INVESTIGATING THE EFFECTS OF PROLONGED MU OPIOID RECEPTOR ACTIVATION UPON OPIOID RECEPTOR HETEROMERIZATION

by

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A thesis submitted to the Graduate Program in Pharmacology & Toxicology
in the Department of Biomedical and Molecular Sciences
In conformity with the requirements for
the degree of Doctor of Philosophy

Queen’s University
Kingston, Ontario, Canada
March, 2017

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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 of M/DOR formation remain limited in large part due to the reliance of existing M/DOR findings upon contrived heterologous systems. This thesis investigated the physiological relevance of M/DOR generation following prolonged MOR activation.

To address M/DOR in endogenous tissues, suitable model systems and experimental tools were established. This included a viable dorsal root ganglion (DRG) neuron primary culture model, antisera specifically directed against M/DOR, a quantitative immunofluorescence colocalizational analysis method, and a floxed-Stop, FLAG-tagged DOR conditional knock-in mouse model. The development and implementation of such techniques make it possible to conduct experiments addressing the nature of M/DOR heteromers in systems with compelling physiological relevance.

Seeking to both reinforce and extend existing findings from heterologous systems, it was first necessary to demonstrate the existence of M/DOR heteromers. Using antibodies directed against M/DOR itself as well as constituent monomers, M/DOR heteromers were identified in endogenous tissues and demonstrated to increase in abundance following prolonged mu opioid receptor (MOR) activation by morphine.

The next experiments addressed an aspect of the functional consequences of M/DOR formation in endogenous tissues by investigating the post-internalization trafficking of delta opioid receptor (DOR) in conditions of augmented M/DOR formation. These experiments identified perturbations to DOR trafficking consistent with incorporation into a M/DOR species...
with probabilistic post-internalisation trafficking behaviour intermediate to that of either constituent and responsive to both MOR and DOR agonists in a manner subject to blockade by DOR antagonist.

These studies provide concurrent evidence of the existence and functionality of physiologically-relevant M/DOR heteromers in endogenous tissues.
Co-Authorship

The research upon which this thesis is based was conducted by Edmund W. Ong under the supervision of Dr. Catherine M. Cahill and Dr. Mary Cella Olmstead. In Chapter 2, Edmund W. Ong examined the effect of morphine on heteromer abundance in DRG cultures with the assistance of Mats Junek; Achla Gupta generated heteromer-selective monoclonal antibodies and carried out ELISA and binding assays; Jan Mulder carried out immunohistochemistry studies with animal tissue; Ivone Gomes carried out Western blot analysis and signalling assays with the assistance of Raphael Rozenfeld and Emeline Maillet; Ittai Bushlin carried out ELISA assays with MNTB and RVM membranes; Maribel Lim carried out reverse transcription polymerase chain reaction studies; Dr. Catherine M. Cahill, Dr. Tibor Harkany, and Dr. Lakshmi A. Devi prepared the final manuscript based on data in this chapter. Lihua Xue assisted with DRG neuronal cultures in Chapter 3. Maggie Li, Linsey Wilson, Priscilla Orellana, and Nancy Tang assisted with the behavioural experiments in Chapter 5.
Acknowledgements

Thank you so much to everyone, especially Cathy.

Now turn the page and let’s talk science.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>5HT5A</td>
<td>5-hydroxytryptamine (serotonin) receptor 5A</td>
</tr>
<tr>
<td>ADL5859</td>
<td>N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide;hydrochloride</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARM1000390</td>
<td>N,N-diethyl-4-[phenyl(piperidin-4-ylidene)methyl]benzamide;hydrochloride</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>CTOP</td>
<td>d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2; (4R,7S,10S,13R,16S,19R)-N-[(2S,3R)-1-amino-3-hydroxy-1-oxobutan-2-yl]-19-[[2R]-2-amino-3-phenylpropanoyl]amino]-10-(3-aminopropyl)-7-[1R]-1-hydroxyethyl]-16-[(4-hydroxyphenyl)methyl]-13-(1H-indol-3-ylmethyl)-3,3-dimethyl-6,9,12,15,18-penta-oxo-1,2-dithia-5,8,11,14,17-pentacycloicosane-4-carboxamide</td>
</tr>
<tr>
<td>Cy</td>
<td>carbocyanine</td>
</tr>
<tr>
<td>CYM51010</td>
<td>1-[(4-(Acetylamino)phenyl)methyl]-4-(2-phenylethyl)-4-Piperidinecarboxylic acid ethyl ester</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin; (2S)-2-amino-N-[(2R)-1-[2-[(2S)-1-(2-hydroxyethylamino)-1-oxo-3-phenylpropan-2-yl]-methylamino]-2-oxoethyl]amino]-1-oxopropan-2-yl]-3-(4-hydroxyphenyl)propanamide</td>
</tr>
<tr>
<td>DOP</td>
<td>delta opioid</td>
</tr>
<tr>
<td>DOPr</td>
<td>delta opioid receptor</td>
</tr>
<tr>
<td>DOR</td>
<td>delta opioid receptor</td>
</tr>
<tr>
<td>DPDPE</td>
<td>[D-Pen2,5]-enkephalin; (4S,7S,13S)-13-[[2S]-2-amino-3-(4-hydroxyphenyl)propanoyl]amino]-7-benzyl-3,3,14,14-tetramethyl-6,9,12-trioxo-1,2-dithia-5,8,11-triazacyclotetradecane-4-carboxylic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>EC50</td>
<td>fiftieth percentile maximal effective concentration</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ED90</td>
<td>ninetieth percentile effective dose</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
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</table>
ER  endoplasmic reticulum
ERK  extracellular signal–regulated kinase
FACS  fluorescence-activated cell sorting
FBS  fetal bovine serum
FLAG  Asp-Tyr-Lys-Asp-Asp-Asp-Lys
GDP  guanosine diphosphate
GFP  green fluorescent protein
GPCR  G-protein coupled receptor
GTP  guanosine triphosphate
GTP\(\gamma\)S  guanosine triphosphate gamma S
HA  hemagglutinin
HBSS  hank's balanced salt solution
HCl  hydrochloric acid
HEK293  human embryonic kidney cells 293
Hippoc  hippocampus
Hypoth  hypothalamus
ICC  immunocytochemistry
ICI 174,864  N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH
IHC  immunohistochemistry
IP  intraperitoneal
K/DOR  kappa/delta opioid receptor (heteromer)
KI  knock-in
KO  knock-out
KOP receptor  kappa opioid receptor
KOR  kappa opioid receptor
LAMP1  lysosomal-associated membrane protein 1
M/DOR  mu/delta opioid receptor (heteromer)
MDAN  MOR-agonist-DOR-antagonist
MNTB  medial nucleus of the trapezoid body
MOP  mu opioid
MOPr  mu opioid receptor
MOR  mu opioid receptor
mRNA  messenger RNA
N.Acc.  nucleus accumbens
NGS  normal goat serum
norBNI  norbinaltorphimine; 17,17‘-(dicyclopopylmethyl)-6,6‘,7,7‘-6,6‘-imino-7,7‘-bimorphinan-3,4‘,14,14‘-tetrol
NTI  naltirndole
OR  opioid receptor
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PFC  prefrontal cortex
PKC  protein kinase C
RTP  receptor transporter protein
RVM  rostral ventromedial medulla
SDM-25N (4bS,8R,8aS,14bR)-5,6,7,8,14,14b-hexahydro-7-(2-methyl-2-propenyl)-4,8-
methanobenzo[2,3-a]pyrido[4,3-b]carbazole-1,8a(9H)-diol hydrochloride
SEM  standard error of the mean
SNC80 (+)-4-{[(αR)-α-((2S,5R)-4-allyl-2,5-
dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide
TAT  trans-activator of transcription
TBS  tris-buffered saline
TICPΨ 2-[[2-[[2-[2-amino-3-(4-hydroxyphenyl)propanoyl]-3,4-dihydroy-1H-
isoquinolin-3-yl]methylamino]-3-phenylpropanoyl]amino]-3-
cyclohexylpropanoic acid
TIFF tagged image file format
TIPP (2S)-2-(((2S)-2-((3S)-2-((2S)-2-amino-3-(4-
hydroxyphenyl)propanoyl]-3,4-dihydro-1H-isoquinolin-3-
carbonyl]amino]-3-phenylpropanoyl]amino]-3-phenylpropanoic acid
TIPPΨ (2S)-2-(((2S)-2-((3S)-2-((2S)-2-amino-3-(4-
hydroxyphenyl)propanoyl]-3,4-dihydro-1H-isoquinolin-3-
yl[methylamino]-3-phenylpropanoyl]amino]-3-phenylpropanoic acid
TM# transmembrane domain number #
TNT tris-NaCl-tween
U69593 N-methyl-2-phenyl-N-[(5R,7S,8S)-7-pyrrrolidin-1-yl-1-
oxaspiro[4.5]decan-8-yl]acetamide
VTA ventral tegmental area
WFA wisteria floribunda agglutinin
WT wildtype
Zp3 zona pellucida 3
Chapter 1

Introduction: Molecular Perspectives for mu/delta Opioid Receptor

Heteromers as Distinct, Functional Receptors

This work is adapted from the version published in Cells:


1.1 Introduction and Historical Perspective

Opioid drugs are widely used and have remained the first line of therapy for moderate to severe pain for well over a century. A broad range of naturally-occurring opioid poppy (papaver somniferum) alkaloids, semi-synthetic, and fully-synthetic opioid drugs are now in clinical use. For most types of pain, opioids remain the most acutely efficacious analgesics available, despite considerable investment into the development of non-opioid alternatives. Inversely, despite a range of other effects, the clinical use of opioids remains primarily for analgesia; aside from some minor use cases as antitussives and an antidiarrheal, the non-analgesic effects of opioids are viewed as adverse effects. For the clinically-used opioids, these effects are numerous and most prominently include sedation, respiratory depression, nausea, constipation, itch, bradycardia, and addiction. Over the past two decades and driven in part by a growing focus on the treatment of pain, opioid prescription rates have increased dramatically in both Canada and the United States (Fischer, Jones, Krahn, & Rehm, 2011; T. Gomes, Mamdani, Michael Paterson, Dhalla, & Juurlink, 2014; US Centers for Disease Control and Prevention, 2011). While intended, innocently enough, to address the under-treatment of pain (Cole, 2002; Ferrell et al., 2001; Ferrell, McCaffery, & Rhiner, 1992; Resnik, Rehm, & Minard, 2001), an over-reliance on the perceived ‘quick fix’ of opioid analgesics has led to substantial over-prescribing and associated morbidity and mortality (Dhalla, Mamdani, Gomes, & Juurlink, 2011; Herzig, Rothberg, Cheung,

Opioid actions are mediated via the opioid receptors (ORs), a family of at least three G-protein coupled receptors (GPCRs): mu opioid receptor (MOR), delta opioid receptor (DOR), and kappa opioid receptor (KOR). A fourth receptor type, the nociceptin receptor, is now often considered part of the OR family; it was previously named the opioid receptor-like receptor. Though the nociceptin receptor shares considerable sequence homology with the other ORs, there is little cross-affinity in ligands. ORs (and GPCRs in general) are large integral membrane polypeptides with complex tertiary structures featuring seven transmembrane domains and both intra- and extracellular loops and termini. ORs are expressed throughout the central, peripheral, and enteric nervous systems as well as peripheral tissues (Wittert, Hope, & Pyle, 1996). All of the OR types have distinct and heterogeneous expression patterns among tissues.

ORs are functional at the cell surface, where they associate with complex intracellular signalling machinery primarily via coupling to heterotrimeric G-proteins. Opioid agonists bind to and activate ORs, which in turn activate G-proteins, thereby propagating intracellular signalling cascades, and usually inhibiting cell responsiveness. The overwhelming majority of clinically-used opioid analgesics act at MOR, though ORs broadly share in their mechanism of action. Primarily, this involves action via $G_{i/o}$ with ultimate effects including inhibition of adenylyl cyclase (and so, cyclic adenosine monophosphate signalling), inhibition of N- and P/Q-type voltage-gated calcium channels (inhibiting calcium signalling), and activation of inwardly rectifying potassium channels (depolarizing the cell), with additional effects on protein kinase A, protein kinase C,
mitogen-activated protein kinase, and transcription factors (Connor & Christie, 1999). Additional OR signalling mediated via G\_\_ (Chakrabarti, Chang, & Gintzler, 2010; L. Wang & Gintzler, 2002) and, separately, beta-arrestins (Aguila et al., 2012; Cahill, Walwyn, Taylor, Pradhan, & Evans, 2016; T. A. Johnson et al., 2016; A. A. Pradhan et al., 2016) have been identified, though a full understanding of their roles and the circumstances in which they occur is still developing.

ORs and their ligands were originally classified by pharmacological type (MOR, DOR, KOR) and subtype, of which general consensus held there to be eight: MOR\_1,2,3; DOR\_1,2; KOR\_1,2,3. This pharmacological understanding failed to harmonize with the identification, in the early-to-mid 1990s, of only three OR cDNAs—one each for MOR, DOR, and KOR. Interestingly, multiple splice variants of MOR have since been reported, including truncated forms (for review, see (Pasternak, 2004)). Some of these splice variants may have implications for clinical opioid effectiveness, such as MOR-1K (Gris et al., 2010). Nevertheless, these variants have pharmacologies either equivalent to prototypical MOR or markedly different (e.g., MOR-1K has been reported to be excitatory and active intracellularly, but not surface-available). As such, their existence fails to account for OR subtype pharmacology.

Pharmacological investigations of the ORs also revealed an array of synergisms and interactions between the ORs (for review, see (Costantino, Gomes, Stockton, Lim, & Devi, 2012)). Such ‘spooky’ action at a sibling and the discrepancy between the pharmacologically and genetically identifiable OR species led to interest in whether ORs, like several other GPCRs, undergo heteromerization. In this process, multiple different receptors associate to form complexes (heteromers; for reviews, see (Bouvier, 2001; Susan R. George, O’Dowd, & Lee, 2002; Lee, 2003)). In some cases, such as the GABA B receptor, such complexing is required for functionality (Gurevich & Gurevich, 2008; Terrillon & Bouvier, 2004), while in others it serves to alter functionality, such as the interactions of dopamine D2 and adenosine A2A receptors (Vidi, Chemel, Hu, & Watts, 2008).
OR heteromerization was first described by Jordan and Devi (1999), who showed KOR and DOR to be capable of interacting to form functional K/DOR heteromers. Following that first report, the existence of OR heteromers has been demonstrated by a large, and still growing, body of literature utilizing a range of experimental techniques including immunoblotting and co-immunoprecipitation, immunocytochemistry, and bioluminescence and Förster resonance energy transfer (Décaillot, Rozenfeld, Gupta, & Devi, 2008; Fan et al., 2005; S R George et al., 2000; I. Gomes et al., 2000; I. Gomes, Filipovska, Jordan, & Devi, 2002; Hasbi et al., 2007; D. Wang, Sun, Bohn, & Sade, 2005). These proximity and interaction assay findings are convincing and have been thoroughly reviewed (Costantino et al., 2012; Stockton & Devi, 2012). OR heteromers are also a leading explanation for the generation of multiple pharmacological subtypes from only three mRNAs.

Of the species identified, the M/DOR heteromer has emerged as the subject of greatest interest. This is in large part because most clinically used opioid drugs act on MOR, and preclinical results have demonstrated both MOR and DOR to have analgesic effects when activated. The heteromer generated by their interaction holds promise as a therapeutic target and, as discussed below, appears to be involved in changes to the opioid system following chronic MOR agonist exposure (e.g., chronic opioid therapy).

**Nomenclature**

A definitive consensus regarding the appropriate nomenclature for receptor heteromers and their disambiguation from non-heteromer receptor-receptor interactions has yet to emerge. The abbreviated form of M/DOR, K/DOR, etc. is used to refer to the receptor heteromers or the form MOR-DOR, KOR-DOR, etc. when referring to non-heteromer receptor-receptor interaction. For published research chapters, all nomenclature is maintained in the as-published form.
1.2 Formation

1.2.1 Molecular Nature of Mu-Delta Interaction

Understanding the details of an interaction’s quaternary structure is typically challenging and notably so in the case of OR heteromers. The constituent monomers are relatively large membrane-bound proteins with individually complex tertiary structures; they undergo many conformational changes (which are themselves poorly understood) as part of their normal function; and they are individually fully competent, meaning that their interacting state may occur but is not required. A definitive identification of structural nature of the interaction would typically require a method such as crystallography of the complete heteromer. That result remains elusive due to the complexities involved.

Indeed, the crystallographic structures of the OR monomers have only recently been reported (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012) for commentary, see (Filizola & Devi, 2012, 2013)). These findings will soon permit the refinement of the currently most useful techniques for understanding the molecular nature of the OR heteromers: computational modelling and experimental disruption.

1.2.1.1 Computational

Most conceptualizations of OR heteromers are as contact dimers. That is, two adjacent independent polypeptides abutting each other. Their overall interaction is a product of several specific interactions between residues of each monomer and results in conformational changes to each. Current computational studies have sought to identify both the specific interacting residues and broadly how the secondary and tertiary structures of each receptor interact.

Even considering only broad domain-domain interactions, there are a large number of potential interacting configurations for heteromers of receptors each with seven transmembrane domains. Filizola and colleagues (Filizola, Olmea, & Weinstein, 2002) predicted probable configurations by first identifying correlated mutations; residues on each MOR and DOR, which
tend to undergo apparently coordinated mutations due to a need to maintain interactions. The resulting set of probabilistically identified residues was then sorted for residues that would be sterically available for interactions. Using models of the receptors’ transmembrane domains based on the crystallographic structure of rhodopsin, the authors retained only those residues exposed on the outer surface of those domains. The locations of this final set of interacting residues were then used to identify the probable domain-domain interactions in M/DOR. Nearly all of the interacting residues were in transmembrane domain 1 (TM1) of MOR and TM4, TM5, and TM6 of DOR.

A later study by Liu and colleagues (Liu, Kai, Jin, & Wang, 2009) constructed 3D models of the complete sequences MOR and DOR. This computationally-intensive work addressed a major limitation of Filizola et al.’s earlier study, which was limited to considering only the transmembrane domains. However, even these full-length models were still not based upon OR crystallographic structures, which had not yet been produced. They were, instead, homology models; each section of the models was based on the known structures of other proteins with similar sections. The resulting models were then subjected to several simulated interactions in a variety of configurations to find the most likely (sterically and energetically favorable) interacting structure. The authors concluded that the most favorable interaction was between TM1 of MOR and TM4 of DOR and also involved both carboxyl terminal domains. These findings are in agreement with those of experiments which disrupted MOR-DOR associations via alterations to either TM1 of MOR or the carboxyl tails of either OR (see below).

It is notable that both of these computational studies arrived at similar conclusions, identifying the same regions of MOR and DOR as interacting, while using distinct methodologies. However, both studies were undertaken prior to the generation of OR crystallographic structures and are limited by their extrapolation to MOR and DOR of data of the structures of other proteins. In particular, both analyses are heavily reliant on the crystallographic
structure of rhodopsin, which limits the importance of their consilience. Much more convincing validations of these analyses, their models, and their findings are now possible using the recently identified crystallographic structures of the OR monomers. Data and modelling derived from these structures are expected to greatly improve understandings of OR heteromerization (also see (Filizola & Devi, 2013)).

1.2.1.2 Experimental Disruption

Several studies have provided insight into the nature of the heteromeric interactions as a result of specific interventions which disrupted the heteromer. In regards to interactions between transmembrane domains, He et al. (2011) constructed a mutant MOR with a duplicate TM3 in place of TM1. When coexpressed with DOR in HEK293 cells, non-mutated MOR formed M/DOR heteromers identifiable by coimmunoprecipitation, while the mutant MOR did not interact with DOR. Conversely, the authors created a construct, which expressed only TM1 of MOR with a GFP tag. When coexpressed with DOR in HEK293 cells, this TM1-GFP construct formed an interaction with DOR, which was detectable by coimmunoprecipitation. This suggested that TM1 of MOR was both required for MOR-DOR heteromerization and sufficient, independently, to physically interact with DOR. Interestingly, the authors extended upon the latter finding by generating a MOR(TM1)-TAT fusion protein which was delivered intracellularly following in vivo administration and could be used to competitively disrupt M/DOR heteromers (discussed below).

Separately, the carboxyl-terminal domains of both MOR and DOR appear to play a role in determining the pharmacology of the M/DOR heteromer. Fan et al. (Fan et al., 2005) and others reported that M/DOR exhibits pharmacology (ligand binding and activation) unique from that of either constituent alone. In particular, these authors noted that coexpression of MOR and DOR altered the binding profiles of DAMGO ([D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin; a MOR agonist) and DPDPE ([D-Ala2]-deltorphin II; a DOR agonist) as compared to expression of MOR
or DOR alone, respectively. The authors then constructed truncated mutants of MOR and DOR, removing carboxyl-terminal amino acids (23 or 35 in MOR; 15 or 28 in DOR). When truncated DOR was coexpressed with MOR, DAMGO binding was normalized. Similarly, when truncated MOR was coexpressed with DOR, DPDPE binding was normalized. Notably, while c-terminal truncation altered heteromer pharmacology, it had little impact on coimmunoprecipitation. This is consistent with MOR-DOR interactions also involving the transmembrane domains.

A subsequent study from this group (O’Dowd, Ji, O’Dowd, Nguyen, & George, 2012) used multiple mutant variants of MOR and DOR to identify specific amino acids involved in the MOR-DOR interaction. By moving from mutants with longer truncations to versions with single amino acid changes, the authors identified a three residue sequence (GGG) near the carboxyl terminus of DOR which was required for MOR-DOR interaction. No such required sequence was found for the MOR c-terminal tail. Additionally, this study also identified a three residue sequence (SVR) on the third intracellular loop of both MOR and DOR, which was also required for MOR-DOR interaction. This region has previously been shown to be involved with MOR coupling to G-proteins. These findings, which differ slightly from those of Fan’s earlier study, may be attributable to the method used to detect MOR-DOR interactions, which relied upon cotrafficking of MOR and DOR in response to a nuclear localization sequence inserted into one of the pair. Different methodologies used in the literature to test heteromerization probe different related aspects of the interaction, including here: physical association (e.g., coimmunoprecipitation and involving TM1 of MOR), pharmacology (e.g., ligand binding, g-protein coupling, and involving the c-terminal tails of MOR and DOR), and cotrafficking (involving specific carboxyl-terminal residues of DOR and possibly G-protein interactions).

Summary, Molecular Nature of Mu-Delta Interaction

Taken together, the present studies investigating the molecular nature of the MOR-DOR interaction suggest that a set of several residues in several domains of each MOR and DOR
interact. Interacting residues appear to be present in transmembrane domains, intracellular loops, and carboxyl tails. Further, overlapping subsets of these residues/domains are involved with different aspects of the interaction. That is, it is constructive to view the MOR-DOR interaction as having multiple facets rather than as a binary (independent or interacting) state.

It is anticipated that the recently identified crystallographic structures of the OR monomers will lead to the refinement of and improved confidence in understandings of OR heteromerization.

1.2.2 Coexpression of MOR and DOR

MOR-DOR interactions resulting in the formation of M/DOR heteromers obviously require coexpression of MOR and DOR. Such coexpression has, for some time, been reported to be common and widespread (Cheng, Liu-Chen, & Pickel, 1997). However, a recent study reported substantially less coexpression of MOR and DOR. Using a transgenic mouse expressing a knock-in DOR linked to enhanced green fluorescent protein (eGFP), Scherrer and colleagues (Scherrer et al., 2009) immunolabeled eGFP and MOR. They reported that colocalization of DOR-linked eGFP and MOR signals occurred in less than five percent of dorsal root ganglia (DRG) neurons labeled for either OR. However, subsequent studies addressing this inconsistency have continued to report extensive coexpression of MOR and DOR. Wang and colleagues (H.-B. Wang et al., 2010) used single-cell PCR, in situ hybridization, and immunofluorescence to demonstrate coexpression of MOR and DOR in DRG neurons. Further, these authors also demonstrated single-cell electrophysiological responses to both MOR- and DOR-selective ligands. This latter finding is mirrored by work by Chieng and Christie (2009) who used electrophysiological recordings from neurons of the central nucleus of the amygdala to show widespread single-cell responsiveness to both MOR- and DOR-selective ligands. Similarly, Beaudry and colleagues (Beaudry, Dubois, & Gendron, 2011) showed that both MOR- and DOR-selective agonists acted on primary afferents to inhibit formalin- and capsaicin-evoked substance
P release. Interestingly, subsequent development of this transgenic construct by the same group has produced an animal expressing both DOR-eGFP, as well as MOR linked to red fluorescent protein. A comprehensive cataloguing of MOR and DOR expression in this double fluorescent knock-in has reported widespread and substantial coexpression of MOR and DOR in a number of brain structures and in DRG neurons (Eric Erbs et al., 2015). Notably, in the same DRG neurons in which the single-tag model reported five percent MOR-DOR coexpression, the double-tag model reported forty percent coexpression.

A compelling line of reasoning to explain these inconsistent results was presented by Stockton and Devi (Stockton & Devi, 2012) and focuses on three separate findings. First, that the labelling observed with DOR-eGFP is substantially different from that observed with DOR-myc when both are immunolabeled via their linked tag. The eGFP-linked DOR is found primarily on the cell surface, while the myc-tagged DOR is distributed at intracellular sites (H.-B. Wang, Guan, Bao, & Zhang, 2008). Second, that the DOR-eGFP mice are known to overexpress DOR-eGFP in comparison to wild-types (Scherrer et al., 2006). Finally, third, that increased cell surface DOR leads to a decrease in MOR maturation ((Décaillot et al., 2008), discussed below). In this argument, DOR-eGFP expression and surface localization are excessive and result in suppressed MOR expression. This would result in a bias among cells coexpressing MOR and DOR towards being detected as expressing only DOR. Such a bias would be further compounded by the high detection limit of the fluorescence microscopy methodology used; imaging parameters appropriate for capturing the intense, amplified signals from high-MOR-expressing cells could fail to distinguish low-to-moderate-MOR-expressing cells (potentially including the suppressed-MOR-expressing coexpressing cells) from background. This would ultimately result in, and account for, the underreporting of MOR-DOR coexpression.

Summary, Coexpression of MOR and DOR
The extent of neuronal coexpression of MOR and DOR remains the subject of some contention as a result of apparently contradictory experimental findings. The degree to which MOR and DOR are coexpressed and the circumstances which influence their coexpression must obviously affect the physiological role of M/DOR. Current findings regarding both the abundance and lack of MOR-DOR coexpression have methodological limitations, which have slowed the emergence of a consensus regarding the extent of coexpression.

1.2.3 Induction of M/DOR Formation

Though widely described in heterologous systems, the relevance of endogenous OR heteromers was, for some time, difficult to establish. Using newly-generated antibodies against the M/DOR heteromer, we and our co-authors demonstrated the existence of endogenous M/DOR heteromers (Gupta et al., 2010). These monoclonal antibodies against M/DOR were generated using a subtractive immunization strategy and screened for reactivity to M/DOR, as well as MOR, DOR, and the expression system used. This process produced a monoclonal antibody, which specifically recognized an epitope of M/DOR. Using this antibody, we and our co-authors identified endogenous M/DOR in neurons from primary DRG cultures, as well as in cells of the medial nucleus of the trapezoid body (MNTB) and the rostral ventral medulla (RVM). These structures are involved in peripheral sensory transmission, audition, and pain perception, respectively. M/DOR were not detected in either MOR- or DOR-knock-out animals.

Further, we and our co-authors showed endogenous M/DOR abundance to vary in response to extracellular conditions, indicating MOR-DOR interactions to be regulated at the cellular level. Chronic, but not acute, morphine treatment of cultured DRG neurons resulted in increased MOR-DOR colocalization and increased M/DOR abundance, as detected both immunofluorescently and by ELISA. Similarly, chronic in vivo morphine administration resulted in increased M/DOR abundance in the cortex, MNTB, RVM, hypothalamus, nucleus accumbens, and ventral tegmental area. Similar results were found following chronic treatment with naltriben,
a DOR antagonist. These findings demonstrated both the \textit{in vivo} presence of M/DOR and the regulation of M/DOR in response to exogenous ligands.

Interestingly, we, and others, have previously demonstrated that chronic morphine treatment causes a translocation of DOR from intracellular sites to the plasma membrane of treated neurons (Cahill, Morinville, et al., 2001; Chieng & Christie, 2009; Gendron et al., 2006). This process requires MOR presence (Morinville et al., 2003) and does not alter DOR mRNA levels (Morinville et al., 2003). It now appears that the changes in DOR distribution described following chronic morphine are, in fact, part of an upregulation of M/DOR formation. Notably, the stability of DOR mRNA levels suggests this regulation to be post-translational. That is, existing MOR and DOR are induced to interact.

**Summary, Induction of M/DOR formation**

M/DOR have been directly detected in neuronal structures involved in sensory transmission, audition, and pain perception. M/DOR abundance varies in response to extracellular conditions; specifically, chronic, but not acute, morphine treatment increases M/DOR abundance. The regulation of M/DOR formation may involve control over the interaction of existing ORs, rather than \textit{de novo} synthesis.

**1.2.4 Subcellular Location of Formation**

The subcellular location, or locations, at which M/DOR formation takes place has been difficult to discern. Law and colleagues (2005) attempted to directly address the question using a heterologous system to co-express wild-type MOR or DOR with surface-transport deficient mutants of DOR or MOR, respectively. That is, one of the MOR-DOR pair was a mutant incapable of being trafficked to the cell surface. The authors then used fluorescence-activated cell sorting (FACS) to identify surface expression of each OR. This study found that neither wild-type OR was able to rescue its surface trafficking deficient partner. The authors therefore conclude that M/DOR formation occurs only at the cell surface. Alternatively, these results may also
indicate that the formation of a M/DOR heteromer from a surface trafficking deficient mutant results in a surface trafficking deficient heteromer.

Summary, Subcellular Location of Formation

The location of heteromer formation remains unclear.

