KAPPA OPIOID RECEPTOR REGULATION OF ERK1/2 MAP KINASE SIGNALING CASCADE: MOLECULAR MECHANISMS MODULATING COCAINE REWARD

A dissertation presented

by

Khampaseuth Rasakham

to

The Department of Psychology

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the field of

Psychology

Northeastern University
Boston, Massachusetts
August, 2008
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements
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ABSTRACT

Activation of the Kappa Opioid Receptor (KOR) modulates dopamine (DA) signaling, and Extracellular Regulated Kinase (ERK) Mitogen-Activated Protein (MAP) kinase activity, thereby potentially regulating the rewarding effects of cocaine. The central hypothesis to be tested is that the time-and drug-dependent KOR-mediated regulation of ERK1/2 MAP kinase activity occurs via distinct molecular mechanisms, which in turn may determine the modulation (suppression or potentiation) by KOR effects on cocaine conditioned place preference (CPP). Three studies were performed to test this hypothesis.

Study 1 examined the effects of U50,488 and salvinorin A on cocaine reward. In these studies, mice were treated with equianalgesic doses of agonist from 15 to 360 min prior to daily saline or cocaine place conditioning. At time points corresponding with peak biological activity, both agonists produced saline-conditioned place aversion and suppressed cocaine-CPP, effects blocked by the KOR antagonist nor-BNI. However, when mice were place conditioned with cocaine 90 min after agonist pretreatment, U50,488-pretreated mice demonstrated a 2.5-fold potentiation of cocaine-CPP, whereas salvinorin A-pretreated mice demonstrated normal cocaine-CPP responses. These behavioral results corresponded to the results of Western Blot analysis of ERK1/2 MAP kinase activity in isolated mouse brain, which suggested that U50,488 increased ERK1/2 MAP kinase activity only at 90 min after administration. In contrast, salvinorin A produced no change at any time point tested. Consistent with this, pretreatment with the ERK1/2 MAP kinase inhibitor SL327 prevented U50,488-induced potentiation of cocaine-CPP. Together, the data suggest that the divergent effects of the KOR agonists were attributed to differential agonist signaling of ERK1/2 MAP kinase activity.

To further determine the molecule mechanisms underlying the differential signaling induced by KOR agonists, study 2 examined four structurally distinct KOR agonists in vitro for their ability to induce the activation of G-proteins, activation of ERK1/2 MAP kinase, and internalization of the KOR using human embryonic kidney (HEK293) cells that expressed KOR fused to an enhanced Green Fluorescent Protein (eGFP) tag. With the exception of salvinorin A,
equivalent concentrations (1 µM) of each agonist tested internalized the KOR and induced a late-phase activation of ERK1/2 MAP kinase. Immunocytochemical studies revealed that U50,488, but not salvinorin A, induced late phase activation in cytosolic vesicles. Inhibitor experiments verified that early phase activation of ERK1/2 MAP kinase involved the activation of protein kinase C (PKC), whereas late phase activation of ERK1/2 MAP kinase required internalization of the KOR. Together, this suggests that diverse mechanisms mediate the differential activation of ERK1/2 MAP kinase.

The third study examined the mechanisms by which nor-BNI and other KOR antagonists mediate an observed suppression of ERK1/2 MAP kinase. Surprisingly, nor-BNI and arodyn induced a concentration-dependent suppression of vehicle stimulated ERK1/2 MAP kinase activation in HEK293 cells that expressed KOR-eGFP but not in nontransfected (NT) HEK293 cells. Nor-BNI suppression of ERK1/2 MAP kinase was not due to a suppression of basal KOR activity, as it effectively suppressed insulin-induced activation of ERK1/2 MAP kinase in KOR-eGFP/HEK293. Of interest, nor-BNI-mediated suppression of ERK1/2 MAP kinase did not require conventional G_{i/o} protein or phosphatidylinositol 3-kinase (PI3K) signaling, but instead occurred through a KOR-induced activation of protein kinase B (also known as Akt). Together, these results show that a KOR-mediated suppression of ERK1/2 MAP kinase occurs via an unconventional Akt-dependent pathway, suggesting a novel signaling mechanism for the kappa opioid receptor.

In conclusion, the present thesis has demonstrated that KOR modulation of cocaine reward is ligand-, time-, and ERK1/2 MAP kinase dependent. These studies offer new insight into the mechanisms by which different ligands may modulate signal transduction and receptor regulatory processes. Moreover, the results suggest a mechanistic basis for divergent behavioral outcomes observed among KOR ligands that are known to act upon the same receptor site, with the potential to produce new therapeutic approaches in the treatment of cocaine abuse.
ACKNOWLEDGEMENTS

I have been fortunate to have the support of many great people who have greatly inspired me to pursue my passion for science. My family, friends, teachers, and supervisors have helped me survive through this long and arduous process and without them I would not be here today. The kind words and shoulders I’ve leaned on have provided me with the strength to continue on through the many times I felt compelled to give up. This work would not have been possible without everyone.

First, the present body of work could not have been completed without the patience, support, guidance, and scientific vigor instilled in me by my advisor and scientific father Dr. Jay P. McLaughlin. As a mentor he has helped mold me to become a confident and rigorous scientist, through his teachings of critical thinking and design, perseverance, and patience. Beyond the bench he has taught me the importance of educating the next generation of scientists and has taught me how to teach. It is through his words and by example that I have excelled in teaching science in the classroom, laboratory, and to individuals outside of the scientific community. It is with the greatest gratitude that I thank him for his commitment to my abilities.

I would also like to thank my committee members for their dedication, efforts, and patience in helping me build this body of work. Thank you Drs. Denise Jackson, Martin Block, Alexandros Makriyannis, and Donald O’Malley for providing me the insight I needed to complete this project. I wish to also extend my gratitude to the labs Dr. O’Malley and Dr. Makriyannis for providing me with access to their facilities to complete my experiments.

The contributions by many people outside of my committee also deserve a special gratitude. I am grateful for the support of the Psychology department faculty and staff, who have helped me make it through these years. Also, the many students that I have had the opportunity to teach deserve my gratitude as they have taught me more about patience than I could ever learn elsewhere. Through their insightful questions and discussions I have become a better scientist. A special thanks to my second hand, Sarah S. Roderick who has worked with me through the many tough long hours of experiments. Moreover, everyone in the McLaughlin lab,
all have helped make the impossible moments work and have helped me enjoy the rough times. Amanda N Carey, the “Stats queen,” thanks for all your help and support through this. Our lab wouldn’t be possible without you.

Much of this work could not possible without the help of people outside of the Northeastern University community. I want to extend my gratitude to everyone in the lab of Drs James A. Joseph and Barbara Shukkit-Hale at the Human Nutrition Research Center at Tufts University. Thank you for allowing me to use your equipment and facilities. I wouldn’t have survived without it. My teacher, undergraduate advisor, and friend, Dr Rachel Galli deserves the utmost gratitude. Her enthusiasm, guidance, and support helped me realize my potential, which has led me to pursue graduate school. My former supervisor, Dr Michael G. Schlossmacher, taught me all about biochemistry and molecular biology. He has been an inspiration and without his teachings I could not be here today.

Most importantly, I want to thank my family and friends. The love and support of many friends, especially Kaliyan Chap, Yordanos Tilahun, and Bishal Thapa, has kept me inspired and motivated to pursue my passion for science. My older siblings Cindy, Sonya, and Sam ensured I stuck through this and kept me disciplined. My younger siblings Justin, Lily, Paul, and Melissa have kept the motivation in me to become an inspiration. I also thank many members of my extended family, my aunt Lakhonsey Insixiengmay, for support. Finally, I dedicate this work to my parents Khamphonh (Katherine) (Oct 2, 1948 - Oct 17, 1992) and Bountheung (Ted) Rasakham. It is from all their hardwork, love, support, and confidence that have inspired, motivated, and instilled in me the foundations that have made me who I am today. My dreams would not have been possible without their struggle to leave our homeland Laos to come to America for the pursuit of happiness for their children.
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### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>5HT</td>
<td>5-hydroxytryptophan (serotonin)</td>
</tr>
<tr>
<td>5-HT2AR</td>
<td>Serotonin type 2A receptor</td>
</tr>
<tr>
<td>5HT2C</td>
<td>Serotonin type 2C receptor</td>
</tr>
<tr>
<td>7TM</td>
<td>Seven transmembrane</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Arodyn</td>
<td>Ac[Phe$^{1,2,3},$Arg$^4,D$-Ala$^8]$dynorphin A-(1-11) amide</td>
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<tr>
<td>AT1A</td>
<td>Angiotensin type 1A</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Baclofen</td>
<td>4-amino-3-(4-chlorophenyl)-butanoic acid</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca++</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CaM</td>
<td>Calcium$^{++}$ calmodulin protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<tr>
<td>CB$_1$</td>
<td>Cannabinoid receptor</td>
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<tr>
<td>CCL19</td>
<td>Chemokine (C-C motif) ligand 19</td>
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<td>CCL 20</td>
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<td>CCR7</td>
<td>Chemokine receptor 7</td>
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<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>Cl$^-$</td>
<td>Chloride ion</td>
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</table>
CP-55940  (-)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
CPP  conditioned place preference
CNS  central nervous system
CO₂  Carbon Dioxide
COS  CV-1 Origin SV40
CREB  cAMP response element binding protein
CTAP  D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH₂
CTOP  D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂
Cyclazocine  2,6-Methano-3-benzazocin-8-ol, 3-(cyclopropylmethyl)-1,2,3,4,5,6-hexahydro-6,11-dimethyl
δ  Delta
D₁  Dopamine Receptor Subtype 1
D₂  Dopamine Receptor Subtype 2
D₃  Dopamine Receptor Subtype 3
DA  Dopamine
DAMGO  [D-Ala², NMe-Phe⁴, Gly-ol⁵]-enkephalin
DAT  Dopamine transporter
DELFIA  Dissociation-enhanced lanthanide fluorescence immunoassay
DELT  [D-Ala²]deltorphin II
DMEM  Dubelco’s Modified Eagle’s Media
DMSO  Dimethyl sulfoxide
DOI  2,5-dimethoxy-4-iodoamphetamine
DOR  Delta opioid receptor
DPDPE  D-Penicillamine(2,5)-enkephalin
ECL  Enhanced chemiluminescence
ECS  Electric convulsive shock
EDTA  Ethylenediaminetetraacetic acid
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<tr>
<th>Term</th>
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<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EL</td>
<td>Extracellular loops</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular regulated kinase 1 and 2</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma(γ)-aminobutyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanidine diphosphate</td>
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<tr>
<td>GIRK</td>
<td>G protein-activated inwardly rectifying K⁺</td>
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<td>GNTI</td>
<td>5'-Guanidinyl-17-(cyclopropylmethyl)-6,7-dehydro-4,5a-e poxy-3,14-dihydroxy-6,7-2',3'-indolomorphinan</td>
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<td>Gö6983</td>
<td>3-[1-3-(Dimethylamino)propyl]-5-methoxy-1H-indol-3-yl -4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione</td>
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<tr>
<td>GPCR</td>
<td>G protein-couple receptor</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor-receptor bound protein 2</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein receptor kinase</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase-3 beta</td>
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<tr>
<td>GTP</td>
<td>Guanidine triphosphate</td>
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<tr>
<td>H⁺</td>
<td>Hydrogen ion</td>
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<tr>
<td>HEK293</td>
<td>Human embryonic kidney-293</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
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<tr>
<td>HSD</td>
<td>Honest Significant Difference</td>
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<tr>
<td>ICI, 174-864</td>
<td>N,N-diallyl-Tyr-Aib-Aib-Phe-Leu-OH</td>
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<tr>
<td>IEG</td>
<td>Immediate early gene</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Intracellular loops</td>
</tr>
<tr>
<td>IP</td>
<td>Inositol phosphate</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory postsynaptic current</td>
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</table>
JDTic: (3R)-7-hydroxy-N-{1S}-1-[(3R,4R)-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl][methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide

JNK: c-Jun N-terminal kinase

JNK/SAPK: c-Jun N-terminal kinase/stress-activated protein kinase

κ: Kappa

K⁺: Potassium ion

KCl: Potassium chloride

KH₂PO₄: Potassium dihydrogen phosphate

Ketocyclazocine: (2R,6S,11S)-3-(cyclopropylmethyl)-3,4,5,6-tetrahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocin-1(2H)-one

KOR: Kappa opioid receptor

LSD: Least Significant Difference

LY294002: 2-(4-Morpholiny1)-8-phenyl-4H-1-benzopyran-4-one

MAP: Mitogen activated protein

MEK: Mitogen activated protein kinase kinase

Memantine: 1-amino-3,5-dimethyl-adamantane

MgCl₂: Magnesium Chloride

MOR: Mu opioid receptor

Morphine: (5α,6α)-7,8-didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol

MSN: Medium spiny neuron

Na⁺: Sodium ion

Na₃HPO₄: Sodium Hydrogen Phosphate

NAc: Nucleus Accumbens

NaCl: Sodium Chloride

Nalorphine: (5 alpha,6 alpha)-7,8-didehydro-4,5-epoxy-17-(2-propenyl)morphinan-3,6-diol

Naloxone: (−)-17-Allyl-4, 5α-epoxy-3,14-dihydroxymorphinan-6-one
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<td>Naltrexone</td>
<td>17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6-one</td>
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<tr>
<td>NHE</td>
<td>NA⁺/H⁺ exchanger</td>
</tr>
<tr>
<td>NHERF</td>
<td>NA⁺/H⁺ exchange regulator 1</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>NLX</td>
<td>Naloxone</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartic acid</td>
</tr>
<tr>
<td>nor-BNI</td>
<td>Norbinaltorphimine</td>
</tr>
<tr>
<td>NP</td>
<td>Nonspecific</td>
</tr>
<tr>
<td>NP-40</td>
<td>Nonylphenyl-polyethylene glycol</td>
</tr>
<tr>
<td>NT</td>
<td>Nontransfected</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen gas</td>
</tr>
<tr>
<td>p38/HOG</td>
<td>p38/high osmolarity glycerol</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffered saline – tween 20</td>
</tr>
<tr>
<td>pERK</td>
<td>Phosphorylated extracellular regulated kinase</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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Salvinorin A  (2S,4aR,6aR,7R,9S,10aS,10bR)-methyl-9-acetoxy-2-(furan-3-yl)-6a,10b-dimethyl-4,10-dioxo-dodecahydro-1H-benzo[f]isochromene-7-carboxylate

SB206,553  5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole

SD  Standard Deviation

SDS  Sodium Dodecyl Sulfate

SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM  Standard error of the mean

SII  Sar1-Ile4, Ile8-AngII

sIPSC  spontaneous inhibitory postsynaptic current

SKF-10047  [2S-(2a,6a,11R*)]-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol

SL-327  a-[Amino[(4-aminophenyl)thio)methylene]-2-(trifluoromethyl)benzeneacetonitrile

SLC6  solute carrier family 6

SOS  Son of Sevenless

SR141716A  N-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide

TBS-T  Tris-buffered saline-Tween 20

TH  Tyrosine hydroxylase

TM  Transmembrane

μ  Mu

U0126  1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene

U50,488  (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide

U69,593  (5α,7α,8α)-(−)-N-methyl-N-(7-(1-pyrrolidinyl)-1-oxaspiro(4,5) dec-8-yl)burzeneacetamide
<table>
<thead>
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<th>VTA</th>
<th>Ventral tegmentum area</th>
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<tr>
<td>WT</td>
<td>Wild type</td>
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INTRODUCTION

In 2006 nearly six million Americans age 12 and older reported abusing cocaine at least once in their lifetime (National Survey on Drug Use and Health, Substance Abuse and Mental Health Services Administration, 2007). The continuing rise in cocaine abuse is evident by the increase in cocaine-related emergency department visits increasing from 19% in 2004 to 31% in 2005 (Substance Abuse and Mental Health Services Administration, 2006, 2007). Surprisingly, there is currently no United States Food and Drug Administration (FDA) approved medication available for the specific treatment of cocaine abuse or the prevention of relapse in abstinent patients (National Institute on Drug Abuse Research Reports, 2004). Thus, given the continuing rise and social impact of cocaine abuse and addiction development of therapeutic approaches remains an important task.

Cocaine and other drugs of abuse, e.g. amphetamine and morphine, are thought to produce their rewarding effects by enhancing dopamine (DA) signaling in the mesolimbic reward circuitry (DiChiara and Imperato, 1988b; Wise, 1990; Robinson and Berridge, 1993). Cocaine primarily exerts its action by binding to the dopamine transporter (DAT) thus inhibiting the ability for DAT to induce reuptake and terminate DA signaling (Ritz et al., 1987).

It has been proposed that compounds that reduce DA signaling may prove useful in opposing the rewarding effects of drugs of abuse (Wise, 1990; Prisinzano et al., 2005). To date, considerable behavioral and biochemical evidence indicate that the endogenous dynorphin peptide-kappa opioid receptor (KOR) system may be a potential target (Kreek, 1996; Prisinzano et al., 2005, discussed further in Chapter 1). Early microdialysis studies demonstrated that kappa opioid agonists could suppress DA release in nucleus accumbens (NAc), striatum, and cortex (DiChiara and Imperato, 1988a; Spanagel et al., 1992; Thompson et al., 2000; Zhang et al., 2004a and b), a biochemical effect consistent with the aversive and dysphoric properties of KOR agonists (Kumor et al., 1986; Suzuki et al., 1992; Pliakas et al., 2001; Zhang et al., 2005). Accordingly, in preclinical models acute KOR agonist treatment suppressed cocaine-induced indicators of behavioral reward, including cocaine-conditioned place preference (Crawford et al.,...
1995; McLaughlin et al., 2006) and cocaine self-administration (Negus et al., 1997; Schenk et al., 1999).

While the studies mentioned above show promising directions for KOR agonists as anti-cocaine medications to prevent drug-taking behavior there are several limitations to their use. Cocaine and other drugs of abuse produce complex neuroadaptive alterations in the brain over time (Nestler et al., 1993; Nestler, 2004), at least some of which involve increased endogenous KOR signaling. Chronic cocaine exposure or “binge” cocaine treatment in rodents induced an up-regulation of the endogenous dynorphin-KOR system in NAc and caudate putamen (Spangler et al., 1993; Unterwald et al., 2001). Similarly, *post mortem* analysis of brains from chronic cocaine users showed an increase in gene and protein expression of dynorphin and KOR in the caudate putamen (Hurd and Herkenham, 1993; Mash and Staley, 1999; Frankel et al., 2008). These neuroadaptive changes in the dynorphin-KOR system after chronic cocaine exposure correlate to the production of the dysphoric effects observed during abstinence and withdrawal, a key cause of relapse to drug-seeking behavior (Shippenberg et al., 2007). Thus, while acute KOR agonist treatment may be beneficial in opposing the immediate effects of cocaine, long-term treatment with KOR agonists for cocaine addiction could be anticipated to augment the effects of withdrawal and potentially exacerbate cocaine abuse.

Verifying these concerns, limited behavioral studies using prolonged KOR agonist treatment have shown a potentiation of cocaine-seeking behavior in primates and rodents (Negus, 2004; McLaughlin et al., 2006). Supporting the behavioral studies, microdialysis studies have demonstrated that prolonged and repeated exposure to KOR agonists produces a paradoxical enhancement of DA signaling (Heidbreder et al., 1998; Fuentealba et al., 2006). The molecular and cellular mechanisms underlying this paradoxical temporal effect are not clearly understood, although a number of neuroadaptive mechanisms have been implicated (Nestler, 2004). For instance, repeated cocaine treatment robustly activates mitogen activated protein (MAP) kinase pathways (Lu et al., 2006; Girault et al., 2007), a molecular event found to mediate cocaine reward (Valjent et al., 2000, 2004, 2006) and facilitate reinstatement of drug-seeking behavior in rodent models that exhibited extinction (Lu et al., 2005).
The present thesis attempts to understand the conditions under which therapeutic KOR agonists might become detrimental, paradoxically potentiating cocaine reward and abuse. To examine this, we investigate the specific temporal activity of MAP kinase signaling mediated by KOR activity by 1) examining the differential signaling produced by different KOR ligands and 2) examining the molecular mechanisms of KOR-mediated regulation of MAP kinase signaling.

Several studies have demonstrated that KOR activates MAP kinase pathways \textit{in vitro} (Belcheva et al., 1998; Brucas et al., 2007). KOR belongs to a family of G-protein-coupled receptors (GPCR) that are well understood to couple to and signal through pertussis-toxin sensitive G\textsubscript{i/o} proteins (discussed further in Chapter 1). However, other mechanisms of GPCR signaling are less clear. In the last decade, research has demonstrated that two major independent pathways, G-protein and \(\beta\)-arrestin-mediated receptor internalization, mediate GPCR activation of MAP kinase pathways (Luttrell, 2003). The two mechanistic pathways of GPCR-mediated activation of the extracellular regulated kinase 1 and 2 (ERK1/2) MAP kinase cascade can be distinguished by their differential temporal and spatial patterns of activation, often described as “pools” of kinases based on their localization within the cell (Ahn et al, 2004; Shenoy et al, 2006). G-proteins and associated enzymatic proteins, e.g. protein kinase A (PKA), induce a rapid activation of ERK1/2 that results predominantly in activation of a nuclear pool of kinases (Ahn et al., 2004; Shenoy et al., 2006). In contrast, \(\beta\)-arrestin-mediated receptor internalization results in a delayed, late-phase activation of ERK1/2 MAP kinase located in endosomal vesicles in the cytosol (Ahn et al., 2004). This “non-conventional” concept by which agonist-induced internalization of GPCRs mediate the activation of ERK1/2 MAP kinase has only been recognized recently (Dewire et al, 2007), and then only with receptors that couple to cholera-toxin sensitive G\textsubscript{s}-proteins, e.g. \(\beta\)-adrenergic (Azzi et al., 2003; Shenoy et al., 2006) and angiotensin type 1A (Tohgo et al., 2002) receptors. However, the physiological and behavioral consequences of activating this latent pool of ERK1/2 MAP kinase remains to be determined.

Moreover, among the numerous effects arising from the activation of ERK1/2 MAP kinase, its signaling has been involved in synaptic plasticity and learning and memory (Sweatt,
Given that addiction to cocaine and other drugs of abuse are hypothesized to be an adaptive form of learning (Girault, 2007), it is feasible that drug addiction may result from synaptic restructuring in the brain mediated by the activation of ERK1/2 MAP kinase signaling. Interestingly, recent studies have demonstrated that dopamine receptor subtypes differentially modulate the cocaine-mediated signaling of ERK1/2 MAP kinase ex vivo (Zhang and Xu, 2006; Jiao et al., 2007). However, whether KOR activation of ERK1/2 MAP kinase may be involved in modulating cocaine reward has not been examined. More importantly, the molecular mechanism(s) by which KOR modulates the activity of ERK1/2 MAP kinase is also not fully understood.

