FACTORS INFLUENCING OPIOID RECEPTOR SIGNALING
TO ADENYLYL CYCLASE

by

Erica Sawyer Levitt

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Pharmacology) in The University of Michigan 2010

Doctoral Committee:

Professor John R. Traynor, Chair
Professor Henry I. Mosberg
Associate Professor Jeffrey R. Martens
Associate Professor Roger K. Sunahara
To my husband Trevor and my mother Barbara

In memory of my father Steve
Acknowledgements

Firstly, I would like to thank my mentor Dr. John Traynor for taking the time to teach me the skills required to become a successful scientist. I appreciate his patience and guidance in learning how to think critically, prepare manuscripts, write grants and deliver presentations. I am very grateful for the true interest he has taken in preparing me for the next steps of my career. Overall, I thank him for providing a challenging and rewarding graduate school experience.

I would like to thank Dr. Jeffrey Martens for providing guidance scientifically as a collaborator and thesis committee member, as well as for the special effort he has taken to help me in my career. I would also like to acknowledge Dr. Henry Mosberg and Dr. Roger Sunahara for their helpful advice and guidance.

To the members of the Traynor lab, present and former, thank you for assistance scientifically and for creating a collaborative work environment. Specifically, I would like to thank Kelly Bosse, Faye Bradbury, Mary Clark, Alexander Delgado, Emily Jutkiewicz, Lauren Purington, Jennifer Thomson and Qin Wang. I would like to thank the undergraduates that have contributed to the work in this thesis, Alexander Harris, Tony McClafferty, Christine Zelnik and Jason Chen.

Special thanks to those in the department who have provided assistance during my graduate career: the Jeffery Martens laboratory, especially Paul Jenkins for help with confocal imaging, lipid raft techniques and reagents; the Paul Hollenberg lab, especially Natasha Snider for providing cannabinoid ligands and advice; the Richard Neubig laboratory, including Sue Wade and Levi Blazer for providing adrenergic ligands and...
advice; the Department of Pharmacology administrative staff, especially Eileen Ferguson and Anna Taylor.

I would like to recognize support and training received from the Pharmacological Sciences Training Program and the University of Michigan Substance Abuse Research Center Training Grant. I also acknowledge funding from the National Institutes of Health National Institute of Drug Abuse including a Ruth Kirschstein pre-doctoral NRSA.

Finally, I would like to thank those outside of science who have supported me. My husband Trevor has been wonderful and I cannot thank him enough for the continuous encouragement and support he has provided. I would like to thank my mom for always believing in me and providing an environment that fostered my interest in learning. Last, but not least, I would like to thank my family and friends for keeping me balanced and helping me accomplish some of my other life goals, such as travel, staying active and keeping a light-hearted personality. I have made some great friends at Michigan, all of whom I am very grateful for and will miss very much.
# Table of Contents

Dedication ........................................................................................................................................ ii  
Acknowledgements ....................................................................................................................... iii  
List of Figures ........................................................................................................................... vii  
List of Tables .............................................................................................................................. ix  
List of Abbreviations ................................................................................................................... x  
Chapter I ........................................................................................................................................ 1  
General Introduction .................................................................................................................. 1  
  Opioid pharmacology .............................................................................................................. 1  
  Opioid receptor signaling ....................................................................................................... 4  
  Receptor coupling models ..................................................................................................... 6  
  Plasma membrane composition and organization ................................................................. 9  
  Cholesterol and/or membrane microdomains in opioid receptor signaling ......................... 15  
  Hypothesis and Aims ............................................................................................................. 23  
Chapter II ..................................................................................................................................... 28  
Differential effect of membrane cholesterol removal on MOR and DOR: A parallel comparison of acute and chronic signaling to adenylyl cyclase .................................................. 28  
  Summary ............................................................................................................................... 28  
  Introduction .......................................................................................................................... 29  
  Materials and Methods ....................................................................................................... 31  
  Results .................................................................................................................................... 36
Discussion ..................................................................................................................... 47

Chapter III ......................................................................................................................... 63

\(G_{i/o}\)-coupled receptors compete for signaling to adenyl cyclase in SH-SY5Y cells and reduce opioid withdrawal-mediated cAMP overshoot ..................................................... 63

Summary ....................................................................................................................... 63

Introduction ................................................................................................................... 64

Materials and Methods ................................................................................................. 65

Results ........................................................................................................................... 69

Discussion ..................................................................................................................... 75

Chapter IV ......................................................................................................................... 91

Discussion and Future Directions ..................................................................................... 91

Future directions related to compartmentalization ....................................................... 92

Future directions related to cholesterol/membrane microdomains ............................... 94

Overall summary and significance .............................................................................. 100

References ....................................................................................................................... 104
List of Figures

Figure 1.1: Schematic of opioid receptor signaling .......................................................... 27

Figure 2.1: MOR, but not DOR, agonist-mediated G protein activation is altered following MβCD treatment .......................................................... 54

Figure 2.2: Cholesterol removal by MβCD induces low-affinity binding in MOR, but not DOR, expressing cells .......................................................... 55

Figure 2.3: MOR, but not DOR, agonist-mediated inhibition of adenylyl cyclase is altered following MβCD treatment .......................................................... 56

Figure 2.4: MβCD treatment blocks MOR, but not DOR, agonist-mediated adenylyl cyclase sensitization .......................................................... 57

Figure 2.5: Effects of MβCD on MOR signaling are due to removal of cholesterol from the membrane .......................................................... 58

Figure 2.6: Alterations in MOR signaling are not due to changes in receptor number ... 59

Figure 2.7: Effect of cytoskeleton inhibitors on MOR-mediated adenylyl cyclase sensitization .......................................................... 60

Figure 2.8: Localization of MOR, but not DOR, in caveolin-enriched fractions, in addition to transferrin receptor-enriched fractions .......................................................... 61

Figure 2.9: DOR is not co-localized with cholera toxin B (CTB) subunit patches ........ 62

Figure 3.1: G_s/o-coupled receptors endogenously expressed in SH-SY5Y cells share a common pool of AC .......................................................... 83

Figure 3.2: MOR and DOR share pertussis toxin-sensitive G proteins ....................... 84

Figure 3.3: DAMGO and SNC80 activation of G protein is additive at concentrations or time-points when G protein is not limiting .......................................................... 85

Figure 3.4: MOR and DOR share AC during chronic agonist administration .......... 86

Figure 3.5: DAMGO-mediated cAMP overshoot is reduced by heterologous inhibition of shared AC by agonist to DOR, NOPr or α2AR. .......................................................... 87
Figure 3.6: Inhibition of cAMP by MOR or DOR agonists is similar for sensitized or non-sensitized AC......................................................................................................................... 88

Figure 3.7: Lack of heterologous tolerance between MOR and DOR.............................. 89

Figure 3.8: Schematic depicting the accessibility of $G_{i/o}$-coupled receptors to portions of the total AC pool................................................................................................................. 90

Figure 4.1: Serpentine model of MOR. ........................................................................... 103
## List of Tables

Table 1.1: Ki (nM) for selected ligands at human MOR, DOR and KOR .......................... 25

Table 1.2: Membrane microdomains in opioid receptor localization and signaling........ 26

Table 3.1: Receptor density in differentiated SH-SY5Y cells ........................................ 80

Table 3.2: Competition between indicated agonists (1 µM) for acute inhibition of AC.. 81

Table 3.3: Competition between 1 µM SNC80 or nociceptin/OFQ and other G<sub>i/o</sub>-coupled receptor agonists for acute inhibition of AC................................................................. 82
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[35S]GTPγS</td>
<td>guanosine-5'-O-(3-[35S]thio)triphosphate</td>
</tr>
<tr>
<td>5-HT_{1A}</td>
<td>serotonin 1A receptor</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>8-hydroxy-2-(di-n-propylamino) tetralin hydrochloride</td>
</tr>
<tr>
<td>α_{2}AR</td>
<td>alpha2-adrenergic receptor</td>
</tr>
<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CB_{1}</td>
<td>cannabinoid receptor 1</td>
</tr>
<tr>
<td>CTAP</td>
<td>D-Phe-[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH₂</td>
</tr>
<tr>
<td>CTB</td>
<td>cholera toxin B subunit</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DOR</td>
<td>delta-opioid receptor</td>
</tr>
<tr>
<td>DPDPE</td>
<td>[D-Pen²⁵]-enkephalin</td>
</tr>
<tr>
<td>DPN</td>
<td>diprenorphine</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FLAG epitope</td>
<td>DYKDDDDK</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>ICI 174,864</td>
<td>N, N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH</td>
</tr>
<tr>
<td>J113397</td>
<td>1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one</td>
</tr>
<tr>
<td>LC</td>
<td>locus coeruleus</td>
</tr>
<tr>
<td>MB₆CD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>MBS</td>
<td>MES buffered saline</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethanesulfonic acid</td>
</tr>
<tr>
<td>MOR</td>
<td>mu-opioid receptor</td>
</tr>
<tr>
<td>N/OQF</td>
<td>nociceptin/orphanin FQ peptide receptor</td>
</tr>
<tr>
<td>NOPr</td>
<td>nociceptin/orphanin FQ peptide receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNC80</td>
<td>(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide</td>
</tr>
<tr>
<td>TfR</td>
<td>transferrin receptor</td>
</tr>
</tbody>
</table>
Chapter I

General Introduction

Opioid pharmacology

Opioids, such as morphine, have been used in human medicine for centuries because of their analgesic properties. Unfortunately, opioid drugs also carry unwanted side effects, such as constipation and respiratory depression, which complicate their use. Furthermore, abuse of opioids, including prescription narcotics, is a problem. Past-year non-medical use of the prescription opioid analgesic Vicodin® by high-school seniors is reported to be 10 percent (Monitoring the Future, 2008). This is second only to marijuana, which carries an annual use rate of 32 percent by high-school seniors.

There are three prototypical opioid receptors, designated mu (MOR), delta (DOR) and kappa (KOR). Most of the clinically used opioid drugs exert their action primarily through MOR, which leads to significant analgesia, sedation, respiratory depression, constipation and euphoria (Dhawan et al., 1996; Traynor, 1996). As demonstrated by studies in MOR knock-out mice (Matthes et al., 1996), the desired analgesic properties of morphine and the undesired side effects and abuse liability are all mediated by MOR, making separation of these effects difficult. Activation of KOR can produce moderate analgesia, but significant dysphoria limits the usefulness of KOR agonists as therapeutics (Pfeiffer et al., 1986). However, some drugs classified as mixed agonist/antagonists, such as pentazocine and nalorphine, do have partial agonist activity at KOR (Remmers et al., 1999) and MOR (Traynor and Nahorski, 1995). These mixed agonist/antagonists could theoretically have lower abuse liability due to a balance of dysphoria and euphoria from KOR and MOR activation, respectively, but clinically this has not been observed. Compounds targeting DOR are not used clinically; however, in rodent models DOR
agonists produce some antinociception (Bilsky et al., 1995) and display antidepressant-like effects (Broom et al., 2002b). Furthermore, the DOR agonist SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) is not self-administered by rhesus monkeys (Negus et al., 1998; Stevenson et al., 2005), indicating a decreased abuse liability. An obstacle to the development of DOR agonists as therapeutics is the tendency of DOR activation to cause convulsions. However, the convulsive effects occur at higher doses than those required to elicit antinociception (Broom et al., 2002c) and are not required for antidepressant-like effects (Broom et al., 2002a).

A unique aspect of opioid pharmacology is the existence of a plethora of ligands (both endogenous and exogenous) for only three cloned receptors. The first endogenous opioid peptides discovered were the enkephalins (Tyr-Gly-Gly-Phe-[Leu or Met]) (Hughes et al., 1975). The initial tetrapeptide sequence Tyr-Gly-Gly-Phe is essential for interaction with the receptor binding pocket (Schwyzer, 1986). Other endogenous opioid peptide families were subsequently discovered; the endorphins include β-endorphin, α-endorphin and γ-endorphin, and the dynorphins include dynorphin(1-17), dynorphin(1-13) and dynorphin(1-9). These begin with the sequence of either Leu- or Met-enkephalin but continue to various lengths which may contribute to selectivity. Contrary to logic, the differing structures of the three classes of endogenous opioid peptides (enkephalin, endorphin and dynorphin) do not confer specificity for the three opioid receptors. Rather, the enkephalins and β-endorphin bind well to both MOR and DOR (Lord et al., 1977; Chang and Cuatrecasas, 1979; Akil et al., 1981), while the dynorphins have some selectivity for KOR (Chavkin et al., 1982; James et al., 1982). The discrepancy between the number of endogenous opioid peptides and the number of opioid receptor types prompted the hypothesis that various receptor conformations exist that are differentially recognized by opioid ligands (Rothman et al., 1988; George et al., 2000; Gomes et al., 2000), which could be the result of receptor-receptor, receptor-lipid or receptor-protein interactions.

Opioids were first used by man in the form of naturally occurring opium, derived from the sap of a poppy bud, *Papaver somniferum*. Opium is a crude mixture of many
alkaloids, the most abundant of which is morphine (Watkyn-Thomas, 1912), which was isolated in 1806 by Serturner and named after the Greek god Morpheus, the god of dreams. Natural compounds that can be derived directly from the opioid poppy include morphine and codeine. Opioids include natural opiates as well as semi-synthetic derivatives (e.g. hydrocodone, oxycodone, buprenorphine) and fully synthetic compounds (e.g. fentanyl, remifentanil and meperidine). While most of these opioids have some preference for MOR, selective ligands have been developed for all three opioid receptors based initially on the structures of the endogenous opioid peptides. Especially important for this thesis are the MOR selective agonist DAMGO ([D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin) (Handa et al., 1981) and the DOR selective agonists SNC80 (Calderon et al., 1994) and DPDPE ([D-Pen²,⁵]-enkephalin) (Mosberg et al., 1983). Selective KOR agonists include U50,488 and U69,593 (Vonvoigtlander et al., 1983). Naloxone, a derivative of morphine, is an antagonist for all three opioid receptors. Antagonism of specific receptor types can be achieved using the MOR antagonist CTAP (D-Phe-[Cys-Tyr-D-Trp-Arg-Thr-Arg-Thr-Pen]-Thr-NH₂) (Pelton et al., 1986) or the DOR antagonist naltrindole (NTI) (Portoghese et al., 1988). In certain instances, the DOR inverse agonist ICI 174,864 (N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH) (Cotton et al., 1984) is used instead of NTI due to increased DOR selectivity (Hirning et al., 1985). Nor-binaltorphimine (nor-BNI) is a commonly used KOR antagonist (Portoghese et al., 1987). Affinities of the above compounds for each receptor type are listed in Table 1.1.

The first opioid receptor cloned was DOR, which was identified using a cDNA library prepared from NG-108 cells that express high amounts of this receptor type (Evans, 1992; Kieffer, 1992). Cloning of the highly homologous MOR and KOR from a rat brain cDNA library soon followed (Chen et al., 1993a; Chen et al., 1993b). An additional receptor, the nociceptin/orphanin FQ peptide receptor (NOPr), was identified based on structural similarity to the other opioid receptors (Bunzow et al., 1994; Mollereau et al., 1994). NOPr, however, does not bind typical opioid ligands and the endogenous peptide ligand for NOPr, nociceptin/OFQ, lacks the initial Tyr residue that is required for binding of the other endogenous peptides to MOR, DOR and KOR (Bunzow et al., 1994; Mollereau et al., 1994; Meunier et al., 1995; Reinscheid et al., 1995).
Regardless, NOPr is now considered a member of the opioid receptor family on the basis of structural homology (Harmar et al., 2009).

**Opioid receptor signaling**

All opioid receptors (including NOPr) contain seven membrane-spanning domains and are classic, family A G protein-coupled receptors (GPCRs) that couple to inhibitory heterotrimeric G\(_{i/o}\) proteins composed of an \(\alpha\), \(\beta\) and \(\gamma\) subunit. The binding of agonist promotes or stabilizes conformations of the receptor that are recognized by G proteins and facilitates exchange of GDP for GTP on the G\(\alpha\) subunit, leading to downstream signaling by both the G\(\alpha\) and G\(\beta\gamma\) subunits. Opioid receptor signaling is summarized in Figure 1.1. The primary effect of the activated G\(\alpha_{i/o}\) subunit is inhibition of adenylyl cyclase (AC), a family of enzymes that catalyzes the conversion of ATP to cAMP (Katada and Ui, 1982; Taussig et al., 1993). The G\(\beta\gamma\) heterodimer can activate many downstream effectors, including inwardly-rectifying potassium (GIRK) channels, mitogen-activated protein kinases (MAPK), phospholipase C (PLC), non-receptor tyrosine kinases (e.g. Src family), and N-type calcium channels (Clapham and Neer, 1993; Milligan and Kostenis, 2006; Dupre et al., 2009). Furthermore, G\(\beta\gamma\) plays a modulatory role in AC activity, as it strongly stimulates some isoforms of AC (AC 2, 4 and 7), but inhibits other isoforms (AC 1, 3 and 8) (Sunahara and Taussig, 2002; Sadana and Dessauer, 2009).

AC plays a critical role in opioid receptor signaling both acutely and during chronic opioid treatment. Mice lacking AC 5, which is highly expressed in the striatum, display reduced behavioral responses to morphine, including locomotor activity, analgesia, reward and withdrawal (Kim et al., 2006). Upregulation of the cAMP pathway, especially AC 1 and 8, in the locus coeruleus (LC) during chronic opioid treatment is thought to contribute to opioid withdrawal (Zachariou et al., 2008). Acute administration of an opioid agonist leads to inhibition of cAMP production. After prolonged opioid exposure, homeostatic mechanisms in the cell facilitate sensitization of AC, allowing cAMP levels to normalize. Following withdrawal of the opioid agonist and
stimulation of the sensitized AC, cAMP production is enhanced. This cAMP “overshoot” is considered a cellular model of withdrawal and was originally described for opioids acting at DOR (Sharma et al., 1975), but has since been described for many Gi/o-coupled receptors including MOR, α2AR, D2-dopamine, M4-muscarinic and somatostatin (Thomas, 1987; Watts, 2002; Clark and Traynor, 2006; Divin et al., 2009).

There are nine mammalian isoforms of AC and not all AC isoforms participate equally in opioid signaling. Only those AC isoforms acutely inhibited by opioids (AC 1, 5, 6 and 8) are sensitized following chronic opioid treatment, and this effect is independent of the agent used to stimulate AC (forskolin, ionomycin or Gαs-coupled receptor agonist) (Avidor-Reiss et al., 1997). The isoform-specific sensitization is similar for Gi/o-coupled D2-dopamine and M4-muscarinic receptors (Nevo et al., 1998). The mechanism of cAMP overshoot is not clearly understood, but it does not require synthesis of new proteins (Avidor-Reiss et al., 1995) and does not depend on the antagonist precipitating withdrawal (Divin et al., 2009). Various kinases have been implicated in the mechanism, including Raf-1, PKC, and Src (Varga et al., 2002; Beazely and Watts, 2005; Zhang et al., 2009), but there is probably redundancy within these pathways.

One prominent hypothesis for the mechanism of overshoot is increased stimulation of AC by Gαs, which is not due to increased Gαs protein levels (Ammer and Schulz, 1995). Evidence suggests that Gαi and Gαs can bind simultaneously to the C1 and C2 domains of AC, respectively (Taussig et al., 1994; Ammer and Schulz, 1998; Dessauer et al., 1998; Chen-Goodspeed et al., 2005). One study has demonstrated enhanced Gαs binding to AC following chronic opioid treatment (Ammer and Schulz, 1997). However, in a different cell line the same authors did not observe direct Gαs binding to AC during opioid withdrawal, although functional receptor-coupled Gαs was still indirectly required for cAMP overshoot (Ammer and Schulz, 1998). The difference between these studies may be due to the expression patterns of AC isoforms in the cells utilized because isoform-specific requirements for Gαs in cAMP overshoot have been reported. Gαs is required for D2-dopamine receptor-mediated sensitization of AC 5, but not AC 1 (Vortherms et al., 2006). In addition, a shift in the membrane localization of Gαs, Gαi or MOR may be an important step in the development of opioid-induced AC
sensitization (Ammer and Schulz, 1997; Bayewitch et al., 2000; Mouledous et al., 2005). This will be further addressed in subsequent sections.

**Receptor coupling models**

The transduction of signal from GPCR to G protein has been described by multiple models over the years. It was originally proposed that receptor and G protein, or effector, were freely diffusible entities in the lipid bilayer and that the interaction between receptor and effector occurred by random collisions (Tolkovsky and Levitzki, 1978). This “collision-coupling” model was further refined to propose “encounters” between active receptor and G protein that adjusted for the rate of dissociation of the G protein and agonist from the receptor, thus defining the duration of time required for the interaction (Stickle and Barber, 1992). Both models were based on data from β-adrenergic receptor activation of AC in turkey erythrocytes, in which it was assumed that the rate of G protein activation was proportional to AC activation. A strength of these models is the opportunity for signal amplification, as multiple G proteins can recognize a single activated receptor.

An alternate situation to the collision-coupling model is that G protein and receptor are associated even in the absence of agonist, thus “pre-coupled”, allowing rapidity of GPCR signal propagation. Fluorescence and bioluminescence resonance energy transfer (FRET and BRET) approaches have been employed to study pre-coupling of various GPCRs to G proteins (Gales et al., 2005; Hein et al., 2005; Nobles et al., 2005; Gales et al., 2006). These studies suggest that the β2-adrenergic receptor (β2AR) is associated with G protein subunits Gαs, Gαi1, Gβ1 and Gγ2 under basal (no drug) conditions, which may be the reason for constitutive activity of this receptor (Gales et al., 2005). These β2AR/G protein complexes have been shown to form early in protein processing prior to trafficking to the plasma membrane (Dupre et al., 2006). The BRET between β2AR and Gαs or Gαi2 proteins was significantly increased upon agonist binding in a concentration-dependent manner that paralleled agonist-stimulated cAMP production (Gales et al., 2005). It is unclear if the increase in signal upon agonist exposure is due to
increased association with G protein or rearrangement of a preassembled receptor/G protein complex, although the authors suggest the latter. This may be the case since agonist addition decreased BRET between $\alpha_{2A}$AR and $\alpha_{i1}$ depending on the location of the tag on the G protein and was not due to dissociation of the G protein from receptor (Gales et al., 2006). In contrast, using FRET, others have concluded that $\alpha_{2A}$AR and G proteins are not pre-coupled, but rather interact by rapid collision coupling following agonist application (Hein et al., 2005). DOR, a receptor that shows endogenous constitutive activity, may also form preassembled complexes with G proteins, although the affinity of G protein for receptor was increased when the receptor was occupied by agonist (Alves et al., 2003; Audet et al., 2008). Interpretation of these data is further complicated by the lack of direct structural evidence of a receptor bound to G protein; however, models do exist (Oldham and Hamm, 2008).

Although MOR has not been shown to be pre-coupled to G proteins, evidence suggests that the probability of MOR and G protein interaction is enhanced by co-localization into the same membrane domains (Remmers et al., 2000; Alt et al., 2001). Such compartmentalization would provide a restricted collision coupling model in which diffusion of signaling proteins is limited to a confined space or compartment. In this thesis, “compartment” will be used to describe any organization in the plasma membrane that reduces diffusion thereby enhancing (or diminishing) the likelihood that two proteins will interact. This type of interaction has been proposed previously for GPCR signaling (Neubig, 1994), largely based on the finding that mobility of G proteins is strikingly limited (Kwon et al., 1994). In agreement with compartmentalization, the diffusion of MOR was shown to be restricted to sub-micrometer permeable domains as determined by fluorescence recovery after photobleaching (FRAP) in SH-SY5Y cells (Sauliere et al., 2006). Furthermore, in single particle tracking experiments, MOR displayed short-term diffusion confined to a domain, which also diffuses (Daumas et al., 2003; Suzuki et al., 2005). This behavior was termed “walking confined diffusion” or “hop diffusion”. In either case, these results indicate that MOR is confined to a compartment with spatial barriers. However, these compartments were not permanent, since agonist treatment changed the diffusion of MOR to “directed diffusion” which the authors contributed to
internalization (Daumas et al., 2003). Compartmentalization of receptors may also be type dependent, since adenosine A2\(\underline{A}\) receptors had restricted mobility similar to MOR, but M\(\_1\) muscarinic receptors have been reported to display simple random diffusion (Charalambous et al., 2008; Hern et al., 2010).

Many modes of compartmentalization have been proposed, including oligomerization of opioid (Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2004) and other GPCRs (Milligan, 2009) as well as membrane microdomains (Allen et al., 2007). Compartmentalization could also occur by protein scaffolding to the cytoskeleton or to specialized proteins, such as A-Kinase Anchoring Proteins (AKAPs) (Hall and Lefkowitz, 2002; Kreienkamp, 2002; Milligan, 2005; Dessauer, 2009). In support of compartmentalization, large protein complexes have been identified that contain receptor (i.e. \(\beta_2\)AR), G protein and multiple effectors, including Ca,1.2, Kir3.2 and AC 5/6 (Davare et al., 2001; Lavine et al., 2002). These complexes also included both kinase (PKA) and phosphatase (PP2A) that could serve to regulate the local environment (Davare et al., 2001). The spatial organization of proteins in a cell has significance for many signal transduction pathways, not just those modulated by GPCRs, and has been recently reviewed (Scott and Pawson, 2009).

A functional consequence of compartmentalization other than enhancing the speed of signaling, may be to separate different types of receptors leading to signaling specificity. In this case, co-application of agonists to separated receptors would lead to an additive response. In N18TG2 neuroblastoma cells, agonists to endogenous DOR and cannabinoid (CB\(_1\)) receptors activated G proteins in an additive manner (Shapira et al., 1998; Shapira et al., 2000). In contrast, receptors located in the same compartment would only have access to a limited pool of effectors, and therefore maximal activation of both receptor types would lead to competition for shared effectors and a less than additive response. Such competition was originally observed between G\(_s\)-coupled \(\beta\)-adrenergic receptors and prostaglandin receptors (Pike and Lefkowitz, 1981) or glucagon receptors (Murayama and Ui, 1984). However, competition can also be observed for G\(\alpha_{\iota/\delta}\)-coupled receptors. A less than additive response has been described for concomitant MOR and DOR activation in SH-SY5Y cells (Alt et al., 2002). Similarly, DOR and CB\(_1\) receptors
cotransfected in COS-7 cells shared G proteins (Shapira et al., 2000) and MOR and α2AR endogenously expressed in SH-SY5Y cells were observed to access the same AC enzymes (Lameh et al., 1992).

