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NMDA RECEPTOR ACTIVATION-DEPENDENT ANTIDEPRESSANT-RELEVANT BEHAVIORAL AND SYNAPTIC ACTIONS OF KETAMINE

Abbreviated title: NMDAR activation-dependent actions of ketamine

Authors: Panos Zanos[1,2,5,6,*], Kyle A. Brown[1], Polymnia Georgiou [1,3], Peixiong Yuan[4], Carlos A. Zarate Jr[4], Scott M. Thompson[1,5,6], Todd D. Gould[1,7,8,9,*]

Affiliations:

[1] Department of Psychiatry, School of Medicine, University of Maryland, Baltimore, MD, USA.
[2] Department of Psychology, University of Cyprus, Nicosia, 2109, Cyprus.
[3] Department of Biology, University of Cyprus, Nicosia, 2109, Cyprus.
[4] Experimental Therapeutics and Pathophysiology Branch, Intramural Research Program, National Institute of Mental Health, National Institutes of Health, Bethesda, Maryland, USA.
[5] Department of Physiology, School of Medicine, University of Maryland, Baltimore, MD, USA.
[6]Current address: Department of Psychiatry, University of Colorado School of Medicine, Aurora, CO, USA.

[7] Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, MD, USA.
[8] Department of Anatomy & Neurobiology, School of Medicine, University of Maryland, Baltimore, MD, USA.
[9] Veterans Affairs Maryland Health Care System, Baltimore, MD, 21201, USA.

***Correspondence to**:

Todd D. Gould
gouldlab@me.com
MSTF 936
685 W. Baltimore Street,
University of Maryland,
Baltimore, MD, 21201
+1 (410) 706-5585
AND
Panos Zanos
zanos.panos@ucy.ac.cy
FEB 02
1 Panepistimiou Avenue,
University of Cyprus,
Nicosia, Cyprus
+357-22892243

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ABSTRACT

Ketamine is a well-characterized *N*-methyl-D-aspartate receptor (NMDAR) antagonist, although the relevance of this pharmacology to its rapid (within hours of administration) antidepressant actions, which depend on mechanisms convergent with strengthening of excitatory synapses, is unclear. Activation of synaptic NMDARs is necessary for the induction of canonical long-term potentiation (LTP) leading to a sustained expression of increased synaptic strength. We tested the hypothesis that induction of rapid antidepressant effects requires NMDAR activation, by utilizing behavioral pharmacology, western blot quantification of hippocampal synaptoneurosomal protein levels, and *ex vivo* hippocampal slice electrophysiology in male mice. We found that ketamine exerts an inverted U-shaped dose-response in antidepressant-sensitive behavioral tests, suggesting that an excessive NMDAR inhibition can prevent ketamine's antidepressant effects. Ketamine's actions to induce antidepressant-like behavioral effects, up-regulation of hippocampal AMPAR subunits GluA1 and GluA2, as well as metaplasticity measured *ex vivo* using electrically-stimulated LTP, were abolished by pretreatment with other non-antidepressant NMDAR antagonists, including MK-801 and CPP. Similarly, the antidepressant-like actions of other putative rapid-acting antidepressant drugs (*2R,6R*)-hydroxynorketamine (ketamine metabolite), MRK-016 ($GABA_A\alpha5$ negative allosteric modulator), and LY341495 ($mGlu_{2/3}$ receptor antagonist) were blocked by NMDAR inhibition. Ketamine acted synergistically with an NMDAR positive allosteric modulator to exert antidepressant-like behavioral effects and activation of the NMDAR subunit GluN2A was necessary and sufficient for ketamine-like antidepressant-like behavioral effects. We conclude that NMDAR activation is necessary for the beneficial effects of ketamine and other rapid-acting antidepressant compounds. Promoting NMDAR signaling or other approaches that enhance NMDAR-dependent LTP-like synaptic potentiation may be an effective antidepressant strategy.

SIGNIFICANCE

The anesthetic and antidepressant drug ketamine is well-characterized as an N-methyl-D-aspartate receptor (NMDAR) antagonist; though, the relevance and full impact of this pharmacology to its antidepressant actions is unclear. We found that NMDAR activation is necessary for the beneficial effects of ketamine and several other putative antidepressant compounds. As such, promoting NMDAR signaling, or other approaches that enhance NMDAR-dependent LTP-like synaptic potentiation in vivo may be an effective antidepressant strategy directly, or acting synergistically with other drug or interventional treatments.

INTRODUCTION

Depressive symptoms, including low mood, anhedonia, and suicidal ideation, can be improved within hours following initiation of pharmacological treatment with racemic (*R*,*S*)-ketamine (ketamine), and similar effects are also observed with ketamine's (*S*)-ketamine enantiomer (Berman et al., 2000; Zarate et al., 2006b; Murrough et al., 2013; Daly et al., 2018; Fava et al., 2020). Ketamine is also effective in patients who are resistant to the beneficial effects of chronic standard treatments (Zarate et al., 2006b; Murrough et al., 2013; Fava et al., 2020). Despite this potential, the development of novel rapid-acting antidepressants is hampered by an incomplete understanding of the mechanism(s) by which ketamine exerts its therapeutic actions (Gould et al., 2019; Krystal et al., 2019).

Ketamine is a well-characterized *N*-methyl-D-aspartate receptor (NMDAR) antagonist, acting non-competitively as an open channel blocker (Anis et al., 1983). Ketamine has long been used as an anesthetic agent, with evidence supporting NMDAR inhibition as a primary mechanism underlying these anesthetic effects, as well as its dissociative side-effects and abuse potential (Zanos et al., 2018b). Hypotheses regarding ketamine's antidepressant mechanism of action have mainly focused on potential NMDAR inhibition-mediated processes. In particular, it has been hypothesized that ketamine acts rapidly to treat depression by *(i)* preferential inhibition of NMDARs localized to GABAergic interneurons leading to selective disinhibition of excitatory glutamatergic neurons, increased glutamate release, and the resultant increase in synaptic strength (Duman, 2014; Krystal et al., 2019), or *(ii)* transient ketamine-mediated inhibition of spontaneously activated NMDARs, which results in a homeostatic reset of synaptic strength (Kavalali and Monteggia, 2020, 2022). Both these hypotheses converge on mechanisms

that lead to an increase in synaptic strength of excitatory neuronal circuits, which are weakened in affective disorders (Zanos et al., 2018a; Thompson, 2022).

However, clinically, other NMDAR open channel blockers, such as memantine and AZD-6765, do not exert the full antidepressant profile of ketamine (Zarate et al., 2006a; Smith et al., 2013; Zarate et al., 2013; Sanacora et al., 2014; Sanacora et al., 2017), nor do antagonists selective for the GluN2B subunit of the NMDAR (Preskorn et al., 2008; Ibrahim et al., 2012; Paterson et al., 2015; Cerecor, 2016), or the NMDAR glycine co-agonist site (Park et al., 2020). These human observations suggest that NMDAR inhibition cannot fully explain the rapid and robust antidepressant actions of ketamine. Alternative hypotheses for ketamine's antidepressant actions include distinct targets, such as opioid receptors (Zhang et al., 2021; Hess et al., 2022; Wulf et al., 2022), or a principal role of ketamine's biologically active metabolites including (*2R,6R*)-hydroxynorketamine (HNK), which is a weak NMDAR antagonist, to increase glutamate release probability (Zanos et al., 2016; Pham et al., 2018; Fukumoto et al., 2019; Aleksandrova et al., 2020; Riggs et al., 2020; Riggs et al., 2022; Wulf et al., 2022).

While there is considerable evidence of a correlation between rapid antidepressant actions and potentiation of excitatory synapses in affect-regulating neuronal circuits, ketamine actions to inhibit NMDAR function is difficult to reconcile, especially considering the critical role of NMDAR activity in the induction of synaptic potentiation. Activation of NMDARs is necessary for induction of canonical long-term potentiation (LTP) of excitatory synapses, leading to maintained upregulation of synaptic strength. Specifically, increased glutamatergic neurotransmission results in membrane depolarization mediated by postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) activation, the opening of the NMDAR following the release of the Mg^{2+} block, and Ca^{2+} influx that facilitates postsynaptic

plasticity including upregulation of synaptic AMPARs (Huganir and Nicoll, 2013). Such a mechanism converges with evidence that ketamine can indirectly increase glutamatergic neurotransmission (Moghaddam et al., 1997; Duman, 2014), resulting in downstream acute activation and subsequent maintained upregulation of AMPARs (Maeng et al., 2008). We thus tested the hypothesis that induction of rapid antidepressant effects requires NMDAR activation by using other NMDAR antagonists (i.e., MK-801 and CPP) prior to administration of ketamine and other putative rapid-acting antidepressant drugs. We also examined the role of synaptic metaplasticity and GluN2A expression in mediating the effects of ketamine.

MATERIALS AND METHODS

Animals

Male CD-1 mice (Charles River, Wilmington, MA), eight weeks of age, were housed in groups of 4-5 per cage upon arrival. Animals were acclimated to the vivarium (University of Maryland, Baltimore, MD) for at least one week after arrival and were maintained on a 12 hrs light/dark cycle. For the social defeat experiments, 8-9 week old male C57BL/6J mice (University of Maryland, Baltimore veterinary resources breeding colony) and retired male CD-1 breeders (Charles River Laboratories, NC, USA) were used. Food and water were provided *ad libitum*. All experiments were approved by the University of Maryland Baltimore Animal Care and Use Committee and were completed in accordance with the latest National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Materials

(*R,S*)-ketamine HCl and the NMDAR channel blocker MK-801 (Sigma-Aldrich, St. Louis, MO) were dissolved in 0.9% saline and administered intraperitoneally in a volume of 7.5 mL/kg of body mass. The negative allosteric modulator of $GABA_A$ receptors containing α5 subunits (GABA-NAM) MRK-016 (Tocris Bioscience, R&D Systems) was prepared in 100% DMSO and injected at the volume of 1.25 ml/kg i.p. (final volume was 40-50 μL). The competitive NMDAR antagonist (±)-CPP (Tocris Bioscience, R&D Systems), the GluN2A-preferring NMDAR antagonist PEAQX hydrochloride (NIMH Chemical Synthesis and Drug Supply Program), the positive NMDAR modulator rapastinel (Allergan Pharmaceuticals), and the mGlu2/3 receptor antagonist LY341495 disodium salt (Tocris Bioscience, R&D Systems) were dissolved in 0.9% saline and administered intraperitoneally in a volume of 7.5 mL/kg of body mass. The GluN2A-selective NMDAR positive modulator GNE-5729 was synthesized by

WuXi (Shanghai, China) and characterized by the National Center for Advancing Translational Sciences (NCATS) and was dissolved in a mixture of 10% DMSO, 10% cremaphor EL, 80% saline solution and given at the volume of 4 mL/kg. Ketamine's metabolite (*2R,6R*)-HNK was synthesized and characterized by NCATS, dissolved in 0.9% saline, and was administered intraperitoneally in a volume of 7.5 mL/kg of body mass. All additional chemicals and reagents used in this study, unless otherwise noted, were of analytical or higher grade obtained from Sigma-Aldrich.

Behavioral assays

All injections were performed by a male experimenter based on the findings of Georgiou et al. (2022).

Forced-swim test (FST). During the FST, mice were subjected to a 6-min swim session in clear Plexiglass cylinders (30 cm height x 20 cm diameter) filled with 15 cm of water (23 ± 1°C). The FST was performed in normal light conditions (800 Lux). Sessions were recorded using a digital video camera. Immobility time, defined as passive floating with no additional activity other than that necessary to keep the animal's head above water, was scored during the last 4 min of the 6-min test by a trained observer blind to the treatment groups. To assess interactions between inhibition of the NMDAR and antidepressant actions of ketamine and other putative rapid-acting antidepressants, an NMDAR antagonist or vehicle was administered 10 minutes prior to ketamine, its active metabolite (*2R,6R*)-HNK, the GABA-NAM MRK-016, the mGlu2/3 receptor antagonist LY341495, or saline, and mice were tested 24 hrs later to avoid acute effects of the drugs (MK-801 has been eliminated and does not have effects 24 hrs post-treatment (Wegener et al., 2011).

