OPIOID-INDUCED SIDE EFFECTS IN BETA-ARRESTIN2 AND G PROTEIN-COUPLED RECEPTOR KINASE KNOCKOUT MICE

DISSERTATION

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By

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*****

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ABSTRACT

Opioid drugs are potent analgesics; however, they also produce several adverse side effects including constipation, antinociceptive tolerance, and physical dependence by activating the mu opioid receptor (μOR), a G protein-coupled receptor (GPCR). There is a substantial literature that suggests that the GPCR regulatory proteins G protein-coupled receptor kinases (GRKs) and beta-arrestins (β-arrestins) play a key role in regulating μOR signaling and responsiveness. In vivo, the loss of β-arrestin2 significantly alters morphine-induced analgesia, antinociceptive tolerance, respiratory suppression, and reward. Moreover, distinct opioid agonists have been shown to differ in their propensity to promote interactions between the μOR and β-arrestins and such agonist directed events may ultimately determine the functional response of the receptor to a particular drug. Therefore, we hypothesize that GRK and β-arrestin2-mediated μOR regulation may determine the extent of opioid-induced side effects including constipation, antinociceptive tolerance, and physical dependence, in a manner that is specifically influenced by different opioid agonists and cellular environments. Using mice genetically lacking individual GRKs and β-arrestin2, we evaluated distinct opioid agonists (morphine, methadone, and fentanyl) for their ability to elicit constipation, antinociceptive tolerance, and physical dependence. We find that β-arrestin2 is important in determining the expression of morphine-induced constipation,
antinociceptive tolerance, and physical dependence, even more so than GRKs. However, while all responses evaluated in response to morphine were affected by the loss of β-arrestin2, only methadone-induced physical dependence was altered in the β-arrestin2-knockout mice, suggesting that β-arrestin2 differentially affects these opioid-mediated responses in an agonist-dependent manner. Collectively, these results provide evidence that distinct opioid agonists can influence μOR regulation and responsiveness and that the contribution of a particular regulatory factor to receptor function can differ based upon the specific cell composition and physiology assessed.
In loving memory of my mother,

Marlene Louise Raehal
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# TABLE OF CONTENTS

Abstract ............................................................................................................................................ ii

Dedication ........................................................................................................................................ iv

Acknowledgments ........................................................................................................................... v

Vita ................................................................................................................................................ viii

List of Tables ................................................................................................................................ xii

List of Figures ................................................................................................................................ xiii

List of Abbreviations ................................................................................................................... xv

Chapters:

1. Introduction ............................................................................................................................. 1
   1.1 Clinical aspects of opioid analgesics ............................................................ 1
   1.2 Opioid receptors ........................................................................................... 2
   1.3 Mu opioid receptor signaling and regulation ............................................. 2
   1.4 GRK and β-arrestin2-mediated regulation of morphine response in vivo .. 6
   1.5 Functional selectivity at the mu opioid receptor ........................................ 9
   1.6 Hypothesis and overview of chapters 2-4 ................................................ 11

2. β-arrestin2 differentially regulates inhibition of gastrointestinal motility in an 
   agonist-dependent and tissue specific manner ......................................................... 17
   2.1 Introduction .............................................................................................................. 17
   2.2 Material and Methods .......................................................................................... 21
   2.3 Results .................................................................................................................. 29
   2.4 Discussion .............................................................................................................. 33
   2.5 Establishment of Ex Vivo Techniques for Future Studies ......................... 37
   2.6 Tables and Figures .............................................................................................. 42
3. β-arrestin2 differentially regulates antinociceptive tolerance and physical dependence in an agonist-dependent manner .......................................................... 54
   3.1 Introduction .................................................................................................. 54
   3.2 Material and Methods .............................................................................. 57
   3.3 Results ...................................................................................................... 60
   3.4 Discussion ............................................................................................... 63
   3.5 Tables and Figures .................................................................................. 70

4. Differential effects of morphine-induced physiological and behavioral responses in mice lacking G protein-coupled receptor kinases (GRKs) ........................................ 77
   4.1 Introduction .............................................................................................. 77
   4.2 Material and Methods .............................................................................. 79
   4.3 Results ..................................................................................................... 84
   4.4 Discussion ............................................................................................... 88
   4.5 Tables and Figures .................................................................................. 93

5. Conclusions ............................................................................................................ 99

6. List of References ................................................................................................ 103
<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Cumulative dosing regimens for morphine, methadone, and fentanyl used to assess antinociceptive tolerance on days 1 and 7 in WT and βarr2-KO mice.</td>
<td>70</td>
</tr>
<tr>
<td>3.2</td>
<td>Summary of ED50 values (mg/kg) (± 95% confidence limits) and potency ratios in WT and βarr2-KO mice for morphine, methadone, and fentanyl in the 54°C hot-plate test.</td>
<td>73</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>Schematic representation of GRK and β-arrestin-mediated homologous desensitization of μOrs</td>
<td>14</td>
</tr>
<tr>
<td>1.2</td>
<td>Schematic representation of GRK/β-arrestin-mediated Internalization</td>
<td>15</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic representation of functional selectivity</td>
<td>16</td>
</tr>
<tr>
<td>2.1</td>
<td>Mouse colon tissue preparations and dissections</td>
<td>42</td>
</tr>
<tr>
<td>2.2</td>
<td>Morphine effects on fecal boli accumulation</td>
<td>43</td>
</tr>
<tr>
<td>2.3</td>
<td>Morphine-induced inhibition of small intestinal transit</td>
<td>44</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of morphine and loperamide on colonic motility</td>
<td>45</td>
</tr>
<tr>
<td>2.5</td>
<td>Effect of centrally administered morphine and loperamide on colonic motility</td>
<td>46</td>
</tr>
<tr>
<td>2.6</td>
<td>Effect of methadone and fentanyl on small intestinal transit</td>
<td>47</td>
</tr>
<tr>
<td>2.7</td>
<td>Effect of methadone and fentanyl on colonic motility</td>
<td>48</td>
</tr>
<tr>
<td>2.8</td>
<td>Colonic pellet propulsion assay in WT mice</td>
<td>49</td>
</tr>
<tr>
<td>2.9</td>
<td>Basal Isc and conductance values in WT mice</td>
<td>50</td>
</tr>
<tr>
<td>2.10</td>
<td>Morphine-mediated inhibition of DMPP-stimulated electrogenic secretion in the colon of WT mice</td>
<td>51</td>
</tr>
<tr>
<td>2.11</td>
<td>Morphine-induced JNK2/3 activation in preparations from WT mouse colon</td>
<td>52</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.12</td>
<td>Confocal microscopy of longitudinal muscle/myenteric plexus and submucosal plexus sections in WT mice</td>
<td>53</td>
</tr>
<tr>
<td>3.1</td>
<td>Morphine, methadone, and fentanyl-induced hot-plate antinociceptive responses in WT and ( \beta )arr2-KO mice</td>
<td>71</td>
</tr>
<tr>
<td>3.2</td>
<td>Morphine, methadone, and fentanyl-induced hot-plate antinociceptive dose-response curves in WT and ( \beta )arr2-KO mice</td>
<td>72</td>
</tr>
<tr>
<td>3.3</td>
<td>Naloxone-precipitated withdrawal responses following chronic morphine infusion</td>
<td>74</td>
</tr>
<tr>
<td>3.4</td>
<td>Naloxone-precipitated withdrawal responses following chronic methadone infusion</td>
<td>75</td>
</tr>
<tr>
<td>3.5</td>
<td>Naloxone-precipitated withdrawal responses following chronic fentanyl infusion</td>
<td>76</td>
</tr>
<tr>
<td>4.1</td>
<td>GRK6 facilitates morphine-induced ( \beta )-arrestin2 recruitment and ( \mu )OR trafficking in vitro</td>
<td>93</td>
</tr>
<tr>
<td>4.2</td>
<td>Morphine-induced antinociception and antinociceptive tolerance profile in GRK6-KO mice</td>
<td>94</td>
</tr>
<tr>
<td>4.3</td>
<td>Morphine-induced locomotor activity and “presensitization” to the stimulating effects of morphine in GRK6-KO mice</td>
<td>95</td>
</tr>
<tr>
<td>4.4</td>
<td>Naloxone-precipitated withdrawal responses in response to chronic morphine treatment in GRK6-KO mice</td>
<td>96</td>
</tr>
<tr>
<td>4.5</td>
<td>Gastrointestinal responses to morphine and cocaine-methiodide in GRK6-KO mice</td>
<td>97</td>
</tr>
<tr>
<td>4.6</td>
<td>Fecal boli accumulation profiles in GRK2-HT, GRK3-KO, GRK4-KO and GRK5-KO mice</td>
<td>98</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>also known as protein kinase B</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP2</td>
<td>clathrin assembly protein 2</td>
</tr>
<tr>
<td>βarrestin</td>
<td>beta-arrestin</td>
</tr>
<tr>
<td>βarr1-KO</td>
<td>beta-arrestin1-knockout</td>
</tr>
<tr>
<td>βarr2-KO</td>
<td>beta-arrestin2-knockout</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>CaMKII</td>
<td>calcium/ calmodulin kinase II</td>
</tr>
<tr>
<td>CCH</td>
<td>carbachol</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>DAMGO</td>
<td>([D-Ala2, N-MePhe4, Gly-ol]-enkephalin)</td>
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<tr>
<td>DMPP</td>
<td>dimethylphenylpiperazinium</td>
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<td>δOR</td>
<td>delta opioid receptor</td>
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<td>ED50</td>
<td>median effective dose</td>
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<td>ENS</td>
<td>enteric nervous system</td>
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<tr>
<td>ERK</td>
<td>1/2extracellular regulated kinases 1 and 2</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GRK</td>
<td>G protein-coupled receptor kinase</td>
</tr>
</tbody>
</table>

xv
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP</td>
<td>guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HEK-293</td>
<td>human embryonic kidney-293</td>
</tr>
<tr>
<td>HT</td>
<td>heterozygous</td>
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<tr>
<td>I.C.V.</td>
<td>intracerebroventricular</td>
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<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-Triphosphate</td>
</tr>
<tr>
<td>I.P.</td>
<td>intraperitoneal</td>
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<tr>
<td>Isc</td>
<td>short-circuit current</td>
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<tr>
<td>JNK</td>
<td>also known as SAPK</td>
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<tr>
<td>kDa</td>
<td>kilodaltons</td>
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<td>κOR</td>
<td>kappa opioid receptor</td>
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<td>LMMP</td>
<td>longitudinal muscle/myenteric plexus</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>μOR</td>
<td>mu opioid receptor</td>
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<td>μOR-KO</td>
<td>mu opioid receptor knockout mice</td>
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<tr>
<td>ORL-1</td>
<td>nociceptin/orphanin FQ receptor</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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<tr>
<td>S.C.</td>
<td>subcutaneous</td>
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<td>SP</td>
<td>submucosal plexus</td>
</tr>
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<td>SPM</td>
<td>submucosal plexus/mucosa</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Clinical aspects of opioid analgesics

Opioids are the most widely used analgesic drugs for treating moderate to severe, acute and chronic pain conditions. There are three broad classes of opioids employed clinically: the naturally occurring opium alkaloids such as morphine (the prototypical opioid), semi-synthetic opioids like oxycodone, and fully synthetic opioids such as methadone and fentanyl, which have chemical structures unrelated to the opium alkaloids (1). Although there are many different opioid drugs available, morphine is the most clinically used opioid for the treatment of pain and the standard against which other analgesics are measured (2).

While opioids are effective pain-relievers, they also produce a number of adverse side effects including, sedation, nausea and vomiting, constipation, respiratory suppression, tolerance, physical dependence, and addiction (2, 3). Tolerance and physical dependence often develop following continuous opioid exposure whereas constipation, the most common side effect observed with opioid use, occurs in response to a single administration of an opioid and persists with repeated drug exposure (4, 5). Opioid-related side effects are often difficult to manage, and their treatment leads to increases
in the overall cost of patient healthcare (2, 3). Given their numerous affects in patients, a major goal in opioid research is to understand the molecular and cellular mechanisms that give rise to opioid-induced analgesia as well as adverse responses.

1.2 Opioid receptors

Opioids produce their biological effects by binding to and activating opioid receptors (6-8). An extensive number of pharmacological, radioligand binding, immunohistochemical and molecular studies have demonstrated that there are four distinct opioid receptor types referred to as mu (\(\mu\)OR), delta (\(\delta\)OR), kappa (\(\kappa\)OR), and nociceptin/orphanin FQ (ORL-1) receptor that belong to the superfamily of G protein-coupled receptors (GPCRs) (1). Each receptor has a unique anatomical distribution in brain, spinal cord, and the periphery, and can differ in their ligand selectivity and pharmacological responses (1). Although each opioid receptor type is widely expressed in the nervous system, morphine and most other clinically used opioid analgesics exert their physiological and behavioral effects primarily through activation of the \(\mu\)OR (1). This has also been supported by the finding that mice lacking the \(\mu\)OR do not experience antinociception, respiratory suppression, constipation, increased locomotor activity, or drug reinforcement in response in response to morphine treatment (9-12).

1.3 Mu opioid receptor signaling and regulation

The clinically observed physiological responses produced by opioids may be determined by how effectively the \(\mu\)OR signals. Like other GPCRs, the \(\mu\)OR modulates the activity of several different second messengers and cellular effectors to produce both acute and long-term changes at the molecular and cellular level. However, \(\mu\)OR activation is determined not only by the initiation of signaling cascades but also by regulatory
mechanisms that control the extent and duration of its signaling. A number of intrinsic regulatory mechanisms including receptor desensitization, internalization, resensitization, and downregulation regulate receptor signaling by preventing receptor overstimulation, promoting signal termination, and regulating cell surface expression of receptors.

Classically, the μOR has been shown to primarily couple to the pertussis toxin-sensitive inhibitory Gi/o class of G proteins to mediate signal transduction in cells (13). Activation of Gi/o proteins results in inhibition of adenylyl cyclase activity and the diminished activation of the adenosine 3',5'-monophosphate (cAMP) and protein kinase A (PKA) (14, 15). Opioid also promote activation of inwardly rectifying potassium channels (15-17) and inhibition of voltage-gated calcium channels (15, 18). A number of in vitro studies also demonstrate that μOR-mediated G protein coupling can regulate the activity of several intracellular signaling cascades which varies depending on the cell type under investigation. These include the mitogen-activated protein (MAP) extracellular-regulated kinases (ERK1/2) and c-jun N-terminal kinases (JNK) signaling pathways (13, 19-21) as well as the phosphatidylinositol (PI)-specific phospholipase Cβ (PLCβ) signaling cascades (22-24). Multiple lines of evidence also support a role for calcium/calmodulin-dependent protein kinase II (CaMKII) in μOR-mediated signaling (25-29). While each of these signaling events have been observed in cellular studies, it is not clear how they relate to actual signaling events occurring in neurons that ultimately produce opioid-induced physiological responses.

Mu opioid receptor signaling can be regulated by several different means. Feedback regulation by second messengers downstream of receptor activation such as PKA, PKC,
ERK and CaMKII have been shown to phosphorylate the $\mu$OR (25, 26, 30-33), resulting in overall decreased $\mu$OR function, which can in turn affect other signaling events. However, a more proximal regulatory mechanism of $\mu$OR signaling is homologous desensitization, a process which results in a loss of receptor signaling and responsiveness to prolonged or repeated administration of an agonist by affecting the degree of coupling between GPCRs and G proteins (34, 35). This process requires the coordinated interactions of two families of proteins with the receptor: GPCR kinases (GRKs) and arrestins. GRKs initiate desensitization by rapidly phosphorylating receptors following agonist activation (Figure 1.1) (36-40). These kinases show a highly selective preference for the activated state of a receptor (41) and the interaction between GRKs and receptors serves to catalytically activate theses kinases, which promotes the phosphorylation of specific amino acid residues on the intracellular loops and the carboxy-terminal tail of GPCRs (42-45).

The mammalian GRKs are a family of serine/threonine kinases which consist of seven distinct members designated GRK1 through GRK7. Based on their sequence, structure, and functional similarities, the GRKs have been divided into three subfamilies: the GRK1 subfamily, which includes GRK1 and GRK7; the GRK2 subfamily which consists of GRK2 and GRK3; and the GRK4 subfamily which contains GRK4, GRK5, and GRK6 (39, 43, 46-48). To date, GRK1 (rhodopsin kinase) and GRK7 (iodopsin kinase) have been identified to be expressed almost exclusively in rod and cone cells in the retina (47, 49). GRK2, GRK3, GRK5, and GRK6 have a vast distribution and are widely expressed throughout the central nervous system (50-54), while GRK4 is predominantly found in the testes with expression also reported in the brain and kidney (55-58).
GRKs are 60-80 kDa proteins that share a three domain protein structure, which include a regulator of G protein signaling (RGS)-like amino terminal domain, a central protein kinase domain, and a variable carboxyl-terminal domain (39, 43, 55, 59-61). Members of the GRK2 subfamily are predominantly cytosolic and translocate to the plasma membrane following receptor activation (62-64). In contrast, GRK4 subfamily members are membrane bound due to the presence of palmitoylated cysteine or polybasic residues which allows these kinases to be anchored the cell membrane (55, 65-67).

GRK-mediated receptor phosphorylation serves to facilitate the subsequent binding of arrestin proteins to receptors, which acts to dampen signaling by preventing further coupling to G proteins despite the continued presence of agonist (36-40, 68) (Figure 1.1). To date, four arrestin proteins, designated arrestin 1 through 4, have been identified (69, 70). The arrestins are 48 kDa proteins which exhibit approximately 80% homology, are predominantly arranged into a \( \beta \)-sheet structure, and contain a large phosphoprotein binding pocket (71). Arrestins 1 and 4 are almost exclusively expressed in rod (72-74) and cone (75, 76) cells in the visual system, and therefore were subsequently termed “visual” arrestins. Arrestin 2 and arrestin 3 were first discovered for their ability to regulate the beta2-adrenergic receptor (77) and have since been classified as beta-arrestin1 (\( \beta \)-arrestin1) and beta-arrestin2 (\( \beta \)-arrestin2), respectively. The two nonvisual \( \beta \)-arrestins are highly expressed in tissues throughout the body (35, 77, 78).

In addition to receptor desensitization, GRK/\( \beta \)-arrestin interactions with GPCRs are integral components of receptor internalization and trafficking (Figure 1.2). Once bound to phosphorylated receptors, \( \beta \)-arrestins can recruit and directly bind the clathrin adaptor
protein AP2 and clathrin itself, which targets receptors to clathrin-coated pits for endocytosis (79, 80). Once internalized, receptors can be resensitized, a process by which receptors are reinserted into the cell membrane, allowing them to be reactivated (36, 37, 40, 68). However, prolonged stimulation can promote events that target receptors for lysosomal degradation, which results in a loss of receptors from the cell surface (36, 37, 40, 68, 78-81).

The balance between activation, desensitization, resensitization, and downregulation can determine the relative potential of the receptor signal. However, the contribution of both receptor activation and regulation to opioid-induced biological responses is not yet clear in vivo. One approach to study how regulation affects opioid responses is to remove individual regulatory proteins and then evaluate how μOR function and drug responsiveness is affected in vivo. It has been difficult to assess the contribution of regulatory elements pharmacologically since there are no chemical inhibitors of GRKs or β-arrestins. Therefore, mice genetically deficient of individual GRKs (GRK2, GRK3, GRK4, GRK5, and GRK6) (82-85) and β-arrestins (β-arrestin1 and β-arrestin2) (86, 87) and their wild-type (WT) littermates have been used to study how these regulatory proteins contribute to the complex effects of opioids at the molecular, cellular, and behavioral level.

