Antidepressant-relevant concentrations of the ketamine metabolite (2R,6R)-hydroxynorketamine do not block NMDA receptor function

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Preclinical studies indicate that (2R,6R)-hydroxynorketamine (HNK) is a putative fast-acting antidepressant candidate. Although inhibition of NMDA-type glutamate receptors (NMDARs) is one mechanism proposed to underlie ketamine's antidepressant and adverse effects, the potency of (2R,6R)-HNK to inhibit NMDARs has not been established. We used a multidisciplinary approach to determine the effects of (2R,6R)-HNK on NMDAR function. Antidepressant-relevant behavioral responses and (2R,6R)-HNK levels in the extracellular compartment of the hippocampus were measured following systemic (2R,6R)-HNK administration in mice. The effects of ketamine, (2R,6R)-HNK, and, in some cases, the (2S,6S)-HNK stereoisomer were evaluated on the following: (*i*) NMDA-induced lethality in mice, (*ii*) NMDAR-mediated field excitatory postsynaptic potentials (fEPSPs) in the CA1 field of mouse hippocampal slices, (*iii*) NMDAR-mediated miniature excitatory postsynaptic currents (mEPSCs) and NMDA-evoked currents in CA1 pyramidal neurons of rat hippocampal slices, and (*iv*) recombinant NMDARs expressed in *Xenopus* oocytes. While a single i.p. injection of 10 mg/kg (2R,6R)-HNK exerted antidepressant-related behavioral and cellular responses in mice, the ED_{50} of (2R,6R)-HNK to prevent NMDA-induced lethality was found to be 228 mg/kg, compared with 6.4 mg/kg for ketamine. The 10 mg/kg (2R,6R)-HNK dose generated maximal hippocampal extracellular concentrations of ~8 μM, which were well below concentrations required to inhibit synaptic and extrasynaptic NMDARs in vitro. (2S,6S)-HNK was more potent than (2R,6R)-HNK, but less potent than ketamine at inhibiting NMDARs. These data demonstrate the stereoselectivity of NMDAR inhibition by (2R,6R;2S,6S)-HNK and support the conclusion that direct NMDAR inhibition does not contribute to antidepressant-relevant effects of (2R,6R)-HNK.

depression | ketamine | hydroxynorketamine | antidepressant | NMDA receptor

Significance

Standard antidepressant treatments require weeks to show effectiveness. A single subanesthetic dose of ketamine rapidly attenuates many clinical signs and symptoms of depression; however, ketamine treatment also has many adverse effects, including dissociation and potential for abuse, which are mediated by NMDA glutamate receptor (NMDAR) inhibition. Previous work has revealed that the ketamine metabolite (2R,6R)-hydroxynorketamine (HNK) induces antidepressant-like responses in rodents while minimizing the adverse effects observed with ketamine. The results of this study, using a multitude of experimental approaches, confirm that antidepressant-relevant concentrations of (2R,6R)-HNK are not sufficient to block NMDARs. This provides a basis for work directed at alternative molecular targets and toward novel drugs that exert rapid antidepressant effects independent of NMDAR inhibition and NMDAR-mediated adverse effects.

Major depressive disorder (MDD) occurs in about 16% of the population over the course of a lifetime (1). It is estimated that MDD affected nearly 7% of all US adults in 2016, and that one-half of those individuals were prescribed typical antidepressant medications as part of their treatment regimen (2). Although such typical antidepressants, including selective serotonin and norepinephrine reuptake inhibitors, tricyclic antidepressants, and monoamine oxidase inhibitors, can sometimes mitigate clinical symptoms of MDD, the onset of action of these drugs is very slow, requiring daily administration over weeks or months for clinical improvement (3). In addition, ~30% of pa-

tients suffering from MDD fail to respond after attempting multiple treatments (3). The use of (*R*,*S*)-ketamine (ketamine) for the treatment of MDD has generated much excitement because it reduces, and in some patients eliminates, many core symptoms of depression, including depressed mood, anhedonia, and suicidal ideation, within hours following i.v. administration of a single subanesthetic dose. Furthermore, ketamine is effective in patients who are refractory to typical antidepressants (4–9).

Although ketamine is a promising alternative to standard clinically used antidepressants, it induces adverse effects at antidepressant doses, particularly dissociation (10). Furthermore, ketamine, a derivative of the illicit drug phencyclidine, is widely abused (11). Ketamine is rapidly and stereoselectively metabolized in the liver to a number of metabolites, including the norketamines, hydroxyketamines, dehydronorketamines, and the hydroxynorketamines (HNKs) (12). Demethylation of the methyl amine on ketamine's central cyclohexyl ring generates the norketamines, which are then hydroxylated on the 4, 5, or 6 position of the cyclohexyl ring to form the HNKs. Following systemic ketamine administration, the 6-HNKs, that is (2*S*,6*S*;2*R*,6*R*)-HNK (Fig. 1), are the major HNK metabolites found in human plasma and in rodent plasma and brain (13–15). Earlier studies in rodents found that ketamine and norketamine exert anesthetic effects, but (2*S*,6*S*;2*R*,6*R*)-HNK does not (16). This finding contributed to the prevailing view that ketamine and possibly norketamine are the clinically active agents, whereas HNK metabolites are pharmacologically inactive (17–19). More recently, the (2*S*,6*S*;2*R*,6*R*)-HNK metabolites, particularly the (2*R*,6*R*)-HNK stereoisomer, were found effective in inducing antidepressant-relevant behavioral and cellular responses in mice (14). (2*S*,6*S*)-HNK was also identified as a potential antidepressant, but with lower potency than (2*R*,6*R*)-HNK (14). Although the antidepressant-relevant effects of (2*R*,6*R*)-HNK were later confirmed by independent research groups using different model systems (20–28), the mechanism underlying these effects is unknown.

Despite the recognized inhibitory action of ketamine on *N*-methyl-D-aspartate receptors (NMDARs), (2*R*,6*R*)-HNK does not appear to inhibit NMDAR function in vitro or to induce adverse effects expected of an NMDAR antagonist in vivo (12, 14, 28–30). Instead, at concentrations associated with antidepressant-relevant effects, (2*R*,6*R*)-HNK has been found to produce robust synaptic potentiation of excitatory synaptic transmission in hippocampal slices
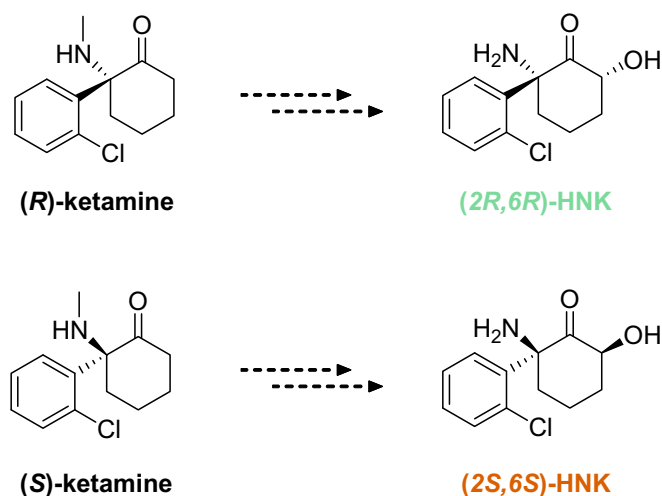
(14). Nevertheless, reports that at high concentrations (2*R*,6*R*)-HNK inhibits NMDAR function led to the suggestion that NMDAR inhibition accounts for (2*R*,6*R*)-HNK's antidepressant-relevant effects (31, 32).

The present study systematically assessed the effects of (2*R*,6*R*)-HNK on NMDAR function. Tests exploring behavioral despair and hyponeophagia in mice were employed to confirm the metabolite's antidepressant-relevant effects. Analytical assays were used to quantify plasma, whole-brain, and extracellular hippocampal levels of (2*R*,6*R*)-HNK following systemic treatment of mice with a dose that produces antidepressant-relevant effects. A series of functional tests including in vivo NMDA-induced lethality and ex vivo electrophysiological measurements of NMDAR activity in hippocampal neurons and in oocytes expressing distinct NMDAR subtypes (GluN1/GluN2A, GluN2B, GluN2C, or GluN2D) were used to determine the potency for (2*R*,6*R*)-HNK to inhibit NMDARs. The results lead to the conclusion that (2*R*,6*R*)-HNK does inhibit NMDAR function, but only at concentrations substantially higher than those produced by doses resulting in antidepressant-relevant effects in mice.