188 *Inescapable shock-induced escape deficits*. The inescapable shock-induced escape deficits (or
189 learned helplessness) paradigm consisted of three different phases: inescapable shock training,
190 escapable shock screening, and the escapable shock test. On Day 1, the animals were placed in
191 one side of two-chambered shuttle boxes (34 cm height x 37 cm width x 18 cm depth; Coulbourn
192 Instruments, PA, USA), with the door between the chambers closed. Following a 5-min
193 adaptation period, 120 inescapable foot-shocks (0.45 mA, 15 sec duration, pseudo-randomized
194 average inter-shock interval of 45 sec) were delivered through the floor. During the escapable
195 shock screening session (Day 2), the mice were placed in one of the two chambers of the
196 apparatus for 5 min. A shock (0.45 mA) was then delivered, and the door between the two
197 chambers was raised simultaneously. Crossing over into the second chamber terminated the
198 shock. If the animal did not cross over, the shock terminated after 3 sec. A total of 30 screening
199 trials of escapable shocks were presented to each mouse with an average of 30-sec delays
200 between each trial. Mice that developed escape deficit behavior (>5 escape failures during the
201 last 10 screening shocks) received the assigned drug in a randomized blinded manner 24 hrs
202 following screening (Day 3) and 24 hrs prior to testing. For the experiments assessing the effects
203 of NMDAR antagonists on the actions of ketamine, (*2R,6R*)-HNK, or LY341495, pretreatment
204 with MK-801 and/or CPP preceded treatment by 10 min. For the sub-effective dose treatment
205 experiments (synergistic effect experiment), rapastinel and ketamine were administered at the
206 same time. During the escapable shock test phase (Day 4), the animals were placed in the shuttle
207 boxes and, after a 5-min adaptation period, a 0.45 mA shock was delivered concomitantly with
208 door opening for the first five trials, followed by a 2-sec delay prior to the door opening for the
209 next 40 trials as we previously established this response was sensitive to ketamine treatment
210 (Zanos et al., 2015). Crossing over to the second chamber terminated the shock. If the animal did

not cross over to the other chamber, the shock was terminated after 24 sec. A total of 45 trials of escapable shocks were presented to each mouse with 30-sec inter-trial intervals. The number of escape failures was recorded for each mouse by computer software (Graphic State *v*3.1; Coulbourn Instruments, Whitehall, PA, USA).

Chronic social defeat stress (CSDS) and sucrose preference. The day prior to the social defeat phase of the experiment, male C57BL/6J mice were singly housed and presented with two identical bottles containing either tap water or 1% (w/v) sucrose solution for assessing their "baseline" sucrose preference. Then, experimental mice were introduced to the home cage (43 cm length x 11 cm width x 20 cm height) of a resident aggressive retired CD-1 breeder (prescreened for aggressive behaviors) for 10 min. Following this physical attack phase, mice were transferred and housed in the opposite side of the resident's cage divided by a perforated Plexiglas divider, to maintain continuous, 24 h, sensory contact. This process was repeated daily for 10 days, with experimental mice being introduced to a novel aggressive CD-1 mouse each day. Following day 10, for assessing the post-defeat sucrose preference, mice were singly housed and presented with two identical bottles containing either tap water or 1% (w/v) sucrose solution. Twenty-four hrs later, sucrose preference was measured and the mice that underwent social defeat stress were assigned to two groups: resilient (sucrose preference >70%) and susceptible (sucrose preference <55%). Only susceptible mice were treated with ketamine and the other compounds described below.

- *Ketamine experiment*: Susceptible mice were treated with saline or ketamine at the doses of 10 or 100 mg/kg and sucrose preference was measured for an additional 24 hrs.
- *MK-801 effects on ketamine's reversal of sucrose preference deficits:* A different cohort of mice underwent the social defeat paradigm (as described above). Susceptible mice

were treated with saline or MK-801 10 prior to treatment with saline or ketamine (10 mg/kg) and sucrose preference was measured for an additional 24 hrs.

Novel-object recognition test (NOR). The NOR test was carried out under dim light conditions (~3-5 Lux). The NOR behavioral testing consisted of three different sessions during the same day. During the habituation phase, the animals explored the NOR apparatus (40cm x 9cm x 23cm) for 10 min in the absence of objects. Immediately after, during the familiarization session and without taking the test mouse outside the boxes, two identical objects were carefully and silently fixed on the floor of the apparatus symmetrically 8.5 cm from the wall, and the animals were allowed to explore the objects for a further 10 min. The objects were either two 50-mL clear glass conical flasks (4.5 cm bottom diameter × 7 cm height) containing blue marbles or two white-painted small glass vials (2.5 cm bottom diameter × 6 cm height). After familiarization with the 'familiar' objects, mice were immediately returned to their home cages. Following a 50-min delay, mice were placed back into the NOR apparatus, in which one of the 'familiar' objects used during the familiarization session was replaced by a 'novel' object (retention phase). Mice were permitted to freely explore the objects for 10 min. During both familiarization and retention sessions, the objects were used in a counterbalanced between-groups manner. All the sessions were videotaped by an overhead digital video camera. The retention sessions were manually scored by a trained observer blind to the experimental groups, using the AnoStar scoring software (Cleversys Inc., VA, US). Mice were considered to be interacting with the objects when their head was facing the object in a pre-set distance of ≤ 1 cm. A discrimination index was calculated as the time a mouse was interacting with the novel object divided by the total time of interaction with both the objects during the retention phase (Bevins and Besheer, 2006).

Open-field test. This experiment was performed at 100 Lux. Mice were placed into individual open-field arenas (50 cm length x 50 cm width x 38 cm height; San Diego Instruments, CA, USA) for a 30-min locomotor assessment. Distance traveled was automatically analyzed using TopScan v2.0 (CleverSys, Inc, VA, USA).

Western blots

To purify synaptoneurosomes, mouse hippocampi were dissected and homogenized in Syn-PER Reagent (ThermoFisher Scientific, Waltham, MA, USA; Cat # 87793) with 1X protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Waltham, MA, USA; Cat # 78440). The homogenate was centrifuged for 10 min at 1,200 x g at 4 ºC. The supernatant was centrifuged at 15,000 x g for 20 min at 4 ºC. After centrifugation, the pellet (synaptosomal fraction) was resuspended and sonicated in N-PER Neuronal Protein Extraction Reagent (ThermoFisher Scientific, Waltham, MA, USA; Cat # 87792). Protein concentration was determined via the BCA protein assay kit (ThermoFisher Scientific, Waltham, MA, USA; Cat # 23227). An equal amount of protein (10-40 µg as optimal for each antibody) for each sample was loaded into NuPage 4-12% Bis-Tris gel for electrophoresis. Gel transfer was performed with the TransBlot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Nitrocellulose membranes with transferred proteins were blocked with 5% milk in TBST (TBS + 0.1% Tween-20) for 1 hr and kept with primary antibodies overnight at 4 ºC. The following primary antibodies were used: GluA1 (Cell Signaling Technology, Danvers, MA, USA; Cat # 13185) and GluA2 (Cell Signaling Technology, Danvers, MA, USA; Cat # 13607). The next day, blots were washed three times in TBST and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (1:5000 to 1:10000) for 1 hr. After the final three washes with TBST, bands were detected using enhanced chemiluminescence (ECL) with the Syngene Imaging System

(G:Box ChemiXX9). After imaging, the blots were incubated in the stripping buffer (ThermoFisher Scientific, Waltham, MA, USA; Cat # 46430) for 10-15 min at room temperature followed by three washes with TBST. The stripped blots were incubated in blocking solution for 1 hr and incubated with the primary antibody directed against the respective protein or GAPDH for loading control. Densitometric analysis of immunoreactive bands for each protein was conducted using Syngene's GenTools software. Protein levels were normalized to GAPDH. Fold change was calculated by normalization to a saline-treated control group for each protein.

Hippocampal slice preparation and electrophysiology

After one week of acclimation, nine-week-old mice were randomly assigned to one of four treatment groups [saline-saline (Sal-Sal); saline-ketamine (Sal-Ket); MK-801-saline (MK-Sal); MK-801-ketamine (MK-Ket)] and, at the same time daily (09:30), animals were treated with either saline or 0.1 mg/kg MK-801 followed by either saline or 10 mg/kg ketamine 10 min later. One mouse within each cage received each of the four treatments. Animals were anesthetized with isoflurane and sacrificed 24 hrs post-treatment.

Hippocampal slice preparation and electrophysiology experiments were conducted generally as previously described (Preston et al., 2019; Brown et al., 2021). Briefly, brains were removed and rapidly submerged in oxygenated (95% O_2 / 5% CO_2), ice-cold dissection artificial cerebrospinal fluid (ACSF; 120mM NaCl, 3mM KCl, 4mM $MgCl_2$, 1mM NaH_2PO_4, 26mM $NaHCO_3$, and 10mM glucose). The brain was mounted on its dorsal surface and sectioned along the horizontal plane with a vibratome to acquire 400 µm slices containing the hippocampus. The hippocampus was subdissected free from the rest of the slice and the CA3 subfield was removed. These slices were then quickly placed in a humidified holding chamber at room temperature (20-22°C). Following 90 min of recovery, slices were transferred to a submersion-type chamber and

continuously perfused (1.5 mL/min; Ismatec Reglo ICC Digital Pump; Cole-Parmer, Vernon Hills, IL) with oxygenated (95% O_2 / 5% CO_2) ACSF (120mM NaCl, 3mM KCl, 1.5mM $MgCl_2$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 26mM $NaHCO_3$, and 10mM glucose) during recording experiments. Schaffer collateral fibers were stimulated by placing a bipolar electrode (100 μs duration at 0.05 Hz; FHC, Bowdoin, ME) in the stratum radiatum of the CA1 subfield and an ACSF-filled glass recording pipette (3-5 MΩ; World Precision Instruments, Sarasota, FL) recorded a field excitatory postsynaptic potential (fEPSP) in the same layer of CA1.

The stimulus intensity was then modified to elicit 35% of the maximal fEPSP slope and paired-pulse fEPSPs (50 ms interpulse interval) were recorded each min for five min. Following paired-pulse recordings, individual stimulus pulses were applied every 20 s for 10 min and baseline fEPSP responses were monitored. After recording baseline responses for 10 min, a high-frequency stimulation (HFS) protocol (4x100 Hz/1 s train at 20 s intervals) induced long-term potentiation, and fEPSP responses were monitored for the subsequent 60 min.

Data Analysis

Experimental groups were executed and analyzed by an experimenter blind to treatment groups. Sample sizes were based upon our prior experience using the same paradigms. Distance traveled, immobility time, and escape failures following administration of different doses of the GluN2A-selective NMDAR positive allosteric modulator GNE-5729, as well as escape failures (learned helplessness) and discrimination index (novel-object recognition) after administration of different doses of ketamine were analyzed via one-way ANOVA. Effects of NMDAR antagonists on the antidepressant-like behavioral (forced-swim test and learned helplessness), biochemical (western blots), or novel-object recognition effects of ketamine or the other putative rapid-acting antidepressant drugs were analyzed using two-way ANOVA, with factors

'pretreatment' x 'treatment'. Social defeat results were analyzed using a repeated measures three-way ANOVA, with factors 'pretreatment' x 'treatment' x 'experimental phase' (repeated factor). ANOVAs were followed by Holm-Sidak's multiple comparisons post-hoc test when significance was reached (i.e., $p<0.05$). Electrophysiology data were digitized at 10 kHz, filtered at 3 kHz, and analyzed with pCLAMP 10.7 software (Axon Instruments, Sunnyvale, CA). Slices with an average baseline fEPSP slope value from 1-5 min that exhibited >10% variation compared to 6-10 min were excluded from the analysis. Between-group comparisons were analyzed via two-way analysis of variance (factor A: treatment; factor B: pretreatment) and treatment means were separated via Holm-Šídák *post-hoc* comparisons. fEPSP values were normalized to the average fEPSP slope response recorded during the last five min of baseline. Individual normalized slope responses represent the average normalized slope value recorded at 20 s intervals successively over a one min period. LTP magnitude was calculated by averaging the normalized fEPSP slope values 56–60 min after HFS. An average of four slices were collected per animal, which were averaged to determine responses for slices from a given animal. Reported *n*-values indicate the number of mice assessed. All statistical analysis and graphic production were completed using GraphPad Prism version 9.2 (GraphPad Software Inc., San Diego, CA). An α level of 0.05 was used as the criterion for statistical significance. All data are presented as mean ± SEM. Statistical outliers (based on priori criteria) were determined and removed from the dataset using the ROUT method (Motulsky et al., 2006), provided by GraphPad Prism; parameter used: Q=1%. Out of a total of 846 mice used, 13 mice were excluded from the analyses based on the outlier ROUT method identification. For a detailed description on the exact statistical analyses used see Table 1.

RESULTS

For full details on the statistical tests used, F values, and *n* numbers for each of the graphs presented, see **Table 1**.

