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Ketamine and Major Ketamine Metabolites Function as Allosteric Modulators of Opioid Receptors[S]

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ABSTRACT

Ketamine is a glutamate receptor antagonist that was developed over 50 years ago as an anesthetic agent. At subanesthetic doses, ketamine and some metabolites are analgesics and fast-acting antidepressants, presumably through targets other than glutamate receptors. We tested ketamine and its metabolites for activity as allosteric modulators of opioid receptors expressed as recombinant receptors in heterologous systems and with native receptors in rodent brain; signaling was examined by measuring GTP binding, β-arrestin recruitment, MAPK activation, and neurotransmitter release. Although micromolar concentrations of ketamine alone had weak agonist activity at μ opioid receptors, the combination of submicromolar concentrations of ketamine with endogenous opioid peptides produced robust synergistic responses with statistically significant increases in efficacies. All three opioid receptors (μ, δ, and κ) showed synergism with submicromolar concentrations of ketamine and either methionine-enkephalin (Met-enk), leucine-enkephalin (Leu-enk), and/or dynorphin A17 (Dyn A17), albeit the extent of synergy was variable between receptors and peptides. *S*-ketamine exhibited higher modulatory effects compared with *R*-ketamine or racemic ketamine, with ~100% increase in efficacy. Importantly, the ketamine metabolite 6-hydroxynorketamine showed robust allosteric modulatory activity at μ opioid receptors; this metabolite is known to have analgesic and antidepressant activity but does not bind to glutamate receptors. Ketamine enhanced potency and efficacy of Met-enkephalin signaling both in mouse midbrain membranes and in rat ventral tegmental area neurons as determined by electrophysiology recordings in brain slices. Taken together, these findings support the hypothesis that some of the therapeutic effects of ketamine and its metabolites are mediated by directly engaging the endogenous opioid system.

SIGNIFICANCE STATEMENT

This study found that ketamine and its major biologically active metabolites function as potent allosteric modulators of μ, δ, and κ opioid receptors, with submicromolar concentrations of these compounds synergizing with endogenous opioid peptides, such as enkephalin and dynorphin. This allosteric activity may contribute to ketamine's therapeutic effectiveness for treating acute and chronic pain and as a fast-acting antidepressant drug.

Introduction

Ketamine is a general anesthetic developed in the 1960s. The S-stereoisomer of ketamine was recently approved by the Food and Drug Administration to treat major depressive disorder (MDD); the R-isomer also has antidepressant activity in animal models, and the racemic mixture is used clinically to treat MDD (Andrade, 2017; Jelen et al., 2021; Passie et al., 2021; Kritzer et al., 2022). For MDD, ketamine is as effective as electroconvulsive shock therapy, eliciting a clinical response in ~50% of patients within hours of the first dose, in contrast to conventional antidepressants, which take weeks for therapeutic effect onset (Berman et al., 2000; Zarate et al., 2006; Machado-Vieira et al., 2009; Cowen and Browning, 2015; Almohammed et al., 2022; Anand et al., 2023). Ketamine is also a powerful analgesic for acute and chronic pain (Niesters et al., 2014; Barrett et al., 2020).

The mechanism of ketamine's anesthetic activity is antagonism of N-methyl-D-aspartate (NMDA) receptors (Zorumski et al., 2016; Jelen and Stone, 2021). Some studies reported that NMDA receptors contribute to ketamine's antidepressant and analgesic activities (Ma et al., 2023; Xue et al., 2023). However, there are several issues. First, doses of ketamine that treat MDD and chronic pain are typically 0.15–0.5 mg/kg

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ABBREVIATIONS: 6-HNK, 6-hydroxynorketamine; aCSF, artificial cerebrospinal fluid; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-amide; DAMGO, [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin; DOR, δ opioid receptor; Dyn A17, dynorphin A17; E_{max}, maximal response or efficacy; IPSC, inhibitory postsynaptic current; KOR, κ opioid receptor; Leu-enk, leucine-enkephalin; MDD, major depressive disorder; Met-enk, methionine-enkephalin; MOR, μ opioid receptor; NK, norketamine; NMDA, N-methyl-D-aspartate; PAM, positive allosteric modulator; RRID, research resource identifier; VTA, ventral tegmental area.

i.v., which is a fraction of the anesthetic dose of 1–2 mg/kg i.v. (Zanos et al., 2018). Second, the antidepressant and analgesic effects often last for days or weeks, whereas anesthesia wears off within minutes when plasma levels drop below ~5 μM (Zanos et al., 2018). Third, the major ketamine metabolite 6-hydroxynorketamine (6-HNK) is a potent antidepressant and analgesic but does not bind to NMDA receptors (Zanos et al., 2016; Yost et al., 2022). Thus, other targets have been proposed to contribute to ketamine's effects; these targets include α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors (Zanos et al., 2016), formation of D-Ser (Singh et al., 2016), and the opioid system (Gupta et al., 2011).

The endogenous opioid system consists of >20 opioid peptides that act through three opioid receptors: μ opioid receptor (MOR), δ opioid receptor (DOR), and κ opioid receptor (KOR) (Mansour et al., 1995; Gomes et al., 2020). Individual opioid peptides have distinct potencies and show variable signaling bias through G protein- versus β-arrestin–mediated signaling at each type of opioid receptor (Fricker et al., 2020; Gomes et al., 2020). Activation of MOR produces analgesia and has antidepressant activity (Gassaway et al., 2014; Samuels et al., 2017; Pollan, 2021; Jelen et al., 2022). Compounds that target DOR and KOR also have analgesic and antidepressant-like effects in mice (van Haaren et al., 2000; Suzuki et al., 2001; Dubois and Gendron, 2010; Gaveriaux-Ruff et al., 2011; Browne and Lucki, 2019; Wulf et al., 2022).

Ketamine weakly binds and activates opioid receptors, with reported K_i or EC_{50} values in the 7–100 μM range (Smith et al., 1980; Finck and Ngai, 1982; Hustveit et al., 1995; Hirota et al., 1999a,b; Bonaventura et al., 2021; Hess et al., 2022). Some studies reported that opioid antagonists block the antidepressant effects of ketamine (Williams et al., 2018 2019; Klein et al., 2020; Zhang et al., 2021), although this was not observed in another study (Marton et al., 2019). Similarly, studies reported that ketamine-induced analgesia in mice is blocked by opioid receptor antagonists (Lawrence and Livingston, 1981; Fidecka, 1987; Petrocchi et al., 2019), whereas other studies did not see reversal by opioid antagonists (Wiley and Downs, 1982; Mikkelsen et al., 1999). In 2011, our laboratory found that low nM concentrations of ketamine potentiate the action of morphine and fentanyl (Gupta et al., 2011). This led us to hypothesize that ketamine is a positive allosteric modulator (PAM) of MOR at submicromolar concentrations, which differs from its direct agonist activity at micromolar concentrations. There are examples of other PAMs that enhance orthosteric ligand signaling at low concentrations and function as direct agonists at higher concentrations (Burford et al., 2013; Abdel-Magid, 2015; Doornbos et al., 2018; Kandasamy et al., 2021; Pryce et al., 2021).

If ketamine's activity as an opioid receptor PAM contributes to its antidepressant and analgesic effects, then ketamine should enhance signaling of endogenous opioid peptides as it does for opioid drugs (Gupta et al., 2011). Here, we investigated the ability of ketamine and ketamine metabolites to function as PAMs enhancing opioid peptide-engaged MOR signaling. We focused on methionine-enkephalin (Met-enk), the most abundant opioid peptide in the brain. We also tested the activity of ketamine at DOR and KOR. Collectively, our studies support the hypothesis that ketamine and its major metabolites are potent allosteric modulators of MOR.

Materials and Methods

Materials. [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO; cat. No. 1171/1), *R*-norketamine (cat. No. 5996/10), *S*-norketamine (cat. No. 6112/10), and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-amide (CTOP) (cat. No. 1578/1) were from Bio-Techne Corporation (Minneapolis, MN). Leucine-enkephalin (Leu-enk; cat. No. 024-21), Met-enk (cat. No. 024-35), and dynorphin A17 (Dyn A17) (cat. No. 021-03) were from Phoenix Pharmaceuticals, Inc (Burlingame, CA). Morphine (cat. No. M8777), RS-ketamine (cat. No.K-2753), 2R,6R-hydroxynorketamine (cat. No. SML1873), 2S,6S-hydroxynorketamine (cat. No. SML1875), protease inhibitor cocktail (cat. No. P2714), phosphatase inhibitor cocktail (cat. No. P0044), GDP (cat. No. G7127), GTPγS (cat. No. G8634), and antibodies recognizing tubulin [cat. No. T8660; research resource identifier (RRID): AB477590] were from Millipore Sigma (St. Louis, MO). *R*-ketamine (cat. No. 26316), *S*-ketamine (cat. No. 26317), and RS-norketamine (cat. No.15787) were from Cayman Chemicals (Ann Arbor, MI). 2R,6R-hydroxynorketamine was also purchased from Cayman Chemicals (cat. No. 19603) and Bio-Techne Corporation (cat. No. 6094) and 2S,6S-hydroxynorketamine from Bio-Techne Corporation (cat. No. 6095), and since only 2R,6R-hydroxynorketamine and 2S,6S-hydroxynorketamine purchased from Millipore Sigma gave consistent results in all our assays, the data presented here are with compounds from Millipore Sigma. [^{35}S]GTPγS (cat. No. NEG030H250UC) was from Perkin-Elmer (Shelton, CT). Antibodies to phospho-ERK1/2 (cat. No. 4370S; RRID: AB_2315112) and total ERK1/2 (cat. No. 4696S; RRID: AB_390780) were from Cell Signaling Technology (Danvers, MA). Rabbit IRDye 800 (cat. No. 926-32211; RRID: AB_621843) and mouse IRDye 680 (cat. No. 926-68070; RRID: AB_10956588) secondary antibodies were from LI-COR Biosciences (Lincoln, NE). F12 media (cat. No. 11765-054), Minimum Essential Medium α (cat. No. 12571-063), streptomycin-penicillin (cat. No. 15140-122), and hygromycin (cat. No. 10687010) were from Gibco/Thermo Fisher (Waltham, MA). FBS (cat. No. FBS-01) was from LDP, Inc (Towaco, NJ). Geneticin (G418; cat. No. G-418-10) was from GoldBio (St. Louis, MO). The PathHunter Chemiluminescence detection kit (cat. No. 93-0001) was from DiscoverX (Eurofins Corporation, Fremont, CA). GF/B filters (cat. No. FP-100) were from Brandel, Inc. (Gaithersburg, MD). Additional reagents for the electrophysiology study included bestatin (Thermofisher; cat. No. 78433), thiorphan (Cayman Chemicals; cat. No. 15600), 6,7-Dinitroquinoxaline-2,3-dione (DNQX) from Hello Bio (cat. No. HB0261), and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 (CTAP) from Fisher Scientific (cat. No. AAJ66219MCR).