Results

(2*R*,6*R*)-HNK, at the Dose of 10 mg/kg, Exerts Antidepressant-Relevant Responses in Mice. Mice received i.p. injections of either (2*R*,6*R*)-HNK (10 mg/kg) or vehicle [0.9% (m/v) NaCl (saline), control] 1 or 24 h before being subjected to the forced swim test (FST), which assesses behavioral despair that is decreased by existing antidepressant drugs. Compared with saline-treated mice, mice treated with (2*R*,6*R*)-HNK showed significantly reduced immobility time at both time points (Fig. 2 *A* and *B*). This response is similar to that induced by ketamine and (2*R*,6*R*)-HNK, as previously reported (14).

The novelty suppressed feeding (NSF) test assesses the time a food-deprived mouse waits until biting a food pellet located in the middle of an illuminated open-field arena. This hyponeophagic response time is decreased by antidepressant drugs (33). We employed this test 30 min after a single 10 mg/kg injection of (2*R*,6*R*)-HNK (i.p.) to understand whether antidepressant-like effects occur at an earlier time point than what has previously been reported (~1 h) (14). Mice that received (2*R*,6*R*)-HNK required a significantly shorter amount of time to bite a food pellet than did saline-treated mice (Fig. 2*C*). (2*R*,6*R*)-HNK administration did not change food consumption of the mice in their home cages, providing evidence that there were no appetite changes following drug administration that motivated approach times [consumption in g/10 min (*n* = 10 mice/treatment): control, 0.3 ± 0.04; (2*R*,6*R*)-HNK, 0.4 ± 0.03; *P* = 0.3518].

Increased expression of mature BDNF (mBDNF) and activation of mTOR complex 1 (mTORC1) are considered important determinants of the effectiveness of antidepressants and can be detected 30 min after administration of ketamine (34, 35). Using immunoblots, we measured relative levels of mBDNF and its precursor, proBDNF, as well as levels of total and phosphorylated (activated) mTOR protein levels [mTOR and p-mTOR (Ser2448), respectively] in hippocampal extracts obtained from mice 30 min after the i.p. injection of 10 mg/kg of (2*R*,6*R*)-HNK or saline. While (2*R*,6*R*)-HNK treatment had no significant effect on the expression of proBDNF or mTOR, it significantly increased mBDNF and p-mTOR levels in the hippocampus (Fig. 2*D*).

Establishment of Antidepressant-Relevant Tissue Concentrations of (2*R*,6*R*)-HNK in Mice. Following i.p. treatment of mice with the 10 mg/kg (2*R*,6*R*)-HNK dose shown to induce antidepressant-relevant effects (Fig. 2 *A*–*D*; also see ref. 14), the highest plasma concentration of (2*R*,6*R*)-HNK was 23.96 ± 0.66 μM at 2.5 min posttreatment. Plasma concentrations rapidly declined to 15.12 ± 0.72 μM at 5 min, 8.71 ± 0.60 μM at 10 min, and below quantification at 60 min postinjection (Fig. 2*E*). In the target organ (brain), the maximum concentration of (2*R*,6*R*)-HNK was 18.70 ± 0.47 μmol/kg 5 min postinjection, with a decline to 10.15 ± 1.20 and 1.20 ± 0.17 μmol/kg, at 10 and 30 min postadministration, respectively, with levels below quantitation 1 h following injection (Fig. 2*F*).

Fig. 1. Metabolism of (*R*,*S*)-ketamine to the two hydroxynorketamine (HNK) stereoisomers, (2*R*,6*R*)-HNK and (2*S*,6*S*)-HNK. The amine group at the chiral center (C2 carbon) of (*R*)-ketamine and (*S*)-ketamine undergoes demethylation, producing (*R*)-norketamine and (*S*)-norketamine, followed by hydroxylation at the C6 carbon *cis* to the amine group to give the (2*R*,6*R*)- and (2*S*,6*S*)-HNKs. (*R*)-Ketamine selectively forms (2*R*,6*R*)-HNK, while (*S*)-ketamine selectively forms (2*S*,6*S*)-HNK. The primary intermediate metabolites, (*R*)- and (*S*)-norketamine, are not depicted.
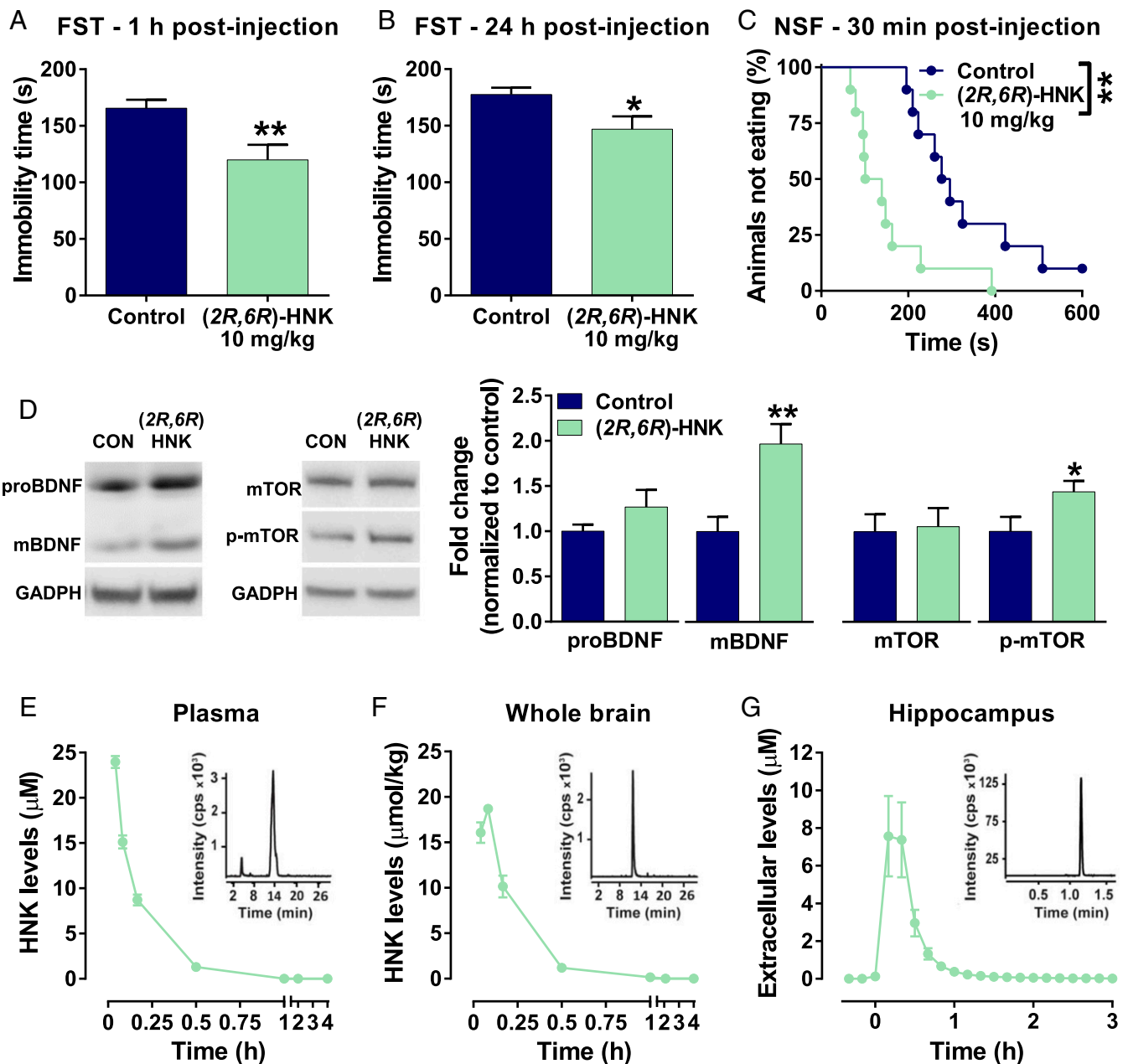
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Fig. 2. Behavioral effects and tissue concentrations following systemic administration of 10 mg/kg (2R,6R)-HNK to mice. Mice received i.p. injections of vehicle (control, i.e., saline) or (2R,6R)-hydroxynorketamine (HNK) at a dose of 10 mg/kg and were tested in the forced swim test (FST) (A) 1 h and (B) 24 h posttreatment, or were tested in the (C) novelty suppressed feeding test (NSF) 30 min posttreatment [$n = 10$ mice/treatment; (A) Student's unpaired t test, $t = 2.98$, df = 18; (B) Student's unpaired t test, $t = 2.40$, df = 18; (C) log-rank, Mantel–Cox test, $\chi^2 = 8.84$]. (D) Western blot analysis of hippocampal extracts revealed that levels of mBDNF (Student's unpaired t test, $t = 3.43$, df = 20; $n = 10$–12 mice/treatment), but not pro-BDNF (Student's unpaired t test, $t = 1.22$, df = 20; $n = 10$–12 mice/treatment) were significantly increased 30 min after treatment of mice with (2R,6R)-HNK (10 mg/kg, i.p.) compared with saline [control (CON)]. Total mTOR levels did not change (Student's unpaired t test, $t = 0.19$, df = 22; $n = 12$ mice/treatment), while the ratio of mTOR phosphorylated at Ser2448, to total mTOR increased 30 min posttreatment with (2R,6R)-HNK (Student's unpaired t test, $t = 2.17$, df = 22; $n = 12$ mice/treatment). Concentrations of (2R,6R)-HNK in the (E) plasma and (F) whole brain following systemic administration of (2R,6R)-HNK (10 mg/kg i.p.) to mice ($n = 4$ mice/treatment/time point). The measured analyte concentrations in the brain were normalized according to tissue weight and are reported as micromoles per kilogram of tissue. (G) Concentrations of (2R,6R)-HNK in the microdialysates from the ventral hippocampus of awake mice collected at a 10-min sampling rate following administration of (2R,6R)-HNK (10 mg/kg, i.p.) corrected for in vivo recovery of 54.8% and for dilution (1:10) of samples collected at low flow rate (0.1 μL/min) with 1 μL/min makeup solvent on the probe outlet ($n = 6$–7 mice/treatment/time point). (E–G, Insets) Representative chromatograms from the 10-min time point from each assay. Data points and error bars represent mean and SEM, respectively. *$P < 0.05$ and **$P < 0.01$.