High doses of ketamine do not exert antidepressant-relevant behavioral actions

We assessed the effects of different doses of ketamine to reverse escape failures following learned helplessness induced by inescapable shocks. This model is sensitive to acute administration of (*R,S*)-ketamine, but not to traditional antidepressants, when tested 24 hrs following drug exposure (see Ramaker and Dulawa, 2017). Mice were given injections of different doses of ketamine (1-100 mg/kg) and tested 24 hrs later in the learned helplessness test (**Fig 1A**). *Post-hoc* analysis revealed that ketamine administration significantly reduced escape failures of susceptible mice at the dose of 10 mg/kg ($p = 0.026$) and indicated a trend to reduce escape failures at the dose of 30 mg/kg ($p = 0.068$). Doses of 1, 3, and 100 mg/kg were ineffective, with 100 mg/kg completely preventing the antidepressant-like actions of ketamine compared with the dose of 10 mg/kg ($p = 0.023$).

To further assess whether ketamine is antidepressant at high doses, we tested the effects of ketamine to reverse sucrose preference deficits induced by 10 days of social defeat stress. Either 10 or 100 mg/kg ketamine, or vehicle, was administered to mice that exhibited <55% sucrose preference following chronic social defeat stress, and sucrose preference was assessed over a 24 hr period after drug administration (**Fig 1B**). While ketamine at the dose of 10 mg/kg reversed the deficit in sucrose preference, the dose of 100 mg/kg was ineffective in ameliorating sucrose preference deficits in chronically stressed, susceptible mice.

NMDAR activity mediates antidepressant-relevant behavioral effects of ketamine

To assess the role of NMDAR activity on ketamine's antidepressant-like behavioral actions, we gave mice an injection of the readily brain penetrant NMDAR channel blocker MK-801 (0.1 mg/kg), followed by the administration of ketamine 10 min later, and tested these mice for reversal of learned helplessness behavior 24 hrs after administration of ketamine (**Fig 2A**). Ketamine's antidepressant-relevant actions were absent in mice that received MK-801 prior to ketamine (**Fig 2A**). Similarly, pretreatment with the competitive NMDAR blocker (±)-CPP (which crosses the blood-brain barrier and exerts centrally-mediated behavioral actions (Lehmann et al., 1987)) completely prevented the effect of ketamine on escape failures in helpless mice (**Fig 2B**). Pretreatment with MK-801 also prevented ketamine's actions on sucrose preference in socially-defeated susceptible mice (**Fig 2C**) and immobility time in the forced-swim test (**Fig 2D**). As in all the other tests, the forced-swimming procedure was conducted 24 hrs after drug administration, a time point long after any locomotor or anesthetic effects of ketamine disappear.

These results might indicate that NMDAR activity is necessary for the antidepressant-like behavioral actions of ketamine, or they could also simply indicate that a higher degree of NMDAR inhibition generally prevents rapid antidepressant activity. We thus tested the hypothesis that ketamine's antidepressant-like action(s) converge with positive allosteric modulators of the NMDAR, via co-administration of sub-effective doses of ketamine (3 mg/kg) with a 1 mg/kg sub-effective dose of GLYX-13 (rapastinel), which was determined by preliminary dose-response experiments. Rapastinel is an NMDAR positive allosteric modulator with EC_{50} effects on NMDAR subunits as measured *in vitro* of 9.8 pM (GluN2A), 9.9 nM (GluN2B), 2.2 pM (GluN2C), and 1.7 pM (GluN2D) (Donello et al., 2019). The antidepressant-related effects of Rapastinel, as well as the pharmacologically-related NMDAR positive

399 allosteric modulator NYX-2925, were blocked with prior administration of the competitive
400 NMDAR antagonist CPP (Burgdorf et al., 2015; Khan et al., 2018). Rapastinel at the dose of 1
401 mg/kg was ineffective to induce any antidepressant-relevant behavioral actions on its own, but
402 the combination with the low (also sub-effective) dose of ketamine induced a synergistic effect
403 to reverse escape failures 24 hrs following administration, suggesting that NMDAR activation
404 possibly contributes to the antidepressant-like effects of ketamine (**Fig 2E**).
405
406 ***Blocking NMDAR activity prevents the antidepressant-relevant behavioral effects of rapid-***
407 ***acting antidepressant drugs with distinct mechanisms***
408 It is possible that our results could indicate only that excessive NMDAR inhibition
409 (arising from a combined administration of ketamine and other NMDAR antagonists (i.e., MK-
410 801 or CPP)) prevents the rapid antidepressant behavioral effects of ketamine. However, these
411 data could also provide a novel mechanism underlying ketamine's rapid antidepressant action
412 involving NMDAR activation. In order to examine the critical role of NMDAR activation as a
413 general mechanism, we assessed whether pre-treatment with the NMDAR inhibitor MK-801
414 prior to administration of other preclinically characterized, putative rapid-acting antidepressants
415 also prevents their antidepressant-relevant behavioral actions.
416 (*2R,6R*)-HNK is a metabolite of ketamine that has been shown to exert ketamine-like
417 antidepressant biobehavioral actions (Zanos et al., 2016; Pham et al., 2018; Fukumoto et al.,
418 2019; Lumsden et al., 2019; Zanos et al., 2019; Aleksandrova et al., 2020; Riggs et al., 2020),
419 without blocking NMDAR function at relevant concentrations (Suzuki et al., 2017; Zanos et al.,
420 2017b; Lumsden et al., 2019). As above, pretreatment with MK-801 at a dose of 0.1 mg/kg
421 completely prevented the antidepressant-like actions of (*2R,6R*)-HNK tested in the forced-swim

test 24 hrs after administration (**Fig 3A**), whereas pretreatment with MK-801 at 0.03 mg/kg did not (**Fig 3B**). The finding that (*2R,6R*)-HNK's effects to change forced swimming behavior required a higher dose of MK-801 compared to that required to reverse ketamine's effects may be reflecting that (*2R,6R*)-HNK itself is not an NMDAR antagonist at the brain concentrations elicited by the dose used (Suzuki et al., 2017; Zanos et al., 2017b; Lumsden et al., 2019).

Negative allosteric modulators of $GABA_A$ receptors containing α5 subunits (GABA-NAM), which promote glutamate release and enhanced excitatory glutamatergic transmission, have been shown to exert ketamine-like rapid and sustained antidepressant-relevant actions in rodent tests (Fischell et al., 2015; Zanos et al., 2017a; Xiong et al., 2018; Troppoli et al., 2021). As we found with ketamine and (*2R,6R*)-HNK, pretreatment with MK-801 (0.1 mg/kg) prevented the anti-immobility actions of the GABA-NAM MRK-016 in the forced-swim test (**Fig 3C**). We also found that MK-801 pretreatment (0.1 mg/kg) prevented the reversal of helpless behavior following inescapable shock induced by both (*2R,6R*)-HNK (**Fig 3D**) and MRK-016 24 hrs after their administration (**Fig 3E**). $mGlu_{2/3}$ receptor antagonists have been shown to exert rapid antidepressant-relevant behavioral actions in rodent tests (Chaki et al., 2004; Yoshimizu et al., 2006; Bespalov et al., 2008; Witkin et al., 2016), likely through increasing glutamatergic neurotransmission in mood-regulating synapses. Similar to these earlier reports we found that the $mGlu_{2/3}$ receptor antagonist, LY341495, reversed helpless behavior in the learned helplessness test. Pretreatment with MK-801 (0.1 mg/kg) prevented this effect (**Fig 3F**). Together, these findings suggest a shared necessity of NMDAR activation for the behavioral antidepressant effects of putative rapid-acting antidepressants with distinct mechanisms of action.

Role for NMDAR-mediated hippocampal plasticity in the effects of ketamine.

Ketamine and its (*2R,6R*)-HNK metabolite have been established to enhance SC-CA1 synaptic strength (Autry et al., 2011; Zanos et al., 2016; Aleksandrova et al., 2020; Riggs et al., 2020; Riggs et al., 2022). Performance in novel object recognition task is mediated, in part, by plasticity of SC-CA1 synapses, and is NMDAR activity-dependent (Clarke et al., 2010; Warburton et al., 2013) (**Fig 4A**). We confirmed the earlier finding that ketamine increases performance in the novel object recognition task (Papp et al., 2017; Willner et al., 2019; Aleksandrova et al., 2020). The administration of ketamine was performed 24 hrs prior to testing to avoid any ataxic and anesthetic effects of the drug. Specifically, ketamine (10 mg/kg) increased the discrimination index in the novel object recognition test compared with saline-treated mice in a 1 hour recognition memory task (**Fig 4A**; $p = 0.006$). In contrast, the high dose of ketamine (100 mg/kg) impaired short-term object recognition memory compared with controls (**Fig 4A**; $p = 0.047$).

To assess the role of NMDAR activity on the pro-cognitive effects of ketamine in the novel-object recognition test we gave mice an injection of MK-801 (0.1 mg/kg), followed by the administration of ketamine 10 min later, and tested these mice in the novel object recognition task 24 hrs after administration of ketamine (**Fig 4B**). Ketamine administration increased the time spent with the novel object compared with saline-treated controls (**Fig 4B**; $p = 0.005$); however ketamine's pro-cognitive actions were absent in mice that received MK-801 prior to ketamine (**Fig 4B**; $p = 0.847$ compared to the respective controls). Similar to high-dose ketamine, the MK-801 treatment resulted in an overall effect to decrease novel object recognition performance ($p = 0.022$; Table 1).

Studies have reported that expression of the AMPA receptor GluA1 and GluA2 subunits in the hippocampus is increased 24 hrs after administration of ketamine to rodents, consistent with the finding that enhanced AMPA receptor activity underlies ketamine's sustained antidepressant-relevant behavioral effects (Maeng et al., 2008; Li et al., 2010; Autry et al., 2011; Zanos et al., 2016). We administered saline or the NMDAR channel blocker MK-801 (0.1 mg/kg), followed by the administration of ketamine 10 min later, and collected hippocampi from these mice at 24 hrs. While ketamine administration induced an enhancement of both GluA1 (**Fig 4C, D**; p = 0.043) and GluA2 (**Fig 4C, E**; p = 0.024) AMPAR subunits, MK-801 pretreatment abolished this effect of ketamine for both the GluA1 (p = 0.682) and GluA2 (p = 0.756) subunits (**Fig 4C-E**).

Ketamine administration to rodents has been found to induce a metaplastic effect on *ex vivo* SC-CA1 synaptic activity, resulting in enhanced long-term potentiation (LTP) (Burgdorf et al., 2013; Graef et al., 2015; Widman et al., 2018). We administered vehicle or ketamine (10 mg/kg) 10 min after administration of vehicle or MK-801 (0.1 mg/kg). Ketamine treatment induced a metaplastic effect on LTP magnitude 24 hrs after ketamine treatment, an effect that was blocked by MK-801 pretreatment (**Fig 4F, G**). *Post-hoc* comparisons revealed a significant increase in LTP magnitude in SAL-KET-treated mice compared to SAL-SAL controls (p = 0.012) (**Fig 4H**). Pretreatment with MK-801 prevented the ketamine-induced enhancement of LTP magnitude (**Fig 4H**; p=0.004; MK-801-KET *vs* SAL-KET). These data suggest that the metaplastic effect of ketamine on LTP magnitude at the SC-CA1 synapse in mouse hippocampal slices requires NMDAR activation.

GluN2A activity mediates antidepressant actions of ketamine

A convergent enhancement in excitatory neurotransmission in brain areas including prefrontal cortex and hippocampus, has been characterized as a key mechanism that underlies ketamine's rapid antidepressant action (Thompson, 2022). Generally, synaptic NMDARs primarily contain GluN2A subunits, which mediate long-term synaptic plasticity and display faster kinetics, whereas GluN2B receptor subunits are expressed extrasynaptically and display slower kinetics (Traynelis et al., 2010; Paoletti et al., 2013). Furthermore, the positive modulator rapastinel that we found augmented the effects of ketamine (**Fig 2E**) has reported selectivity to GluN2A compared to GluN2B (Donello et al., 2019)**.** Therefore, we hypothesized a primary role of GluN2A-containing receptors in ketamine's actions described above. We administered saline or the GluN2A subunit-selective NMDAR antagonist PEAQX (at the doses of either 5 mg/kg or 30 mg/kg), followed by the administration of ketamine 10 min later, and assessed the reversal of helpless behaviors 24 hrs later. Pretreatment with PEAQX at the doses of 5 mg/kg and 30 mg/kg completely prevented the antidepressant-like actions of ketamine to reverse learned helplessness (**Fig 5A**; $p = 0.016$ and $p = 0.022$, respectively, compared with SAL-KET mice).

