THE ROLE OF INTRACELLULAR SIGNALING PATHWAYS ON MU-OPIOID RECEPTOR AGONIST-INDUCED ANTINOCICEPTION AND TOLERANCE DEVELOPMENT

By

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The members of the Committee appointed to examine the dissertation of ERIN NICOLE BOBECK find it satisfactory and recommend that it be accepted.

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Mu-opioid receptor (MOPr) agonists, such as morphine and fentanyl, are the most commonly prescribed drugs to alleviate severe and chronic pain. Although all agonists produce antinociception, they vary in terms of activation of intracellular signaling cascades and tolerance development. Morphine produces little receptor desensitization, β-arrestin recruitment, and receptor internalization, whereas DAMGO and fentanyl produce rapid and robust MOPr desensitization and internalization. Intracellular signaling pathways can be activated by G-protein activation or β-arrestin signaling. Therefore, agonists that cause receptor internalization may lead to activation of different pathways than agonists that strictly use G-protein dependent signaling. Extracellular regulated kinase 1 and 2 (ERK1/2) is a known downstream messenger of β-arrestin. The purpose of the studies in this dissertation was to evaluate the behavioral effects of ligand-biased signaling pathways. Microinjections directly into the ventrolateral periaqueductal gray (vPAG) allowed for direct manipulation of proteins within the descending pain modulation pathway. It was determined that G-protein or internalization
pathways appear to contribute to antinociception depending on the MOPr agonist. Morphine uses a predominately G-protein pathway, whereas DAMGO can use G-protein or internalization mechanisms. Surprisingly, fentanyl seemed to produce antinociception independent of either pathway. These differences were consistent with proposed differences in activation of intracellular signaling cascades, including ERK1/2. Therefore, evaluation of the role of ERK1/2 on antinociception and tolerance was investigated. ERK1/2 was activated following vlPAG microinjection of DAMGO, but not fentanyl, and ERK1/2 inhibition led to attenuation of DAMGO antinociception. Moreover, both the development and expression of tolerance to DAMGO was reversed by inhibition of ERK1/2, but there was no effect of ERK1/2 inhibition on fentanyl tolerance. Although tolerance develops to repeated morphine, DAMGO, or fentanyl microinjections into the vlPAG, the current studies showed that cross-tolerance does not develop between these drugs. This finding suggests that different tolerance mechanisms are engaged by different MOPr agonists and that ERK1/2 plays a unique role depending on the agonist: Counteracting morphine tolerance, contributing to DAMGO tolerance, and having no effect on fentanyl tolerance. These studies reveal that antinociception and tolerance occur via different mechanisms depending on MOPr agonist.
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ABBREVIATIONS

DAMGO—[D-Ala$^2$, N-MePhe$^4$, Gly-ol$^5$]-enkephalin

dyn-DN—dominant negative dynamin peptide

ERK1/2—extracellular signal-regulated kinase 1/2

GPCR—G-protein coupled receptor

GRK—G-protein receptor kinase

JNK—c-Jun N-terminal kinase

MOPr—mu opioid receptor

PKC—protein kinase C

PTX—pertussis toxin

scr-dyn—scrambled dynamin control peptide

vlPAG—ventrolateral periaqueductal gray
CHAPTER 1: GENERAL INTRODUCTION

Opioids such as morphine and fentanyl are the most commonly used drugs to treat severe and chronic pain (Soliman et al., 2001) due to their superior ability to produce antinociception at the mu-opioid receptor (MOPr), compared to agonists at the other opioid receptors (delta and kappa) (Trescot et al., 2008). MOPrs are found in several key brain structures, the spinal cord, and in the periphery, all of which contribute to the antinociceptive effects of opioids (Williams et al., 2001).

Opioids produce antinociception, in part, by activating a descending pain modulatory pathway which includes the periaqueductal gray (PAG), rostral ventromedial medulla (RVM) and the dorsal horn of the spinal cord. Microinjection of opioids into any part of this pathway produces antinociception (Jacquet and Lajtha, 1974; Fang et al., 1989; Rossi et al., 1994; Morgan et al., 1998; Fairbanks and Wilcox, 2000; Meyer et al., 2007; Bobeck et al., 2009). The PAG is divided into subregions defined both anatomically and by the different effects produced following opioid administration. While both the dorsal/lateral and ventrolateral regions produce antinociception, the dorsal/lateral causes an increase in locomotor activity and the ventrolateral PAG (vPAG) causes immobility (Morgan et al., 1998). Opioids produce antinociception by disinhibition of tonically active GABAergic neurons within the PAG leading to activation of output neurons. These neurons then activate a subset of neurons within the RVM, which then inhibits pain transmission in the spinal cord (Moreau and Fields, 1986; Depaulis et al., 1987).
G-protein signaling

The MOPr is a G-protein coupled receptor (GPCR) which couples to inhibitory heterotrimeric G-proteins. Opioids typically produce antinociception by activating inhibitory $G_{i/o}$-proteins which alter intracellular signaling proteins and ion conductance (Goode and Raffa, 1997; Williams et al., 2001; Gintzler and Chakrabarti, 2004). Coupling to G-proteins has been shown to differ depending on the MOPr agonist. Morphine antinociception is greatly diminished following inhibition of $G_{i/o}$-proteins with pertussis toxin (PTX) following intrathecal, intracerbroventricular, or intra-PAG administration (Bodnar et al., 1990; Goode and Raffa, 1997; Gomes et al., 2002). Antinociception induced by other agonists, such as DAMGO and sufentanil, is not dependent on the same G-proteins inhibited by PTX suggesting a different mechanism of antinociception (Goode and Raffa, 1997).

Binding of an agonist to the receptor leads to a conformational change, which dissociates the $G\alpha$ subunit from the $G\beta\gamma$ subunits (Rosenbaum et al., 2009). At this point, the $\alpha$ and $\beta\gamma$ subunits act independently to activate different effector systems. The most commonly studied effector is inhibition of the adenylyl cyclase/cyclic adenosine monophosphate (cAMP) pathway (Williams et al., 2001; Nestler, 2004; Al-Hasani and Bruchas, 2011). Activation of other signaling proteins such as protein kinase C (PKC) and mitogen activated protein kinases (MAPK) following opioid exposure also has been demonstrated (Al-Hasani and Bruchas, 2011). MOPr activation also causes direct interaction of $\beta\gamma$ subunits with ion channels such as inhibition of calcium channels and activation of potassium channels. This alteration in ion currents leads to
hyperpolarization of the membrane potential and decreases subsequent neurotransmitter release in many different systems and brain regions (Williams et al., 2001; Al-Hasani and Bruchas, 2011). It is this alteration in neurotransmitter release in key brain regions (i.e. vIPAG) that leads to antinociception.

Agonists bind and activate the MOPr with varying efficacies. Efficacy is the magnitude of the receptor-mediated effect that is produced by an agonist relative to its receptor occupancy (Clarke and Bond, 1998; Strange, 2008). Relative efficacies of MOPr agonists can be measured using various endpoints, including G-protein, effector activation, or behavioral outputs including antinociception. Moreover, efficacy can vary depending on the particular endpoint. Radioligand [35S]GTPγS and other G-protein binding studies have shown that certain agonists stimulate G-proteins to a greater degree than other agonists. For example, [D-Ala\(^2\), N-MePhe\(^4\), Gly-ol\(^5\)]-enkephalin (DAMGO) and fentanyl are more efficacious than morphine (Traynor and Nahorski, 1995; McPherson et al., 2010; Rivero et al., 2012). This difference in G-protein activation leads to subsequent differences in effector activation. In human embryonic kidney cells, DAMGO causes greater inhibition of cAMP than morphine (Keith et al., 1998). However, other studies have found that DAMGO and fentanyl inhibit cAMP to a similar or a lesser degree than morphine (Hirst and Lambert, 1995; Zaki et al., 2000; Koch et al., 2005). Evaluation of efficacy of agonists to alter ion currents has found similar rank order between agonists at other measures of efficacy, in which DAMGO is most efficacious and morphine is least (Yu et al., 1997; Borgland et al., 2003).

Antinociceptive efficacy also differs between agonists. Using increasing doses of
irreversible antagonists to systematically eliminate functional receptors causes a greater
decrease in morphine-induced antinociception than fentanyl-induced antinociception
(Duttaroy and Yoburn, 1995; Madia et al., 2012). Morphine also has lower efficacy when
compared to spinal administration of DAMGO and sufentanil (Mjanger and Yaksh,
1991). However, the antinociceptive efficacy of fentanyl and morphine are equal in the
vIvPAG (Bobek et al., 2012). These data indicate that differences may exist between
regions in the central nervous system. The mechanism by which antinociception occurs
within the vIvPAG remains unclear and will be addressed in Chapter 2.

**MOPr regulation and β-arrestin signaling**

Recent findings in other receptor systems reveal that other signaling
mechanisms not linked to G-protein activation do exist (Reiter et al., 2012). Despite
binding to the same receptor and activating the same G-protein mediated processes,
agonists can activate of different receptor regulation processes and intracellular
signaling pathways (Kelly et al., 2008; Reiter et al., 2012). This phenomenon is called
ligand-biased signaling. Most of the research comparing various MOPr agonists has
focused on receptor regulation processes such as desensitization and internalization
(Finn and Whistler, 2001; Alvarez et al., 2002; Borgland et al., 2003; Hull et al., 2010;
Melief et al., 2010), whereas behavioral effects of ligand-biased signaling have been
largely overlooked.

Following binding of an agonist to the receptor and G-protein activation, several
processes take place in order to terminate further G-protein signaling. Desensitization is
a rapid decrease in response to the agonist following initial binding caused by phosphorylation of the receptor (Kelly et al., 2008). Phosphorylation at certain residues on the receptor by G-protein regulated kinase (GRK) leads to β-arrrestin recruitment which then initiates internalization of the receptor. Internalization is the process of removing the receptor from the membrane into the cytosol of the cell via the formation of clathrin coated pits and then dynamin pinches off the membrane (Kelly et al., 2008; Al-Hasani and Bruchas, 2011). The receptor has one of two fates after internalization: degradation by lysosomes or resensitization and recycling back to the membrane (Whistler, 2012).

In vitro experiments have revealed that desensitization and internalization can differ between agonists. In general, morphine produces less desensitization and internalization than other high efficacy agonists such as DAMGO and fentanyl (Whistler et al., 1999; Alvarez et al., 2002; Kelly et al., 2008; McPherson et al., 2010). This may be because morphine uses a protein kinase C (PKC)-dependent desensitization mechanism whereas other agonists use a GRK-dependent mechanism (Kelly et al., 2008; Hull et al., 2010). Recent studies have shown that certain MOPr agonists (DAMGO and fentanyl) associate with β-arrrestin stronger than morphine (McPherson et al., 2010; Molinari et al., 2010). Given these differences in GRK mediated desensitization and β-arrrestin recruitment, it is not surprising that morphine produces little internalization, while DAMGO and fentanyl produce rapid internalization (Finn and Whistler, 2001; Alvarez et al., 2002).
These differences in regulation of the receptor may lead to differences in activation of subsequent intracellular signaling pathways and cellular function. The function of MOPr regulation in vivo remains unclear. β-arrestin 2 knockout mice have enhanced antinociception to morphine, but not other opioids (Bohn et al., 1999; Raehal and Bohn, 2011). It has been suggested that this is due to certain agonists’ ability to use β-arrestin 1 in the absence of β-arrestin 2 (Groer et al., 2011). The MOPr agonist dermorphin causes MOPr internalization and produces a decrease in antinociception following inhibition of dynamin (Macey et al., 2010). These results suggest that β-arrestin and MOPr regulation may play a role in antinociception for some agonists. Chapter 2 will test this hypothesis.

