International Journal of Neuropsychopharmacology (2018) 21(9): 847–857

doi:10.1093/ijnp/pyy034
Advance Access Publication: April 4, 2018
Regular Research Article

REGULAR RESEARCH ARTICLE

Structurally Related Kappa Opioid Receptor Agonists with Substantial Differential Signaling Bias: Neuroendocrine and Behavioral Effects in C57BL6 Mice

Amelia D. Dunn*, Brian Reed,* Catherine Guariglia, Alexandra M. Dunn, Joshua M. Hillman, Mary Jeanne Kreek

Laboratory of the Biology of Addictive Diseases, Rockefeller University, New York, New York.

*These authors contributed equally to this work.

Correspondence: Amelia Dunn, BS, BA, 1230 York Ave, Box 243, New York, NY 10065 (adunn@rockefeller.edu).

Abstract

Background: The kappa opioid receptor system has been revealed as a potential pharmacotherapeutic target for the treatment of addictions to substances of abuse. Kappa opioid receptor agonists have been shown to block the rewarding and dopamine-releasing effects of psychostimulants. Recent investigations have profiled the in vivo effects of compounds biased towards G-protein-mediated signaling, with less potent arrestin-mediated signaling. The compounds studied here derive from a series of trialkylamines: N-substituted-N- phenylethyl-N-3-hydroxyphenylethyl-amine, with N-substituents including n-butyl (BPHA), methylcyclobutyl (MCBPHA), and methylcyclopentyl (MCPPHA).
Methods: BPHA, MCBPHA, and MCPPHA were characterized in vitro in a kappa opioid receptor-expressing cell line in binding assays and functional assays. We also tested the compounds in C57BL6 mice, assaying incoordination with rotarod, as well as circulating levels of the neuroendocrine kappa opioid receptor biomarker, prolactin.
Results: BPHA, MCBPHA, and MCPPHA showed full kappa opioid receptor agonism for G-protein coupling compared with the reference compound U69,593. BPHA showed no measurable β-arrestin-2 recruitment, indicating that it is extremely G-protein biased. MCBPHA and MCPPHA, however, showed submaximal efficacy for recruiting β-arrestin-2. Studies in C57BL6 mice reveal that all compounds stimulate release of prolactin, consistent with dependence on G-protein signaling. MCBPHA and MCPPHA result in rotarod incoordination, whereas BPHA does not, consistent with the reported requirement of intact kappa opioid receptor/β-arrestin-2 mediated coupling for kappa opioid receptor agonist-induced rotarod incoordination.
Conclusions: BPHA, MCBPHA, and MCPPHA are thus novel differentially G-protein-biased kappa opioid receptor agonists. They can be used to investigate how signaling pathways mediate kappa opioid receptor effects in vitro and in vivo and to explore the effects of candidate kappa opioid receptor-targeted pharmacotherapeutics.

Keywords: arrestin, GPCR, ligand bias, rotarod, prolactin

Introduction

The endogenous opioid system consists of the mu, delta, and kappa opioid receptors (MOR, DOR, and KOR, respectively) as well as the closely related nociceptin receptor (NOR, also referred to as OPRL1). The opioid receptors, and the KOR in particular, play important roles in reward and addiction and therefore are promising targets for the development of therapeutics. Whereas

Received: November 14, 2017; **Revised:** March 14, 2018; **Accepted:** March 30, 2018

Significance Statement

The kappa opioid receptor (KOR) has recently emerged as a potential therapeutic target for addictive diseases as well as other disorders. It is yet to be determined, however, if full receptor activation or just activating certain downstream pathways is best. This study presents side-by-side comparisons of 3 very similar compounds that activate the KOR, but with different signaling properties (referred to as "bias"). The effect of these differences in signaling is investigated in mice.

approved medications, namely methadone (full MOR agonist) and buprenorphine/naloxone (partial MOR agonist/MOR antagonist to prevent rapid onset in the event of parenteral administration), allow for extended abstinence and recovery from opiate addiction, there are no approved medications for psychostimulant addiction. Efforts to develop new medications, or to utilize existing neurotransmitter-targeting medications, have not been successful to date. Given the immense medical, social, and economic consequences of addiction, and psychostimulant addiction in particular, continued efforts to develop medications to treat patients dependent on cocaine or other psychostimulants is essential.

Exposure to cocaine, which inhibits the biogenic amine neurotransmitter transporters, acutely causes increased extracellular dopamine, serotonin, and norepinephrine and also results in changes in components of the endogenous opioid system. Acutely, cocaine results in increased gene expression of the endogenous KOR ligand dynorphin in the dorsal and ventral striatum in animal models (Daunais et al., 1993; Spangler et al., 1993, 1996). Chronic cocaine exposure also results in changes in MOR and KOR binding (Unterwald et al., 1992, 1994; Zubieta et al., 1996; Bailey et al., 2007). Full KOR agonists have the ability to block the rewarding effects of cocaine (Schenk et al., 1999; Zhang et al., 2004), but by themselves they have been shown to be aversive (Zhang et al., 2005). Kappa antagonists have been shown to block stress-induced reinstatement to cocaine seeking in animal self-administration models, but with no effect on drug-induced reinstatement (Beardsley et al., 2005). In addition to addiction, KOR-directed ligands are attractive potential therapeutics in a range of conditions, including depression, pain, PTSD, pruritis, and others (Van'T Veer and Carlezon, 2013; Jones et al., 2016). Additional investigation is needed to determine the effects of different KOR ligands on models of cocaine use.

Recent advances in the molecular pharmacology of G-protein coupled receptors have revealed that effectors of signaling can differentially activate select intracellular mediators, known as ligand bias (Violin and Lefkowitz, 2007). Ligand bias can be used to describe differences in activation of G-protein subtypes as well as differences in activation between other pathways. In many cases, ligand bias is used to describe differences between G-protein and arrestin signaling pathways. These differences in signaling can in turn yield different in vivo effects for biased vs unbiased compounds, with the potential for favorable clinical properties for biased compounds. This has been shown for biased agonists of the angiotensin receptor (Monasky et al., 2013) and the mu opioid receptor (MOR) (Soergel et al., 2014).

Several recent studies have described kappa opioid receptor (KOR) biased ligands in in vitro studies (including White et al., 2013; Lovell et al., 2015). Further, 2 studies of G-protein biased vs unbiased KOR agonists indicate that ligand bias can yield disparate effects in vivo at the behavioral level (White et al., 2015; Brust et al., 2016). Additional investigations on the effects of ligand bias in the KOR system are needed to explore the therapeutic potential for biased KOR ligands.

Several KOR-selective pharmacophores have been recently reported and characterized (Frankowski et al., 2010, 2012; Nagase et al., 2010; Spetea et al., 2012; Bourgeois et al., 2014; Hirayama et al., 2014; Riley et al., 2014; Scarry et al., 2016). The current study follows from a report (Spetea et al., 2012) of novel compounds based on a diphenethylamine structural backbone known to interact with the dopamine D2 receptor and reported to also have KOR antagonist activity in in vitro rodent tissue binding assays and bioassays (Fortin et al., 1991). The novel compounds in this series were studied in KOR radioligand binding and [^{35}S]GTPγS binding stimulation in vitro, with the same assays performed with membranes prepared from cell lines expressing MOR or DOR, as well as dopamine D1, D2, or D3 receptors (Spetea et al., 2012). The compound that had the highest affinity for KOR was *N*-methylcyclobutyl-*N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine (Figure 1A, MCBPHA, referred to as HS-665, compound 4 in Spetea et al., 2012). MCBHPA was reported to have 90% intrinsic activity in KOR [^{35}S]GTPγS assays, with low affinity and no agonism at the dopamine D2 receptor. This compound was further characterized in an in vivo acetic acid writhing analgesia assay in mice. It was demonstrated to be equally potent as the well-characterized KOR agonist U50,488 in peripheral analgesia, and the effect was blocked by a KOR antagonist

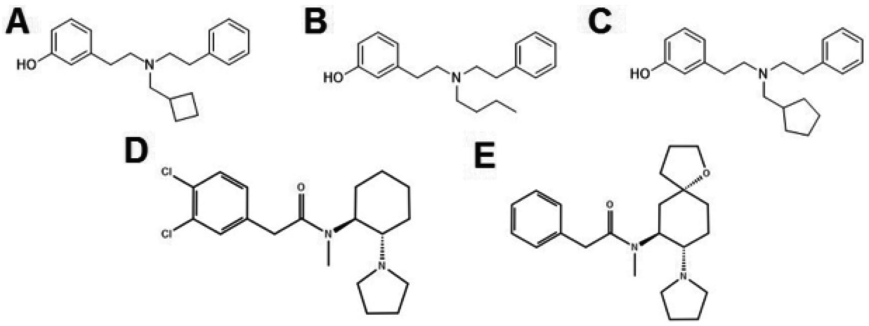

Figure 1. Structures of phenethylamine compounds and control KOR agonist compounds. (A) MCBPHA, *N*-methylcyclobutyl-*N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine. (B) BPHA, *N*-*n*-butyl- *N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine. (C) MCPPHA, *N*-methylcyclopentyl-*N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine. (D) U50,488, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide. (E) U69,593, N-methyl-2-phenyl-N-[(5R,7S,8S)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl]acetamide.