As GluN2A-specific blockade prevented the antidepressant-like effects of ketamine, we then tested whether the GluN2A NMDAR positive modulator GNE-5729 may produce antidepressant-like behavioral effects comparable to those of ketamine. The effect of GNE-5729 at doses ranging from 0.1-3 mg/kg on the locomotor activity of mice was evaluated, where we found that only the dose of 3 mg/kg reduced distance travelled compared with vehicle-treated mice (**Fig 5B inset**; $p = 0.027$). The doses of 0.1, 0.3, and 1 mg/kg GNE-5729 did not alter the locomotion of mice compared with the controls (**Fig 5B**). We next assessed the ability of GNE-5729 to induce antidepressant-like behaviors. GNE-5729 (3 mg/kg) induced a significant decrease in immobility time of mice in the forced-swim test compared with the control mice

tested 24 hr after administration (**Fig 5C**; p = 0.030), indicative of the antidepressant-like efficacy of this GluN2A NMDAR positive allosteric modulator. In the learned helplessness paradigm, GNE-5729 at the dose of 1 mg/kg also significantly decreased escape failures of helpless mice compared to vehicle-treated controls when tested 24 hrs after administration (**Fig 5D**; p = 0.029). None of the other doses changed immobility time or escape failures in the forced-swim and learned helplessness tests, respectively (**Fig 5C, D**). Collectively, these results indicate that GluN2A activation is necessary and sufficient to induce behavioral ketamine-like effects.

DISCUSSION

We hypothesize that the antidepressant effects of ketamine are mediated by the induction of NMDAR activation-dependent synaptic plasticity. Specifically, increases in synchronous neuronal activation and glutamate release lead to acute activation of NMDARs and the induction of persistent changes in synaptic strength and plasticity that underlie maintained therapeutic actions after the drug is no longer present. Such an NMDAR activation mechanism converges with increased glutamate release probability—either through ketamine disinhibition via acting on interneurons or hydroxynorketamine's direct action—resulting in postsynaptic AMPAR activation, membrane depolarization release of NMDAR magnesium block, NMDAR activation, calcium influx and consequential postsynaptic plasticity including upregulation of synaptic AMPARs. We tested the role of NMDAR activation in the antidepressant actions of ketamine 24 hr following administration of the drug (where no relevant brain concentrations of ketamine are present), confirming that ketamine exerts an inverted U-shaped dose-response in previously unassessed behavioral outcomes. Our findings suggest that NMDAR inhibition induced by higher doses of ketamine prevents antidepressant-related effects of ketamine that are triggered by lower doses. New findings include that (i) ketamine's persistent antidepressant-like behavioral actions are blocked following acute pretreatment with either a competitive (CPP) or channel blocking (MK-801) NMDAR antagonist, (ii) an otherwise ineffective low dose of ketamine acts synergistically with an NMDAR positive allosteric modulator to exert a persistent antidepressant-like behavioral effect, (iii) the antidepressant-like actions of other rapid-acting antidepressant drug classes in development (that do not block the NMDAR themselves) are similarly blocked by NMDAR inhibition, (iv) ketamine-induced up-regulation of hippocampal AMPAR subunits GluA1 and GluA2 are blocked by acute NMDAR inhibition, (v) ketamine-

induced metaplasticity measured *ex vivo* using electrically-stimulated LTP is blocked by NMDAR inhibition, and (vi) pharmacological activation of the NMDAR subunit GluN2A is sufficient to exert antidepressant-like behavioral effects.

In humans, the typical dose/route of ketamine administration used in clinical antidepressant studies is 0.5 mg/kg administered during a 40-min intravenous infusion. 1.0 mg/kg can be also effective, while a lower dose appears to be generally less effective (Fava et al., 2020). However, there are no controlled studies to date that have compared these to higher doses. Previous preclinical studies have demonstrated that antidepressant-like effects following administration of ketamine are consistently observed in animal experiments when subanesthetic doses are administered, but these actions are absent at higher doses near or within the range used for anesthesia (Duncan et al., 1998; Li et al., 2010; Chowdhury et al., 2012; Zanos et al., 2016; Chowdhury et al., 2017; Miller et al., 2018; Hibicke et al., 2020; Kim and Monteggia, 2020). To our knowledge, all previous reports have used the forced swimming test rodent behavioral assay. Our current results add to these reports, further showing a U-shaped dose response in the reversal of learned helplessness. We also found that 10 mg/kg ketamine effectively reversed the deleterious effects of chronic social defeat stress on anhedonia as measured by the sucrose preference test, whereas the dose of 100 mg/kg was ineffective. We note that at the 24 hr time point of testing, ketamine and its metabolites are eliminated from mice (below detectable limits by 2 hr), and any anesthetic or other side effects of ketamine have been absent for over 23 hr (Zanos et al., 2018b).

We also found that ketamine's persistent antidepressant-like behavioral actions are blocked following pretreatment with other NMDAR antagonists. In particular, we demonstrated that pre-administration of the non-competitive antagonist MK-801 blocks ketamine's

antidepressant-like effects, as measured in the forced swimming test, learned helplessness test, and recovery of sucrose preference following chronic social defeat stress. Similarly, pretreatment with the competitive NMDAR antagonist CPP also blocked ketamine's effects to reverse learned helplessness. We conclude that NMDAR activation is required for ketamine's ability to induce the antidepressant-relevant behavioral responses to ketamine. We also conclude that the failure of higher doses of ketamine to induce antidepressant-like behavioral responses is due to the block of some critical fraction of NMDARs that is needed for the induction of antidepressant-relevant actions.

Our studies identified that the antidepressant-like actions of other putative rapid-acting antidepressant drugs are blocked by NMDAR inhibition. Specifically, we found that the antidepressant-like behavioral effects of ketamine's metabolite (*2R,6R*)-HNK and the GABA-NAM MRK-016 in the forced swimming and learned helplessness tests, as well as the antidepressant-like effects of the $mGlu_{2/3}$ receptor antagonist LY341495 in the learned helplessness test, were prevented by pre-administration with MK-801. We note that prevention of (*2R,6R*)-HNK's effects to change forced swimming behavior (**Fig 3A, B**) required a higher dose of MK-801 compared to that required to reverse ketamine's effects (**Fig 2D**), potentially reflecting the fact that (*2R,6R*)-HNK is not an NMDAR antagonist at the doses used here (Suzuki et al., 2017; Zanos et al., 2017b; Lumsden et al., 2019). We have previously shown that antidepressant-relevant doses of (*2R,6R*)-HNK increase the probability of glutamate release independent of NMDAR inhibition, potentially via a mechanism convergent with $mGlu_2$ receptor signaling (Zanos et al., 2016; Zanos et al., 2019; Riggs et al., 2020; Riggs et al., 2022). MRK-016 is an α5-selective GABA-NAM that exerts rapid antidepressant-like and anti-anhedonic actions in addition to restoring stress-weakened synapses in the hippocampus, presumably by

591 disinhibition of excitatory activity (Fischell et al., 2015; Zanos et al., 2017a; Xiong et al., 2018;
592 Troppoli et al., 2021). Consistent with this, we found that ketamine acts synergistically with the
593 NMDAR positive allosteric modulator rapastinel to exert an antidepressant-like behavioral
594 effect. These results support the conclusions that rapid-acting antidepressant compounds share a
595 common downstream NMDAR-activation dependent effector mechanism, despite their wide
596 range of independent upstream targets.

597 Canonical SC-CA1 LTP, mediated by NMDAR activation, is maintained by upregulation
598 of postsynaptic GluA1 and GluA2 (Huganir and Nicoll, 2013). The novel object recognition task
599 is established to involve SC-CA1 potentiation and to be NMDAR activity-dependent (Clarke et
600 al., 2010; Warburton et al., 2013). The function of this hippocampal synapse may also have
601 relevance to the cognitive deficits observed in depression and may be reversed by ketamine (see
602 Gill et al., 2021). Here, we demonstrated that a 10 mg/kg dose of ketamine improved novel
603 object discrimination. Furthermore, the effectiveness of 10 mg/kg ketamine in enhancing novel
604 object discrimination was blocked by pre-administration with MK-801. Consistent with a
605 ketamine-mediated activation of NMDAR signaling, we identified that ketamine-induced up-
606 regulation of AMPAR subunits GluA1 and GluA2 in a synaptoneurosome preparation from the
607 hippocampus 24 hrs after administration, was blocked by the prior administration of MK-801.
608 These data are consistent with previous observations that antidepressant-relevant doses of
609 ketamine produce effects on plasticity-mediated cellular signaling pathways that are not
610 observed at higher doses (Duncan et al., 1998; Li et al., 2010; Chowdhury et al., 2012;
611 Chowdhury et al., 2017; Kim and Monteggia, 2020)

612 *In vivo* administration of ketamine has been reported to enhance *ex vivo* SC-CA1 LTP
613 (Burgdorf et al., 2013; Graef et al., 2015; Widman et al., 2018). Similarly, the NMDAR PAMs

rapastinel and NYX-295 induce a similar metaplasticity (Burgdorf et al., 2013; Burgdorf et al., 2015; Khan et al., 2018). Ketamine and its metabolite (*2R,6R*)-HNK ameliorate impaired SC-CA1 LTP measured in anesthetized Wistar-Kyoto rats 3.5 hrs after drug injection (Aleksandrova et al., 2020). Bath application of ketamine has also been reported to induce enhanced synaptic potentiation in hippocampal slices obtained from mice treated with ketamine 7 days prior to slice collection (Kim et al., 2021). Here, we report that the metaplastic effect of ketamine on *ex vivo* electrically-stimulated LTP assessed 24 hr following administration is blocked by the prior administration of MK-801. While it is unclear if SC-CA1 function is directly related to ketamine's antidepressant actions, or if such changes are generally representative of ketamine-induced plasticity of excitatory synapses (Thompson, 2022) these findings suggest that ketamine induces NMDAR-activation-dependent process that enhances LTP induction. We note that while concentrations in the range of 1 and 20μM of ketamine are reported to enhance SC-CA1 synaptic strength in hippocampal slices, a higher concentration of 100μM does not have this effect, which can also be blocked by APV (Kim and Monteggia, 2020; Izumi et al., in press). Ketamine (50uM) also blocks LTP induced in nucleus accumbens slices, which may explain a lack of ketamine *in vivo* dopamine-dependent plasticity in this region (Simmler et al., 2022).

Finally, we report that pharmacological activation of NMDAR subunit GluN2A with GNE-5729 is sufficient to exert antidepressant-like behavioral effects when tested in both the forced swimming and learned helplessness assays of antidepressant efficacy (maximally effective at 3 and 1 mg/kg respectively). We also show that pretreatment with the GluN2A antagonist PEAQX prevents the antidepressant-like effects of ketamine in the learned helplessness assay, suggesting that ketamine requires, and potentially acts via, GluN2A activation to exert its antidepressant behavioral actions.

637 Our results support targeting NMDAR-dependent LTP-like synaptic potentiation as an
638 effective antidepressant strategy. Our findings implicate ketamine-induced SC-CA1 synaptic to
639 be NMDAR-activation dependent, but it remains to be determined whether such changes are
640 necessary, or unique, to this synapse. A focus on the strength of inhibition of NMDAR signaling
641 to develop future treatments of depression may be counterproductively resulting in drugs that
642 prevent increases in NMDAR-activation dependent increases in synaptic strength necessary for
643 efficacy.
644

Figure Legends

Figure 1. High doses of ketamine do not elicit antidepressant-relevant behavioral actions in mice. Mice received an injection of vehicle or different doses of racemic ketamine and were assessed for antidepressant-like responses 24 hr later. **(A)** Ketamine at the dose of 10 mg/kg significantly decreased escape failures in helpless mice, whereas the doses of 1, 3, 30, and 100 mg/kg did not exert significant antidepressant-relevant reductions in escape failures in the learned helplessness paradigm. **(B)** Similarly, ketamine at the dose of 10 mg/kg reversed the decrease in sucrose preference of mice that underwent chronic social defeat stress whereas the high dose of 100 mg/kg did not elicit such an antidepressant-related response. Mice were tested for sucrose versus water preference during the 24 hr period following drug administration. Data are the mean ± SEM. * $p<0.05$; *** $p<0.001$ as indicated by Holm-Šídák *post-hoc* comparisons. See Table 1 for complete details on the statistical analyses and precise group sizes. Abbreviations: CSDS, chronic social defeat stress; KET, racemic ketamine; SAL, saline; Treat, treatment.