Animals. Adult (12 weeks old) male C57BL/6 mice (Jackson Laboratories, Ban Harbor, ME; RRID: IMSR_JAX:000664) weighing 20–25 g were killed using CO_2 from compressed gas according to the protocol approved by the Icahn School of Medicine Institutional Animal Care and Use Committee (LA11-00322), and midbrain regions were extracted from individual mice by gross dissection and used to prepare membranes as described below.

Adult male Sprague-Dawley rats (Envigo, Indianapolis, IN; RRID: RGD_734476) weighing 250–300 g were used for whole-cell electrophysiology recordings. Procedures were conducted in strict accordance with the recommendations of the National Institutes of Health, described in the Guide for the Care and Use of Laboratory Animals. Research protocols were approved by the Institutional Animal Care and Use Committee (University of California at San Francisco, CA), approval ID AN200119-00F.

Cell Culture. CHO cells (American Type Culture Collection cat. No. CCL-61; RRID: CVCL_0214) were grown in F12 media containing 10% (vol/vol) FBS and streptomycin-penicillin. CHO cells stably expressing Flag-tagged mouse MOR (*OPRM1*), mouse DOR (*OPRD1*), or rat KOR (*OPRK1*) were generated previously (Cvejic et al., 1996; Jordan and Devi, 1999; Trapaidze et al., 2000) and grown in F12 media containing 10% (vol/vol) FBS, streptomycin-penicillin, and 500 μg/mL geneticin. The plasmids for Flag-epitope tagged mouse μ and δ opioid

receptors were a gift from Dr. M. von Zastrow, UCSF. The plasmid for untagged rat κ opioid receptor was a gift from Dr. David Grandy, Oregon Health Sciences University, and was tagged with a Flag epitope at the N-terminus as described in Jordan and Devi (1999). Saturation binding assays with [^{3}H]DAMGO show that CHO cells stably expressing Flag-tagged mouse MOR exhibit a K_d of 2 ± 1 nM and a B_{max} of 517 ± 9 fmol/mg protein, with [^{3}H]Deltorphin II show that CHO cells stably expressing Flag-tagged mouse DOR exhibit a K_d of 3 ± 2 nM and a B_{max} of 497 ± 13 fmol/mg protein, with [^{3}H]U69,593 show that CHO cells stably expressing Flag-tagged rat KOR exhibit a K_d of 1 ± 1 nM and a B_{max} of 322 ± 10 fmol/mg protein. MOR UO5S cells expressing human MOR tagged with a ProLink/β-gal donor - fragment at the C-terminal region and β-arrestin tagged with a complementary β-gal activator - fragment ($MOR^{\beta gal}$) were a gift from DiscoverX (Fremont, CA; cat. No. 93-0213C3). These cells were grown in Minimum Essential Medium α containing 10% (vol/vol) FBS, streptomycin-penicillin, 500 μg/mL of geneticin, and 250 μg/mL of hygromycin. Saturation binding assays with [^{3}H]DAMGO show that the cells exhibit a K_d of 6 ± 1 nM and a B_{max} of 690 ± 50 fmol/mg protein.

Measurement of ERK1/2 Phosphorylation. CHO cells expressing MOR (2×10^5 cells/well) were seeded into 24-well poly-D-lysine–coated plates (Corning, Kennebunk, ME; cat. No. 356414). The next day, cells were grown in growth media without FBS for 3 hours followed by treatment with either vehicle, morphine, DAMGO, or Met-enk (0–10^{-6} M) in the absence or presence of 100 nM RS-ketamine for 5 minutes at 37°C. RS-ketamine was added first followed by either morphine, DAMGO, or Met-enk. In a separate set of experiments, cells were treated with vehicle or RS-ketamine (0–10^{-6} M) in the absence or presence of 100 nM Met-enk for 5 minutes at 37°C; RS-ketamine was added first followed by Met-enk.

Cells were lysed with 2% sodium dodecyl sulfate in 50 mM Tris-Cl, pH 6.8, containing protease and phosphatase inhibitor cocktails, and aliquots of lysates were subjected to western blot analysis as described (Gupta et al., 2011, 2016) using antibodies to phosphoERK1/2 (1:1000), to total ERK1/2 (1:1000), and to tubulin (1:5000) as primary antibodies. Anti-rabbit IRDye 800 (1:10,000) and anti-mouse IRDye 680 (1:10,000) were used as secondary antibodies. Protein bands were visualized and densitized using the Odyssey infrared imaging system (LI-COR Biosciences; Lincoln, NE).

Membrane Preparation. Membranes from CHO cells alone, from CHO cells expressing either MOR, DOR, or KOR, or from the midbrain of four individual wild-type C57Bl/6 mice were prepared as described previously (Gomes et al., 2016; Mack et al., 2022). Briefly, cells/midbrain tissue were homogenized in 25 volumes (1 g wet weight per 25 mL) of ice-cold 20 mM Tris-Cl buffer, pH 7.4, containing 250 mM sucrose, 2 mM EGTA, and 1 mM $MgCl_2$, followed by centrifugation at 27,000 *g* for 15 minutes at 4°C. The pellet was resuspended in 25 mL of the same buffer, and the centrifugation step was repeated. The resulting membrane pellet was resuspended in 10 volumes (of original wet weight) of 2 mM Tris-Cl buffer, pH 7.4, containing 2 mM EGTA and 10% glycerol. The protein content of the homogenates was determined using the Pierce BCA protein assay reagent (Rockford, IL), after which homogenates were stored in aliquots at −80°C until use.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding assays were carried out as described previously (Gomes et al., 2020; Mack et al., 2022). In experiments examining if RS-ketamine exhibits signaling at MOR, membranes (20 μg protein) from CHO cells alone or from CHO cells expressing MOR were incubated for 1 hour at 30°C with different concentrations of RS-ketamine in the absence or presence of 1 μM final concentration CTOP (CTOP/assay buffer was added first to the tubes followed by RS-ketamine) in assay buffer A (50 mM Tris-Cl buffer, pH 7.4, containing 100 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EGTA, and protease inhibitor cocktail) containing freshly prepared 30 μM GDP and 0.1 nM [^{35}S]GTPγS. Nonspecific binding was determined in the presence of 10 μM cold GTPγS. Basal values represent values obtained in the presence of GDP and in the absence of ligand. In experiments examining the allosteric effects of different ketamines on opioid-mediated G protein activity, membranes (20 μg protein) from cells expressing either MOR, DOR, or KOR, or from midbrain of each single mouse were incubated with opioids and/or ketamines; ketamines (concentrations described in figure legends) were added first followed by different concentrations of opioids, and assays were carried out as described above. At the end of the incubation period, samples were filtered using a Brandel filtration system and GF/B filters. Filters were washed three times with 3 mL of ice-cold 50 mM Tris-Cl buffer, pH 7.4, and bound radioactivity was measured using a scintillation counter (MicroBeta TriLux; PerkinElmer).

β-Arrestin Recruitment. Cells expressing $MOR^{\beta gal}$ were plated in each well of either a 96-well white clear-bottom plate (Corning, Kennebunk, ME; cat. No. 3903; 10,000 cells/well) or a 384-well white clear-bottom plate (Thermo Scientific, Rochester, NY; cat. No. 142762; 2500 cells/well) in 100 μL media. The next day, cells were rinsed with buffer A and treated with different concentrations of ketamines (concentrations described in figure legends) followed by opioids for 60 minutes at 37°C in buffer A. At the end of the incubation period, the bottoms of the plates were sealed with white vinyl sealing tape, and β-arrestin recruitment was measured using the PathHunter Chemiluminescence detection kit as described in the manufacturer's protocol (DiscoverX).

Slice Preparation and Ex Vivo Whole-Cell Electrophysiology. Rats were anesthetized with isoflurane and their brains removed. Horizontal brain slices (200 μm thick) containing the ventral tegmental area (VTA) were prepared using a vibratome (Campden Instruments). Slices were cut in ice-cold artificial cerebrospinal fluid (aCSF) solution containing (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.4 NaH_2PO_4, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose saturated with 95% O_2/5% CO_2 and then allowed to recover at 33°C for at least 1 hour. Individual slices were visualized under a Zeiss AxioExaminer D1 with differential interference contrast, Dodt, and near-infrared optics using a monochrome Axiocam 506 (Zeiss).