The present thesis examines the time-dependent effects of kappa opioid ligand modulation of cocaine reward by focusing on elucidating the molecular mechanisms by which KOR regulates the activity of ERK1/2 MAP kinase. The central hypothesis tested is that KOR ligands differentially regulate the activity of ERK1/2 MAP kinase via recruitment of diverse molecular and cellular mechanisms. In essence, we believe the differential regulation of ERK1/2 MAP kinase signaling by assorted KOR ligands is crucial, as it determines the magnitude and direction of KOR ligand modulation of reward. To this end, an experimental approach was adopted to test the temporal effects of KOR ligands in vivo in behavioral models both alone and against cocaine reward, then we examined the molecular mechanisms of KOR signaling in vitro using human embryonic kidney (HEK)-293 cells stably expressing the rat kappa opioid receptor. Notably, HEK293 cells were used in these studies as they provide a controlled environment to allow the manipulations required to examine the molecular mechanisms underlying KOR regulation of ERK1/2 MAP kinase. The central hypothesis was tested with the specific aims outline below, corresponding to specific chapters of this thesis:

Chapter 2: To test the hypothesis that agonist-selective activation of ERK1/2 MAP kinase by KOR underlies the paradoxical KOR-mediated potentiation of cocaine reward. These studies examined the differential ability of two KOR agonists (U50,488 and salvinorin A) to suppress or potentiate cocaine reward using a cocaine conditioned place preference (CPP) assay. In parallel,
Western blot analysis of mice brain protein pretreated with KOR agonists determined if KOR-induced activation of ERK1/2 MAP kinase correlates with the potentiation of cocaine CPP.

Chapter 3: To test the hypothesis that KOR activates two distinct sub-cellular pools of ERK1/2 MAP kinase in an agonist- and time-dependent mechanism in HEK293 cells. To determine the molecular mechanisms involved in KOR-induced activation of ERK1/2 MAP kinase, HEK293 cells stably expressing KOR fused to an enhanced green fluorescent protein (KOR-eGFP) were utilized. The studies outlined here aimed to characterize four structurally distinct KOR agonists, i.e., U50,488, salvinorin A, dynorphin A (1-11), and cyclazocine, for their ability to induce activation of ERK1/2 MAP kinase assessed with Western blot analysis, receptor internalization and co-localization of activated ERK1/2 MAP kinase assessed by immunocytochemistry and confocal fluorescent microscopy. Additional experiments examined the mechanistic causes of activation of ERK1/2 MAP kinase induced by U50,488 by testing the effects of chemical inhibitors of G\textsubscript{i/o} proteins, protein kinase C (PKC), and receptor internalization.

Chapter 4: To test the hypothesis that certain KOR antagonists will actually suppress the activity of ERK1/2 MAP kinase in KOR-eGFP HEK293 cells. The studies outlined here characterized the effects of four distinct KOR antagonists, i.e., nor-BNI, arodyn, JDTic, and naloxone on signaling of ERK1/2 MAP kinase. We tested if certain antagonists possess negative intrinsic activity, or in other words, produce the opposite effects of agonists, thereby classifying the antagonist as a putative inverse agonist. Such compounds might suppress ERK1/2 MAP kinase activity, and therefore prove valuable in the treatment of cocaine abuse. Subsequent studies examined the mechanisms by which the select putative KOR inverse agonist, nor-BNI, suppressed ERK1/2 signaling by testing the effects of chemical inhibitors of G\textsubscript{i/o} proteins, phosphatidylinositol-3 kinase (PI3K), and protein kinase B (PKB also termed Akt) as these proteins are known to modulate ERK1/2 MAP kinase activity. Finally, experiments were conducted to examine whether nor-BNI suppressed insulin-mediated signaling of ERK1/2 MAP kinase.
I. COCAINE ABUSE AND ADDICTION

Cocaine is an alkaloid that comes from the leaves of the *Erthyroxylon coca* plant (Karch, 2006). Illicit cocaine is typically processed into two forms, either as a water-soluble hydrochloride salt or water-insoluble freebase, also known as “crack” cocaine. The water-soluble form of cocaine can be injected, smoked, or insufflated whereas “crack” cocaine is generally inhaled by pipe smoking (NIDA Research Reports, 2004).

Cocaine is a psychostimulant classified as a Schedule II drug under the Controlled Substances Act of 1970. Drugs classified into this category are those considered to have medicinal value but also known to have high abuse potential that could lead to physical and psychological dependence. The medicinal value of cocaine stems from its ability to act as a local anesthetic, as it is able to simultaneously block sodium (Na+) ion channels and constrict peripheral blood vessels. As a CNS stimulant, cocaine increases heart rate, blood pressure, produces hyperthermia and convulsions (Liska, 2004). At the cellular level, cocaine is known to inhibit dopamine transporter (DAT) function (see below), which enhances dopamine (DA) signaling that is thought to be responsible, in part, for producing feelings of intense euphoria, increase in self-esteem, well being, and energy (Liska, 2004).

The euphoric effects (“high”) of cocaine make it one of the most potent pharmacological substances known, reinforcing its addictive potential (Wise, 1990; O’Brien, 2001). However, the euphoric effects are only one of the components that contribute to the highly addictive nature of cocaine. Psychological tolerance to the “high” associated with cocaine develops quickly as a higher dose becomes required to achieve the same euphoric effects initially obtained (O’Brien, 2001). Curiously, in contrast to tolerance, repeated cocaine exposure is also known to produce “reverse-tolerance” or sensitization to certain physical effects of cocaine, e.g. locomotion and stereotypy. It has been suggested that sensitization to cocaine may augment drug reward and increase the vulnerability to relapse, which is in contrast to the notion that tolerance produces addictive behavior (Shippenberg et al., 2007). Nevertheless, individuals generally cycle between
periods of abuse and abstinence (cessation of use) (Shippenberg et al., 2007). This too, is problematic, as research has demonstrated that periods of abstinence and withdrawal are marked by dysphoria and irritability, likely contributing to the incubation of cocaine craving (O’Brien, 2001; Lu et al., 2004). During this period the majority of individuals will resume drug-taking behavior to offset the negative feelings (O’Brien, 2001). This is a significant issue for treatment, as even after prolonged periods of abstinence, 80-90% of cocaine addicts relapse to cocaine addiction (Shippenberg et al., 2007).

In addition to psychological dependence, the adverse physiological effects of cocaine usage can include dramatic increases in heart rate and blood pressure. The toxicity of acute and chronic cocaine intake can result in respiratory depression, cardiac arrest, or liver failure. Together, these liabilities have pushed cocaine abuse and addiction to rank among the top 3 health problems in the United States in terms of medical expenditure (Cleck and Blendy, 2008). However, it is notable that while many drugs are currently in human trials (Preti, 2007), to date no specific pharmaco-therapeutic has been approved by the FDA for treating cocaine abuse or addiction.

II. COCAINE: MOLECULAR SUBSTRATES

**DOPAMINE TRANSPORTER AND DOPAMINE SIGNALING**

It has been demonstrated that the subjective effects of cocaine are primarily mediated by cocaine’s action at the DAT (Ritz et al., 1987; Volkow et al., 1997; Chen et al., 2006). The DAT is a presynaptic protein belonging to the family of sodium chloride (Na⁺/Cl⁻) dependent monoamine transporters that are derived from the SLC6 gene family of transporters. DAT plays a major role in regulation of the termination of DA signaling and maintenance of dopamine homeostasis via reuptake of DA from the synaptic cleft (Gether et al., 2006). The removal of synaptic DA occurs along with the cotransport of Na⁺ and Cl⁻ ions mediated by Na⁺/K⁺ ATPase and changes in the ion gradient. As an integral membrane protein, DAT is comprised of twelve transmembrane (TM) alpha helicals and contains multiple sites subject to post-translational modifications in the extra-
and intra-cellular domains that regulate the activity and trafficking of the transporter. For example, several lines of evidence demonstrate that DAT is constitutively phosphorylated at multiple sites (Foster et al., 2002; Lin et al., 2003) and regulation of DAT trafficking and cell surface expression is modulated by multiple protein kinases (Foster et al., 2002; Gulley and Zhaniser, 2003; Lin et al., 2003). Notably, activation of ERK1/2 MAP kinase enhances DAT cell surface expression and DAT activity (Moron et al., 2003; Bolan et al., 2007). This regulation of the DAT by ERK1/2 MAP kinase is of particular interest given the correlation between ERK1/2 MAP kinase and cocaine reward, and because both cocaine and KOR signaling have been demonstrated to modulate the activity of ERK1/2 MAP kinase (discussed further below).

**COCaine AND MITOGEN ACTIVATED PROTEIN KINASE**

Considering cocaine addiction as a chronic disease, one might expect neuroadaptive changes in protein and gene expression to occur, which could affect synaptic plasticity and contribute to the development and the long-lasting persistence of this drug-seeking behavior (Nestler, 2004; Lu et al., 2006; Thomas et al., 2008). Cocaine and other drugs of abuse activate ERK1/2 MAP kinase in brain regions associated with reward (Valjent et al., 2000) and the pharmacological blockade of ERK1/2 MAP kinase effectively inhibited cocaine-induced expression of immediate early genes (IEG, reviewed in Thomas et al., 2008). In rats exposed to repeated cocaine treatments, ERK1/2 MAP kinase activation in NAc was attenuated by NAc infusions of the MEK1/2 inhibitor, U0126 (Mattson et al., 2005). As MEK1/2 is the upstream regulator of ERK1/2 MAP kinase, this effectively inhibits ERK1/2 MAP kinase (see Figure 1.4). In mouse amygdala, increased expression of c-Fos and JunB induced by repeated cocaine, but not acute cocaine treatment, was also inhibited by pretreatment with the systemic MEK1/2 inhibitor, SL-327, as was both the acute and chronic cocaine induced expression of Zif268 (Radwanska et al., 2005). Moreover, the pharmacological inhibition of ERK1/2 MAP kinase has been demonstrated to inhibit previously learned cocaine preference and cocaine-induced hyperlocomotor activity (Valjent et al., 2000; Valjent et al., 2006; Lu et al., 2005).
It has been suggested that ERK1 and ERK2 are differentially modulated by cocaine in different brain regions (Girault et al., 2007), although a clear understanding of this is lacking. For instance, Berhow et al. (1996) demonstrated that cocaine enhanced activity of ERK1 MAP kinase in the ventral tegmentum area (VTA). In contrast, ERK2 MAP kinase may also mediate the effects of cocaine, as mice with a genetic deletion of the ERK1 gene demonstrated enhanced cocaine-conditioned place preference and locomotor activity, effects the authors attribute to a compensatory increase in ERK2 activity (Ferguson et al., 2006; Girault et al., 2007). ERK1 knockout mice also demonstrated a significant increase in cocaine-induced expression of c-Fos and the opioid peptide enkephalin in the striatum compared to their wild-type (WT) littermates (Ferguson et al., 2006). However, a conclusive distinction between ERK1 and ERK2 activity has yet to be defined.

**NEUROTRANSMITTER SYSTEMS MODULATING DOPAMINERGIC SIGNALING**

It is well established that cocaine affects the activity of the mesolimbic DA reward circuitry (DiChiara and Imperato, 1988b; Nestler et al., 1993; Thomas et al., 2008). This mesolimbic reward circuitry primarily consists of DA neurons originating in the A10 region or VTA with efferent projections to the NAc and prefrontal cortex (PFC). Input from other brain regions have been implicated in modulation of the activity of this pathway, such as afferents from amygdala to the VTA and NAc (Wright et al., 1996; Radwanska et al., 2005). Moreover, efferents to several other brain regions, e.g. striatum, extend from this circuitry and are further affected by cocaine (reviewed in Pierce and Kumaresan, 2006). Importantly, several neurotransmitter systems found along this pathway play a role in modulation of DA signaling and are potential targets for anti-cocaine medication (Preti, 2007). Receptors for \(\gamma\)-aminobutyric acid (GABA) are primarily localized on medium spiny neurons (MSN or interneurons), which project into the NAc to provide indirect tonic inhibition of basal DA signaling (Spanagel and Weiss, 1999; Preti, 2007). Accordingly, baclofen, a GABA-B receptor agonist, has been demonstrated to reduce cocaine-seeking behavior, presumably through enhancing the inhibitory tone of the MSN (Roberts, 2005). In contrast, glutamatergic, N-methyl-D-aspartic acid (NMDA) receptors facilitate DA signaling, as
activation of NMDA receptors enhances DA release (Shimizu et al., 1990) and memantine, a non-competitive NMDA receptor antagonist, inhibits cocaine self-administration (reviewed in Vetulani, 2001).

Opioid peptide receptor systems are another major neurotransmitter system involved in modulating DA signaling within the mesolimbic circuitry (Ding et al., 1996). However, endogenous opioid peptides and opioid receptors produce differential modulation of DA signaling and tone (Torres and Horowitz, 1999). There are three major types of opioid receptors known to exist: µ (mu or MOR), δ (delta or DOR), and κ (kappa or KOR). While each has unique pharmacological characteristics and selective endogenous peptide agonists, all are G-protein-coupled receptors (GPCR) that couple to and signal via $G_o$ or $G_i$ proteins. The endogenous opioid compounds known as endorphins, enkephalins, and dynorphins are peptide cleavage products processed from larger precursor molecules (Höllt, 1986). Notably, while all opioid receptors commonly share sequence, structure, and signal transduction cascades they have been demonstrated to differentially modulate DA signaling and cocaine-mediated behaviors.

Neurochemical studies have demonstrated that activation of MOR or DOR enhances DA release, while KOR activation reduces DA release (Wood et al., 1980; Lubetzki et al., 1982; DiChiara and Imperato, 1988a, b; Spanagel et al., 1992; Noel and Gratton, 1995). Evidence from immunolabeling and electrophysiology studies suggest that the differential modulation of DA signaling by opioid receptors are due to their location (Spanagel and Weiss, 1999). For example, MORs, localized on GABAergic interneurons, mediate enhanced DA signaling by inducing a disinhibition of DA tone (Spanagel and Weiss, 1999), a fact confirmed by transgenic MOR knockout mice that displayed a significantly greater frequency of spontaneous inhibitory postsynaptic currents (sIPSCs) in GABAergic neurons but not dopaminergic (DAergic) neurons localized in the VTA (Mathon et al., 2005). Injection of MOR agonist DAMGO into the VTA enhanced basal DA release in NAc whereas the MOR antagonist, CTOP, reduced basal DA release (Spanagel et al., 1992). In line with this, studies have demonstrated that infusion of CTOP into VTA suppressed cocaine self-administration (Corrigall et al., 1999). Likewise, stimulation of rat striatal slices with the DOR peptide agonists, met-enkephalin and leu-
enkephalin increased basal DA release (Lubetski et al., 1982), and VTA stimulation with the DOR agonist, DPDPE, potentiated DA release assessed by in vivo microdialysis (Devine et al., 1993). Cocaine-induced locomotor activity was potentiated by the administration of the DOR agonist, DPDPE and blocked by the DOR antagonist, naltrexone (Waddell and Holtzman, 1998). Moreover, DOR agonists, such as DPDPE, and DELT have been demonstrated to produce rewarding effects assessed with conditioned place preference assays (Suzuki et al., 1997).

In contrast, acute treatment with KOR agonists attenuates the effects of cocaine via inhibition of DA release in vitro (Spanagel et al., 1992; Thompson et al., 2000; Fuentealba et al., 2006) and in vivo (Zhang et al., 2004a; Zhang et al., 2004b). Moreover, acute KOR agonist treatment suppressed cocaine-induced alterations in locomotor activity (Heidbreder et al., 1993; Collins et al., 2001), inhibited cocaine-conditioned place preference (Crawford et al., 1995; McLaughlin et al., 2006) and reduced cocaine self-administration (Negus et al., 1997; Schenk et al., 1999). This is thought to be due to activation of KOR localized on presynaptic DA nerve terminals in cells that also expressed DAT (Svingos et al., 2001). Electrophysiology studies demonstrated that KOR activation mediates hyperpolarization (Ford et al., 2007) presumably by activating G-protein-activated inwardly rectifying K+ (GIRK) ion channels (Margolis et al., 2003).

Moreover, studies in vitro (Spanagel et al., 1992) and in vivo with transgenic mice lacking the KOR (Chefer et al., 2005) have demonstrated that endogenous activation of KOR is involved in tonic inhibition of DA signaling. KOR knockout mice were found to exhibit an increase in basal DA signaling and cocaine-evoked DA release in NAc (Chefer et al., 2005). Interestingly, while KOR knockout mice displayed an increase in basal locomotor activity they failed to develop cocaine-induced locomotor sensitization (Chefer et al., 2005). The inability of KOR knockout mice to develop cocaine sensitization could be attributed to the differential time-dependent effects of the endogenous kappa opioid system to modulate the effects of cocaine. If true, this is especially relevant, as it limits the hope of using KOR agonists as a therapeutic for cocaine abuse and the few behavioral studies examining this issue raise serious concerns. In fact, prolonged treatment with the synthetic KOR agonist, U50,488, has been shown to paradoxically enhance cocaine-seeking behavior as assessed using self-administration and cocaine-conditioned place...
preference (Negus, 2004; McLaughlin et al., 2006, respectively). Moreover, repeated and prolonged administration with a similar synthetic KOR agonist, U69,593, has been demonstrated to produce a paradoxical enhancement of DA signaling (Heidbreder et al., 1998; Fuentealba et al., 2006). The paradoxical enhancement by KOR agonists on DA signaling and modulation of cocaine is poorly understood. Thus, until better understandings of how the kappa opioid systems regulate reward circuitry is reached, the potential development of opioids as therapeutic targets as medications for drug abuse will remain impeded.

III. OPIOIDS: PHARMACOLOGY OF OPIOIDS

**OPIOIDS AND OPIATES**

The first documented use of opioids dates back to ancient Sumerian society in the third century BC. Opium, derived from the juice of the poppy plant, *Papaver somniferum*, is comprised of more than 20 different alkaloids. Pure alkaloids isolated from the poppy plant are classified as opiates, or more generally as opioids. Note that, “opiate” is a term used to describe compounds that are derived from opium, e.g. morphine and codeine, whereas “opioid” describes compounds that include all opiates, endogenous peptides, as well as semi-synthetic, and synthetic compounds, all of which possess affinity for and/or activity at opioid receptors (Reisine and Pasternak, 2001).

Opioids are the most potent pain-relieving (analgesic) compounds that exist to date and thus, are widely used for pain management. However, in addition to analgesia, opioids produce numerous pharmacological effects, such as respiratory depression, sedation, tolerance, dependence, constipation, and nausea, as well as modulation of mood and reward (Reisine and Pasternak, 2001).

Historically, the synthesis of a large range of structurally distinct opioids preceded the discovery of a precise binding site. However, as pharmacological studies demonstrated the diverse actions of various opioid agonists, researchers came to propose the existence of multiple opioid receptor types (Martin et al., 1976; Martin and Sloan, 1977; Martin, 1984). Prior to the
discovery of distinct opioid receptors, the classification of opioids was based upon whether they possessed a *morphine-like* or *nalorphine-like syndrome*, broadly termed *agonist* or *antagonist* effects, respectively (Martin, 1967). The opioid-induced syndrome was a compilation of the pharmacological effects of a drug in a whole organism as measured by its ability to produce analgesia, tolerance, dependence, respiratory depression, and change in body temperature. The term *antagonist* was initially given to these drugs because of their ability to oppose the effects of morphine-induced analgesia and respiratory depression (Hart, 1941; Hart, 1943; Eckenhoff et al., 1951; Martin, 1967). This ultimately proved misleading as nalorphine-like drugs were found to produce agonistic analgesia, tolerance and withdrawal. A report by Martin et al., 1976 was the first to provide a more conclusive pharmacological classification by suggesting that the unique opioid-syndromes could be the result of actions at distinct opioid receptor sites. These opioid receptor sites were defined by the drugs that produced these syndromes, namely μ (for morphine), κ (for ketocyclazocine), and σ (for SKF 10047 or N-allylnormetazocine) (Martin et al., 1976). A fourth type of opioid receptor soon followed, named δ (for deferens) (Lord et al., 1977), as it was discovered by enkephalin activity measured *ex vivo* in mouse vas deferens. Binding studies in brain membranes and *in vitro* efficacy studies in guinea pig ileum and vas deferens of mouse, rat, and rabbit later confirmed the existence of these receptors (Goldstein et al., 1979; reviewed in Paterson et al., 1984; Pert and Snyder, 1973; Simon et al., 1973). To date, the isolation, cloning, and sequencing of the receptors confirms the existence of three major type of opioid receptors as stated previously, which were found to be nearly 60% homologous and shared the same features of receptors that belonged to the seven transmembrane (7TM) G protein-coupled receptor family (Chen et al., 1993; Kieffer et al., 1992; Knapp et al., 1995; Yasuda et al., 1993).

Likewise, three major families of endogenous opioid ligands, enkephalins, endorphins, and dynorphins, have been described to date (Reisine & Pasternak, 2001). All of these endogenous opioid peptides are derived from a single larger precursor molecule and share an N-terminal sequence that is comprised of either a met-enkephalin or leu-enkephalin, i.e. tyrosine-glycine-glycine-phenylalanine-methionine or tyrosine-glycine-glycine-phenylalanine-leucine,
respectively (Höllt, 1986; Reisine and Pasternak, 2001). The enkephalins were the first endogenous opioids to be discovered (Hughes et al., 1975). Derived from cleavage of proenkephalin, the pentapeptides met- and leu-enkephalin possess high affinity and selectivity for DOR (Hughes et al., 1975; Höllt, 1986; Reisine and Bell, 1993). Later studies discovered that the met-enkephalin sequence was found within β-endorphin (amino acids 61-65), a cleavage product of proopiomelanocortin (POMC) (Li and Chung, 1976; Höllt, 1986). Isolated from porcine pituitary, the leu-enkephalin containing opioid peptide dynorphin was found to be a cleavage product of prodynorphin (proenkephalin B) and demonstrated high affinity and selectivity at the KOR (Goldstein et al., 1979; Höllt, 1986; Reisine and Bell, 1993). Since their discoveries, more than seven leu-enkephalin containing peptides have been demonstrated to be products derived from the cleavage of prodynorphin, some of which include dynorphin A (1-17), dynorphin B, and α- and β-neoendorphin (Höllt, 1986; Reisine and Pasternak, 2001). Schoffelmeer et al., (1991) demonstrated that endogenous β-endorphin peptide possessed high affinity for MOR and potently induced a MOR-mediated decrease in noradrenaline and acetylcholine release that could be blocked by the MOR antagonist CTAP. However, the selectivity of β-endorphin for MOR was only 2.5-fold over DOR, prompting many to seek a higher affinity and selective endogenous MOR ligand. Using a synthetic library of peptides derived from the brain peptide Tyr-W-MIF-1, two endogenous endomorphin peptides, endomorphin-1 and endomorphin-2 showed high affinity for the MOR in competition binding assays and were found to produce analgesic effects more potent than morphine (Zadina et al., 1997). However, the acceptance of this finding has been slow, as the biosynthetic precursor of the endomorphins has still not been discovered (Ronai et al., 2006).