(Spetea et al., 2012). In an additional recent study, MCBPHA, as well as a structurally related methylcyclopropyl derivative, was further characterized in vitro and in vivo (Spetea et al., 2017). Of the other remaining compounds reported in the 2012 study, one, *N*-butyl-*N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine (BPHA; Figure 1B), was found to exhibit high affinity at KOR in radioligand binding, no appreciable activity at the dopamine receptors, and partial [^{35}S]GTPγS agonist activity (45.5% intrinsic activity in KOR [^{35}S]GTPγS assay). Further, this compound was found to exhibit approximate 40-fold selectivity for KOR compared with MOR (and over 200-fold selectivity for KOR vs DOR) (Spetea et al., 2012).

The goal of this study was to further characterize the properties of these 2 prior published compounds, BPHA and MCBPHA, with respect to KOR G-protein coupled receptor ligand bias and in vivo kappaergic effects. In addition to BPHA and MCBPHA, we also characterized a structurally related diphenylethylamine derivative -*N*-methylcyclopentyl-*N*-phenylethyl-*N*-3-hydroxyphenylethyl-amine (MCPPHA, Compound 3 in Erli et al. 2017; Figure 1C) that was reported to have full agonist activity in the [^{35}S]GTPγS assay (Erli et al., 2017). We report here the results of in vitro studies characterizing MCPPHA, MCBPHA, and BPHA with respect to differential KOR signaling via β-arrestin-2 vs G-protein-mediated signaling compared with the full agonist U69,593 (Figure 1E). We also conducted in vivo studies in C57BL6 mice comparing MCPPHA, MCBPHA, and BPHA with U50,488 (Figure 1D). We explored whether KOR-mediated endpoints, including prolactin release and rotarod incoordination, are affected by these compounds in the whole animal.

Materials and Methods

Compounds

[^{3} H]-(-)-U69,593, [^{3} H][D-Ala^{2}, *N*-MePhe^{4}, Gly-ol]-enkephalin ([^{3} H]DAMGO), [^{3} H][D-Pen^{2,5}]-Enkephalin ([^{3} H]DPDPE), and [^{35} S]GTPγS were procured from Perkin Elmer. (±)-Trans-U50,488, (-)-U69,593, DAMGO, and DPDPE were obtained from Sigma. BPHA, MCBPHA, and MCPPHA were synthesized by a contract research organization, WuXi Apptech, using methods adapted from those reported previously by the group of Schmidhammer (Spetea et al., 2012). Mass spectrometric and NMR signals were in agreement. LY2444296 [(S)-3-fluoro-4-(4-((2-(3-fluorophenyl) pyrrolidin-1-yl) methyl)phenoxy)benzamide] was also synthesized by WuXi, with a small portion generously donated by Eli Lilly, which was used to confirm that the WuXi synthesized compound was molecularly identical (as determined by reversed-phase high-performance liquid chromatography retention time). For in vivo studies, BPHA, MCBPHA, MCPPHA, U50,488, and LY2444296 were dissolved in 10% ethanol, 10% Tween-80, and 80% water vehicle. Vehicle had no effect on any of the measures studied and is shown as a control where appropriate.

Animals

Male C57BL6 mice (9–13 weeks, 20–30g) were used in all studies herein. Mice were housed, 4 to a cage, in sound attenuated chambers with individual light controls, in stress-minimized rooms, with a 12-hour-light/-dark cycle, and food and water were provided ad libitum. All animals were housed for at least 1 week, with daily handling, prior to studies. Animals were housed and euthanized in a manner approved by The Rockefeller University Institutional Animal Care and Use Committee.

In Vitro Assays

Opioid Receptor Binding

Membranes from cells stably expressing KOR, MOR, or DOR constructs (PathHunter U2OS hOPRK1, CHO-K1 rOPRM1, and CHO-K1 OPRD1 β-arrestin-2 cell line, DiscoverX) were used. Cells were scraped from tissue culture plates, homogenized with a tissue tearor homogenizer in membrane buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA; pH 7.4), and centrifuged at 20000 g for 30 minutes at 4°C and frozen at -80°C until use. Prior to use, the pellets were resuspended in binding buffer (50 mM Tris-HCl, pH 7.4), homogenized with a dounce homogenizer, and 50 μg incubated with 1.0 nM of the appropriate tritiated ligand ([^{3} H]U69,593, [^{3} H]DAMGO, or [^{3} H]DPDPE for kappa, mu, or delta binding, respectively) and the appropriate concentration of compound for 60 minutes at 30°C. Nonspecific binding of radioligand to KOR, MOR, or DOR was determined in the presence of 10 μM norbinaltorphimine, naloxone, or naltrindole, respectively. Membranes with bound tritiated ligand were collected on Whatman GF/B filter paper (Brandel) utilizing a Brandel harvester. Bound tritiated ligand was quantified using a TriCarb-2900TR scintillation counter (Packard) following addition of 4 mL ReadySafe scintillation fluid (Beckman Coulter).

[^{35}S]GTPgammaS Binding

Membranes from U2OS cells (PathHunter U2OS hOPRK1) stably expressing human kappa opioid receptors were used. Cells were scraped from tissue culture plates, homogenized with a tissue tearor homogenizer in membrane buffer (10 mM Tris, 100 mM NaCl, and 1 mM EDTA; pH 7.4), and centrifuged at 20000 g for 30 minutes at 4°C and frozen at -80°C until use. Prior to use, the pellets were resuspended in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA; pH 7.4) and homogenized with a dounce homogenizer and 50 μg incubated with 0.1 nM [^{35} S]GTPγS, 10 nM GDP, and the appropriate concentration of agonist for 20 minutes at 30°C. Membranes with bound [^{35} S]GTPγS were collected on Whatman GF/B filter paper (Brandel) utilizing a Brandel harvester. Bound [^{35} S]GTPγS was quantified using a TriCarb-2900TR scintillation counter (Packard) following addition of 4 mL ReadySafe scintillation fluid (Beckman Coulter).

β-Arrestin-2 Signaling

Experiments were conducted using the PathHunter Detection Kit obtained from DiscoverX. Cells stably expressing KOR, MOR, or DOR constructs (PathHunter U2OS hOPRK1, CHO-K1 rOPRM1, and CHO-K1 OPRD1 β-arrestin-2 cell line, DiscoverX) were plated in 96- or 384-well plates. Cells were stimulated with the compounds for 90 minutes at 37°C followed by incubation for 60 minutes in the presence of galactosidase substrate, yielding chemiluminescent product. Chemiluminescence was measured using a Synergy Neo microplate reader (BioTek). Antagonism assays were done in the same manner in the presence of 300 nM U69,593, 1 uM DAMGO, or 1 μM DPDPE for KOR, MOR, or DOR assays, respectively.

Competitive Model Analysis

Competitive model analysis was performed as described in Stahl et al., 2015. Dose response curves for [^{35}S]GTPγS and β-arrestin-2 assays were used as well as antagonist β-arrestin-2 dose response curves (as described above) for each test ligand. All curves were fit by nonlinear regression in GraphPad Prism 7.0. U69,593 was used as the reference ligand.

