

ARCHIVAL REPORT

Glutamate *N*-methyl-D-aspartate Receptor Antagonists Rapidly Reverse Behavioral and Synaptic Deficits Caused by Chronic Stress Exposure

Nanxin Li, Rong-Jian Liu, Jason M. Dwyer, Mounira Banasr, Boyoung Lee, Hyeon Son, Xiao-Yuan Li, George Aghajanian, and Ronald S. Duman

Background: Despite widely reported clinical and preclinical studies of rapid antidepressant actions of glutamate *N*-methyl-D-aspartate (NMDA) receptor antagonists, there has been very little work examining the effects of these drugs in stress models of depression that require chronic administration of antidepressants or the molecular mechanisms that could account for the rapid responses.

Methods: We used a rat 21-day chronic unpredictable stress (CUS) model to test the rapid actions of NMDA receptor antagonists on depressant-like behavior, neurochemistry, and spine density and synaptic function of prefrontal cortex neurons.

Results: The results demonstrate that acute treatment with the noncompetitive NMDA channel blocker ketamine or the selective NMDA receptor 2B antagonist Ro 25-6981 rapidly ameliorates CUS-induced anhedonic and anxiogenic behaviors. We also found that CUS exposure decreases the expression levels of synaptic proteins and spine number and the frequency/amplitude of synaptic currents (excitatory postsynaptic currents) in layer V pyramidal neurons in the prefrontal cortex and that these deficits are rapidly reversed by ketamine. Blockade of the mammalian target of rapamycin protein synthesis cascade abolishes both the behavioral and biochemical effects of ketamine.

Conclusions: The results indicate that the structural and functional deficits resulting from long-term stress exposure, which could contribute to the pathophysiology of depression, are rapidly reversed by NMDA receptor antagonists in a mammalian target of rapamycin dependent manner.

Key Words: Antidepressant, depression, ketamine, rapamycin, spines, synaptogenesis

Major depressive disorder (MDD) is a debilitating and recurring psychiatric disorder that affects up to 17% of the American population (1). Despite a wide range of antidepressants available, only one third of the patients show significant mood improvement in response to an initial antidepressant treatment (2). Moreover, there is a time lag of weeks to months with currently available medications, further highlighting a major unmet need for novel rapid-acting and more efficacious antidepressant agents. Recent studies with ketamine, a noncompetitive glutamate *N*-methyl-D-aspartate (NMDA) receptor antagonist, may address this critical need. Ketamine produces rapid antidepressant responses (within hours) in treatment-resistant MDD patients (3–5). However, the widespread use of ketamine is limited by the potential for toxicity and abuse, and studies are being conducted in animal models to elucidate the mechanisms underlying the actions of ketamine and to develop safe, rapid-acting agents.

Preclinical studies have demonstrated antidepressant actions of ketamine in rodent behavioral despair models, including the forced swim test (FST) and learned helplessness (LH) paradigm (6–10).

From the Laboratory of Molecular Psychiatry (NL, R-JL, JMD, MB, X-YL, GA, RSD), Center for Genes and Behavior, Departments of Psychiatry and Neurobiology, Yale University School of Medicine, New Haven, Connecticut; and Neural Science Center (BL), Korea Institute of Science and Technology; and Department of Biochemistry and Molecular Biology (HS), College of Medicine, Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul, Korea.

Address correspondence to Ronald S. Duman, Ph.D., Yale University School of Medicine, Laboratory of Molecular Psychiatry, Center for Genes and Behavior, Departments of Psychiatry and Neurobiology, 34 Park Street, Room S308, New Haven, CT 06508; E-mail: ronald.duman@yale.edu.

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However, FST and LH are responsive to acute or subchronic antidepressant treatments and do not provide a rigorous test of the rapid actions of ketamine (11). In the current study, we used a chronic unpredictable stress (CUS) paradigm, which results in anhedonia, a core symptom of depression, that is responsive to chronic (3 weeks) but not acute or short-term antidepressant treatment (12). In addition, chronic stress exposure also causes atrophy of neurons in rodent prefrontal cortex and hippocampus (13–20), effects that could contribute to decreased volume of these regions reported in brain imaging studies of MDD patients (14,21–24). Another aim of the current study is to determine if ketamine can rapidly reverse the neuronal atrophy and functional deficits caused by CUS exposure.

We have recently reported that NMDA antagonists rapidly increase the density and function of spine synapses in the prefrontal cortex (PFC) and that these effects, as well as the behavioral responses in the FST and LH, are mediated by activation of the mammalian target of rapamycin (mTOR) (25). The mTOR pathway has been implicated in activity-dependent synaptic plasticity and is localized in neuronal dendrites and spines, where it controls the synthesis of proteins that are required for new synapse formation (26). *N*-methyl-D-aspartate antagonist stimulation of mTOR-mediated synaptogenesis provides a mechanism for rapid reversal of stress-mediated and/or depression-mediated deficits (25).

In the present study, we report the ability of a single dose of NMDA antagonists to rapidly reverse the behavioral and synaptic deficits caused by long-term CUS exposure in an mTOR-dependent manner. These results highlight mTOR and upstream signaling pathways as pivotal targets for development of novel rapid-acting and efficacious antidepressant agents.

Methods and Materials

Animals

Male Sprague-Dawley rats weighing 175 to 250 g were pair-housed and maintained in standard conditions with a 12-hour

light/dark cycle and ad libitum access to food and water. Animal use and procedures were in accordance with the National Institutes of Health guidelines and approved by the Yale University Animal Care and Use Committees.