**KAPPA OPIOID RECEPTORS AND THEIR SIGNALING: G PROTEINS, RECEPTOR REGULATION, AND SIGNAL TRANSDUCTION PATHWAYS**

The rat kappa opioid receptor (KOR) is comprised of 380 amino acids containing several sites within the intra- and extra-cellular loops that have been demonstrated to undergo post-translational modifications involved in modulation of KOR activity (Liu-Chen, 2004; see Figure 1.1). It has been demonstrated that N-linked glycosylation at two putative N-terminal sites of
cloned human KOR is required for the synthesis and trafficking of the receptor to cell surface (Li et al., 2007). Interestingly, elimination of N-linked glycosylation enhanced U50,488-induced desensitization and internalization of the KOR (Li et al., 2007) suggesting a role for receptor glycosylation not only in synthesis but modulating downstream receptor regulatory processes. GRK-mediated phosphorylation of rat KOR at serine 369 was found to desensitize KOR signaling of K⁺ ion channels (Appleyard et al., 1999) and was required for internalization of KOR (McLaughlin et al., 2003). Tyrosine phosphorylation has also been implicated in the regulation of the coupling of KOR to G-proteins, where enhancement of this coupling was shown to regulate agonist efficacy (Appleyard et al., 2000).

G protein-coupled receptors (GPCR) are a large family of receptors that signal via second messenger systems to control a variety of physiological processes. Over 40% of therapeutic drugs are thought to modulate the activity of GPCRs (Brink et al., 2004). Traditionally, binding of orthosteric ligands are known to modulate the intrinsic activity of GPCRs by inducing conformational shifts in the receptor that alter the affinity for and activation of G-proteins that subsequently propagate signals to a variety of intracellular effector systems (Kenakin, 2004; Greasley and Clapham, 2006). As noted above, opioid ligands are generally classified into two major categories: agonist or antagonist, although a number of opioid ligands can possess more than one form of activity, dependent on dose and receptor subtype (Martin, 1984). Nevertheless, the classical definition of ligands describe agonists as those that enhance receptor-mediated activation of G proteins, while antagonists elicit no response alone, but rather block agonists or inverse agonists from exerting their effects (Negus, 2006; see Figure 1.2).

The discovery of distinct opioid receptor sites helped to clarify the pharmacological classification of opioids, but it was quickly realized that opioids varied widely in their selectivity (receptor preference), affinity (binding potential), and efficacy (activity). Terms such as “partial agonist”, “competitive antagonism”, “agonist-antagonist”, and “mixed agonist/antagonist” (receptor dualism) were soon utilized to describe these effects (Martin, 1984). Drugs classified as partial agonists are drugs unable to produce maximal efficacy, whereas agonist/antagonist compounds are those ligands that produce agonistic effects at low doses but at higher doses behaved as
antagonists. In contrast, mixed agonists or mixed antagonists were those that interacted with more than one opioid binding site (i.e., displayed receptor dualism) (Martin, 1984). Proceeding from the early binding study findings, it became clear that some opioids could not be exclusively classified as agonists or antagonists, as they demonstrated varying degrees of efficacy and promiscuity (non-selectivity) between opioid binding sites.

In recent years, these definitions have proven progressively more limited to define new observations. For example, DOR found in neuroblastoma glioma hybrid cell lines exhibited agonist-independent activity that was found to be negatively regulated by compounds described as inverse agonists, as these drugs produced the opposite effect of agonists (Costa et al., 1988; Costa and Herz, 1989). The ligand ICI,174-864 remains the best characterized of these compounds, reducing adenylyl cyclase (AC) activity in a concentration-dependent, naloxone-reversible manner (Chui et al., 1996). Interestingly, some researchers have estimated that 85% of classified antagonists may actually be inverse agonists but simply remain improperly examined as such (Kenakin, 2004; Greasley and Clapham, 2006). The distinction between antagonists and inverse agonists appears increasingly important in the face of emerging studies of functional selectivity, which suggest that they could produce distinct pharmacological outcomes (Wang et al., 2001; Chanrion et al., 2008). Moreover, adding to the complexity of opioid classification, a growing body of research has demonstrated that opioids acting upon the same receptor may also induce differential intracellular signaling and receptor regulatory pathways, a recently developed pharmacological concept often termed functional selectivity, biased agonism, or stimulus-directed trafficking (Urban et al., 2007). These findings suggest an increasingly complex classification of opioids lies ahead as new compounds and new methods for researching them become available. The present thesis examines the KOR system in attempts to elucidate the molecular mechanisms involved in differential signaling produced by structurally distinct ligands and their potential implications for behavior. Thus, the proceeding sections will focus the discussion on KOR regulation and signal transduction.
Figure 1.1. **Schematic representation of the 7TM kappa opioid receptor (KOR).** KOR spans the membrane seven times with its N-terminal located on the extracellular side and C-terminal on the intracellular side of the cell. Putative glycosylation sites are located on the N-terminus (indicated by Y). Extracellular loops are indicated EL and intracellular loops (IL). Taken from University of Minnesota Department of Medicinal Chemistry at [www.opioid.umn.edu/serp.html](http://www.opioid.umn.edu/serp.html).
Figure 1.2.  **Dose response curves of three major ligands** (agonist, neutral antagonists, and inverse agonists). The X-axis (abscissa) represents drug concentration (dose) and Y-axis (ordinate) represents drug effect (efficacy). Adapted from Negus, 2006.
Like other members from the family of GPCRs, KOR activation is agonist-dependent and thought to involve the uncoupling of inhibitory heterotrimeric G\textsubscript{i} or G\textsubscript{o} proteins from cognate receptors that results in the dissociation of G-proteins into G\textsubscript{α} and G\textsubscript{βγ} subunits (Law et al., 2000; Gainetdinov et al., 2004; Figure 1.3). In the inactive state, the G\textsubscript{α} subunit is bound to guanidine diphosphate (GDP), which is then switched for a guanidine triphosphate (GTP) upon activation. Active GTP bound G\textsubscript{αi} subunits, in turn, negatively modulate AC activity (Bhoola and Pay, 1986; De Montis et al., 1987; also reviewed in Law et al., 2000; Gainetdinov et al., 2004). Concurrently, dissociated G\textsubscript{βγ} subunits open K\textsuperscript{+} and calcium (Ca\textsuperscript{2+}) ion channels, and activate phospholipase C (PLC), and MAP kinase cascades (for review see Law et al., 2000 and Liu-Chen, 2004). KOR regulation is predominately agonist-dependent and regulated in a time-dependent manner, involving activation (seconds), desensitization and internalization (minutes to hours), and down-regulation (hours to days) (Gainetdinov et al., 2004; Liu-Chen, 2004).

Moreover, agonist-induced signaling is desensitized and terminated via receptor phosphorylation by a G-protein receptor kinase (GRK) at the C-terminus tail (Appleyard et al., 1999; McLaughlin et al., 2003), and the KOR is either recycled or down-regulated. Receptor down-regulation is marked by a complete loss of available receptors through a process mediated by proteasomal degradation, which is triggered by ubiquitination of the internalized KOR (Li et al., 2008).

Internalization has been traditionally thought to be involved in mediating termination of receptor signaling (Gainetdinov et al., 2004). While internalization of the KOR is required for down-regulation, it is important to note that it has also recently been associated with signaling through alternate signal transduction cascades, namely ERK1/2 MAP kinase (reviewed in Liu-Chen, 2004). However, to date, there are no in vivo studies that demonstrate a behavioral phenotype to support this hypothesis.
Figure 1.3.  **Kappa opioid receptor signal transduction.** KOR couples to heterotrimeric G protein comprised of $\alpha$, $\beta$, and $\gamma$ subunits. Ligand binding (yellow upside-down triangle) initiates conformational change in receptor, which causes the dissociation of the heterotrimeric G protein. The $G\alpha$ subunit inhibits adenylyl cyclase activity (AC). $G\beta\gamma$ subunits modulate $K^+$ and $Ca^{++}$ ion channels. $\beta$-arrestin recruitment results in the termination of G protein signaling by sequestering receptor from cell surface into internalized vesicles (internalization). Notably, ERK1/2 MAP kinase is activated via phosphorylation (PO$_4$) presumably mediated by G proteins and internalization.
Mitogen activated protein (MAP) kinase pathways are a series of serine/threonine kinases arranged in serial pathways that produce a cascade of signaling that are involved in a host of cellular processes, including the regulating cell function, cell proliferation, cell differentiation, and cell death (Pearson et al., 2001; Qi and Elion, 2005). The MAP kinase signaling cascades are ubiquitous and are found in every cell of the body functioning to regulate nuclear and cytosolic proteins (Cobb and Goldsmith, 1995). Regulation of these cascades is widespread, with a number of positive and negative influences converging on the activating proteins to dictate activation state moment-to-moment. As such, these cascades are important in the coordination of cellular function, acting as molecular coincidence detectors to adjust cellular activity to both internal and external events. To date, five distinct cascades have been described, but of these, the ERK1/2 MAP kinase, the p38/HOG, and the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) are the best characterized of the MAP kinase pathways (Guan, 1994; Qi & Elion, 2005). The p38/HOG kinase and JNK/SAPK pathways are predominately activated by stress, whereas the ERK1/2 MAP kinase pathway is predominately activated by growth hormones (Guan, 1994; Obata et al., 2000). It is notable that extensive cross-talk between pathways has been described (Guan, 1994; Obata et al., 2000; Kogkopoulou et al., 2006), in particular between p38 and ERK1/2 MAP kinase pathways (Kogkopoulou et al., 2006).

The activation of ERK1/2 MAP kinase stimulated by insulin and growth factors has been extensively studied and well characterized (Cobb & Goldsmith, 1995, Figure 1.4). Typically, the activation of a receptor tyrosine kinase (RTK) induces autophosphorylation and mediates the recruitment of a signaling complex comprised of growth factor-receptor bound protein 2 (GRB2), SOS, and Ras. SOS protein induces the activation of Ras protein, which in turn activates Raf (MAP kinase kinase kinase). Activated Raf phosphorylates and activates an ERK kinase (MEK1/2 or MAP kinase Kinase), which in turn activates ERK1/2 MAP kinase (Cobb and Goldsmith, 1995; Pearson et al., 2001). The signaling of ERK1/2 MAP kinase, as well as other MAP kinase pathways, is tightly controlled. Negative feedback regulation of ERK1/2 MAP kinase
Figure 1.4. **Receptor tyrosine kinase (RTK) activation of ERK1/2 MAP kinase.** Growth factor stimulation of RTK induces autophosphorylation and recruitment of GRB2, SOS, and Ras. Activated Ras targets Raf, which in turn activates MEK1/2. Note inhibitors, SL327 and U0126 target MEK1/2, which directly activates ERK1/2 MAP kinase. Subsequently, ERK1/2 MAP kinase activates transcription factors Elk1, Sap1a, and Net (From Riken Biosresource Center DNA Bank http://www.brc.riken.jp/lab/dna/en/GENESETBANK/mapk_ras.html).
occurs via interaction with several proteins, including protein kinase B (PKB or Akt) (Zimmermann & Moelling, 1999; Guan et al., 2000; Pearson et al., 2001) and phosphatases, which dephosphorylate (and thereby deactivate) kinases (Harding et al., 2005). Negative feedback regulation is crucial, as overactive signaling of MAP kinase has been associated with tumor growth and the progression of cancer (Qi and Elion, 2005).

The mechanisms underlying GPCR-mediated activation of ERK1/2 MAP kinase have only recently begun to be understood. Two major signaling pathways, a conventional G protein mediated pathway and an unconventional β-arrestin-mediated internalization mechanism, have been implicated in GPCR-mediated activation of MAP kinase (Luttrell, 2003). Research has shown that the two pathways of GPCR activation of ERK1/2 can be distinguished by their differential temporal and spatial patterns of activation (Ahn et al., 2004; Shenoy et al., 2006). G proteins and associated enzymatic proteins, e.g. protein kinase A (PKA), induce a rapid activation of ERK1/2 that is predominantly nuclear (Ahn et al., 2004; Shenoy et al., 2006), while β-arrestin-mediated receptor internalization underlies the delayed, late-phase activation located in endosomal vesicles in the cytosol (Ahn et al., 2004). This “unconventional” method by which agonist-induced internalization of GPCRs mediate the activation of MAP kinase is a relatively new concept, and has only been explored with receptors that couple to Gs-proteins, such as β-adrenergic (Azzi et al., 2003) and angiotensin type 1A (Tohgo et al., 2002) receptors.

Several studies have reported that peptide and non-peptide (synthetic) KOR agonists can activate MAP kinase pathways, and suggest multiple mechanisms are involved (Belcheva et al., 1998; Jordan et al., 2000). Collectively, however, these data are inconsistent. For instance, some reports suggest that KOR internalization is required for activation of p38 (Brucas et al., 2006) and ERK1/2 (Ignatova et al., 1999) while others suggest KOR internalization is not required (Li et al., 1999; Jordan et al., 2000). Further reports also show that KOR-mediated activation of ERK1/2 requires a G protein mediated event involving PKC, Ras, and G\(\beta\gamma\)-protein activity (Belcheva et al., 1998; Belcheva et al., 2005). Given the data obtained from studies of Gs-GPCRs implicating two distinct temporal mechanisms in GPCR activation ERK1/2, it is possible that the aforementioned mechanisms of KOR activation of ERK1/2 may also occur in a similar
bimodal manner. Surprisingly, there are no studies to date examining this possibility. This has become increasingly important given recent reports suggesting that the dysphoria induced by KOR agonists may be due solely to activation of nuclear MAP kinases (Brucas et al., 2007; Land et al., 2008). These and other findings (Moron et al., 2003) suggest that KOR regulation of MAP kinase activation may also be key in both understanding the full pharmacology of the receptor as well as developing effective KOR agonists free of detrimental side effects as therapeutics for cocaine abuse.

**SUMMARY**

Cocaine abuse and addiction is a major public health concern, yet no FDA approved pharmacotherapeutic treatment is available. KOR agonists have been demonstrated to have potential as therapeutic agents to combat cocaine abuse and addiction (Prisinzano et al., 2005). However, several limitations exist and repeated KOR agonist treatment may paradoxically enhance cocaine-seeking behavior. To advance the development of KOR ligands as therapeutics, a mechanistic understanding of the paradox is needed. Of interest, several studies demonstrate that the activation of ERK1/2 MAP kinase is a molecular determinant of cocaine reward, abuse, and relapse to drug-seeking behavior (Valjent et al., 2000 Radwanska et al., 2005). As KOR agonists are able to both hyperpolarize neurons (Magolis et al., 2003) and induce activation of ERK1/2 MAP kinase in vitro (Belcheva et al., 1998), these results together suggest a means by which KOR agonists may produce their differential time-mediated modulation of cocaine reward. Given that GPCRs can mediate the activation of distinct temporal and spatial pools of ERK1/2 MAP kinase, further understanding of how KOR modulates ERK1/2 MAP kinase activity may help explain how the divergent in vivo pharmacological effects occur over time.
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The psychostimulant cocaine produces the perception of reward via enhancement of DA signaling (Spanagel et al., 1992). The endogenous kappa opioid system is thought to modulate reward by hyperpolarizing midbrain dopaminergic neurons indirectly reducing neuronal DA signaling (Spanagel et al., 1992; Margolis et al., 2003; Chefer et al, 2005). Thus, agonists activating the KOR may be useful in the treatment of cocaine abuse (Prisinzano et al., 2005). Indeed, acute administration of KOR agonists has been found to suppress behavioral demonstrations of the rewarding effects of cocaine (Crawford et al., 1995; McLaughlin et al., 2006). However, the utility of long-term KOR agonist treatment is limited by several adverse effects, such as sedation and dysphoria (Pfeiffer et al., 1986), although it is thought that tolerance may develop to these effects. More concerning are recent studies demonstrating that repeated KOR agonist administration results in a paradoxical increase in chemically- and electrically-evoked DA levels in the NAc (Fuentealba et al., 2006). Consistent with the neurophysiological effects, behavioral reports show that prolonged exposure to KOR agonists can increase drug craving and drug-seeking behavior (Negus, 2004). A recent study found that administration of a single low dose (5 mg/kg) of the KOR agonist (trans)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide (U50,488) initially suppressed but then paradoxically potentiated cocaine-conditioned place preference (CPP); an effect dependent on the interval between agonist and cocaine administration (McLaughlin et al., 2006). However, an underlying neurobiological mechanism to explain the distinct time-dependent behavioral effects was not determined.

It is feasible that the KOR-mediated activation of the ERK1/2 MAP kinase could account for the paradoxical potentiation, as this signal transduction pathway is a molecular marker for
drug-induced plasticity (Valjent et al., 2000). Notably, agonist-induced stimulation of the KOR has been reported to activate ERK in vitro (Belcheva et al., 2005). However, studies examining the effects of KOR agonists on ERK activity in vivo and a correlation of this effect to behavioral changes in drug reward are lacking.

The use of a recently discovered KOR agonist, salvinorin A, may provide a test for this idea. Salvinorin A is the active ingredient of the hallucinogenic plant *Salvia divinorum* and, like U50,488, possesses high affinity and agonist efficacy for the KOR in vitro (Chavkin et al., 2004). Yet, despite having similar potencies in G protein mediated signaling, U50,488 and salvinorin A appear to differ significantly in other aspects. For instance, although salvinorin A binds within the same binding pocket as other KOR agonists, U69,593 and dynorphin, receptor modeling suggest that salvinorin A may interact with a distinct set of residues within this region (Vortherms et al., 2007). While speculative, this result was supported by the demonstration that salvinorin A was ~40% less effective than U50,488 at inducing internalization and down-regulation of the KOR (Wang et al., 2005). As agonist-induced receptor internalization is required for the activation and signaling of a distinct temporal pool of ERK1/2 MAP kinase (Leftkowitz & Shenoy, 2005) it is feasible that salvinorin A could produce qualitatively distinct effects on receptor signaling and regulation from other agonists, and that this may result in significant differences on behavior.

In the present studies, it is hypothesized that U50,488 induces a time-dependent paradoxical potentiation of cocaine reward that is a result of a KOR-mediated activation of ERK1/2 MAP kinase. In contrast, as salvinorin A-induced internalization of the KOR is impaired it is theorized that it would lack the ability to activate ERK signaling. If proven, it would be expected that salvinorin A would not potentiate cocaine reward overtime in the same manner as U50,488. To test this hypothesis, we established and compared the time-dependent modulation of equipotent doses of the two structurally distinct KOR agonists on cocaine reward using the conditioned place preference (CPP) assay. We also determined the activating effect of U50,488 and salvinorin A on ERK1/2 MAP kinase using Western blot analysis to measure ERK1/2 phosphorylation, a marker of activation, in brain following agonist pretreatment ex vivo. Finally,
we examined the effects of ERK1/2 MAP kinase inhibitor SL-327 on U50,488-mediated potentiation of cocaine-CPP.
MATERIALS AND METHODS

Animals and Housing

Male C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME) weighing 21–30 g (8–12 weeks old) were used in these experiments. Mice were group-housed, 2–4 per cage, in self-standing plastic cages (28 cm L × 16 cm W × 13 cm H) within the Nightingale Hall animal core facility at Northeastern University were acclimated in the environment for a minimum of 1 week prior to experiments. Housing rooms were illuminated on a 12-h light–dark cycle with lights on at 0700 h. Food pellets and water were available *ad libitum*. Animals were housed and cared for in accordance with the 1996 National Institute of Health *Guide for the Care and Use of Laboratory Animals* for procedures approved by the Institutional Animal Care Committee, with regular inspection by veterinary staff to ensure compliance. C57Bl/6J mice were selected for this study because of their established responses to cocaine place conditioning (Brabant et al., 2005; McLaughlin et al., 2003; 2006).

Chemicals

U50,488 and norbinaltorphimine (nor-BNI) were obtained from the NIDA Drug Chemical Supply (Bethesda, MD). Salvinorin A was provided by Dr. Thomas Prisinzano, after isolation as described previously (Butelman et al. 2007). Cocaine and SL-327 were obtained from Sigma-Aldrich Chemicals (St. Louis, MO), as were all other drugs if not otherwise noted. SL-327, U50,488 and salvinorin A were dissolved in 30% DMSO/70% saline/0.9% prior to injection; all other drugs used were dissolved in 0.9% saline.
Antinociceptive Testing Using the 55°C Warm-Water Tail-Withdrawal Assay

The tail-withdrawal assay was selected over other appropriate antinociceptive measures (e.g., the acetic-acid writhing test) for the assay’s innate capacity for repeated measures, allowing the monitoring of agonist effect from onset to completion in the same animal. The response latency for the mouse to withdraw its tail following immersion in 55°C water was taken as the end point (Vaught & Takemori, 1979), modified as previously described (McLaughlin et al., 2003). A cutoff time of 15 seconds (s) was used to prevent tissue damage.

Conditioned Place Preference

C57Bl/6J mice were used in a three-compartment place conditioning apparatus as described previously using a biased conditioning design (McLaughlin et al., 2003; 2006; Carey et al., 2007). Briefly, mice were tested for place preference by placing individual animals in the small central compartment and allowing them to freely explore the entire apparatus for 30 minutes (min). Movement through the chambers was detected by the breaking of infrared beams emitted from imbedded sensors, recorded by an automated computerized system (San Diego Instruments, San Diego, CA) to measure the time spent in each compartment. The apparatus itself is balanced, with animals demonstrating a statistically equivalent amount of time in each of the three compartments prior to place conditioning (606±22 s in the left compartment, 631±22 s in the right compartment, and 563±17 s in the central compartment; one-way ANOVA, F(2,123)=2.82, p = 0.06).