Whole-cell patch-clamp recordings were made at 33°C using 2.5–5 MΩ pipettes containing (in mM) 128 KCl, 20 NaCl, 1 $MgCl_2$, 1 EGTA, 0.3 $CaCl_2$, 10 HEPES, 2 MgATP, and 0.3 Na_3GTP (pH 7.2, osmolarity adjusted to 275). Signals were amplified using an integrated patch amplifier with SutterPatch software (Sutter Instrument) filtered at 1 kHz and collected at 10 kHz. Voltage clamp recordings were made at holding voltage = −70 mV. Series resistance and input resistance were tracked throughout the experiment (0.1 Hz) with 4-mV, 200-millisecond hyperpolarizing steps. $GABA_A$ receptor–mediated inhibitory postsynaptic potentials were pharmacologically isolated with DNQX (10 μM). Stimulating electrodes were placed 80–250 μm anterior or posterior to the soma of the recorded neuron. To measure drug effects on evoked inhibitory postsynaptic currents (IPSCs), paired pulses (50-millisecond interval) were delivered once every 10 seconds. At least 7 minutes of baseline-evoked IPSCs were collected in control aCSF or 10 nM ketamine. Met-enk was then added to the aCSF perfusion for 7 minutes. In a subset of experiments, 500 nM CTAP was then added to the Met-enk solution for an additional 7–10 minutes.

The IPSC amplitude was calculated by comparing the peak postsynaptic current to a 2-milliseconds interval just before stimulation. All drugs were bath applied.

Data Analysis. Each experiment was carried out three independent times with triplicates unless otherwise stated. Data were analyzed using GraphPad Prism 10 software. Each data set was fit in GraphPad Prism 10 using sigmoidal or bell-shaped concentration-response models to determine which one fits best with confidence intervals of 95% for potency (EC_{50}) and maximal response or efficacy (E_{max}). Statistical analysis was carried out in GraphPad Prism 10 using either Student's *t* test, one-way ANOVA with Tukey's multiple comparison test (indicated as preferred tests in GraphPad Prism 10), or two-way ANOVA with Sidak's or Tukey's multiple comparison test in GraphPad Prism 10 (indicated as preferred tests in GraphPad Prism 10), with $P < 0.05$ considered to be significant. Since the

studies in this manuscript are exploratory, the described P values are descriptive.

Whole-cell recording data were analyzed in IGOR (Wavemetrics). Drug effects were quantified by comparing the mean evoked IPSC amplitude during the 4 minutes of baseline just preceding drug application and the mean response amplitudes during minutes 4–7 of drug application. $P < 0.05$ was considered significant.

Results

Modulation of MOR-Mediated ERK1/2 Phosphorylation by RS-Ketamine. We previously used ERK1/2 phosphorylation as a readout for MOR activation and found that a combination of morphine and RS-ketamine in a 1:1 ratio caused a greater response than either drug alone (Gupta et al., 2011). Here, we extend this finding with a peptidic synthetic ligand, DAMGO, and an endogenous peptide, Met-enk. First, to confirm earlier findings, studies were carried out with morphine. As previously found, treatment with 100 nM RS-ketamine plus morphine produced a significant increase in ERK1/2 phosphorylation compared with cells treated with morphine alone (***$P > 0.001$; Fig. 1A; Supplemental Fig. 1). The increase was especially dramatic at the lowest concentration of morphine tested (0.1 nM). Next, we examined the effect of 100 nM RS-ketamine on DAMGO, a classic synthetic peptidic agonist. The results revealed that as for morphine,

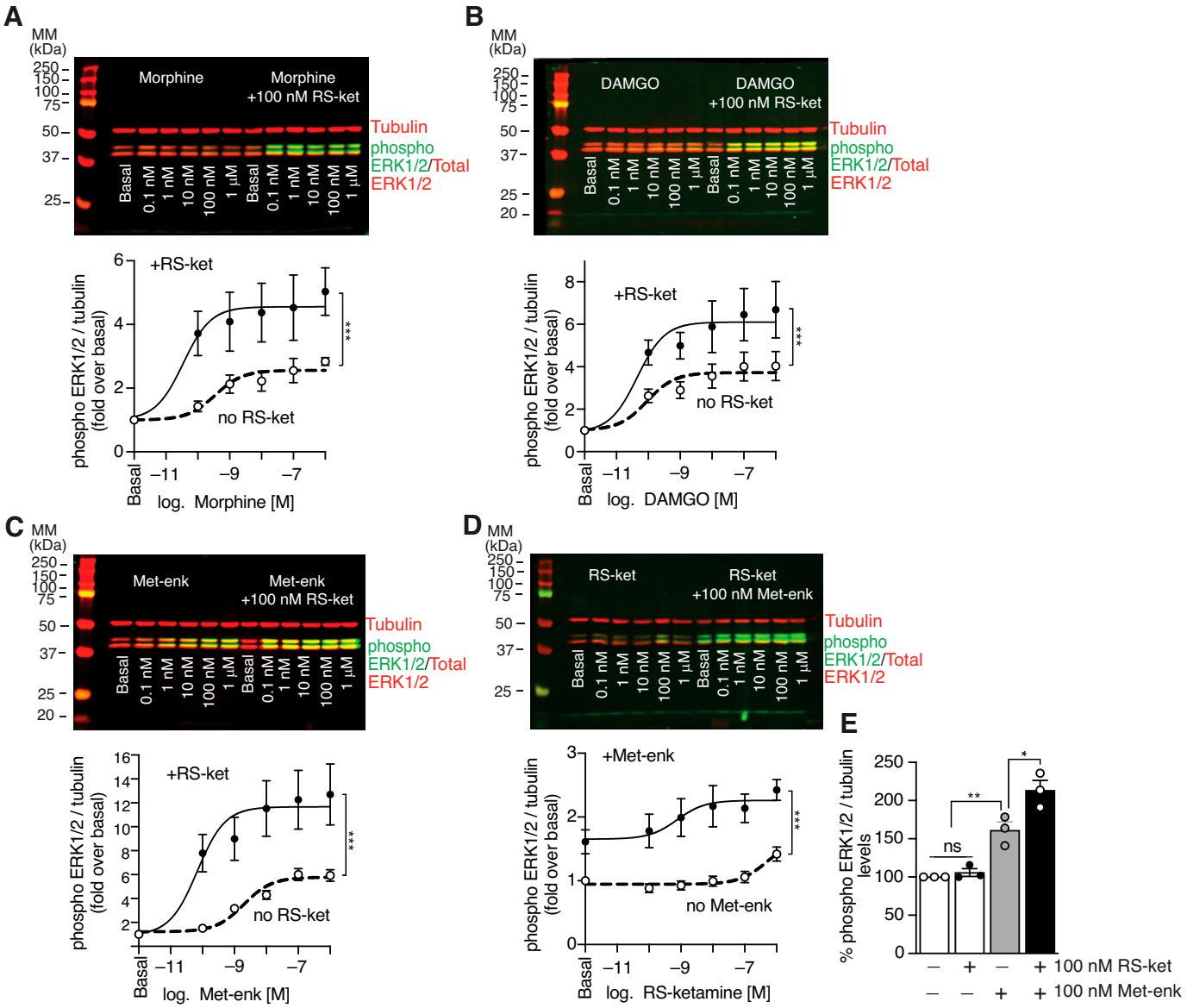

Fig. 1. RS-ketamine (RS-ket) enhances MOR-mediated ERK1/2 phosphorylation. CHO cells expressing MOR were treated for 5 minutes at 37°C with either vehicle alone (basal) or with 10^{-10} to 10^{-6} M morphine (A), DAMGO (B), or Met-enkephalin (Met-enk; C) in the absence or presence of 100 nM RS-ket or with RS-ketamine (10^{-10} to 10^{-6} M) in the absence or presence of 100 nM Met-enk (D). Cell lysates were subjected to western blot analysis as described in *Methods*. Representative blots are shown in the figure (also see Supplemental Fig. 1). Data (A–D) represents mean ± S.D. $n = 3$; ***$P < 0.001$ for treatment effect; two-way ANOVA with Sidak's multiple comparison test (statistical analysis in Supplemental Table 8). (E) Comparison of phosphoERK1/2 levels obtained under basal conditions, with 100 nM RS-ketamine, 100 nM Met-enk, and a combination of the two. Basal values were taken as 100%. Each dot represents the mean of an individual experiment. Data represent mean ± S.D. *$P < 0.05$; **$P < 0.01$ one-way ANOVA with Tukey's multiple comparison test (statistical analysis in Supplemental Table 8). MM, molecular mass; n.s., not significant.

RS-ketamine significantly enhanced signaling by DAMGO (***$P < 0.001$; Fig. 1B; Supplemental Fig. 1).