For each analysis, the data was fit to the equations below. The LogK value is the affinity constant of the ligand, either reference

or test. LogR is a measure of efficacy and affinity of the ligand, derived from the operational model of partial agonism (Black and Leff, 1983). LogRA is a measure of the difference between the LogR value of the test ligand and the LogR value of the reference ligand. In these equations, X is the concentration of the test ligand, while A is the concentration of the reference ligand (held constant in the assays where the test ligand is treated as an antagonist). Finally, n is the transducer slope factor that was held to 1 for analysis. For the [^{35}S]GTPγS assays, the U69,593 curve was first fit to the reference ligand equation. These parameters were then used to fit the data from the test ligand. For the β-arrestin-2 assays, the U69,593 curve was first fit to the reference ligand equation. These parameters were then used to fit the data from the test ligand both using the agonist and antagonist equations, when data was available. Initial parameters and constraints were held as described in Stahl et al. 2015.

Reference ligand:

$$Y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{1 + 10^{(X - LogK_{ref})}}{10^{(X + LogR)}}\right)^{n}}$$

Test ligand as an agonist:

$$Y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{1 + 10^{(X - LogK_{test})}}{10^{(X + LogR + LogRA)}}\right)^{n}}$$

Test ligand as an antagonist:

$$Y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{1 + 10^{(A - LogK_{ref})} + 10^{(X - LogK_{test})}}{10^{(A + LogR)} + 10^{(X + LogR + LogRA)}}\right)^{n}}$$

In Vivo Assays

Prolactin Assay

Mice were injected i.p. with varying doses of MCBPHA, BPHA, or MCPPHA (or vehicle) 30 minutes prior to sampling. LY2444296 or vehicle pretreatment, if applicable, was given by i.p. injection 60 minutes prior to sampling. Vehicle for all experiments and all compounds except for U50,488 consisted of 10% ethanol, 10% Tween-80, and 80% distilled deionized water. In the case of U50,488 injections, sterile saline was used as the vehicle. Trunk blood was collected by rapid decapitation, followed within 2 hours by preparation of serum. Serum prolactin levels were determined using a commercially available enzyme-linked immunoassay (AbCam) following dilution of serum 5-fold in assay buffer.

Rotarod Assay

Rotarod experiments were conducted with mice using a dedicated rodent rotarod apparatus, with up to 5 animals tested concurrently (IITC Life Science). Rotarod rotation rate begins at 3 rpm, and ramps to 30 rpm over the course of 300 seconds, at which time the assay is terminated and animals removed to their home cage. Animals were acclimated to the rotarod on at least 2 occasions prior to the day of the test. On the day of the test, baseline times for each animal to fall off the rotarod were recorded. Mice were then injected intraperitoneally with vehicle or compound, and rotarod measurements conducted, beginning 0–2 minutes after injection, and then subsequently at select time points thereafter. Animals that failed to remain on the rotarod for at least 150 seconds during baseline testing were removed from the analysis. Additional experiments, with pretreatment of animals with the short-acting kappa antagonist, LY2444296, prior to kappa agonist administration, were also conducted.

Data Analysis

In vitro binding, [^{35}S]GTPγS stimulation, and β-arrestin-2 coupling experiments were analyzed using Origin 5.0 software (OriginLab), with sigmoidal fitting to determine parameters of half-maximal concentrations and maximal intrinsic activity. In the case of binding experiments, sigmoidal IC_{50} determinations were converted to K_i values using the Cheng-Prusoff equation (Yung-Chi and Prusoff, 1973). The K_d values used were obtained from saturation binding analyses for [^{3}H]U69,593, [^{3}H]DAMGO, and [^{3}H]DPDPE for the respective KOR, MOR, and DOR cell lines, using Scatchard analysis. For intrinsic activity calculations, values were normalized to that obtained with concurrent reference ligand stimulation. The reference ligand used for all in vitro assays was U69,593. All in vitro determinations were conducted in a minimum of 3 separate experiments In vivo rotarod and prolactin experiments were analyzed using Statistica 13.0 statistical software (Dell Statistica). For rotarod experiments, 2-way ANOVAs with repeated measures (condition/dose x time, with repeated measures on time) were used to examine effects and/or interactions of condition/dose and time. For prolactin experiments, 1-way ANOVAs were used to examine effect of condition. In both cases, Newman-Keuls posthoc tests were utilized to examine significant differences.

Results

In Vitro Characterization

MCBPHA (HS-665), BPHA, and MCPPHA all inhibited binding of 1 nM [^{3}H]U69,593 to KOR membranes prepared from U2OS-KOR cells (Table 1; Figure S1). Binding to MOR and DOR was also inhibited, but with considerably lower affinity compared to binding at KOR in all cases (Table 1).

BPHA, MCBPHA, and MCPPHA exhibited full agonism in the [^{35}S]GTPγS assay (defined as maximal stimulation by U69,593) (Figure 2A; Table 2). MCPPHA and MCBPHA also exhibited partial agonism in the β-arrestin-2 recruitment assay, while BPHA yielded no stimulation of β-arrestin-2 signaling (Figure 2B; Table 2). None of the compounds exhibited agonism in the arrestin assays for MOR and DOR (Table S1).

These results indicate that BPHA, MCBPHA, and MCPPHA are KOR agonists with bias for G-protein signaling over β-arrestin-2 signaling, albeit with different degrees of bias. To quantify the difference in bias between compounds, we fit the data to the "competitive model" (Stahl et al., 2015). This model was derived from the traditional operational model of partial agonism (Black and Leff, 1983) to better model agonists with very partial activity. LogRA values from the competitive model describe the difference in efficacy and potency between a test ligand and the reference ligand (U69,593) in a single signaling assay (Table 3). The ΔLogRA between 2 pathways is a quantitative measure of the bias of the ligand between those 2 pathways. The bias can also be described using the bias factor, which is defined as the antilog of ΔLogRA (Schmid et al., 2013). The units of both the ΔLogRA and bias factor are undefined, as is the limit. An unbiased ligand would have similar LogRA values across the 2 pathways. This would yield a ΔLogRA of 0, and therefore a bias factor of 1.

Both the [35S]GTPγS and β-arrestin-2 data for the compounds studied here were fit to this model, using U69,593 as the reference ligand (supplementary Figs. S2 and S3). The previously well-characterized full agonist U50,488 was also fit to this model as a control. LogRA values were calculated for each signaling assay, comparing the activity of each agonist to that of U69,593 in that assay (Table 3). The ΔLogRA values were then calculated for each agonist, comparing activity between the [35S]GTPγS and β-arrestin-2 pathways. In this case, a positive ΔLogRA value indicates G-protein bias. BPHA, MCBPHA, and MCPPHA have ΔLogRA values of 1.8 (0.3 to 3.2), 1.6 (1.2 to 2.0), and 1.3 (0.8 to 1.7), respectively (Table 3). These in turn yield bias factors of 59.3, 42.4,

Table 1. In Vitro Opioid Receptor Binding of Compounds

	R Group	KOR Binding Ki (nM)	MOR Binding Ki (nM)	Selectivity KOR:MOR	DOR Binding Ki (nM)	Selectivity KOR:DOR
MCBPHA	Methylcyclobutyl	0.98 (±0.16)	270 (± 20)	270	3000 (± 1400)	3100
BPHA	n-Butyl	8.4 (±1.4)	360 (± 30)	29	2800 (± 700)	340
MCPPHA	Methylcyclopentyl	0.23 (±0.03)	130 (± 30)	550	1700 (± 200)	7600

All KOR assays were performed in commercially available DiscoverX U2OS cells expressing KOR. MOR and DOR assays were performed using commercially available DiscoverX CHO cells expressing each receptor. Binding was assayed by inhibiting [3H]U69,593, [3H]DAMGO, or [3H]DPDPE to KOR, MOR, or DOR, respectively. Sigmoidal curve fits were performed to determine IC_{50} values, which were then converted to K_i values based on the K_d values determined for [3H]U69,593, [3H]DAMGO, or [3H]DPDPE in membranes prepared from KOR, MOR, or DOR expressing cell-lines (6.6±0.6 nM, 8.3±0.9 nM, and 11±5 nM, respectively). Nonspecific binding was determined in the presence of 10 μM norBNI, naloxone, or naltrindole, respectively. The values presented represent the mean of at least 3 separate experiments, with SEM shown in parentheses.