In the present study, a preconditioning bias prior to place conditioning was established. Place conditioning began immediately following cocaine (10 mg/kg, s.c.) or saline (0.9% saline, 0.3 ml/30 g body weight, s.c.) administration, whereupon mice were consistently confined for 30 min in the appropriate outer compartment. A dose of 10 mg/kg cocaine was selected for this study as it has been shown previously to produce a reliable conditioned-place preference response in C57Bl/6J mice (Brabant et al., 2005). Conditioning with saline followed 4 hours (h)
later in a similar manner, but paired to the opposite chamber. This conditioning cycle was repeated once on each of two days, as demonstrated previously to be effective in producing a significant place preference response (McLaughlin et al., 2003; 2006). Data are plotted as the difference in time spent in the eventual conditioning-drug paired (e.g., cocaine)- and vehicle-paired compartments, such that by convention the initial bias generates a negative value, and a positive value reflects a conditioned preference for the initial drug-paired side. Note that conditioned place aversion, where animals avoid the initial drug-paired compartment, is also detectable under this method when animals spend significantly more time in the initially preferred side as compared to both preconditioning and saline-conditioned responses.

To examine the effect of pretreatment with the KOR agonists, U50,488 or salvinorin A on cocaine-CPP, mice were tested for their initial preferences then pretreated with vehicle (30% DMSO in 0.9% saline) or U50,488 (10 mg/kg i.p.) or salvinorin A (1 or 3 mg/kg, i.p.) prior to the daily start of place conditioning. Doses of salvinorin A were selected on the basis of the pharmacokinetics obtained in the antinociceptive assays (see page 50), such that it was equipotent and comparable to U50,488. In these experiments, pretreated mice were subsequently administered saline or cocaine at various times 15-360 min after agonist administration, and confined to the initial drug-paired compartment for 30 min of place conditioning. Some animals were administered the KOR antagonist nor-BNI (10 mg/kg i.p./day, - 120 min prior to place conditioning), a dose shown previously to be KOR selective under these testing conditions (McLaughlin et al, 2003). Additional mice were administered the ERK1/2 MAP kinase inhibitor SL-327 (50 mg/kg i.p./day, either -60 or -140 min prior to place conditioning).

**Isolation and Western Blot analysis of Mouse Brain Protein**

Mice were sacrificed, the cerebellums discarded, and the remaining brain excised. Individual brains were homogenized on ice in 10X weight volume of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.01% Triton X-100, 0.1 mM PMSF, and a cocktail of protease and phosphatase inhibitors (leupeptin 10 µg/ml, 10 µg/ml aprotinin, 2 mM sodium
orthovanadate, 5 µg/ml okadaic acid) using a Dounce tissue homogenizer. Protein concentration was estimated based on bovine serum albumin standards using the Bradford assay (Bradford, 1976). A total concentration of 15 µg of protein was resolved by SDS-PAGE using 10% bis-acrylamide non-reducing gels and transferred to nitrocellulose. Blots were blocked in 10% nonfat dry milk with TBS-T (0.1% Tween 20 in Tris-buffered saline, pH 8.0) and then incubated overnight at 4°C with primary antibody (Cell Signaling Technologies, Beverly, MA) in 1% bovine serum albumin (BSA)/TBS-T. Primary antibodies used were specific for the phosphorylated form of ERK1/2 MAP kinase at a dilution of 1:1000 and non-specifically recognized ERK1/2 MAP kinase regardless of phosphorylation state at a dilution of 1:2000. The phosphorylated-specific antibody was used to measure the population of activated ERK1/2 MAP kinase protein, as phosphorylation correlates with kinase activation, whereas the non-specific antibody determined total amounts of ERK1/2 MAP kinase protein as it labels all forms of the protein. Blots were then probed with HRP-conjugated goat-anti-rabbit secondary antibody (Jackson ImmunoLaboratories, West Grove, PA) (1:5000) in 1%BSA/TBS-T buffer. Antibody labeling was visualized with an enhanced chemiluminescence detection kit (ECL, Amersham) on Kodak X-OMAT film. Antibody labeling of both ERK1/2 MAP kinase antibodies labeled only two bands, consistent with the expected weights of the ERK1 and ERK2 at 44 and 42 kilodaltons, respectively.

**Data Analysis**

Student’s paired samples t-tests comparing baseline and post-treatment tail-withdrawal latencies were used to determine statistical significance for all tail-withdrawal data. Signal intensity in Western blots was quantified with NIH ImageJ 1.62 software (National Institutes of Health) standardized against matching, non-specific total ERK protein labeling from vehicle treated control protein within the same experiment and was analyzed by One-way analysis of variance (ANOVA) with significant differences analyzed by Tukey’s HSD *post hoc* tests. Significant differences in behavioral data were analyzed by ANOVA (one-way or repeated measures), with significant results further analyzed with the Fisher’s LSD or Tukey’s HSD *post hoc* tests for
significant pairwise comparisons within and between groups using SPSS 14.0 (Chicago, IL). Dependent variables were expressed and analyzed as the difference in time spent in the initial drug- and saline-paired compartments. Data for CPP groups were analyzed for cocaine or vehicle conditioning, with the additional factors of U50,488, salvinorin A, nor-BNI, SL-327 or vehicle pretreatment. Seconds spent in the drug-paired, saline-paired, and neutral central compartments were additionally analyzed separately. All data are presented as means±SEM, with significance set at $p<0.05$. 
RESULTS

Kappa opioid receptor agonists mediate antinociception in a dose- and time-dependent manner

Salvinorin A (0.5-6 mg/kg, i.p.) increased the tail withdrawal latency of C57Bl/6J mice (Figure 2.1) in a dose-dependent manner that was blocked by the selective KOR antagonist nor-BNI (10 mg/kg) (Figure 2.1, square). After a single administration of 1 mg/kg of salvinorin A the antinociceptive time course was similar to that produced by 5 mg/kg of U50,488, with peak antinociception measured 30 min after administration returning to baseline by 50 min (Figure 2.2). Salvinorin A (3 mg/kg) produced antinociceptive effects that were similar to a higher dose of U50,488 (10 mg/kg); having peak effects 40 min after administration which subsided by 90 min after administration (Figure 2.2). Notably, vehicle treatment did not affect antinociceptive responses at any time point (Figure 2.2, triangles).

Additionally, mice that received a second equivalent dose of either salvinorin A (3 mg/kg, i.p.) or U50,488 (10 mg/kg, i.p.) 95 min after the initial injection showed reduced antinociceptive responses ($p<0.05$, vs. initial peak response), indicative of acute KOR desensitization. We further examined the duration of acute agonist-induced desensitization by administering a second equivalent dose of either agonist 6 hours after initial administration. The subsequent administration of U50,488 (10 mg/kg) or salvinorin A (3 mg/kg) resulted in peak antinociception (9.82±1.78 s and 14.6±0.34 s) 40 min after administration that was not significantly different from the initial peak response. Overall, these results established the basis for an equipotent comparison of the time-dependent activity between the two agonists in subsequent conditioned place preference testing.
Figure 2.1. **Dose-response of Salvinorin A-induced antinociception in the mouse 55°C warm-water tail withdrawal test.** Response latencies were obtained from C57Bl/6J mice both before (open triangle, left of dashed line) and 40 min after administration of a single graded dose of salvinorin A (Sal A, dark circles). Antinociception induced by a high dose of salvinorin A (6 mg/kg, i.p.) was blocked by pretreatment with the kappa opioid receptor selective antagonist, nor-BNI (open square, 10 mg/kg, i.p., -90 min). n=8-10 mice for all points. * = p<0.05, significantly different than baseline tail-withdrawal response as determined by Student’s paired-samples t-test.
Figure 2.2. **Time course of kappa agonist-induced antinociception.** The time course of agonist-induced antinociception was obtained with tail withdrawal latencies measured before and after administration of U50,488 (5 mg/kg i.p., open inverted triangles, or 10 mg/kg i.p., open circles), salvinorin A (1 mg/kg i.p., filled diamonds, or 3 mg/kg i.p., filled squares) or vehicle (30% DMSO in 0.9% saline, open triangles) obtained in 10 min intervals for up to 90 min. *= p<0.05, significantly different than baseline tail-withdrawal response as determined by Student’s t-test. Data points each represent n=9 mice.
**Exposure to U50,488 modulates cocaine-conditioned place preference in a time-dependent manner**

To confirm the earlier finding that direct activation of KOR may produce a time-dependent modulation of cocaine-CPP, we examined the effects of U50,488 on place preference at twice the dose (10 mg/kg, i.p.) used previously (McLaughlin et al, 2006). U50,488 or vehicle was administered from 30 to 360 min prior to cocaine or saline place conditioning. Mice pretreated 30 min with vehicle prior to saline conditioning demonstrated no significant change from initial CPP responses (Figure 2.3, left bar), whereas vehicle pretreated mice conditioned with cocaine demonstrated a significant increase in CPP (Figure 2.3, left-most filled bar; $F_{1,80}=7.53$, $p<0.01$). Notably, mice pretreated 30 min with U50,488 (10 mg/kg, i.p.) prior to saline place conditioning demonstrated conditioned place aversion as indicated by a significant decrease in the difference in time spent in the initial saline-paired box ($-496.75.4$; $F_{1,64}=6.22$, $p<0.05$). Moreover, U50,488 pretreated mice also spent significantly less time in the saline-paired box than mice that were pretreated with vehicle ($p<0.01$, Student’s $t$ test).

U50,488 pretreatment also modulated the direction and magnitude of the subsequent cocaine-CPP response (Figure 2.3, $F_{5,104}=10.56$, $p<0.001$). Tukey’s HSD post hoc test demonstrated that cocaine conditioning during peak U50,488-induced analgesia (30 min after administration) prevented the normal cocaine-CPP response (Figure 2.3, left-center bar; $p<0.05$ vs vehicle pretreated cocaine). Cocaine conditioning following U50,488 pretreatment corresponding to the decline of antinociception (~75 min, see Figure 2.2) resulted in a slight reduction in cocaine-CPP that was not significantly different from either saline or cocaine place conditioned mice pretreated with vehicle ($p>0.05$; Figure 2.3). However, mice that were cocaine place conditioned 90 min after U50,488 pretreatment showed a potentiation of cocaine-CPP significantly greater than that of all other place-conditioned groups of mice ($p<0.05$; Figure 2.3).

The potentiating effect of this dose of U50,488 pretreatment lasted less than 6 hr, as pretreatment with U50,488 6 hr prior to cocaine place conditioning resulted in a response that was not
significantly different from the cocaine-CPP response of vehicle-pretreated mice ($p>0.05$; Figure 2.3, rightmost bar).

**Exposure to salvinorin A dose-dependently suppresses, but does not potentiate, cocaine-conditioned place preference**

We next examined the effects of salvinorin A on modulation of conditioned place preference (Figure 2.4). Mice pretreated with salvinorin A (1 mg/kg, i.p.) 15 min prior to saline place conditioning demonstrated the expected conditioned place aversion, spending significantly less time in the salvinorin A-saline paired chamber when compared to their initial preconditioned responses and to the final preferences of vehicle-pretreated mice ($F_{9,167}=7.19$, $p<0.001$; repeated-measures ANOVA, Figure 2.4, striped center bar). Mice pretreated with salvinorin A (1 mg/kg, i.p.) were subsequently place conditioned with cocaine at time points corresponding to either the peak- or post-analgesic effects of this dose of salvinorin A (-15 min and -75 min, respectively, indicated by right-center and rightmost bar, Figure 2.4). Salvinorin A pretreated mice demonstrated a cocaine-CPP response that was significantly greater than preconditioning responses ($F_{1,167}=20.19$, $p<0.001$) and salvinorin A-saline conditioned mice ($F_{4,178}=5.07$, $p<0.01$, Figure 2.4). However, Fisher’s LSD *post hoc* test showed no significant difference between salvinorin A pretreated cocaine and vehicle pretreated cocaine place conditioned animals ($p>0.05$). Moreover, cocaine place conditioning 75 min after salvinorin A administration (see Figure 2.2) did not potentiate cocaine-CPP as was previously observed with an equipotent dose of U50,488 pretreatment (Figure 2.3; McLaughlin et al, 2006).

We then examined the effect of a higher dose of salvinorin A, 3 mg/kg, equipotent to the dose of U50,488 used in this study (10 mg/kg; Figure 2.1 and Figure 2.2). Mice administered salvinorin A (3 mg/kg i.p.) 15 min prior to cocaine place conditioning demonstrated a cocaine-CPP response that was significantly less than vehicle-pretreated cocaine-CPP responses ($F_{1,155}=5.17$, $p<0.05$; repeated-measures ANOVA), but no different than preconditioning or final saline place-conditioned responses ($p>0.05$; Figure 2.5, center black bar, normal cocaine-CPP response is indicated by dotted line). Pretreatment with nor-BNI (10 mg/kg, i.p.) prior to
Figure 2.3. Cocaine-conditioned place preference is potentiated by a time-dependent pretreatment with U50,488. Final place preference is plotted as the difference in time spent in the first-drug paired side of the apparatus (in seconds). A positive value represents the time spent in the first-drug (cocaine or saline) paired chamber. Vehicle-pretreated mice place conditioned with saline alone demonstrated no significant change in place preference from initial responses (open leftmost bar), whereas cocaine place conditioned mice demonstrated a significant place preference (dark bar second from left). In contrast, mice administered U50,488 30, 75, 90 or 360 min prior to cocaine demonstrated time-dependent modulation of cocaine-conditioned place preference responses. Bars represent n=11-24 mice. * = p<0.05, significantly different from both matching preconditioning and saline-saline place conditioned responses; † = p<0.05, significantly different from all other place preference responses of conditioned mice as determined by one-way ANOVA followed by Tukey’s HSD post hoc test.
Figure 2.4. **Aversive effects of 1 mg/kg salvinorin A and suppression of cocaine-CPP.**

Final place preference is plotted as the difference in time spent (in seconds) in the first-drug paired side of the apparatus. Positive values represent the time spent in the first-drug (cocaine or saline) paired chamber, whereas negative values represent less time spent in the first-drug paired chamber. Cocaine-conditioned mice demonstrated a significant place preference (left-most bar).

While salvinorin A (Sal A, 1 mg/kg, i.p.) administered 15 min prior to saline conditioning resulted in conditioned place aversion (left middle bar), salvinorin A administered 15 or 75 min prior to cocaine place conditioning did not significantly suppress cocaine-conditioned place preference responses (right bars). Bars represent n=11-24 mice. *p<0.05, significantly different from matching preconditioning responses; †= significantly different from cocaine-CPP responses; α=significantly different from saline CPP response as determined by one-way ANOVA followed by Fisher LSD *post hoc* test.
salvinorin A blocked the suppression of cocaine-CPP produced when salvinorin A was administered 15 min before cocaine conditioning (p<0.05; Figure 2.5, left-center grey bar). Notably, no differences between nor-BNI pretreatment and vehicle-treated cocaine place conditioned mice were observed (p>0.05; left center gray bar compared to dotted line, respectively).

Cocaine place conditioning following pretreatment with salvinorin A (3 mg/kg, i.p) at time points corresponding to the decline (-75 min) or abatement of salvinorin A-induced antinociception (-90 or -120 min) were also examined. While shorter salvinorin A pretreatments time-dependently reduced cocaine-CPP (-15 or -75 min; F[11,199]=6.99, p<0.05; Figure 2.5), longer pretreatments did not potentiate cocaine-CPP (-90 or -120 min; p>0.05; Figure 2.5). Moreover, additional testing demonstrated that salvinorin A (3 mg/kg) pretreatments 6 hours prior to cocaine-conditioning also had no effect on subsequent cocaine-CPP response (196±62.3 sec; p>0.05). Thus, while salvinorin A dose- and time-dependently suppressed cocaine-CPP, it did not induce a subsequent potentiation of cocaine-CPP.

**U50,488, but not salvinorin A, activates ERK 1/2 MAP Kinase through the kappa-opioid receptor in a time-dependent manner**

To examine the effect of KOR agonists on activation of ERK in mouse brain we performed semi-quantitative Western blot analysis. Phosphorylation-specific and non-specific (total) antibody labeling was compared to determine activated (pERK) and total levels of ERK, respectively. Notably, no significant differences in concentration of total ERK1 or ERK2 isoforms between samples were observed (Figure 2.6, bottom pair of bands). The immunoreactive bands corresponding to phosphorylated protein from each agonist treatment condition were normalized to total ERK of vehicle treated controls and graphed (as described in Materials and Methods) (Figure 2.6). Analysis of each isoform by One-way ANOVA showed significant effects of agonist on pERK1 and pERK2 levels (F[8,51]=5.39, p<0.01 and F[8,184]=8.87, p<0.01, respectively). ERK1 phosphorylation was enhanced relative to vehicle control when brains were analyzed 90 min after U50,488 treatment (1.39±1.34) but not at any other time point after U50,488 administration.
Figure 2.5. **Aversive effects of 3 mg/kg salvinorin A and suppression of cocaine-CPP.**

A high dose of salvinorin A, 3 mg/kg, i.p., was administered at four different time points (-15, -75, -90, -120 min), before daily place conditioning with cocaine and saline ("Cocaine-CPP" groups) or saline in both compartments ("Saline CPP"). Post-conditioning preference results collected blind are plotted in seconds to highlight time spent on the first drug-paired side of the apparatus. Mice pretreated with salvinorin A 15 min prior to saline place conditioning showed no change from initial place preference (striped bar), but this dose of salvinorin A suppressed cocaine-CPP (dashed horizontal line) in a time-dependent manner (black bars). Mice administered nor-BNI (10 mg/kg, i.p.) 90 min prior to a 15 min pretreatment with salvinorin A (3 mg/kg) demonstrated a normal CPP response after place conditioning with cocaine (left grey bar). However, salvinorin A pretreated mice did not demonstrate potentiation of cocaine place preference over that shown by cocaine alone at any time point or dose. Bars represent n=11-23 mice. *= p<0.05, significantly different from preconditioning as determined by a multivariate ANOVA followed by Fisher’s LSD *post hoc* test.)
Salvinorin A treatment did not produce significant change in pERK1 at any time point. Treatment with nor-BNI prior to U50,488 showed a significant reduction in pERK1 ($p<0.05$ vs. U50,488 at – 90 min). Curiously, nor-BNI alone showed a significant reduction in pERK1 ($p<0.05$). Further analysis of pERK2 isoform showed a similar pattern to pERK1, with U50,488 treatment showing an increase in phosphorylation of ERK2 ($1.22\pm0.08$) only at 90 min after treatment, but this effect was insignificant ($p>0.05$). Salvinorin A treatment did not produce statistically significant effects on pERK2 at any time point after administration. Again, nor-BNI treatment alone resulted in a significant reduction in pERK2 isoform when compared to vehicle alone, although this was highly variable and thus, not significant ($p<0.05$). Moreover, the significant reduction in pERK2 isoform was still evident even when nor-BNI treatment was followed by U50,488 treatment for 90 min (Figure 2.6).

**Prolonged exposure to U50,488 results in a KOR-mediated potentiation of cocaine-CPP blocked by the KOR-selective antagonist, nor-BNI, or the MAP kinase inhibitor, SL-327**

To test the hypothesis that U50,488 directly activated the KOR to produce the potentiation of the cocaine-CPP response, mice were administered nor-BNI (10 mg/kg i.p.) 2 h prior to vehicle or U50,488 pretreatment and cocaine conditioning. One-way ANOVA showed that pretreatment with nor-BNI prevented the U50,488-induced potentiation while nor-BNI alone had no significant effect on cocaine-CPP ($F_{5,106}=4.842$, $p<0.05$; Figure 2.7, white bars).

The Western blot results in brain suggest that U50,488 may be activating ERK1/2 MAP kinase through the KOR to potentiate cocaine-CPP. We therefore tested the effect of inhibiting ERK1/2 MAP kinase prior to U50,488 treatment on cocaine-CPP. Mice were administered the selective ERK1/2 MAP kinase inhibitor SL-327 (50 mg/kg, i.p.) 50 min prior to the pretreatment with vehicle or U50,488, i.e., 2.3 hr before subsequent cocaine conditioning. SL-327 pretreatment suppressed the potentiation of cocaine-CPP produced by U50,488 ($p<0.05$ versus U50,488 at -90 min, Figure 2.7). It is important to note that acute inhibition (-60 min) of ERK1/2 MAP kinase with SL-327 has been shown to suppress cocaine-CPP (Valjent et al., 2006). In control experiments, we duplicated this result, with a 60 min pretreatment prior to place
conditioning this resulted in an SL-327 inhibition of cocaine-CPP (-197.5±67.9 s) that was not significantly different from initial place preference responses (-137±43.4 s). However, the inhibitory effect as a 2.3 hour pretreatment used here did not significantly alter cocaine-CPP alone (Figure 2.7, right most grey bar). Together, these data further support the hypothesis that U50,488-induces potentiation of cocaine-CPP through a KOR-mediated activation of ERK1/2 MAP kinase.
a

Phospho-ERK1/2 MAP Kinase Ab

Nonphospho-ERK1/2 MAP Kinase Ab

b

% Control Labeling (+/- SEM)

Vehicle  Salvinorin A  U50,488  U50,488 + nor-BNI

ERK1 MAP kinase

ERK2 MAP kinase

Veh  -40  -90  -120  -40  -90  -360  -90  nor-BNI  nor-BNI
Figure 2.6. U50,488, but not salvinorin A, activated ERK1/2 MAP Kinase through activation of the kappa opioid receptor. (a) Representative Western blots of mouse brain protein (15 μg/lane) incubated with phosphorylated-specific ERK1/2 MAPK antibody (1:1000, upper row) or non-specific ERK1/2 MAPK antibody (1:2000, lower row). Protein was isolated from mice as described in “Materials and Methods” after being pretreated with vehicle (-90 min), salvinorin A (3 mg/kg, i.p., -90 min), U50,488 (10 mg/kg, i.p.,-90 min bars), nor-BNI (10 mg/kg, i.p., -180 min prior to harvest) or a combination as listed. (b) Summary graph of normalized phosphorylated-specific ERK1 (black bars) and ERK2 (white bars) MAP kinase antibody labeling of agonist-treated mouse brain. Graph summarizes the analysis of protein blots using NIH-ImageJ densitometry software analysis of the ECL image, standardized against matching, untreated control protein from the same experiment. Samples reflect protein isolated from mice pretreated with vehicle (-90 min), salvinorin A (3 mg/kg, i.p., -40, -90 or -120 min), U50,488 (10 mg/kg, i.p.,-40 or -90 min), nor-BNI (10 mg/kg, i.p., -180 min prior to harvest) or a combination as listed. Data points represent the means±SEM of 8-17 independent experiments. *= p<0.05, statistically significant against intensity of vehicle-treated mouse brain protein.
Figure 2.7. **U50,488-mediated potentiation of cocaine-CPP is blocked by the KOR-selective antagonist, nor-BNI, or the ERK1/2 MAP kinase inhibitor, SL-327.** Mice were administered U50,488 (10 mg/kg i.p.) 90 min prior to daily cocaine place conditioning (10 mg/kg, s.c.; striped left-most bar), producing a potentiation of cocaine-CPP (dashed horizontal line). Pretreatment with the KOR antagonist nor-BNI (10 mg/kg, i.p.) 90 min prior to either U50,488 (striped white bar) or vehicle (center white bar) administration and subsequent cocaine conditioning produced a cocaine-CPP response that was not significantly different from that of cocaine alone. Likewise, administration of SL-327 (50mg/kg i.p., -50 min) preceding a 90 min pretreatment with U50,488 (striped grey bar) or vehicle (right-most grey bar) before cocaine place conditioning prevented U50,488-induced potentiation of cocaine-CPP. Results are plotted as difference in seconds spent in the cocaine-paired chamber to highlight time spent on the cocaine-paired side of the apparatus. Bars represent n=12-24 mice. *= p<0.05, significantly different from response of mice conditioned with cocaine 90 min after U50,488 pretreatment as determined by one-way ANOVA followed by Fisher’s LSD post hoc test.
DISCUSSION

In the present study we sought to examine the mechanism by which kappa opioid agonists modulate cocaine reward. We hypothesized that KOR-mediated potentiation of cocaine reward occurs in an agonist-, time-, and ERK1/2 MAP kinase signaling dependent manner. Supporting our hypothesis we have demonstrated the following: 1) the synthetic agonist, U50,488, suppressed and potentiated cocaine reward in a time-dependent manner, 2) at equipotent and kinetically comparable analgesic doses the naturally occurring agonist, salvinorin A, suppressed but did not potentiate cocaine reward, and 3) a KOR-mediated activation of the ERK signaling pathway is required to induce potentiation of cocaine reward.