1.4 GRK and β-arrestin2-mediated regulation of morphine responses in vivo
Morphine is the most widely used opioid drug clinically; and therefore, a number of studies have evaluated whether certain GRKs or β-arrestins contribute to morphine-mediated responses in vivo. The physiological importance of β-arrestin2 regulation of the μOR in response to morphine has been extensively evaluated using mice lacking β-
arrestin2. Consistent with their classically described role as desensitizing agents, many morphine responses are enhanced in the absence of β-arrestin2. β-arrestin2-knockout (βarr2-KO) mice display enhanced and prolonged morphine-induced antinociception in paradigms which evaluate supraspinal (hot-plate) and spinal (tail-flick) antinociceptive responses to a noxious thermal stimulus (87, 88). This finding also correlates with the observation that μOR agonist stimulated G protein coupling is elevated in βarr2-KO mouse brain regions (periaqueductal gray, brainstem) as well as spinal cord, indicating that β-arrestin2 acts as a desensitizing component of μOR signaling in vivo (87-89). The βarr2-KO mice also display dramatically attenuated antinociceptive tolerance after acute and chronic morphine treatment (88, 89), which suggests that antinociceptive tolerance may be a behavioral manifestation of receptor desensitization. Moreover, morphine also produces enhanced striatal dopamine release, hypothermia, and drug reinforcement in the βarr2-KO mice compared to WT mice (34, 90). Investigation into behaviors in the absence of drug revealed that basal tail-flick nociceptive response latencies are prolonged, which can be blocked by the opioid antagonist naltrexone (89), suggesting that the μOR/β-arrestin2 interaction may not only be important for regulating the morphine-activated receptor, but may also help to establish the basal tone of receptor signaling.

Although GRKs and β-arrestins have traditionally been viewed as negative regulators of GPCR signaling, they have also been shown to function as scaffolding molecules that mediate GPCR signaling by facilitating interactions between signaling proteins and the receptor. For example, GRKs have been shown to act as adaptors for recruiting key signaling intermediates such as GIT1 and hedgehog, which bring these signaling partners into a complex with beta2-adrenergic (67) and smootherened receptors (91),
respectively. Furthermore, β-arrestins can act as adaptors between receptors and a number of intracellular signaling proteins including ERK1/2 (92-99), JNK (95, 100), and Akt (101, 102). GPCRs which display β-arrestin-mediated signaling in cell culture include the vasopressin, angiotensin, beta2-adrenergic, and the serotonin 2A receptors (94-96, 98, 103-105). Additionally, β-arrestins can mediate alpha2-adrenergic, dopamine D2, and serotonin 2A receptor signaling in vivo (101, 102, 105, 106).

A recent paper by Zheng et al. (99) has presented the first line of evidence that the μOR can signal via β-arrestins in cellular culture studies, but this has yet to be demonstrated in vivo. Interestingly, while many of the morphine-induced responses in βarr2-KO mice support the classically defined role of β-arrestins as negative regulators of GPCR signaling, other physiological and behavioral responses to morphine do not. Morphine is known to activate locomotor activity in mice; however, the βarr2-KO mice display less activation of locomotion compared with their WT counterparts despite increased extracellular dopamine levels in striatum (90). Morphine-induced respiratory suppression is also less severe in the βarr2-KO mice following acute treatment. Therefore, the βarr2-KO mice represent a model system with which to evaluate whether β-arrestin2 is acting as a positive mediator of μOR signaling in vivo.

While the contribution of β-arrestin2 to morphine responses have been extensively characterized, only a few studies have evaluated the role of individual GRKs to μOR regulation in vivo. Although GRK3 can promote μOR desensitization in vivo, mice lacking GRK3 do not display altered morphine-induced antinociception or antinociceptive tolerance (107). Furthermore, differences in morphine-induced antinociception have not been observed in GRK2-HT, GRK4-KO, or GRK5-KO mice (34). Therefore, additional
studies in these mouse lines may reveal if specific GRKs are involved in other morphine-induced responses such as antinociceptive tolerance, physical dependence, and constipation.

1.5 Functional selectivity at the mu opioid receptor

Several in vitro and in vivo studies demonstrate that there are qualitatively distinct differences in the ability of certain opioid agonists to regulate receptor phosphorylation, β-arrestin-recruitment, and receptor internalization that cannot be explained by differences in drug efficacy with respect to G protein coupling or adenyllyl cyclase inhibition (33, 34). The observation that different ligands can promote diverse functional responses mediated by activation of a single receptor has given rise to a concept which has been termed “functional selectivity” (also referred to as “ligand-directed signaling” or “biased agonism”) (Figure 1.3) (56, 108-110). This concept is based on the idea that the chemical characteristics of the ligand may alter receptor conformations in a manner that will cause the receptor to preferentially activate specific signaling pathways to mediate distinct cellular and biological responses. Thus, one ligand may activate one signaling pathway whereas another different ligand, acting at the same receptor, may activate a completely different pathway. In addition, the cellular complement of regulatory and signaling proteins expressed could further define the manner in which a receptor responds to a particular ligand. Therefore, the nature of receptor-protein interactions may dictate the signal transduction pathway based on the properties of the ligand bound. Functional selectivity has now been described for several GPCRs including the μOR, serotonin 2A, beta2-adrenergic, vasopressin V2, and dopamine receptors (108, 111).
Several groups have demonstrated functional selectivity at the μOR in vitro. Morphine, etorphine, methadone and fentanyl can activate μOR signaling with similar efficacy; however, they differ in their ability to promote receptor desensitization and internalization. For example, morphine does not promote robust receptor phosphorylation, β-arrestin recruitment or μOR internalization while other opioid agonists such as etorphine, methadone and fentanyl do (112-119). The inability of morphine to induce β-arrestin2 recruitment can however be overcome by the overexpression of GRK2 (115, 117, 119). Studies in mouse embryonic fibroblasts lacking endogenous β-arrestin1 and β-arrestin2 suggest that the morphine-bound μOR preferentially interacts with β-arrestin2 (117). This concept is strengthened by the finding that the enhanced morphine analgesia in βarr2-KO mice could not be recapitulated in mice lacking β-arrestin1, indicating that β-arrestin2, rather than β-arrestin1, may preferentially regulate the morphine-bound μOR in vivo (117). Comparable differences in opioid responses have also been observed in mice lacking GRK3, wherein GRK3-KO mice display reduced antinociceptive tolerance after treatment with fentanyl but not with morphine (107).

In addition to the agonist, the relative responsiveness of the μOR is also influenced by the cellular environment in which the receptor is expressed. In nucleus accumbens preparations, the μOR does not internalize in neuron cell bodies but does internalize in dendritic projections in the same neuron in response to morphine treatment (120). Furthermore, chronic morphine has been shown to induce desensitization of the μOR as measured by adenylyl cyclase inhibition in thalamus and periaqueductal gray brain regions but not in caudate putamen or nucleus accumbens (121). Therefore, even if the μOR is regulated by β-arrestin2-mediated desensitization in some neurons, this may not
hold true for other cell types. These results suggest that μORs can be differentially regulated in different cellular environments.

1.6 Hypothesis and Overview of Chapters 2-4

The current dissertation presents work which addresses the hypothesis that GRK- and β-arrestin2-mediated μOR regulation can determine the extent of opioid-induced side effects including constipation, analgesic tolerance, and physical dependence, in a manner that is specifically influenced by different opioid agonists and cellular environments.

In Chapter 2, we evaluate the contribution of β-arrestin2 to μOR-mediated constipation in response to the three distinct μOR agonists (morphine, methadone, and fentanyl) using βarr2-KO mice. The data in this chapter demonstrate that morphine, but not methadone or fentanyl-induced constipation, is attenuated in the absence of β-arrestin2, indicating that β-arrestin2 influences the development of constipation in an agonist-dependent manner. The reduction in constipation in the βarr2-KO mice in response to morphine suggests that β-arrestin2 may be mediating μOR function when morphine is bound. Furthermore, using several different methods for assessing gastrointestinal motility, considering the contribution of both central and peripheral receptors, we find that loss of morphine-induced constipation in the βarr2-KO mice appears to be due to altered regulation of μORs expressed in the enteric nervous system at the level of the colon. Preliminary results from the development of methods to assess opioid effects on propulsion, secretion, and μOR signaling and localization in the colon are also presented in this chapter.
Chapter 3 assesses the role of β-arrestin2 in regulating antinociceptive tolerance and physical dependence in response to morphine, methadone and fentanyl in the βarr2-KO mice. The results presented in this chapter reveal that unlike morphine, methadone and fentanyl-induced antinociceptive tolerance is unaffected by the loss of β-arrestin2. Interestingly however, we find that βarr2-KO mice dependent on either morphine or methadone, but not fentanyl, display significantly less severe naloxone-precipitated withdrawal jumps and paw tremors compared to their WT littermates. These results suggest that β-arrestin2 may also be playing a pro-signaling role in this response as well. However, it is also possible that the loss of β-arrestin2 is affecting regulation of other GPCRs that are involved in the expression of these withdrawal signs.

Finally, Chapter 4 examines whether GRKs make a similar contribution to morphine-induced antinociception, antinociceptive tolerance, locomotor activity, physical dependence, and constipation in vivo using mice deficient of individual GRKs. We find that acute morphine treatment induces greater locomotor activation in GRK6-KO mice and “presensitizes” these mice to the locomotor stimulating effects induced by chronic morphine treatment. However, several other morphine-mediated responses, including thermal antinociception, antinociceptive tolerance, and physical dependence, were not affected by ablation of the GRK6 gene, suggesting that GRK6 may play a role in regulating some, but not all morphine-mediated responses. Furthermore, while GRK6 contributes to morphine constipation, other GRKs including GRK2, GRK3, GRK4, and GRK5 do not, suggesting that GRK6 may specifically regulate morphine-induced constipation.
The studies presented in this dissertation seek to provide a clearer understanding of how μOR regulation by GRKs and β-arrestins affect opioid-mediated side effects and have contributed to our knowledge regarding the diversity and complexity of GPCR regulation in vivo. Importantly, understanding which cellular and molecular events are involved in opioid-mediated physiological and behavioral effects may ultimately provide insight into the development of new drugs that may more effectively treat pain without producing adverse side effects.
**Figure 1.1.** Schematic representation of GRK and β-arrestin-mediated homologous desensitization of μORs. Rapidly following receptor activation, GRKs phosphorylate amino acid residues in the carboxyl-terminal tail of the receptor. This acts to promote the binding of β-arrestins, which in turn prevents further G protein coupling and receptor signaling. (Adapted from Bohn et al, *Neuromolecular Medicine*, 2004)
Figure 1.2. Schematic representation of GRK/β-arrestin-mediated Internalization. In addition to inducing μOR desensitization, GRK/β-arrestin interactions promote receptor internalization by targeting them to clathrin coated pits. Once internalized, receptors can either be recycled back to the plasma membrane (resensitized) or targeted for lysosomal degradation (downregulated). (Adapted from Bohn et al, *Neuromolecular Medicine*, 2004)
Figure 1.3. Schematic representation of functional selectivity. With functional selectivity, some ligands (L1) selectively modulate the activity of one effector pathway (E1) whereas other ligands (L2), acting at the same receptor (R), preferentially modulate a different effector pathway (E2) within the same cell. The thickness of the lines with arrows indicates the relative degree of activation of one effector pathway with respect to the other. (Adapted from Simmons, Molecular interventions, 2008)
CHAPTER 2

β-ARRESTIN2 DIFFERENTIALLY REGULATES INHIBITION OF GASTROINTESTINAL MOTILITY IN AN AGONIST-DEPENDENT AND TISSUE-SPECIFIC MANNER

2.1 Introduction

Approximately 40 to 50% of patients given opioids chronically for pain management develop constipation, making it one of the most common observed side effects of opioids (122). Constipated individuals experience infrequent and difficult bowel movements and current treatments for opioid-induced bowel dysfunction are often insufficient (123). In many cases, constipation becomes so severe and problematic that it presents challenges for patient compliance and is often one of the top reasons why patients discontinue opioid therapy (3, 123).

An extensive number of reports show that the constipatory actions of opioids primarily result from activation of opioid receptors expressed in the enteric nervous system (ENS) which is located within the walls of the gastrointestinal tract. The ENS is comprised of two major ganglionic nerve plexuses: the myenteric plexus, located between the longitudinal and circular smooth muscle layers, and the submucosal plexus which runs between the circular muscle and the mucosa (124). Neurons in these plexuses form microcircuits that regulate basic gastrointestinal functions including initiating and
coordinating intestinal propulsion, secretion and absorption to and from the intestinal lumen, and local blood flow (124).

Each opioid receptor type is expressed in the ENS (8, 125-130). Although application of selective \( \mu \)OR, \( \kappa \)OR, \( \delta \)OR and ORL-1 agonists can suppress gastrointestinal motility, a number of pharmacological studies (131) and experiments using genetically-modified mice lacking the \( \mu \)OR (11) indicate that the constipatory actions of morphine and other clinically used opioids are principally mediated through the \( \mu \)OR. Treatment with \( \mu \)OR-selective agonists such as DAMGO, morphine, loperamide, methadone, and fentanyl have been shown to inhibit small and/or large intestinal motility in several animal models including guinea pig, rat and mouse (131-138). Furthermore, small intestinal transit is not inhibited in \( \mu \)OR-KO mice treated with morphine, or the \( \delta \)OR and \( \kappa \)OR-selective agonists DPDPE and U50,488H, suggesting that opioid-induced inhibition of gastrointestinal transit is primarily a \( \mu \)OR-mediated function (11). Moreover, basal gastrointestinal motility is lower in \( \mu \)OR-KO mice compared to WT controls, suggesting that the \( \mu \)OR may also be involved in basal motility (11).

At the cellular level, opioid-mediated suppression of neurotransmission has been correlated with the inhibitory effects of opioids on gastrointestinal function. Studies from guinea pig intestine suggest that the \( \mu \)OR is expressed on ascending cholinergic motoneurons and interneurons as well as descending nitric oxide and vasoactive intestinal polypeptide (VIP) containing neurons in the myenteric plexus, which are involved in regulating muscle contractility (139). Electrophysiological recordings in enteric neurons have demonstrated that the primary function of the \( \mu \)OR within the ENS is to suppress neuronal excitability and neurotransmission (140). Postsynaptically,
opioids induce hyperpolarization of enteric neurons by increasing potassium channel conductance (17, 141, 142), which suppresses the activity of both the stimulatory musculomotor neurons that innervate the longitudinal muscle and the inhibitory musculomotor neurons that innervate the circular muscle (141). Furthermore, opioids interrupt neurotransmission by acting presynaptically to inactivate voltage-gated calcium channels, thus inhibiting the release of neurotransmitters including acetylcholine and substance P from interneurons (18, 142-144). Collectively, hyperpolarization of musculomotor neurons and interneurons delays intestinal transit by causing non-propulsive contractility of the longitudinal muscle, decreased segmentation activity of the circular muscle, and slowing of gastric emptying and biliary activity (140). Moreover, opioids also suppress intestinal mucosal ion transport by acting presynaptically at secretomotor neurons to suppress the release of acetylcholine and vasoactive intestinal peptide (VIP) in the submucosal plexus (131, 138, 140). This causes a net fluid reduction in the lumen of the small intestine and leads to the formation of hard, dry stools in the large intestine (140).

While the constipatory effects of opioids result primarily from activation of opioid receptors in the ENS, their inhibitory effects on gastrointestinal motility also result, in part, from their actions in the CNS, especially at doses which produce analgesia. When administered centrally, morphine decreases intestinal transit and fluid secretion in several species including guinea pigs, rats and mice (132, 134, 137, 145-149). Furthermore, when given intrathecally, opioids can also inhibit intestinal motility (147, 150). While the exact central sites involved in mediating the inhibitory actions of opioids on gastrointestinal motility are not clear, several studies indicate that opioids can influence the gastrointestinal function at the level of the CNS by modulating the activity of parasympathetic and sympathetic pathways that innervate the gastrointestinal tract (135, 151-153). Opioids may also act through central
mechanisms by stimulating postganglionic nerve fibers, which heavily innervate the ENS to release the neurotransmitter norepinephrine, suppressing neurotransmission at both slow and fast enteric neuron excitatory synapses within enteric microcircuits and inhibits firing of submucosal secretomotor neurons (154-159).

The effect of μOR regulation in the gastrointestinal system has been extensively studied in tissue preparations from guinea pig ileum, with most efforts focusing upon opioid actions on G protein coupling and adenylyl cyclase inhibition. Following chronic morphine treatment adenylyl cyclase activity is dramatically enhanced and the μOR is less effective in suppressing the elevated cyclase-mediated signaling (160-162). Furthermore, immunohistochemical studies reveal that the μOR can be internalized and recycled in neurons in the gut in an agonist dependent manner, suggesting functional selectivity may occur with μORs expressed in the ENS (114, 163, 164). However, the contribution of β-arrestins to regulation of μORs involved in modulating gastrointestinal function is unknown.

In the present study, we evaluated the role of β-arrestin2 in opioid-induced constipation using βarr2-KO mice. Several opioid agonists were tested, including morphine, methadone, fentanyl to determine if there are agonist-dependent differences with respect to their ability it inhibit gastrointestinal responses in the absence of β-arrestin2. Gastrointestinal function was assessed using three different gastrointestinal function assays which measured fecal boli production, small intestinal transit, and colonic motility; considering both central and peripheral sites of action. We find that morphine-induced constipation is reduced in the βarr2-KO mice when compared to their WT littermates, which appears to be mediated by altered regulation of μORs in the ENS specifically at the level of the colon. In contrast to
morphine, methadone and fentanyl-induced inhibition of gastrointestinal motility is unaffected in the absence of β-arrestin2.

In addition to assessing the effects of β-arrestin2 deletion on opioid-induced constipation in vivo, ex vivo studies were initiated to determine whether differences in physiological and biochemical responses exist at the tissue and cellular levels of the colon when β-arrestin2 is not present. Ex vivo methods to measure colon propulsion and secretion in the absence of CNS innervations were implemented and optimized. Furthermore, in order to determine if μOR-mediated signaling is altered in the βarr2-KO mice, organotypic cultures of colonic neuronal plexuses were adopted and modified to measure the activity of several kinases, including JNK, as well as to identify the neuronal and cellular populations that may be affected by opioid agonist drug treatment. The preliminary results from these studies in WT mice are presented in this chapter.

2.2 Materials and Methods

*Mice.* Male WT and βarr2-KO mice (22-30 g), between the ages of 3-6 months, were used for these studies. Mice were generated by heterozygote breeding (87). Some studies employed a small number (less than 30%) of animals derived from homozygous crossing (homozygous breeders are offspring of heterozygous parents). However, the data obtained from these animals did not differ from those obtained in heterozygously bred animals and were combined with this population. Mice were housed in groups of five in Plexiglas chambers in a temperature-controlled room and maintained on a 12 hour reversed light/dark cycle and mice had free access to food and water before any experiments unless otherwise noted. Mice were examined during the light phase of their circadian cycle and used only once for each dose and each drug tested. All studies
were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with approved animal protocols by The Ohio State University.

*Drugs and Solutions.* Morphine sulfate pentahydrate, (±)-methadone hydrochloride, fentanyl citrate, naloxone hydrochloride, dimethylphenylpiperazinium iodide (DMPP), and carbachol were purchased from Sigma (St. Louis, MO, USA). Loperamide hydrochloride was purchased from Tocris Bioscience (Ellisville, MO, USA). Morphine, methadone and fentanyl were all dissolved in 0.9% physiological saline, while loperamide was dissolved in a 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma, St. Louis, MO, USA) solution for subcutaneous (s.c.) injections which were given in a volume of 10 μL/g at the back of the neck. For intracerebroventricular (i.c.v.) injections, morphine and loperamide were prepared in sterile distilled water and were performed as previously described (Raehal et al., 2005). Briefly, mice were lightly anesthetized with isoflurane, and a 10 mm incision was made along the midline of the scalp. An injection was made using a 25 μL Hamilton syringe at a point 2 mm caudal and 2 mm lateral from bregma using a 27-gauge needle at a depth of 3 mm in a volume of 5 μl. For ex vivo studies morphine, naloxone and DMPP were dissolved in Kreb’s buffer. All drugs were freshly prepared prior to use.