1.3 Outwards Trafficking

1.3.1 Protein Chaperone

As evidenced by the responsiveness of M/DOR abundance to cellular conditions without altering individual OR transcription/translation, M/DOR formation is a controlled process. Further, given that increased M/DOR abundance following chronic morphine exposure is accompanied by translocation of DOR from internal to cell-surface sites, it is likely that M/DOR formation and this DOR outwards trafficking are linked processes. While Law and colleagues (2005) suggest that heteromer formation occurs only at the cell surface, they did not address the possibility that M/DOR formation may occur internally but resultant heteromers are retained intracellularly. Indeed, this is the case for DOR; over 60% of newly synthesized receptors are retained in the endoplasmic reticulum (ER) and ultimately degraded (Petaja-Repo et al., 2001) and a majority of the remainder are delivered to intracellular sites, with only a paucity going to the cell surface (Cahill, McClellan, et al., 2001). In their 2008 study, Decaillot and colleagues (2008) show that a similar pattern of intracellular retention occurs with M/DOR and that its surface availability is dependent on chaperoning. The authors used HEK293 and Neuro2A expression systems to show that coexpression of MOR and DOR leads to reduced cell surface MOR. This reduction in surface MOR correlated with increased MOR and DOR colocalization with the Golgi apparatus, but not the ER. That is, coexpression of MOR and DOR led to the formation of M/DOR, which was retained in the Golgi. The authors then coexpressed MOR and DOR with RTP4, a member of the RTP family of chaperone proteins, which have been described to facilitate export of proteins, which accumulate in the Golgi (Saito, Kubota, Roberts, Chi, &
Matsunami, 2004). Coexpression with RTP4 resulted in a redistribution of MOR and DOR, presumably as M/DOR, out of the Golgi to the cell surface. Notably, RTP4 had no effect on MOR when expressed alone, nor did RTP2 (another family member) have any effect. The authors then showed that RTP4 coimmunoprecipitates with MOR and DOR (but not c-terminal truncated MOR) and that RTP4 coexpression decreases MOR ubiquitination. Interestingly, while RTP4 coexpression normalizes (i.e., increases) MOR surface abundance, the authors also showed it to decrease DAMGO (a MOR agonist) signalling, further suggesting that the increase in MOR surface abundance is in the form of M/DOR. (M/DOR functionality is discussed in detail in 1.4.)

**Summary, Protein Chaperone**

M/DOR can form intracellularly but is largely retained in the Golgi, ubiquitinated, and degraded. RTP4, a known chaperone protein, can interact with M/DOR to protect it from ubiquitination and chaperone its export from the Golgi to the cell surface. RTP4, and possibly other such chaperone proteins, thus, act to regulate M/DOR availability (Figure 1.1).

These findings, together with those showing increased M/DOR abundance following chronic morphine in the absence of changes in DOR mRNA, show that M/DOR availability, rather than formation, is the major regulated step controlling M/DOR abundance. That is, M/DOR forms readily, but is retained during post-ER transport (and ultimately degraded). This retention controls M/DOR availability and, thus, abundance.

**1.3.2 Perspective from DOR Results**

To date, the work by Decaillot and colleagues remains the only description of M/DOR chaperoning. However, it shares many conceptual and mechanistic similarities with reports of DOR chaperoning. Using metabolic pulse-chase labelling of DOR expressed in HEK293 cells, Petaja-Repo and colleagues (2002) assessed DOR export from the ER compartment where it is typically retained. The authors identified several ligands, both agonists and antagonists, which acted as pharmacological chaperones of DOR. Of the compounds tested, all of the non-peptidic
Under basal conditions M/DOR is normally retained in the Golgi (grey) and cell-surface availability is low. The augmentation of M/DOR surface availability involves transport chaperones, including RTP4 and, potentially, membrane-permeable ligands.
(i.e., small molecule) ligands but none of the peptidic ligands acted as DOR chaperones. The authors conclude that the pharmacological chaperoning of DOR requires membrane permeability in order for the ligands to bind to immature DOR retained in the ER. It is speculated that ligand binding assists in proper folding of DOR, and thus its continued maturation and export. Similarly, the same group went on to demonstrate (Petäjä-Repo et al., 2006) a role for palmitoylation, a post-translational lipid modification, of DOR in its ER export and trafficking to the cell surface. By preventing palmitoylation, the authors inhibited DOR transport to the cell surface. The authors also demonstrated, by inhibiting post-ER transport, that palmitoylation of immature DOR occur around the time of export from the ER. This leads to the conclusion that palmitoylation is a required step in the maturation of DOR and its successful release from the ER.

Despite the parallels, the potential role of DOR transport-regulating mechanism in M/DOR transport has not been investigated. In particular, the ability of non-peptidic ligands to act as DOR pharmacological chaperones has interesting implications for the role of chronic morphine treatment in increasing M/DOR abundance.

Summary, Perspective from DOR Results

The outwards trafficking of M/DOR appears to have many similarities to that of DOR, which is also retained intracellularly (albeit in the ER rather than the Golgi) and who’s outwards trafficking may be modulated by chaperones. Membrane-permeable ligands can act as DOR chaperones. DOR export is also modulated by palmitoylation. The roles of these systems in M/DOR export are unknown.

1.4 Function

As a result of controls on their availability, M/DOR appear to be present in consequential amounts only in certain states (e.g., prolonged opioid use). Interestingly, these states often present difficulties for classical opioid therapy (i.e., reductions in efficacy). Considerable attention is being paid to M/DOR’s possible role in these changes and as its use as a novel therapeutic target.
Research along both of these paths is elucidating the functional consequences of M/DOR existence, though interpretation of these results is made difficult by the variability of M/DOR availability.

1.4.1 Surface Expression

Current understandings of the effects of M/DOR availability on MOR and DOR surface expression are incomplete. As discussed earlier, Decaillot and colleagues (2008) used a heterologous system to demonstrate that coexpression of DOR with MOR resulted in a reduction of surface MOR compared to cells expressing MOR alone. This change was attributed to the formation of M/DOR, with the resulting heteromers being retained in the Golgi. A substantial proportion of the MOR expressed was unavailable, which resulted in the reduction in MOR surface expression. These findings are contrasted by work by Walwyn and colleagues (2009) using primary cultures of DRG neurons from wild-type and DOR knock-out (KO) animals. Measuring immunofluorescent labelling of MOR surface expression by flow cytometry, the authors show reduced MOR surface expression in DOR KO neurons. While prima facie contradictory, these results likely represent M/DOR effects on MOR surface expression in different conditions of M/DOR availability. In a simple expression system, MOR and DOR are produced at a near 1:1 ratio in large quantities, due to the use of CMV promoters. This appears to be ideal for M/DOR formation, with so much MOR diverted to M/DOR (and thus substantially retained in the Golgi, in part due to the lack of chaperones) that MOR surface expression is greatly reduced. In the more physiologically relevant DRG neuronal culture, MOR and DOR expression levels are far lower, M/DOR formation would likely be far less common, M/DOR chaperones would be available, and normal MOR regulatory mechanisms are present. As a result, a small pool of M/DOR is on the surface in wildtypes and absent in DOR KOs. Surface MOR, meanwhile, remains normally and independently regulated in both states. Total surface MOR labelling (M/DOR + MOR) is then reduced in DOR KOs.
Summary, Surface Expression

The apparently contradictory findings regarding the effects of M/DOR formation on MOR surface expression can be explained by methodological differences. Under basal physiological conditions, the surface population of M/DOR is likely small, but sufficient that in DOR KO neurons, the apparent surface population of MOR is reduced by the loss of the M/DOR pool. The remaining MOR surface population remains under normal controls, present in physiologically relevant neurons but not in expressions system. This is further evidence that M/DOR availability is specifically regulated and not simply the result of stochastic MOR-DOR contacts. That is, M/DOR and MOR are independently regulated populations.

1.4.2 Ligand Binding

Much of the interest concerning the M/DOR heteromer stems from its unique pharmacology. Having both MOR and DOR binding sites and affinities for both MOR and DOR ligands, M/DOR pharmacology is, nevertheless, not simply a combination of the profiles of MOR and DOR. Fan and colleagues (2005) showed that the ligand binding profile of M/DOR is distinct from those of MOR and DOR. The authors used competitive radioligand binding to assess the affinities of three OR agonists in cells expressing MOR alone, DOR alone, or MOR and DOR. DAMGO (a MOR agonist) and DPDPE (a DOR agonist) were shown to have high affinity in matched single OR-expressing cells, despite having far lower affinities than Deltorphin II (DELT, a DOR agonist) in MOR-DOR coexpressing cells. Thus, while binding the same overall class of ligands (opioids), M/DOR has a unique pharmacological profile. Prior to the discovery of widespread OR heteromerization, ORs were commonly classified into pharmacological subtypes. There was general consensus on the existence of eight such subtypes (MOR1-3, DOR1-2, KOR1-3), each with unique pharmacological profiles. The subtype classifications have fallen out of use since the identification of only a single cDNA (and, so, mRNA) for each OR and the failure to identify post-translational modifications, which might account for the various subtypes. It now
appears that various OR heteromers may, in fact, be those pharmacologically distinct subtypes. Portoghese and Lunzer (2003) identify the DOR1 subtype as a D/KOR heteromer by studying the \textit{in vivo} actions of the KOR antagonist norbinaltorphimine (norBNI). norBNI was previously held to be nonspecific as it inhibited the actions of DAMGO and DPDPE. The authors show that norBNI’s antagonism of DAMGO is a result of norBNI blocking the actions of dynorphin-A, of which release is promoted by DAMGO. Blockade of dynorphin-A by dynorphin-A antiserum results in little added antagonistic effect of norBNI on DAMGO. However, this is not the case for the DOR1 agonist DPDPE. Even in the presence of dynorphin-A antiserum, norBNI retains its full antagonistic action. Conversely, norBNI has little effect on the DOR2 agonist DELT. The identity of DOR1 as D/KOR is reinforced by work from the same group using KO animals (Ansonoff, Portoghese, & Pintar, 2010). norBNI is able to antagonise DPDPE in wild-type but not KOR KO animals. The authors suggest that KO of KOR prevents the formation of D/KOR, and thus norBNI’s ability to antagonise DPDPE, while DPDPE itself retains efficacy at other DOR sites. In this case, DPDPE appears to be heteromer-active but not heteromer-specific.

**Summary, Ligand Binding**

M/DOR’s overall pharmacological profile is unique and responsiveness to any given ligand is different from that of MOR or DOR. Still, M/DOR’s pharmacology overlaps that of MOR and DOR. These findings are a useful example of the difficulties involved in understanding the pharmacology of M/DOR. Even in the most artificial heterologous systems, it coexists with both MOR and DOR. Therefore, much of the work investigating M/DOR ligands is only able to identify M/DOR-active ligands. The identification of heteromer-specific ligands is more demanding and frequently involves the synthesis and screening of novel compounds. For that reason, much attention has been paid to the use of ligand combinations, as well as bivalent ligands, to exploit heteromers (discussed below).
1.4.2.1 M/DOR-Active Ligands

A large number of ligands to MOR or DOR have been reported to show activity at M/DOR and a summary is shown in Table 1. In some cases, these reports have also sought to reconcile previous categorizations of ligand by OR subtype activity with M/DOR. These studies are, however, limited by the difficulties in isolating M/DOR effects from MOR or DOR actions, as discussed previously.

1.4.2.2 M/DOR Specific/Selective Ligands

The vast majority of the ligands reported to have activity at M/DOR are also active at either MOR or DOR. There is typically some difference in affinity or potency at the different receptor species. From this, some authors have concluded some of these ligands to be M/DOR selective (Yekkirala, Kalyuzhny, & Portoghese, 2010), though most reports describe activities at M/DOR equal to or less than that at MOR or DOR. The identification of ligands, which are M/DOR specific, may not be possible given the nature of M/DOR. Even the identification of truly selective M/DOR ligands is difficult and limited by the challenge of interpreting findings derived from systems with both MOR and DOR present in addition to M/DOR. Two groups have now described high-throughput assays intended to screen compounds for M/DOR selectivity. (These screening assays are discussed in greater detail below.) Gomes and colleagues (2013) report identifying such compounds with their assay. Briefly, the authors use four cell lines stably expressing MOR, DOR, MOR and DOR, or 5HT5A. In these cell lines, receptor activation results in the unmasking of beta-galactosidase activity, which then activates a chemiluminescent reporter. This provides the authors with cells, which report activity at MOR, DOR, MOR, and/or M/DOR, or 5HT5A. (In cells expressing MOR and DOR, the reporter is linked to MOR, meaning those cells report activity at both MOR and M/DOR.) The authors used these reporter cells to screen a small molecule library of several hundred thousand compounds for activity at M/DOR,
<table>
<thead>
<tr>
<th>Ligand</th>
<th>Monomer action</th>
<th>Reported heteromer action</th>
<th>Methods</th>
<th>Source</th>
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<td>G-protein activation screening assay</td>
<td>(van Rijn, Harvey, Brissett, DeFriel, &amp; Whistler, 2013)</td>
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<td>Calcium mobilization</td>
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<td>Deltorphin II</td>
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<td>KOR agonist</td>
<td>none</td>
<td>Calcium mobilization</td>
<td>(Yekkirala et al., 2010)</td>
</tr>
<tr>
<td>Ligand</td>
<td>Monomer action</td>
<td>Reported heteromer action</td>
<td>Methods</td>
<td>Source</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>--------------------------</td>
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<td>--------</td>
</tr>
<tr>
<td>Naltriben</td>
<td>DOR antagonist</td>
<td>Blocks M/DOR endocytosis but not signalling</td>
<td>Biotin protection, calcium mobilization</td>
<td>(Milan-Lobo &amp; Whistler, 2011)</td>
</tr>
<tr>
<td>DAMGO &amp; Deltorphin II</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(I. Gomes et al., 2004)</td>
</tr>
<tr>
<td>DAMGO &amp; Deltorphin II</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>none</td>
<td>Antinociception</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>DAMGO &amp; DPDPE</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>none</td>
<td>Antinociception</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>DAMGO &amp; TIPPPψ</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>DAMGO &amp; TICPψ</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>Antinociception</td>
<td>(Yekkirala et al., 2012)</td>
</tr>
<tr>
<td>DAMGO &amp; TIPP</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>Antinociception</td>
<td>(Yekkirala et al., 2012)</td>
</tr>
<tr>
<td>DAMGO &amp; Naltriben</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>DAMGO &amp; ICI 174,864</td>
<td>MOR agonist &amp; DOR inverse agonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Morphine &amp; Deltorphin II</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Morphine &amp; TIPPPψ</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding, cAMP inhibition, antinociception</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Morphine &amp; Naltriben</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTPγS binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Ligand</td>
<td>Monomer action</td>
<td>Reported heteromer action</td>
<td>Methods</td>
<td>Source</td>
</tr>
<tr>
<td>--------</td>
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</tr>
<tr>
<td>Morphine &amp; Naltrindole</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR antagonism</td>
<td>Calcium mobilization, antinociception</td>
<td>(I. Gomes et al., 2013)</td>
</tr>
<tr>
<td>Morphine &amp; ICI 174,864</td>
<td>MOR agonist &amp; DOR inverse agonist</td>
<td>M/DOR agonism</td>
<td>GTP(_\gamma)S binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Fentanyl &amp; Deltorphin II</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>M/DOR agonism</td>
<td>GTP(_\gamma)S binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Fentanyl &amp; TIPP(\psi)</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTP(_\gamma)S binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Fentanyl &amp; Naltrindole</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR antagonism</td>
<td>Calcium mobilization, antinociception</td>
<td>(I. Gomes et al., 2013)</td>
</tr>
<tr>
<td>Methadone &amp; Deltorphin II</td>
<td>MOR agonist &amp; DOR agonist</td>
<td>M/DOR agonism</td>
<td>GTP(_\gamma)S binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Methadone &amp; TIPP(\psi)</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR agonism</td>
<td>GTP(_\gamma)S binding</td>
<td>(Szentirmay et al., 2013)</td>
</tr>
<tr>
<td>Methadone &amp; Naltrindole</td>
<td>MOR agonist &amp; DOR antagonist</td>
<td>M/DOR antagonism</td>
<td>Calcium mobilization, antinociception</td>
<td>(I. Gomes et al., 2013)</td>
</tr>
<tr>
<td>MDAN 16 to 21</td>
<td>Novel bivalent ligand</td>
<td>M/DOR agonism</td>
<td>Antinociception</td>
<td>(Daniels, Kulkarni, Xie, Bhushan, &amp; Portoghese, 2005)</td>
</tr>
<tr>
<td>CYM51010</td>
<td>Novel ligand</td>
<td>M/DOR agonism</td>
<td>M/DOR activation screening assay</td>
<td>(I. Gomes et al., 2004)</td>
</tr>
</tbody>
</table>
but not MOR, DOR, or 5HT5A. From this library, 94 compounds were identified with an EC50 in M/DOR expressing cells of ≤10 µM and five times higher than the EC50 in MOR or DOR expressing cells. Of the 94 compounds meeting selectivity criteria in high-throughput screening, the author chose 14 for follow-up validation. Of these 14, six show higher efficacy in M/DOR expressing cells compared to MOR or DOR expressing cells. This hit rate likely reflects the high-throughput assay’s detection of compounds with higher M/DOR potency but not higher efficacy. The authors proceeded to characterise one of the identified compounds, CYM51010. They show a reduction in CYM51010 activity by coadministration of a M/DOR antibody (described above) but not an anti-Flag antibody. Indeed, coadministration of CYM51010 with both the M/DOR antibody and naltrexone (a MOR antagonist), completely blocks CYM51010 activity. In vivo, CYM51010 is antinociceptive and can be blocked by the M/DOR antibody. The susceptibility of CYM51010 to antagonism by the M/DOR antibody is an important finding in discriminating a ligand with activity at M/DOR from one active at only MOR or DOR. This technique addresses one of the major limitations of other studies seeking to identify M/DOR-active ligands in which changes in activity in M/DOR expressing cells could not distinguish between those changes being a result of M/DOR activity or a result of changes in MOR or DOR availability or coupling.

**Summary, M/DOR Specific/Selective Ligands**

Most of the M/DOR active ligands identified to date are also MOR or DOR ligands, which is to be expected because of the similar/shared binding sites and the availability of MOR and DOR ligands. While the activities of these ligands at M/DOR differ from the activities of the same ligands at either MOR or DOR, they generally cannot be said to be M/DOR selective or specific. The identification of true M/DOR selective-specific ligands depends on recently-begun projects which have implemented novel screening assays. A preliminary report from one group has identified one novel M/DOR selective agonist, CYM51010.
1.4.2.3 Ligand Combinations

One of the more conceptually straightforward approaches to exploiting a pharmacological target with two binding sites has been to simply use two ligands. In their 2004 work, Gomes and colleagues (I. Gomes et al., 2004) show in cell lines coexpressing MOR and DOR, that coadministration of DELT with morphine or DAMGO produces increased GTPγS binding versus administration of morphine or DAMGO alone. This, of course, could be explained by additive effects at MOR and DOR separately. However, the authors proceed to demonstrate similar increases in signalling follow MOR agonist coadministration with TIPPψ (peptidic DOR antagonist), naltriben (non-peptidic DOR antagonist), and ICI 174,864 (DOR inverse agonist). They go on to show the same effects in mouse spinal cord membranes and then demonstrate the augmentation of morphine antinociception, in vivo, following TIPPψ coadministration. These findings lead to the conclusion that simple occupancy of DOR binding sites by any ligand is sufficient to augment MOR signalling.

These findings are reinforced by the work of Szentirmay and colleagues (2013), who investigated the in vivo coadministration of DAMGO with DOR ligands. Interestingly, they conducted these experiments in both naive and chronic morphine treated animals. Chronic morphine treatment causes both morphine tolerance (and cross-tolerance to other MOR agonists) and the increased availability of M/DOR (discussed above). In morphine treated mice, the potency of DAMGO antinociception is, unsurprisingly, greatly reduced (that is, there is tolerance). Coadministration of DELT (DOR agonist) had no effect on DAMGO potency, while DPDPE (DOR agonist) coadministration actually reduced DAMGO potency even further. Conversely, coadministration of either TIPP or TICPψ (both DOR antagonists) increase DAMGO potency, restoring it to control (non-morphine-tolerant) levels.

The mechanisms responsible for this phenomenon were addressed by the work of Rozenfeld and Devi (2007), in which they describe the unique downstream coupling of M/DOR (discussed in detail below). Briefly, the authors show that M/DOR constitutively recruits β-
arrestin2 and that subsequent DAMGO-evoked signalling results in an altered pattern of ERK phosphorylation. Coadministration of DAMGO with TIPPψ is shown to revert ERK phosphorylation to the pattern observed with MOR alone. There is, though, no evidence that ligand combinations dissociate M/DOR. Rather, MOR/DOR ligand combinations appear to shift M/DOR from β-arrestin2-mediated signalling to non-β-arrestin2-mediated signalling. This could be interpreted as a normalization of signalling from M/DOR-typical to MOR-typical. This would also explain the augmentation of MOR activity. In the specific case of the restoration of MOR activity following chronic morphine, an appealing explanation is that chronic morphine causes an increase in M/DOR availability. The shift in surface receptor complement from MOR to M/DOR results in decreased overall MOR responsiveness. This change contributes to observed MOR tolerance. Subsequent administration of MOR agonist - DOR antagonist combination can normalise M/DOR signalling. This presents as a reversal of tolerance.

This narrative is contrasted by a study which reports blockade of MOR signalling by DOR antagonist (Yekkirala et al., 2012). This work used HEK293 cells expressing either MOR alone or MOR and DOR. In MOR expressing cells, DOR antagonist naltrindole (NTI) is unable to induce calcium mobilization evoked by morphine, fentanyl, or methadone (all MOR agonists). In cells coexpressing MOR and DOR, however, NTI was able to block the effects of all three MOR agonists. In vivo assessments of antinociception produced by these MOR agonists also demonstrated the ability of NTI to shift dose-response curves rightward in a dose-dependent manner. That is, increasing doses of NTI resulted in increasing reductions in MOR agonist antinociception. These results are difficult to reconcile with other reports of ligand combinations acting on M/DOR. The most apparent difference in the models used is that these findings are from opioid-naive cells and animals, where as other reports showing augmentation of MOR activity by DOR antagonist show those effects in opioid tolerant states. It is also possible that
these results reflect pharmacological chaperoning of DOR by the non-peptidic NTI which did not occur with peptidic TIPP, TIPPψ, or TICPψ.

Summary, Ligand Combinations

The majority of current work aiming to rationally manipulate M/DOR has used combinations of MOR and DOR ligands. Activity by MOR agonists at M/DOR is less than that at MOR. Coadministration of a MOR agonist with a DOR ligand can result in augmented MOR activity at M/DOR. Peptidic DOR antagonists appear to be the most effective.

Shifts in surface receptor complement towards more M/DOR and less MOR may contribute to MOR agonist tolerance. High efficacy activation of M/DOR by appropriate ligand combinations can manifest as a reduction of tolerance (versus low efficacy activation by single MOR ligands).

1.4.2.4 Bivalent Ligands

A particularly interesting method to exploit M/DOR via ligand combinations has been the design of so-called bivalent ligands. These compounds are constructed of two distinct pharmacophores joined by a spacer into a single molecule. Essentially, these are single molecule ligand combinations. The use of bivalent ligands targeted against OR heteromers was first described, and subsequently developed, for D/KOR (Bhushan, Sharma, Xie, Daniels, & Portoghese, 2004; Daniels, Kulkarni, et al., 2005). This technique was translated to M/DOR by Daniels and colleagues (Daniels, Lenard, et al., 2005), who designed their strategy based upon the reports of the M/DOR efficacy of MOR agonist-DOR antagonist combinations (see 1.4.2.3 above). The resulting bivalent ligands were named MDANs (MOR-agonist-DOR-antagonist) and used the oxymorphone (MOR agonist) and naltrindole (DOR antagonist) pharmacophores. Based on previous experience with D/KOR and M/DOR structural models, the authors expected that MOR-DOR heteromerization should result in MOR and DOR binding pockets fixed in space relative to each other. That is, the binding pockets should remain a set distance away from each
other, as dictated by the structure of the heteromer. This distance necessitates the spacer in MDANs, linking the two pharmacophores and holding them at a set distance away from each other. As the authors did not know the precise spacing of the binding pockets, they constructed a series of MDANs with spacers varying from 16 to 21 atoms in length. The authors then tested the in vivo antinociceptive efficacy of these ligands. Administered acutely, none of the MDANs is as potent as a control ligand containing only a MOR-agonist pharmacophore (a ‘monovalent’ ligand) or the parent compound, oxymorphone. This finding is consistent with previous reports of ligand combinations in naive animals (a state which appears to have low M/DOR availability). However, when administered chronically, the bivalent ligands cause less tolerance and physical dependence than the monovalent control, the monovalent control combined with a separate DOR antagonist, or morphine. Interestingly, MDANs with the longest spacers cause no detectable tolerance nor significant physical dependence.

In a follow-on study, the rewarding properties of the longest MDANs were investigated (Lenard, Daniels, Portoghese, & Roerig, 2007). These MDANs have spacers of 19 and 21 atoms (MDAN-19 and MDAN-21, respectively). MDAN-16 was included as a control. As reported, conditioned place preference, a measure of drug reward, develops to morphine, fentanyl (a MOR agonist), and the monovalent control, but not to MDAN-19 or MDAN-21. Results for MDAN-16 were equivocal and all ligands were administered at a dose four-fold higher than their ED90 dose for antinociception. The place preference induced by the monovalent control could be blocked by coadministration of naloxone (a MOR antagonist) but not by coadministration of a DOR antagonist. This demonstrates that inability of MDAN-19 and -21 to induce a place preference is not simply a matter of ligand combination, but rather that it is dependent on the bivalent nature of the ligands. The authors also demonstrate that MDAN-19 and -21 are similarly unable to reinstate a previously extinguished place preference to morphine, whereas reinstatement does occur with both morphine and the monovalent control. Together, these results show that MDAN-19 and -21
are less rewarding than typical MOR agonists. These measures of reward are widely used as animal models of abuse liability. Overall, long-spacer MDANs are suggested to be effective analgesics with reduced tolerance, physical dependence, and psychological dependence.

**Summary, Bivalent Ligands**

The construction of bivalent ligands is a refinement of the ligand combination concept. Bivalent ligands allow more specific targeting of heteromers. Optimizations of pharmacophore spacing have yielded insights into the relative orientation of heteromer binding pockets. The M/DOR bivalent ligands studied to date have involved a single MOR agonist pharmacophore (oxymorphone) linked to a single DOR antagonist pharmacophore (naltrindole; these compounds are termed MDAN for Mu-Delta-Agonist-aNtagonist). One can imagine that future studies using the bivalent ligand concept could investigate different specific pharmacophore combinations.

MDANs have less acute efficacy than monovalent MOR agonists, both in terms of antinociception and reward. Administered chronically, they cause less tolerance and physical dependence.

**1.4.3 Downstream Coupling**

The intracellular signalling cascades to which M/DOR couples are poorly understood. Broadly, M/DOR acts similarly to its constituent ORs: M/DOR activation causes activation of G-proteins, inhibition of intracellular calcium release, and overall inhibition of cellular excitability. In the majority of work addressing M/DOR, these effects are used as accessible telltales for the overall concept of receptor functionality. The lack of attention to M/DOR downstream coupling is both understandable, given the often intimidating complexities of intracellular signal propagation, and lamentable, because it is now abundantly clear that M/DOR is a distinct functional species that behaves differently, often very much so, than other OR species. The differences in the physiological actions of M/DOR certainly involve changes in downstream coupling. Research which has addressed M/DOR downstream coupling remains limited in both quantity and scope.
Fan and colleagues (2005) sought to identify the G-protein to which M/DOR couples. They used a heterologous system to generate cells expressing MOR, DOR, or MOR and DOR (and so, presumably, M/DOR). In cells expressing MOR and DOR, pertussis toxin failed to abolish the inhibition of adenylyl cyclase activity caused by DELT (a DOR agonist). This is in contrast to cells expressing only MOR or DOR, in which pertussis toxin did abolish OR functionality. MOR and DOR are commonly held to signal via coupling with the pertussis toxin-sensitive Gαi. These data suggest that M/DOR signals, at least in part, via coupling with a pertussis toxin-insensitive G-protein. The authors then radiolabeled activated G-proteins with GTPγ35S and immunoprecipitated Gαi3 and Gz (a pertussis-insensitive G-protein capable of inhibiting adenylyl cyclase). In doing so, they showed that in cells expressing only MOR or DOR, only Gαi3 was activated. In cells expressing MOR and DOR, both Gαi3 and Gz were activated. Therefore, it appears that MOR and DOR couple only to Gαi3, whereas M/DOR couples, in part or in whole, with Gz.

As discussed above, Rozenfeld and Devi (2007) investigated M/DOR association with β-arrestin2. These authors also used a heterologous system to generate cells expressing MOR, DOR, or MOR and DOR (and so, presumably, M/DOR). In cells expressing MOR and DOR, β-arrestin2 appears to colocalize with MOR and DOR. This contrasts with what appears to be nearly no colocalization in cells expressing only MOR or DOR. More convincingly, β-arrestin coimmunoprecipitates with MOR and DOR in coexpressing cells, but not with either MOR or DOR when expressed alone. The authors note that the association of β-arrestin2 with MOR and DOR even in the absence of ligand binding or activation suggests that M/DOR constitutively recruits β-arrestin2. Given that β-arrestin2 has previously been shown to be involved in the ERK signalling pathway, the authors then assessed the time course of ERK phosphorylation following MOR agonist treatment in cells expressing either MOR and DOR or MOR alone. They showed that ERK phosphorylation occurs at later time points in cells expressing MOR and DOR,
compared to cells expressing MOR alone. That is, the time course of ERK phosphorylation is right-shifted. Interestingly, this shift in ERK phosphorylation is proportionate to the ratio of MOR to DOR expressed; relatively more MOR results in less of a shift (i.e., closer to pattern of phosphorylation seen with MOR alone), while shifting MOR expression closer to a 1:1 ratio with DOR results in a progressively greater right-shift in ERK phosphorylation. This suggests that M/DOR activation results in a predominately late-phase ERK phosphorylation, in contrast to the predominately early-phase phosphorylation seen following MOR activation. Using the PKC inhibitor calphostin C, the authors then showed that the early phase of ERK phosphorylation seen with MOR is PKC-dependent; early ERK phosphorylation is inhibited by calphostin C. Late phase ERK phosphorylation as seen with M/DOR, however, is not PKC-dependent. Though not PKC-dependent, the shift to late-phase ERK phosphorylation does appear to be β-arrestin2-dependent. Knock-down of β-arrestin2 expression by siRNA in cells expressing MOR and DOR results in restoration of ERK phosphorylation to the pattern observed in cells expressing only MOR. As described above, the authors also demonstrated that this β-arrestin2-mediated signalling occurs in response to single-ligand M/DOR activation but is abolished in favor of MOR-typical signalling in response to some ligand combinations.

These studies both offer interesting glimpses of M/DOR downstream coupling and its differences from that of MOR and DOR. In particular, the work of Rozenfeld and Devi (2007) highlights that not only does M/DOR have distinct coupling, but that that coupling can change in an agonist-dependent manner. Indeed, the emerging concept of unique agonist-dependent effects now appears to apply to ORs generally. That two drugs of the same class may act via the same receptor and have different downstream effects - sometimes slightly but sometimes substantially – is challenging to understand but presents promising avenues for therapeutic exploitation. However, these studies specifically and the current understandings of M/DOR intracellular signalling more generally remain narrow in scope. The identification of associations with a
handful of intracellular signalling components and changes in a few downstream effects are interesting and important progress but remain well short of understanding the full cascades and variations involved.