Although typically intracellular signaling is evoked by G-proteins coupled to the receptor, recent studies on other GPCRs have determined that β-arrestin can induce activation of a distinct signaling cascade that may continue regardless of G-protein signaling (Shenoy et al., 2006). A key downstream effector of this β-arrestin signaling is extracellular regulated kinase 1/2 (ERK1/2). ERK1/2 is in the family of MAPKs that manage cellular responses, including proliferation, apoptosis, gene transcription, and channel phosphorylation (Al-Hasani and Bruchas, 2011).

Several cellular studies have begun to evaluate the role of ERK1/2 following exposure to different MOPr agonists. A study of the MOPr using cultured neurons showed that morphine does not activate ERK1/2 while fentanyl does in a β-arrestin dependent manner (Macey et al., 2006). Yet other studies using heterologous cell systems found ERK1/2 is activated by a PKC-mediated mechanism following morphine
and by a β-arrestin mechanism following DAMGO or fentanyl exposure (Belcheva et al., 2005; Zheng et al., 2008; Zheng et al., 2011). Differential ERK1/2 activation corresponds with the ability of these agonists to recruit β-arrestin and internalize the receptor (Finn and Whistler, 2001; Alvarez et al., 2002; McPherson et al., 2010). Nonetheless, activation of ERK1/2 appears to occur in a ligand-biased manner which will be evaluated further following vIPAG microinjection of morphine, DAMGO, and fentanyl in Chapter 3.

**Opioid tolerance**

The effectiveness of MOPr agonists is greatly reduced over time due to the development of antinociceptive tolerance. Tolerance develops rapidly to morphine following as little as one injection in a variety of different injection sites (Huidobro et al., 1976; Larcher et al., 1998; Bobeck et al., 2012). The exact mechanism for opioid tolerance is unknown and current theories are controversial. Great focus has been placed on the intermediate efficacy MOPr agonist, morphine. Many cellular changes are produced by chronic morphine administration including changes in receptor desensitization, intracellular signaling, and synaptic transmission (Williams et al., 2001). Investigations of tolerance mechanisms involving G-protein and β-arrestin signaling cascades have been conducted, but only a few studies address the behavioral role of these signaling differences.

An interesting finding is that the magnitude of tolerance development differs between agonists. For example, high efficacy MOPr agonists, such as fentanyl, produce
less tolerance than low efficacy MOPr agonists, such as morphine (Madia et al., 2009). Cross-tolerance from one MOPr agonist to another has been shown to be minimal or to not occur following prolonged administration in rodents or humans (Duttaroy and Yoburn, 1995; Madia et al., 2009; Slatkin, 2009). These studies suggest that different mechanisms are driving tolerance to various agonists. However, a previous study from our lab found that cross-tolerance to intra-vlPAG morphine does develop following pretreatment with intra-vlPAG DAMGO (Meyer et al., 2007), which argues that similar mechanisms may be involved in the vlPAG. Chapter 4 will further evaluate cross-tolerance between morphine, DAMGO, and fentanyl microinjections into the vlPAG.

The most studied G-protein activated pathway involved in tolerance is the adenyl cyclase-cAMP pathway. Following prolonged morphine exposure there is an upregulation of cAMP, in contrast to the acute inhibitory effect of opioids (Gintzler and Chakrabarti, 2004; Nestler, 2004; Al-Hasani and Bruchas, 2011). It has been proposed that this upregulation in cAMP is caused by cellular adaptive changes to overcome the initial inhibition of adenyl cyclase (Nestler, 1996). Another hypothesis is that $G_{i/o}$-proteins are uncoupled from the receptor and there is a switch to coupling with $G_s$-proteins (Gintzler and Chakrabarti, 2004). Very few in vivo studies have evaluated the role of the AC pathway in morphine tolerance, but inhibition of the AC pathway during morphine pretreatment has been shown to block the development of morphine tolerance (Gabra et al., 2008) and dependence (Li et al., 2006).

Repeated morphine administration also leads to alterations of several other intracellular signaling pathways, including PKC and ERK1/2. PKC is a G-protein-
dependent protein that is activated following exposure to MOPr agonists (Granados-Soto et al., 2000; Zheng et al., 2008). Inhibition of several isoforms of PKC have been shown to decrease the development and expression of tolerance to systemic morphine (Smith et al., 2006; Song et al., 2010). PKC mediation of tolerance induced by a single injection (i.e., acute tolerance) appears to be specific for morphine as well, because blocking PKC has no effect on acute tolerance to the high efficacy MOPr agonist, DAMGO (Hull et al., 2010).

The role of a key β-arrestin activated protein, ERK1/2, in morphine tolerance has also been investigated. Not only is β-arrestin important for internalization it also activates a different set of signaling proteins that are not activated by G-proteins (Shenoy et al., 2006). In vitro experiments have found ligand-biased activation of ERK1/2 following MOPr agonist exposure where fentanyl and DAMGO show greater ERK1/2 activation than morphine (Belcheva et al., 2005; Macey et al., 2006; Zheng et al., 2008). The role of ERK1/2 in opioid tolerance remains unclear given that inhibition of ERK1/2 activation during the development of morphine tolerance can either increase, decrease or have no effect on tolerance depending on route of administration (Mouledous, 2007; Macey et al., 2009; Horvath et al., 2010). Surprisingly, little is known about whether this pathway is important for tolerance to repeated exposure of these other MOPr agonists that preferentially activate ERK1/2 in cellular studies. This will be evaluated in Chapter 3.

Previous studies evaluating acute tolerance, a decrease in effectiveness of the drug after a single administration, have determined that agonists have distinct
mechanisms which are typically linked to the ability of the agonist to recruit \( \beta \)-arrestin. GRK inhibition attenuates acute tolerance to DAMGO and fentanyl, which correlates with the ability of those agonists to internalize the MOPr (Hull et al., 2010; Melief et al., 2010). However, morphine does not rapidly internalize the receptor and uses a G-protein mediated tolerance mechanism, such as PKC or c-Jun N-terminal kinase (JNK) (Hull et al., 2010; Melief et al., 2010). Activation of many signaling cascades have been found to contribute to morphine tolerance (Williams et al., 2001), but it is unclear whether these same changes occur following long-term exposure to other opioids.

Agonist-dependent differences in the development of tolerance have been suggested to inversely correlate with the agonist’s intrinsic efficacy and ability to desensitize and internalize MOPr. Phosphorylation of the receptor by GRKs leads to desensitization of G-protein signaling and has long been considered the mechanism of opioid tolerance. The fact that agonists such as morphine that do not induce GRK mediated desensitization produce greater tolerance than agonists that do (i.e. DAMGO) (Williams et al., 2001; Hull et al., 2010; Al-Hasani and Bruchas, 2011) desensitization does not appear to be related to antinociceptive tolerance. Similarly MOPr agonists that rapidly recruit \( \beta \)-arrestin and induce internalization produce less tolerance than morphine which does not induce MOPr internalization (Alvarez et al., 2001; Finn and Whistler, 2001; Arttamangkul et al., 2008). Interestingly, \( \beta \)-arrestin2 knockout mice show a decrease in the development of tolerance to morphine, but not fentanyl (Raehal and Bohn, 2011). Genetic modifications of the MOPr that allow morphine to function similarly to these other agonists in regards to MOPr internalization and recycling, leads to
diminished tolerance (Whistler, 2012).

It has been shown that the vPAG contributes to the development of tolerance to various MOPr agonists, including morphine, DAMGO and fentanyl (Tortorici et al., 1999; Morgan et al., 2006; Meyer et al., 2007; Bobeck et al., 2012). Therefore, the vPAG is an important structure in which to study the cellular mechanisms behind the development of tolerance and other opioid actions. Given the known differences in G-protein versus β-arrestin signaling, it is hypothesized that agonists that preferentially activate G-proteins will have different mechanisms that drive antinociception and tolerance than agonists that use a β-arrestin signaling cascade.

In summary, the following studies will investigate the actions of morphine, DAMGO, and fentanyl within the vPAG to determine: 1) the importance of G\textsubscript{i/o}-proteins and internalization in antinociception; 2) the role of ERK1/2 in antinociception and tolerance; and 3) whether cross-tolerance develops between agonists. These agonists were chosen because of their clinical use and extensive use in cellular experiments.
CHAPTER 2:
DIFFERENTIAL ANTINOCICEPTION MECHANISMS FOR MORPHINE, DAMGO, AND FENTANYL WITHIN THE VLPAG

ABBREVIATED TITLE: ANTINOCICEPTION MECHANISMS
Opioids such as morphine bind and activate the MOPr which leads to activation of G-proteins and second messenger signaling proteins. Desensitization of the G-protein signaling is mediated by phosphorylation of the receptor by GRK or other kinases (Kelly et al., 2008; Reiter et al., 2012). Phosphorylation at the serine 375 site on the C-terminal recruits β-arrestin, which then either initiates internalization of the receptor or activation of a distinct signaling cascade (Rodriguez-Munoz et al., 2007; Reiter et al., 2012). Internalization of the receptor occurs by the pinching off of the clathrin coated pit by dynamin, which includes the portion of the membrane containing the activated receptor (Reiter et al., 2012). It is known that these two processes occur simultaneously and may be different depending on the agonist, but their role in mediating antinociception is unclear.

Inhibitory G-proteins have been shown to be important in MOPr signaling and morphine-induced antinociception (Goode and Raffa, 1997; Gintzler and Chakrabarti, 2004). Coupling to G-proteins has been shown to differ between agonists where inhibition of Gi/o-proteins by pertussis toxin (PTX) causes a reduction in morphine-induced antinociception (Bodnar et al., 1990; Goode and Raffa, 1997; Gomes et al., 2002), but only decreases antinociception slightly at equal antinociceptive doses of DAMGO and has no effect on sufentanil-induced antinociception when administered via intracerebroventricular injections (Goode and Raffa, 1997). Another aspect of MOPr actions that differs between agonists is the ability to internalize the receptor: agonists such as morphine cause little internalization whereas the high efficacy agonists DAMGO and fentanyl produce rapid internalization (Finn and Whistler, 2001; Alvarez et al., 2002). The role of MOPr internalization in antinociception is unclear. Blocking
internalization by injecting a dominant negative dynamin peptide (dyn-DN) attenuates dermorphin antinociception (Macey et al., 2010), so it appears that internalization may be important for antinociception to some agonists. These studies indicate that both signaling pathways, G-protein and internalization, contribute to antinociception to some agonists. This hypothesis was tested by comparing antinociception induced by microinjections of three distinct opioids after blocking G-protein or internalization-dependent signaling.

Given the known differences between MOPr agonists in the ability to internalize the receptor, it is hypothesized that morphine uses a predominately G-protein-dependent pathway whereas DAMGO and fentanyl use an internalization-dependent pathway. To test this hypothesis, morphine, DAMGO, and fentanyl were microinjected into the vlPAG following inhibition of either G_{i/o}-proteins or internalization and antinociception and locomotor activity were evaluated.

**Methods**

**Subjects**

The subjects were 124 male Sprague-Dawley rats (230 – 360 g) from Harlan Laboratories (Livermore, CA). Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and implanted with a guide cannula (23 gauge; 9 mm long) aimed at the vlPAG using stereotaxic techniques (AP: +1.7 mm, ML: ±0.6 mm, DV: -4.6 mm from lambda). Two screws were used to anchor the cannula to the skull with dental cement. A 9 mm stylet was inserted into the guide cannula following surgery. Rats were handled daily and allowed to recover for 1 week before testing. Rats were housed in groups until surgery
and were housed individually on a reverse light cycle (lights off at 7:00 AM) after surgery. Food and water were available at all times except during experimental testing. All procedures were approved by the Washington State University Animal Care and Use Committee and conducted in accordance with the guidelines for animal use described by the International Association for the Study of Pain.

**Behavioral testing**

Nociception was assessed using the hot plate test which measures the latency for the rat to lick a hind paw when placed on a 52.5°C hot plate. The rat was removed if no response occurred within 50 s. Those animals that had baseline latencies greater than 25 s were not included in further experimental testing. Activity was measured by placing the rat in an open field (1.0 x 0.6 m) where the number of 15.24 cm X 15.24 cm squares crossed in 30 s was counted.

