

## 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



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### ABSTRACT

Kappa opioid receptors and their endogenous neuropeptide ligand, dynorphin A, are densely localized in limbic and cortical areas comprising the brain reward system, and appear to play a key role in modulating stress and mood. Growing literature indicates that kappa receptor antagonists may be beneficial in the treatment of mood and addictive disorders. However, existing literature on kappa receptor antagonists has used extensively JDTic and nor-BNI which exhibit long-lasting pharmacokinetic properties that complicate experimental design and interpretation of results. Herein, we report for the first time the *in vitro* and *in vivo* pharmacological profile of a novel, potent kappa opioid receptor antagonist with excellent selectivity over other receptors and markedly improved drug-like properties over existing research tools. LY2456302 exhibits canonical pharmacokinetic properties that are favorable for clinical development, with rapid absorption ( $t_{max}$ : 1–2 h) and good oral bioavailability ( $F = 25\%$ ). Oral LY2456302 administration selectively and potently occupied central kappa opioid receptors *in vivo* ( $ED_{50} = 0.33$  mg/kg), without evidence of mu or delta receptor occupancy at doses up to 30 mg/kg. LY2456302 potently blocked kappa-agonist-mediated analgesia and disruption of prepulse inhibition, without affecting mu-agonist-mediated effects at doses >30-fold higher. Importantly, LY2456302 did not block kappa-agonist-induced analgesia one week after administration, indicating lack of long-lasting pharmacodynamic effects. In contrast to the nonselective opioid antagonist naltrexone, LY2456302 produced antidepressant-like effects in the mouse forced swim test and enhanced the effects of imipramine and citalopram. LY2456302 reduced ethanol self-administration in alcohol-preferring (P) rats and, unlike naltrexone, did not exhibit significant tolerance upon 4 days of repeated dosing. LY2456302 is a centrally-penetrant, potent, kappa-selective antagonist with pharmacokinetic properties favorable for clinical development and activity in animal models predictive of efficacy in mood and addictive disorders.

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*Non-standard abbreviations:* CREB, cyclic adenosine monophosphate-response element binding protein; DAMGO, [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin; DPDPE, [d-Pen<sup>2</sup>, d-Pen<sup>5</sup>]-enkephalin; ERK, extracellular regulated kinase; GR103545, methyl (3R)-4-[(3,4-dichlorophenyl)acetyl]-3-(pyrrolidin-1-ylmethyl)piperazine-1-carboxylate; JDTic, (3R)-7-hydroxy-N-[(1S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide; LY2048978, 4-[4-[(pentylamino)methyl]phenoxy]benzamide; LY2456302, [(S)-3-fluoro-4-(4-[(2-(3,5-dimethylphenyl)pyrrolidin-1-yl)methyl]phenoxy)benzamide]; MAP kinase, mitogen-activated protein kinase; MR2034, (2R,11R)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-[[[(R)-tetrahydrofuran-2-yl]methyl]-2 $\alpha$ ,6 $\alpha$ -methano-3-benzazocin-8-ol]; PPI, prepulse inhibition; U-69593, N-methyl-2-phenyl-N-[(5R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide.

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## 1. Introduction

Major depression, characterized by negative mood, reduced motivation, and sometimes anhedonia and decreased energy, affects nearly 5% of people worldwide each year. Current antidepressants work well for some patients, but ~60% suffer from unresolved residual symptoms or inadequate treatment response (Thase et al., 2001). As a result, some patients may abuse or misuse alcohol or other drugs in an attempt to reduce their depressive symptoms, often termed “self-medication” (Bolton et al., 2009). A recent study estimated that 16% of depressed patients also have a diagnosable addiction disorder (Sher et al., 2008). Such comorbidity puts patients at greater risk. Comorbid substance use in depressed patients is associated with greater symptom severity, inadequate treatment response, poorer prognosis (including increased risk of suicide), and persistence of depressive symptoms (Thase et al., 2001; Blanco et al., 2012). Therefore, a tremendous need exists for pharmacotherapies effective in treating both depressive symptoms and alcohol dependence.

Kappa opioid receptors and their endogenous neuropeptide ligand, dynorphin A, are densely localized in limbic and cortical areas comprising the brain reward and stress systems, and play a key role in modulating neurotransmission in these areas (Mansour et al., 1987, 1994; Margolis et al., 2006). In preclinical models, stress produces a prodepressive phenotype that is believed to be associated with the activation of kappa opioid receptors and subsequent downstream signaling events (Pliakas et al., 2001; Newton et al., 2002; McLaughlin et al., 2003; Shirayama et al., 2004; Land et al., 2008). Consistent with this hypothesis, kappa receptor agonists produce anxiogenic- and prodepressive-like effects in animals and humans (Pfeiffer et al., 1986; Todtenkopf et al., 2004; but see also Harden et al., 2012), whereas kappa receptor antagonists reliably exhibit antidepressant-like effects in animal models predictive of efficacy in the domains of mood and affect (Mague et al., 2003; Land et al., 2009; Carr et al., 2010). Unfortunately, there have been no reports of selective kappa receptor antagonists administered in clinical populations.

Kappa-selective antagonists also reduce ethanol intake and reinstatement in a number of preclinical paradigms (Deehan et al., 2012; Walker and Koob, 2008). While nonselective opioid antagonists such as naltrexone, an FDA-approved medication for alcohol dependence, are efficacious in animal models of alcoholism, they do not produce reliable antidepressant- or anxiolytic-like effects in animals or humans, likely due to functional opposition between mu and kappa receptors (Margolis et al., 2003; Spanagel et al., 1992). Similarly, antidepressants are weakly and inconsistently effective at reducing alcohol consumption in depressed patients with comorbid addictive disorders (Kranzler et al., 2006; Pettinati et al., 2010). Because kappa antagonists demonstrate efficacy in animal models predictive of efficacy in mood and addictive disorders, they have the potential to treat depressed patients with comorbid alcohol dependence.

Molecules targeting specific biological mechanisms are powerful tools for elucidating biological function. Most current knowledge of the kappa opioid system comes from studies on the prototypical antagonists, norbinaltorphimine (nor-BNI) and (3R)-7-Hydroxy-N-((1S)-1-((3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl)methyl)-2-methylpropyl)-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide (JDTic), which have unusual pharmacokinetic properties, including delayed onset of centrally-mediated effects (24–48 h) and very long duration of pharmacodynamic effects (28+ days; Munro et al., 2012; Patkar et al., 2013), that complicate research design and interpretation of the results. Long-duration activity of an antagonist can result in biological consequences that are different from that of short-term receptor

blockade; differential biochemical modifications occur at the receptor level and at down-stream targets as a result of prolonged gating of agonist from the receptor. In order to expand and crystallize our current understanding of the biological basis of kappa opioid receptor function, the purpose of the present experiments was two-fold: first, to pharmacologically characterize (S)-3-fluoro-4-(4-((2-(3,5-dimethylphenyl)pyrrolidin-1-yl)methyl)phenoxy)benzamide (LY2456302) as an improved research tool for studying the kappa opioid receptor system; and second, to examine its antidepressant-like effects and its ability to decrease ethanol consumption.

## 2. Materials and methods

### 2.1. Drugs and reagents

(S)-3-fluoro-4-(4-((2-(3,5-dimethylphenyl)pyrrolidin-1-yl)methyl)phenoxy)benzamide (LY2456302; Diaz Buezo et al., 2009; Mitch et al., 2011), LY2048978, JDTic, and GR103545 were synthesized at Lilly Research Laboratories. Naltrexone HCl, DAMGO acetate, DPDPE hydrate, naltriben methanesulfonate hydrate, U-69593, morphine sulfate, imipramine HCl, chlordiazepoxide HCl, phencyclidine HCl, and formalin solution (10%, diluted to 5%) were purchased from Sigma Aldrich (St. Louis, MO). LY2456302, LY2048978, JDTic, and naltrexone were dissolved in water with the addition of 85% lactic acid. U-69593 was dissolved in 12.5% 2-hydroxypropyl- $\beta$ -cyclodextrin (formalin study) or sterile water (prepulse inhibition studies). Morphine, phencyclidine, chlordiazepoxide, and imipramine were prepared in sterile water. For *in vitro* assays, DAMGO, U-69593, DPDPE, and naltriben were prepared in a buffer consisting of 20 mM HEPES, pH 7.4, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol. Drugs were mixed fresh on the day of dosing. Doses were administered to rats in a volume of 1 or 2 ml/kg; doses were administered to mice in a volume of 10 ml/kg. Doses, routes of administration, and pre-treatment times are indicated separately for each experiment. Unless otherwise indicated, experiments were conducted in non-fasted animals. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals under protocols approved by a local animal care and use committee.

### 2.2. *In vitro* receptor binding and functional activity

*In vitro* opioid receptor binding and [<sup>35</sup>S]-GTP $\gamma$ S binding experiments were conducted as previously described (Mitch et al., 2011). Briefly, radioligand displacement studies with [<sup>3</sup>H]diprenorphine were carried out using membranes prepared from CHO cells expressing cloned human  $\kappa$  and  $\mu$  opioid receptors or HEK293 cells expressing the cloned  $\delta$  opioid receptor. Concentrations causing 50% inhibition (IC<sub>50</sub>) of [<sup>3</sup>H]-diprenorphine binding were determined from 11-point concentration response curves in assay buffer containing sodium and guanosine diphosphate (GDP). Naltrexone was included as a control at 10  $\mu$ M to define nonspecific binding and was also tested as a comparator molecule in concentration response curves.

### 2.3. Rat and mouse plasma exposure and unbound fraction in plasma and brain

Three male cannulated rats were administered a single 1 mg/kg intravenous (IV) and 10 mg/kg oral (PO) dose of LY2456302 to determine the pharmacokinetic parameters. Plasma samples were collected at 0.08 (IV only), 0.25, 0.5, 1, 2, 4, 8, 12 and 24 h post-dose and analyzed by liquid chromatography coupled to tandem mass spectral detection (LC–MS/MS) to determine the concentrations of LY2456302. Male mice ( $n = 3$  per time point) were administered a single 10 mg/kg PO dose of LY2456302 to determine the pharmacokinetic parameters. Plasma samples were collected at 0.5, 1, 2, 4, 8, and 24 h post-dose and analyzed by LC–MS/MS to determine the concentrations of LY2456302. The plasma and brain binding of LY2456302 was determined by equilibrium dialysis at 1  $\mu$ M.