Figure 2. Blocking NMDAR activity prevents the antidepressant-relevant behavioral effects of ketamine. (A) Mice received an injection of vehicle or the NMDAR channel blocker MK-801 (0.1 mg/kg) and 10 min later they received an injection of vehicle or racemic ketamine (10 mg/kg). MK-801 pretreatment prevented the antidepressant-relevant behavioral actions of ketamine in the learned helplessness paradigm. **(B)** Similarly, pretreatment with the competitive NMDAR blocker (±)-CPP (10 mg/kg) blocked ketamine's antidepressant-relevant actions in the learned helplessness paradigm. **(C)** MK-801 (0.1 mg/kg) pretreatment also prevented the anti-anhedonic actions of ketamine in mice that underwent chronic social defeat stress, as measured

668 by the sucrose preference test. **(D)** MK-801 (0.03 mg/kg) pretreatment prevented ketamine's
669 actions on immobility time in the forced-swim test. **(E)** Co-administration of sub-effective doses
670 of ketamine with the NMDAR positive modulator rapastinel induced a synergistic reduction of
671 escape failures in helpless mice in the learned helplessness paradigm. In all paradigms mice were
672 tested 24 hr following drug administration. Data are the mean ± SEM. * $p<0.05$; ** $p<0.01$; ***
673 $p<0.001$ as indicated by Holm-Šídák *post-hoc* comparisons. See Table 1 for complete details on
674 the statistical analyses and precise group sizes. Abbreviations: CSDS, chronic social defeat
675 stress; KET, racemic ketamine; SAL, saline; Treat, treatment.
676
677 **Figure 3. Blocking NMDAR activity prevents the antidepressant-relevant behavioral effects**
678 **of other rapid-acting antidepressant compounds. (A-B)** Mice received an injection of vehicle
679 or the NMDAR channel blocker MK-801 and 10 min later were given an additional injection of
680 vehicle or ketamine's metabolite (*2R,6R*)-hydroxynorketamine (HNK; 10 mg/kg) and tested in
681 the forced swim test 24 hr later. While **(A)** the dose of 0.1 mg/kg MK-801 completely prevented
682 the antidepressant-like behavioral actions of (*2R,6R*)-HNK, **(B)** 0.03 mg/kg MK-801 did not
683 prevent (*2R,6R*)-HNK's actions to decrease immobility time in the forced-swim test. MK-801
684 pretreatment (0.1 mg/kg) prevented the antidepressant-relevant behavioral actions of **(C)** the
685 negative allosteric modulator of $GABA_A$ receptors containing α5 subunits (GABA-NAM) MRK-
686 016 in the forced-swim test. MK-801 pretreatment (0.1 mg/kg) prevented the antidepressant-like
687 effects of **(D)** (*2R,6R*)-HNK, **(E)** MRK-016 and **(F)** the mGlu2/3 receptor antagonist LY341495
688 in the learned helplessness paradigm 24 hr following drug administration. Data are the mean ±
689 SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ as indicated by Holm-Šídák *post-hoc* comparisons. See

690 Table 1 for complete details on the statistical analyses and precise group sizes. Abbreviations:
691 KET, racemic ketamine; SAL, saline.
692
693 **Figure 4. Blocking NMDAR activity prevents the pro-cognitive and synaptic actions of**
694 **ketamine. (A)** Mice received an injection of vehicle or different doses of racemic ketamine and
695 were tested for short-term novel object recognition memory 24 hr after drug administration.
696 Ketamine (10 mg/kg) enhanced the discrimination index, indicative of a pro-cognitive effect,
697 whereas a higher dose of ketamine (100 mg/kg) impaired object recognition memory. **(B)**
698 Administration of the NMDAR channel blocker MK-801 (0.1 mg/kg) 10 min prior to ketamine
699 (10 mg/kg) prevented the pro-cognitive effect of ketamine in the novel-object recognition test.
700 **(C)** Representative western blot images for GluA1 and GluA2 AMPAR subunits from
701 hippocampal synaptoneurosomes. **(D,E)** Pretreatment with MK-801 prevented the ketamine-
702 induced enhancement of synaptoneurosomal levels of GluA1 and GluA2 AMPAR subunits. **(F)**
703 Traces composed of representative sweeps from 5 min pre-tetanus (grey) and 56–60 min post-
704 tetanus (black) from SAL-SAL, SAL-KET, MK-801-SAL, and MK-801-KET treatment groups.
705 **(G,H)** Pretreatment with MK-801 prevented the metaplastic effect of ketamine on long-term
706 potentiation magnitude at the SC-CA1 synapse. Data are the mean ± SEM. * $p<0.05$; ** $p<0.01$;
707 *** $p<0.001$ as indicated by Holm-Šídák *post-hoc* comparisons. See Table 1 for complete details
708 on the statistical analyses and precise group sizes. Abbreviations: HFS, high-frequency
709 stimulation; KET, racemic ketamine; LTP, long-term potentiation; MK, MK-801; SAL, saline.
710
711 **Figure 5. The antidepressant-like actions of ketamine are mediated through GluN2A**
712 **activity. (A)** Mice received an injection of vehicle or the GluN2A-selective NMDAR negative

allosteric modulator PEAQX (5 or 30 mg/kg) followed by an injection of vehicle or ketamine (10 mg/kg) 10 min later, and then were tested for reversal of helpless behavior 24 hr later. PEAQX pretreatment, at both doses administered, prevented the antidepressant-relevant actions of ketamine to decrease escape failures of helpless mice. **(B)** The GluN2A-selective NMDAR positive allosteric modulator GNE-5729 induced a decrease in locomotor activity of mice in the open-field test only at the highest dose administered (3 mg/kg). **(C)** In the forced-swim test, GNE-5729 at the dose of 3 mg/kg significantly reduced immobility time of mice, indicative of an antidepressant response. **(D)** Similarly, the dose of 1 mg/kg of GNE-5729 significantly reduced escape failures of helpless mice. Data are the mean ± SEM. * $p<0.05$; *** $p<0.001$ as indicated by Holm-Šídák *post-hoc* comparisons. See Table 1 for complete details on the statistical analyses and precise group sizes. Abbreviations: KET, ketamine; SAL, saline; VEH, vehicle.

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TDG has received research funding from Roche Pharmaceuticals, and served as a consultant for FSV7 LLC, during the preceding three years. CAZ is listed as a co-inventor on a patent for the use of ketamine in major depression and suicidal ideation. PZ, CAZ and TDG are listed as co-authors in patents and patent applications related to the pharmacology and use of (*2R,6R*)-HNK in the treatment of depression, anxiety, anhedonia, suicidal ideation and post-traumatic stress disorders. All other authors report no conflict of interest. CAZ has assigned his patent rights to the US Government, but will share a percentage of any royalties that may be received. PZ and TDG has have assigned patent rights to the University of Maryland, Baltimore, but will share a percentage of any royalties that may be received by the University of Maryland, Baltimore. SMT is listed as an inventor on a patent for the use of GABA-NAMs for treatment of depression, filed by the University of Maryland, Baltimore.

REFERENCES

Aleksandrova LR, Wang YT, Phillips AG (2020) Ketamine and its metabolite, (2R,6R)-HNK, restore hippocampal LTP and long-term spatial memory in the Wistar-Kyoto rat model of depression. Mol Brain 13:92.

Anis NA, Berry SC, Burton NR, Lodge D (1983) The dissociative anaesthetics, ketamine and phencyclidine, selectively reduce excitation of central mammalian neurones by N-methyl-aspartate. Br J Pharmacol 79:565-575.

Autry AE, Adachi M, Nosyreva E, Na ES, Los MF, Cheng PF, Kavalali ET, Monteggia LM (2011) NMDA receptor blockade at rest triggers rapid behavioural antidepressant responses. Nature 475:91-95.

Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS, Krystal JH (2000) Antidepressant effects of ketamine in depressed patients. Biol Psychiatry 47:351-354.

Bespalov AY, van Gaalen MM, Sukhotina IA, Wicke K, Mezler M, Schoemaker H, Gross G (2008) Behavioral characterization of the mGlu group II/III receptor antagonist, LY-341495, in animal models of anxiety and depression. European Journal of Pharmacology 592:96-102.

Bevins RA, Besheer J (2006) Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat Protoc 1:1306-1311.

Brown KA, Carpenter JM, Preston CJ, Ludwig HD, Clay KB, Harn DA, Norberg T, Wagner JJ, Filipov NM (2021) Lacto-N-fucopentaose-III ameliorates acute and persisting hippocampal synaptic plasticity and transmission deficits in a Gulf War Illness mouse model. Life Sciences 279:119707.

Burgdorf J, Zhang XL, Nicholson KL, Balster RL, Leander JD, Stanton PK, Gross AL, Kroes RA, Moskal JR (2013) GLYX-13, a NMDA receptor glycine-site functional partial agonist, induces antidepressant-like effects without ketamine-like side effects. Neuropsychopharmacology 38:729-742.

Burgdorf J, Zhang XL, Weiss C, Gross A, Boikess SR, Kroes RA, Khan MA, Burch RM, Rex CS, Disterhoft JF, Stanton PK, Moskal JR (2015) The long-lasting antidepressant effects of rapastinel (GLYX-13) are associated with a metaplasticity process in the medial prefrontal cortex and hippocampus. Neuroscience 308:202-211.

Cerecor (2016) Cerecor Reports Top-Line Data from CERC-301 Phase 2 Study for Major Depressive Disorder. In: Cerecor, Inc.

Chaki S, Yoshikawa R, Hirota S, Shimazaki T, Maeda M, Kawashima N, Yoshimizu T, Yasuhara A, Sakagami K, Okuyama S, Nakanishi S, Nakazato A (2004) MGS0039: a potent and selective group II metabotropic glutamate receptor antagonist with antidepressant-like activity. Neuropharmacology 46:457-467.

Chowdhury GM, Behar KL, Cho W, Thomas MA, Rothman DL, Sanacora G (2012) (1)H-[(1)(3)C]-nuclear magnetic resonance spectroscopy measures of ketamine's effect on amino acid neurotransmitter metabolism. Biol Psychiatry 71:1022-1025.

Chowdhury GM, Zhang J, Thomas M, Banasr M, Ma X, Pittman B, Bristow L, Schaeffer E, Duman RS, Rothman DL, Behar KL, Sanacora G (2017) Transiently increased glutamate cycling in rat PFC is associated with rapid onset of antidepressant-like effects. Mol Psychiatry 22:120-126.

Clarke JR, Cammarota M, Gruart A, Izquierdo I, Delgado-Garcia JM (2010) Plastic modifications induced by object recognition memory processing. Proc Natl Acad Sci U S A 107:2652-2657.

Daly EJ, Singh JB, Fedgchin M, Cooper K, Lim P, Shelton RC, Thase ME, Winokur A, Van Nueten L, Manji H, Drevets WC (2018) Efficacy and Safety of Intranasal Esketamine Adjunctive to Oral Antidepressant Therapy in Treatment-Resistant Depression: A Randomized Clinical Trial. JAMA Psychiatry 75:139-148.

Donello JE, Banerjee P, Li YX, Guo YX, Yoshitake T, Zhang XL, Miry O, Kehr J, Stanton PK, Gross AL, Burgdorf JS, Kroes RA, Moskal JR (2019) Positive N-Methyl-D-Aspartate Receptor Modulation by Rapastinel Promotes Rapid and Sustained Antidepressant-Like Effects. Int J Neuropsychopharmacol 22:247-259.

Duman RS (2014) Pathophysiology of depression and innovative treatments: remodeling glutamatergic synaptic connections. Dialogues Clin Neurosci 16:11-27.

Duncan GE, Moy SS, Knapp DJ, Mueller RA, Breese GR (1998) Metabolic mapping of the rat brain after subanesthetic doses of ketamine: potential relevance to schizophrenia. Brain Res 787:181-190.

Fava M, Freeman MP, Flynn M, Judge H, Hoeppner BB, Cusin C, Ionescu DF, Mathew SJ, Chang LC, Iosifescu DV, Murrough J, Debattista C, Schatzberg AF, Trivedi MH, Jha MK, Sanacora G, Wilkinson ST, Papakostas GI (2020) Double-blind, placebo-controlled, dose-ranging trial of intravenous ketamine as adjunctive therapy in treatment-resistant depression (TRD). Mol Psychiatry 25:1592-1603.

Fischell J, Van Dyke AM, Kvarta MD, LeGates TA, Thompson SM (2015) Rapid Antidepressant Action and Restoration of Excitatory Synaptic Strength After Chronic Stress by Negative Modulators of Alpha5-Containing GABAA Receptors. Neuropsychopharmacology 40:2499-2509.

Fukumoto K, Fogaca MV, Liu RJ, Duman C, Kato T, Li XY, Duman RS (2019) Activity-dependent brain-derived neurotrophic factor signaling is required for the antidepressant actions of (2R,6R)-hydroxynorketamine. Proc Natl Acad Sci U S A 116:297-302.