**Microinjections**

All drugs were purchased from Tocris Bioscience except where noted. Morphine sulfate (a gift from the National Institute on Drug Abuse), DAMGO, fentanyl citrate (Sigma-Aldrich), PTX, scr-dyn, and dyn-DN were dissolved in sterile saline. Drugs were administered through a 31-gauge injection cannula extending 2 mm beyond the guide cannula. One day prior to testing, the injector was inserted into the guide cannula without drug administration to habituate the rat to the microinjection procedure.

**Experiment 1: G-protein induced antinociception**

To test whether G\textsubscript{i/o}-protein activation is required for antinociception, after baseline testing rats were microinjected into the vIPAG with a G\textsubscript{i/o} protein inhibitor (PTX; 5 or 50 ng/0.4 μL or 0.4 μL saline) 24 hours prior to obtaining cumulative dose-
response for morphine, DAMGO, or fentanyl. To determine whether there is a change in
nociception following PTX, hot plate latencies were measured prior to the opioid dose-
response. The morphine procedure involved microinjection of cumulative third-log doses
(1, 2.2, 4.6, 10, 22 μg/0.4 μL) at 20 min intervals followed by hot plate and open field
testing 15 min after each injection. Third-log doses of DAMGO (0.046, 0.1, 0.22, 0.46 &
1 μg/0.4 μL) were administered at 12 min intervals and hot plate and open field testing
were conducted 10 min after each injection. Fentanyl microinjections (0.46, 1, 2.2, 4.6 &
10 μg/0.4 μL) were administered at 4 min intervals with behavioral testing 2 min after
each injection. The timing and doses of PTX and opioids were chosen based on
previous studies following microinjections in the vlPAG (Bodnar et al., 1990; Bobeck et
al., 2009).

**Experiment 2: Internalization induced antinociception**

To test whether antinociception is linked to an internalization process,
internalization was blocked by a dominant negative dynamin peptide (dyn-DN; 2 μg/0.4
μL) or the control scrambled peptide (scr-dyn; 2 μg/0.4 μL) microinjected into the vlPAG
20 min prior to MOPr agonist administration. Hot plate testing was conducted 15 min
following injection prior to cumulative dose-response determination for morphine,
DAMGO, or fentanyl (see Experiment 1).

**Histology and Data Analysis**

Following testing, rats received a lethal dose of halothane. Brains were removed
and stored in formalin (10%) and sliced coronally (100 μm) at least 2 days later to
determine the location of the injection site (Paxinos and Watson, 2005). Only those
injection sites in or adjacent to the vlPAG were included in data analysis (see Figure 1).
Dose-response curves were plotted using Prism 6 (GraphPad Software, Inc; La Jolla, CA) and the half maximal antinociceptive effect ($D_{50}$) was calculated for each group using nonlinear regression. The minimum was set as the mean baseline score and the maximum was set as the cutoff value (50 s). ANOVA or t-tests were conducted to determine statistical significance ($\alpha < 0.05$). A Bonferroni post-hoc analysis was used when necessary. Data are presented as mean ± SEM unless otherwise stated.

**Results**

*Experiment 1: G-protein induced antinociception*

There was no significant difference in baseline hot plate latencies among rats tested with each opioids ($F(2, 66) = 2.30; p = 0.11$) or inhibitor treatment ($F(2, 66) = 0.40; p = 0.67$). Nociception was not altered following PTX treatment alone ($F(2, 72) = 0.50; p = 0.61$). Pretreatment with 5 ng/0.4 μL or 50 ng/0.4 μL of PTX caused a rightward shift in the morphine dose-response (Figure 2A; $F(2, 144) = 4.93; p < 0.05$). Similarly, PTX caused a rightward shift in the DAMGO dose-response (Figure 2B; $F(2, 150) = 5.43; p < 0.05$). However, only the high dose (50 ng /0.4 μL) was significantly different from saline treated (see Table 1 for $D_{50}$s). Pretreatment with PTX did not alter the fentanyl dose-response curve (Figure 2C; $F(2, 138) = 2.05; p = 0.13$). $D_{50}$ values for all opioids are presented in Table 1.

A two-way repeated measures ANOVA for each opioid showed a significant decrease in activity with increasing doses of morphine (Figure 3A; $F(5, 110) = 3.59; p < 0.05$), DAMGO (Figure 3B; $F(5, 120) = 6.89; p < 0.05$), and fentanyl (Figure 3C; $F(5, 105) = 11.50; p < 0.05$). PTX pretreatment did not alter this decrease in activity for
morphine \( F(2, 22) = 0.26; p = 0.77 \), DAMGO \( F(2, 24) = 1.46, p = 0.25 \) or fentanyl \( F(2, 21) = 3.20; p = 0.06 \).

**Experiment 2: Internalization induced antinociception**

Baseline hot plate latencies did not differ significantly between opioid \( F(2, 43) = 1.50; p = 0.23 \) or pretreatment with dynamin inhibitor \( F(1, 43) = 0.04; p = 0.84 \). Nociception was not altered by microinjection of dyn-DN into the vIPAG \( t(47) = 1.14; p = 0.26 \). Dyn-DN did not alter morphine-induced antinociception compared to scr-dyn \( F(1, 98) = 1.81; p = 0.18 \). Pretreatment with dyn-DN caused a rightward shift in the DAMGO dose-response curve \( F(1,85) = 4.77; p < 0.05 \). Interestingly, pretreatment with dyn-DN had the opposite effect (leftward shift) on the fentanyl dose-response curve \( F(1, 98) = 10.47; p < 0.05 \). \( D_{50} \) values are shown in Table 2.

A two-way repeated measures ANOVA for each opioid revealed a significant decrease in activity over time for DAMGO \( F(5, 70) = 6.24; p < 0.05 \) and fentanyl \( F(5, 75) = 17.07; p < 0.05 \), but not for morphine \( F(5, 75) = 1.74; p = 0.13 \). Pretreatment with dyn-DN 20 min prior to opioid cumulative dosing did not alter this decrease in activity compared to scr-dyn for morphine \( F(1, 15) = 0.01; p = 0.94 \) or DAMGO \( F(1, 14) = 1.97, p = 0.18 \). However, dyn-DN caused a significant decrease in activity compared to scr-dyn for fentanyl \( F(1, 15) = 7.38; p < 0.05 \). A Bonferroni post-hoc test revealed that dyn-DN resulted in significantly lower activity scores than scr-dyn at 1 μg and 2.2 μg of fentanyl \( p < 0.05 \).
Discussion

The current study determined that PTX and dyn-DN had ligand-biased effects on antinociception, implying that different mechanisms underlie antinociception to each agonist (G-protein versus internalization). Blockade of $G_{i/o}$-proteins with PTX decreased morphine and DAMGO, but not fentanyl, induced antinociception. Administration of dyn-DN had no effect on morphine antinociception, whereas it had opposite effects on DAMGO (attenuation) and fentanyl (enhancement) antinociception. These data indicate that antinociception is mediated through different signaling pathways for different MOPr agonists.

A potential contributing factor to differences in antinociception is that the affinity and efficacy at the MOPr differs between agonists. It has been shown that MOPr agonists bind and activate different splice variants of the MOPr which may be linked to the ligand-biased effects seen in this study. Fentanyl, but not morphine, antinociception is blocked following deletion of a particular exon on the MOPr (Pan et al., 2009), suggesting that certain agonists preferentially activate certain receptor variants. It is unknown what isoforms are present in the vlPAG. It is also possible that certain agonists activate heterodimers (such as MOPr/DOPr) and other agonists do not (Costantino et al., 2012). This difference could lead to differences in downstream signaling and behavioral outcomes (i.e. antinociception).

Morphine has lower efficacy than DAMGO and fentanyl when using $[^35S]$GTPγS assays (Ammer and Schulz, 1993; Traynor and Nahorski, 1995; McPherson et al., 2010; Madia et al., 2012). Morphine also has a lower antinociceptive efficacy than fentanyl and DAMGO. Morphine antinociception following systemic or intrathecal
administration is decreased via irreversible antagonists to a much greater degree than fentanyl or DAMGO antinociception (Mjanger and Yaksh, 1991; Madia et al., 2009). Differences in G_{i/o}-protein induced antinociception may contribute to this difference in efficacy. However, a recent study from our lab showed that morphine and fentanyl have equal antinociceptive efficacies within the vlPAG (Bobeck et al., 2012), suggesting that these agonists cause differences in intracellular signaling, not in receptor activation within the vlPAG.

It was determined that only the highest dose (50 ng) of PTX altered DAMGO antinociception while morphine antinociception was attenuated at a lower dose (5 ng). Previous studies evaluating the role of G_{i/o}-protein on antinociception have found similar results to the current study with intracerebroventricular administration of PTX and opioids. PTX administration or blockade of G_{i/o}-proteins with antisense caused a greater decrease in morphine antinociception than DAMGO or sufentanil (a fentanyl analog) (Raffa et al., 1994; Goode and Raffa, 1997). An explanation for why fentanyl was not altered by blockade of G_{i/o}-proteins in the current study is that fentanyl preferentially activates G_s-proteins and not G_{i/o}-proteins. Blockade of G_s-proteins with cholera toxin decreases sufentanil antinociception, but not morphine antinociception (Goode and Raffa, 1997).

It is well established that certain agonists cause robust MOPr phosphorylation, desensitization, and internalization. Morphine is typically inferior to DAMGO and fentanyl on all of these aspects of receptor regulation. DAMGO and fentanyl cause phosphorylation of the receptor via GRK whereas morphine uses a PKC mediated mechanism (Kelly et al., 2008). Phosphorylation by GRK at the serine 375 site on the C
terminus of the MOPr leads to desensitization and enhanced β-arrestin recruitment (Rodriguez-Munoz et al., 2007; McPherson et al., 2010; Molinari et al., 2010). In many different tissue preparations it has been found that morphine is very weak at inducing MOPr internalization compared to other agonists including DAMGO and fentanyl (Whistler et al., 1999; Borgland et al., 2003; Celver et al., 2004; McPherson et al., 2010; Melief et al., 2010). These MOPr regulation processes have been proposed to contribute to tolerance and dependence despite minimal behavioral evidence. The purpose of the current study was to link MOPr internalization and antinociception by using an inhibitor of dynamin—that prevents removal of the receptor from the membrane.

Similar to G-protein inhibition, blockade of internalization caused a ligand-biased effect on antinociception. Given that morphine does not readily internalize the receptor, it is not surprising that dyn-DN had no effect on morphine antinociception. It was hypothesized that blocking internalization would decrease antinociception similarly for DAMGO and fentanyl since both agonists readily internalize the receptor. However, the current study found opposite effects for these two MOPr agonists. DAMGO-induced antinociception was attenuated by inhibiting dynamin, similar to a previous study using the internalizing MOPr agonist, dermorphin (Macey et al., 2010). In contrast, inhibition of dynamin caused an enhancement of fentanyl-induced antinociception. A major difference between fentanyl and the other agonists is the duration of antinociception. Fentanyl produces a rapid (3 min) and short-lived (< 30 min) peak effect in comparison to morphine, DAMGO, and dermorphin, which show a peak effects of 15-30 min and which last at least an hour following the vlPAG administration (Bobeck et al., 2009;
Macey et al., 2010). The short antinociceptive effect of fentanyl may be caused by rapid internalization and recycling of the receptor, which leads to desensitization of the G-protein signaling. By inhibiting dynamin, this may slow the desensitization process since the receptor is unable to internalize, thus enhance antinociception. It is possible that internalization terminates the signaling that is driving antinociception. Inhibition of dynamin allows for this signaling to continue.