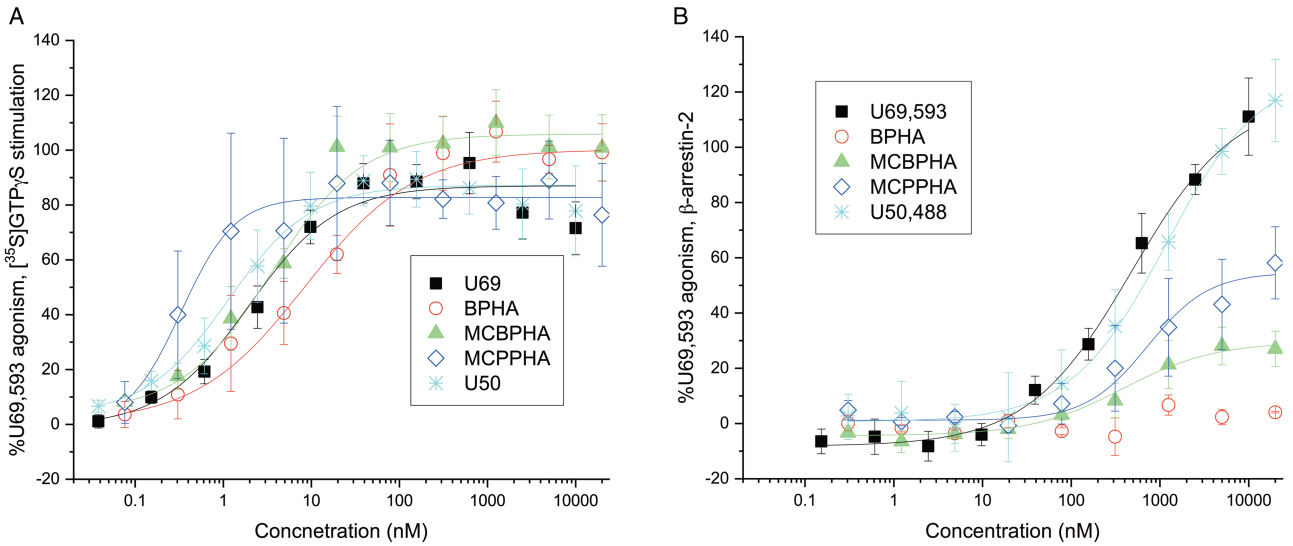

Figure 2. In vitro effects of compounds on KOP-r signaling. Dose response curves with average values of a minimum of 3 separate experiments for each compound. The plots shown are for illustrative purposes; calculated and reported values of EC_{50} and % stimulation (Table 2) were generated from respective fits of 3 separate experiments. (A) Increasing doses of U50,488, BPHA, MCBPHA, and MCPPHA stimulated [35S]γS binding to U2OS-KOR cell membranes. (B) U50,488, MCBPHA, and MCPPHA caused arrestin recruitment, with varying degress of efficacy, normalized to 100% of arrestin recruitment by 10 μM U69,593. BPHA did not cause any arrestin recruitment at any dose up to 20 μM. Values are presented as mean ± SEM. For both assays, U69,593 stimulation curves are shown for comparison.

Table 2. In Vitro Opioid Receptor Activation of Compounds

	R Group	[35S]GTPγS EC50 (nM)	[35S]GTPγS % max	β-Arrestin EC50 (nM)	β-Arrestin % max
MCBPHA	Methylcyclobutyl	1.8 (±0.5)	110(±20)	380 (±90)	30 (±12)
BPHA	n-Propyl	14 (±3)	94 (±23)	No Stim	No Stim
MCPPHA	Methylcyclopentyl	0.64 (±0.16)	100 (±10)	720 (±60)	55 (±4)

All KOR assays were performed in commercially available DiscoverX U2OS cells expressing KOR. [35S]GTPγS stimulation was used to assay G-protein activity, and maximal efficacy was compared to 10 μM U69,593 in KOR cells. Arrestin max efficacy was compared with 10 μM U69,593 in KOR cells. The values presented represent the average EC_{50} and % efficacy values from at least 3 separate experiments, with SEM shown in parentheses. For [35S]GTPγS, U69,593 EC_{50} was 3.7±0.4 nM (% stimulation by definition was 100%), and as a reference, U50,488 EC_{50} was 1.5±0.4 nM, % stimulation was 99±2%. For arrestin, U69,593 EC_{50} was 410±90 nM (% stimulation by definition was 100%); and as a reference, U50,488 EC_{50} was 1000±100 nM, % stimulation was 120±10%.

Table 3. Quantification of Ligand Bias Using the Competitive Model of Partial Agonism.

	U50,488	BPHA	MCBPHA	MCPPHA
LogRA GTPγS	0.5 (±0.1)	-0.4 (±0.1)	0.5 (±0.2)	1.0 (±0.2)
LogRA Arrestin	-0.1 (±0.6)	-2.2 (±1.3)	-1.2 (±0.2)	-0.3 (±0.3)
ΔLogRA (GTPγS – Arrestin)	0.6 (-0.1 to 1.2)	1.8 (0.3 to 3.2)	1.6 (1.2 to 2.0)	1.3 (0.8 to 1.7)
Bias Factor	4.0	59	42	18

Bias factors greater than 1 indicate G-protein bias. These best-fit parameters were determined by non-linear regression in Graphpad Prism and are presented plus or minus the standard error in parentheses.

and 18.0 (Table 3), indicating that they are all G-protein biased agonists with varying degrees of bias Note, this range in bias factors is similar to that recently reported for a structurally related class of MOR agonists (Schmid et al., 2017). U50,488 has a ΔLogRA value of 0.6 (-0.1 to 1.2), with confidence range inclusive of 0. A bias factor of 4.0 suggests that U50,488 is slightly G-protein biased compared to U69,593 in our assays, but less biased than BPHA, MCBPHA, or MCPPHA.

In Vivo Characterization

Male C57BL6 mice were used to investigate the effects of these compounds on release of prolactin into the systemic circulation, a biomarker of KOR activity. We first verified that a KOR antagonist was able to block the serum prolactin-releasing effects of U50,488. The KOR antagonist used, LY2444296, is a novel, short-acting (<48 hours), selective kappa antagonist that has been shown in rodents to block behavioral effects of kappa agonists (Valenza et al., 2017). U50,488 was chosen over U69,593 as the reference KOR agonist in vivo due to solubility reasons. Pretreatment of animals with 3 mg/kg LY2444296 blocks the effects of 10 mg/kg U50,488 in stimulating prolactin release (Figure 3A). Importantly, KOR antagonism by LY2444296 itself has no effect on prolactin levels, indicating that there is no contribution of endogenous dynorphin/KOR tone on circulating prolactin in the stress-minimized conditions in which the mice in this study were maintained. BPHA and MCBPHA were studied concomitantly for their effects on prolactin (Figure 3B). For reasons of compound availability at the time, MCPPHA was studied in a separate experiment (Figure 3C). Each of the 3 compounds resulted in increased prolactin, and this rise was antagonized by pretreatment with the KOR antagonist LY2444296.

In rotarod studies, BPHA (30 mg/kg) resulted in no motor incoordination, in contrast to the full, unbiased KOR agonist U50,488 (Figure 4A). MCPPHA and MCBPHA both resulted in submaximal incoordination on the rotarod assay, with MCPPHA resulting in longer lasting effects, which may result from enhanced arrestin signaling (Figure 4A). We show the dose response curves for MCBPHA and MCPPHA (Figures S4A and S4B). A 30-minute pretreatment with the short-acting antagonist LY2444296 at a KOR-selective dose (1 mg/kg) blocked the motor incoordination effects of U50,488 (Figure 4B), MCBPHA (Figure 4C), and MCPPHA (Figure 4D).

Discussion

In several publications involving these compounds, Spetea et al. examined the functional activation of G-protein signaling and several in vivo assays (Spetea et al., 2012, 2017; Erli et al., 2017). In the original publication of this chemical scaffold, Spetea et al. described MCBPHA (HS665 in their report) as a full agonist in a [^{35}S]GTPγS assay and BPHA (compound 5 in their report) as a partial agonist compared to U69,593 (Spetea et al., 2012). We found BPHA to be a full agonist in this assay, with over 100% efficacy compared with U69,593 (Table 2). There are differences in the cell system (U2OS vs HEK cells), although we found similar full agonism in [^{35}S]GTPγS stimulation by BPHA in HEK-KOR cell membranes (data not shown). Other parameters examined here are in agreement with Spetea et al. (2012), such as KOR, MOR, and DOR binding by BPHA and MCBPHA as well as efficacy of MCBPHA in stimulating [^{35}S] GTPγS binding. [^{35}S]GTPγS data for MCPPHA (referred to as compound 3 in Spetea et al. 2017) is also in agreement with recent findings (Spetea et al., 2017).