Using microdialysis in freely moving mice, we measured (2R,6R)-HNK levels in the extracellular compartment of the hippocampus after an i.p. injection of 10 mg/kg (2R,6R)-HNK. Extracellular levels of (2R,6R)-HNK in the hippocampus reached a maximum (7.57 ± 2.13 μM) 10 min after the systemic administration (Fig. 2G). Clearance from the hippocampal extracellular space was slower than from plasma and whole-brain tissue. Approximately 30 min posttreatment, 39.1% of the measured highest concentrations remained in the hippocampal extracellular space (2.96 ± 0.79 μM), whereas only 15% and 12% of maximum (2R,6R)-HNK remained in the plasma and whole

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brain, respectively (Fig. 2 *E* and *F*). Extracellular concentrations of (2R,6R)-HNK in the hippocampus decreased to 0.37 ± 0.09 μM (5% of maximum) and 0.054 ± 0.015 μM (0.7% of maximum) by 1 and 2 h, respectively, after the systemic treatment of mice. We conclude that the concentrations in the extracellular compartment of the hippocampus treated systemically with the (2R,6R)-HNK dose of 10 mg/kg that induces antidepressant-relevant effects in mice are ≤10 μM.

Antidepressant-Relevant Doses of (2R,6R)-HNK Are Insufficient to Prevent NMDA-Induced Lethality. Prevention of lethality induced by systemic administration of NMDA is a historical measure of in vivo potency of drugs that inhibit NMDAR function (36–38). Here, mice were treated with a single i.p. injection of ketamine, (2R,6R)-HNK, or (2S,6S)-HNK, and 5 min later, injected with the LD_{99} of NMDA (250 mg/kg) (36). The doses of ketamine, (2R,6R)-HNK, and (2S,6S)-HNK that protected 50% of mice from NMDA-induced lethality (i.e., ED_{50}) were 6.4, 227.8, and 18.6 mg/kg, respectively (Fig. 3A and Table 1). The calculated mean time to death at each of these ED_{50} values was ∼30 min [28.3, 24.0, and 31.7 min for ketamine, (2S,6S)-HNK, (2R,6R)-HNK, respectively]. At doses that had no effect on NMDA-induced lethality, the mean time to death was <20 min.

The highest brain concentrations measured following treatment of a separate group of mice with the estimated ED_{50} values of ketamine (6.4 mg/kg, i.p.), (2R,6R)-HNK (227.8 mg/kg, i.p.), and (2S,6S)-HNK (18.6 mg/kg, i.p.) were 13.66 (5 min), 830.4 (5 min), and 30.8 (10 min) μmol/kg, respectively (Fig. 3B). The area under the curve of brain concentrations vs. time between the first and last sampling time (AUClast) revealed that total brain levels over time were 3.05, 302.6, and 10.11 μM/kg·h for ketamine, (2R,6R)-HNK, and (2S,6S)-HNK, respectively. Thus, based on the brain concentrations produced by the ED_{50} values of the test compounds, ketamine is estimated to be 60- to 100-fold more potent than (2R,6R)-HNK in this in vivo measure of NMDAR function.

Antidepressant-Relevant Concentrations of (2R,6R)-HNK Are Insufficient to Inhibit Evoked NMDAR-Mediated Field Excitatory Postsynaptic Potentials in the Mouse Hippocampus. In nominally Mg^{2+}-free ACSF at room temperature, ketamine, (2R,6R)-HNK, and (2S,6S)-HNK inhibited NMDAR-mediated field excitatory postsynaptic potentials (fEPSPs) in the CA1 field of mouse hippocampal slices, as evidenced by a concentration-dependent reduction of fEPSP slopes (Fig. 4). While ketamine inhibited NMDAR-mediated fEPSPs with an IC_{50} of 4.5 μM, (2R,6R)-HNK inhibited these fEPSPs with a nearly 50-fold higher IC_{50} (211.9 μM; Fig. 4D and Table 1). The IC_{50} for (2S,6S)-HNK to inhibit fEPSPs was 47.2 μM (Fig. 4E and Table 1). Likewise, recordings obtained at 32–35 °C revealed that, at these physiologically relevant temperatures, 10 and 100 μM ketamine significantly reduced NDMAR-mediated fEPSP slopes by $76.3\% \pm 7.2$ and $88.7\% \pm 6.1$, respectively, whereas 10 and 100 μM (2R,6R)-HNK had no significant effect on these synaptic responses (*SI Appendix*, Fig. S1).

Antidepressant-Relevant Concentrations of (2R,6R)-HNK Are Insufficient to Inhibit NMDAR-Mediated Miniature Excitatory Postsynaptic Currents in Rat CA1 Pyramidal Neurons. NMDAR-mediated miniature excitatory postsynaptic currents (mEPSCs) were recorded from rat CA1 pyramidal neurons in the presence and absence of a range of concentrations of ketamine and (2R,6R)-HNK in nominally Mg^{2+}-free ACSF (Fig. 5A). Ketamine and (2R,6R)-HNK reduced the mean amplitude of the mEPSCs in a concentration-dependent manner (Fig. 5B), with IC_{50} values of 6.4 and 63.7 μM, respectively (Table 1). The median event amplitudes measured from neurons following control superfusion were 10.57 ± 0.59 pA. Based on the 5-pA threshold for event detection, the largest possible reduction of event amplitude is ∼52% of control explaining why the inhibition reached a plateau at ∼60% of control.