### 2.4. *In vivo* receptor occupancy

The ability of orally administered LY2456302 to occupy brain mu, delta, and kappa opioid receptors *in vivo* was assessed in male Sprague-Dawley (SD) rats (Harlan, Indianapolis, IN), weighing 250–300 g,  $n = 4$ /dose. Kappa opioid occupancy was measured in male NIH-Swiss mice (Harlan, Indianapolis, IN), weighing 25–30 g,  $n = 4$ /dose. Receptor occupancy (RO) was determined 90 min after a PO dose of LY2456302 by measuring displacement of unlabeled tracers by LC–MS/MS (Need et al., 2007). Tracers for mu, kappa, and delta receptors, were naltrexone (10  $\mu$ g/kg), naltriben (10  $\mu$ g/kg), and GR103545 (1.5  $\mu$ g/kg), respectively, administered as a single IV injection (Need et al., 2007). In a separate study, RO was determined by LC–MS/MS in male SD rats (Harlan, Indianapolis, IN, 250–300 g,  $n = 4$ /dose) at 1, 4, 8, and 48 h after a 10 mg/kg PO dose of LY2456302. Total binding was represented by levels of tracers in the striatum for delta and kappa receptors, and thalamus for the

mu receptor. The cerebellum, which contains significantly lower densities of mu, kappa, or delta receptors, was used for measuring nonspecific binding (Mansour et al., 1994). Receptor occupancies were calculated by using the ratio method described by Wadenberg et al. (2000), but substituting the tracer concentrations determined by LC/MS/MS for the radiolabeled tracer levels determined with scintillation spectrometry. The following equation was employed:

$$100 * \{1 - [(Ratio_t - 1) / (Ratio_c - 1)]\} = \% \text{ Occupancy}$$

Each 'Ratio' refers to the ratio of tracer in a brain area rich in target receptor relative to the tracer detected in the cerebellum. 'Ratio<sub>t</sub>' refers to animals treated with test drug, while 'Ratio<sub>c</sub>' refers to the average ratio in vehicle-treated animals. ED<sub>50</sub> curves, where reported, were calculated from experimental values using 4-parameter non-linear regression curve fit. The lower and upper bounds were not fixed in these calculations.

#### 2.4.1. Tissue preparation and analysis for in vivo tracer characterization

Rat brain tissue samples were weighed and placed in conical centrifuge tubes on ice. Four volumes (w/v) of acetonitrile containing 0.1% formic acid was added to each tube. These samples were then homogenized using an ultrasonic probe and centrifuged. Supernatant was diluted with sterile water in HPLC injection vials for LC/MS/MS analysis. Analysis of potential tracers were carried out using an Agilent model 1200 HPLC (Agilent Technologies, Palo Alto, CA) and an API 3000 mass spectrometer (Applied Biosystems, Foster City, CA, USA). The chromatographic separation employed Zorbax C18 column (Agilent Technologies, Wilmington, DE, USA) gradient from 20% to 90% acetonitrile/water, each with 0.1% formic acid. The total HPLC run time was 3.5 min with an additional 2.0 min re-equilibration time. Detection of small molecule tracers was accomplished by monitoring ion transitions 342.0/324.1, 416.0/398.2, and 414.0/343.0 mass/charge for naltrexone, naltriben, and GR103545, respectively. Standards were prepared by adding known quantities of analyte to brain tissue samples from nontreated-rats and processed as described above.

#### 2.5. Reversal of kappa-agonist-induced analgesia

The functional selectivity of LY2456302 at kappa vs. mu opioid receptors was determined *in vivo* by measuring reversal of the analgesic effects of the kappa agonist U-69593 and mu agonist morphine, previously demonstrated in the formalin model of acute sensitization. This procedure has been described in detail previously (Shannon and Lutz, 2000) and utilizes the commercially available startle behavior chambers (Model SR-Lab, San Diego Instruments, San Diego, CA) that detect movements by means of an accelerometer. Briefly, male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN), weighing approximately 200–210 g, were administered vehicle or LY2456302 and 30 min later, were administered vehicle, subcutaneous (SC) morphine (10 mg/kg) or U-69593 (1 mg/kg),  $n = 8$  animals per treatment group. A novel, nonselective opioid antagonist LY2048978 (Statnick et al., under review) was used as the quality control in the U-69593 and morphine reversal studies with LY2456302 at a PO dose of 3 and 0.6 mg/kg, respectively (Table 1). Naltrexone was used as the quality control in the U-69593 and morphine reversal studies (doses of 0.2 and 0.02 mg/kg SC, respectively). The dose and route of administration of LY2048978 or naltrexone used as quality controls had been previously established as approximate ED<sub>50</sub> doses for antagonizing the analgesic effects of U-69593 and morphine. Animals were then individually placed in confinement cylinders (i.d. 8.5 cm; length 16 cm) on the recording equipment for a 30-min acclimation. Formalin (50 μl of a 5% solution in saline) was then administered SC into the plantar surface of the right hindpaw, and the animals were immediately placed back into the confinement cylinders. In a separate experiment, the long-lasting functional effects of LY2456302 at the kappa opioid receptor were determined by pretreating animals with a single dose of vehicle (1 ml/kg PO), LY2456302 (3 mg/kg PO), nor-BNI (10 mg/kg SC), or JDTC (10 mg/kg SC) 1-wk prior to U-69593 (1 mg/kg SC), which was administered 30 min prior to the formalin test (performed as previously described).

**Table 1**  
*In vitro* binding and functional activity at human mu, kappa, and delta opioid receptors.

Compound	Receptor binding affinity, Ki (nM) <sup>a</sup>			Functional antagonist Activity, Kb (nM) <sup>b</sup>		
	Mu	Kappa	Delta	Mu	Kappa	Delta
LY2456302	24.0 (5.45)	0.807 (0.237)	155 (33.0)	17.4 (6.3)	0.813 (0.285)	110 (34.0)
Nor-BNI	32.4 (8.58)	0.153 (0.07)	6.56 (1.58)	32.9 (16.7)	0.798 (0.48)	14.1 (7.24)
JDTC	10.5 (5.04)	0.031 (0.014)	153 (49.5)	6.58 (3.14)	0.098 (0.069)	168 (67.2)
Naltrexone	0.627 (0.12)	1.87 (0.22)	13.8 (2.7)	0.245 (0.03)	1.94 (0.41)	9.45 (1.45)
LY2048978	0.287 (0.06)	0.471 (0.23)	1.05 (0.27)	0.337 (0.13)	0.919 (0.39)	2.63 (1.01)

<sup>a</sup> Radioligand binding with [<sup>3</sup>H]-diprenorphine, using membranes from HEK or CHO cells expressing human mu, kappa, or delta opioid receptors, respectively. Data represent the geometric mean (±SEM) from at least three independent experiments performed on separate occasions.

<sup>b</sup> Inhibition of agonist-stimulated [<sup>35</sup>S]GTPγS binding in membranes from HEK or CHO cells expressing human mu, kappa, or delta opioid receptors, respectively. Agonists used to determine functional antagonist activity were DAMGO (mu), U-69593 (kappa), and DPDPE (delta). Data represent the geometric mean (±SEM) from at least three independent experiments performed on separate occasions.

The magnitude of movements was then monitored continuously for 60 min in 1 s bins. The number of events, defined as the number of 1 s bins with a change in dynamic force that exceeds an empirically determined threshold value (20 arbitrary load units, which was previously determined in pilot experiments to be greater than that produced by animals quietly sniffing and breathing) were totaled in 5 min intervals. The movements detected by the system included licking, flinching, hopping and turning as a consequence of the formalin injection. Data reported are the Phase II component of the formalin response.

#### 2.5.1. Data analysis

The time course of the number of nocifensive events was totaled in 5-min-intervals, but only the Phase II component of the formalin response is reported (11–45 min). Treatment groups were compared to appropriate control groups using ANOVA followed by Dunnett's *t*-test. Data are expressed as means + SEM, with a *p* value of <0.05 considered significant. All computations, including outlier plots of residuals, were performed using JMP (SAS Institute Inc., Cary, NC).

#### 2.6. Reversal of kappa-agonist-induced disruption of prepulse inhibition of startle responding

Prepulse inhibition (PPI) is a measure of sensorimotor gating and is thought to reflect pre-attentive processing. Although deficits in PPI are often used in preclinical models and clinical assessment of schizophrenia, PPI dysfunction occurs in other psychiatric and neurological disorders involving deficits of attention (Braff et al., 2001). Kappa and mu receptor agonists have been demonstrated to disrupt cognition in humans (Kamboj et al., 2005; Pfeiffer et al., 1986) and can disrupt PPI in rodents (Bortolato et al., 2005; Meng et al., 2010; but see Tejada et al., 2010). We tested LY2456302 for its effects on PPI alone and for its ability to reverse PPI deficits induced by U-69593 and morphine. A total of 136 male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing approximately 250 g, were used in the two experiments. Rats were maintained on *ad libitum* food and water housed 2 per cage on a 12:12 light:dark cycle; testing occurred during the light phase of the light:dark cycle.

LY2456302 was first evaluated alone for its effects on PPI. Rats were equally divided into 5 treatment groups ( $n = 8$ /group) and dosed with either vehicle, LY2456302 (3, 10, or 30 mg/kg), or phencyclidine hydrochloride (PCP; 3 mg/kg); PCP served as a positive control for detecting disruption of PPI. Rats were counter-balanced by dose-group across chambers. One hour after a PO dose of LY2456302 and 15 min after a SC dose of PCP, rats were tested for startle and PPI responses.

In the next experiments, we determined the functional kappa receptor selectivity, relative to mu-receptor-mediated effects, by assessing the ability of LY2456302 to antagonize disruption of PPI by U-69593 and morphine, respectively. In the U-69593 challenge study, rats were divided into 5 groups of 8 and dosed with vehicle, 0.1, 0.3, or 1.0 mg/kg (PO) LY2456302, 60 min prior to testing. Each rat was then dosed with 3 mg/kg U-69593 or vehicle (SC), 15 min before testing. To evaluate LY2456302 functional activity against mu opioid receptor activation, higher doses of LY2456302 (10 and 30 mg/kg, PO) were administered to rats ( $n = 14$  per group) 60 min prior to 20 mg/kg morphine sulfate (SC) challenge. Testing began 15 min later and the ability of LY2456302 to reverse morphine-induced PPI deficits was recorded. For the purposes of comparison, the ability of the mu-preferring antagonist, naltrexone, to reverse kappa and mu agonists was also evaluated. In separate studies, naltrexone was dosed (0.1–1 mg/kg, SC) 30 min prior to testing for antagonism of U-69593- and morphine-induced PPI disruption.

#### 2.6.1. Experiment 1: prepulse inhibition procedure for effects of LY2456302 alone

Following a 10-min acclimation period, the first trial of a 54-trial session began. The session consisted of randomized trial types of 11 startle-alone, 3 prepulse trial types of 11 each, 5 background-alone control trials and 5 prepulse-alone trials. The startle-alone trial was a 40-ms, 115-decibel (dB) burst of white noise. Prepulse trials consisted of a 40-ms, 115-dB white noise burst preceded by a 20-ms 65-, 70-, or 80-dB prepulse of white noise with an inter-stimulus interval (onset to onset) of 75 ms. The inter-trial interval (ITI) varied from 10 to 20 s, during which, the background

noise was maintained consistently at 60 dB. Startle Amplitude for each trial was measured via accelerometer (Hamilton Kinder, model SM100RP; San Diego, CA) where average force in Newtons was recorded over a 120-ms period initiated during the presentation of the 115-dB startle stimulus for each trial. Inhibition of startle reactivity was measured as each rat's percent change from mean prepulse startle reactivity to mean baseline startle reactivity using the formula: [(Mean Baseline Startle – Mean Prepulse Startle)/Mean Baseline Startle] × 100.

#### 2.6.2. Experiment 2: prepulse inhibition procedure for U-69593 and morphine reversal

Following a 5-min acclimation period the first trial of a 45-trial session began. The first 5 trials consisted of 115-dB startle-alone bursts; the remaining 35 trials were a pseudo-random combination of prepulse and startle trials. In addition, there were 5 background-alone control trials. Startle-alone trials consisted of 40-ms, 115-dB white noise bursts. Prepulse trials consisted of a 40-ms, 115-dB noise burst preceded by a 20-ms 65 dB prepulse of sound with an onset to onset inter-stimulus interval of 75 ms. The ITI varied from 15 to 45 s, during which, background noise was maintained at 60 dB. All other variables and methods were identical to Experiment 1.

