

(*R,S*)-Ketamine Metabolites (*R,S*)-norketamine and (2*S*,6*S*)-hydroxynorketamine Increase the Mammalian Target of Rapamycin Function

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ABSTRACT

Background: Subanesthetic doses of (*R,S*)-ketamine are used in the treatment of neuropathic pain and depression. In the rat, the antidepressant effects of (*R,S*)-ketamine are associated with increased activity and function of mammalian target of rapamycin (mTOR); however, (*R,S*)-ketamine is extensively metabolized and the contribution of its metabolites to increased mTOR signaling is unknown.

Methods: Rats ($n = 3$ per time point) were given (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine and their effect on the mTOR pathway determined after 20, 30, and 60 min. PC-12 pheochromocytoma cells ($n = 3$ per experiment) were treated with escalating concentrations of each compound and the impact on the mTOR pathway was determined.

Results: The phosphorylation of mTOR and its downstream targets was significantly increased in rat prefrontal cortex tissue by more than ~ 2.5 -, ~ 25 -, and ~ 2 -fold, respectively, in response to a 60-min postadministration of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine ($P < 0.05$, ANOVA analysis). In PC-12 pheochromocytoma cells, the test compounds activated the mTOR pathway in a concentration-dependent manner, which resulted in a significantly higher expression of serine racemase with ~ 2 -fold increases at 0.05 nM (2*S*,6*S*)-hydroxynorketamine, 10 nM (*R,S*)-norketamine, and 1,000 nM (*R,S*)-ketamine. The potency of the effect reflected antagonistic activity of the test compounds at the α_7 -nicotinic acetylcholine receptor.

Conclusions: The data demonstrate that (*R,S*)-norketamine and (2*S*,6*S*)-hydroxynorketamine have potent pharmacological activity both *in vitro* and *in vivo* and contribute to the molecular effects produced by subanesthetic doses of (*R,S*)-ketamine. The results suggest that the determination of the mechanisms underlying the antidepressant and analgesic effects of (*R,S*)-ketamine requires a full study of the parent compound and its metabolites. (ANESTHESIOLOGY 2014; 121:149-59)

(*R,S*)-KETAMINE is a rapid and long-lasting antidepressant agent used in the treatment of major depressive disorder and bipolar depression.¹⁻³ Data from studies in rats suggest that the rapid effect produced by (*R,S*)-ketamine is due to the increased phosphorylation of the mammalian target of rapamycin (mTOR) and corresponding increases in the phosphorylated forms of the extracellular signal-regulated kinases (pERK1/ERK2), protein kinase B (pAkt), eukaryotic initiation factor 4E binding protein (p4E-BP1), and p70S6 kinase (pp70S6K)⁴ and an increase in the number and function of new spine synapses in the prefrontal cortex.^{4,5}

(*R,S*)-Ketamine is extensively transformed into the *N*-demethylated metabolite (*R,S*)-norketamine, two diastereomeric hydroxyketamines, a series of diastereomeric hydroxynorketamines, including (2*S*,6*S*;2*R*,6*R*)-hydroxynorketamine, and (*R,S*)-dehydronorketamine⁶⁻⁸; figure 1. In the rat, (*R,S*)-ketamine is rapidly converted to (*R,S*)-norketamine

What We Already Know about This Topic

- Anesthetic effects of ketamine are primarily due to (*R,S*)-ketamine; its metabolite (*R,S*)-norketamine contributes to this effect but its metabolite (2*S*,6*S*;2*R*,6*R*)-hydroxynorketamine does not

What This Article Tells Us That Is New

- Antidepressant effects of subanesthetic doses of (*R,S*)-ketamine may be due to a combination of interrelated effects at the α_7 -nicotinic acetylcholine receptor (α_7 -nAChR) produced by (*R,S*)-ketamine and its metabolites
- One effect is increased protein expression *via* the mammalian target of rapamycin signaling pathway, which is initiated by antagonism of α_7 -nAChR and is reflected by increased monomeric serine racemase expression

and (2*S*,6*S*;2*R*,6*R*)-hydroxynorketamine, which occurs within 2 min after an intravenous administration.⁹ Similarly, administration of (*R,S*)-norketamine leads to the detection

This article is featured in "This Month in Anesthesiology," page 3A. Corresponding article on p. 4. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). The first two authors contributed equally to this work (R.K.P. and N.S.S.).

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of both (*R,S*)-norketamine and (*2S,6S;2R,6R*)-hydroxynorketamine, whereas only (*2S,6S;2R,6R*)-hydroxynorketamine is present after intravenous administration of (*2S,6S;2R,6R*)-hydroxynorketamine.⁹ The rapid and extensive metabolic transformation of (*R,S*)-ketamine is also observed in studies using human microsomal preparations¹⁰ and in clinical studies.^{2,3,11}

(*R,S*)-Ketamine was developed as an anesthetic agent, and initial pharmacodynamic studies demonstrated that both (*R,S*)-ketamine and (*R,S*)-norketamine produce anesthesia,⁹ which is associated with noncompetitive inhibition of the *N*-methyl-D-aspartate receptor (NMDAR).¹² (*2S,6S;2R,6R*)-Hydroxynorketamine lacks anesthetic activity,⁹ and the individual enantiomers of the compound, (*2S,6S*)-hydroxynorketamine and (*2R,6R*)-hydroxynorketamine, are not active at the NMDAR,¹³ $\alpha_3\beta_4$ -nicotinic acetylcholine receptor ($\alpha_3\beta_4$ -nAChR),¹³ or muscarinic and opioid receptors (unpublished data from competitive binding screen provided by Bryan Roth, M.D., Ph.D., NIMH Psychoactive Drug Screening, Chapel-Hill, North Carolina, September 2013). However, (*2S,6S*)-hydroxynorketamine and (*2R,6R*)-hydroxynorketamine are potent ($IC_{50} < 100$ nM) and selective inhibitors of the α_7 -nAChR.¹³ We have recently demonstrated that treatment of 1321N1 astrocytoma cells with the α_7 -nAChR-selective inhibitor methyllycaconitine promotes the *de novo* protein synthesis of monomeric serine racemase (m-SR) due to increased levels of pERK1/2, pAkt, and pmTOR.¹⁴ A similar effect was observed with PC-12 cells.¹⁴ Because (*R,S*)-norketamine, (*2S,6S*)-hydroxynorketamine, and (*2R,6R*)-hydroxynorketamine are α_7 -nAChR inhibitors,¹³ it is possible that the synaptogenesis and antidepressive effects observed in the rat after (*R,S*)-ketamine administration may be due, in part, to the activity of these metabolites. This

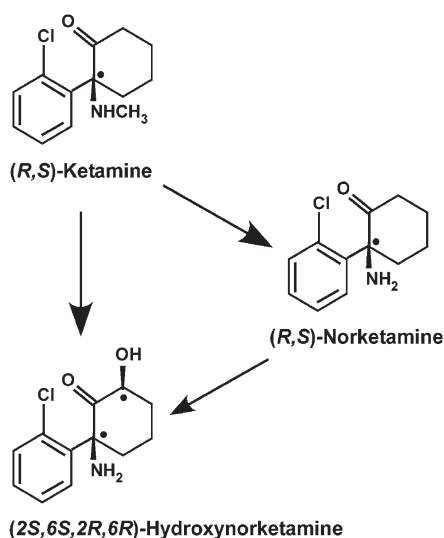


Fig. 1. Structures of (*R,S*)-ketamine, (*R,S*)-norketamine, and (*2S,6S;2R,6R*)-hydroxynorketamine. (*R,S*)-Ketamine undergoes hepatic metabolism to be transformed into (*2S,6S;2R,6R*)-hydroxynorketamine directly and/or via (*R,S*)-norketamine as an intermediate.