The endogenous opioid peptide Met-enk also showed synergism with RS-ketamine (Fig. 1C; Supplemental Fig. 1), and the RS-ketamine–mediated increase was most pronounced at low concentrations of Met-enk. For example, 0.1 nM Met-enk alone produced a negligible response, but when combined with 100 nM RS-ketamine there was an eightfold increase in signaling over basal (Fig. 1C). The E_{max} for all three opioids was higher in the presence of 100 nM RS-ketamine (Supplemental Table 1). The EC_{50} values for each opioid were dramatically lower in the presence of 100 nM RS-ketamine, although statistical analyses failed to reach significance (Supplemental Table 1). It should be pointed out that RS-ketamine alone showed a small increase in phosophoERK1/2 levels but only at high concentrations (1 μM), whereas in the presence of 100 nM Met-Enk there was an enhancement of phosphoERK1/2 levels with submicromolar concentrations of RS-ketamine (Fig. 1D; Supplemental Table 1). Comparison of the effect of 100 nM RS-ketamine alone, or 100 nM Met-enk alone, with that of a combination of RS-ketamine and Met-enk clearly indicates that the combination of ligands produces a greater increase compared with either ligand alone (Fig. 1E). Moreover, because submicromolar concentrations of RS-ketamine alone have no effect, the increases seen with the combination of submicromolar RS-ketamine and Met-enk are much greater than additive changes. Together, these results confirm our earlier study and extend it by showing that submicromolar concentrations of RS-ketamine potently synergize with opioid peptides.

Modulation of MOR-Mediated G Protein Activity by RS-Ketamine. Activation of opioid receptors can lead to activation of G protein–dependent and β-arrestin–dependent pathways of signaling (McLennan et al., 2008; Zheng et al., 2008a,b; Al-Hasani and Bruchas, 2011). To directly examine the effect of RS-ketamine on G protein signaling, increases in [^{35}S]GTPγS binding were measured in CHO cells expressing MOR and compared with CHO cells alone. In CHO cells alone (without MOR), RS-ketamine did not cause measurable signaling (Fig. 2 A), whereas in cells with MOR a small increase in signal (~20% over basal) was observed at high concentrations (10 μM), and this was completely blocked by the MOR antagonist CTOP (Fig. 2B; Supplemental Table 2). These data fit with a previous study that found that ketamine alone had partial agonist activity at MOR, with an EC_{50} of ~9 and ~34 μM for the *S*- and *R*-stereoisomers, respectively (Bonaventura et al., 2021).

Next, the ability of RS-ketamine to enhance signaling by the classic MOR agonist DAMGO was examined. Because nanomolar concentrations of RS-ketamine do not increase signaling in the absence of opioid agonists (Fig. 2B), we examined the effects of these concentrations on [^{35}S]GTPγS binding mediated by DAMGO (Fig. 2C). RS-ketamine at a concentration as low as 1 nM was able to enhance maximal signaling by DAMGO with an increase in the potency (Fig. 2C; Supplemental Table 2). Next, the ability of RS-ketamine to synergize with the endogenous opioid peptide Met-enk was examined (Fig. 2, D and E). Met-enk responses in the absence or presence of different concentrations of RS-ketamine show a concentration-dependent enhancement of Met-enk efficacy (Fig. 2, D and E; Supplemental Fig. 2, D and E; Supplemental Table 2). For example, 1 nM RS-ketamine caused a ~64% increase and 100 nM RS-ketamine a ~78% increase in the [^{35}S]GTPγS binding mediated by 1 μM Met-enk (taken as 100%; Fig. 2E; Supplemental Fig. 2E; Supplemental Table 2). Morphine responses in the absence and presence of different concentrations of RS-ketamine also show that nanomolar concentrations of RS-ketamine increase the efficacy for morphine, with 100 nM RS-ketamine increasing the efficacy of signaling by 100 nM morphine by ~131% (Fig. 2, F and G; Supplemental Fig. 2, F and G; Supplemental Table 2). Taken together, these results show that RS-ketamine enhances G protein signaling by MOR mediated by peptidic (DAMGO, Met-Enk) and nonpeptidic (morphine) agonists.

Modulation of MOR-Mediated β-Arrestin Recruitment by RS-Ketamine. Next, we examined the effect of RS-ketamine on opioid peptide- or morphine-mediated β-arrestin recruitment using the enzyme-fragment complementation technology developed by DiscoverX. In their MOR^{βgal} cell line, MOR is tagged at the C-terminus with a β-galactosidase fragment, and β-arrestin is tagged with the enzyme acceptor fragment; activation of the receptor selectively recruits β-arrestin, leading to β-galactosidase activity, providing a rapid, sensitive, and selective readout of MOR activation (Gomes et al., 2020). In this assay, RS-ketamine elicits a weak signal (~17% above basal) at the maximum concentration tested, 10 μM (Fig. 2H; Supplemental Table 2). Because signaling by submicromolar concentrations of RS-ketamine was not different from basal signaling in this assay (Fig. 2H; Supplemental Fig. 2H; Supplemental Table 2), the effects of these concentrations on β-arrestin recruitment by Met-enk and morphine were examined (Fig. 2, I–L; Supplemental Fig. 2, I–L). The effect of various concentrations of RS-ketamine on Met-enk response curves revealed enhancement of the efficacy for β-arrestin recruitment at most of the concentrations tested (Supplemental Fig. 2J). For example, the addition of 1 nM RS-ketamine caused a ~78% increase in the E_{max} of Met-enk–mediated β-arrestin recruitment, whereas higher concentrations of RS-ketamine were less effective (Fig. 2J; Supplemental Fig. 2J; Supplemental Table 2). RS-ketamine effects on morphine-mediated β-arrestin recruitment were not as robust as that seen with Met-enk, and enhancement of RS-ketamine–mediated signaling was dependent on the concentration of morphine used (Fig. 2, K and L; Supplemental Fig. 2, K and L; Supplemental Table 2). Also, morphine alone increased β-arrestin recruitment to a much lesser extent than Met-enk, with an E_{max} of ~40% over basal (Fig. 2L; Supplemental Fig. 2L; Supplemental Table 2). These results indicate that β-arrestin recruitment to MOR is greatly enhanced by the combination of ketamine with Met-enk but not with morphine.

RS-Ketamine Modulation of Leu-Enkephalin- and Dynorphin A17–Mediated G Protein Signaling by MOR, DOR, and KOR. To examine if potentiated signaling by RS-ketamine at CHO-MOR could also be seen with other endogenous opioid peptides, we examined signaling by Leu-enk and Dyn A17, each of which has previously been shown to activate MOR (Gomes et al., 2020). RS-ketamine increased the efficacy of Leu-Enk signaling (Fig. 3B), albeit to a lesser extent than Met-Enk (Fig. 3A; Supplemental Figs. 2 and 3). For example, treatment with 1 nM RS-ketamine leads to a ~20% increase in signaling mediated by 1 μM Leu-enk as compared with a ~64% increase in signaling mediated by 1 μM Met-enk (Fig. 3, A and B; Supplemental Figs. 2 and 3; Supplemental Table 2