Experiment 1 data used a two-way, mixed design ANOVA with Dose group and Prepulse Intensity as independent variables. Experiment 2 used one-way ANOVAs using a between-subjects design with Dose group as the factor. Following a significant ANOVA, individual group differences were determined using Bonferroni's Multiple Comparison Test (alpha set at 0.05). In both experiments, the first 5 trials were used to habituate the startle response and were not used in the statistical analyses. Statistical analyses were performed in GraphPad Prism.

#### 2.7. Mouse forced swim test

Male NIH-Swiss mice (Harlan, Indianapolis, IN) weighing 20–25 g were housed in plastic cages (40.6 × 20.3 × 15.2 cm) with 10–12 mice/cage in a vivarium for at least 7 days. Animals were removed from the vivarium to the testing area in their homecages and allowed to adapt to the new environment for at least 1 h before testing. The forced swim test was performed using the original method described by Porsolt et al. (1979). Briefly, mice were placed individually in clear plastic cylinders (10 cm in diameter, 25 cm in height) filled to 6 cm with 22–25 °C water for 6 min. The duration of immobility was recorded during the last 4 min of a 6-min trial. A mouse was regarded as immobile when floating motionless or making only those movements necessary to keep its head above the water. Dose–effect functions for LY2456302 were performed after administration of LY2456302 (1–10 mg/kg PO, 60 min before the test) alone, and in combination with a sub-effective dose of imipramine (5 mg/kg IP, 30 min before the test) or a sub-effective dose of citalopram (3 mg/kg IP, 30 min before the test). A synergy analysis was conducted using the method of Bliss Independence (Greco et al., 1995; Fitzgerald et al., 2006). Using the endpoint of % inhibition, an ANOVA was applied to test the coefficient of the interaction term in a 2 × 2 full factorial model of the two compounds. Imipramine was used as a comparator standard (15 mg/kg IP, 30 min pretreatment). In a separate experiment, the ability of naltrexone (1–10 mg/kg PO, 60 min before the test) was tested in the mouse forced swim test.

#### 2.8. Ethanol self-administration in alcohol-preferring (P) rats

##### 2.8.1. Homecage ethanol self-administration

In a chronic free-choice drinking paradigm, LY2456302 was assessed and compared with the mu-preferring opioid antagonist naltrexone in selectively-bred Alcohol-Preferring (P) rats (generously supplied by the Indiana University School of Medicine and maintained at Taconic Inc, Germantown, NY). Subjects were female P rats with 24-h *ad libitum* access to 15% (v/v) ethanol, water, and food in the homecage. Water and ethanol intake (in mL), food intake (in g), and homecage locomotor activity (infrared counts) were measured once every 5 min throughout the 12-h dark cycle and recorded for later analysis using the TSE LabMaster system (TSE Systems, Bad Homburg, Germany). A "bout" was defined as total ethanol intake summed over consecutive 5 min bins. Rats were not drug naïve, but a standard washout period of 4–7 days was instituted between subsequent studies. In the first experiment, rats were given vehicle (PO), 10 mg/kg naltrexone, or LY2456302, at doses of 3 or 10 mg/kg, 60 min before onset of the 12-h dark phase of the light–dark cycle, using a between-subjects design. In a subsequent study, a 1 mg/kg dose of LY2456302 was compared with vehicle in a between-subjects design. The third experiment was subdivided into two experiments in which repeated dosing of naltrexone was compared with repeated dosing of LY2456302. In the naltrexone repeated dosing experiment, vehicle was administered to all rats on Monday, followed by vehicle or 30 mg/kg PO naltrexone on four consecutive days (T–F), using a between-subjects design. On each dose day, compounds were administered 60 min before onset of the 12-h dark phase of the light–dark cycle, and intake was measured over the entire 12 h of the dark cycle. Using the same rats that received vehicle during the sub-chronic naltrexone study, we then assessed the effects of repeated dosing of 10 mg/kg LY2456302. In this experiment, vehicle was administered to all rats on Monday, followed by vehicle or 10 mg/kg (PO) LY2456302, dosed

60 min before onset of the 12-h dark phase of the light–dark cycle on four consecutive days, using a between-subjects design.

##### 2.8.2. Progressive ratio operant ethanol self-administration

A separate cohort of P rats was used in a progressive ratio operant model of ethanol self-administration, which is a model used to measure the motivation to consume ethanol. All training and testing was conducted in 12 standard sized rat operant chambers (Med-Associates, St. Albans, VT) measuring 30.5 cm × 24.1 cm × 21 cm (L × W × H), within sound attenuating boxes. The chambers consisted of clear Plexiglas front and back walls, modular aluminum side-walls, a metal bar floor and Plexiglas ceiling. A food cup was located in the center of one side wall with retractable levers on either side of the food cup, and stimulus light directly above each lever. A liquid dipper device allowed the delivery of 0.1 mL of 15% ethanol (v/v) into the food cup. A computer running the MED-IV software package (Med-Associates, St. Albans, VT) controlled stimulus presentations and recorded lever presses. Female P rats were trained to lever press for ethanol reinforcement during operant sessions on 5 days per week. Once animals were trained to lever press on a fixed ratio (FR1) schedule of reinforcement, the response requirement for each reinforcement was slowly increased to FR2 and then FR3 over 1–2 weeks. When rats demonstrated a stable level of responding on the FR3 schedule, they were transferred to a progressive ratio schedule, in which the response requirement increased in each experimental session as follows: the response requirement for ethanol delivery increased from 1 to 2 after 3 reinforcer presentations, and then incremented by 2 more after every 3 ethanol presentations (Rodd et al., 2003). Experimental sessions terminated after 60 min. The amount of ethanol consumed was recorded in mL (and converted to g/kg), as well as the number of responses on the active and inactive levers, and the breakpoint, which was defined as the highest FR value reached during the session. The experiment was conducted using a within-subject design, with 3–4 days washout between subsequent doses. Separate cohorts were used to evaluate naltrexone and LY2456302. On drug days, rats received vehicle, 1, 3, or 10 mg/kg LY2456302 (PO) or vehicle, 0.3, 1, or 3 mg/kg naltrexone (IP).

##### 2.9. Dopamine efflux in the nucleus accumbens

Adult male Sprague-Dawley rats (250–270 g, Taconic Farms, Germantown, NY) were anesthetized with isoflurane and surgically implanted with guide cannula above the Nucleus Accumbens-shell. Coordinates used were relative to Bregma (in mm): AP 1.7, ML 0.8, DV –0.6. Following surgery rats were single housed in shoebox cages in a temperature and humidity controlled colony maintained on a 12/12 light/dark cycle (lights on at 06:00 h). Rats were allowed to recover from surgery for 5–7 days prior to experimentation. All experiments began at approximately 07:00 h.

Microdialysis probes (2 mm, BASi, West Lafayette, IN) were prepared by flushing with 100 µl of sterile water. Probes were inserted 18–24 h prior to the experiment. On the day of the experiment, the rats were weighed and placed in round bottom cages and tethered to a counterbalance arm. The probe inlet tubing was connected to a syringe pump that delivered aCSF (in mM: 145 NaCl, 2.7 KCl, 1.0 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 2.0 Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2–7.4) at a flow rate of 1.5 µL/min. Animals were perfused for 2.5 h prior to any collections. Following this equilibration, samples were collected every 30 min. Three baseline samples were collected, the animals were dosed and samples were collected for an additional 3 h (A total of 9 samples per rat were collected). Samples were collected into vials containing 10 µl of preservation cocktail (3 mM L-cysteine, 10 mM EDTA in 0.1 M acetic acid) and maintained at 4 °C in a fraction collector. At the end of the experiment samples were stored at –80 °C until they could be analyzed. The entire experiment was conducted over three different days with groups of 8, 12 and 12 animals. Rats assigned to each dose group were run on each of the days.

##### 2.9.1. HPLC

An ESA 542 pump delivered mobile phase (75 mM sodium dihydrogen phosphate monohydrate, 3.0 mM 1-octanesulfonic acid sodium salt, 100 µL/L triethylamine, 25 µM EDTA, and 8% acetonitrile; pH = 5) through a reverse phase column (3.2 × 150 mm; C-18, 3 µM particle size) at 0.6 ml/min. Samples (20 µL) were injected onto the column with the aid of an autosampler and DA was quantified using a Coulachem III detector. Cell potentials were: guard cell potential = +350 mV, E1 = +220 mV and E2 = –290 mV. DA was detected on E2.

##### 2.9.2. Histology

At the end of experiment, animals were sacrificed with CO<sub>2</sub> inhalation and tetrazolium red dye (5 mg/ml; Sigma–Aldrich, St. Louis, MO) was perfused through the probe to mark the probe track. The brain was removed and frozen at –20 °C and later sliced on a cryostat at 40 µm to verify probe location.

##### 2.9.3. Data analysis

Data were normalized to percent of baseline (average of the three samples prior to dosing) and analyzed using a two-way mixed ANOVA (Toeplitz Matrix, SAS RM Unix ver 2.2) with factors of Dose and Time. Baseline DA levels were compared using a one-way ANOVA on the untransformed data. Two of the 36 animals were excluded from the study because the probes were not patent and one of the 36 animals was

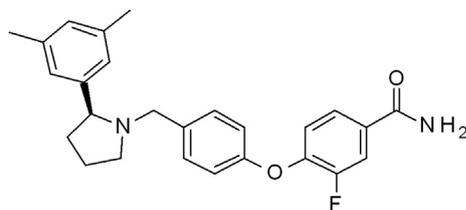


Fig. 1. Chemical structure of LY2456302.

excluded because the probe location was found to be outside the Nucleus Accumbens-shell. Of the remaining 33 rats, 3 rats had one sample (out of 9 continuous data points) missing due to problems with the chromatography.

## 2.10. Side effect profiling

### 2.10.1. Rotarod motor performance

Any treatment for psychiatric disorders should ideally be relatively devoid of motor or cognitive side-effects. A common and validated task to assess motor disruption in rodents is the rotarod task. In our version of this task, rats are minimally trained to maintain balance on a rotating circular rod for 180 s. Subjects were male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing approximately 200 g, housed four per cage and maintained on *ad libitum* food and water,  $n = 9-10$  per group. Food was removed in the late afternoon of the day prior to testing. Drug groups were assigned to the 4 rotarod chambers in a counter-balanced design. Forty rats received 4, 60-s trials on a rod (2.75 in. diameter; San Diego Instruments, San Diego, CA) spinning at 4 revolutions per minute (RPM). The initial training phase ended once the rat stayed on the rotarod for a full 60-s trial. If a rat did not stay on the rod for 60 s after 4 trials, it was removed from the study. Two hours later, a single 60-s trial was given and only rats that completed this trial qualified for the drug testing phase of the study. Qualifying rats were dosed immediately following this trial and returned to the holding cage for 60 min. Following the 60-min pretreatment, rats received a single 180-s trial at 4 RPM. The duration of time (in s) spent on the rotarod during the drug trial was recorded for each rat. LY2456302 was administered at 10 and 30 mg/kg PO in a 2-ml/kg dose volume. Chlordiazepoxide was administered at 10 mg/kg PO (2-ml/kg dose volume). Data were analyzed using a one-way ANOVA and Dunnett's test (alpha set at 0.05) with vehicle-treated rats serving as the control group. Statistical analyses were performed in JMP (SAS Institute Inc., Cary, NC).