possibility was examined in this study through the administration of (*R,S*)-ketamine, (*R,S*)-norketamine, and (*2S,6S*)-hydroxynorketamine to male Wistar rats. The resultant brain tissue concentration profiles of the tested compounds and corresponding changes in the levels of pmTOR and related proteins were determined. The data were compared with the results obtained in PC-12 cells. The latter studies were undertaken to clarify the independent contribution of each of the tested compounds due to the rapid and significant *in vivo* conversion of (*R,S*)-ketamine into (*R,S*)-norketamine and (*2S,6S;2R,6R*)-hydroxynorketamine, and of (*R,S*)-norketamine to (*2S,6S;2R,6R*)-hydroxynorketamine. In the current study, (*2S,6S*)-hydroxynorketamine was used instead of (*2R,6R*)-hydroxynorketamine or the racemic mixture, a decision based on data indicating that this isomer was the most potent of the three compounds in the suppression of intracellular D-serine levels (unpublished data from preliminary *in vitro* study obtained following previously described protocol¹⁴ provided by Irving W. Wainer, Ph.D., D.H.C., Baltimore, Maryland, August 2013) and its availability in our laboratory.

Materials and Methods

Materials

(*R,S*)-Ketamine, (*R,S*)-norketamine, and (*2S,6S*)-hydroxynorketamine were prepared as previously described.¹¹ Methyllycaconitine and (*S*)-nicotine were obtained from Sigma-Aldrich (St. Louis, MO). Deionized water was obtained from a Milli-Q system (Millipore, Billerica, MA). All other chemicals used were of analytical grade.

Studies in the Wistar Rat

Animals. Male Wistar rats were obtained from Harlan (Livermore, CA) at 9 to 10 weeks of age and housed one to three rats per cage, in polycarbonate hanging cages. All rats (271 to 354 g of body weight) had *ad libitum* access to Harlan Teklad Certified Rodent Chow #2018C and reverse osmosis purified water. Animal rooms were maintained at 68° to 73°F temperature with 20 to 60% relative humidity and a 12-h light–dark cycle. All animal procedures in this study were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (1996) and the Animal Welfare Standards incorporated in 9 CFR Part 3, 1991. All study protocols were reviewed and approved by SRI's Institutional Animal Care and Use Committee (SRI International, Menlo Park, CA).

Administration of (*R,S*)-ketamine, (*R,S*)-norketamine, and (*2S,6S*)-hydroxynorketamine. The animals ($n = 3$ for each experiment) received either a single intraperitoneal injection of (*R,S*)-ketamine, 40 mg/kg in saline (5 ml/kg), or a single intravenous injection of either (*R,S*)-norketamine, 20 mg/kg in saline (5 ml/kg), or (*2S,6S*)-hydroxynorketamine, 20 mg/kg in saline (5 ml/kg), administered *via* a jugular vein catheter. After (*R,S*)-ketamine injection, the rats were euthanized with an overdose of pentobarbital at 10, 30, 60, and 240 min, whereas rats treated with (*R,S*)-norketamine and

(2*S*,6*S*)-hydroxyketamine were euthanized with an overdose of pentobarbital at 10, 20, 60, and 240 min postadministration. In all cases, whole brains were promptly collected, rinsed with phosphate-buffered saline, and stored frozen at -70°C until analysis.

Preparation of Brain Tissue Samples. The frozen whole brains were thawed on ice and longitudinally dissected. One of the hemispheres was used for the determination of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine concentrations. A portion of the prefrontal cortex of the other hemisphere was used for Western blotting. Brain tissue obtained from drug-free male Wistar rats was used as control tissue samples.

Analysis of Brain Tissue Concentrations of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine. The brain tissue sample was weighed (average weight 900 mg) and suspended in 990 μl of water:methanol (3:2, v/v) and 10 μl of the internal standard 3,4,5,6-tetradeuterophenyl- (*R,S*)-ketamine-HCl (10 $\mu\text{g}/\text{ml}$ in methanol) (Cerilliant, Round Rock, TX). The mixture was homogenized on ice with a polytron homogenizer, centrifuged at 21,000*g* for 30 min, and the supernatant was collected. The analytes were isolated using 1 ml Oasis HLB solid-phase extraction cartridges (Waters Corp., Waltham, MA). The cartridges were preconditioned with 1 ml of methanol, followed by 1 ml of water, and then 1 ml of ammonium acetate (10 mM, pH 9.5). The supernatants were added to the cartridges, followed by 1 ml of water and (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine were eluted with 1 ml of methanol. The eluent was transferred to an autosampler vial for analysis. The samples were assayed using a previously reported liquid chromatographic method using mass spectrometric detection, which had been validated for use with clinical samples.¹¹ The method was cross-validated using whole brains obtained from drug-free Wistar rats and spiked drug concentrations ranging from 0.025 to 25 μM . The measured analyte concentrations were normalized using the weight of each tissue sample and reported as $\mu\text{M}/\text{g}$ tissue. Based on 1 g of tissue, the lowest levels of quantification were 0.16 $\mu\text{M}/\text{g}$ tissue for (*R,S*)-ketamine, 0.18 $\mu\text{M}/\text{g}$ tissue for (*R,S*)-norketamine, and 0.16 $\mu\text{M}/\text{g}$ tissue for (2*S*,6*S*)-hydroxynorketamine.

Western Blotting. Cells and brain tissues (50 mg cortex) were lysed in radioimmunoprecipitation buffer containing EDTA and EGTA (Boston BioProducts, Ashland, MA) and supplemented with a protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail sets I and II (EMD Millipore, Billerica, MA). Brain tissues were homogenized using PRO200 (PRO Scientific Inc., Oxford, CT) hand homogenizer for 15 s on ice, and soluble extracts were collected after centrifugation at 14,000*g* for 20 min at 4°C . Protein concentrations were determined in the clarified lysates using the bicinchoninic acid reagent (Thermo Fisher Scientific, Waltham, MA). Proteins (20 μg per well) were separated on 4 to 12% precast gels (Invitrogen, Carlsbad,

CA) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and then electrophoretically transferred onto polyvinylidene fluoride membranes (Invitrogen). Western blotting experiments were performed according to standard methods, which involved a blocking step in 5% nonfat milk/0.1% Tween-20 in phosphate-buffered saline and incubation with the primary antibody of interest, followed by incubation with a secondary antibody conjugated with the enzyme horseradish peroxidase. The detection of immunoreactive bands was performed by using the ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ). The quantification of bands was done by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalization to β -actin. The primary antibodies for the phosphorylated forms of ERK1/2 (pERK1/2; Thr202/Tyr204; cat. # 4376S), Akt (pAkt; Ser473; cat. # 9271S), mTOR (pmTOR; Ser2448; cat. # 2971), 4E-BP1 (p4E-BP1; Thr37/46; cat. # 2855), p70S6K (pp70S6K; Thr389; cat. # 9234), and total forms of ERK1/2 (cat. # 9108S), Akt (cat. # 4685S), 4E-BP1 (cat. # 9452), p70S6K (cat. # 2708), and mTOR (cat. # 2972) were obtained from Cell Signaling Technology (Beverly, MA). The primary antibodies for the determination of SR (cat. # ab45434) and β -actin (cat. # ab6276) were purchased from Abcam, Inc. (Cambridge, MA). The antibodies were used at a dilution recommended by the manufacturers.