**Plasma membrane composition and organization**

One of the proposed modes of compartmentalization is the presence of membrane microdomains, also called lipid rafts. These are regions of the plasma membrane enriched in cholesterol and sphingolipids (Simons and Ikonen, 1997; Pike, 2006). The identification of certain signaling proteins, including GPCRs and G proteins, with markers of these domains led to the hypothesis that microdomains may serve as signaling platforms (Simons and Ikonen, 1997). However, the existence and importance of membrane microdomains is highly controversial (Munro, 2003), although recent reports using sophisticated microscopy and spectroscopy techniques provide more convincing support (Lingwood et al., 2008; Eggeling et al., 2009; Kaiser et al., 2009). Despite this controversy, many groups have attempted to study the role of microdomains in opioid receptor signaling. Furthermore, the lipids of the plasma membrane, and in particular cholesterol, are important for the structure and function of the proteins that are embedded within or associated with the membrane, such as opioid receptors, adenylyl cyclase and G proteins. The progress and pitfalls in identifying the role of cholesterol and membrane microdomains in opioid receptor signaling will be discussed below.

**Plasma membrane lipids**

The mammalian plasma membrane is comprised of three main types of lipids: phospholipids, cholesterol and sphingolipids. Although the relative amount of these lipids varies with cell type, phospholipids are usually the most abundant of the three. Cholesterol is often the second most prevalent species and represents 35 % of the plasma membrane lipids in N2A neuroblastoma cells (Charalampous, 1979). Less than 10 % of the plasma membrane lipids of N2A cells were sphingomyelin, the most common type of sphingolipid (Charalampous, 1979).
Phospholipids and sphingolipids have similarities in their structures as both contain a polar head group, a carbon backbone and two fatty acid chains. However, the differences between these two molecules provide heterogeneity in membranes. Phospholipids contain a glycerol backbone, one of several polar head groups and two moderately long (16 - 18 carbons) fatty acid chains, one of which is usually unsaturated with a cis double bond (Ramstedt and Slotte, 2002). Sphingolipids contain a long chain sphingoid base, an amide-linked fatty acid chain and, in the case of sphingomyelin, a phosphocholine head group. The amide-linked acyl chain varies in length from 16 – 24 carbons and is usually fully saturated (Ramstedt and Slotte, 2002). In brain gray matter, the most common fatty acid attached to sphingomyelin is the fully saturated stearic acid (18:0) (Koval and Pagano, 1991). Thus, both acyl chains of sphingomyelin are usually saturated, in contrast to most phospholipid molecules, which have at least one unsaturated cis double bond.

Another key difference between phospholipids and sphingolipids is their ability to interact with cholesterol. Considerable evidence shows that cholesterol interacts preferentially with sphingomyelin over other phospholipids, such as phosphatidylcholine and phosphatidylethanolamine (Demel et al., 1977; Ohvo-Rekila et al., 2002). This may have physiological relevance, since homeostatic regulation of sphingomyelin and cholesterol levels is known to be interdependent and coordinated (Slotte, 1999). However, the structural basis for this interaction is less clear. Unlike phospholipids that interact with cholesterol in a manner very sensitive to chain length and degree of unsaturation, cholesterol interacts favorably with sphingomyelin even in the presence of long, unmatched acyl chains (Ohvo-Rekila et al., 2002). The sphingolipid-cholesterol interaction is partly due to van der Waals forces between the normally saturated acyl chains of sphingomyelin and the rigid hydrophobic rings of cholesterol (Ramstedt and Slotte, 2002). It has been suggested that the sphingomyelin-cholesterol interaction is enhanced by a hydrogen bond formed between the amide group of sphingomyelin and the 3-OH of cholesterol (Bittman et al., 1994). However, authors of atomic-scale molecular dynamics simulations reached conflicting conclusions about the importance of direct hydrogen-bonding on the sphingolipid-cholesterol interaction, although they do agree that
one neighboring cholesterol molecule is sufficient to induce an interaction with sphingomyelin (Aittoniemi et al., 2007; Zidar et al., 2009). Thus, cholesterol interacts favorably with sphingomyelin although the structural basis for this interaction is unclear.

**Lipid organization in model membranes**

Bilayers containing solely phospholipids will form two temperature-dependent phases, with a shift from an ordered, solid-like gel state to a liquid-disordered ($l_d$) state as temperature increases past the melting temperature ($T_m$) of the lipid. The $T_m$ of a lipid is dependent on the length and saturation of its fatty acid chains, with saturated fatty acids having a higher $T_m$ than polyunsaturated fatty acids, because cis double bonds interfere with lateral packing ability. Addition of cholesterol to the bilayer induces an intermediate state, increasing the fluidity of lipids below and decreasing the fluidity of lipids above their $T_m$. At concentrations of cholesterol above 30 mol %, the transition between gel and $l_d$ phases is obscured and a liquid-ordered ($l_o$) phase prevails, characterized by increased organization and tighter packing of fatty acid chains leading to a thicker bilayer that retains rapid lateral diffusion (Demel et al., 1977; Ipsen et al., 1987; Lawrence et al., 2003; Zidar et al., 2009). This $l_o$ phase is considered a simplified representation of the lipid raft or membrane microdomain state. In mixed lipid bilayers, cholesterol will induce $l_o$ domain formation preferentially with sphingomyelin (Demel et al., 1977; Zidar et al., 2009). Together, these results provide evidence used to define lipid rafts or membrane microdomains as highly ordered regions enriched in cholesterol and sphingolipids (Pike, 2006).

**Detergent-resistant membranes**

The two physiologically relevant bilayer phases, the $l_d$ and $l_o$ phases, can be separated in model membranes based on solubility in non-ionic detergents, such as Triton X-100 (London and Brown, 2000). The $l_o$ phase is relatively resistant to solubilization with Triton X-100 because van der Waals forces between tightly packed lipids are stronger than lipid-Triton interactions (Shogomori and Brown, 2003). Thus, in model membranes, non-ionic detergent-resistant extracts are a reliable representation of the $l_o$
state. In cells, Triton X-100-insoluble membranes can be extracted that are enriched in cholesterol and sphingolipids (Brown and Rose, 1992). However, these membranes may not truly represent the physiological state because addition of detergent at the low temperature (4°C) utilized in these experiments could easily perturb the membrane (London and Brown, 2000; Shogomori and Brown, 2003). Thus, detergent-resistant membranes should not be considered identical to membrane microdomains. Despite these drawbacks, separation of non-ionic detergent-resistant membranes is an accessible technique that has commonly been used to study the lipid and protein content of putative membrane microdomains.

Do membrane microdomains exist in the plasma membrane?

The membrane microdomain theory began as a hypothesis to explain the observed sphingomyelin and cholesterol-enriched l_0 phases of model membranes in terms of the heterogeneous environment of the plasma membrane (Simons and Ikonen, 1997). The tools initially available to study microdomains in cells, such as separation of detergent-resistant membranes, were crude and have led to misinterpretation and controversy, limiting the progress of this field. New technological advances, primarily in microscopy and spectroscopy, have allowed researchers to begin to overcome these challenges and the current status of the membrane microdomain theory has been reviewed very recently (Lingwood and Simons, 2010). Convincing evidence toward the existence of membrane microdomains comes from experiments using stimulated emission depletion (STED) far-field fluorescence nanoscopy to detect the movement of single molecules (lipid or protein) across regions of a living cell membrane < 50 nm in diameter (Eggeling et al., 2009). Using STED nanoscopy, it was observed that phosphoethanolamine (a phospholipid not associated with microdomains) displayed a single, rapid diffusion pattern. In contrast, sphingomyelin exhibited two types of diffusion; free diffusion and hindered diffusion, which was due to a brief trapping of the molecules that was dependent upon cholesterol and consistent with membrane microdomains (Eggeling et al., 2009).
In cells, two types of membrane microdomains that are enriched in cholesterol and sphingolipids have been proposed to exist. Planar domains have proven elusive to observe, but caveolae, which are nonclathrin-coated, flask-shaped invaginations of the plasma membrane, are obvious in electron micrographs (Yamada, 1955). The primary structural component of caveolae is the cholesterol-binding protein caveolin (Rothberg et al., 1992). Although caveolae have not been identified in neurons, these cells do express caveolin, which may play a role as a scaffolding protein or in the formation of synapses (Cameron et al., 1997; Head and Insel, 2007). Thus, caveolae, which are also enriched in cholesterol, are well-accepted structures and may account for some of the experimental observations attributed to microdomains.

**Protein localization in membrane microdomains**

If membrane microdomains exist and can selectively recruit certain proteins as proposed by Simons and Ikonen (1997), what are the criteria for such incorporation? The presence of saturated fatty acid modifications has been shown to help target certain proteins to detergent-resistant membranes (Melkonian et al., 1999). This was first observed for glycosylphosphatidylinositol (GPI)-anchored proteins that bind to the extracellular side of the membrane (Brown and Rose, 1992), but has since been identified for acylated proteins, such as Src family kinases and G proteins, which attach to the intracellular side of the membrane (Harder et al., 1998; Moffett et al., 2000). For G proteins, myristoylation and palmitoylation of Gαi1 is necessary for partitioning to Triton-resistant regions of a reconstituted lipid bilayer (Moffett et al., 2000). In contrast, prenylated Gβγ2, and Gαi1 with a cis-unsaturated fatty acid instead of palmitate, do not incorporate into Triton-resistant membranes (Moffett et al., 2000). Other aspects of protein structure must also be important for targeting because some palmitoylated proteins do not resolve in detergent (Triton)-resistant membranes (Melkonian et al., 1999). For example, family A GPCRs, such as the LH receptor, are often palmitoylated, but this palmitoylation rarely drives membrane microdomain targeting (Chini and Parenti, 2009).
Another possibility is that proteins concentrate in caveolin-enriched domains due to protein-protein interactions with caveolin itself. For example, $G_{\alpha_s}$, $G_{\alpha_2}$ and $G_{\alpha_0}$ were pulled-down with a GST-caveolin fusion protein (Li et al., 1995). The portion of caveolin responsible for this and other binding interactions was identified in the cytoplasmic amino-terminal tail, and this domain was used as bait to elucidate a caveolin-binding motif ($\Phi X \Phi X X \Phi \Phi$ or $\Phi X X \Phi X X \Phi \Phi$; $\Phi = F, W$ or $Y$) (Couet et al., 1997). However, this motif is fairly broad and may be present in proteins that do not associate with caveolin. Furthermore, proteins that are directed in this manner may be specific to caveolae and not generalizable to membrane microdomains.

Finally, proteins may associate with membrane microdomains due to interaction with cholesterol. The best example of this is the cholesterol-binding protein caveolin, which is found in caveolae and is used as a putative membrane microdomain marker. There may also be potential for cholesterol to interact with GPCRs directly. This will be further discussed below.

**Cholesterol and GPCR structure**

Despite the controversy regarding membrane microdomains, it is well-established that cholesterol is an important plasma membrane constituent. In addition, cholesterol may directly interact with transmembrane proteins, such as GPCRs, since cholesterol was necessary for successful crystallization of $\beta_2$AR and three cholesterol molecules per receptor were resolved in the crystal structure (Cherezov et al., 2007). A second $\beta_2$AR crystal structure allowed the elucidation of a cholesterol consensus motif (CCM) as follows: $[4.39-4.43(R,K)][4.50(W,Y)][4.46(I,V,L)][2.41(F,Y)]$ (Hanson et al., 2008). Twenty-one percent of human class A GPCRs contain this consensus motif and 44% have all but the aromatic residue at 2.41 (Hanson et al., 2008). However, in the crystal structure of the $A_{2\alpha}$ adenosine receptor, which contains the strict CCM, this region contained a lipid (modeled as stearic acid) instead of cholesterol, though cholesterol was necessary for crystallization (Jaakola et al., 2008). Thus, this consensus site may not be as specific to cholesterol as originally proposed, and may be better described as a region important for hydrophobic interactions with membrane lipids.
Both of the β2AR structures were solved in the presence of an inverse agonist, so cholesterol binding may favor an inactive conformation of this receptor. In support of this, binding of the inverse agonist timolol, but not the full agonist isoproterenol was enhanced by the addition of cholesterol to purified receptors (Hanson et al., 2008). Functionally, cholesterol depletion increases the ability of β2AR agonists to stimulate AC, indicating an increase of active conformation receptors following cholesterol depletion (Rybin et al., 2000; Pontier et al., 2008). Indeed, under basal conditions, β2AR is found in caveolin-containing, cholesterol-rich domains of the plasma membrane but moves out of caveolin-containing fractions following agonist treatment (Rybin et al., 2000). Cholesterol may help stabilize closely related GPCRs in a similar manner. For example, the α1A-adrenergic receptor, which shares the strict cholesterol consensus motif with β2AR, is also stabilized in an inactive conformation by cholesterol (Lei et al., 2009).

**Cholesterol and/or membrane microdomains in opioid receptor signaling**

*Cholesterol and/or membrane microdomains in MOR signaling*

A role for cholesterol and membrane viscosity in opioid receptor signaling was appreciated even before the membrane microdomain theory became popular. The addition of cis-unsaturated fatty acids to synaptosomal membranes from rat brain reduced membrane viscosity and proportionally reduced MOR and DOR agonist binding (Remmers et al., 1990; Lazar and Medzihradsky, 1992). The cis fatty acid isomers were more potent than similar trans isomers at inhibiting MOR agonist binding and in decreasing membrane viscosity (Remmers et al., 1990). The effects of membrane viscosity were specific to agonist binding, as binding of the antagonist naltrexone was less affected by either cis or trans fatty acid addition, indicating that a high-affinity conformation of MOR and/or DOR that can bind agonist is more sensitive to changes in the bulk membrane environment (Remmers et al., 1990). The inhibition of MOR and DOR agonist binding could be reversed by adding cholesteryl hemisuccinate to the membranes, which normalized membrane viscosity (Lazar and Medzihradsky, 1992).

To further study the high-affinity receptor conformation, experiments were performed in the presence of 5 μM GTPγS with or without 150 mM NaCl, which
uncouples G proteins from MOR and prevents high-affinity agonist binding. The addition of cholesteryl hemisuccinate reduced the ability of sodium to decrease high-affinity MOR agonist binding (Lazar and Medzihradsky, 1993). Addition of cis-unsaturated oleic acid to cholesterol-enriched membranes reversed the increase in viscosity and restored the ability of sodium to modulate MOR agonist binding. These results indicate that increasing the viscosity of the membrane and/or adding cholesterol can stabilize MOR in a high-affinity state and prevent the transition to a low-affinity state.

A more specific role for cholesterol in stabilization of MOR in a high-affinity state was recently elucidated. In yeast expressing MOR and Gαi2, MOR agonist binding is only observed when endogenous ergosterol is removed and replaced with cholesterol, while antagonist binding is observed regardless of sterol status (Lagane et al., 2000). Since cholesterol and ergosterol have similar effects on membrane viscosity (Gimpl et al., 1997), these results indicate that the high-affinity MOR conformation requires the presence of cholesterol. Similarly, MOR expressed in CHO cells required cholesterol for stabilization of a high-affinity conformation and for coupling to G proteins (Gaibelet et al., 2008).

Despite the apparent role of cholesterol in coupling MOR to G proteins, conflicting data exist about the effect of cholesterol removal on MOR agonist-mediated G protein activation, which are summarized in Table 1.2. In all cases described below, cholesterol depletion was achieved using the cholesterol-sequestering agent methyl-β-cyclodextrin (MβCD), which selectively removes cholesterol from the membrane (Gaibelet et al., 2008). However, MβCD treatment conditions varied, which could be the cause of conflicting results. For instance, using the same cells (CHO cells stably expressing MOR), depletion of cholesterol either increased (Huang et al., 2007b) or decreased (Gaibelet et al., 2008) the ability of the MOR agonist DAMGO to stimulate [35S]GTPγS binding. In the study that showed an increase in DAMGO activity, the cells were treated with 15 mM MβCD for 1 h at 37°C prior to preparation of membranes for the [35S]GTPγS binding assay (Huang et al., 2007b). In contrast, in the study that demonstrated a decreased DAMGO response, membranes were prepared first, then
treated with 10 mM MβCD for 30 min at 20°C prior to use in the [35S]GTPγS binding assay (Gaibelet et al., 2008). Similarly, when Huang et al. treated membranes prepared from rat caudate putamen with MβCD (15 mM for 1 h at 37°C), they also showed a reduction in DAMGO-stimulated [35S]GTPγS binding (Huang et al., 2007b). These results could lead to the interpretation that cholesterol removal from cells does not equate to cholesterol removal from membranes. However, this interpretation does not account for all conflicting results since treatment of HEK293 cells stably expressing MOR with 1 mM MβCD for 1 h reduced the ability of morphine to phosphorylate ERK or to inhibit AC, which could be reversed by adding back cholesterol (Zheng et al., 2008a). Therefore, the study showing an increase in [35S]GTPγS binding following MβCD treatment of cells could be an anomaly.

Conflicting results also exist regarding the localization of MORs with membrane microdomain markers, which again could be a consequence of different methods employed. The most stringent method uses Triton X-100 to isolate detergent-resistant membranes, which, as described above, roughly resembles the composition of putative membrane microdomains. Using this method in CHO cells, greater than 95 % of MOR was found in cholesterol-poor domains (Gaibelet et al., 2008). Another method originally designed to isolate caveolae consists of homogenization of cells in 500 mM sodium carbonate buffer at pH 11 followed by sonication. Using this method, others have found a proportion of MOR in fractions with the membrane microdomain marker caveolin when isolated from CHO cells, HEK293 cells or rat brain membranes (Zhao et al., 2006; Huang et al., 2007b).

As suggested by single particle tracking, membrane microdomains are likely transient and thus any “localization” to these domains would be a snapshot in time and easily altered by activation state of the receptor, as mentioned earlier for β₂AR. However, studies disagree on the effect of agonist treatment on MOR distribution. In one study, treatment with etorphine or morphine did not change the fractional distribution of MOR (Huang et al., 2007b), while in another study, etorphine, but not morphine, caused a shift of MOR out of caveolin-containing fractions (Zheng et al., 2008a). This agonist-dependent difference was not attributed to MOR internalization by etorphine. The
opposite shift has also been reported for MOR that was found in cholesterol-poor regions under resting conditions and shifted to cholesterol-rich fractions following DAMGO treatment (Gaibelet et al., 2008). On the other hand, the above studies do agree that MOR localization in fractions containing membrane microdomain markers is dependent on G protein-coupling. For instance, the agonist-mediated redistribution of MOR to cholesterol-enriched domains was attenuated when membranes were treated with the nucleotide analogue GppNHp (Gaibelet et al., 2008). Furthermore, down-regulation of \( \text{G}_{\alpha_{i2}} \) by antisense or expression of a MOR mutant lacking the sequence \(^{276}\text{RRITR}^{280}\), which prevents interaction with G proteins, resulted in the detection of MOR mostly outside of the caveolin-containing fractions. This was in contrast to wild-type MOR, which was found in caveolin-containing fractions, indicating that there may have been some precoupling of MOR to G proteins under basal conditions (Zheng et al., 2008a).

Thus, while conflicting evidence suggests MOR can function without microdomain localization, there may still be a role of membrane microdomains and/or cholesterol in optimizing MOR signaling.

### Cholesterol and/or membrane microdomains in DOR signaling

Similar to MOR, conflicting reports exist about the role of cholesterol and/or membrane microdomains for DOR signaling, as summarized in Table 1.2. In NG 108-15 cells, DORs were found in two different fractions of the plasma membrane that varied in G protein concentration (Ott et al., 1989). More recently, using 1 % Triton X-100 to separate insoluble plasma membrane domains, DOR was excluded from caveolin-containing fractions (Eisinger and Ammer, 2009). Other evidence suggests that DOR is only found in cholesterol-enriched fractions if very low (< 0.3%) concentrations of Triton X-100 are used, which likely does not completely separate soluble and resistant membranes (Andre et al., 2008). These results agree with studies showing that unliganded DOR incorporates preferentially into phosphatidylcholine rich domains of model membranes (Alves et al., 2003), but disagree with studies using a non-detergent separation method that found DOR in caveolin- or cholesterol-enriched fractions from CHO cells, NG 108-15 cells and cardiac myocytes (Patel et al., 2006; Huang et al., 2008).
These differences can be attributed to the stringency of the separation method utilized, as proper separation was not always confirmed using non-raft controls, such as transferrin receptor.

Conflicting reports also abound regarding the effect of agonist treatment on DOR distribution. In model membranes, agonist-bound DOR incorporated preferentially into sphingomyelin-rich domains (Alves et al., 2003). This was attributed to the previously observed elongation of DOR, identified by plasmon-waveguide resonance (PWR) spectroscopy, following agonist (Deltorphin II) binding which may cause DOR to distribute to thicker \( l_0 \) domains (Salamon et al., 2002; Alves et al., 2003). In the study, G proteins bound with 30-fold higher affinity to DOR in sphingomyelin-enriched membranes than to DOR in phosphocholine-rich membranes, suggesting that the active conformation was stabilized in the sphingomyelin-enriched domains (Alves et al., 2003). In HEK293 cells, addition of the DOR agonist deltorphin II lead to a two-fold increase in the amount of DOR in cholesterol-enriched fractions and, similar to MOR, this redistribution of DOR was prevented by the addition of GppNHp (Andre et al., 2008). However, in a different study also using HEK293 cells, addition of etorphine and morphine did not change the distribution of DOR from transferrin receptor-containing fractions (Eisinger and Ammer, 2009). The complete opposite situation was also observed. In a study using the non-detergent separation method, DOR resided in fractions with caveolin under basal conditions and partially partitioned out of caveolin-enriched fractions following agonist treatment (Huang et al., 2007a).

As with MOR, removal of cholesterol has been shown to have differing effects on DOR signaling. In one study, removal of cholesterol using \( \text{MβCD} \) decreased the ability of the DOR agonist DPDPE to stimulate \([^{35}\text{S}]\text{GTPyS}\) binding (Andre et al., 2008) even though DOR was found primarily in Triton-soluble membranes. The study that previously found DOR in cholesterol-enriched fractions also showed that \( \text{MβCD} \) treatment of NG 108-15 cells or caudate putamen membranes reduced the ability of DPDPE to stimulate \([^{35}\text{S}]\text{GTPyS}\) binding. However, in this study, treatment of CHO cells stably expressing DOR with \( \text{MβCD} \) increased DPDPE-stimulated \([^{35}\text{S}]\text{GTPyS}\)
binding (Huang et al., 2007a). These conflicting results were attributed to the presence of caveolin in CHO cells, but not NG 108-15 cells or caudate putamen.

**Cholesterol and/or membrane microdomains in signaling of KOR and NOPr**

The localization and effect of cholesterol removal on KOR and NOPr has not been as extensively studied. The most recent study identified KOR in cholesterol and caveolin-enriched fractions of a gradient prepared from CHO cells using a non-detergent method (Xu et al., 2006). Treatment of cells with MβCD to deplete cholesterol enhanced the ability of the KOR agonist U50,488H to stimulate \[^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding, which is identical to findings published by the same group regarding the effect of MβCD treatment on CHO cells expressing MOR or DOR (Huang et al., 2007a; Huang et al., 2007b). MβCD treatment also enhanced ERK1/2 phosphorylation by the KOR agonist U50,488H (Xu et al., 2006). The effects of MβCD on \[^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding and ERK1/2 phosphorylation were reversible when cholesterol was reintroduced to the cells. These results are counterintuitive when considering a previously published study that reported a significant decrease in high-affinity KOR binding to \[^3\text{H}]\text{U69,593}\) following incorporation of the cis-unsaturated oleic acid to synaptosomal membranes, which increased membrane fluidity and would theoretically also destabilize membrane microdomains (Lazar and Medzihradsky, 1992). Less is known about the role of cholesterol or membrane interactions on NOPr signaling. In a single study, NOPr was found in Triton-resistant membranes that also contained flotillin (Butour et al., 2004). This localization was not altered by NOP pretreatment and these receptors were functionally coupled to G proteins.

**Cholesterol in opioid withdrawal**

Many AC isoforms have been identified in cholesterol- or caveolin-enriched fractions of plasma membrane, including AC 3, 5, 6 and 8 (Fagan et al., 2000; Ostrom et al., 2001; Ostrom et al., 2002; Smith et al., 2002; Crossthwaite et al., 2005; Zhao et al., 2006). These are virtually the same AC isoforms (1, 5, 6 and 8) that are inhibited by Ga\(_{i/o}\) and sensitized by chronic opioid treatment. Furthermore, many of the signaling
proteins implicated in the mechanism of AC sensitization have been identified in caveolin- or cholesterol-containing fractions, including Src family kinases (Liang et al., 2001; Mukherjee et al., 2003), Raf-1 (Mineo et al., 1996), and PKC (Weerth et al., 2007).

Multiple lines of evidence suggest that cholesterol plays a modulatory role in AC activity. Depletion of cholesterol by MβCD enhances activation of AC by forskolin or a Gαs-coupled receptor agonist (Rybin et al., 2000; Pontier et al., 2008). The enhancement in AC activity following MβCD treatment is not nearly as pronounced as the overshoot that can be produced following opioid withdrawal so, theoretically, cAMP overshoot could still be observed after cholesterol removal. However, MβCD pretreatment prevents AC sensitization by morphine in HEK293 cells stably expressing MOR (Zhao et al., 2006). It has been suggested that AC sensitization develops in membrane microdomains following the activation and recruitment of Src kinases by chronic morphine treatment (Zhang et al., 2009). The Src kinases are proposed to phosphorylate MOR allowing the receptor to switch to stimulatory signaling (Zhang et al., 2009). Recently, dephosphorylated Gαs has been shown to couple to MOR in caveolae (Chakrabarti et al., 2010).

Chronic treatment with agonists for MOR, KOR or M₄-muscarinic receptors that cause AC sensitization increased the amount of Gβ₁ and Gαᵢ found in 1% sodium cholate-insoluble particulate with a parallel decrease in the cholate-soluble fraction (Bayewitch et al., 2000). This solubility shift was not seen with Gαs, was blocked by PTX, and was reversible on a time-scale similar to the loss of AC sensitization (Bayewitch et al., 2000). These results suggest that a shift of Gαᵢ and/or Gβγ into cholate-resistant membranes contributes to AC sensitization or that AC sensitization occurs in membrane microdomains. For the latter, one might have expected a shift in Gαs into detergent-resistant membranes as well, since increased Gαs input has been suggested as one mechanism of AC sensitization. If anything, the opposite shift may occur since chronic morphine treatment depalmitoylates Gαs (Ammer and Schulz, 1997) and palmitoylation helps target G proteins into detergent-resistant membranes. Furthermore, depalmitoylated Gαs may be more effective at stimulating AC (Ammer and Schulz, 1997). In contrast, another study found that long-term (24 h) morphine treatment
reduced the amount of $\alpha_{i2}$, $\alpha_{i3}$, $\beta_1$ and $\beta_2$ in 1% Triton-resistant membranes (Mouledous et al., 2005), but this may represent degradation rather than a solubility shift. These results suggest that AC sensitization may occur outside of membrane microdomains where depalmitoylated $\alpha_s$ would partition, while $\alpha_i$ is sequestered into rafts and away from AC. These results are consistent with the movement of high-affinity, $\alpha_i$ protein bound MOR into membrane microdomains described above. However, it conflicts with evidence that dephosphorylated $\alpha_s$ has been shown to couple to MOR in caveolae after treatment with the agonist sufentanil (Chakrabarti et al., 2010).