The cumulative distributions of mEPSC amplitudes recorded in the presence of ≥10 μM ketamine and ≥50 μM (2R,6R)-HNK were also significantly shifted toward smaller amplitudes in comparison with control (Fig. 5C). Analysis of the frequency of events revealed that the reduction of mEPSC frequency by ketamine and (2R,6R)-HNK
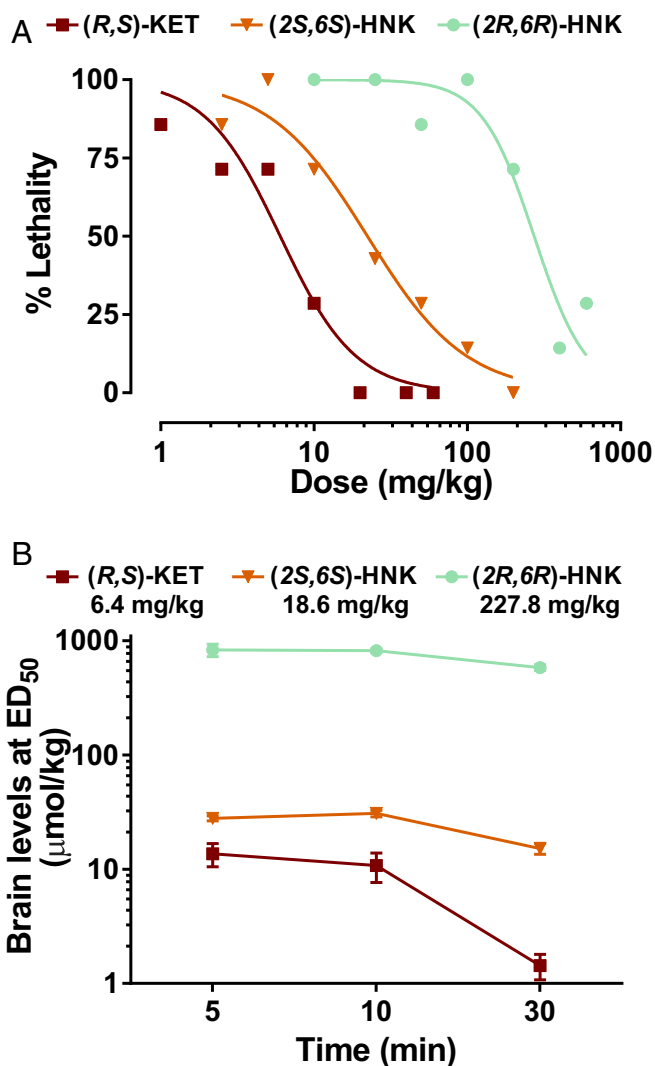

Fig. 3. Dose–response relationship for (R,S)-ketamine, (2R,6R)-HNK, and (2S,6S)-HNK to prevent NMDA-induced lethality. Mice received an i.p. injection of ketamine (KET), (2R,6R)- hydroxynorketamine (HNK), or (2S,6S)-HNK. Five minutes after the treatment, mice received an i.p. injection of 250 mg/kg NMDA. (A) Percent lethality at 24 h post-NMDA (n = 6 mice/dose). (R,S)-ketamine, (2R,6R)-HNK, and (2S,6S)-HNK dose dependently prevented lethality. The effective doses of ketamine, (2R,6R)-HNK, and (2S,6S)-HNK that protected 50% of the population from NMDA-induced lethality (i.e., ED_{50}) were 6.4, 227.8, and 18.63 mg/kg, respectively. (B) Whole-brain measurements following systemic administration of ED_{50} doses of ketamine (6.4 mg/kg), (2R,6R)-NHK (227.8 mg/kg), and (2S,6S)-HNK (18.63 mg/kg) normalized according to tissue weight (n = 3–4 mice/treatment/time point). Data points and error bars represent mean and SEM, respectively.

mirrored the reduction of the mEPSC amplitudes, suggesting that, in the presence of effective concentrations of the test compounds, many events became too small to be detected.

Antidepressant-Relevant Concentrations of (2R,6R)-HNK Are Insufficient to Inhibit NMDA-Induced Whole-Cell Current in Rat CA1 Pyramidal Neurons. Whole-cell currents induced by the admixture of NMDA (50 μM) and glycine (10 μM) delivered via a U-tube system were recorded from rat CA1 pyramidal neurons. Representative sample recordings obtained in the absence and in the presence of each test compound in Mg^{2+} (1 mM)-containing ACSF are shown in Fig. 6A. Ketamine reduced the total charge carried by NMDA-induced currents in a concentration-dependent manner (Fig. 6B). The IC_{50} for ketamine was estimated to be 45.9 μM (Fig. 6B and

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Table 1). Likewise, (2S,6S)-HNK suppressed the NMDA-evoked currents (Fig. 6B); however, with only two test concentrations, the data could not be fitted for an IC_{50} calculation. In contrast, (2R,6R)-HNK tested at concentrations ranging from 50 to 1,000 μM had no significant effect on NMDA-induced currents (Fig. 6).

Antidepressant-Relevant Concentrations of (2R,6R)-HNK Are Insufficient to Inhibit NMDARs Regardless of Subunit Composition. Rat GluN1/GluN2 RNA was injected into *Xenopus* oocytes to form recombinant heterodimeric receptors of GluN1 and either GluN2A, GluN2B, GluN2C, or GluN2D. An admixture of glutamate-plus-glycine (100 μM each, 100G/G) was applied to the oocytes to establish the maximum current amplitude. (2R,6R)-HNK or (2S,6S)-HNK was then applied to individual cells at ascending concentrations in combination with glutamate/glycine, and the difference in current was recorded as a percent inhibition from maximum.

(2R,6R)-HNK and (2S,6S)-HNK concentration dependently reduced the amplitudes of glutamate/glycine-evoked currents in oocytes expressing different NMDAR subtypes. Based on the analysis of the concentration–response relationships, (2S,6S)-HNK inhibited the different NMDAR subtypes with markedly higher potency than did (2R,6R)-HNK (Fig. 7 and Table 1). The rank order of potency for the two compounds to block the distinct NMDAR subtypes also differed. For (2R,6R)-HNK, the rank order of potency was as follows: GluN1/GluN2C receptors (IC_{50}, 202 μM) ~ GluN1/GluN2B (IC_{50}, 258 μM) ~ GluN1/GluN2D (IC_{50}, 287 μM) > GluN1/GluN2A receptors (IC_{50}, 498 μM). In contrast, for (2S,6S)-HNK, the rank order of potency was as follows: GluN1/GluN2D (IC_{50}, ~13 μM) = GluN1/GluN2C (IC_{50}, ~15 μM) > GluN1/GluN2B (IC_{50}, ~21 μM) > GluN1/GluN2A (IC_{50}, ~43 μM). Under similar conditions, the rank order of potency for ketamine was previously reported to be as follows: GluN1/2B (IC_{50}, 0.9 μM) > GluN1/2C (IC_{50}, 1.7 μM) ~ GluN1/2D (IC_{50}, 2.4 μM) > GluN1/2A (IC_{50}, 3.3 μM) (39).

The voltage dependence of the inhibitory effect of (2S,6S)-HNK on NMDARs was also explored. (2S,6S)-HNK inhibition of each NMDAR subtype was voltage dependent (*SI Appendix*, Fig. S2). Specifically, less current passed through each receptor subtype in the presence of different concentrations (3–30 μM) of the metabolite relative to control when the cells were voltage clamped at progressively more negative holding voltages. The magnitude of the inhibitory effect of (2S,6S)-HNK on the four NMDAR subtypes decreased markedly as the membrane potentials became less negative, with the effect of all test concentrations in each NMDAR subtype becoming negligible at membrane potentials more positive than −10 mV.

Discussion

Ketamine has emerged as an alternative treatment for depression due to its fast onset of action and effectiveness in treating patients who are refractory to typical pharmacotherapies; however, the beneficial antidepressant effects of ketamine are accompanied by detrimental adverse effects, including dissociation and abuse potential, limiting its clinical utility (12). We and others reported that, in a number of preclinical models, the ketamine metabolite (2R,6R)-HNK induces antidepressant-relevant effects at similar doses as ketamine, without ketamine's adverse effects at these doses (14, 20–28). It has been debated, however, whether NMDAR inhibition, the mechanism proposed to underlie the antidepressant effects of ketamine, contributes to the antidepressant effects of (2R,6R)-HNK (14, 31, 32, 40). The results of the present study reveal that the rank order of potency for inhibition of NMDAR function is ketamine > (2S,6S)-HNK > (2R,6R)-HNK regardless of animal species and type of NMDAR-mediated response measured in vivo or in vitro (mouse fEPSPs; rat mEPSCs and NMDA-evoked responses; glutamate-evoked responses in *Xenopus* oocytes expressing distinct rat NMDAR subtypes; and NMDA-induced lethality). These findings support the hypothesis that direct inhibition of NMDARs by (2R,6R)-HNK is not a determinant of the antidepressant-relevant effects of this ketamine metabolite.

