scepticism of the existence of such receptor complexes in vivo, due to the general lack of tools available for detection of such complexes. There have been major advances in this tool kit that provide validation of M/DOR existence and the capacity of producing physiological effects. The crystal structures of MOR and DOR support the possibility of direct interaction between the two receptor types. Using an unbiased coarse-grained molecular dynamics simulation of freely diffusing opioid receptors in an explicit lipid–water environment, Provasi and colleagues identified the formation of M/DOR heteromers during the simulation. Importantly, once formed, the complex did not dissociate (Provasi et al. 2015). Further, in this latter study the minimum distance between each crystal structure within the heteromer was identified as 10 Å. This finding complements research showing that functional activity of bivalent ligands with linked mu agonist and delta antagonist pharmacophores have the greatest activity with a linkage spacer length of 22 Å (Lenard et al. 2007; Daniels et al. 2005; Yekkirala et al. 2013).

Further evidence that M/DOR exist in vivo used co-immunoprecipitation techniques similar to previous studies with various heterologous cell systems. For example, co-immunoprecipitation of spinal cord tissue revealed the existence of constitutively expressed M/DOR heteromers (Xie et al. 2009; Gomes et al. 2004; He et al. 2011). Because of the questionable specificity of DOR antibodies required for such studies, a novel approach of subtraction immunization was taken to produce an M/DOR specific antibody (Gomes et al. 2014). Using this antibody, in vivo expression of M/DOR was visualized in various brain structures (Gupta et al. 2010). The subcellular co-localization together with co-immunoprecipitation studies strengthens the existence of M/DOR heteromers, especially in subcortical networks involved in eating, sexual behavior, and response to aversive stimuli (Erbs et al. 2015).

It is not clear if M/DOR are synthesized within intracellular compartments and are trafficked to the membrane as a functional unit or formed at the plasma membrane. It is generally well accepted that the majority of DORs leaving the endoplasmic reticulum do not mature or traffic to the plasma membrane. This results in low expression of functional DORs on the cell surface. Rather, DORs are primarily degraded in lysosomal pathways. The formation of M/DOR may be one mechanism to enhance DOR maturation and trafficking. A Golgi chaperone, receptor transport protein 4 (RTP4), was shown to regulate the expression and cell surface trafficking of M/DOR heteromers (Décaillot et al. 2008). Chaperoning resulted in an increase in the cellular signalling of these receptors. A recent elegant review is available on molecular and pharmacological chaperones for GPCRs (Williams and Devi 2010). Cell surface trafficking of DOR is also evident in models of chronic inflammatory pain (Cahill et al. 2003; Morinville et al. 2004a; Gendron et al. 2007) or after prolonged morphine treatment (Cahill et al. 2001; Hack et al. 2005; Lucido et al. 2005; Morinville et al. 2004b). Prolonged morphine treatment also increases the abundance of M/DOR in various brain regions as detected by heteromeric antibodies (Gupta et al. 2010). Subsequently, it was proposed that morphine acts as a pharmacochaperone bringing the M/DOR heteromer to the cell surface (Costantino et al. 2012). In contrast, other studies provide evidence that M/DOR heteromers form at the cell surface (Law et al. 2005). This alternative is supported by studies showing that DOR and MOR can interact via transmembrane domains in coarse-grained molecular dynamics simulations (Provasi et al. 2015). Since data support both formation of heteromers within the receptor maturation process and their formation at the cell surface, it would not be unreasonable to suggest that both processes may occur depending on the physiological processes that engage formation of the heteromer.

4 Functional Evidence: *Pharmacological Subtypes – Bias Ligand Signalling or Heteromers?*

Pharmacological studies have proposed DOR-1 and DOR-2 subtypes. [D-Pen^2,D-Pen^5]enkephalin (DPDPE, DOR-1 agonist) and [D-Ala^2,Glu^4]deltorphin (Deltorphin II, DOR-2 agonist) both elicit antinociception in various pain models

but repeated intracerebroventricular administration of either ligand was shown not to produce cross-tolerance to the other agonist (Mattia et al. 1991). Moreover, opposite effects on ethanol consumption were produced using delta subtype (DOR-1 and DOR-2) selective ligands (van Rijn and Whistler 2009).