Membrane cholesterol and/or microdomains in vivo

There is evidence that AC sensitization and subsequent withdrawal-induced cAMP overshoot may mediate certain opioid withdrawal symptoms in vivo. In mice lacking AC 5, which is highly expressed in the striatum, some morphine withdrawal signs are attenuated, including weight loss, sniffing, teeth chattering, ptosis and body tremor (Kim et al., 2006). Furthermore, morphine-dependent mice lacking AC 1 and 8 showed significantly less withdrawal-induced jumping, paw tremor and diarrhea (Zachariou et al., 2008). Hyperactivity of noradrenergic LC neurons has been observed following opioid withdrawal (Aghajanian, 1978). This hyperactivity may be partially mediated by AC sensitization since in vitro it can be suppressed by PKA inhibitors and is enhanced by forskolin or an active cAMP analog (Ivanov and Aston-Jones, 2001). In addition, withdrawal-induced hyperactivity is not observed for neurons isolated from the LC of morphine-dependent, AC 1 and 8 knock-out mice (Zachariou et al., 2008).

Results regarding the role of cholesterol in cAMP overshoot must be interpreted carefully when attempting to apply them to the opioid withdrawal syndrome observed in vivo. In healthy adults, brain cholesterol is very tightly regulated, so cholesterol levels are likely to only vary on a subcellular level. Neurons contain many subcellular compartments in the form of synapses, which have been hypothesized to form with the help of cholesterol-binding to proteins including caveolin and synaptophysin (Thiele et al., 2000; Head and Insel, 2007). Although chronic morphine treatment of rats increased the cholesterol to phospholipid ratio in hippocampus and caudate (Heron et al., 1982b),
these results do not indicate a mechanism or cellular location of the cholesterol increase. There is some evidence that membrane-fluidizing “natural lipids” decreased morphine withdrawal symptoms in mice (Heron et al., 1982a). However, attempts to modulate brain cholesterol or membrane viscosity as a therapeutic would be very non-specific and dangerous. Cholesterol is obviously very important to brain functioning since the brain, which is only 2% of the total body mass, contains 25% of the body’s unesterified cholesterol and almost all of the cholesterol in the brain is synthesized de novo (Dietschy and Turley, 2001). Total brain cholesterol levels are tightly regulated and cholesterol turnover is very slow with an estimated half-life of approximately 5 years (Bjorkhem and Meaney, 2004). However, there may be a therapeutic potential for cholesterol and/or membrane microdomain modifiers in some pathological conditions such as Niemann-Pick type C (NPC) and Alzheimer’s disease when the turnover of cholesterol is increased (Dietschy and Turley, 2001). In the case of NPC, a build-up of cholesterol occurs which has been experimentally treated with cyclodextrins (Liu et al., 2009). Furthermore, other neurodegenerative diseases such as Krabbe disease and Huntington’s disease have altered metabolism of cholesterol and/or sphingolipids (Korade and Kenworthy, 2008; White et al., 2009). Thus, in addition to the importance of understanding the role of cholesterol and membrane microdomains in the organization of cell signaling, there may be applications to diseases characterized by altered metabolism of cholesterol or sphingolipids in the brain.

**Hypothesis and Aims**

The overall aim of this thesis is to investigate factors that can influence the interaction between GPCR and G protein; namely, the lipid environment (especially the role of cholesterol) and competition between multiple receptors, using opioid receptors as a model.

**Aim 1:** In the first data chapter, I test the hypothesis that coupling of agonist-bound MOR to G protein is dependent on cholesterol concentration. Experiments were performed in HEK293 cells stably expressing FLAG-tagged MOR or DOR to answer the following
questions: Is cholesterol differentially important for MOR or DOR signaling? Does cholesterol stabilize certain receptor conformations? Does localization of MOR and DOR to membrane microdomains correlate with the role of cholesterol in signaling?

Aim 2: In the second data chapter, experiments were performed to examine the hypothesis that compartmentalization of MOR receptors controls interactions with other co-expressed GPCRs. Differentiated human neuroblastoma SH-SY5Y cells were employed to answer the following questions regarding the organization of endogenously expressed $G_{i/o}$-coupled receptors, including MOR and DOR: Is there compartmentalization of signaling in SH-SY5Y cells that leads to sharing of adenylyl cyclase? Does compartmentalization occur during acute and chronic signaling? Can activation of receptors that share adenylyl cyclase with MOR prevent MOR-mediated cAMP overshoot?
Table 1.1: Ki (nM) for selected ligands at human MOR, DOR and KOR
Literature values for ligand binding as reported previously for receptors expressed in CHO cells. SNC80 binding experiments were performed in mouse brain membranes.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>MOR (³H-DAMGO)</th>
<th>DOR (³H-DPDPE)</th>
<th>KOR (³H-U69,593)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO</td>
<td>0.5 ± 0.05</td>
<td>300.0 ± 58.6</td>
<td>305.5 ± 46</td>
</tr>
<tr>
<td>SNC80*</td>
<td>881.5</td>
<td>1.78</td>
<td>441.8</td>
</tr>
<tr>
<td>DPDPE</td>
<td>503.6 ± 10</td>
<td>1.7 ± 0.1</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>U50,488</td>
<td>290.0 ± 14.3</td>
<td>&gt; 10,000</td>
<td>0.2 ± 0.05</td>
</tr>
<tr>
<td>Naloxone</td>
<td>1.4 ± 0.05</td>
<td>67.5 ± 40</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>CTAP</td>
<td>2.3 ± 0.65</td>
<td>365 ± 82</td>
<td>&gt; 10,000</td>
</tr>
<tr>
<td>Naltrindole</td>
<td>6.3 ± 2.3</td>
<td>0.2 ± 0.05</td>
<td>10.1 ± 0.65</td>
</tr>
<tr>
<td>ICI 174,864⁺</td>
<td>18900 ± 1100</td>
<td>703 ± 1</td>
<td>n.d.</td>
</tr>
<tr>
<td>Nor-BNI</td>
<td>21.0 ± 5</td>
<td>5.7 ± 0.9</td>
<td>0.2 ± 0.05</td>
</tr>
</tbody>
</table>

Values obtained from (Toll et al., 1998) except *SNC80 from (Bilsky et al., 1995), and ³IC₅₀ for ICI 174,864 from (Maeda et al., 2000). n.d., not determined
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell/tissue type</th>
<th>Method</th>
<th>Basal association</th>
<th>Agonist shift</th>
<th>Effect of cholesterol removal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>HEK293</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>Out (etorphine) n.c. (morphine)</td>
<td>↓ AC, ↓ ERK (morphine) ↓ AC, n.c. ERK (etorphine)</td>
<td>(Zheng et al., 2008a)</td>
</tr>
<tr>
<td>MOR</td>
<td>CHO</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>n.c. (etorphine, morphine)</td>
<td>↑ $[^{35}$S]GTP$\gamma$S (etorphine, morphine, DAMGO)</td>
<td>(Huang et al., 2007b)</td>
</tr>
<tr>
<td>MOR</td>
<td>Rat CPu</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>n.c. (morphine)</td>
<td>↓ $[^{35}$S]GTP$\gamma$S (morphine, DAMGO)</td>
<td>(Huang et al., 2007b)</td>
</tr>
<tr>
<td>MOR</td>
<td>CHO</td>
<td>Triton X-100</td>
<td>Non-raft</td>
<td>In (DAMGO)</td>
<td>↓ $[^{35}$S]GTP$\gamma$S (DAMGO)</td>
<td>(Gaibele et al., 2008)</td>
</tr>
<tr>
<td>DOR</td>
<td>CHO</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>Out (etorphine)</td>
<td>↑ $[^{35}$S]GTP$\gamma$S (DPDPE), ↓ DPDPE binding</td>
<td>(Huang et al., 2007a)</td>
</tr>
<tr>
<td>DOR</td>
<td>NG 108-15</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>Out (etorphine)</td>
<td>↓ $[^{35}$S]GTP$\gamma$S (DPDPE), ↓ DPDPE binding</td>
<td>(Huang et al., 2007a)</td>
</tr>
<tr>
<td>DOR</td>
<td>Rat CPu</td>
<td>Na$_2$CO$_3$</td>
<td>Raft</td>
<td>n.d.</td>
<td>↓ $[^{35}$S]GTP$\gamma$S (DPDPE, SNC80)</td>
<td>(Huang et al., 2007a)</td>
</tr>
<tr>
<td>DOR</td>
<td>HEK293</td>
<td>Triton X-100</td>
<td>Non-raft</td>
<td>n.c. (etorphine, morphine)</td>
<td>n.d.</td>
<td>(Eisinger and Ammer, 2009)</td>
</tr>
<tr>
<td>DOR</td>
<td>HEK293</td>
<td>Triton X-100</td>
<td>Non-raft</td>
<td>In (delt II)</td>
<td>↓ $[^{35}$S]GTP$\gamma$S, ↓ delt II binding</td>
<td>(Andre et al., 2008)</td>
</tr>
<tr>
<td>DOR</td>
<td>POPC/SM bilayer</td>
<td>PWR</td>
<td>POPC (non-raft)</td>
<td>SM (“in”) (DPDPE)</td>
<td>n.d.</td>
<td>(Alves et al., 2003)</td>
</tr>
<tr>
<td>KOR</td>
<td>CHO</td>
<td>Na$_2$CO$_3$</td>
<td>In</td>
<td>n.d.</td>
<td>↑ $[^{35}$S]GTP$\gamma$S, ↑ ERK (U50,488), ↑ U50 binding</td>
<td>(Xu et al., 2006)</td>
</tr>
<tr>
<td>NOPr</td>
<td>HEK293</td>
<td>Triton X-100</td>
<td>In</td>
<td>n.c. (NOP)</td>
<td>n.d.</td>
<td>(Butour et al., 2004)</td>
</tr>
</tbody>
</table>

n.c., no change; n.d., not determined; CTB, Cholera Toxin B subunit; CPu, caudate putamen, POPC, palmitoyloleoylphosphatidylcholine; SM, sphingomyelin; PWR, plasmon-waveguide resonance spectroscopy; delt II, deltorphin II
Figure 1.1: Schematic of opioid receptor signaling

Agonist binding to opioid receptors stimulates the exchange of GTP for GDP, thus activating G proteins resulting in downstream effects from $\text{G}^{\alpha}$ and $\text{G}^{\beta\gamma}$ subunits. The main effect of $\text{G}^{\alpha}_{i/o}$ is inhibition of AC. $\text{G}^{\beta\gamma}$ can activate G protein-coupled inwardly-rectifying potassium (GIRK) channels, mitogen-activated protein kinases (MAPK), phospholipase C (PLC) and non-receptor tyrosine kinases (e.g. Src family). $\text{G}^{\beta\gamma}$ inhibits N-type calcium channels. $\text{G}^{\beta\gamma}$ modulates AC in an isoform-specific manner, inhibiting some isoforms (e.g. AC 1) while activating other isoforms (e.g. AC 2).
Chapter II

Differential effect of membrane cholesterol removal on MOR and DOR: A parallel comparison of acute and chronic signaling to adenylyl cyclase

Summary

According to the lipid raft theory, the plasma membrane contains small domains enriched in cholesterol and sphingolipid which may serve as platforms to organize membrane proteins. Using methyl-β-cyclodextrin (MβCD) to deplete membrane cholesterol, many G protein-coupled receptors have been shown to depend on putative lipid rafts for proper signaling. Here, we examine the hypothesis that treatment of HEK293 cells stably expressing the FLAG-tagged mu opioid receptor (HEK FLAG-MOR) or delta opioid receptor (HEK FLAG-DOR) with MβCD will reduce opioid receptor signaling to adenylyl cyclase. The ability of the MOR agonist DAMGO to acutely inhibit adenylyl cyclase or to cause sensitization of adenylyl cyclase following chronic treatment was attenuated with MβCD. These effects were due to the removal of cholesterol, since replenishment of cholesterol restored DAMGO responses back to control values, and were confirmed in SH-SY5Y cells endogenously expressing MOR. The effects of MβCD may be due to uncoupling of MOR from G proteins, but were not due to decreases in receptor number and were not mimicked by cytoskeleton disruption. In contrast to the results in HEK FLAG-MOR cells, MβCD treatment of HEK FLAG-DOR cells had no effect on acute inhibition or sensitization of adenylyl cyclase by DOR agonists. The differential responses of MOR and DOR agonists to cholesterol depletion

suggest that MOR are more dependent on cholesterol for efficient signaling than DOR and can be partly explained by localization of MOR, but not DOR, in cholesterol- and caveolin-enriched membrane domains.

**Introduction**

Membrane cholesterol can alter the function of integral proteins, such as G protein-coupled receptors, through cholesterol-protein interactions and by changes in membrane viscosity (Gimpl et al., 1997). In addition, cholesterol interacts with other lipids found in the bilayer, particularly sphingolipids (Slotte, 1999), which allows for tight and organized packing that can precipitate the formation of specialized domains within the plasma membrane (Simons and Ikonen, 1997). These domains have become an area of intense research interest, and have been termed lipid or membrane rafts (Pike, 2006). The study of membrane rafts in intact cells is controversial, due in part to the limitations of the current methods used to study rafts (Munro, 2003; Shogomori and Brown, 2003). Regardless, the membrane environment formed in regions of high cholesterol and sphingolipids may be such that certain proteins have an affinity for these regions, especially proteins with a propensity to interact with cholesterol.

Many G protein-coupled receptors and signaling proteins have been found to prefer cholesterol-enriched domains leading to the hypothesis that these domains can organize signaling molecules in the membrane in order to enhance or inhibit specific signaling events (Allen et al., 2007). This includes MOR (Head et al., 2005; Zhao et al., 2006), DOR (Patel et al., 2006; Huang et al., 2007a) and KOR (Xu et al., 2006). In addition, Gαi (Li et al., 1995; Li et al., 1996; Moffett et al., 2000; Foster et al., 2003; Quinton et al., 2005; Xu et al., 2006), Gαo (Li et al., 1995) and adenylyl cyclase isoforms 3 (Ostrom et al., 2002), 5/6 (Fagan et al., 2000; Ostrom et al., 2002; Zhao et al., 2006) and 8 (Smith et al., 2002) have been found to associate with cholesterol and/or the cholesterol binding protein caveolin. Activated opioid receptors couple to Gαi/o proteins and acutely inhibit the activity of adenylyl cyclase. Longer term exposure to opioid agonists causes sensitization of adenylyl cyclase and a rebound overshoot of cAMP production upon withdrawal of the agonist (Watts, 2002). Consequently, we sought to
assess the role of cholesterol depletion on the ability of MOR and DOR agonists to inhibit, and cause sensitization of, adenylyl cyclase.

There are conflicting data for the effect of changes in membrane cholesterol on opioid signaling. For example, an increase in plasma membrane microviscosity by addition of cholesteryl hemisuccinate to SH-SY5Y cell membranes increased MOR coupling to G proteins (Emmerson et al., 1999). Conversely, removal of membrane cholesterol from CHO cells has been shown to either decrease (Gaibelet et al., 2008) or increase (Huang et al., 2007b) the coupling of MOR to G proteins, as measured by $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding stimulated by the MOR agonist DAMGO. Furthermore, the effect of cholesterol removal on DOR agonist-stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ binding varies by cell type (Huang et al., 2007a; Andre et al., 2008). In these previous studies, the variety of cell types utilized and the conflicting results makes comparisons between opioid receptor types difficult. The objective of this study was to directly compare the role of membrane cholesterol in modulating acute and chronic MOR and DOR signaling in the same cell systems using identical methods, including 1) depletion of cholesterol by the cholesterol-sequestering agent methyl-$\beta$-cyclodextrin (M$\beta$CD), 2) separation of cholesterol-enriched membranes by sucrose gradient ultracentrifugation and 3) clustering of lipid raft patches in whole cells with cholera toxin B subunit.

In initial experiments using human embryonic kidney (HEK) cells heterologously expressing MOR or DOR, we found that DOR was located in caveolin-poor fractions following 1% Triton X-100 homogenization and sucrose gradient ultracentrifugation. This differs from studies using a detergent-free method to identify lipid raft fractions (Patel et al., 2006; Huang et al., 2007a). In contrast, we found that MOR was found in both caveolin-poor and caveolin-rich fractions, in accordance with previous literature (Head et al., 2005; Zhao et al., 2006). This differential localization of opioid receptors led us to test the hypothesis that, in contrast to MOR, DOR would not be dependent on cholesterol for signaling. The results show that MOR, but not DOR, have a dependence on cholesterol for signaling to adenylyl cyclase and that this effect is much more pronounced following chronic exposure to opioids.
Materials and Methods

**Materials** - SNC80 ((+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide), DPDPE ([D-Pen\(^{2,5}\)]-enkephalin) and naltrindole hydrochloride were obtained from the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). Lovastatin hydroxy acid was obtained from Cayman Chemical (Ann Arbor, MI). \(^{[3H]}\)diprenorphine, \(^{[3H]}\)DAMGO ([D-Ala\(^2\), N-Me-Phe\(^4\), Gly\(^5\)-ol]-enkephalin) and \(^{[35S]}\)GTP\(_{\gamma}\)S were obtained from Perkin-Elmer Life Sciences (Boston, MA). Tissue culture media, geneticin, fetal bovine serum and trypsin were from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Cell culture** - Human embryonic kidney 293 cells stably transfected with the N-terminal FLAG-tagged DOR (HEK FLAG-DOR) or MOR (HEK FLAG-MOR) were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 0.8 mg/ml geneticin and 10% fetal bovine serum at 37°C in 5% CO\(_2\). Receptor expression in HEK FLAG-DOR (8.4 ± 1.5 pmol/mg protein; n = 5) and HEK FLAG-MOR (9.7 ± 1.3 pmol/mg protein; n= 5) cells was similar (p > 0.05). Receptor expression was measured by saturation binding of the opioid antagonist \(^{[3H]}\)diprenorphine as described previously (Emmerson et al., 1999). SH-SY5Y cells were grown as above but without geneticin. SH-SY5Y cells were differentiated by adding 10 µM retinoic acid (Calbiochem, La Jolla, CA) 3 – 5 days prior to assay.

**Cholesterol modulation** - HEK FLAG-MOR or HEK FLAG-DOR cells were grown to confluence in DMEM + 10% FBS. Media was replaced with serum-free DMEM with or without 2% (15 mM) methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich, St. Louis, MO) for 1 h, 37°C. SH-SY5Y cells were treated with 5 mM MβCD for 10 min. For cholesterol replenishment, cells were incubated with or without a 2% MβCD-cholesterol complex (MβCD-CH) in serum-free DMEM for 2 h following cholesterol depletion. MβCD-cholesterol complexes were formed in an 8:1 molar ratio as described previously (Christian et al., 1997). Briefly, cholesterol was dissolved in a 1:1 ratio by volume of chloroform/methanol in a glass tube. Following evaporation of the solvent, the dried cholesterol was reconstituted with a suitable volume of serum-free DMEM.
containing 2% MβCD, vortexed, sonicated for 30 s and incubated overnight at 37°C with shaking. For lovastatin experiments, cells were treated with serum-free Opti-MEM containing 10 µM lovastatin hydroxy acid or DMSO vehicle for 48 h. Cholesterol content from cell lysates was determined using the Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Cholesterol content was normalized to protein content, as determined by the method of Bradford (Bradford, 1976).

Stimulation of $[^{35}S]$GTPγS binding- Membranes were prepared from HEK FLAG-DOR or FLAG-MOR cells following treatment with or without 2% MβCD as described previously (Clark et al., 2003). Final membrane pellets were resuspended in 50 mM Tris-HCl buffer, aliquoted and stored at -80°C. Protein concentration was measured using the Bradford assay (Bradford, 1976).

Membranes (30 µg protein) were incubated with 0.1 nM $[^{35}S]$GTPγS for 60 min at 25°C, with or without various concentrations of the DOR agonist SNC80 or the MOR agonist DAMGO in $[^{35}S]$GTPγS binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl, 1mM EDTA, 2 mM dithiothreitol and 30 µM GDP). Membranes with bound $[^{35}S]$GTPγS were collected on GF/C filters (Whatman, Middlesex, UK) using a Brandel harvester (MLR-24, Gaithersburg, MD) and rinsed three times with cold wash buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 100 mM NaCl). Dried filters were saturated with EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted in a Wallac 1450 MicroBeta (PerkinElmer, Waltham, MA).

Cyclic AMP accumulation assays- Inhibition of adenylyl cyclase activity was measured in HEK FLAG-DOR or FLAG-MOR cells grown to confluence in 24-well poly-D-lysine coated plates. Cells were washed with serum-free DMEM and incubated with various concentrations of the DOR agonist SNC80 or the MOR agonist DAMGO in the presence of 5 µM forskolin and 1 mM 3-Isobutyl-1-methylxanthine (IBMX) in serum-free media for 10 min at 37°C. The assay was stopped by replacing the media with 1 ml ice-cold 3% perchloric acid. After at least 30 min at 4°C, a 400 µl aliquot of sample was neutralized with 2.5 M KHCO₃ and centrifuged at 13,000 x g. Cyclic AMP was measured from the supernatant using a $[^{3}H]$cAMP assay system (GE Healthcare,
Buckinghamshire, UK) following the manufacturer’s instructions. Inhibition of cAMP formation was calculated as a percent of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

For adenylyl cyclase sensitization experiments, HEK cells were rinsed with serum-free DMEM and incubated in the presence or absence of SNC80, DPDPE or DAMGO in serum-free DMEM for 30 min at 37°C. SH-SY5Y cells, plated in uncoated 24-well plates (5 x 10^5 cells / well) and differentiated with 10 μM retinoic acid for 3 – 5 days prior to sensitization experiments, were incubated in the presence or absence of 1 μM DAMGO for 60 min at 37°C. The drug-containing media was then removed and replaced with serum-free media containing 5 μM forskolin, 1 mM IBMX and 10 μM of the opioid antagonist naloxone for HEK FLAG-MOR cells and SH-SY5Y cells, or 10 μM of the DOR antagonist naltrindole for HEK FLAG-DOR cells, to precipitate cAMP overshoot. After 10 min at 37°C, the assay was stopped with ice cold 3% perchloric acid and cAMP accumulation was quantified as described above. Overshoot was calculated as a percent of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

Radioligand binding assays- In competition binding assays, membranes (5 - 12 μg protein) from HEK FLAG-MOR or DOR cells treated with or without 2% MβCD were incubated for 1 h with shaking at 25°C with 0.2 nM [3H]diprenorphine and increasing concentrations of unlabeled ligand (DAMGO or SNC80) in 50 mM Tris-HCl, pH 7.4. Where indicated, a final concentration of 10 μM GTPγS and 100 mM NaCl was added to the incubation buffer. For saturation binding assays, membranes (20 μg protein) from low-expressing HEK FLAG-MOR cells (1.6 ± 0.1 pmol/mg protein) were incubated with increasing concentrations of [3H]diprenorphine [0.08 – 5 nM] or [3H]DAMGO [0.06 – 12 nM] in 50 mM Tris buffer, pH 7.4, for 1 h with shaking at 25°C. For whole cell binding, HEK FLAG-MOR cells (1 x 10^5 cells/tube) were incubated for 1 h in a 37°C shaking water bath with 4 nM [3H]diprenorphine ± 10 μM CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2) in serum-free DMEM. Protein content from a representative aliquot was determined by the method of Bradford (1976). For all binding assays, non-specific binding was determined using 10 μM naloxone. All assays were stopped by rapid filtration through GF/C filters using a Brandel harvester (MLR-24, Gaithersburg,
MD) and rinsed three times with ice-cold 50 mM Tris-HCl wash buffer, pH 7.4. Bound radioactivity was determined by liquid scintillation counting as described in $[^{35}S]GTP\gamma S$ methods.

Separation of Detergent Resistant Membranes- HEK FLAG-DOR or FLAG-MOR cells were grown to confluence in 10 cm$^2$ dishes, washed and resuspended in ice-cold phosphate buffered saline (PBS). Cells were pelleted and homogenized with a Dounce homogenizer in 100 µl MES buffered saline (MBS) containing 1% Triton X-100. The homogenate was placed on the bottom of a 2.2 ml ultracentrifuge tube, adjusted to 40% by addition of 53.3% sucrose in MBS and overlaid with 900 µl of 30% sucrose and 900 µl of 5% sucrose in MBS for a discontinuous gradient. The samples were centrifuged at 200,000 x g in a Beckman-Coulter (Fullerton, CA) Optima Max-E Ultracentrifuge using a swinging bucket rotor (TLS-55) for 16-24 h at 4°C. Equal volume (183 µl) fractions were collected from the top. Equal volume aliquots were taken from each fraction, mixed with sample buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.008% bromophenol blue, 50 mM DTT), separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL) for western blotting. Antibodies used were monoclonal anti-FLAG M1 (1:2000; Sigma-Aldrich, St. Louis, MO), polyclonal anti-caveolin (1:2000; B.D. Transduction Laboratories, Rockville, MD) and monoclonal anti-human transferrin receptor (TfR) (1:2000; Zymed Laboratories, Carlsbad, CA). Secondary antibodies used were goat anti-mouse HRP or goat anti-rabbit HRP (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). Above antibodies were diluted in 5% milk in TBS-0.05% Tween 20 (+1 mM CaCl$_2$ for FLAG M1 antibody). SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) was used to detect immunoreactivity. For detection of adenylyl cyclase (AC) 5/6, samples were separated on a 6% polyacrylamide gel and transferred to an Immobilon-P polyvinylidene fluoride membrane (Millipore, Billerica, MA). AC 5/6 was detected by rabbit anti-AC 5/6 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in 1% BSA in TBS-0.05% Tween 20.
Cholera toxin B-induced Patching- HEK FLAG-DOR or HEK FLAG-MOR cells were plated on poly-D-lysine coated coverslips in a 6-well plate (1x10^6 cells/well) 24 h prior to patching. Cells were incubated with AlexaFluor 488-conjugated cholera toxin B subunit (1 μg/μl in DMEM, 10% goat serum; Invitrogen, Carlsbad, CA) for 45 min at 4°C to label endogenous ganglioside GM1. Lipid raft aggregation, or patching, was induced as described previously (Fra et al., 1994) by incubating with goat anti-CTB antibody (1:250 in DMEM, 10% goat serum; Calbiochem, San Diego, CA) for 30 min at 4°C, followed by 20 min at 37°C. Cells were fixed with 4% paraformaldehyde for 20 min and incubated in monoclonal anti-FLAG M1 antibody (1:1000 in PBS, 5% milk; Sigma-Aldrich, St. Louis, MO) for 1 h followed by AlexaFluor 594-conjugated goat anti-mouse antibody (1:1000 in PBS, 5% milk; Invitrogen, Carlsbad, CA) to stain the FLAG-tagged MOR or DOR. For transferrin receptor (TfR) and caveolin staining, cells were permeabilized with 0.1% Triton X-100 for 10 min and incubated with monoclonal anti-TfR antibody (1:200 in PBS, 5% milk; Zymed, Carlsbad, CA) or polyclonal anti-caveolin antibody (1:200 in PBS, 5% milk; B.D. Transduction Laboratories, Rockville, MD). Coverslips were mounted on slides using ProLong Gold (Invitrogen, Carlsbad, CA). Fluorescent images of 0.5 μm Z planes were captured using an Olympus FV-500 confocal microscope.