Morphine, DAMGO, and fentanyl caused a comparable decrease in locomotor activity. This is similar to previous studies comparing morphine and fentanyl in the spinal cord and other species (Durant and Yaksh, 1986; Kamata et al., 2011). Inhibition of G_{i/o}-proteins did not alter the inhibition of locomotion caused by opioids. However, the decrease in locomotor activity was strengthened by inhibition of internalization following fentanyl administration, likely due to preventing termination of the G-protein signaling, which matches the effect seen in antinociception.

In conclusion, certain MOPr agonists such as morphine and DAMGO produce antinociception via a G_{i/o}-protein dependent mechanism. However, antinociception may be enhanced or decreased via internalization of the receptor. These data reveal a functional outcome caused by ligand-biased signaling at the MOPr.
Figure 1: Location of injection sites within the vlPAG. Experiment 1 is shown on left side of each section. Open circles are saline pretreated, closed diamonds are 5 ng/0.5 µL PTX, and closed circles are 50 ng/0.5 µL PTX. Experiment 2 is on the right. Open squares are scr-dyn (2 µg/0.5 µL) and closed squares are dyn-DN (2 µg/0.5 µL). All placements were located on the right side of the brain, but are separated here for clarity.
Figure 2: Differential alteration in antinociception following G-protein inhibition. Pretreatment with 5 ng/0.4 μL or 50 ng/0.4 μL of PTX prior to (A) morphine, (B) DAMGO, and (C) fentanyl dose-response. PTX significantly decreased antinociception for morphine (5 ng & 50 ng) and DAMGO (50 ng), but did not alter fentanyl antinociception.
Figure 3: Opioid mediated decrease in activity is not dependent on G-proteins. Pretreatment with 5 ng/0.4 μL or 50 ng/0.4 μL of PTX prior to (A) morphine, (B) DAMGO, and (C) fentanyl dose-response. All opioids caused a decrease in locomotor activity with higher doses regardless of PTX pretreatment.
Figure 4: Ligand-biased effects on antinociception following inhibition of internalization. Pretreatment with dyn-DN (2 μg/0.4 μL) 20 min prior to opioid dose-response had no effect on (A) morphine, decreased antinociception to (B) DAMGO, and enhanced antinociception to (C) fentanyl compared to scr-dyn (2 μg/0.4 μL).
Figure 5: Inhibition of internalization altered activity following fentanyl, but not morphine or DAMGO exposure. Rats were pretreated with dyn-DN (2 μg/ 0.4 μL) or a scrambled peptide 20 min prior to (A) morphine, (B) DAMGO, or (C) fentanyl dose cumulative dosing. Pretreatment with dyn-DN caused a significant decrease in fentanyl-induced reduction of activity.
Table 1: Comparison of D$_{50}$ values following G-protein inhibition

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine D$_{50}$ ± C.I. (n)</th>
<th>DAMGO D$_{50}$ ± C.I. (n)</th>
<th>Fentanyl D$_{50}$ ± C.I. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.98 ± 1.32 (9)</td>
<td>0.087 ± 0.044 (10)</td>
<td>1.03 ± 0.30 (8)</td>
</tr>
<tr>
<td>5ng PTX</td>
<td>9.11 ± 4.33 (7)*</td>
<td>0.160 ± 0.088 (8)</td>
<td>1.50 ± 0.50 (8)</td>
</tr>
<tr>
<td>50ng PTX</td>
<td>7.85 ± 2.59 (9)*</td>
<td>0.517 ± 0.343 (8)*</td>
<td>1.67 ± 0.64 (8)</td>
</tr>
</tbody>
</table>

Notes:
D$_{50}$s are presented in μg doses
C.I. – 95% confidence interval
* – statistically different from saline
Table 2: Comparison of D50 values following inhibition of internalization

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine D50 ± C.I. (n)</th>
<th>DAMGO D50 ± C.I. (n)</th>
<th>Fentanyl D50 ± C.I. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scr-dyn</td>
<td>2.02 ± 0.70 (9)</td>
<td>0.040 ± 0.015 (8)</td>
<td>1.63 ± 0.69 (9)</td>
</tr>
<tr>
<td>dyn-DN</td>
<td>2.83 ± 0.96 (8)</td>
<td>0.123 ± 0.070 (7)*</td>
<td>0.73 ± 0.13 (8)*</td>
</tr>
</tbody>
</table>

Notes:
D50s are presented in μg doses
C.I. – 95% confidence interval
* – statistically different from scr-dyn
CHAPTER 3:

INHIBITION OF ERK1/2 ALTERS ANTINOCICEPTION AND TOLERANCE TO DAMGO, BUT NOT TO FENTANYL
MOPr agonists cause activation and inhibition of many different intracellular signaling pathways following binding to the MOPr. The best characterized route is through G-proteins which activate downstream effectors, such as adenylyl cyclase and protein kinase C (PKC). However, signaling proteins may also be activated in a G protein-independent, β-arrestin-dependent mechanism. β-arrestin activation and recruitment to the receptor leads to activation of a separate group of signaling proteins (e.g., ERK1/2) that are not activated by G-proteins. Moreover, β-arrestin initiates receptor internalization (Shenoy et al., 2006; DeFea, 2010). It has been shown extensively in other receptor systems that certain agonists preferentially activate only G-proteins while other agonists also activate β-arrestins and their respective downstream effectors (Lefkowitz and Shenoy, 2005; Shenoy and Lefkowitz, 2005; Drake et al., 2008). Recent studies on the opioid receptors have shown that some agonists (DAMGO and fentanyl) recruit β-arrestin equal to their activation of G-proteins, while morphine preferentially activates G-proteins while minimally recruiting β-arrestin (McPherson et al., 2010; Molinari et al., 2010). It is likely that this difference leads to activation of different intracellular signaling pathways and it was hypothesized that ERK1/2 is activated following DAMGO and fentanyl, but not morphine exposure.

The role of intracellular signaling pathways on opioid tolerance has been studied extensively, yet research has focused on morphine and evaluation of other opioids is lacking (Williams et al., 2001; Christie, 2008). Despite minimal activation of ERK1/2 in vitro, inhibition of ERK1/2 has been shown to prevent or enhance the development of morphine tolerance depending on injection site (Macey et al., 2009; Wang et al., 2010).
Given that acute tolerance mechanisms appear to be different between MOPr agonists, tolerance to prolonged administration are likely different as well. Blockade of G-protein associated signaling proteins (JNK or PKC) prevents acute tolerance to morphine, but not DAMGO or fentanyl. Conversely, blockade of internalization-dependent signaling pathways prevent tolerance to fentanyl and DAMGO, but not morphine (Hull et al., 2010; Melief et al., 2010). Differences in tolerance within the internalization pathway following longer administration have yet to be investigated.

Repeated microinjections into the vlPAG (a key brain region involved in pain modulation) of morphine, DAMGO, or fentanyl produce antinociceptive tolerance (Morgan et al., 2006; Meyer et al., 2007; Bobeck et al., 2012). Given that DAMGO and fentanyl activate ERK1/2 in cellular studies, it was hypothesized that ERK1/2 is activated following DAMGO and fentanyl administration in the vlPAG and tolerance will be attenuated following inhibition of ERK1/2.

**Methods**

**Subjects**

145 male Sprague-Dawley rats between 220 – 360 g were used. General procedures and stereotaxic surgery targeting the vlPAG were performed as in Chapter 2. Only injection placements within the boundary of the vlPAG were used (see Chapter 2 Figure 1).

*Experiment 1: ERK1/2 activation following opioid microinjection into the vlPAG*

The purpose of this experiment was to evaluate ERK1/2 activation within the vlPAG following MOPr agonist exposure in an intact animal. Rats received
microinjections of saline, morphine, DAMGO, or fentanyl into the vPAG. To test whether ERK1/2 was activated via G-protein or internalization-dependent mechanism, a subset of rats were microinjected with PTX (50 ng/0.4 μL) or dyn-DN (2 μg/0.4 μL) prior to opioid administration. PTX or saline was administered one day prior to opioid administration, whereas dyn-DN or the scramble control peptide dynamin-scr was injected 20 minutes prior based on previous vPAG studies (Bodnar et al., 1990; Macey et al., 2010).

For immunohistochemistry, rats were deeply anesthetized with pentobarbital (150 mg/kg i.p.) 25 min after opioid injection and then perfused transcardially through the ascending aorta with 10 mL heparinized saline followed by 400-600 mL of 4% paraformaldehyde in 0.1M phosphate buffer (PB). Brains were postfixed in 4% paraformaldehyde for 30 min and then stored in 0.1M PB for up to 15 hours. Immunohistochemistry was performed on coronal brain slices (40 μm) containing the vPAG. Every second section was incubated in 1% sodium borohydride in PB for 30 min followed by another 30 min incubation with 0.3% H₂O₂ in 0.1M PB. The blocking reagent was then used: 0.5% bovine serum albumin in 0.1M Tris buffered saline for 30 min. The tissue was incubated in primary rabbit antibody against phospho-p44/42 MAPK (ERK 1/2) (1:400; Cell signaling, Beverly, MA) in Tris buffered saline containing 0.1% bovine serum albumin and 0.25% TritonX-100, for 42 hours at 4°C. Bound ERK1/2 antibody was visualized by incubating tissue in biotinylated goat-anti rabbit IgG secondary antibody (1:400 Vector Laboratories, Burlingame, CA) secondary antibody for 30 min. This was followed by incubation in Avidin-Biotin (Elite Vectastain ABC kit; Vector Laboratories) for 30 min and then diaminobenzidine-hydrogen peroxidase (DAB-H₂O₂)
for 3 min. Brain slices were mounted, dehydrated and then coverslipped with DPX mounting medium (Sigma-Aldrich, St. Louis, MO). Three sections per animal containing vIPAG were analyzed. Images were taken with an Olympus DP71 digital camera mounted on an Olympus BX51 microscope. Sections containing injection site and sections within 150 µm away were quantified within a 300X300 µm² region. Depending on location of injection, up to 3 separate boxes were used to equal 9000 µm² area. To avoid damaged tissue, the region was chosen 50 µm away from injection site. The number of pERK-positive cells was assessed using ImageJ particle analysis (National Institutes of Health; Bethesda, MA).

**Experiment 2: The role of ERK1/2 inhibition on opioid antinociception and tolerance**

The purpose of this experiment was to evaluate the role of ERK1/2 on antinociception and tolerance to DAMGO and fentanyl. A MEK inhibitor (U0126) which prevents phosphorylation of ERK1/2, was microinjected into the vIPAG 20 min prior to MOPr agonist administration (Macey et al., 2009). The inhibitor was dissolved in 20% DMSO/80% saline and MOPr agonists were dissolved in saline. On Trial 1, hot plate and open field tests were conducted 20 min after DAMGO microinjection and 3 min after fentanyl microinjection. Tolerance was induced via twice daily microinjection for two days of DAMGO or fentanyl. To evaluate the role of ERK1/2 on the development of tolerance, a subset of rats received U0126 (100 ng/0.5 µL) or 20% DMSO in saline (0.5 µL) 20 min prior to each pretreatment injection. Tolerance was assessed on Trial 5 using a cumulative dosing procedure where third-log doses of DAMGO (0.046, 0.1, 0.22, 0.46, & 1 µg/0.4 µL) or fentanyl (0.46, 1, 2.2, 4.6, 10 µg/0.4 µL) were administered. To evaluate the expression of tolerance, a subset of rats received U0126
(100 ng/0.5 μL) or 20% DMSO (0.5 μL) 20 min prior to cumulative dosing procedure in rats made pretreated with repeated injections of DAMGO or fentanyl. Hot plate and open field tests were conducted as in Chapter 2.