The existence of DOR subtypes does not easily comport with molecular studies where only one transcript for DOR has been identified and splice variants for the DOR have not been described. However, there are many mechanisms that one could envisage to create alternative behaviors of different DOR ligands. One explanation for pharmacological subtypes is the existence and functional activity of heteromers (van Rijn and Whistler 2009), where the M/DOR heteromer was proposed to account for the DOR-1 subtype (van Rijn and Whistler 2009). Other studies suggest that DOR-2 subtype accounts for heteromers, where antagonism of DOR-2, but not DOR-1, reduced the development of morphine tolerance following chronic morphine treatment in a model of inflammatory pain (Beaudry et al. 2015). Using electrophysiological techniques on a slice preparation of the ventral tegmental area, DPDPE and Deltorphin II were shown to elicit opposing depolarization or hyperpolarization effects in the same neuron, which was not predicted by MOR agonist-induced effects, topographical localization, or whether it was positive for tyrosine hydroxylase or not (Margolis et al. 2017). While these data may argue against M/DOR heteromers explaining the DOR subtype phenomenon in this midbrain structure, this latter study identified that: (1) MOR agonist-induced effects could be augmented by a DOR antagonist and vice versa, (2) DOR agonist effects could be augmented with MOR selective antagonist CTAP, and finally (3) most VTA neurons expressed both DOR and MOR (Margolis et al. 2017). Together, these data support previous findings that DOR antagonists increase the potency and intrinsic efficacy of MOR agonists in cells co-expressing both receptors (Gomes et al. 2000, 2004). MOR ligands are capable of allosterically enhancing DOR radioligand binding and vice versa, which suggests strong positive cooperativity between the two receptor units. These data support the concept that DOR ligands (including antagonists) will allosterically enhance MOR ligand binding leading to the potentiation of MOR-mediated effects including antinociception.

In cultured cells, M/DOR heteromers have unique signalling properties compared to either MOR or DOR alone: signalling switched from a G-protein dependent (monomeric) to an independent (heteromeric) pathway (Rozenfeld and Devi 2007). MOR or DOR monomeric receptor activation couples to G-protein signalling cascades, and there has been a concerted effort to develop ligands that only couple through this G-protein signalling rather than β-arrestin, as the latter is proposed to account for unwanted pharmacological effects such as respiratory depression (Siuda et al. 2017). In contrast, the M/DOR heteromer led to a constitutive recruitment of β-arrestin-2 to the receptor complex resulting in changes in the spatiotemporal regulation of ERK1/2 signalling. However, treatment with an MOR or DOR ligand switched signalling to a non-β-arrestin-2-mediated signalling. Thus, the heteromer and the bias lines of drug development are trying to achieve the same fate – less β-arrestin signalling.

The identification that M/DOR heteromers primarily signal via β-arrestins led to the development of MOR agonists with DOR antagonist properties that were devoid of β-arrestin-2 recruitment activity. These compounds promised to have a unique pharmacology that would produce less respiratory depression, less GI dysfunction, and lower propensity to induce tolerance and dependence compared to morphine. Such compounds were synthesized based on endomorphin structure (Cai et al. 2014) or drug library screening for β-arrestin recruitment (Gomes et al. 2013). CYM51010 was identified through the latter method. The involvement of M/DOR heteromers in CYM51010-induced antinociception following spinal administration was confirmed by co-administration of a heteromeric antibody that acts as a functional antagonist at the receptor complex (Gomes et al. 2013). Importantly, this chemical elicited antinociception but reduced tolerance and physical dependence compared to morphine. Others took the approach to identify M/DOR selective ligands with the hypothesis that the heteromer would produce analgesia but be devoid of many side effects (Pinello et al. 2010). For example, 6′-guanidinonaltrindole was reported to produce analgesia following spinal administration (but not into the brain) via the unique property of selectively activating only M/DOR heteromers but not either MOR or DOR alone (Waldhoer et al. 2005). Chemists also synthesized bivalent ligands with MOR agonist and DOR antagonist pharmacophores, which with specific spacers (21 atoms) allowed for potent analgesic activity but devoid of tolerance and dependence (Daniels et al. 2005). Small molecule chemicals with similar pharmacology of MOR agonist and DOR antagonist properties were also reported to produce analgesia with less analgesic tolerance and dependence (Ananthan et al. 2012). The possibility of those drugs with MOR agonist and DOR antagonist properties have less side effect profile led to the development of eluxadoline (Breslin et al. 2012), which is now FDA approved for treatment of diarrhea associated with irritable bowel syndrome (Levio and Cash 2017). Eluxadoline-induced reductions in gastrointestinal transit were reduced in constitutive DOR knockout mice (Fujita et al. 2014). Using M/DOR heteromer antibodies as functional antagonists, Fujita and colleagues showed that eluxadoline-mediated signalling could be partially blocked (Fujita et al. 2014). Together, these data suggest that eluxadoline effects on gut motility are mediated, in part, by M/DOR heteromers. Figure 1 depicts a cartoon comparing DOR and MOR monomeric and M/DOR heteromeric formation, trafficking, signalling, and pharmacological effects.