Quantification of colocalization was performed using the RG2B colocalization plug-in to ImageJ (version http://rsb.info.nih.gov). The minimum threshold pixel intensity was set to 50 for both channels and the minimum ratio for pixel intensity between the channels was 50%. Colocalization pixels from individual Z planes were displayed as a stack of Z projections with maximum pixel intensity. Co-localization was reported as the average pixel density of co-localized pixels per cell.

Statistical Analysis- All data were analyzed using GraphPad Prism 4 software (San Diego, CA). All data points represent at least three separate experiments in duplicate and are presented as mean ± standard error of the mean (S.E.M.), unless otherwise noted. The effect of treatment on agonist responses at various concentrations was analyzed by 2-way ANOVA with Bonferroni’s post-hoc test. EC50 values were calculated from individual concentration-effect curves using fixed slope sigmoidal dose-
response curve fit analysis in GraphPad Prism. Ki and Bmax/Kd values were calculated from individual binding experiments using one or two-site competition or one-site hyperbola binding curve fit linear regression analysis, respectively. EC50, Ki, Bmax and Kd values are expressed as mean ± S.E.M. and compared for statistical significance by unpaired, two-tailed Student’s t-test. Cholesterol repletion experiments were compared using 1-way ANOVA with Bonferroni’s post-hoc test. All other statistical comparisons were made with unpaired, two-tailed Student’s t-test, unless otherwise indicated. For all tests significance was set at p < 0.05.

Results

Effect of cholesterol depletion on opioid receptor coupling to G protein

HEK293 cells stably expressing either the FLAG-MOR or FLAG-DOR were treated with the cholesterol-sequestering agent MβCD (2%) for 1 h at 37°C. This reduced cholesterol to 40 ± 5.6 % of control, consistent with previously published results of 40 - 60 % reductions (Rodal et al., 1999; Xu et al., 2006; Pontier et al., 2008), and induced rounder cell morphology, although cells were still viable by trypan blue exclusion. This treatment has been shown to eliminate caveolae as determined by electron microscopy (Rodal et al., 1999), and has been commonly used to disrupt lipid rafts in order to study effects on opioid signaling (Patel et al., 2006; Xu et al., 2006; Zhao et al., 2006; Huang et al., 2007a). Therefore, we used this pharmacological tool to directly compare the effects of cholesterol depletion on MOR and DOR signaling.

Agonist-activated opioid receptors couple to Gαi/o proteins and induce the exchange of GTP for GDP, which can be measured by the increase in binding of the guanine nucleotide analog [35S]GTPγS. Basal levels of [35S]GTPγS binding were similar in membranes from untransfected HEK293 cells and HEK cells expressing MOR, but were higher in membranes from cells expressing DOR (Figure 2.1a), which are thought to be tightly coupled to G proteins and show constitutive activity (Costa et al., 1988).

Treatment of either HEK FLAG-MOR cells or untransfected HEK293 cells with 2 % MβCD for 1 h reduced basal [35S]GTPγS binding by 38 ± 5.5 % or 39 ± 2.2 %, respectively (Figure 2.1a), suggesting an opioid receptor-independent effect. In contrast,
treatment of HEK FLAG-DOR cells with MβCD reduced basal levels of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding by \(61 \pm 1.7\%\). However, the DOR inverse agonist RTI-5989-25 (Zaki et al., 2001) was able to reduce \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding by \(18 \pm 3.8\%\) in control HEK FLAG-DOR cells (data not shown). Therefore, in HEK FLAG-DOR cells approximately one-third of the decrease in basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding caused by MβCD may be due to a loss of constitutively active receptors. The remaining decrease, which is similar to the decrease in HEK FLAG-MOR or untransfected HEK293 cells, is likely due to a reduction in available, unoccupied Go proteins themselves or a loss of constitutive activity of other G protein-coupled receptors endogenous to HEK293 cells.

Due to this decrease in basal \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding, data were graphed as fmol of agonist-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) bound/mg protein rather than as percent change over basal, as has been reported previously (Huang et al., 2007a; Huang et al., 2007b; Andre et al., 2008). Treatment of HEK FLAG-DOR or FLAG-MOR cells with MβCD did not alter maximal stimulation of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) by the DOR agonist, SNC80 \((p = 0.557)\), or the MOR agonist, DAMGO \((p = 0.200)\), respectively (Figure 2.1b and c). The potency of SNC80 to stimulate \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding was also similar in control \((\text{EC}_{50} = 4.9 \pm 2.4\text{ nM})\) and MβCD-treated cells \((\text{EC}_{50} = 5.2 \pm 0.8\text{ nM})\) \((p = 0.898; \text{Figure 2.1b})\). In contrast, MβCD treatment caused a significant rightward shift in the ability of DAMGO to stimulate \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding (Figure 2.1c) \([\text{treatment: } F(1, 92) = 9.838, p = 0.002]\). This was reflected by a 4-fold increase in \(\text{EC}_{50}\) from 16.8 \(\pm\) 9.3 nM in control cells to 73.0 \(\pm\) 26.5 nM in MβCD-treated cells; although variability precluded significance \((p = 0.116)\). Even though this difference in G protein activation between MOR and DOR in response to cholesterol removal is small, it does disagree with previous reports using heterologous systems that show an effect of MβCD on DOR agonist-stimulated \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding (Huang et al., 2007a; Huang et al., 2007b; Andre et al., 2008). This could be due to cell types utilized or methods of data analysis since the basal level of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding does change. Regardless, the trend shown here suggests that cholesterol aids in efficient coupling of MOR to G proteins, but is not necessary for efficient DOR coupling.
The reduced ability of the MOR agonist DAMGO to stimulate \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding may be due to a loss of receptor-G protein coupling following cholesterol depletion. To test this hypothesis, we used a ligand binding approach. Receptors were labeled with the non-selective opioid antagonist \(^3\text{H}\)-diprenorphine and displaced by the DOR agonist SNC80 or the MOR agonist DAMGO, as appropriate. The DOR agonist SNC80 was best fit by a single, high-affinity binding site model (Ki = 0.78 ± 0.21 nM), although the slope of the displacement curve was less than unity (-0.77 ± 0.09) suggesting a population of DOR with slightly different agonist affinities. Regardless, this high-affinity binding was retained even after removal of cholesterol from the plasma membrane of HEK FLAG-DOR cells with M\(\beta\)CD (Ki = 0.87 ± 0.09 nM; slope = -0.72 ± 0.12) (Figure 2.2a). Similarly, the MOR agonist DAMGO displayed mainly high-affinity binding in control membranes (Ki = 4.9 ± 1.2 nM; slope = -0.76 ± 0.03). In contrast, DAMGO displacement (slope = -0.45 ± 0.05) was best fit to two affinity sites following removal of cholesterol from HEK FLAG-MOR cells (Ki high = 1.9 ± 0.3 nM; Ki low = 244 ± 49 nM) (Figure 2.2b). This M\(\beta\)CD-induced low-affinity site in HEK FLAG-MOR cells had a similar affinity to the site induced by uncoupling the receptor from G proteins with sodium ions and GTP\(\gamma\)S (Ki = 768 ± 82 nM). Moreover, the Ki induced by sodium ions and GTP\(\gamma\)S was not significantly reduced further in membranes from cells treated with M\(\beta\)CD (Ki = 1887 ± 712 nM). These results confirm the \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) experiments and indicate that removal of membrane cholesterol by M\(\beta\)CD generates two affinity states of MOR, likely due to uncoupling a proportion of MOR from G proteins, thus resulting in a loss of stimulatory activity of agonist on G proteins.

**Effect of cholesterol depletion on opioid inhibition of adenylyl cyclase**

To examine if a decrease in G protein activation by agonists translates to decreases in downstream responses within the cell, inhibition of adenylyl cyclase by MOR and DOR agonists was measured following cholesterol removal. Treatment of HEK FLAG-DOR cells with 2% M\(\beta\)CD did not alter the ability of the DOR agonist SNC80 to inhibit adenylyl cyclase (Figure 2.3a), (EC\(_{50}\) of control = 1.05 ± 0.32 nM; M\(\beta\)CD-treated = 1.51 ± 0.07 nM; p = 0.229). In contrast, M\(\beta\)CD treatment of HEK
FLAG-MOR cells significantly affected acute inhibition of adenylyl cyclase by the MOR agonist DAMGO (Figure 2.3b) [treatment: F(1,54) = 17.39, p = 0.0001] with a significant 4-fold rightward shift in the potency of DAMGO from an EC\textsubscript{50} of 2.49 ± 1.01 nM in control cells to 9.24 ± 1.15 nM in MβCD treated cells (p = 0.023). Together, these results are consistent with \[^{35}\text{S}]\text{GTP}\gamma\text{S} and binding results, indicating that cholesterol or cholesterol-dependent membrane effects modulate efficient MOR signaling more than DOR signaling.

In order to measure inhibition of adenylyl cyclase by opioid agonists, the enzyme is stimulated directly with forskolin. Consistent with previous reports (Rybin et al., 2000; Pontier et al., 2008), the level of cAMP produced by forskolin was enhanced approximately 2-fold by MβCD treatment in both HEK FLAG-DOR (control = 0.83 ± 0.07 pmol cAMP/\textmu g protein; MβCD-treated = 2.3 ± 0.35 pmol cAMP/\textmu g protein, p = 0.0004) and FLAG-MOR cells (control = 1.3 ± 0.06 pmol cAMP/\textmu g protein; MβCD-treated = 2.3 ± 0.14 pmol/\textmu g protein, p < 0.0001) (Figure 2.3c). This could be due to decreased inhibitory input on adenylyl cyclase by the lower basal G\alpha\textsubscript{i/o} protein activity which was observed in \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding experiments. To investigate if this was due to decreased constitutive activity of opioid receptor, cells were treated with pertussis toxin overnight to block receptor-G\alpha\textsubscript{i/o} protein coupling. Pertussis toxin treatment (100 ng/ml) did not lead to a significant increase of basal or forskolin-stimulated adenylyl cyclase activity in control or MβCD-treated HEK FLAG-DOR cells (data not shown), suggesting that the decreased baseline activity of the G protein following MβCD treatment is either independent of coupling to the receptor or is not responsible for the increased forskolin response.

**Effect of cholesterol depletion on opioid-induced adenylyl cyclase sensitization**

Chronic administration of opioid agonists results in a dependent state in both animal and cell models, which is characterized by withdrawal following removal of the agonist. In cells, the chronic inhibition of adenylyl cyclase by agonists for G\alpha\textsubscript{i/o}-coupled receptors causes a sensitization of the enzyme. Upon withdrawal of the agonist (usually
by addition of a competitive antagonist) and subsequent stimulation of adenylyl cyclase by forskolin, cAMP production is increased over that of forskolin alone, termed cAMP overshoot (Watts, 2002). Treatment of HEK FLAG-DOR cells with 2% MβCD for 1 h, prior to chronic (30 min) exposure to varying concentrations of either the non-peptidic DOR agonist, SNC80 (1 – 100 nM), or the peptidic DOR agonist, DPDPE (0.1 – 10 nM), did not alter the degree of cAMP overshoot precipitated by the DOR antagonist naltrindole at any of the agonist concentrations tested (Figure 2.4a and b). However, similar treatment of HEK FLAG-MOR cells with MβCD reduced the resultant cAMP overshoot induced by incubation with various concentrations (10 – 1000 nM) of the MOR agonist, DAMGO (Figure 2.4c), [treatment: F(1,32) = 16.39, p = 0.0003], consistent with results published previously (Zhao et al., 2006). These results suggest that cholesterol removal by MβCD selectively blocks MOR, rather than DOR, agonist-induced sensitization of adenylyl cyclase. Furthermore, this alteration in chronic signaling is much more robust than effects of MβCD on the acute signaling to Gαi/o proteins and adenylyl cyclase.

To determine if the effect of MβCD on MOR-induced adenylyl cyclase sensitization was restricted to a heterologous expression system, we repeated the sensitization experiments with SH-SY5Y neuroblastoma cells that endogenously express MOR. SH-SY5Y cells were differentiated with retinoic acid prior to sensitization experiments to create a more neuronal model (Pahlman et al., 1984; Encinas et al., 2000). SH-SY5Y cells were treated with 5 mM MβCD in serum-free DMEM for 10 min, which depleted cholesterol by 30.6 ± 4.4 % (Control = 43.32 ± 2.2 µg/mg protein; MβCD = 29.94 ± 2.0 µg/mg protein, p = 0.002, n = 5), similar to a previous report (Cheema and Fisher, 2008). This treatment paradigm had no effect on cAMP production by 5 µM forskolin (Control = 1.27 ± 0.12 pmol cAMP/µg protein; MβCD = 1.27 ± 0.16 pmol cAMP/µg protein). However, adenylyl cyclase sensitization by 1 µM DAMGO was significantly attenuated in SH-SY5Y cells treated with MβCD (Figure 2.4d, p < 0.01). Therefore, the robust effect of MβCD on MOR agonist-mediated adenylyl cyclase
sensitization is not limited to HEK293 cells, but also occurs in this neuronal model expressing endogenous receptors.

*Alteration of MOR signaling by MβCD is due to removal of cholesterol*

Despite the common use of MβCD for its cholesterol-sequestering properties, it may also have other non-specific effects on a cell due in part to changes in cell morphology. To ensure that the effects on MOR signaling observed following MβCD treatment were related to the removal of cholesterol from the membrane, membrane cholesterol was replenished using 2% MβCD pre-conjugated to cholesterol (MβCD-CH) in an 8:1 MβCD : cholesterol molar ratio (Christian et al., 1997; Xu et al., 2006; Zhao et al., 2006). In these experiments, HEK FLAG-MOR cells were treated first with 2% MβCD for 1 h to deplete membrane cholesterol. Cholesterol was then reintroduced to some cells by incubation with serum-free media containing 2% MβCD-CH for 2 h, while other MβCD-treated cells were incubated for 2 h with serum-free media alone. As expected, while cholesterol levels in MβCD-treated cells remained low, cholesterol levels in cells incubated with MβCD-CH returned to a level similar to that of control cells (p > 0.05), (control = 21.1 ± 3.3 µg cholesterol/mg protein, MβCD-treated = 7.8 ± 1.0 µg cholesterol/mg protein, MβCD-CH treated = 30.6 ± 3.4 µg cholesterol/mg protein; n = 6).

The replenishment of cholesterol restored acute inhibition of adenylyl cyclase by 10 nM DAMGO (Figure 2.5a) and sensitization of adenylyl cyclase by 100 nM DAMGO (Figure 2.5b) to levels similar to control cells. Restoration of membrane cholesterol by MβCD-CH also allowed cell morphology to return to normal. Incubation with serum-free media or MβCD-CH for 2 h produced similar levels of forskolin-stimulated cAMP as control cells (control = 1.73 ± 0.10 pmol cAMP/µg protein; MβCD = 2.16 ± 0.33; MβCD-CH = 1.53 ± 0.20 pmol cAMP/µg protein), indicating that effects of MβCD on forskolin response are transient.

To further verify that the effect of MβCD on MOR-mediated overshoot was due to reduction in cellular cholesterol, we used the cholesterol-lowering drug lovastatin, which inhibits the rate limiting enzyme in cholesterol synthesis, 3-hydroxy-3-methyl-
glutaryl-CoA reductase (HMG-CoA reductase). HEK FLAG-MOR cells were treated with the activated, open ring form of lovastatin (lovastatin hydroxy acid) for 48 h to lower cholesterol by $28.3 \pm 0.07$ % ($p = 0.02$) as compared to vehicle treated cells. Under this treatment paradigm, lovastatin abolished the ability of the MOR agonist DAMGO (100 nM) to elicit overshoot (Figure 2.5c; $p < 0.001$). To prevent cellular uptake of cholesterol from the serum present in the normal growth media (DMEM with 10% FBS), cells were grown in lovastatin or DMSO vehicle containing Opti-MEM for 48 hours. Cells were 70-80% viable as assessed by trypan blue exclusion after 48 h in Opti-MEM. Furthermore, the overshoot elicited by the MOR agonist DAMGO was similar in cells cultured in Opti-MEM or regular growth media (DMEM with 10% FBS) (Figure 2.5c). Additionally, there was no effect of media type or lovastatin treatment on the forskolin response (DMEM/FBS = $1.06 \pm 0.03$ pmol cAMP/µg protein, Opti-MEM+DMSO = $1.30 \pm 0.04$ pmol cAMP/µg protein, Opti-MEM+lovastatin = $1.02 \pm 0.12$ pmol cAMP/µg protein).

Similarly, differentiated SH-SY5Y cells were treated with lovastatin hydroxy acid for 48 h to measure the effect on overshoot mediated by endogenous MOR. Lovastatin treatment reduced cholesterol by $17 \pm 2.4$ % compared to vehicle-treated controls, which was not as robust as the reduction observed with MβCD or with lovastatin in HEK cells. However, there was a significant reduction in the ability of the MOR agonist DAMGO (1 µM, 1 h) to cause adenylyl cyclase overshoot (Figure 2.5d). Production of cAMP by 5 µM forskolin was similar in lovastatin and vehicle-treated SH-SY5Y cells (vehicle = $0.93 \pm 0.07$ pmol/µg protein; lovastatin = $0.89 \pm 0.10$ pmol/µg protein). Together, the data obtained from replenishment of cholesterol and reduction of cholesterol using the synthesis inhibitor lovastatin indicate that MβCD prevents MOR agonist sensitization of adenylyl cyclase by a mechanism dependent on lowered cholesterol.

**Receptor number is not responsible for alterations in MOR signaling by MβCD**

Both the HEK FLAG-MOR and HEK FLAG-DOR cells used in this study express a high number of opioid receptors ($9.7 \pm 1.3$ pmol/mg protein and $8.4 \pm 1.5$ pmol/mg protein, respectively), so it is unlikely that the difference observed between
MOR and DOR agonists is due to different expression levels. Furthermore, HEK FLAG-MOR cells treated with 2% MβCD for 1 h retained a similar level of cell surface MOR as vehicle treated cells (92.2 ± 3.1%; p = 0.107; n = 2, in triplicate) as determined by immunoassays directed toward the extracellular FLAG epitope performed as described previously (Divin et al., 2009). Of the total MOR in HEK FLAG-MOR cells, 51 ± 4% of the receptors are on the cell surface as determined by competition binding in whole cells with the membrane impermeable MOR antagonist CTAP and the cell permeable antagonist ³H-diprenorphine (Figure 2.6a). To further confirm that the high receptor number is not responsible for the alteration in MOR signaling to adenylyl cyclase, we repeated experiments using an HEK FLAG-MOR clone that expressed six times less MOR (1.6 ± 0.1 pmol/mg protein). Similar to results in the high-expressing clone, treatment of this lower receptor-expressing HEK FLAG-MOR clone with 2% MβCD reduced the ability of the MOR agonist DAMGO to acutely inhibit adenylyl cyclase (Figure 2.6b; p < 0.05) and greatly blocked its ability to cause adenylyl cyclase sensitization (Figure 2.6c; p < 0.01). Furthermore, treatment of these cells with MβCD did not reduce total receptor number as determined by saturation binding of the opioid antagonist ³H-diprenorphine (Bₘₐₓ of Control = 1560 ± 108 fmol/mg; MβCD = 1580 ± 64 fmol/mg; Figure 2.6d). Similarly, treatment of SH-SY5Y cells with 5 mM MβCD for 10 min did not alter maximal (4 nM) ³H-diprenorphine binding (Control = 879 ± 51 fmol/mg protein; MβCD = 858 ± 43 fmol/mg protein; p > 0.05; n = 2, in triplicate).

The antagonist ³H-diprenorphine can recognize all affinity states of the MOR, but as an agonist ³H-DAMGO will only label high-affinity sites at the concentrations used in saturation binding assays. Therefore, in HEK FLAG-MOR cells the maximum number of receptors bound by ³H-DAMGO (944 ± 85 fmol/mg protein; Figure 2.6e) was expectedly less than those bound by ³H-diprenorphine. Furthermore, treatment of these cells with MβCD significantly reduced the Bₘₐₓ of ³H-DAMGO to 205 ± 24 (p = 0.002), indicating a loss of MOR in a high-affinity state. This is consistent with the increase in low-affinity agonist binding observed in competition binding assays following cholesterol depletion by MβCD. These results support the previous observations from a high receptor-expressing model and suggest that the effects of MβCD on decreasing
MOR-G protein coupling and downstream signaling to adenylyl cyclase is not merely the result of a loss of receptors.

**Effect of cytoskeletal disruption on MOR-induced adenylyl cyclase sensitization**

Disruption of either actin or tubulin has been shown to perturb caveolae and raft microdomains in rodent cardiac myocytes and S49 T-lymphoma cells (Head et al., 2006). To address the effects of caveolae and raft disruption using cytoskeletal inhibitors, HEK FLAG-MOR cells were treated with demecolcine to prevent microtubule polymerization or cytochalasin D to disrupt actin filament organization. Demecolcine is a close analog of colchicine, which has been shown to disrupt rafts (Head et al., 2006). Demecolcine (1 µg/ml) for 16 - 20 h produced rounding cell morphology similar to 1 h MβCD treatment, but did not inhibit MOR agonist-induced sensitization of adenylyl cyclase (Figure 2.7a). In contrast, cytochalasin D (20 µM, 1.5 h), which has previously been shown to disrupt rafts (Head et al., 2006), caused slimming of the cells and decreased the ability of DAMGO to sensitize adenylyl cyclase [treatment: F(1,34) = 42.14, p < 0.0001].

Cytochalasin D altered the efficacy of DAMGO to produce sensitization at maximal concentrations (p < 0.01 at 0.1 µM and p < 0.001 at 1 µM DAMGO), but did not shift the potency of DAMGO-mediated sensitization (EC$_{50}$ after DMSO vehicle = 13.4 ± 8.4 nM and after cytochalasin D = 17.8 ± 5.7 nM, p = 0.676; Figure 2.7b). Neither demecolcine nor cytochalasin D treatment affected cAMP produced by forskolin alone (control = 1.14 ± 0.09 pmol/µg vs. demecolcine = 1.13 ± 0.07 pmol/µg; DMSO = 1.06 ± 0.05 pmol/µg vs. cytochalasin D = 1.08 ± 0.08 pmol/µg). These data demonstrate that although the effect of MβCD on DAMGO-mediated signaling was due to removal of cholesterol, it could not be mimicked using a tubulin inhibitor, even though tubulin inhibitors have been shown to disrupt rafts (Head et al., 2006). However, the actin inhibitor cytochalasin D did attenuate MOR overshoot, indicating a potential role for actin in adenylyl cyclase sensitization.
Differential membrane localization of opioid receptors and adenylyl cyclase

The disparate importance of cholesterol for MOR, but not for DOR signaling described above could be explained by direct interaction of the receptor with cholesterol or differing localization of receptors and signaling proteins in putative cholesterol-enriched membrane rafts. Membrane rafts have been identified by their buoyancy and insolubility in non-ionic detergents, such as Triton X-100 (Brown and London, 1998). To assess the localization of MOR and DOR in putative membrane rafts in HEK293 cells, a detergent-based method was employed similar to that described by Brady and colleagues (Brady et al., 2004). HEK FLAG-DOR or HEK FLAG-MOR cells were homogenized in 1% Triton X-100 and separated on a 5/30/40% discontinuous sucrose gradient, after which twelve fractions were collected from the top. Fractions 7 - 9 contained a cloudy band that floated at the interface between the 5 % and 30 % sucrose layers, consistent with the definition of membrane rafts. These fractions were also enriched in the cholesterol-binding protein, caveolin (Figure 2.8). Transferrin receptor, which is found in clathrin-coated pits and is excluded from caveolin-enriched fractions (Janes et al., 1999; Brady et al., 2004), was used as a marker to identify the cholesterol-poor, detergent-soluble membrane fractions. These solubilized membranes are found in the dense 40 % sucrose fractions located at the bottom of the discontinuous gradient as evidenced by transferrin receptor immunoreactivity found in fractions 11 and 12 (Figure 2.8). The separation between the caveolin-containing fractions and the transferrin receptor-containing fractions indicates the quality of the preparation and efficient separation of the putative raft and non-raft membrane types. Under these conditions, DOR was observed only in the transferrin receptor-containing fractions (Figure 2.8a and e), while the MOR was found in both caveolin-containing and transferrin receptor-containing fractions (Figure 2.8b and f). DOR was not observed in the caveolin-containing fractions even after overexposure of the blot (data not shown). Although this separation method is inherently non-quantitative for the amount of protein in detergent-resistant fractions (Brown, 2006), analysis of the signal in each fraction from multiple experiments illustrated the less exclusive distribution of MOR in both caveolin-enriched and non-enriched fractions compared to DOR (Figure 2.8e and f).
Many of the signaling proteins that DOR activates have been identified in cholesterol-enriched membranes, including $G\alpha_i$ (Xu et al., 2006) and adenylyl cyclase isoforms 5/6 (Ostrom et al., 2002). Consequently, we tested the hypothesis that DOR would move into cholesterol-enriched membranes, and therefore associate with these signaling proteins upon agonist stimulation. Such movement into rafts has been reported previously for muscarinic M2 and purinergic A1 receptors (Allen et al., 2007). However, treatment of HEK FLAG-DOR cells with a 10 $\mu$M concentration of the DOR agonist DPDPE for 5 min did not change the localization of the receptors (Figure 2.8c and e).

Because DOR signaling to adenylyl cyclase is unaffected by cholesterol depletion, this raises the question of which adenylyl cyclase isoform DOR is coupled to, especially as adenylyl cyclase enzymes that are inhibited by DOR have been found in rafts, including AC 5/6 (Ostrom et al., 2002). However, it is likely that the propensity for the adenylyl cyclases to localize only to cholesterol-enriched domains is not absolute and that cyclases are also present in other areas of the plasma membrane. In HEK293 cells, endogenous AC 5/6 was detected in transferrin receptor-containing fractions, in addition to the caveolin-containing fractions, following detergent solubilization (Figure 2.8d). Therefore, DOR would not need to reside in high-cholesterol regions of the membrane to effectively signal to adenylyl cyclase.

