Title:

Antidepressant potential of (R)-ketamine in rodent models: Comparison with (S)-ketamine

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Abbreviations:
AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionate; ANOVA, analysis of variance; CSF, cerebrospinal fluid; CORT, corticosterone; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide; NMDA, N-methyl-D-aspartic acid

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Abstract

The rapid-acting and long-lasting antidepressant effects of (R,S)-ketamine have recently gained much attention. Although (S)-ketamine has been studied as an active isomer, recent evidence has suggested that (R)-ketamine exhibits longer-lasting antidepressant effects than (S)-ketamine in rodents. However, the antidepressant potential of (R)-ketamine has not been fully addressed. In the present study, we compared the antidepressant effects of (R)-ketamine with those of (S)-ketamine in animal models of depression, including a model that is refractory to current medications. Both (R)-ketamine and (S)-ketamine exhibited antidepressant effects at 30 min as well as at 24 h after administration in forced swimming and tail suspension tests in mice. At 48 h after administration, however, (R)-ketamine still exerted a significant antidepressant effect in the tail suspension test, while the effect of (S)-ketamine was no longer observed. Moreover, (R)-ketamine, but not (S)-ketamine, significantly reversed the depressive-like behavior induced by repeated treatments with corticosterone in rats at 24 h after a single administration. This effect was attenuated by an α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor antagonist, suggesting the involvement of AMPA receptor stimulation in the effects. Both (R)-ketamine and (S)-ketamine exhibited practically the same exposure levels in plasma, brain and cerebrospinal fluid in mice and rats, and both compounds were rapidly eliminated from plasma (<4-8 h). The present results confirmed the previous findings that (R)-ketamine exerted longer-lasting antidepressant effects than (S)-ketamine in animal models of depression. Moreover, we demonstrated, for the first time, that (R)-ketamine exerted a sustained antidepressant effect even in a model that is refractory to currently prescribed antidepressants.
Introduction

Several lines of evidence have shown that the non-competitive \( N \)-methyl-D-aspartate (NMDA) receptor antagonist \((R,S)\)-ketamine exerts rapid and long-lasting antidepressant effects in depressed patients, including those with treatment-resistant depression (Berman et al., 2000; Krystal et al., 2013; Zarate et al., 2006). In addition to its rapid antidepressant effects, \((R,S)\)-ketamine rapidly reduces suicidal ideation in depressed patients (DiazGranados et al., 2010). Despite these ground-breaking findings, the routine use of \((R,S)\)-ketamine in daily practice is prevented by some adverse effects, including psychotomimetic and dissociative symptoms after \((R,S)\)-ketamine injection as well as the potential for abuse following chronic treatment with \((R,S)\)-ketamine (Freedman, 2016; Newport et al., 2016).

\((R,S)\)-Ketamine is a racemic mixture containing the \((R)\)-ketamine enantiomer and the \((S)\)-ketamine enantiomer in equal parts. Of these, \((S)\)-ketamine has been regarded as the active isomer because of its higher affinity for the NMDA receptor and greater anesthetic potency, compared with \((R)\)-ketamine (Domino, 2010; Kohrs and Durieux, 1998). Indeed, the intravenous injection of \((S)\)-ketamine has been recently reported to exert rapid antidepressant effects in patients with treatment-resistant depression (Singh et al., 2016), indicating that \((S)\)-ketamine is the active isomer for antidepressant effects as well. Of note, the intranasal injection of \((S)\)-ketamine is currently under development for the treatment of patients with treatment-resistant depression. However, similar to \((R,S)\)-ketamine, \((S)\)-ketamine can also lead to transient dissociative and psychotic symptoms in depressed patients (Singh et al., 2016), although one case report has suggested that \((S)\)-ketamine, unlike \((R,S)\)-ketamine, does not induce psychotic symptoms (Paul et al.,
Recently, \((R)\)-ketamine has been reported to exert more potent and longer-lasting antidepressant effects than \((S)\)-ketamine in animal models, including the chronic social defeat stress model (Yang et al., 2015; Zanos et al., 2016; Zhang et al., 2014). Very importantly, \((R)\)-ketamine does not cause the adverse effects that are observed with \((S)\)-ketamine treatment in rodents such as psychotomimetic behaviors, neurotoxicity and abuse potential (Yang et al., 2015), even after repeated treatment (Yang et al., 2016). In addition, \((R)\)-ketamine, unlike \((S)\)-ketamine, did not increase dopamine release in the striatum in monkeys (Hashimoto et al., 2016), providing additional evidence for the lack of an association with psychotic symptoms and abuse liabilities. Based on these findings, \((R)\)-ketamine may exert \((R,S)\)-ketamine-like antidepressant effects but without adverse effects, potentially making it a more useful antidepressant than \((R,S)\)-ketamine (Hashimoto, 2016a, 2016b).