*Fecal Boli Accumulation Assay.* Mice were treated with saline or morphine (5, 10, 20 mg/kg, s.c.) and then individually placed in a small Plexiglas box (15.2 cm x 16.5 cm x 12.0 cm) lined with filter paper. Expelled fecal boli were collected in a plastic weight boat and weighed at 1 hour intervals over a 6 hour period. Prior to the test, mice were provided food and water ad libitum.
**Charcoal Meal Assay.** Small intestinal transit was measured using the charcoal meal test as previously described with some modification (11). Forty-eight hours prior to testing, a mesh wire insert was placed in the bottom of each cage to suspend the mice above their bedding and prevent the ingestion of feces or bedding. Mice were habituated to the modified cage for 24 hours in the presence of food and water and then fasted for 24 hours with free access to water. For the experiment, mice were given an injection of saline or morphine (1, 3, 10 mg/kg, s.c.) 20 minutes prior to receiving a charcoal meal containing a 5% aqueous suspension of charcoal (2-12 micron; Sigma-Aldrich, St. Louis, MO, USA) in a 10% gum arabic (Acros Organics, Morris Plains, NJ, USA) solution by oral gavage. Methadone (1, 3 mg/kg, s.c.) and fentanyl (0.03, 0.06, 1.0 mg/kg, s.c.) were injected 5 minutes prior to administering the charcoal meal. Mice were then sacrificed by cervical dislocation at 30 minutes post-gavage and the small intestine from the pyloric sphincter to the ilealcecal junction was isolated and the mysentery removed. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine and the percent of gastrointestinal transit for each treatment group was calculated as follows: % gastrointestinal transit= [(charcoal bolus distance (cm))/ (small intestine length (cm))] x 100%.

**Colonic Bead Expulsion Assay.** Large intestinal transit was measured using a bead expulsion test as previously described with some modification (165). Mice were first habituated and fasted in the same manner as described for the small intestinal transit studies and then were administered either saline (0.9%, s.c.), (2-hydroxypropyl)-β-cyclodextrin (20%, s.c.), morphine (1, 3, 10, 20 mg/kg, s.c.), loperamide (0.3, 0.6, 1.0 mg/kg, s.c.), methadone (1, 10 mg/kg, s.c.) or fentanyl (0.1, 0.25 mg/kg, s.c.). Twenty minutes following morphine or loperamide injection, or 5 minutes after methadone and
fentanyl treatment, a 3 mm glass bead (Fisher Scientific, Pittsburgh, PA, USA) was
gently inserted 2 cm into the distal rectum using 2 mm round, flexible, plastic tubing.
Mice were individually placed into a small, Plexiglas box (14.0 cm x 12.7 cm x 15.2 cm)
for observation and the time to bead expulsion was recorded. On the rare occasion that
mice did not expel their bead without manipulation or produced feces before expelling
the bead, the mouse was excluded from the study.

Colonic Pellet Expulsion Assay. Peristaltic propulsion of the colon was measured ex
vivo using previously described methods for mouse and guinea pig (166, 167). Mice
were sacrificed by cervical dislocation, the intestines were removed, and the large
intestine from the ilealcecal junction to the rectum was isolated. The lumen was gently
washed free of fecal material and the most distal 0.5 cm of the colon was removed. The
colon was then pinned by the attached mysentery at regular intervals to a Sylgard-
coated plate (Dow Corning, USA) and incubated with circulating Kreb’s buffer (in mM:
NaCl, 119; KCl, 4.7; KH2PO4, 1.2; MgSO4, 1.2; CaCl2, 2.5; NaHCO3, 24; glucose, 11 at
pH 7.4 gassed with 95% O2/5% CO2) at 37-38°C. Following a 15 minute equilibration
period, a shellacked-coated fecal boli or a 3 x 5 mm plastic pellet was inserted 15 mm
into the proximal end of the colon. The distance traveled and the time required for the
pellet to exit the distal end of the colon was recorded using a video camera and the
propulsion rate was calculated by dividing the distance traveled over time. Prior to drug
treatment, the basal velocity for each tissue was determined using two successive trials
given at 5 minute intervals. After an additional 5 minute equilibration period, tissues
were pretreated with morphine (1μM) for 10 minutes and then the pellet was inserted
and the distance traveled over time was measured. After 20 minutes, the tissue was
exposed to Kreb’s buffer containing naloxone (1μM) to demonstrate that the measured affects were mediated by specific activation of opioid receptors.

**Ussing Chamber Studies.** Intestinal secretion was measured using Ussing chambers as previously described (138, 168). Mice were sacrificed by cervical dislocation, the intestines were removed, and the large intestine from the ilealcecal junction to the rectum was isolated and placed in ice-cold Kreb’s buffer (composition in mM: NaCl, 118; KCl, 4.8; MgCl₂·6H₂O, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose 10; and CaCl₂·2H₂O, 2.5 buffered at pH 7.4). The tissue was opened along the mesenteric border and pinned taught to a Sylgard-coated plate containing ice-cold Kreb’s buffer. Intact mucosal preparations containing the mucosa and submucosal plexus (SPM) were isolated using blunt microdissection. The colon was divided into four sections of equal length (with the most distal 0.5 cm of the colon being discarded) (See Figure 2.1 for details about region divisions and microdissections). Each section was mounted between two half chambers with a cross-sectional area of 0.725 cm². The serosal side of the tissue was exposed to 5 mL of Kreb’s buffer maintained at 37°C and aerated with 95% O₂ and 5% CO₂. The mucosal bathing solution was identical except mannitol (10 mM) was substituted for glucose (169). Each chamber was connected to pairs of current and voltage Ag/AgCl electrodes with one electrode on each side of the tissue via Krebs-4% agar bridges connected to a voltage-clamp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL, USA). The current necessary to change the transepithelial potential difference by 8 mV was used to monitor tissue conductance as a determinant of tissue viability and was calculated using Ohm’s law. During the experiment, Isc was monitored and recorded using a digital data acquisition system (Lab Trax 2.0 software, World Precision Instruments, Sarasota, FL, USA).
Once mounted, the tissue preparations were allowed to equilibrate in the chambers for at least 30 to 40 minutes prior to drug treatment. Vehicle or morphine (10 μM) was then added to the serosal bathing solution followed 10 minutes by DMPP (60 μM). At the end of the experiment, tissues were challenged with carbachol (20 μM) to measure tissue viability. All measurements were normalized to the cross-sectional area of the tissue preparations and tissues that responded with less than a 20 μA/cm² carbachol-induced increase in Isc were excluded from analysis.

Immunoblotting. Mice were sacrificed by cervical dislocation, the intestines were removed, and the large intestine from the ilealcecal junction to the rectum was isolated and placed in ice-cold Kreb’s buffer. The colon was opened along the mesenteric border and washed, then pinned taught to a Sylgard-coated plate containing ice-cold Kreb’s buffer. The colon was divided into four sections of equal length and LMMP and SP whole mount preparations were isolated using blunt dissection (Figure 2.1). The tissues preparations were then placed in warmed organotypic culture media (DMEM/F12 media containing 10% v/v FBS, 5% penicillin/streptomycin, and 2.5 ug/ml amphotericin B) and incubated at 37°C for 1 hour (114; 163; 164). Saline or morphine (10 μM) was then added to the media and tissues were incubated at 37°C for an additional 30 minutes.

Following drug treatment, tissues were snap frozen using liquid nitrogen. Tissue samples were pulverized into a fine powder using a stainless steel multi-sample biopulverizer (BioSpec Products, Bartlesville, OK, USA) that was thoroughly chilled with liquid nitrogen. The resulting powder was then further homogenized using a motorized pestle in ice-cold RIPA lysis buffer (composition in mM: Tris-HCl pH 7.4, 50; EDTA, 2; NaCl, 150; NaF, 1; Na3VO4, 1; PMSF, 1; 0.1% SDS, 1% NP-40, 0.25% deoxycholate,
and a protease inhibitor cocktail tablet (Roche, Indianapolis, IN, USA)). Samples were allowed to solubilize for 2 hours on ice followed by centrifugation at 10,000xg for 10 minutes at 4°C. The protein concentration of the supernatant fraction was quantitated using a DC protein assay (BioRad, Hercules, CA, USA). Equal amounts of proteins were separated by SDS-PAGE under denaturing conditions, transferred to PVDF membranes, and incubated in the presence of primary antibodies (anti-phospho-SAPK/JNK: 1:750 and anti-SAPK/JNK 1:1000 (Cell Signaling, Danvers, MA, USA) overnight at 4°C. Membranes were then incubated in the presence of the appropriate secondary antibodies conjugated to HRP for 1 hour at room temperature and proteins were detected using enhanced chemiluminescence reagents visualized using a Kodak 2000R image station (Kodak, New Haven, CT, USA). Densitometry was assessed using the Kodak 1D Image Analysis software to quantitate and phospho-JNK was normalized to the total amount of JNK per lane and to the degree of stimulation compared with saline treated controls in each blot.

**Immunohistochemistry.** Immunohistochemistry was assessed using methods previously described for assessing opioid effects in guinea pig ileum, with some modifications (114). Mice were sacrificed by cervical dislocation, the intestines were removed, and the large intestine from the ilealcecal junction to the rectum was isolated and placed in warmed oxygenated Krebs’ buffer. The tissue samples were washed with warmed phosphate-buffered saline (PBS; 0.01M, pH 7.2-7.4) and incubated at 37°C for 30 minutes in organotypic culture media. The colon was then opened along the mesenteric border, pinned taught to a Sylgard-coated plate, and then incubated in organotypic culture media in the absence of serum (10% FBS) at 37°C for 30 minutes. Serum was then added back for 10 minutes.
Colon cultures were next fixed by treating with a 4% paraformaldehyde solution containing 20% sucrose overnight at 4°C. Tissues were washed three times over a 30 minute period with 1X PBS and the longitudinal muscle-myenteric plexus (LMMP) and submucosal plexus (SP) was isolated using microdissection (Figure 2.1) and processed as whole-mounts for immunohistochemistry. To reduce nonspecific antibody binding and permeabilize the tissue, tissues were incubated in 1X PBS containing 0.3% Triton-X and 5% normal goat serum for 1 hour at room temperature. Preparations were then incubated in PBS containing 1% bovine serum albumin, 0.3% Triton X-100, and primary antibodies of interest (anti-HuCD conjugated to strepavidin: 1:100 (Invitrogen, Carlsbad, CA, USA); anti-Map2: 1:20,000 (Cell Signaling, Danvers, MA, USA); phospho-JNK 1:200 (Cell Signaling, Danvers, MA, USA)) for a period of 48 hours at 4°C. Tissue preparations were washed three times with 1X PBS containing 0.3% Triton X-100, then incubated with fluorescently-conjugated secondary antibodies for 1 hour at room temperature, and then washed again with 1X PBS and coverslipped with Immunomount mounting medium (Invitrogen, Carlsbad, CA, USA). Fluorescence labeling was then examined using a confocal microscope with green-helium and multi-argon lasers (Olympus, Tokyo, Japan).

**Statistical Analysis.** Results for each experiment are expressed as the mean ± S.E.M. Studies evaluating time course or dose-response effects between genotypes were analyzed using a standard two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis when appropriate. When two groups were compared for a single response, a Student’s t test was used. All statistics were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).
2.3 Results

The μOR has been shown to be critical in mediating the inhibitory actions of opioids on gastrointestinal function. Therefore, we asked whether β-arrestin2 plays a role in opioid-induced constipation in vivo. The inhibitory effect of morphine on defecation was evaluated by measuring fecal boli output over a 6 hour period in response to saline (s.c.) and morphine (5, 10, 20 mg/kg, s.c.). Saline treatment resulted in a similar profile of fecal boli production in both the WT and βarr2-KO mice (Figure 2.2A), suggesting that the two genotypes are not intrinsically different in their normal gastrointestinal function. As shown in Figure 2.2B, two-way ANOVA revealed that treatment with a moderate analgesic dose of morphine (10 mg/kg) produced significant differences in fecal boli between the genotypes. Morphine induced an initial suppression of defecation in both groups of mice; however, the βarr2-KO mice fully recover to saline levels after 2 hours, while the WT mice continue to display suppressed defecation throughout the test period. Furthermore, the βarr2-KO mice produce more fecal boli than the WT mice over the 6 hour test period at several doses of morphine tested (Figure 2.2C). To determine if the observed differences in defecation were due to differences in eating behaviors between the two genotypes, food consumption was monitored for grams of food consumed in 24 hours normalized per mouse and there was no significant difference between WT and βarr2-KO mice (Figure 2.2D).

To study the inhibitory effects of morphine on intestinal function in greater detail, small intestinal transit was assessed by measuring the distance traveled by an orally administered charcoal meal following administration of saline (s.c.) or morphine (1, 3, 10 mg/kg, s.c.) in fasted mice. There were no significant differences in transit of the meal following saline treatment, and while morphine dose-dependently decreased transit in
both the WT and βarr2-KO mice, there were no significant differences between the genotypes at any of the doses tested (Figure 2.3). Since significant differences were apparent in fecal boli production, we asked whether morphine would differentially affect colonic motility in the βarr2-KO mice using a bead expulsion test. Saline treated mice expelled their beads in approximately 5 minutes, while morphine produced a dose-dependent increase in bead retention times (Figure 2.4A). However, in this assay, the βarr2-KO mice displayed significantly shorter delays in bead expulsion times at the lower doses of morphine (1, 3 and 10 mg/kg, s.c.) tested compared to WT mice.

Morphine acts at opioid receptors both peripherally and centrally to effect gastrointestinal motility. In order to ascertain whether the differences in colonic motility were due to peripheral site of action, the peripherally-restricted μOR agonist loperamide was employed. Loperamide does not cross the blood brain barrier and acts to inhibit motility primarily by activating the μOR (170-172). Loperamide (0.1, 0.6, 0.1 mg/kg, s.c.) delayed bead expulsion times in the WT mice, yet had no significant effect in the βarr2-KO mice when compared to vehicle treated animals (Figure 2.4B). Moreover, loperamide (0.1 nmol, i.c.v.) also induced similar colon transit delays in the WT and βarr2-KO mice when injected centrally (Figure 2.5A). Likewise, morphine (1.5, 10 nmol, i.c.v.) also inhibited colon motility to the same extent in both genotypes when administered centrally (Figure 2.5B).

Differences in agonist-induced antinociception have been previously observed in the βarr2-KO mice. Therefore, we determined whether there were also differences in morphine, methadone or fentanyl effects on gastrointestinal function in the absence of β-arrestin. In the small intestinal transit assay, methadone and fentanyl dose-dependently
inhibited small intestinal transit. Like morphine, there were no differences between the WT and KO mice following treatment with methadone (Figure 2.6A) or fentanyl (Figure 2.6B). However, unlike morphine, there were no differences in colonic bead transit times between the WT and βarr2-KO mice in response to methadone (Figure 2.7A) or fentanyl (Figure 2.7B).

Opioids have been shown to induce constipation by affecting both propulsion and secretion in the gastrointestinal tract. To begin to understand how β-arrestins may affect these processes in the colon, we have adapted ex vivo techniques which will allow us to determine if the loss of β-arrestin2 specifically influences μOR-mediated changes in the coordinated actions of the muscle layers which mediate propulsion, or if it specifically affects secretion, or whether both processes are altered. Given that our in vivo results indicate that β-arrestin2 may differentially regulate μORs in the ENS, these ex vivo approaches will also us to further evaluate opioid effects on the ENS in the βarr2-KO mice in the absence of input from the CNS.

To begin to determine how morphine induces its inhibitory effects on colonic activity, we have initiated studies using a pellet propulsion assay to measure the effects of morphine on colonic propulsion using the WT mice. For these studies, intact colon from the mouse was isolated and placed into an organ bath. Morphine or naloxone was then applied to the bathing solution to determine how they affect the movement of an artificial pellet through the colon. As shown if Figure 2.8B, when morphine (1 μM) was added to the bath, distance traveled by a 3 mm x 5 mm plastic bead were significantly reduced compared to basal conditions. Furthermore, application of 1 μM naloxone, while not significant, reduced the effect of morphine at the dose tested. Moreover, the calculated
propulsion rate of the bead was reduced in response to morphine compared to basal conditions, an effect which was reduced by naloxone (Figure 2.8C).

In addition to their effects on propulsion, opioids act to inhibit neurogenically-induced secretion. To assess the effects of morphine on colonic secretion ex vivo, we used Ussing flux chambers to measure net ion transport taking place across the intestinal epithelium which mediates secretion in the gut. Using these chambers we demonstrate that morphine can inhibit secretion induced by the nicotinic agonist DMPP, in colonic SPM preparations from WT mice. As shown in Figure 2.9, under basal conditions, the short circuit current (Isc) and tissue conductance decreased from the proximal (section 1) to the distal end (section 4) of the colon. Treatment with a high concentration of DMPP (60 μM) increased the Isc over basal levels in each colon section (Figure 2.10A). Furthermore, morphine (10 μM) significantly reduced the DMPP-mediated increases in Isc by more than 50% in section 2 (a proximal region of the colon) at the single dose of each drug tested (Figure 2.10, B and C). Following inhibition with morphine, treatment with carbachol which induces secretion independent of neural input, also increased Isc in all colon tissues (data not shown), indicating that the tissues were still viable after 1.5 to 2 hours in the bathing solution.

Using multiple approaches to assess opioid-induced inhibition on gastrointestinal function in the βarr2-KO mice revealed that constipation is less in the absence of β-arrestin2, suggesting that this molecule may be playing a pro-signaling role downstream of μOR activation. We have adapted organotypic cultures of LMMP and SP colonic preparations to begin to study whether the differences in opioid-induced signaling is affected in the absence of β-arrestin2. We have conducted initial immunoblotting
experiments using the LMMP and SP organotypic culture preparations form WT mice to ascertain if the activity of signaling kinases such as JNK, which has been shown to be activated by stimulation of the $\mu$OR and by $\beta$-arrestin-mediated signaling in cell culture, is activated by morphine in mouse colon. As shown in Figure 2.11, in these initial studies we find that in the myenteric plexus there is an increase in phosphorylated-JNK2/3 levels upon morphine treatment. Interestingly we find that serum treatment, which can also non-specifically increase phospho-JNK levels does so primarily in myenteric neurons that co-stain with neuronal marker HuC/D, indicating that JNK activation occurs in enteric neurons and not other types of cells in the preparations (Figure 2.12, A and B).

2.4 Discussion

The data presented here reveal that morphine-induced constipation is diminished in the absence of $\beta$-arrestin2 (Figures 2.1 and 2.3), an effect which appears to be mediated by altered regulation of $\mu$ORs expressed in the ENS (Figures 2.4 and 2.5) at the level of the colon. Moreover, while disruption of $\mu$OR regulation by removal of $\beta$-arrestin2 affects the ability of morphine to induce constipation, it does not impact methadone or fentanyl effects on gastrointestinal function (Figures 2.6 and 2.7). These results further support the hypothesis that $\beta$-arrestin2 regulation of the $\mu$OR can determine the expression of opioid-induced constipation and that there may be functional selectivity among opioid agonists in respect to their ability to induced constipation in the absence of $\beta$-arrestin2.