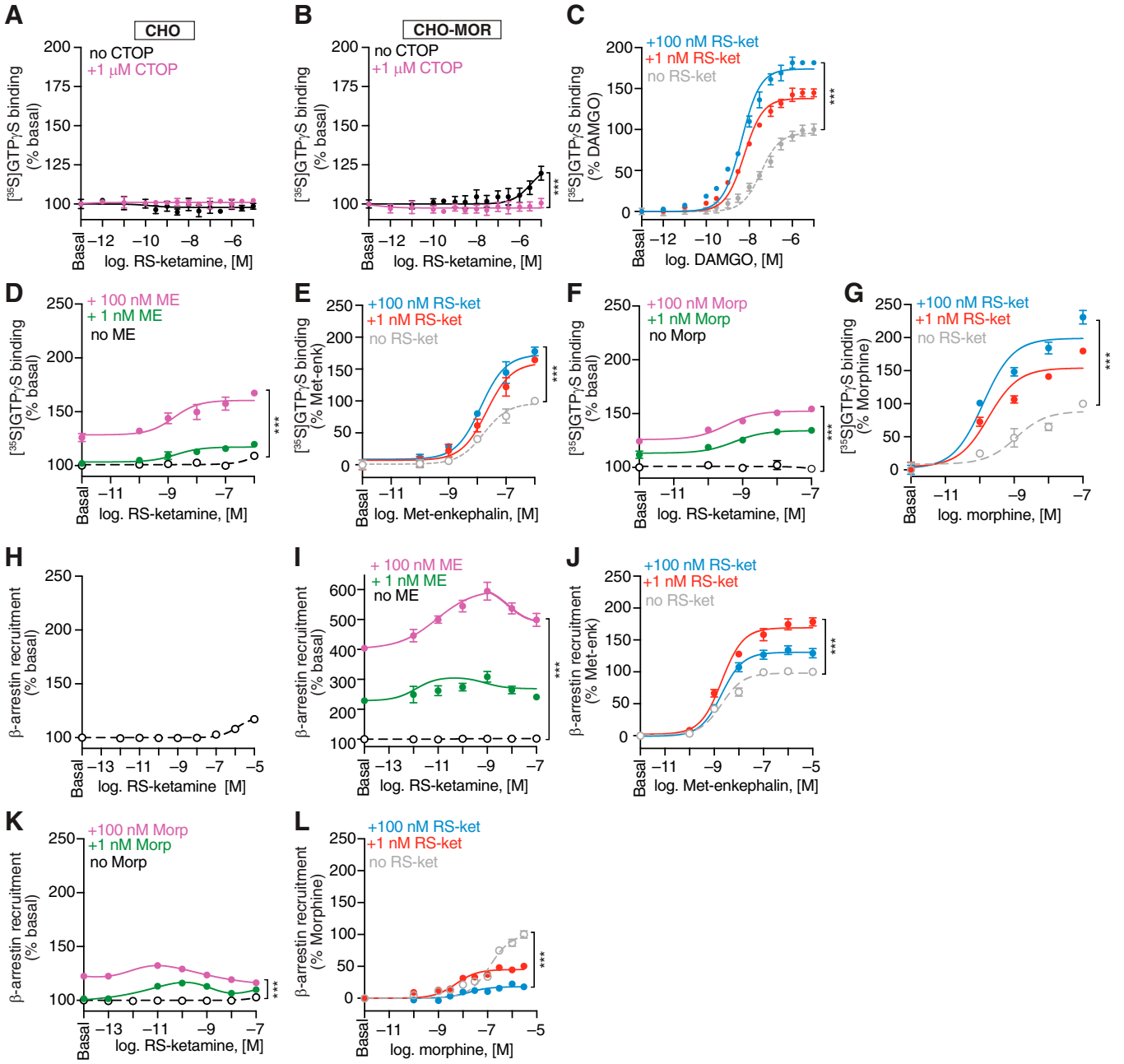

Fig. 2. RS-ketamine (RS-ket) modulates MOR-mediated signaling. (A and B) CHO cells (A) or CHO cells expressing Flag-MOR (CHO-MOR; B) were subjected to a [^{35}S]GTPγS binding assay with 0–10 μM RS-ketamine in the absence or presence of 1 μM CTOP as described in *Methods*. (C) CHO-MOR cells were subjected to a [^{35}S]GTPγS binding assay with 0–10 μM DAMGO in the absence or presence of 1 or 100 nM RS-ket as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 10 μM DAMGO as 100%. (D and F) CHO-MOR cells were subjected to a [^{35}S]GTPγS binding assay with 0–100 nM or 1 μM RS-ketamine in the absence or presence of 1 or 100 nM of either Met-enkephalin (ME; D) or morphine (Morp; F) as described in *Methods*. (E and G) CHO-MOR cells were subjected to a [^{35}S]GTPγS binding assay with 0–1 μM Met-enkephalin (E) or 0–100 nM morphine (G) in the absence or presence of 1 nM or 100 nM RS-ket as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin (E) or 100 nM morphine (G) as 100%. (H) Cells expressing MOR$^{\beta gal}$ were treated with 0–10 μM RS-ketamine, and β-arrestin recruitment was measured as described in *Methods*. Data represents mean ± S.D.; $n = 3$. (I and K) Cells expressing MOR$^{\beta gal}$ were treated with 0–100 nM RS-ketamine in the absence or presence of 1 or 100 nM of either ME (I) or Morp (K), and β-arrestin recruitment was measured as described in *Methods*. (J and L) Cells expressing MOR$^{\beta gal}$ were treated with different concentrations of either 0–10 μM Met-enkephalin (J) or 0–3 μM morphine (L) in the absence or presence of 1 nM or 100 nM RS-ket, and β-arrestin recruitment was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 10 μM Met-enk or 3 μM morphine as 100%. Data represent mean ± S.D. $n = 3$; ***$P < 0.001$ for treatment effect; two-way ANOVA with Sidak's (B and K) or Tukey's (C–G, I, J, and L) multiple comparison tests (statistical analysis in Supplemental Table 8). Original data for figure shown in Supplemental Fig. 2.

and 3). Treatment with RS-ketamine also increased the efficacy of Dyn A17 signaling at MOR (Fig. 3C; Supplemental Fig. 3; Supplemental Table 3). In the case of [^{35}S]GTPγS binding mediated by 1 μM Dyn A17, the addition of 1 nM RS-ketamine caused a ~43% increase, and 100 nM RS-ketamine caused a ~66% increase in signaling (Fig. 3C; Supplemental Fig. 3; Supplemental Table 3). Together, these results show that RS-ketamine increases the efficacy of these endogenous

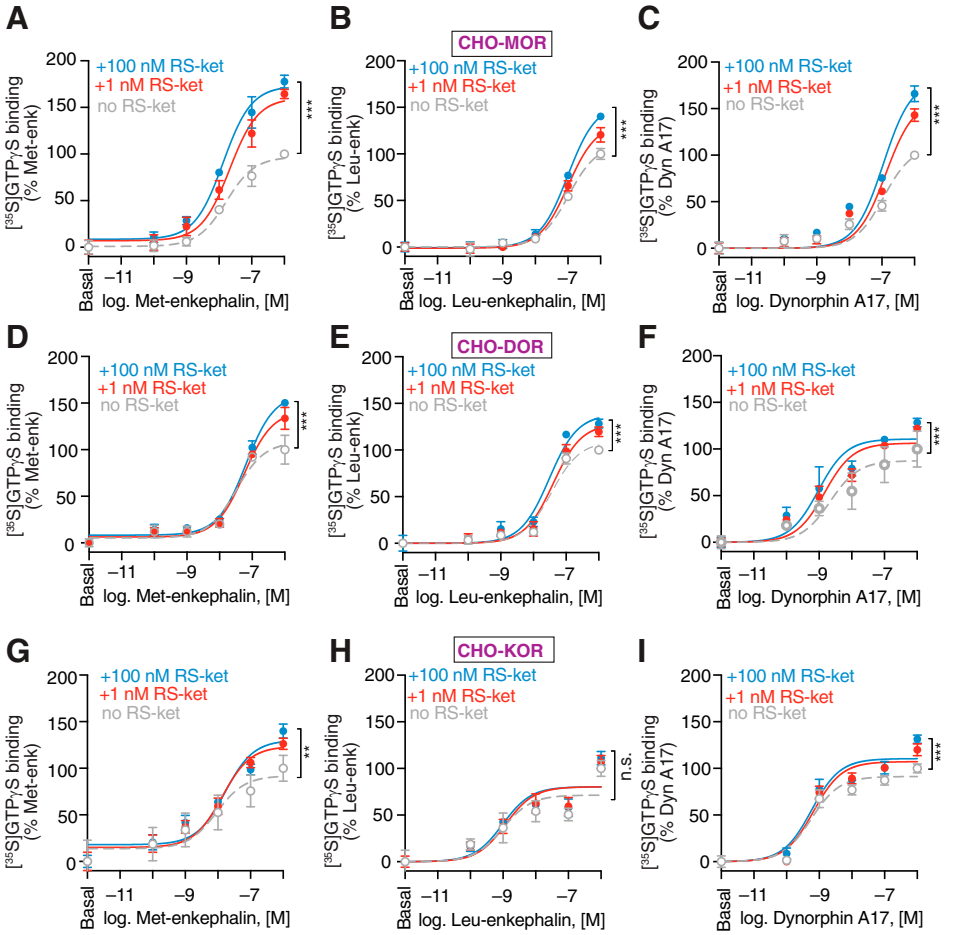

Fig. 3. Comparison of the effects of RS-ketamine (RS-ket) on Met-enkephalin-, Leu-enkephalin-, and dynorphin A17–mediated G protein activity at MOR, DOR, and KOR. CHO cells stably expressing Flag-MOR (A–C), Flag-DOR (D–F), or Flag-KOR (G–I) were subjected to a [^{35}S]GTPγS binding assay with different concentrations of 0–1 μM Met-enkephalin (A, D, and G), 0–1 μM Leu-enkephalin (B, E, and H), or 0–1 μM Dynorphin A17 (C, F, and I) in the absence or presence of 1 nM or 100 nM RS-ket as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response at 1 μM of either Met-enkephalin (A, D, and G), Leu-enkephalin (B, E, and H), or Dynorphin A17 (C, F, and I) as 100%. Data represent mean ± S.D. $n = 3$; $^{**}P < 0.01$; $^{***}P < 0.001$ for treatment effect; two-way ANOVA with Tukey's (A, B, and E–I) or Sidak's (C and D) multiple comparison tests (statistical analysis in Supplemental Table 8). Original data for figure shown in Supplemental Fig. 2 and 3.

opioid peptides at MOR in the following order: Met-enk > Dyn A17 > Leu-enk.

There is evidence that the analgesic and/or antidepressant effects of ketamine can be mediated in part through DOR and KOR (Pacheco et al., 2014; Wulf et al., 2022). Therefore, we examined synergism of RS-ketamine and opioid peptides in CHO cells stably expressing DOR or KOR using [^{35}S]GTPγS binding as a measure of receptor activation (Fig. 3; Supplemental Fig. 3). In general, the EC_{50} values for the opioid peptides in the absence of RS-ketamine were similar to previously published results, although there were some differences that could be due to the different cell lines used (Gomes et al., 2020).

In CHO-DOR cells, submicromolar concentrations of RS-ketamine alone did not substantially influence [^{35}S]GTPγS binding (Supplemental Fig. 3E; Supplemental Table 4) but did enhance signaling by 1 μM Met-enk, Leu-enk, or Dyn A17 (Fig. 3, D–F; Supplemental Fig. 3, E–J). For 1 μM Met-enk, the addition of 1 nM RS-ketamine caused a ∼34% increase in signaling and 100 nM RS-ketamine a ∼50% increase in signaling (Fig. 3D; Supplemental Fig. 3, E and F; Supplemental Table 4), whereas for 1 μM Leu-enk, 1 nM RS-ketamine caused a ∼20% increase and 100 nM RS-ketamine a ∼28% increase in signaling (Fig. 3E; Supplemental Fig. 3, G and H; Supplemental Table 4). For 1 μM Dyn A17, 1 nM RS-ketamine caused a ∼22% increase and 100 nM RS-ketamine a ∼28% increase in signaling (Fig. 3F; Supplemental Fig. 3, I and J; Supplemental Table 4).