However, evidence for the antidepressant effects of \((R)\)-ketamine remains limited, and the usefulness of \((R)\)-ketamine as an antidepressant has still not been established adequately. Moreover, some issues regarding the antidepressant effects of \((R)\)-ketamine, such as its efficacy for treatment-resistant depression and the neural mechanisms underlying the antidepressant effects, remain to be solved. To address these issues, the antidepressant effects of \((R)\)-ketamine must first be confirmed and then compared with those of \((S)\)-ketamine in other animal models of depression, including one that is refractory to conventional antidepressants. Here, we compared the antidepressant effects of \((R)\)-ketamine with those of \((R,S)\)-ketamine and \((S)\)-ketamine in rodent models of depression. Moreover, we also compared the drug levels of
(R)-ketamine with (S)-ketamine in plasma and brain using a well validated method to determine whether the differential effects of both compounds are attributable to differences in their pharmacokinetic profiles.

Also, we additionally measured the drug levels in cerebrospinal fluid (CSF), which may reflect the drug levels responsible for the actions in the central nervous system.
Materials and Methods

Animals

Eight or nine-week-old male C57BL/6J mice (Charles River Laboratories, Yokohama, Japan), 5-week-old male ICR mice (Charles River Laboratories, Yokohama, Japan) and male Sprague-Dawley (SD) rats (4-weeks-old at the beginning of the experiments, 84-102 g; Charles River, Yokohama, Japan) were used for this study. After treatment with corticosterone (CORT) or vehicle for 21 consecutive days, 7-week-old rats (198-299 g) were used for the forced swimming test. The animals were maintained under controlled temperature (23.3 ± 3˚C) and humidity (50 % ± 20%) conditions with a 12-h light/dark cycle (lights on at 7:00 AM). Food and water were provided ad libitum except during the tests. All the experiments were conducted in accordance with the criteria of the Taisho Pharmaceutical Co., Ltd. Animal Care. Committee and met the Japanese Experimental Animal Research association standards, as defined in the Guidelines for Animal Experiments (1987).

Drugs

Racemic ketamine ((R,S)-ketamine) (Veterinary Ketalar® 50; Sankyo Yell Pharmaceutical Co., Ltd., Tokyo, Japan) was diluted with saline. (R)-Ketamine hydrochloride and (S)-ketamine hydrochloride were prepared by the recrystallization of (R,S)-ketamine and D-(-)-tartaric acid (or L-(+)-tartaric acid), as described previously (Zhang et al., 2014). The purity of these stereoisomers was determined using high-performance liquid chromatography (CHIRALPAK®IA, column size: 250 × 4.6 mm, mobile phase:
methyl tert-butyl ether / diethylamine (100/0.1); Daicel Corporation, Tokyo, Japan). (R,S)-Ketamine, (R)-ketamine or (S)-ketamine was dissolved in saline. Desipramine and paroxetine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and dissolved in 0.3% polyoxyethylene glycol sorbitan monooleate (Tween80)/saline solution and distilled water, respectively. 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinazoline-7-sulfonamide (NBQX) was purchased from Tocris Cookson Ltd. (Bristol, UK) and was suspended in saline. CORT was purchased from Sigma-Aldrich Co. (St. Louis, MO) and suspended in 0.3% Tween80/saline solution.

**Forced swimming test in mice**

Male C57BL/6J mice were used. The forced swimming test was performed using a previously reported method, with some modification (Fukumoto et al, 2016). For the test, the mice were placed in a swim tank for 6 min on day 1 to induce a state of helplessness, then placed back in the swim tank for 6 min on day 2 to measure the immobility time. The swimming sessions were conducted by placing the mice in cylinders (24 cm height × 17 cm diameter) containing water (25 ± 1 ºC) up to a height of 13 cm, so that the mice could not support themselves by touching the bottom of the tank with their paws. The forced swimming test was conducted between 8:00 AM and 17:00 PM. The water in the cylinders was changed after every trial. The test sessions were videotaped from the front of the cylinders for later scoring by a scorer. The total duration of immobility during a 6-min test session was measured by an observer who was blinded to the treatment conditions. Immobility was defined as floating in the water without struggling and only making
movements necessary to keep the head above the water. (R)-Ketamine or (S)-ketamine was administered intraperitoneally (i.p.) at 30 min or 24 h prior to the test. Separate cohorts of mice were used to test at each time point. Because the swim session was conducted twice (day 1 and day 2) in the present experimental schedule, we did not conduct 48 h pretreatment with the compound in the forced swimming test to avoid influence of pre-swim. Paroxetine, which was used as a positive control, was administered i.p. at 30 min prior to the test. All the drugs were injected at a volume of 10 mL/kg body weight.