Systemic administration of 10 mg/kg (2R,6R)-HNK to adult male CD-1 mice suppressed behavioral despair and hyponeophagia in the

FST (1 and 24 h following treatment) and the NSF test (30 min after treatment), respectively. This is in line with previous studies reporting antidepressant-related behavioral effects of (2R,6R)-HNK similar to those of ketamine in mice and rats (14, 20, 27, 28). We have previously reported antidepressant-relevant behavioral effects of (2R,6R)-HNK at doses ranging between 3 and 10 mg/kg (i.p.) in the FST (1 h, 24 h, and 3 d after administration), NSF (1 h after administration), reversal of learned helplessness behavior (24 h after administration), and chronic corticosterone-induced anhedonia, all in CD-1 mice. Additionally, reversal of social defeat-induced social interaction deficits was observed in C57BL/6J mice 24 h after i.p. administration of 20 mg/kg (2R,6R)-HNK (14). BALB/cJ mice treated with (2R,6R)-HNK, delivered via an i.p. injection (10 mg/kg) or directly to the medial prefrontal cortex, also exhibited 24 h later antidepressant-related behaviors in the FST consistent with those induced by similar treatments with ketamine (20). (2R,6R)-HNK, administered i.p. (10 or 30 mg/kg) or directly to the medial pre-frontal cortex, resulted in sustained antidepressant-like effects on a number of outcomes assessed in C57BL/6J mice (27). In addition, Chou et al. (24) reported that rats exhibited antidepressant-relevant behaviors 1 h and up to 21 d after a single i.p. administration of (2R,6R)-HNK (10 mg/kg). We note one research group has reported being unable to detect antidepressant-relevant behavioral effects of (2R,6R)-HNK in rodent behavioral tests (41, 42).

Prevention of NMDA-induced lethality, a well-established measure of the in vivo potency of NMDAR antagonists (36–38), was used here to assess the potency of ketamine, (2R,6R)-HNK, and (2S,6S)-HNK to inhibit NMDAR function in the same mouse strain utilized for our behavioral tests. Although significant effects on behavioral despair and hyponeophagia measures were observed in mice treated with 10 mg/kg (2R,6R)-HNK, this dose had no effect on NMDA-induced lethality. (2R,6R)-HNK doses greater than 200 mg/kg were required to prevent the lethal effect of NMDA (Fig. 3A). In contrast, NMDA-induced lethality was prevented in ~60–70% of the tested mice pretreated with previously reported antidepressant doses of ketamine (10 mg/kg, i.p.) or (2S,6S)-HNK (25 mg/kg, i.p) (14). Thus, while an antidepressant-relevant dose of (2R,6R)-HNK is well below doses needed to inhibit NMDARs in vivo, antidepressant doses of ketamine and (2S,6S)-HNK overlap those required to inhibit an NMDAR-mediated response in vivo. The ED_{50}s required to prevent NMDA-induced lethality resulted in peak brain concentrations of ~14, 830, and 31 μmol/kg for ketamine, (2R,6R)-HNK, and (2S,6S)-HNK, respectively, indicating that remarkably high in vivo concentrations of (2R,6R)-HNK are necessary for NMDAR inhibition. These data strongly argue that NMDAR inhibition in vivo is not a shared characteristic leading to the antidepressant-relevant actions of ketamine and (2R,6R)-HNK.

In the nominal absence of extracellular Mg^{2+}, ketamine, (2R,6R)-HNK, and (2S,6S)-HNK reduced the slope of NMDAR-mediated fEPSPs in the CA1 field of mouse hippocampal slices, with the rank order of potency being ketamine > (2S,6S)-HNK > (2R,6R)-HNK (Table 1). Under similar experimental conditions, ketamine was found to be approximately 10-fold more potent than (2R,6R)-HNK in reducing the amplitude of the mEPSCs recorded from CA1 pyramidal neurons in rat hippocampal slices. Neither mEPSCs nor fEPSPs were significantly blocked by 10 μM (2R,6R)-HNK, a concentration comparable to the hippocampal extracellular C_{max} generated by an antidepressant-relevant dose of this metabolite (Fig. 2G). In contrast, at concentrations of ketamine that result in antidepressant-like efficacy (i.e., 10 μM; ref. 14), mEPSC amplitudes and fEPSP slopes were suppressed by >50%.