### 2.10.2. Ethanol-induced hypothermia

High doses of ethanol are known to reduce core body temperature in rats and humans (Kalant and Le, 1983). In order to get a preliminary assessment of potential pharmacodynamic interactions with ethanol, the effects of LY2456302 were assessed in combination with ethanol at doses that produce hypothermia. Subjects were male Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing approximately 250 g, housed four per cage and maintained on *ad libitum* food and water. Forty-eight rats (8 rats per group) were first dosed with either vehicle or 30 mg/kg LY2456302 (PO), followed immediately by an IP dose of either 0.9% saline or 1.5 g/kg (20% w/v) of ethanol in water. Immediately after the second dose, an initial rectal temperature ( $^{\circ}\text{C}$ ) was taken and recorded as the dependent measure using a rat rectal probe (RET-2, Physitemp Instruments Inc., Clifton, NJ). The rats were placed in their homecage for 60 min and a second rectal temperature was then taken. Body temperature data were analyzed via one-way ANOVA followed by a Dunnett's test post-hoc analysis using the ethanol-alone group as the control comparison.

## 3. Results

### 3.1. *In vitro* receptor binding and functional activity

LY2456302 (Fig. 1) was tested in filtration radioligand binding and GTP- $\gamma$ - $^{35}\text{S}$  functional assays using membranes prepared from HEK or CHO cells expressing cloned human mu-, kappa-, and delta-opioid receptor subtypes (Mitch et al., 2011). LY2456302 bound with high affinity to the human kappa opioid receptor with a 30-fold higher affinity over the human mu opioid receptor and 190-fold higher affinity over the human delta opioid receptor (Table 1; Mitch et al., 2011). By comparison, naltrexone had a higher *in vitro* binding affinity for the mu opioid receptor and lower affinities for kappa (3-fold) and delta (22-fold) opioid receptors. LY2456302 is a high-affinity antagonist at the human kappa opioid receptor with a 21-fold higher affinity over the human mu opioid

Table 2

Mean ( $\pm$ SEM)<sup>a</sup> pharmacokinetic and oral bioavailability data<sup>b</sup> for LY2456302.

Parameter	1 mg/kg IV	10 mg/kg PO
AUC (0– $t_{\text{last}}$ ; ng h/mL)	186 (6.7)	466 (117)
$C_{\text{max}}$ (ng/mL)	334 (30.3)	95 (31.8)
$t_{\text{max}}$ (h)	0 (0)	2.0 (0)
$t_{1/2}$ (h)	1.8 (0.1)	3.8 (1.8)
CL (mL/min/kg)	89.3 (3.1)	
% F		25.4 (0.1)

<sup>a</sup>  $n = 3$  cannulated rats/dose.

<sup>b</sup> Abbreviations: AUC(0– $t_{\text{last}}$ ) = area under the concentration vs. time curve from time zero to time  $t$ , where  $t_{\text{last}}$  is the last time point with a measurable concentration;  $C_{\text{max}}$  = maximum observed drug concentration;  $t_{\text{max}}$  = time to maximum observed drug concentration;  $t_{1/2}$  = half-life; CL = apparent total body clearance of drug; F = bioavailability.

receptor and nearly 135-fold higher affinity over the human delta opioid receptor in the GTP- $\gamma$ - $^{35}\text{S}$  assay (Table 1; Mitch et al., 2011). In contrast, naltrexone is a mu-preferring nonselective opioid antagonist, with 8-fold and 39-fold selectivity over kappa and delta receptors, respectively, in the GTP- $\gamma$ - $^{35}\text{S}$  assay. These results demonstrate the potency and selectivity of LY2456302 for the human kappa opioid receptor.

### 3.2. *In vitro* broad receptor profiling

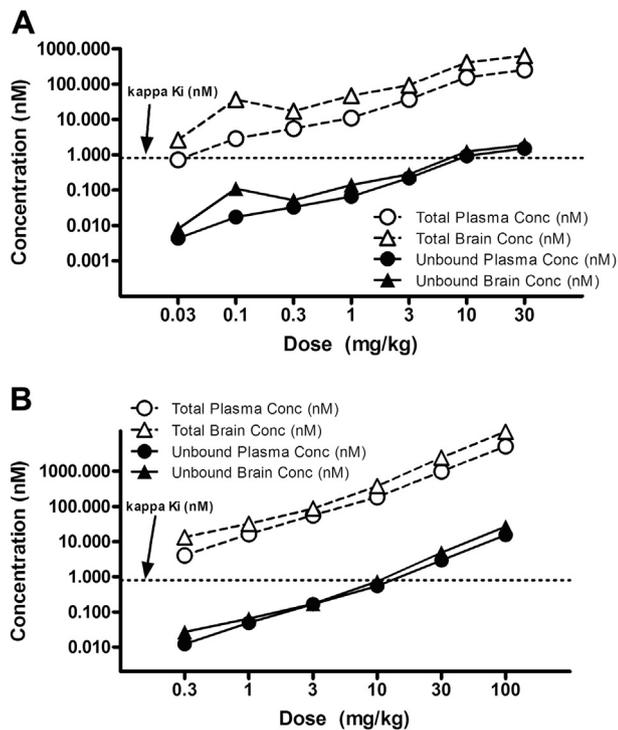
To assess receptor selectivity, LY2456302 was submitted to a panel of common cell surface receptor targets that were assayed under standard techniques (CEREP, Poitiers, France). At a concentration of 1  $\mu\text{M}$ , only mu opioid receptor activity exceeded 50% inhibition which was previously noted during in-house tests (data not shown). LY2456302 showed no appreciable affinity for several non-opioid cell surface G-protein-coupled receptor targets, including monoaminergic, muscarinic, cholinergic, and adrenergic receptors or ion channel/transporter binding targets or the central benzodiazepine binding site.

### 3.3. Rat and mouse plasma exposure and unbound fraction in plasma and brain

Pharmacokinetic and oral bioavailability data in rats for LY2456302 are summarized in Table 2. Plasma clearance following a single 1 mg/kg IV dose in rats was  $89.3 \pm 3.1$  mL/min/kg (Table 2). Mean maximum plasma concentrations following a single 10 mg/kg PO dose occurred at 2 h in rats. The mean plasma elimination half-life ( $t_{1/2}$ ) was  $3.8 \pm 1.8$  h following oral dosing. The absolute oral bioavailability in rats was  $25.4 \pm 0.1\%$ . In mice, the mean maximum plasma concentrations following a single 10 mg/kg oral dose occurred at 1 h post dose. The mean plasma elimination half-life ( $t_{1/2}$ ) was 2.2 h following oral dosing. Unbound fraction in plasma was determined to be 0.003 and 0.006 in mouse and rat, respectively. Unbound fraction in brain was determined to be 0.002 and 0.003 in mouse and rat, respectively. Dose–response curves of the total and unbound plasma and brain concentrations in rats and mice, respectively, are plotted in Fig. 2.

### 3.4. *In vivo* receptor occupancy

LY2456302 showed a dose-dependent increase in occupancy at kappa receptors in both rats and mice with a calculated  $\text{ED}_{50}$  of 0.33 mg/kg and greater than 90% occupancy at 10 mg/kg PO (Fig. 3A). The  $\text{ED}_{50}$  in mice was not calculated because the lowest dose tested (0.3 mg/kg) showed greater than 50% occupancy (Fig. 3B). The increasing levels of kappa receptor occupancy were driven by roughly proportional increases in plasma and brain

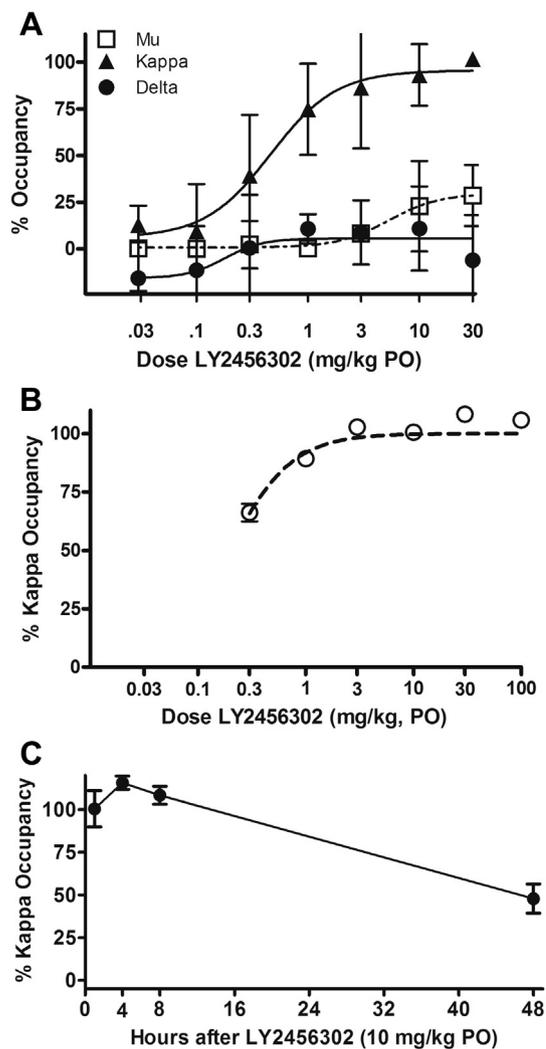


**Fig. 2.** Total and unbound concentrations of LY2456302 by dose. Dose–exposure curves in brain and plasma of male Sprague-Dawley rats (A) and male NIH-Swiss mice (B) following oral administration of LY2456302. Solid lines indicate total concentrations measured; dashed lines indicate unbound concentrations;  $n = 3$  per time point. Dotted horizontal line indicates binding affinity of LY2456302 at recombinant human kappa opioid receptors expressed in CHO cells from Table 1.

exposure of LY2456302 in both rats and mice (Supplemental Table S1). In contrast, LY2456302 showed minimal to no occupancy at mu and delta receptors up to 30 mg/kg PO in rats (not tested in mice). High levels of kappa receptor occupancy were maintained for up to 8 h, but declined to less than 50% 48 h after administration (Fig. 3C). These data demonstrate that LY2456302 selectively occupies kappa opioid receptors in rat striatum.