**Results**

*Experiment 1: ERK1/2 activation following opioid microinjection into the vlPAG*

Microinjection into the vlPAG of morphine and DAMGO, but not fentanyl, caused an increase in pERK1/2 immunoreactivity (Figure 1). DAMGO microinjection caused an increase in pERK1/2 positive cells compared to saline ($F(3, 11) = 4.62; p < 0.05$). Pretreatment with dyn-DN caused a decrease in the number of pERK1/2 cells, although it was not a statistically significant decrease compared to rats treated with DAMGO alone (Bonferroni, $p = 0.10$). Morphine microinjection caused a significant increase in pERK1/2 positive cells ($F(3, 11) = 14.87; p < 0.05$). A Bonferroni post-hoc test revealed that morphine caused significantly greater ERK1/2 activation compared to rats pretreated with saline, morphine+dyn-DN, and morphine+PTX ($p < 0.05$). Fentanyl failed to cause an increase in pERK1/2 labeled cells ($F(3, 14) = 1.24; p = 0.33$).

*Experiment 2: The role of ERK1/2 inhibition on opioid antinociception and tolerance*

Given that there was a differential activation of ERK1/2 following microinjection of DAMGO and fentanyl, it is hypothesized that blocking ERK1/2 will have different effects on behavior. Microinjection of DAMGO caused an increase in hot plate latency on Trial 1 compared to saline pretreated rats ($F(3, 52) = 59.24; p < 0.05$) which was not altered by prior injection of U0126 (Figure 2A; Bonferroni; $p > 0.05$). Similarly, microinjection of fentanyl increased hot plate latency ($F(3, 39) = 14.38; p < 0.05$) which was not altered
by U0126 (Figure 2B; Bonferroni; p > 0.05). DAMGO and fentanyl decreased locomotor activity at 20 and 3 min respectively (Figure 3; $F(3, 51) = 6.21; p < 0.05; F(3, 55) = 3.48; p < 0.05$). This decrease was prevented by pretreatment with U0126 20 min prior to either MOPr agonist (Bonferroni; p > 0.05 compared to saline controls).

There was no significant difference between baseline hot plate latencies conducted on Trial 5 prior to DAMGO cumulative dosing procedure ($F(5, 46) = 0.970; p = 0.446$). ERK inhibition caused a rightward shift in the DAMGO dose-response curve in rats that were pretreated with saline for 2 days (Figure 4A; $F(1, 98) = 34.10; p < 0.05$). Four pretreatment injections of DAMGO caused a rightward shift in the dose-response curve ($F(1, 98) = 21.81; p < 0.05$), which was reversed with administration of U0126 20 min prior to the DAMGO dose-response (Figure 4B; $F(1, 86) = 4.94; p < 0.05$). Similarly, pretreatment with U0126 20 min prior to DAMGO during tolerance induction caused a reversal of the rightward shift seen with DAMGO alone (Figure 5; $F(1, 104) = 4.86; p < 0.05$). $D_{50}$ values are shown in Table 1. DAMGO caused a dose-dependent decrease in activity on Trial 5 ($F(5, 165) = 3.68; p < 0.05$) which was not altered by pretreatment Trials 1-4 ($F(3, 33) = 1.44; p = 0.25$).

There was no significant difference between baseline hot plate latencies conducted on Trial 5 prior to fentanyl cumulative dosing procedure ($F(5, 53) = 0.78; p = 0.57$). In contrast to DAMGO, U0126 had no effect on the fentanyl dose-response on Trial 5 for saline (Figure 6A; $F(1, 128) = 0.04; p = 0.83$) or fentanyl pretreated rats (Figure 6B; $F(1, 110) = 0.70; p = 0.40$). A rightward shift in the fentanyl dose-response was found for those rats pretreated with fentanyl for 2 days as would be expected with the development of tolerance ($F(1, 122) = 7.14; p < 0.05$) which was not altered by co-administration of
U0126 (Figure 7; $F(1, 116) = 2.53; p = 0.12$). Similar to DAMGO, microinjection of fentanyl into the vlPAG caused a dose-dependent decrease in activity ($F(5, 170) = 15.66; p < 0.05$) that was not altered by pretreatment with U0126 or fentanyl ($F(3, 34) = 0.79; p = 0.51$).

**Discussion**

The current study found ligand-biased effects on ERK1/2 activation and its role in antinociception and tolerance. It was determined that DAMGO microinjection into the vlPAG led to an increase in pERK1/2 labeled cells, which is likely dependent on internalization. Morphine also caused an increase in pERK1/2 positive cells, which was prevented by inhibition of $G_{i/o}$-proteins or internalization. Similarly, ERK1/2 is important for DAMGO, but not fentanyl, antinociception and tolerance. Blockade of ERK1/2 phosphorylation in saline pretreated rats caused a rightward shift in the DAMGO dose-response which reveals that it is a key protein required to produce antinociception. Repeatedly blocking ERK1/2 activation in combination with DAMGO pretreatment led to a decrease in the development of tolerance and blockade of ERK1/2 activation in DAMGO tolerant animals decreased the expression of tolerance. Inhibition of ERK1/2 activation did not alter antinociception or tolerance to fentanyl, however, it did reverse the fentanyl-induced decrease in locomotor activity.

Cellular studies have shown that ERK1/2 is activated following acute administration of fentanyl and DAMGO, but not morphine (Macey et al., 2006; Zheng et al., 2011). Given these studies, it is surprising that fentanyl did not cause activation of ERK1/2 in the vlPAG nor did blockade of ERK1/2 alter fentanyl antinociception. It is
possible that fentanyl does not activate ERK1/2 in the vIPAG even though it has been shown to be activated in heterologous cell systems and striatal cultured neurons (Macey et al., 2006; Zheng et al., 2008). Acute morphine-induced ERK1/2 activation is brain-region specific: regions such as anterior cingulate and locus ceruleus show an increase in pERK1/2, whereas the nucleus accumbens and central amygdala show a decrease in pERK1/2 (Eitan et al., 2003). Prolonged morphine treatment showed an increase in pERK1/2 within the vIPAG, but a decrease in a cell line (Bilecki et al., 2005; Macey et al., 2009). Although ERK1/2 is typically considered to be activated in a β-arrestin dependent manner, some agonists such as morphine may activate ERK1/2 via a different signaling mechanism. For example, morphine activates ERK1/2 in a PKC and/or calmodulin dependent mechanism, whereas DAMGO and fentanyl use a β-arrestin dependent pathway (Belcheva et al., 2005; Zheng et al., 2011). The current study using PTX and dyn-DN indicate that morphine activates ERK1/2 via both mechanisms whereas DAMGO only uses an internalization process.

Previous studies have shown that ERK1/2 contributes to nociception associated with inflammation, such as in the formalin test (Dai et al., 2011). In contrast, there is no alteration in mechanical or thermal nociception in ERK1 knockout mice (Alter et al., 2010). Activation of ERK1/2 (phosphorylation) has been found in the PAG, dorsal root ganglion, spinal cord, and amygdala following a variety of noxious stimuli and chronic pain models (Gioia et al., 2003; Ji et al., 2009). DAMGO suppresses c-fiber activation of pERK in nerve ligated animals, suggesting a role of ERK in analgesia (Kawasaki et al., 2006). These studies indicate that ERK1/2 may be involved in certain types of pain and that opioids may function to reverse ERK1/2 activation.
Inhibition of ERK1/2 activation had no effect on hot plate latency in the current study. Administration of U0126 20 min prior to DAMGO microinjection also did not alter antinociception on Trial 1, although U0126 caused a rightward shift in the DAMGO dose-response in vehicle treated animals on Trial 5. The dose of DAMGO used on Trial 1 may have been too high to be altered by the inhibitor. The timing is also different between these two paradigms. It is unlikely that multiple injections of the vehicle altered ERK1/2 activation, because multiple injections had no effect on fentanyl antinociception or tolerance. Morphine antinociception was not altered in a previous study using this same paradigm (Macey et al., 2009).

Opioids showed distinct effects on opioid-induced antinociception versus locomotor suppression. The Trial 1 decrease in locomotor activity due to DAMGO and fentanyl was prevented by inhibition of ERK1/2, which suggests that ERK1/2 may be involved in the sedative effects of these agonists. It is surprising that activity was not altered similarly following cumulative dosing on Trial 5. Therefore, this effect on activity may be dependent on dose or timing differences between these paradigms.

Studies investigating the role of ERK1/2 on morphine tolerance have found mixed results depending on injection site. Co-administration of intrathecal morphine with a MEK inhibitor attenuated the development of tolerance to morphine (Wang et al., 2010). Another study found that morphine tolerance was not altered by ERK1/2 inhibition following systemic administration (Mouledous, 2007). Inhibition of ERK1/2 activation enhances tolerance to morphine within the vIPAG (Macey et al., 2009) revealing that activation of ERK1/2 may counteract tolerance. The present study shows that ERK even has distinct effects within the vIPAG depending on the opioid injected:
ERK1/2 counteracts (morphine), enhances (DAMGO), or has no effect (fentanyl) on tolerance to MOPr agonists in the vlPAG. A previous study on acute tolerance found that tolerance to DAMGO, but not morphine or fentanyl, was prevented by GRK inhibition (Hull et al., 2010). These results are similar to the current study, since ERK1/2 is thought to be downstream of GRK/β-arrestin (Macey et al., 2006; Shenoy et al., 2006). The current study is the first to explicitly evaluate the role of ERK1/2 in tolerance to these other MOPr agonists.

It is unclear how phosphorylated ERK1/2 alters opioid function. Two possibilities include ERK-mediated gene transcription or changes in synaptic transmission. ERK1/2 alters several epigenetic markers and transcription factors including c-fos, brain-derived neurotrophic factor, and cAMP response element binding proteins (CREB) in several brain regions following morphine withdrawal (Wang et al., 2012; Ciccarelli et al., 2013). Acute exposure to opioids inhibits adenylyl cyclase which subsequently inhibits cAMP and CREB. Conversely, long-term opioid exposure causes an upregulation of adenylyl cyclase-cAMP-CREB pathway (Cao et al., 2010). ERK1/2 activation may contribute to upregulation of this pathway through activation of PKC after morphine withdrawal, although the exact mechanism remains unknown (Martin et al., 2011).

ERK1/2 also has been implicated in altering synaptic transmission. ERK1/2 inhibition has been found to directly increase synaptic vesicle exocytosis via calcium channels (Subramanian and Morozov, 2011). Given that opioids in the vlPAG inhibit GABA release in order to produce antinociception (Morgan et al., 2003; Heinricher et al., 2009), an increase in GABA release would require a higher dose of the agonist to
overcome the unbalanced GABA to produce antinociception, which would resemble the rightward shift seen in tolerance.