**Cholera toxin B-induced patching**

Cholera toxin B subunit (CTB)-induced patching was used to further examine the membrane localization characteristics of MOR and DOR in intact cells using a method that does not rely on cell disruption or detergent solubilization. This method has been used previously to identify raft-associated proteins (Janes et al., 1999). With an estimated size of 10 – 200 nm (Pike, 2006) putative membrane rafts are too small for observation by conventional light microscopy, but can be clustered into patches by CTB, which binds with high affinity as a pentamer to the raft-associated ganglioside GM1 (Janes et al., 1999). Further clustering with an anti-CTB antibody aggregated the CTB-GM1 complexes into patches visible by confocal microscopy (Figure 2.9a).
Staining of the FLAG epitope on the extracellular N-terminus of DOR following patching showed that DOR on the cell surface were poorly co-localized with CTB-clustered patches. In fact, upon quantification of over 25 cells from at least 3 separate experiments, DOR co-localized with CTB to the same degree as the non-raft marker protein transferrin receptor (Figure 2.9b). In contrast, MOR co-localized with many CTB patches, as indicated by arrows (Figure 2.9a). Upon quantification, MOR were colocalized with CTB to a higher degree than DOR (MOR = 7.89 ± 2.22 vs. DOR = 2.00 ± 0.56 pixel density/ cell, p = 0.01) or transferrin receptors (1.93 ± 0.46 pixel density/ cell, p = 0.01), but to a significantly lesser degree than caveolin (17.85 ± 2.41 pixel density/ cell, p = 0.005). Together, these localization and cell signaling experiments indicate that in HEK293 cells MOR tends to depend on cholesterol, and can partly interact with cholesterol-rich domains of the cell membrane, more than DOR.

Discussion

In this study we have used FLAG-tagged MOR and DOR expressed in HEK293 cells to ask questions concerning the localization and signaling of these two highly homologous plasma membrane proteins. Two conclusions can be drawn from this study. Firstly, MOR signaling is sensitive to the cholesterol content of the cell membrane, with a much greater effect on signaling following chronic agonist exposure. This conclusion was reached by removal and replenishment of plasma membrane cholesterol using the cholesterol sequestering agent MβCD and by cholesterol synthesis inhibition by lovastatin. Furthermore, the decrease in MOR signaling appears to be due to an uncoupling of the MOR from its G protein and is not due to a decrease in receptor number. The effect of MβCD on adenylyl cyclase sensitization is also not a product of using a heterologous expression system or a high receptor number as shown by experiments using SH-SY5Y cells and HEK cells expressing a lower level of MOR. Under the same conditions, DOR signaling and G protein coupling are not affected. Secondly, using fractionation of the cell membrane and CTB patching of whole cells, we demonstrate the localization of DOR outside of cholesterol-enriched domains, but a less exclusive localization of MOR in both cholesterol-enriched and cholesterol-poor
membrane regions. This differential distribution pattern supports the differential sensitivity of MOR and DOR signaling to cholesterol.

There are two hypotheses to explain the differential dependence of MOR and DOR on membrane cholesterol. Firstly, the partial localization of MOR in detergent-insoluble domains may result from a higher propensity for these receptors to bind to cholesterol or caveolin than DOR. Although both MOR and DOR contain one of the proposed caveolin binding motifs (ΦXΦXXXXΦ; Φ = F, W or Y) (Couet et al., 1997) at the interface of TM7 and the C-terminal tail, MOR contains more aromatic and hydrophobic residues in the area immediately distal to this region. This difference may be especially important for interactions with cholesterol in the membrane because it is in the same region of the receptor that was shown to interact with cholesterol in the β2AR crystal structure (Cherezov et al., 2007), and includes a conserved palmitoylated cysteine. Even though DOR contains a caveolin-binding domain, this may not be enough for the receptor to bind tightly to caveolin, since the binding domain is a rather general sequence that could be found on many proteins. For example, Gαi2 contains a caveolin binding domain (Couet et al., 1997), but has not always been shown to associate with caveolin (Head et al., 2005).

Alternatively, DOR may not need the extra membrane rigidity provided by cholesterol to support signaling because it is known to couple tightly to G proteins (Costa et al., 1988; Polastraon et al., 1992). In fact, Andre et al. (2008) conclude that DOR can form a high affinity state that is stabilized by G-proteins rather than cholesterol. MOR, however, is less tightly coupled to G proteins (Chakrabarti et al., 1997) and may depend on cholesterol to aid in this coupling even in non-raft regions of the plasma membrane. This is supported by evidence that cholesterol alone can stabilize MOR in a high-affinity state (Gaibelet et al., 2008), and increasing membrane viscosity with a cholesterol analog has been shown to improve the potency of the MOR agonist DAMGO to stimulate \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding (Emmerson et al., 1999).

The heterogeneous distribution of MOR in caveolin-enriched and caveolin-poor fractions may explain the rather small effect of cholesterol depletion on acute MOR signaling (either \[^{35}\text{S}]\text{GTP}\gamma\text{S} binding or adenylyl cyclase inhibition). However, this
effect was greatly amplified when cells were treated chronically with a MOR agonist to induce adenylyl cyclase sensitization. The complexity of adenylyl cyclase sensitization (Watts, 2002) may explain the more dramatic effect of cholesterol depletion (either by MβCD or lovastatin) on adenylyl cyclase sensitization versus acute inhibition of adenylyl cyclase by DAMGO. Besides the previously mentioned G proteins, many of the other signaling pathways postulated to play a role in adenylyl cyclase sensitization have also been shown to be associated with cholesterol-enriched domains, such as Raf-1 (Mineo et al., 1996), PKC (Weerth et al., 2007) and Src kinase (Mukherjee et al., 2003). Recently, Src kinase has been implicated as an important mediator of MOR adenylyl cyclase sensitization in putative rafts (Zhang et al., 2009). In addition, many of the adenylyl cyclase isoforms that can be sensitized [AC 5, 6 and 8 (Watts, 2002)] have also been found in cholesterol-enriched domains [AC 5/6 (Fagan et al., 2000; Ostrom et al., 2002; Zhao et al., 2006) and 8 (Smith et al., 2002)]. Exceptions to this include AC 1, which can be sensitized by a variety of agonists (Avidor-Reiss et al., 1997; Nevo et al., 1998; Rhee et al., 2000) but has not been found in rafts (Ostrom et al., 2002), and AC 3, which is preferentially found in cholesterol-enriched membranes (Ostrom et al., 2002) but is not sensitized by several agonists, including the MOR agonist morphine (Avidor-Reiss et al., 1997; Nevo et al., 1998). Additionally, sensitization caused by chronic agonist treatment may require a redistribution of the heterotrimeric G protein to detergent-insoluble domains. In a study using CHO-MOR cells, chronic morphine treatment increased the amount of $G_{\alpha i}$ and $G_{\beta 1}$ in the cholate-insoluble cellular fraction (with a corresponding decrease of G protein in the cholate-soluble fraction) in a time-dependent manner that was similar to the time-course of adenylyl cyclase sensitization (Bayewitch et al., 2000). This redistribution of $G_{\alpha i}$ and $G_{\beta 1}$ was reversed following removal of morphine in a similar time frame as the loss of sensitization. Therefore, cholesterol depletion may prevent the development of sensitization by preventing the shift of heterotrimeric G proteins to detergent-insoluble microdomains.

Although a percentage of MOR opioid receptors were found in detergent-insoluble domains, most were found in putative non-raft domains. Since the effect of cholesterol depletion by MβCD or lovastatin on adenylyl cyclase sensitization was very
robust, this may suggest a loss of cholesterol function on MOR, rather than, or in addition to, disruption of rafts. The cytoskeleton, including both actin and tubulin, has also been shown to be important for the formation of lipid rafts and caveolae (Head et al., 2006). Therefore, the lack of effect of the tubulin inhibitor demecolcine on MOR agonist-mediated adenylyl cyclase overshoot would point away from a raft mechanism. Even so, with the actin inhibitor cytochalasin D we do see a decrease in the ability of the MOR agonist DAMGO to cause overshoot. However, differences between MβCD and cytochalasin D, including morphology changes and the lack of effect of cytochalasin D on forskolin-stimulated cAMP production, argue against a contributing role of actin disruption in the mechanism of action of MβCD. Therefore, it appears most likely that cholesterol and actin are both important independently for MOR overshoot. To our knowledge this is the first report of a role for actin in adenylyl cyclase sensitization, although, there is evidence of increased actin cycling following withdrawal from chronic morphine treatment in rats (Toda et al., 2006).

The increase in forskolin-stimulated cAMP production observed immediately following cholesterol removal by MβCD has been observed by others (Rybin et al., 2000; Pontier et al., 2008). However, this enhanced forskolin effect is lost if a 2 h incubation in serum-free media is included following cholesterol removal, even though cholesterol levels remain depleted. Furthermore, chronic inhibition of cholesterol synthesis using lovastatin for 48 h did not affect the forskolin response. Therefore, the effect of cholesterol removal on forskolin appears transient as the cell has to rapidly adapt to the loss of cholesterol. In addition, basal and isoproterenol activation of adenylyl cyclase through Gαs have been shown to increase following cholesterol depletion by MβCD or cholesterol oxidase (Rybin et al., 2000; Pontier et al., 2008). Thus, the increased forskolin response could be due to increased synergistic activation of adenylyl cyclase with Gαs. Results from experiments by us and others (Pontier et al., 2008) using pertussis toxin suggest that the increase in forskolin or isoproterenol response does not involve receptor-activated Gαi/o proteins.

The results from the sucrose gradient membrane separation experiments are consistent with reports of the localization of MOR in caveolin-enriched membrane
fractions as well as caveolin-poor fractions (Zhao et al., 2006; Gaibelet et al., 2008). In contrast, we found DOR only in the same cellular fractions as transferrin receptor, a non-raft marker. This finding differs from previously published reports of DOR localization in putative raft fractions (Patel et al., 2006; Huang et al., 2007a). These conflicting data may relate to the method used for isolation of membrane microdomains, about which there is much debate (Shogomori and Brown, 2003; Allen et al., 2007). Previous studies of DOR localization used high molarity sodium carbonate buffer, pH 11, for homogenization prior to sucrose gradient ultracentrifugation to separate cholesterol-enriched from cholesterol-deficient membranes. Although this method has been commonly employed to study protein content in putative lipid rafts (Song et al., 1996; Allen et al., 2007), there are some problems intrinsic to this method. The primary concern with the non-detergent method is a lack of specificity for isolating putative raft proteins, since even non-plasma membrane proteins, such as mitochondrial proteins, are found in the cholesterol-enriched fractions (Foster et al., 2003). Using the sodium carbonate method, we have also identified DOR in more buoyant, caveolin-containing fractions. However, these fractions also contained significant amounts of the non-raft marker transferrin receptor indicating a lack of separation between putative raft and non-raft membranes (data not shown). Thus, we are not confident using this method to identify raft proteins. Previous studies (Patel et al., 2006; Huang et al., 2007a) did not include a negative control, such as transferrin receptor, for comparison.

The detergent method used in the present study also has limitations, but is more stringent for identification of cholesterol-binding proteins (Foster et al., 2003). Recently, DOR localization has been studied using the detergent Triton X-100 in various concentrations (Andre et al., 2008). Although this study identified a small percentage (~5 %) of DOR in raft-like fractions, the majority of DOR were found in the non-raft pellet, which generally agrees with our findings. In addition, our identification of DOR in caveolin- and cholesterol-poor fractions was supported by the lack of association of DOR with raft domains as labeled by CTB-patching of intact cells. Furthermore, these localization results are consistent with the DOR signaling data in this study.
demonstrating that cholesterol depletion by MβCD did not affect agonist-mediated DOR signaling.

Although the evidence presented here suggests that DOR is excluded from cholesterol-enriched regions, many signaling molecules, including Gαi/o proteins (Li et al., 1995; Li et al., 1996; Moffett et al., 2000; Foster et al., 2003; Quinton et al., 2005; Xu et al., 2006), known to be activated by DOR have been found in cholesterol-enriched fractions or associated with caveolin. One possible explanation we have excluded is that DOR moves into cholesterol-enriched fractions upon agonist treatment. A more likely possibility is that although many signaling proteins are found in rafts, this relationship is not exclusive and these proteins are also functioning outside of raft domains. For example, Gαi proteins have also been found in cholesterol-poor fractions in cardiomyocytes (Head et al., 2005) and in these HEK cells (data not shown) so that DOR could signal through these Gαi proteins. In addition, adenylyl cyclase isoforms 3 and 5/6 are primarily, but not exclusively, found in caveolin-containing fractions (Ostrom et al., 2002). In our preparations, adenylyl cyclase 5/6 is found in transferrin receptor-containing fractions. Moreover, DOR may also signal through adenylyl cyclase 1 which has not been found in cholesterol-enriched domains (Ostrom et al., 2002; Ostrom and Insel, 2004), is readily sensitized (Watts, 2002) and is present in HEK293 cells (Premont, 1994).

In conclusion, these studies demonstrate differences in signaling and localization properties of MOR and DOR, using identical methods in the same HEK cell system, and are supported by studies in SH-SY5Y neuroblastoma cells that endogenously express opioid receptors. DOR, which were not found in cholesterol-enriched membranes, were not as affected by changes in the microenvironment of the membrane. In contrast, MOR, to which agonists cause robust dependence clinically, is significantly altered by cholesterol modulation, with a greater effect on chronic signaling than acute signaling. Although these effects are due to cholesterol removal, these studies cannot confirm that they are due to lipid raft disruption and may suggest a need for cholesterol to provide efficient MOR signaling in non-raft regions of the plasma membrane. Regardless, these findings have implications for cellular signaling in general, especially in light of the
recent, somewhat controversial (LaRosa et al., 2005; Pitt, 2005) lowering of clinical cholesterol guidelines (Smith et al., 2006).
Figure 2.1: MOR, but not DOR, agonist-mediated G protein activation is altered following MβCD treatment. A, basal [35S]GTPγS binding in membranes from HEK FLAG-DOR, HEK FLAG-MOR or untransfected HEK293 cells treated with (hatched bars) or without (open bars) 2% MβCD in serum-free media for 1 h. **p < 0.01, ***p < 0.001 versus respective control by unpaired two-tailed Student’s t-test. B, stimulation of [35S]GTPγS binding by the DOR agonist SNC80 (1 nM – 10 µM) was unchanged in membranes from HEK FLAG-DOR cells treated with MβCD. EC50 values: Control = 4.9 ± 2.4 nM; MβCD = 5.2 ± 0.8 nM (p = 0.898). C, stimulation of [35S]GTPγS binding by the MOR agonist DAMGO (1 nM – 10 µM) was shifted in membranes from HEK FLAG-MOR cells treated with MβCD. EC50 values: Control = 16.8 ± 9.3 nM; MβCD = 73.0 ± 26.5 nM (p = 0.116). Data are presented as mean ± S.E.M. from 3–4 separate experiments, in duplicate.
Figure 2.2: Cholesterol removal by MβCD induces low-affinity binding in MOR, but not DOR, expressing cells. Competition binding experiments in membranes prepared from HEK FLAG-DOR (A) or HEK FLAG-MOR (B) cells treated with (open circles) or without (filled circles) 2% MβCD for 1 h. Unlabeled agonist (SNC80 or DAMGO) was added in increasing concentrations in competition with 0.2 nM ³H-diprenorphine (DPN). Non-specific binding was determined using 10 µM naloxone. A, Ki for SNC80 using one-site competition linear regression: Control = 0.78 ± 0.21 nM; MβCD = 0.87 ± 0.09 nM (p = 0.767). Slope of concentration displacement curve was unchanged: Control = -0.77 ± 0.09; MβCD = -0.72 ± 0.12 (p = 0.788). In B, 100 mM NaCl and 10 µM GTPγS was added in the Tris incubation buffer as indicated to uncouple G proteins. Ki for DAMGO: Control = 4.9 ± 1.2 nM, one-site model; MβCD = 1.9 ± 0.3 nM (Ki high), 244 ± 49 nM (Ki low), two-site model. Slope of concentration displacement curve was reduced: Control = -0.76 ± 0.03; MβCD = -0.45 ± 0.05 (p = 0.0004). Ki after NaCl and GTPγS addition: Control = 768 ± 82 nM; MβCD = 1887 ± 712 nM. Data are presented as a percent of the total specifically bound ³H-diprenorphine in absence of competing agonist and are from 3 separate experiments, in duplicate. Ki and slopes were compared using two-tailed, unpaired Student’s t-tests.
Figure 2.3: MOR, but not DOR, agonist-mediated inhibition of adenylyl cyclase is altered following MβCD treatment. A and B, Concentration-response curves for acute adenylyl cyclase inhibition by the DOR agonist SNC80 (0.1 nM – 1 µM), or the MOR agonist DAMGO (1 nM – 1 µM) in HEK FLAG-DOR (A) or HEK FLAG-MOR (B) cells pretreated with (open circles) or without (filled circles) 2% MβCD for 1 h. Data are presented as a percent of the forskolin-stimulated cAMP accumulation in the absence of agonist (n=3, in duplicate). A, EC$_{50}$ values for SNC80 were not changed: Control = 1.05 ± 0.32 nM; MβCD = 1.51 ± 0.07 nM (p = 0.229). B, MβCD caused a 4-fold rightward shift in the potency of DAMGO: Control = 2.49 ± 1.01 nM; MβCD = 9.24 ± 1.15 nM (p = 0.023). C, cAMP production by 5 µM forskolin in HEK FLAG-DOR or FLAG-MOR cells treated with (hatched bars) or without (open bars) 2% MβCD for 1 h. ***p < 0.001 compared to respective control by unpaired two-tailed Student’s t-test. Data presented as mean ± S.E.M from 6 separate experiments, in duplicate.
Figure 2.4: MβCD treatment blocks MOR, but not DOR, agonist-mediated adenylyl cyclase sensitization. Adenylyl cyclase overshoot was precipitated with antagonist (naltrindole [A & B] or naloxone [C]) in the presence of 5 μM forskolin following chronic (30 min) treatment with the non-peptidic DOR agonist SNC80 (A), the peptidic DOR agonist DPDPE (B) or the MOR agonist DAMGO (C) in HEK FLAG-DOR (A & B) or HEK FLAG-MOR (C) cells. Pretreatment with 2% MβCD (hatched bars) for 1 h prior to agonist exposure prevented overshoot produced by the MOR agonist DAMGO (*p < 0.05 by 2-way ANOVA with Bonferroni’s post-hoc test), but not by either DOR agonist. In D, retinoic acid-differentiated SH-SY5Y cells were treated with 5 mM MβCD (hatched bar) for 10 min prior to incubation with 1 μM DAMGO for 60 min. Overshoot was precipitated with naloxone as above. *p < 0.05 by unpaired two-tailed Student’s t-test. Dashed lines indicate cAMP production with forskolin alone. Data are presented as mean ± S.E.M from 3 separate experiments, in duplicate.
Figure 2.5: Effects of MβCD on MOR signaling are due to removal of cholesterol from the membrane. A, acute adenylyl cyclase inhibition by 10 nM DAMGO in HEK FLAG-MOR cells following cholesterol depletion without (MβCD + veh) or with cholesterol replenishment (MβCD + CH). B, adenylyl cyclase overshoot following chronic (30 min) treatment with 100 nM DAMGO in HEK FLAG-MOR cells following cholesterol depletion without (MβCD + veh) or with replenishment (MβCD + CH). Dashed lines indicate cAMP production with forskolin alone. *p < 0.05, **p < 0.01 compared to control (veh + veh) following 1-way ANOVA and Bonferroni’s post-hoc test. Forskolin stimulation was similar across all groups in each experiment (p > 0.05 by two-way ANOVA and Bonferroni’s post-test: veh+veh = 1.75 ± 0.13; MβCD+veh = 2.06 ± 0.22; MβCD+CH = 1.53 ± 0.11 pmol cAMP/µg protein). In C and D, HEK FLAG-MOR or SH-SY5Y cells, respectively, were grown in regular growth media (DMEM/FBS), Opti-MEM containing DMSO vehicle (Opti + DMSO) or Opti-MEM containing the cholesterol synthesis inhibitor lovastatin hydroxy acid (10 µM) (Opti + Lova) for 48 h. Opti-MEM had no effect on overshoot compared to normal growth media (DMEM/FBS). C, adenylyl cyclase overshoot following chronic (30 min) treatment with 100 nM DAMGO in HEK FLAG-MOR cells grown in each of the above conditions. ***p < 0.001 compared to regular media (DMEM/FBS) or Opti-MEM + DMSO controls by one-way ANOVA with Bonferroni’s post-test. Forskolin stimulation was similar among groups (DMEM/FBS = 1.06 ± 0.03, Opti + DMSO = 1.30 ± 0.04, Opti + Lova = 1.02 ± 0.12 pmol cAMP/µg protein). D, adenylyl cyclase overshoot following chronic (60 min) treatment with 1 µM DAMGO in differentiated SH-SY5Y cells grown in each of the above conditions plus 10 µM retinoic acid. ***p < 0.001 vs. DMEM/FBS, ++p < 0.01 vs. Opti + DMSO by one-way ANOVA and Bonferroni’s post-test. DMEM/FBS and Opti + DMSO were not different (p > 0.05). Forskolin stimulation was not different in Opti + DMSO (0.93 ± 0.07 pmol/µg protein) and Opti + Lova (0.89 ± 0.10 pmol/µg protein). Dashed lines indicate cAMP production with forskolin alone. All data are presented as mean ± S.E.M. (n = 3 or 4, in duplicate).
Figure 2.6: Alterations in MOR signaling are not due to changes in receptor number. A, surface expression of MOR was determined in whole cells (1 x 10^5 cells/tube) by displacement of 4 nM ^3^H-diprenorphine (DPN) by the cell impermeable antagonist CTAP (10 µM). Non-specific binding was determined using the cell permeable antagonist naloxone (10 µM). In B and C, HEK FLAG-MOR cells expressing only 1.6 fmol/mg receptor were treated with (hatched bars) or without (open bars) 2% MβCD for 1 h prior to acute cyclase inhibition (B) or adenylyl cyclase overshoot (C) experiments with 10 nM DAMGO or 100 nM DAMGO, respectively. Data are graphed as percent of forskolin-stimulated cAMP, represented by the hashed line (n = 3, in duplicate). *p < 0.05; **p < 0.01 by unpaired, two-tailed Student’s t-test. D and E, saturation binding experiments with the opioid antagonist ^3^H-diprenorphine (DPN) (D) or the MOR opioid agonist ^3^H-DAMGO (E) in membranes from low-expressing HEK FLAG-MOR cells treated with (closed circles) or without (open circles) 2% MβCD for 1 h. Control cells (open circles) in E were incubated with vehicle for 3 h. Non-specific binding was determined using 10 µM naloxone. Data are presented as mean ± S.E.M (n = 3, in duplicate). D, ^3^H-diprenorphine binding was unchanged following cholesterol depletion: B\text{max}: control = 1560 ± 108 fmol/mg; MβCD = 1580 ± 64 fmol/mg; Kd: control = 0.20 ± 0.07 nM; MβCD = 0.18 ± 0.01 nM. E, ^3^H-DAMGO B\text{max} decreased (control = 944 ± 85 fmol/mg; MβCD = 205 ± 24 fmol/mg; p = 0.0019 by unpaired two-tailed Student’s t-test) with no change in Kd (control = 0.86 ± 0.33 nM; MβCD = 0.60 ± 0.24 nM).
Figure 2.7: Effect of cytoskeleton inhibitors on MOR-mediated adenylyl cyclase sensitization. HEK FLAG-MOR cells were treated with (filled circles) or without (open circles) the tubulin inhibitor demecolcine (1 µg/ml, overnight) (A) or the actin inhibitor cytochalasin D (20 µM, 1.5 h) (B) prior to induction of adenylyl cyclase sensitization by the MOR agonist DAMGO as described in Methods. Data are presented as mean ± S.E.M. (n = 3, in duplicate). *p < 0.05, **p < 0.01, **p < 0.001 by 2-way ANOVA with Bonferroni’s post-hoc test. Forskolin stimulation was not affected by cytoskeleton disruption in either experiment (p > 0.05 by two-way ANOVA and Bonferroni’s post-hoc test): control = 1.14 ± 0.09 pmol/µg vs. demecolcine = 1.13 ± 0.07 pmol/µg; DMSO = 1.06 ± 0.05 pmol/µg vs. cytochalasin D = 1.08 ± 0.08 pmol/µg.
Figure 2.8: Localization of MOR, but not DOR, in caveolin-enriched fractions, in addition to transferrin receptor-enriched fractions. Detergent-resistant membranes were prepared from HEK FLAG-DOR (A, C & D) or HEK FLAG-MOR (B) cells as described in Methods. Equal volume-loaded samples were assessed for localization of opioid receptor (using anti-FLAG M1 antibody), adenylyl cyclase 5/6 (AC 5/6), caveolin (a raft marker), or transferrin receptor (TfR, a non-raft marker) as indicated. C, treatment of HEK FLAG-DOR cells with the DOR agonist, DPDPE (10 µM, 5 min), prior to homogenization did not change the localization of DOR compared to untreated cells (A). In E and F, the mean pixel density of the FLAG-DOR or FLAG-MOR (55 kD band) in each fraction of a separation experiment was quantified and compared to the total mean pixel density in all lanes for an experiment. Note that equal volume loading and presence of intracellular receptors in soluble fractions (11 and 12) precludes truly representative quantification. Results are presented as a percent of the total pixel density in each fraction from 2-7 separate experiments.
Figure 2.9: DOR is not co-localized with cholera toxin B (CTB) subunit patches. 

A, HEK FLAG-DOR or FLAG-MOR cells were plated on poly-D-lysine coated coverslips 24 h prior to patching with cholera toxin B subunit (CTB) conjugated to Alexa 488 at 4°C to label endogenous ganglioside GM1, enriched in lipid rafts. Lipid raft clustering was induced by incubating with goat anti-CTB for 30 min at 4°C, followed by 20 min at 37°C. Cells were fixed with 4% paraformaldehyde and DOR or MOR were stained using mouse anti-FLAG M1 antibody followed by goat anti-mouse-Alexa 594. Fluorescent images of 0.5 µm Z planes were captured using confocal microscopy. Scale bar = 10 µm. 