Georgiou P et al. (2022) Experimenters' sex modulates mouse behaviors and neural responses to ketamine via corticotropin releasing factor. Nat Neurosci 25:1191-1200.

Gill H, Gill B, Rodrigues NB, Lipsitz O, Rosenblat JD, El-Halabi S, Nasri F, Mansur RB, Lee Y, McIntyre RS (2021) The Effects of Ketamine on Cognition in Treatment-Resistant Depression: A Systematic Review and Priority Avenues for Future Research. Neurosci Biobehav Rev 120:78-85.

Gould TD, Zarate CA, Jr., Thompson SM (2019) Molecular Pharmacology and Neurobiology of Rapid-Acting Antidepressants. Annu Rev Pharmacol Toxicol 59:213-236.

Graef JD, Newberry K, Newton A, Pieschl R, Shields E, Luan FN, Simmermacher J, Luchetti D, Schaeffer E, Li YW, Kiss L, Bristow LJ (2015) Effect of acute NR2B antagonist treatment on long-term potentiation in the rat hippocampus. Brain Res 1609:31-39.

Hess EM, Riggs LM, Michaelides M, Gould TD (2022) Mechanisms of ketamine and its metabolites as antidepressants. Biochem Pharmacol 197:114892.

Hibicke M, Landry AN, Kramer HM, Talman ZK, Nichols CD (2020) Psychedelics, but Not Ketamine, Produce Persistent Antidepressant-like Effects in a Rodent Experimental System for the Study of Depression. ACS Chem Neurosci 11:864-871.

Huganir RL, Nicoll RA (2013) AMPARs and synaptic plasticity: the last 25 years. Neuron 80:704-717.

839 Ibrahim L, Diazgranados N, Jolkovsky L, Brutsche N, Luckenbaugh D, Herring WJ, Potter WZ,
840 Zarate CAJ (2012) A randomized, placebo-controlled, crossover pilot trial of the oral
841 selective NR2B antagonist MK-0657 in patients with treatment-resistant major
842 depressive disorder. J Clin Psychopharmacol 32:551-557.
843 Izumi Y, Hsu FF, Conway CR, Nagele P, Mennerick SJ, Zorumski CF (in press) Nitrous Oxide,
844 a Rapid Antidepressant, Has Ketamine-like Effects on Excitatory Transmission in Adult
845 Hippocampus Biological Psychiatry.
846 Kavalali ET, Monteggia LM (2020) Targeting Homeostatic Synaptic Plasticity for Treatment of
847 Mood Disorders. Neuron 106:715-726.
848 Kavalali ET, Monteggia LM (2022) Rapid homeostatic plasticity and neuropsychiatric
849 therapeutics. Neuropsychopharmacology.
850 Khan MA, Houck DR, Gross AL, Zhang XL, Cearley C, Madsen TM, Kroes RA, Stanton PK,
851 Burgdorf J, Moskal JR (2018) NYX-2925 Is a Novel NMDA Receptor-Specific
852 Spirocyclic-beta-Lactam That Modulates Synaptic Plasticity Processes Associated with
853 Learning and Memory. Int J Neuropsychopharmacol 21:242-254.
854 Kim JW, Monteggia LM (2020) Increasing doses of ketamine curtail antidepressant responses
855 and suppress associated synaptic signaling pathways. Behav Brain Res 380:112378.
856 Kim JW, Autry AE, Na ES, Adachi M, Bjorkholm C, Kavalali ET, Monteggia LM (2021)
857 Sustained effects of rapidly acting antidepressants require BDNF-dependent MeCP2
858 phosphorylation. Nat Neurosci 24:1100-1109.
859 Krystal JH, Abdallah CG, Sanacora G, Charney DS, Duman RS (2019) Ketamine: A Paradigm
860 Shift for Depression Research and Treatment. Neuron 101:774-778.
861 Lehmann J, Schneider J, McPherson S, Murphy DE, Bernard P, Tsai C, Bennett DA, Pastor G,
862 Steel DJ, Boehm C, et al. (1987) CPP, a selective N-methyl-D-aspartate (NMDA)-type
863 receptor antagonist: characterization in vitro and in vivo. J Pharmacol Exp Ther 240:737-
864 746.
865 Li N, Lee B, Liu RJ, Banasr M, Dwyer JM, Iwata M, Li XY, Aghajanian G, Duman RS (2010)
866 mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA
867 antagonists. Science 329:959-964.
868 Lumsden EW et al. (2019) Antidepressant-relevant concentrations of the ketamine metabolite
869 (2R,6R)-hydroxynorketamine do not block NMDA receptor function. Proc Natl Acad Sci
870 U S A 116:5160-5169.
871 Maeng S, Zarate CA, Jr., Du J, Schloesser RJ, McCammon J, Chen G, Manji HK (2008) Cellular
872 mechanisms underlying the antidepressant effects of ketamine: role of alpha-amino-3-
873 hydroxy-5-methylisoxazole-4-propionic acid receptors. Biol Psychiatry 63:349-352.
874 Miller OH, Grabole N, Wells I, Hall BJ (2018) Genome-wide translating mRNA analysis
875 following ketamine reveals novel targets for antidepressant treatment. bioRxiv:254904.
876 Moghaddam B, Adams B, Verma A, Daly D (1997) Activation of glutamatergic
877 neurotransmission by ketamine: a novel step in the pathway from NMDA receptor
878 blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex.
879 J Neurosci 17:2921-2927.
880 Murrough JW, Perez AM, Pillemer S, Stern J, Parides MK, aan het Rot M, Collins KA, Mathew
881 SJ, Charney DS, Iosifescu DV (2013) Rapid and longer-term antidepressant effects of
882 repeated ketamine infusions in treatment-resistant major depression. Biol Psychiatry
883 74:250-256.

884 Paoletti P, Bellone C, Zhou Q (2013) NMDA receptor subunit diversity: impact on receptor
885 properties, synaptic plasticity and disease. Nat Rev Neurosci 14:383-400.
886 Papp M, Gruca P, Lason-Tyburkiewicz M, Willner P (2017) Antidepressant, anxiolytic and
887 procognitive effects of subacute and chronic ketamine in the chronic mild stress model of
888 depression. Behav Pharmacol 28:1-8.
889 Park LT, Kadriu B, Gould TD, Zanos P, Greenstein D, Evans JW, Yuan P, Farmer CA,
890 Oppenheimer M, George JM, Adeojo LW, Snodgrass HR, Smith MA, Henter ID,
891 Machado-Vieira R, Mannes AJ, Zarate CA (2020) A Randomized Trial of the N-Methyl-
892 d-Aspartate Receptor Glycine Site Antagonist Prodrug 4-Chlorokynurenine in Treatment-
893 Resistant Depression. Int J Neuropsychopharmacol 23:417-425.
894 Paterson B, Fraser H, Wang C, Marcus R (2015) A randomized, double- blind, placebo-
895 controlled, sequential parallel study of CERC-301 in the adjunctive treatment of subjects
896 with severe depression and recent active suicidal ideation despite antidepressant
897 treatment. . In: National Network of Depression Centers Annual Conference. Ann Arbor,
898 Michigan.
899 Pham TH, Defaix C, Xu X, Deng SX, Fabresse N, Alvarez JC, Landry DW, Brachman RA,
900 Denny CA, Gardier AM (2018) Common Neurotransmission Recruited in (R,S)-
901 Ketamine and (2R,6R)-Hydroxynorketamine-Induced Sustained Antidepressant-like
902 Effects. Biol Psychiatry 84:e3-e6.
903 Preskorn SH, Baker B, Kolluri S, Menniti FS, Krams M, Landen JW (2008) An innovative
904 design to establish proof of concept of the antidepressant effects of the NR2B subunit
905 selective N-methyl-D-aspartate antagonist, CP-101,606, in patients with treatment-
906 refractory major depressive disorder. J Clin Psychopharmacol 28:631-637.
907 Preston CJ, Brown KA, Wagner JJ (2019) Cocaine conditioning induces persisting changes in
908 ventral hippocampus synaptic transmission, long-term potentiation, and radial arm maze
909 performance in the mouse. Neuropharmacology 150:27-37.
910 Ramaker MJ, Dulawa SC (2017) Identifying fast-onset antidepressants using rodent models. Mol
911 Psychiatry 22:656-665.
912 Riggs LM, Thompson SM, Gould TD (2022) (2R,6R)-hydroxynorketamine rapidly potentiates
913 optically-evoked Schaffer collateral synaptic activity. Neuropharmacology 214:109153.
914 Riggs LM, Aracava Y, Zanos P, Fischell J, Albuquerque EX, Pereira EFR, Thompson SM,
915 Gould TD (2020) (2R,6R)-hydroxynorketamine rapidly potentiates hippocampal
916 glutamatergic transmission through a synapse-specific presynaptic mechanism.
917 Neuropsychopharmacology 45:426-436.
918 Sanacora G, Smith MA, Pathak S, Su HL, Boeijinga PH, McCarthy DJ, Quirk MC (2014)
919 Lanicemine: a low-trapping NMDA channel blocker produces sustained antidepressant
920 efficacy with minimal psychotomimetic adverse effects. Mol Psychiatry 19:978-985.
921 Sanacora G, Johnson MR, Khan A, Atkinson SD, Riesenberg RR, Schronen JP, Burke MA,
922 Zajecka JM, Barra L, Su HL, Posener JA, Bui KH, Quirk MC, Piser TM, Mathew SJ,
923 Pathak S (2017) Adjunctive Lanicemine (AZD6765) in Patients with Major Depressive
924 Disorder and History of Inadequate Response to Antidepressants: A Randomized,
925 Placebo-Controlled Study. Neuropsychopharmacology 42:844-853.
926 Simmler LD, Li Y, Hadjas LC, Hiver A, van Zessen R, Luscher C (2022) Dual action of
927 ketamine confines addiction liability. Nature 608:368-373.
928 Smith EG, Deligiannidis KM, Ulbricht CM, Landolin CS, Patel JK, Rothschild AJ (2013)
929 Antidepressant augmentation using the N-methyl-D-aspartate antagonist memantine: a

randomized, double-blind, placebo-controlled trial. The Journal of clinical psychiatry 74:966-973.

Suzuki K, Nosyreva E, Hunt KW, Kavalali ET, Monteggia LM (2017) Effects of a ketamine metabolite on synaptic NMDAR function. Nature 546:E1-E3.

Thompson SM (2022) Plasticity of synapses and reward circuit function in the genesis and treatment of depression. Neuropsychopharmacology.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62:405-496.

Troppoli TA, Zanos P, Georgiou P, Gould TD, Rudolph U, Thompson SM (2021) Negative Allosteric Modulation of Gamma-Aminobutyric Acid A Receptors at alpha5 Subunit-Containing Benzodiazepine Sites Reverses Stress-Induced Anhedonia and Weakened Synaptic Function in Mice. Biol Psychiatry.

Warburton EC, Barker GR, Brown MW (2013) Investigations into the involvement of NMDA mechanisms in recognition memory. Neuropharmacology 74:41-47.

Wegener N, Nagel J, Gross R, Chambon C, Greco S, Pietraszek M, Gravius A, Danysz W (2011) Evaluation of brain pharmacokinetics of (+)MK-801 in relation to behaviour. Neurosci Lett 503:68-72.

Widman AJ, Stewart AE, Erb EM, Gardner E, McMahon LL (2018) Intravascular Ketamine Increases Theta-Burst but Not High Frequency Tetanus Induced LTP at CA3-CA1 Synapses Within Three Hours and Devoid of an Increase in Spine Density. Front Synaptic Neurosci 10:8.

Willner P, Gruca P, Lason M, Tota-Glowczyk K, Litwa E, Niemczyk M, Papp M (2019) Validation of chronic mild stress in the Wistar-Kyoto rat as an animal model of treatment-resistant depression. Behav Pharmacol 30:239-250.

Witkin JM, Monn JA, Schoepp DD, Li X, Overshiner C, Mitchell SN, Carter G, Johnson B, Rasmussen K, Rorick-Kehn LM (2016) The Rapidly Acting Antidepressant Ketamine and the mGlu2/3 Receptor Antagonist LY341495 Rapidly Engage Dopaminergic Mood Circuits. Journal of Pharmacology and Experimental Therapeutics 358:71-82.

Wulf HA, Browne CA, Zarate CA, Lucki I (2022) Mediation of the behavioral effects of ketamine and (2R,6R)-hydroxynorketamine in mice by kappa opioid receptors. Psychopharmacology (Berl).