In CHO-KOR cells, submicromolar concentrations of RS-ketamine alone had a negligible effect on [^{35}S]GTPγS binding (Supplemental Fig. 3K; Supplemental Table 5). RS-ketamine at 1 nM enhanced signaling by Met-enk and Dyn A17, whereas there was no significant impact on Leu-enk signaling (Fig. 3, G–I; Supplemental Fig. 3, K–P; Supplemental Table 5). RS-ketamine at 1 nM caused a ∼26% increase and at 100 nM a ∼40% increase in [^{35}S]GTPγS binding mediated by 1 μM Met-enk (Fig. 3G; Supplemental Fig. 3, K and L; Supplemental Table 5). No such increases were seen for Leu-enk (Fig. 3H; Supplemental Fig. 3, M and N; Supplemental Table 5). For 1 μM Dyn A17, the increase was ∼20% with 1 nM and ∼31% with 100 nM RS-ketamine (Fig. 3I; Supplemental Fig. 3, O and P; Supplemental Table 5). Together, these results show that RS-ketamine can enhance opioid peptide–mediated signaling at MOR, DOR, and KOR (with the

exception of Leu-enk at KOR) and are most robust with MOR.

Modulation of MOR Mediated [^{35}S]GTPγS Binding and β-Arrestin Recruitment by Ketamine Enantiomers. The response curves with the racemic mixture, RS-ketamine, were compared with those for the individual stereoisomers in the [^{35}S]GTPγS binding assay. Both *R*- and *S*-ketamine produced a small increase above basal signaling in the [^{35}S]GTPγS binding assay at micromolar concentrations but not at submicromolar concentrations (Supplemental Fig. 4A; Supplemental Table 6). Met-enk concentration-response curves in the absence and presence of different concentrations of *R*- or *S*-ketamine show that both isomers enhanced Met-enk responses (Fig. 4, A and B; Supplemental Fig. 4, B–E; Supplemental Table 6). The enhancement was more robust with *S*-ketamine compared with *R*-ketamine (Fig. 4, A and B; Supplemental Fig. 4, B–E; Supplemental Table 6). The addition of 1 nM *S*-ketamine caused a ∼186% increase whereas 1 nM *R*-ketamine a ∼72% increase in [^{35}S]GTPγS binding mediated by 1 μM Met-enk (Fig. 4, A and B; Supplemental Table 6).

In the β-arrestin recruitment assay in the absence of opioid peptide, the profile of *S*-ketamine is similar to that of RS-ketamine with a small signal at the maximum tested concentration (10 μM), whereas *R*-ketamine produced a negligible response (Supplemental Fig. 4F; Supplemental Table 6). Met-enk response curves in the absence and presence of different concentrations of *R*- or *S*-ketamine show that *R*-ketamine had no effect on β-arrestin recruitment (Fig. 4C; Supplemental Fig. 4, G and H), whereas *S*-ketamine caused a marked enhancement of Met-enk responses, with peak enhancement seen with 1 nM *S*-ketamine (Fig. 4D; Supplemental Fig. 4, I and J; Supplemental Table 6). Taken together, these results suggest that *S*-ketamine is more effective than *R*-ketamine in the enhancement of G protein activity and β-arrestin recruitment at MOR.

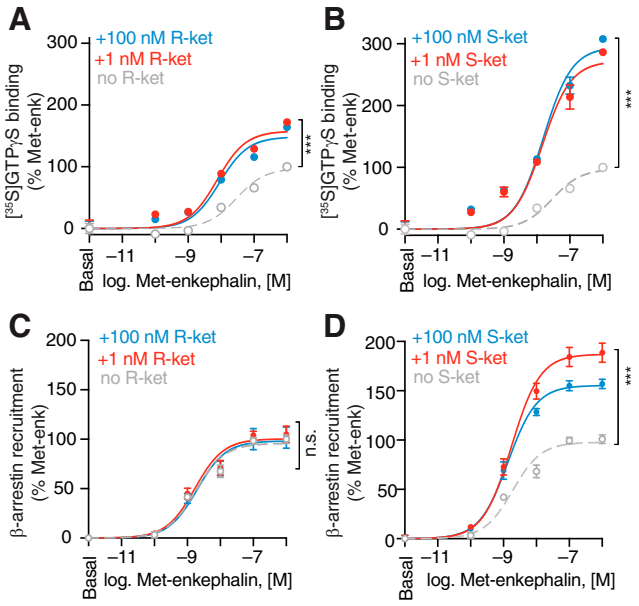

Fig. 4. Effects of ketamine enantiomers (*R*-ketamine and *S*-ketamine) on signaling mediated by Met-enkephalin at MOR. CHO-MOR cells were treated with 0–1 μM Met-enkephalin in the absence or presence of 1 nM or 100 nM of either *R*-ketamine (R-ket; A) or *S*-ketamine (S-ket; B), and [^{35}S]GTPγS binding was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin as 100%. Cells expressing MOR$^{\beta gal}$ were treated with 0–1 μM Met-enkephalin (C and D) in the absence or presence of 1 nM or 100 nM of either *R*-ketamine (R-ket; C) or *S*-ketamine (S-ket; D), and β-arrestin recruitment was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin as 100%. Data represent mean ± S.D. $n = 3$. ***$P < 0.001$ for treatment effect; two-way ANOVA with Sidak's (A) or Tukey's (B–D) multiple comparison tests (statistical analysis in Supplemental Table 8). Original data for figure shown in Supplemental Fig. 4.

Modulation of MOR Activity by Ketamine Metabolites. The ketamine metabolite 6-HNK is active as an analgesic and antidepressant but does not bind to NMDA receptors (Zanos et al., 2016); therefore, a key question is whether it interacts with opioid receptors. We tested stereoisomers of 6-HNK and also the intermediate metabolite norketamine (NK) in the [^{35}S]GTPγS binding and β-arrestin recruitment assays with MOR. In both assays, without Met-enk the ketamine metabolites produce negligible effects at submicromolar concentrations (Supplemental Fig. 5; Supplemental Table 7).

In the [^{35}S]GTPγS binding assay, Met-enk signaling was concentration-dependently enhanced by various ketamine metabolites (Fig. 5, A–D; Supplemental Fig. 5, A–F). The NK isomers enhanced the efficacy for Met-enk to different extents; 1 nM *R*-NK increased the efficiency of Met-enk signaling by ∼23%, and *S*-NK increased the efficiency by ∼71% (Fig. 5C; Supplemental Fig. 5; Supplemental Table 7). Met-enk efficacy was increased by the NK compounds in the following order: *S*-NK > RS-NK > *R*-NK, and a small decrease in EC_{50} was observed with *R*-NK (Fig. 5C; Supplemental Fig. 5C; Supplemental Table 7). With HNKs, we found that both isomers enhanced the efficacy of Met-Enk signaling to a similar extent; 10 nM RR-HNK increased the efficacy by ∼40%, and 10 nM SS-HNK increased the efficacy by ∼47% (Fig. 5D; Supplemental Fig. 5; Supplemental Table 7).

In the β-arrestin recruitment assay, Met-enk concentration-response curves in the absence and presence of different concentrations of the ketamine metabolites showed enhancement of Met-enk–mediated β-arrestin recruitment, with the enhancement being more robust for RS-NK, *S*-NK, and RR-HNK compared with *R*-NK and SS-HNK (Fig. 5, E–I; Supplemental Fig. 5). Submicromolar concentrations of ketamine metabolites increase the efficacy for Met-enk to varying extents (Fig. 5, E–I; Supplemental Fig. 5; Supplemental Table 7). For example, 1 nM RS-NK enhanced Met-Enk efficacy by ∼71%, *R*-NK by ∼20%, and *S*-NK by ∼110% (Fig. 5, E–G; Supplemental Fig. 5; Supplemental Table 7). Although ketamine metabolites showed minimal β-arrestin recruitment when tested alone at submicromolar concentrations, when tested at micromolar levels some metabolites produced a small response (Supplemental Fig. 5). When combined with Met-Enk, both HNK isomers enhanced the efficacy of Met-Enk, with 1 nM RR-HNK increasing the efficacy by ∼65% and 1 nM SS-HNK increasing the efficacy by ∼24% (Fig. 5H, I; Supplemental Fig. 5, O–R; Supplemental Table 7). Taken together, the finding that ketamine stereoisomers and metabolites are all able to enhance the efficacy of Met-enk supports the idea that opioid receptors contribute to the therapeutic activity of ketamine and its major metabolites as analgesics and antidepressants.