B, quantification of colocalization with CTB from at least 25 cells from 3 separate experiments is presented as mean pixel density of colocalization per cell ± S.E.M. *p < 0.05, ***p<0.001 compared to TfR or DOR by two-tailed unpaired Student’s t-test; ‡ p = 0.005 compared to caveolin by two-tailed unpaired Student’s t-test.
Chapter III

\textbf{G}_{i/o}-coupled receptors compete for signaling to adenylyl cyclase in SH-SY5Y cells and reduce opioid withdrawal-mediated cAMP overshoot}

\textbf{Summary}

Organization of G protein-coupled receptors and cognate signaling partners at the plasma membrane has been proposed to occur \textit{via} multiple mechanisms including membrane microdomains, receptor oligomerization and protein scaffolding. Here, we investigate the organization of six types of \textit{G}_{i/o}-coupled receptors endogenously expressed in SH-SY5Y cells. The most abundant receptor in these cells was MOR, activation of which occluded acute inhibition of AC by agonists to DOR, NOPr, \alpha_2-adrenergic (\alpha_2AR), cannabinoid (CB_1) and serotonin 1A (5-HT_1A) receptors. We further demonstrate that all receptor pairs share a common pool of AC. The MOR agonist DAMGO also occluded the ability of DOR agonist to stimulate G proteins. However, at lower agonist concentrations and at shorter incubation times when G proteins were not limiting, the relationship between MOR and DOR agonists was additive. The additive relationship was confirmed by isobolographic analysis. Chronic co-administration of MOR and DOR agonists caused cAMP overshoot that was not additive, suggesting that sensitization of AC mediated by these two receptors occurs by a common pathway. Furthermore, heterologous inhibition of AC by agonists to DOR, NOPr and \alpha_2AR reduced the expression of cAMP overshoot in DAMGO-dependent cells. However, this cross-talk did not lead to heterologous tolerance. These results indicate that multiple receptors could be tethered into complexes with cognate signaling proteins and that access to shared AC by multiple receptor types may provide a means to prevent opioid withdrawal.
Introduction

Opioid receptors are members of the G protein-coupled receptor (GPCR) family and signal via activation of AC-inhibitory (G_{i/o}) GTP-binding proteins. It has been suggested that the probability of opioid receptor/G protein interaction is enhanced by compartmentalization in the membrane (Alt et al., 2001), allowing rapidity of GPCR signal propagation (Hur and Kim, 2002). Various modes of organization in the plasma membrane have been proposed to describe these compartments, including dimerization of opioid receptors (George et al., 2000; Gomes et al., 2004) and other GPCRs (Milligan, 2009), membrane microdomains (Allen et al., 2007) or protein scaffolds (Hall and Lefkowitz, 2002). However, mathematical modeling of experimental findings supporting compartmentalization has claimed that these data can be explained by a collision coupling model (Tolkovsky and Levitzki, 1978; Stickle and Barber, 1992) without the need to invoke compartments (Brinkerhoff et al., 2008).

Compartments also prevent interactions between two proteins by constraining cross-talk and/or sharing of effector molecules, thus leading to signaling specificity. In NG108-15 cells, muscarinic receptors and DOR did not share G proteins with α_{2}AR, as measured by agonist binding (Graeser and Neubig, 1993). In this scenario, co-administration of agonists for separately compartmentalized receptors would result in an additive response as each receptor type activated its own pool of effectors. For instance, in N18TG2 neuroblastoma cells, agonists to endogenous DOR and CB_{1} receptors activated G proteins in an additive manner (Shapira et al., 1998; Shapira et al., 2000). On the other hand, in SH-SY5Y cells, co-administration of a MOR agonist and a DOR agonist produced the same level of G protein activation as the MOR agonist alone, indicating that MOR and DOR activate the same G proteins (Alt et al., 2002). Similarly, DOR and CB_{1} receptors cotransfected in COS-7 cells shared G proteins (Shapira et al., 2000) and MOR and α_{2}AR endogenously expressed in SH-SY5Y cells were observed to access the same AC enzymes (Lameh et al., 1992).

The conflicting data on DOR and CB_{1} receptor competition in N18TG2 and COS-7 cells can potentially be explained by differences in the level of expression of receptors. At high density, receptors compete for a limiting pool of G proteins, whereas, at low
receptor concentrations, G proteins are in excess and agonists for two receptor types activate G proteins in an additive manner regardless of compartmentalization (Brinkerhoff et al., 2008). However, at low receptor levels, artificially reducing G protein number (using pertussis toxin; PTX) did not increase competition (Graeser and Neubig, 1993; Shapira et al., 2000), suggesting that receptor number is more predictive of competition than G protein number (Brinkerhoff et al., 2008).

Competition between only two GPCR types would be observed if the receptors were able to freely diffuse along the cell membrane to access all available G proteins or if they were corralled together, i.e. in a membrane microdomain, by scaffolding proteins or by dimerization. By considering competition between multiple receptor types, the chance of all receptors sharing the same compartment decreases, and it should therefore be easier to differentiate between receptors that are somehow constrained together and those that are not. The goal of the experiments presented here was to determine the degree of competition or effector sharing between multiple inhibitory GPCRs endogenously expressed in SH-SY5Y cells and the consequences of this competition for signaling to AC. We show that agonist-occupied MOR can access all AC available to these other G\textsubscript{\textalpha} \textalpha \textsubscript{\textbeta} \textbeta \textgamma \textgammacoupled GPCRs suggesting a lack of compartmentalization and/or the presence of complexes containing multiple receptors. Moreover, depending on the level of receptor expression, agonists at non-MOR GPCRs are able to attenuate the cAMP overshoot observed following withdrawal from exposure to a chronic MOR agonist, thus suggesting a mechanism for the prevention of opioid withdrawal.

**Materials and Methods**

*Materials*—[^3]H[DAMGO ([D-Ala\textsuperscript{2}, N-Me-Phe\textsuperscript{4}, Gly\textsuperscript{5}-ol]-enkephalin), [^3]HDPDPE ([D-Pen\textsuperscript{2,5}]-enkephalin), [^3]Hnociceptin/OFQ, [^3]Hyohimbine, [^3]HCP 55,940 and [\textsuperscript{35}S]GTP\textgamma S (guanosine-5'-O-(3-[\textsuperscript{35}S]thio)triphosphate) were obtained from Perkin-Elmer Life Sciences (Boston, MA). SNC80 ((+)-4-[(\alpha R)-\alpha-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide) was obtained from the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). DAMGO, DPDPE, naloxone, CTAP (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-
Thr-NH₂), nociceptin/orphanin FQ (nociceptin/OFQ), UK 14304, clonidine, forskolin and IBMX (3-isobutyl-1-methylxanthine) were from Sigma-Aldrich (St. Louis, MO). CP 55,940 and WIN 55212-2 were from Cayman Chemical (Ann Arbor, MI). ICI 174,864 (N, N-diallyl-Tyr-Aib-Aib-Phe-Leu-Oh) and J113397 (1-[(3R,4R)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one) were from Tocris Bioscience (Ellisville, MO). Retinoic acid was obtained from Calbiochem (La Jolla, CA). Pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). Tissue culture media, fetal bovine serum and trypsin were from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of analytical grade.

Cell culture- Human neuroblastoma SH-SY5Y cells, a subclone of SK-N-SH cells, were obtained from ATCC (Manassas, VA), grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37°C in 5% CO₂, and used within passages 34 – 44 from subcloning to maintain consistent neuroblast properties between experiments. All experiments were performed in SH-SY5Y cells differentiated with 10 µM retinoic acid for 4 – 7 days prior to assay.

Radioligand binding assays- Membranes were prepared from retinoic acid-differentiated SH-SY5Y cells, as described previously (Alt et al., 2002). Final membrane pellets were resuspended in 50 mM Tris-HCl buffer, aliquoted and stored at -80°C. Protein concentration was measured using the Bradford assay (Bradford, 1976).

Receptor density was determined by incubating membranes (50 µg) for 60 min at 25°C with shaking in 50 mM Tris-HCl, pH 7.4 buffer containing saturating concentrations of radiolabeled ligand as follows: 12 nM [³H]DAMGO for MOR, 16 nM [³H]DPDPE for DOR, 1 nM [³H]nociceptin/OFQ for nociceptin/orphanin FQ peptide receptor (NOPr), 10 nM [³H]yohimbine for α₂AR or 6 nM [³H]CP 55,940 for CB₁. Non-specific binding was determined with unlabeled naloxone (MOR and DOR), J113397 (NOPr), UK 14304 (α₂AR) or WIN 55212-2 (CB₁). All plasticware was precoated with Sigma Cote (Sigma-Aldrich, St. Louis, MO) and 0.1% BSA was included for [³H]CP 55,940 binding. Assays were stopped by rapid filtration through GF/C filters presoaked in 0.1% polyethyleneimine using a Brandel harvester (MLR-24, Gaithersburg, MD) and
rinsed three times with ice-cold 50 mM Tris-HCl wash buffer, pH 7.4. Dried filters were saturated with EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH) and radioactivity was counted in a Wallac 1450 MicroBeta (Perkin-Elmer, Waltham, MA).

**Stimulation of \[^{35}S\]GTP\(\gamma\)S binding-** Membranes were prepared from retinoic acid-differentiated SH-SY5Y cells, as described for radioligand binding assays. In some experiments, cells were treated overnight with agonist (SNC80 or DAMGO) or for 24 h with PTX (100 ng/ml) prior to membrane preparation.

Membranes (50 \(\mu\)g protein) were incubated with 0.1 nM \[^{35}S\]GTP\(\gamma\)S for 60 min (unless otherwise indicated) at 25°C, with or without various concentrations of SNC80 and/or DAMGO in \[^{35}S\]GTP\(\gamma\)S binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 100 mM NaCl, 1mM EDTA, 2 mM dithiothreitol and 30 \(\mu\)M GDP). Membranes with bound \[^{35}S\]GTP\(\gamma\)S were collected on GF/C filters (Whatman, Middlesex, UK) using a Brandel harvester (MLR-24, Gaithersburg, MD) and rinsed three times with cold wash buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 100 mM NaCl). Bound radioactivity was determined by liquid scintillation counting as described in radioligand binding methods.

**Cyclic AMP accumulation assays-** For inhibition of AC, SH-SY5Y cells were plated in 24-well plates (5 x 10\(^5\) cells / well) and differentiated with 10 \(\mu\)M retinoic acid 4 days prior to assay. Cells were incubated with 1 \(\mu\)M of the indicated agonist(s) in the presence of 5 \(\mu\)M forskolin and 1 mM IBMX in DMEM/10% FBS for 10 min at 37°C. The assay was stopped by replacing the media with 1 ml ice-cold 3% perchloric acid. After at least 30 min at 4°C, a 400 \(\mu\)l aliquot of sample was neutralized with 2.5 M KHCO\(_3\) and centrifuged at 13,000 x g. Cyclic AMP was measured from the supernatant using a \[^{3}H\]cAMP assay system (GE Healthcare, Buckinghamshire, UK) following the manufacturer’s instructions. Inhibition of cAMP formation was calculated as percent inhibition of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

For AC sensitization experiments, differentiated SH-SY5Y cells were incubated overnight in the presence or absence of DAMGO and/or DPDPE in DMEM/10% FBS at 37°C. Drug-containing media was replaced with media containing 5 \(\mu\)M forskolin, 1 mM IBMX and 1 \(\mu\)M MOR antagonist CTAP for DAMGO-treated cells, 30 \(\mu\)M forskolin, 1
mM IBMX and 1 µM DOR antagonist ICI 174,864 for DPDPE-treated cells, or 5 µM forskolin, 1 mM IBMX and 100 µM opioid antagonist naloxone for chronic DAMGO and DPDPE-treated cells, to precipitate cAMP overshoot. In some experiments, DAMGO-containing media was replaced with media containing 5 µM forskolin, 1 mM IBMX, 1 µM MOR antagonist CTAP and 1 µM G\(_{i/o}\)-coupled receptor agonist. After 10 min at 37°C, the assay was stopped with ice cold 3% perchloric acid and cAMP accumulation was quantified as described above. Overshoot was calculated as either percent cAMP overshoot or as a percent of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

*Isobologram analysis* - An isobologram for agonists (SNC80 and DAMGO) with different maxima and therefore a variable potency ratio was constructed based on the following equation (Tallarida, 2006):

\[
b = B_i - \frac{B_{50}}{\frac{E_b}{E_c}\left(1 + \frac{A}{a}\right) - 1}
\]

The parameters for the equation were based on values from the individual concentration-effect curves for DAMGO and SNC80. As the more efficacious drug in these experiments, DAMGO was assigned as Drug B and SNC80 as Drug A (Tallarida, 2006). \(B_{50}\) and \(A_c\) represent the EC\(_{50}\) of DAMGO and SNC80, respectively. \(E_b\) and \(E_c\) represent the maximal effect (fmol \([^{35}S]\)GTP\(\gamma\)S bound/mg) produced by DAMGO and SNC80, respectively. The equation was solved for either \(a\) or \(b\) (concentration of SNC80 or DAMGO in nM, respectively) at the concentration of DAMGO that produced 50 % of its maximal effect (\(B_i\)). Therefore, in this case \(B_i = B_{50}\). The derived \((a, b)\) coordinates were fit to exponential one phase decay in GraphPad Prism 5 (San Diego, CA) to produce the line of additivity. Concentration-effect curves for DAMGO were then obtained in the presence of set concentrations of SNC80 (1, 5, 10, 20 or 30 nM). The concentration of DAMGO, when in combination with SNC80, needed to produce 50 % of its maximum effect was determined and plotted on the isobologram as mean ± S.E.M. for 3 separate experiments. Since the error for all points overlaps the line of additivity they were
assumed to not be statistically different than the line and no further statistical analysis was conducted.

Statistical Analysis- All data were analyzed using GraphPad Prism 5 software (San Diego, CA). Data points represent at least three separate experiments in duplicate and are presented as mean ± standard error of the mean (S.E.M.), unless otherwise noted. The addition of single agonist concentrations was analyzed by one-way ANOVA with Bonferroni’s post-hoc test. Percent competition values were compared to 100 by one-sample t-test. Effects on agonist responses at various concentrations were analyzed by 2-way ANOVA with Bonferroni’s post-hoc test. EC50 values were calculated from individual concentration-effect curves using non-linear three parameter log [agonist]-response curve fit analysis in GraphPad Prism and compared for statistical significance by unpaired, two-tailed Student’s t-test. For all tests significance was set at p < 0.05.

Results

\( G_{i/o} \)-coupled receptors expressed in SH-SY5Y cells

Human neuroblastoma SH-SY5Y cells were differentiated with retinoic acid (10 µM for 4 – 7 days) to produce a neuronal-like phenotype. Differentiation increased MOR density from 232 ± 33 fmol/mg protein to 305 ± 42 fmol/mg protein, as identified by the specific MOR agonist \(^{3}\)H-DAMGO, and increased the level of AC inhibition by DAMGO, as reported previously (Yu and Sadee, 1988; Zadina et al., 1994). In differentiated SH-SY5Y cells, agonists for the following receptors were shown to inhibit AC: MOR, DOR, NOPr, \( \alpha_2 \)AR, CB1 and 5-HT1A (Figure 3.1a). However, the ability of a maximal concentration (1 µM) of these agonists to inhibit AC was not equal. The most effective agonist was the MOR agonist DAMGO, followed closely by the DOR agonist SNC80 and the NOPr agonist nociceptin/OFQ. The following agonists had similar activity, but caused significantly less inhibition than DAMGO: UK 14304 (\( \alpha_2 \)AR), clonidine (\( \alpha_2 \)AR), CP 55,9140 (CB1), 8-OH-DPAT (5-HT1A). All of the agonists used are commonly regarded as full agonists, except clonidine and 8-OH-DPAT, which display partial agonist activity in certain assays (Varrault and Bockaert, 1992; Parsley et
However, in this assay, clonidine caused the same degree of cAMP inhibition as the full α₂AR agonist UK 14304.

The endogenous expression level of the above identified receptors was determined in membranes from differentiated SH-SY5Y cells using maximal concentrations of selective radioligands for each receptor. Receptor densities are listed in Table 3.1 and follow the same rank order as the ability of selective agonists for each receptor to inhibit AC.

\textit{G\textsubscript{i/o}-coupled receptors compete for AC}

To evaluate the level of interaction between MOR and other G\textsubscript{i/o}-coupled receptors in SH-SY5Y cells, maximal concentrations of G\textsubscript{i/o}-coupled receptor agonists were combined with a maximal concentration of DAMGO. When added to DAMGO, none of the agonists were able to inhibit AC to a greater degree than DAMGO alone (Figure 3.1b), indicating that MORs are able to access and inhibit the same AC enzymes as other G\textsubscript{i/o}-coupled receptors. We next addressed whether agonists to α₂AR, CB\textsubscript{1} or 5-HT\textsubscript{1A} receptors, which produced lower maximal inhibition than DAMGO, would compete with each other for AC. Even these less efficacious agonists were not able to inhibit AC to any greater extent in combination than when applied alone (Figure 3.1c).

The extent of competition between receptors for AC can be calculated using the following equation (Brinkerhoff et al., 2008):

\[
\% \text{ competition} = \left[ \frac{(R_1 + R_2) - R_{1+2}}{(R_1 + R_2) - \max(R_1, R_2)} \right] \times 100
\]

where \(R_1\) and \(R_2\) are two different receptor types giving a theoretical maximum response \((R_1 + R_2)\), which is compared to the experimentally determined effect of \(R_{1+2}\) and the effect of the most efficacious agonist alone \([\max(R_1, R_2)]\). If two receptors do not compete, the theoretical additive and experimental additive will be equivalent so there will be zero % competition. When there is complete (100%) competition between two receptors, the experimental addition of both agonists does not increase the response over the most efficacious agonist alone. Using the data from experiments in SH-SY5Y cells described above, complete competition was observed for all receptor pairs (Table 2; \(p >\)}
0.05 compared to 100 % by one sample t-test). In addition, the DOR agonist SNC80 or the NOPr agonist nociceptin/OFQ occluded responses from the other less effective agonists, resulting in competition that was not significantly different from 100 % (Table 3.3; p > 0.05, one sample t-test). Therefore, at maximal agonist concentrations, G\textsubscript{i/o}-coupled receptors, including the opioid receptors, compete for a shared pool of AC.

**MOR and DOR share G proteins**

Interactions between receptors could be occurring at the level of AC or G protein. To evaluate this we studied the interaction between MOR and DOR for stimulation of G proteins as measured by binding of [\textsuperscript{35}S]GTP\textsubscript{γ}S. Maximum concentrations of DAMGO stimulated a greater degree of [\textsuperscript{35}S]GTP\textsubscript{γ}S binding than SNC80, consistent with their relative degrees of AC inhibition and the greater expression of MOR compared to DOR in these cells. When added together, DAMGO and SNC80 stimulation of [\textsuperscript{35}S]GTP\textsubscript{γ}S binding was similar to binding stimulated by DAMGO alone (p > 0.05 comparing DAMGO to DAMGO/SNC80 by one-way ANOVA with Bonferroni’s post-test) and significantly less than the theoretical additive (p < 0.01 comparing R1 + R2 to DAMGO/SNC80 by one-way ANOVA with Bonferroni’s post-test) (Figure 3.2a), giving a percent competition between DAMGO and SNC80 of 88 ± 2 %, similar to the level of competition between these two agonists for AC. These results indicate significant sharing of G proteins between MOR and DOR in differentiated SH-SY5Y cells.

It has been proposed that MOR and DOR heterodimerize and that these oligomers can activate PTX-resistant G proteins (George et al., 2000). However, in the differentiated SH-SY5Y cells used in our experiments, [\textsuperscript{35}S]GTP\textsubscript{γ}S binding stimulated by the combination of DAMGO and SNC80 was completely eliminated by PTX treatment (Figure 3.2b; PTX 100 ng/ml, 24 h), indicating that the combination of agonists still signals through PTX-sensitive G\textsubscript{αi/o} proteins. In addition, PTX did not alter spontaneous [\textsuperscript{35}S]GTP\textsubscript{γ}S binding. Similarly, AC inhibition by the combination of DAMGO and SNC80 was also blocked by 24 h pretreatment with PTX (data not shown).
Isobolographic analysis of interactions between MOR and DOR agonists

Combinations of maximally effective concentrations of agonists resulted in less than additive effects and predict competition for a common effector; however, at lower agonist concentrations it should be possible to identify additive, sub-additive and synergistic interactions. To this end, the concentration dependence of DAMGO to stimulate \[^{35}\text{S}]\text{GTP} \gamma \text{S}\ binding was determined in the presence of a sub-maximal concentration of SNC80. Addition of 30 nM SNC80 with DAMGO did not significantly change the potency of DAMGO to stimulate \[^{35}\text{S}]\text{GTP} \gamma \text{S}\ binding (Figure 3.3a; EC\textsubscript{50} of DAMGO alone = 121 ± 32 nM, EC\textsubscript{50} of DAMGO + 30 nM SNC80 = 64 ± 12 nM, p > 0.05), and at maximal concentrations of DAMGO the level of \[^{35}\text{S}]\text{GTP} \gamma \text{S}\ binding was similar in the presence or absence of SNC80 (at 1 \mu M and 10 \mu M DAMGO, p > 0.05 by two-way ANOVA with Bonferroni’s post-test) and significantly less than the theoretical additive (at 10 \mu M DAMGO, p < 0.05 by two-way ANOVA with Bonferroni’s post-test; Figure 3.3a). However, at lower concentrations of DAMGO, combination with 30 nM SNC80 was similar to the theoretical additive (Figure 3.3a), indicating an additive interaction between MOR and DOR agonists until G proteins become limiting, at which point DAMGO is occlusive.

Additive actions of agonists or deviations from additivity can be observed graphically using an isobologram (Tallarida, 2006). An isobologram for combinations of DAMGO and SNC80 that produced 50 % of the maximal DAMGO effect was constructed based on values from the individual concentration-effect curves for DAMGO and SNC80 using the equation described in Methods (Tallarida, 2006). The line of additivity is not linear because DAMGO and SNC80 have different maxima and therefore a variable potency ratio. \[^{35}\text{S}]\text{GTP} \gamma \text{S}\ concentration-effect curves for DAMGO were performed in the presence of low concentrations of SNC80. The concentration combination that was required to produce 50 % of the maximal DAMGO effect was plotted on the isobologram. At these low concentrations, SNC80 produced an additive interaction when combined with DAMGO, as indicated by points falling on the line of additivity (Figure 3.3b). This agrees with results above and indicates that MOR and
DOR share a common set of G proteins that are activated additively at low concentrations but sub-additively when G proteins become limiting.

The binding of \( [^{35}\text{S}]\text{GTP}\gamma\text{S} \) is time-dependent with a \( t_{1/2} \) of approximately 20 min for DAMGO or SNC80-stimulated binding. Therefore, at time points shorter than 20 min, G proteins should not be limiting. When \( [^{35}\text{S}]\text{GTP}\gamma\text{S} \) binding was measured after a 10 or 20 min incubation, maximal concentrations of DAMGO and SNC80 were similar to the theoretical additive (Figure 3.3c) with competition between receptors only 12 ± 8.6 % after 10 min, increasing to 33 ± 11% after 20 min. These results are in agreement with an additive interaction of these agonists at less than saturating concentrations when G proteins are not limiting.

**Heterologous inhibition of AC prevents opioid receptor-mediated cAMP overshoot**

Chronic administration of opioid agonists and other G\(_{i/o}\)-coupled receptor agonists causes a homeostatic sensitization of AC resulting in an overshoot of cAMP production upon addition of a competitive antagonist (Sharma et al., 1975). To determine if MOR and DOR accessed the same systems responsible for AC sensitization during chronic treatment, cells were treated overnight with the peptidic DOR agonist DPDPE (10 µM) in the presence or absence of DAMGO. DPDPE alone produced an overshoot response which was enhanced in the presence of 10 nM, but not 100 nM DAMGO (Figure 3.4).

Since MOR and DOR accessed the same pool of AC in both the acute and chronic opioid state, we hypothesized that overshoot of cAMP occurring upon precipitation of withdrawal from chronic MOR agonist treatment would be prevented by acute addition of a DOR agonist. To test this hypothesis, differentiated SH-SY5Y cells were treated overnight with DAMGO (100 nM) and withdrawal was precipitated with the MOR antagonist CTAP in the presence or absence of 1 µM SNC80. The addition of SNC80 attenuated the AC overshoot response (Figure 3.5). Moreover, this attenuation was also observed with addition of the NOPr agonist nociceptin/OFQ and the \( \alpha_2\)AR agonists UK 14304 and clonidine (Figure 3.5). Agonists that did not inhibit AC as efficiently (CP 55,9140 and 8-OH-DPAT) were unable to attenuate MOR-mediated cAMP overshoot.
To determine the concentration-relationship of this effect, DAMGO-mediated cAMP overshoot was precipitated by CTAP in the presence or absence of varying concentrations of SNC80. The addition of SNC80 reduced DAMGO-mediated overshoot in a concentration-dependent manner (Figure 3.6a). Furthermore, SNC80 inhibited cAMP production with a similar potency in vehicle or DAMGO-treated cells (vehicle = 14.6 ± 7.8 nM, DAMGO-treated = 13.8 ± 7.2 nM). The effect of SNC80 was via DOR because MOR was blocked by the selective antagonist CTAP in both vehicle and DAMGO-treated cells, and in separate experiments 1 µM CTAP did not affect AC inhibition by 100 nM SNC80 (100 nM SNC80 = 59 ± 3 % cAMP inhibition, 100 nM SNC80 + 1 µM CTAP = 55 ± 2 % cAMP inhibition; n = 6, p > 0.05).

Reciprocally, DOR-mediated cAMP overshoot was attenuated in a concentration-dependent manner by DAMGO (Figure 3.6b). In these experiments, cells were treated overnight with DPDPE and specific DOR-mediated cAMP overshoot was precipitated using the selective DOR antagonist ICI 174,864 (1 µM) in the absence or presence of increasing concentrations of DAMGO. In addition to preventing overshoot, DAMGO inhibited AC with a similar potency in vehicle or DPDPE-treated cells (EC50: vehicle-treated = 32.2 ± 12.5 nM, DPDPE-treated = 29.0 ± 7.1 nM). 1 µM ICI 174,864 did not shift the ability of DAMGO to stimulate [35S]GTPγS binding in SH-SY5Y cells (EC50: Control = 263 ± 30 nM; with ICI 174,864 = 283 ± 35 nM, p = 0.70, n = 2).

Lack of heterologous tolerance between MOR and DOR

The similar EC50 values for AC inhibition in opioid-treated and naïve cells for both MOR and DOR agonists suggest a lack of cross-tolerance between these two receptors in SH-SY5Y cells. To confirm this, agonist-stimulated [35S]GTPγS binding was measured in membranes from SH-SY5Y cells that were treated overnight with vehicle, DAMGO or SNC80. DAMGO stimulated [35S]GTPγS binding in vehicle-treated SH-SY5Y membranes in a concentration dependent manner (Figure 3.7a, EC50 = 86 ± 16 nM). Both the maximum effect (vehicle-treated = 121 ± 3.7 % stimulation; DAMGO-treated = 29 ± 2.9 % stimulation, p < 0.0001) and EC50 (vehicle-treated = 86 ± 16 nM; DAMGO-treated = 260 ± 62 nM, p < 0.05) of DAMGO-stimulated [35S]GTPγS binding
was significantly attenuated in membranes from cells treated overnight with 1 µM DAMGO, indicating the development of tolerance (Figure 3.7a). In comparison, SNC80-stimulated $[^{35}S]$GTPγS binding was similar in membranes from vehicle or DAMGO-treated cells (Figure 3.7b, vehicle-treated EC$_{50}$ = 29.8 ± 12.5 nM; DAMGO-treated EC$_{50}$ = 34.6 ± 10.8 nM; vehicle-treated max = 53.1 ± 4.7 % stimulation; DAMGO-treated max = 56.5 ± 3.5 % stimulation). The reverse treatment paradigm produced similar results.