Tail suspension test in mice

Male ICR mice were used. The tail suspension test was performed according to a previously described method (Steru et al., 1985), with some modifications. Mice were suspended by the tail from a metal rod using adhesive tape. The rod was fixed 45 cm above the surface of a table in a sound-isolated room. The mouse was positioned at least 15 cm away from the nearest object. The test session was recorded for 6 min, and the immobility time was determined by an observer who was blinded to the treatment conditions. The mice were considered immobile only when they hung passively and were completely motionless. (R)-Ketamine, (S)-ketamine or (R,S)-ketamine was administered i.p. at 30 min, 24 h or 48 h prior to the test. Separate cohorts of mice were used to test at each time point. Desipramine, used as a positive control, was administered i.p. at 30 min prior to the test. All the drugs were injected at a volume of 10 mL/kg body weight.
Repeated CORT treatment model of rats

The forced swimming test using repeated CORT treated rats was performed according to a previously reported method (Iijima et al., 2010). Male SD rats were injected daily with CORT (20 mg/kg) or the vehicle alone for 21 days. All the injections were delivered subcutaneously (s.c.) once per day between 7:00 AM and 11:00 AM for 21 consecutive days.

The forced swimming test was performed 48 h after the final injection of CORT. The forced swimming test was performed according to a previously reported method (Johnson et al., 2006). In the present study, the rats were tested in the swim tank only once, as we sought to assess helplessness induced by prior exposure to CORT injections, rather than prior exposure to the swim task itself. The swimming sessions were conducted by placing the rats in cylinders containing water at a temperature of 25°C and a depth of 30 cm, so that the rats could not support themselves by touching the bottom with their feet. The forced swimming test was conducted for 10 min between 9:00 AM and 13:00 PM. The water in the cylinders was changed after every trial. The test sessions were videotaped from the front of the cylinders for later scoring by an observer who was blinded to the treatment conditions. Immobility was regarded as floating in the water without struggling and only making movements necessary to keep its head above the water.

(R)-Ketamine, (S)-ketamine or (R,S)-ketamine was administered i.p. twice at 24 h and 30 min or once at 24 h prior to the forced swimming test. The effect of NBQX was examined in cases treated with (R)-ketamine once at 24 h prior to the test; NBQX was administered s.c. at 5 min prior to the i.p. administration of (R)-ketamine. All the drugs were injected at a volume of 2 mL/kg body weight.
Measurement of spontaneous locomotor activity in mice and rats

Spontaneous locomotor activity was determined as previously reported (Yang et al., 2015). Male SD rats or male C57BL/6J mice were used. The animals were housed individually in transparent acrylic cages (for rats, 47 x 28.5 x 29.5 cm; for mice, 30 cm in diameter, 30 cm in height), and (R)-ketamine or (S)-ketamine was injected i.p. after habituation (60 min) to the cage. After the injection, spontaneous locomotor activity was immediately recorded every 10 min for 60 min using a SCANET apparatus (Neuroscience Inc., Tokyo, Japan) placed in a soundproof box.

Determination of (R)-ketamine and (S)-ketamine in plasma, brain and CSF in mice and rats

Sample collection and preparation

(R)-Ketamine or (S)-ketamine was administered i.p. to male C57BL/6J mice (10 or 30 mg/kg) and male SD rats (3 or 10 mg/kg). To investigate the plasma concentration-time profiles of each ketamine enantiomer, blood was collected sequentially at each sampling time point (0.25, 0.5, 1, 2, 4, 8, and 24 h) from an individual animal. The resulting blood samples were treated with ethylenediamine-\(N,N,N',N'\)-tetraacetic acid dipotassium salt dihydrate (EDTA-2K) as an anticoagulant and were immediately centrifuged to prepare the plasma specimens. To investigate the drug distribution in the brain and CSF, blood was taken into a tube containing EDTA-2K to prepare the plasma samples, and the animal was then immediately sacrificed by exsanguination to collect the brain and CSF specimens. The excised brain was rinsed in
ice-cold saline and then homogenized with 4 volumes of distilled water to prepare a brain homogenate specimen. The CSF (mouse: pooled from 3 animals, rat: individual) was diluted with saline four times (mice) or twice (rats) to prepare a CSF specimen. These biological specimens were stored at -30°C until bioanalysis.

Bioanalysis

A 50-µL aliquot of the biological specimen except for mouse plasma and CSF was mixed with 100 µL of acetonitrile/methanol (9:1, v/v) containing \(^2\)H\(_4\)-norketamine (Sigma-Aldrich, St. Louis, MO) as an internal standard (I.S.). The mouse plasma and CSF specimens at volumes of 5 µL and 10 µL, respectively, were subjected to the above-mentioned pretreatment. Each sample was stirred for approximately 30 s using a vortex mixer and centrifuged (preset value, 3700 ×g, 10 min). A 0.5-µL aliquot of the resulting supernatant was subjected to an enantioselective LC-MS/MS analysis. The analytical system was constructed using a Shimadzu LC-30AD high-performance liquid chromatography system (Shimadzu, Tokyo, Japan) and a TripleQuad5500 mass spectrometer (AB SCIEX, Foster City, CA). The MS data were acquired and processed using Analyst version 1.6 software (AB SCIEX, Foster City, CA). Chromatographic separation was performed at 25°C on a CHIRALPAK AS-3R analytical column (4.6 mm i.d.×100 mm, 3 µm particles, Daicel, Tokyo, Japan) with a guard column of CHIRALPAK AS-3R (4.0 mm i.d.×10 mm, 3 µm particles) using 1 mM ammonium hydrogen carbonate/acetonitrile (54:46, v/v) as a mobile phase at a flow rate of 1.0 mL/min. The selected reaction monitoring transition of ketamine was m/z 238 → m/z 125, and the I.S. was m/z 228 → m/z 129. The lower limit of quantification (LLOQ) in plasma was 1 ng/mL (mice) or 0.1
ng/mL (rats); the LLOQ in the brain was 0.5 ng/g for both species; the LLOQ in CSF was 2 ng/mL (mice) or 0.2 ng/mL (rats).