**Summary, Downstream Coupling**

M/DOR has distinct downstream coupling that can involve signalling via Gz and/or β-arrestin2. Subsequent ERK phosphorylation is temporally shifted in M/DOR signalling versus that of MOR or DOR. Such distinct coupling can change in an agonist-dependent manner. Activation by certain ligand combinations appears to shift M/DOR downstream coupling to that of MOR or DOR (Figure 1.2). These findings correspond with the previously described cell- and system-level effects of ligand combinations/bivalent ligands.

**1.5 Inwards Trafficking**

In addition to availability and functionality at the surface, OR inwards trafficking is fundamental to the function of ORs. OR desensitization, internalization, and recycling to the plasma membrane have significant functional consequences, affecting both analgesia and tolerance (Groer et al., 2007; L. He, Kim, & Whistler, 2009; Kim et al., 2008; A. A. A. Pradhan et al., 2010) (for reviews (Cahill, Holdridge, & Morinville, 2007; P. Y. Law & Loh, 1999; Martini & Whistler, 2007; Zuo, 2005)). ORs routinely internalise upon agonist exposure. Such internalization is likely agonist-directed and, together with post-internalization handling, modulates cell-level OR functionality. Following endocytosis, receptors may undergo recycling back to the plasma membrane, which is thought to underlie re-sensitization, or may undergo targeting to the degradation pathways, a key process in receptor down-regulation. As these fates are mutually exclusive, GPCRs are generally categorized as either ‘class A’ (recycled) or ‘class B’ (degraded) (Drake, Shenoy, & Lefkowitz, 2006). This division appears to arise from the relative stability of the GPCR-arrestin interaction. Individual receptors are directed to one or the other pathway based upon ubiquitination signals, with greater ubiquitination marking the receptor
Figure 1.2  Downstream coupling.

MOR and DOR monomers, when activated by their respective ligands, signal via activation of Gi3, which leads, via PKC, to early phase ERK phosphorylation. M/DOR ligand-induced signalling occurs via Gz and β-arrestin2 and involves a shift to late phase ERK phosphorylation. Activation of M/DOR by ligand combinations (MOR agonism, DOR antagonism) results in monomer-like downstream coupling resulting in PKC-dependent early phase ERK phosphorylation.
for degradation. The post-internalization handling of M/DOR remains unresolved, however, as its constituents are trafficked differently. While both MOR and DOR are internalized constitutively and in response to activation by agonists, MOR is recycled to the cell surface (class A), whereas DOR is trafficked to lysosomes (class B) and degraded (P. Y. Law & Loh, 1999; Tanowitz & Von Zastrow, 2002; Whistler et al., 2002).

Two recent studies have begun to elucidate the post-internalization trafficking of M/DOR. He and colleagues (2011) report that MOR and DOR cointernalize in response to agonist exposure. Using a HEK293 expression system, the authors immunolabeled surface MOR and DOR (and so, presumably, M/DOR). Following deltorphin I, deltorphin II, SNC80 (all DOR agonists), or DAMGO (a MOR agonist), MOR and DOR were both observed to internalise more than in control conditions and appeared to be located in similar areas within the cell. The authors went on to use a LysoTracker probe to additionally label acidic organelles and report that following treatment with deltorphin I, but not DAMGO, MOR and DOR appeared to colocalize with these lysosome-like acidic organelles. Immunoblotting was then used to show that deltorphin I, but not DAMGO, increased ubiquitination of coimmunoprecipitated MOR and DOR (a signal for trafficking to degradation).

Similarly, Milan-Lobo and Whistler (2011) also used a HEK293 expression system and report that in cells coexpressing MOR and DOR, both methadone and DAMGO (both MOR agonists) induced the endocytosis of DOR. This effect was not observed in cells expressing only DOR. The authors used serial immunoprecipitations to identify the internalized species and report that both methadone and DAMGO induced the endocytosis of MOR and M/DOR, but not DOR. The authors then showed that this methadone- and DAMGO-induced endocytosis was blocked by pretreatment of the cells with naltriben (a DOR antagonist). Interestingly, although naltriben was able to block methadone- and DAMGO-induced endocytosis, it was only moderately effective at inhibiting methadone and DAMGO signalling. Indeed, naltriben was more effective at inhibiting
methadone and DAMGO signalling in cells expressing only MOR than in the cells coexpressing MOR and DOR. This is interesting, as it is further evidence that MOR-agonist/DOR-antagonist ligand combinations act to stabilize M/DOR while still permitting signalling. Precise interpretations are difficult, however, as it is unclear whether naltriben’s effects on MOR agonist signalling owed to effects at MOR or M/DOR. Additionally, these findings also raise the question of naltriben’s selectivity at the concentrations used.

Puzzlingly, the findings of Milan-Lobo and Whistler (2011) do not fully agree with those of He and colleagues’ (2011) with regards to the post-internalization fate of M/DOR. Milan-Lobo and Whistler used biotinylation to assess the degradation of internalized receptors and serial immunoprecipitation to identify the species of those degraded receptors. In response to methadone treatment, both MOR and M/DOR were endocytosed. Internalized MOR were relatively stable, with only ~25% degraded after two hours’ agonist treatment. Internalized M/DOR, however, underwent significantly greater degradation, with ~85% degraded after two hours. This contrasts with He and colleagues’ report that treatment with DAMGO did not result in either increased ubiquitination or lysosome-like acidic organelle colocalization or MOR and DOR. This discrepancy may owe to the different ligands used by the two groups, methadone and DAMGO (summarized in Table 2). If this were the case, it would suggest that M/DOR undergoes agonist-directed post-internalization trafficking. It is also possible that this inconsistency reflects a methodological limitation of He and colleagues’ study, which relied on a coarse and somewhat subjective measure of OR-lysosome associations. Finally, it is conceivable that M/DOR post-internalization trafficking does not cleanly fit the model of either MOR or DOR but is, rather, intermediate, with trafficking to degradation occurring probabilistically, not obligately (Figure 1.3). In such a case, the alternative fate is recycling. In a state of continued agonist exposure, recycled receptors would soon repeat the same probabilistic sorting. Therefore, the original pool of receptors would inevitably, albeit gradually, be fully depleted by trafficking to degradation.
Table 2  Ligands reported to traffick M/DOR.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Internalization</th>
<th>Degradation</th>
<th>Recycling</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltorphin I</td>
<td>Increased</td>
<td>Increased</td>
<td>-</td>
<td>(S.-Q. He et al., 2011)</td>
</tr>
<tr>
<td>Deltorphin II</td>
<td>Increased</td>
<td>-</td>
<td>-</td>
<td>(S.-Q. He et al., 2011)</td>
</tr>
<tr>
<td>SNC80</td>
<td>Increased</td>
<td>-</td>
<td>-</td>
<td>(S.-Q. He et al., 2011)</td>
</tr>
<tr>
<td>DAMGO</td>
<td>Increased</td>
<td>No change</td>
<td>-</td>
<td>(S.-Q. He et al., 2011)</td>
</tr>
<tr>
<td>DAMGO</td>
<td>Increased</td>
<td>-</td>
<td>-</td>
<td>(Milan-Lobo &amp; Whistler, 2011)</td>
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<tr>
<td>DAMGO &amp; Naltriben</td>
<td>No change</td>
<td>-</td>
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<td>(Milan-Lobo &amp; Whistler, 2011)</td>
</tr>
<tr>
<td>Methadone</td>
<td>Increased</td>
<td>Increased</td>
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<td>(Milan-Lobo &amp; Whistler, 2011)</td>
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<tr>
<td>Methadone &amp; Naltriben</td>
<td>No change</td>
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<td>(Milan-Lobo &amp; Whistler, 2011)</td>
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“-” denotes untested conditions
Figure 1.3  MOR, DOR, and M/DOR are internalized in response to agonist activation.

The sorting of receptors to either recycling or degradation is probabilistic and varies by both receptor species and ligand. Monomeric MOR and DOR have high probabilities of recycling and degrading, respectively. M/DOR is intermediate to the monomers and is subject to greater ligand biases.
Thus, post-internalization trafficking intermediate to those of MOR and DOR would appear, ultimately, to be ‘class B’ trafficking to degradation but would occur at a slower rate. If this were the case, the discrepancy between these findings might simply be a matter of time point, with early assessments concluding little degradation and later assessments finding near-complete degradation. Further, the amount of M/DOR associated with lysosomal compartments at any given time would be less than expected for class B receptors. Studies using measures of lysosomal colocalization would be biased towards concluding a lack of trafficking to degradation. This illustrates the need for further and more detailed investigation of M/DOR post-internalization trafficking. In particular, both studies to address this topic have relied upon the use of HEK293 expression systems, which, while convenient, are limited by a lack of physiological relevance.

**Summary, Inwards Trafficking**

MOR and DOR undergo different post-internalization trafficking: MOR are ultimately recycled and DOR are ultimately degraded. The handling of M/DOR is, therefore, difficult to predict because of the differential behavior of its constituents. Recent studies using different methodologies have reported inconsistent findings regarding M/DOR post-internalization trafficking. Some ligands induce M/DOR internalization. Post-internalization, M/DOR appear more likely to be degraded than MOR. The literature regarding M/DOR inwards trafficking remains limited.

**1.6 Conclusions and Outlooks**

M/DOR, and OR heteromers more generally, are extant and physiologically relevant receptor species. A plethora of evidence demonstrates them to participate in the cellular environment as would be expected of any distinct receptor type. They are not, obviously, a *de novo* novel receptor. However, conceptualizing M/DOR functionality in terms of a distinct receptor provides a useful framework with which to understand current findings. The functional
availability of M/DOR is complexly regulated and responsive to cellular conditions. When present at the cell surface, M/DOR exhibits unique pharmacology; both ligand binding and responsiveness and downstream coupling are clearly distinct from other ORs, including the constituents.

Notably, M/DOR appears to have a range of actions that vary in a ligand- (or ligands-) dependent manner. As such, current exploitations of M/DOR often involve combinations of ligands or pharmacophores.

The recent identification of OR crystallographic structures and the continued development of M/DOR specific tools hold considerable promise for the further understanding of M/DOR and its exploitation as a viable therapeutic target. Definitive OR crystallographic structures will permit true modelling of heteromer-forming OR-OR interactions, as well as providing a vastly improved understanding of ligand binding to all species of OR. This is of particular importance to those interested in the therapeutic potential of M/DOR, as such understandings will improve the rational design of new ligands, both for M/DOR and ORs more broadly (see (Filizola & Devi, 2013)). Further driving the therapeutic targeting of M/DOR is the recent development of assays to screen for M/DOR active and selective compounds (I. Gomes et al., 2013; van Rijn et al., 2013). Both assays were designed to allow for high-throughput screening and together demonstrate the interest in designing better M/DOR ligands.

Concurrently, fundamental understandings of M/DOR are being advanced by the availability of new, M/DOR-specific tools. The generation of specific M/DOR monoclonal antibodies is facilitating studies with physiologically relevant and in vivo models (Gupta et al., 2010). This is an important step in moving M/DOR work beyond the constraints of heterologous expression systems and is part of uncovering the physiological role of M/DOR. Additionally, the development of polypeptides to competitively dissociate M/DOR provides an experimental functionality akin to knock-out or knock-down of classical receptor species (S.-Q. He et al.,
This is an ability which has not been available to M/DOR research because of the nature of heteromers; that is, one cannot knock-out M/DOR without also knocking-out MOR or DOR and thoroughly confounding any results.

With the development and availability of better tools for addressing M/DOR, including M/DOR-selective antibodies and ligands, the use of more appropriate model systems, including primary neuronal cultures, ex vivo tissues, and whole animals, is becoming increasingly feasible. These advancements are helping to drive the shift from addressing M/DOR as a solitary receptor to working to identify its roles in the wider cellular and system contexts. This shift is required for, and will ultimately determine, the usefulness of M/DOR as a therapeutic target.

1.7 Research Statement and Objectives

There is ample evidence that M/DOR heteromerization is a real phenomenon, though it occurs largely in response to certain cellular states. One of the most relevant and well-evidenced such states occurs following prolonged opioid agonist exposure, in particular to MOR agonists. Understandings of the physiological relevance of M/DOR formation, however, remain limited in large part due to the reliance of existing M/DOR findings upon contrived heterologous systems. The following thesis addresses the GENERAL HYPOTHESIS that M/DOR heteromers are a real, distinct, and physiologically-relevant species of OR which are generated following prolonged MOR activation.

In order to address the limitations of existing M/DOR evidence produced using heterologous systems, OBJECTIVE ONE broadly aimed to establish suitable models and experimental tools with which to address OR heteromerization while maximizing physiological relevance. This included a viable DRG neuron primary culture model to permit experimentation at individual-cell level, an antiserum specifically directed against M/DOR in order to detect it in cultured neurons and fixed brain slices, a quantitative colocalizational analysis method for the assessment of receptor trafficking, and a floxed-Stop, FLAG-tagged DOR conditional knock-in
mouse model to surmount DOR antibody controversies and permit experimentation at whole- animal and isolated-tissue levels. The development and implementation of these models and methods make it possible to conduct experiments addressing the nature of M/DOR heteromers in systems with increasingly compelling physiological relevance.

Using these tools and seeking to both reinforce and extend existing findings from heterologous systems, it was first necessary to demonstrate the existence of M/DOR heteromers. Therefore, **OBJECTIVE TWO** aimed to identify M/DOR heteromers under physiological conditions and to determine if their abundance increased following prolonged MOR activation.

Addressing M/DOR physiological relevance only begins with demonstrating their existence. Knowing that M/DOR are present and establishing at least one of the conditions in which their formation is triggered necessarily leads to the far larger question of the functional consequences of M/DOR formation; that is, how do they function and what is their role in the cell? In a start to answering these questions, **OBJECTIVE THREE** aimed to investigate the post-internalization trafficking of DOR following prolonged MOR activation, a condition in which there would be augmented DOR incorporation into M/DOR. Since MOR and DOR are understood to undergo differential post-endocytic trafficking, this study addressed whether M/DOR remains unitary post-internalization and whether one or the other constituent of M/DOR would retain dominance in directing trafficking.

These studies aim to demonstrate concordant evidence of M/DOR heteromers, confirming earlier observations from heterologous systems, and to extended understanding of the functional consequences of M/DOR formation in terms of both precipitating conditions and receptor behaviour. In doing so, these studies also further understandings of OR heteromerization in general. Moreover, prolonged MOR activation, a trigger for M/DOR formation, is most commonly encountered in the clinical setting as medium-to-long term opioid use. Such use cases are increasingly relevant and commonly include the development of tolerance to opioid analgesia.
(often necessitating dose escalation or challenging opioid rotation strategies). The presence of a pharmacologically distinct OR species specifically in those cases may represent a useful, and hitherto unaddressed, treatment target.
Chapter 2
Increased Abundance of Opioid Receptor Heteromers After Chronic Morphine Administration

This work is published in Science Signaling:


2.1 Introduction

Morphine, the choice analgesic in the treatment of chronic pain, elicits its effects through opioid receptors. Repeated or continuous use of morphine leads to the development of tolerance and physical dependence. Opioid receptors are members of the heterotrimeric guanosine 5’-triphosphate–binding protein (G protein)–coupled receptor (GPCR) superfamily characterized by the presence of seven transmembrane regions. To date, three subtypes of the opioid receptor have been identified: μ, δ, and κ. Functional and physical interactions between these receptor subtypes have been noted (Maggio, Novi, Scarselli, & Corsini, 2005; Milligan, 2007; Prinster, Hague, & Hall, 2005; Rios, Jordan, Gomes, & Devi; Terrillon & Bouvier, 2004). Heteromerization between μ and δ opioid receptors leads to distinct receptor pharmacology in that doses of δ receptor ligands (agonists and antagonists) too low to trigger signalling can potentiate the binding and signalling of μ receptor agonists, an effect not seen in cells expressing only μ receptor homomers (I. Gomes et al., 2000, 2004). In addition, although homomers of μ or δ opioid receptors signal through pertussis toxin–sensitive inhibitory G proteins, Ga, the μ-δ heteromer either couples to a pertussis toxin–insensitive G protein, Gz (S R George et al., 2000), or exhibits a switch in receptor coupling from G protein to β-arrestin2 (Rozenfeld & Devi, 2007, 2010). In addition, μ-δ heteromerization could play a role in morphine-mediated analgesia because the analgesic effects
of morphine are mediated through ρ receptors (Kieffer, 1999). Moreover, low doses of δ receptor antagonists can potentiate morphine-mediated analgesia (I. Gomes et al., 2004). For these reasons, μ-δ heteromers are considered to be a choice target for the development of new therapies to treat chronic pain (Rozenfeld, Décaillot, IJzerman, & Devi, 2006). However, relatively little information is available about the biochemical and signalling properties of the endogenous heteromers and their regulation under pathological conditions, mainly as a result of the lack of appropriate tools to study heteromers in situ.

In the case of GPCRs, antibodies have been used as tools for receptor characterization, as reagents for their purification and tissue localization, and as probes for mapping their functional domains (Gupta, Heimann, Gomes, & Devi, 2008). We reasoned that heteromer-specific antibodies would be useful tools to study endogenous heteromers in tissue, to probe their regulation in situ, and to delineate the mechanisms of regulation. Using a subtractive immunization strategy (Gupta et al., 2008; Salata et al., 1992; Sleister & Rao, 2001, 2002) in which antibody-producing cells to unwanted antigens are eliminated through cyclophosphamide treatment, leading to the enrichment of cells producing antibodies to the desired antigen (in this case, a region shared by the heteromer), we generated μ-δ heteromer–selective antibodies. Using these heteromer-selective antibodies, we show that conditions that lead to the development of morphine tolerance correlate with increased abundance of the μ-δ heteromer in regions of the brain involved in pain perception. This suggests that the μ-δ heteromer could play a role in the development of morphine tolerance. Because the μ-δ heteromer exhibits unique pharmacology in that low non-signalling doses of δ receptor ligands can potentiate μ receptor–mediated binding and signalling, as well as morphine antinociception (S R George et al., 2000; I. Gomes et al., 2000, 2004, Rozenfeld & Devi, 2007, 2010), these results identify this heteromer as a target for the development of new therapeutics in the treatment of chronic or neuropathic pain.
2.2 Materials and Methods

2.2.1 Cell culture and transfection

HEK293 and SK-N-SH cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. CHO cells were grown in F12 media containing 10% FBS and 1% penicillin-streptomycin. HEK293 cells were transfected with either μ and δ opioid receptors, μ and α2A adrenergic receptors, μ and CB1 cannabinoid receptors, δ and α2A receptors, δ and CB1 receptors, or chimeric constructs to μ and δ opioid receptors; CHO cells were transfected with HA-tagged μ and Flag-tagged δ opioid receptors. CHO cells stably expressing Flag-tagged μ and Myc-tagged δ opioid receptors were generated as previously described (I. Gomes et al., 2000). These cells were transfected with a chimeric G16/Gi3 to assess intracellular Ca2+ concentrations. All transfections were carried out with Lipofectamine as per manufacturer’s protocol (Invitrogen).

2.2.2 Subtractive immunization

For induction of tolerance to immunogenic epitopes in HEK293 membranes, female balb/c mice (6-8 weeks old, 25-35 g body weight) were injected intraperitoneally (i.p.) with 5 mg HEK293 membranes and 15 min later with cyclophosphamide (100 mg/kg body weight, i.p.). The cyclophosphamide injection was repeated after 24 and 48 hours respectively. Mice were bled every 15 days and antibody titers checked by ELISA against HEK293 membranes. This protocol was repeated at 2 week intervals until stable background titers were obtained with HEK293 membranes. Mice were then given an i.p. injection of membranes from HEK293 cells coexpressing μ-δ receptors (5 mg) in complete Freund’s adjuvant. Booster i.p. injections of HEK293 membranes coexpressing μ-δ receptors were administered every 15 days. Antibody titers were checked by ELISA against HEK293 membranes from untransfected cells and from cells coexpressing μ-δ receptors. Spleens from animals giving a high titer with HEK293 membranes coexpressing μ-δ receptors were fused with SP-20 myeloma cells to generate
monoclonal antibodies as described (I. Gomes, Gupta, Singh, & Sharma, 1999). Clones secreting monoclonal antibodies were screened by ELISA against untransfected HEK293 membranes, and HEK293 membranes expressing μ, δ or coexpressing μ-δ receptors as described (Gupta et al., 2007) using 1:10 hybridoma supernatant and 1:500 horseradish peroxidase labeled anti-mouse IgG. Hybridoma supernatant from positive clones was concentrated using Centricon 10 and stored at a concentration of 10 μg protein/μl. Antibody specificity for the μ–δ heteromer was determined by ELISA with antibodies that were preincubated with membranes from HEK293 cells, or HEK293 cells expressing μ, δ, or μ and δ receptors. Membranes (10 μg) were incubated with 1:10 dilution of hybridoma supernatant in the presence of protease inhibitor cocktail (Sigma) for 3 hours at 4°C followed by centrifugation at 15,000 x g for 20 min and the supernatant was used for the ELISA assay using cells coexpressing μ and δ receptors.

2.2.3 Generation of polyclonal antibodies
Polyclonal antibodies to μ opioid, δ opioid, α2A adrenergic and CB1 cannabinoid receptors were generated as described previously (Gupta et al., 2007).

2.2.4 Membrane preparation
Membranes were prepared as described previously (I. Gomes, Filipovska, & Devi, 2003) from HEK293 cells (alone or expressing μ, δ, or μ and δ receptors), the cortex of wild-type (WT), μ KO, δ KO, or μ-δ KO mouse brain, from different brain regions of rats injected subcutaneously with morphine (5mg/kg), NTB (0.1mg/kg), or saline for 9 days, or from mice treated either acutely (single injection, 10mg/kg) or chronically (intermittent escalating dose protocol, 10mg/kg on day 1 to 100 mg/kg on day 5).

2.2.5 ELISAs
ELISAs were carried out as previously described (Gupta et al., 2007) either with cells (2 × 10^5 per well) expressing individual receptors or with cells coexpressing μ and δ opioid
receptors that were treated with 1 or 10 μM different opioid ligands for 0 to 72 hours, with cultured DRG neurons, or with membranes (10 μg) prepared from different brain regions of wild-type, μ knockout, δ knockout, and μ-δ double-knockout mice or from mice that were treated either acutely or with escalating doses of morphine. Data obtained with μ-δ heteromer–selective antibodies in ELISA assays are expressed as μ-δ abundance in the figures.

2.2.6 Binding assays

CHO or SK-N-SH cells (2 × 10^5 per well) coexpressing μ and δ opioid receptors were incubated with 5 μg of antibodies for 10 min at room temperature (RT), and then with 10 nM [^3H]DAMGO in the presence of antibodies and in the presence or absence of 10 nM TIPPψ (I. Gomes et al., 2003).

2.2.7 GTP-γ-S binding assays

Membranes (10 μg) were incubated with 5 μg of antibodies for 10 min at RT, and then with 1 μM DAMGO in the presence or absence of 10 nM TIPPψ and in the presence of antibodies. [^35S]GTP-γ-S binding was determined as described (I. Gomes et al., 2004).

2.2.8 Adenylyl cyclase assays

Membranes (10 μg) were incubated with 5 μg of antibodies for 10 min at RT, and then with 1 μM DAMGO in the presence or absence of 10 nM TIPPψ and in the presence of the antibodies. Adenylyl cyclase activity was determined as described (Heimann et al., 2007; Unterwald, Cox, Kreek, Cote, & Izenwasser, 1993).

2.2.9 Gai16-facilitated Ca2+ release

CHO cells coexpressing a chimeric G16-Gi3 protein and μ and δ opioid receptors were plated onto poly-l-lysine–coated, 96-well clear-bottom plates (40,000 per well). The next day, the growth medium was removed, and the cells were washed twice in Hanks’ balanced salt solution containing 20 mM Hepes. Cells were incubated with Fluo-4 NW calcium dye (3 μM in 100 μl)
for 1 hour at 37°C. The cells were preincubated for 30 min with or without 5 μg of antibodies. Deltorphin II (1 μM) or CTOP (10 nM) was added to the wells (in the presence of the antibodies) by the robotic arm of the FLEX Station, and intracellular Ca2+ concentration was measured for 300 s at excitation and emission wavelengths of 494 and 516 nm, respectively.

2.2.10 Immunoprecipitation and Western blotting

CHO cells coexpressing hemagglutinin-tagged μ and Flag-tagged δ receptors or DRGs with endogenous μ and δ receptors [isolated from embryonic rat pups at embryonic day 16 (Breit, Gagnidze, Devi, Lagacé, & Bouvier, 2006)] were immunoprecipitated with the μ-δ heteromer–selective antibody as described (I. Gomes et al., 2004). μ and δ receptors were detected in the immunoprecipitates by means of polyclonal antibodies to the epitope tags (CHO cells) or to the individual receptors (DRGs). The δ receptor antibodies were from Chemicon or Proteimax, and the μ receptor antibodies were a gift from T. Cote [Uniformed Services University of the Health Sciences (USUHS)].

2.2.11 Colocalization of μ, δ, and μ-δ receptors in DRG neurons

DRG neurons from adult rats were grown in culture for 4 days. They were then treated with either vehicle (saline) or morphine (10 μM, 48 hours) before fixation with 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min at 37°C. Immunocytochemical labelling for μ and δ opioid receptors was accomplished with antibodies against the μ receptor (Neuromics) and the δ receptor (Alamone) and Alexa 488– and Alexa 594–conjugated secondary antibodies, respectively. Photomicrographs were captured by a Leica TCS SP2 multiphoton confocal microscope (63× primary magnification; Leica Microsystems), and images were acquired and digitized for quantitative analysis with Leica Confocal Software.
2.2.12 Animal studies

Animal studies were carried out according to protocols approved by the Mount Sinai School of Medicine Animal Care and Use Committee. Mice lacking μ opioid (I. Gomes et al., 2003) (n = 6) or δ opioid receptors (Heimann et al., 2007) (n = 6) were back-crossed onto a C57Bl6/J background, bred, and maintained as described (Filliol et al., 2000; Matthes et al., 1996). Wild-type littermates (n = 8) were used as controls in pharmacological challenge experiments. Morphine was systemically administered according to a chronic intermittent escalating dose protocol (from 10 mg/kg on day 1 to 100 mg/kg on day 5) or acutely (a single injection of 10 mg/kg). For tissue collection, each mouse brain was placed in a mouse brain mold (Braintree Scientific) and the brainstem was coronally sliced at 1-mm thickness. The ventral portion of the 1-mm slice located between −4.60 and −5.60 mm relative to bregma included MNTB and RVM. Bilateral MNTB and midline RVM regions were punched with a 14-gauge tissue needle (Scientific Commodities), pooled, and stored at −80°C until use. For immunohistochemistry, animals were perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) that was preceded by a short prerinse with physiological saline. Tissues were cryoprotected in 30% sucrose in phosphate buffer.

2.2.13 Immunohistochemistry

Coronal sections of wild-type and knockout mice treated or not with morphine were prepared on a cryostat microtome at a 16-μm thickness and thaw-mounted onto fluorescence-free glass slides. Sections at identical anterior-posterior coordinates from wild-type and μ or δ knockout mice treated or not with drug were mounted onto each glass slide to allow simultaneous histochemical processing. Similar simultaneous tissue processing was performed when comparing the effects of acute and chronic morphine treatments, with sections spanning MNTB and RVM, from a control, acute, or chronic morphine-treated mouse tissue mounted onto each glass slide. Specimens were rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4), blocked in PBS
containing 10% normal donkey serum and 0.3% Triton X-100, and incubated in diluted (>1:4000) supernatant from the 1E12D1 hybridoma secreting clone (expressing monclonal μ-δ heteromer–selective antibody) that was diluted in PBS–0.3% Triton X-100 and 0.1% NaN3 at 4°C for 16 to 24 hours. Immunoreactivity was visualized with the tyramide signal amplification system (TSA-Plus; NEN Life Science Products): Sections were washed in TNT buffer [0.1 M tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20] for 10 min, followed by 30 min of incubation with horseradish peroxidase–conjugated donkey immunoglobulin G against mouse (1:500; Jackson ImmunoResearch). After three washes in TNT, sections were exposed to fluorescein- or carbocyanine 3 (Cy3)–conjugated biotinyl tyramide diluted in amplification diluent (1:100) for 15 min at RT and thereafter washed twice in TNT buffer. After single staining to reveal μ-δ heteromers, sections were repeatedly washed in PBS and exposed to rabbit antibody against golgin subfamily A5 (GOLGA5; 1:1000; Atlas Antibodies) (Filliol et al., 2000; Matthes et al., 1996; Mulder et al., 2007) or goat antibody against parvalbumin (1:11,000; Swant); biotinylatated Wisteria floribunda agglutinin (WFA) was used to visualize perineuronal nets (20 μg/ml; Sigma) (38) overnight at 4°C. After repeated rinses in TNT buffer, immunoreactivities were revealed by Cy2-, Cy3-, or Cy5-conjugated donkey antibodies against rabbit or goat (1:300; Jackson ImmunoResearch). WFA binding was revealed by Cy2-tagged streptavidin (1:5000; Jackson).

Specimens were imaged with a confocal laser-scanning microscope (model 510, Zeiss) equipped with appropriate excitation and emission filters for maximum separation of Cy2 (505 to 530 nm, band pass), Cy3 (560 to 610 nm, band pass) and Cy5 signals (>650 nm, long pass). Images were taken with identical pinhole, detector gain and offset, and amplification gain settings to allow direct comparisons of degree of immunoreactivity, which was measured from primary unmodified confocal laser-scanning microscope images with National Institutes of Health ImageJ optical software (version 1.41). The mean grayscale optical density and the maximum of pixel intensity were measured within individual neurons obtained from two to four animals per
treatment group. Results were analyzed with one-way analysis of variance (ANOVA) with Bonferroni post hoc correction. Digital images were color-coded for optimal visualization of signals and underwent linear optimization of brightness and contrast with Adobe Photoshop CS3 (Adobe Systems).

2.3 Results

2.3.1 Generation of μ-δ heteromer–selective antibodies

We used a subtractive immunization strategy (Salata et al., 1992) to generate antibodies that selectively recognize the endogenous μ-δ heteromer but do not recognize either μ or δ receptors (Table 3). Mice were first made tolerant to unwanted epitopes on membrane proteins by the simultaneous administration of human embryonic kidney 293 (HEK293) cell membranes and cyclophosphamide, which causes the destruction of antibody-generating activated B cells (Salata et al., 1992; Sleister & Rao, 2001, 2002). Once a low titer to HEK293 membrane proteins was achieved, mice were immunized with membranes from HEK293 cells coexpressing μ-δ receptors (Figure 2.1A). The spleens of mice with high antibody titers were used to generate monoclonal antibodies. The supernatants from the resultant hybridoma clones were screened with HEK293 membranes alone, membranes from cells expressing only μ or δ receptors, and membranes from cells coexpressing both μ and δ receptors. This led to the identification of various antibody-secreting clones (Table 3), including the 1E12D1 clone that gave a high signal with membranes from cells coexpressing μ and δ receptors, but not with membranes from cells expressing only μ or δ receptors (Table 3).