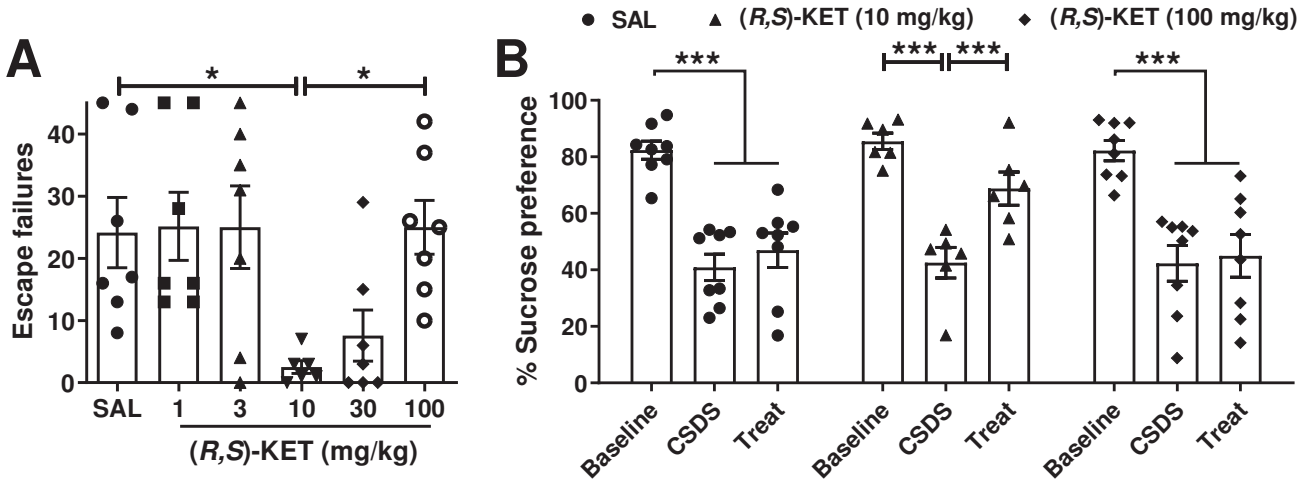
Xiong Z, Zhang K, Ishima T, Ren Q, Chang L, Chen J, Hashimoto K (2018) Comparison of rapid and long-lasting antidepressant effects of negative modulators of alpha5-containing GABAA receptors and (R)ketamine in a chronic social defeat stress model. Pharmacol Biochem Behav 175:139-145.

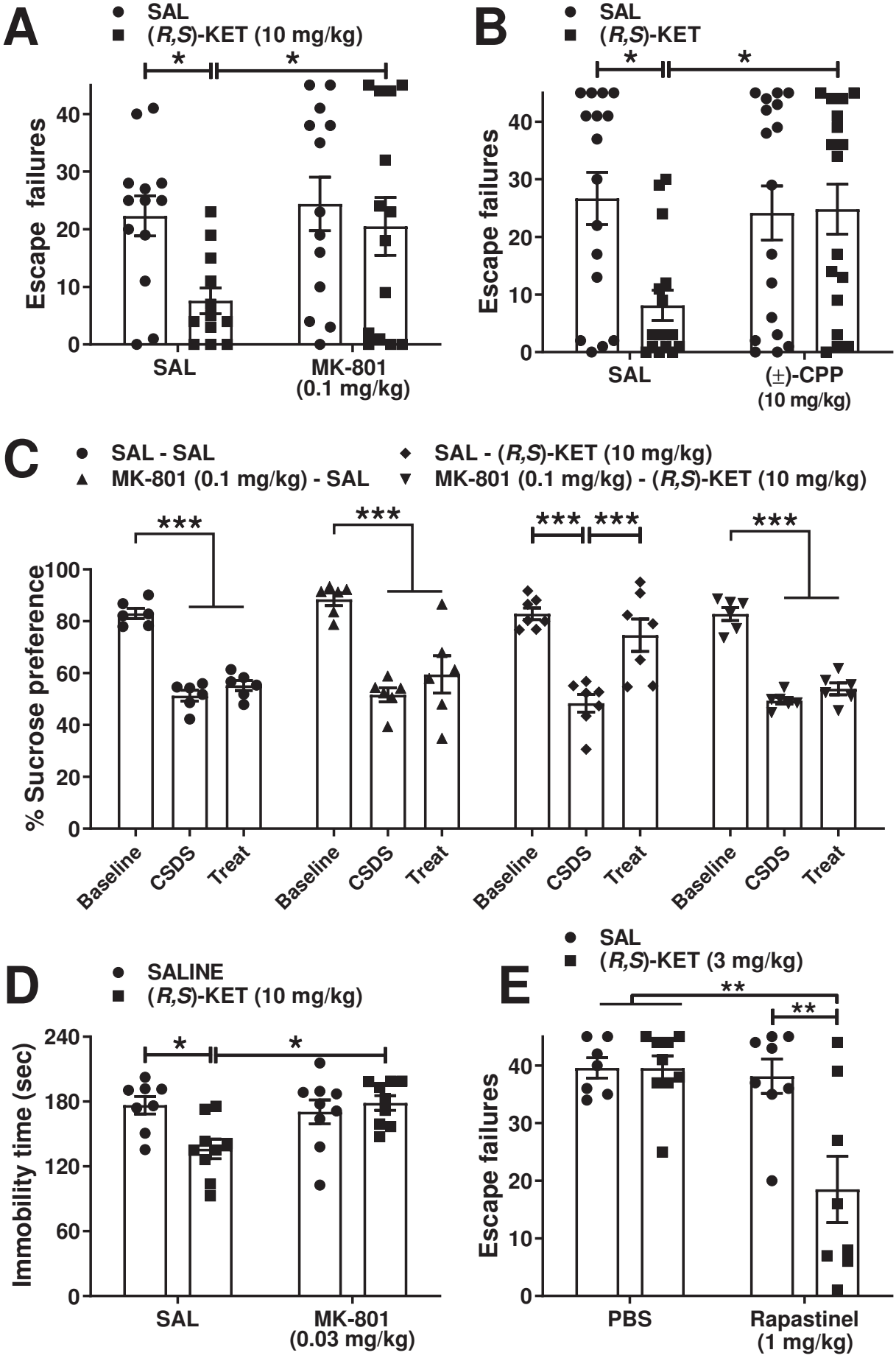
Yoshimizu T, Shimazaki T, Ito A, Chaki S (2006) An mGluR2/3 antagonist, MGS0039, exerts antidepressant and anxiolytic effects in behavioral models in rats. Psychopharmacology 186:587.

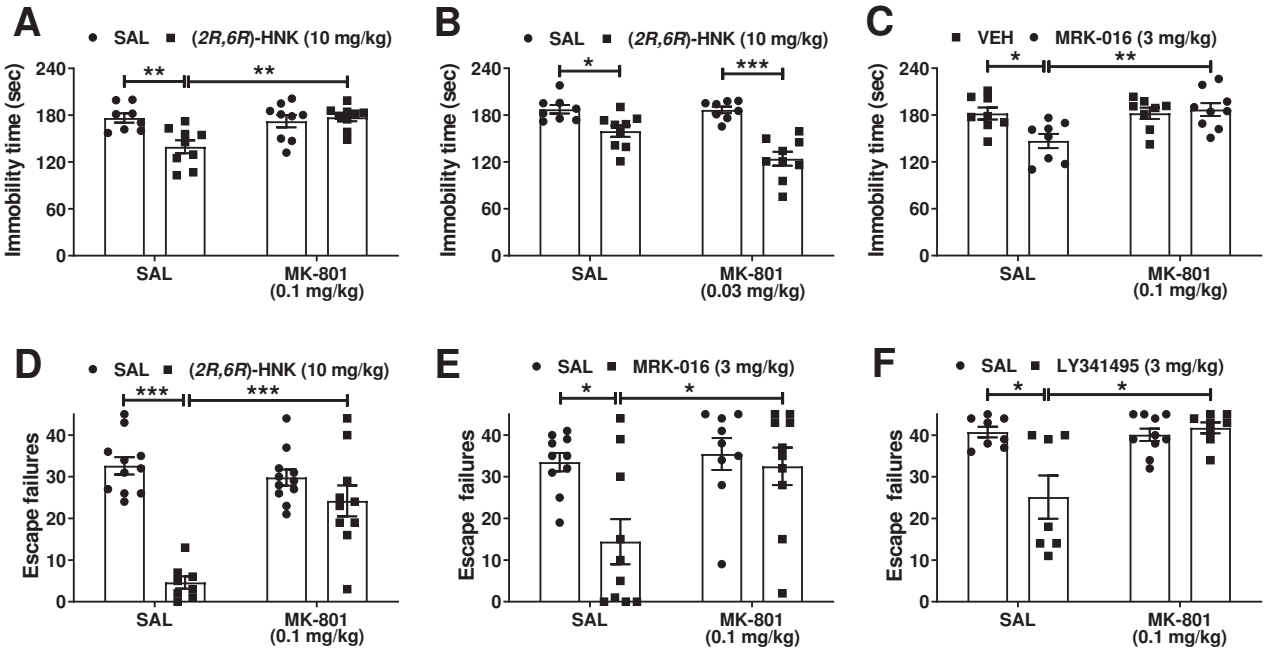
Zanos P, Thompson SM, Duman RS, Zarate CA, Jr., Gould TD (2018a) Convergent Mechanisms Underlying Rapid Antidepressant Action. CNS Drugs 32:197-227.

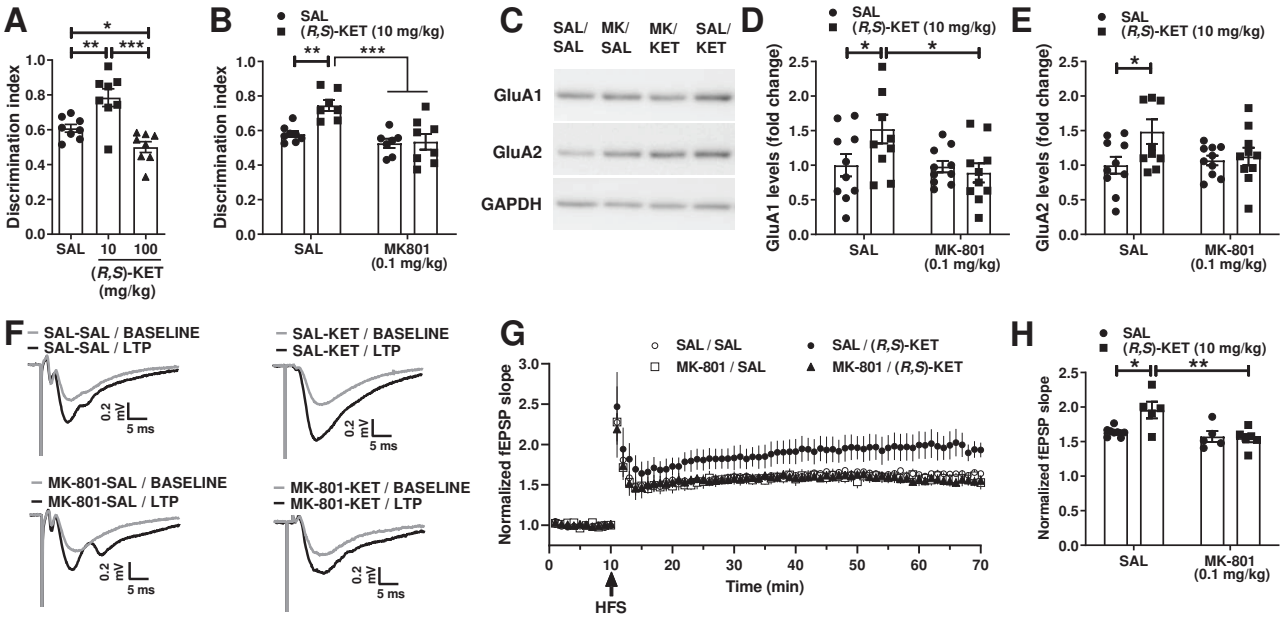
Zanos P, Nelson ME, Highland JN, Krimmel SR, Georgiou P, Gould TD, Thompson SM (2017a) A Negative Allosteric Modulator for alpha5 Subunit-Containing GABA Receptors Exerts a Rapid and Persistent Antidepressant-Like Action without the Side Effects of the NMDA Receptor Antagonist Ketamine in Mice. eNeuro 4.