Statistical analysis

All the data were analyzed using a one-way analysis of variance. Subsequent comparisons between the vehicle and treatment groups were performed using the Student’s t-test or the Dunnett’s test. All the data were analyzed using SAS System Version 9.2 (SAS Institute Japan Ltd., Tokyo, Japan).
Results

Antidepressant effects of (R)-ketamine and (S)-ketamine in the mouse forced swimming test

Both (R)-ketamine and (S)-ketamine significantly reduced the immobility time in the forced swimming test in mice at 30 min after i.p. administration, with (S)-ketamine effective at a lower dose than (R)-ketamine \([F(4, 35) = 11.93, P < 0.01]\) (Fig. 1a). Both (R)-ketamine and (S)-ketamine significantly reduced the immobility time at 24 h after administration with the same potency, indicating the sustained antidepressant effects of both compounds \([F(4, 35) = 5.91, P < 0.01]\) (Fig. 1b). Of note, the i.p. administration of paroxetine, at 30 min prior to the test, exerted significant antidepressant effects in this model \([F(3, 28) = 4.15, P < 0.05]\) (Fig. 1c).

Antidepressant effects of (R)-ketamine, (S)-ketamine and (R,S)-ketamine in mouse tail suspension test

Both (R)-ketamine and (S)-ketamine significantly reduced the immobility time in the tail suspension test in mice at 30 min after i.p. administration, with (S)-ketamine effective at a lower dose than (R)-ketamine \([F(4, 75) = 7.49, P < 0.01]\) (Fig. 2a). Both (R)-ketamine and (S)-ketamine significantly reduced the immobility time at 24 h after administration with practically the same potency, as did (R,S)-ketamine \([F(5, 72) = 5.70, P < 0.01]\) (Fig. 2b). In contrast, (R)-ketamine and (R,S)-ketamine \([F(5, 74) = 2.71, P < 0.05]\) still exerted a significant antidepressant effect in this model at 48 h after administration, while (S)-ketamine no longer exerted an antidepressant effect (Fig. 2c), indicating that (R)-ketamine exhibited longer-lasting effects.
antidepressant effects than (S)-ketamine. Of note, the i.p. administration of desipramine, at 30 min prior to the test, exerted significant and dose-dependent antidepressant effects in this model [F(3, 56) = 5.18, \( P < 0.01 \)] (Fig. 2d).

**Effects of (R)-ketamine, (S)-ketamine and (R,S)-ketamine administered at 24 h and 30 min prior to the test on the chronic CORT injections-increased immobility time during the forced swimming test.**

Repeated s.c. injections of CORT for 21 days, at a dose 20 mg/kg, significantly increased the immobility time during the forced swimming test in rats compared with a vehicle-treated group [F(1, 14) = 17.39, \( P < 0.01 \)] (Fig. 3a). (R)-Ketamine (10 mg/kg), (S)-ketamine (10 mg/kg) or (R,S)-ketamine (10 mg/kg), administered at 24 h and 30 min prior to the test, significantly reduced the increase in the immobility time produced by the chronic CORT treatments [F(5, 40) = 2.64, \( P < 0.05 \)] (Fig. 3a).

**Effects of (R)-ketamine, (S)-ketamine and (R,S)-ketamine administered at 24 h prior to the test on chronic CORT injections-increased immobility time during the forced swimming test.**

Repeated s.c. injections of CORT for 21 days, at a dose 20 mg/kg, significantly increased the immobility time in the forced swimming test in rats, compared with a vehicle-treated group [F(1, 14) = 16.34, \( P < 0.01 \)] (Fig. 3b). (R)-Ketamine (10 mg/kg) and (R,S)-ketamine (10 mg/kg), administered at 24 h prior to the test, significantly reduced the increase in the immobility time produced by the chronic CORT treatments [F(5, 40) = 2.64, \( P < 0.05 \)] (Fig. 3b). In contrast, (S)-ketamine (3 and 10 mg/kg) administered at 24 h prior
to the test did not affect the CORT treatment-increased immobility time (Fig. 3b). The effect of (R)-ketamine on CORT treatment-increased immobility was significantly prevented by pretreatment with NBQX (10 mg/kg) [F(3, 28) = 8.92, P < 0.01] (Fig. 4a), while NBQX (10 mg/kg) per se did not affect CORT treatment increased immobility [F(1, 14) = 1.51, P = 0.24] (Fig. 4b).