In conclusion, ERK1/2 plays different roles in antinociception and tolerance to MOPr agonists in the vIPAG. DAMGO antinociception is dependent on activation of ERK1/2, while morphine and fentanyl antinociception are not. Activation of ERK1/2 counteracts tolerance to morphine, enhances tolerance to DAMGO, and has no effect on tolerance to fentanyl. This adds to the growing body of research on ligand-biased signaling at the MOPr.
Figure 1: ERK1/2 activation following opioid exposure. Representative photomicrographs of pERK1/2 immunoreactivity in vIPAG following pretreatment of saline (A), DAMGO (B), and DAMGO+dyn-DN. Quantification of pERK1/2 immunoreactivity 25 min following microinjection of 5 µg/0.4 µL morphine (D), 0.5 µg/0.4 µL DAMGO (E), and 3 µg/0.4 µL fentanyl into the vIPAG. A subset of rats were pretreated with dyn-DN (2 µg/0.4 µL) 20 min prior or PTX (50ng/0.4 µL) 24 hours prior to opioid pretreatment. *-statistically different from saline, #- statistically different from all other groups (p < 0.05). Morphine and DAMGO caused a significant increase in pERK1/2, which was prevented by pretreatment with dyn-DN or PTX for morphine.
Figure 2: Antinociception on Trial 1 following vPAG microinjection. (A) Rats were microinjected with vehicle (20% DMSO) or U0126 (100ng/0.5 μL) 20 min prior to saline (0.4 μL), DAMGO (0.5 μg/0.4 μL) and tested on the hot plate test 20 min later. (B) Microinjection of vehicle or U0126 20 min prior to saline or fentanyl (3μg/0.4 μL) followed by the hot plate test 3 min later. Data are presented as mean ± SEM. *-significant from saline or U0126+Saline (p < 0.05). These data show that DAMGO and fentanyl cause an increase in hot plate latency which is not altered by ERK1/2 inhibition.
Figure 3: ERK1/2 inhibition prevents reduction in activity by opioids. A) Rats were microinjected with vehicle (20% DMSO) or U0126 (100ng/0.5 μL) 20 min prior to saline (0.4 μL), DAMGO (0.5 μg/0.4 μL). Activity was measured for 30s on the open field test 20 min after injection. (B) Microinjection of vehicle or U0126 20 min prior to saline or fentanyl (3μg/0.4 μL) followed by the open field test 3 min later. Data are presented as mean ± SEM. * - significant from saline (p < 0.05). DAMGO and fentanyl cause a decrease in activity which is blocked by prior inhibition of ERK1/2.
Figure 4: ERK1/2 inhibition on the expression of DAMGO tolerance. Rats were pretreated with 0.4 µL saline (A) or 0.5 µg/0.4 µL DAMGO (B) twice daily for two days. On Day 3, rats received an injection of vehicle (20% DMSO) or U0126 (100 ng/0.5 µL) 20 min prior to DAMGO dose-response. ERK inhibition caused a decrease in DAMGO antinociception and reversed the expression of tolerance in DAMGO-tolerant rats.
Figure 5: ERK1/2 prevents the development of DAMGO tolerance. Rats were pretreated with twice daily microinjections of saline (0.4 μL) or DAMGO (0.5 μg/0.4 μL). A subset of rats received a microinjection of U012620 min prior to each DAMGO microinjection. On Trial 5, all rats recived cumulative doses of DAMGO. ERK1/2 inhibition in combination with DAMGO prevented the development of tolerance to DAMGO.
Figure 6: ERK1/2 inhibition does not alter fentanyl tolerance. Rats were pretreated with 0.4 µL saline (A) or 3 µg/0.4 µL fentanyl (B) twice daily for two days. On Trial 5, rats received an injection of vehicle (20% DMSO) or U0126 (100 ng/0.4 µL) 20 min prior to fentanyl dose-response. Fentanyl antinociception or expression of tolerance was not altered by ERK1/2 inhibition.
Figure 7: ERK1/2 inhibition does not alter the development of tolerance to fentanyl. Rats were pretreated with twice daily microinjections of saline (0.4 μL) or fentanyl (3 μg/0.4 μL). A subset of rats received a microinjection of U0126 (100 ng/0.4 μL) 20 min prior to each fentanyl microinjection. On Trial 5, all rats received cumulative doses of fentanyl. Fentanyl tolerance was not dependent on ERK/12 activation.
Table 1: Comparison of $D_{50}$ values following ERK inhibition

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>$DAMGO \ D_{50} \pm \ C.I. \ (n)$</th>
<th>$Fentanyl \ D_{50} \pm \ C.I. \ (n)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.087 ± 0.033 (9)</td>
<td>2.16 ± 0.44 (11)</td>
</tr>
<tr>
<td>Opioid</td>
<td>0.340 ± 0.125 (8)*</td>
<td>3.42 ± 0.90 (10)*</td>
</tr>
<tr>
<td>U0126+Sal</td>
<td>0.530 ± 0.252 (10)*</td>
<td>1.79 ± 0.48 (8)</td>
</tr>
<tr>
<td>U0126+Opioid</td>
<td>0.152 ± 0.064 (10)#</td>
<td>2.62 ± 0.52 (10)</td>
</tr>
<tr>
<td>Saline+T5 U0126</td>
<td>0.482 ± 0.179 (8)*</td>
<td>2.24 ± 0.58 (11)</td>
</tr>
<tr>
<td>Opioid+T5 U0126</td>
<td>0.196 ± 0.044 (7)#</td>
<td>3.96 ± 0.82 (9)*</td>
</tr>
</tbody>
</table>

Notes:
* - statistically different from saline ($p < 0.05$)
# - statistically different from opioid ($p < 0.05$)
CHAPTER 4:

SELECTIVE CROSS-TOLERANCE BETWEEN SYSTEMIC OR vIPAG ADMINISTRATION OF DIFFERENT OPIOIDS IN THE RAT
Opioids are the most commonly used drugs to treat chronic pain. A downside of using opioids for chronic pain is that repeated administration leads to the development of tolerance, a decrease in the responsiveness of the drug following repeated exposure. Opioid rotation is a commonly used technique clinically to enhance pain relief and overcome the development of tolerance to a particular agonist (Slatkin, 2009). However, the degree of cross-tolerance remains debatable. Studies investigating the mechanism of tolerance have been conducted in animals which have revealed mixed results on the magnitude of cross-tolerance. Continuous systemic fentanyl administration produces minimal cross-tolerance to morphine (Madia et al., 2009; Sirohi et al., 2009): however, pretreatment with morphine produces cross-tolerance to fentanyl (Romero et al., 2010). Studies comparing cross-tolerance between morphine and DAMGO have also had mixed results. We have previously shown that rats pretreated with DAMGO in the vIPAG are cross-tolerant to morphine microinjection (Meyer et al., 2007), but this is not true after spinal administration, in which minimal cross-tolerance between these agonists was observed (Russell et al., 1987).

It is well established that the degree of MOPr activation, desensitization, β-arrestin recruitment, and subsequent internalization differs depending on the opioid (Finn and Whistler, 2001; Williams et al., 2001; Alvarez et al., 2002; Virk and Williams, 2008). These differences in signaling may cause differences in tolerance development. Previous studies have linked G-protein mediated pathways as contributing to acute tolerance to morphine and β-arrestin dependent pathways as contributing to acute tolerance to higher efficacy agonists such as fentanyl and DAMGO (Hull et al., 2010;
Melief et al., 2010). Given these known differences in MOPr regulation and intracellular signaling, it was hypothesized that cross-tolerance to repeated opioid administration would not develop between opioids. To test this hypothesis, rats were treated with subcutaneous injections of saline, morphine, or fentanyl and then received a different opioid on Trial 5. To investigate the role of the vlPAG in cross-tolerance, microinjections of saline, morphine, DAMGO, or fentanyl were administered twice a day for two days and cross-tolerance to a different agonist was evaluated on Trial 5.

**Methods**

**Subjects**

The subjects were 130 male Sprague-Dawley rats (240 – 330 g) from Harlan Laboratories (Livermore, CA). A subset of rats (n = 72) were anesthetized with pentobarbital (60 mg/kg i.p.) and implanted with a guide cannula aimed at the vlPAG using stereotaxic techniques. All surgical and testing procedures were the same as in Chapter 2. Only injection sites within the vlPAG were included in analysis (See Chapter 2, Figure 1).

**Experiment 1: Cross-tolerance between systemic morphine and fentanyl**

The purpose of this experiment is to examine cross-tolerance between morphine and fentanyl using the same multiple systemic injection paradigm since previous studies were not consistent in tolerance induction paradigms. Tolerance was induced by twice daily subcutaneous injections for 2 days, with saline (1 mL/kg), morphine (5 mg/kg/mL), or fentanyl (80 µg/kg/mL). On Trial 1, antinociception and activity were assessed at 5 & 30 min following the injection. Cross-tolerance was evaluated on Trial 5 by injecting the
same or a different MOPr agonist using a cumulative dosing procedure (e.g., morphine in rats pretreated with fentanyl). This procedure involved injecting 5 third-log doses of morphine (1, 2.2, 4.6, 10, & 22 mg/kg) at 20 min intervals. Hot plate and open field tests were conducted 15 min after each injection. Similarly, 5 third-log doses of fentanyl (4.6, 10, 22, 46, & 100 µg/kg) were administered at 15 min intervals and behavioral testing was conducted 10 min after each injection. To confirm that tolerance development occurred to morphine and fentanyl occurred under these conditions, a subset of rats received the same MOPr agonist on all trials.

**Experiment 2: Cross-tolerance between vlPAG morphine, DAMGO, and fentanyl**

To assess the role of the vlPAG in cross-tolerance between MOPr agonists, tolerance was induced via twice daily microinjections for 2 days with saline (0.4 µL), morphine (5 µg/0.4 µL), DAMGO (0.5 µg/0.4 µL), or fentanyl (3 µg/0.4 µL) (Morgan et al., 2006; Bobeck et al., 2012). Antinociception and locomotor activity were assessed on Trial 1 using the hot plate test at 30 (morphine), 20 (DAMGO), or 3 (fentanyl) min post-injection. On Trial 5, rats received a cumulative dose-response of a different agonist to assess cross-tolerance. The cumulative dosing procedure involved microinjecting third-log doses of morphine (1, 2.2, 4.6, 10 & 22 µg/0.4 µL) at 20 min intervals. The hot plate and open field tests were conducted 15 min following each injection. Cumulative third-log doses of DAMGO (0.046, 0.1, 0.22, 0.46, & 1 µg/0.4 µL) were administered at 12 min intervals with behavioral testing 10 min after each injection. Fentanyl cumulative dosing was done in a similar fashion except doses (0.46, 1, 2.2, 4.6, 10 µg/0.4 µL) were administered at 4 min intervals and tested 2 min after each injection. These doses and procedure were based on previous studies assessing antinociception and tolerance to
these agonists following microinjections in vIPAG (Meyer et al., 2007; Bobeck et al., 2009; Bobeck et al., 2012).

Histology and Data Analysis

Following testing, rats received a lethal dose of Halothane. Brains were removed and stored in formalin (10%) and sliced coronally (100 μm) at least 2 days later to determine the location of the injection site (Paxinos and Watson, 2005). Only data from rats with injection sites in or adjacent to the vIPAG were included in data analysis. Location of injection sites was similar to Experiment 1 (see Figure 2.1). Dose-response curves were plotted using Prism 6 (GraphPad Software, Inc) and the half maximal antinociceptive effect (D$_{50}$) was calculated for each group. ANOVA was used to determine statistical significance (α < 0.05). A Bonferroni post-hoc analysis was used when necessary. Data are presented as mean ± SEM unless otherwise stated.

Results

Experiment 1: Cross-tolerance between systemic morphine and fentanyl

Subcutaneous injection of morphine and fentanyl caused a significant increase in hot plate latency compared to saline controls on Trial 1 (Figure 1A; F$_{(2, 55)}$ = 302.8; p < 0.05). Fentanyl produced a rapid and robust antinociceptive effect at 5 and 30 min following injection (Bonferroni, p < 0.05). Morphine antinociception was not as rapid and produced a significant increase in hot plate latency only at 30 min post injection (Bonferroni, p < 0.05). Morphine and fentanyl both caused a decrease in locomotor activity compared to saline controls (Figure 1B; F$_{(2, 40)}$ = 29.09; p < 0.05). This difference
was significant for fentanyl at 5 and 30 min (Bonferroni, p < 0.05) and only at 30 min for morphine (Bonferroni, p < 0.05).

Pretreatment with 4 injections of morphine caused a rightward shift in the morphine (Figure 2A; F(1, 110) = 20.36; p < 0.05) and fentanyl (Figure 2B; F(1, 105) = 26.98; p < 0.05) dose-response curves. However, fentanyl pretreatment did not cause a rightward shift in the morphine dose response curve (Figure 2A; F(1, 116) = 0.077; p > 0.05) despite causing a significant shift in fentanyl potency (Figure 2B; F(1, 112) = 10.93; p < 0.05). D_{50} values are presented in Table 1 for comparison.