The 1E12D1 antibody–secreting clone recognized an epitope in membranes from wild-type animals, but not from animals lacking μ or δ receptors, and in cells coexpressing μ and δ receptors, but not in cells coexpressing μ or δ receptors in combination with other GPCRs (Figure 2.2, A to C, and Figure 2.1B). Preincubation of the antibody with membranes from HEK293 cells
### Table 3 ELISA characterization of select hybridoma clones’ binding to target epitopes.

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>HEK293 O.D. 490 nm</th>
<th>HEK293 μ O.D. 490 nm</th>
<th>HEK293 δ O.D. 490 nm</th>
<th>HEK293 μ−δ O.D. 490 nm</th>
</tr>
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<tbody>
<tr>
<td>1A4</td>
<td>0.13 ± 0.004</td>
<td>0.68 ± 0.04</td>
<td>0.04 ± 0.008</td>
<td>0.65 ± 0.03</td>
</tr>
<tr>
<td>2B1</td>
<td>0.15 ± 0.007</td>
<td>0.051 ± 0.02</td>
<td>0.65 ± 0.032</td>
<td>0.63 ± 0.03</td>
</tr>
<tr>
<td>1E12D1</td>
<td>0.10 ± 0.007</td>
<td>0.21 ± 0.01</td>
<td>0.22 ± 0.013</td>
<td>1.80 ± 0.04</td>
</tr>
</tbody>
</table>

Membranes from un transfected HEK293 cells or cells expressing μ, δ, or μ and δ receptors were plated on 96-well poly-L-lysine coated plates and subjected to ELISA by incubation with 1:10 hybridoma supernatant and 1:500 horseradish peroxidase labeled anti-mouse IgG. The receptor recognition was measured using o-phenylenediamine as substrate. Results are mean ± SEM (n=3 experiments). 1A4, μ monoclonal Ab; 2B1, δ monoclonal Ab; 1E12D1, μ-δ monoclonal Ab.
Figure 2.1  Generation and characterization of μ–δ heteromer-selective antibodies

(A) Subtractive immunization scheme. Balb/c mice were made tolerant to immunogenic epitopes by administration of HEK293 membranes and 100 mg/kg cyclophosphamide. Mice were immunized with membranes coexpressing μ and δ receptors and monoclonal antibodies were generated. Mean antibody titers ± SEM for each individual mouse is
presented. (B) Cells coexpressing either μ and δ, μ and a2A, μ and CB1, δ and a2A, or δ and CB1 receptors were subjected to ELISA using μ−δ heteromer-selective antibodies. Abundance of individual receptors was determined in parallel by ELISA with receptor specific polyclonal antibodies. (C) The abundance of endogenous μ-δ heteromers in SKN-SH cells was determined by ELISA using μ−δ heteromer-selective antibodies preincubated without or with membranes from HEK293 cells alone or HEK293 cells expressing μ, δ, or μ−δ receptors. (D) μ−δ heteromer-selectivity was determined by ELISA with HEK293 cells expressing μ and δ receptors at 1:1, 5:1, and 1:5 ratios. Results in (B-E) are means ± SEM (n=3-4 experiments). (E) μ−δ heteromers from CHO cells coexpressing HA-tagged μ and Flag-tagged δ receptors or from DRGs endogenously expressing μ and δ receptors were immunoprecipitated with the μ−δ heteromer-selective antibody and subjected to Western blot analysis with polyclonal antibodies to the epitope tags (CHO cells) or to the individual receptors (DRGs). A representative blot of 3 is shown.
expressing both μ and δ receptors, but not those from cells expressing the receptors individually, substantially decreased the recognition of an epitope in SK-N-SH cells, presumably the endogenous μ and δ receptors (Figure 2.1C). In addition, the μ-δ heteromer antibody exhibited maximal recognition when μ and δ receptors were expressed at a 1:1 ratio, but not at a 5:1 or a 1:5 ratio (Figure 2.1D). Furthermore, the antibody recognized wild-type μ and δ receptors better than μ-δ chimeric constructs (Table 4). These results indicate that the 1E12D1 monoclonal antibody exhibited μ-δ heteromer selectivity. Using these heteromer-selective antibodies, we could isolate μ-δ heteromers from cells expressing recombinant μ-δ receptors, as well as endogenous μ-δ heteromers from primary dorsal root ganglion (DRG) neurons (Figure 2.1E).

2.3.2 Increased abundance of endogenous μ-δ heteromers after chronic morphine treatment

Because chronic opiate treatment has been reported to lead to increased opioid receptor abundance (Lucido, Morinville, Gendron, Stroh, & Beaudet, 2005), we examined the effect of chronic morphine treatment on the heteromerization and subcellular distribution of μ and δ opioid receptors in cultured DRG neurons. Immunofluorescent labelling of cultured DRG neurons with antibodies to μ and δ opioid receptors showed μ-δ colocalization in neurons after vehicle or prolonged morphine treatment (Figure 2.3A). Increased abundance and colocalization was detected at the plasma membrane, as well as in intracellular compartments. Quantification of the extent of colocalization of the two receptors demonstrated significantly increased probability of μ and δ heteromer formation in morphine-treated DRG neurons relative to that of vehicle-treated controls (Figure 2.3B) in all cell sizes. Chronic, but not acute, treatment led to a significant increase in μ-δ heteromer immunoreactivity that was also detected by enzyme-linked immunosorbent assay (ELISA; Figure 2.3, B and C).

Next, we determined whether chronic morphine treatment triggers the formation of μ-δ heteromers using a chronic escalating dose of morphine administration paradigm (Décaillot, Che, Fricker, & Devi, 2006) that leads to the development of antinociceptive tolerance.
Figure 2.2    Detection of μ-δ heteromers with heteromer-selective monoclonal antibodies (Ab).

(A) Receptor abundance was determined in cortical membranes from wild-type (WT), μ knockout (KO), δ KO, or μ-δ double-KO mice with monoclonal antibodies to μ-δ, μ, or δ receptors by ELISA. (B and C) Cells endogenously (B) or stably (C) coexpressing μ-δ receptors were treated with or without morphine (1 μM) for 48 hours. Heteromer abundance was determined by ELISA with μ-δ heteromer-selective antibodies. Results are means ± SEM (n = 3 experiments). *P < 0.05. (D to G) Repeated morphine treatment increased μ-δ heteromer immunoreactivity in MNTB. Immunoreactivity in μ KO or δ KO mice after morphine treatment was below the detection limits. OR, opioid receptor. (H) Individual neuronal profiles were outlined (n = 15 to 20 per group) and μ-δ heteromer immunoreactivity, as assessed by mean optical density (OD), was determined. Morphine treatment increased μ-δ heteromer immunoreactivity in MNTB neurons from WT mice (***P < 0.01 relative to untreated control), but not in those from μ or δ KO mice. Scale bar = 25 μm.
HEK293 cells were cotransfected with full length wild-type μ and δ receptor cDNAs or with a combination of chimeric μ-δ constructs. The degree of receptor recognition was determined by ELISA. Results are means ± SE (n=3 experiments. **P<0.001 compared to μ/δ, Dunnett’s test).
Figure 2.3  Subcellular distribution and colocalization of $\mu$ and $\delta$ opioid receptors.

(A) DRG neurons were isolated from adult rats and grown in culture for 4 days prior to experimentation. DRG neurons were treated with either vehicle (saline) or morphine (10
μM, 48 hours) before fixation and immunocytochemical labelling for μ (green) and δ (red) receptors. Image overlay illustrated in white maps shows that opioid receptor colocalization is increased after prolonged morphine treatment. Representative images shown. (B) Colocalization was quantified using Pearson's colocalization coefficient. μ−δ colocalization was significantly increased in morphine-treated neurons compared to vehicle controls ($P < 0.05$). Data shown is representative of 4 independent experiments. (C) DRG neurons were treated with either vehicle (saline) or morphine (1 μM, 30 min or 48 hours) and μ−δ heteromer abundance was quantified by ELISA using μ−δ receptor selective antibodies. Results are means ± SEM ($n=3$ experiments). Scale bars = 10 μm. ***$P<0.001$. 

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Immunohistochemistry with the μ-δ heteromer–selective antibody revealed increased μ-δ heteromer immunoreactivity after morphine administration in the medial nucleus of the trapezoid body (MNTB), an auditory relay nucleus (Figure 2.2, D to G), and the rostral ventral medulla (RVM), a key relay nucleus of pain perception (Figure 2.4, A to E), relative to saline treatment in wild-type mice. The Allen Brain Atlas (http://www.brainatlas.org/), a genome-wide map of gene expression in the mouse brain created by high-throughput in situ hybridization analysis, shows that messenger RNAs (mRNAs) encoding the μ and δ receptors are abundant in these nuclei. μ-δ heteromer immunofluorescence in MNTB neurons was higher in wild-type mice than in mice lacking either μ or δ receptors (Figure 2.2, D to H). In MNTB neurons, μ-δ heteromer immunoreactivity was detected in putative glycinergic neurons that stained for parvalbumin, a calcium-binding protein found in neurons that use either γ-aminobutyric acid or glycine (or both) as neurotransmitters (Antal et al., 1991; Klausberger & Somogyi, 2008; Laing, Todd, Heizmann, & Schmidt, 1994) (Figure 2.4, F to I). A single (acute) administration of morphine (30-min treatment) did not significantly alter the abundance of μ-δ heteromer immunoreactivity in either MNTB or RVM as determined by immunohistochemistry (Figure 2.5, A to K) or ELISA (Figure 2.5L). In contrast, chronic escalating morphine treatment induced μ-δ heteromer formation, as indicated by both the significantly increased density and the intensity of μ-δ heteromer immunoreactivity in MNTB neurons (Figure 2.5, A to K). Within MNTB, immunoreactivity was localized to glycinergic neurons ensheathed by perineuronal nets, a specialized form of extracellular matrix that provides active neurons with a well-hydrated, strongly anionic microenvironment that facilitates fast-spiking neuronal activity and the rapid transport of cations (Härtig et al., 2001) (Figure 2.5, A to K). Overall, our results indicate that chronic morphine treatment selectively increases μ-δ heteromer assembly in brainstem neurons.

To quantify the morphine-induced increase in abundance of μ-δ heteromers, we carried
Figure 2.4  Chronic morphine treatment induces increased μ−δ heteromer immunoreactivity in the brainstem

(A-D) A fine meshwork of μ−δ heteromer-stained small-caliber processes is visible in the absence of morphine treatment in the RVM. Perisomatic μ−δ immunoreactivity increased after morphine challenge (arrows, inset) in wild-type, but not knock-out mice. Scale bar = 18 μm. (E) μ−δ immunoreactivity along a putative axon (arrowheads) of an RVM neuron.

(F-I) Subsets of MNTB neurons containing components of perineuronal nets [as detected by Wisteria floribunda agglutinin (WFA)] and parvalbumin positive (arrowheads) putative glycinergetic cells (Härtig et al., 2001) showed μ−δ heteromer immunoreactivity. Scale bar = 10 μm.
Figure 2.5 Chronic morphine treatment induces μ-δ heteromer abundance in different brain regions.

(A to D) Chronic (5 days of escalating dose regime), but not acute (30 min), morphine treatment significantly increased μ-δ heteromer immunoreactivity in perineuronal net-bearing MNTB neurons detected by WFA. Arrowheads indicate neurons bearing μ-δ
immunoreactivity that are ensheathed by perineuronal nets. Scale bars, 25 μm. (D) Mean grayscale optical density measured within individual neurons of two to four mice. (E to K) Quantitative analysis of the maximal fluorescence intensity of μ-δ immunoreactivity in individual perineuronal net-bearing neurons of MNTB after acute or chronic morphine treatment. Numbers in parentheses indicate the number of neurons. Scale bars, 18 μm. **P < 0.01. (L) ELISA with membranes from MNTB, RVM, or cortex shows that a 5-day chronic escalating dose of morphine but not a single dose of morphine (acute, 10 mg/kg) or saline significantly increased μ-δ receptor abundance. Results are means ± SEM (n = 3 experiments). ***P < 0.001 relative to saline. (M) ELISA with membranes from different mice brain regions shows that treatment with morphine (5 mg/kg) or NTB (0.1 mg/kg) for 9 days significantly increased μ-δ receptor abundance. Results are means ± SEM (n = 3 experiments). *P < 0.05; **P < 0.01; ***P < 0.001. Hippoc, hippocampus; Hypoth, hypothalamus, N.Acc., nucleus accumbens; PFC, prefrontal cortex; VTA, ventral tegmental area.
out ELISA assays with brain membranes from saline- and morphine-treated animals. Animals treated chronically with morphine showed increased heteromer abundance in the cortex, MNTB, and RVM (Figure 2.5L), as well as in the hypothalamus, nucleus accumbens, and ventral tegmental area, relative to saline-treated controls (Figure 2.5M). We have previously shown that δ antagonists can potentiate morphine analgesia. Therefore, we examined whether chronic treatment with naltriben (NTB), an alkaloid δ antagonist, enhances μ-δ heteromer abundance. Similar to chronic morphine treatment, chronic treatment with NTB caused increased μ-δ heteromer abundance in different brain regions (Figure 2.5M). In addition, treatment of cells expressing recombinant or native μ and δ receptors with morphine for 48 hours significantly increased the abundance of the μ-δ heteromer (Figure 2.2, B and C and Figure 2.6B). Thus, a component of the morphine-mediated up-regulation of μ-δ heteromer formation can be recapitulated in cell culture systems. Therefore, we used Chinese hamster ovary (CHO) cells expressing recombinant μ and δ receptors and SK-N-SH cells expressing native μ and δ receptors to determine whether only morphine induced increased μ-δ heteromer abundance. Treatment of CHO cells for 48 hours with alkaloid but not peptide ligands (either antagonists or agonists) increased μ-δ heteromer abundance (Figure 2.7A). The time course analysis in SK-N-SH cells revealed that this increase was seen within 2 hours of treatment and peaked around 8 hours (Figure 2.7B). This increase was also seen in cells expressing recombinant receptors, thereby suggesting a posttranslational mode of regulation of the heteromer.

### 2.3.3 Inhibition of agonist binding and signalling by the μ-δ heteromer–selective antibody

We examined the ability of the μ-δ heteromer–selective antibody to selectively inhibit the ability of low doses of the δ antagonist TIPPψ [(2S)-2-[(2S)-2-[(3S)-2-[(2S)-2-amino-3-(4-hydroxyphenyl)propanoyl]-3,4-dihydro-1H-isoquinolin-3-yl]methylamino]-3-phenylpropanoyl]amino]-3-phenylpropanoic acid] to potentiate the binding and signalling of the μ agonist DAMGO (Tyr-d-Ala-Gly-MePhe-Gly-ol) (I. Gomes et al., 2000, 2004). The μ-δ
Figure 2.6  Chronic morphine treatment increases μ–δ heteromer abundance.

(A) SK-N-SH cells or CHO cells stably expressing μ and δ receptors were treated without or with morphine (1 μM) for 48 hours. Binding assays were carried using [3H]DAMGO (10 nM) in the absence or presence of TIPPψ (10 nM). Results are means ± SEM (n=3 experiments). *p<0.05; **p<0.01; + p<0.05  (B) SK-N-SH cells or CHO cells stably expressing μ and δ receptors were treated with saline or morphine (1 μM) for 48 hours. Cortical membranes were prepared from mice treated with saline or an escalating dose of morphine. The abundance of μ, δ or μ–δ receptors in cells or membranes was quantified by ELISA with μ, δ, or μ–δ receptor selective antibodies. Results are means ± SEM (n=3 experiments). *p<0.05; ***p<0.001.
Figure 2.7  Chronic alkaloid treatment increases μ-δ heteromer abundance.

(A) CHO cells stably expressing μ and δ receptors were treated with the indicated ligands (1 μM) for 48 hours and μ-δ heteromer abundance was determined by ELISA. Results are means ± SEM (n = 3 to 4 experiments). **P < 0.01. (B) Morphine-treated (1 μM) SK-N-SH cells were probed by ELISA with monoclonal antibodies to μ, δ, or μ-δ receptors. Results are means ± SEM (n = 3 to 4 experiments).
heteromer–selective antibody significantly decreased δ antagonist–mediated increases in the binding of μ agonist (Figure 2.9A and Figure 2.8A). We used the [35S]guanosine 5′-O-(3′-thiotriphosphate) ([35S]GTP-γ-S) binding and adenylyl cyclase activity assays to measure the effect of the heteromer-selective antibody on signalling events downstream of receptor activation. Activation of opioid receptors leads to exchange of guanosine diphosphate (GDP) for guanosine 5′-triphosphate (GTP) at the associated G protein (Gαi) subunit; this can be measured with a radiolabeled non-hydrolyzable analog of GTP, [35S]GTP-γ-S. This exchange, in turn, leads to the activation of the Gαi subunit followed by inhibition of adenylyl cyclase activity and, consequently, decreases in intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) concentrations. Using these signalling assays, we found that the heteromer antibody significantly blocked the δ antagonist–mediated increases in μ receptor agonist–mediated signalling in membranes from wild-type animals, but not those from animals lacking μ or δ receptors (Figure 2.9, B and C, and Figure 2.8, B to D).

We have previously shown that μ receptor antagonists can potentiate δ agonist binding and signalling in cells coexpressing μ and δ receptors (I. Gomes et al., 2000). To determine whether the heteromer-selective antibody could block μ antagonist–mediated increases in δ agonist signalling, we used an assay in which cells are transfected with μ and δ receptors along with a chimeric G16-Gi3 protein. The activation of this chimeric G protein after binding of a receptor agonist results in increases in intracellular Ca2+ concentrations that can be measured with a Ca2+-binding dye. We found that the heteromer-selective antibody blocked μ-δ heteromer–mediated [deltorphin II, a δ agonist, in the presence of CTOP (d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH2), a μ antagonist] increases in intracellular Ca2+ concentrations in cells coexpressing these receptors (Figure 2.9D). These results indicate that our μ-δ heteromer antibody exhibits receptor heteromer selectivity and can be used to probe μ-δ heteromer–mediated effects.
Figure 2.8  μ-δ heteromer–selective antibody blocks heteromer-mediated increases in binding and signalling.

(A) CHO cells coexpressing Flag-tagged μ and Myc-tagged δ receptors were pretreated with either the μ-δ heteromer–selective antibody or monoclonal antibodies to Flag or Myc.
Binding of the μ receptor agonist [3H]DAMGO (10 nM) to cells was measured in the presence or absence of the δ receptor antagonist TIPψ (10 nM). Results are means ± SEM (n = 3 experiments). (B and C) Mouse cortical membranes were preincubated with or without μ-δ heteromer antibodies and G protein activity, as assessed by [35S]GTP-γ-S binding (expressed as G protein activity) (B), or adenylyl cyclase activity (C) in response to DAMGO (1 μM) was determined in the presence or absence of TIPψ (10 nM). Results are means ± SEM (n = 3 experiments). (D) HEK293 cells coexpressing a G16-Gi3 chimera and μ and δ opioid receptors were preincubated with or without μ-δ heteromer antibodies, and then treated with the δ opioid agonist deltorphin II (1 μM) in the presence or absence of the μ opioid antagonist CTOP (10 nM), and intracellular Ca2+ concentrations were determined. Results are means ± SEM (n = 3 experiments), *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2.9  μ–δ heteromer selective antibodies block heteromer-mediated binding and signalling.

(A) HEK293 cells coexpressing Flag-tagged μ and Myc-tagged δ opioid receptors were pre-treated with either antibodies against μ, δ, the μ–δ heteromer, Flag, or Myc epitopes.
Binding assays were carried in the presence of the antibodies using the μ receptor agonist [3H]DAMGO (10 nM) in the absence or presence of the δ receptor antagonist TIPPψ (10 nM). Results are means ± SEM (n=3 experiments). (B and C) Mouse cortical membranes were preincubated without or with μ or δ antibodies and the extent of [35S]GTPγS binding (expressed as G-protein activity) (B) or adenyl cyclase activity (C), in the presence of the antibodies, in response to treatment with 1 μM DAMGO in the absence or presence of 10 nM TIPPψ was determined. Results are means ± SEM (n=3 experiments). (D) Cortical membranes from wild-type (WT), μ KO, or δ KO mice were preincubated without or with either μ, δ or μ−δ heteromer antibodies. Membranes were then subjected to a [35S]GTPγS binding assay with 1 μM DAMGO in the absence or presence of 10 nM TIPPψ. Results are means ± SEM (n=3 experiments). *p<0.05; **p<0.01; ***p<0.001.
2.4 Discussion

Various *in vitro* studies have demonstrated that the heteromerization of GPCRs alters their pharmacological and functional properties (Maggio et al., 2005; Milligan, 2007; Prinster et al., 2005; Rios et al.; Terrillon & Bouvier, 2004). However, the evaluation of the *in situ* distribution of heteromers—and their functional relevance and modulation under pathological conditions—has been hampered by the lack of appropriate tools. We generated heteromer-selective antibodies using a subtractive immunization strategy in which cyclophosphamide was used to kill cells that generated antibodies to undesired antigens (in this case, HEK293 membranes), thereby increasing the exposure of the desired antigen (μ-δ heteromers) to antibody-producing cells. Our heteromer-selective antibody enabled the detection and isolation of μ-δ heteromers and blocked heteromer-mediated signalling. Thus, a subtractive immunization strategy could be used to generate antibodies against other GPCR heteromers and enable studies examining the abundance and regulation of receptor multimers in normal and pathological states.

The heteromer-selective antibody generated in this study blocked heteromer-mediated ligand binding and signalling and hence could be used to determine the biological contribution of heteromers compared to homomers after receptor activation. These findings are relevant to opiate action because the relative ratio of μ-δ heteromers to μ homomers could play a role in modulating the response to an opiate, and studies have shown that the heteromer transduces signalling through a pathway that is distinct from that of receptor homomers (Rozenfeld & Devi, 2010). μ-δ receptors display decreased G protein coupling and signalling relative to that of μ homomers and exhibit a β-arrestin–mediated μ receptor response (Rozenfeld & Devi, 2007). This, in turn, leads to changes in the spatiotemporal dynamics of signalling, as assessed by the phosphorylation status and localization of extracellular signal–regulated kinase (ERK). In cells expressing μ homomers, ERK shows peak phosphorylation at 3 to 5 min after stimulation and the activated kinase localizes primarily to the nucleus, whereas in cells expressing μ-δ heteromers, ERK shows peak
phosphorylation at 15 min and the activated kinase is retained in the cytoplasm (Rozenfeld & Devi, 2007). This causes differential activation of transcription factors (Rozenfeld & Devi, 2007) that could be responsible for the differences in gene expression that occur with the development of morphine tolerance. Thus, the switch to the “β-arrestin–dependent” signalling cascade could contribute to the changes in morphine response that underlie tolerance. In addition, the absence of morphine tolerance in animals lacking δ receptors (Zhu et al., 1999) or in animals with reduced surface abundance of δ receptors resulting from deletion of the gene encoding preprotachykinin (Guan et al., 2005) is consistent with a requirement of μ-δ heteromer–mediated signalling in the development of morphine tolerance. Furthermore, mouse knockouts lacking β-arrestin2 or δ receptors do not develop morphine tolerance (Bohn, Gainetdinov, Lin, Lefkowitz, & Caron, 2000; Zhu et al., 1999). Thus, μ-δ heteromers, through their association with β-arrestin2, could contribute to the development of tolerance to morphine. Although our data showing increased μ-δ heteromer abundance after chronic morphine administration would support this notion, further studies are needed to examine how interactions between β-arrestin2 and μ-δ heteromers contribute to the development of morphine tolerance.

We found that μ-δ heteromer abundance was increased in RVM after chronic morphine treatment. RVM, a brain region containing both μ and δ opioid receptor mRNAs, is involved in antinociception through facilitation of the descending inhibitory pain pathways. Malfunction in this circuitry is thought to play a role in neuropathic pain, a condition characterized by the presence of hyperalgesia (supersensitivity to painful stimuli) and tactile allodynia (painful sensation by normally nonpainful stimuli) (Porreca, Ossipov, & Gebhart, 2002; Ren & Dubner, 2002). Therefore, if neuropathic pain increases the likelihood of μ-δ heteromer formation in RVM, this could account for the lack of analgesic potency of morphine in the treatment of neuropathic pain (Przewlocki & Przewlocka, 2005) because the μ-δ heteromer signals through β-arrestin2 (Rozenfeld & Devi, 2010) and deletion of β-arrestin2 gene in mice leads to a
potentiation, as well as a prolongation of the analgesic effects of morphine (Bohn et al., 1999). Therefore, further studies are needed to examine the effects of neuropathic pain on μ-δ heteromers in RVM. Glycinergic neurons of MNTB, an auditory relay nucleus, also showed increased μ-δ heteromer abundance after chronic morphine administration. However, not much is known about the role of these receptors in auditory processing. Because chronic morphine administration causes increased μ-δ heteromer abundance in MNTB, studies are needed to examine the role of these receptors in acute and chronic pain states.

We observed colocalization of μ and δ receptors in cultured DRG neurons that is increased upon prolonged treatment with morphine. In a study using mice with a knock-in of δ opioid receptor tagged with enhanced green fluorescent protein (δEGFP), immunostaining with antibodies against GFP and μ receptors showed that δEGFP colocalizes with μ opioid receptors in less than 5% of DRG neurons (Scherrer et al., 2009). This degree of colocalization could be an underestimate because these mice show increased abundance of δ opioid receptors (Scherrer et al., 2006), and the GFP antibody exhibits higher avidity for GFP than the μ antibody does toward μ receptors. This may result in an overestimation of δ opioid receptor abundance relative to that of μ receptors. In addition, the GFP tag at the C terminus increases the cell surface localization of the δ opioid receptor (H.-B. Wang et al., 2008). Together with the evidence that increased abundance of δ opioid receptor attenuates the maturation of the μ opioid receptor (Décaillot et al., 2008), these results suggest that the low degree of colocalization between δEGFP and the μ opioid receptor (Scherrer et al., 2009) could be a result of alterations in δ opioid receptor maturation. Thus, as supported by our immunostaining data, μ and δ opioid receptors may colocalize in DRGs as well as in other regions of the brain.

In summary, we report the generation of a μ-δ heteromer–selective antibody that enabled us to examine chronic morphine treatment–mediated up-regulation of μ-δ heteromers in endogenous tissue. The subtractive immunization strategy used in the generation of the μ-δ
heteromer–selective antibodies could be used to generate antibodies selective for other GPCR heteromers, which would be useful in studies examining the role of GPCR heteromers in physiological and pathophysiological conditions.
Chapter 3

Prolonged morphine treatment alters δ opioid receptor post-
internalization trafficking

This work is published in The British Journal of Pharmacology:


3.1 Introduction

Opioids mediate their pharmacological effects via activation of three types of GPCRs: μ opioid receptor (MOP receptor), δ opioid receptor (DOP receptor) and κ opioid receptor (KOP receptor) (Alexander et al., 2013). The availability of opioid receptors at the cell surface is under complex control and is regulated by a number of processes that allow the cell to respond to external stimuli. Opioid receptors traffic regularly to and from the cell surface (Décaillot et al., 2008) (for review, see (Bernard, Décossas, Liste, & Bloch, 2006; Hanyaloglu & von Zastrow, 2008; von Zastrow, 2010; Williams et al., 2013)) and such trafficking is fundamental to their function. Desensitization, internalization and recycling of opioid receptors to the plasma membrane have significant functional consequences, affecting both analgesia and tolerance (Groer et al., 2007; L. He et al., 2009; Kim et al., 2008; A. A. A. Pradhan et al., 2010) (for review, see (Cahill et al., 2007; P. Y. Law & Loh, 1999; Martini & Whistler, 2007; Zuo, 2005).

Receptor internalization upon agonist exposure is a well-documented phenomenon for many GPCRs including opioid receptors. Following endocytosis, receptors may undergo recycling back to the plasma membrane, which is thought to underlie re-sensitization, or may undergo targeting to the degradation pathways, a key process in receptor down-regulation. Many GPCRs have been categorized as either ‘class A’ (recycled) or ‘class B’ (degraded) depending upon the relative stability of the GPCR–arrestin interaction (Drake et al., 2006). Such differential
Trafficking involves a number of different intrinsic and extrinsic regulatory mechanisms, including ubiquitination, specific sequence direction and Src (for review, see (Hanyaloglu & von Zastrow, 2008; Hislop & von Zastrow, 2011; Nagi & Piñeyro, 2011)). Both MOP and DOP receptors are internalized constitutively and in response to activation by agonists. They are, however, trafficked differently after internalization: the MOP receptor is recycled to the cell surface (class A), whereas the DOP receptor is trafficked to lysosomes (class B) and degraded (P. Y. Law & Loh, 1999; Tanowitz & Von Zastrow, 2002; Whistler et al., 2002).

A growing body of literature exists revealing that expression of analgesic tolerance following prolonged morphine exposure requires cell surface expression of DOP receptors (Abul-Husn, Sutak, Milne, & Jhamandas, 2007; S R George et al., 2000; I. Gomes et al., 2004; S.-Q. He et al., 2011; Rozenfeld, Abul-Husn, Gomez, & Devi, 2007; Zhu et al., 1999). We and others have demonstrated that prolonged morphine treatment causes the trafficking of DOP receptors from intracellular compartments to neuronal cell membranes (Cahill, Morinville, et al., 2001; Gendron et al., 2006; Lucido et al., 2005; Morinville et al., 2003; Morinville, Cahill, Aibak, et al., 2004). This effect does not occur with acute treatment and appears to be distinct from the trafficking of DOP receptors elicited by acute nociceptive input (Gendron et al., 2006; Morinville et al., 2003). Prolonged morphine-induced DOP receptor trafficking is also dependent on MOP receptors (Morinville et al., 2003). What remains unclear and has not been investigated is whether the fate of opioid receptors following prolonged stimulation with morphine differs from the internalization pathways that ensue following short-term agonist exposure.