Effect of (R)-ketamine and (S)-ketamine on locomotor activity in mice and rats

Both (R)-ketamine (10 and 30 mg/kg, i.p.) and (S)-ketamine (10 and 30 mg/kg, i.p.) significantly increased the locomotor activity of mice [F(4, 35) = 31.88, P < 0.01] (Table 1). In addition, although (R)-ketamine (30 mg/kg, i.p.) and (S)-ketamine (30 mg/kg, i.p.) significantly increased the locomotor activity of rats [F(4, 35) = 18.07, P < 0.01], neither (R)-ketamine (10 mg/kg, i.p.) nor (S)-ketamine (10 mg/kg, i.p.) affected the locomotor activity of rats (Table 1).

Determination of (R)-ketamine and (S)-ketamine in plasma, brain and CSF in mice and rats

The exposure levels of (R)-ketamine or (S)-ketamine in the rodent plasma, brain and CSF were determined after a single i.p. administration of each ketamine enantiomer to mice (10 or 30 mg/kg) and rats (3 or 10 mg/kg). As shown in Figs. 5a and b, in each treatment group, the plasma concentrations of (R)-ketamine and (S)-ketamine reached the maximum concentration at the first sampling time point (0.25 h) and rapidly declined in parallel in each species to below the LLOQ, at least after the post-dose sampling times of 4 h (mice) or 8 h (rats). Chiral inversion of the ketamine enantiomers was not observed.
No significant differences in the concentrations of (R)-ketamine and (S)-ketamine were observed in either the brain or the CSF (Table 2), as well as in the plasma concentration time profiles. At 0.5 h post-dose, the brain and CSF levels of both ketamine enantiomers were measureable in all the animals. The brain specimens exhibited higher levels than the plasma samples, with brain-to-plasma concentration ratios of 1.3 - 2.6 (mice) and 1.7 - 2.2 (rats), while the CSF levels were lower than the plasma levels, with CSF-to-plasma concentration ratios of 0.2 - 0.5 (mice) and 0.3 - 0.4 (rats). At 24 h post-dose, both the (R)-ketamine and (S)-ketamine concentrations in the tissues were below the LLOQ in both species except for in mouse brain after a dose of 30 mg/kg, in which only (S)-ketamine was measurable at a trace levels near the LLOQ.
Discussion

In the present study, we provided additional evidence that (R)-ketamine exhibited longer-lasting antidepressant effects than (S)-ketamine in rodent models of depression. In addition, we, for the first time, demonstrated that (R)-ketamine reversed depressive-like behavior in an animal model that is refractory to conventional antidepressants, while (S)-ketamine did not show a sustained effect in this model. Moreover, we also demonstrated that the effect of (R)-ketamine in the treatment-resistant model was mediated through the stimulation of the \( \alpha \)-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor. Finally, we confirmed differential antidepressant effects between (R)-ketamine and (S)-ketamine that cannot be ascribed to differences in their pharmacokinetic profiles.

In two despair models in mice, the forced swimming test and the tail suspension test, both (R)-ketamine and (S)-ketamine reduced the immobility time at 30 min after administration (acute effects), while (S)-ketamine showed effects at a lower dose (10 mg/kg, i.p.) than (R)-ketamine. However, even at 10 mg/kg (i.p.), (S)-ketamine and (R)-ketamine transiently increased locomotor activity in mice after acute administration, which may have large effects on the outcomes in these animal models. Therefore, the acute effects of both compounds may be partly ascribed to increased locomotor activity, and we cannot conclude that (S)-ketamine had more potent acute effects than (R)-ketamine in these despair models. It should be noted that (R)-ketamine has been reported to show acute antidepressant effects at lower doses than (S)-ketamine in other animal models (Zanos et al., 2016), suggesting that the potency of the acute effects
may depend on the test that is used or the testing conditions. Moreover, we obtained results indicating that
the exposure levels of both compounds in the brain and CSF were approximately the same after
administration. Thus, the difference in potency between \((R)\)-ketamine and \((S)\)-ketamine cannot be ascribed
to differences in their pharmacokinetic profiles.

In addition to the acute effects, both \((R)\)-ketamine and \((S)\)-ketamine showed sustained antidepressant
effects at 24 h after administration in these models. The pharmacokinetic results showed that both
compounds no longer existed in the brain or CSF at 24 h after administration. Therefore, secondary
changes, presumably adaptive synaptic plasticity, induced by the compound rather than the direct actions of
the compound during the test may have been involved in the sustained antidepressant effects. Moreover, in
the tail suspension test, we obtained the result that \((R)\)-ketamine still showed antidepressant effects at 48 h
after administration, as did \((R,S)\)-ketamine, while \((S)\)-ketamine no longer showed the effects. Therefore,
\((R)\)-ketamine may have longer-lasting antidepressant effects in the animal model than \((S)\)-ketamine, which
is consistent with previously reported results (Yang et al., 2015; Zanos et al., 2016; Zhang et al., 2014).
Since \((R)\)-ketamine has a lower affinity at the NMDA receptor than \((S)\)-ketamine, this result raises the
possibility that mechanisms other than NMDA receptor blockade may be involved in the longer-lasting
antidepressant effects of \((R)\)-ketamine.