On Trial 5, there was a significant difference in baseline locomotor activity scores between saline and opioid pretreated animals (F(2, 56) = 3.98; p < 0.05) data not shown. A post-hoc test determined morphine to have significantly greater activity compared to saline controls (Bonferroni, p < 0.05). A two-way ANOVA found a significant decrease in activity with increasing doses of morphine (F(5, 130) = 14.35; p < 0.05) regardless of pretreatment group (F(2, 26) = 1.64; p = 0.21). In contrast, fentanyl caused a dose-dependent reduction in activity (F(4, 116) = 11.93; p < 0.05) that was significantly different for those pretreated with fentanyl or morphine and saline pretreated rats (F(2, 29) = 6.05; p < 0.05).

**Experiment 2: Cross-tolerance between vlPAG morphine, DAMGO, and fentanyl**

Microinjection of morphine, DAMGO, and fentanyl into the vlPAG caused a significant increase in hot plate latency compared to saline controls (30, 20, and 3 min post-injection, respectively) (Figure 3A; F(3, 65) = 50.27; p < 0.05). The degree of antinociception was equal for all three opioids (Bonferroni; p > 0.05). Morphine, DAMGO, and fentanyl caused a decrease in locomotor activity compared to saline.
treated rats as indicated by the open field test (Figure 3B; \( F(3, 68) = 11.13; p < 0.05 \)). This decrease was similar for morphine at 30 min, DAMGO at 20 min, and fentanyl at 3 min (Bonferroni, \( p > 0.05 \)).

Repeated microinjections of fentanyl did not cause a change in morphine potency on Trial 5 (Figure 4A; \( F(1, 92) = 2.210; p = 0.1406 \)). Likewise, two days of pretreatment with morphine or DAMGO did not alter fentanyl potency compared to rats pretreated with saline (Figure 4B; \( F(2, 144) = 0.918, p = 0.402 \)). Similarly, two days of pretreatment with morphine or fentanyl did not alter DAMGO potency compared to saline controls (Figure 4C; \( F(2, 180) = 0.233, p = 0.793 \)). \( D_{50} \) values are presented in Table 2.

Evaluation of locomotor activity on Trial 5 revealed minimal differences between pretreatment groups. There was an overall dose-dependent decrease in activity for morphine (Figure 5; \( F(5, 70) = 27.17; p < 0.05 \)), DAMGO (\( F(5, 140) = 5.29; p < 0.05 \)), and fentanyl (\( F(5, 110) = 22.54; p < 0.05 \)). There was a significant difference in those rats pretreated with saline versus opioid followed by DAMGO dose-response (\( F(2, 140) = 3.68; p < 0.05 \)), but not fentanyl (\( F(2, 110) = 2.86; p = 0.08 \)) or morphine (\( F(1, 70) = 0.30; p = 0.59 \)). A two-way repeated measures ANOVA comparing the saline pretreated rats revealed a significant difference between morphine, DAMGO, and fentanyl (Figure 5; \( F(2, 24) = 6.93; p < 0.05 \)). A Bonferroni post-hoc revealed that DAMGO did not cause a decrease in activity at the 3 highest doses compared to morphine or fentanyl (\( p < 0.05 \)).
Discussion

The current study showed that tolerance develops to repeated injections of morphine and fentanyl, but cross-tolerance between morphine and fentanyl is much less likely to occur. Systemic administration showed that cross-tolerance develops from morphine to fentanyl, but not from fentanyl to morphine. No cross-tolerance was evident following microinjections into the vPAG between three different opioids (morphine, DAMGO, and fentanyl) despite using a paradigm previously shown to produce tolerance to each drug (Tortorici et al., 1999; Morgan et al., 2006; Meyer et al., 2007; Bobeck et al., 2012). These data suggest that tolerance is being driven by different mechanisms for each of these MOR agonists.

It is interesting that cross-tolerance only developed in one direction following systemic administration (morphine to fentanyl). However, this is similar to previous studies showing that pretreatment with fentanyl causes little or no cross-tolerance to morphine (Paronis and Holtzman, 1992; Duttaroy and Yoburn, 1995; Pawar et al., 2007; Sirohi et al., 2009). Cross-tolerance has been demonstrated using certain injection paradigms including cross-tolerance following a single injection as well as continuous administration. Cross-tolerance develops in both directions between single morphine and fentanyl injections (Melief et al., 2010). Continuous systemic administration of morphine causes cross-tolerance to fentanyl (Romero et al., 2010). In contrast, spinal administration of morphine leads to minimal cross-tolerance to DAMGO (Russell et al., 1987). There appears to be a difference in the magnitude of the cross-tolerance that develops based on whether the opioids are administered in the systemically versus
centrally. The current study picked a key brain region important for pain modulation and opioid functions, the vlPAG, to systematically evaluate the role of cross-tolerance.

The lack of cross-tolerance from fentanyl to morphine following systemic administration is consistent with pretreatment with vlPAG microinjections reported here. We also show that cross-tolerance to fentanyl does not develop following morphine pretreatment. Our recent findings that the antinociceptive efficacy of morphine and fentanyl at the MOPr are not different within the vlPAG (Bobeck et al., 2012), suggests that lack of cross-tolerance is driven by differences in: 1) receptor subtypes, 2) receptor regulation (i.e. desensitization and internalization), 3) intracellular signaling, or 4) pharmacokinetics.

The first possible explanation is that these different opioids activate different receptor subtypes. Fentanyl and DAMGO are considered full MOPr agonists, whereas morphine also shows effects on delta and kappa opioid receptors (DOPr and KOPr respectively). This may cause differential activation of heterodimers (Costantino et al., 2012), which may lead to activation of a separate set of signaling proteins. One potential protein is ERK1/2, which is activated in a β-arrestin dependent manner in the presence of MOPr and DOPr heterodimers (Rozenfeld and Devi, 2007). These agonists may also activate differential MOPr splice variants. Previous studies have shown that analgesia produced by different opioids is mediated by particular exons on the receptor (Rossi et al., 1995). For example, knockout of exon 11 on the MOPr decreases antinociception to fentanyl, but has no effect on antinociception to morphine (Pan et al., 2009). It is also possible that different splice variants are present within the vlPAG.
compared to spinal cord or other brain regions which may contribute to differences in
tolerance development to different agonists.

Another difference between these agonists that may lead to a difference in cross-
tolerance is receptor regulation properties. Fentanyl and DAMGO cause
phosphorylation of the MOPr via G-protein receptor kinase (GRK) leading to robust β-
arrestin recruitment and subsequent internalization of the receptor. Morphine causes a
much slower desensitization via protein kinase C (PKC) which leads to little or no
internalization (Finn and Whistler, 2001; Alvarez et al., 2002; Kelly et al., 2008). Studies
evaluating the magnitude of tolerance to different agonists using β-arrestin knockout
mice have shown that tolerance is decreased following chronic administration of some
agonists, but not others (Raehal and Bohn, 2011). One possible explanation for lack of
cross-tolerance from morphine to DAMGO, despite previous findings of cross-tolerance
from DAMGO to morphine occurs (Meyer et al., 2007), is that DAMGO acts via multiple
mechanisms to produce analgesia whereas morphine is limited to one. Hypothetically, if
tolerance is driven by activation of G-proteins and subsequent downstream signaling
proteins, then cross-tolerance may not be observed if the other agonist (i.e. DAMGO)
also uses a β-arrestin dependent pathway to produce antinociception

These initial differences may lead to differences in activation of signaling
cascades and tolerance by different mechanisms. One protein that has shown to be
differentially activated following different MOPr agonists is ERK1/2. After acute (< 30
min) administration in cell lines or cultured neurons, ERK1/2 is only activated following
fentanyl or DAMGO, not morphine (Belcheva et al., 2005; Bilecki et al., 2005; Macey et
al., 2006). Similarly, GRK is thought to be activated only following certain agonists and
blockade of GRK has been shown to prevent acute tolerance to agonists such as fentanyl and DAMGO, without affecting acute tolerance to morphine (Hull et al., 2010; Melief et al., 2010). Conversely, inhibition of proteins downstream of G-protein activation such as PKC or c-Jun N-terminal kinase (JNK) led to a decrease in acute tolerance to morphine, but not fentanyl or DAMGO (Hull et al., 2010; Melief et al., 2010).

Lastly, lack of cross-tolerance between these agonists may be influenced by pharmacokinetics. The longer duration of morphine antinociception may be due to its low lipid solubility and long half-life compared to fentanyl (Golembiewski et al., 2005; Lotsch, 2005). This increases the duration of bioavailability for morphine compared to fentanyl (Bernards et al., 2003). Another difference between the two drugs is how they are metabolized. Morphine is metabolized into morphine-6-glucuronide, which also has analgesic effects. Fentanyl does not have any active metabolites (Golembiewski et al., 2005). These differences may lead to prolonged receptor activation following morphine which may lead to the development of tolerance. The antinociceptive time course following microinjection of DAMGO is more similar to that following morphine, which may explain our previous findings that cross-tolerance developed to morphine after DAMGO pretreatment (Meyer et al., 2007; Bobeck et al., 2009). As has been reported before, fentanyl is more potent than morphine when administered systemically (Bartok and Craft, 1997). However, to produce antinociception in the vIPAG, a very high dose of fentanyl was used that is similar in potency to morphine.

The current study reveals that the cross-tolerance between MOPr agonists in the vIPAG is minimal. The idea that different tolerance mechanisms exists is valuable
clinically and may open up new pain treatment options which would allow opioids to be more efficacious with long-term administration.
Figure 1: Antinociception and activity following systemic morphine and fentanyl on Trial 1. Rats were injected with saline (1 mL/kg), morphine (5 mg/kg/mL), or fentanyl (80 μg/kg/mL). Hot plate (A) and open field (B) tests were conducted at 5 and 30 min post injection. Data are presented as mean ± SEM. *- statistically different from saline (p < 0.05). Fentanyl produces an increase in hotplate latency and a decrease in activity at 5 and 30 min, whereas morphine only increases hotplate latency and decreases activity at 30 min post injection.
Figure 2: Tolerance and Cross-tolerance between systemic morphine and fentanyl (Trial 5). Rats were pretreated with twice daily injections for two days of saline (1mL/kg), morphine (5mg/kg/mL), or fentanyl (80μg/kg/mL). On Trial 5, a cumulative dosing procedure was conducted with morphine or fentanyl. Tolerance develops to morphine and fentanyl following 4 systemic injections, but cross tolerance only develops in one direction (morphine to fentanyl).
Figure 3: Antinociception and activity following vIPAG morphine and fentanyl. Rats were injected with saline (0.4 μL), morphine (5 μg/0.4 μL), DAMGO (0.5 μg/0.4 μL), or fentanyl (3 μg/0.4 μL). Hot plate (A) and open field (B) tests were conducted at 3, 20, or 30 min post injection for fentanyl, DAMGO, and morphine respectively. Data are presented as mean ± SEM. * - statistically different from saline (p < 0.05). Morphine, DAMGO, and fentanyl all increase hot plate latency and decrease activity following vIPAG administration.
Figure 4: Lack of cross-tolerance between vlPAG morphine, DAMGO, and fentanyl. Rats were received twice daily microinjections for two days of saline (0.4 μL), morphine (5 μg/0.4 μL), DAMGO (0.5 μg/0.4 μL), or fentanyl (3 μg/0.4 μL). On Trial 5, a cumulative dosing procedure was used of either (A) morphine, (B) DAMGO, or (C) fentanyl. Cross-tolerance did not develop between any groups following vlPAG administration.
Figure 5: Lack of DAMGO-induced decrease in activity. Rats received twice daily microinjections for two days of saline (0.4 μL) on Trial 5, they received cumulative third-log doses of morphine, DAMGO, or fentanyl. Morphine and fentanyl caused a reduction in locomotor activity, but DAMGO did not.
Table 1: Tolerance and Cross-tolerance following systemic opioids