In the presence of 1 mM extracellular Mg^{2+}, NMDA-plus-glycine–evoked whole-cell currents in CA1 pyramidal neurons of the rat hippocampus were insensitive to (2R,6R)-HNK concentrations as high as 1 mM. These NMDAR-mediated whole-cell currents were blocked by the test compounds with the same order of potency as that observed for NMDAR-mediated synaptic responses in the nominal absence of Mg^{2+}, that is, ketamine > (2S,6S)-HNK > (2R,6R)-HNK. It is noteworthy, however, that the IC_{50} for ketamine to block whole-cell currents evoked by NMDA-plus-glycine was found to be ~50 μM, which is markedly greater than the IC_{50} for
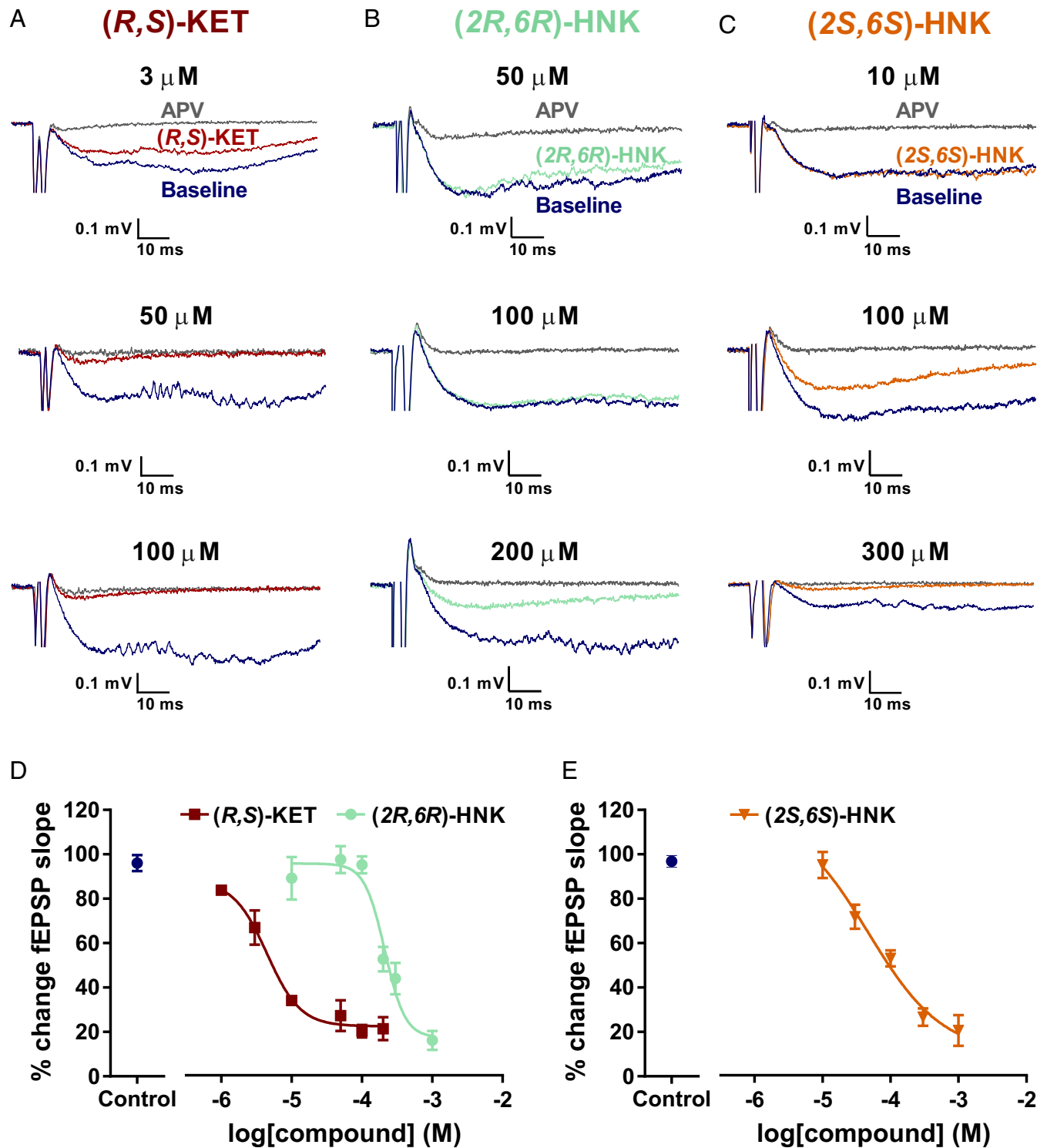
Fig. 4. Concentration–response relationship for (R,S)-ketamine, (2R,6R)-HNK, and (2S,6S)-HNK to inhibit NMDAR fEPSPs in the CA1 field of mouse hippo-campal slices. NMDAR-mediated fEPSPs were recorded before and after superfusion of slices with various concentrations of ketamine (KET), (2R,6R)-HNK, and (2S,6S)-HNK. (A–C) Sample recordings of fEPSPs obtained before and during exposure to the slices to KET, (2R,6R)-HNK, or (2S,6S)-HNK are shown. Traces in blue represent baseline potentials. Traces in red, green, and orange represent fEPSPs recorded in the presence of ketamine, (2R,6R)-HNK, or (2S,6S)-HNK, respectively. Traces in gray represent fEPSPs recorded after application of APV. Graphs of changes in fEPSP slope as a function of concentrations of (D) KET and (2R,6R)-HNK and (E) (2S,6S)-HNK. The respective vehicle control values are plotted in blue. Data points and error bars represent mean and SEM, respectively [n = 4–7 slices/test compound concentration; (R,S)-KET and (2R,6R)-HNK control, n = 36; (2S,6S)-HNK control, n = 19 (controls for each concentration were run separately for blinding purposes)]. The IC$_{50}$ values of ketamine, (2R,6R)-HNK, and (2S,6S)-HNK were found to be 4.5, 211.9, and 47.2 μM, respectively (Table 1).
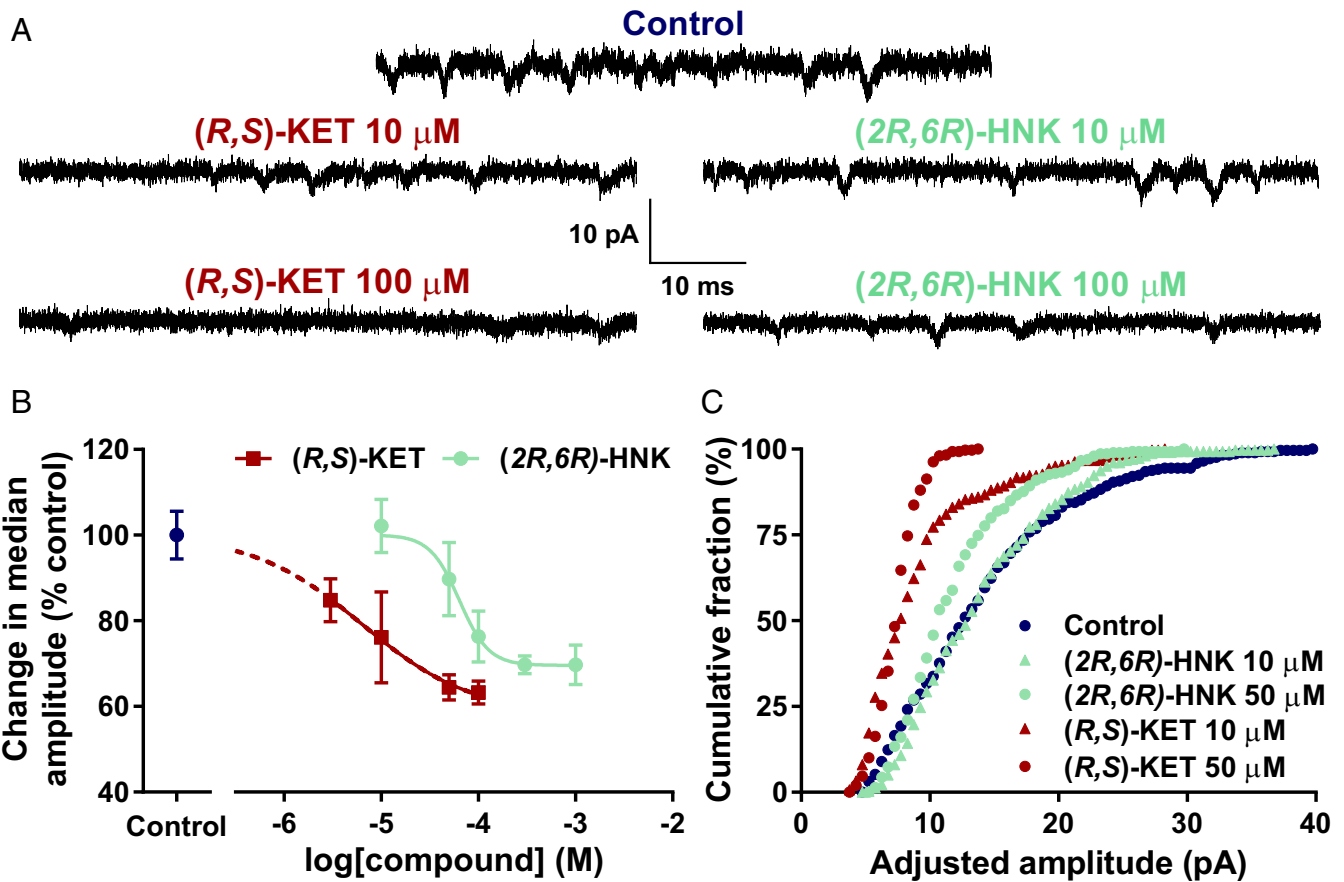
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Fig. 5. Concentration–response relationship for (R,S)-ketamine and (2R,6R)-hydroxynorketamine (HNK) to inhibit NMDAR mEPSCs in rat hippocampal slice CA1 pyramidal neurons. NMDAR mEPSCs were recorded before and after perfusion of slices with various concentrations of ketamine (KET) and (2R,6R)-HNK. (A) Sample recordings of mEPSCs recorded in the absence (control) and in the presence of different concentrations of KET and (2R,6R)-HNK. (B) Graphs of changes in median EPSC amplitude as a function of compound concentrations. All results were normalized to control, as described in *Materials and Methods*. Data points and error bars represent mean and SEM, respectively ($n = 3–8$ neurons/test compound concentration; control, $n = 26$: controls for each concentration were run separately for blinding purposes). IC_{50} values were estimated to be 6.4 μM for ketamine and 63.7 μM for (2R,6R)-HNK (Table 1). (C) Cumulative distribution of adjusted amplitudes of mEPSCs recorded in the presence of vehicle (control) or different concentrations of KET and (2R,6R)-HNK. Adjusted amplitude was determined by multiplying every event by its cell's respective inhibition ratio (postsuperfusion median/presuperfusion median). All events from all cells were pooled together by compound and concentration and then randomized. Subsequently, 300 events were randomly selected from the total pool for each group to generate the cumulative histograms.

ketamine to inhibit other electrophysiological NMDAR-mediated responses measured in this study (Table 1). This could be accounted for in part by the presence of different concentrations of extracellular Mg^{2+} among the experiments. As ketamine is a noncompetitive open-channel blocker at NMDARs (12), extracellular Mg^{2+} affects the potency with which it blocks the receptors (43).

Experiments carried out in *Xenopus* oocytes demonstrated that NMDARs of defined subunit compositions were also markedly more sensitive to inhibition by ketamine than (2R,6R)-HNK, with the antagonistic potencies of the two compounds differing by more than 100-fold. The potencies of (2S,6S)-HNK to block GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D were intermediate between ketamine and (2R,6R)-HNK. Of the four NMDARs, GluN1/GluN2A receptors were the least sensitive to inhibition by ketamine, (2S,6S)-HNK, and (2R,6R)-HNK (Table 1). At an antidepressant-relevant ketamine concentration [i.e., 10 μM, as previously determined (14)], in the nominal absence of extracellular Mg^{2+}, the activity of GluN1/GluN2A, GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D receptors was inhibited by >50% (39). In contrast, at an antidepressant-relevant (2R,6R)-HNK concentration (i.e., 8 μM, as determined in the present study), the activity of all NMDAR subtypes remained unaffected. Nearly 60- and 30-fold higher concentrations of (2R,6R)-HNK were needed to block the activity of GluN1/GluN2A receptors and the other three NMDAR

subtypes (GluN1/GluN2B, GluN1/GluN2C, and GluN1/GluN2D), respectively.