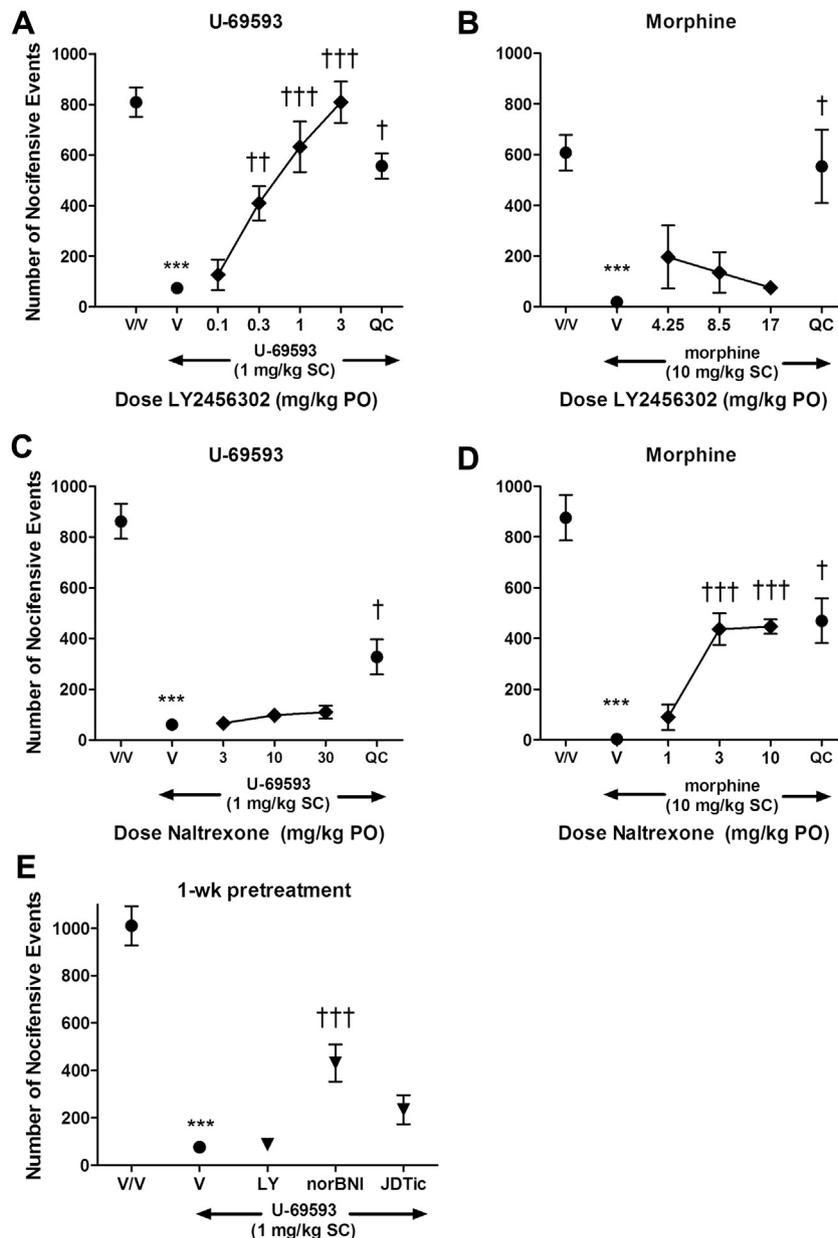
### 3.5. Reversal of agonist-induced analgesia

To determine the functional selectivity of LY2456302 *in vivo*, the ability of LY2456302 and naltrexone to reverse mu- and kappa-agonist-induced analgesia was evaluated. U-69593 significantly decreased the number of nocifensive events produced by administration of 5% formalin ( $F(6,65) = 17.33, p < 0.001$ ; Dunnett's post-hoc,  $p < 0.001$ ; Fig. 4A). LY2456302 (0.1–3.0 mg/kg; PO) produced a dose-dependent reversal of the analgesic effects of U-69593, with a dose of 0.3 mg/kg producing a significant reversal, and a calculated  $ED_{50} = 0.4$  mg/kg PO ( $F(6,65) = 17.33, p < 0.001$ ; significant reversal indicated by daggers). The quality control (3 mg/kg PO LY2048798) also significantly reversed U-69593-induced analgesia by about 50%, as expected ( $p < 0.05$ , Dunnett's post-hoc test). Morphine significantly decreased the number of nocifensive events produced by formalin administration,  $F(5,42) = 7.93, p < 0.0001$  (Fig. 4B). However, LY2456302 (4.25–17.0 mg/kg) did not attenuate the morphine-induced analgesic effects ( $p > 0.05$ ; Fig. 4B). The quality control (0.6 mg/kg PO LY2048798) significantly reversed morphine-induced analgesia ( $p < 0.05$ , Dunnett's post-hoc test; Fig. 4B). Based on an  $ED_{50}$  calculation for kappa agonist reversal, the lack of effect at 17 mg/kg for mu-agonist-induced analgesia indicates at least 43-fold selectivity of LY2456302 for kappa- over



**Fig. 3.** *In vivo* opioid receptor occupancy. (A) Dose–occupancy curves for orally administered LY2456302 at mu, kappa and delta opioid receptors determined in a triple tracer receptor occupancy assay in male Sprague-Dawley rats. (B) Dose–occupancy curve for orally administered LY2456302 at kappa opioid receptors in male NIH-Swiss mice. (C) Time-course of kappa opioid receptor occupancy by 10 mg/kg LY2456302 in male Sprague-Dawley rats. Points represent the mean ( $\pm$ SEM) displacement of IV administration of tracer doses of naltrexone (10  $\mu$ g/kg), GR103545 (1.5  $\mu$ g/kg), or naltrexone (10  $\mu$ g/kg) bound to mu, kappa and delta receptors, respectively, as measured by LC/MS/MS;  $n = 4$ /dose.

mu-mediated functional effects *in vivo*. The mu-preferring antagonist naltrexone (3–30 mg/kg, PO) did not produce a significant reversal of the analgesic effects of U-69593 ( $p > 0.05$ ), although the positive control dose of naltrexone (0.2 mg/kg SC) was significant ( $p < 0.05$ , Dunnett's post-hoc test; Fig. 4C). However, against morphine-induced analgesia, naltrexone (1–10 mg/kg PO) produced a significant dose-dependent reversal ( $F(5,18) = 25.22, p < 0.0001$ ; Fig. 4D). The quality control (0.02 mg/kg SC naltrexone) also significantly reversed morphine-induced analgesia ( $p < 0.05$ , Dunnett's post-hoc test; Fig. 4B). In a separate experiment designed to measure long-lasting functional effects of the antagonists, LY2456302, nor-BNI, and JDTic were administered 1-wk prior to the formalin test. In that experiment, U-69593 produced significant analgesia ( $F(4,31) = 12.93, p < 0.0001$ ; Fig. 4E). As expected, 1-wk pretreatment with nor-BNI produced significant reversal of U-69593-induced analgesia ( $p < 0.0005$ , Dunnett's post-hoc test; Fig. 4E). In contrast, LY2456302 and JDTic failed to block U-69593-induced analgesia after 1-wk pretreatment ( $ps > 0.05$ ; Fig. 4E).



**Fig. 4.** Reversal of kappa- and mu-agonist-induced analgesia during Phase II of the formalin test in male Sprague-Dawley rats. Oral administration of LY2456302 potently and dose-dependently blocked the analgesic effects of U-69593 (1 mg/kg, SC) in the rat formalin assay, with a dose of 0.3 mg/kg producing a significant reversal and a calculated  $ED_{50} = 0.4$  mg/kg (A). QC = LY2048978 3 mg/kg PO. (B) In contrast, LY2456302 did not significantly reverse the analgesic effects of morphine (10 mg/kg, SC) at oral doses up to 17 mg/kg (a dose representing >40-fold selectivity over the calculated  $ED_{50}$  value for reversal of U-69593-mediated effects). QC = LY2048978 0.6 mg/kg PO. (C and D) In contrast, oral administration of the nonselective, mu-preferring opioid antagonist naltrexone produced a significant and dose-dependent reversal of morphine-induced analgesia, with significant reversal at 3 mg/kg (D; QC = naltrexone 0.02 mg/kg SC), but did not reverse U-69593-induced analgesia at oral doses up to 30 mg/kg (C; QC = naltrexone 0.2 mg/kg SC). (E) nor-BNI significantly reversed U-69593-induced analgesia after 1-wk pretreatment. In contrast, LY2456302 and JDtTic failed to reverse the analgesic effects of U-69593 following 1-wk pretreatment, indicating lack of long-duration functional effects at the kappa opioid receptor. Each point represents the mean ( $\pm$ SEM) number of nocifensive events after injection of 5% formalin into the paw;  $n = 8-12$ , except panel D ( $n = 4$  per group). QC = quality control: \*\*\* $p < 0.001$  vs. respective V/V control. † $p < 0.01$  vs. respective V control. †† $p < 0.001$  vs. respective V control.

### 3.6. Reversal of agonist-induced disruption of prepulse inhibition

#### 3.6.1. Experiment 1: prepulse inhibition procedure for effects of LY2456302 alone

First, the effect of PCP (3 mg/kg) or LY2456302 (3, 10, or 30 mg/kg) on PPI was evaluated. Analysis revealed main effects of Prepulse Intensity and Treatment [ $F(2,105) = 63.12$ ,  $p < 0.0001$  and  $F(4,105) = 9.29$ ,  $p < 0.0001$ , respectively]. Bonferroni's post-hoc comparisons on the main effect determined that PPI increased with higher prepulse intensities (regardless of Treatment) and rats

treated with PCP exhibited significantly lower PPI levels than did either vehicle or LY2456302 groups (Fig. 5A). LY2456302, when administered alone up to 30 mg/kg PO, did not significantly affect PPI relative to vehicle controls.

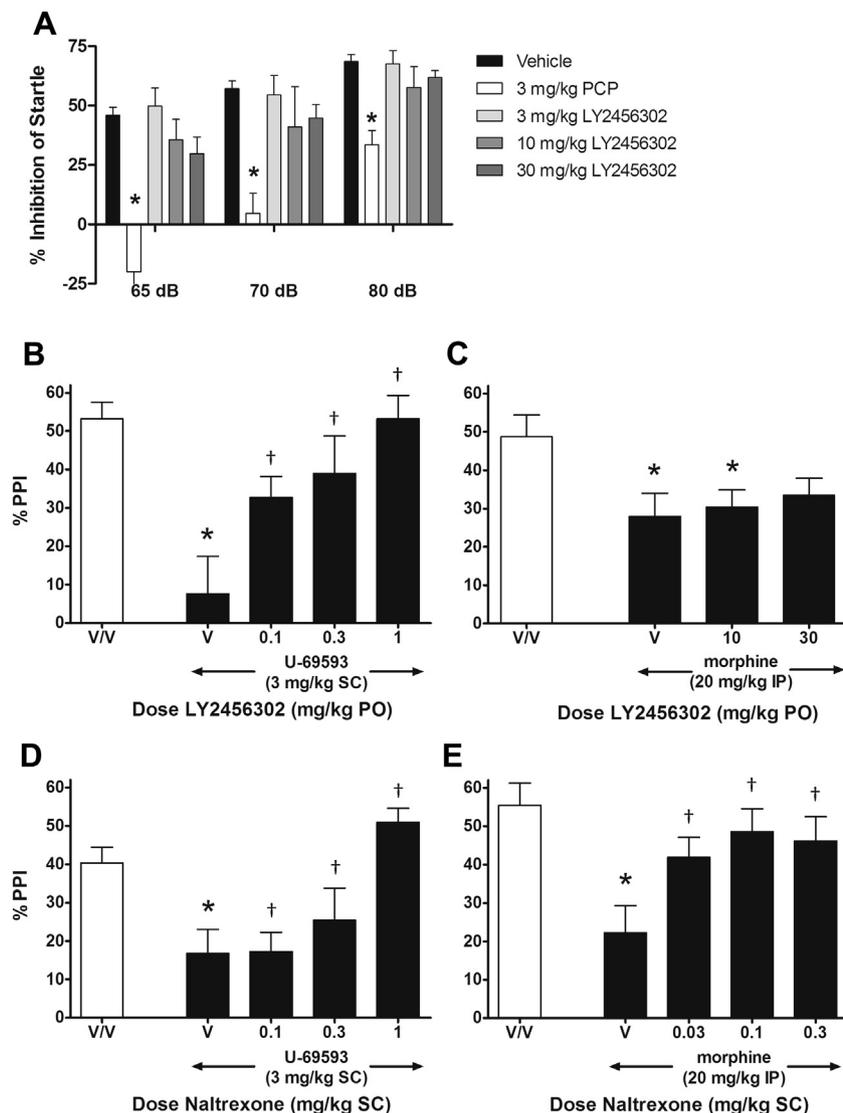
#### 3.6.2. Experiment 2: prepulse inhibition procedure for U-69593 and morphine reversal