The divergent behavioral consequence that was observed between the KOR agonists was evident at time points when agonists were no longer producing antinociception. Although this implies the loss of KOR activity, there are alternative possibilities that may potentially confound the present results. First, the U50,488-induced potentiation of cocaine reward may be due to agonist-induced desensitization of the KOR, thereby resulting in the removal of an endogenous, tonic suppression of reward. For example, previous work has shown that prolonged administration of KOR agonists produced a KOR desensitization lasting up to 2 weeks (McLaughlin et al., 2004). However, results here suggest that this is unlikely, as agonist-induced desensitization of the KOR was over 6 hours later, a time course insufficient to desensitize the KOR during subsequent days of agonist treatment and cocaine place conditioning. Second, it is possible that the agonist selectivity for the KOR in mediating perception of reward differs from those that mediate KOR analgesia. However, KOR-selective antagonist pretreatment blocked salvinorin A-induced analgesia and conditioned place aversion as well as U50,488-induced potentiation of cocaine-CPP, confirming KOR selectivity. Third, some studies suggest that the analgesic dose of opioids may be dissociated from reward-modulating doses (De Vry et al., 1989). However, the present results are consistent with other studies that contradict De Vry et al., demonstrating that the analgesic doses of opioids correlate with their rewarding doses (Sala et al., 1992).
Emerging studies show that while agonist-induced receptor internalization has traditionally been thought to shut off agonist-induced signaling it may actually be involved in rerouting of signal transduction, i.e. ERK1/2 MAP kinase signaling (Lefkowitz & Shenoy, 2005). Again, salvinorin A was reported to be ~40% less effective than U50,488 at inducing internalization of the KOR expressed in CHO cell lines (Wang et al., 2005). In the present study, salvinorin A did not enhance the activation of ERK1 and 2 signaling in the brain as did U50,488. This activation of ERK by U50,488 correlated with its ability to potentiate cocaine reward. However, it must be noted that the use of whole brains limits our interpretation of whether the agonists are affecting the reward circuitry. In the present study we chose to detect global changes in the brain as to coincide with our behavioral pharmacological studies, in which blockade of ERK was achieved through systematic administration of SL-327. Future studies assessing changes in discrete brain regions would provide a more conclusive interpretation of whether the KOR agonists are differentially affecting ERK signaling particularly in the brain reward circuitry.

Previous reports show that salvinorin A has an ultra-short duration of action in analgesic assays (McCurdy et al., 2006; Ansonoff et al., 2006), which is inconsistent with results in the present studies. However, the present results are consistent with its long-lasting effects on suppression of DA release in the caudate putamen and NAc, which were shown to last up to 180 min after administration (Zhang et al., 2005; Carlezon et al., 2006). The aversive effects of salvinorin A presented here are also consistent with other reports demonstrating that salvinorin A induces conditioned place aversion (Zhang et al., 2005) and an increase in the rate of intracranial self-stimulation in trained rodents (Carlezon et al., 2006). Furthermore, the present results are also in line with Gehrke et al., 2008 demonstrating that repeated administration of salvinorin A modulates cocaine-related behaviors distinct from other KOR agonists. The possible explanations for the observed discrepancies in analgesic assays may be due to differences in route of administration, compound solubility and/or sample purity (Wolowich et al, 2006). For example, isolation of salvinorin A from *Salvia divinorum* may inadvertently include the inactive deacetylated metabolite salvinorin B, potentially diluting the subsequent dose. Moreover, with
multiple reactive ester groups, salvinorin A may demonstrate structural instability with the production of metabolites (Hooker et al., 2008). The potential metabolic modification of salvinorin A is significant, as substitution of the esters at the 2-position reduce or abolish activity at the KOR (Chavkin et al., 2004; Ansonoff et al., 2006). Furthermore, recent work indicates that a photooxidation product of salvinorin A (salvidivin A) has KOR antagonist activity (Simpson et al., 2007), an issue that cannot be resolved by the present data. Further work is needed to evaluate the contribution other metabolites of salvinorin A make to the pharmacology of this agonist, and if this may explain the observed discrepancies.

In summary, the study indicates that KOR activation and ERK1/2 MAP signaling is required for U50,488-induced potentiation of cocaine reward. The study highlights that two structurally distinct KOR agonists may produce diverse behavioral effects suggesting that this may be due to a functional selectivity or “biased agonism” elicited through the KOR in regulation of ERK1/2 signaling. The findings here suggest that further examination of agonist-mediated receptor signaling and regulation may demonstrate that other KOR agonists acting upon the same receptor may also promote distinct pharmacological effects in vivo and in vitro.
REFERENCES


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Conventional GPCR signaling is a series of events beginning with an agonist binding to the receptor to induce a confirmation change that results in activation of G-proteins and subsequent regulation of enzymatic proteins and ion channel effector systems. Regulation and termination of agonist-induced signaling has been thought to occur through a time-controlled process involving receptor desensitization and internalization (occurring within minutes to hours), followed by agonist-induced down-regulation of the receptor (occurring within hours to days). However, there is a growing realization that the traditional role of receptor desensitization and internalization solely as a terminator of signaling is an oversimplification (Gainetdinov et al., 2004).

A leading example of this comes from the study of GPCR induced activation of ERK1/2 MAP kinase, which is now recognized to occur by two major pathways that can be distinguished by their differential temporal and spatial patterns (Ahn et al., 2004; Shenoy et al, 2006). In the first, conventional pathway, GPCR-activated G-proteins and associated enzymatic proteins, e.g. protein kinase A (PKA), induce a rapid activation of ERK1/2 that is predominantly nuclear (Ahn et al., 2004; Shenoy et al., 2006). However, in the second unconventional pathway β-arrestin-mediated receptor internalization mediates a delayed, late-phase activation of MAP kinase located in endosomal vesicles in the cytosol (Ahn et al., 2004). Although physically independent, the contribution of these pathways to overall signaling is unclear, since both mechanisms can occur simultaneously (Ahn et al., 2004), complicating the elucidation of distinct mechanisms activating the distinct spatio-temporal pools of ERK1/2 MAP kinase (Dewire et al., 2007). To date, distinguishing the time-dependent mechanisms has entailed utilizing a combination of inhibitors of G-protein and internalization, ectopic expression of dominant negative mutants of
proteins associated with internalization, biased agonists (Azzi et al., 2003; Ahn et al., 2004), and mutant receptors (Ahn et al., 2004).

Both peptide and non-peptide (synthetic) KOR agonists are known to activate MAP kinase and suggest multiple KOR-mediated mechanisms are involved (Belcheva et al, 1998; Jordan et al., 2000). Collectively, however, these data are mechanistically inconsistent. For instance, some reports suggest that KOR internalization is required for activation of p38 (Brucas et al, 2006) and ERK1/2 (Ignatova et al., 1999) while others suggest KOR internalization is not required (Li et al., 1999; Jordan et al., 2000), but that ERK1/2 MAP activation requires PKC (Belcheva et al., 1998). Given the data obtained from studies of Gs-coupled GPCRS demonstrating two distinct temporal mechanisms for the GPCR activation of ERK1/2, it is feasible that a similar dual mechanism regulates the ERK1/2 MAP kinase activation by KOR. As demonstrated in chapter 2, the signaling differences arising from this dual mechanism may have significant behavioral consequences. Despite this, surprisingly, to date there are no studies that have clearly examined this possibility.

In the present study, the molecular mechanism(s) underlying agonist- and time-dependent signaling of ERK1/2 by KOR were characterized using HEK293 cells stably expressing rat KOR fused to a green fluorescent protein (KOR-eGFP). Notably, the use of HEK293 cells in these studies enables a controlled environment to: 1) eliminating the confound of endogenous signaling in the whole animal and 2) allow for the manipulations required to examine the molecular mechanisms underlying KOR regulation of ERK1/2 MAP kinase. Here, we utilized four structurally distinct agonists, 1) the endogenous peptide dynorphin A (1-11) containing an amide group at the C-terminus, 2) the mixed agonist/antagonist cyclazocine, 3) the neoclerodane diterpenoid salvinorin A, and 4) the commonly used arylacetamide U50,488. As introduced in chapter 2, salvinorin A is of particular interest because it is the first naturally occurring non-nitrogenous KOR agonist found to be highly efficacious at inducing G-protein mediated signaling (Wang et al., 2005; Chavkin et al., 2004), yet, it lacks the ability to induce internalization and down-regulation of the KOR (Wang et al., 2005). Moreover, as demonstrated in chapter 2, it was found to lack the ability to activate ERK1/2 MAP kinase in vivo. We hypothesized that agonist
induction of a late-phase activation of ERK1/2 requires potent sustained KOR internalization. Furthermore, we examined the differential mechanism(s) involved in the kinetics of agonist-induced biphasic activation of ERK1/2 using U50,488. Notably, the results here confirm that early-phase activation by U50,488 is G-protein, PKC-mediated while late-phase activation requires receptor internalization.
MATERIALS AND METHODS

Reagents and Drugs

Salvinorin A was provided as a generous gift from Dr Thomas E. Prinsinzano (University of Kansas, Lawrence, KS). Dynorphin A (1-11) was provided as a gift from Dr Jane V. Aldrich (University of Kansas). Dynorphin A (1-11) used in the present study contains an amide at its C-terminus that prevent its degradation. However, it has been shown to produce similar biological activity as the full-length endogenous peptide dynorphin A (1-17) (Bennett et al., 2002). Norbinaltorphimine (Nor-BNI) was obtained from the NIDA drug supply. Antibodies (total ERK1/2 and phospho-specific ERK1/2 map kinase) and chemiluminescent reagents were obtained from Cell Signaling (Beverly, MA) as detailed in chapter 2. Phorbol 12-myristate 13-acetate and all other chemicals were obtained from Sigma (St. Louis, MO).

Cell Culture

HEK293 cells were stably transfected with KOR-eGFP cloned into the pTarget vector (Appleyard et al, 1999) using Lipofectamine2000 (Invitrogen) according to manufacturer’s recommendation. Wild type (WT) Chinese hamster ovary non-transfected (CHO) cells were obtained from Invitrogen. CHO stably expressing flagged tagged human KOR (CHO-KOR) were provided as a gift from Dr Lee-Yuan Liu-Chen (Temple University, Philadelphia, PA) generated previously (Zhu et al., 1995). Wild type non-transfected HEK293 and CHO cells were grown in Dulbecco’s Modified Eagle’s Media (DMEM) in 10% fetal bovine serum with 100 units/ml of penicilllin and streptomycin. Selection of stable KOR-eGFP transfected HEK293 cells and CHO-hKOR was obtained using media supplemented with 500 µg/ml geneticin. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% O₂.
Cell Membrane Preparation

Membrane preparation was performed as described previously (Zhu et al., 1997). Briefly, CHO-KOR and CHO-WT cells were washed twice with 1X phosphate buffered saline (PBS) and harvested in Versene solution (0.54 mM EDTA, 0.14 mM NaCl, 2.7 mM KCl, 8.1 mM Na$_2$HPO$_4$, 1.46 mM KH$_2$PO$_4$, 1 mM glucose), centrifuged at 500 x g for 5 min at 4°C, and the cell pellet washed once with cold PBS followed by centrifugation at 500 x g for 5 min at 4°C. The cell pellet was then resuspended in 50 mM Tris-HCl, pH 7.4 containing 1 mM ethylene glycol-bis(β-aminoethyl)ether N,N,N′,N′-tetraacetic acid, 5 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM sodium pyrophosphate. Following resuspension cells were centrifuged at 46,000 x g for 30 min at 4°C. The final pellet was then resuspended in 50 mM Tris, pH 7.0 and kept on ice until experimental use on the same day.

GTP-Europium Binding Assay

GTP-europium binding was performed per manufacturer’s instructions (Perkin Elmer) with modifications. CHO cell Membranes were diluted to 100 μg/ml in 50 mM HEPES buffer. Membranes (10 μg) were pre-incubated in GTP-europium binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl$_2$, and 1 mM EDTA) with 10 μM GDP in the presence of agonists (0-10 μM) for 30 min at 30°C in 96 well plates (Acrowell). After pre-incubation, 10 μl of 100 nM GTP-europium were added and the plate was incubated for an additional 40 min at 30°C. Nonspecific binding was obtained in the presence of 10 μM GTPγS. Reaction was terminated by rapid filtration under vacuum filtration (Millipore), washed 2 X 300 μl ice-cold 1X GTP wash buffer. Plates were counted in 2104 DELFIA fluorometer (Perkin Elmer).
Western Blot Analysis

Stable KOR-eGFP HEK293 cells were plated and grown on 35 mm dishes to approximately 80% confluency. Cells were serum-starved for 18-24 hours before stimulation to reduce basal kinase activity. Cells were treated with chemicals and agonists as indicated in text. Notably, as salvinorin A contains many reactive ester groups that are susceptible to cleavage, for time course studies media containing salvinorin A was switched for fresh media with salvinorin A every 30 min. Immediately after treatment, cells were washed one time with cold PBS, placed in ice, and lysed in ice for 3 min with 500 µl of lysis buffer (50 mM Tris HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.3% (v/v) NP-40, 1 mM PMSF and a cocktail of protease and phosphatase inhibitors (leupeptin 10 µg/ml, 10 µg/ml aprotinin, 2 mM sodium orthovanadate, 5 µg/ml okadaic acid).

Cells were scraped off, collected, and homogenized by passing cells through a 25 G/8 gauge syringe-needle five times. Homogenates were then centrifuged at \( \leq 10,000 \times g \) at 4°C for 5 min.

Cell lysates were aliquoted for protein assay using the Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. Protein extracts were prepared in Laemmli buffer and approximately 10 µg of total protein were subjected to 10% Tris-glycine SDS-PAGE followed by transfer onto nitrocellulose membranes. Immunoblotting was performed as described previously in chapter 2.

Immunofluorescence

Cells were grown on 35 mm glass coverslip dishes (MakTek) for 2-3 days prior to drug treatment. When cells reached greater than 80% confluency cells were treated with agonists for various time periods as indicated in the text. Immediately after agonist treatment cells were washed 2 times with cold PBS then fixed with 4% Paraformaldehyde in 1X PBS, washed with 1X PBS, permeabilized by treatment with PBS/0.3% Triton-x (PBSTx) for 20 min at 22°C, then blocked in 5% normal goat serum/1% BSA in PBSTx for 1 hour at 22°C. Cells were then incubated with primary antibody (Phospho-ERK1/2) diluted in blocking solution at 1:250 for 1 hour at 22°C. After
primary incubation cells were washed 3X PBS and incubated in secondary antibody (goat anti-rabbit IgG conjugated to rhodamine red) diluted in blocking solution (1:500) and incubated for 1 hour at 22°C. Cells were then washed 3X PBS and were visualized by confocal microscopy. For internalization studies, after cells were fixed with 4%PFA, washed 3 times in PBS prior to imaging with confocal microscopy.

Confocal Image Analysis

Cells were visualized using a Laser Scanning Confocal microscope MRC-600 (Bio-Rad) attached to Zeiss microscope and images were captured at 40X magnification. KOR-eGFP was visualized at 488 nM (producing green staining) and immunolabeled Phospho-ERK1/2 MAP kinase at 560 nM (producing red staining). Images were captured from the same planes and analyzed using NIH ImageJ software program. Where appropriate images captured from dual wavelengths (i.e. 488 nM and 560 nM) were merged using NIH ImageJ to determine the degree of co-localization.

Data Analysis

Western blots and cell images were analyzed using the NIH ImageJ software program. For western blot data the area density of immunoreactive bands was quantified. Phospho-ERK1/2 (pERK1/2) immunoreactive bands were normalized to total nonspecific (NP) ERK1/2 MAP kinase immunoreactive bands of vehicle treated controls. Internalization of KOR-eGFP was analyzed by measuring the pixel intensity inside the cell and adjusted relative to background as described previously in McLaughlin et al, 2003. An experimenter blind of drug treatment condition measured receptor internalization and data obtained was determined to be reliable between individual experimenters. The effect of each agonist on activation of ERK1/2 and receptor internalization was analyzed by One-way ANOVA with significant differences further analyzed by Fisher's LSD post hoc tests. Statistical analysis was performed using SPSS 14 software.
RESULTS

Kappa Opioid Receptor agonists stimulate GTP-europium binding

This study examined four structurally distinct agonists: dynorphin A (1-11), cyclazocine, U50,488, and salvinorin A. Dynorphin A (1-11) is a peptide KOR agonist (Jordan et al., 2000). Cyclazocine is a mixed agonist/antagonist previously demonstrated to produce KOR-mediated effects in vivo (Kumor et al., 1986). The synthetic arylacetamide U50,488 was examined given its common usage in kappa opioid research. Finally, we examined the unique neoclerodane diterpenoid salvinorin A as studies have demonstrated its selectivity and efficacy at KOR (Chavkin et al., 2004) and its lacking ability to induce KOR internalization (Wang et al., 2005).

To confirm that each agonist is able to initiate KOR-mediated G-protein signaling we performed GTP-europium binding in membranes prepared from CHO WT and CHO-KOR cells. As shown in figure 3.1 each agonist enhanced GTP-europium binding in a concentration-dependent manner in membranes prepared from CHO-hKOR cells. Notably, no change in activity was observed in samples taken from nontransfected CHO WT cells (data not shown). Relative to U50,488, the maximal fold-increase in GTP-europium binding for each of the other agonists are as follows: dynorphin A (1-11), 1.56 ± 3.75; salvinorin A, 3.03 ± 0.61; and cyclazocine, 3.47 ± 0.35.

Concentration-dependent activation of ERK1/2 MAP kinase by KOR agonists in KOR-eGFP HEK293 cells

To determine whether each agonist can stimulate the activation of ERK1/2 we treated stable KOR-eGFP HEK293 cells with varying concentrations (0 to 10 µM) of the agonists for 5 min at 37°C. This time point was chosen based upon previous studies that demonstrate this time phase is mediated by G-proteins (Ahn et al., 2004). All agonists produced a concentration-dependent enhancement of ERK1/2 MAP kinase activation over vehicle control (Figure 3.2A, B).
FIGURE 3.1. **KOR agonists enhance GTP-europium binding in CHO-KOR cells.** Fresh cell membranes (10 µg) were incubated in the presence of vehicle (1% DMSO) or in the presence of agonists U50,488, salvinorin A, dynorphin A (1-11), or cyclazocine (at 0, 0.1, 1, 10) at 30°C as described in “materials and methods.” Specific values for GTP-europium binding was subtracted from fluorescent units obtained in the presence of cold GTPγS (basal binding). Data are expressed as % basal GTP-europium binding (±) standard deviation of triplicate determination for each concentration.
The response by each of the agonists was blocked by pretreatment with the opioid receptor antagonist, naloxone (10 µM x 60 min) (Figure 3.2C and E, white bars). Moreover, in non-transfected (NT) HEK293 cells no significant effect on ERK1/2 MAP kinase activation was observed, demonstrating a KOR-mediated effect of the agonists (Figure 3.2D and E, grey bars).

**Late-phase activation of ERK1/2 MAP kinase by KOR occurs in an agonist-dependent manner in KOR-eGFP HEK293 cells**

We examined the time-course of ERK1/2 activation induced by each of the structurally distinct agonists. At equivalent concentrations (1 µM) all agonists produced significant increase in activation of ERK1/2 that was time-dependent (Figure 3.3A, B). Consistent the results in figure 3.3, all agonists produced significant activation of ERK1/2 MAP kinase after a 5 min incubation (U50,488, F(9,80)=4.27, p<0.05; dynorphin A (1-11), F(9,43)=5.04, p<0.05; cyclazocine, F(9,56)=10.49, p<0.05; salvinorin A, F(9,34)=3.93, p<0.05). U50,488 produced a rapid increase in pERK1/2 that was significant across time when cells were harvested after 2, 5, and 20 min exposure periods. Dynorphin A (1-11) and cyclazocine increased activation of ERK1/2 in a biphasic manner. Dynorphin A (1-11) induced significant activation when cells were harvested after 5, 10, 20, 30, 45 min of drug treatment. Similarly, cyclazocine induced significant activation of ERK1/2 after 5, 10, and 45 min of exposure. Interestingly, although salvinorin A produced initial activation and a significant effect over time, Fisher's LSD demonstrated significant decreases in ERK1/2 phosphorylation after 10, 45, and 60 min of exposure.