Studies in PC-12 Cells

Maintenance of PC-12 Cells. The PC-12 pheochromocytoma cell line derived from rat adrenal medulla was obtained from American Type Culture Collection (Manassas, VA). RPMI-1640, trypsin solution, phosphate-buffered saline, fetal bovine serum, sodium pyruvate (0.1 M), L-glutamine (0.2 M), and penicillin/streptomycin solution (containing 10,000 units/ml penicillin and 10,000 $\mu\text{g}/\text{ml}$ streptomycin) were obtained from Quality Biological (Gaithersburg, MD), horse serum (heat inactivated) was purchased from Biosource (Rockville, MD), and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (1 M, pH 7.4) was obtained from Mediatech, Inc. (Manassas, VA). The PC-12 cells were maintained in RPMI-1640 supplemented with 1 mM HEPES buffer, 10% horse serum, 5% fetal bovine serum, 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin/streptomycin.

Effect of (*R,S*)-ketamine, (*R,S*)-norketamine, (2*S*,6*S*)-hydroxynorketamine, Methyllycaconitine, and (*S*)-nicotine on Monomeric SR Expression in PC-12 Cells. The studies were carried out using a previously described approach.¹⁴ In brief, PC-12 cells were seeded on 100-mm tissue culture plates and maintained at 37°C under humidified 5% CO_2 in air until they reached greater than 70% confluence. The original media was replaced with medium containing the test compounds and the plates were incubated for an additional 36 h. The medium was removed, and the cells were collected

for analysis. The concentrations used for (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine were as indicated below. In some experiments, PC-12 cells were preincubated with (*S*)-nicotine (2 μ M) for 1 h followed by the addition of vehicle, (*R,S*)-ketamine (1 μ M), (*R,S*)-norketamine (10 nM), (2*S,6S*)-hydroxynorketamine (0.1 nM), or methyllycaconitine (50 nM) and the incubation continued for an additional 36 h.¹⁴ Determination of m-SR protein level was carried out by Western blot analysis on one set of dishes, and the same experiment was repeated on 3 separate days (*n* = 3).

Effect of (*R,S*)-ketamine, (*R,S*)-norketamine, (2*S,6S*)-hydroxynorketamine, Methyllycaconitine, and (*S*)-nicotine on pmTOR, pAkt, pERK, p4E-BP1, and pp70S6K Levels in PC-12 Cells. PC-12 cells were seeded on 100-mm tissue culture dishes and maintained at 37°C under humidified 5% CO₂ in air until they reached greater than 70% confluence. The original media was replaced with serum-free medium and the plates were incubated for an additional 12 h, unless otherwise indicated. Then, the media were replaced with serum-free medium containing the test compounds and the plates were incubated for an additional 1 h. (*R,S*)-Ketamine was used at the concentrations of 0 to 10 μ M; (*R,S*)-norketamine at 0 to 1 μ M, and (2*S,6S*)-hydroxynorketamine at 0 to 0.1 μ M. The effect of (*S*)-nicotine on (*R,S*)-ketamine-, (*R,S*)-norketamine-, (2*S,6S*)-hydroxynorketamine-, and methyllycaconitine-induced changes in mTOR, Akt, ERK, 4E-BP1, and p70S6K phosphorylation levels was then investigated in PC-12 cells. In this experiment, PC-12 cells were incubated for 1 h in the presence of (*R,S*)-ketamine (1 μ M), (*R,S*)-norketamine (10 nM), (2*S,6S*)-hydroxynorketamine (0.1 nM), or methyllycaconitine (50 nM) alone and combined with (*S*)-nicotine (2 μ M).¹⁴ Determination of the expression of total and phosphorylated forms of the signaling proteins was carried out on one set of dishes, and the same experiment was repeated on 3 separate days (*n* = 3).

Statistical Analysis

Data are presented as “average relative change \pm SD.” All statistical analyses were performed using one-way ANOVA and the Dunnett test for *post hoc* multiple comparisons. GraphPad Prism 4 software package (GraphPad Software, Inc., La Jolla, CA) was used to carry out statistical analyses. *P* values of 0.05 or less were considered statistically significant.

Results

Brain Tissue Concentrations of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine

After the intraperitoneal administration of (*R,S*)-ketamine, the brain tissue samples contained (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S;2R,6R*)-hydroxynorketamine as well as four additional diastereomeric hydroxynorketamines. A representative chromatogram from a 60-min sample is presented in figure 2A. The brain tissue concentration of (*R,S*)-ketamine peaked at 137 ± 6 μ M/g in the 10-min postadministration sample and then rapidly declined to 0.6 ± 0.1

μ M/g in the 240-min sample, table 1. In these samples, the concentration of (2*S,6S;2R,6R*)-hydroxynorketamine exceeded that of (*R,S*)-norketamine at the 60- and 240-min time points (table 1).

After the intravenous administration of (*R,S*)-norketamine, the brain tissue samples contained (*R,S*)-norketamine, (2*S,6S;2R,6R*)-hydroxynorketamine, and three additional diastereomeric hydroxynorketamines, figure 2B. (*R,S*)-Norketamine concentrations peaked at 88 ± 8 μ M/g in the 10-min postadministration sample and then rapidly declined to 1.0 ± 0.1 μ M/g in the 240-min sample, table 1. (2*S,6S;2R,6R*)-Hydroxynorketamine was also present in the 10-min sample and the concentration of this metabolite exceeded that of (*R,S*)-norketamine in the 240-min sample, table 1.

After intravenous administration of (2*S,6S*)-hydroxynorketamine, the brain tissue samples contained only this compound and no additional diastereomeric hydroxynorketamines were observed (fig. 2C). The peak brain tissue concentration of (2*S,6S*)-hydroxynorketamine was 127 ± 4 μ M/g in the 10-min sample and was essentially maintained in the 20-min sample, with the 240-min sample retaining ~10% of the peak concentration, table 1.

Effect of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine on the In Vivo Phosphorylation of mTOR and Related Proteins in Rat Brain Tissue

The phosphorylation of mTOR and related proteins was determined in rat brain tissue obtained 30, 60, and 240 min after the administration of (*R,S*)-ketamine, but not in the 10-min postadministration samples as the amount of tissue was insufficient to carry out these studies. Western blot analysis was performed using primary antibodies specific for the phosphorylated forms of mTOR, 4E-BP1, p70S6K, ERK1/2, and Akt. The results demonstrated that (*R,S*)-ketamine administration produced a time-dependent increase in the expression of pmTOR (~2.5-fold) and pp70S6K (~2.5-fold), which reached a maximum at the 30- and 60-min time points, followed by a gradual decline (figs. 3A and D). Similar findings were reported in a recent study where the increase in the phosphorylated forms of these proteins (~1.5-fold) peaked at 30 to 60 min postadministration of (*R,S*)-ketamine in Wistar rats, before returning to baseline levels.⁴ In this study, ANOVA analysis showed that administration of (*R,S*)-ketamine for 30 and 60 min led to significant increases in pmTOR and pp70S6K levels (*P* < 0.05), whereas increases in the average expression of p4E-BP1 (~1.5-fold), pERK1/2 (~2-fold), and pAkt (~1.3-fold) did not reach statistical significance (figs. 3A and D, and Supplemental Digital Content 1 figure S1, <http://links.lww.com/ALN/B51>, which illustrates the ratios of phosphorylated to total Akt and ERK protein levels at various incubation periods with (*R,S*)-ketamine and its metabolites).