As a mediator of GPCR desensitization, $\beta$-arrestin2 regulates the degree of coupling between the $\mu$OR and G proteins, which has been demonstrated in certain brain regions and in the spinal cord in the $\beta$arr2-KO mice (87, 88). In this simple scenario in which $\beta$-
arrestin2 only acts as a desensitizing element would indicate that all behavioral responses to morphine, including constipation, should be enhanced. However, our results suggest that β-arrestin2 may be facilitating μOR signaling in the colon. A number of studies have now demonstrated that -β-arrestins can also promote GPCR signaling independent of G proteins (93-96, 98, 103, 104, 173) and recent evidence in cellular cultures provides evidence that the μOR may signal via β-arrestins in an agonist-dependent manner (99). Unlike opioid receptors expressed in the brain, it is possible that opioid receptors involved in mediating morphine’s inhibitory actions on colonic motility are in a cellular environment in which the β-arrestin2 molecule plays an important role in initiating G protein-independent signal transduction in response to receptor activation. In such a scenario, removal of the β-arrestin2 molecule could prevent downstream signaling and the subsequent biological response. The loss of β-arrestin2 has already been shown to disrupt alpha2-adrenergic, D2 dopamine, and serotonin 2A receptor signaling in vivo (101, 102, 105, 106). Interestingly, we have also observed that βarr2-KO mice also display significantly attenuated respiratory suppression in response to morphine, suggesting that β-arrestin2 could be positively mediating μOR signaling in the colon.

Using three different approaches to assess the effects of morphine on gastrointestinal function, we find there are differences in morphine-induced inhibition of fecal boli production (Figure 2.2) and colonic motility (Figure 2.4), but not small intestinal transit (Figure 2.3) between WT and βarr2-KO mice. The fecal boli accumulation studies may be the definitive test for morphine-induced constipation as the animals had free access to food and water prior to the test and were simply monitored for their ability to produce fecal waste following drug treatment as compared to saline. We considered that food
deprivation could potentially confound the effects on the small intestinal transit times. However, the colonic bead expulsion studies, which were also performed under fasting conditions, paralleled the whole gut transit assay at the lower doses of morphine, suggesting that this was not the case. Interestingly, a recent study by Ross et al. (174) show that morphine-induced effects in the small intestine of mice decreases over time, whereas morphine actions on the colon do not, indicating that there are inherent differences in μOR regulation between these regions of the gastrointestinal tract. Therefore, it is likely then that the difference in small and large intestinal responses in the βarr2-KO mice may be due to differences of μOR regulation or the contribution of centrally expressed μORs to gastrointestinal function in these two distinct intestinal regions.

Opioids act at μORs expressed within the CNS as well as in the ENS to inhibit gastrointestinal motility. Our study with the μOR agonist loperamide, which is limited to peripheral sites of action, recapitulates the finding with morphine in the colonic bead expulsion studies. Moreover, when both morphine and loperamide are administered centrally, there are no differences in bead expulsion times between the WT and βarr2-KO mice, further suggesting that the differences in genotype may be due to altered μOR regulation in the ENS. Additional studies of colonic motility in which μOR antagonists are administered centrally prior to systemic administration of morphine will be completed by giving systemic injections of opioid agonists following pretreatment with centrally administered opioid receptor antagonists and measuring fecal boli accumulation and colonic transit in the WT and βarr2-KO mice. Such studies may shed light on the contribution of β-arrestin2 interactions with peripheral versus central μORs to the development of constipation.
While morphine has a reduced effect on inhibition of colonic motility in the absence of β-arrestin2, methadone and fentanyl treatment produces similar effects on small intestinal transit and colonic motility in both genotypes. This difference in agonist response in the βarr2-KO mice between morphine versus methadone and fentanyl was also observed with antinociception (117). One possible explanation for the differences among these agonists is that β-arrestin1 may be compensating for the loss of β-arrestin2 when methadone or fentanyl is used, but not morphine. Cell culture studies support this hypothesis as methadone and fentanyl robustly recruit β-arrestin1 and β-arrestin2 to the μOR, whereas morphine only weakly recruits β-arrestin2 to the μOR (117). Another alternative explanation is that methadone and fentanyl may more effectively reach the CNS and as a result, their activation of μORs in the CNS may overshadow differences between genotypes induced by these agonists in the periphery. There is some evidence for this as methadone has been shown to cross the blood brain barrier more effectively than morphine (175) and has a lower affinity for the p-glycoprotein transporter than morphine as well (176). Moreover, centrally administered morphine and loperamide produce equivalent delays in colon bead expulsion times in the WT and βarr2-KO mice, suggesting the CNS-mediated μOR regulation of intestinal transit may not require β-arrestin2.

While the lack of morphine-induced constipation in the KO mice suggests that β-arrestin2 may be playing a pro-signaling role at the μOR it is possible that morphine may act at multiple μOR subtypes that are regulated differently by β-arrestin2. A number of studies have suggested that opioid control of constipation and respiratory suppression might be due to activation of a different subset of μORs (μ2OR, type 2 μOR) as opposed
to those that are believed to mediate antinociception (μ1OR, type 1 μOR) (177). Furthermore, we have also considered that the differences observed in morphine-induced constipation between the βarr2-KO mice and their WT littermates may not be due to direct regulation of the μOR, but rather could also be due to β-arrestin2 regulation of other GPCRs, such as dopamine and alpha2-adrenergic receptors which have been shown to inhibit gastrointestinal motility (154, 156, 178-181). While morphine has not yet been shown to promote dopamine release in the ENS, it does promote dopamine release in the CNS (182). Previously, Beaulieu et al. (101) described decreased D2 dopamine receptor function in the absence of β-arrestin2 in the striatum following treatment with psychostimulants. It may be possible that the ablation of β-arrestin2 could potentially also decrease D2 dopamine receptor/β-arrestin2 interactions in gut, which could impact the physiological effects observed here by interrupting a downstream β-arrestin2-mediated signaling pathway. In addition, opioids can promote the release of norepinephrine which can activate alpha2-adrenergic receptors in the ENS to inhibit secretion (156) and βarr2-KO mice respond less to an alpha2-adrenergic receptor agonist in the rotorod test, suggesting that β-arrestin2 may be positively mediating signal transduction via these receptors (106). Therefore, further studies examining the contribution of dopamine, and other neurotransmitter systems, on morphine-induced constipation and signal transduction in the gut are warranted in the βarr2-KO mice.

2.5 Establishment of Ex Vivo Techniques for Future Studies

Our in vivo results demonstrate that the loss of β-arrestin2 can prevent morphine-induced constipation, an effect which is most likely due to altered regulation of μORs expressed on enteric neurons in the colon. In order to ascertain the mechanisms by which the loss of β-arrestin2 impacts the effect of morphine on colonic function, we have
begun to implement several ex vivo techniques that will allow us to assess whether colonic propulsion, secretion, and signaling is altered in the βarr2-KO mice compared to their WT littermates in the absence of central μOR input. A significant amount of time and effort was spent optimizing many of the techniques necessary to measure these effects; therefore, only preliminary results in WT mice are presented in this chapter. The development of these methods was critical and will serve as the basis of a number of future studies.

To assess opioid-induced effects on colonic motility in the absence of CNS innervations, we used an assay wherein the propulsion of an artificial fecal pellet or bead through the intact colon was measured in an organ bath. This method has previously been used to study the effect of opioids on colonic propulsion in intact colon from guinea pig (167). Using this approach, we show that morphine inhibits colonic propulsion in the WT mice (Figure 2.8). Moreover, the inhibitory effect of morphine could be reduced by naloxone indicating that it is an opioid receptor mediated affected. We found that the most critical parameters which affect propulsion are the size of the pellet, bath temperature and sufficient oxygenation of the buffer. Future experiments will include further optimizing the bathing system, determining the most appropriate pellet size and composition, and completing morphine concentration response curves in both the WT and βarr2-KO mice. Based on our in vivo studies, we expect that morphine will produce less suppression of propulsion in the βarr2-KO mice, whereas methadone and fentanyl will produce equivalent degrees of inhibition of colonic propulsion in both genotypes.

To evaluate colonic secretion, we have implemented methods for assessing morphine-induced inhibition of secretion using Ussing flux chambers. This technique measures
changes in chloride ion flux as a function of short-circuit current (Isc) and serves as a measure of mucosal secretion activity (183). A decrease in Isc indicates that drug treatment is preventing chloride ion transport, signifying decreased mucosal secretion activity. Under basal conditions we find that Isc and conductance a measure of a tissues capability for ion flow) decrease in a region-dependent manner wherein the more proximal regions (section 1 and 2) of the colon have higher Isc and conductance values than more distal regions (sections 3 and 4) (Figure 2.9). Similar differences have been previously observed in normal mouse colon where contractility was shown to decrease from the proximal to distal end of the colon, an effect which may be due to the reduced number of neurons in the distal colon compared to the proximal colon (184-186)

Previously, morphine has been shown to inhibit basal Isc and chloride ion flow in mouse jujenum (138). However, we have found that under unstimulated conditions, the effect of morphine is very minimal and difficult to detect in mouse colon. We determined that we can overcome this problem by utilizing the selective nicotinic receptor agonist DMPP. Activation of nicotinic receptors in the submucosal plexus excites secretomotor neurons and stimulates secretion (140, 187). When administered prior to nicotine, agonists that produce inhibitory post synaptic potentials such as morphine, decrease the overall degree of excitatory stimulation evoked by nicotinic receptor activation (140). Our preliminary studies demonstrate that application of an initial high dose of DMPP (60 μM) induces an increase in Isc in all SPM preparations evaluated (Figure 2.10). Moreover, pretreatment with morphine (10 μM) for 10 minutes effectively blocks DMPP-stimulation of Isc. These results suggest that we will be able to use this approach to compare the effects of morphine and other opioids on colonic secretion in the βarr2-KO mice to determine the role β-arrestin2 plays in μOR mediation of secretion. We predict that
morphine will not inhibit secretion in the βarr2-KO mice, but methadone and fentanyl will. However, before these studies can be completed, DMPP concentration response curves will need to be determined in the WT mice to identify the EC50 concentration which should be used in these assays. Moreover, additional controls including the use of μOR antagonists (selective and non-selective) and nicotinic receptor antagonists will be used to demonstrate that the effects of morphine and DMPP are due to μOR and nicotinic receptor activation, respectively. Finally, inhibitors of excitatory neurotransmission, such as tetrodotoxin, will be used to demonstrate that secretion induced by nicotinic receptor stimulation is due to neurogenically-mediated transmission. Based on our hypothesis, we expect that morphine, but not methadone or fentanyl-induced inhibition of secretion will be unaffected in the βarr2-KO mice when compared to their WT littermates.

Relatively little is currently know about μOR-mediated signaling in enteric neurons. Our in vivo data support a hypothesis wherein β-arrestin2 plays a pro-signaling role downstream of μOR activation in the colon. As mentioned previously, β-arrestins have been shown to activate MAP kinases in cell culture studies. Therefore, we have employed experimental approaches that will allow us to test whether μOR activation of signaling molecules such as JNK, ERK1/2 and Akt is altered in the absence of β-arrestin2. Using organotypic cultures, we demonstrate that we can measure morphine-induced changes in JNK phosphorylation in LMMP and SP preparations from WT mice. Using immunoblotting techniques, we find that morphine leads to activation of p-JNK2/3 in all four regions of the myenteric plexus (Figure 2.11). Moreover, we observed that JNK is activated in response to serum specifically in neurons in the myenteric plexus (figure 2.12), suggesting that we can detect changes in signaling molecules using confocal microscopy in enteric neurons. Once both methods are firmly established
potential differences in morphine, methadone, and fentanyl activation of JNK, ERK1/2, and Akt will be assessed in the WT and βarr2-KO mice. Based on our in vivo studies, we predict that morphine, but not methadone- or fentanyl-induced activation or inhibition of these kinases will be disrupted in the βarr2-KO mice.

Taken together with our previous findings, the results of the experiments presented in this chapter suggest that while the analgesic properties of morphine are enhanced in βarr2-KO mice, the removal of β-arrestin2 may be protective against morphine-induced constipation. The lack of morphine-induced constipation suggests that unlike for antinociception, β-arrestin2 may be acting as a signaling molecule at μORs in the gut. Furthermore, there are differences in agonist-induced constipation in the absence of β-arrestin2 which may be due to differences in differential β-arrestin2 regulation of μORs expressed in the ENS versus the CNS as well as differences in pharmacokinetic properties among the drugs tested. However, our results indicate that inhibition of β-arrestin2 represents a novel strategy through which μOR-mediated constipation may be prevented.
Figure 2.1. Mouse colon tissue preparations and dissections. **A.** Schematic of the mouse gastrointestinal tract. The large intestine from the ileocecal junction to the rectum was isolated for Ussing Flux chamber, immunoblotting and immunohistochemical studies. **B.** Preparation of the colon for microdissection. The most distal 0.5 cm of the colon was discarded and the remaining tissue was divided into 4 sections of equal length. **C.** Microdissection of the neuronal layers of the colon. For Ussing chamber studies, the submucosal plexus with the mucosa (SPM) attached was used. For immunoblotting and immunohistochemical studies, preparations of the longitudinal muscle and myenteric plexus (LMMP) and the isolated submucosal plexus without the mucosa was used (Image C was derived from schoarpedia.com).
Figure 2.2. Morphine effects on fecal boli accumulation. WT and βarr2-KO mice were treated with saline (s.c.) or morphine (5, 10 or 20 mg/kg, s.c.) and fecal boli were collected and weighed from each mouse every hour for 6 hours. A. Both genotypes produce equivalent amounts of fecal boli in response to saline (two-way ANOVA for genotype: F(1,96)=0.9, p=0.3462, for dose: F(5,96)=3.04, p=0.0136; n=9). B. Morphine (10 mg/kg, s.c.) significantly suppresses fecal boli production in WT but not βarr2-KO mice (two-way ANOVA for genotype: F (1,120)=36.39, p<0.001, for dose: F (5,120)=6.86, p<0.001; WT vs KO, **p<0.01, Bonferroni post-hoc analysis; n=11). C. Morphine dose-dependently suppresses total fecal boli accumulation over the 6 hour test period in WT mice, while fecal boli accumulation from βarr2-KO mice is similar to control levels at every dose tested (two-way ANOVA for genotype: F(1,120)=36.39, p<0.001, for dose: F(3,58)=2.81, p=0.0472; WT vs KO, ***p<0.001, Bonferroni post-hoc analysis; n=6-11). D. There are no differences in food consumption behaviors between the two genotypes (p=0.5871; Student’s t test, n=3). (Dr. Laura Bohn collected a portion of this data).
Figure 2.3. Morphine-induced inhibition of small intestinal transit. Small intestinal transit was measured in fasted WT and βarr2-KO mice 30 minutes after receiving an orally administered charcoal meal. The meal was given 20 minutes after saline (s.c.) or morphine (1, 3, 10 mg/kg, s.c.) treatment. Data represent the mean ± S.E.M. Saline produces similar % transit values in both WT and βarr2-KO mice (p=0.5690, Student’s t test). While morphine dose-dependently reduces the % transit of the charcoal meal, there are no significant differences between the two genotypes at any dose tested (two-way ANOVA for genotype: F(1,28)=0.2263, p=0.638; for dose: F(3,28)=124.21, p<0.0001; n=4-6).
Figure 2.4. Effect of morphine and loperamide on colonic motility. Fasted WT and βarr2-KO mice were treated with either saline (s.c.) or morphine (1, 3, 10, 20 mg/kg, s.c.), vehicle 20% (2-hydroxypropyl)-β-cyclodextrin (s.c.) or loperamide (0.3, 0.6, and 1 mg/kg, s.c.) and the time required for a bead inserted 2 cm into the distal colon 20 minutes after drug treatment was measured. Data represent the mean ± S.E.M. A. Morphine dose-dependently inhibits colon transit in both genotypes; however, the βarr2-KO mice are less adversely affected compared to their WT littermates (two-way ANOVA for genotype: F(1,65)=11.98, p=0.001; for dose: F(4,65)=178.96, p<0.0001; WT vs KO, *p<0.05, ***p<0.0001, Bonferroni post-hoc analysis; n=4-11). Saline produces similar bead expulsion times in WT and βarr2-KO mice (p=0.4849, Student’s t test; n=4). B. Loperamide inhibits colonic bead expulsion time in WT mice in a dose-dependent manner; however, loperamide does not inhibit bead expulsion times in the βarr2-KO mice at any of the doses tested (two-way ANOVA for genotype: F(1,45)=12.12, p=0.0011; for dose: F(3,45)=4.58, p=0.007; WT vs KO, **p<0.01, Bonferroni post-hoc analysis; n=5-8). There were no differences in colonic bead expulsion times in response to vehicle between the two genotypes (p=0.2694, Student’s t test; n=6-7).
Figure 2.5. Effect of centrally administered morphine and loperamide on colonic motility. Fasted WT and βarr2-KO mice were treated with either vehicle (5 μL, i.c.v), morphine (1.5, 10 nmol, i.c.v.) or loperamide (0.1 nmol, i.c.v.). The time required for expulsion of a bead inserted 2 cm into the distal colon 20 minutes after drug treatment was measured. Data represent the mean ± S.E.M. A. Both vehicle and the single dose of loperamide tested inhibit bead expulsion times to an equal extent in WT and βarr2-KO mice (vehicle: WT vs KO: p=0.7254; Student’s t test; n=3; loperamide: WT vs KO: p=0.7933, Student’s t test; n=5-6). B. There are no significant differences in colonic bead expulsion times in response to centrally administered vehicle or morphine between the two genotypes (vehicle: WT vs KO: p=0.6709; Student’s t test; n=5; morphine two-way ANOVA for genotype: F_{(1,25)}=0.07, p=0.7897, for dose: F_{(2,25)}=30.00, p<0.0001; n=5-6).
Figure 2.6. Effect of methadone and fentanyl on small intestinal transit. Small intestinal transit was measured in fasted WT and βarr2-KO mice 30 minutes after receiving an orally administered charcoal meal. The meal was given 5 minutes after saline (s.c.) or methadone (1, 3 mg/kg, s.c.) treatment. Data represent the mean ± S.E.M. There were no significant differences in % transit between the two genotypes in response to (A) methadone (two-way ANOVA for genotype: F_{1,12}=0.12, p=0.7347; for dose: F_{1,12}=1.64, p=0.2245; n=4) or (B) fentanyl (two-way ANOVA for genotype: F_{1,41}=3.06, p=0.0875; for dose: F_{1,41}=22.49, p<0.0001; n=3-7) treatment.
Figure 2.7. Effect of methadone and fentanyl on colonic motility. Fasted WT and βarr2-KO mice were treated with either saline (s.c.), methadone (1, 10 mg/kg, s.c.) or fentanyl (0.1, 0.25 mg/kg, s.c.). The time required for expulsion of a bead inserted 2 cm into the distal colon 5 minutes after drug treatment was measured. Data represent the mean ± S.E.M. A. Methadone dose-dependently inhibits bead expulsion times to a similar extent in both genotypes (two-way ANOVA for genotype: F(1,23)=2.33, p=0.1405; for dose: F(2,23)=265.87, p<0.0001; n=4-6). B. There are no significant differences in colonic bead expulsion times in WT and βarr2-KO mice following treatment with fentanyl (two-way ANOVA for genotype: F(1,26)=0.06, p=0.8136; for dose: F(2,26)=60.00, p<0.0001; n=4-7).
Figure 2.8. Colonic pellet propulsion assay in WT mice. Colon propulsion rates were assessed ex vivo in WT mice by measuring the distance traveled by a synthetic fecal boli inserted 1.5 cm from the ilealcecal junction over time in the absence or presence of drug. Data represent the mean ± S.E.M. A. Still shots were taken from video recording with time stamp annotation. The leading edge of the pellet is indicated by the line in the photograph. B. Treatment with morphine (1 μM) for 10 minutes significantly reduces the distance traveled by the bead compared to basal conditions (*p<0.05, **p<0.01; Student's t test, n=3). Application of naloxone (1 μM), while not significant, shows a trend towards reversing the effect of morphine (p>0.05; Students t test; n=3). C. The propulsion rate of the bead is significantly reduced over a 40 second period in response to morphine compared to basal conditions (**p<0.01; Student’s t test, n=3). Again, there was a trend for naloxone reversal of morphine’s effect (p>0.05; Students t test; n=3). MOR=morphine; NAL= naloxone.
Figure 2.9. Basal short circuit (Isc) and conductance values in WT mice. The colon was isolated, divided into four sections of equal length (section 1 is the most proximal end and section 4 is the most distal end), and dissected SPM layers were mounted on Ussing chambers. Data represent mean ± S.E.M. A. Basal Isc was measured 30 to 40 minutes after tissues were mounted. The basal Isc decreased in a region dependent manner, wherein proximal colon tissues displayed higher basal Isc values compared to more distal tissues (n=2-9). B. The conductance of each tissue was determined by measuring the voltage and Isc when the tissue was voltage clamped at 8.0 mV and a 0 mV. Basal tissue conductance was lower in the distal colon compared to the proximal colon (n=2-9).
Figure 2.10. Morphine-mediated inhibition of DMPP-stimulated electrogenic secretion in the colon of WT mice. **A.** Maximum change in Isc evoked by application of DMPP (60 μM) to the serosal bath. DMPP increases the Isc in all four colon sections (n=1-5). **B.** Representative tracings depicting the Isc response to application following a 10 minute pretreatment with vehicle of morphine (10 μM) in section 2 preparations. **C.** Comparison of Isc responses of section 2 tissues treated with different combinations of vehicle, morphine (10 μM), and DMPP (60 μM) for 10 minutes. Morphine significantly inhibits DMPP-evoked increases in Isc in the proximal region of the colon (VEH vs VEH + DMPP, **p=0.0002;** VEH + DMPP vs MOR + DMPP, †p=0.0497; n=4-5). (VEH= vehicle, MOR= morphine, CCH=carbachol)
Figure 2.11. Morphine-induced JNK2/3 activation in preparations from WT mouse colon. Submucosal plexus (SP) and longitudinal muscle/myenteric plexus (LMMP) organotypic culture tissue preparations from WT mice were treated with either vehicle or morphine (10 μM) for 30 minutes. Sections were washed and lysates of sequential sections of each plexus (labeled 1-4; 1 most proximal to 4 most distal) were prepared and analyzed by western blot. A. Densitometric analysis of two experiments (n=2 lysates, each lysate contained combined tissue from 2 mice) shows the mean ± S.E.M. of phospho-JNK2/3 (P-JNK) levels normalized to total JNK2/3 (T-JNK) levels. B. Sample immunoblots are shown.
Figure 2.12. Confocal microscopy of longitudinal muscle/myenteric plexus and submucosal plexus sections in WT mice (objective noted). A. Neuronal marker staining of Map2 staining in myenteric (40X) and HuC/D staining in submucosal (100X) neuronal ganglions. B. Preliminary studies to test phospho-JNK antibody. Organotypic cultures were serum starved for 30 minutes, then serum was added back for 10 minutes as indicated. Images were sequentially obtained (green then red) and composited. “Serum free” and “+ serum” were prepared and imaged in parallel, receiving the same staining reagents and imaged with same microscope/laser settings. Green: phospho-JNK (1:200); Red: NeuN (1:500) (neuronal nuclear marker). (Dr. Laura Bohn did the imaging for these studies).
CHAPTER 3