**Agonist induction of prolonged activation of ERK1/2 MAP kinase corresponds to the potency of agonist-induced Internalization**

Given previous reports suggesting differences among agonists to induce internalization we examined the time-course of agonist-induced KOR internalization and measured the potency of each agonist to induce KOR internalization using confocal microscopy. We compared the time-course of receptor internalization induced by all agonist as treated above (Figure 3.4) and
Figure 3.2. KOR agonists stimulate a concentration-dependent activation ERK1/2 after 5 min stimulation in KOR-eGFP HEK293 cells. HEK293 cells expressing KOR-eGFP were treated with vehicle control 1% DMSO (V), U50,488 (U), Salvinorin A (S), Dynorphin A (1-11), (D) or Cyclazocine (C) (at 0.1, 1, 10, or 100 µM) for 5 minutes at 37°C. (A) Representative Western blots of Phospho-specific (P-p44/42) immunoblots represent antibody labeling of activated phosphorylated ERK1/2 MAP kinase (NP-p44/42) represent total ERK1/2 MAP kinase antibody labeling which was used as a loading control. (B) Summary plot graph of mean ± SEM agonist-induced activation of ERK 1/2 MAP kinase relative to vehicle control. (C) Representative Western blot of KOR-eGFP HEK293 cells pretreated with naloxone (NLX, 10 µM x 60 min) prior to treatment with agonist for 5 min at 37°C. (D) Representative Western blot of nontransfected (NT) HEK293 cells treated with agonist for 5 minutes at 37°C. (E) Summary plot of mean ± SEM of ERK1/2 activation induced by agonist treatment alone (1 µM x 5 min at 37°C, black bars), naloxone pretreated (white bars), and agonist treatment in NT HEK293 cells (gray bars). All data represent at least 2 independent experiments with replicate Western blots. * = p<0.05 statistically significant from vehicle control. Dashed line indicates vehicle control.
assessed KOR internalization by quantifying the intensity of fluorescent green vesicles inside the cell (McLaughlin et al., 2003). As shown in figure 3.4A and 3.4B U50,488, dynorphin A (1-11), and cyclazocine all induced significant internalization of the rat KOR, in a time-dependent manner (Figure 3.4B). U50,488-induced significant KOR internalization that peaked after 10 min of exposure ($F_{(9,44)}=6.32, p<0.05$). Fisher’s LSD post hoc tests revealed that U50,488-induced significant internalization at 5, 10, 15, 30, and 90 min of incubation. Dynorphin A (1-11)-induced a significant degree of internalization after 2 min of incubation that continued to 45 min of exposure ($F_{(9,33)}=2.59, p<0.05$). Cyclazocine-induced KOR internalization was more variable, with significant effects seen after 15, 30, and 90 min of incubation ($F_{(9,32)}=2.40, p<0.05$). Lastly, although salvinorin A induced some KOR internalization, unlike the other KOR agonists, it did not reach statistical significance in comparison to vehicle control ($F_{(9,27)}=0.83, p>0.05$).

**U50,488 and salvinorin A produce differential localization patterns of activated ERK1/2 MAP kinase**

As salvinorin A internalizes the KOR poorly, we determined whether U50,488 and salvinorin A may vary in their ability to activate specific subcellular pools of ERK1/2 MAP kinase. Immunocytochemical labeling of phospho-ERK1/2 MAP kinase after treatment of cells with either agonists (1 µM) for 5 or 15 min was performed. After 5 min both U50,488 and salvinorin A incubation resulted in activated ERK1/2 MAP kinase that was localized at the plasma membrane (Figure 3.5A). Interestingly, a 15 min incubation with salvinorin A produced a slight degree of phospho-ERK1/2 labeling suggestive of ERK1/2 activation, but this labeling localized at the plasma membrane. In contrast, after a 15 min incubation U50,488 induced activation of ERK1/2 MAP kinase was localized predominantly in the cytosol demonstrated as punctate-labeled vesicles colocalizing with internalized KOR-eGFP signals (Figure 3.5A,B).
Figure 3.3. Treatment with KOR agonists activates ERK 1/2 MAP kinase in KOR-eGFP HEK293 cells in a time-dependent manner. KOR-eGFP HEK293 cells were treated with vehicle control 1% DMSO, or U50,488, salvinorin A, dynorphin A (1-11), or cyclazocine (at 1 µM) for indicated time periods (in min) at 37°C. (A) Representative Western blot analysis and (B) Summary plot of mean ± SEM of agonist-induced activation of pERK relative to vehicle control. Data represent at least 2 independent experiments with replicate Western blots. * = p<0.05, statistically significant difference from vehicle control as determined by One-Way ANOVA followed by Fisher’s LSD post hoc test for individual agonist. Dashed line indicates vehicle control.
Figure 3.4. Time-course of agonist-induced internalization of KOR-eGFP: potency of internalization corresponds to long-lasting activation of pERK1/2. KOR-eGFP HEK293 cells were pretreated with vehicle control 1% DMSO, U50,488, salvinorin A, dynorphin A (1-11), or cyclazocine (at 1 µM) at 37°C for indicated time periods (in min) at 37°C. (A) Representative images of cells treated with either vehicle, U50,488, salvinorin A, dynorphin A (1-11), or cyclazocine for 30 min at 37°C. Images were captured at 40X magnification. White arrows highlight internalized receptor vesicles. White scale bar indicates 10 µm. Note: U50,488, dynorphin A (1-11), and cyclazocine show a more robust internalization with lack of cell surface expression compared to salvinorin A. The degree of agonist-induced KOR-eGFP internalization was quantified using NIH ImageJ (see methods). (B) Summary plot of mean ± SEM agonist-induced receptor internalization relative to vehicle control. Data represent at quantification from at least 10 cells from at least 2 independent experiments. a, b, c, d = p<0.05, statistically significant difference from vehicle control as determined by One-Way ANOVA followed by Fisher’s LSD post hoc test for individual agonist for U50,488, salvinorin A, cyclazocine, and dynorphin A (1-11), respectively. Dashed line indicates vehicle control.
Figure 3.5. U50,488 and Salvinorin A produce differential localization patterns of activated ERK1/2 MAP kinase. KOR-eGFP HEK293 cells were pretreated with vehicle control 1% DMSO, U50,488, or salvinorin A (1 µM) at 37°C for (A) 5 min or (B) 15 min followed by immunocytochemical labeling of pERK1/2. KOR-eGFP was visualized at 488 nM (A, B) and immunolabeled pERK1/2 at 560 nM (producing red staining). Images captured from dual wavelengths (i.e. 488 nM and 560 nM) were merged using NIH ImageJ.
Distinct mechanisms are involved in the temporal pattern of U50,488-induced activation of ERK1/2 MAP kinase

To demonstrate if KOR activation of ERK1/2 MAP kinase is mediated by distinct mechanisms at different time points we tested the effects of inhibitors of G\textsubscript{i/o}-protein, PKC, and internalization on the time course of U50,488-induced activation of ERK1/2. We chose to examine the effects of inhibitors on U50,488-induced activation at up to 20 min as beyond that U50,488 no further magnitude of effect was observed (see Figure 3.3A, B). Pretreatment of cells with pertussis toxin (PTX, 100 ng/mL x overnight), which inhibits receptor coupling of G\textsubscript{i/o}-proteins via ADP ribosylation of the G\textsubscript{αi/o} subunit, blocked the activation of ERK1/2 at all time points of stimulation (Figure 3.6A, B) resulting in an insignificant U50,488-induced activation of ERK1/2 ($F_{(5,26)}=0.743$, $p<0.05$). Unexpectedly, the pertussis toxin blockade of the late phase of activation of ERK1/2 corresponded to impairment of U50,488-induced internalization, possibly due to an irreversible uncoupling of the receptor and lack of receptor signaling in the first place.

Opioid activation of ERK1/2 MAP kinase has been shown to be mediated by PKC (Belcheva et al., 2005). As activation of PKC is known to be downstream of G-protein activation we examined the effects of inhibition of PKC on U50,488-induced activation of ERK1/2 MAP kinase. As shown in figure 3.6A and 3.6B pretreatment of KOR-eGFP HEK293 cells with a PKC inhibitor, Gö6983 (0.1 µM x 30 min), completely abolished U50,488-induced activation of ERK1/2 at 2 min and resulted in a reduction of activated ERK1/2 at 5 min compared to vehicle control ($F_{(5,52)}=6.78$, $p<0.05$). However, Gö6983 had no effect on the late-phase (20 min) U50,488-induced activation of ERK1/2. Notably, phorbol 12-myristate 13-acetate (P, 10 µM x 30 min) was able to induce activation of ERK1/2, a positive control indicating an intact PKC activity in these cells (Figure 3.6A, top left corner blot).

Lastly, we then examined the effect of inhibiting internalization on the temporal course of U50,488-induced increase in activation of ERK1/2 by pretreating cells with hypertonic sucrose (0.4 M x 20 min) (Heuser & Anderson, 1989) prior to U50,488 treatment (Figure 3.6A, B). One-way ANOVA revealed that U50,488-induced significant activation of ERK1/2 ($F_{(9,72)}=6.53$, $p<0.05$). Fisher’s LSD post hoc test demonstrated significant activation at only 2 and 5 min of
exposure with U50,488. Further analysis revealed that U50,488-induced activation at early time points (2 and 5 min) was significantly enhanced by sucrose pretreatment (Two-tailed, unpaired, Student's t test, \( p<0.05 \) vs U50,488 treatment alone at respective time point).
Figures 3.6. Distinct mechanisms are involved in the temporal pattern of U50,488-induced activation of ERK1/2 MAP kinase. KOR-eGFP HEK293 cells were either pretreated G\textsubscript{i/o} protein inhibitor, pertussis toxin (PTX, 100 ng/mL overnight), PKC inhibitor, Gö6983 (0.1 µM x 30 min), or hypertonic sucrose (0.4 M x 20 min) prior to U50,488 (1 µM) for indicated time periods (in minutes) at 37ºC. \( \text{(A)} \) Representative Western blot analysis and \( \text{(B)} \) Summary plot of mean ± SEM of U50,488-induced activation of pERK relative to vehicle control. Data represent at least 2 independent experiments with replicate Western blots. * = statistically significant difference from vehicle control \( (p<0.05) \) as determined by One-Way ANOVA followed by Fisher’s LSD post hoc test for individual agonist. Dashed line indicates vehicle control. Top left hand blot first three lanes are cells not pretreated with PTX, indicating positive controls of cells treated with either U50,488 alone \( (U, 1 \mu M \times 5 \text{ min}) \) or phorbol ester 12-myristate 13-acetate \( (P) \) \( (10 \mu M \times 30 \text{ min}) \).
DISCUSSION

The principal finding of this work presented in this chapter is that agonist-induced KOR regulation of ERK1/2 MAP kinase is biphasic, and that late-phase activation of ERK1/2 MAP kinase by select KOR agonists is mediated by the ability of the agonist to induce receptor internalization. In these studies we examined the time course of agonist-induced trafficking and signaling of ERK1/2 produced by four structurally distinct agonists. We then elucidated the role of temporally distinct mechanisms by examining whether inhibition of G-protein, PKC, or internalization could affect U50,488-induced biphasic activation of ERK1/2.

Characterization of opioid receptor activation of ERK1/2 and elucidation of the molecular mechanisms involved has been reported (reviewed in Law et al., 2000; Belcheva et al., 1998; Bohn et al., 2000; Belcheva et al., 2005). However, in the present study, we have demonstrated that despite having similar capabilities for G-protein signaling the tested KOR agonists varied in their ability to induce receptor internalization consequently affecting the agonists’ ability to induce late-phase activation of ERK1/2. The agonist-selective regulation of receptor signaling and trafficking has been reported previously (Li et al, 1999; Jordan et al, 2000) but not until recently has functional-selectivity or biased agonism received much attention (Urban et al, 2007).

The present findings demonstrate a relationship between the time-course of agonist-induced KOR internalization and activation of ERK1/2 MAP kinase. The present findings contradict previous studies by Li et al., 1999 and Jordan et al., 2000, that did not demonstrate a relationship between agonist induced activation of ERK1/2 MAP kinase and internalization of the KOR. The discrepancy between studies could be attributed to cell model systems and technical differences utilized to measure receptor internalization and activation of ERK1/2. This may be relevant as agonist-induced receptor internalization varies between cell types. For instance, agonist-induced internalization of β-adrenergic receptors expressed in COS cells achieved similar levels as that expressed in HEK293 only when β-arrestin was overexpressed (Zhang et al., 1996). Moreover, the techniques used to measure internalization between studies also makes the comparisons difficult, as studies by Li et al, 1999 and Jordan et al, 2000 assessed internalization using radioligand binding assays, whereas we and Ignatova et al, 1998 measured
receptor internalization using fluorescence and immunofluorescence methods, respectively. Quantification of receptor internalization by measuring fluorescent-labeled internalized opioid receptors has been done previously (McLaughlin et al., 2003; Trapaidze et al., 1996) and has been found to yield similar results to that assessed by radioligand binding (Trapaidze et al., 1996). However, it must be noted that it is possible that the eGFP tag at the C-terminus of the KOR may have confounded the present results, e.g. by altering the affinity for β-arrestin, thereby resulting in reduced agonist-induced internalization. This suggests that all agonists would produce significant reduction in internalization. However, in the present study we found that only salvinorin A lacked the ability to induce internalization of eGFP-tagged receptors, a result consistent with Wang et al., 2005. The impairment of salvinorin A to induce KOR internalization matched its inability to stimulate late-phase activation of ERK1/2. Alternatively, the short-lasting effect of salvinorin A could be due to several ester groups that could have been cleaved into inactive metabolites (Wang et al., 2008). However, this seems unlikely as we ensured that cells were exposed to a stable level of salvinorin A, by switching and replenishing media with fresh drug every 30 minutes. Therefore, the short lasting-effect of salvinorin A cannot be attributed to loss of activity at KOR.

Notably, in the present study we compared the localization of activated ERK1/2 induced by U50,488 and salvinorin A. Despite being a full agonist (Chavkin et al., 2004), salvinorin A was shown to be ~40% less effective than U50,488 at inducing internalization and down-regulation of the human KOR (Wang et al., 2005), providing us with a useful pharmacological tool for the study of KOR internalization and ERK1/2 activation. Our data demonstrated that brief incubation with U50,488 and salvinorin A produced activation of ERK1/2, localized to the plasma membrane. However, after a longer exposure (15 min) only U50,488-induced activation of ERK1/2 in cytosolic vesicles.

Our data implicate that distinct mechanistic pathways underlie the time-dependent KOR-mediated activation of ERK1/2 induced by the agonist U50,488. Consistent with previous studies we found that inhibitors of Gι/0-protein and PKC blocked U50,488-induced activation of ERK1/2 (Belcheva et al., 1998; Belcheva et al., 2005). Similar to previous studies, we also found that
inhibition of internalization by pretreating cells with hypertonic sucrose did not block U50,488-induced ERK1/2 activation at early time points (Jordan et al., 2000). However, we found that sucrose inhibited U50,488-induced late-phase activation of ERK1/2 similar to that reported for the Ca\sup{2+}-sensing receptor (Holstein et al., 2004). Unexpectedly, while sucrose pretreatment blocked late-phase, the early-phase activation of ERK1/2 was enhanced suggesting that inhibition of internalization may have enhanced G-protein/PKC mediated activation of ERK1/2. This cannot be attributed the effects of sucrose alone as in control cells pretreated with sucrose alone we did not observe significant activation of ERK1/2.

There are limitations in generalizing the current findings of U50,488-induced activation of ERK1/2 to other KOR agonists. Recent work by Zheng et al., 2008 has demonstrated that while the kinetics of activation of ERK1/2 by morphine and etorphine were similar the mechanistic pathway and the functional output were dramatically distinct. Morphine utilized a PKC-dependent pathway to activate ERK1/2 MAP kinase that subsequently modulated the activity of cytosolic proteins. In contrast, etorphine activation of ERK1/2 MAP kinase required β-arrestin-mediated receptor internalization that ultimately increased gene expression of β-arrestin and G-protein receptor kinase (GRK) (Zheng et al., 2008). In the present study, the functional differences between the “pools” of ERK1/2 induced by distinct agonists or mechanistic pathways were not examined. Nevertheless, the present study emphasizes that distinct mechanisms mediate the time-course of KOR agonist U50,488-induced activation of ERK1/2.

In summary, we have demonstrated that despite similarities in G-protein signaling, structurally distinct kappa opioid agonists vary in their temporal course of activation of ERK1/2 and regulation of receptor trafficking. These data suggest that differential temporal effects of agonist-dependent activation of ERK1/2 are governed by distinct mechanisms that are controlled via receptor regulatory processes. Together, the results in the present study highlight the important need to characterize the differential effects of ligands by screening a multitude of signal transduction and receptor regulatory pathways that can be elicited by ligands. While this may lead to a more complicated reclassification of ligands, e.g. as dual efficacy (Shenoy et al., 2006) or biased agonists, this may shed light into distinguishing the clinical utility of various receptor
ligands (Urban et al., 2007; Daniels et al., 2005; Schmid et al., 2008). Moreover, a further evaluation of multiple types of receptor ligands in signaling to unconventional signaling pathways could expose a yet to be identified mechanism of drug action.
REFERENCES


CHAPTER 4

SELECTIVE KAPPA OPIOID RECEPTOR ANTAGONISTS SUPPRESS ERK1/2 MAP KINASE SIGNALING IN HEK293 CELLS: EVIDENCE FOR INVERSE AGONISM MEDIATED BY AKT

The concept of functional selectivity posits that distinct receptor ligands can initiate differential signaling efficiencies dependent on the receptor-coupled effector systems it prefers (Kenakin, 2007; Urban et al., 2007). Opioid receptors display basal spontaneous (agonist-independent) activation of opioid receptors (Wang et al., 2000; Wang et al., 2001; Wang et al., 2007). In fact, the DOR was the first GPCR found to exhibit high basal activity (Costa et al., 1988; Costa et al., 1989). This led to the discovery of negative intrinsic efficacy (or inverse agonism), as the opioid antagonist ICI,174-864 inhibited basal DOR-mediated GTPase activity in endogenous expression systems (Costa and Herz, 1989). Later studies demonstrated that ICI,174-864 also displayed inverse agonist actions at endogenously expressed DOR and at cloned DOR using adenylyl cyclase and [35S] GTPγS binding assays as endpoints of analysis (Chiu et al., 1996; Szekeres and Traynor, 1997). However, inverse agonism has not been demonstrated at the KOR, and in no case has a behavioral phenotype been determined.

Nor-binaltorphimine (nor-BNI) is an established KOR-selective competitive antagonist comprised of two naltrexone molecules connected by a pyrrole ring (Portoghese et al., 1979; Portoghese et al., 1987; Takemori et al, 1988). For years, nor-BNI has been the prototype antagonist used to study KOR pharmacology (Metcalf and Coop., 2005). However, considerable evidence suggests that nor-BNI may be an inverse agonist (Simen and Miller, 1998; Hampson et al, 2000; Mizoguchi et al., 2002; Wang et al., 2007; Zhang et al., 2007). Notably, nor-BNI enhanced basal Ca++ ion currents in a manner similar to that induced by the DOR inverse agonist, ICI,174-864 (Simen and Miller, 1998). In hippocampal neurons nor-BNI also enhanced inactivating K+ outward ion currents via a mechanism independent from G\text{i/o}-proteins but dependent on G\text{s}-proteins and PKC (Hampson et al., 2000). However, others have demonstrated that nor-BNI did not simply antagonize, but actually suppressed receptor-mediated activation of
Gi/o-proteins measured in [35S] GTPγS binding assays in the pons/medulla isolated from MOR knockout mice (Mizoguchi et al., 2002) and in vitro in KOR transfected HEK293 cells (Wang et al., 2007). However, full characterization of this effect and a mechanism to account for it remains unstudied.

A possible mechanistic basis for KOR-mediated inverse agonism may come from the activity of protein kinase B (PKB also termed Akt). Akt has been demonstrated to be a negative regulator of ERK1/2 MAP kinase (Galetic et al., 2003). Akt can be activated either by insulin or growth factors via phosphatidylinositol 3-kinase (PI3K) (Sale and Sale, 2008). Upon activation, Akt binds to and inactivates the upstream ERK1/2 MAP kinase regulator Raf (Zimmermann and Moelling, 1999; Guan et al., 2000), suppressing activation of this kinase cascade. Interestingly, a negative relationship between activity of Akt and ERK1/2 MAP kinase has been demonstrated in vivo after acute opioid treatment (Muller & Unterwald, 2004).

In this study, we examined if nor-BNI and three other structurally distinct KOR antagonists, JDTic, arodyn, and naloxone, could exhibit inverse agonist actions on ERK1/2 MAP kinase signaling in HEK293 cells that stably express rat KOR. We used JDTic because it produces selective and potent antagonism of KOR with similar kinetics as nor-BNI (Carroll et al., 2004). Arodyn, a KOR-selective peptide antagonist, was used because it was recently shown to produce inverse agonist effects in adenyly cyclase assays (Bennett et al., 2002). Lastly, naloxone was utilized because while it is non-selective at opioid receptors, it is reported to lack inverse agonist activity at KOR in the absence of agonist pretreatment (Wang et al., 2007). We found that nor-BNI and arodyn produced a KOR-mediated suppression of ERK1/2 MAP kinase activity in a concentration-dependent manner. The inverse agonist effects of nor-BNI were receptor-mediated as it was not observed in nontransfected HEK293 cells and it could be suppressed by the neutral antagonist naloxone. Nor-BNI suppression of ERK1/2 MAP kinase appears to be independent from Gi/o-proteins but requires Akt signaling but not through its upstream regulator, PI3K. Furthermore, we found that nor-BNI could suppress insulin-induced activation of ERK1/2 MAP kinase in a KOR-dependent manner.
MATERIALS AND METHODS

Reagents and Drugs

Arodyn, (Ac[\text{Phe}^{1,2,3}\text{,Arg}^{4}\text{,D-Ala}^{8}]\text{dynorphin A-}(1-11) \text{amide}), was provided as a gift from Dr Jane V. Aldrich (University of Kansas, Lawrence, KS). JDTic, (3R)-7-hydroxy-N-(1S)-1-[(3R,4R)-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl[methyl]-2-methyl[propyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide was provided as a gift from Dr F. Ivy Carroll (Research Triangle International, North Carolina). Norbinaltorphimine (Nor-BNI) was obtained from the NIDA drug supply. Insulin was obtained from Sigma (St. Louis, MO). The AKT inhibitor, 1L 6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate (Akt inhibitor-1), and the PI3 kinase inhibitor, 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) was obtained from Calbiochem (Gibbstown, New Jersey). Antibodies (total ERK1/2 and phospho-specific ERK1/2 map kinase) and chemiluminescent reagents were obtained from Cell Signaling (Beverly, MA).

Cell Culture

As described in chapter 3, HEK293 cells and nontransfected (NT) HEK293 were grown in Dulbecco’s Modified Eagle’s Media (DMEM) in 10% fetal bovine serum with 100 units/ml of penicillin and streptomycin. Selection of stable KOR-eGFP transfected HEK293 cells was obtained using media supplemented with 500 µg/ml geneticin. All cells were grown at 37°C in a humidified atmosphere of 5% CO₂/95% O₂.