Administration of (*R,S*)-norketamine produced significant increases in the levels of phosphorylated forms of mTOR, pp70S6K, and p4E-BP1 in the 20- and 60-min

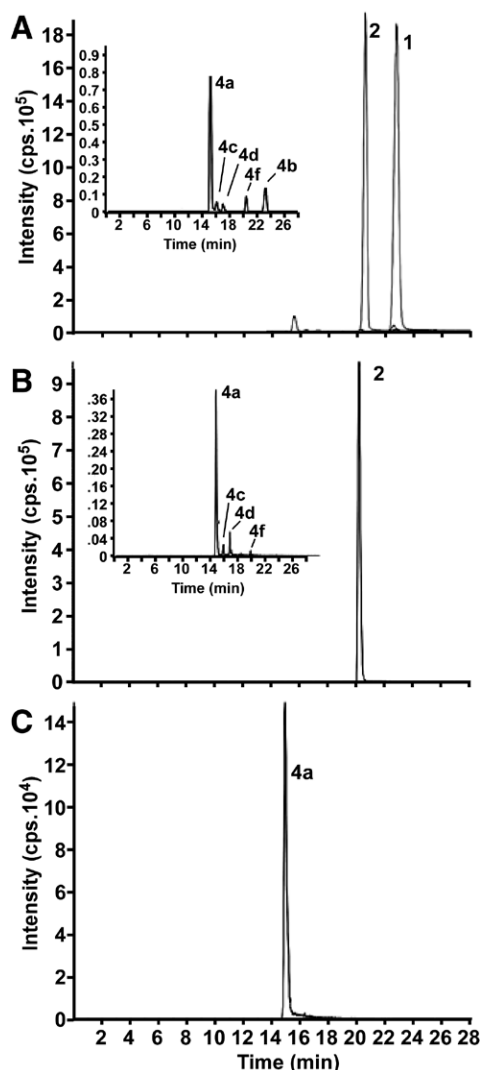


Fig. 2. A representative chromatogram from a 60-min brain sample obtained (A) after a single intraperitoneal injection of (*R,S*)-ketamine, 40 mg/kg in saline (5 ml/kg), (B) after a single intravenous injection of (*R,S*)-norketamine, 20 mg/kg in saline (5 ml/kg), and (C) after a single intravenous injection of (2*S,6S*)-hydroxynorketamine, 20 mg/kg in saline (5 ml/kg). The labeled peaks correspond to (*R,S*)-ketamine (1), (*R,S*)-norketamine (2), (2*S,6S*;2*R,6R*)-hydroxynorketamine (4a), (2*S,6R*;2*R,6S*)-hydroxynorketamine (4b), (2*S,5S*;2*R,5R*)-hydroxynorketamine (4c), (2*S,4S*;2*R,5R*)-hydroxynorketamine (4d), and (2*S,4R*;2*R,4S*)-hydroxynorketamine (4f).

samples (figs. 3B and E). The 15- and 25-fold increases in the pmTOR levels were much higher than the ~2.5-fold increase observed after the administration of (*R,S*)-ketamine and may be a result of larger initial exposure to (*R,S*)-norketamine relative to the concentration of the compound produced by the administration of (*R,S*)-ketamine (figs. 2A and B, table 1). Although the relative pAkt levels were not affected, there was significant increase in the relative levels of pERK1/2 in response to (*R,S*)-norketamine administration (figs. 3B and E and Supplemental Digital Content 1 figure S1, <http://links.lww.com/ALN/B51>). The ~6-fold increase in pERK

level was also significantly greater than the ~2-fold (figs. 3A and D) and 1.5-fold⁴ increases produced by (*R,S*)-ketamine.

Administration of (2*S,6S*)-hydroxynorketamine produced time-dependent increases in pmTOR (~2.0-fold), p4E-BP1 (~2.0-fold), and pp70S6K (~2.5-fold) levels, which reached significance in the 20- and 60-min samples (figs. 3C and F). These effects were similar to that produced by (*R,S*)-ketamine (figs. 3A and D). Even though the relative levels of pERK1/2 and pAkt were increased in the 20- and 60-min samples, they did not reach statistical significance (Supplemental Digital Content 1 figure S1, <http://links.lww.com/ALN/B51>).

Effect of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine on the Expression of m-SR in PC-12 Cells

The treatment of PC-12 cells with (*R,S*)-ketamine (0 to 10,000 nM), (*R,S*)-norketamine (0 to 1,000 nM), and (2*S,6S*)-hydroxynorketamine (0 to 100 nM) produced concentration-dependent increases in the expression of the m-SR protein, with maximum at 600, 10, and 0.05 nM, respectively, and gradual decline at higher concentrations of the test compounds (fig. 4 and Supplemental Digital Content 1 figure S2, <http://links.lww.com/ALN/B51>, which illustrates m-SR protein levels at various concentrations of (*R,S*)-ketamine and its metabolites). ANOVA analysis showed significant increases at 600 and 1,000 nM of (*R,S*)-ketamine, 10 and 25 nM of (*R,S*)-norketamine, and between 0.05 and 0.25 nM of (2*S,6S*)-hydroxynorketamine.

Effect of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine on the Phosphorylation of mTOR and Related Proteins in PC-12 Cells

PC-12 cells were incubated for 60 min with a range of concentrations of (*R,S*)-ketamine (50 to 10,000 nM), (*R,S*)-norketamine (0.05 to 1,000 nM), and (2*S,6S*)-hydroxynorketamine (0.01 to 100 nM). The incubation time was chosen based on the data obtained in the *in vivo* studies in which significant increases in the levels of phosphorylated forms of mTOR, 4E-BP1, p70S6K, ERK1/2, and Akt were observed 60 min after administration of (*R,S*)-ketamine and its metabolites. All three compounds produced significant and concentration-dependent increases in the phosphorylation of these signaling intermediates (figs. 5–7 and Supplemental Digital Content 1 figures S3–S5, <http://links.lww.com/ALN/B51>, which illustrate quantitative densitometric analyses of the relative phosphorylation levels of these signaling proteins at various concentrations of (*R,S*)-ketamine and its metabolites). Cell treatment with (*R,S*)-ketamine (400 to 600 nM) elicited significant increases in protein phosphorylation, which gradually lost significance at concentrations greater than 2,000 nM (fig. 5 and Supplemental Digital Content 1 figure S3, <http://links.lww.com/ALN/B51>). At 600 nM of (*R,S*)-ketamine, the relative increases in phosphorylation over baseline ranged from 1.5- to 3-fold (fig. 5B). Incubation

Table 1. Brain Tissue Concentrations of (*R,S*)-Ket, (*R,S*)-norKet, and (2*S*,6*S*;2*R*,6*R*)-HNK after the Administration of (*R,S*)-Ket, (*R,S*)-norKet, and (2*S*,6*S*)-HNK to Male Wistar Rats

Administered Compound	Time (min)	Rat Brain Tissue Concentrations (μM/g)		
		(<i>R,S</i>)-Ket	(<i>R,S</i>)-norKet	(2 <i>S</i> ,6 <i>S</i> ;2 <i>R</i> ,6 <i>R</i>)-HNK
(<i>R,S</i>)-Ket	10	137 ± 6	21 ± 8	30 ± 5
	30	52 ± 44	34 ± 6	21 ± 8
	60	19 ± 9	16 ± 4	25 ± 5
	240	0.6 ± 0.1	1.3 ± 0.3	11 ± 5
(<i>R,S</i>)-norKet	10	—	88 ± 8	16 ± 3
	20	—	51 ± 4	16 ± 3
	60	—	20 ± 7	8.9 ± 0.7
	240	—	1.0 ± 0.1	2.1 ± 0.4
(2 <i>S</i> ,6 <i>S</i>)-HNK	10	—	—	127 ± 4*
	20	—	—	122 ± 17*
	60	—	—	26 ± 9*
	240	—	—	12 ± 14*

n = 3 at each time point.

* Reported values are of (2*S*,6*S*)-HNK.