The repeated administration of CORT induces depressive-like behavior in rodents, such as increased
immobility during the forced swimming test; this behavior is not improved by acute treatment with
conventional antidepressants (Iijima et al., 2010). Thus, this model may be regarded as a model of depression refractory to current medications, although there is a report showing that chronic treatment with conventional antidepressants reversed the repeated CORT treatment-induced anhedonia (Gourley and Taylor, 2009). We observed that (R,S)-ketamine reversed the depressive-like behavior in this model (Koike et al., 2013), reflecting the clinical finding that (R,S)-ketamine is effective for patients with treatment-resistant depression (Zarate et al., 2006). In the present study, both (R)-ketamine and (S)-ketamine significantly reversed the repeated CORT treatment-increased immobility time when administered twice at 24 h and 30 min prior to the test, as did (R,S)-ketamine. Because both (R)-ketamine and (S)-ketamine did not affect locomotor activity at the doses tested in rats, the acute antidepressant effects observed in this model might not have been due to changes in locomotor activity. In contrast, when dosed once at 24 h before the forced swimming test, (R)-ketamine significantly reversed the depressive-like behavior in this model, as did (R,S)-ketamine, while (S)-ketamine did not affect the increased immobility time. Therefore, (R)-ketamine had a long-lasting effect in the treatment resistant model as well, while (S)-ketamine only showed acute antidepressant effects. Also, because (R,S)-ketamine has been reported to exert sustained antidepressant effects in patients with treatment-resistant depression (Zarate et al., 2006), (R)-ketamine may be responsible for the long-lasting effect of (R,S)-ketamine in treatment-resistant depression. Pharmacokinetic studies indicated that the drug levels of both (R)-ketamine and (S)-ketamine in the plasma, brain and CSF in rats were practically the same after administration, and both compounds did not exist in the brain and CSF at 24 h after administration in rats. Therefore, the differences in the sustained
antidepressant effects of (R)-ketamine and (S)-ketamine might not be due to differences in the pharmacokinetic profiles of the two compounds, and secondary changes, rather than direct actions, induced by (R)-ketamine may be involved in the sustained effects. The present result contrasts that of a recent report indicating that (S)-ketamine exerted long-lasting anti-anhedonic effects for 32 days in CORT treated rats (Soumier et al., 2016). However, in their study, (S)-ketamine was injected before repeated CORT treatment, while both (R)-ketamine and (S)-ketamine were injected after CORT treatment in the present study. Therefore, the present study investigated only the therapeutic effects, and not the prophylactic effects.

In the present study, the sustained antidepressant effect of (R)-ketamine was attenuated by pretreatment with NBQX, an AMPA receptor antagonist. Therefore, AMPA receptor stimulation is involved in the sustained antidepressant effects of (R)-ketamine, which is in line with previous findings in a chronic social defeat stress model of mice (Yang et al., 2015). Given that the sustained antidepressant effects of (R,S)-ketamine and the increase in the synthesis of synaptic proteins induced by (R,S)-ketamine are mediated through AMPA receptor stimulation (Koike et al., 2011, 2014; Li et al., 2010) and that AMPA receptor potentiation has been reported to have a pivotal role to exert antidepressant effects (Alt et al., 2006), it is plausible to speculate that (R)-ketamine may exert the sustained antidepressant effects through changes in synaptic plasticity, which are mediated via the AMPA receptor.

Precise mechanisms explaining the differences in the antidepressant effects of (R)-ketamine and (S)-ketamine remain to be elucidated. Although a metabolite of (R)-ketamine, (2R,6R)-hydroxynorketamine,
has recently been reported to be responsible for the long-lasting antidepressant effects of \((R,S)\)-ketamine (Zanos et al., 2016), \((R)\)-ketamine itself may be responsible for the long-lasting antidepressant effects, because the local injection of \((R)\)-ketamine into brain nuclei reportedly exerts sustained antidepressant effects (Shirayama et al., 2016) and we recently reported that antidepressant effects of \((R)\)-ketamine is much greater than those of \((2R,6R)\)-hydroxynorketamine (Yang et al., in press). In either case, because both \((R)\)-ketamine and its metabolite have a lower affinity for the NMDA receptor than \((S)\)-ketamine (Kohrs and Durieux, 1998; Zanos et al., 2016), mechanisms other than the NMDA receptor blockade may be involved in the antidepressant effects of \((R)\)-ketamine. In the present study, while both \((R)\)-ketamine and \((S)\)-ketamine exerted long-lasting antidepressant effects even when they were no longer present in the brain and CSF, only \((R)\)-ketamine exerted sustained antidepressant effects in the repeated CORT treatment model in which reduced synaptic plasticity has been reported (Pazini et al., 2016). Therefore, the difference in the potency of the effects on synaptic plasticity may be responsible for the differences in the sustained antidepressant effects. This assumption may be underpinned by the report that \((R)\)-ketamine reversed a social defeat stress-induced reduction in dendritic spine density and brain-derived neurotrophic factor levels more robustly than \((S)\)-ketamine on the eighth day after the single administration (Yang et al., 2015).