The dependence of the morphine-induced DOP receptor trafficking on MOP receptors may simply reflect a role for MOP receptor signalling. However, notably similar MOP receptor-dependent DOP receptor trafficking occurs following chronic, but not acute, pain (Cahill et al., 2007; Gendron et al., 2006; Holdridge, Armstrong, Taylor, & Cahill, 2007; Holdridge & Cahill, 2007; Morinville, Cahill, Kieffer, Collier, & Beaudet, 2004). In this case, obligate MOP receptor
signalling seems less likely. An alternative hypothesis is that tolerance is due to the functional interaction of MOP with DOP receptors in a hetero-oligomeric complex. A requirement for MOP receptors in order to form MOP/DOP receptor heteromers would explain the MOPr dependence of both morphine- and pain-induced DOP receptor trafficking on MOP receptors. That is, the DOP receptors trafficked to the cell surface in both cases are in the form of MOP/DOP receptor heteromers. The existence of opioid receptor heteromers has been demonstrated by immunoblotting and co-immunoprecipitation (I. Gomes et al., 2000), immunocytochemistry (Décaillot et al., 2008; Gupta et al., 2010), and bioluminescence and Foerster resonance energy transfer (Hasbi et al., 2007). Opioid receptor heteromers are functional receptors (P.-Y. Law et al., 2005). There is ample evidence that MOP/DOP receptor heterodimers exist in vivo. MOP and DOP receptors are co-expressed in dorsal root ganglia (DRG) neurons (Beaudry et al., 2011; Chieng & Christie, 2009; Eric Erbs et al., 2015; Fields, Emson, Leigh, & Gilbert, 1980; H.-B. Wang et al., 2010, 2008) (but see (Scherrer et al., 2009)) and on axon terminals (Arvidsson, Dado, et al., 1995; Arvidsson, Riedl, et al., 1995) within the superficial dorsal horn, which supports the possibility of a MOP/DOP receptor species. More convincing are the demonstrations that there is a direct physical interaction between the two receptors in the spinal cord (Eric Erbs et al., 2015; I. Gomes et al., 2004) and the immunohistochemical labelling of MOP/DOP receptor heteromers in the brain and on DRG neurons (Gupta et al., 2010). We have also demonstrated that prolonged morphine treatment increases the abundance of MOP/DOP receptor heteromers in DRG neurons and several brain regions (Gupta et al., 2010). Opioid heteromers identified in heterologous systems have pharmacological profiles unique from that of any constituent alone (Fan et al., 2005) and similar to that of previously described opioid receptor subtypes (Jordan & Devi, 1999; Portoghese & Lunzer, 2003; Waldhoer et al., 2005). In vivo, MOP-DOP receptor heteromerization appears to be required for full functional competence of MOP receptors (Walwyn et al., 2009).
In this study, we investigated the post-internalization trafficking of DOP receptors following prolonged morphine treatment, a condition which we suspect induces MOP–DOP receptor interactions. We used cultured sensory neurons and measured the co-localization of DOPr and several post-endocytotic compartments following acute treatment with MOP or DOP receptor agonists. The use of this neuronal model enabled us to assess endogenous DOP receptor trafficking in a non-transfected, non-immortalized model. We showed that prolonged morphine treatment alters DOP receptor agonist-induced trafficking in response to both DOP and MOP receptor agonists.

3.2 Methods

3.2.1 DRG cultures

The lumbar DRGs of two adult, male Sprague Dawley rats (≈200 g; Charles River, Quebec, Canada) were used to produce each culture. All experimental protocols were approved by the Queen's University Animal Care Committee and complied with the policies and directives of the Canadian Council on Animal Care; appropriate measures were taken to reduce the pain or discomfort of experimental animals. Each rat was deeply anaesthetized using halothane and killed by decapitation. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010; McGrath, Drummond, McLachlan, Kilkenny, & Wainwright, 2010). The body was sprayed with 70% ethanol and wiped with clean, 70% ethanol-soaked gauze. An incision was made through the superficial layers dorsal to the spinal column. The column was transected in the thoracic and sacral regions and the spinal cord was removed by spinal ejection with sterile, ice-cold HBSS. The length of spinal column was removed and placed in sterile, ice-cold HBSS. In a sterile laminar flow hood, the remaining muscle on the column was debulked and the column bisected lengthwise along the coronal plane. Twelve lumbar DRGs were isolated from each cord and placed in cold Neurobasal-A medium containing 100 000 U L\(^{-1}\) penicillin/100 mg L\(^{-1}\)
streptomycin. The DRGs were cut using scissors to open their connective tissue envelopes and incubated in a shaking water bath at 37°C with 0.25% collagenase-D (Roche, Laval, Quebec, Canada) for 2 h. After collagenase digestion, the DRGs were spun down at 900× g for 2 min and transferred to medium containing 0.25% trypsin for 30 min. After enzymatic dissociation, the DRGs were tritiated using fire-polished pipettes. The dissociated cells were again spun down and transferred to the final culture medium, Neurobasal-A augmented with 10% FBS, 0.5 mM l-glutamine, 0.1 μg·mL⁻¹ nerve growth factor 7 s and containing 100 000 U L⁻¹ penicillin/100 mg L⁻¹ streptomycin. The medium and cells were passed through a 70 μm filter and pre-plated on an untreated 12 cm plastic Petri dish and placed in a 37°C, 5% CO2 incubator for 2 h to reduce glial cell population in the culture. After pre-plating, the cells still in solution were collected and plated on 12-round glass coverslips in a 24-well plate. The coverslips were pre-coated with poly-d-lysine and laminin to facilitate cell adherence. Cultures were incubated at 37°C with 5% CO2 for a total of 4 days before experimentation.

At the time of experimentation, the cultured cells were firmly adhered to the glass coverslips. The overall cell density was approximately 50–70% confluence. The majority of cells were glia, but neurons were plentiful (>100 per 12 mm round coverslip) and readily identifiable by morphology; neurons extended higher from the glass coverslip, with notably rounder cell bodies than glial cells (Figure 3.1A). These morphologically identifiable neurons were the same as those identified by microtubule-associated protein 2 immunofluorescent labelling (Figure 3.1B), which also revealed the growth of numerous fine, distinct processes. The cultured neurons varied in size, with cell bodies between 10 and 40 μm in diameter (Figure 3.1C). Both the range and the frequency of the observed neuronal cell body sizes were in line with previous findings for similar DRG neuronal cultures (von Banchet et al., 2007).
Figure 3.1 Cultured DRG neurons were morphologically identifiable. (A) Neurons (black arrows) are easily distinguished from co-cultured glial cells in a lower focal plane (white arrows). (B) The same cells morphologically identified as neurons showed positive immunolabelling for microtubule-associated protein 2, a common neuronal marker. Scale bar shows 20 μm. (C) Cultured DRG neurons vary in size. A subset of DRG neurons was analysed for size; the cell body diameter at the plane corresponding to the centre of the nucleus was measured. Bars show quartiles. (D) Anti-DOP receptor antibody specificity was validated by labelling DRG neuronal cultures from DOP receptor knockout (KO) and wild-type animals.
3.2.2 DOP receptor knockout DRG cultures

Cultures were also prepared using DOP receptor knockout and corresponding wild-type littermate mice (Filliol et al., 2000) for use in antibody validation. This was conducted as described earlier with the following exceptions: DRGs were collected from one mouse to produce each culture; for each culture, cells were plated on four wells of a 24-well plate.

3.2.3 Prolonged morphine treatment

For morphine-treated cells, morphine sulfate (Sandoz Canada, Quebec, Canada) was added to the culture medium for the final 48 h of incubation at a final concentration of 10 μM. Vehicle was added to the culture medium of control cells.

3.2.4 Acute agonist/antagonist treatment

Cells were treated with one of three opioid agonists: DAMGO, DELT or SNC80. The original growth medium was removed and replaced with medium containing DAMGO, DELT or SNC80 at 1 μM. The medium was formulated as follows: Neurobasal-A augmented with 10% FBS, 0.5 mM l-glutamine and 100 000 U L−1 penicillin/100 mg L−1 streptomycin. The cells were incubated with the acute agonists for 1 h at 37°C. Control cells were vehicle-treated. For acute antagonist co-treatment experiments, cells treated with DAMGO, DELT, SNC80 and vehicle were co-treated with SDM-25N at 50 nM. SDM-25N is a small-molecule DOP receptor antagonist that exhibits greater selectivity for DOP over MOP receptors (200- to >2000-fold selectivity) than naltrindole (McLamore et al., 2001). SDM-25N has been characterized in binding, GTPγS and smooth muscle assays, and has been used in vivo, in brain slices and in cultured cells (Blomeley & Bracci, 2011; Chen et al., 2007; Southern et al., 2013). The dose used was chosen based on those reports to maximize DOP receptor selectivity.
3.2.5 Immunofluorescence cytochemistry

Cultured cells, on coverslips, were washed with cold Tris-buffered saline (TBS) to remove the culture medium and any drugs. Cells were then fixed by immersion in 4% paraformaldehyde for 10 min at 37°C and washed with cold TBS. Fixed cells were blocked by incubating in 300 µl of TBS supplemented with 5% BSA, 5% normal goat serum (NGS) and 0.1% Triton X-100 at room temperature for 2 h. After being blocked, the cells were incubated for 48 h at 4°C in 300 µl TBS supplemented with 1% NGS and 0.1% Triton X-100 and containing the primary antisera at appropriate concentrations. The cells were double-labelled using rabbit anti-DOP receptor diluted 1:1500 (MyBioSource, San Diego, CA, USA; MBS316175, lot 1B03801, directed against DOP receptor residues 360–372) and one of mouse anti-Rab5 diluted 1:750 (Sigma-Aldrich, St. Louis, MO, USA; R7904, lot 129K4799, directed against Rab5 residues 2–14), mouse anti-Rab11 diluted 1:500 (Millipore, Billerica, MA, USA; 05-853, lot 2011576) or goat anti-LAMP1 (lysosomal-associated membrane protein 1) diluted 1:750 (Santa Cruz, Dallas, TX, USA; SC8098, lot D0113, directed against LAMP1 C-terminus). Cells were then washed with TBS and incubated in 300 µl of identically supplemented TBS containing the appropriate secondary antibodies conjugated to Alexa fluorophores diluted 1:200 for 2 h at room temperature. Alexa-488 conjugated donkey anti-rabbit was used to fluorescently label DOP receptors and Alexa-594 conjugated to either goat anti-mouse or donkey anti-goat was used to fluorescently label one of the internalization compartments of interest. Finally, the cells were washed with TBS and mounted on Superfrost Plus slides (Fisher Scientific, Ottawa, Ontario, Canada) using aquamount (Polyscience, Niles, IL, USA). The slides were kept in the dark at 4°C until imaging. Anti-DOP receptor antibody specificity was validated by labelling DRG neuronal cultures from DOP receptor knockout and wild-type animals (Figure 3.1D).
3.2.6 Confocal microscopy

Immunoreactive neurons were imaged using a Leica SP2 scanning laser confocal microscope (Leica Microsystems, Wetzlar, Germany). Neurons were identified morphologically and imaged at 100× magnification with 488 and 543 nm wavelength excitation. Emissions corresponding to each Alexa-488 and Alexa-568 were detected by independent photomultiplier tubes to yield an image pair, with one image showing the fluorescently labelled DOP receptors and the other image showing the fluorescently labelled internalization compartment of interest. Approximately 20 randomly selected fields of view containing 1–2 neurons were imaged from each unique drug pair–antibody pair condition per experiment. One image pair was captured per field of view at a focal plane transecting the nucleus of the neuron(s). Images were digitally captured as 8-bit uncompressed TIFF files using Leica Confocal Software (Leica Microsystems) on a connected computer and saved for offline analysis.

3.2.7 Co-localization analysis

Confocal image pairs were analysed using ImageJ (v1.45s; NCBI). Eight-bit image pairs were merged as red and green channels to a single RGB image. Neurons of interest were selected free-hand. Co-localization analysis was performed using the PSC Colocalization plugin (v0.9.01; Andrew French, University of Nottingham, Nottingham, UK) to measure co-localization as previously described (French, Mills, Swarup, Bennett, & Pridmore, 2008). A threshold value of 40 was used and the Pearson's co-localization coefficient was recorded for each neuron analysed. Approximately 15–25 neurons were analysed for each unique drug pair–antibody pair condition per experiment. Across all conditions and all experiments, a total of 3018 neurons were analysed. For each experiment, the Pearson's coefficients recorded for each antibody pair were normalized such that the mean of the values for the prolonged vehicle-treated, acute vehicle-treated condition was zero. Subsequent analyses used these normalized co-localization scores. The results from three (DOP receptor-Rab11 agonist only and all antagonist experiments) or four (DOP receptor-
Rab5 and DOP receptor-LAMP1 agonist only) experiments were pooled. Pooled Pearson's coefficients were analysed by two-way ANOVA followed by Tukey's HSD (honest significant difference) test using R (v2.15.0 x86_64; R Foundation for Statistical Computing, Vienna, Austria). Data are presented as mean ± 95% confidence interval using Prism 5 for Windows (v5.0.1; GraphPad Software, San Diego, CA, USA).

3.2.8 Reagents

Neurobasal-A, HBSS, FBS, nerve growth factor 7 s, laminin and Alexa-conjugated secondary antibodies were purchased from Life Technologies (Burlington, Ontario, Canada). Penicillin/streptomycin, trypsin, l-glutamine and poly-d-lysine were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). [D-Ala2, NMe-Phe4, Gly-ol5]-enkephalin (DAMGO), [D-Ala2]-deltorphin II (DELT), (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide (SNC80) and (4bS,8R,8aS,14bR)-5,6,7,8,14,14b-hexahydro-7-(2-methyl-2-propenyl)-4,8-methanobenzofuro[2,3-a]pyrido[4,3-b]carbazole-1,8a(9H)-dil hydrochloride (SDM-25N) were purchased from Tocris (Bristol, UK). Milli-Mark pan neuronal marker (MAB2300, Lot 2383597) was purchased from Millipore.

3.3 Results

3.3.1 Assessment of DOP receptor co-localization with post-endocytic compartments

We measured DOP receptor co-localization with markers for three post-endocytic compartments: Rab 5, marks early endosomes; Rab 11, marks recycling endosomes; and LAMP 1, marks lysosomes. DOP receptor co-localization with these compartments was assessed following prolonged and acute opioid receptor agonist treatment, with and without acute DOP receptor antagonist.
3.3.2 Deltorphin II, but not SNC80, induces DOP receptor recycling

We first examined DOP receptor internalization trafficking following acute treatment with a DOP receptor agonist in prolonged vehicle-treated neurons (Figure 3.2). In vehicle-treated neurons, acute DELT increased DOP receptor co-localization with recycling endosomes ($P < 0.0001$) and decreased DOP receptor co-localization with lysosomes ($P = 0.0033$). There was no effect on DOP receptor co-localization with early endosomes ($P = 0.0713$). Acute SNC80 had no effect on DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes $P = 0.9885$; DOP receptor-recycling endosomes $P = 0.2638$; DOP receptor-lysosomes $P = 0.6132$).

3.3.3 DAMGO does not affect DOP receptor trafficking

We also examined DOP receptor internalization trafficking following acute MOP receptor agonist treatment in prolonged vehicle-treated neurons (Figure 3.3). In vehicle-treated neurons, the MOP receptor agonist DAMGO had no effect on DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes $P = 0.9174$; DOP receptor-recycling endosomes $P = 0.9989$; DOP receptor-lysosomes $P = 0.9457$).

3.3.4 Constitutive DOP receptor trafficking is augmented following morphine treatment

We assessed the effect of prolonged morphine treatment on DOP receptor internalization trafficking (Figure 3.4A). Prolonged morphine treatment increased DOP receptor co-localization with early endosomes ($P = 0.0002$) and lysosomes ($P = 0.0013$). There was no effect on DOP receptor co-localization with recycling endosomes ($P = 0.7834$).

3.3.5 SDM-25N inhibits DOP receptor trafficking augmentation following morphine treatment

We treated neurons acutely with the DOP receptor antagonist SDM-25N (Figure 3.4B) and observed that prolonged morphine treatment increased DOP receptor co-localization with early endosomes ($P = 0.0415$) but decreased DOP receptor co-localization with lysosomes.
Figure 3.2  Deltorphin II, but not SNC80, induces DOP receptor recycling.

Cultured DRG neurons underwent prolonged treatment with vehicle and acute treatment with vehicle, DELT or SNC80. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. In vehicle-treated neurons, acute DELT, but not SNC80, increased DOP receptor co-localization with recycling endosomes and decreased DOP receptor co-localization with lysosomes. Data are shown as mean ± 95% confidence interval. *P < 0.05.
Figure 3.3   DAMGO does not affect DOP receptor trafficking.

Cultured DRG neurons underwent prolonged treatment with vehicle and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. In vehicle-treated neurons, DAMGO had no effect on DOPr co-localization with any of these compartments. Data are shown as mean ± 95% confidence interval.
Figure 3.4 Constitutive DOP receptor trafficking is augmented following morphine treatment.

(A) Cultured DRG neurons underwent prolonged treatment with vehicle or morphine and acute treatment with vehicle. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. Prolonged morphine treatment increased DOP receptor co-localization with early endosomes and lysosomes. (B) Neurons underwent prolonged treatment with vehicle or morphine and acute treatment with SDM-25N or vehicle. Prolonged morphine and acute DOP receptor antagonist increased DOP receptor co-localization with early endosomes and decreased DOP receptor co-localization with lysosomes. Data are shown as mean ± 95% confidence interval. *P < 0.05.
There was no effect on DOP receptor co-localization with recycling endosomes \((P = 0.0579)\).

### 3.3.6 Deltorphin II and SNC80 induce DOP receptor recycling following morphine treatment

We next examined DOP receptor internalization trafficking following acute DOP receptor agonist treatment in prolonged morphine-treated neurons (Figure 3.5B). Following morphine treatment, acute DELT had no effect on DOP receptor co-localization with early endosomes \((P = 0.9430)\), increased DOP receptor co-localization with recycling endosomes \((P < 0.0001)\) and decreased DOP receptor co-localization with lysosomes \((P = 0.0118)\) compared with prolonged morphine, acute vehicle-treated neurons. Similarly, following prolonged morphine treatment, acute SNC80 had no effect on DOP receptor co-localization with early endosomes \((P = 0.9099)\), increased DOP receptor co-localization with recycling endosomes \((P = 0.0102)\) and decreased DOP receptor co-localization with lysosomes \((P = 0.0129)\) compared with prolonged morphine, acute vehicle-treated neurons.

### 3.3.7 SDM-25N blocks deltorphin II- and SNC80-induced DOP receptor recycling following morphine treatment

Using prolonged morphine-treated neurons, we co-treated acutely with SMD-25N and each DOP receptor agonist (Figure 3.5C). When co-administered with the DOP receptor antagonist, acute DELT decreased DOP receptor co-localization with early endosomes \((P = 0.0048)\) and had no effect on DOP receptor co-localization with either recycling endosomes \((P = 0.9310)\) or lysosomes \((P = 0.9999)\) compared with prolonged morphine, acute vehicle, acute SDM-25N-treated neurons. SNC80, when acutely co-administered with the DOP receptor antagonist, had no effect on DOP receptor co-localization with any of these compartments (DOP receptor-early endosomes \(P = 0.6857\); DOP receptor-recycling endosomes \(P = 0.9674\); DOP
Figure 3.5  Deltorphin II and SNC80 induce DOP receptor recycling following morphine treatment.

(A) Example confocal photomicrographs of two neurons. The upper neuron was treated with prolonged morphine and acute vehicle. The lower neuron was treated with prolonged
morphine and acute DELT. Both neurons were immunolabelled for DOP receptor (green, left) and Rab 11 (recycling endosomes, red, centre). False-colour maps of co-localization were generated for each neuron, illustrating per-pixel DOP receptor co-localization with recycling endosomes (heat map, right). Scale bars show 10 μm. (B) Cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle, DELT or SNC80. Co-localization was measured by Pearson’s co-localization coefficient following the 60 min acute treatment. Following morphine treatment, both acute DELT and SNC80 increased DOP receptor co-localization with recycling endosomes and decreased DOP receptor co-localization with lysosomes. (C) Neurons underwent prolonged treatment with morphine and acute treatment with SDM-25N and vehicle, DELT or SNC80. Following morphine treatment and DOP receptor antagonist co-treatment, acute DELT decreased DOP receptor co-localization with early endosomes. Neither DELT nor SNC80 had any other effects on DOP receptor co-localization with the compartments. Data are shown as mean ± 95% confidence interval. *P < 0.05.
Figure 3.6  DAMGO induces DOP receptor internalization and recycling following morphine treatment.

(A) Example confocal photomicrographs of two neurons. The upper neuron was treated with prolonged vehicle and acute DAMGO. The lower neuron was treated with prolonged...
morphine and acute DAMGO. Both neurons were immunolabelled for DOP receptors (green, left) and Rab 11 (recycling endosomes, red, centre). False-colour maps of co-localization were generated for each neuron, illustrating per-pixel DOP receptor co-localization with recycling endosomes (heat map, right). Scale bars show 10 μm. (B) Cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson's co-localization coefficient following the 60 min acute treatment. Following morphine treatment, acute DAMGO increased DOP receptor co-localization with early endosomes and recycling endosomes. (C) Neurons underwent prolonged treatment with morphine and acute treatment with SDM-25N and vehicle or DAMGO. Following morphine treatment and DOP receptor antagonist co-treatment, acute DAMGO only decreased DOP receptor co-localization with early endosomes. Data are shown as mean ± 95% confidence interval. *P < 0.05.
receptor-lysosomes $P = 0.0529$) compared with prolonged morphine, acute vehicle acute SDM-25N-treated neurons.

### 3.3.8 DAMGO induces DOP receptor internalization and recycling following morphine treatment

Finally, we examined DOP receptor internalization trafficking following acute MOP receptor agonist treatment in prolonged morphine-treated neurons (Figure 3.6B). After morphine treatment, acute DAMGO increased DOP receptor co-localization with early endosomes ($P < 0.0001$) and recycling endosomes ($P < 0.0001$) compared with prolonged morphine, acute vehicle-treated neurons. There was no effect on DOP receptor co-localization with lysosomes ($P = 0.9738$). We also conducted preliminary time-course experiments, which revealed DAMGO-induced DOP receptor trafficking was early and sustained (Table 5).

### 3.3.9 SDM-25N blocks DAMGO-induced DOP receptor trafficking following morphine treatment

We acutely co-treated prolonged morphine neurons with SDM-25N and DAMGO (Figure 3.6C). When co-administered with the DOP receptor antagonist, DAMGO decreased DOP receptor co-localization with early endosomes ($P = 0.0196$) and had no effect on DOP receptor co-localization with recycling endosomes ($P = 1.0000$) or lysosomes ($P = 0.6691$) compared with prolonged morphine, acute vehicle, acute SDM-25N-treated neurons.

### 3.4 Discussion and conclusions

In this study, we have used co-localization analysis of immunolabelling to quantify endogenous DOP receptor association with three post-endocytic compartments following various ligand treatments, both prolonged and acute. Co-localization with the compartment markers is interpreted, directly, as co-localization with those compartments and, more abstractly, with the processes in which those compartments participate, namely internalization, degradation and recycling. This abstraction is helpful in understanding the results, but it is important to recognize
Table 5  DAMGO induction of DOP receptor recycling is early and sustained following morphine treatment

<table>
<thead>
<tr>
<th>Prolonged</th>
<th>Acute</th>
<th>Time (minutes)</th>
<th>DOPr-Rab5</th>
<th>DOPr-Rab11</th>
<th>DOPr-Lamp1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>Vehicle</td>
<td>0.17 ± 0.03</td>
<td>-0.05 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>0.15 ± 0.02</td>
<td>0.23 ± 0.03 *</td>
<td>0.01 ± 0.03 *</td>
</tr>
<tr>
<td>Morphine</td>
<td>DAMGO</td>
<td>30</td>
<td>0.20 ± 0.04</td>
<td>0.17 ± 0.04 *</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.36 ± 0.05 *</td>
<td>0.18 ± 0.04 *</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

In a preliminary experiment, cultured DRG neurons underwent prolonged treatment with morphine and acute treatment with vehicle or DAMGO. Co-localization was measured by Pearson’s co-localization coefficient following 15, 30 and 60 min acute treatments. Data are reported as mean ± 95% confidence interval. n = 1–4 independent cultures; 15–25 neurons imaged per culture.

*P < 0.05 compared with acute vehicle-treated neurons.
that the results remain proxies for these overarching processes. These processes, in their totalities, are broader and more complex than the telltale signs we measure (for review, see [Hanyaloglu & von Zastrow, 2008; von Zastrow, 2010; Williams et al., 2013]). However, these results do present a broad survey of post-internalization DOP receptor trafficking in which interesting comparisons can be made.

As class B GPCRs, constitutively internalized DOP receptors are trafficked via early endosomes ultimately to lysosomes where they are degraded (Figure 3.7A). Our results, however, indicate a departure from the constitutive trafficking pathway in many cases following acute agonist-induced internalization (Figure 3.7B). Consistent with previously published results obtained in immortalized neurons and expression systems, DOP receptors were found to be associated with lysosomes following acute agonist treatment. However, acute administration of the DOP receptor peptide agonist DELT to control, prolonged vehicle-treated sensory neurons induced DOP receptor recycling. This increase in DOP receptor co-localization with subcellular compartments associated with receptor re-sensitization pathways was accompanied by a reduction in DOP receptor trafficking to lysosomes. This rules out the possibility that the observed increase in DOP receptor recycling was due to a global increase in DOP receptor internalization trafficking, as that explanation would require a concomitant, substantial increase in DOP receptor trafficking to lysosomes. Rather, these data show that DELT induced a shift in DOP receptor trafficking from the constitutive, lysosome-terminated pathway to recycling of the receptor back to the cell surface. DOP receptor post-internalization trafficking appears not to be a pre-determined track, but rather open to agonist influence. That is, the determinant of post-endocytic sorting is not DOP receptor per se but instead the event which triggered internalization.

It should be noted that while we observed increased DOP receptor recycling and decreased degradation, we did not detect any change in DOP receptor internalization (DOP receptor-early endosome co-localization). There are two possible explanations. Firstly, that there
Figure 3.7  Prolonged morphine alters DOP receptor post-internalization trafficking.

(A) Schematic summary of constitutive DOP receptor trafficking: internalization via early endosomes and ultimate degradation at lysosomes. Recycling is not considered to play a major role. (B) Schematic summary of experimental findings. In prolonged vehicle-treated neurons, DELT induced DOP receptor recycling and reduced degradation; no other acute condition affected DOP receptor trafficking. Prolonged morphine treatment (acute vehicle) augmented DOP receptor constitutive trafficking. Following prolonged morphine treatment, both DELT and SNC80 induced DOP receptor recycling and reduced degradation. Notably, DAMGO induced DOP receptor internalization and recycling in prolonged morphine-treated, but not control, neurons.
was, in fact, no net change in DOP receptor internalization. Agonist-induced DOP receptor internalization may have replaced constitutive DOP receptor internalization. On normal DOP receptor-expressing neurons, the vast majority of DOP receptor are at intracellular sites. There is a paucity of DOP receptors on the cell surface (Cahill, Morinville, et al., 2001; Petaja-Repo, Hogue, Laperriere, Walker, & Bouvier, 2000). The DELT-induced internalization of DOP receptors may pre-empt its normal, constitutive internalization either by design (i.e. constitutive internalization is only triggered in the absence of induced internalization) or by competition (i.e. there is only a sufficient pool of surface receptors for one internalization process). This would lead to no net change in DOP receptor internalization and co-localization with early endosomes, while preserving down-stream changes in trafficking. Secondly, we may have failed to detect a change in DOP receptor internalization. The methodology we used to assess DOP receptor trafficking provides a measure of DOP receptors associated with post-endocytic compartments at a specific time point. In this case, all measurements were made on neurons fixed after 60 min of acute agonist exposure. It is possible that there was a DELT-induced change in DOP receptor internalization, which happened and ended within that time. That is, the DOP receptor internalization had already occurred and DOP receptor-early endosome associations had returned to baseline levels by the time we washed and fixed the cells. This would have resulted in our temporally failing to detect the change.

Acute administration of SNC80 to control neurons had no effect on DOP receptor trafficking. It seems unlikely that SNC80-induced internalization simply replaced constitutive internalization exactly, yet this result does not, at first, seem to agree with the well-established ability of SNC80 to induce DOP receptor internalization (A. A. A. Pradhan et al., 2010). The most likely explanation is that while SNC80 can induce DOP receptor internalization, there are simply too few DOP receptors on the surface of normal neurons for the non-peptide agonist's effects to be detectable. In this case, DELT appears to be better able to induce DOP receptor
trafficking. Such variability in the ability to induce receptor internalization and trafficking is well established for both DOP (A. A. A. Pradhan et al., 2010) and MOP receptor (Martini & Whistler, 2007) agonists. More broadly, these findings also fit with the growing body of work describing ligand-specific sorting in opioid receptors (Audet et al., 2008, 2012; Audet, Paquin-Gobeil, Landry-Paquet, Schiller, & Piñeyro, 2005; Hong et al., 2009; Marie, Lecoq, Jauzac, & Allouche, 2003) (for review, see (Nagi & Piñeyro, 2011; Piñeyro & Archer-Lahlou, 2007; Williams et al., 2013)). These results may also reflect DELT and SNC80 acting on different DOP receptor populations. As a peptidic ligand, DELT would largely be restricted to acting on surface DOP receptors, whereas SNC80, a small molecule, would not have that limitation. As a result, SNC80's additional actions on intracellular DOP receptors may contribute to the differences observed.

Since prolonged morphine treatment causes MOP receptor-dependent trafficking of DOP receptors to neuronal cell membranes (Cahill, Morinville, et al., 2001; Gendron et al., 2006; Lucido et al., 2005; Morinville et al., 2003; Morinville, Cahill, Aibak, et al., 2004), we examined whether endogenous DOP receptor post-internalization trafficking in neurons was altered by prolonged morphine treatment. Constitutive DOP receptor trafficking was augmented following prolonged morphine treatment; we observed increased DOP receptor internalization and trafficking to lysosomes. This is consistent with an increase in the cell surface DOP receptor population. With more surface DOP receptors, there are more DOP receptors constitutively internalized, whereupon they follow the typical pathway to degradation in lysosomes. With acute administration of the DOP receptor antagonist SDM-25N, we continued to observe increased DOP receptor internalization but decreased trafficking to lysosomes. As SDM-25N was administered acutely, without acute agonist treatment, it is not surprising that effects of prolonged morphine were still apparent, in the form of increased DOP receptor internalization. It is interesting, though, that lysosomal trafficking of DOP receptors decreased. These apparently
opposite changes may represent a combination of stabilization of DOP receptors by SDM-25N against the background of an increased DOP receptor surface population.

Acute administration of DELT to morphine-treated neurons induced DOP receptor recycling and decreased DOP receptor degradation (Figure 3.7B). This is a preservation of the same effect as observed in control neurons. Once again, DELT induced DOP receptor trafficking different from the constitutive pathway. Interestingly, we were again unable to detect a change in DOP receptor internalization following acute DELT compared to control neurons, which received acute vehicle and the same prolonged treatment. Once again both possible explanations are valid. There may be no net change in DOP receptor internalization; constitutive internalization was augmented following prolonged morphine, and DELT-induced internalization may have replaced some of the constitutive traffic. Alternatively, we may still be temporally failing to detect the DELT-induced changes in internalization.