We extended the prior studies by investigating signaling through the β-arrestin-2 signaling pathway for all 3 ligands, with calculations of ligand signaling bias, using the model of Stahl et al. 2015. Qualitatively, ligand bias is understood as enhanced signaling via one intracellular signaling pathway compared with another. G-protein-mediated signaling compared with arrestin-mediated signaling for any given ligand can be defined by differences in potency or efficacy compared with a reference ligand (Stahl et al., 2015). The term "extreme bias" has been used to describe compounds with signaling efficacy in one pathway, but no signaling efficacy in the other. Nalmefene, a well-known MOR antagonist, was described as a KOR partial agonist in humans (Bart et al., 2005) and in vitro using G-protein pathway endpoints. It has recently been shown to be an extremely G-protein-biased KOR agonist by the group of Bohn (Stahl et al., 2015) as well as in unpublished studies by our group. To quantify bias for this kind of ligand, a model must be used that can accommodate quantifying ligand activity that is very low. Quantification of ligand bias has been described in multiple ways. Bias quantification is based on the Black and Leff model of partial agonism (Ehlert, 2008; Kenakin et al., 2012); however, this model becomes less accurate when the partial agonist shows very little activity in the assay. Thus, the competitive model becomes particularly useful in characterizing bias in "extremely" biased ligands.

In this study, BPHA showed full efficacy in the [^{35}S]GTPγS assay and no efficacy in β-arrestin-2 signaling, indicating it to be an "extremely" G-protein biased KOR agonist (Tables 2 and 3; Figure 2B). All 3 ligands are full agonists in [^{35}S]GTPγS stimulation, with varying efficacy recruiting β-arrestin-2. Our calculations utilizing the model reported by Stahl et al., 2015 demonstrate all compounds to be G-protein biased (Tables 2 and 3; Figure 2B). BPHA has the highest bias factor, as it has no efficacy in β-arrestin-2 signaling. MCBPHA and MCPPHA have lower bias factors, as they both have partial efficacy in β-arrestin-2 signaling.

In prior reports of in vivo activity, Spetea et al. demonstrated efficacy of both MCBPHA (HS665 in Spetea et al. 2012, compound 1 in Erli et al. 2017) and MCPPHA (compound 3 in Erli et al. 2017) in an analgesia assay in mice. Erli et al. also reported that neither of these compounds caused locomotor defects as measured in the rotarod assay at concentrations up to 10 mg/kg. Here, we demonstrate that both MCBPHA and MCPPHA cause motor incoordination as measured by the rotarod at higher doses. BPHA, however, does not cause such effects up to the dose of 30 mg/

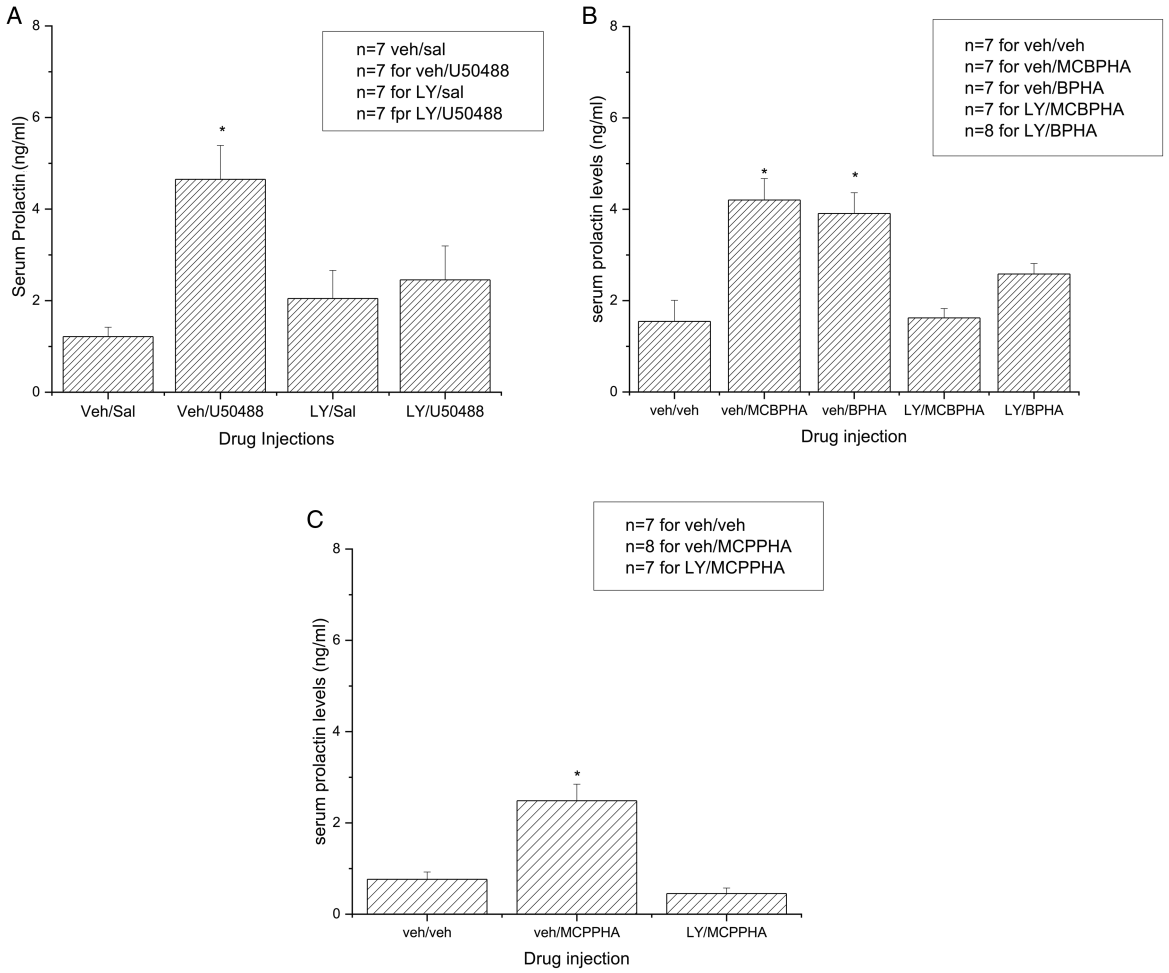

Figure 3. Prolactin release after compound administration. Results of serum prolactin measurement from C57BL6 mice experiments. (A) In experiment 1, vehicle or LY2444296 (3 mg/kg) was injected 30 minutes prior to saline or U50,488 (10 mg/kg). LY2444296 had no effect on prolactin, but prevents the increase caused by U50,488 (*$P < .0001$, compared with vehicle). (B) In experiment 2, LY2444296 (3 mg/kg) blocked the effect of MCBPHA (10 mg/kg) and BPHA (10 mg/kg) (*$P < .001$, compared with vehicle). (C) In experiment 3, LY2444296 (3 mg/kg) blocked the effect of MCPPHA (10 mg/kg) on prolactin release (*$P < .0001$). All values are presented as mean ± SEM. n = 7–8 animals per group for all experiments.

kg. These findings indicate that this class of compounds is likely crossing the blood brain barrier, leading to centrally mediated KOR-specific behavioral effects. We have not investigated in detail the pharmacokinetic profiles of these compounds, but substantial behavioral effects are possible in some receptor systems when only a minute fraction of a drug enters the brain, as is the case with morphine (Oldendorf et al., 1972). There is the possibility that differential effects observed in vivo with these compounds in the rotarod incoordination assay reflect differences in bioavailability, reflecting differences in blood-brain barrier permeability and/or metabolism. The similarity in structure suggests this is not necessarily the case, but they are empirical questions that will require further experiments.