Each electrophysiological experiment in this study supported an NMDAR inhibition rank order of ketamine > (2S,6S)-HNK > (2R,6R)-HNK, a conclusion in agreement with the published inhibitory constants (K_i) for ketamine, (2S,6S)-HNK, and (2R,6R)-HNK of <5, 10–20, and >100 μM respectively, to displace [³H]MK-801 binding to the PCP/ketamine binding site of the NMDAR in rat forebrain homogenates (12, 14, 29, 30, 44, 45). However, there are discrepancies among the IC_{50} values estimated on the basis of reduction of fEPSP slopes, mEPSC amplitudes, and amplitudes of glutamate-plus-glycine–evoked whole-cell currents in *Xenopus* oocytes expressing distinct NMDAR subtypes (Table 1). These differences may be explained by the different preparations used, distinct cellular location and receptor subtypes assessed, and the use of varying extracellular Mg^{2+} concentrations, as previously mentioned.

There is a consensus that NMDAR inhibition underlies the adverse effects of ketamine (12). Considering that NMDAR inhibition is only observed at (2R,6R)-HNK concentrations/doses well above those associated with (2R,6R)-HNK's antidepressant-relevant effects, the adverse effects of (2R,6R)-HNK would be predicted to occur at much higher doses than those of ketamine. Indeed, while hyperactivity is observed in mice treated with 10 mg/kg ketamine (14), it is not detected in mice treated with (2R,6R)-HNK doses up to
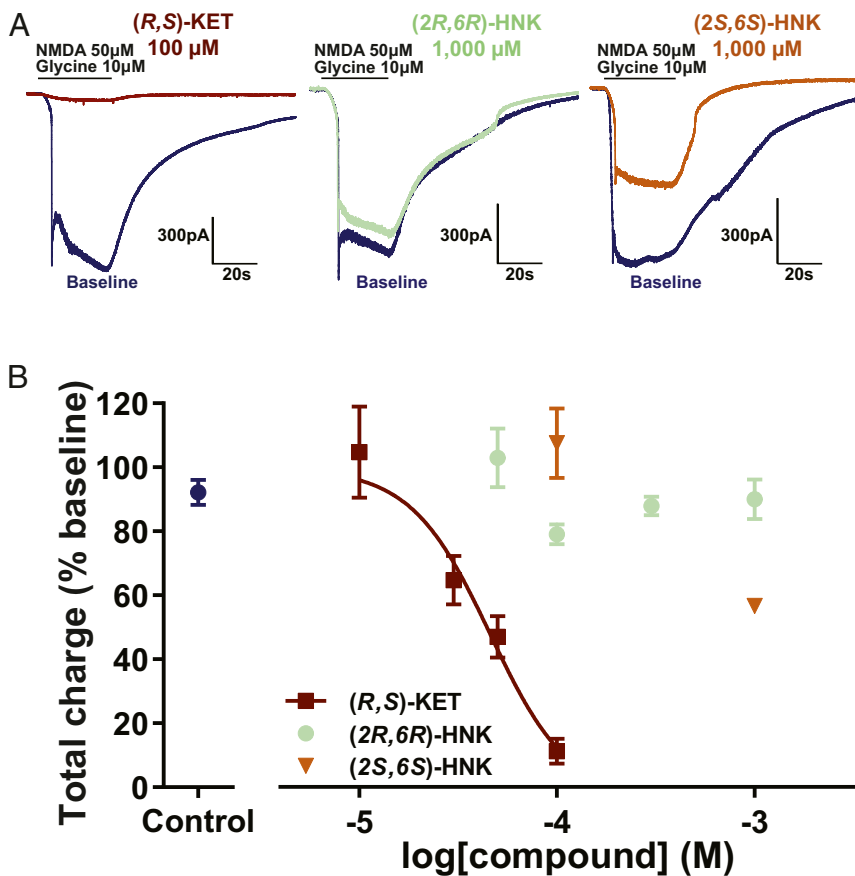
Fig. 6. Concentration-dependent effects of (*R,S*)-ketamine, (2*R*,6*R*)-hydroxynorketamine (HNK), and (2*S*,6*S*)-HNK on NMDA-induced whole-cell currents in rat hippocampal slice CA1 pyramidal neurons. (*A*) Sample recordings of NMDA-induced whole-cell currents with baseline measurements (maximum current following agonist pulse, blue) overlaid with currents in the presence of the maximum concentrations of each test compound [red, ketamine; green, (2*R*,6*R*)-HNK; orange, (2*S*,6*S*)-HNK]. (*B*) Concentration–response relationship for inhibition of the whole-cell currents by the test compounds. Data points and error bars represent mean and SEM, respectively (n = 4–13 neurons/test compound concentration; control, n = 24: controls for each concentration were run separately for blinding purposes). The IC_{50} value for ketamine was calculated to be 45.9 μM.

125 mg/kg (14). Similarly, 30 mg/kg ketamine induced sensory dissociation deficits, as measured by prepulse inhibition, while doses of (2*R*,6*R*)-HNK up to 375 mg/kg had no such effect (14). Likewise, impaired coordination has been observed in mice treated with antidepressant-relevant doses of ketamine and (2*S*,6*S*)-HNK, but not in mice treated with (2*R*,6*R*)-HNK doses up to 125 mg/kg (14). These potencies in behavioral models align with our current findings regarding the potencies of these compounds to inhibit NMDARs.

The abuse liability of NMDAR antagonists, which is also associated with NMDAR inhibition, is predicted to be less with (2*R*,6*R*)-HNK. Specifically, noncompetitive NMDAR antagonists, including ketamine and phencyclidine (46), produce discriminative stimulus effects in drug discrimination protocols and manifest cross-drug substitution profiles at an antidepressant-relevant dose range. On the other hand, in CD-1 mice trained to discriminate 10 mg/kg ketamine, (2*R*,6*R*)-HNK administration at doses of 10 and 50 mg/kg did not produce ketamine-related discrimination responses, whereas phencyclidine did (14). Furthermore, mice did not self-administer antidepressant-relevant doses of (2*R*,6*R*)-HNK under the same conditions for which they self-administered ketamine (14).

The exact mechanism of action underlying (2*R*,6*R*)-HNK's antidepressant effects is still not completely understood. Many signaling and physiological mechanisms, which are considered critical to the antidepressant effects of ketamine and are not dependent on NMDAR inhibition, are similarly affected by (2*R*,6*R*)-HNK both in vivo and in vitro. For example, decreased phosphorylation of the eukaryotic elongation factor 2 (at 1 and 24 h) and increased mBDNF, GluA1, and GluA2 levels (at 24 h) in hippocampal synaptoneurosome fractions have been observed following administration of 10 mg/kg (2*R*,6*R*)-HNK to mice (14). Likewise, ketamine and (2*R*,6*R*)-HNK (10 mg/kg, i.p.) increased cortical electroencephalographic gamma rhythms in mice (14). Cortical 5-hydroxytryptamine levels and basal glutamate release were also significantly increased

24 h after i.p. administration of 10 mg/kg (2*R*,6*R*)-HNK or ketamine to mice (20). In vitro, similar to ketamine, 10 μM (2*R*,6*R*)-HNK was reported to translocate Gα$_s$ from lipid raft domains to nonraft domains and increase intracellular cAMP (21), up-regulate AMPA receptor (AMPAR) subunit mRNA expression in cell culture at a concentration of 0.4 μM (25), and enhance structural plasticity in mouse mesencephalic and human induced pluripotent stem cell-derived dopaminergic neurons via AMPAR-driven BDNF and mTOR signaling at a concentration of 0.5 μM (23). According to results obtained in the present study, mBDNF and phosphorylated mTOR levels in hippocampal extracts were also significantly increased 30 min after administration of 10 mg/kg (2*R*,6*R*)-HNK to mice. The earlier report that (2*R*,6*R*)-HNK treatment lacked effect on mTOR phosphorylation or mature BDNF levels at a later time point (i.e., 1 h postinjection) in mice (14) can be reconciled by the fact that, in that study, mTOR phosphorylation and BDNF expression were assessed in synaptoneurosome fractions instead of total extracts. In addition, the possibility cannot be ruled out that immediate changes in mBDNF and mTOR activation occur in a narrow time window following a treatment (34, 35) and may, therefore, have been missed in the earlier study. Indeed, Fukumoto et al. (27) recently reported increases in mTOR phosphorylation at 30 min, but not 60 min, postinjection in the medial prefrontal cortex and also found that (2*R*,6*R*)-HNK antidepressant-relevant responses are mTORC1 and BDNF activity dependent.