Western Blot Analysis

Performed exactly as described in chapter 3.
Data Analysis

Western blots and cell images were analyzed using the NIH ImageJ software program. The area density of immunoreactive bands were quantified. Phospho-ERK1/2 (pERK1/2) immunoreactive bands were normalized to total non-specific (NP) ERK1/2 MAP kinase immunoreactive bands of vehicle treated controls. The concentration-response effects of antagonist in KOR-eGFP HEK293 cells and nontransfected HEK293 cells was analyzed separately using One-way ANOVA with significant differences ($p<0.05$) further analyzed by Fisher’s LSD post hoc tests. Statistical analysis was performed using SPSS 14 software.
RESULTS

Select Kappa opioid receptor antagonists suppress 1% DMSO stimulated activation of ERK1/2 MAP kinase in a concentration-dependent manner in KOR-eGFP HEK293 cells but not nontransfected HEK293 cells

We treated KOR-eGFP and wild type nontransfected (NT) HEK293 cells with four structurally distinct opioid antagonists nor-BNI, JDTic, arodyn, and naloxone (0, 0.1, 1, or 10 µM x 60 min) then harvested the cell lysates for Western blot analysis of ERK1/2 MAP kinase activation. As shown in figure 4.1A, the selective KOR antagonist, nor-BNI, and the peptide antagonist, arodyn, produced a significant concentration-dependent decrease of vehicle (1% DMSO)-stimulated activation of ERK1/2 MAP kinase ($F_{(3,54)} = 5.40, p < 0.05$ and $F_{(3,60)} = 4.93, p < 0.05$, respectively). Fisher's LSD post hoc test revealed that both nor-BNI and arodyn produced significant suppression at concentrations of 1 and 10 µM. In contrast, the antagonist, JDTic and non-selective opioid antagonist, naloxone, had not significant effects on activation of ERK1/2 levels ($F_{(3,20)} = 0.81, p > 0.05$ and $F_{(3,21)} = 1.71$, respectively). Notably, stimulation of NT HEK293 cells with any of the antagonists did not significantly alter ERK1/2 MAP kinase phosphorylation state at any concentration tested (arodyn, $F_{(3,19)} = 0.69, p > 0.05$; JDTic, $F_{(3,24)} = 2.17, p > 0.05$; nor-BNI, $F_{(3,18)} = 0.30, p > 0.05$; and naloxone, $F_{(3,27)} = 0.87, p > 0.05$) (Figure 4.1B), demonstrating a KOR-mediated effect. Moreover, the reduction in ERK1/2 MAP kinase signaling induced by nor-BNI was reversed when KOR-eGFP HEK293 cells were pretreated with naloxone (10 µM x 10 min) followed by co-incubation in the presence of naloxone (10 µM) for an additional 60 min ($p > 0.05$ compared to vehicle control, Figure 4.1C).

Insulin-induced activation of ERK1/2 MAP kinase is suppressed in a concentration-dependent manner by nor-BNI

In the experiments above, nor-BNI behaved as an inverse agonist by suppressing activation of ERK1/2 MAP kinase. This could be due to enhanced constitutive activity of the
### Table A: KOR-EGFP HEK293

<table>
<thead>
<tr>
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<th>Nor-BNI</th>
<th>JDTic</th>
<th>Arodyn</th>
<th>Naloxone</th>
</tr>
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<tr>
<td>Concentration (μM)</td>
<td>0.1</td>
<td>1</td>
<td>10</td>
<td>0.1</td>
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![Image of gel blots showing relative expression levels](image)

- P-p44/42
- NP-p44/42

### Graph B

#### KOR-EGFP HEK293

- Mean fold change relative to control (± SEM)
  - Nor-BNI
  - JDTic
  - Arodyn
  - Naloxone

#### NT HEK293

- Mean fold change relative to control (± SEM)
  - Nor-BNI
  - JDTic
  - Arodyn
  - Naloxone

### Graph C

- Mean fold change relative to control (± SEM)
  - DMSO
  - nor-BNI
  - nor-BNI nix

![Image of Bar Chart](image)
Figure 4.1. Select KOR antagonists suppress vehicle-stimulated activation of ERK1/2 MAP kinase in KOR HEK293 cells but not nontransfected HEK293 cells. Nontransfected HEK293 cells and cells expressing KOR-eGFP were treated with vehicle control 1% DMSO (V) in combination with either distilled water, nor-BNI, arodyn, JDTic, or naloxone (0.1, 1, or 10 µM) for 60 minutes at 37°C. (A) Representative Western blots of Phospho-specific (P-p44/42) immunoblots represent antibody labeling of activated ERK 1/2 MAP kinase (ERK1/2) and NP-p44/42 represent total ERK1/2 MAP kinase antibody labeling which was used as a loading control. (B) Summary plot graph of mean ± SEM antagonist-mediated activation of ERK1/2 MAP kinase relative to vehicle control. (C) Summary plot of KOR HEK293 cells pretreated with naloxone (NLX, 10 µM x 60 min) prior to coincubation with nor-BNI for an additional 60 minutes at 37°C. All data represent at least 2 independent experiments with replicate Western blots. * = p<0.05, statistically significant difference from vehicle control as determined by One-Way ANOVA followed by Fisher’s LSD post hoc test. Dashed line indicates vehicle control.
KOR. To address this concern, we next examined whether nor-BNI inactivation of KOR could affect the activity of another signaling pathway, specifically insulin receptor tyrosine kinase. As demonstrated in figure 4.2A and B, nor-BNI pretreatment significantly suppressed insulin-induced (1 µg/ml x 20 min) activation of ERK1/2 MAP kinase in a concentration-dependent manner ($F_{(6, 38)}=2.90 \ p<0.05$). This effect was KOR-mediated as nor-BNI had no effect in NT HEK293 cells ($F_{(6, 40)}=5.30 \ p<0.05$). Moreover, naloxone pretreatment (10 µM x 10 min) of KOR-eGFP HEK293 cells followed by coincubation blocked the effect of nor-BNI ($p>0.05$ compared to vehicle control) (figure 4.2A and B).

**Inhibition of $G_{i/o}$-proteins does not affect negative intrinsic efficacy of ERK1/2 MAP kinase signaling mediated by nor-BNI**

Since KOR couples to $G_{i/o}$-proteins, we examined whether the concentration-dependent nor-BNI suppression of ERK1/2 MAP kinase could be affected by pertussis-toxin treatment (0.1 µg/ml x overnight). Pertussis-toxin treatment did not block nor-BNI suppression of ERK1/2 MAP kinase relative to vehicle-treated alone ($F_{(3,22)}=5.92, \ p<0.05$). Further analysis between non-treated and pertussis toxin pretreated cells at each dose revealed no significant difference (Fisher’s LSD *post hoc* test, $p>0.05$ for each concentration).

**Suppression of ERK1/2 MAP kinase by nor-BNI requires activation of Protein Kinase B (Akt) but not Phosphatidylinositol-3 Kinase (PI3K)**

We tested the role of Akt and its upstream regulator phosphatidylinositol-3 kinase (PI3K) on nor-BNI-mediated suppression of ERK1/2 MAP kinase. One-way ANOVA revealed a significant effect of pretreatment ($F_{(6,105)}=10.92, \ p<0.05$). Further analysis demonstrated that pretreatment with the Akt inhibitor, 1L 6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate, (10 µM x overnight) blocked the suppressive effects of nor-BNI (10 µM) on ERK1/2 MAP kinase activity ($p<0.05$ compared to nor-BNI alone, figure 4.4A, B). Interestingly, the inhibition of an upstream regulator of Akt, PI3K, by pretreatment of cells with LY
294002 (10 µM x 30 min) did not affect nor-BNI reduction in ERK1/2 MAP kinase ($p>0.05$, figure 4.4A, B). Notably, the PI3K and Akt inhibitors alone had no effect on ERK1/2 MAP kinase ($p>0.05$) (Figure 4.4, left bars).
Figures 4.2.  **Nor-BNI suppresses Insulin-induced activation of ERK1/2 MAP kinase in KOR but not NT HEK293 cells.** KOR-eGFP and NT HEK293 cells were pretreated with nor-BNI (0.1, 1, or 10 μM), naloxone (10 μM) (nlx), or naloxone (10 μM x 10 min) plus coincubation with nor-BNI (10 μM) for 60 minutes at 37°C then stimulated with insulin (1 μg/ml x 20 min) at 37°C. **(A)** Representative Western blots and **(B)** Summary plot of mean ± SEM of activity of ERK1/2 MAP kinase adjusted relative to vehicle non-insulin treated controls. All data represent at least 2 independent experiments with replicate Western blots. * = p < 0.05, statistically significant difference from insulin alone and § = statistically significant difference from insulin pretreated with nor-BNI (10 μM) as determined by One-Way ANOVA followed by Fisher’s LSD *post hoc* test. Dashed line indicates vehicle control.
Figure 4.3. **Inhibition of $G_{i/o}$-protein by pertussis toxin treatment does not affect nor-BNI-mediated suppression of ERK1/2 MAP kinase.** KOR-eGFP HEK293 cells were either pretreated with the $G_{i/o}$ protein inhibitor, pertussis toxin (PTX, 100 ng/mL overnight) or vehicle prior to nor-BNI (at 0.1, 1, or 10, $\mu$M) for 60 minutes at 37°C. (A) Representative Western blots and (B) Summary plot of mean ± SEM of norBNI-induced suppression of pERK relative to vehicle control. Data represent at least 2 independent experiments with replicate Western blots. Dashed line indicates vehicle control.
Figure 4.4  Nor-BNI inhibition of ERK1/2 MAP kinase is mediated by a mechanism involving Akt activity independent of PI3K activity. KOR-eGFP HEK293 cells were pretreated with either the Akt inhibitor, 1L 6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate, (10 µM x overnight), the PI3K inhibitor LY294002 (10 µM x 30 min), or vehicle prior to nor-BNI (10 µM) for 60 minutes at 37°C. (A) Representative Western blots and (B) Summary plot of mean ± SEM of norBNI-induced suppression of pERK relative to vehicle control. Data represent at least 2 independent experiments performed in duplicate with replicate Western blots. * = p<0.05, statistically significant difference from vehicle control as determined by One-Way ANOVA followed by Fisher’s LSD post hoc test. C = nontreated control KOR-eGFP HEK293 cells. Dashed line indicates vehicle control.
**DISCUSSION**

In the present study, we have demonstrated that the nor-BNI and arodyn acted as putative inverse agonists, nor-BNI suppressed vehicle and insulin-induced activation of ERK1/2 MAP kinase via a KOR- and Akt-dependent, but PI3K-independent mechanism. However, in these studies we found that JDTic and naloxone did not produce any significant effects on ERK1/2 MAP kinase activation via the KOR.

It is estimated that as many as 85% of ligands classified as antagonists may actually be inverse agonists (Kenakin, 2004; Greasley and Clapham, 2006). The lack of adequate classification can be attributed to the inability of some GPCRs to exert spontaneous (constitutive) activity or as a result of differences in intracellular milieu in expression systems studied (Negus, 2006). For example, in HEK293 cells, delineation of inverse agonists at the serotonin type 2C (5HT2C) receptors could only be demonstrated when Gq-proteins were overexpressed, as its coexpression enhanced basal 5HT2C-mediated inositol phosphate (IP) formation (Chanrion et al., 2008). Moreover, early studies of inverse agonist activity at MOR could only be demonstrated under appropriate conditions that required chronic opioid agonist exposure to enhance basal opioid receptor signaling (Wang et al., 2000; Wang et al., 2001; Raehal et al., 2005).

Transactivation of RTKs by opioid receptors has been demonstrated to be a mechanism by which agonist-induced activation of opioid receptors activate ERK1/2 MAP kinase (Belcheva et al., 2001; Belcheva et al., 2003). Therefore, we examined whether the putative inverse agonist nor-BNI could suppress insulin-mediated activation of ERK1/2 MAP kinase. Our data demonstrated that nor-BNI induced a concentration-dependent suppression of insulin-induced activation of ERK1/2 MAP kinase that required the KOR, as no effect was observed in non-transfected HEK293 cells and naloxone inhibited nor-BNI. These results are consistent with studies of the cannabinoid receptor (CB1) inverse agonist SR 141716A, which has also been found to suppress spontaneous receptor activity and insulin, as well as insulin-like growth factor stimulated activation of ERK1/2 MAP kinase via inhibition of Gi protein (Boualboula et al., 1997).

In addition to ERK1/2 MAP kinase, KOR regulates the activity of the p38 and JNK MAP kinase pathways (Brucas et al., 2006; Kam et al., 2004). Although, they are of particular interest
examining these additional pathways are beyond the scope of the present studies. Notably, as extensive cross talk between pathways has been described (Guan, 1994; Obata et al., 2000; Kogkopoulou et al., 2006), future studies will be required for further examination.

In summary, we have demonstrated that the KOR antagonists nor-BNI and arodyn behaved as inverse agonists by inducing a KOR-mediated suppression of ERK1/2 MAP kinase while naloxone and JDTic demonstrated antagonistic actions. Moreover, although naloxone has been previously characterized as a neutral antagonist at MOR (Raehal et al., 2005; Wang et al., 2007), the present study indicates that naloxone is a neutral antagonist at KOR since it blocked the effects of nor-BNI. Interestingly, in this study, we have demonstrated a potential mechanism by which nor-BNI mediates the KOR-induced suppression of ERK1/2 MAP kinase, namely through the activity of Akt. Current studies that examine the KOR interaction with CaM and subsequent modulation of Akt activity will be required to further understand this novel mechanism by which KOR regulates ERK1/2 MAP kinase activity.
REPRESENTATIONS


CHAPTER 5

SUMMARY AND DISCUSSION

The goal of this dissertation was to investigate the molecular mechanisms underlying kappa opioid receptor signaling influences on cocaine reward. Importantly, a major theme was to identify functional selectivity of distinct opioid ligands in induction of receptor regulatory processes, signal transduction, and behavior, so as to identify optimal candidates as therapeutics for treating cocaine abuse. To this end, KOR ligands were characterized in their time-dependent modulation of cocaine-conditioned place preference, time-dependent activation of ERK1/2 MAP kinase ex vivo and in vitro, time-dependent induction of receptor internalization, putative inverse agonist suppression of ERK1/2 MAP kinase, and lastly, in their ability to mediate distinct regulatory and mechanistic pathways of ligand-dependent modulation of ERK1/2 MAP kinase.

In chapter 2, the KOR agonists, U50,488 and salvinorin A were both found to suppress cocaine-CPP when mice were conditioned with cocaine at a time point of peak analgesic activity of both agonists. However, when mice were place-conditioned when the analgesic effects of both agonists abated, U50,488, but not salvinorin A pretreatment induced potentiation of cocaine-conditioned place preference. These effects correlated with the subsequent demonstration that U50,488 (but not salvinorin A) enhanced the activity of ERK1/2 MAP kinase in brain within a similar time course. Pharmacological inhibition of ERK1/2 MAP kinase blocked the U50,488-induced potentiation of cocaine-CPP, as did blockade of KOR by pretreatment with a selective antagonist, nor-BNI. Together, the results suggest the paradoxical KOR agonist-mediated potentiation of cocaine reward resulted from the signaling of late-phase activation of ERK1/2 MAP kinase.

Studies in chapter 3 were performed in HEK293 cells to examine and fully characterize the functionally selective activity of structurally distinct KOR agonists on ERK1/2 MAP kinase as well as the molecular and the cellular mechanisms that underlie agonist-induced activation of
ERK1/2 MAP kinase. Four structurally distinct agonists all activated G-proteins with varying efficacy. However, the agonists differed in their ability and magnitude of activation of ERK1/2 MAP kinase and induction of receptor internalization. Moreover, agonist-induction of a late-phase activation of ERK1/2 MAP kinase was associated with KOR internalization. Comparisons between U50,488 and salvinorin A (as these compounds were screened in behavioral assays in chapter 2) revealed a differential subcellular localization pattern of ERK1/2 MAP kinase during late-phase activation. Specifically, an early-phase activation of ERK1/2 MAP kinase was associated with the plasma membrane, possibly interacting with the receptor. However, whereas U50,488 induced a late-phase activation of ERK1/2 MAP kinase localized in endosomal vesicles, in contrast, salvinorin A did not produce any activation, a finding consistent with Western blot analysis in chapter 2. The role of distinct mechanistic pathways in U50,488-induced activation of ERK1/2 MAP kinase was tested by pretreating cells with inhibitors of upstream-mediated signaling, i.e. G-protein and PKC, and downstream-mediated signaling, i.e. receptor internalization. It was demonstrated that U50,488-induced activation of ERK1/2 MAP kinase is mediated by two distinct mechanisms, whereas salvinorin A was limited to a brief G-protein mediated event. Together, these data provided a molecular basis for distinct actions of KOR agonists in modulation of cocaine reward, with the initial suppression of cocaine-CPP attributed to the the hyperpolarizing effects of KOR agonists and the prolonged late-phase activation of ERK1/2 MAP kinase, producing the potentiating effect on cocaine reward. From a therapeutic perspective, this suggests that developing KOR agonists that do not elicit a late phase-activation of ERK1/2 MAP kinase could eliminate the unwanted side effect of paradoxical potentiation of cocaine reward.

In chapter 2, it was unexpectedly found that the KOR antagonist nor-BNI suppressed ERK1/2 MAP kinase activation ex vivo. This is a characteristic property of ligands classified as inverse agonists. Therefore, studies in chapter 4 were performed in vitro to further examine and characterize potential inverse agonist activity of KOR antagonists and to determine the molecular mechanisms mediating the suppression of ERK1/2 MAP kinase. In these studies nor-BNI and a previously characterized inverse agonist arodyn demonstrated a KOR-dependent suppression of
ERK1/2 MAP kinase. The nor-BNI mediated suppression could not be blocked by inhibition of $G_{i/o}$ protein, but inhibitor studies demonstrated a role for an Akt- (but not PI3K) dependent pathway. Alternatively, it was possible the inverse agonism was due to suppression of constitutive activity of the highly expressed KOR-eGFP in HEK293. However, this seems unlikely, as neither naloxone nor JDTic, both potent KOR antagonists, could suppress ERK1/2 MAP kinase activity. To further discount the possibility that nor-BNI mediated suppression of ERK1/2 MAP kinase was not due to a suppression of constitutive activation of KOR or freely activated $G_{i/o}$ proteins, the effects of nor-BNI on insulin-induced activation of ERK1/2 MAP kinase was tested. It was demonstrated that nor-BNI could negatively regulate insulin-mediated activation of ERK1/2 MAP kinase via a KOR-dependent manner. The data suggest that a subset of KOR antagonists display inverse agonist activity in the ERK1/2 MAP kinase signaling pathway through alternative mechanisms independent from $G_{i/o}$-protein signaling.

I. FUNCTIONAL SELECTIVITY OF KOR LIGANDS AND OTHER MOLECULAR CONSIDERATIONS

Recent studies of other receptor systems have identified that different ligands acting upon the same receptor have the capacity to initiate differential signaling and receptor regulatory pathways (Kohout et al 2004; Groer et al, 2007; Xu et al, 2008), coined agonist-selective signaling or biased agonism (Urban et al, 2007). For example, despite having high efficacy, morphine has been demonstrated to lack the ability to induce $\beta$-arrestin-mediated internalization of the MOR (Whistler and Von Zastrow, 1998). Consequently, morphine activates ERK1/2 MAP kinase via a PKC pathway, unlike etorphine, which uses a predominantly $\beta$-arrestin-mediated pathway to activate ERK1/2 MAP kinase (Zheng et al, 2008). Indeed, considerable evidence suggests that depending on the pathway a ligand recruits, the kinetics, subcellular distribution, and biological output of ERK1/2 MAP kinase activity may vary (Luttrell, 2003; Harding et al, 2005; Ebisuya et al, 2005). This diversity among ligands suggest that the current understanding of GPCR pharmacology is inadequate implicating that no longer are second messenger signal transduction mechanisms exclusively mediated by G-proteins, but in fact, could involve several
molecular mechanisms, including those governing receptor regulation (Daaka et al, 1998; DeFea et al, 2000; Luttrell DK and Luttrell LM, 2003; Tohgo et al, 2003; Huang et al, 2004a; Urban et al, 2006). Agonist-dependent signaling and regulation has only begun to receive attention with evidence suggesting that diverse agonists can induce differential regulation of ERK1/2 MAP kinase activity (Ahn et al, 2004; Kohout et al, 2004; Daniels et al, 2005), receptor desensitization (Kohout et al, 2004; Trester-Zedlitz, 2005), and receptor internalization (Jordan et al, 2000). For instance, two endogenous ligands for the chemokine receptor 7 (CCR7) were found to vary in their ability to elicit activation of ERK1/2 MAP kinase due to their differential abilities to induce desensitization and recruitment of β-arrestin (Kohout et al., 2004). Both the endogenous ligands for CCR7, CCL19 and CCL20 were able to produce equal potencies in Ca\textsuperscript{2+} mobilization, a mechanism mediated by G proteins. However, only CCL19 was able to robustly induce receptor desensitization, receptor phosphorylation, and β-arrestin2 recruitment. Furthermore, at a maximal dose of stimulation CCL19 was nearly 3.7 fold more potent than CCL20 in inducing activation of ERK1/2 MAP kinase. However, the functional and behavioral consequences of diverse signaling and regulation mediated by these endogenous ligands have not been completely explored.

Very few studies have explored the effects of agonists and differential signaling of ERK1/2 MAP kinase activity in mediating diverse behavioral outputs, and of the few studies available none have examined opioid mediated behaviors. For example, studies in vitro demonstrate that two angiotensin type 1a receptor (AT\textsubscript{1}) ligands elicit diverse signal transduction cascades (Ahn et al, 2004) associated with biobehavioral effects (Daniels et al, 2005). Intracerebroventricular injections of the AT\textsubscript{1} receptor of the endogenous ligand angiotensin II (AngII) controls water as well as NaCl intake, whereas, the AngII analog, Sar\textsuperscript{1} Ile\textsuperscript{8}, Ile\textsuperscript{8}-AngII (SII) binds to the AT\textsubscript{1}R, but has no effect on water intake but mediates NaCl intake (Daniels et al., 2005). The divergent behavioral consequences of these high affinity ligands are attributed to differences in intracellular signaling, with AngII being able to stimulate PLC, PKC, and activation of ERK1/2 MAP kinase, while SII only able to stimulate activation of ERK1/2 MAP kinase. These differences are similar to a recent report comparing the endogenous ligand, serotonin, to a
synthetic serotonin type 2A receptor (5-HT2AR) hallucinogenic agonist, 2,5-dimethoxy-4-iodoamphetamine (DOI), on 5HT2AR signaling to ERK1/2 MAP kinase, receptor regulation, and head twitching behavior in WT and β-arrestin knockout mice (Schmid et al., 2008). Interestingly, the authors found that while both agonists produced head-twitching behavior, characteristic of hallucinogenic effects, each drug did so through different signaling mechanisms. While both agonists-induced activation of ERK1/2 MAP kinase in primary cell neurons as well as in ex vivo tissue extracts from frontal cortex, serotonin was found to induce significantly greater activation of ERK1/2 MAP kinase than DOI. More importantly, serotonin was demonstrated to predominantly signal ERK1/2 MAP kinase via a β-arrestin dependent mechanism and partly via PLC, conversely DOI utilized a completely PLC-dependent pathway.