HNK = hydroxynorketamine; (*R,S*)-Ket = (*R,S*)-ketamine; (*R,S*)-norKet = (*R,S*)-norketamine.

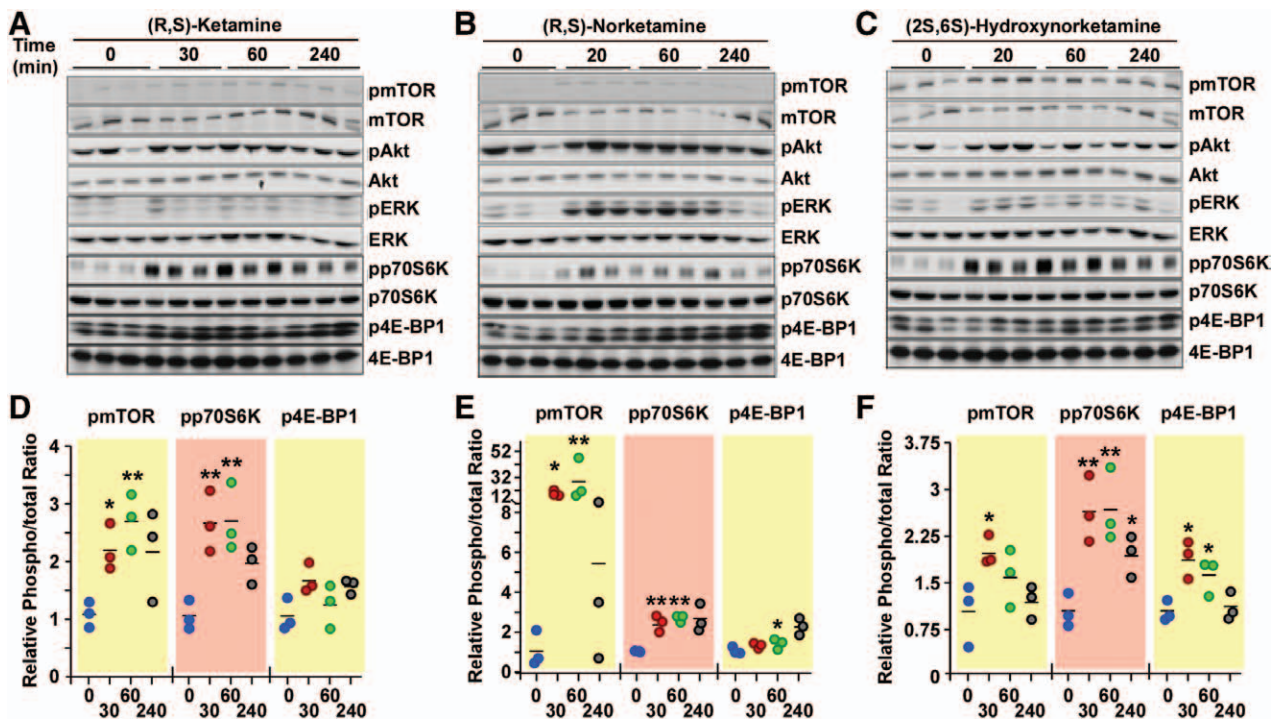


Fig. 3. Levels of phospho-active forms of mammalian target of rapamycin (mTOR), extracellular signal-regulated kinases (ERK1/2), protein kinase B (Akt), eukaryotic initiation factor 4E binding protein (4E-BP1), and p70S6 kinase (p70S6K) in rat brain (cortex) tissues at different time intervals (0–240 min) after administration of either (*R,S*)-ketamine, (*R,S*)-norketamine, or (2*S*,6*S*)-hydroxynorketamine. The mode of administration and dosage protocol of the test compounds are described in the legend of figure 2. (A–C) Representative immunoblots using the indicated primary antibodies. (D–F) Scatter plots illustrating the relative levels of phosphorylated and total forms of mTOR, p70S6K, and 4E-BP1 in response to (*R,S*)-ketamine (D), (*R,S*)-norketamine (E), and (2*S*,6*S*)-hydroxynorketamine (F) are shown (n = 3 independent experiments). *, **P < 0.05, 0.01 (ANOVA) compared with controls.

with 1 to 250 nM of (*R,S*)-norketamine produced significant increases in pmTOR levels, which lost significance at concentrations greater than 250 nM (fig. 6 and Supplemental Digital Content 1 figure S4, <http://links.lww.com/ALN/B51>). At 25 nM of (*R,S*)-norketamine, the relative increases

in phosphorylation over baseline ranged from 2.5- to 5-fold (fig. 6B). Finally, treatment of PC-12 cells with 0.01 to 10 nM of (2*S*,6*S*)-hydroxynorketamine resulted in significant increases in pmTOR levels, with marked reduction at concentrations greater than 1 nM (fig. 7, Supplemental

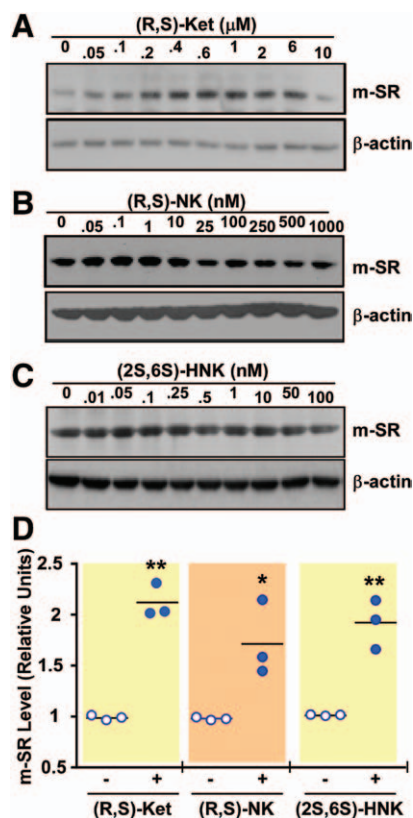


Fig. 4. Expression of monomeric serine racemase (m-SR) protein in PC-12 cells after 36 h incubation with different concentrations of (A) (*R,S*)-ketamine (Ket, 0–10 μM), (B) (*R,S*)-norketamine (NK, 0–1 μM), and (C) (2*S*,6*S*)-hydroxynorketamine (HNK, 0–0.1 μM). (A–C) Representative Western blot analysis with primary antibodies raised against SR and β-actin. (D) Scatter plots illustrating the relative levels of m-SR in response to 600 nM (*R,S*)-Ket, 10 nM (*R,S*)-NK, and 0.05 nM (2*S*,6*S*)-HNK after quantification and normalization with β-actin (*n* = 3 independent experiments). *, ***P* < 0.05, 0.01 (ANOVA) compared with control cells.

Digital Content 1 figure S5, <http://links.lww.com/ALN/B51>. At 0.50 nM of (2*S*,6*S*)-hydroxynorketamine, the relative increases in phosphorylation over baseline ranged from ~2.8- to ~4.2-fold (fig. 7B).