Trafficking in response to SNC80 changed following prolonged morphine treatment compared with control neurons. Acute administration of SNC80 to morphine-treated neurons induced DOP receptor recycling and decreased DOP receptor degradation (Figure 3.7). This unmasking of distinct SNC80-induced trafficking is consistent with the hypothesis that such activity was not observed in control neurons because of the relative paucity of surface DOP receptors. The increase in available DOP receptors following prolonged morphine permits SNC80 to induce DOP receptor trafficking. SNC80's trafficking effects appear to be the same as DELT's; DOP receptor trafficking shifts from the constitutive lysosome-terminated pathway to recycling. In a further similarity to DELT, we observed no change in DOP receptor internalization after acute SNC80, and the same explanations are possible. As would be expected, DOP receptor agonist-induced trafficking was absent when DELT and SNC80 were co-administered with SDM-25N.
It is unlikely that alterations in trafficking in response to DELT and SNC80 are as a result of pharmacological chaperoning of DOP receptors given the similarity of the responses. Previous studies identified that a high percentage of DOP receptors are targeted directly from endoplasmic reticulum to lysosomes with only a small percentage ever maturing and reaching the plasma membrane (Petaja-Repo et al., 2001, 2000). However, non-peptide DOP receptor agonists have been shown to act as chaperones, promoting DOP receptor maturation and thus increasing plasmalemma-associated receptors (Petäjä-Repo et al., 2006). While chaperoning by SNC80 could play a role in the availability of surface DOP receptors for SNC80 to then internalize, DELT would not have such effects given the need for any pharmacological chaperone to be membrane permeable (Petäjä-Repo et al., 2002). Furthermore, in such a case, one would expect the effects of DELT and SNC80 in control neurons to be reversed; that is, the membrane-permeable SNC80, but not DELT, should have chaperoned DOP receptors to the surface to then internalize it. In both cases, the opposite occurred.

Acute administration of DAMGO, a MOP receptor agonist, to control neurons had no effect on DOP receptor internalization. It is possible that such a lack of effect reflects a temporal limitation, but in such a case, DAMGO effects on DOP receptor trafficking would have to normalize within 60 min despite continued agonist application.

Acute administration of DAMGO to morphine-treated neurons, however, induced DOP receptor internalization and recycling. This is a change from control neurons, where DAMGO had no detected effect, consistent with the postulated induction of MOP–DOP receptor interactions by prolonged morphine treatment. Indeed, preliminary findings indicate that the induced DOP receptor recycling is both early and sustained. *Prima facie*, DAMGO would be expected to have no effect on DOP receptor trafficking as it has negligible activity at that receptor. However, the presence of a MOP/DOP receptor species at the cell surface following prolonged morphine could account for the existence of a DOP receptor species upon which
DAMGO may act. Interestingly, in this case, we did observe increased DOP receptor internalization but did not observe any change in DOP receptor degradation following 60 min DAMGO exposure. Our preliminary findings, however, indicate that DAMGO may reduce early (15 min) DOP receptor degradation, whereas the increase in internalization occurs following sustained (60 min) exposure. This may reflect DAMGO inducing the recycling of a largely different pool of receptors than those undergoing constitutive trafficking. In this case, DAMGO-responsive DOP receptor would be immediately shifted away from degradation to recycling, resulting in the early reduction in DOP receptor degradation. With continued exposure, the surface population becomes enriched in DAMGO-responsive DOP receptors, ultimately leading to the late increase in DOP receptor internalization and sustained increase in DOP receptor recycling. Such recycling may also involve recycling from superficial endosomes, identified as the Rab5-labelled compartment, consistent with the existence of a distinct pool of MOP/DOP receptors. Further, surface enrichment in DAMGO-responsive DOP receptors may also involve DAMGO-induced export of new or intracellularly reserved DOP receptors. Ultimately, whereas DOP receptor internalization in response to DELT or SNC80 may occur to some extent in place of constitutive internalization, DAMGO internalization may be occurring in addition to it. This is consistent with the presence, post-prolonged morphine, of an additional MOP/DOP receptor species with distinct trafficking characteristics. Notably, the responsiveness of DOP receptor trafficking to DAMGO was abolished by co-treatment with SDM-25N. Together, these results demonstrate that following prolonged morphine, but not in basal conditions, there is surface availability of a DOP receptor species upon which DAMGO may act to induce internalization trafficking and at which such action may be blocked by a DOP receptor antagonist. These findings support previous reports that prolonged morphine treatment induces an increase in cell surface available DOP receptor and that prolonged morphine treatment induces MOP–DOP receptor interactions, potentially in the form of a MOP/DOP receptor species. These MOP–DOP
receptor interactions appear to alter responsiveness to opioid receptor ligands and subsequent post-internalization DOP receptor trafficking.

These findings are consistent with other reports of MOP–DOP receptor interaction effects upon trafficking. Milan-Lobo and Whistler (2011) reported that in a heterologous system, the MOP receptor agonist methadone internalizes both MOP and MOP/DOP receptors. Additionally, He et al. (2011) reported that in a heterologous system, DOP receptor agonists induced endocytosis of both DOP and MOP receptors. In this case, DOP receptor agonist-induced endocytosis of MOP receptors was attenuated by disrupting putative MOP–DOP receptor interactions. In both cases, these studies concluded that the existence of a MOP–DOP receptor interaction permitted the apparently paradoxical agonist-induced receptor internalization. These reports agree with both our observation of DAMGO-induced DOP receptor internalization and our interpretation that the internalized DOP receptors were in the form of MOP/DOP receptor heteromers. Furthermore, and in agreement with the finding of altered post-internalization trafficking of MOP/DOP receptors, Milan-Lobo and Whistler reported that an internalized MOP/DOP receptor was handled differently from an internalized MOP receptor, tending towards degradation (Milan-Lobo & Whistler, 2011). Notably, Milan-Lobo and Whistler reported a shift towards degradation for MOP/DOP receptors, while we observed a shift towards recycling. This is, however, not surprising as we each compared against different standards. That MOP/DOP receptors would undergo greater degradation than MOP receptors and also greater recycling than DOP receptors is sensible; MOP/DOP receptor's trafficking behaviour appears to be unique from and intermediate to that of either component individually.

It is important to recognize that we measured DOP receptor sorting to these post-endocytic compartments after 60 min ligand treatment. This provides a ‘snapshot’ of DOP receptor post-endocytic trafficking across these compartments at that time point but not a summation or time course of trafficking over that time period. Indeed, a comparison of the time
courses of post-endocytic trafficking in certain ligand treatment conditions is an interesting future direction.

In conclusion, we have demonstrated that in physiologically relevant neurons expressing endogenous receptors, prolonged morphine treatment augments constitutive DOP receptor trafficking and alters agonist-induced DOP receptor trafficking. Notably, the DOP receptor internalizes and traffics in response to DAMGO, a MOP receptor agonist. These effects are inhibited or absent when agonists are co-administered with a DOP receptor antagonist. These observations are consistent with the hypothesis that prolonged morphine treatment induces the formation of MOP–DOP receptor interactions and subsequent cell surface availability of a MOP/DOP receptor species. The pharmacology and trafficking of such a species appear to be unique compared to those of its individual constituents and could represent a novel therapeutic target.
Chapter 4

Tracking Drug-induced Changes in Receptor Post-internalization

Trafficking by Colocalizational Analysis

This work is published in The Journal of Visualized Experiments:

Ong E, Cahill C. (2015), (101): e52824; doi:10.3791/52824

4.1 Introduction

Receptors, especially G protein coupled receptors (GPCRs), are routinely trafficked intracellularly, to and from the cell surface (Drake et al., 2006). These complexly orchestrated and tightly controlled processes dictate cells’ available receptor complements and regulate receptor temporal activity, desensitization, and resensitization (Hanyaloglu & von Zastrow, 2008; Hislop & von Zastrow, 2011; Nagi & Piñeyro, 2011). Importantly, these processes are responsive to cellular environments including drug-induced receptor activity or inactivity. That is, the actions of ligands at receptors can alter intracellular trafficking of those receptors, thereby altering cell responsiveness. In this manner, external ligands exert yet more effects upon cell function, even beyond classical messenger-to-effector cascades (von Zastrow, 2010; Whistler et al., 2002).

Examining such changes in induced receptor trafficking is difficult. All available techniques involve limitations. Biotin protection assays have been used to monitor surface receptor populations. These receptors are biotinylated and a timecourse of immunoprecipitations is performed to quantify the reduction in biotinylated receptors over time. This technique essentially monitors the gradual degradation of an initial, labeled, population of receptors (Milan-Lobo & Whistler, 2011), and is very useful in constructing time courses of this process. Unfortunately, this assay is unable to monitor any process other than degradation of the original pool of receptors, such as internalization, recycling, or new receptors. Also, the addition of an
antibody in the 150kDa range to a receptor in the 50kDa range can alter the receptor’s trafficking (Scherrer et al., 2006; H.-B. Wang et al., 2008), and this technique may be difficult to use with low expression-level receptors.

Other procedures use various methods to identify intracellular trafficking compartments (e.g., endosomes, etc.) and assess their colocalization with the receptors of interest. This includes the use of heterologous systems expressing fluorescent-protein-tagged chimeric constructs of the receptors and compartment markers (e.g., Rab-family GTPases). This potentially enables the use of live-cell imaging, removing issues related to fixation and permeabilization. While powerful, such a strategy suffers from the same limitations of heterologous systems in general: tag and expression level effects on trafficking behavior and incompatibility with more physiologically representative cell types. More popularly, dyes are used to easily label intracellular compartments (e.g., lysosomes, ostensibly) (S.-Q. He et al., 2011). Dyes, however, can lack specificity (all acidic organelles in the case of dyes for lysosomes) and do not assess trafficking through other compartments. Still, these techniques allow considerable control over the system and experimental conditions and may benefit from the colocalization analysis methods presented here, below.

The method we present here refines the tracking of receptor trafficking by colocalization. Using immunocytochemistry (ICC) to label appropriate markers, it is possible to identify multiple distinct intracellular compartments. This also allows the use of physiologically-relevant primary cell cultures in place of heterologous systems. This ICC protocol involves fixing the cells of interest prior to labelling; this permits labelling at a specific timepoint following drug treatment(s). This produces a ‘snapshot’ of global receptor-compartment associations at that timepoint. With multiple timepoints, a timecourse of trafficking changes can also be constructed. Briefly, cells are drug-treated, labeled for the receptor and intracellular compartment of interest, confocally imaged, and the photomicrographs are analyzed to mathematically quantify
colocalization of the receptor and compartment (French et al., 2008). In our use, we examined the colocalization of a receptor with Rab5, Rab11, and Lysosomal-associated membrane protein 1 (LAMP1). These markers identify early endosomes, recycling endosomes, and lysosomes, respectively. These colocalization measures act as proxies for the overarching processes of internalization, recycling, and degradation (Ong, Xue, Olmstead, & Cahill, 2015).

As with all techniques, some limitations should be considered. Due to the need to image every individual neuron analyzed, this technique can become quite labor-intensive depending on the number of conditions and timepoints involved. All immunolabelling must also contend with the effects on cellular ultrastructure, protein localization, and epitope accessibility caused by fixation and permeabilization (Schnell, Dijk, Sjollema, & Giepmans, 2012). Though originally optimized for use with primary cultures of primary sensory neurons, this method is broadly compatible with other monolayer plated culture models. The use of a mathematically quantified measure of colocalization is, notably, far more methodologically rigorous than previous techniques used to assess receptor trafficking changes, which have often relied on vague, subjective measures such as visually-inspected multi-channel overlays (Décaillot et al., 2008).

This technique is particularly useful for its broad compatibility with in vivo interventions (prior to primary culture generation), in vitro interventions (during culture growth), and various labelling targets (Gupta et al., 2010). As such, it may be adapted to many different research questions.

4.2 Protocol

Note: This protocol is broadly compatible with various monolayer-plated cell/tissue culture models, drug treatment regimens, and labelling targets. Thus in actual use, many specific parameters will vary based on experimental design. Here, references to these user-defined parameters are generic. Example conditions, as used to obtain the representative results, are included in italics.
4.2.1 Solutions

1. Prepare washing buffer by mixing 0.1M Tris-Buffered Hypertonic (300mM) Saline and 0.05% Polysorbate 20; pH 7.4 at room temperature.

2. Prepare blocking buffer. To 0.05M Tris-Buffered Hypertonic (300mM) Saline add 0.05% Polysorbate 20, 3% Bovine Serum Albumin (BSA) and 0.1% cold fish skin gelatin and adjust the pH to 7.4 at room temperature.

3. Prepare antibody diluent by adding 0.05% Polysorbate 20, 1% BSA, 0.1% cold fish skin gelatin to 0.05M Tris-Buffered Hypertonic (300mM) Saline and adjust the pH to 7.4 at 4 degrees Celsius.

Note: The pH of Tris buffers is temperature-dependent. That is, a change in temperature will change the pH of the solution. For consistency, these buffers should always be pH-adjusted at the temperature at which they will be used.

4.2.2 Cell culture

Note: Appropriate cell culture protocols will vary based on cell type(s) used. These procedures must be separately optimized. Detailed cell culture methodologies are readily available, including (Seibenhener & Wooten, 2012). A similar procedure was used to obtain the representative results, with the notable differences:

1. Culture dorsal root ganglia neurons from adult animals as described (Ong et al., 2015).

   Use adult-neuron growth medium to culture the cells. Reduce the glial cell density by preplating, rather than glutamate. This results in a mixed neuron-glial culture grown on 12-round glass coverslips with at least 30-60 neurons per plate and accompanying glial cells grown to approximately 40% confluency. It should be noted that neurons in a primary culture will not replicate and will be pushed off of the plate by co-cultured glia if the glia are allowed to grow excessively. In order to avoid affecting the neurons, physical reduction of glial numbers is preferable to chemical/pharmacological methods.
Note: For the purposes of this protocol, we assume that the cells of interest are grown to their experimentally-desired state and monolayer-plated. We find that plating on 12-round glass coverslips in 24 well plates to be most convenient. All volumes and procedures described are appropriate for one coverslip in one well of a 24 well plate.

4.2.3 Drug treatment of cultured cells

Note: Multiple sequential, or overlapping, drug treatments are possible. The drugs, doses/concentrations, and durations of exposure used will depend on the specific experiment.

1. Remove the original growth medium (Seibenhener & Wooten, 2012) and replace it with medium containing the drug(s) of interest at the chosen concentration(s). In this case, use 10µM of morphine solubilized in saline or use saline as control.

Note: It is important that each independent replicate (generally a single 24-well plate culture) contain the same common control condition. This will permit normalization of data across replicates, which corrects for inter-trial variability.

2. Incubate the cells in the original growth conditions (Seibenhener & Wooten, 2012) for 48 hours.

3. For subsequent drug treatments, repeat steps 3.1 and 3.2 with Deltorphin 1µM, SNC80 1µM, or vehicle and incubate for 60 minutes (Ong et al., 2015). Solubilize deltorphin II in water and solubilize SNC80 at 10mM in 40µM HCl and Sonicate, diluted to 1mM in water.

Note: This protocol assumes the drug(s) have been solubilized such that they may be dissolved at the desired concentration(s) in the chosen growth medium. Appropriate procedures for such solubilization will vary depending on the drug in question and must be separately optimized.
4. Wash the cells gently, three times, with ~1ml of washing buffer per wash. Perform each wash by gently aspirating the liquid from the well, then promptly, and gently, refilling the well with fresh solution, as desired (in this case with washing buffer).

4.2.4 Fixation and Immunocytochemistry

Note: The specific labelling targets will vary by experiment. *In our use, we labelled delta opioid receptors (DOR) and Rab5, Rab 11, and LAMP1, as discussed.* The specific antibodies used will depend on the specific experiment. Optimum labelling conditions are dependent on the specific antibody(ies) used. A much fuller discussion of this topic is below.

1. Fix the cells by immersion in ~300ul 4% paraformaldehyde in 0.1M phosphate buffered saline for 10 minutes at 37 degrees Celsius.

   Note: It is important that the 4% paraformaldehyde be freshly prepared due to the autofluorescence caused by the use of previously-frozen paraformaldehyde.

2. Wash the cells gently, three times, with ~1ml of washing buffer per wash, as described in 4.2.3.

3. Incubate the cells in 300ul blocking buffer for two hours at room temperature.

4. Incubate the cells with primary antibodies in 300ul of antibody diluent for 48 hours at 4 degrees Celsius. *In our use, primary antibodies against DOR (rabbit anti-DOR) and one of: Rab5 (mouse anti-Rab5), Rab 11 (mouse anti-Rab11), and LAMP1 (goat anti-LAMP1).*

5. Wash the cells gently, three times, with ~1ml of washing buffer per wash, as described in 4.2.3.

6. Incubate the cells with secondary antibodies conjugated to different fluorophores (see discussion below) in 300ul of antibody diluent for one hour at room temperature. In this, and all subsequent steps, protect the cells from light. *To obtain the representative results,*
*use green-emitting fluorophore-conjugated anti-rabbit and either a red-emitting fluorophore-conjugated anti-mouse or red-emitting fluorophore-conjugated anti-goat.*

7. Wash the cells gently, three times, with ~ 1ml of washing buffer per wash, as described in 4.2.3.

8. Mount the coverslips, cell-side-down, on microscope slides with anti-fading mounting medium. Remove the 12-round coverslips from 24 well plates by leaving ~ 1ml of washing buffer in each well, holding the plate at ~ 45 degrees, and lever the coverslip off of the well floor using fine forceps. The coverslip will come to rest against the well wall, from where it can easily be removed using the forceps.

### 4.2.5 Microscope Settings

1. Using a high magnification objective (100x), first locate a representative cell to be imaged using epifluorescence.

2. Configure the image capture settings to record 8-bit uncompressed TIFF images of each labelled channel (for detection of each fluorophore used e.g. 488nm and 594nm), to image each channel sequentially (either by line or frame), and to average 4 to 6 scans for the final image. Optimal image resolution will be microscope-specific, but 1024x1024 typically yields good results.

3. Switch to the confocal imaging path and focus to a z-plane through the center of the cell.

4. Optimize pinhole (typically 1 Airy), laser power, and photomultiplier tube voltage (gain) and offset for each channel. Save/record these settings. Use the same microscope settings for imaging all the cells labelled for any given target pair in the same replicate.

Note: This process will usually result in sufficient photobleaching that this cell should not be imaged for analysis.
4.2.6 Imaging

1. Using a high magnification objective (e.g. 100x), first locate a cell to be imaged using epifluorescence.

2. Switch to the confocal imaging path and focus to a z-plane through the center of the cell. If available, use software zoom/crop to restrict the scan area to the cell of interest.

3. Capture images for each labelled channel using the settings previously established. Repeat steps 6.1 to 6.3 to image 15 or more cells per condition per replicate to ensure a sufficient sample.

4.2.7 Colocalization Analysis

1. Open the pair of images (e.g. ‘green’ and ‘red’) of a cell in ImageJ. Use the Image > Color > Merge Channels… command to generate an RGB image.

2. Draw a selection around the cell of interest. Use the ImageJ plugin “PSC Colocalization” ((French et al., 2008); available from https://www.cpib.ac.uk/tools-resources/software/psc-colocalization-plugin/) to quantify colocalization of the targets in the selected cell.)

3. Record the desired colocalization measure (typically Pearson’s r (Bolte & Cordelières, 2006)). Repeat steps 7.1 to 7.3 for each imaged cell.

4.2.8 Data normalization

1. Calculate the mean of the recorded colocalization values of the common control conditions of each replicate of each labelling condition.

Note: For example, the mean of the colocalization values of neurons in the “48-hour vehicle, 60-minute vehicle” drug condition in each of three independent replicates in each of three labelling conditions (receptor and each of three compartments).
2. Multiply the means by (-1) to yield the offset for each replicate in each labelling condition. Add the offset for each replicate in each labelling condition to each colocalization value in that replicate.

Note: This will normalize the data such that the mean colocalization measure for the common control condition is 0 in each replicate of each labelling condition.

3. Pool the data from all replicates of each labelling condition.

Note: Changes in colocalization can now be analyzed across replicates. Statistical analyzes will depend upon experimental design but will typically involve analysis-of-variance (ANOVA) followed by appropriate post-hoc tests (e.g. Tukey). For statistical resources, see e.g. (Field, Miles, & Field, 2012).

4.3 Representative Results

Using this technique, it is possible to quantify changes in receptor post-internalization trafficking following both chronic/prolonged and acute drug treatments. After drug treatments, fixation, and labelling, high-resolution two-channel photomicrographs are captured of each cell of interest. Representative images may be combined with false-color colocalization to generate illustrative figures (Figure 4.1). Subsequent colocalizational analysis, as described, yield quantitative scores of target-target colocalization (e.g. receptor-compartment marker). These data may then be used to compare changes in, in this case, receptor trafficking induced by the drug treatment(s) (Figure 4.2).

4.4 Discussion

We have optimized this protocol for the analysis of primary cultures of adult dorsal root ganglion neurons (primary sensory neurons). It can also be used, with little or no modification, for monolayer-plated cultured cells broadly. The colocalizational analysis is also possible in tissue slices and other such preparations (French et al., 2008), however the drug treatment and tissue fixation/preparation components would not be appropriate.
Primary cultures of dorsal root ganglia sensory neurons were exposed to prolonged (48 h) and acute (1 h) drug treatments (left axis labels). Only one prolonged condition is shown as representative results. The cells were then immunolabeled for delta opioid receptors (DOR) and a marker of recycling endosomes (Rab 11) with distinct primary-secondary (fluorophore conjugated) antibody pairs (top axis labels). The cells were imaged by two-channel sequential confocal microscopy (left column, center column). Representative images show the two labelled targets in two neurons. One neuron was treated with prolonged morphine followed by acute vehicle (top). The other neuron was treated with prolonged morphine followed by acute deltorphin II (DELT, a DOR agonist; bottom). In addition to subsequent
quantitative colocalizational analysis, these representative images were processed to generate false-color colocalization maps (right column). Scale bars show 10 μm. Adapted from (Ong et al., 2015) under the provisions of CC BY-NC 3.0.
Figure 4.2  Quantitative colocalization scores can be used to compare changes in receptor post-internalization trafficking.

Primary cultures of dorsal root ganglia sensory neurons were exposed to prolonged (48 h) and acute (1 h) drug treatments (x-axis labels). Only one prolonged condition is shown as representative results. The cells were then immunolabelled for DOR and markers of early endosomes, recycling endosomes, and lysosomes. After imaging colocalization scores were determined, normalized, and pooled across 3 - 4 independent replicates. Data were then analyzed by two-way analysis of variance (ANOVA) with Tukey’s HSD post-hoc. Data are presented as mean +/- 95% confidence interval. * denotes $p < 0.05$. As is evident, this technique allowed the identification of changes in
DOR post-internalization trafficking induced by acute treatment with deltorphin II, but not SNC80 (two different DOR agonists).
Adapted from (Ong et al., 2015) under the provisions of CC BY-NC 3.0.
Of interest, the ICC methods presented here can also be used, with appropriate fixation and tissue preparation, to perform double-labelled immunohistochemistry in tissue slices, irrespective of subsequent analysis (e.g., (Mattioli, Milne, & Cahill, 2010)).

This protocol is broadly compatible with different labelling targets. Each coverslip of plated cells will be double-labelled. Typically, this will be for the receptor of interest and a marker of one of the compartments of interest. There will typically be multiple labelling conditions in order to examine receptor colocalization with multiple different compartments.

Antibodies are inherently variable. Appropriate labelling protocols will vary between different antibodies. This includes appropriate antibody concentrations, buffer recipes, and timepoints. It should also be noted that different lots of the same antibody are best considered to be different antibodies. As such, labelling methods and antibody specificity should be validated, and if necessary optimized, for each combination of antibody and target tissue. The methods used here are a useful starting point. When validating labelling, it is important to perform appropriate controls: this includes samples processed without the addition of secondary antibodies (to control for autofluorescence) and without primary antibodies (to control for non-specific secondary labelling).

There are many factors which influence the design of labelling methods. Some considerations of note affecting the methods presented here include the use of Tris buffers, hypertonic saline, Polysorbate, BSA, and cold fish skin gelatin. Phosphate buffers can cause higher non-specific labelling and may interact with some lesser-used antibody conjugates. However, Tris buffers are, as noted, temperature sensitive. Hypertonic salt concentrations reduce non-specific labelling by disrupting ionic interactions. It is possible to further increase salt concentrations beyond what is specified in this protocol, if desired. Polysorbate 20 is used as a relatively gentle surfactant/detergent. This is preferred over Triton X-100, which has been reported to significantly disrupt, or even dissolve, membranes and thereby distort subcellular
structure. The inclusion of low-concentration Polysorbate 20 in all buffers is useful in reducing non-specific labelling, as it improves the thoroughness of washes. BSA is a commonly used blocking agent intended to occupy non-specific protein binding sites in the target tissue. Some have reported that BSA may aggregate and lead to non-specific punctate labelling. If this issue arises, it is possible to replace BSA with non-fat dry milk or additional cold fish skin gelatin. Cold fish skin gelatin is a non-mammalian protein source also intended to occupy non-specific protein binding sites while presenting low reactivity to antibodies directed against mammalian proteins (Vogt, Phillips, Henderson, Whitfield, & Spierto, 1987).

Though not specified in this protocol, it is typical to add, to the blocking buffer, normal serum from the species in which the secondary antibody was raised. Typical concentrations are 1-3%. As discussed below, care in species selection must be exercised to avoid cross-reactivities.

When detecting two or more fluorescently-labelled targets, the choice of appropriate fluorophore combinations is particularly important. It is essential to have good spectral separation in order to avoid crosstalk. Further, the choice of fluorophores will depend on the configuration of the microscope to be used (available filters, laser lines, etc.). Fluorophore-conjugated antibody suppliers will offer advice on the best combinations of their products (e.g. (I. Johnson & Spence, 2010)). We find 488nm- and 594nm-excited fluorophores to be best pair for double-labelling and colocalizational analysis.

Secondary antibodies are typically species-reactive. That is, they will label any antibodies produced by the target species. Double-labelling ICC therefore requires that care be taken in choosing the host species of the primary and secondary antibodies in order to avoid cross-reactivity. For example, if the primaries are raised in rabbit and goat, it would not be appropriate to use a goat-raised secondary. If antibody availability does not allow for such species separation, there are protocols available to accomplish labelling with multiple same-host primary antibodies.
(e.g. (Morris & Stanley, 2003)), though substantially more optimization and validation should be expected.

As this method quantifies colocalization in photomicrographs, it is fundamentally necessary that all microscopy be consistent, of high quality, and accurately representative of the samples imaged. This includes both the hardware used (optics quality, etc.) and the particular procedures and parameters chosen. There are excellent resources available for guidance on appropriate microscopy (North, 2006; Pawley, 2006), including microscopy specifically for colocalizational analysis (Bolte & Cordelières, 2006; French et al., 2008; Zinchuk, Zinchuk, & Okada, 2007).

Though of considerable methodological superiority to visual overlay methods, quantitative measures of colocalization do not as nicely lend themselves to the generation of illustrative figures for publication. Such illustrative figures are often helpful for readers and their absence may be criticized by reviewers. We have found that the inclusion of false-color ‘heatmaps’ visualizing colocalization to be helpful in constructing figures. The ImageJ plugin “Colocalization Colormap” (available from https://sites.google.com/site/colocalizationcolormap/) is useful in generating these images. It is important to note, however, that these images would be strictly illustrative in the context of the technique described here.

4.5 Video

The video component of this article can be found at http://www.jove.com/video/52824/
Chapter 5

Validation of a Floxed-Stop, FLAG-Tagged Oprd1 Conditional Knock-In Mouse

5.1 Introduction

Opioid receptors are a family of seven transmembrane domain G-protein coupled receptors (GPCRs). The family was historically considered to consist of three types of opioid receptor: the mu opioid receptor (MOR), delta opioid receptor (DOR), and kappa opioid receptor (KOR) (Alexander et al., 2013). A related GPCR with substantial sequence homology to the opioid receptors, the nociceptin receptor (previously referred to as the opioid receptor-like receptor), is now considered part of the opioid receptor family, though it exhibits little ligand cross-affinity with MOR, DOR, and KOR (Butour, Moisand, Mazarguil, Mollereau, & Meunier, 1997). Opioid receptor effects are generally inhibitory in nature, with the receptor primarily signalling via G"/G/). Additional beta-arrestin-mediated signalling from opioid receptors has recently been identified, though the precise contribution of those signalling pathways to overall opioid receptor effects remains the subject of study (Aguila et al., 2012; Cahill et al., 2016; T. A. Johnson et al., 2016; A. A. Pradhan et al., 2016). Opioid receptors are expressed mostly in neural tissues. The three opioid receptor types are widely expressed with varied and distinct expression patterns. The functional effects of opioid receptor signalling are similarly varied. Clinical uses are largely restricted to MOR activation, with the desired analgesic ‘on-target’ effects involving MOR on neurons of sensory transmission pathways and affective processing centres. Nearly all clinically-used opioids are principally MOR agonists. MOR signalling at these and other sites (enteric nervous system, respiratory centre) is responsible for the majority of the ‘off-target’ side effects of clinically used opioid (Trang et al., 2015).
Opioid receptors were first identified in 1973 (Pert & Snyder, 1973), their coding genes identified in the early 1990s (Evans, Keith, Morrison, Magendzo, & Edwards, 1992; Kieffer, Befort, Gaveriaux-Ruff, & Hirth, 1992; Mansour, Burke, Pavlic, Akil, & Watson, 1996; J. B. Wang et al., 1993), and their crystal structures identified in 2012 (Granier et al., 2012; Manglik et al., 2012; Thompson et al., 2012; Wu et al., 2012). During this time, as specific understandings of the opioid receptors grew, a growing array of tools and techniques has become available for their study. As with numerous other targets of study throughout the biological sciences, antibodies raised to specifically bind opioid receptors became a key tool, supplanting radiolabeled ligands as the preferred method to identify opioid receptors in isolated tissues. Antibody-based techniques, however, have always been limited by the inconsistent quality of antibodies against any target. Many antibodies are limited in either their sensitivity or specificity. Obtaining high-quality antibodies against GPCRs such as opioid receptors is particularly challenging (Gendron, Cahill, Zastrow, Schiller, & Pineyro, 2016; Michel, Wieland, & Tsujimoto, 2009; Takeda et al., 2015; Vicente-Sanchez, Segura, & Pradhan, 2016). One method used to mitigate antibody quality issues has been the use of gene constructs coding for the target of interest, here an opioid receptor, in combination with an epitope tag. The most commonly used epitope tags are short, highly unique, amino acid sequences which have proven to be excellent antibody targets: HA (hemagglutinin, nine residues), myc (derived from e-Myc, ten residues), and FLAG (purpose-designed, eight residues). The major drawback of using these recombinant, epitope-tagged receptors has been that they have typically only been available in heterologous systems using immortalized cell lines. These models generally lacked the native opioid receptor promoters and many of the systems modulating opioid receptor expression and availability. It was often unclear if resulting observations of opioid receptor behavior in heterologous systems were physiologically relevant.