β-ARRESTIN2 DIFFERENTIALLY REGULATES ANTINOCICEPTIVE TOLERANCE
AND PHYSICAL DEPENDENCE IN AN AGONIST-DEPENDENT MANNER

3.1 Introduction
Long-term administration of opioids is associated with the development of drug tolerance and physical dependence, processes that have been linked to the development of opioid addiction (188). Tolerance is characterized by a reduced responsiveness to an opioid agonist, which often results in the need to increase drug doses to achieve a desired effect (189, 190). All opioids produce analgesic tolerance in the clinical setting, yet the degree of tolerance that develops varies among different opioid drugs (191). Physical dependence develops when chronic opioid exposure produces cellular and physiological changes that result in the appearance of a withdrawal syndrome following abrupt drug cessation or treatment with an opioid antagonist (188). While the overall expression of tolerance and physical dependence may involve complex neuronal adaptations and many different signaling components, considerable evidence suggests that regulation of μOR activity contributes to tolerance and physical dependence induced by opioids.

B-arrestins are key regulators of μOR signaling as they desensitize activated μORs by blocking G protein coupling and also promote receptor internalization by recruiting the
endocytotic machinery to the receptor (36, 37, 40, 78, 79). Several in vitro studies have demonstrated that there is a paradoxical relationship between μOR activation and β-arrestin-mediated desensitization in the presence of different opioid agonists. Morphine, does not promote robust receptor phosphorylation, β-arrestin2 recruitment or μOR internalization in cell culture while other highly efficacious μOR agonists such as etorphine, methadone and fentanyl do (112-117). Moreover, antisense oligo studies further indicate that β-arrestins play a role in the development of tolerance as intrathecal administration of β-arrestin2-specific antisense oligonucleotides can delay the onset of morphine antinociceptive tolerance in mice (192). Therefore, the ability of an agonist to effectively promote μOR/β-arrestin interactions may determine the degree of tolerance that develops following opioid treatment.

Alterations in μOR signaling pathways have long been correlated with opioid-induced physical dependence. Acutely, opioids inhibit the activity of adenylyl cyclases and the cAMP pathway (193). However, studies show that following persistent μOR activation there is a reduced ability of opioids to inhibit adenylyl cyclase activity in several brain regions presumably due to compensatory mechanisms that attempt to re-establish basal signaling and neuronal excitability (88, 121, 193-195). As a result, there is an increase in adenylyl cyclase activity and a subsequent upregulation of cAMP-dependent signaling, a response that has been proposed to play a major role in opioid tolerance and withdrawal (196). Therefore, regulation of μOR signaling could contribute to opioid tolerance and physical dependence.

Given that β-arrestins play a key role in μOR signaling, desensitization, and trafficking, we have studied the contribution of β-arrestin2 to the development of tolerance and
physical dependence using βarr2-KO mice. Following acute or chronic treatment with morphine, βarr2-KO mice do not develop antinociceptive tolerance in the hot-plate test, a paradigm that primarily assesses supraspinally mediated pain responsiveness to a thermal stimulus (88). However, βarr2-KO mice do develop tolerance to morphine, although to a lesser degree compared to their WT littermates in the tail-flick test, which assess spinally mediated pain responses (89). On the other hand, βarr2-KO mice develop physical dependence to an equal extent as their WT counterparts in response to chronic administration of a significantly high dose of morphine via a subcutaneously implanted 75 mg morphine pellet (88), which suggested that β-arrestin2 contributes to the development of antinociceptive tolerance to morphine, but not physical dependence.

Acute antinociceptive hot-plate studies with βarr2-KO mice have demonstrated that β-arrestin2 is required for morphine-induced antinociception, but not for methadone and fentanyl. Since agonist-specific differences in antinociception have been observed in the βarr2-KO mice, we investigated whether the loss of β-arrestin2 affects antinociceptive tolerance. Additionally, since prior studies assessing morphine dependence were only preformed with a high dose of treatment regimen, we lowered the doses of the drug and tested different agonists to see if differences in physical dependence could be discerned. To this end, mice were chronically infused with morphine, methadone, and fentanyl using subcutaneously implanted osmotic pumps, and tolerance was determined by measuring antinociceptive responses in the hot-plate test. Physical dependence was also assessed by measuring antagonist-precipitated withdrawal responses following chronic infusion with all three drugs. We find that the βarr2-KO mice develop very little antinociceptive tolerance compared to their WT counterparts with chronic morphine administration, whereas tolerance develops to the same degree in WT and βarr2-KO
mice following chronic treatment with methadone or fentanyl. In addition, the βarr2-KO mice develop less physical dependence following chronic infusions with lower doses of morphine and methadone, but not at the doses of fentanyl tested. These results indicate that μOR regulation by β-arrestin2 may differentially affect the development of antinociceptive tolerance and physical dependence in an agonist-dependent manner.

3.2 Materials and Methods

*Mice.* Male βarr2-KO mice and their WT littermate controls (3-8 months old and weighing between 22 and 37 grams) were generated from breeding mice heterozygous for β-arrestin2 as previously described (87). To increase mouse numbers, some studies employed a small number (less than 20%) of mice derived from homozygous breeding. The data obtained from these mice did not differ from those obtained in heterozygously bred animals and were combined with this population. Mice were housed by genotype in groups of five in Plexiglas chambers in a temperature-controlled room and maintained on a 12 hour reversed light/dark cycle. All behavioral studies were conducted during the light phase of the animal’s circadian cycle. Mice had free access to food and water before any experiments, were tested in parallel, and used only once for each experimental assay. All studies were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and with approved animal protocols by The Ohio State University Animal Care and Use Committee.

*Drugs and Solutions.* Morphine sulfate pentahydrate and (±)-methadone hydrochloride was either purchased from Sigma-Aldrich (St Louis, MO, USA) or obtained from the National Institute on Drug Abuse Drug Supply Program (Rockville, MD, USA). Fentanyl
citrate was obtained from Sigma and naloxone hydrochloride was obtained from Tocris Bioscience (Ellisville, MO, USA). For subcutaneous (s.c.) and intraperitoneal (i.p.) injections, all drugs were dissolved in 0.9% physiological saline. Subcutaneous injections were given at a volume of 10 μL/g body weight. When administered using osmotic pumps, drugs were dissolved in sterile distilled water. All drugs were freshly prepared prior to use.

**Antinociceptive Testing.** Antinociception was assessed using a hot-plate analgesia meter (Columbus Instruments, Columbus, OH, USA) (87, 88). Mice were placed on a uniformly heated surface maintained at 54°C and antinociceptive responses were evaluated by measuring the latency for each mouse to either flick or lick its fore- or hindpaws. Basal latencies were measured prior to any drug treatment and a 30 second cutoff latency time was used to prevent tissue damage. Nociceptive latencies were measured at the time of peak effect for each drug (30 min after morphine and methadone injection or 10 min following fentanyl administration). Antinociception was reported as the percentage of maximum possible effect (% MPE) and calculated using the following formula: % MPE = 100% x [(drug response latency – basal latency)/ (cutoff time – basal latency)].

**Tolerance Studies.** Antinociceptive tolerance was induced using osmotic pumps, which continuously infused morphine (48 mg/kg/day, s.c.), methadone (96 mg/kg/day, s.c.) or fentanyl (3.2 mg/kg/day, s.c.) over a 5 or 7 day period. Morphine and methadone were delivered at a rate of 1 μl/hr using Alzet mini-osmotic pumps (Model 2001, Durect Corporation, Cupertino, CA, USA), while fentanyl was supplied at a rate of 0.5 μl/hr using Alzet micro-osmotic pumps (Model 1007D, Durect Corporation, Cupertino, CA,
USA). A single pump was implanted just beneath the skin on the back of each mouse under light isoflurane anesthesia. Osmotic pumps were selected for continuous drug administration because they allow for direct comparison among the agonists tested at a number of doses while maintaining a common route of administration. Antinociceptive tolerance was assessed using two different approaches. In one set of studies, antinociception was measured at several time points following osmotic pump implantation over a 5 day period. In another set of experiments, dose response curves were established using a cumulative dosing scheme (Table 3.1) 2 hours before and 7 days after pump implantation.

*Antagonist-Precipitated Withdrawal.* Following 7 days of continuous morphine (12, 24, 48 mg/kg/day, s.c.), methadone (48, 72 mg/kg/day, s.c.), or fentanyl (0.8, 1.6, 3.2 mg/kg/day, s.c.) infusion using Alzet osmotic pumps, mice were administered naloxone (0.5 mg/kg, i.p.). Mice were then placed in Plexiglas cylinders (14.5 cm x 40.5 cm) lined with filter paper and were observed for the manifestation of several withdrawal responses over a 30 minute period (88). The total number of jumps, wet dog shakes, and paw tremors were counted in 5 minute intervals. The occurrence of diarrhea and mastication were also measured at 5 minute intervals and scored: a score of 0 was assigned if the behavior was absent and a score of 1 was given if the behavior was present. The percent occurrence of these two signs was calculated by dividing the number of observed occurrences by 6 (the total number of occurrences possible) and then multiplying by 100%. Weight loss was also determined by subtracting measured body weight after withdrawal from body weight prior to precipitating withdrawal. A global withdrawal score summarizing the results from the withdrawal signs collectively was calculated by multiplying withdrawal values for each mouse by a constant to make each sign of equal weight and then adding each average value as previously described (197).
Signs were weighted as follows: jumping x 0.8; wet dog shakes x 1; paw tremors x 0.35; diarrhea x 1.5; mastication x 1.5.

Statistical Analysis. Results for each experiment are expressed as mean ± S.E.M. ED50 values and 95% confidence intervals (CI) were calculated for cumulative dose-response curves using nonlinear regression. Studies evaluating time course or dose-response effects between genotypes were analyzed using a standard two-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis when appropriate. In some cases, time course effects for each genotype were analyzed using a one-way ANOVA, followed by Bonferroni post-hoc analysis when appropriate. When two groups were compared for a single response, a Student’s t test was used. All statistics were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3.3. Results

Opioid-induced tolerance to morphine, methadone, and fentanyl following continuous infusion for 5 days was assessed by measuring antinociceptive responses in βarr2-KO mice and their WT littermate controls using the 54°C hot-plate test. During chronic morphine (48 mg/kg/day, s.c.) infusion, the βarr2-KO mice displayed significantly greater antinociception than their WT controls at every time point evaluated (Figure 3.1A). Furthermore, while the WT mice exhibited a significant reduction in response over the same period, the βarr2-KO did not show a significant reduction in response across days. In contrast, chronic infusion with methadone (96 mg/kg/day, s.c.) produced an equivalent amount of antinociception in the WT and βarr2-KO mice at every time point tested, and antinociceptive responsiveness significantly diminished in both genotypes at the same rate and to an equal extent over the 5 day treatment period (Figure 3.1B). Moreover,
WT and βarr2-KO mice showed similar degrees of antinociception in response to chronic fentanyl (3.2 mg/kg/day, s.c.) infusion at every time point measured, and their responses decreased at a similar rate and to the same extent, with responses returning to baseline 3 days after pump implantation (Figure 3.1C).

To further characterize differences in agonist-induced antinociceptive tolerance in the βarr2-KO mice, we examined the potency of each drug in a dose-response scheme wherein responsiveness to drug treatment on day 1 before pump implantation was compared to that on day 7. As illustrated in Figure 3.2A, morphine produced greater antinociception on day 1 in the βarr2-KO mice compared to WT controls. Following 7 days of continuous morphine (48 mg/kg/day, s.c.) infusion, the morphine dose-response curves shifted significantly to the right in both groups of mice. However, based on calculated ED50 values (Table 3.2), the βarr2-KO mice showed a smaller shift (1.3-fold) in their antinociceptive responses as compared to WT mice (2.9-fold shift). In contrast to morphine, methadone produced an equivalent degree of antinociception in WT and βarr2-KO on day 1 at every dose tested (Figure 3.2B). Furthermore, following chronic methadone (96 mg/kg/day, s.c.) infusion, the methadone dose-response curves for both genotypes significantly shifted to the right to the same degree, wherein WT and βarr2-KO mice showed a significant 2.2-fold shift and 2.1-fold shift in their ED50 values, respectively (Table 3.2; Figure 3.2B). Similar to methadone, there were no significant differences in antinociceptive responses between the WT and βarr2-KO mice on day 1 and based on calculated ED50 values, both genotypes exhibited a significant rightward shift in their ED50 values on day 7 in response to chronic fentanyl (3.2 mg/kg/day, s.c.) administration (2.4-fold shift for WT mice and 2.1-fold for the KO mice) (Table 3.2; Figure 3.3C).
The degree of opioid-induced physical dependence produced by chronic morphine, methadone, and fentanyl was determined in the WT and βarr2-KO mice by measuring antagonist-precipitated withdrawal responses 7 days after implantation of osmotic pumps. As shown in Figure 3.3A, naloxone (0.5 mg/kg, i.p.) precipitated withdrawal responses in both genotypes following chronic morphine (12, 24 or 48 mg/kg/day, s.c.) treatment. However, the βarr2-KO mice displayed a significant attenuation in the number of withdrawal jumps and paw tremors compared to WT controls, while no differences in wet dog shakes, diarrhea, mastication, and weight loss were observed between the genotypes. Furthermore, the calculated global withdrawal score, which accounts for withdrawal behaviors (except for weight loss) collectively, was significantly lower in the βarr2-KO mice (Figure 3.3B). The WT and βarr2-KO mice also display naloxone-precipitated (0.5 mg/kg, i.p.) withdrawal behaviors in response to chronic methadone (72 or 96 mg/kg/day, s.c.) infusion. Two-way ANOVA analysis revealed that like morphine, βarr2-KO mice show a significantly lower number of jumps and paw tremors, but a greater number of wet dog shakes, compared to their WT littermates. However, there were no discernable differences in diarrhea, mastication, or weight loss between the genotypes (Figure 3.4A). Furthermore, the calculated global withdrawal score was also significantly less in the chronic methadone treated βarr2-KO mice (Figure 3.4B). Unlike morphine and methadone, both WT and βarr2-KO mice displayed similar naloxone-induced (0.5 mg/kg, i.p.) jumps, wet dog shakes, paw tremors, diarrhea, mastication, and weight loss following chronic fentanyl (0.8, 1.6 or 3.2 mg/kg/day, s.c.) infusion at each of the doses tested (Figure 3.5A). Consistent with this, there were also no differences in the calculated global withdrawal score between the genotypes (Figure 3.5B).
3.4 Discussion

The current study examined the contribution of β-arrestin2 to antinociceptive tolerance and physical dependence in response to distinct μOR agonists by assessing antinociception and withdrawal responses in mice chronically infused with morphine, methadone, or fentanyl. We find that βarr2-KO mice develop very little antinociceptive tolerance in response to chronic morphine treatment as WT mice, whereas chronic methadone and fentanyl produced tolerance in both genotypes equally. In addition, when compared to the WT controls, βarr2-KO mice develop less physical dependence to morphine and methadone, but not fentanyl. These results support our hypothesis that β-arrestin2 regulation of the μOR can determine the expression of analgesic tolerance and physical dependence in an agonist-dependent manner.

We have previously reported that βarr2-KO mice fail to develop antinociceptive tolerance in the hot-plate test to both repeated injections of morphine and to subcutaneously implanted 75 mg morphine pellet (88). Similar to these results, the βarr2-KO mice maintain the same degree of antinociceptive responsiveness when morphine is chronically infused; whereas the WT mice show significantly diminished responsiveness to the drug by day 5 (Figure 3.1). We also employed a cumulative dosing regimen for each drug in which the doses of drug tested on day 7 produced equiefficacious responses in both genotypes to determine how the potency of morphine changes following chronic morphine infusion. However, since morphine is more potent in βarr2-KO mice as compared to WT mice calculated ED50 values from day 1 and day 7 were then compared to determine if a significant shift in potency occurred following chronic drug infusion, and hence whether tolerance had developed. Using this approach, we find that both WT and βarr2-KO mice develop antinociceptive tolerance to morphine, but
not to the same degree (Figure 3.2). Based on comparison of calculated ED50 values, morphine is 2.9-fold less potent in the WT mice and only 1.3-fold less potent in the \( \beta \)arr2-KO mice after continuous treatment, demonstrating that \( \beta \)arr2-KO mice are much less susceptible to the development of morphine tolerance. Although the same cumulative-dosing schemes for morphine on day 1 and day 7 were used in previous study (Bohn et al., 2000), the results obtained in this study may have differed from the previous one because the mice were exposed to a higher concentration of morphine continuously, whereas in the earlier study used repeated daily injections. However, when assessed collectively, our tolerance studies are consistent with our previous studies indicating that the loss of \( \beta \)-arrestin2 hinders the development of antinociceptive tolerance to morphine.