RS-Ketamine Modulates MOR Activity in Brain. The assays described above used opioid receptors heterologously expressed in various cell lines to measure [^{35}S]GTPγS

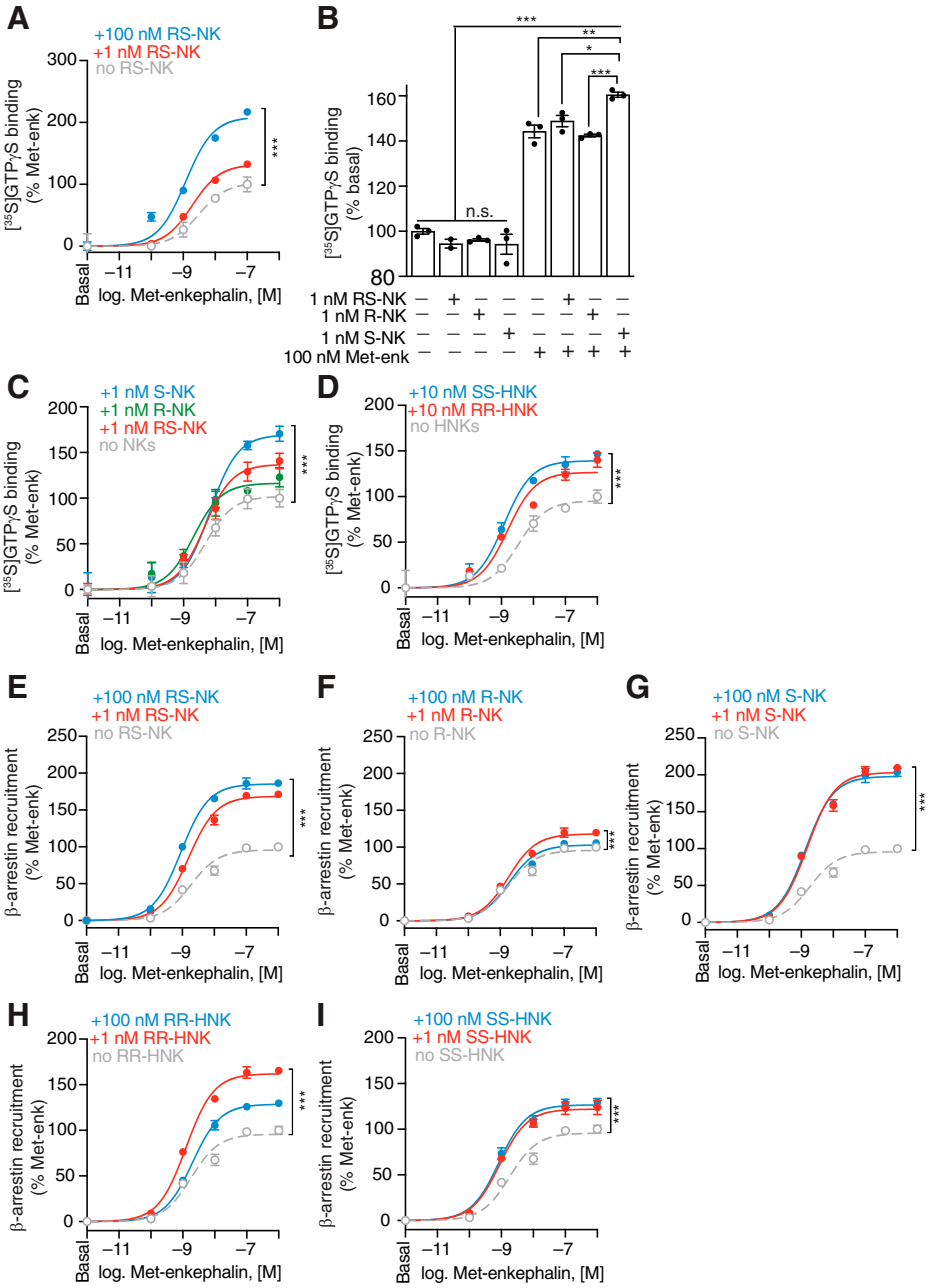

Fig. 5. Effects of ketamine metabolites on signaling mediated by Met-enkephalin at MOR. CHO-MOR cells were treated with 0–1 μM Met-enkephalin in the absence or presence of 1 nM or 100 nM of RS-norketamine (RS-NK; A), and [^{35}S]GTPγS binding was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin as 100%. A comparison of the effect of 1 nM RS-NK, *R*-norketamine (R-NK), or *S*-norketamine (S-NK) on 100 nM Met-enkephalin (Met-enk)-mediated G protein activity (B); basal values in the absence of any ligand were taken as 100%. CHO-MOR cells were treated with 0–1 μM Met-enkephalin in the absence or presence of 1 nM of RS-, *R*-, or *S*-norketamine (C) or in the absence or presence of 10 nM RR- or SS-hydroxynorketamine (RR-HNK or SS-HNK) (D), and [^{35}S]GTPγS binding was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin as 100%. Cells expressing MOR$^{\beta gal}$ were treated with 0–1 μM Met-enkephalin in the absence or presence of 1 nM or 100 nM of either RS-NK (E), R-NK (F), S-NK (G), RR-HNK (H), or SS-HNK (I), and β-arrestin recruitment was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 1 μM Met-enkephalin as 100%. Data represent mean ± S.D. $n = 3$. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; one-way ANOVA (B) or two-way ANOVA with Tukey's multiple comparison test (A and C–I) (statistical analysis in Supplemental Table 8). Original data for figure shown in Supplemental Fig. 5.

binding, β-arrestin recruitment, and MAPK phosphorylation. We also used the TRUPATH biosensor assay (Olsen et al., 2020) to detect modulation of Met-enk responses by RS-ketamine; however, this assay did not yield reproducible results. Because all of the assays above were with cell lines heterologously expressing opioid receptors and/or in engineered systems, it is important to test whether ketamine enhances opioid peptide–mediated signaling with native receptors in the brain. We first used midbrain membranes from wild-type mice and examined the effect of RS-ketamine on DAMGO-mediated [^{35}S]GTPγS binding. Both 1 and 100 nM RS-ketamine increased the potency and efficacy of DAMGO

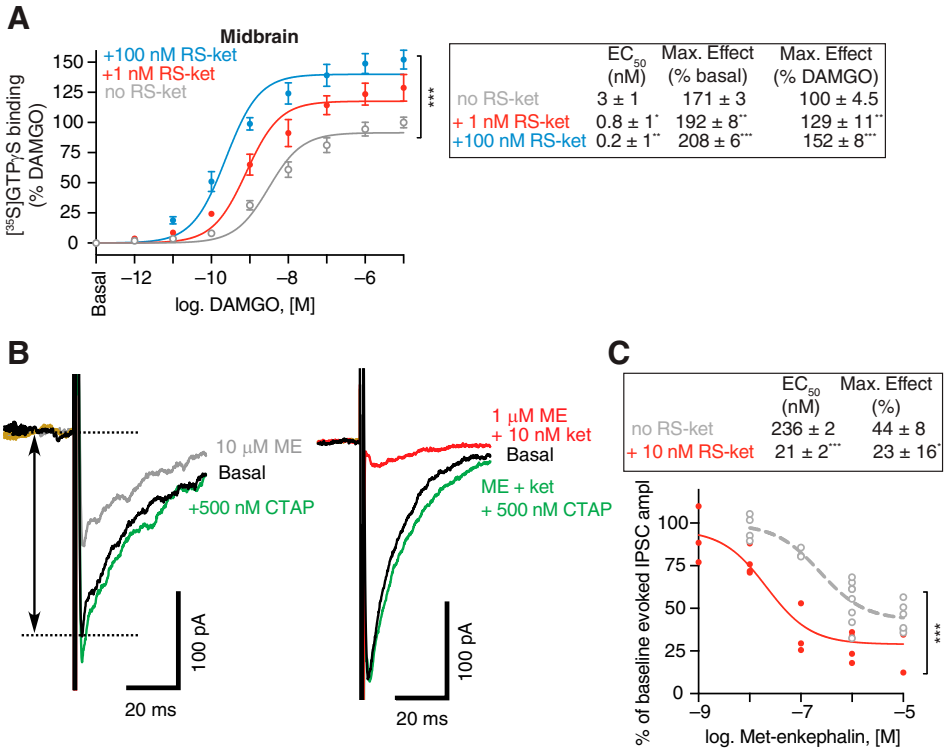

Fig. 6. RS-ketamine (RS-ket) modulates MOR activity in brain. (A) Midbrain membranes from individual wild-type C57BL/6 mice were treated with 0–10 μM DAMGO in the absence or presence of 1 nM or 100 nM of RS-ket, and [^{35}S]GTPγS binding was measured as described in *Methods*. Data were normalized by taking basal values in the absence of any drug as 0% and the response with 10 μM DAMGO as 100%. Data are mean ± S.D. n = 4 animals each in triplicate. (B and C) Whole-cell electrophysiology recordings of electrically evoked IPSCs were made in VTA neurons. (B) Example traces from two different neurons, with 10 μM Met-enk (ME) in aCSF (left) and 1 μM ME + 10 nM RS-ketamine (ket) in aCSF (right) in the absence or presence of 500 nM CTAP. (C) Concentration-response curves for Met-enkephalin–induced inhibition of electrically evoked IPSCs in the absence and presence of 10 nM RS-ket. ***$P < 0.001$ for treatment effect; two-way ANOVA with Tukey's (A) or Sidak's (C) multiple comparison test (statistical analysis in Supplemental Table 8). For insets, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ one-way ANOVA Tukey's multiple comparison test (A) or Student's t test (C). Original data for (A) is in Supplemental Fig. 3Q.

signaling (Fig. 6A; Supplemental Fig. 3Q). The EC_{50} of DAMGO was reduced from 3 nM to 0.2 nM by 100 nM RS-ketamine, and the E_{max} was increased by 52% (Fig. 6A).

Next, MOR agonist induced inhibition of GABA release onto VTA neurons, an action of MOR that is strongly associated with the in vivo rewarding effects of opioids, was examined. For this, ex vivo whole-cell recordings were carried out in acutely prepared rat brain slices to test whether ketamine modifies Met-enk responses at MOR in the VTA. Representative traces from recordings without ketamine show responses to saturating concentrations of Met-enk that are completely reversed by the MOR-selective antagonist CTAP (500 nM), indicating that the response is fully mediated by MOR (Fig. 6B, left panel). In a second example neuron, the combination of 1 μM Met-enk and 10 nM RS-ketamine produced a greater response than 10 μM Met-enk alone (Fig. 6B, right panel). This augmented response was also fully reversed by CTAP (Fig. 6B, right panel). Concentration-response curves for Met-enk in the absence or presence of 10 nM RS-ketamine show that RS-ketamine increased both the potency and E_{max} of Met-enk at this synaptic site (Fig. 6C). Specifically, the EC_{50} for Met-enk was shifted 10-fold by ketamine (Fig. 6C). The maximal inhibition was also increased: calculated as percentage of baseline IPSC amplitude, ~44% of the IPSC persisted with Met-enk alone, but only ~23% remained in the presence of 10 nM ketamine. Together, these results indicate that the PAM effect of RS-ketamine characterized in [^{35}S]GTPγS binding and β-arrestin recruitment assays can also occur at endogenously expressed MORs in brain.