Questions regarding the usefulness of antibody-based techniques became particularly prominent in the study of DOR following the development of a DOR-eGFP mouse line. This line
expresses DOR fused with enhanced green fluorescent protein (eGFP) at the C-terminal of the receptor and under the control of the native DOR promoter (Scherrer et al., 2006). This was initially intended to produce fluorophore-tagged DOR which could be directly visualized by fluorescence microscopy. However, DOR expression proved too low for eGFP detection. This model is now used much like an epitope-tagged DOR with antibodies against the GFP portion of the recombinant receptor protein. Nevertheless, this has proven to be a highly productive model, if a controversial one. Early reports using the DOR-eGFP mouse line highlighted a lack of correspondence between the eGFP labelling and the purported DOR labelling of a number of commercially-available DOR antibodies (Scherrer et al., 2009). However, the initial characterization of this mouse demonstrated significant overlap with cellular labelling using DOR antibodies and DOR binding (Scherrer et al., 2006), and later work with this mouse from the same group reported contradictory findings regarding DOR expression patterns (Eric Erbs et al., 2015). Indeed, the findings of this 2009 paper were highly controversial (Chieng & Christie, 2009; Eric Erbs et al., 2015; Stockton & Devi, 2012; H.-B. Wang et al., 2010). Among other issues raised, it has been noted that eGFP is a poor choice for an epitope tag owing to its large size (26.9 kDa) and effects on receptor trafficking (H.-B. Wang et al., 2008) not seen with smaller tags.

In order to address the deficits seen in these earlier DOR tools and resolve some of the outstanding discrepancies, we and our collaborators undertook to develop a new transgenic mouse line. This mouse line was designed to include both a FLAG epitope tag on DOR and to permit the selective expression of DOR by use of a floxed Stop cassette. By inserting this gene construct immediately upstream of exon 1 of Oprd1 (the DOR gene), both design goals were accomplished in a single recombination, and expression of Oprd1 was left under the control of the native promoter and expression machinery. We undertook a program of experimental validation of this floxed-Stop, FLAG-tagged Oprd1 conditional knock-in, using genotyping, behavioural
phenotyping, and epitope immunoprobing. Simultaneously, our collaborators have been validating the line’s ligand binding, virally-induced selective expression, and nociceptive responses (Appendix B).

Behaviourally, we expected divergent phenotypes from the two construct genotypes. FLAG-DOR should act identically to WT, with functional DOR being expressed as normal; that is, DOR responsiveness should be neither augmented nor reduced. Stop-DOR should be, effectively, a full DOR knock-out and should respond as such: no DOR-induced effects.

Molecularly, our expectations were the same (DOR in FLAG-DOR the same as WT and absent in Stop-DOR) with the additional expectation that DOR in FLAG-DOR should carry the FLAG epitope tag and be detectable using anti-FLAG antibodies.

5.2 Methods

5.2.1 A floxed-Stop, FLAG-tagged Oprd1 conditional knock-in

A conditional knock-in, FLAG-tagged DOR mouse line was produced and provided by Dr. Jim Boulter. Briefly, a floxed-Stop FLAG-tag construct was inserted immediately upstream of the first coding exon of OPRD1 on a C57Bl/6J background (Figure 5.1A and Figure 5.1B). As inserted, the Stop cassette in this construct suppresses DOR expression. Animals homozygous for the Stop-DOR allele are functionally DOR knock-outs. Alternatively, the floxed Stop cassette may be excised at a cell-level by expression of Cre recombinase. In this manner, conditional knock-in animals or animal lines may be generated. For our purposes, Oprd1 Stop/Stop animals were crossed with animals expressing Cre recombinase under the control of the zona pellucida 3 (Zp3) promoter (Figure 5.1C). Zp3 is active in oocytes. As a result, the Stop cassette is excised from all cells, and the change is heritable. The FLAG-DOR allele yields normal expression of DOR under the control of its native promoter but including an N-terminal FLAG epitope tag (sequence DYKDDDDK) (Figure 5.1D).
Figure 5.1 Oprd1 gene constructs for WT, Stop-DOR, and FLAG-DOR lines.

Representational diagrams (not to scale) showing the gene constructs for each genotype and summarising the development strategy. The floxed-Stop FLAG construct was introduced immediately upstream of the wildtype (A) Oprd1 exon 1 to yield the Stop-DOR allele (B) in which DOR is not expressed. The floxed Stop cassette was heritably excised by crossing with animals homozygous for Cre recombinase under the control of the Zp3 promoter (C). This resulted in the FLAG-DOR allele from which DOR is expressed with the addition of an N-terminal FLAG tag.
5.2.2 Animals

All procedures involving animals were conducted in accordance with the National Institutes of Health’s *Guide for the Care of Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. The FLAG-DOR on C57BL/6J background line was maintained by pair breeding of Oprd1 +/FLAG heterozygous mice. The Stop-DOR on C57BL/6J background line was maintained by pair breeding of Oprd +/Stop heterozygous mice. These lines were maintained separately using the same procedures. Multiple breeding pairs were maintained in order to avoid directly consanguineous matings. Mice were backcrossed with C57BL/6J wildtype mates (The Jackson Laboratory) every third generation to counter background drift. After weening, animals were housed in single-sex groups of two to five and kept on a 12-hour light/dark cycle with *ad libitum* access to food and water. All behavioural testing was conducted during the animals’ dark phase. All experiments were conducted using adult mice of at least 8 weeks of age. Wildtype Oprd1 +/+ littermates were used as controls for FLAG-DOR and Stop-DOR lines.

5.2.3 Genotyping – DNA sample preparation

Ear punch tissue samples were collected from all animals at the time of weening. Punches were individually incubated in 100 μl DirectPCR Lysis Reagent (Tail) (Viagen Biotech) containing freshly prepared 500 μg/ml Proteinase K (New England Biolabs). Digestion was conducted at 55 °C overnight with rotation using a rotating hybridization oven. Resulting lysates were incubated at 85 °C for 45 minutes in a water bath in order to inactivate the Proteinase K. The lysates were then stored at -20 °C.

5.2.4 Genotyping – Primer sets

Two genotyping approaches were used due to the initial approach’s failure to yield useable results. This first approach, Approach A, used distinct primer sets for each possible construct allele designed to yield binary present/absent detections of wildtype, Stop-DOR, and
FLAG-DOR alleles. Approach B used a single common primer set to yield differential-length products based on the presence or absence of the construct insert. This approach also required the strict segregation of FLAG-DOR and Stop-DOR lines as it was unable to distinguish between the two. Genotyping approach A utilized a common upstream (5’) primer and specific downstream (3’) primers to detect the different construct variants – wildtype, Stop-DOR, and FLAG-DOR. The primer sequences were: upstream, 5’-TGG GTA TCC TGG TCT ACA AAG-3’ (forward); wildtype, 5’-GCG GCG CCA TGG AGC TGG TGC-3’ (reverse); STOP-DOR, 5’-GTC TGG ATC TGA CAT GGT AAG-3’ (reverse); FLAG-DOR, 5’-GCC ATG GAT TAC AAA GAT GAC-3’ (reverse). The resulting product sizes were: wildtype, 313 bp; Stop-DOR, 520 bp; FLAG-DOR, 327 bp. Genotyping approach B utilized a single common primer set: 5’-TGG CCT CCG TTT TCC GCG C-3’ (forward) and 5’-CTG GGA AAG GCG TCC GAG AGG T-3’ (reverse). The resulting product was then 150 bp from the wildtype DOR allele and 174 bp from the FLAG-inserted DOR allele, with the 24 bp difference being the size of the FLAG sequence. Notably, the 174 bp product denoted the presence of the construct insert but was ambivalent regarding the presence of the Stop cassette.

5.2.5 Genotyping – Polymerase Chain Reaction (PCR) and Agarose gel electrophoresis

Approach A required running two PCR reactions per sample: one with the wildtype primer set and a second with the appropriate construct primer set (Stop-DOR or FLAG-DOR). Approach B required only a single reaction per sample. All reactions were conducted using EmeraldAmp GT PCR Master Mix (Takara Bio USA) based on the manufacturer’s specifications. Each reaction was conducted in a total volume of 25 μl consisting of 12.5 μl EmeraldAmp, 0.5 μl DNA sample, forward and reverse primers at a final concentration of 0.2 μM each, and balance sterile distilled water. Initial PCR conditions were 98 °C for 10 seconds, 60 °C for 30 seconds, and 72 °C for 45 seconds with a repeat of 30 cycles using a MyCycler thermal cycler (Bio-Rad Laboratories).
PCR products were immediately collected on ice and separated by agarose gel electrophoresis using a Sub-Cell GT electrophoresis cell (Bio-Rad Laboratories). 100 ml gels of either 1.5% (Approach A) or 2% (Approach B) agarose were made using TBE buffer (89 mM tris pH 7.6, 89 mM boric acid, 2 mM EDTA) containing 15 μl GelGreen fluorescent DNA stain (Biotium). 20 μl of PCR product was loaded per well and run at 100 volts. A GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific) was included for reference. For Approach A, total running time was approximately 30 to 45 minutes, which was sufficient to separate residual primers and unincorporated nucleotides from the much larger PCR products. For Approach B, total running time was considerably longer, often approaching 120 minutes and utilizing the full length of the gel. This was necessary in order to achieve good separation between the 150 bp and 174 bp products. After electrophoresis, gels were imaged using a Gel Doc XR+ ultraviolet imager via QuantityOne (Bio-Rad Laboratories).

5.2.6 Drugs

(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamidine (SNC80) was purchased from Tocris and solubilized by sonication at 20 mg/ml in distilled water containing 6% v/v 1 M HCl. For in vivo use in mice, SNC80 was diluted twenty-fold to a final concentration of 1 mg/ml and filter-sterilized using a 20 μm syringe-tip filter. Morphine sulfate was prepared in saline to a final concentration of 1 mg/ml and similarly sterilized.

5.2.7 Blinding

For all behavioural experiments involving multiple drug or genotype conditions, experiments were conducted and analysed by experimenters blinded to the experimental groups.
5.2.8 Open field locomotion

Locomotor activity was assessed using 30 cm by 30 cm opaque-sided Plexiglas chambers equipped with overhead camera video tracking. Lighting in the experimental room was indirect and even and was maintained at 40 lux. Animals were allowed to habituate to the experimental room for at least 30 minutes prior to any procedures. Animals were habituated to the locomotor chambers twice on the days preceding testing. Notably, open field testing was used only as a measure of locomotion. Anxiety-like behaviour was not assessed using this test. While open field behaviour can be used to assess anxiety-like behaviour, the conditions required are distinct from those used to primarily assess locomotor activity. Testing was conducted 20 minutes after drug administration and lasted 15 minutes. These timepoints were based upon the timecourse pharmacokinetics of intraperitoneally-administered SNC80, which shows peak effects from 20 to 40 minutes after administration (Bilsky et al., 1995). Two separate testing sessions were conducted on consecutive days: the first with 10 ml/kg saline as control, and the second with SNC80. Total horizontal distance travelled was determined using Ethovision XT v8 video analysis software (Noldus Information Technology).

5.2.9 Elevated-plus maze

The elevated-plus maze (EPM) test is a measure of anxiety-like behaviour and/or risk taking. Animals are presented with a novel environment (the maze) to explore. The maze is an elevated platform with both protected (closed) sections and unprotected (open) sections. Specifically, the EPM consisted of a platform of four arms (11 inches by 3 inches each) radiating at right angles from a 3 inch by 3 inch center. The + shaped platform stood on legs elevating it 21.5 inches above the floor. Of the arms, two on opposing sides were enclosed by walls 8 inches high except for where they joined the center section. The remaining arms were open. In the EPM, animals are intended to perceive the closed arms as safe (low aversion) and the open arm as dangerous (high aversion). The sectional differentiation is a manipulation of rodents’ preferences.
for enclosed, protected spaces. A reduction in anxiety-like behaviour should manifest as increased time spent exploring the open arms. Two variant test conditions were used in optimising the test procedure. In Condition A, all surfaces of the maze were untreated opaque black polycarbonate with a smooth surface. Lighting in the room was indirect from the perimeter (reflected off the walls) and was maintained at 40 lux. Due to the maze’s enclosing walls, the closed arms were in shadow. In Condition B, all surfaces of the maze were painted matte white and the room was indirectly lit from directly above the maze and reflected off the ceiling such that the maze was evenly illuminated at 1 lux. In both conditions, animals were allowed to habituate to the experimental room for at least 30 minutes prior to any procedures. Animals were not habituated to the elevated-plus maze and were tested only once. Testing was conducted 20 minutes after drug administration and lasted 5 minutes. Overhead camera video tracking was used to record testing, and animal positions relative to the maze throughout the test period were determined using Ethovision XT v8 video analysis software (Noldus Information Technology).

5.2.10 Light/dark box

The light/dark test is also a measure of anxiety-like behaviour. Similar to the EPM, animals are presented with a novel environment to explore. The environment (the light/dark box) contains sections which are low aversion (“safe”, dark) and high aversion (“dangerous”, light). In this case, the differentiation is a manipulation of rodents’ preferences for dark spaces. The light/dark boxes were 30 cm by 30 cm opaque-sided Plexiglas chambers subdivided into three sections. One section was white-walled and open to the room at its top. One section was opaquely black-walled and closed above. The third section was a tunnel connecting the other two, grey walled and closed above. The light side was brightly illuminated at 4000 lux. Animals were allowed to habituate to the experimental room for at least 30 minutes prior to any procedures. Animals were not habituated to the light/dark box and were tested only once. Testing was conducted 20 minutes after drug administration and lasted 10 minutes. Overhead camera video
tracking was used to record testing, and animal time in the light side throughout the test period
was determined using Ethovision XT v8 video analysis software (Noldus Information
Technology).

5.2.11 Fluorescent immunohistochemistry

Immunofluorescent labelling of FLAG-tagged DOR was conducted using the high-
sensitivity Image-iT FX kit (Thermo Fisher Scientific) in conjunction with a monoclonal biotin-
conjugated mouse anti-FLAG primary antibody (Sigma, BioM2, F9291, lot SLBF5390) at
dilutions from 1:120 to 1:960 and either a 1:2000 streptavidin-conjugated Alexa 594 secondary
reagent (Thermo Fisher Scientific S11227, lot 412444) or a 1:2000 goat anti-mouse conjugated
Alexa 594 secondary antibody (Thermo Fisher Scientific A11005, lot 1219862). IHC
experiments were also tested with and without endogenous biotin blocking using an
Avidin/Biotin blocking kit (Vector Laboratories) according to the manufacturer’s directions.

Wildtype Oprd1 +/+ , Stop-DOR Oprd1 Stop/Stop, and FLAG DOR Oprd1 FLAG/FLAG
mice were sacrificed by transcardial perfusion with ice-cold 4% paraformaldehyde in 0.1 M
phosphate buffer. Brains were collected, post-fixed for 30-minutes, and cryoprotected with 30%
sucrose in 0.1 M phosphate buffer for 48 hours. Cryoprotected brains were snap-frozen and cut
into 40 μm coronal sections using a freezing stage sledge microtome (Leica). Slices were
repeatedly washed with cold tris-buffered saline washing buffer (0.1M tris, 0.9% NaCl, 0.05%
Tween 20, pH 7.4) prior to incubation with Image-iT FX Signal Enhancer. Following subsequent
washing, slices were blocked for two hours at room temperature (blocking buffer: 0.05 M tris,
0.9% NaCl, 0.05% Tween 20, 3% BSA, 0.1% cold fish skin gelatin, pH 7.4). Primary antibody
incubation proceeded overnight at 4 °C (antibody diluent: 0.05 M tris, 0.9% NaCl, 0.05% Tween
20, 1% BSA, 0.1% cold fish skin gelatin, pH 7.4). Slices were then washed and incubated with
secondary Alexa 594 conjugate for 60 minutes at 4 °C. Finally, labelled slices were washed and
mounted on Superfrost Plus microscope slides (Thermo Fisher Scientific) using ProLong Gold
antifade reagent (Thermo Fisher Scientific). The slides were kept in the dark at -20 °C until visualization using a Leica DM-LFSA epifluorescent microscope.

5.2.12 Membrane preparation

Brains of wildtype Oprd1 +/+, Stop-DOR Oprd1 Stop/Stop, and FLAG DOR Oprd1 FLAG/FLAG mice were collected fresh and snap-frozen. The olfactory bulbs of each brain were isolated and collected into individual borosilicate glass vials with 3 ml ice cold homogenization buffer (50 mM tris, 4 mM EDTA, pH 7.4) containing protease inhibitors (Pierce Protease Inhibitor Tablets, Thermo Fisher Scientific). Tissue samples were homogenized using a motorized homogenizer (Polytron). Homogenized samples were transferred to centrifuge tubes and large debris was removed by centrifugation at 1000 g for 20 minutes at 4 °C. The supernatant was collected, the pellet resuspended in 1 ml of homogenization buffer, and centrifuged again at 1000 g for 20 minutes at 4 °C. Once again, the supernatant was collected with the first supernatant. The combined supernatants were centrifuged at 16000 g for 30 minutes at 4 °C to isolate membranes from soluble proteins. The resulting supernatant was discarded and the pellet resuspended in 300 μl of membrane preparation sample buffer (50 mM tris, 0.2 mM EDTA, pH 7.4) containing protease inhibitors.

5.2.13 Immunoblotting

Immunoblotting was carried out using the Novex NuPAGE SDS-PAGE gel system (Thermo Fisher Scientific). Immunoblotting samples were made from membrane preparations. Protein concentrations of membrane preparations from wildtype, Stop-DOR, and FLAG-DOR olfactory bulbs were determined by triplicate measurement using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and equalized by dilution with 0.1M phosphate buffer as required. 6.5 parts of each sample was then combined with 2.5 parts of NuPAGE LAD Sample Buffer (4x) and 1 part of NuPAGE Reducing Agent (10x) before heating at 70 °C for 10 minutes to yield the final denatured samples for immunoblotting. Final protein concentrations were 0.95 mg/ml.
Samples were loaded, 15 μl per well, into 15-well 4-12% NuPAGE Bis-Tris mini-gels. Reference lanes were loaded with 5 μl of either PageRule Plus prestained protein ladder (Thermo Fisher Scientific) or MagicMark XP western protein standard (Thermo Fisher Scientific). Gels were run at 100 volts using an X-Cell SureLock electrophoresis mini-cell (Thermo Fisher Scientific) filled with NuPAGE MES SDS running buffer, per the manufacturer’s specifications. After separation by electrophoresis, proteins were transferred from gels to nitrocellulose membranes with pore size 0.45 μm (Thermo Fisher Scientific) using a wet transfer technique at 0.25 amps for 90 minutes. After transfer, gels were stained using PageBlue protein stain (Thermo Fisher Scientific) for verification.

Protein containing membranes were blocked overnight at 4 °C in washing buffer (0.05 M tris, 0.9% NaCl, 1 mM CaCl₂, pH 8.0) containing 5% non-fat dry milk. After blocking, membranes were probed with primary antibodies: 10 μg/ml monoclonal mouse anti-FLAG M1 (Sigma, F3040, lot SLBK1592V), 5 μg/ml monoclonal biotin-conjugated mouse anti-FLAG BioM2 (Sigma, F9291, lot SLBF5390), monoclonal mouse anti-GFAP (Millipore, MAB360, lot 2271174), 1:2000 rabbit anti-beta actin (Abcam, ab8227, lot GR245058-1), rabbit anti-DOR (MyBioSource, MBS316175, lot 1B03801, directed against DOR residues 360–372), 1:1000 monoclonal mouse anti-tyrosine hydroxylase (Millipore, MAB318, lot 2585892). Primary antibody incubation was carried out at 4 °C overnight with shaking. For some high sensitivity probing, primary antibody incubation was 60 to 90 minutes at room temperature, as described below. Following primary antibody incubation and washing, membranes were incubated with appropriate secondary antibodies: either horseradish peroxidase-conjugated goat anti-mouse (Thermo Fisher Scientific, G-21040, lot 1120116) or horseradish peroxidase-conjugated goat anti-rabbit (Thermo Fisher Scientific, G-21234, lot 1148960) for 60 minutes at room temperature. Finally, chemiluminescent substrate was added: one of Amersham ECL (GE Healthcare), LI-COR WesternSure ECL (LI-COR Biosciences), or SuperSignal West Dura (Thermo Fisher
Scientific) per manufacturer’s guidelines and as described below. Resulting chemiluminescent signals were recorded by a LI-COR Odyssey Fc digital CCD imaging system using a 45-minute integration exposure.

5.3 Results

5.3.1 Stop-DOR and FLAG-DOR mouse lines

Stop-DOR (Oprd1 Stop/Stop, Oprd1 +/Stop, Oprd1 +/+ ) and FLAG-DOR (Oprd1 FLAG/FLAG, Oprd1 +/FLAG, Oprd1 +/+ ) mouse lines were generated and maintained as described above. From each line, construct homozygous mice (Oprd1 Stop/Stop and Oprd1 FLAG/FLAG) were used for the experiments discussed below, with wildtype homozygous littermates (Oprd +/+ ) from each line serving as controls. Construct heterozygous littermates were used for breeding. No reported experimental results involved construct heterozygous animals.

As both mouse lines were propagated from construct heterozygous breeding pairs, it was necessary to genotype each animal. As described above, two genotyping approaches were used. Approach A used distinct primer sets for each possible construct allele (WT, Stop-DOR, FLAG-DOR). This required primers designed to anneal to unique portions of the constructs themselves. Unfortunately, the options for primer design were constrained by the substantial overlap in sequence between the full construct-containing Stop-DOR variant and the Stop cassette-excised FLAG-DOR variant. The resulting primer sets had notably non-optimal annealing temperatures, with excessive differences within pairs. While a collaborating group reported successful genotyping using this approach, we were unable to obtain clear or consistent amplification of any of the possible construct alleles (Figure 5.2A). These results persisted despite exhaustive attempts to optimize reaction conditions, including the modification of the primer sets to improve annealing temperature differences.

Genotyping Approach B used a single common primer set which yielded different-length products depending on whether or not the construct insert was present. The primer set spanned
Figure 5.2  Mouse line genotyping.

Typical results from genotyping Approach A (A) and Approach B (B). For Approach A, the image has been purposefully overexposed to demonstrate the lack of effective target amplification. For Approach B, results are shown for the FLAG-DOR mouse line; results for the Stop-DOR mouse line would be equivalent.
the start of the coding region of Oprd1 exon 1. The presence of the construct insert resulted in the addition of the FLAG-tag coding sequence to the amplified span. This approach allowed for considerably more flexibility in primer design and, hence, a more effective primer set. We were able to readily obtain consistent amplification of the target sequences, despite using the same reagents, equipment, and techniques for both approaches. Notably, wildtype C57Bl/6J samples from mice obtained directly from the supplier yielded the expected results under Approach B but not Approach A (data not shown). Further, the differential-length products were clearly discernable following electrophoretic separation (Figure 5.2B). As a result, all genotyping of Stop-DOR and FLAG-DOR lines was conducted using Approach B. These results demonstrate that the target sequences overlapping the beginning of the coding region of Oprd1 exon 1 are present (primers anneal and produce the expected product), and are consistent with insertion of the FLAG tag coding sequence in the anticipated location (construct product lengthened as expected).

5.3.2 DOR agonist-induced hyperlocomotion is intact in FLAG-DOR mice and absent in Stop-DOR mice

Systemic DOR agonists are well-recognised to induce hyperlocomotion via a DOR-dependent mechanism (Vicente-Sanchez et al., 2016). We assessed the hyperlocomotive effect of SNC80, a small molecule DOR agonist. Control experiments using saline administration revealed no difference in baseline locomotor activity between genotypes (data not shown). Initial SNC80 locomotor testing showed no difference between WT, FLAG-DOR, or Stop-DOR animals in distance travelled following 10 mg/kg intraperitoneal SNC80 (Figure 5.3A; one-way ANOVA $F(2,28) = 0.715, p = 0.498, N = 7-16$ per group). To determine if inter-animal differences obscured possible drug effects, locomotor activity following SNC80 was normalised to each animal’s activity following control saline administration. Again, no significant difference was
Initial SNC80 hyperlocomotion experiments showed no difference between genotypes in either absolute distance travelled (A; one-way ANOVA $F(2,28) = 0.715, p = 0.498, N = 7-16$ per group) or in saline-normalised distance travelled (B; one-way ANOVA $F(2,28) = 1.733, p = 0.196, N = 7-16$ per group) following 10 mg/kg I.P. SNC80. These negative findings appeared attributable to an age-related decline in SNC80-induced hyperlocomotion among WT and FLAG-DOR, but not Stop-DOR, animals (C; simple linear regression of distance on age: FLAG-DOR ($F(1,6) = 6.561, p = 0.043, R^2 = 0.522$); WT ($F(1,14) = 7.073, p = 0.019, R^2 = 0.336$); Stop-DOR ($F(1,5) = 0.529, p = 0.500, R^2 = 0.096$)). Overlays show mean ± SEM.
observed between groups (Figure 5.3B; one-way ANOVA $F(2,28) = 1.733, p = 0.196, N = 7-16$ per group), though a moderate increase in activity over saline baselines appeared to be present in all groups. As a positive control, a subset of the same animals was also tested following morphine, a small molecule MOR agonist which also induces hyperlocomotion. In this case, robust hyperlocomotion was observed in all genotypes (data not shown). Subsequent analysis of SNC80 locomotor testing data revealed a pronounced age-related drop-off in SNC80 hyperlocomotive effects among WT and FLAG-DOR animals (Figure 5.3C; simple linear regression of distance on age: FLAG-DOR ($F(1,6) = 6.561, p = 0.043, R^2 = 0.522$); WT ($F(1,14) = 7.073, p = 0.019, R^2 = 0.336$); Stop-DOR ($F(1,5) = 0.529, p = 0.500, R^2 = 0.096$)). This drop-off appeared to begin at seven months’ age and to continue progressively. As an incidental result of colony management decisions (a small number of breeding pairs) and experimental planning (to test all animals at one time), most of the animals tested were above this age (due to the need to breed a sufficient number of animals).

We repeated the same experiments using animals less than seven months’ age. We also adjusted the dose of SNC80 from 10 mg/kg to a fixed 0.25mg dose to assess whether the animals’ masses affected SNC80’s hyperlocomotive effects (essentially a proxy measure of whether SNC80’s volume of distribution varies with overall animal size). Animals in this cohort had a mean mass of 29.9 g (range 21.7 g to 40.0 g). In this case, we observed clear intergroup differences in locomotor activity (Figure 5.4A). In terms of raw distance travelled, FLAG-DOR mice showed significantly greater SNC80-induced hyperlocomotion when compared to Stop-DOR mice (one-way ANOVA $F(2,15) = 4.416, p = 0.031$; Tukey post hoc $p < 0.05$; $N = 4-8$ per group). No significant difference was noted between WT and either other group. However, when SNC80-induced hyperlocomotion was normalised to saline baselines on a per animal basis, both WT and FLAG-DOR groups showed significantly greater hyperlocomotion than Stop-DOR mice (Figure 5.4B; one-way ANOVA $F(2,15) = 11.90, p = 0.001$; Tukey post hoc $p < 0.05$ for both;
SNC80 hyperlocomotion experiments using younger animals (less than 7 months’ age) showed FLAG-DOR mice to experience significantly more SNC80-induced hyperlocomotion than Stop-DOR mice in terms of absolute distance travelled (A) following 0.25 mg I.P. SNC80. When expressed as saline-normalised distance travelled, both WT and FLAG-DOR groups experienced greater SNC80-induced hyperlocomotion than Stop-DOR (B). The hyperlocomotive effects of this fixed SNC80 dose varied negatively with animal mass in WT and FLAG-DOR, but not Stop-DOR, animals (C; simple linear regression of distance on weight FLAG-DOR ($F(1,6) = 5.108, p = 0.065, R^2 = 0.460, \text{slope} = -4.351 \pm 1.925$); WT ($F(1,4) = 41.64, p = 0.003, R^2 = 0.912, \text{slope} = -7.840 \pm 1.215$); Stop-DOR ($F(1,2) = 3.563, p =$)
0.200, $R^2 = 0.641$, slope $2.595 \pm 1.375$). This finding confirmed both the phenotypic differences between the groups and the commonly understood whole-animal volume of distribution of SNC80. Overlays show mean $\pm$ SEM. * $p < 0.05$
Notably, in this experiment, Stop-DOR animals did not show any SNC80-induced increase in locomotor activity over their saline baselines. Further, hyperlocomotive effects for WT and FLAG-DOR animals, but not Stop-DOR, appeared to decline with increasing animal weight (Figure 5.4C). Simple linear regressions of distance on weight (FLAG-DOR $(F(1,6) = 5.108, p = 0.065, R^2 = 0.460)$; WT $(F(1,4) = 41.64, p = 0.003, R^2 = 0.912)$; Stop-DOR $(F(1,2) = 3.563, p = 0.200, R^2 = 0.641)$) yielded slopes of $-4.351 \pm 1.925$ (FLAG-DOR), $-7.840 \pm 1.215$ (WT), and $2.595 \pm 1.375$ (Stop-DOR). This provides evidence that SNC80’s effects vary with animal weight (that is, SNC80’s volume of distribution is dependent on the overall size of the animal). Interestingly, there did still appear to be age-related reductions in SNC80-induced hyperlocomotion among the WT and FLAG-DOR animals, though these reductions were not large enough to obscure intergroup differences and any effects would have been confounded with weight-related effects (data not shown). Overall, there was good correspondence between WT and FLAG-DOR responses to SNC80, both in terms of locomotor activity and the variance of those effects by weight, and Stop-DOR responses were consistent with the expectations for animals lacking functional DOR.

### 5.3.3 DOR agonist-induced anxiolysis is intact in FLAG-DOR mice and absent in Stop-DOR mice

DOR agonists are also known to be anxiolytic (Perrine, Hoshaw, & Unterwald, 2006), and DOR knock-out is known to be anxiogenic (Filliol et al., 2000). We assessed the effects of 10 mg/kg intraperitoneal SNC80 in WT, FLAG-DOR, and Stop-DOR mice on anxiety-like behaviour using the elevated- plus maze (EPM). In the EPM, reduced anxiety manifests as animals spending less time in the closed arms and more time exploring the open arms. We tracked animals’ locations on the maze following SNC80 administration. We expected WT and FLAG-DOR genotype mice to show reduced anxiety-like behaviour when administered SNC80 compared to Stop-DOR genotype mice.
Figure 5.5  Overly aversive elevated-plus maze conditions obscure SNC80's anxiolytic effects.