Effect of (*S*)-nicotine on the Expression of m-SR and the Phosphorylation of mTOR and Related Proteins in PC-12 Cells Treated with (*R,S*)-ketamine, (*R,S*)-norketamine, (2*S*,6*S*)-hydroxynorketamine, and Methyllycaconitine

We have recently shown that the incubation of PC-12 cells with the selective α₇-nAChR antagonist methyllycaconitine significantly increases the expression of m-SR, whereas treatment with the α₇-nAChR agonist (*S*)-nicotine alone has no effect but can attenuate the response to methyllycaconitine.¹⁴ Here, the data illustrates that (*S*)-nicotine attenuated the increase in m-SR expression elicited by (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*)-hydroxynorketamine in PC-12 cells, figure 8. Moreover, (*S*)-nicotine markedly attenuated the response of (*R,S*)-ketamine, (*R,S*)-norketamine,

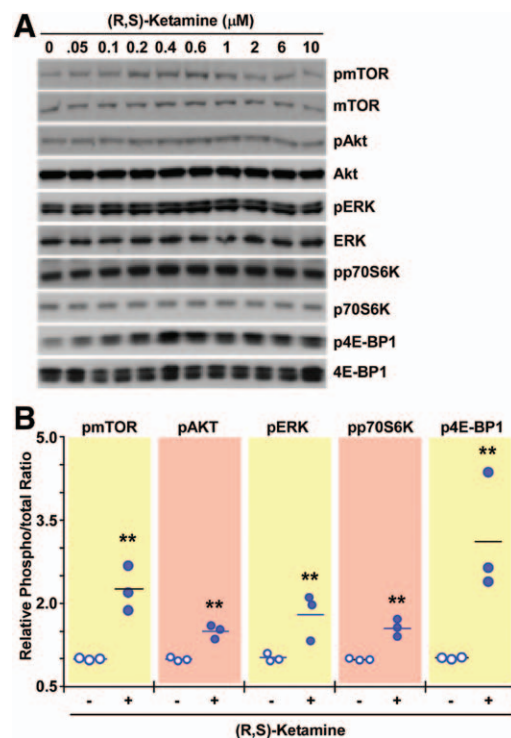


Fig. 5. Effect of (*R,S*)-ketamine on the levels of phospho-active forms of mammalian target of rapamycin (mTOR), protein kinase B (Akt), extracellular signal-regulated kinases (ERK1/2), p70S6 kinase (p70S6K), and eukaryotic initiation factor 4E binding protein (4E-BP1) in PC-12 cells. (A) Cells were treated with different concentrations of (*R,S*)-ketamine (0–10 μM) for 1 h and processed for Western blot analysis. (B) Scatter plots illustrating the relative ratio of phosphorylated versus total forms of mTOR, Akt, ERK1/2, p70S6K, and 4E-BP1 in response to cell treatment with 600 nM of (*R,S*)-ketamine are shown (*n* = 3 independent experiments). ***P* < 0.01 (ANOVA) compared with control cells.

(2*S*,6*S*)-hydroxynorketamine, and methyllycaconitine toward pmTOR, p4E-BP1, pp70S6K, pERK1/2, and pAkt levels, figure 9. Treatment of PC-12 cells with (*S*)-nicotine alone had no effect on the phosphorylation of these signaling intermediates.

Discussion

The initial studies of (*R,S*)-ketamine in the rat demonstrated that the compound was rapidly and extensively metabolized, forming (*R,S*)-norketamine, (2*S*,6*S*;2*R*,6*R*)-hydroxynorketamine, and additional diastereomeric hydroxynorketamines.^{6–9} This was also observed in the current study, figure 2A. In an earlier study, the anesthetic effects of (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S*,6*S*;2*R*,6*R*)-hydroxynorketamine were examined in the rat by determining the duration of anesthesia and spontaneous locomotor activity during the postanesthetic recovery phase.⁹ The results indicated that the anesthetic effects were primarily due to (*R,S*)-ketamine and that (*R,S*)-norketamine contributed also to this effect.⁹ Moreover, the data indicated that

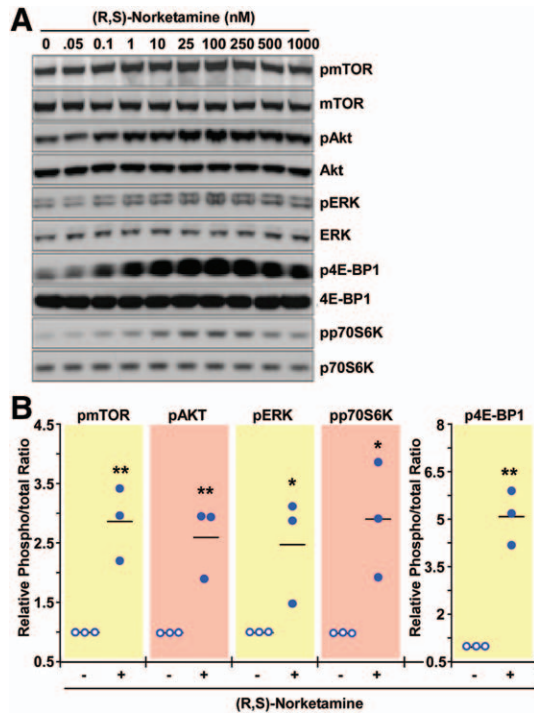


Fig. 6. Effect of (*R,S*)-norketamine on the levels of phospho-active forms of mammalian target of rapamycin (mTOR), protein kinase B (Akt), extracellular signal-regulated kinases (ERK1/2), p70S6 kinase (p70S6K), and eukaryotic initiation factor 4E binding protein (4E-BP1) in PC-12 cells. (A) Cells were treated with different concentrations of (*R,S*)-norketamine (0–1 μ M) for 1 h and processed for Western blot analysis. (B) Scatter plots illustrating the relative ratio of phosphorylated *versus* total forms of mTOR, Akt, ERK1/2, p70S6K, and 4E-BP1 in response to cell treatment with 25 nM of (*R,S*)-norketamine are shown ($n = 3$ independent experiments). *, ** $P < 0.05$, 0.01 (ANOVA) compared with control cells.

(2*S,6S*;2*R,6R*)-hydroxynorketamine had no anesthetic effect, and on the basis of this observation, further investigations of the pharmacological activities of (2*S,6S*;2*R,6R*)-hydroxynorketamine and other postnorketamine metabolites have not been undertaken. This concept still persists even when sub-anesthetic doses of (*R,S*)-ketamine and (*S*)-ketamine are used and the pharmacological endpoints are the treatment of pain and depression. The results from the current study demonstrate that (2*S,6S*)-hydroxynorketamine has pharmacological activity that contributes to the effects produced by the administration of subanesthetic doses of (*R,S*)-ketamine in the rat.

In this study, the administration of (*R,S*)-ketamine to male Wistar rats produced an increase in the relative levels of the phosphorylated forms of mTOR, Akt, ERK1/2, p70S6K, and 4E-BP1 in the prefrontal cortex, consistent with earlier observations.^{4,5} The administration of (*R,S*)-norketamine and (2*S,6S*)-hydroxynorketamine produced similar effects. The data indicate that all the three compounds activate the mTOR signaling pathway, which has been associated with the rapid antidepressant effects of