Discussion

Major findings of the present study are that ketamine and its metabolites synergize with endogenous opioid peptides to increase opioid receptor–mediated signaling but do not directly activate opioid receptors at 100 nM or lower concentrations. These results build on our previous finding that treatment of cells expressing MOR with a combination of ketamine and morphine led to a 2- to 3-fold increase in ERK1/2 phosphorylation relative to either drug alone (Gupta et al., 2011). By extending this finding to endogenous opioid peptides, we provide a potential mechanism for the analgesic and antidepressant actions reported for ketamine. This ketamine interaction is not limited to MOR; it was also observed with DOR and KOR, albeit with different efficacies. Our finding that stereoisomers of ketamine and its major metabolites share this effect is important because all of these compounds are known to have analgesic and antidepressant activity (Zanos et al., 2018). Our finding that ketamine augments Met-enk's actions at MOR in mouse brain membranes and rat brain slices extends the results to endogenous receptors. Collectively, these studies advance our understanding of the physiological actions of ketamine that likely account for some of its therapeutic effects.

Previously, opioid peptides and drugs were found to exhibit differential signaling at opioid receptors (Raehal et al., 2011; Thompson et al., 2016; Ho et al., 2018; Stoeber et al., 2018; Civciristov et al., 2019; Gomes et al., 2020). This was also found in the present study comparing the synergism between ketamine and three different classes of opioids: an opiate (morphine), a synthetic peptide (DAMGO), and native opioid peptides (Met-enk, Leu-enk, and DynA17). The magnitude of the synergism was most dramatic with Met-enk, especially with low concentrations of ketamine and Met-enk. Our finding that three different opioid peptides showed different efficacies with ketamine also fits with previous studies that found signaling differences between peptides (Thompson et al., 2016; Gomes et al., 2020). The term "bias" is often used to describe differential signaling seen with agonists and positive allosteric modulators (Livingston and Traynor, 2018; Kandasamy et al., 2021; Slosky et al., 2021; Ramos-Gonzalez et al., 2023). An example of this was observed in the present study; *S*-ketamine was synergistic with Met-enk in both G protein activity and β-arrestin recruitment assays, whereas *R*-ketamine significantly enhanced only G protein activity and not β-arrestin recruitment activity of Met-enk. The subtle differences in the way *S*- and *R*-ketamine affect the opioid system may contribute to their analgesic and antidepressant activities, which are similar but not identical (Bonaventura et al., 2021; Jelen et al., 2021).

The present results are consistent with the proposal that ketamine is a PAM for opioid receptors. PAMs can enhance binding affinity by modulating the rate constants for association (k_{on}) and dissociation (k_{off}) of the orthosteric agonist, they can enhance the efficacy of the orthosteric agonist, and they can prevent receptor downregulation triggered by sustained exposure to orthosteric agonists (Valant et al., 2012). Although k_{on} and k_{off} rates were not measured in the present study, the observation of a significant change in EC_{50} under some conditions is consistent with these rates being altered by ketamine. Direct evidence of an altered signaling efficacy was observed for most combinations of ketamine and opioid peptides, and because ketamine alone at submicromolar concentrations had no effect on signaling in the absence of opioids, it can be considered an opioid receptor PAM. However, due to its weak effects as a direct agonist at micromolar concentrations, technically ketamine should be considered a combined agonist/PAM.

The potent activity of ketamine as an opioid receptor PAM may explain previous controversial data. Several studies reported that micromolar concentrations of ketamine affect opioid receptor activity, but relatively low concentrations of ketamine are required for behavioral effects (Browne et al., 2018, 2020; Browne and Lucki, 2019; Zhang et al., 2021; Wulf et al., 2022; Adzic et al., 2023; Zhou et al., 2023; Levinstein and Michaelides, 2024). Evidence that the opioid system is involved in mediating ketamine's therapeutic effects came from studies testing the effect of opioid antagonists, such as naloxone or naltrexone. Some studies reported that antagonists blocked the analgesic effects of ketamine (Lawrence and Livingston, 1981; Fidecka, 1987; Petrocchi et al., 2019), although other studies did not see reversal by the opioid antagonists (Wiley and Downs, 1982; Mikkelsen et al., 1999; Yost et al., 2022). Similarly, some clinical studies reported that naltrexone blocked the antidepressant effect of ketamine (Williams et al., 2018, 2019; Klein et al., 2020; Zhang et al., 2021), although this effect was not observed in another study (Marton et al., 2019). Naltrexone blocked the antidepressant action of ketamine in mice, and the authors concluded that the "opioid system is necessary … for antidepressive actions of ketamine in rodents" (Klein et al., 2020). However, because morphine did not provide comparable antidepressant activity to ketamine, the authors stated that the opioid system was "not sufficient" for ketamine's antidepressant action (Klein et al., 2020). This apparent paradox can be explained by ketamine functioning as an opioid receptor PAM rather than a direct agonist. Morphine is an orthosteric agonist that activates all MORs regardless of whether endogenous peptides are present. In contrast, PAMs amplify endogenous signals, only driving a response when the orthosteric ligand is present. Thus, the biological effects of PAMs are usually distinct from those of orthosteric receptor agonists (Livingston and Traynor, 2018).

Studies examining ketamine's analgesic effects in mice are consistent with an action as an opioid receptor PAM (Petrocchi et al., 2019). Specifically, Petrocchi et al. (2019) demonstrated that a nonselective opioid receptor antagonist (naloxone) as well as selective MOR and DOR antagonists blocked ketamine-induced peripheral antinociception. Importantly, they found that bestatin significantly potentiated ketamine-induced peripheral antinociception (Petrocchi et al., 2019). Bestatin inhibits a key enzyme involved in opioid peptide degradation and prolongs the half-life of extracellular opioid peptides (Chaillet et al., 1983). Thus, the synergism between bestatin and ketamine is consistent with a role for ketamine as a PAM of peptide-engaged opioid receptors.

Our finding that stereoisomers of ketamine and its major metabolites (norketamine and 6-hydroxynorketamine) show opioid receptor PAM activity is important for two reasons. First, each of these compounds has analgesic and antidepressant activity (Zanos et al., 2016, 2018). Although ketamine and norketamine act as noncompetitive NMDA receptor antagonists, 6-hydroxynorketamine does not (Zanos et al., 2016, 2018). This is strong evidence that NMDA activity cannot fully account for the analgesic and antidepressant effects. Second, the activity of ketamine metabolites can potentially explain why antidepressant and analgesic effects last considerably longer than the elimination half-life of ketamine, which is typically 2 to 3 hours (Niesters et al., 2014; Zanos et al., 2018; Orhurhu et al., 2019). Norketamine has a longer half-life, approximately 12 hours, and 6-hydroxynorketamine also has a long elimination half-life (Zanos et al., 2018). Ketamine metabolites are detectable in plasma >24 hours after administration and may be present at nM levels in the brain or other tissues for days due to their hydrophobicity and/or binding to tissue proteins (Zanos et al., 2018). The antidepressive and analgesic concentrations of ketamine typically produce peak plasma levels of ~1 μM (Zanos et al., 2018), which is orders of magnitude higher than the ~1 nM levels of ketamine and metabolites that were found in the present study to synergize with opioid peptides. It takes approximately 10 half-lives for levels to drop three orders of magnitude, from 1 μM to 1 nM, assuming linearity. Thus, the biological activity of the metabolites together with their ultra-high potency as opioid receptor PAMs is consistent with the days-long therapeutic effects. Other mechanisms may also contribute, such as the reported upregulation of opioid peptides and receptors in rat brain following ketamine treatment (Jiang et al., 2024).

In summary, we found a potential mechanism for the antidepressant and analgesic effects of ketamine. By acting as PAMs at opioid receptors, ketamine and its metabolites amplify the activity of endogenous opioid peptides. Because this activity only affects opioid receptors that are stimulated by the nearby release of endogenous opioid peptides, this is a distinct target from conventional opioid agonists. PAMs of opioid receptors are being developed for clinical use due to their potential to have fewer side effects than orthosteric opioid agonists (Livingston et al., 2018; Livingston and Traynor, 2018; Kandasamy et al., 2021). It remains to be determined if the allosteric binding site targeted by these other PAMs is where ketamine binds or if there are multiple allosteric sites on these receptors. Because low nanomolar concentrations of ketamine do not drive opioid receptor signaling in the absence of orthosteric agonists, but much higher micromolar concentrations show weak agonist activity in our studies, it is possible that there are multiple binding sites on the opioid receptors. A recent molecular modeling study predicted that MOR binds 6-hydroxynorketamine in the orthosteric pocket, but experimentally the metabolite had only modest effects on GTPγS binding and appeared to function as an inverse agonist (Joseph et al., 2021). Further studies are needed to directly examine binding of ketamine and metabolites to opioid receptors.

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Data Availability

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Material.

Authorship Contributions

Participated in research design: Gomes, Margolis, Fricker, Devi.

Conducted experiments: Gomes, Gupta, Margolis.

Performed data analysis: Gomes, Margolis.

Wrote or contributed to the writing of the manuscript: Gomes, Margolis, Fricker, Devi.

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