Under initial testing conditions, there was no difference between genotypes in the anxiolytic effects of 10 mg/kg I.P. SNC80 as measured by cumulative time spend on the closed arms of the elevated plus maze (A). This negative result was ascribed to overly aversive testing conditions based on the lack of difference between WT animals receiving either saline or SNC80 (B). Test conditions were altered to reduce overall aversion so that animals spent substantial time on both open and closed arms (C, closed arm time shown). Overlays show mean ± SEM.
Using the revised elevated plus maze conditions, no significant difference in closed arm time was observed between genotypes (A) due to a high rate of animals falling off the maze. Using falls as an alternative outcome measure, WT and FLAG-DOR animals showed significantly less anxiety-like behaviour following 10 mg/kg I.P. SNC80 (B). No difference in anxiety-like behaviour following SNC80, as measured by light side time, was observed using the Light/Dark test (C). Overlays show mean ± SEM. * $p < 0.05$
In our initial testing, there was no difference between WT, FLAG-DOR, or Stop-DOR closed arm times (Figure 5.5A; one-way ANOVA $F(2,21) = 0.822, p = 0.453, N = 8$ per group). In fact, all animals spent nearly all of the test time within the closed arms. To assess whether any SNC80 effects could be observed, we conducted EPM testing on an additional group of WT animals administered saline. There was no difference in closed arm times between this saline WT group and the earlier SNC80 WT group (Figure 5.5B; $t(14) = 1.109, p = 0.286$). This strongly suggested that the initial test conditions (Condition A) were so aversive as to obscure any SNC80 effects at this SNC80 dose.

The maze and accompanying test conditions were revised to be less aversive (Condition B), with the goal of obtaining lower closed arm times. EPM Condition B was assessed using naïve animals, which showed substantially reduced closed arm times (Figure 5.5C). Testing of SNC80-induced anxiolysis in WT, FLAG-DOR, and Stop-DOR animals was repeated using EPM Condition B. Post-SNC80 closed arm times appeared, on average, lower among WT and FLAG-DOR animals compared to Stop-DOR. However, there was no statistically significant difference between the groups owing to the extreme variability in closed arm times (Figure 5.6A; one-way ANOVA $F(2,27) = 2.394, p = 0.110, N = 9-11$ per group). Rather than indicating a lack of SNC80 effects, this variability seemed to be caused by excessive anxiolysis: animals became so willing to explore the open arms, that they frequently fell off of the maze entirely at various times during the testing period. In fact, by considering falling off the EPM to itself be a measure of anxiolytic effect, there was a clear, statistically significant difference between groups ($\chi^2(2, N = 30) = 6.228, p = 0.044$). WT and FLAG-DOR animals experienced greater SNC80-induced anxiolysis (Figure 5.6B).

SNC80 anxiolytic effects were also tested using the Light-Dark test, in which lower anxiety-like behaviour maps to increased time in the illuminated, light side of the test chamber. WT, FLAG-DOR, and Stop-DOR animals were again tested following intraperitoneal
administration of 10 mg/kg SNC80. Though *prima facie* suggestive, the results do not show any significant difference between groups (Figure 5.6C, one-way ANOVA F(2,27) = 2.473, p = 0.103, N = 9-11 per group).

5.3.4 The FLAG-DOR epitope tag is not detectable by fluorescent immunohistochemistry

Immunohistochemical detection of the FLAG tag epitope was attempted using two different, well-validated anti-FLAG antibodies in fixed brain slices from FLAG-DOR animals. The FLAG tag was not detectable, despite the use of highly sensitive reagent systems and considerable protocol optimisation. A variety of sections, which included several brain structures established to have abundant DOR expression, were probed in each tested condition. Despite these efforts, no positive labelling was observed.

5.3.5 The FLAG-DOR epitope tag is not detectable by immunoblotting

In an attempt to detect the FLAG tag epitope using a more sensitive technique, immunoblotting was carried out using membrane preparations from the isolated olfactory bulbs of FLAG-DOR and Stop-DOR animals. The olfactory bulb contains some of the densest DOR expression of any brain structure. The resulting membrane preparations would be expected to be DOR- (and, hence, FLAG-) rich.

As with the immunohistochemical experiments, two anti-FLAG antibodies were used: Sigma’s M1 and M2. Both are extensively well-validated (Einhauer & Jungbauer, 2001; L. Zhang, Hernan, & Brizzard, 2001). Initial immunoblots were probed with M1 at both 5 μg/ml and 10 μg/ml and with M2 at 5 μg/ml, per the manufacturer’s specifications. Chemiluminescent detection via standard-sensitivity Amersham ECL seemed to show positively-labelled bands (Figure 5.7A, first column). However, the labelling detected was exceedingly faint and appeared potentially non-specific, given possible bands in the Stop-DOR samples.

In order to make a more definitive detection, immunoblotting was repeated using the same conditions, but chemiluminescent detection was performed with high-sensitivity ECL (LI-
Figure 5.7  FLAG epitope tags were not detectable by immunoblotting.

Repeated attempts by immunoblotting failed to detect the FLAG tag in olfactory bulb membrane preparations from FLAG-DOR animals (A). Different anti-FLAG antibodies or antibody concentrations (amount per ml diluent) are shown in the rows; different chemiluminescent substrate reagents are shown in the columns. Positive controls were unambiguously detected as expected (B). All immunoblots performed had the same lane allocations (C).
COR WesternSure). This did not detect any labelling in the sample lanes (Figure 5.7A, middle column). A subsequent repetition using very-high-sensitivity ECL (SuperSignal West Dura) also failed to detect the FLAG tag (Figure 5.7A, right column).

Throughout this process, positive controls were readily detectable on membranes after they had been probed for FLAG. β-arrestin, a common control target, was easily identified using this protocol, as were GFAP and tyrosine hydroxylase (Figure 5.7B).

5.4 Discussion

In this study, we used genotyping, behavioural phenotyping, and epitope immunoprobing to undertake a program of experimental validation of a novel floxed-Stop, FLAG-tagged Oprd1 conditional knock-in mouse model. In our findings we have demonstrated, via multiple behavioural modalities, the functional presence of DOR in the FLAG-DOR line, in which the floxed Stop cassette has been heritably excised. We also demonstrated the functional absence of DOR in the Stop-DOR line, in which the floxed Stop cassette is intact. In the process of conducting these behavioural validations, we also identified and resolved methodological limitations in both locomotor and anxiety-like behavioural assays; most notably, this contributes the novel finding that the hyperlocomotive effects of the DOR agonist SNC80 are subject to an age-dependent drop-off in mice. Similarly, we also identified limitations in genotyping strategies. Of concern, our molecular validations failed to detect the presence of the FLAG tag in tissue from the FLAG-DOR line. Together, these findings indicate that the mouse model we have co-developed is, in practice, merely a simple conditional knock-in.

Successful genotyping of the DOR construct mouse lines was more challenging than anticipated due to the failure of Approach A and its variant-specific primer sets to yield amplification products. We suspect that the failure of this strategy stems from constraints placed upon primer design by the highly defined and relatively short genomic sequences which were distinct between DOR construct variants. While this may be interpreted as object lesson on the
problems caused by primers with divergent annealing temperatures, it is worth considering that Approach A was designed by the collaborating group which was responsible for the initial design and creation of the DOR construct mouse model. Additionally, another collaborating group was successful in genotyping with Approach A. It is, therefore, somewhat puzzling that we were unable to obtain any useable results with Approach A while seeing, frankly, excellent results with Approach B. In seeking to optimise our application of Approach A, we went so far as to use the same reagents (in some cases literally the same vial) and techniques as our collaborators who reported that approach to work, and we attempted extensive optimization of reaction conditions. The most salient remaining variable we are aware of is equipment: we used different thermocyclers. In addition to being the only apparent variable, thermocycler choice is particularly suspicious because ours was a relatively dated unit, while our collaborators had recently replaced theirs with a new unit. Exactly what effect thermocycler choice may have had is unclear. Being an essentially solid state device and having reported no errors, it seems unlikely that our thermocycler was grossly malfunctioning, especially given our later success with it. Vendor marketing materials would suggest that the newer unit may achieve better temperature control and faster transitions. Though much of such assertions is likely puffery, it is possible that amplification procedures reliant upon less-than-ideal primer sets are noticeably affected by thermocycler choice. Indeed, there are literature reports of amplification variability between thermocyclers and even between different locations within the same thermocycler block (Q. He, Viljanen, & Mertsola, 1994; MacPherson, Eckstein, Scoles, & Gajadhar, 1993; Saunders, Dukes, Parkes, & Cornett, 2001). This raises the broader possibility that conflicting findings from or failures to reproduce PCR-based experiments may be explained by differences in equipment. That minor, but real, differences in experimental apparatus may lead to differences in results is not, in the abstract, surprising. That such differences may persist among products as apparently commodified and interchangeable as thermocyclers, however, is interesting.
As expected, SNC80 induced hyperlocomotion among WT and FLAG-DOR animals, but not Stop-DOR animals. This is consistent with DOR being expressed and functional in WT and FLAG-DOR, but not Stop-DOR, lines. In the process of reaching this finding, we also established that SNC80’s hyperlocomotor effects are weight and age dependent. Weight dependence is unsurprising and simply indicates that SNC80’s volume of distribution varies with the overall size (and, thus, mass) of the animal. We tested this aspect of SNC80’s effects as a mostly incidental addition to investigating the age-dependency identified in our initial locomotor testing. The age-related drop-off in hyperlocomotive effects is the more interesting of our accidental findings. Pain responses are known to change with age (Hess, Joseph, & Roth, 1981; Yezierski, 2012), the behaviours of the opioid system are known to undergo changes during development (Hodgson, Hofford, Wellman, & Eitan, 2009; Terranova & Laviola, 2001), and an age-related decline in opioid system function has been described (Hamm & Knisely, 1985; Hess et al., 1981). Most relevantly, a progressive age-related dysfunction of cortical DOR has been implicated in age-related increases in anxiety- and depressive-like behaviours in rodent models (Narita et al., 2006). The age-related reduction in DOR-induced hyperlocomotion we observed here fits with the overall trend towards reduced opioidergic function with age. Literature reports from the basic sciences of age effects on opioidergic function are, however, scattered and typically focused on specific effects. As such, the exact extent of and mechanisms for these changes in the opioid system are unclear. Clinical reports, however, are seemingly contradictory: age has been reported to accompany increased MOR binding (Zubieta, Dannals, & Frost, 1999), reduced tolerance as measured by dose escalation (Buntin-Mushock, Phillip, Moriyama, & Palmer, 2005), greater opioid analgesic sensitivity (Macintyre & Jarvis, 1996; Viganó, Bruera, & Suarez-Almazor, 1998; Woodhouse & Mather, 1997), and greater opioid side-effect sensitivity (Cepeda et al., 2003). It is possible that the apparent divergence of these literature reports is instead reflective of a split between DOR and MOR age-related effects. Because clinically-used opioid drugs are
overwhelmingly MOR agonists, the clinical literature reflects changes in MOR function. This raises the possibility that age-related declines in DOR function are offset by augmented MOR function. This is in-line with related literature findings of a degree of interdependence between MOR and DOR functionality (Walwyn et al., 2009; X. Zhang & Bao, 2012). This would also explain our observation of continued, robust morphine-induced hyperlocomotion in the aged animals showing reduced SNC80-induced effects.

At first glance, interpretation of measures of anxiety-like behaviour in the phenotypes might seem confounded because, as discussed, DOR agonism is anxiolytic (Perrine et al., 2006) and DOR ablation is anxiogenic (Filliol et al., 2000). One would therefore expect Stop-DOR animals to show greater anxiety-like behaviour at baseline than the other genotypes. This effect would then be overlaid upon the SNC80-induced anxiolysis present only in the genotypes expressing functional DOR (WT and FLAG-DOR). Fortunately for our purposes, the combined effects of DOR agonism and ablation are effectively consilient in this case. The expected phenotypic differences between the genotypes in question should be amplified when considering SNC80’s effects on anxiety-like behaviour.

Indeed, as with locomotor results, testing of SNC80-induced anxiolysis suggests expressed, functional DOR in WT and FLAG-DOR, but not Stop-DOR, animals. The sensitivity of tests of anxiety-like behaviour to the overall averseness of the test has been well established; these tests, after all, essentially probe the balance between animals’ drive to explore an environment and that environment’s aversiveness (Carobrez & Bertoglio, 2005; Martínez, Cardenas, Lamprea, & Morato, 2002). What is more surprising is the great variety of test conditions and outcome measures advocated or reported by users of these tests (Dawson & Tricklebank, 1995; Fernandes & File, 1996; Hogg, 1996; Komada, Takao, & Miyakawa, 2008; Pellow, Chopin, File, & Briley, 1985; Walf & Frye, 2007). Especially notable is that, in addition to variability due to the test conditions themselves, these reports also highlight effects of nearly
all other aspects of animal use ranging from vendor and strain, to housing conditions and prior handling. It should not come as a surprise that testing of the highly integrative phenomena such as anxiety (and related affect) could be sensitive to a wide range of inputs. That is, anxiety as a component of overall affect is informed the breadth of experience and, so, is responsive to that same breadth of experience. What emerges from the literature, then, is less a unified prescriptive formula for these tests but rather a catalogue of variables which may be manipulated so as to optimise the tests to the particular circumstances at hand. Two groups conducting the same test in slightly different models will likely require different test conditions. These literature reports and our findings here also suggest that these tests effectively have a limited dynamic range: if conditions are too aversive, all animals will show the same behaviour (a response to aversiveness), but if conditions are insufficiently aversive, there will also be no differentiation and all animals will show the same behaviour (a behaviour in the absence of aversiveness). Because of the nature of the species being tested and the strong effect of aversion on anxiety-like behaviour, the former limit is more likely to be observed than the latter. This complication to the testing of anxiety-like behaviour reinforces the need for the use of positive controls. Simply because a particular test paradigm has worked in the past does not mean it will be appropriate for the new questions being asked with it, even when used by the same lab group with a similar experimental model.

The failure of FLAG epitope detection by either immunohistochemistry or immunoblotting raises the obvious question of what happens to the tag. We know that the construct sequence including both the Stop cassette and FLAG tag was inserted where expected. The Stop cassette is functional as anticipated and an additional DNA sequence corresponding in length to that of the FLAG epitope is inserted in the intended region (as was detected by genotyping). It is possible that some error in gene targeting inserted an incorrect sequence. While seemingly unlikely, it would be prudent to directly verify, by sequencing, the code around the 5’
start of Oprd1 exon 1. Presuming that the epitope coding sequence was introduced as expected, our findings that it was excised somewhere between DNA and the final, mature polypeptide. mRNA PCR would be able to ascertain whether the FLAG-coding sequence remained intact after transcription. It seems most likely that the FLAG tag would be translated intact and subsequently excised. DOR is subject to considerable post-translational maturation control and modification (P. Y. Law & Loh, 1999; Petäjä-Repo et al., 2006). It is conceivable that during these processes, the FLAG tag was removed. Puzzlingly, however, there are no reports of epitope tag excision from DOR. DOR has been successfully expressed with N-terminal HA and myc tags (H.-B. Wang et al., 2008) as well as both N- and C-terminal (separately) GFP (Scherrer et al., 2006; H.-B. Wang et al., 2008). Albeit, only the C-terminal eGFP has been used in a whole-animal model. It is also unclear what mechanism might remove the FLAG tag, though some action of exopeptidases would be suspected.

It should also be noted that a transgenic mouse model expressing MOR with an N-terminal FLAG tag has been developed and is both functional (Arttamangkul et al., 2008) and commercially available (Jackson B6.Cg-Tg(Th-Oprm1)4Jtw/J). This mouse model is, however, substantially different from that investigated here. The FLAG-MOR sequence is not a manipulation of the existing native Oprm1 gene, but rather an entirely novel expression construct. It uses the tyrosine hydroxylase promotor upstream of a modified influenza hemagglutinin signal peptide sequence followed by FLAG and MOR sequences obtained from cDNA and containing a bovine growth hormone poly-A sequence. Mice carrying this gene construct were then crossed with Oprm1 -/- MOR knockouts in order to remove natively-expressed MOR. The authors particularly note that the hemagglutinin signal peptide is required for trafficking of the nascent receptor to the endoplasmic reticulum. And while essential, that signal peptide is cleaved in the process of receptor maturation. Given the substantial exogenous modifications and optimisations
made to this FLAG-MOR mouse model, it is difficult to postulate what specifically accounts for the viability of that FLAG tag.

Ultimately, the fate of the FLAG tag is likely an academic question. That the tag is effectively absent is the practical conclusion. At the same time, the floxed-Stop component of the construct appears effective: as we have demonstrated, functional DOR is present when the Stop cassette is excised (FLAG-DOR) and absent when the cassette is unaltered (Stop-DOR). The lack of the tag is merely limiting for the overall value of the model. A conditional DOR knock-in model remains novel and useful, especially for questions of sufficiency not addressable using the existing conditional DOR knock-out (Gaveriaux-Ruff et al., 2011). Without the tag, however, many of the questions and controversies which drove the development of this mouse model will remain unanswerable.
Chapter 6

General Discussion

6.1 Principal Findings

It has been well established that it is possible for MOR and DOR to heteromerize as M/DOR. This conclusion had, however, been limited by the nature of the findings supporting it: because of the nature of M/DOR as a subject of research, a preponderance of early evidence for its 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 favourability for the detection of OR heteromers and a wealth of approaches to intricately dissect their functionality. A great deal of information about M/DOR has been gained using these models. Their deficit lay in the uncertain physiological relevance of those precisely engineered conditions. That is, ORs are typically expressed in limited quantities in neuronal tissue with each OR type under tight and differential translational and trafficking control. Meanwhile, these models often expressed these receptors in very large quantities (e.g. using a CMV promoter) 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 behaviours occur in normal, physiological systems was a matter of some debate. The global aim of this thesis was to address the physiological relevance gap in our understanding of M/DOR heteromers. We hypothesized that M/DOR heteromers are a real, distinct, and physiologically-relevant species of OR. Further, based upon findings from studies of DOR in which prolonged MOR activation led to the translocation of DOR from intracellular to cell-surface locations (Cahill, Morinville, et al., 2001; E. Erbs et al., 2016; Morinville et al., 2003) and concomitant DOR antagonism was found to reduce MOR agonist tolerance (Abdelhamid,
Sultana, Portoghese, & Takemori, 1991; Kest, Lee, McLemore, & Inturrisi, 1996; Zhu et al., 1999), we hypothesized that M/DOR heteromer formation is induced by prolonged MOR activation. We tested both the existence and functionality of M/DOR by developing and implementing models and techniques new in the study of OR heteromers which allowed us to address M/DOR in physiologically relevant conditions.

Using novel M/DOR-selective antibodies, we directly identified the heteromer in DRG neurons, brain regions, and brainstem regions. We demonstrated increased M/DOR abundance in these various regions following prolonged morphine treatment. Both the direct identifications and morphine-induced increases in abundance were likewise also demonstrated using, separately, MOR-DOR immunofluorescent colocalization and heterologous expression systems using the same antibodies and methodologies. This contributes convergent confirmation of the validity of these techniques and the myriad findings made using them in the study of OR heteromers. Indeed, we went on to demonstrate that these M/DOR-selective antibodies blocked OR binding and signalling. The use of antibodies to sterically hinder ligand binding is a common technique, and here it demonstrated the functionality of M/DOR.

We refined our technique of quantitative immunofluorescent colocalizational analysis of DRG neurons, as described in Chapter 4, and applied it to the question of M/DOR trafficking. MOR and DOR are understood, as ‘class A’ and ‘class B’ GPCRs respectively (Drake 2006) to undergo different post-internalization trafficking: MOR is recycled to the cell surface, while DOR is trafficked to lysosomal degradation. This presented an opportunity to examine the functional consequences of M/DOR formation by assessing the changes in both constitutive and agonist-induced post-internalization trafficking of DOR. We used prolonged morphine exposure to induce M/DOR formation and functional availability and compared DOR post-internalisation trafficking between low-M/DOR and high-M/DOR abundance states. Initial expectation was either that DOR trafficking would remain lysosomal (indicating either heteromer dissolution or DOR-direction of...
M/DOR trafficking) or that DOR would shift to recycling (indicating MOR-direction of M/DOR trafficking). Our findings suggested M/DOR trafficking to, in fact, be intermediate between strictly DOR-driven or MOR-driven paths. We further demonstrated biased ligand-directed trafficking of DOR which included recycling. Together, our findings suggested a conceptualization of post-internalisation trafficking which was probabilistic rather than proscriptive. That is, MOR has a higher probability of recycling and DOR has higher probability of degrading. Given this model of GPCR trafficking, it is sensible that M/DOR would have behaviour intermediate to and distinct from that of its constituents. These findings further reinforce the developing understanding of M/DOR as, effectively, a distinct OR species rather than merely as a coincident assemblage of MOR and DOR functioning monomerically.

It is also worth recognition that the effects upon DOR trafficking we observed following prolonged morphine treatment continue to provide ever greater evidence for the physiological relevance of M/DOR. We demonstrated both a shift to more MOR-like trafficking following DOR agonist-induced internalization and a novel responsiveness of DOR to a MOR agonist (presumably acting at M/DOR) which could be abolished by co-treatment with a DOR antagonist. These findings argue for both M/DOR existence and distinct functionality in endogenous tissues.

Finally, we aimed to address the shortcomings of methods reliant on DOR antibodies, which have been the subject of considerable controversy. By developing a floxed-Stop, Flag-tagged Oprd1 knock-in mouse model, we hoped to develop a powerful, flexible, and physiologically-relevant tool for the study of ORs. Key in the design of this mouse model was a maintenance of expression control of the tag-DOR chimera by endogenous DOR mechanisms and the use of a small, minimally disruptive epitope tag (H.-B. Wang et al., 2008). Successful development of this mouse model would have permitted future development of, and crossing with, a corresponding small-epitope tagged MOR mouse model to permit direct dual-tag experimentation with M/DOR in a maximally endogenous model. Unfortunately, despite our
validation experiments demonstrating functionally present and wildtype-equivalent DOR in the full knock-in (FLAG-DOR) line and functional DOR absence in the Stop-DOR line (effectively a full knock-out), we were unable to detect the FLAG tag. These findings and our genotyping results indicate that the gene construct and targeting functioned as intended but that the FLAG tag was excised or suppressed at some point. This effectively renders the mouse model a floxed-Stop Oprd1 knock-in. While this remains a useful tool, it is disappointing with regard to our original intentions and will be of limited value for work addressing OR heteromerization; direct molecular identification of receptors is far more useful in the investigation of heteromers than selective expression.

6.2 Implications

In this thesis, we provide concordant evidence of the existence and functionality of M/DOR heteromers in endogenous tissues. Through the refinement and execution of physiologically-relevant experimental tools, we contribute to the advancement of OR heteromer understanding beyond the confines of earlier, more contrived model systems while also reinforcing and complementing those preceding findings. From our and others’ work, it has become abundantly clear that OR heteromerization is a real and relevant phenomenon and that M/DOR is a real product of that process. In the process of our work, the attention has shifted from the mere existence of heteromers towards a more determined effort to understand the processes by which they are formed and regulated, as well as their behaviour as receptors. We contributed to the latter question by addressing M/DOR post-internalization trafficking, and, as discussed in Chapters 1, 2, and 3, our findings are in concordance with other literature reports: OR heteromer functionality is best conceptualized in terms of a distinct OR species. This mirrors, and may explain, earlier understandings of OR pharmacological subtype diversity – that is, at least eight subtypes from the three classical OR types.
Understanding OR heteromers as distinct OR species naturally raises the prospect of OR heteromers as therapeutic targets. The discussion passages of nearly all of 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 only target for effective opioid analgesia with acceptable adverse effects. While actions on DOR and KOR can produce analgesia, no ligand has yet been identified which can do so safely. (Tested DOR agonists have produced seizure effects (Chung et al., 2015) and KOR agonists are psychoactive (Babu, McCurdy, & Boyer, 2008)). A reliance on MOR agonism carries with it adverse effects due to the same MOR agonism. 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 OR heteromers as distinct targets may offer alternatives for opioid analgesia, but considerable work remains to be done in advancing OR heteromer understandings to the point of realizing translational potentials.

At the same time, a particularly promising aspect of M/DOR behaviour for its therapeutic usefulness deserves special attention: control of M/DOR formation. As we repeatedly observed in this thesis, M/DOR formation and functional availability is complexly regulated and responsive to cellular conditions. Under basal conditions, little M/DOR appears to be functionally available at neuronal cell surfaces. Rather, specific conditions induce the regulated formation and surface presentation of the heteromer. We have demonstrated prolonged MOR activation as being one such state. Its clinical analogue is the opioid tolerant state observed as a result of long-term opioid therapy. The formation and surface trafficking of a distinct OR species (M/DOR) may act as a cellular response to this state, and insofar as available MOR are replaced with M/DOR, this response may constitute a mechanism of MOR tolerance. That is, the cell actively regulates available OR complements in response to exogenous signals. While mechanistically fascinating,
this would also seem to strengthen the case for M/DOR as a therapeutic target. Specifically, a
target which becomes available in precisely those circumstances in which MOR analgesia is most
challenged.

Successful pharmacological targeting of M/DOR will require a focused medicinal
chemistry program. Early efforts towards this end have been reported, including novel ligands,
eexisting ligands newly characterized in terms of M/DOR, and various ligand combinations (as
discussed in Chapter 1). Ultimately, entirely novel ligands hold the greatest promise, especially
given the recent identifications of OR crystal structures. While no crystallography of OR
heteromers has been reported, the monomeric structures should allow substantial refinement of
existing interaction models. Opioid medicinal chemistry has already been greatly advanced by the
availability of these structures, though the focus to date has largely been upon MOR biased
agonism in an effort to reduce non-analgesic effects at MOR by differentially engaging
downstream signalling cascades (T. A. Johnson et al., 2016; Link & Müller, 2016; Manglik et al.,
2016; Melkes, Hejnova, & Novotny, 2016; Sounier et al., 2015; Winpenny, Clark, & Cawkill,
2016). Two such compounds appear to have already reached relatively advanced development –
PZM21 (Manglik et al., 2016) and TRV130 (oliceridine), the latter of which has begun Phase
Three clinical testing (ClinicalTrials.gov Identifier NCT02820324). M/DOR may present an even
more promising target for these approaches; being composed of both MOR and DOR
components, M/DOR may have more diverse downstream coupling and a greater potential for
ligand-biased effects. Indeed, such biased agonism at M/DOR may be the effect of some of the
ligand combinations and bivalent ligands investigate at the heteromer. While somewhat inelegant
and likely more prone to off-target effects at monomeric MOR and DOR than novel structure-
designed ligands, these approaches appear to simultaneously manipulate the conformational states
of both MOR and DOR components in order to exert effects not observed in single binding
pocket interactions. These effects have been proposed (Gendron et al., 2016; Rozenfeld & Devi,
2010) to underlie MOR tolerance reducing effects of concomitant DOR antagonism (Abdelhamid et al., 1991; Kest et al., 1996; Zhu et al., 1999). It is also possible that such mixed effects at M/DOR may be involved in the pharmacodynamics of buprenorphine, a clinically-used opioid analgesic which appears to be a partial MOR agonist (Virk, Arttamangkul, Birdsong, & Williams, 2009), weak partial DOR agonist (Negus et al., 2002), and KOR antagonist (Leander, 1988), though the precise nature of buprenorphine pharmacology is sometime contentious (Butler et al., 2013). It is possible that buprenorphine acts against both MOR and DOR binding sites (albeit, different individual molecules of buprenorphine), effectively accomplishing a ligand combination from a single agent.

6.3 Outlook

Clearly, much remains to be understood about M/DOR and OR heteromers in general. This should come as little surprise, however, as it is becoming equally clear that OR heteromers are best understood as distinct receptor species with a complexity to match. Considerable work was, and continues to be, performed in understanding the OR monomers. Investigation of the heteromers will likely follow complementary paths.

An essential component of understanding the OR heteromers will be in understanding their endogenous behaviour. This will require focus on the use of physiologically-relevant models, as we have done in this thesis. Such a focus is particularly challenging for the OR heteromers because of the potentially confounding effect of OR monomers on the available tools. Small epitope-tagged native ORs under native expression control remains a promising aspiration, but, as we have demonstrated, a challenging one. The availability or generation of highly-specific antibodies is similarly promising and challenging, though antibody-based methods are often now subject to greater scepticism than in the past (Burry, 2011; Schnell et al., 2012).

The conditions for OR heteromer formation is a particularly interesting and accessible question, especially for M/DOR. Using established tools and techniques, it should be possible to
address whether prolonged MOR activation is the only state to induce M/DOR functional availability. The same DOR trafficking effects which led us to investigate prolonged MOR activation occur in chronic, but not acute, pain states (Cahill et al., 2007; Cahill, Morinville, Hoffert, O’Donnell, & Beaudet, 2003; Gendron et al., 2006; Morinville, Cahill, Kieffer, et al., 2004). This would suggest that such chronic pain states also induce M/DOR, a possibility with similarly intriguing clinical implications – a distinct OR species available in opioid tolerant and chronic pain states.

The concept of OR heteromerization has moved rapidly from its origins as a theoretical possibility. With ever accumulating evidence, considerations of OR heteromers are now moving to their functional impact upon physiological systems. These shifts in perspective and focus bring with them new and more difficult challenges of methodology. Surmounting those challenges will help to uncover new understandings of receptor neurobiology and, possibly, new clinical resources.
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Appendix A


Supplementary Figures
Figure S1      Original image, Figure 3.5A, upper left
Figure S2  Original image, Figure 3.5A, upper centre
Figure S3  Original image, Figure 3.5A, lower left
Figure S4  Original image, Figure 3.5A, lower centre
Figure S5  Original image, Figure 3.6A, upper left
Figure S6  Original image, Figure 3.6A, upper centre
Figure S7    Original image, Figure 3.6A, lower left
Figure S8: Original image, Figure 3.6A, lower centre
Appendix B

Validation of a Floxed-Stop, FLAG-Tagged Oprd1 Conditional Knock-In Mouse

Supplementary Figure
Figure S1  Characterization of DOR binding and G-protein activation in FLAG-DOR and Stop-DOR mice.

By selectively crossing Stop-DOR mice with mice expressing Cre recombinase under the control of various promoters, it is possible to obtain differential DOR expression including (A) whole animal, (B) microglial, or neuronal. (C) Radiolabelled ligand binding to DOR is present in WT and FLAG-DOR (KI) brains and absent in Stop-DOR (KO) brains. (D) In FLAG-DOR brains, DOR agonist Deltorphin II (DLTII) induces G-protein activation as detected by $^{35}$SGTPγS incorporation.