One of the limitations of the present study is that we did not address safety issues regarding \((R)\)-ketamine. Indeed, when we investigated the effects of \((R)\)-ketamine and \((S)\)-ketamine on locomotor activity in mice, increased locomotor activity was observed at doses of both 10 and 30 mg/kg, i.p. Although increased
locomotor activity does not necessarily reflect psychotomimetic liability in human, this finding markedly contrasts the previous finding that (R)-ketamine (5, 10, 20 mg/kg, i.p.) did not affect locomotor activity, while (S)-ketamine (10 and 20 mg/kg, i.p.) markedly increased locomotor activity in mice (Yang et al., 2015). In both experiments, the same strain of mice and the same time schedule were used. Although the reason for this discrepancy is not known, differences in the experimental apparatuses that were used or in specific treatment conditions might have led to the different outcomes. Further studies are required to assess the safety profiles of (R)-ketamine, including the abuse potential and neurotoxicity. Another limitation of the present study is that long-lasting antidepressant effects of (R,S)-ketamine in rodent models are not universally observed (Bechtholt-Gompf et al., 2011; Popik et al., 2008). Therefore, data obtained in animal models have to be confirmed in clinical studies.

In conclusion, we confirmed that (R)-ketamine exerted longer-lasting antidepressant effects than (S)-ketamine in animal models of depression. In addition, we provide evidence, for the first time, that (R)-ketamine, but not (S)-ketamine exerted sustained antidepressant effects in a treatment refractory model in a manner that is mediated through stimulation of the AMPA receptor. Therefore, (R)-ketamine might have the potential to exert rapid and sustained antidepressant effects in patients with depression, including those with treatment resistant depression, although these assumptions need to be confirmed in clinical settings.
Authorship Contributions

Participated in research design: Yamaguchi, Hashimoto, Chaki

Conducted experiments: Fukumoto, Toki, Iijima

 Contributed new reagents or analytic tools: Hashihayata

Performed data analysis: Fukumoto, Toki, Iijima

Wrote or contributed to the writing of the manuscript: Toki, Iijima, Yamaguchi, Hashimoto, Chaki
References


Koike H, Iijima M, Chaki S (2011) Involvement of AMPA receptor in both the rapid and sustained antidepressant-like effects of ketamine in animal models of depression. *Behav Brain Res* **224**:107-111.


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Science 329:959-964.


Yang C, Qu Y, Abe M, Nozawa D, Chaki S, Hashimoto K. (R)-Ketamine shows greater potency and longer lasting antidepressant effects than its metabolite (2R, 6R)-hydroxynorketamine. *Biol Psychiatry* in press.


**Footnotes**

Figure legends

Fig. 1 Effect of (R)-ketamine or (S)-ketamine in mouse forced swimming test

(R)-Ketamine or (S)-ketamine was administered i.p. at 30 min (a) or 24 h (b) prior to the forced swimming test. Paroxetine was administered i.p. at 30 min prior to the forced swimming test (c). The vertical axis shows the immobility time (seconds). All data are expressed as the mean ± SEM (n=8). **P < 0.01 compared with each vehicle (Dunnett’s test); #P < 0.05 compared with (R)-ketamine (30 mg/kg, i.p.) (Student’s t-test).

Fig. 2 Effect of (R)-ketamine, (S)-ketamine or (R,S)-ketamine in mouse tail suspension test

(R)-Ketamine, (S)-ketamine or (R,S)-ketamine was administered i.p. at 30 min (a), 24 h (b) or 48 h (c) prior to the tail suspension test. Desipramine was administered i.p. at 30 min prior to the tail suspension test (d). The vertical axis shows the immobility time (seconds). All data are expressed as the mean ± SEM ((a): n=16, (b): n=12-14, (c): n=13-14, (d): n=15). *P < 0.05, **P < 0.01 compared with each vehicle (Dunnett’s test); #P < 0.05, ##P < 0.01 compared with each vehicle (Student’s t-test).

Fig. 3 Effect of (R)-ketamine, (S)-ketamine and (R,S)-ketamine on repeated CORT injections-induced immobility time during the forced swimming test

(R)-Ketamine, (S)-ketamine or (R,S)-ketamine was administered twice at 24 h and 30 min prior to the test
(a) or once at 24 h prior to the test (b). The vertical axis shows the immobility time (seconds). All the data are expressed as the mean ± SEM ((a): n=7-8, (b): n=8). ++$P < 0.01$ compared with the 0.3% tween80/saline-treated vehicle (Student’s t test); *$P < 0.05$, **$P < 0.01$ compared with the CORT-treated vehicle (Dunnett’s test); #$P < 0.05$, ##$P < 0.01$ compared with the CORT-treated vehicle (Student’s t test).