In the kappa agonist challenge study, U-69593 produced a profound disruption of prepulse inhibition relative to vehicle-treated animals which was potently and dose-dependently

reversed by LY2456302 [ $F(4,35) = 6.38, p < 0.0006$ ]. Bonferroni's post-hoc comparisons revealed a significant attenuation of U-69593 effects at all 3 doses of LY2456302 tested (Fig. 5B). In contrast, LY2456302 at doses of 10 and 30 mg/kg did not significantly reverse PPI disruption induced by morphine, indicating excellent *in vivo* selectivity for kappa antagonism over mu [ $F(3,52) = 3.20, p < 0.03$ ]. Morphine significantly disrupted PPI relative to vehicle and LY2456302 failed to attenuate this effect (Fig. 5C). For comparison purposes, the effect of naltrexone on U-69593- and morphine-induced PPI disruption was also measured. Naltrexone potently blocked PPI disruptions produced by both U-69593 and morphine [ $F(4,35) = 6.91, p < 0.0003$  and  $F(4,35) = 4.23, p < 0.007$ , respectively]. Consistent with its mu-prefering receptor profile, a 1 mg/kg naltrexone dose significantly reversed U-69593 PPI disruption (Fig. 5D), whereas a dose as low as 0.03 mg/kg naltrexone blocked PPI deficits induced by morphine (Fig. 5E; Bonferroni-corrected post-hoc tests).

### 3.7. Mouse forced swim test

LY2456302 reduced immobility time in a dose-dependent manner, achieving efficacy at 10 mg/kg comparable to that of the tricyclic antidepressant imipramine (15 mg/kg, IP;  $F(4,31) = 6.79, p < 0.001$ ; Fig. 6A). In marked contrast to LY2456302, naltrexone did not significantly affect the time spent immobile ( $p > 0.05$ ; Fig. 6B). In a subsequent study, sub-active doses of LY2456302 (1 and 3 mg/kg) were combined with a sub-active dose of imipramine (5 mg/kg), producing significant antidepressant-like activity in both of the combination groups ( $F(6,49) = 19.99, p < 0.0001$ ; Fig. 6C). The maximal reduction of immobility produced by concurrent administration of 3 mg/kg of LY2456302 and 5 mg/kg of imipramine was comparable with that produced by 15 mg/kg of imipramine (Fig. 6C). In order to determine whether the combination with imipramine represented a better than additive effect (i.e. synergy), a synergy



**Fig. 5.** Prepulse inhibition. Oral administration of LY2456302 alone (3–30 mg/kg) did not disrupt prepulse inhibition (PPI), whereas phencyclidine (3 mg/kg, SC) significantly blunted PPI at all prepulse intensities, consistent with literature reports (A);  $*p < 0.05$  vs. respective vehicle control. (B–E) In reversal studies, oral administration of LY2456302 significantly and dose-dependently reversed the PPI deficits induced by U-69593 (3 mg/kg, SC; panel B), but not PPI deficits induced by morphine (20 mg/kg, IP) at doses up to 30 mg/kg (C), indicating selectivity of kappa- over mu-mediated effects. In contrast, the nonselective opioid antagonist naltrexone reversed morphine-induced PPI disruption with greater potency than U-69593-induced deficits (D; minimally efficacious dose = 0.03 vs. 1 mg/kg, respectively), consistent with its mu-prefering *in vitro* receptor profile. Data represent the mean (+SEM) inhibition of startle responding produced by the low intensity prepulse stimulus (65 dB;  $n = 8$  per group).  $*p < 0.05$  vs. respective V/V control.  $†p < 0.05$  vs. respective V/U or V/M control.

analysis was conducted using the method of Bliss Independence (Greco et al., 1995; Fitzgerald et al., 2006). Using the endpoint of % inhibition, an ANOVA was applied to test the coefficient of the interaction term in a  $2 \times 2$  full factorial model of the two compounds. Given that the interaction term was significant (full ANOVA results below), the determination that the effect was synergistic was made by comparing the effect size to the expected effect size if the two compounds were simply additive. In this case, the effect size was larger than what would have been expected if the agents were simply additive. Specifically, when 3 mg/kg LY2456302 was combined with an inactive dose of imipramine, the combination produced synergistic effects ( $F(1,21) = 17.53$ ,  $p < 0.0001$ ). The combination of 1 mg/kg LY2456302 with imipramine did not reach statistical significance for synergy ( $p = 0.0684$ ). In another study, a sub-active dose of LY2456302 (3 mg/kg) was combined with a sub-active dose of citalopram (3 mg/kg), producing significant antidepressant-like activity in the combination group ( $F(3,31) = 5.9$ ,  $p < 0.01$ ; Fig. 6D). When 3 mg/kg LY2456302 was combined with an inactive dose of citalopram, the combination produced synergistic effects ( $F(1,21) = 6.87$ ,  $p = 0.0483$ ).

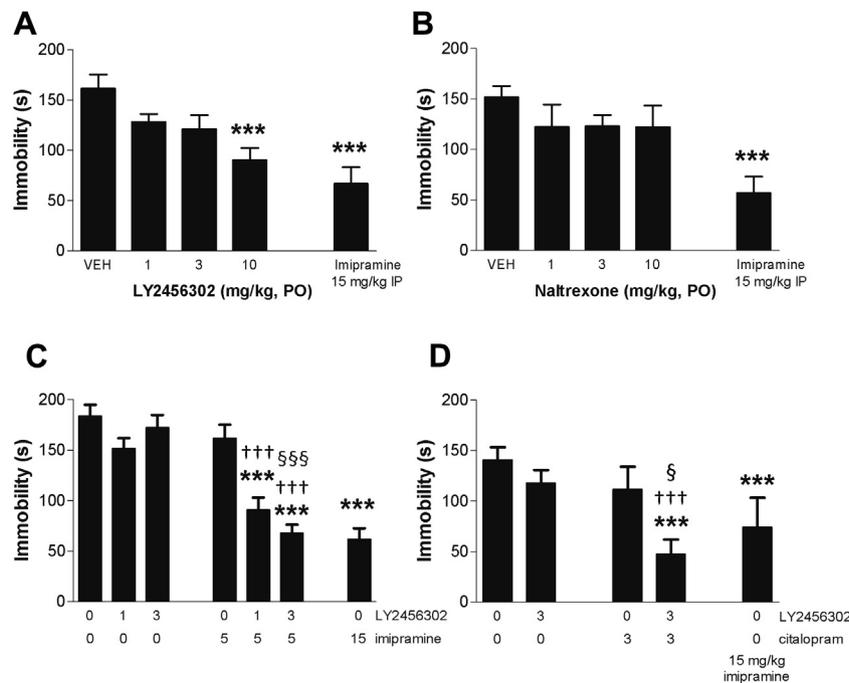
### 3.8. Ethanol self-administration in P rats

The ability of LY2456302 to reduce ethanol self-administration in P rats, an animal model of alcoholism, was assessed and compared with the mu-preferring opioid antagonist naltrexone. In the first experiment, rats received vehicle, 10 mg/kg naltrexone, or 3 or 10 mg/kg LY2456302. Following acute doses, a significant main effect of dose was observed on ethanol self-administration [ $F(3, 36) = 16.68$ ,  $p < 0.0001$ ; Fig. 7A]. LY2456302, at 3 mg/kg, exhibited

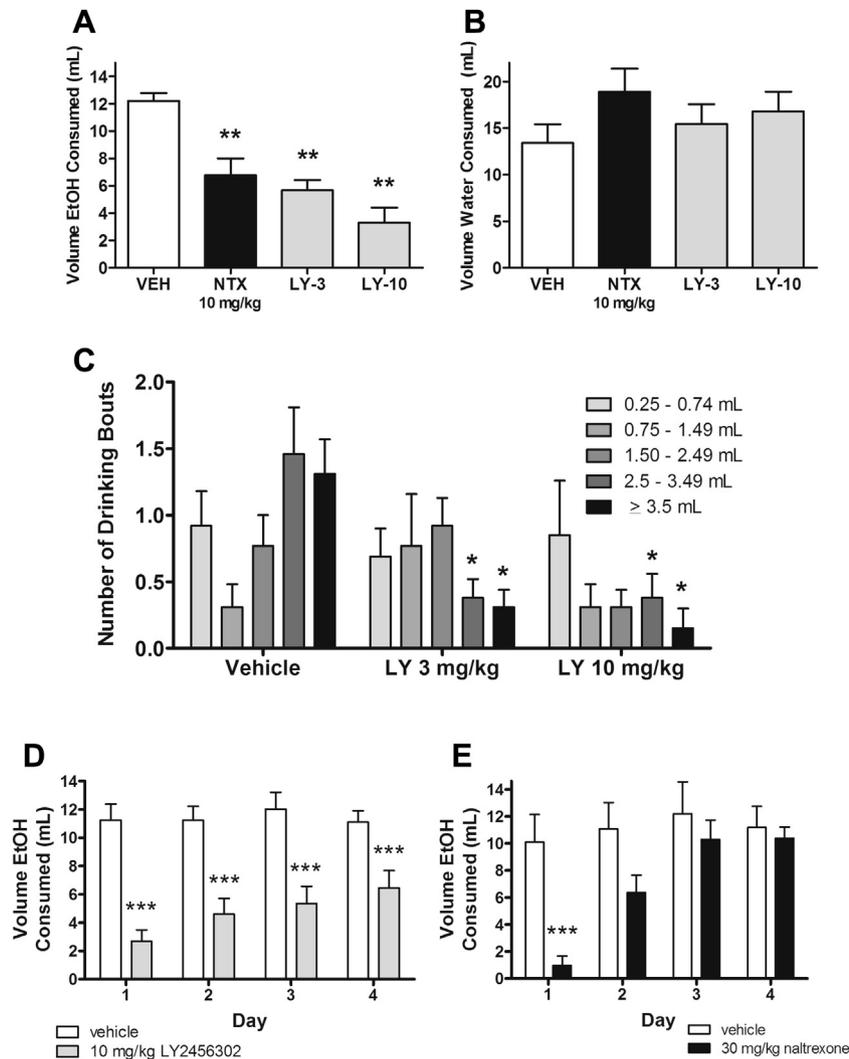
comparable efficacy to a 10 mg/kg dose of naltrexone (Fig. 7A). Neither LY2456302 nor naltrexone significantly altered water intake ( $p > 0.05$ ; Fig. 7B), food intake or locomotor activity ( $p_s > 0.05$ ; data not shown). Consistent with previous reports on the drinking patterns of P rats (Kampov-Polevoy et al., 2000), large bouts (2.5 mL or greater; Fig. 7C) of ethanol intake were seen under vehicle treatment conditions. Besides reducing total 12-h ethanol intake, LY2456302 selectively reduced the number of large bouts of ethanol intake (2.5 mL–3.49 mL, and 3.5+ mL) without affecting more moderate ethanol intake (i.e., bouts of 1.5 mL or less; Wilcoxon Signed Ranks Test,  $p < 0.05$ ; Fig. 7C).