Treatment of SH-SY5Y cells overnight with 1 µM SNC80 produced marked homologous tolerance, indicated by a significant reduction in $[^{35}S]$GTPγS binding by maximal concentrations of SNC80 (Figure 3.7c, vehicle-treated = 57.2 ± 3.4 % stimulation; SNC80-treated = 14.6 ± 4.8 % stimulation, p < 0.0001). However, the potency and efficacy for DAMGO to stimulate $[^{35}S]$GTPγS binding was not affected by SNC80 pretreatment (Figure 3.7d, vehicle-treated EC$_{50}$ = 69 ± 8.5 nM; SNC80-treated EC$_{50}$ = 79 ± 1.6 nM; vehicle-treated max = 106.7 ± 7.5 %; SNC80-treated max = 100.7 ± 5.9 %, p > 0.05). Unstimulated binding of $[^{35}S]$GTPγS in the absence of agonist was similar in vehicle, DAMGO or SNC80-pretreated cells (vehicle-treated = 9.8 ± 0.6 fmol/mg; DAMGO-treated = 11.6 ± 1.4 fmol/mg; SNC80-treated = 11.4 ± 0.6 fmol/mg, p > 0.05 one-way ANOVA with Bonferroni’s post-test). Together these results confirm a lack of cross-tolerance between MOR and DOR in differentiated SH-SY5Y cells, similar to previous reports in undifferentiated cells (Zadina et al., 1994; Alt et al., 2002).

**Discussion**

In this study, we have shown that AC inhibition by agonists to DOR, NOPr, $\alpha_2$AR, CB$_1$ and 5-HT$_{1A}$ receptors in differentiated SH-SY5Y cells was occluded by a maximal concentration of the MOR agonist DAMGO, suggesting that all of these receptors compete for and inhibit the same AC enzymes. The competition, as shown by MOR and DOR, began at the G protein, was additive when the G protein was not limiting, and reached an occlusive ceiling at maximal agonist concentrations. Similar competition occurred during chronic agonist exposure such that acute administration of agonists to DOR, NOPr and $\alpha_2$AR prevented the expression of AC sensitization.
following chronic DAMGO-treatment. However, the cross-talk between MOR and DOR did not lead to heterologous tolerance.

The rank order of AC inhibition by a maximum concentration of full agonists acting at $G_{i/o}$-coupled receptors in SH-SY5Y cells was $\text{MOR} > \text{DOR} \geq \text{NOPr} > \alpha_2\text{AR} \geq \text{CB}_1 = 5\text{-HT}_1\text{A}$. This order is likely determined by the relative receptor expression, which follows a similar pattern. Thus, all of these $G_{i/o}$-coupled receptors shared a common pool of AC, but the proportion of the AC pool utilized by each receptor was determined by receptor density. A conceptual schematic representing the relative pool of AC accessed by each receptor type is shown in Figure 3.8, where the pool of AC shrinks as receptor density and the ability to inhibit AC decreases. The most active agonist, the MOR agonist DAMGO, had access to the greatest amount of AC and at a maximal concentration occluded effects by agonists to DOR, NOPr, $\alpha_2\text{AR}$, CB$_1$ and 5-HT$_1$A receptors. DOR and NOPr agonists were slightly less effective than DAMGO to inhibit AC, but still occluded responses from $\alpha_2\text{AR}$, CB$_1$ and 5-HT$_1$A receptors. Agonists to $\alpha_2\text{AR}$, CB$_1$ and 5-HT$_1$A receptors inhibited AC the least and even these receptors were not additive with each other, suggesting that the pool is always limiting even for receptors with lower expression levels.

The ability of all receptors to share AC indicates that barriers to prevent free diffusion in the membrane, such as receptor dimers, membrane microdomains, and protein scaffolds (George et al., 2000; Alt et al., 2001; Hall and Lefkowitz, 2002; Gomes et al., 2004; Allen et al., 2007) do not segregate these receptors from the common pool of AC. Two alternative hypotheses could explain these results. Free access of receptors to all G proteins and AC as predicted by the collision coupling model would allow receptors to share a common pool of AC and has recently been discussed as an alternative explanation to negative cooperativity data that was attributed to dimerization (Chabre et al., 2009). However, such a scenario would seem unlikely given the evidence that MOR diffusion is restricted to sub-micrometer domains (Sauliere et al., 2006). Secondly, there could be complexes of multiple receptors isolated with signaling molecules. Most of the $G_{i/o}$-coupled receptors expressed in SH-SY5Y cells have been reported to heterodimerize with MOR, including DOR (George et al., 2000; Gomes et al., 2004), NOPr (Wang et al., 2004),
2005), α₂AR (Jordan et al., 2003) and CB₁ (Rios et al., 2006), while DOR has been shown to form heterodimers with α₂AR (Rios et al., 2004). In addition, preformed signaling complexes containing GPCR and multiple effectors have been identified (Davare et al., 2001). If such complexes contained multiple GPCRs with shared G protein and AC, and the proportion of complexes containing a certain receptor type was related to the receptor density, then these large signalosomes could account for the results shown above. In SH-SY5Y cells, such complexes would always include MOR and could include at least five other G_{i/o}-coupled receptor types (Figure 3.8).

Agonists for DOR, NOPr and α₂AR were able to inhibit AC in the naïve and opioid-dependent state. The rank order of the effectiveness of agonists to inhibit AC remained the same in control and DAMGO-dependent cells, so that the most efficacious agonists (SNC80 and nociceptin/OFQ) significantly prevented DAMGO-mediated cAMP overshoot. One exception was the α₂AR agonist UK 14304, which was equally effective as SNC80 and nociceptin/OFQ at preventing DAMGO-mediated overshoot, but considerably less efficacious at AC inhibition in the naïve cell. An increase in α₂AR density following chronic DAMGO exposure could explain the enhanced UK 14304 response. However, in rats, α₂AR density in various brain regions was either decreased or unchanged following chronic morphine treatment (Smith et al., 1989). Alternatively, the α₂AR signaling system may become more efficient after chronic MOR agonist treatment. One mechanism may involve the regulators of G protein signaling (RGS) family. Chronic MOR agonist treatment can down-regulate certain RGS proteins (Traynor, 2010). Since RGS proteins are negative regulators of signaling, this down-regulation could permit increased signaling through α₂AR. In addition, a lack of effect on NOPr signaling by the RGS proteins endogenously expressed in SH-SY5Y cells could explain the robust signaling through NOPr in SH-SY5Y cells that would not be predicted based on NOPr expression levels.

AC sensitization occurs following chronic MOR occupation and is thought to be important for the manifestation of withdrawal (Sharma et al., 1975). Specifically, upregulation of the cAMP/AC/ protein kinase A (PKA) pathway in the locus coeruleus (LC) has been identified as a mediator of opioid dependence and withdrawal, most
recently by (Zachariou et al., 2008). Thus, drugs that inhibit the cAMP pathway and can counter AC sensitization, such as the \( G_{i/o} \)-coupled receptor agonists presented here, would have therapeutic potential in the treatment of opioid withdrawal. For instance, the clinical utility of the \( \alpha_2 \)AR agonist clonidine in opioid withdrawal has been known for some time (Gold et al., 1978) and \( \alpha_2 \)AR agonists are often used “off label” to treat or prevent opioid withdrawal (Gowing et al., 2009). Furthermore, the \( \alpha_2 \)AR agonist lofexidine, which has been reported to cause fewer side effects and is available in the U.K., could become the first non-opiate FDA-approved treatment of opioid withdrawal (Yu et al., 2008).

It is thought that clonidine prevents opioid withdrawal symptoms by reversing hyperactivity of noradrenergic neurons in the LC (Aghajanian, 1978). There are two prevalent proposed mechanisms for withdrawal-induced hyperactivity of LC neurons in opioid-dependence. The first is an enhanced input of excitatory glutamate into the LC (Aston-Jones et al., 1997). The second is an intracellular sensitization mediated by upregulation of the cAMP pathway, and is supported by data that in vitro, withdrawal-induced hyperactivity is suppressed by inhibitors of PKA and is enhanced by forskolin or an active cAMP analog (Ivanov and Aston-Jones, 2001). Thus, our findings that clonidine can heterologously inhibit cAMP and prevent DAMGO-mediated cAMP overshoot, supports a mechanism for clonidine in opioid withdrawal.

NOP and CB\(_1\) receptors are also co-expressed with MOR in LC neurons, where NOPr was found to activate the same population of \( K^+ \) channels as MOR and \( \alpha_2 \)AR (Connor et al., 1996; Scavone et al., 2009). Furthermore, intracerebroventricular injection of nociceptin/OFQ prevented naloxone-precipitated withdrawal symptoms in morphine-dependent rats, and the non-peptidic NOPr agonist Ro 64-6198 reduced the expression of morphine-withdrawal jumping in mice when administered just prior to precipitation of withdrawal (Kotlinska et al., 2000; Kotlinska et al., 2003). This is again consistent with our findings that administration of nociceptin/OFQ during antagonist-precipitated withdrawal reduced cAMP overshoot and supports an intracellular mechanism of competitive inhibition of shared AC. Although the CB\(_1\) agonist CP 55,940 did not prevent MOR-mediated cAMP overshoot in SH-SY5Y cells, CB\(_1\) receptors, if co-
expressed with MOR in sufficient quantities, would be predicted to prevent morphine-withdrawal signs through a similar intracellular mechanism of AC inhibition. This is pertinent because levels of CB₁ in the brain are generally high and acute administration of cannabinoid agonists Δ⁹-tetrahydrocannabinol or anandamide to morphine-dependent rodents prevented withdrawal symptoms including jumping, weight loss, wet dog shakes and diarrhea, although the mechanism or site of action was not determined (Hine et al., 1975; Vela et al., 1995).

In contrast to α₂AR, NOPr and CB₁ receptors, the role of DOR in attenuating MOR-mediated withdrawal may be less relevant in vivo. Although we have shown that DOR shares AC with MOR, and the DOR agonist SNC80 inhibits DAMGO-mediated cAMP overshoot in SH-SY5Y cells, an in vivo intracellular mechanism between MOR and DOR to prevent morphine-withdrawal will obviously depend on co-expression in a single neuron. DOR is not expressed in the LC and although other brain regions are also important in withdrawal (Christie et al., 1997), the co-expression of MOR and DOR on neurons in other regions is debatable (Gomes et al., 2004; Scherrer et al., 2009). Furthermore, administration of the DOR agonist BW373U86 just prior to naloxone-precipitated withdrawal in morphine-dependent rats did not reduce withdrawal symptoms (Lee et al., 1993).

In conclusion, these studies have shown that all identified G<sub>1i/o</sub>-coupled receptors endogenously expressed in differentiated SH-SY5Y cells shared a common pool of AC so that spatial barriers did not prevent specific receptor access. The interaction likely begins at the G-protein level for all receptors, but certainly does for MOR and DOR. At this stage, we cannot distinguish between a lack of compartmentalization and the presence of signalosomes that contain several receptor types and signaling proteins. However, biophysical data on MOR membrane diffusion would tend to support a model in which there is organization of receptors (Sauliere et al., 2006). Regardless of the model, heterologous inhibition of shared AC by DOR, NOPr and α₂AR agonists prevented the expression of cAMP overshoot in MOR agonist-dependent cells. Thus, these studies support an intracellular mechanism for the prevention of morphine-withdrawal symptoms by acute administration of α₂AR, NOPr or CB₁ receptor agonists.
Table 3.1: Receptor density in differentiated SH-SY5Y cells.
Receptor numbers in cell membranes were determined using selective radioligands at a maximal concentration as described in Methods. Results are presented as mean fmol radioligand bound/mg protein ± S.E.M. (n = 6).

<table>
<thead>
<tr>
<th>Receptor Type</th>
<th>Radioligand</th>
<th>Receptor density (fmol/mg protein ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOR</td>
<td>$^{3}$H-DAMGO</td>
<td>305 ± 42</td>
</tr>
<tr>
<td>DOR</td>
<td>$^{3}$H-DPDPE</td>
<td>191 ± 21</td>
</tr>
<tr>
<td>CB$_1$</td>
<td>$^{3}$H-CP 55,940</td>
<td>60 ± 25</td>
</tr>
<tr>
<td>$\alpha_{2}$AR</td>
<td>$^{3}$H-UK 14304</td>
<td>43 ± 11</td>
</tr>
<tr>
<td>NOPr</td>
<td>$^{3}$H-nociceptin/OFQ</td>
<td>30 ± 11</td>
</tr>
</tbody>
</table>

$^{*}$5-HT$_{1A}$ not tested
Table 3.2: Competition between indicated agonists (1 µM) for acute inhibition of AC.

Percent competition was calculated as described in Results (Brinkerhoff et al., 2008) using experimental data shown in Figure 3.1. The percent competition from three individual experiments was compiled and is presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Agonist $R_1$ (receptor)</th>
<th>Agonist $R_2$ (receptor)</th>
<th>% competition (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAMGO (MOR)</td>
<td>SNC80 (DOR)</td>
<td>78 ± 8</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>Nociceptin/OFQ (NOPr)</td>
<td>97 ± 12</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>UK 14304 ($\alpha_2$AR)</td>
<td>115 ± 10</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>Clonidine ($\alpha_2$AR)</td>
<td>106 ± 22</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>CP 55,9140 (CB)</td>
<td>121 ± 24</td>
</tr>
<tr>
<td>DAMGO (MOR)</td>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>97 ± 13</td>
</tr>
<tr>
<td>UK 14301 ($\alpha_2$AR)</td>
<td>CP 55,9140 (CB)</td>
<td>95 ± 19</td>
</tr>
<tr>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>Clonidine ($\alpha_2$AR)</td>
<td>95 ± 16</td>
</tr>
<tr>
<td>8-OH-DPAT (5-HT$_{1A}$)</td>
<td>CP 55,9140 (CB)</td>
<td>83 ± 6</td>
</tr>
</tbody>
</table>
Table 3.3: Competition between 1 μM SNC80 or nociceptin/OFQ and other G<sub>i/o</sub>-coupled receptor agonists for acute inhibition of AC.

Inhibition of AC by 1 μM of agonist(s) alone or in combination was performed as described for Figure 3.1 and in Methods. Percent competition was calculated from three individual experiments, in duplicate, as described for Table 3.2.

<table>
<thead>
<tr>
<th>Agonist R&lt;sub&gt;1&lt;/sub&gt; (receptor)</th>
<th>Agonist R&lt;sub&gt;2&lt;/sub&gt; (receptor)</th>
<th>% competition (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNC80 (DOR)</td>
<td>Nociceptin/OFQ (NOPr)</td>
<td>79 ± 18</td>
</tr>
<tr>
<td>SNC80 (DOR)</td>
<td>UK 14304 (α&lt;sub&gt;2&lt;/sub&gt;AR)</td>
<td>84 ± 30</td>
</tr>
<tr>
<td>SNC80 (DOR)</td>
<td>Clonidine (α&lt;sub&gt;2&lt;/sub&gt;AR)</td>
<td>72 ± 21</td>
</tr>
<tr>
<td>SNC80 (DOR)</td>
<td>CP 55,9140 (CB)</td>
<td>88 ± 32</td>
</tr>
<tr>
<td>SNC80 (DOR)</td>
<td>8-OH-DPAT (5-HT&lt;sub&gt;1A&lt;/sub&gt;)</td>
<td>119 ± 12</td>
</tr>
<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>UK 14304 (α&lt;sub&gt;2&lt;/sub&gt;AR)</td>
<td>108 ± 15</td>
</tr>
<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>Clonidine (α&lt;sub&gt;2&lt;/sub&gt;AR)</td>
<td>119 ± 11</td>
</tr>
<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>CP 55,9140 (CB)</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>Nociceptin/OFQ (NOPr)</td>
<td>8-OH-DPAT (5-HT&lt;sub&gt;1A&lt;/sub&gt;)</td>
<td>124 ± 10</td>
</tr>
</tbody>
</table>
Figure 3.1: Gi/o-coupled receptors endogenously expressed in SH-SY5Y cells share a common pool of AC. A, acute cAMP inhibition of 5 µM forskolin-stimulated AC by 1 µM of the indicated agonist alone. Agonist (receptor) = DAMGO (MOR), SNC80 (DOR), OFQ (NOPr), UK 14304 (α2AR), clonidine (α2AR), CP 55,940 (CB1), 8-OH-DPAT (5-HT1A). ***p < 0.001 compared to DAMGO by one-way ANOVA with Bonferroni’s post-test. B, co-incubation with 1 µM DAMGO occludes inhibition by 1 µM of all indicated agonists. All bars are not statistically different from 1 µM DAMGO alone (p > 0.05 by one-way ANOVA with Bonferroni’s post-test). C, lower efficacy agonists (1 µM) were also not additive when co-administered in the indicated pairs (p > 0.05 for all pairs when compared to the most efficacious agonist of the pair by one-way ANOVA with Bonferroni’s post-test). Data are presented as mean ± S.E.M. (n = 4, in duplicate) of percent cAMP inhibition, where stimulation by 5 µM forskolin alone is represented as 0%.
Figure 3.2: MOR and DOR share pertussis toxin-sensitive G proteins. A, stimulation of $[^{35}S]$GTP$_{i}S$ binding in membranes from SH-SY5Y cells after 60 min incubation with 1 µM DAMGO or SNC80 alone or in combination (DAMGO/SNC80). Incubation with DAMGO and SNC80 in combination (DAMGO/SNC80) did not significantly increase $[^{35}S]$GTP$_{i}S$ binding more than DAMGO alone ($p > 0.05$ by one-way ANOVA with Bonferroni’s post-test), and stimulated significantly less $[^{35}S]$GTP$_{i}S$ binding than the theoretical additive of the individual responses ($R_1 + R_2$) (**$p < 0.01$ by one-way ANOVA with Bonferroni’s post-test). B, pretreatment of SH-SY5Y cells with pertussis toxin (PTX, 100 ng/ml) for 24 h prior to membrane preparation blocked stimulation of $[^{35}S]$GTP$_{i}S$ binding by 1 µM DAMGO, SNC80 or the combination (DAMGO/SNC80). Pertussis toxin treatment did not alter spontaneous $[^{35}S]$GTP$_{i}S$ binding in the absence of agonist. Data are presented as mean ± S.E.M. (n = 3, in triplicate).
Figure 3.3: DAMGO and SNC80 activation of G protein is additive at concentrations or time-points when G protein is not limiting.  
A, concentration-dependent stimulation of \([^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding in SH-SY5Y membranes following 60 min incubation with various concentrations of DAMGO alone (filled squares) or DAMGO with 30 nM SNC80 (open squares). The EC\(_{50}\) of DAMGO is not significantly altered by the addition of 30 nM SNC80 (DAMGO alone = 121 ± 32 nM, DAMGO + 30 nM SNC80 = 64 ± 12 nM, \(p = 0.14\) by two-tailed Student’s \(t\)-test). Co-incubation of DAMGO with 30 nM SNC80 produces additive \([^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding similar to the theoretical additive curve (triangles), which diverges only when DAMGO becomes occlusive at maximal concentrations (*\(p < 0.05\) at 10 \(\mu\)M for “DAMGO + 30 nM SNC80” compared to the “theoretical additive” by two-way ANOVA with Bonferroni’s post-test; \(n = 4\), in duplicate).  
B, Isobologram for agonists with a variable potency ratio calculated as described in Methods. Stimulation of \([^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding by DAMGO was conducted in the presence of indicated concentrations of SNC80. Concentration combinations that produced 50 % of the maximum effect of DAMGO alone are plotted from 3 separate experiments as mean ± S.E.M. Points on the line indicate additivity between DAMGO and SNC80.  
C, stimulation of \([^{35}\text{S}]\text{GTP} \gamma \text{S}\) binding in SH-SY5Y membranes following incubation with 1 \(\mu\)M DAMGO or SNC80 alone or in combination for 10 or 20 min, before the incubation reaches steady state. At both time-points, co-incubation with DAMGO and SNC80 (DAMGO/SNC80) is greater than DAMGO alone (*\(p < 0.05\) by one-way ANOVA with Bonferroni’s post-test) and similar to the theoretical additive (R1 + R2) (p > 0.05 by one-way ANOVA with Bonferroni’s post-test; \(n = 2\), in triplicate).
Figure 3.4: MOR and DOR share AC during chronic agonist administration. SH-SY5Y cells were treated overnight with vehicle (open bars) or 10 µM DPDPE (hatched bars) in the presence or absence of the MOR agonist DAMGO (10 nM or 100 nM) to induce dependence. Withdrawal was precipitated with the opioid antagonist naloxone (100 µM) in the presence of 5 µM forskolin. Data are presented as mean ± S.E.M. (n = 4, in duplicate) of the percent of forskolin-stimulated cAMP, where forskolin alone is 100% and is indicated by the dashed line. Overnight incubation with DPDPE produced overshoot on its own and enhanced the overshoot produced by 10 nM, but not 100 nM DAMGO. ***p < 0.001 compared to the vehicle with the same concentration of DAMGO.
Figure 3.5: DAMGO-mediated cAMP overshoot is reduced by heterologous inhibition of shared AC by agonist to DOR, NOPr or $\alpha_2$AR. AC sensitization was allowed to develop by incubating SH-SY5Y cells overnight with 100 nM DAMGO. To precipitate withdrawal, DAMGO-containing media was replaced with media containing 5 µM forskolin, 1 mM IBMX and 1 µM CTAP in the presence or absence of 1 µM of non-MOR agonist, as indicated, for 10 min. Data are presented as mean ± S.E.M. of percent cAMP overshoot, where stimulation by forskolin alone is represented as 0%. Three of six experiments, in duplicate, were compiled that produced > 100% DAMGO overshoot in the absence of non-MOR agonist. **p < 0.01, ***p < 0.001 compared to DAMGO overshoot without non-MOR agonist by one-way ANOVA with Bonferroni’s post-test.
Figure 3.6: Inhibition of cAMP by MOR or DOR agonists is similar for sensitized or non-sensitized AC. A, SH-SY5Y cells were incubated with vehicle (filled squares) or the MOR agonist DAMGO (100 nM, open squares) overnight to induce dependence. Withdrawal was precipitated with the MOR antagonist CTAP (1 µM) in the presence of 5 µM forskolin. Acute cAMP inhibition was produced by including various concentrations of the DOR agonist SNC80 in the precipitating media. The concentration-response of SNC80 to inhibit cAMP was similar in control and DAMGO-dependent cells (EC\textsubscript{50}: vehicle-treated = 14.6 ± 7.8 nM, DAMGO-treated = 13.8 ± 7.2 nM, p > 0.05 by two-tailed student’s t-test). B, Cells were incubated with vehicle (filled squares) or the DOR agonist DPDPE (10 µM, open squares) overnight to induce dependence. Receptor-specific withdrawal was precipitated with the DOR antagonist ICI 174,864 (1 µM) in the presence of 30 µM forskolin. Various concentrations of the MOR agonist DAMGO were included in the precipitating media to acutely inhibit cAMP. The concentration-response of DAMGO to inhibit cAMP was similar in control and DPDPE-withdrawn cells (EC\textsubscript{50}: vehicle-treated = 32.2 ± 12.5 nM, DPDPE-treated = 29.0 ± 7.1 nM, p > 0.05 by two-tailed Student’s t-test). Data are presented as mean pmol cAMP/mg protein ± S.E.M. (n = 3 or 4, in duplicate). cAMP produced by forskolin alone is indicated by the dashed line.
Figure 3.7: Lack of heterologous tolerance between MOR and DOR. SH-SY5Y cells were incubated with 1 µM of the MOR agonist DAMGO (A, B) or the DOR agonist SNC80 (C, D) for 24 h prior to membrane preparation. $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding in membranes from treated cells was stimulated by incubation for 60 min with various concentrations of DAMGO (A, D) or SNC80 (B, C). Chronic treatment with 1 µM DAMGO reduced the potency of DAMGO (EC$_{50}$: vehicle-treated = 86 ± 16 nM; DAMGO-treated = 260 ± 62 nM, p = 0.04), but not SNC80 (EC$_{50}$: vehicle-treated = 29.8 ± 12.5 nM; DAMGO-treated EC$_{50}$ = 34.6 ± 10.8 nM, p > 0.05). Similarly, chronic treatment with 1 µM SNC80 almost completely abolished SNC80-mediated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding, but did not alter the potency of DAMGO-mediated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding (EC$_{50}$: vehicle-treated = 69 ± 8.5 nM; SNC80-treated = 79 ± 1.6 nM, p > 0.05). EC$_{50}$ statistical comparisons were made by two-tailed Student’s $t$-test.
Figure 3.8: Schematic depicting the accessibility of $G_{i/o}$-coupled receptors to portions of the total AC pool. The amount of the AC pool utilized by each receptor type is related to receptor expression. The most highly expressed receptor MOR shares AC with all other receptor types. The other receptor types share AC in a manner predicted by receptor density such that three receptor groupings exist – one that contains MOR only, one that contains MOR, DOR and NOPr, and one that contains MOR, DOR, NOPr, $\alpha_2$AR, CB$_1$ and 5-HT$_{1A}$. 
Chapter IV
Discussion and Future Directions

The studies presented in this thesis have described cellular and molecular aspects of GPCR signaling using opioid receptors as a model, either heterologously expressed in HEK293 cells or endogenously expressed in SH-SY5Y cells. I have provided evidence that MOR and DOR have a differential requirement for cholesterol to sustain signaling. MOR required cholesterol for coupling to G proteins and efficient signaling to AC, while DOR signaling was unaffected by removal of cholesterol. Hypotheses regarding the basis for this differential dependence on cholesterol were proposed previously in this thesis, and approaches to test these hypotheses are presented below.

In addition, a portion of the MOR population was associated with markers of cholesterol-enriched membrane microdomains. In contrast, DOR was not associated with these markers, even after agonist treatment. Thus, MOR and DOR are likely not compartmentalized together within cholesterol-enriched membrane microdomains. Regardless, MOR and DOR were observed to compete for a common pool of G proteins and AC in SH-SY5Y cells. Moreover, this was not limited to MOR and DOR, as all $G_{i/o}$-coupled receptors endogenously expressed in SH-SY5Y cells were able to access a shared pool of AC. This applied to both an acute measure (AC inhibition) and a chronic measure (cAMP overshoot) of receptor activation. Given that multiple receptors can share AC and reduce MOR-mediated cAMP overshoot, receptors that are co-expressed on MOR-containing neurons may be utilized in the treatment of opioid withdrawal symptoms.