We have previously reported that agonist-induced antinociception is differentially affected by the loss of \( \beta \)-arrestin2. Similarly, while \( \beta \)arr2-KO mice develop a small degree of antinociceptive tolerance to morphine compared to WT controls, chronic methadone and fentanyl infusion produces the same degree of tolerance in both genotypes (Figure 3.1). Given that both methadone and fentanyl can promote \( \beta \)-arrestin1 and \( \beta \)-arrestin2 interactions with the \( \mu \)OR in cell culture, it is likely that in the absence of \( \beta \)-arrestin2, \( \beta \)-arrestin1 may compensate to induce receptor desensitization and antinociceptive tolerance. This hypothesis could be addressed by assessing antinociceptive tolerance in mice lacking both \( \beta \)-arrestin1 and \( \beta \)-arrestin2, but genetic ablation of both \( \beta \)arrestins is embryonic lethal (82). However, if the \( \beta \)arr1/\( \beta \)arr2 double knockout mice were viable, we would expect that the KO mice would not develop antinociceptive tolerance to methadone or fentanyl. Such studies may be possible to
address as genetic techniques, such as siRNA silencing of β-arrestin1 in adult βarr2-KO mice becomes more feasible.

Initial studies in the βarr2-KO mice in which a large quantity of morphine was administered over 3 days using a 75 mg subcutaneously implanted slow-release morphine pellet suggested that β-arrestin2 does not contribute to the development of morphine-induced physical dependence (88). However, our results reveal that with lower doses of morphine, multiple withdrawal signs are reduced in the βarr2-KO mice (Figure 3.3). Both naloxone-precipitated jumps and paw tremors are significantly attenuated in the βarr2-KO mice compared to WT controls. Furthermore, the calculated global score, which accounts for the withdrawal responses collectively, was also lower in the βarr2-KO mice. Of all the withdrawal responses measured, jumping is considered the most sensitive and reliable indicator of the degree of dependence that develops in response to chronic opioid treatment in mice (198) and therefore also contributes the most weight to the global withdrawal score. Although not all responses are altered in the βarr2-KO mice, the overall reduction in withdrawal jumping suggests that β-arrestin2 could contribute to the development of morphine dependence or alternatively, it could affect other GPCRs other than the μOR that are involved in the expression of this particular withdrawal sign.

Consistent with the previous morphine dependence study, we find that at the highest dose of morphine tested (48 mg/kg/day, s.c.), there are no differences in any of the withdrawal signs measured. A study by Feng et al. (199) measured the concentration of free morphine in mouse serum in mice implanted with either a 75 mg morphine pellet or an osmotic pump that delivered a 25 mg/kg/day dose of morphine and showed that
morphine concentrations are 27-fold higher in the pellet implanted mice. At high enough doses of morphine, other regulatory proteins may be recruited or other signaling pathways or neurotransmitter systems could be activated which may overshadow any differences in withdrawal responses revealed at the lower doses of morphine. For example, increased dopamine levels have been observed following naloxone-precipitated withdrawal in the striatum in rats made dependent on morphine (200). Moreover, activation of dopamine receptors during withdrawal has been shown to enhance locomotor activity (201). Therefore, increases in dopamine release during opioid withdrawal may be significant enough to saturate dopamine receptors in the striatum and mask any differences between the WT and βarr2-KO mice at high doses of morphine. We have also considered that a high dose of morphine could induce β-arrestin1 interactions with the μOR, which could then compensate for β-arrestin2 and produce equivalents amount of jumping and other withdrawal signs in both genotypes.

Although the number of naloxone-precipitated jumps and paw tremors were less in the βarr2-KO mice following chronic morphine and methadone treatment, other withdrawal responses such as diarrhea, mastication, and weight loss were similar between WT and βarr2-KO mice. While it is not clear why these other withdrawal responses were not affected by the loss of β-arrestin2, it is possible that the doses of morphine tested were too high to reveal differences in these responses or that these responses are mediated to a greater extent by other neurotransmitter systems not affected by the loss of β-arrestin2. Furthermore, tissue specific regulation of the μOR may affect these other behaviors. For example, the effects of opioids on gastrointestinal function are mediated by both peripherally and centrally expressed μORs. We have previously observed that with lower doses of systemically injected morphine, the βarr2-KO mice exhibit less
constipation compared to their WT littermates. However, when administered centrally, differences in gastrointestinal function are no longer observed between the genotypes, suggesting that regulation of central μORs by β-arrestin2 involved in modulating gut function is different from those expressed in the periphery. Therefore, in response to continuous drug infusion at the doses tested, the activation of central μORs may be significant and therefore, no differences would be observed in the occurrence of naloxone-precipitated diarrhea or weight loss (which includes loss of urine and fecal matter).

Interestingly, βarr2-KO mice chronically treated with methadone also exhibited significantly attenuated naloxone-induced withdrawal jumping and paw tremors (Figure 3.4). Though they did display a greater number of wet dog shakes, their overall global withdrawal score was significantly lower than the WT mice. This result was somewhat surprising given that the ablation of β-arrestin2 has no affect on methadone-induced antinociception, antinociceptive tolerance, or constipation. While naloxone-induced withdrawal jumps and paw tremors are reduced with chronic morphine and methadone infusion in the KO mice, no differences in these responses were observed between the genotypes following chronic fentanyl exposure. At the two highest doses of fentanyl (1.6 and 3.2 mg/kg/day, s.c.), both genotypes exhibited a similar number of withdrawal jumps, suggesting that a ceiling effect may have been reached while very few jumps were elicited in the mice at the 0.8 mg/kg/day dose which may have been too low in the WT mice to reveal differences. Therefore, it is possible that differences may become apparent for this response when intermediate doses between 0.8 and 1.6 mg/kg/day are evaluated. In addition, fentanyl-bound μORs may be regulated differently than morphine
or methadone μORs in brain regions associated with the development of physical
dependence and/or the expression of antagonist-precipitated withdrawal responses.

Therefore, similar to morphine-induced constipation and respiratory suppression, we find
that withdrawal responses are not enhanced, but rather are significantly reduced in the
absence of β-arrestin2. A simplistic interpretation would be that these decreased
responses in the βarr2-KO mice suggest that β-arrestin2 may be playing a pro-signaling
role in μOR expressing neurons that are involved the development of opioid physical
dependence and/or antagonist-precipitated withdrawal behaviors. Interestingly,
naloxone-precipitated withdrawal signs are enhanced in spinophilin-KO mice that have
been chronically treated with morphine (202). Spinophilin is an intracellular protein
which has been shown to bind to GPCRs in a manner that counteract interactions
between βarrestin and GPCRs in vitro and in vivo (106), suggesting there may be a
dynamic interaction between spinophilin and β-arrestin2 during chronic morphine
treatment. Therefore, in the absence of spinophilin, perhaps β-arrestin2-mediated
signaling is enhanced and physical dependence develops to a greater degree.
Furthermore, mice lacking RGS9, a protein that negatively regulates μOR-mediated G
protein signaling (203, 204), also display enhanced morphine-induced physical
dependence compared to their WT littermates (205). Collectively, these results suggest
that μOR regulation is important in the development of opioid physical dependence.
However, the precise mechanism by which these proteins act to modulate dependence
remains unclear.

In conclusion, we find that β-arrestin2 contributes to the development of morphine, but
not methadone or fentanyl-induced antinociceptive tolerance. Furthermore, the loss of
β-arrestin2 affects the degree of physical dependence that develops in an agonist dependent manner. While the results of our antinociceptive tolerance studies further support that β-arrestin2 behaves as a negative regulator of morphine-induced tolerance, the attenuation in naloxone-induced withdrawal in the βarr2-KO mice suggests that β-arrestin2 may promote opioid-induced physical dependence and/or the expression of antagonist-precipitated withdrawal behaviors. Furthermore, the environment in which the μOR is expressed may influence how the receptor is regulated as μORs involved in antinociceptive tolerance appear to be regulated differently than those involved in physical dependence and that the underlying molecular mechanisms that lead to opioid-induced antinociceptive tolerance and physical dependence are distinct.
Table 3.1. Cumulative dosing regimens for morphine, methadone, and fentanyl used to assess antinociceptive tolerance on days 1 and 7 in WT and βarr2-KO mice.

<table>
<thead>
<tr>
<th>DRUG</th>
<th>DAY 1 (mg/kg, s.c.)</th>
<th>DAY 7 (mg/kg, s.c.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>WT: 1, 5, 10, 20</td>
<td>WT: 10, 20, 40, 80</td>
</tr>
<tr>
<td></td>
<td>KO: 1, 5, 10, 20</td>
<td>KO: 1, 5, 10, 20</td>
</tr>
<tr>
<td>Methadone</td>
<td>WT: 2.5, 5, 7.5</td>
<td>WT: 5, 7.5, 15</td>
</tr>
<tr>
<td></td>
<td>KO: 2.5, 5, 7.5</td>
<td>KO: 5, 7.5, 15</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>WT: 0.1, 0.25, 0.5</td>
<td>WT: 0.1, 0.35, 0.85, 1.6</td>
</tr>
<tr>
<td></td>
<td>KO: 0.1, 0.25, 0.5</td>
<td>KO: 0.1, 0.35, 0.85, 1.6</td>
</tr>
</tbody>
</table>
Figure 3.1. Morphine, methadone, and fentanyl-induced hot-plate antinociceptive responses in WT and βarr2-KO mice. Mice were continuously infused with drug over a 5 day period and paw withdrawal latencies were measured at various time points in response to an applied thermal (54°C) stimulus. Data represent the mean ± S.E.M. A. Chronic morphine (48 mg/kg/day, s.c.) produces greater responses in βarr2-KO mice at every time point tested (two-way ANOVA for genotype: F(1,24)=27.89, p<0.0001; for time: F(2,24)=4.97, p=0.0157; WT vs KO, *p<0.05, **p<0.01, Bonferroni post-hoc analysis; n=5). There are no differences in responses across days in the KO mice (ANOVA, p=0.2311; n=5) whereas WT mice show a significant reduction in responsiveness over time (ANOVA, p=0.033, Day 1 vs Day 5, †p<0.05, Bonferroni post-hoc analysis; n=5). B. Chronic methadone (96 mg/kg/day, s.c.) produces equivalent antinociceptive responses at each time point measured in WT and βarr2-KO mice (two-way ANOVA for genotype: F(1,49)=0.67, p=0.418, for time: F(3,49)=20.02, p<0.001; n=8), which decreases at the same rate over time (ANOVA for WT, p=0.0002; for KO p=0.0001, Bonferroni 8 hrs vs all other days, **p<0.01, ***p<0.0001) C. Fentanyl also induces similar antinociceptive responses in both genotypes at all time points (two-way ANOVA for genotype: F(1,32)=0.56, p=0.4597, for time: F(3,32)=40.56, p<0.001; n=5) and tolerance develops to the same degree (ANOVA for WT, p<0.0001; for KO p<0.0001, Bonferroni 4 hrs vs all other days, ***p<0.0001).
Figure 3.2. Morphine, methadone, and fentanyl-induced hot-plate antinociceptive dose-response curves in WT and βarr2-KO mice. Antinociceptive responses were determined using a cumulative dosing scheme before and after 7 days of chronic drug infusion. Data represent the mean ± S.E.M. ** A. On day 1, βarr2-KO mice display significantly greater antinociception than WT mice (two-way ANOVA for genotype: $F_{(1,56)}=24.96$, $p<0.0001$, for time: $F_{(3,56)}=140.41$, $p<0.001$; WT vs KO, **$p<0.01$, ***$p<0.001$; Bonferroni post-hoc analysis; n=8). Following 7 days of chronic morphine (48 mg/kg/day, s.c.) infusion, morphine is 2.9-fold less potent in the WT and 1.3-fold less potent in the βarr2-KO mice. ** B. Methadone produces similar responses in both genotypes on day 1 (two-way ANOVA for genotype: $F_{(1,39)}=0.80$, $p=0.3759$, for time: $F_{(2,39)}=82.51$, $p<0.0001$; n=5-8) and is 2.3-fold less potent in WT and 2.1-fold less potent in βarr2-KO mice following 7 days of chronic methadone (96 mg/kg/day, s.c.). ** C. There is no significant difference in response to fentanyl on day 1 between the WT and βarr2-KO mice (two-way ANOVA for genotype: $F_{(1,24)}=0.91$, $p=0.3486$, for time: $F_{(2,24)}=244.96$, $p<0.0001$; n=5) and fentanyl is 2.4-fold less potent in the WT mice and 2.1-fold less potent in βarr2-KO mice following 7 days of chronic fentanyl (3.2 mg/kg/day, s.c.) treatment. For comparison purposes, calculated ED values with confidence intervals are presented in Table 3.2.
Table 3.2. Summary of ED50 values (mg/kg) (± 95% confidence limits) and potency ratios in WT and βarr2-KO mice for morphine, methadone, and fentanyl in the 54°C hot-plate test.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Genotype</th>
<th>Day 1 ED50 (95% CI)</th>
<th>Day 7 ED50 (95% CI)</th>
<th>Potency Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine</td>
<td>WT</td>
<td>8.00 (6.96-9.20)</td>
<td>23.46 (20.44-26.93)</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>βarr2-KO</td>
<td>5.32 (4.65-6.07)</td>
<td>6.98 (6.12-7.96)</td>
<td>1.3</td>
</tr>
<tr>
<td>Methadone</td>
<td>WT</td>
<td>5.02 (4.48-5.62)</td>
<td>11.39 (6.85-18.95)</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>βarr2-KO</td>
<td>4.59 (3.95-5.33)</td>
<td>9.85 (7.918-12.24)</td>
<td>2.1</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>WT</td>
<td>0.18 (0.16-0.21)</td>
<td>0.43 (0.33-0.55)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>βarr2-KO</td>
<td>0.20 (0.18-0.23)</td>
<td>0.41 (0.32-0.53)</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Figure 3.3. Naloxone-precipitated withdrawal responses following chronic morphine infusion. Withdrawal was precipitated using naloxone (0.5 mg/kg, i.p.) after 7 days of chronic morphine (12, 24, 48 mg/kg/day, s.c.) infusion. Data are presented as the mean ± S.E.M. A. Naloxone elicits significantly less jumps (two-way ANOVA for genotype: $F_{(1,45)}=6.86$, $p=0.012$; for dose: $F_{(2,45)}=6.28$, $p=0.0039$, Bonferroni post-hoc analysis, **$p<0.01$; n=7-11) and paw tremors (two-way ANOVA for genotype: $F_{(1,45)}=6.86$, $p=0.012$; for dose: $F_{(2,45)}=6.28$, $p=0.0039$; n=7-11) in the βarr2-KO mice compared to WT mice. However, there is no difference in wet dog shakes, diarrhea, mastication, or weight loss (two-way ANOVA for genotype: $p>0.05$; n=7-11). B. The calculated global withdrawal score is significantly lower in the βarr2-KO mice (two-way ANOVA for genotype: $F_{(1,45)}=11.70$, $p=0.0013$; for dose: $F_{(2,45)}=12.80$, $p<0.0001$, Bonferroni post-hoc analysis, **$p<0.01$; n=7-11).
Figure 3.4. Naloxone-precipitated withdrawal responses following chronic methadone infusion. Withdrawal was precipitated using naloxone (0.5 mg/kg, i.p.) after 7 days of chronic methadone (48, 72 mg/kg/day, s.c.) infusion. Data are presented as the mean ± S.E.M. A. Naloxone elicits significantly less jumps (two-way ANOVA for genotype: \( F(1,27)=4.47, p=0.0438 \); for dose: \( F(1,27)=16.30, p=0.0004 \), Bonferroni post-hoc analysis, \(*p<0.05\); \( n=7-8 \)) and paw tremors (two-way ANOVA for genotype: \( F(1,27)=7.24, p=0.0121 \); for dose: \( F(1,27)=0.12, p=0.7312 \); Bonferroni post-hoc analysis, \(*p<0.05\); \( n=7-8 \)) in the βarr2-KO mice compared to WT mice. However, there is no difference in diarrhea, mastication, or weight loss (two-way ANOVA for genotype: \( p>0.05 \); \( n=7-8 \)). B. The calculated global withdrawal score is significantly lower in the βarr2-KO mice (two-way ANOVA for genotype: \( F(1,27)=7.66, p=0.0101 \); for dose: \( F(1,27)=9.57, p=0.0046 \), Bonferroni post-hoc analysis, \(*p<0.05\); \( n=7-8 \)).
Figure 3.5. Naloxone-precipitated withdrawal responses following chronic fentanyl infusion. Withdrawal was precipitated using naloxone (0.5 mg/kg, i.p.) after 7 days of chronic fentanyl (0.8, 1.6, 3.2 mg/kg/day, s.c.) infusion. Data are presented as the mean ± S.E.M. **A.** There are no significant differences in any of the withdrawal response measured between the two genotypes (two-way ANOVA for genotype: *p* >0.05; *n*=6-11). **B.** The calculated global withdrawal score is not significantly different between WT and βarr2-KO mice at any of the doses of fentanyl tested (two-way ANOVA for genotype: *p* >0.05; *n*=6-11).
CHAPTER 4

DIFFERENTIAL EFFECTS OF MORPHINE-INDUCED PHYSIOLOGICAL AND BEHAVIORAL RESPONSES IN MICE LACKING G PROTEIN-COUPLED RECEPTOR KINASES (GRKs)

4.1 Introduction

G protein-coupled receptor kinases (GRKs) are a family of intracellular proteins that act in concert with β-arrestins to desensitize GPCRs. There is a significant body of work which shows that GRKs can impact the overall responsiveness of GPCRs (41). There are seven distinct GRK subtypes, GRK1 through GRK7 which have different expression patterns throughout the body. GRK1 and GRK7 are expressed exclusively found in the visual system (47, 49), and therefore the majority of studies of GRK-mediated GPCR regulation have focused on the five other GRK, GRK2 through GRK6. Studies using cell culture and heterologous expression systems have shown that both GRK2 and GRK3 can phosphorylate and desensitize the μOR (34, 115, 116, 119, 206, 207). Alterations in GRK levels in response to opioids have also been observed in vivo. Rats chronically treated with the opioid agonist sufentanil display increased GRK2, GRK3 and GRK6 expression in cerebral cortex compared to controls (52), whereas decreased expression of GRK2 and GRK6 has been observed in postmortem brain of opiate addicts (208). Therefore, multiple GRKs may be involved in regulating the morphine-bound μOR.
Mouse models in which individual GRK genes are ablated have provided a useful tool for elucidating the contribution of specific GRKs to opioid drug responses in vivo. Mice lacking GRK3 develop significantly less antinociceptive tolerance in response to chronic fentanyl treatment compared to their WT littermates although their acute antinociceptive responses to fentanyl did not differ between genotypes (107). GRK2-KO mice are embryonically lethal and therefore the biological significance of GRK2 regulation of opioid effects in vivo has not been conclusively studied. However, the different GRKs may not necessarily perform interchangeable functions and there may be some specificity in regard to their individual ability to regulate certain GPCRs under specific conditions (41). Moreover, while GRK2 and GRK3 are cytosolic proteins, GRKs 4, 5, & 6 are palmitoylated or contain polybasic domains, allowing them to reside at the cellular membrane; therefore, their contributions to μOR regulation may fundamentally differ from GRK2 and GRK3. The current study was undertaken to investigate the specific role of GRK6 in regulating morphine-activated μOR across a wide range of biological responses. However, the contribution of GRK2, GRK3, GRK4, and GRK5 to morphine-induced constipation was also determined.