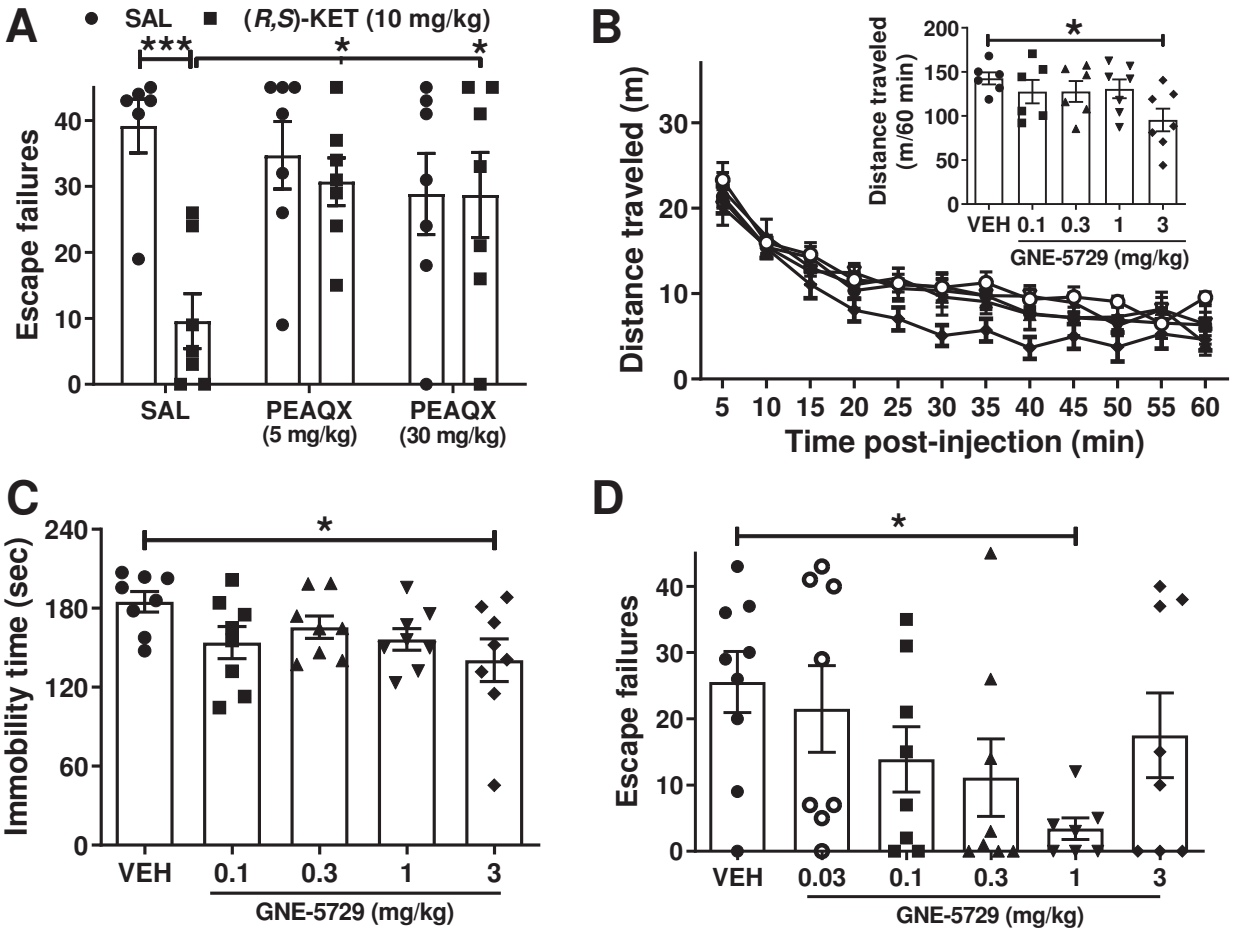
975 Zanos P, Piantadosi SC, Wu HQ, Pribut HJ, Dell MJ, Can A, Snodgrass HR, Zarate CA, Jr.,
976 Schwarcz R, Gould TD (2015) The Prodrug 4-Chlorokynurenine Causes Ketamine-Like
977 Antidepressant Effects, but Not Side Effects, by NMDA/GlycineB-Site Inhibition. J
978 Pharmacol Exp Ther 355:76-85.
979 Zanos P, Moaddel R, Morris PJ, Riggs LM, Highland JN, Georgiou P, Pereira EFR,
980 Albuquerque EX, Thomas CJ, Zarate CA, Jr., Gould TD (2018b) Ketamine and Ketamine
981 Metabolite Pharmacology: Insights into Therapeutic Mechanisms. Pharmacol Rev
982 70:621-660.
983 Zanos P, Highland JN, Stewart BW, Georgiou P, Jenne CE, Lovett J, Morris PJ, Thomas CJ,
984 Moaddel R, Zarate CA, Jr., Gould TD (2019) (2R,6R)-hydroxynorketamine exerts mGlu2
985 receptor-dependent antidepressant actions. Proc Natl Acad Sci U S A 116:6441-6450.
986 Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, Alkondon M, Yuan P, Pribut
987 HJ, Singh NS, Dossou KSS, Fang Y, Huang XP, Mayo CL, Albuquerque EX, Thompson
988 SM, Thomas CJ, Zarate CA, Gould TD (2017b) Zanos et al. reply. Nature 546:E4-E5.
989 Zanos P, Moaddel R, Morris PJ, Georgiou P, Fischell J, Elmer GI, Alkondon M, Yuan P, Pribut
990 HJ, Singh NS, Dossou KS, Fang Y, Huang XP, Mayo CL, Wainer IW, Albuquerque EX,
991 Thompson SM, Thomas CJ, Zarate CA, Jr., Gould TD (2016) NMDAR inhibition-
992 independent antidepressant actions of ketamine metabolites. Nature 533:481-486.
993 Zarate CA, Jr., Singh JB, Quiroz JA, De Jesus G, Denicoff KK, Luckenbaugh DA, Manji HK,
994 Charney DS (2006a) A double-blind, placebo-controlled study of memantine in the
995 treatment of major depression. The American journal of psychiatry 163:153-155.
996 Zarate CA, Jr., Singh JB, Carlson PJ, Brutsche NE, Ameli R, Luckenbaugh DA, Charney DS,
997 Manji HK (2006b) A randomized trial of an N-methyl-D-aspartate antagonist in
998 treatment-resistant major depression. Arch Gen Psychiatry 63:856-864.
999 Zarate CA, Jr., Mathews D, Ibrahim L, Chaves JF, Marquardt C, Ukoh I, Jolkovsky L, Brutsche
1000 NE, Smith MA, Luckenbaugh DA (2013) A randomized trial of a low-trapping
1001 nonselective N-methyl-D-aspartate channel blocker in major depression. Biol Psychiatry
1002 74:257-264.
1003 Zhang F, Hillhouse TM, Anderson PM, Koppenhaver PO, Kegen TN, Manicka SG, Lane JT,
1004 Pottanat E, Van Fossen M, Rice R, Porter JH (2021) Opioid receptor system contributes
1005 to the acute and sustained antidepressant-like effects, but not the hyperactivity motor
1006 effects of ketamine in mice. Pharmacol Biochem Behav 208:173228.
1007











Figure/Statistical test		Number of mice (as appear in graph)	Factorial effects				Interaction effect	
Overall effects for Figure 1								
			Factor 'treatment'					
1A	one-way ANOVA	n = 7,7,7,6,7,7	$F_{[5,35]}$ = 4.10;	*p=0.005*				
			Factor 'treatment'		**Factor 'CSDS phase'**		***Factor 'treatment' x 'CSDS phase'***	
1B	two-way RM ANOVA	n = 8,6,7	$F_{[2,19]}$ = 1.288;	*p=0.299*	$F_{[2,38]}$ = 84.300;	*p<0.0001*	$F_{[4,38]}$ = 2.737;	*p=0.043*
Overall effects for Figure 2								
			Factor 'pre-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
2A	two-way ANOVA	n = 13,13,13,14	$F_{[1,48]}$ = 3.333;	*p=0.074*	$F_{[1,48]}$ = 5.134;	*p=0.028*	$F_{[1,48]}$ = 1.742;	*p=0.193*
2B	two-way ANOVA	n = 16,16,17,17	$F_{[1,62]}$ = 2.900;	*p=0.094*	$F_{[1,62]}$ = 4.625;	*p=0.035*	$F_{[1,62]}$ = 5.317;	*p=0.025*
			Factor 'treatment' x 'phase'		***Factor 'pre-treatment' x 'treatment'***		***Factor 'pre-treatment' x 'treatment' x 'phase'***	
2C	three-way RM ANOVA	n = 6,6,7,6	$F_{[2,42]}$ = 2.316;	*p=0.111*	$F_{[1,21]}$ = 6.452;	*p=0.019*	$F_{[2,42]}$ = 3.278;	*p=0.048*
			Factor 'pre-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
2D	two-way ANOVA	n = 8,9,9,9	$F_{[1,31]}$ = 4.109;	*p=0.051*	$F_{[1,31]}$ = 3.230;	*p=0.082*	$F_{[1,31]}$ = 7.313;	*p=0.011*
			Factor 'co-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
2E	two-way ANOVA	n = 7,9,8,8	$F_{[1,28]}$ = 10.020;	*p=0.004*	$F_{[1,28]}$ = 7.631;	*p=0.010*	$F_{[1,28]}$ = 7.606;	*p=0.010*
Overall effects for Figure 3								
			Factor 'pre-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
3A	two-way ANOVA	n = 8,9,8,9	$F_{[1,30]}$ = 6.804;	*p=0.014*	$F_{[1,30]}$ = 43.040;	*p<0.0001*	$F_{[1,30]}$ = 6.202;	*p=0.019*
3B	two-way ANOVA	n = 8,9,9,9	$F_{[1,31]}$ = 5.795;	*p=0.022*	$F_{[1,31]}$ = 5.261;	*p=0.029*	$F_{[1,31]}$ = 9.041;	*p=0.005*
3C	two-way ANOVA	n = 8,8,9,8	$F_{[1,29]}$ = 6.209;	*p=0.019*	$F_{[1,29]}$ = 3.499;	*p=0.072*	$F_{[1,29]}$ = 6.077;	*p=0.020*
3D	two-way ANOVA	n = 11,8,11,10	$F_{[1,36]}$ = 11.060;	*p=0.002*	$F_{[1,36]}$ = 44.570;	*p<0.0001*	$F_{[1,36]}$ = 19.760;	*p<0.0001*
3E	two-way ANOVA	n = 8, 10, 7, 10	$F_{[1,35]}$ = 5.753;	*p=0.022*	$F_{[1,35]}$ = 6.958;	*p=0.012*	$F_{[1,35]}$ = 3.737;	*p=0.061*
3F	two-way ANOVA	n = 8,7,10,8	$F_{[1,29]}$ = 9.865;	*p=0.004*	$F_{[1,29]}$ = 7.547;	*p=0.010*	$F_{[1,29]}$ = 11.540;	*p=0.002*
Overall effects for Figure 4								
			Factor 'treatment'					
4A	one-way ANOVA	n = 8,8,8	$F_{[2,21]}$ = 15.28;	*p<0.0001*				
			Factor 'pre-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
4B	two-way ANOVA	n = 8,7,7,8	$F_{[1,26]}$ = 16.410;	*p<0.001*	$F_{[1,26]}$ = 7.356;	*p=0.012*	$F_{[1,26]}$ = 5.937;	*p=0.022*
4D	two-way ANOVA	n = 10,10,10,10	$F_{[1,36]}$ = 4.468;	*p=0.042*	$F_{[1,36]}$ = 1.963;	*p=0.170*	$F_{[1,36]}$ = 3.944;	*p=0.054*
4E	two-way ANOVA	n = 10,10,10,10	$F_{[1,36]}$ = 1.253;	*p=0.270*	$F_{[1,36]}$ = 4.389;	*p=0.043*	$F_{[1,36]}$ = 2.729;	*p=0.107*
4H	two-way ANOVA	n = 8,5,5,6	$F_{[1,20]}$ = 11.630;	*p=0.003*	$F_{[1,20]}$ = 4.616;	*p=0.044*	$F_{[1,20]}$ = 6.246;	*p=0.021*
Overall effects for Figure 5								
			Factor 'pre-treatment'		***Factor 'treatment'***		***Factor 'pre-treatment' x 'treatment'***	
5A	two-way ANOVA	n = 6,7,7,7,7,7	$F_{[2,35]}$ = 1.320;	*p=0.280*	$F_{[1,35]}$ = 7.287;	*p=0.011*	$F_{[2,35]}$ = 4.796;	*p=0.014*
			Factor 'treatment'					
5B	one-way ANOVA	n = 6,6,6,7,7	$F_{[4,27]}$ = 2.469;	*p=0.069*				
5C	one-way ANOVA	n = 8,8,8,8,8	$F_{[4,35]}$ = 2.469;	*p=0.088*				
5D	one-way ANOVA	n = 9,8,8,8,7,8	$F_{[5,42]}$ = 2.110;	*p=0.083*				