**Fig. 4** Effect of an AMPA receptor antagonist on antidepressant effects of (R)-ketamine in a repeated CORT injection-induced model.

The effect of NBQX pretreatment on the effect of (R)-ketamine (a) and the effect of NBQX itself on the immobility time (b) were investigated. (R)-ketamine was administered at 24 h prior to the test, and NBQX was administered at 24.5 h prior to the test. The vertical axis shows the immobility time (seconds). All the data are expressed as the mean ± SEM ((a): n=8, (b): n=8). ++$P < 0.01$ compared with the 0.3% tween80/saline-treated vehicle (Student’s t test); **$P < 0.01$ compared with the CORT-treated vehicle (Student’s t-test); ##$P < 0.01$ compared with the CORT-treated vehicle + (R)-ketamine (Dunnett’s test).

**Fig. 5** Plasma concentration-time profiles of (R)-ketamine and (S)-ketamine following a single intraperitoneal administration to mice (a) and rats (b)

(R)-Ketamine or (S)-ketamine was administered to the animals, and plasma specimens were obtained at the designated time points and subjected to an enantioselective LC-MS/MS analysis. Data are presented as the mean ± SD of 3 animals. The lower limit of quantification (LLOQ) was 1 ng/mL for mice and 0.1 ng/mL.
for rats.
### Table 1  Effect of (R)-ketamine and (S)-ketamine on locomotor activity in mice and rats

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle</td>
<td>2217.5 (±139.7)</td>
<td>1025.9 (±294.9)</td>
</tr>
<tr>
<td>(R)-ketamine 10 mg/kg (i.p.)</td>
<td>5029.5 (±782.1) *</td>
<td>1869.9 (±289.5)</td>
</tr>
<tr>
<td>(R)-ketamine 30 mg/kg (i.p.)</td>
<td>16654.5 (±1106.5) **</td>
<td>4458.3 (±807.5) **</td>
</tr>
<tr>
<td>(S)-ketamine 10 mg/kg (i.p.)</td>
<td>6018.0 (±804.9) *</td>
<td>1826.6 (±278.0)</td>
</tr>
<tr>
<td>(S)-ketamine 30 mg/kg (i.p.)</td>
<td>12368.5 (±1106.5) **</td>
<td>9547.1 (±1572.5) **</td>
</tr>
</tbody>
</table>

*p<0.05, **p<0.01 vs vehicle (Dunnett’s test), n=8
Table 2 Plasma, brain and CSF concentrations of (R)-ketamine and (S)-ketamine following a single intraperitoneal administration to mice and rats.

<table>
<thead>
<tr>
<th>Species</th>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Time (hours)</th>
<th>Concentration (ng/mL or ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>Mouse</td>
<td>(R)-ketamine</td>
<td>10</td>
<td>0.5</td>
<td>210±83.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
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<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.5</td>
<td>958±155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td></td>
<td>(S)-ketamine</td>
<td>10</td>
<td>0.5</td>
<td>376±34.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>0.5</td>
<td>1770±203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>0.823±0.792</td>
</tr>
<tr>
<td>Rat</td>
<td>(R)-ketamine</td>
<td>3</td>
<td>0.5</td>
<td>57.9±4.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.5</td>
<td>289±38.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td></td>
<td>(S)-ketamine</td>
<td>3</td>
<td>0.5</td>
<td>105±19.3</td>
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<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.5</td>
<td>303±73.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
</tr>
</tbody>
</table>

Data are presented as the mean ± SD of 3 animals.

The LLOQ in plasma was 1 ng/mL (mice) or 0.1 ng/mL (rats); the LLOQ in brain was 0.5 ng/g for both spices; the LLOQ in cerebrospinal fluid was 2 ng/mL (mice) or 0.2 ng/mL (rats).

a): Pooled sample of three mice.
Fig. 1

(a) Immobility time (sec)

(b) Immobility time (sec)

(c) Immobility time (sec)
Fig. 2

For the sake of clarity, please note that the figure shows the immobility time (sec) for different treatments: vehicle, (R)-ketamine, (S)-ketamine, (R,S)-ketamine, and Desipramine. The effects are measured in mg/kg, i.p. for each substance.

- **(a)**: Vehicle vs. (R)- and (S)-ketamine
- **(b)**: Vehicle vs. (R)-, (S)-, and (R,S)-ketamine
- **(c)**: Vehicle vs. (R)- and (S)-ketamine
- **(d)**: Vehicle vs. Desipramine

Significance levels are indicated as follows:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
- ###: p < 0.001
Fig. 3
Fig. 4

(a) 

(b) 

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Fig. 5

(a) (b)