In light of these studies, the behavioral data presented in this thesis suggests that acute activation of KOR suppresses cocaine reward via an upstream receptor-mediated mechanism that is independent of its regulation of ERK1/2 MAP kinase while prolonged activation of KOR (which is agonist-dependent) potentiates cocaine reward via a receptor-mediated activation of ERK1/2 MAP kinase. Although, not in complete parallel to a recent report by Ferguson et al (2006) that has suggested that only ERK2 is involved in mediating cocaine reward, we found that prolonged KOR activation by U50,488 treatment activated both isoforms, but only ERK1 was found to be significantly increased in vivo. Consistent with this, pretreatment of mice with a MEK1/2 inhibitor prior to prolonged U50,488 treatment prevented the potentiation of cocaine reward (Fig 2.7) without preventing the normal cocaine-CPP response. Importantly, the longer SL-327 treatment by itself did not affect the normal cocaine-CPP response either (Figure 2.7). Unexpectedly, we found that mice treated with nor-BNI alone showed reduced levels of activated ERK1 and ERK2, however, without having any effect on cocaine-CPP. The lack of effect of nor-BNI on cocaine-CPP is unclear but may be dependent on cocaine dose (Kuzmin et al, 1998).

The mechanism by which acute KOR agonist treatment suppresses cocaine reward has been accepted to be the result of a KOR-induced hyperpolarization of DA neurons (Margolis et al., 2003). Additionally, as KOR is localized on DA neurons that also express DAT (Svingos et al., 2001) KOR may also reduce extracellular DA signaling via an indirect mechanism to enhance
the capacity for DAT uptake (Heidbreder et al., 1998; Thompson et al., 2000; Fuentealba et al., 2006). This appears to be relevant given studies that have demonstrated that acute KOR agonists upregulate DAT activity, while prolonged treatment with KOR agonists are ineffective at modulating DAT activity (Thompson et al., 2000; Fuentealba et al., 2006). Recall that DAT activity is modulated by ERK1/2 MAP kinase (Lin et al., 2003; Moron et al., 2003; see also discussion in chapter 1), whereby inhibition of ERK1/2 MAP kinase enhances DAT transport capacity and cell surface expression. Given that prolonged activation of KOR (agonist-dependent) mediates potentiation of cocaine reward via activation of ERK1/2 MAP kinase it is possible that while acute KOR agonists enhance DAT activity the prolonged activation of KOR may cause dysregulation of DAT activity via a KOR activation of ERK1/2 MAP kinase. GPCR-mediated activation of ERK1/2 MAP kinase to regulate the activity of DAT has been demonstrated with DA subtype receptors (D2 and D3) (Bolan et al., 2007; Zapata et al., 2007). Stimulation of D2 receptors increased DAT activity mediated by Gi/o protein and ERK1/2 MAP kinase independent of PI3K (Bolan et al., 2007). In contrast, D3 receptor modulation of DAT activity was dependent on Gi/o protein, ERK1/2 MAP kinase, and PI3K. Of particular interest is that while acute D3 stimulation (<1 min) increased DAT surface activity and expression prolonged DA agonist stimulation (30 min) reduced trafficking to the cell surface and enhanced internalization of DAT (Zapata et al., 2007). It is feasible that KOR-mediated activation of ERK1/2 MAP kinase may regulate DAT cell surface expression and activity in a similar manner. The KOR agonist-mediated paradoxical potentiation might be accounted for by a late-phase phase induced by internalization of the receptor and activation of ERK1/2 MAP kinase, which could result in co-internalization of the DAT. Future studies should examine whether these proteins directly interact with each other using coimmunoprecipitation experiments to isolate and verify proteins within the complex. Moreover, given that agonists possess differential signaling and receptor regulatory capabilities that vary in magnitude and duration, this may also consequently result in diverse agonist-mediated regulation of the DAT.

It is notable that there are reports that have demonstrated that ligand-induced internalization could be dissociated from ERK1/2 signaling in CHO cells that express rat KOR (Li
et al., 1999 and Jordan et al., 2000). KOR peptides have been found to promote potent receptor internalization while synthetic ligands, such as U50,488 were ineffective (Jordan et al., 2000). Even after a 90 min period of incubation with U50,488 (0.1 µM) rat KOR expressed in CHO cells did not undergo internalization (Jordan et al, 2000) while prolonged incubation with U50,488 (for up to 4.5 hours) produced long-lasting activation of ERK1/2 MAP kinase. Expression of dominant negative mutants of dynamin (dynamin K44E) and β-arrestin (β-arrestin 319-418), adaptor proteins required for KOR internalization, abolished agonist-induced KOR internalization, but did not affect U50,488-induced activation of ERK1/2 at a time point corresponding to the onset of internalization, i.e. producing internalization of ~26% of total KOR in CHO cell lines (Li et al, 1999). However, others have demonstrated that expression of mutant dynamin K44E abolished agonist-induced activation of ERK1/2 after 10 and 60 min of treatment with 1 µM U69,593, as measured by in vitro phosphorylation of myelin basic protein (MBP) in HEK293 cells (Ignatova et al, 1998), similar to that observed in the present studies. The discrepancy between studies can be attributed to differences in cell lines and by differences in treatment conditions. In studies that observed a U50,488-induced a prolonged activation of ERK1/2 MAP kinase lasting up to 4.5 hours, cells were treated with agonists by supplementing media with protease inhibitors to prevent the degradation of the various peptides (Jordan et al., 2000). It is possible that the addition of protease inhibitors to the cells during agonist treatment may have potentially confounded their results. This would disrupt the normal turnover of ERK1/2 and thus would result in a longer-lasting activation of ERK1/2.

KOR agonist-induced potentiation of cocaine reward via ERK1/2 MAP kinase activation may also be due to downstream activation of transcription factors and genes involved in regulation of cocaine reward. Acute salvinorin A treatment was found to inhibit cocaine-induced activation of c-Fos expression in striatum but not in the NAc, while repeated treatment with salvinorin A-induced a paradoxically increase in cocaine-induced c-Fos expression in the striatum (Chartoff et al., 2008). This is consistent with data presented in this dissertation that demonstrated that salvinorin A induces early, but not late-phase, activation of ERK1/2 MAP kinase. This would suggest that salvinorin A modulation of cocaine-induced c-Fos expression is
independent of ERK1/2 MAP kinase. Consistent with this idea, Radwanska et al., 2005 demonstrated that c-Fos activation by cocaine treatment can occur independent from ERK1/2 MAP kinase.

The *in vitro* findings suggested that distinct mechanisms govern agonist-induced activation of ERK1/2 MAP kinase and inverse-agonist suppression of ERK1/2 MAP kinase. This diversity may be due to the coupling of regulatory protein inside the cell (discussed further below). However, one potential mechanism for this diversity could be explained by initial binding of ligands based upon concepts of pre-existing states of the receptor.

Conventional GPCR receptor theory posits the existence of multiple receptor states that are based upon the coupling of the receptor, G-protein, and ligand. For nearly a decade, the extended ternary complex model was used to explain the pharmacological actions of agonist and antagonist activity upon binding (Samama et al., 1993). However, in this model G-proteins can only interact with an active receptor, occurring only in the presence of a ligand. Recent evidence has supported the expansion of this old model into the new cubic ternary complex, which allow for the interaction between G-protein and the inactivated receptor, in the absence of ligand (Figure 5.1; Kenakin, 2001). As the receptor can exist between multiple states, differential signaling induced by ligands will occur based upon the state of the receptor the ligand favorably prefers to bind (Kenakin, 2001). For instance, it has been suggested that agonists preferentially bind to receptors pre-coupled to active G proteins, but inverse agonists may prefer the inactive state, whereas antagonists have no preference (Brink et al., 2004).

The present evidence suggests KOR are involved in the stimulation of multiple responses independent of G-protein. The present work is in line with a growing number of studies demonstrating that agonists could promote functionally selective signaling of G-proteins and independent effector systems (Berg et al., 1998; Chanrion et al., 2008; reviewed in Urban et al., 2007). For instance, the 5HT2A inverse agonist, SB206,553 suppressed IP formation but did not inversely regulate intracellular Ca**+** accumulation (Chanrion et al., 2008). Thus, not only does receptor state affect ligand signaling but it appears that ligands may also “prefer”-have greater efficacy through- one effector system over another (Kenakin, 2007).
It remains to be determined how a ligand would direct receptor activation of one pathway over another. One possibility is the recruitment and binding of regulatory proteins located inside the cell (Tilakaratne and Sexton, 2005). In the studies presented in this dissertation, the KOR agonist U50,488 induced a late-phase activation that was mediated by internalization of the receptor. In previous reports, internalization of the KOR required the scaffolding action of β-arrestin (Li et al., 1999). It has been demonstrated that β-arrestin serves as an adaptor protein that recruits multiple proteins involved in mediating the activation of ERK1/2 MAP kinase (Lefkowitz and Shenoy, 2005). Thus, the present findings of functional selectivity of KOR agonists may possibly mediated by their ability to promote the recruitment of β-arrestin to activate ERK1/2 MAP kinase. In contrast, nor-BNI displayed inverse agonist activity at ERK1/2 MAP kinase. The data suggested a KOR-mediated, G-protein independent, Akt-dependent mechanism is involved in mediating the effects of nor-BNI. This novel pathway has not been examined and therefore, the mechanism by which nor-BNI promotes signaling through to Akt is unclear. It is speculated that this may be occurring via CaM interaction with the third intracellular loop of the KOR (Wang et al., 2001). Previous studies have demonstrated that the interaction of CaM reduces basal opioid receptor activity via blockade of G-protein activation (Wang et al., 1999; Wang et al., 2000). Blockade of CaM interaction with the MOR or DOR by either calcium chelation or chemical inhibitors, was shown to effectively increase basal opioid receptor activity and potentiate acute morphine-induced signaling (Wang et al., 2000). Recent studies have demonstrated that CaM is involved in mediating growth factor stimulation of Akt independent of PI3K (Deb et al., 2004) possibly via direct binding to Akt (Dong et al., 2007). Much work remains to be done in future studies to confirm this observation and extend the understanding of the cellular mechanism governing it.

Another possible mechanism by which Akt activation may suppress ERK1/2 MAP kinase activation is through a GPCR-mediated activation of the NA+/H+ exchange regulator 1 (NHERF), which has previously been shown to interact directly with Akt to promote inactivation of Raf (Wang et al., 2008). Mutations in the regulatory domain of Raf abolished NHERF-mediated reduction of ERK1/2 MAP kinase phosphorylation (Wang et al., 2008). Interestingly, NHERF
binds to the C-terminal tail of KOR and regulates intracellular pH independent from G<sub>i/o</sub> protein in CHO and opossum kidney proximal tubule epithelium cells (Li et al., 2002; Huang et al., 2004B). Likewise, cannabinoid receptor (CB<sub>1</sub>) activation also has been demonstrated to regulate the NHE-1 subtype of NA<sup>+</sup>/H<sup>+</sup> exchangers through a mechanism dependent on G<sub>i/o</sub> protein and ERK1/2 MAP kinase. Interestingly, the inverse agonist, SR 141716A, not only blocked the effects of the agonist CP-55940 but was also found to reduce basal NHE-1 activity (Bouaboula et al., 1999).

Previous studies have demonstrated that NHERF expression did not affect U50,488-induced activation of ERK1/2 MAP kinase or desensitization but instead enhanced the recycling rate of KOR following prolonged exposure to U50,488 (Li et al., 2002; Huang et al., 2004). Moreover, in opossum kidney proximal tubule epithelium cells higher levels of NHERF expression were required for the U50,488 mediated, naloxone-sensitive, change in intracellular pH. The effects of nor-BNI were not determined in these studies (Huang et al., 2004). However, given the study by Bouaboula et al., 1999 it is feasible to speculate that nor-BNI alone would reduce basal NHERF regulation of intracellular pH (Na<sup>+</sup>/H<sup>+</sup>). Much as agonist-dependent internalization of the KOR is required for rerouting of receptor signal transduction to a select pool of ERK1/2 MAP kinase, perhaps putative inverse agonists, such as nor-BNI, may cause a rerouting of NHERF activity to regulate the activity of Akt, which would in turn suppress ERK1/2 MAP kinase activity (Wang et al., 2008). Supporting this, parathyroid receptor association with NHERF has been shown to result in a switching of receptor signaling from G<sub>s</sub> to G<sub>i/o</sub>-mediated signaling (Mahon et al., 2004). Thus, it is proposed that ligands that induce differential signaling independent of G-proteins require the recruitment of adaptor proteins to enable the scaffolding actions of ligand-receptor mediated signaling to the appropriate preferable effector system. Determining the proteins involved and their order of cascading activity will require extensive study, but one that may finally provide insight as to why certain drugs within the same class and acting upon the same receptor may produce distinct biological outcomes.
II. THERAPEUTIC IMPLICATIONS FOR COCAINE TREATMENT

The findings presented in the present thesis are in line with previous studies (Crawford et al., 1995; McLaughlin et al., 2006a) that have demonstrated the acute benefits of KOR agonists on suppressing cocaine-induced behaviors. However, in animal studies presented here prolonged exposure to KOR agonists produces a paradoxical enhancement of drug-seeking behavior, limiting the long-term therapeutic use of KOR agonists (Negus, 2004; McLaughlin et al., 2006). Thus, the use of KOR agonists could be used beneficially during the initiation of abstinence, to acutely suppress cocaine cravings. However, as salvinorin A lacked the ability to potentiate cocaine reward, matched by its impairment in inducing an internalization-mediated signaling of ERK1/2 MAP kinase, this exposes a possible mechanism by which to distinguish potential therapeutic agonists. KOR agonists that produce the similar impairments as salvinorin A may potentially yield similar behavioral pharmacological effects. If proven, the development of potentially effective KOR ligands could be easily predicted through in vitro assays utilizing high-throughput screening methods.

As the disease progresses and cocaine cravings persist, the upregulation of the KOR system that occurs during this period has been demonstrated to contribute to the detrimental effects of cocaine (Spangler et al., 1993; Unterwald et al., 2001; Hurd & Herkenham, 1993; Frankel et al., 2008), a neurobiological effect related to the dysphoric and anhedonic effects associated with cocaine withdrawal (Johanson and Fischman, 1989). It has been proposed that the type of KOR ligand that will be effective in opposing cocaine is dependent on the stage of addiction (Shippenberg et al., 2001). KOR antagonists (or inverse agonists) would be required to oppose the effects by long-term maintenance of drug abstinence and prevention of relapse. The findings presented here, indicate this, as the putative KOR inverse agonist, nor-BNI, did not have any effect by itself, instead, it was found found to inhibit the enhanced drug-seeking behavior elicited through prolonged activation of the KOR.

Prolonged activation of the KOR has been demonstrated to play a major role in mediating stress- and depressive-like behaviors (McLaughlin et al., 2006a, b). Stress is known to potentiate the rewarding properties of drugs of abuse and precipitate reinstatement of drug-seeking and
drug-taking behavior (Piazza et al, 1990; Will et al, 1998; Haile et al, 2001; Cleck and Blendy, 2008). Accordingly, repeated forced-swim stress and social defeat stress dramatically increased the reinforcing effects of cocaine by a dynorphin-dependent mechanism that required KOR activation (McLaughlin et al, 2006a, b). In contrast, KOR antagonist treatment or genetic disruption of the endogenous KOR systems has been demonstrated to attenuate stress-induced reinstatement of cocaine seeking behavior, but not cocaine-primed reinstatement (Beardsley et al., 2005; Carey et al., 2007; Redila and Chavkin, 2008). Overall, this suggests that opposing the KOR system may be therapeutically relevant to prevent stress-induced relapse in recovering addicts.

III. BROADER THERAPEUTIC IMPLICATIONS FOR STUDYING KOR LIGAND SIGNALING

In addition to modulation of cocaine-mediated behaviors, KOR activation underlies a diverse array of physiological and behavioral processes, including analgesia, diuresis, psychotomimesis, anxiety, and depression (for review see Liu-Chen, 2004; Bodnar, 2007). As such, a growing interest has been in the development selective kappa opioid antagonists for therapeutic treatment of depression, anxiety, and schizophrenia (Metcalf and Coop, 2005).

As noted, KOR agonists have been demonstrated to suppress DA release that is associated with their aversive and dysphoric properties (Kumor et al., 1986; Suzuki et al., 1992; Pliakas et al., 2001; Zhang et al., 2004a, b; Zhang et al., 2005). Accordingly, animal studies have demonstrated that activation of the KOR mediates stress- and depressive-like behaviors (McLaughlin et al., 2003; 2006a, b). Conversely, KOR antagonists and disruption of the genes that code for the KOR or the endogenous agonist dynorphin have been demonstrated to reduce immobility and increase swimming behaviors in forced swim test assay, an animal model of learned helplessness (Mague et al., 2003; Reindl et al., 2008; McLaughlin et al., 2003; 2006). Together, these findings suggest that blockade of the KOR could represent a potentially new class of antidepressants.
However, not all KOR antagonists are effective at preventing depressive-like behaviors. For example, the opioid antagonist naloxone was found to actually enhance stress-induced learned helplessness in an escape-avoidance task (Pignatiello et al., 1989; Besson et al., 1995; Tejedor-Real et al., 1995). This discrepancy among opioid antagonists is poorly understood. However, in light of the neutral antagonistic actions of naloxone found in the present thesis, the effectiveness of select KOR antagonists (such as nor-BNI) in preventing learned helplessness, may in fact be mediated by the ability to suppress ERK1/2 MAP kinase signaling, or in other words act as an inverse agonist. This idea is supported mechanistically by previous studies have shown that blockade of ERK1/2 MAP kinase reduces depressive-like behavior (Einat et al., 2003). Interestingly, chronic treatment with the antidepressant fluoxetine reduced hippocampal and frontal cortex expression levels of activated ERK1/2 MAP kinase (Fumagalli et al., 2005) and electric convulsive shock (ECS), another form of treatment for depression, produced a transient activation of ERK1/2 MAP kinase followed by a sustained increase in gene expression of MAP kinase phosphatases (MKP) (Kodama et al., 2005). Given that the activity of ERK1/2 MAP kinase is negatively regulated by phosphatases involved in dephosphorylation (deactivating) of ERK1/2 MAP kinase (Harding et al., 2005) and by protein kinase B (PKB or Akt) (Zimmermann and Moelling, 1999; Guan et al., 2000), and the present findings here, these results posit a broader implication for those KOR antagonists that possess the ability to suppress ERK1/2 MAP kinase (inverse agonists), indicating a novel class of antidepressants.

Additionally, KOR agonists have been demonstrated to produce psychotomimetic effects (Liu-Chen, 2004; Prisinzano, 2005). From this, KOR antagonists (or inverse agonists) have also been suggested to be useful for treatment of schizophrenia and bipolar disorders (Metcalf and Coop, 2005). The long-lasting KOR antagonist, GNTI, was found to inhibit the hyperlocomotor and stereotypic behavior induced by NMDA receptor agonist, MK-801 (Qi et al., 2006). DAT knockout mice display an unusual hyperactivity along with stereotypic movements when exposed to novel environments (Beaulieu et al., 2004). It is interesting that the mood stabilizer lithium has been demonstrated to attenuate hyperactivity (Beaulieu et al., 2007), through a mechanism attributed to modulation of Akt activity (Beaulieu et al., 2004). Interestingly, genetic studies in
humans suggest that schizophrenia is marked by a dysregulation in Akt activity (Tan et al., 2008). Consistent with this, mice that have a deficiency in the Akt1 gene show impaired working memory under neurochemical challenges with DA receptor agonists when compared to their wild type littermates (Lai et al., 2006). Moreover, Akt mediated activity induced by lithium has been demonstrated to inhibit the activity of the downstream kinase glycogen synthase kinase-3 beta (GSK3β) (Klein and Melton, 1996), which is becoming a major therapeutic target of interest in the treatment of schizophrenia, bipolar disorders, and depression (Gould and Maji, 2005). Given the present findings that the KOR antagonist nor-BNI acts as an inverse agonist to mediate the suppression of ERK1/2 MAP kinase via a mechanism dependent on the activation Akt, the effects of nor-BNI on GSK3β should be investigated. The potential signaling cascades that could be affected by KOR inverse agonists are a direction for future interest. It is of interest to examine the KOR inverse agonist mediated suppression of ERK1/2 MAP kinase and regulation of Akt/GSK3β signaling as this may expose a potential common mechanism among effective mood stabilizers and could present a method to screen potential novel therapeutics for a broad variety of mood disorders.

In conclusion, the present dissertation has examined the diversity of intracellular signaling mediated by the KOR and its implication on one form of behavior, cocaine reward. As many physiological and behavioral processes are modulated by the endogenous KOR system understanding and determining the importance of the precise intracellular signaling proteins involved will be a major challenge, as not all drugs produce the same effects. Targeting the precise cascades of therapeutic drug action will be an important task. For now, the goal is to acknowledge and appreciate this diversity and its potential role in the development of effective therapeutics.
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Figure 5.1. **Models of receptor complex.** The extended ternary complex developed by Samama et al., 1993 depicts that receptors can exit in six different states by which ligands can bind. In this model ligands can only bind to the receptor when it is in an activate states (R$_a$) but not when it is inactive (R$_i$). The cubic ternary complex model extends from this and includes an inactivate receptor. R, receptor; R$_a$, inactive receptor; R$_i$, inactivate receptor; G, G protein; A, ligand; K, association constants for ligand to receptor, $\beta$K$_G$, association constant receptor to G-protein; L, allosteric constant; $\alpha$, modifier of affinity in receptor active state; $\gamma$, modifier of ligand bound, respectively. L, allosteric constant and $\alpha$, and $\gamma$ the modifiers of affinity once the receptor is active or ligand bound, respectively (From Kenakin 2001)