We also demonstrate for the first time KOR-mediated prolactin releasing effects for these compounds. For determination of KOR specificity, we utilized the recently developed short-acting KOR antagonist LY2444296. LY2444296 is a close analog of the compound LY2456302, which has been investigated in the clinic (Lowe et al., 2014; Rorick-Kehn et al., 2014; Reed et al., 2017). Prolactin and rotarod effects of the compounds studied here were blocked by LY2444296 (Figures 3B and 4C-D). These experiments used a dose of LY2444296 that inhibits effects of U50,488 in releasing prolactin and causing rotarod incoordination but does not inhibit morphine-induced analgesia in a hot plate assay in C57BL6 mice (data not shown), suggesting it is a KOR-specific dose. A recent report demonstrated that RB-64, a G-protein-biased KOR agonist, had reduced potency in inducing arrestin-mediated signaling but similar maximal efficacy (White et al., 2015). This G-protein-biased KOR agonist did not have an effect on the rotarod, indicating for the first time that arrestin-mediated signaling was important for KOR-mediated rotarod incoordination. This is supported by the in vitro arrestin signaling results and the in vivo behavioral rotarod studies for the compounds under study here. BPHA has no β-arrestin-2 signaling activity and results in no rotarod incoordination. In contrast, MCPPHA and MCBPHA are partial agonists for β-arrestin-2 signaling and result in significant motor incoordination. While additional signaling mechanisms that could contribute to KOR-induced incoordination have not been explored, the extent of incoordination correlates with the extent of arrestin activity within these structurally related compounds (Figure 4A). The antagonism by BPHA of U69,593 beta-arrestin coupling in vitro (Figure S3) suggests this compound would antagonize U50,488-induced rotarod incoordination and is a possibility for future examination, albeit potentially requiring higher doses than those utilized this study.

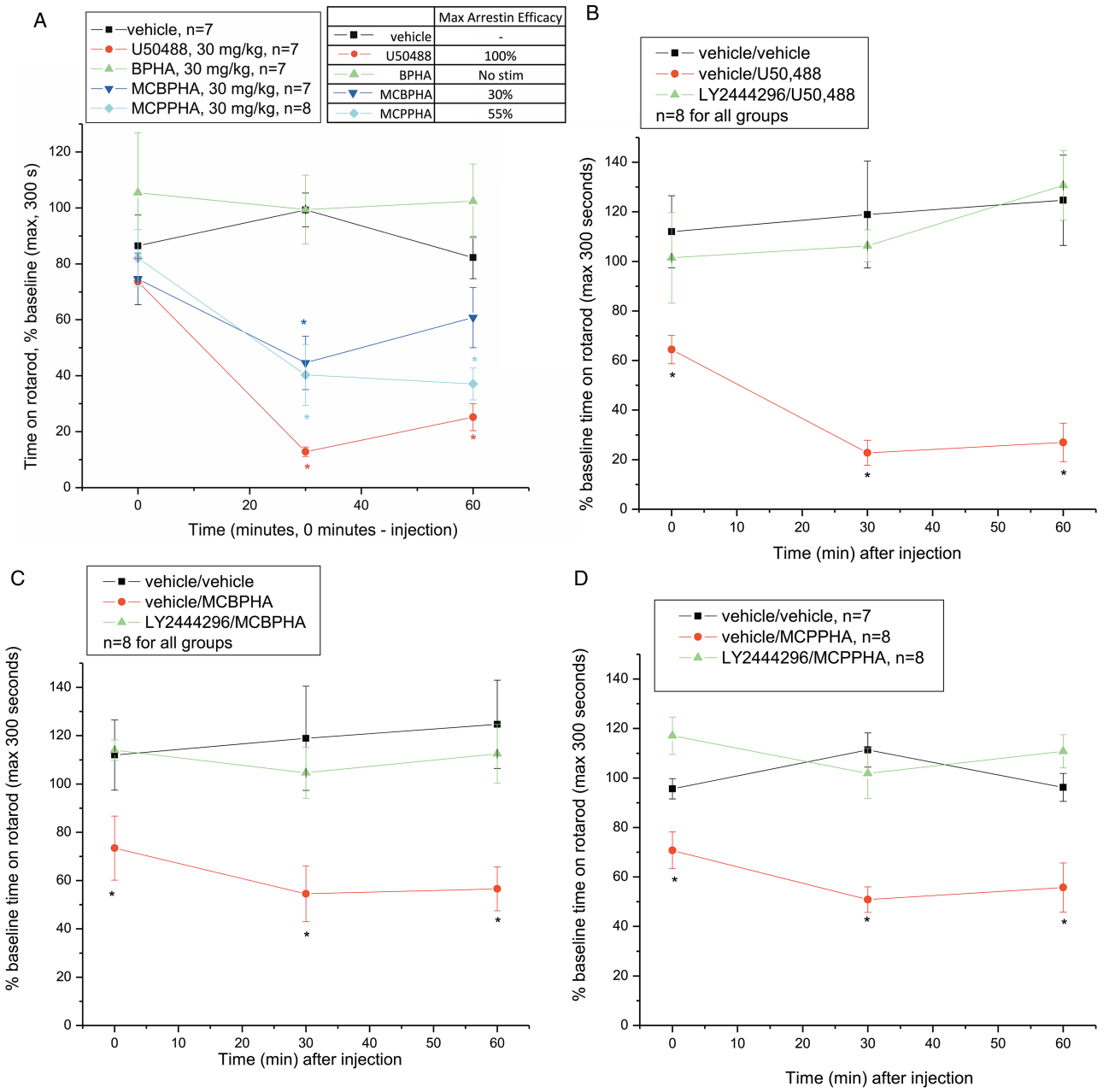

Figure 4. Rotarod incoordination caused by compound administration. (A) Rotarod incoordination following 30 mg/kg compound vs vehicle. MCBPHA, MCPPHA, and U50,488 caused a significant decrease in time spent on the rotarod, while BPHA had no effect. *P < .01 compared with vehicle. All values shown are normalized to the baseline rotarod times for each individual mouse. The baseline values across groups are not significantly different – vehicle: 203 (±11) seconds; U50,488: 225 (±15) seconds; BPHA: 210 (±14) seconds; MCBPHA: 223 (±15) seconds; MCPPHA: 204 (±9) seconds. (B-D) The short acting kappa selective antagonist LY2444296 (1 mg/kg) blocked rotarod incoordination caused by 30 mg/kg U50,488 (B), MCBPHA (C), and MCPPHA (D). All values are presented as mean ± SEM. *P < .05compared with vehicle. n = 7–8 animals per group for all experiments.

There have been 2 other recent reports on the in vivo effects of G-protein biased KOR agonists (White et al., 2015; Brust et al., 2016). Only a single biased compound was profiled for in vivo investigation in each study. Both of these studies reported disparate KOR-mediated behavioral and neurochemical effects of biased vs unbiased compounds. Our investigation, while similar, utilized 3 different structurally related but differentially biased (based on differences in β-arrestin-2 signaling efficacy) compounds to learn more about the relationship between ligand bias (as measured in vitro) and KOR-mediated effects in vivo. The correlation of β-arrestin-2 signaling efficacy in vitro with rotarod incoordination in vivo amongst these 3 structurally similar compounds strongly supports the hypothesis that KOR-mediated incoordination requires arrestin signaling. In vitro data collected in heterologous cell lines often does not accurately reflect in vivo effects, and in fact ligand bias has been shown to vary across cell lines (McLennan et al., 2008). The rotarod and β-arrestin-2 data for MCBPHA, MCPPHA, and BPHA suggest that in this system, the in vivo data may reflect in vitro findings. Verification of ligand bias in the mouse brain is needed; however, currently there are no assays available for measuring arrestin recruitment in vivo directly (Bohn et al., 2015).

The mechanism by which β-arrestin-2 mediates KOR effects on incoordination as assessed by the rotarod assay is not well understood. There was an investigator-observed sedative effect of the KOR agonists in C57BL6 mice, which is consistent with observations of the effects of KOR agonists in humans (Rimoy et al., 1994) and nonhuman primates (Butelman et al., 2009). The rotarod incoordination assay has been utilized as a measure to quantify KOR sedation in rodents (Giardina et al., 1994). Although rotarod incoordination can also reflect gross disruptions of dopaminergic neurons in the striatum (Fahim et al., 2013), given the timecourse of the effects of KOR agonists on rotarod behavior it is more likely that the sedation is caused by a different mechanism. This is supported by evidence published for salvinorin A, separately by our laboratory, on the effects of systemic salvinorin A on extracellular dopamine in the caudate putamen (Zhang et al., 2005), and the effects of systemic salvinorin A on rotarod incoordination (White et al., 2015). At a dose of 3.2 mg/kg, salvinorin A results in immediate decrease of extracellular dopamine by over 50% in the caudate putamen for at least 3 hours after administration (Zhang et al., 2005). With a dose of 3 mg/kg salvinorin A, the effects on rotarod incoordination have been reported to begin returning to normal within 30 minutes after injection (White et al., 2015). Together, these findings suggest that dopamine levels in the striatum may not correlate with the sedative effects of KOR agonists.