Ex vivo studies revealed that antidepressant-relevant concentrations of (2*R*,6*R*)-HNK produce a robust potentiation of AMPAR-mediated excitatory synaptic transmission in slices from the hippocampus (14) and the midbrain ventrolateral periaqueductal gray of rats (24). By 24 h after administration of 10 mg/kg (2*R*,6*R*)-HNK or ketamine to mice, induction of long-term potentiation was impaired in the nucleus accumbens and AMPAR-mediated responses were depressed in ventral tegmental area dopaminergic neurons (22). The
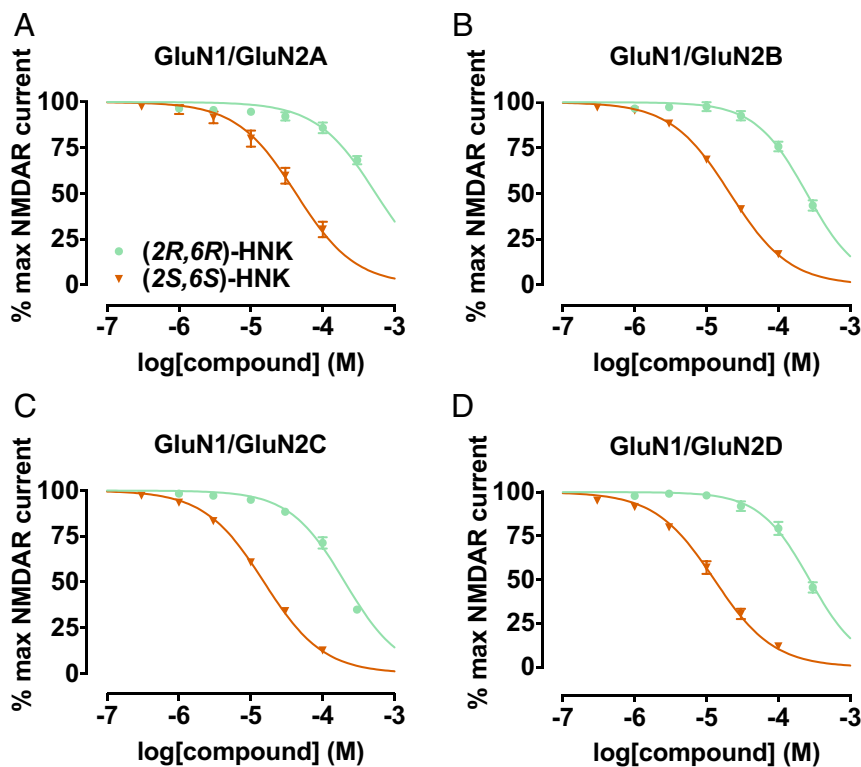
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Fig. 7. Concentration-dependent inhibition of glutamate NMDAR subtypes by (2R,6R)-hydroxynorketamine (HNK) and (2S,6S)-HNK. *Xenopus laevis* oocytes coexpressing rat GluN1 with either rat (*A*) GluN2A, (*B*) GluN2B, (*C*) GluN2C, or (*D*) GluN2D were activated with L-glutamate and glycine (100 μM each) and exposed to increasing concentrations of (2S,6S)-HNK or (2R,6R)-HNK to determine the IC_{50} for each NMDAR subtype. (2S,6S)-HNK inhibited each NMDAR subtype to a greater degree than its isomeric counterpart (2R,6R)-HNK (Table 1). Data points and error bars represent mean and SEM, respectively (n = 3–20 oocytes/receptor subtype/test compound).

finding that administration of an AMPAR antagonist to mice blocked the antidepressant behavioral effects of (2R,6R)-HNK suggests that modulation of AMPAR activity plays a role in the antidepressant-relevant effects of (2R,6R)-HNK (14).

Although there are a multitude of treatment options for depression, typical pharmacotherapies require daily administration over multiple weeks before improvement is expected, with many patients failing to find an effective therapy (3). The discovery that a single administration of ketamine rapidly relieves depressive symptoms has brought upon hopes for new therapies; however, ketamine's many adverse effects and abuse potential due to NMDAR inhibition pose serious challenges for its clinical use. Overall, we found that at antidepressant-relevant concentrations/doses, (2R,6R)-HNK is unable to inhibit NMDARs, potentially accounting for its reduced adverse behavioral effects compared with ketamine. The NMDAR-independent antidepressant actions of the ketamine's metabolite (2R,6R)-HNK and of other compounds that may share (2R,6R)-HNK's antidepressant-relevant mechanisms, may provide safe, fast-acting, and effective alternatives to the currently approved pharmacological treatments for MDD.

Materials and Methods

Detailed methods are described in *SI Appendix, SI Materials and Methods*.

Animals. Male CD-1 mice (Charles River Laboratories) were 8–10 wk of age at the time of experiments. Male Sprague-Dawley rats (8 d old on arrival; Charles River Laboratories) were acclimated with a nursing dam until postnatal day 21 when they were weaned.

Drugs. (2R,6R)- and (2S,6S)-HNK hydrochlorides (Fig. 1) were synthesized at the National Center for Advancing Translational Sciences. Absolute and relative stereochemistries were confirmed by small-molecule X-ray crystallography. Ketamine-HCl was purchased from Sigma-Aldrich.

Tissue Measurements of (2R,6R)-HNK Levels. To obtain brain and plasma levels of (2R,6R)-HNK, mice were anesthetized with 3% isoflurane and subsequently decapitated 2.5, 5, 10, 30, 60, 120, or 240 min following (2R,6R)-HNK (10 mg/kg, i.p.) administration. Microdialysis experiments were carried out on awake mice from cannula implanted into the ventral hippocampus.

Prevention of NMDA-Induced Lethality. Mice received a single i.p. injection of ketamine (1, 2.5, 5, 10, 20, 40, or 60 mg/kg), (2R,6R)-HNK (10, 25, 50, 100, 200, 400, or 600 mg/kg), or (2S,6S)-HNK (2.5, 5, 10, 25, 50, 100, or 200 mg/kg). Five

Table 1. ED_{50} and IC_{50} for NMDAR inhibition by ketamine, (2R,6R)-HNK, and (2S,6S)-HNK

Experiment	Ketamine	(2R,6R)-HNK	(2S,6S)-HNK
ED_{50}, mg/kg			
NMDA-induced lethality	6.4	227.8	18.6
IC_{50}, μM			
NMDAR-mediated fEPSP slope	4.5	211.9	47.2
NMDAR-mediated mEPSC amplitude	6.4	63.7	N/A
NMDA-induced whole-current charge	45.9	>1,000	>1,000
GluN1A/2A-mediated current amplitude	3.3*	498	43
GluN1A/2B-mediated current amplitude	0.9*	258	21
GluN1A/2C-mediated current amplitude	1.7*	202	15
GluN1A/2D-mediated current amplitude	2.4*	287	13

*Refers to previously published data obtained under similar conditions (39).

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minutes after each treatment, mice received an i.p. injection of 250 mg/kg NMDA, and the number of mice that survived 24 h was recorded.

Hippocampal Slice and *Xenopus laevis* Oocyte Electrophysiology. See detailed methods described in *SI Appendix, SI Materials and Methods*.

Experimental Design Statistical Analysis. All in vitro and in vivo tests and data analyses were performed by experimenters who were blind to treatment assignments. To analyze the effects of (2*R*,6*R*)-HNK in the FST, unpaired Student's t tests were used for each time point. For assessment of the NSF results, Kaplan–Meier survival analysis was used followed by the Mantel–Cox log-rank test. Probit analysis was used to determine the ED_{50} of each test compound to prevent NMDA-induced lethality in mice (i.e., the dose required to reduce lethality by 50%). Latencies for lethality against drug doses were plotted as second-order polynomial (quadratic) curves. The polynomial equa-

tion $Y = B_0 + B_1*X + B_2*X^2$ (quadratic equation; where Y is latency for lethality, and X is dose of the drug) was used to determine the time needed for the animals to die following the NMDA injection at the ED_{50}. Unless otherwise noted, IC_{50} values were estimated from four-parameter Hill fits using GraphPad Prism, version 7.04 (GraphPad Software). All data are available per request.

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