5 Conclusions

In this review, we provide concordant and compelling evidence of the existence and functionality of M/DOR heteromers in endogenous tissues. Through the refinement and execution of physiologically relevant experimental tools, there is now an advancement of M/DOR heteromer understanding beyond the confines of earlier, more contrived model systems while also reinforcing and complementing those preceding findings. Attention has now shifted from the mere existence of heteromers towards a more determined effort to understand the processes by which they are

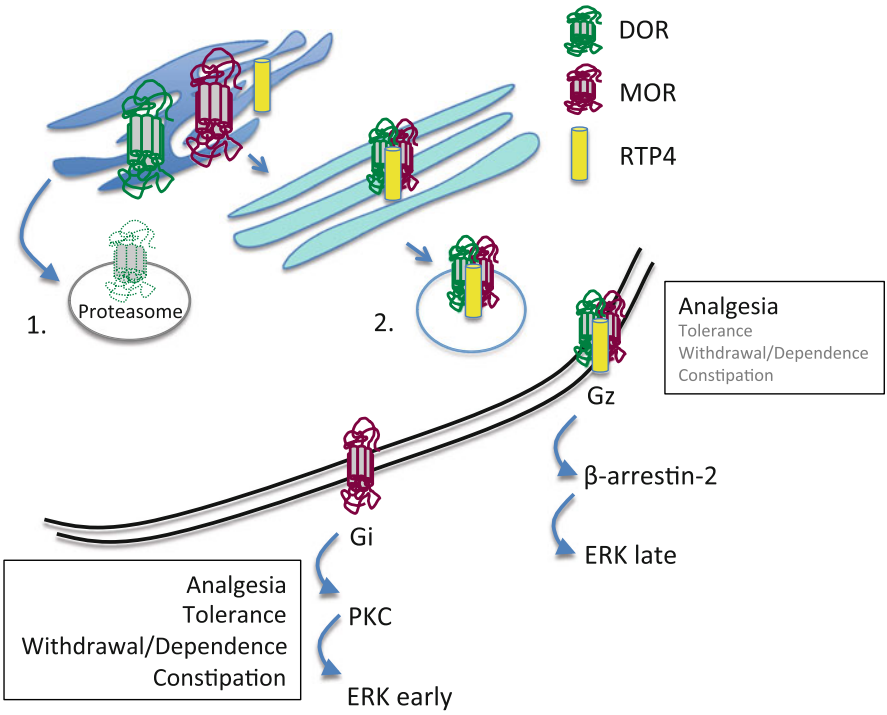

Fig. 1 Proposed model of mu/delta opioid receptor (M/DOR) heteromeric formation and signaling to plasma membranes. 1. DORs in the endoplasmic reticulum are often misfolded and targeted to degradation pathways. A high portion of MOR mature through the endoplasmic reticulum and Golgi network which allows for high cell surface expression. 2. M/DOR heteromers are formed in the endoplasmic reticulum and stabilized by RTP4 chaperone to allow for translocation to plasma membranes. Once at the plasma membrane, MOR signal predominantly through G-protein Gi/o coupling, which in turn generates PKC phosphorylation and early activation of mitogen activated protein kinase ERK activation. In contrast, M/DOR heteromers predominantly activate β-arrestin pathways although have been shown to activate Gz proteins in cell culture models. The heteromer also activates mitogen activated protein kinase ERK activation but the temporal and spatial activation is different than MOR alone, where activation of ERK is minutes later. Following agonist stimulation, MOR produces many pharmacological effects including analgesia, and prolonged treatment produces cellular adaptations or allostasis that contributes to the development of constipation, physical dependence, and analgesic tolerance, whereas activation of the heteromer also produces robust analgesia but appears to induce fewer negative effects caused by allostatic adaptations

formed and regulated as well as their behavior as receptors. Thus, although functional interactions between MOR and DORs may arise, such as competition for downstream effector systems, the research highlighted above confirms that physical interaction exists in the formation of heteromeric complexes.

Understanding M/DOR heteromers as distinct opioid receptor species naturally raises the prospect of these heteromers as therapeutic targets. The relevant literature certainly make this assertion, and justifiably so. Clinical opioid pharmacology has

always been limited by a reliance, albeit necessary, on MOR agonism. Under basal conditions, MOR is the most obvious target of opioid analgesic drug development. While actions on DOR can produce analgesia, many are associated with seizures (Chung et al. 2015). A reliance on MOR agonism carries with it adverse effects. Indeed, the side effects of primary concern for opioid analgesics in clinical use – sedation, respiratory depression, nausea, constipation, itch, bradycardia, and addiction – are all mediated by action at MOR. The availability of M/DOR heteromers as distinct targets may offer alternatives for opioid analgesia, but considerable work remains to be done in advancing our understanding of heteromers to the point of realizing translational potentials.

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