Relative to other GRKs, GRK6 is somewhat highly expressed in brain regions and gastrointestinal tissues associated with many of the physiological responses elicited by morphine (53, 85). Therefore, we determined whether GRK6 regulates morphine-mediated responses, including antinociception, analgesic tolerance, locomotor activity, physical dependence and constipation is altered in the absence of GRK6 using GRK6-KO mice. Morphine-induced constipation was also assessed in GRK2-HT, GRK3-KO, GRK4-KO, and GRK5-KO mice to determine if any of these GRKs also influence the effect of morphine on gastrointestinal function. We find that GRK6 can regulate the
morphine-activated μOR in cell culture by facilitating morphine-induced β-arrestin2 recruitment and receptor internalization. In vivo, however, only morphine-induced locomotor activity, sensitization, and constipation were altered in GRK6-KO mice. Furthermore, morphine-induced constipation was unaltered in the absence of GRK2, GRK3, GRK4 and GRK5, suggesting that GRK6 is the primary GRK involved in regulation of μORs involved in modulating gastrointestinal function. These findings suggest that while GRK6 can contribute to μOR regulation in vitro, its effects on morphine-induced responses in vivo are more complex and depend upon the immediate cellular environment and the physiology being assessed.

4.2 Materials and Methods

Animals. WT and GRK6-KO mice were generated from crossing heterozygous GRK6 animals as previously described (85). Genotyping was performed on DNA extracted from tissue punches taken from the ear of each mouse to prevent damage to the tail. Mice used in these studies were age-matched (3-8 months old), males weighing between 25 and 35 grams housed in groups of five in Plexiglas chambers in a temperature-controlled room and maintained on a 12 hour reversed light/dark cycle. Mice had free access to food and water before any experiments unless otherwise noted. Mice were examined during the light phase of their circadian cycle and used only once. All studies were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with approved animal protocols by The Ohio State University Animal Care and Use Committees.

Drugs and solutions. Morphine sulfate pentahydrate (Sigma-Aldrich, St. Louis, MO), naloxone hydrochloride (Tocris Bioscience, Ellisville, MO), and cocaine methiodide
(generously supplied by the National Institute on Drug Abuse Drug Program, Rockville, MD) were dissolved in 0.9% physiological saline and were given at a volume of 10 μL/g body weight for systemic injections. Morphine was injected subcutaneously (s.c.) or intraperitonially (i.p.) while naloxone and cocaine methiodide were administered i.p. For studies using osmotic pumps, morphine was dissolved in sterile distilled water. All drug solutions were freshly prepared prior to use.

**β-arrestin2-GFP translocation.** HEK-293 cells were transiently transfected with N-terminally haemagglutinin-tagged μOR (HA-μOR) cDNA (5 μg) and βarr2-GFP (green fluorescent protein) cDNA (2 μg) as previously described (119). Some cells were also transfected with 5 μg of GRK6A cDNA (65). Cells were incubated with anti-HA antibody conjugated to 594-Alexafluor (Molecular Probes, Carlsbad, CA) for 30 minutes in serum free media at 37°C prior to agonist treatment and live cell imaging. Images were collected by confocal microscopy (Olympus Fluoview 300) as previously described (119).

**Receptor Internalization.** HEK-293 cells were transiently transfected with μOR cDNA (2 μg) tagged on the C-terminus with yellow fluorescent protein (YFP). Some cells were also transfected with 5 μg of GRK6A cDNA. Cells were serum deprived for 30 minutes prior to live cell imaging as described above.

**Antinociceptive Testing.** Antinociception was evaluated by measuring response latencies to a thermal stimulus using a hot-plate test (56°C) or a warm-water tail-flick (54°C) assay as previously described (87, 88). Nociceptive latencies were assessed by measuring the time required for the mouse to either flick or lick its fore- or hindpaws (hot-
plate) or to the first sign of a rapid flick of the tail (tail-flick). For both assays, basal latencies were measured immediately prior to treatment and test latencies were measured at various times following morphine (5, 10, or 20 mg/kg, i.p.) injection. A cutoff latency of 30 seconds for the hot-plate and 15 seconds for the tail-flick test were used to avoid tissue damage. Antinociception was reported as the percentage of maximum possible effect (% MPE) and calculated using the following formula: % MPE = 100 x [(drug response latency – basal latency)/ (cutoff time –basal latency)].

_Tolerance Studies._ Antinociceptive tolerance was assessed by treating mice with morphine (5, 10, 20 mg/kg, i.p.) once daily for 5 days. Basal latencies were determined in the hot-plate and warm water tail-flick assays prior to morphine treatment on day 1, and then latencies were assessed every other day 30 minutes following morphine treatment at the time of peak drug effect.

_Locomotor Activity._ Locomotor activity measurements were made using the Versamax Animal Activity Monitoring System [20 x 20 cm²] (Accuscan Instruments, Columbus, OH) as previously described (90). Mice were individually placed into the activity monitoring boxes for 30 minutes to habituate the mice to the new environment, removed, injected with morphine (5, 10, 20 mg/kg, i.p.), and immediately placed back into the boxes for 120 minutes of monitoring. For morphine locomotor sensitization experiments, mice were chronically treated with morphine (10 mg/kg, i.p.) once daily for 6 days. On days 1 and 7, locomotor activity was assessed as described above. Data were collected using Versadat software (Accuscan Instruments, Columbus, OH, USA) and analyzed for the total number of beam breaks made by each animal in 5 minute increments.
Induction of Physical Dependence and Withdrawal. Mice were made physically dependent on morphine (12, 24 mg/kg/day, s.c.) using Alzet mini-osmotic pumps which continuously infused morphine at a rate of 1μL/hr for 7 days (Model 2001; Durect Corporation, Cupertino, CA, USA). A single pump filled with morphine was implanted subcutaneously on the back of each mouse under light isoflurane anesthesia. To assess the extent of dependence, mice were administered naloxone (0.05, 0.5 mg/kg, i.p), placed in Plexiglas cylinders (14.5 cm x 40.5 cm) lined with filter paper, and observed for the manifestation of different withdrawal signs over a 30 minute period (88). The number of jumps, wet dog shakes, and paw tremors elicited by each mouse were counted in 5 minute intervals. The occurrence of diarrhea and mastication were also measured at 5 minute intervals and scored: a score of 0 was assigned if the behavior was absent, and a score of 1 was given if the behavior was present. The percent occurrence of these two signs was calculated by dividing the number of observed occurrences by 6 (the total number of occurrences possible) and then multiplying by 100%. Weight loss was also determined by subtracting measured body weight after withdrawal from body weight prior to precipitating withdrawal. A global withdrawal score summarizing the results from the withdrawal signs collectively was calculated by multiplying withdrawal values for each mouse by a constant to make each sign of equal weight and then adding each average value. Signs were weighted as follows: jumps x 0.8; wet dog shakes x 1; paw tremors x 0.35; diarrhea score x 1.5; mastication score x 1.5 (weight loss was not included in the global score (197).

Fecal Boli Accumulation Assay. Whole gut motility was assessed by measuring the amount of fecal boli produced over a 6 hour period. Mice were given an injection of saline (s.c.), morphine (10 mg/kg, s.c.), methadone (10 mg/kg, s.c.), or fentanyl (10 mg/kg, s.c.) and individually placed into small Plexiglas boxes (15.2 cm x 16.5 cm x 12.0
cm) lined with filter paper. Fecal boli were collected and weighed using an analytical balance every hour for 6 hours.

Charcoal Meal Assay. Small intestinal transit was measured using an orally administered charcoal meal as previously described (11). Forty-eight hours prior to the experiment, mice were habituated to a modified cage containing a mesh wire insert in the presence of food and water and then fasted for 24 hours with free access to water. For the experiment, mice were given an injection of saline (s.c.) or morphine (1, 5 mg/kg, s.c.) 20 minutes prior to receiving an administered a charcoal meal consisting of 5% charcoal (2-12 μm powder, Sigma-Aldrich, St. Louis, MO, USA), 10% gum arabic (Acros Organics, Morris Plains, NJ, USA) in sterile water by oral gavage at a volume of 10 μL/g body weight. Thirty minutes later, mice were sacrificed by cervical dislocation and the small intestine from the pyloric sphincter to the ileal cecal junction was isolated and the mysentery removed. The distance traveled by the leading edge of the charcoal meal was measured relative to the total length of the small intestine and the percent of gastrointestinal transit for each treatment group was calculated as follows: % gastrointestinal transit= [(charcoal bolus distance)/ (small intestine length)] * 100%.

Colonic Bead Expulsion Assay. Large intestine transit was measured using a bead expulsion assay as previously described (165). Mice were first habituated in the same manner as the small intestinal transit assay. For the test, mice were given an injection of saline (s.c.) or morphine (1, 5, or 10 mg/kg, s.c.) and 5 minutes later, a 3 mm glass bead (Thermo Fisher Scientific, Pittsburg, PA, USA) was inserted 2 cm into the distal rectum using 2 mm round, flexible, plastic tubing. Mice were placed into small Plexiglas boxes (13.9 cm x 12.7 cm x 15.2 cm) lined with filter paper for observation and the time to bead
expulsion was measured for each animal. Mice that did not expel their bead within a reasonable time period (4 hours) or produced feces before expelling the bead were eliminated from data analysis.

Statistical Analysis. Results for each experiment are expressed as mean ± S.E.M. Studies evaluating time course or dose-response effects between genotypes were analyzed using a standard two-way analysis of variance (ANOVA) test, followed by Bonferroni post-hoc analysis where appropriate. When two groups were compared for a single response, a Student’s $t$ test was used. All statistics were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

4.3 Results
To determine if GRK6 is capable of regulating the $\mu$OR in vitro, we used confocal microscopy to assess both the translocation of $\beta$arr2-GFP to the plasma membrane of HEK-293 cells as well as internalization of $\mu$OR-YFP, with or without overexpression of GRK6A which is the variant predominantly expressed in brain (209). In the absence of GRK6 overexpression, morphine treatment induced weak $\beta$arrestin2 translocation (Figure 4.1A) and receptor internalization (Figure 4.1B) in cells expressing the $\mu$OR. Overexpression of GRK6, however, was capable of augmenting morphine-induced $\beta$arr2-GFP translocation (Figure 4.1A) and $\mu$OR-YFP internalization (Figure 4.1B). Importantly, cells not expressing the $\mu$OR did not display $\beta$arrestin2 recruitment with or without morphine treatment (data not shown).

The contribution of GRK6 to morphine-mediated thermal antinociceptive responses was assessed in GRK6-KO mice and their WT littermates using hot-plate (56°C) and warm
water tail-flick (54°C) assays. Basal response latencies did not differ between the WT and GRK6-KO mice in either test (Figure 4.2A and 4.2B, insets). At a moderate dose of morphine (10 mg/kg, i.p.) both genotypes displayed similar time-dependent antinociceptive responses in the hot-plate (Figure 4.2A) and the tail-flick (Figure 4.2B) test. Furthermore, no differences were observed between WT and GRK6-KO mice in response to several doses of morphine (5, 10, and 20 mg/kg, i.p.) measured at 30 minutes post drug injection in the hot-plate (Figure 4.2C) and tail-flick (Figure 4.2D). Moreover, morphine-induced tolerance developed to an equal extent and at a similar rate in the WT and GRK6-KO mice in response several doses of morphine in the hot-plate (Figure 4.2E) and the tail-flick test (Figure 4.2F).

Morphine exerts a stimulatory effect on locomotor activity in mice. Therefore, GRK6-KO mice and their WT littermates were monitored for morphine-induced locomotor activity. Both WT and GRK6-KO mice became less active to a similar extent during the 30 minute habituation period. Following acute treatment with morphine (10 mg/kg, i.p.), both genotypes showed a marked increase in locomotor activation; however, the GRK6-KO mice showed a significantly greater increase in locomotor activity as measured by the number of beam breaks compared to WT controls (Figure 4.3A). Moreover, the GRK6-KO mice displayed enhanced locomotor activity over the total 120 minute test period at several doses of morphine (Figure 4.3B).

Chronic morphine treatment in mice can also lead to the development of sensitization to the locomotor response in which repeated drug treatment leads to enhanced locomotor behaviors over that observed following the first treatment with drug (210). Following single morphine injections over 6 days, WT and GRK6-KO mice were both responsive to the locomotor stimulating effects of morphine on day 7 for (Figure 4.3C). However, as
shown in Figure 4.3D, WT mice show a significantly enhanced locomotor response on day 7, as compared to day 1, while GRK6-KO mice display a similar locomotor activity profile on day 7 as on the first day of treatment.

Prolonged exposure to morphine leads to the development of physical dependence, which can be assessed by measuring antagonist-precipitated withdrawal behaviors. Both WT and GRK6-KO mice displayed prominent withdrawal signs following naloxone (0.05 and 0.5 mg/kg, i.p.) administration, indicating that they had become dependent on morphine at the doses tested. However, with the exception of the number of paw tremors, there were no significant differences by two-way ANOVA in the number or severity of the withdrawal signs measured between the two genotypes in mice treated with 24 mg/kg/day morphine and two different doses of naloxone (0.05 and 0.5 mg/kg, i.p.) (Figure 4.4A). As shown in Figure 4.4C, two-way ANOVA analysis revealed that GRK6-KO mice treated with 12 mg/kg/day morphine and 0.5 mg/kg naloxone showed significant differences in the occurrence of mastication, while there were no differences with any of the other withdrawal signs measured. Furthermore, there were no differences in overall global scores which simultaneously account for all withdrawal parameters measured between the two genotypes at the doses of naloxone (Figure 4.4B) or morphine (Figure 4.4D) tested.

Morphine treatment also affects gastrointestinal function in a manner that produces constipation. Therefore, GRK6-KO mice were monitored for differences in their gastrointestinal responses to morphine. Whole gut motility was initially assessed by measuring fecal boli production over 6 hours in response to saline (s.c.) or morphine (10 mg/kg, s.c.) treatment. Both WT and GRK6-KO mice produced equivalent amounts of feces over the 6 hour period in response to saline, indicating that normal gastrointestinal
function in not altered in the absence of GRK6 (Figure 4.5A). However, the GRK6-KO mice produced significantly more fecal boli over the test period compared to their WT littermates following morphine treatment. To determine if differences in eating behaviors was responsible for these differences, food consumption was determined for each cage of animals in a 24 hour period among 3 cages containing the same genotype. There were no significant differences in food intake between the WT and GRK6-KO mice (WT 4.57 ± 0.32 g/3 days vs KO 4.12 ± 0.36 g/3 days; p=0.4460, Student’s t test; n=8/genotype; data not shown).

To further assess how the loss of GRK6 is affecting gastrointestinal responses, the effect of morphine on inhibition of small intestinal transit in WT and GRK6-KO mice was evaluated using a charcoal meal assay. There were no significant differences in small intestinal transit measured between the two genotypes in response to saline (Figure 4.5B). While morphine dose-dependently reduced small intestinal transit, there were no significant differences between GRK6-KO mice and WT controls at any of the doses tested. Since differences in the overall amount of fecal boli were apparent, potential differences in large intestinal transit were assessed using a colonic bead expulsion assay. There were no differences in bead expulsion times in response to saline between the two genotypes. However, while morphine dose-dependently delayed bead expulsion in the WT and GRK6-KO mice, the GRK6-KO mice expelled their beads more rapidly than WT controls (Figure 4.5C), which is consistent to the results observed in the whole gut transit study.

In addition to opioid receptors, activation of dopamine receptors has been shown to inhibit gastrointestinal motility (178, 179, 211). GRK6 has been previously been shown to play a role in regulating D2 dopamine receptors in vivo (85). Therefore, we assessed
possible dopamine contributions to gastrointestinal transit in the GRK6-KO mice using the colonic bead expulsion assay. Cocaine methiodide, a quaternary salt that does not cross the blood brain barrier at low concentrations (212), was used to increase dopamine level in the enteric nervous system. While WT and GRK6-KO mice displayed similar expulsion times following saline treatment, cocaine methiodide (5 mg/kg, i.p.) significantly delayed bead transit times in WT, but not GRK6-KO mice (Figure 4.5D).

In addition to evaluating fecal boli accumulation in the GRK6-KO mice, morphine-induced fecal production was also assessed in GRK2-HT, GRK3-KO, GRK4-KO, and GRK5-KO mice. As shown in Figure 4.6 (A through D), when challenged with saline or morphine (10 mg/kg, s.c.), the GRK2-HT, GRK4-KO, and GRK5-KO show similar fecal boli accumulation profiles as their WT littermates, suggesting that loss of these individuals GRKs does not affect morphine-induced constipation. The GRK3-KO mice produce significantly less fecal boli in response saline compared to WT controls. However, there were no differences between the genotypes following morphine treatment.

4.4 Discussion
In the present study we demonstrate that GRK6 can promote μOR/β-arrestin2 interactions with the morphine-bound receptor in cell culture by facilitating morphine-induced β-arrestin2-GFP recruitment and receptor internalization (Figure 4.1), which is analogous to studies in which GRK2 overexpression was shown to facilitate morphine-induced β-arrestin2-GFP recruitment and μOR internalization (115-117, 119). However, the role of GRK6 in regulating the responsiveness of the μOR to morphine in vivo appears to be diverse. Moreover, no differences in morphine-induced constipation were
observed in GRK2-HT, GRK3-KO, GRK4-KO, and GRK5-KO mice. These results further support the hypothesis that GRK-mediated regulation of the μOR depends on the complement of regulatory proteins expressed and can differ in different cellular environments.

In vivo, morphine-induced locomotor activity (Figure 4.3), sensitization (Figure 4.3), and constipation (Figure 4.5) were altered in GRK6-KO mice. However, there were no significant differences in morphine-induced constipation in GRK2-HT, GRK3-KO, GRK4-KO, and GRK5-KO, suggesting that the loss of these GRKs is not sufficient enough to alter μOR regulation of gastrointestinal function (Figure 4.6). Moreover, the loss of β-arrestin2 does no impact thermal antinociceptive responses in the GRK6-KO mice following acute morphine treatment in both the hot-plate and warm water tail-flick assays (Figure 4.2). There were also no differences in the onset of antinociceptive tolerance (Figure 4.2) or the development of physical dependence (Figure 4.4) following chronic morphine treatment, suggesting that GRK6 does not play a predominant role in regulating these biological responses to morphine. Taken together with previous studies, our findings suggest that μOR may be regulated by multiple GRKs rather than predominantly by a single GRK, and the extent of regulation by each GRK may vary among cell types.

While genetic ablation of GRK6 does not appear to impact on morphine-induced in thermal antinociception, tolerance and physical dependence, the loss of GRK6 does affect the locomotor stimulating effects of morphine. Acute morphine treatment resulted in enhanced locomotor activity at several doses in GRK6-KO mice compared to their WT littermates (Figure 4.3). While WT mice become sensitized to the locomotor-stimulating
effects of morphine following chronic treatment, GRK6-KO mice display the same locomotor activation profile following chronic treatment as they did in response to acute treatment, indicating that the loss of GRK6 “presenstizes” these mice to the locomotor activating effects induced by morphine. The locomotor activity profiles observed in the GRK6-KO mice in response to morphine resemble those seen following treatment with the psychostimulants cocaine and d-amphetamine (85); wherein GRK6-KO mice displayed enhanced locomotor activation and “presensitization” to the locomotor activating effects of these drugs compared to WT mice (85). In this study, the locomotor stimulating effects of these drugs are correlated to an increase in extracellular dopamine levels in the striatum (85). Furthermore, the enhanced locomotor activity in the GRK6-KO mice compared to WT animals correlated with an increase in G protein-coupling to dopamine D2 receptors in striatal membranes in the absence of GRK6 (85). Interestingly, it is well established that opioid treatment indirectly causes increased dopamine signaling within the striatum and nucleus accumbens, brain regions involved in mediating morphine-induced locomotion (213, 214). Therefore, the increase in locomotor activation and “presensitization” observed in the GRK6-KO mice in response to morphine may be due to enhanced D2 dopamine receptor activity in the striatum of GRK6-KO mice, rather than a direct result of altered regulation of the μOR.