Although I have ruled out co-association of receptors within cholesterol-enriched membrane microdomains as a mechanism by which receptors compete for AC (at least for MOR and DOR), I have proposed two hypotheses to explain the observed competition. Firstly, free diffusion of receptors would allow each receptor type access to
G protein and AC. Secondly, MOR, DOR and other G\textsubscript{i/o}-coupled receptors could coexist in large protein complexes that contain multiple receptor types and signaling molecules, which have been termed signalosomes. These two alternative possibilities cannot be differentiated based on the work presented in this thesis. Strategies to address this and other unanswered questions that have arisen from this work are presented below.

**Future directions related to compartmentalization**

*Differentiating between free diffusion and multi-receptor signalosomes*

One of the major unanswered questions arising from work described in this thesis is whether the ability of all G\textsubscript{i/o}-coupled receptors expressed in SH-SY5Y cells to share AC is due to competition between multiple receptors in a signalosome or due to free diffusion of receptors. Although I argue that studying a large number of receptors increases the likelihood that interactions result from unconstrained diffusion, I cannot rule out the possibility that multiple receptors may be somehow tethered together (i.e. compartmentalized). Especially since biophysical studies have shown restricted MOR membrane diffusion in SH-SY5Y cells that would tend to support a model in which there is organization of receptors (Sauliere et al., 2006; Sauliere-Nzeh Ndong et al., 2010). In fact, assuming AC was not limiting, the lack of additive cAMP inhibition observed between the lower efficacy agonists also supports a compartmentalization model. One approach to differentiate between these two possible scenarios would be to evaluate the existence of large multimeric complexes containing multiple receptor types.

The literature contains many reports of interactions between two proteins, such as receptor-receptor or receptor-effector pairs. Many of these reports employed BRET or FRET, to study close (< 100 Å) interactions. These techniques require the use of two fluorescent or bioluminescent tags, which limits the detection of interactions to only two proteins at one time and prevents study in a truly native environment. Therefore, these techniques are not ideal to identify interactions between multiple proteins. To study multimeric complexes, immunoprecipitation would be more appropriate and has been employed to identify β\textsubscript{2}AR signaling complexes (Davare et al., 2001). Though
immunoprecipitation is not sensitive enough to identify protein-protein interactions, it is a valuable tool to detect proteins that may be in a complex.

Ideally, complexes of endogenously expressed receptors would be identified using antibodies to the native receptor. For example, Davare et al. (2001) identified β2AR complexes from rat brain using an antibody to the endogenous β2AR. Unfortunately, there is a general lack of availability of quality antibodies to the GPCRs used in this study. Thus, to study receptor complexes in SH-SY5Y cells, one of the receptors could be heterologously expressed in a tagged form. The tagged receptor could then be immunoprecipitated and the presence of other endogenous G_{i/o}-coupled receptors identified in the supernatant or the pelleted complex by radioligand binding. Radioligand binding has been performed in detergent extracts (Huang et al., 2007a) and in the soluble and pelleted fractions of immunoprecipitates (Mehta et al., 2007). Associated signaling molecules, such as AC isoforms and G protein subunits, could be determined by Western blot. Receptors present in the immunoprecipitated pellet would be assumed to be in complex with the tagged receptor, whereas receptors in the supernatant would not be in complex. In this way it would be possible to determine the receptor composition of multimeric complexes. I would expect such experiments to show that MOR will exist in complexes with all other receptors based on the functional data presented here and the fact that MOR has been shown to heterodimerize individually with DOR, NOPr, α2AR and CB1 receptors (Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2004; Wang et al., 2005; Rios et al., 2006; Vilardaga et al., 2008).

Based on evidence in this thesis, I hypothesized that the number of complexes occupied by a receptor and the fraction of the AC pool available to a receptor type is proportional to receptor expression levels. This could be tested by expressing increasing levels of receptor (by increasing the concentration of transfected cDNA) or decreasing receptor levels (with RNAi), then determining the effect on AC inhibition and the composition of receptor immunoprecipitates. For example, co-expression of increasing amounts of α2AR should result in increased recovery of α2AR in a FLAG-MOR precipitated pellet. If the increased recovery is specific to the pellet and not the supernatant, then it can be assumed that α2AR is in complex with MOR. If this was true
for multiple receptor types, it would indicate that the presence of receptors in complexes is determined by receptor expression levels, although there is the caveat that this could depend on specific characteristics of the receptor type. Functionally, this could be determined by expressing higher amounts of other receptors (e.g. α₂AR, CB₁ or 5-HT₁A receptors) in SH-SY5Y cells, which should allow agonists at these receptors to more effectively inhibit AC. If AC inhibition mediated by the overexpressed receptor is more pronounced than AC inhibition by MOR, then a maximally effective concentration of agonist to the overexpressed receptor should occlude the response by a MOR agonist. Although not direct proof, this would be consistent evidence that increasing receptor concentration increases the number of complexes the receptor occupies, and in turn, the amount of accessible AC.

If the ability of GPCRs to share AC is due to unrestricted diffusion and independent of complex formation, then simply increasing the efficiency of the receptor to signal without increasing receptor number should enhance the amount of AC the receptor can access. DOR, but not MOR, signaling is modulated by RGS4 endogenously expressed in SH-SY5Y cells, and down-regulation of RGS4 by shRNA enhances both the potency and efficacy of AC inhibition by DOR agonists (Wang et al., 2009). Selectively enhancing DOR agonist-mediated inhibition of AC following RGS4 down-regulation should allow a DOR agonist to inhibit AC to a greater extent than a MOR agonist. Then, as the higher efficacy system, DOR agonists should occlude responses by a MOR agonist. Combined with the finding that RGS4 does not co-immunoprecipitate with MOR in HEK293 cells (Wang et al., 2009), this would provide evidence for a free diffusion model rather than MOR, DOR and RGS4 containing complexes.

**Future directions related to cholesterol/membrane microdomains**

**Cholesterol as a modulator of functional selectivity**

It has been reported that cholesterol can stabilize active and inactive conformations of GPCRs depending on the receptor type. For example, evidence presented here and elsewhere (Gaibelet et al., 2008) suggests that cholesterol stabilizes a high-affinity conformation of MOR, whereas β₂AR (Rybin et al., 2000; Hanson et al.,
(2008) and α1AR (Lei et al., 2009) are held in an inactive conformation by cholesterol. In my study and the study by Gaibelet et al. (2008), the decrease in DAMGO binding was mimicked when G proteins were decoupled from receptors with sodium and/or guanine nucleotide.

It is now commonly accepted that a single type of receptor can display multiple intracellular responses depending on the environment of the receptor and the ligand activating the receptor. This phenomenon has been described using multiple terms including functional selectivity, agonist-directed signaling, biased agonism and protean agonism (Urban et al., 2007). Functional selectivity has been identified for MOR signaling, trafficking and desensitization (Whistler et al., 1999; Zheng et al., 2008b; Bailey et al., 2009). For functional selectivity to occur, receptors must be able to form more than one “active” conformation. If cholesterol affects some agonist-recognizable conformations of MOR more than others, then the presence of cholesterol or location of MOR in cholesterol-enriched or -depleted membrane microdomains could modulate functional selectivity.

The hypothesis that membrane microdomains confer functional selectivity of MOR has been recently reviewed (Simmons, 2008). This is based primarily on evidence from two studies of morphine and etorphine signaling to ERK and AC in putative raft and non-raft membrane microdomains (Zheng et al., 2008a; Zheng et al., 2008b). Etorphine, which binds to G protein-coupled and -uncoupled forms of MOR, (Lee et al., 1999), stimulates ERK via β-arrestins and partitions MOR out of caveolin-containing membranes in a β-arrestin-dependent manner (Zheng et al., 2008a; Zheng et al., 2008b). This etorphine-mediated ERK stimulation was not altered by cholesterol removal, which the authors interpreted as evidence that the β-arrestin-mediated activation of ERK by etorphine occurs in non-raft membranes (Zheng et al., 2008a). In contrast, morphine-mediated ERK stimulation was found to be G protein-dependent and was attenuated following cholesterol depletion (Zheng et al., 2008a; Zheng et al., 2008b). This cholesterol-dependent, G protein-coupled conformation of MOR is likely also recognized by etorphine, since cholesterol depletion attenuates etorphine-mediated AC inhibition, a G protein-dependent response (Zheng et al., 2008a). Together, the authors of these
studies conclude that morphine and etorphine-mediated activation of G proteins requires MOR to be in membrane microdomains, while etorphine activation of ERK occurs in non-raft membranes.

I propose a related, but more conservative, explanation that accounts for the role of cholesterol binding to the receptor itself, irrespective of cholesterol-enriched membrane microdomains. I propose that etorphine recognizes MOR in both a G protein-coupled conformation that is dependent on cholesterol and an additional non-G protein-coupled conformation that is not dependent on cholesterol and not able to bind morphine. This recognition of different MOR conformations with a differential requirement for cholesterol could lead to the observed functional selectivity. This hypothesis requires that the binding of etorphine to MOR is not affected by cholesterol removal. Agonists structurally similar to etorphine, such as buprenorphine and dihydroetorphine, that do not display an affinity shift in the presence of sodium and guanine nucleotide may also recognize this MOR conformation. To investigate this hypothesis, the effect of cholesterol depletion on binding of various agonists including those that exhibit a shift in affinity after uncoupling G protein with sodium ions and guanine nucleotides (morphine, fentanyl, methadone) and those that do not (etorphine, dihydroetorphine, buprenorphine) can be examined to identify agonist-specific differences in the importance of cholesterol in MOR signaling.

Unlike MOR, I found that cholesterol removal did not affect AC sensitization mediated by the peptidic agonist DPDPE or the non-peptidic agonist SNC80 at DOR. DOR has been shown to display multiple agonist-induced conformational states, which have been observed as BRET signal between DOR and heterotrimeric G protein tagged at various positions (tagged-$G\alpha_{i1}$ or $G\gamma_2$) (Audet et al., 2008). Since DPDPE and SNC80 were shown to induce different conformations of DOR in BRET assays (Audet et al., 2008), this suggests that cholesterol is not required for stabilization of at least these two agonist-bound receptor conformations. It would be of interest to measure binding of a range of DOR ligands, including partial agonists (e.g. morphine) and antagonists (e.g. naltrindole) that produced very different conformations in the BRET study (Audet et al., 2008).
Determining cholesterol binding to MOR and DOR

To determine if the differential effects of cholesterol signaling on MOR, but not DOR, are indeed due to the ability of MOR to bind cholesterol with higher affinity, binding of [3H]cholesterol to purified receptors could be performed. Purification of MOR is enhanced by the addition of cholesteryl hemisuccinate (Kuszak et al., 2009). Theoretically, one could measure binding of cholesterol to purified MOR as long as cholesterol binding is competitive and has a reasonable off-rate. Saturation binding of [3H]cholesterol to purified membrane proteins solublized in detergent has been determined previously for SREBP cleavage-activating protein (SCAP) and Niemann-Pick Type C1 protein (NPC1) (Radhakrishnan et al., 2004; Infante et al., 2008). However, the conformation of purified MOR is not equivalent to MOR in membranes, as indicated by a decreased ability to bind antagonist in detergent- and cholesterol-containing micelles (Kuszak et al., 2009), so the binding of [3H]cholesterol to purified MOR may not be truly representative of MOR in the plasma membrane.

The binding of [3H]cholesterol observed in vitro could be confirmed in cells using the photoactivatable analog of cholesterol [3H]photocholesterol, which has been used previously to detect cholesterol binding to synaptophysin (Thiele et al., 2000). [3H]Photocholesterol can be introduced into cells as a complex with MβCD and covalently linked to proteins by UV irradiation. Following covalent linkage, FLAG-tagged MOR or DOR can be immunoprecipitated and the presence of [3H]photocholesterol identified by SDS-PAGE and fluorography.

Molecular determinants of MOR association with cholesterol

The differential effect of membrane cholesterol removal on MOR and DOR signaling was surprising for two highly homologous receptors. A goal of future experiments could be to identify the molecular determinants of the ability of MOR, but not DOR, to be modulated by cholesterol and at least partially associate with markers of cholesterol-enriched membrane microdomains. Several potential targets have been identified that could influence the interaction of MOR with cholesterol, including the cholesterol consensus motif (CCM), binding to caveolin or palmitoylation of the receptor.
The CCM (Hanson et al., 2008) is composed of residues in TM 4, while the caveolin binding motif (Couet et al., 1997) is located at the end of TM 7 and beginning of the C-terminal tail (Figure 4.1). Therefore, the overall importance of these regions in MOR association with cholesterol could be determined using chimeric receptors. Since swapping of entire sections of the receptor could have functional effects unrelated to cholesterol, endpoints that are cholesterol-specific should be utilized, such as [³H]photocholesterol linkage or cholesterol removal-induced changes in agonist binding to wild-type and chimeric receptors at equal expression levels. Furthermore, since MOR partially associated with markers of cholesterol-enriched membrane microdomains, the distribution of chimeric receptors in detergent-resistant membranes could be evaluated. If a chimeric receptor behaves differently than its wild-type counterpart, follow-up experiments can be performed to identify which residues of these possible “motifs” are most predictive of cholesterol interactions.

MOR and DOR both contain two residues of the proposed CCM (4.39-4.43K and 4.50W). However, MOR has an additional Arg at the 4.40 position, which may increase the propensity for MOR to bind cholesterol (Figure 4.1). Strategic point mutations would provide a more exact picture of the requirements of this motif for cholesterol binding, which could be measured using [³H]cholesterol or [³H]photocholesterol, as described above. If the CCM is sufficient to promote MOR association with cholesterol, introduction of 4.40R into DOR should increase [³H]cholesterol or [³H]photocholesterol incorporation. If the CCM is required for MOR interaction with cholesterol, mutation of 4.40R to the corresponding residue in DOR (4.40A) should decrease [³H]cholesterol or [³H]photocholesterol binding to MOR R4.40A. Functionally, if the proposed CCM is required for cholesterol stabilization of MOR in a high-affinity conformation, then MOR R4.40A should display decreased binding affinity to DAMGO that is unaltered by sodium and GTPγS. Finally, a percentage of MOR was found to associate with markers of cholesterol-enriched membrane microdomains. If cholesterol binding to the CCM regulates this association, then mutated DOR containing the MOR CCM may associate with microdomain markers. In contrast, substitution of DOR residues into the MOR CCM could prevent association of MOR with microdomain markers.
As described previously, a caveolin binding motif has been identified \((\Phi X \Phi \times \times \times \Phi; \Phi = F, W \text{ or } Y; \text{ (Couet et al., 1997)})\) and is present in both MOR and DOR, although MOR contains more hydrophobic residues in the area immediately distal to this region. Mutation of these hydrophobic residues (370F and 372I) in MOR to the corresponding DOR residues (370L and 372R) may decrease association of MOR with caveolin, which could be determined by co-immunoprecipitation of caveolin and FLAG-MOR F370L/I372R. However, decreased association with caveolin may not necessarily decrease cholesterol binding to MOR, which could be determined using \(^3\text{H}\)cholesterol or \(^3\text{H}\)photocholesterol. Furthermore, if MOR F370L/I372R can still bind cholesterol, coupling of MOR to G proteins may not necessarily be affected by alterations in caveolin binding. Indeed, caveolin has been observed to suppress G protein activity in some studies (Li et al., 1995; Huang et al., 2007a), so decreased association with caveolin may lead to increased, rather than decreased, signaling by MOR agonists.

The residues identified above flank a C-terminal cysteine, and for many GPCRs, cysteines in the C-terminal tail are often a site of palmitoylation (Qanbar and Bouvier, 2003). It is known that MOR is palmitoylated in an agonist-independent manner, but the site of palmitoylation is not located at either of the two cysteines found in the C-terminal tail (Chen et al., 1998). Palmitoylation of mature DOR occurs at the plasma membrane in a receptor-activation dependent manner (Petaja-Repo et al., 2006); however, the site(s) of DOR palmitoylation are unknown. The C-terminal tail of DOR contains three cysteine residues. Measuring the incorporation of \(^3\text{H}\)palmitate following mutation of these residues to alanine would permit identification of the residue(s) that can be palmitoylated. If one or more of these DOR cysteines are palmitoylated, this palmitate could substitute for cholesterol to allow proper functioning of DOR. Indeed, palmitic acid covalently attached to cysteine 341 in the C-terminal tail of \(\beta_2\text{AR}\) was resolved in its crystal structure at the lipid-packing interface with the cholesterol molecules (Cherezov et al., 2007). In contrast, the \(A_{2A}\)-adenosine receptor structure contained fully saturated stearic acid instead of cholesterol (Jaakola et al., 2008). Perhaps a saturated fatty acid, such as palmitate, binding in an analogous region is sufficient to stabilize DOR. In this case, DOR would not require cholesterol for signaling. On the other hand, MOR, which is not
palmitoylated on the C-terminal tail, may instead bind cholesterol. This hypothesis could be tested by depalmitoylating DOR using hydroxylamine and measuring cholesterol binding (using \(^{[3]H}\)cholesterol) and high-affinity agonist binding. Alternatively, high-affinity agonist binding or \(^{[3]H}\)photocholesterol incorporation could be determined for DOR mutants in which palmitoylated Cys residue(s) are replaced with Ala.

**Overall summary and significance**

*Receptor location requirements for potential therapeutic benefit in opioid withdrawal*

In SH-SY5Y cells, endogenously expressed G\(_{i/o}\)-coupled receptors can access the same pool of AC and attenuate MOR-mediated cAMP overshoot. Altered homeostasis of the AC/cAMP/PKA pathway has been implicated in withdrawal. Thus, receptors that are co-expressed on neurons with MOR could be targets to prevent the manifestation of hyperactive systems, such as cAMP overshoot, that may contribute to withdrawal symptoms. This is a possible mechanism for the clinical utility of the \(\alpha_2\)AR agonist clonidine in opioid withdrawal, and suggests that agonists for other receptors, such as NOPr and CB\(_1\), may be useful to alleviate withdrawal.

The utility of designing therapeutics to treat opioid withdrawal based on an intracellular mechanism in which receptors can dampen responses from a sensitized, shared effector, such as the attenuation of hyperactive LC neurons, obviously requires co-expression of the receptors in the same neuron. As described previously, \(\alpha_2\)AR and NOPr co-expression with MOR in the LC, a brain structure relevant to withdrawal, can prevent opioid withdrawal-induced hyperactivity. However, the LC is not the only region that contributes to the opioid withdrawal syndrome (Christie et al., 1997). In mice that lacked AC 5, which is prominently expressed in the striatum, certain opioid withdrawal symptoms were also attenuated (Kim et al., 2006). Other brain regions that have been implicated in opioid withdrawal include the periaqueductal grey (PAG), dorsal horn of spinal cord, several hypothalamic nuclei, nucleus raphe magnus and the rostral ventromedial medulla (Christie et al., 1997). Therefore, G\(_{i/o}\)-coupled receptors co-expressed with MOR in other relevant brain regions could reduce certain withdrawal symptoms by an intracellular mechanism. For example, MOR and G\(_{i/o}\)-coupled
dopamine D2 receptors are co-expressed in rat striatum (Ambrose et al., 2004). In addition, MOR and NOPr are co-localized on neurons in the rostral ventromedial medulla (Vaughan et al., 2001) and are found in similar brain regions in the guinea pig, such as ventromedial hypothalamus (Sim and Childers, 1997). However, MOR and NOPr are not co-expressed in spinal cord (Monteillet-Agius et al., 1998).

Obviously, not all agonists with therapeutic potential in the treatment of opioid withdrawal would need to directly modulate the intracellular environment of a MOR-containing neuron. Receptors on neurons that converge on MOR-containing neurons could also be targets. In the LC, a major component of withdrawal-induced neuronal hyperactivity is due to enhanced input by excitatory glutamatergic neurons (Aston-Jones et al., 1997). Therefore, receptors that act on neurons in series to MOR-containing neurons could also have physiological benefit. For example, 5-HT1A receptor activation may have an indirect effect in LC (Berrocoso and Mico, 2007) to decrease noradrenergic neuron firing by acting on presynaptic glutamatergic afferent terminals (Bobker and Williams, 1989; Pudovkina et al., 2001).

As discussed previously, evidence suggests that MOR and DOR do not act at the same neuron to prevent opioid withdrawal. However, co-administration of MOR and DOR agonists could still have beneficial effects via actions on neurons in series. DOR has been reported to prevent morphine-withdrawal by an indirect mechanism given that continuous administration of BW373U86 throughout morphine exposure did reduce naloxone-precipitated withdrawal signs in morphine-dependent rats (Lee et al., 1993). In addition, co-administration of MOR and DOR agonists could have other favorable effects, since SNC80 has been shown to synergistically enhance antinociceptive, but not sedative effects of MOR agonists in monkeys (Stevenson et al., 2003; Stevenson et al., 2005). Synergistic or additive interactions have been observed in antinociception assays between MOR and CB1 or α2AR agonists, as well. Morphine and the CB1 agonist CP 55,940 produced synergistic antinociception, while additive interactions existed for morphine and the α2AR agonist dexmedetomidine (Tham et al., 2005).
Relevance of cholesterol in opioid withdrawal

The relationship between cholesterol/membrane viscosity and opioid withdrawal has been reported in studies from over 20 years ago using rodents. Chronic morphine treatment of rats led to a naloxone-reversible increase in plasma cholesterol (Bryant et al., 1987), brain cholesterol, and viscosity of membranes from the hippocampus and caudate (Heron et al., 1982b). However, substance abusers generally have low cholesterol levels due to malnutrition (Gettler, 1991). Thus, treating opioid-addicted patients with cholesterol-lowering drugs, such as statins, is not a likely therapeutic option. However, modulation of membrane viscosity may have some benefit, since treatment of rats systemically with lipids that prevented the morphine-induced increase in membrane viscosity attenuated opioid withdrawal symptoms (Heron et al., 1982a). These general cholesterol or membrane viscosity effects do not indicate the involvement of membrane microdomains, although some biochemical data has identified MOR with microdomain markers isolated from rat brain (Huang et al., 2007b).

In summary, I have found that cholesterol stabilizes MOR but not DOR in a conformation that is capable of binding G proteins and signaling to AC. Similar findings at MOR have been reported by others during preparation of this thesis (Gaibelet et al., 2008; Zheng et al., 2008a). Cholesterol depletion attenuated AC sensitization induced by MOR, but not DOR, agonists. Others have implicated membrane microdomains in the mechanism of MOR-mediated AC sensitization (Zhang et al., 2009). However, MOR and DOR can access a similar AC pool which, based on the differential distribution of these receptors, is not compartmentalized in membrane microdomains. Thus, the role of cholesterol in MOR-mediated AC sensitization is probably independent of membrane microdomain localization. Although modulation of brain cholesterol is an unlikely therapeutic target, modulation of membrane viscosity has been shown to reduce opioid withdrawal signs in rats (Heron et al., 1982a), and may be involved in the mechanism of several neurodegenerative diseases, including Alzheimer’s disease, Krabbe disease and Niemann-Pick Type C (Korade and Kenworthy, 2008).
Figure 4.1: Serpentine model of MOR. Residues that satisfy the requirements of the putative caveolin binding motif (ΦXΦXXXXΦ; Φ = F, W or Y) are found at the end of TM 7 and beginning of the C terminal tail, as outlined with red. These residues are found in both MOR and DOR. The putative cholesterol consensus motif (CCM; [4.39-4.43(R,K)]-[4.50(W,Y)]-[4.46(I,V,L)]-[2.41(F,Y)]; Hanson et al., 2008) is located in TM 4 of MOR (box and insert) and DOR (insert). MOR contains three of these residues (4.43K, 4.50W and 4.40R) highlighted in yellow in the serpentine model and the insert. For comparison, DOR contains two of these CCM residues (4.43K and 4.50W; yellow; insert). Residues that do not satisfy the requirements of the CCM are outlined in green. Serpentine models were adapted from the Center for Opioid Research and Design, University of Minnesota, http://www.opioid.umn.edu/ and are consistent with the original published sequence of MOR (Chen et al., 1993a). Gray residues are conserved between opioid receptor types and black residues are highly conserved among many family A GPCRs.
References


intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *J Biol Chem* **280:**31036-31044.


Liu B, Ramirez CM, Miller AM, Repa JJ, Turley SD and Dietschy JM (2009) Cyclodextrin overcomes the transport defect in nearly every organ of the newborn or mature NPC1 mouse leading to excretion of the sequestered cholesterol as bile acid. *J Lipid Res.*


Melkonian KA, Ostermeyer AG, Chen JZ, Roth MG and Brown DA (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *J Biol Chem** **274**:3910-3917.


dissociation of mu opioid receptor signaling and endocytosis: implications for the

White AB, Givogri MI, Lopez-Rosas A, Cao H, van Breemen R, Thinakaran G and
Bongarzone ER (2009) Psychosine accumulates in membrane microdomains in
the brain of krabbe patients, disrupting the raft architecture. J Neurosci 29:6068-
6077.

Localization of the κ opioid receptor in lipid rafts. J Pharmacol Exp Ther
317:1295-1306.

Biophys Biochem Cytol 1:445-458.

MW, Collins J, McSherry F, Boardman K, Davies DK, O'Brien CP, Ling W,
Kleber H and Herman BH (2008) A Phase 3 placebo-controlled, double-blind,
multi-site trial of the alpha-2-adrenergic agonist, lofexidine, for opioid

Yu VC and Sadee W (1988) Efficacy and tolerance of narcotic analgesics at the mu
opioid receptor in differentiated human neuroblastoma cells. J Pharmacol Exp
Ther 245:350-355.

Zachariou V, Liu R, LaPlant Q, Xiao G, Renthal W, Chan GC, Storm DR, Aghajanian G
and Nestler EJ (2008) Distinct roles of adenyl cyclases 1 and 8 in opiate
dependence: behavioral, electrophysiological, and molecular studies. Biol
Psychiatry 63:1013-1021.

of mu and delta opiate receptors by morphine, selective agonists and antagonists
and differentiating agents in SH-SY5Y human neuroblastoma cells. J Pharmacol
Exp Ther 270:1086-1096.

Zaki PA, Keith DE, Jr., Thomas JB, Carroll FI and Evans CJ (2001) Agonist-, antagonist-
, and inverse agonist-regulated trafficking of the delta-opioid receptor correlates
with, but does not require, G protein activation. J Pharmacol Exp Ther 298:1015-
1020.

Zhang L, Zhao H, Qiu Y, Loh HH and Law PY (2009) Src phosphorylation of micro-
receptor is responsible for the receptor switching from an inhibitory to a