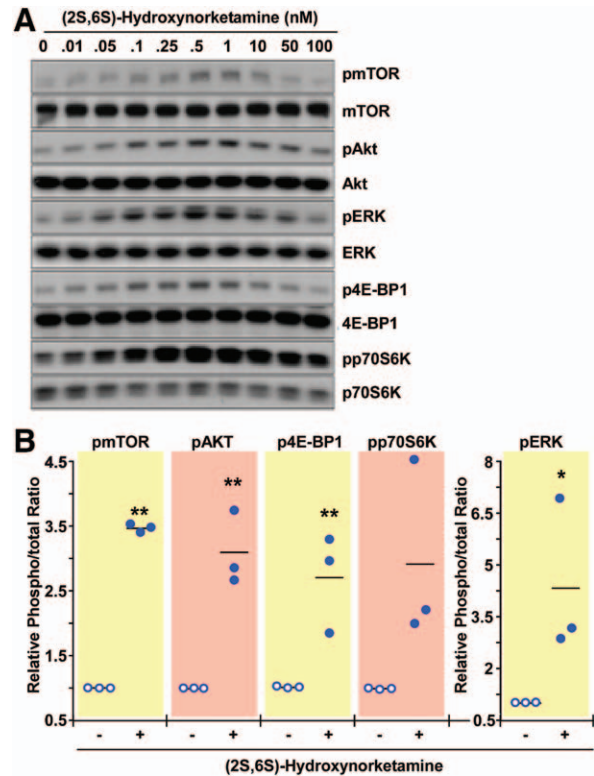


Fig. 7. Effect of (2*S,6S*)-hydroxynorketamine on the levels of phospho-active forms of mammalian target of rapamycin (mTOR), protein kinase B (Akt), extracellular signal-regulated kinases (ERK1/2), p70S6 kinase (p70S6K), and eukaryotic initiation factor 4E binding protein (4E-BP1) in PC-12 cells. (A) Cells were treated with different concentrations of (2*S,6S*)-hydroxynorketamine (0–0.1 μ M) for 1 h and processed for Western blot analysis. (B) Scatter plots illustrating the relative ratio of phosphorylated *versus* total forms of mTOR, Akt, ERK1/2, p70S6K, and 4E-BP1 in response to cell treatment with 0.5 nM of (*R,S*)-hydroxynorketamine are shown ($n = 3$ independent experiments). *, ** $P < 0.05$, 0.01 (ANOVA) compared with control cells.

(*R,S*)-ketamine, including the induction of synaptogenesis and behavioral response.^{4,5} Although the effect of (*R,S*)-norketamine and (2*S,6S*)-hydroxynorketamine on synaptogenesis was not examined, studies using PC-12 cells indicate that the activation of the mTOR signaling pathway by (*R,S*)-ketamine, (*R,S*)-norketamine, and (2*S,6S*)-hydroxynorketamine results in an increase in the *de novo* synthesis of m-SR. Thus, the signaling process initiated by these compounds was successfully translated into an increased protein expression.

We have recently reported that the incubation of 1321N1 astrocytoma cells with the α_7 -nAChR antagonists, methyllycaconitine and (*R,S*)-dehydroxynorketamine, increased the *de novo* protein synthesis of m-SR *via* the mTOR pathway.¹⁴ A similar effect on m-SR expression was observed in PC-12 cells although the involvement of the mTOR pathway was not definitively established.¹⁴ The previous study also demonstrated that the effect on m-SR expression in PC-12 cells

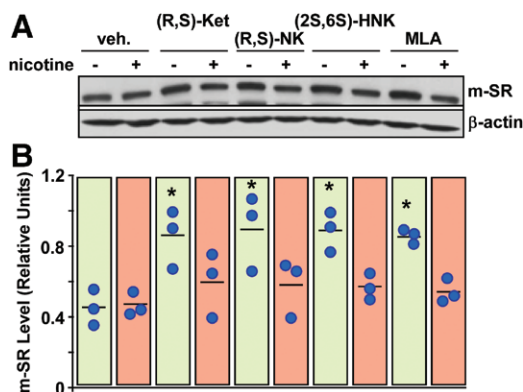


Fig. 8. Effects of (*R,S*)-ketamine (Ket, 1 μ M), (*R,S*)-norketamine (NK, 10 nM), (*2S,6S*)-hydroxynorketamine (HNK, 0.1 nM), and methyllycaconitine (MLA, 50 nM) with or without nicotine (2 μ M) on the levels of monomeric serine racemase (m-SR) protein in PC-12 cells. (A) Representative immunoblots of m-SR and β -actin. (B) Scatter plot illustrating the relative m-SR levels after quantification and normalization with β -actin ($n = 3$ independent experiments). * $P < 0.05$ (ANOVA) compared with control cells.

was primarily due to inhibition of the α_7 -nAChR and not heteromeric $\alpha_3\beta_4$ -nAChRs; both subtypes are expressed in PC-12 cells. In the current study, we have demonstrated that incubation of PC-12 cells with (*R,S*)-ketamine, (*R,S*)-norketamine, (*2S,6S*)-hydroxynorketamine, and methyllycaconitine produced significant increases in the levels of phosphorylated mTOR, 4E-BP1, p70S6K, ERK1/2, and Akt and that of m-SR protein. The concentrations required to produce the maximum effect on the mTOR pathway and m-SR expression were as follows: (*2S,6S*)-hydroxynorketamine (0.50 nM) greater than (*R,S*)-norketamine (25 nM) greater than (*R,S*)-ketamine (600 nM). The fact that treatment with (*S*)-nicotine attenuated the response produced by ketamine and its metabolites supports the involvement of the nAChR. As indicated above, PC-12 cells express both the homomeric α_7 -nAChR and heteromeric nAChRs, in particular, the $\alpha_3\beta_4$ -nAChR. The relative potencies of the test compounds reflect their activities at the α_7 -nAChR as (*2S,6S*)-hydroxynorketamine is a selective and potent inhibitor of the α_7 -nAChR with little effect on $\alpha_3\beta_4$ -nAChR, IC_{50} greater than 300 μ M.¹³ (*R,S*)-Norketamine is also a potent inhibitor of the α_7 -nAChR, although weaker than (*2S,6S*)-hydroxynorketamine, and an effective inhibitor of the $\alpha_3\beta_4$ -nAChR with an IC_{50} of 9 μ M.¹³ Finally, (*R,S*)-ketamine is the weakest of the three compounds as it inhibits (*S*)-nicotine-induced currents in PC-12 cells (IC_{50} 5 μ M),¹⁵ which seems to be due to the inhibition of both α_7 -nAChR (IC_{50} 20 μ M)¹⁶ and $\alpha_3\beta_4$ -nAChR (IC_{50} 3 μ M).¹³ Thus, the data indicate that the observed pharmacological effect is initiated by inhibition of the α_7 -nAChR, which is consistent with the results from our previous study of nAChR antagonists in 1321N1 and PC-12 cells.¹⁴

Inhibition of the homomeric α_7 -nAChR basal activity by (*R,S*)-ketamine and ketamine metabolites stimulates multiple signaling pathways, which, in turn, increases the translation of SR mRNA into monomeric SR protein. Activation of the phosphatidylinositol 3-kinase/Akt pathway is key to mTOR complex (mTORC) 1 activation and regulation of cap-dependent protein synthesis.^{17,18} Here, (*R,S*)-ketamine, (*R,S*)-norketamine, and (*2S,6S*)-hydroxynorketamine were found to promote mTORC1 function, as determined by the increased phosphorylation of the translation regulatory protein 4E-BP1 and the 70-kDa isoform of S6K1, which are frequently used as surrogate markers of mTOR kinase activity.¹⁹ AKT phosphorylation on Thr308 and Ser473 contribute to the stimulation of mTORC1, and AKT phosphorylation on Ser473 is a recognized effector of mTORC2 whose activity is considered to be upstream of mTORC1.¹⁷ This process is depicted in figure 10. Our results suggest that mTORC2 mediates the translational activity of (*R,S*)-ketamine and its metabolites because of their ability to promote AKT Ser473 phosphorylation, but how mTORC2 is regulated is unclear. Sin1 (stress-activated protein kinase-interacting protein 1) and rictor maintain mTORC2 integrity and mediates mTORC2 function.^{18,19} It is possible that stimulation of mTORC2 by (*R,S*)-ketamine, (*R,S*)-norketamine, and (*2S,6S*)-hydroxynorketamine might come from enhanced binding of rictor and Sin1 to the mTOR kinase.