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine $D_{50} \pm$ C.I. (n)</th>
<th>Fentanyl $D_{50} \pm$ C.I. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5.0 ± 0.64 (11)</td>
<td>0.030 ± 0.006 (9)</td>
</tr>
<tr>
<td>Morphine</td>
<td>8.7 ± 1.6 (8)*</td>
<td>0.055 ± 0.007 (10)*</td>
</tr>
<tr>
<td>Fentanyl</td>
<td>4.8 ± 0.90 (9)</td>
<td>0.046 ± 0.008 (11)*</td>
</tr>
</tbody>
</table>

Notes:
D50s are presented in μg doses
C.I. – 95% confidence interval
* – statistically different from saline
Table 2: Cross-tolerance following opioid microinjection in the vIPAG

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine $D_{50} \pm \text{C.I. (n)}$</th>
<th>DAMGO $D_{50} \pm \text{C.I. (n)}$</th>
<th>Fentanyl $D_{50} \pm \text{C.I. (n)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3.1 ± 0.76 (8)</td>
<td>0.14 ± 0.041 (11)</td>
<td>2.3 ± 0.54 (8)</td>
</tr>
<tr>
<td>Morphine</td>
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<td>0.15 ± 0.066 (9)</td>
<td>1.7 ± 0.55 (7)</td>
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<tr>
<td>DAMGO</td>
<td>6.6 ± 2.2*</td>
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<td>2.11 ± 0.65 (10)</td>
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<tr>
<td>Fentanyl</td>
<td>4.0 ± 0.87 (8)</td>
<td>0.16 ± 0.048 (11)</td>
<td></td>
</tr>
</tbody>
</table>

Notes:
D50s are presented in µg doses
C.I. – 95% confidence interval
* – statistically different from saline
# – previously published (Meyer et. al., 2007)
MOPr agonists have been shown to differ in G-protein activation, intracellular signaling and MOPr regulation properties. For example, DAMGO strongly activates G-proteins and also causes rapid internalization and desensitization (Finn and Whistler, 2001; Borgland et al., 2003; Arttamangkul et al., 2008). Fentanyl has a similar profile but to a slightly lesser degree (McPherson et al., 2010). However, morphine is relatively weak in regard to all of these properties (G-protein activation, desensitization, and internalization) (Arttamangkul et al., 2008; McPherson et al., 2010). These differences have been implicated as the cellular mechanism underlying the development of tolerance to each drug, despite minimal behavioral evidence. One hypothesis it that agonists that cause MOPr internalization produce less tolerance (Whistler, 2012), although previous studies in our lab have shown that tolerance develops in the vlPAG to several agonists regardless of their ability to internalize the receptor (Morgan et al., 2006; Meyer et al., 2007; Bobeck et al., 2012). The studies of this dissertation are among the first to demonstrate that ligand-biased signaling at the MOPr alters the antinociceptive and tolerance inducing properties of opioids at the behavioral level.

The role of these cellular properties in vivo remains poorly understood. A few studies have evaluated the role of G-proteins in antinociception and have determined that G_{i/o}-proteins are especially important for morphine-induced antinociception following intrathecal, intracerebroventricular, or intra-PAG administration (Bodnar et al., 1990; Goode and Raffa, 1997; Gomes et al., 2002). These inhibitory proteins are not as
important for DAMGO or sufentanil antinociception following intracerebroventricular injections (Goode and Raffa, 1997). Chapter 2 expanded on these findings by conducting a full dose-response analysis within the vlPAG comparing morphine, DAMGO, and fentanyl. The results in the vlPAG confirmed previous studies showing that morphine antinociception was attenuated by both low and high doses of PTX, DAMGO antinociception was only altered at the highest PTX dose and fentanyl antinociception was not altered by PTX. Even though previous studies have determined that these MOPr agonists differ in antinociceptive efficacy (Mjanger and Yaksh, 1991; Goode and Raffa, 1997; Madia et al., 2009), fentanyl and morphine have equal antinociceptive efficacies in the vlPAG, given that microinjection of the irreversible antagonist, β-funaltrexamine, caused an equal decrease in morphine and fentanyl antinociception (Bobeck et al., 2012). This difference in inhibition at the MOPr with an irreversible antagonist versus inhibition of G\textsubscript{i/o}-protein with PTX suggests that fentanyl acts via a different signaling mechanism to produce antinociception.

To investigate another potential antinociception mechanism, Chapter 2 evaluated the role of internalization. Unexpectedly, dynamin inhibition, which prevents pinching off of the membrane and internalization of the receptor, enhanced fentanyl antinociception. These data suggest that fentanyl antinociception is terminated by internalization of the MOPr, so that internalization prolongs signaling and subsequent antinociception. The current studies revealed that fentanyl does not produce antinociception via G\textsubscript{i/o}-proteins or internalization. It is possible that fentanyl activates G\textsubscript{s}-proteins in order to produce its antinociceptive effects (Goode and Raffa, 1997). In contrast to fentanyl, inhibition of
dynamin attenuated DAMGO-induced antinociception. Our lab has previously shown that inhibition of dynamin also decreases antinociception to the MOPr internalizing agonist, dermorphin (Macey et al., 2010). ERK1/2 inhibition in Chapter 3 also led to a reduction in DAMGO-induced antinociception. This finding supports the argument that ERK1/2 is a downstream signaling protein of the internalization process and suggests that ERK1/2 has a direct effect on neurotransmitter release or ion channels (Subramanian and Morozov, 2011). Morphine antinociception was not altered by inhibition of dynamin. Since morphine does not readily induce receptor internalization, it not surprising that morphine antinociception is not altered by inhibition of internalization (Finn and Whistler, 2001; Borgland et al., 2003; Arttamangkul et al., 2008). In sum, it can be concluded from these studies that ligand-biased signaling is associated with differences in antinociception mechanisms within the vlPAG. Morphine uses a G\textsubscript{\textalpha}/G\textbeta protein dependent mechanism. DAMGO uses both a G\textsubscript{\textalpha}/G\textbeta-protein and internalization dependent mechanisms (dynamin and ERK1/2). Further studies are necessary to determine the mechanism of fentanyl-induced antinociception, but the current studies rule out a G\textsubscript{\textalpha}/G\textbeta-protein and internalization process.

Many studies have evaluated the activation of signaling proteins following opioid administration and have found an upregulation of many different proteins in various brain regions (Williams et al., 2001; Macey et al., 2009; Cao et al., 2010; Al-Hasani and Bruchas, 2011). Proteins that are differentially activated following acute versus chronic administration in key brain regions responsible for pain modulation (i.e. vlPAG) or reward (i.e. ventral tegmental area) have been implicated in tolerance and addiction
(Williams et al., 2001; Lesscher et al., 2003; Macey et al., 2009). Many studies have evaluated changes in protein activation involved in morphine tolerance with an overwhelming focus on G-protein activated proteins. This is the first study to examine the role of non-G-protein activated proteins on antinociception and tolerance following repeated exposures to other MOPr agonists besides morphine. One such β-arrestin signaling protein that is increased following repeated morphine administration in the vlPAG and spinal cord is ERK1/2 (Macey et al., 2009; Horvath et al., 2010).

The current studies (Chapter 3) expand on previous studies by evaluating ERK1/2 activation following an acute vlPAG microinjection of other MOPr agonists. It was demonstrated that ERK1/2 is activated within the vlPAG following morphine and DAMGO, but not fentanyl microinjection. DAMGO acts via a dynamin dependent mechanism, but morphine also activated ERK1/2 through $G_{i/o}$-proteins. Previous studies using heterologous cell systems have shown that ERK1/2 activation can originate via G-protein (PKC) or β-arrestin depending on the agonist (Zheng et al., 2008; Zheng et al., 2011). In contrast to the current findings, most studies do not report an increase in ERK1/2 following acute morphine exposure within brain tissue (Eitan et al., 2003; Macey et al., 2006; Macey et al., 2009). Given that previous studies have shown that ERK1/2 is activated following fentanyl administration under several different experimental conditions (Lesscher et al., 2003; Zheng et al., 2008; Macey et al., 2009), it is surprising that ERK1/2 was not activated in the current study following microinjection into the vlPAG. The rapid actions of fentanyl compared to DAMGO and
morphine may have contributed to this difference in ERK1/2 activation, especially since β-arrestin activation of ERK1/2 takes time to develop (Costantino et al., 2012).

The behavioral consequence of this difference in ligand-biased activation of ERK1/2 has not been previously evaluated. The role of ERK1/2 in antinociception and tolerance to the MOPr agonists DAMGO and fentanyl was assessed in Chapter 3. It was determined that DAMGO, but not fentanyl antinociception was attenuated following inhibition of ERK1/2, confirming a functional role of its activation, or lack thereof, found in the immunohistochemistry experiment. Activation of ERK1/2 following fentanyl administration in areas such as the ventral tegmental area implicates the role of ERK1/2 in the addiction properties of the drug (Lesscher et al., 2003), despite a lack of a role of ERK1/2 in the nociceptive properties studied here.

Previous studies evaluating the role of ERK1/2 in morphine tolerance have had mixed results. Although pERK1/2 has been shown to increase after repeated morphine administration (Berhow et al., 1996; Macey et al., 2009), inhibition of ERK1/2 during tolerance development can either decrease, enhance, or have no effect on tolerance depending on the location of opioid administration (Mouledous, 2007; Macey et al., 2009; Wang et al., 2010). Within the vlPAG, ERK1/2 inhibition enhances morphine tolerance, signifying that ERK1/2 activation counteracts morphine tolerance (Macey et al., 2009). In contrast, the current study (Chapter 3) found that DAMGO-induced tolerance is attenuated by inhibition of ERK1/2. This opposing effect to morphine reveals that DAMGO-induced activation of ERK1/2 is driving tolerance. The lack of fentanyl-induced ERK1/2 activation in the vlPAG using immunohistochemistry was
supported by the lack of effect of ERK1/2 in fentanyl tolerance. Differential mechanisms have been implicated previously following acute tolerance paradigms in which tolerance to morphine was linked to a G-protein mediated pathway, whereas DAMGO-induced tolerance was linked to a β-arrestin pathway (Hull et al., 2010; Melief et al., 2010).

These proposed mechanistic differences in tolerance support cellular studies showing differences in signaling and MOPr regulation. Microinjection into the vlPAG of morphine, DAMGO, and fentanyl produces antinociception and tolerance (Morgan et al., 2006; Meyer et al., 2007; Bobeck et al., 2012). The current study (Chapter 4) determined that cross-tolerance did not develop between these MOPr agonists, which also supports the hypothesis regarding differences in the mechanism of tolerance.

Previous studies measuring cross tolerance between systemic morphine and fentanyl have determined that minimal cross-tolerance developed, but others have shown that it does occur (Madia et al., 2009; Sirohi et al., 2009; Melief et al., 2010; Romero et al., 2010). Similar differences have been shown for morphine and DAMGO, showing that cross-tolerance between morphine to DAMGO does not develop in both directions (Russell et al., 1987; Meyer et al., 2007). These data support the findings in Chapter 2: DAMGO can activate both pathways to produce antinociception (G-proteins and internalization), which suggests that changes in both of these pathways can occur following repeated opioid exposure, to lead to the development of tolerance. If antinociception and tolerance were mediated by a single pathway, it would be expected that cross-tolerance would develop between all agonists.
In conclusion, antinociception can be driven by multiple mechanisms, such as G-protein signaling and internalization. Certain agonists (i.e. DAMGO) are able to activate multiple signaling pathways, which decreases the likelihood of tolerance development via a single mechanism. These are the first experiments to directly show a relationship between a β-arrestin activated protein, ERK1/2, and antinociception and tolerance to MOPr agonists other than morphine. However, ERK1/2 activation is important for tolerance to some MOPr agonists that induce internalization, but not others. It is likely that the differences in G-protein versus β-arrestin signaling lead to a minimal cross-tolerance between these MOPr agonists. These studies contribute to our knowledge about ligand-biased signaling and show that different mechanisms are involved in antinociception and tolerance to various MOPr agonists.
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