Given the role that GRKs play in desensitizing GPCRs, it is interesting that morphine-induced constipation was reduced in the GRK6-KO mice, an affect that appears to be mediated at the level of the colon (Figure 4.6). This behavioral response suggests that GRK6 may be acting to mediate μOR signaling in the gastrointestinal tract rather than as a desensitizing agent. We observed a similar reduction in morphine-induced constipation and colonic motility in βarr2-KO mice. Following GRK-mediated
phosphorylation of an activated GPCR, β-arrestins interact with the receptor and, for some GPCRs, can serve as scaffolding molecules for components of signaling cascades such as the mitogen activated (MAP) kinases (215). Several in vivo studies have demonstrated that β-arrestin2 can function as a positive mediator of receptor signaling and that these signaling pathways have physiological effects (105). Importantly, GRK6 has been shown to facilitate β-arrestin2-mediated signaling pathways. For example, in the heart, GRK6 can phosphorylate angiotensin II receptors to facilitate a G protein-independent, β-arrestin2-mediated pathway leading to inotropy (216). Therefore, GRK6 may also be involved in positively regulating morphine-mediated constipation by facilitating activation of a similar pathway in colon.

We have also considered that the differences observed in morphine-induced constipation between the GRK6-KO mice and their WT littermates may not be due to direct regulation of the μOR, but rather could also be due to GRK6 regulation of other neurotransmitter systems that affect gastrointestinal motility. Since it is known that the dopamine transporter is expressed in the gastrointestinal tract (178) and that the activation of peripheral dopamine receptors can decrease gastrointestinal motility (178, 179, 211), we evaluated the possible contribution of the dopamine system by using cocaine methiodide, a peripherally restricted dopamine transporter blocker. As shown in Figure 4.6, this treatment delayed colonic motility in WT but not the GRK6-KO mice. While these results indicate an altered regulation of dopamine signaling in the gastrointestinal tract of mutant mice, the exact mechanism of such alterations is not immediately clear. One possible explanation could be that in the absence of GRK6, dopamine D2 receptor signaling in the gut is disrupted. Previously, Beaulieu et al. (101) described decreased dopamine D2 receptor function in the absence of β-arrestin2 in the
striatum following treatment with psychostimulants. The deletion of the GRK6 gene could potentially decrease D2 dopamine receptor/β-arrestin2 interactions in gut, which could impact the physiological effects observed here by interrupting a downstream β-arrestin2-mediated signaling pathway. Further studies examining the contribution of dopamine, and other neurotransmitter systems, on morphine-induced constipation and signal transduction in the gut are underway.

In summary, we have shown that morphine-induced responses like locomotor activity, sensitization, and constipation are affected by the loss of GRK6, while other responses such as thermal antinociception, antinociceptive tolerance, physical dependence and conditioned place preference are not. It is not clear whether the differences in responses are due to direct GRK6-mediated regulation of the μOR, or to altered regulation of other neurotransmitter systems such as dopamine. Therefore, additional studies will need to be conducted to further elucidate the potential contribution of other systems. Importantly, the results of these studies lend further support to the idea that the cellular environment and exact GRK repertoire play a key role in determining GPCR responsiveness and highlight that studying receptors in their endogenous setting is critical to understanding the mechanisms involved in receptor signaling and drug effects.
4.5 Tables and Figures

Figure 4.1. GRK6 facilitates morphine-induced β-arrestin2 recruitment and μOR trafficking in vitro. **A.** HEK-293 cells expressing HA-tagged μOR and βarr2-GFP (green) were labeled with an HA antibody conjugated to Alexafluor 594 (red) and monitored via live cell confocal microscopy (100X objective). Morphine treatment (10 μM, 10 minutes) alone did not cause detectable recruitment of βarr2-GFP to the μOR. In contrast, morphine was able to induce recruitment of βarr2-GFP to the μOR in cells overexpressing GRK6, as indicated by arrows (inset: 2X magnification). **B.** HEK-293 cells expressing μOR-YFP display robust receptor internalization following morphine treatment (10 μM, times indicated) only in cells overexpressing GRK6 as indicated by arrows (inset: 2X magnification). Images are representative of several images collected from 3 independent transfections (Dr. Laura Bohn completed this experiment).
Figure 4.2. Morphine-induced antinociception and antinociceptive tolerance profiles in GRK6-KO mice. Mice were treated with morphine (5, 10, 20 mg/kg, i.p.) and antinociception was determined at various time points after treatment using a hot-plate (56°C) and warm-water tail-flick (54°C) test. Data are presented as the mean ± S.E.M. A. & B. Morphine (10 mg/kg, i.p.) produces an equivalent degree of antinociception in both genotypes in both tests (two-way ANOVA for genotype: \( p > 0.05 \); n=6-7) and basal response latencies (insets) are not significantly different between the genotypes (\( p > 0.05 \), Student’s \( t \) test; n=17). C. & D. Morphine produces similar antinociceptive profiles in WT and GRK6-KO mice assessed 30 minutes after drug injection in both tests (two-way ANOVA for genotype: \( p > 0.05 \); n=5-7). E. & F. Chronic morphine treatment produces tolerance at the same rate and to the same extent at several doses in both the hot-plate and tail-flick test (two-way ANOVA for genotype at all doses: \( p > 0.05 \); n=5-7). (Laura Bohn collected the data presented in panel A and B)
Figure 4.3. Morphine-induced locomotor activity and “presensitization” to the stimulating effects of morphine in GRK6-KO mice. Locomotor activity was measured before and after morphine (5, 10, or 20 mg/kg, i.p.) treatment. Data are presented as the mean ± S.E.M. 

A. GRK6-KO mice display a significant increase in the number of beam breaks per 5 minutes with a moderate dose of morphine (10 mg/kg, i.p.) compared to WT controls (two-way ANOVA for genotype: F(1,480)=171.04, *p*<0.0001; for time: F(23,480)=7.77, *p*<0.0001; n=11). There are no differences in habituation responses between both genotypes (two-way ANOVA for genotype: *p*>0.05; n=34-36). 

B. Acute morphine produces a dose-dependent increase in the total number of beam breaks made by GRK6-KO mice over the 120 min test period compared to WT littermates (two-way ANOVA for genotype: F(1,480)= 7.11, *p*<0.001; for time: F(3,63)=29.47, *p*<0.0001; Bonferroni post-hoc analysis, WT vs GRK6-KO, *p*<0.05, **p*<0.01; n=7-12). 

C. Mice were treated with morphine (10 mg/kg, i.p.) once daily for 6 days and locomotor activity was assessed on day 7. Chronic morphine treatment produces different locomotor profiles in WT and GRK6-KO mice (for genotype, F(1,480)= 5.759, *p*<0.0168; for time, F(23,480)= 9.262, *p*<0.0001; n=11). 

D. The total number of beam breaks made in 120 minutes is significantly higher in the GRK6-KO mice on day 1 compared to WT controls following acute morphine treatment (WT day 1 vs KO day 1, **p*<0.01, Student’s *t* test; n=11). By day 7, WT mice show an increase in the total number of beam breaks in 120 minutes compared to day 1 (WT day 1 vs WT day 7, #*p*<0.05, Student’s *t* test; n=11) while GRK6-KO mice display similar locomotor activity profiles on days 1 and 7 (KO day 1 vs KO day 7, *p*>0.05, Student’s *t* test; n=11). (Dr. Laura Bohn and Cullen Schmid helped to collect some of these data).
Figure 4.4. Naloxone-precipitated withdrawal responses in response to chronic morphine treatment in GRK6-KO mice. Withdrawal was precipitated using naloxone (0.05 or 0.5 mg/kg, i.p.) after 7 days of chronic morphine (12 or 24 mg/kg/day) infusion. Data are presented as the mean ± S.E.M. 

A. Morphine (24 mg/kg/day, s.c.) produces a similar amount of withdrawal behaviors (two-way ANOVA for genotype: \( p > 0.05 \); \( n=8-12 \)) in both genotypes following treatment with two different doses of naloxone, except for the number of paw tremors two-way ANOVA for genotype: \( F(1,39)=5.52, p=0.0239; n=8-12 \) which is higher in the GRK6-KO mice.

B. The global score is not significantly different between WT and GRK6-KO mice treated with 24 mg/kg/day morphine or naloxone (0.05 or 0.5 mg/kg, i.p.) (two-way ANOVA for genotype: \( F(1,39)=3.06, p=0.0882; n=8-12 \) which is higher in the GRK6-KO mice.

C. Morphine (12 and 24 mg/kg/day, s.c.) produces the same degree of withdrawal behaviors in response to naloxone (0.5 mg/kg, i.p.) in both genotypes (two-way ANOVA for genotype: \( p > 0.05; n=6-17 \) except the occurrence of mastication (for genotype: \( F(1,36)=5.80, p=0.0212; \) for dose: \( F(1,36)=0.1, p=0.7369; n=6-17 \) is greater in GRK6-KO mice.

D. There are no significant differences in global withdrawal scores between WT and GRK6-KO mice (two-way ANOVA for genotype: \( F(1,36)=2.29, p=0.1393; \) for dose: \( F(1,36)=1.36, p=0.2507; n=5-17 \).
Figure 4.5. Gastrointestinal responses to morphine and cocaine-methiodide in GRK6-KO mice. A. Mice were treated with saline (s.c.) or morphine (s.c.) and fecal boli were weighed at 1 hour intervals over a 6 hour period. Data represent the mean ± S.E.M. There are no significant differences in fecal boli production between GRK6-KO and WT mice in response to saline treatment (two-way ANOVA for genotype: $F_{(1,168)}=0.01$, $p=0.9185$; for time: $F_{(5,168)}=7.33$, $p<0.0001$; $n=15$). While fecal boli is initially suppressed in both genotypes, the GRK6-KO mice recover to control values faster than their WT counterparts (two-way ANOVA for genotype: $F_{(1,144)}=5.95$, $p=0.0159$; for time: $F_{(5,144)}=13.19$, $p<0.0001$; $n=12-13$). B. Small intestinal transit was evaluated following saline (s.c.) or morphine (s.c.) treatment using a charcoal meal assay. There were no significant differences in small intestinal transit between the two genotypes in response to either saline ($p>0.05$, Student’s $t$ test; $n=3$) or morphine (two-way ANOVA for genotype: $F_{(1,144)}=5.95$, $p=0.0159$; for time: $F_{(5,144)}=13.19$, $p<0.0001$; $n=12-13$). B. Small intestinal transit was evaluated following saline (s.c.) or morphine (s.c.) treatment using a charcoal meal assay. There were no significant differences in small intestinal transit between the two genotypes in response to either saline ($p>0.05$, Student’s $t$ test; $n=3$) or morphine (two-way ANOVA for genotype: $F_{(1,144)}=5.95$, $p=0.0159$; for time: $F_{(5,144)}=13.19$, $p<0.0001$; $n=12-13$). C. Large intestinal transit was determined using a colonic bead expulsion assay following treatment with saline (s.c.) or morphine (s.c.). Morphine dose-dependently inhibits colon transit in both genotypes; however, the GRK6-KO mice are less adversely affected compared to their WT counterparts across doses (for genotype: $F_{(1,58)}=11.51$, $p=0.0013$; for dose: $F_{(3,58)}=264.61$, $p<0.0001$; WT vs KO *$p<0.05$, Bonferroni post-hoc analysis; $n=4-8$). D. Cocaine methiodide (cocaine MI, 5 mg/kg, i.p.) significantly delays colonic bead transit in WT mice compared to GRK6-KO mice (##$p<0.01$, Student’s $t$ test; $n=5$) while GRK6-KO mice exhibit similar bead expulsion times with both saline and drug treatment ($p>0.05$, Student’s $t$ test; $n=5$).
Figure 4.6. Morphine-induced inhibition of fecal boli production is GRK2-HT, GRK3-KO, GRK4-KO and GRK5-KO mice. Mice were treated with saline (s.c.) or morphine (10 mg/kg, s.c.) and fecal boli was collected and weighed every hour over a 6 hour period. Data represent the mean ± S.E.M.

A. GRK2-HT mice and produce an equivalent amount of fecal boli in response to saline (two-way ANOVA for genotype: $F_{(1,72)}=1.06$, $p=0.3077$; for time: $F_{(5,72)}=2.71$, $p=0.0268$; $n=7$) and morphine (two-way ANOVA for genotype: $F_{(1,72)}=1.73$, $p=0.1926$; for time: $F_{(5,72)}= 17.08$, $p<0.0001$; $n=7$) compared to WT controls.

B. GRK3-KO mice produce less fecal boli in response to saline (two-way ANOVA for genotype: $F_{(1,192)}=11.95$, $p=0.0007$; for time: $F_{(5,192)}= 3.27$, $p=0.0074$; $n=16$) but an equal amount as WT mice following morphine (two-way ANOVA for genotype: $F_{(1,222)}= 0.26$, $p=0.6098$; for time: $F_{(5,222)}= 23.40$, $p<0.0001$; $n=20$) treatment.

C. Both GRK4-KO and WT mice produce a similar amount of fecal boli in response to saline (two-way ANOVA for genotype: $F_{(1,54)}= 5.54$, $p=0.0120$; $n=4-7$) and morphine (two-way ANOVA for genotype: $F_{(1,60)}= 0.12$, $p=0.7282$; for time: $F_{(5,60)}=26.15$, $p<0.0001$; $n=5-7$). D. GRK5-KO mice and their WT littermates produce an equal amount of fecal boli over the test period in response to saline (two-way ANOVA for genotype: $F_{(1,72)}=0.44$, $p=0.5074$; for time: $F_{(5,72)}= 1.94$, $p=0.0988$; $n=7$) and morphine treatment (two-way ANOVA for genotype: $F_{(1,72)}=6.02$, $p=0.051$; for time: $F_{(5,72)}=8.17$, $p<0.0001$; $n=7$).
CHAPTER 5

CONCLUSIONS

The findings presented in this dissertation demonstrate that disruption of μOR regulation, by removal of β-arrestin2 or GKR6, determines the overall effectiveness of opioid drugs in vivo. Consistent with the classically defined role of β-arrestins as negative regulators of signaling, we have previously shown that morphine-induced antinociception is enhanced and prolonged in βarr2-KO mice when compared to WT littermates. In addition, βarr2-KO mice are resistant to the development of antinociceptive tolerance to chronic morphine. Moreover, the increased antinociception and decreased antinociceptive tolerance in the βarr2-KO mice correlates with impaired μOR desensitization in brain regions associated with processing pain (88). However, in this simple scenario in which β-arrestin2 only acts as a desensitizing element would indicate that all behavioral responses to morphine should be enhanced in the βarr2-KO mice. However, we find that several morphine-induced side effects including constipation, physical dependence, and respiratory suppression are not more severe but rather are diminished in a mouse model that displays enhanced morphine analgesia, suggesting that β-arrestins may play a more complex role in mediating μOR signaling in vivo.
The reduction in morphine constipation and physical dependence in the βarr2-KO mice suggests a new potential mechanism by which β-arrestin2 may affect overall receptor signaling and drug responsiveness in vivo. A myriad of studies have demonstrated that β-arrestins also function as scaffolding molecules to mediate GPCR signaling by facilitating interactions between signaling proteins and the receptor. While cell culture studies provide evidence that that β-arrestins have the potential to mediate μOR signaling in an agonist-dependent manner (99), this has not yet been shown in vivo. However, reductions in behaviors in the βarr2-KO mice for other GPCRs including the dopamine D2, and serotonin 2A receptors have been correlated with a reduction in β-arrestin2-mediated signaling (101, 102, 105), suggesting that the loss of β-arrestin2 could potentially affect μOR signaling in vivo as well. Future studies in which μOR signaling in measured in colonic neuronal tissues from βarr2-KO mice and their WT littermates will be useful in determining whether β-arrestin2 can facilitate μOR signaling in at least a subset neurons in vivo.

Given that not all morphine-mediated side effects were enhanced in the βarr2-KO mice suggests that β-arrestin2 may influence μOR-mediated responses by multiple means. Our results also indicate that while the μOR may be regulated by the classical desensitization paradigm by βarrestin2 in some neurons, this may not hold true for other cell types, suggesting that the μOR can be differentially regulated in different cellular environments. In this scenario, μOR signaling may differ in certain cell types wherein the receptor’s fate may be determined by the cellular complement of proteins within the receptor’s immediate environment. For example, μORs expressed in periaqueductal gray neurons, which are negatively regulated by βarrestin2 (87, 88) may not be subject
to the same regulatory mechanisms as μORS expressed in other cell types such as enteric neurons in the gut. In the absence of β-arrestin2 morphine antinociception and physical dependence is affected; however, these responses are not affected in mice lacking GRK6. Furthermore, the loss of both β-arrestin2 and GRK6 suppresses morphine-induced constipation, demonstrating that the complement of certain regulatory and scaffolding proteins within a given cellular environment may play a major role in determining overall μOR responsiveness in particular cell types.

The current studies also provide further evidence that the relative responsiveness of the μOR in vivo is not only dependent on agonist occupancy but can vary with distinct opioid agonists. Several groups have demonstrated in vitro that while morphine, methadone, and fentanyl can activate μOR signaling with similar efficacy they differ in their ability to promoter receptor desensitization and internalization. Furthermore, differences in antinociceptive tolerance in response to distinct agonists had also been observed, suggesting that the same μOR mediated response can be differentially determined by the agonist bound to the receptor in vivo. Similar differences in agonist-induced antinociceptive responses had already been described in these mice (117). In the current dissertation we show that β-arrestin2 also differentially effects the opioid-induced side effects constipation, antinociceptive tolerance, and physical dependence in an agonist-dependent manner as well. Studies in cell culture suggest that this difference may be due to the fact that β-arrestin2 preferentially interacts with the morphine-bound μOR, whereas methadone and fentanyl can promote interactions between both β-arrestin1 and β-arrestin2. Interestingly however, both morphine and methadone produce less physical dependence, suggesting that only β-arrestin2 may regulate μOR signaling in cell populations that are involved in mediating this response. Taken together, these
results suggest that different physiological responses may be differentially affected by
the μOR regulatory components as well as depend on the specific opiate agonist used.

It is apparent that the current understanding of GPCR signaling is rapidly expanding past
the classical models of G protein-coupling and β-arrestin-mediated desensitization.
Furthermore, μOR regulation profiles are dynamic; dependent not only on the site of
expression, but also upon drug exposure. Upon considering both the cell culture and
animal studies in parallel, it is apparent that opioid receptor regulation can have
profound impacts on overall agonist responsiveness. The complexity governing such
diverse potential regulatory mechanisms emphasizes the need to study receptor
signaling in the endogenous environment as this may ultimately determine the
physiological response to the drug. Importantly, as these complexities are revealed,
new avenues for the development of novel therapeutic targets or drugs may become
available. For example, if the μOR signals to a particular kinase in the colon that
underlies its ability to induce constipation, then modulation of such a pathway, perhaps
by blocking β-arrestin2, might represent a clinical therapeutic target for treating
constipation. Moreover, developing a modulator of morphine-mediated μOR/β-arrestin2
interactions may prove to have beneficial therapeutic value in enhancing and prolonging
the analgesic effects of morphine, while at the same time preventing morphine-induced
side effects such as analgesic tolerance, physical dependence, constipation, and
respiratory suppression.
LIST OF REFERENCES


109


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