A single subanesthetic dose of (*R,S*)-ketamine, 10 mg/kg, has been shown to relieve depression-like behaviors in rats suffering from neuropathic pain but not spared nerve injury-induced hypersensitivity.²⁰ The authors suggested that there are differences in the underlying mechanism of the antidepressant function of (*R,S*)-ketamine and its analgesic properties. Although the data from this study do not include behavioral studies of pain or depression, the results are consistent with the proposed hypothesis. A single low dose of (*R,S*)-ketamine increases the phosphorylated forms of the proteins associated with the mTOR pathway and subsequently stimulates *de novo* protein synthesis, a process that has been associated with the antidepressant activity of (*R,S*)-ketamine.^{4,5} However, these effects of (*R,S*)-ketamine are likely to be time- and concentration dependent. The results from this study suggest that the higher doses of (*R,S*)-ketamine required to achieve analgesia as well as the repetitive or continuous dosage protocols used in the treatment of neuropathic pain syndromes, such as Complex Regional Pain Syndrome, might negate or perhaps even decrease phosphorylation of the proteins associated with the mTOR pathway and protein expression. An alternative mechanism may lie in the regulation of SR activity rather than its expression. Indeed, previous studies in our laboratory have indicated that the incubation of PC-12 cells with increasing concentrations of methyllycaconitine or (*R,S*)-dehydroxynorketamine decreases the intracellular concentration of the NMDAR coagonist D-serine, a product of SR-mediated racemization of L-serine, despite higher expression of

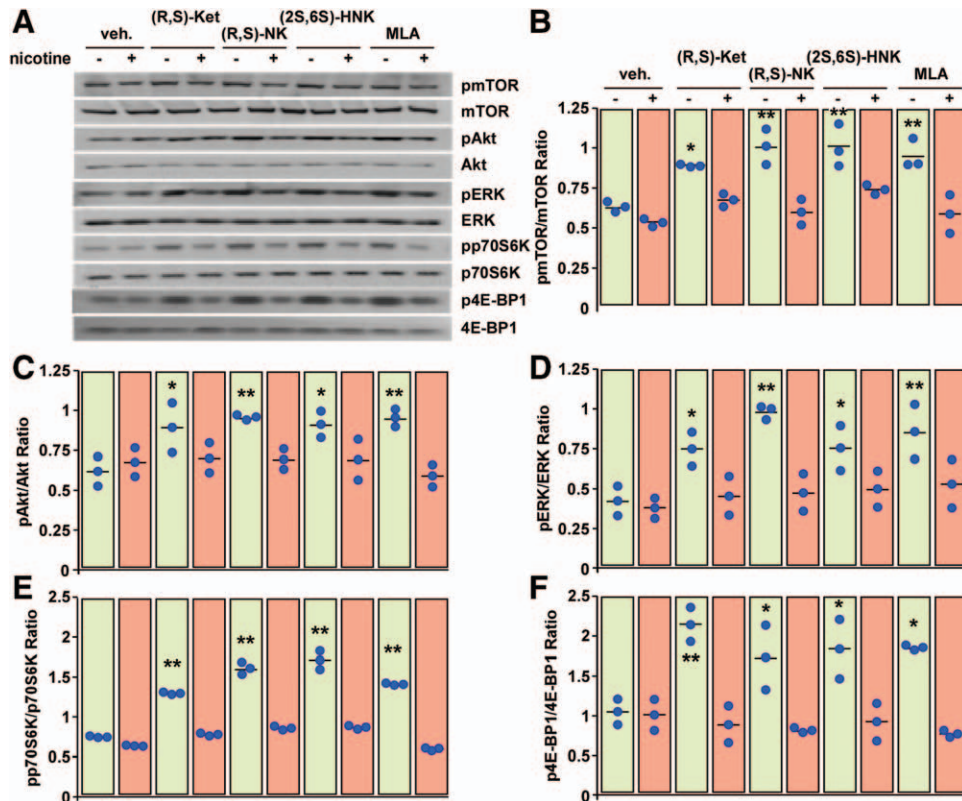


Fig. 9. Effects of (*R,S*)-ketamine, (*R,S*)-norketamine (NK), (*2S,6S*)-hydroxynorketamine (HNK), and methyllycaconitine with or without nicotine on the levels of phospho-active forms of mammalian target of rapamycin (mTOR), protein kinase B (Akt), extracellular signal-regulated kinases (ERK1/2), p70S6 kinase (p70S6K), and eukaryotic initiation factor 4E binding protein (4E-BP1) in PC-12 cells. (A) Cells were treated with (*R,S*)-Ket (1 μ M), (*R,S*)-NK (10 nM), (*2S,6S*)-HNK (0.1 nM), or methyllycaconitine (MLA, 50 nM) with or without nicotine (2 μ M) for 1 h and then processed for Western blot analysis. (A) Representative immunoblots. (B–F) Scatter plots illustrating the relative ratio of phosphorylated versus total forms of mTOR (B), Akt (C), ERK1/2 (D), p70S6K (E), and 4E-BP1 (F) are shown ($n = 3$ independent experiments). *, ** $P < 0.05$, 0.01 (ANOVA) compared with control cells.

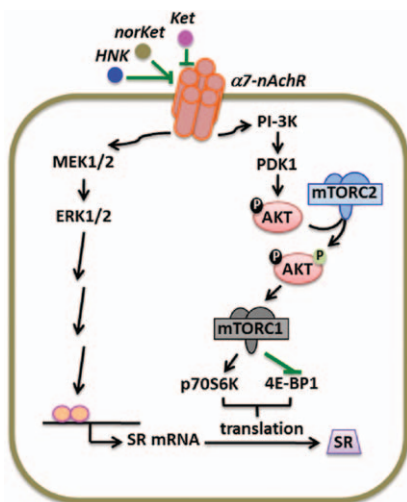


Fig. 10. Schematic representation of the modulation of serine racemase (SR) expression functioning via mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK) pathways. HNK = (*2S,6S*)-hydroxynorketamine; Ket = (*R,S*)-ketamine; mTORC1 = mTOR complex 1; mTORC2 = mTOR complex 2; nAChR = nicotinic acetylcholine receptor; NorKet = (*R,S*)-norketamine.

m-SR.¹⁴ A similar decrease in intracellular D-serine concentrations was observed after incubation of PC-12 cells with the voltage-gated calcium channel $\alpha_2\delta$ inhibitors gabapentin and (*S*)-pregabalin,²¹ which are used in the treatment of neuropathic pain, without affecting m-SR protein level. The inhibition of SR activity has also been associated with a decrease in NMDAR activity, and SR inhibitors are being explored for use in the treatment of some central nervous system disorders.^{22,23}

The results of the study suggest that the therapeutic effects produced by subanesthetic doses of (*R,S*)-ketamine may be the result of a combination of independent but inter-related pharmacological effects at the α_7 -nAChR produced by the parent drug and its metabolites. One of the effects is increased protein expression via the mTOR pathway, which is initiated by antagonism of α_7 -nAChR, and is reflected by the observed increase in m-SR expression. The second effect is an “indirect” inhibition of NMDAR activity resulting from a reduction in the intracellular Ca^{2+} flux. These two interconnected mechanisms are reflected in the previously observed and apparently contradictory effects produced by methyllycaconitine and (*R,S*)-dehydroxynorketamine in

1321N1 and PC-12 cells in which m-SR expression was increased, whereas the m-SR function, expressed as intracellular D-serine concentration, was reduced.¹⁴ The interrelation and importance of the effects produced by (*R,S*)-ketamine metabolites and the related mechanisms are under investigation, and the results will be reported elsewhere.

Acknowledgments

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Competing Interests

Drs. Wainer, Moaddel, Bernier, and Torjman are listed as coinventors on a patent application for the use of ketamine metabolites in the treatment of bipolar disorder and major depression. They have assigned their rights in the patent to the U.S. government but will share a percentage of any royalties that may be received by the government. The other authors declare no competing interests.

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