In a subsequent study, the ability of LY2456302 or naltrexone to attenuate ethanol intake following 4 consecutive days of daily administration was evaluated. Naltrexone (30 mg/kg) significantly decreased ethanol intake relative to vehicle control on the first day, but not on subsequent days (main effect of Treatment:  $F(1,36) = 14.32$ ,  $p < 0.001$ ; main effect of Day:  $F(3,36) = 5.681$ ,  $p < 0.005$ ; Interaction:  $F(3,36) = 2.9$ ,  $p < 0.05$ ; Fig. 7D), indicating rapid tachyphylaxis. Water and food intake were unaffected by naltrexone ( $p > 0.05$ ; data not shown). In contrast, 10 mg/kg LY2456302 significantly decreased ethanol intake on all four test days (main effect of Treatment:  $F(1,60) = 29.93$ ,  $p = 0.001$ ; main effect of Day:  $p = 0.06$ ; Interaction:  $p = 0.0791$ ; Fig. 7E). Water and food intake were unaffected by LY2456302 ( $p > 0.05$ ; data not shown).

The ability of LY2456302 to reduce progressive ratio operant responding for ethanol was assessed in P rats. Oral administration of LY2456302 reduced ethanol-seeking behavior, with a statistically significant reduction in active lever presses for ethanol and breakpoint at 10 mg/kg ( $F(3,36) = 3.56$ ,  $p = 0.024$  and  $F(3,36) = 3.4$ ,  $p < 0.05$ , respectively; Fig. 8) and g/kg ethanol consumed



**Fig. 6.** Forced swim test. (A) Oral administration of LY2456302 dose-dependently produced an antidepressant-like response, as indicated by reduced immobility responses in the mouse forced swim test. Intraperitoneally administered imipramine (15 mg/kg) served as the positive control. \*\*\* $p < 0.001$  vs. respective vehicle control. (B) Oral administration of naltrexone did not significantly attenuate immobility in the mouse forced swim test. Bars represent mean (+SEM) of  $n = 8$  mice per group, except imipramine ( $n = 4$  per group). \*\*\* $p < 0.001$  vs. respective vehicle control. (C) Co-administration of sub-active doses of LY2456302 (1 and 3 mg/kg) and a sub-active dose of imipramine (5 mg/kg) lead to a significant reduction of immobility. Statistical analysis indicated that the combination of 3 mg/kg LY2456302 plus 5 mg/kg imipramine constituted a synergistic effect of the combination. \*\*\* $p < 0.001$  vs. respective V/V control. ††† $p < 0.001$  vs. respective imipramine-alone control. §§§ $p < 0.001$  statistically synergistic effect relative to 3 mg/kg LY2456302 and 5 mg/kg imipramine. (D) Co-administration of a sub-active dose of LY2456302 (3 mg/kg) and a sub-active dose of citalopram (3 mg/kg) lead to a significant reduction of immobility. Statistical analysis indicated that the combination of 3 mg/kg LY2456302 plus 3 mg/kg citalopram constituted a synergistic effect. \*\*\* $p < 0.001$  vs. respective V/V control. ††† $p < 0.001$  vs. respective citalopram-alone control. § $p < 0.05$  statistically synergistic effect relative to 3 mg/kg LY2456302 and 3 mg/kg citalopram.



**Fig. 7.** Ethanol self-administration in Alcohol-Preferring (P) rats. Oral administration of LY2456302 (3 or 10 mg/kg) or naltrexone (10 mg/kg) significantly attenuated continuous (24-h) ethanol (15% v/v EtOH) self-administration in female P rats with a chronic history of high ethanol intake. Data represent the mean ethanol (A) or water (B) intake in mL (+SEM) from 13 rats per group, summed over the 12 h dark cycle. \*\* $p < 0.001$  for all dose-groups having lower ethanol intake relative to vehicle control. (C) LY2456302 affected the pattern of drinking by P rats. Specifically, 3 and 10 mg/kg LY2456302 preferentially reduced large drinking bouts exceeding 2.5 mL, without significantly affecting smaller bouts of ethanol intake. A "bout" was defined as any series of consecutive 5-min bins where ethanol was consumed and bout magnitude was calculated as the total ethanol consumed (in mL) during the 5-min bins comprising the bout. \* $p < 0.05$  vs. vehicle treatment. (D) LY2456302 significantly reduced ethanol intake on each of the 4 days of treatment. Data represent the mean ethanol intake (mL + SEM) from 11 rats per group, summed over the 12 h dark cycle. \*\*\* $p < 0.0001$  vs. vehicle-treated controls on the respective test day. (E) Naltrexone (30 mg/kg) significantly reduced ethanol intake on the first day of administration, but tolerance was observed on the 2nd day of dosing. Data represent the mean ethanol intake (mL + SEM) from 11 rats per group, summed over the 12 h dark cycle. \*\*\* $p < 0.0001$  vs. vehicle-treated controls on the respective test day.

( $F(3,36) = 3.99$ ,  $p = 0.01$ ; data not shown), relative to vehicle. In contrast to LY2456302 and consistent with the mu-preferring *in vitro* profile, naltrexone potentially reduced both ethanol seeking and consumption in the current experiment ( $F(3,30) = 26.94$ ,  $p < 0.001$ ; Fig. 8).

### 3.9. *In vivo* microdialysis

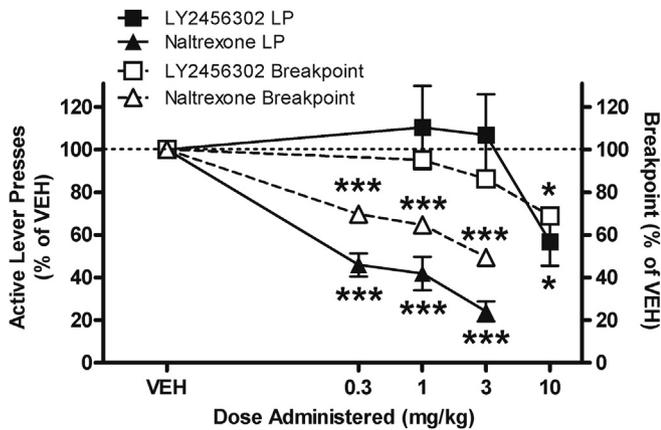
Kappa receptor agonists have been shown to decrease basal mesolimbic dopamine levels, and compounds that significantly elevate mesolimbic dopamine are rewarding in animals and abused in humans (Di Chiara and Imperato, 1988). Therefore, the effect of LY2456302 on extracellular DA levels in the nucleus accumbens shell (NAs) was examined using this one index of abuse potential in rats. LY2456302 did not alter extracellular DA levels at any of the doses tested (3, 10 or 30 mg/kg, PO; Fig. 9). Absolute baseline DA levels did not differ between any of the doses. The average baseline

DA level was  $1.07 \pm 0.15$  (mean  $\pm$  SEM) nM. To put the data into perspective, a 10 mg/kg SC dose of morphine produces a ~230% increase in DA release in the NAs for approximately 1–3 h post-administration (data not shown).

### 3.10. Side effect profiling

#### 3.10.1. Rotarod motor performance

Whereas 10 mg/kg chlordiazepoxide significantly reduced the number of sec rats were able to maintain balance on the rotarod relative to vehicle, LY2456302 did not affect motor performance on the rotarod at either 10 or 30 mg/kg PO (mean number of seconds remaining on the rotating rod ( $\pm$ SEM); 180 s maximum): vehicle =  $173.3 \pm 6.67$ , chlordiazepoxide =  $70.9 \pm 18.95$ , 10 mg/kg LY =  $149.7 \pm 18.74$ , 30 mg/kg LY =  $172.9 \pm 7.11$ ; overall ANOVA:  $F(3,31) = 5.8192$   $p < 0.003$ ; Dunnett's post-hoc: chlordiazepoxide vs. vehicle =  $p < 0.05$ ).



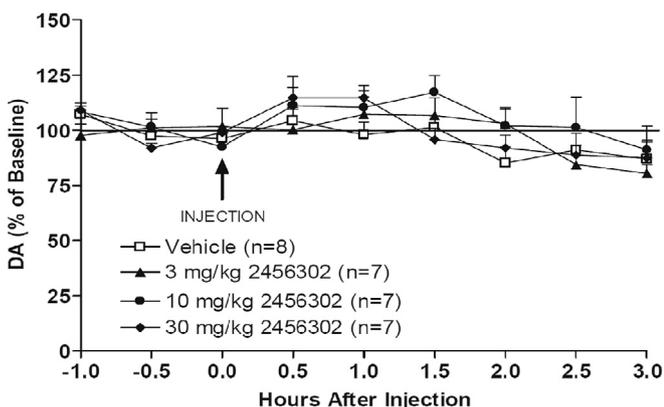
**Fig. 8.** Progressive ratio operant responding for ethanol in Alcohol-Preferring (P) rats. Both naltrexone and LY2456302 dose-dependently attenuated lever presses for ethanol (15% v/v; left y-axis) and breakpoints (right y-axis) in P rats maintained on a progressive ratio operant schedule of reinforcement. Data represent the mean ( $\pm$ SEM) number of lever presses on the active lever from  $n = 13$  subjects. Lever presses on the inactive lever were not affected at any dose of either naltrexone or LY2456302 ( $p > 0.05$ ; data not shown). \* $p < 0.05$  vs. respective vehicle treatment. \*\*\* $p < 0.001$  vs. respective vehicle treatment.

### 3.10.2. Ethanol-induced hypothermia

Consistent with previous literature reports, 1.5 g/kg ethanol produced a significant hypothermic response in rats, an effect which was not significantly affected by 30 mg/kg LY2456302, which did not alter thermoregulation on its own (core body temperature group means in degrees C ( $\pm$ SEM): vehicle =  $37.89 \pm 0.11$ , 30 mg/kg LY2456302 =  $37.51 \pm 0.16$ , 1.5 g/kg ethanol =  $36.48 \pm 0.16$ , 30 mg/kg LY2456302 + 1.5 g/kg ethanol =  $36.56 \pm 0.27$ ; overall ANOVA:  $F(3, 28) = 14.42$ ,  $p < 0.01$ ; Dunnett's post-hoc: both ethanol vs. vehicle and LY + ethanol vs. vehicle =  $p < 0.05$ ).

## 4. Discussion

LY2456302 is a structurally-unique, high-affinity, selective antagonist at the kappa opioid receptor (Diaz Buezo et al., 2009; Mitch et al., 2011), with approximately 30- and 200-fold selectivity over mu and delta opioid receptors, respectively, as assessed in *in vitro* binding and functional assays. LY2456302 had no



**Fig. 9.** *In vivo* microdialysis in the nucleus accumbens shell in rats. Oral administration of LY2456302 did not significantly alter basal extracellular dopamine levels in the nucleus accumbens shell at any dose tested, suggesting low propensity for abuse liability, based on this one index of abuse potential. Data are expressed as percent of baseline (mean  $\pm$  SEM) from  $n = 7-8$  rats per dose. Baseline dopamine values did not differ between any of the groups ( $p > 0.90$ ; overall mean =  $1.07 \pm 0.15$  nM).

appreciable affinity at several non-opioid cell surface receptor targets, including monoaminergic, muscarinic, cholinergic, and adrenergic receptors, as well as the central benzodiazepine binding site, ion channels, or transporters. LY2456302 selectively and potently occupied central kappa opioid receptors *in vivo*, with an  $ED_{50}$  of 0.33 mg/kg PO in rats without evidence of mu or delta receptor occupancy at doses up to 30 mg/kg.