Further studies, potentially involving intracerebral microinfusions, will be required to determine the brain regions and precise neurochemical mechanism of KOR-mediated sedation. A review of the sedative effects of the KOR agonist spiradoline, one of the few KOR agonists to have been studied in humans, speculated that a KOR-mediated antihistamine effect underlies the sedative properties (Wadenberg, 2003). Further studies will be required, but the data presented by the current studies, as well as previous studies with G-protein biased KOR agonists (White et al., 2015; Brust et al., 2016), strongly suggest that β-arrestin-2-mediated signaling plays a key role.

It has long been established that prolactin can serve as a biomarker of kappa opioid receptor agonism (Butelman et al., 1999; Kreek et al., 1999), but it is has not previously been investigated with respect to G-protein-biased KOR ligands. The mechanism for KOR regulation of prolactin is via KOR-mediated disinhibition of pituitary lactotrophs, which are tonically inhibited by tuberoinfundibular dopaminergic neurons. KOR agonists block dopamine release from tuberoinfundibular neurons in addition to lowering extracellular dopamine levels in the striatum (Manzanares et al., 1991). It has been reported that ligands lacking β-arrestin-2 signaling in vitro do not cause KOR-mediated decreases in dopamine release in the nucleus accumbens, suggesting that β-arrestin-2 signaling is necessary for this process (Brust et al., 2016). Were β-arrestin-2 similarly required for inhibition of dopamine release from tuberoinfundibular neurons, we would anticipate a similar gradation of the effects of the three compounds on prolactin release to that observed for the effects in rotarod incoordination. The fact that each compound with differential arrestin signaling and rotarod incoordination results in similar prolactin release suggests that prolactin release, as a biomarker of KOR activity, does not require arrestin signaling and thus is potentially mediated by KOR G-protein signaling. As mentioned above in the introduction, there are potentially other signaling pathways that could also be involved, and the signaling components present in in vitro heterologously expressing cells may not adequately reflect those mediating KOR signaling in vivo, and in particular in the tuberoinfundibular dopaminergic neurons.

Conclusions

The current findings include the description of an extremely G-protein-biased compound (BPHA) and 2 G-protein biased compounds with differential arrestin efficacy (MCBPHA and MCPPHA) and their effects in vivo. These compounds add to the growing pharmacological toolbox to explore the role of KOR signaling in diverse animal models of addiction, depression, PTSD, pain, pruritis, and other disorders. BPHA, MCBPHA, MCPPHA, and related compounds may prove to be useful tools for delineating the role of differential G-protein bias in the behavioral effects of KOR agonists in animals. This could prove essential for the development of novel KOR ligands as potential pharmacotherapeutics.

Funding

This work was supported by the Robertson Therapeutic Development Fund and the Dr. Miriam and Sheldon Adelson Medical Research Foundation.

Acknowledgments

We gratefully acknowledge Dr. Eduardo Butelman, Dr. Bruce Conway, Dr. Amy Ripka, and Dr. Joseph Vacca for intellectual input. Additionally, we thank Margaret Hofstedt and Jose Erazo for technical contributions. We thank Dr. Edward Stahl for assistance with quantifying bias using the competitive model. We thank Dr. Linda Rorick-Kehn for facilitating the generous provision by Eli Lilly and Co. of a small portion of LY2444296. We thank the Rockefeller University High-Throughput and Spectroscopy Resource Center for access to their instrumentation and guidance by the director, Dr. Fraser Glickman.

Statement of Interest

None.

References

Bailey A, Gianotti R, Ho A, Kreek MJ (2007) Downregulation of kappa-opioid receptors in basolateral amygdala and septum of rats withdrawn for 14 days from an escalating dose “Binge” cocaine administration paradigm. Synapse 61:820–826.

Bart G, Schluger JH, Borg L, Ho A, Bidlack JM, Kreek MJ (2005) Nalmefene induced elevation in serum prolactin in normal human volunteers: partial kappa opioid agonist activity? Neuropsychopharmacology 30:2254–2262.

Beardsley PM, Howard JL, Shelton KL, Carroll FI (2005) Differential effects of the novel kappa opioid receptor antagonist, jdtic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. Psychopharmacology (Berl) 183:118–126.

Black JW, Leff P (1983) Operational models of pharmacological agonism. Proc R Soc Lond B Biol Sci 220:141–162.

Bohn LM, Zhou L, Ho JH (2015) Approaches to assess functional selectivity in gpcrs: evaluating G protein signaling in an endogenous environment. Methods Mol Biol 1335:177–189.

Bourgeois C, Werfel E, Schepmann D, Wünsch B (2014) Combination of cyclohexane and piperazine based κ-opioid receptor agonists: synthesis and pharmacological evaluation of trans, trans-configured perhydroquinoxalines. Bioorg Med Chem 22:3316–3324.

Brust TF, Morgenweck J, Kim SA, Rose JH, Locke JL, Schmid CL, Zhou L, Stahl EL, Cameron MD, Scarry SM, Aubé J, Jones SR, Martin TJ, Bohn LM (2016) Biased agonists of the kappa opioid receptor suppress pain and itch without causing sedation or dysphoria. Sci Signal 9:ra117.

Butelman ER, Harris TJ, Kreek M (1999) Apparent efficacy of kappa-opioid receptor ligands on serum prolactin levels in rhesus monkeys. Eur J Pharmacol 383:305–309.

Butelman ER, Prisinzano TE, Deng H, Rus S, Kreek MJ (2009) Unconditioned behavioral effects of the powerful kappa-opioid hallucinogen salvinorin A in nonhuman primates: fast onset and entry into cerebrospinal fluid. J Pharmacol Exp Ther 328:588–597.

Cheng Y, Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.

Daunais JB, Roberts DC, McGinty JF (1993) Cocaine self-administration increases preprodynorphin, but not c-fos, mRNA in rat striatum. Neuroreport 4:543–546.

Ehlert FJ (2008) On the analysis of ligand-directed signaling at G protein-coupled receptors. Naunyn Schmiedebergs Arch Pharmacol 377:549–577.

Erli F, Guerrieri E, Ben Haddou T, Lantero A, Mairegger M, Schmidhammer H, Spetea M (2017) Highly potent and selective new diphenethylamines interacting with the κ-opioid receptor: synthesis, pharmacology, and structure-activity relationships. J Med Chem 60:7579–7590.

Fahim MA, Shehab S, Nemmar A, Adem A, Dhanasekaran S, Hasan MY (2013) Daily subacute paraquat exposure decreases muscle function and substantia nigra dopamine level. Physiol Res 62:313–321.

Fortin M, Degryse M, Petit F, Hunt PF (1991) The dopamine D2 agonists RU 24213 and RU 24926 are also KAPPA-opioid receptor antagonists. Neuropharmacology 30:409–412.

Frankowski KJ, et al. (2012) Discovery of small molecule kappa opioid receptor agonist and antagonist chemotypes through a HTS and hit refinement strategy. ACS Chem Neurosci 3:221–236.

Frankowski KJ, Ghosh P, Setola V, Tran TB, Roth BL, Aubé J (2010) N-alkyl-octahydroisoquinolin-1-one-8-carboxamides: a novel class of selective, nonbasic, nitrogen-containing κ-opioid receptor ligands. ACS Med Chem Lett 1:189–193.

Giardina G, Clarke GD, Dondio G, Petrone G, Sbacchi M, Vecchietti V (1994) Selective kappa-opioid agonists: synthesis and structure-activity relationships of piperidines incorporating on oxo-containing acyl group. J Med Chem 37:3482–3491.

Hirayama S, Wada N, Nemoto T, Iwai T, Fujii H, Nagase H (2014) Synthesis and pharmacology of a novel κ opioid receptor (KOR) agonist with a 1,3,5-trioxazatriquinane skeleton. ACS Med Chem Lett 5:868–872.

Jones MR, Kaye AD, Kaye AJ, Urman RD (2016) The emerging therapeutic roles of κ-opioid agonists. J Opioid Manag 12:101–107.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. ACS Chem Neurosci 3:193–203.