In contrast to the prototypical literature kappa antagonists, nor-BNI and JD1c, LY2456302 exhibits rapid absorption, good oral bioavailability ( $F = 25\%$ , 1–2 h  $t_{max}$ ) and a more typical rate of clearance (2–4 h plasma half-life). Nor-BNI and JD1c have demonstrated pharmacokinetic and pharmacodynamic effects for multiple weeks (Melief et al., 2011; Munro et al., 2012; Patkar et al., 2013), and exhibit sustained receptor occupancy for up to 4 weeks after a single administration (Rorick-Kehn et al., unpublished observations). In contrast, kappa receptor occupancy of LY2456302 was reduced to  $<50\%$  within 48 h after administration, indicating more canonical pharmacodynamic effects. We confirmed this by testing the ability of LY2456302 to produce long-duration pharmacodynamic effects in the rat formalin test. One week after administration, LY2456302 failed to reverse U-69593-induced analgesia (Fig. 4E), whereas nor-BNI produced the expected long-duration antagonism, consistent with literature reports. *In vivo* assays indicated that LY2456302 had excellent functional selectivity over mu-mediated effects, with no evidence of appreciable mu receptor occupancy or associated pharmacodynamic effects at doses up to 30 mg/kg. LY2456302 potently blocked U-69593-mediated analgesia at doses as low as 0.3 mg/kg, without affecting morphine-mediated analgesia up to 17 mg/kg ( $>40$ -fold higher than the kappa  $ED_{50}$ ). Similarly, oral administration of LY2456302 reversed U-69593-induced disruption of prepulse inhibition without affecting morphine-induced disruption up to 30 mg/kg. In contrast, consistent with its mu-preferring nonselective opioid antagonist *in vitro* profile, naltrexone preferentially blocked mu-agonist-mediated analgesia and PPI disruption, while blockade of kappa-agonist-mediated behaviors was observed at higher doses. Taken together, these findings document a novel, drug-like small molecule with good *in vitro* and *in vivo* potency and selectivity. The high oral bioavailability of LY2456302 combined with its ability to selectively occupy and functionally block kappa opioid receptors, without producing long-duration pharmacodynamic effects, confirm that this molecule is a novel and valuable research tool for further preclinical and clinical investigation.

LY2456302 demonstrated efficacy in the mouse forced swim test, an animal model predictive of antidepressant-like efficacy, at doses that saturate kappa receptors but maintain selectivity over mu receptors. The magnitude of effect at the 10 mg/kg dose of LY2456302 was similar to that produced by an optimal dose of the tricyclic antidepressant, imipramine. These data are consistent with previous reports that kappa antagonists produced antidepressant-like effects in animal models (Mague et al., 2003; McLaughlin et al., 2003; Carr et al., 2010). The mechanism by which LY2456302 produces efficacy similar to imipramine is unknown, but may be related to blockade of CREB phosphorylation in the nucleus accumbens. Indeed, desipramine and fluoxetine were reported to block stress-induced dynorphin mRNA and CREB phosphorylation in the nucleus accumbens (Chartoff et al., 2009). Additional evidence for the role of kappa opioid receptor blockade in the antidepressant-like effects of LY2456302 comes from the finding that the mu-preferring opioid antagonist naltrexone did not exhibit significant antidepressant-like effects. Indeed, there has been little evidence of antidepressant activity in individuals taking naltrexone. To the contrary, naltrexone has been reported to produce dysphoria in both humans and rodents (Mendelson et al., 1978; Hollister et al., 1981; West and Wise, 1988). The

concordance of these data with those obtained with long-acting kappa antagonists (nor-BNI and JDTic) adds important and needed support of the hypothesis that kappa opioid receptor blockade might result in antidepressant actions.

Interestingly, when sub-eficacious doses of LY2456302 were combined with a sub-eficacious dose of imipramine or citalopram, the combination produced robust antidepressant-like efficacy and even synergy in some cases, suggesting perhaps that LY2456302 may augment antidepressant efficacy in patients who do not achieve adequate reduction of depressive symptoms on standard monoaminergic antidepressant medications. These data are particularly interesting in light of recent reports that augmentation with the functional kappa antagonist, ALKS-5461, produced antidepressant effects in treatment-resistant depressed patients in a small Phase 1b study and subsequent Phase 2 trial (Fava et al., 2012; Harrison, 2013). Despite the complex pharmacology of ALKS-5461, which is a combination of buprenorphine and a proprietary mu antagonist (ALKS-33), it is tempting to speculate that the potent kappa antagonist properties of that combination contribute to its reported antidepressant efficacy.

Our understanding of the role of the kappa opioid system in mediating mood has grown substantially in the last decade. Kappa-selective opioid agonists, including U-69593 and U-50488, produce anhedonia and a prodepressive phenotype in rodent models, including intracranial self-stimulation and forced swim test (McLaughlin et al., 2003; Mague et al., 2003; Todtenkopf et al., 2004). In humans, kappa agonist administration (e.g., MR2034 or salvinorin A) produces robust dissociative effects and cognitive disruption that partially, but not completely, overlap with symptoms associated with classic serotonergic hallucinogens (Maclean et al., 2013; Pfeiffer et al., 1986). The effects of MR2034 were blocked by naloxone, indicating opioid-specific pharmacological effects (Pfeiffer et al., 1986). Moreover, the prodepressive phenotype produced by stress or kappa receptor activation in rodents is blocked by kappa antagonists or by genetic deletion of dynorphin or the kappa opioid receptor (Todtenkopf et al., 2004; Mague et al., 2003; McLaughlin et al., 2003; Beardsley et al., 2005; Carr et al., 2010). The mechanism by which kappa antagonists produce antidepressant-like effects has not been fully elucidated, but may involve the transcription factor cAMP-response element binding protein (CREB) and MAP kinase signaling cascades (Pliakas et al., 2001; Kreibich and Blendy, 2004; Bruchas et al., 2011).

In Alcohol-Preferring (P) rats, LY2456302 potentially reduced ethanol self-administration under free-access conditions, without affecting food/water intake or locomotor activity. P rats represent a robust model system in which to study alcohol-seeking behavior as they meet all perceived criteria for an animal model of alcoholism (Cicero, 1980). For instance, P rats voluntarily consume high amounts of ethanol which cannot be attributed to taste or caloric properties of ethanol. P rats will work for ethanol access in operant paradigms and, when allowed chronic, voluntary access to ethanol, will develop tolerance and dependence (Waller et al., 1982). Consistent with previous reports (Kampov-Polevoy et al., 2000), P rats showed high levels of ethanol binge-drinking behaviors (>2.5 mL per bout) after vehicle administration, which were blocked by 3 and 10 mg/kg LY2456302. After sub-chronic dosing, LY2456302 exhibited significantly less tolerance than naltrexone in its ability to reduce high ethanol drinking behavior. In P rats maintained on a progressive ratio operant schedule of reinforcement, LY2456302 significantly reduced active lever responding, breakpoints, and ethanol consumption following a 10 mg/kg dose, whereas naltrexone significantly attenuated behaviors at 0.3–3 mg/kg, consistent with its mu-preferring antagonist profile. It is notable that LY2456302 more potently attenuated ethanol self-administration in P rats with continuous

free access to ethanol in the homecage, relative to rats maintained on a progressive ratio operant schedule with limited daily access to ethanol ( $\leq 1$  h/day for 5 days/week). Although not directly measured here, it is tempting to speculate that the rats with free-access to ethanol in the homecage developed ethanol dependence, whereas the rats with limited access to ethanol only in the operant chambers did not. The data presented here are consistent with previous reports that nor-BNI significantly attenuated ethanol self-administration in ethanol-dependent, but not non-dependent, Wistar rats (Walker and Koob, 2008). Although it is not known whether P rats exhibit an inherent alteration in the kappa opioid system or whether the chronic ethanol drinking history of the free-choice P rats produced changes in kappa opioid receptor density or function, the OPRK1 gene has been associated with increased risk for alcohol dependence (Edenberg et al., 2008; Xuei et al., 2006; Butelman et al., 2012).

It has been well-established that an increase in nucleus accumbens dopamine release is a common mechanism among drugs of abuse (Di Chiara and Imperato, 1988). Previous literature indicates that kappa agonists decrease dopamine cell firing in the VTA and dopamine release in nucleus accumbens (Spanagel et al., 1992; Margolis et al., 2003), whereas reverse microdialysis of nor-BNI increased extracellular levels of dopamine in the nucleus accumbens (Spanagel et al., 1992). Moreover, constitutive deletion of kappa receptors resulted in elevated basal dopamine tone and enhanced cocaine-stimulated dopamine release in the nucleus accumbens (Chefer et al., 2005). Therefore, robust effects on dopamine efflux could be a potential abuse liability issue in the development of kappa antagonist medications. Importantly, LY2456302 did not affect basal extracellular dopamine levels in the nucleus accumbens, suggesting low abuse potential. These data are consistent with previous observations that kappa antagonists did not, by themselves, affect intracranial self-stimulation thresholds (a model used as a surrogate to measure activation of the brain reward circuit) at doses that produced antidepressant-like efficacy (Todtenkopf et al., 2004). Although no changes were observed in dopamine efflux, stimulated dopamine release is just a single index of potential abuse liability. Further studies will be required to fully explore the abuse liability potential of LY2456302.

#### 4.1. Conclusions

The preclinical data from the present series of experiments add key data to the emerging literature showing that selective kappa opioid antagonists produce antidepressant-like efficacy and reduce excessive ethanol consumption. Our data and those from the scientific literature suggest promise for a kappa opioid antagonist as an efficacious novel therapeutic for the treatment of major depressive disorder. In addition, the present findings, combined with literature data, also support the idea that blockade of kappa opioid receptors might be a viable treatment for alcohol use disorders by promoting reduction of drinking and/or initiation of abstinence. To date, no medications have been approved to treat patients who suffer from both major depressive disorder and alcohol use disorder. Considering the high prevalence rates of comorbidity between major depression and alcohol use disorder, and the negative health and social consequences of the comorbid condition, a significant unmet medical need exists. The data presented here support the development of selective kappa opioid antagonists as novel therapeutics for the treatment of major depressive disorder and alcohol use disorder. In addition, the approach could also afford the unprecedented opportunity for medication development for the comorbid depressed alcoholic patient.

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## Authorship contributions

*Participated in research design:* Rorick-Kehn, Statnick, Witkin, Kahl, Cramer, Mitch, D McKinzie, Forster.

*Conducted experiments:* Rorick-Kehn, Crile, Shaw, Sahr, J McKinzie, Eberle, Adams, Li, Kahl, D McKinzie, Forster.

*Contributed new reagents or analytic tools:* Quimby, Diaz, Jimenez, Pedregal, Mitch, Anderson, D McKinzie.

*Performed data analysis:* Rorick-Kehn, Crile, Shaw, Sahr, J McKinzie, Eberle, Adams, Li, Kahl, Anderson, Cramer, D McKinzie, Witkin, Forster.

*Wrote or contributed to the writing of the manuscript:* Rorick-Kehn, Witkin, Statnick, Wong, Statnick, Kahl, Anderson, Cramer, J McKinzie, Eberle, Knopp, D McKinzie.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2013.09.021>.

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