Kreek MJ, Schluger J, Borg L, Gunduz M, Ho A (1999) Dynorphin A1-13 causes elevation of serum levels of prolactin through an opioid receptor mechanism in humans: gender differences and implications for modulation of dopaminergic tone in the treatment of addictions. J Pharmacol Exp Ther 288:260–269.

Lovell KM, Frankowski KJ, Stahl EL, Slauson SR, Yoo E, Prisinzano TE, Aubé J, Bohn LM (2015) Structure-activity relationship studies of functionally selective kappa opioid receptor agonists that modulate ERK ½ phosphorylation while preserving G protein over βarrestin2 signaling bias. ACS Chem Neurosci 6:1411–1419.

Lowe SL, Wong CJ, Witcher J, Gonzales CR, Dickinson GL, Bell RL, Rorick-Kehn L, Weller M, Stoltz RR, Royalty J, Tauscher-Wisniewski S (2014) Safety, tolerability, and pharmacokinetic evaluation of single- and multiple-ascending doses of a novel kappa opioid receptor antagonist LY2456302 and drug interaction with ethanol in healthy subjects. J Clin Pharmacol 54:968–978.

Manzanares J, Lookingland KJ, Moore KE (1991) Kappa opioid receptor-mediated regulation of dopaminergic neurons in the rat brain. J Pharmacol Exp Ther 256:500–505.

McLennan GP, Kiss A, Miyatake M, Belcheva MM, Chambers KT, Pozek JJ, Mohabbat Y, Moyer RA, Bohn LM, Coscia CJ (2008) Kappa opioids promote the proliferation of astrocytes via gbetagamma and beta-arrestin 2-dependent MAPK-mediated pathways. J Neurochem 107:1753–1765.

Monasky MM, Taglieri DM, Henze M, Warren CM, Utter MS, Soergel DG, Violin JD, Solaro RJ (2013) The β-arrestin-biased ligand TRV120023 inhibits angiotensin II-induced cardiac hypertrophy while preserving enhanced myofilament response to calcium. Am J Physiol Heart Circ Physiol 305:H856–H866.

Nagase H, Watanabe A, Nemoto T, Yamaotsu N, Hayashida K, Nakajima M, Hasebe K, Nakao K, Mochizuki H, Hirono S, Fujii H (2010) Drug design and synthesis of a novel kappa opioid receptor agonist with an oxabicyclo[2.2.2]octane skeleton and its pharmacology. Bioorg Med Chem Lett 20:121–124.

Oldendorf WH, Hyman S, Braun L, Oldendorf SZ (1972) Blood-brain barrier: penetration of morphine, codeine, heroin, and methadone after carotid injection. Science 178:984–986.

Reed B, Butelman ER, Fry RS, Kimani R, Kreek MJ (2018) Repeated administration of Opra Kappa (LY2456302), a novel, short-acting, selective KOP-r antagonist, in persons with and without cocaine dependence. Neuropsychopharmacology 43:928.

Riley AP, Groer CE, Young D, Ewald AW, Kivell BM, Prisinzano TE (2014) Synthesis and κ-opioid receptor activity of furan-substituted salvinorin A analogues. J Med Chem 57:10464–10475.

Rimoy GH, Wright DM, Bhaskar NK, Rubin PC (1994) The cardiovascular and central nervous system effects in the human of U-62066E. A selective opioid receptor agonist. Eur J Clin Pharmacol 46:203–207.

Rorick-Kehn LM, et al (2014) LY2456302 is a novel, potent, orally-bioavailable small molecule kappa-selective antagonist with activity in animal models predictive of efficacy in mood and addictive disorders. Neuropharmacology 77:131–144.

Scarry SM, Lovell KM, Frankowski KJ, Bohn LM, Aubé J (2016) Synthesis of kappa opioid antagonists based on pyrrolo[1,2-α] quinoxalinones using an N-arylation/condensation/oxidation reaction sequence. J Org Chem 81:10538–10550.

Schenk S, Partridge B, Shippenberg TS (1999) U69593, a kappa-opioid agonist, decreases cocaine self-administration and decreases cocaine-produced drug-seeking. Psychopharmacology (Berl) 144:339–346.

Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, Morgenweck J, Cameron MD, Bannister TD, Bohn LM (2017) Bias factor and therapeutic window correlate to predict safer opioid analgesics. Cell 171:1165–1175.e13.

Schmid CL, Streicher JM, Groer CE, Munro TA, Zhou L, Bohn LM (2013) Functional selectivity of 6'-guanidinonaltrindole (6'-GNTI) at κ-opioid receptors in striatal neurons. J Biol Chem 288:22387–22398.

Soergel DG, Subach RA, Burnham N, Lark MW, James IE, Sadler BM, Skobieranda F, Violin JD, Webster LR (2014) Biased agonism of the μ-opioid receptor by TRV130 increases analgesia and reduces on-target adverse effects vs morphine: a randomized, double-blind, placebo-controlled, crossover study in healthy volunteers. Pain 155:1829–1835.

Spangler R, Ho A, Zhou Y, Maggos CE, Yuferov V, Kreek MJ (1996) Regulation of kappa opioid receptor mRNA in the rat brain by "Binge' pattern cocaine administration and correlation with preprodynorphin mRNA. Brain Res Mol Brain Res 38:71–76.

Spangler R, Unterwald EM, Kreek MJ (1993) 'Binge' cocaine administration induces a sustained increase of prodynorphin mRNA in rat caudate-putamen. Brain Res Mol Brain Res 19:323–327.

Spetea M, Berzetei-Gurske IP, Guerrieri E, Schmidhammer H (2012) Discovery and pharmacological evaluation of a diphenethylamine derivative (HS665), a highly potent and selective κ opioid receptor agonist. J Med Chem 55:10302–10306.

Spetea M, Eans SO, Ganno ML, Lantero A, Mairegger M, Toll L, Schmidhammer H, McLaughlin JP (2017) Selective κ receptor partial agonist HS666 produces potent antinociception without inducing aversion after i.c.v. administration in mice. Br J Pharmacol 174:2444–2456.

Stahl EL, Zhou L, Ehlert FJ, Bohn LM (2015) A novel method for analyzing extremely biased agonism at G protein-coupled receptors. Mol Pharmacol 87:866–877.

Unterwald EM, Horne-King J, Kreek MJ (1992) Chronic cocaine alters brain mu opioid receptors. Brain Res 584:314–318.

Unterwald EM, Rubenfeld JM, Kreek MJ (1994) Repeated cocaine administration upregulates kappa and mu, but not delta, opioid receptors. Neuroreport 5:1613–1616.

Valenza M, Butelman ER, Kreek MJ (2017) Effects of the novel relatively short-acting kappa opioid receptor antagonist LY2444296 in behaviors observed after chronic extended-access cocaine self-administration in rats. Psychopharmacology (Berl) 234:2219–2231.

Van't Veer A, Carlezon WA Jr (2013) Role of kappa-opioid receptors in stress and anxiety-related behavior. Psychopharmacology (Berl) 229:435–452.

Violin JD, Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 28:416–422.

Wadenberg ML (2003) A review of the properties of spiradoline: a potent and selective kappa-opioid receptor agonist. CNS Drug Rev 9:187–198.

White KL, Robinson JE, Zhu H, Diberto JF, Polepally PR, Zjawiony JK, Nichols DE, Malanga CJ, Roth BL (2015) The G protein – biased k -opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities in vivo. J Pharmacol Exp Ther 352:98–109.

White KL, Scopton AP, Rives ML, Bikbulatov RV, Polepally PR, Brown PJ, Kenakin T, Javitch JA, Zjawiony JK, Roth BL (2013) Identification of novel functionally selective κ-opioid receptor scaffolds. Mol Pharmacol 85:83–90.

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ (2004) Effect of the endogenous kappa opioid agonist dynorphin A(1-17) on cocaine-evoked increases in striatal dopamine levels and cocaine-induced place preference in C57BL/6J mice. Psychopharmacology (Berl) 172:422–429.

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ (2005) Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. Psychopharmacology (Berl) 179:551–558.

Zubieta JK, Gorelick DA, Stauffer R, Ravert HT, Dannals RF, Frost JJ (1996) Increased mu opioid receptor binding detected by PET in cocaine-dependent men is associated with cocaine craving. Nat Med 2:1225–1229.