CUS Procedure

Animals were exposed to a variable sequence of mild and unpredictable stressors for 21 days, a procedure that we have found produces depressive-like behavioral changes (27,28). A total of 10 different stressors were used (2 stressors per day, see Figure 1A). The stressors included rotation on a shaker, placement in a 4°C ambient, lights off for 3 hours (10:00 AM–1:00 PM), lights on overnight, strobe light overnight, aversive odor, 45° tilted cages, food and water deprivation, crowded housing, and isolation housing.

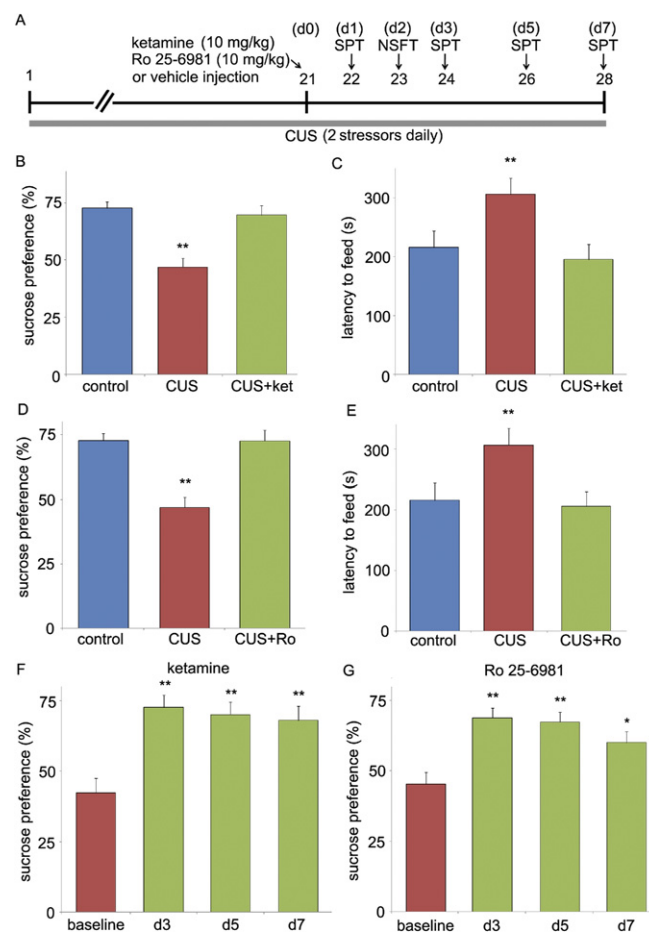


Figure 1. *N*-methyl-D-aspartate receptor antagonists produce rapid antidepressant responses in a chronic unpredictable stress (CUS) paradigm. (A) Schematic demonstrating the time line for CUS exposure, drug administration, and behavioral testing. Numbers in parentheses represent days after drug administration. Rats were exposed to CUS and administered ketamine or Ro 25-6981 (both at 10 mg/kg intraperitoneal) on day 21. The sucrose preference test was conducted 1 day later (B, D) and novelty-suppressed feeding test 2 days after drug treatment (C, E). Ketamine and Ro 25-6981 administration in CUS rats reversed the decreased sucrose preference and increased latency to feed to the level of nonstressed control rats. The sucrose preference test was also conducted at 3, 5, and 7 days after ketamine or Ro 25-6981 (F, G). Baseline was measured on day 21 before drug injections. Values represent mean ± SEM ($n = 6$ per group). ** $p < .01$, * $p < .05$, analysis of variance). ket, ketamine; NSFT, novelty-suppressed feeding test; Ro, Ro 25-6981; SPT, sucrose preference test.

Drug Administration and Surgical Procedure

Animals received a single acute intraperitoneal (IP) injection of vehicle, ketamine, or Ro 25-6981 on day 21 of CUS treatment. Based on previous studies (25), the dose used for both drugs was 10 mg/kg. Tissue was collected for molecular assays or animals were tested in behavioral paradigms as described below. For experiments involving central administration of inhibitors, rats were implanted with guide cannulae (22 ga) into the lateral ventricles (coordinates from bregma: -9 mm anterior/posterior, -1.5 mm medial/lateral, -3.3 mm dorsal/ventral from dura). The surgical procedures were carried out under Nembutal anesthesia (IP 55 mg/kg; Butler Schein, Chicago, Illinois). Postoperative care consisted of perisurgical administration of carprofen (5 mg/kg; Butler Schein) and topical triple antibiotic ointment. During recovery, animals carried a dummy cannula. After a 7-day recovery period, rapamycin (.2 nmol in 2 μ L; Sigma, St. Louis, Missouri) or a vehicle (dimethyl sulfoxide, DMSO; Sigma) was delivered 30 minutes before drug injections at the rate of .25 μ L/min, with an injection cannula (26 ga) protruding .5 mm beyond the guide cannula. These doses were chosen based on previous reports demonstrating effective and selective inhibition of the respective targets (25,29). The injection cannula stayed in the guide cannula for 1 minute after infusions.

Behavioral Tests

Sucrose Preference Test. For the sucrose preference test (SPT), rats were exposed to a palatable sucrose solution (1%; Sigma) for 48 hours, followed by 4 hours of water deprivation and a 1 hour exposure to two identical bottles, one filled with sucrose solution and the other with water. This procedure was adapted from previous studies and has been used previously in our laboratory (30,31). Sucrose and water consumption were determined by measuring the change in the volume of fluid consumed. Sucrose preference was defined as the ratio of the volume of sucrose versus total volume of sucrose and water consumed during the 1-hour test.

Novelty-Suppressed Feeding Test. The novelty-suppressed feeding test (NSFT) was performed as previously described (31). Before testing, rats were food-deprived overnight. Rats were placed in an open field (76.5 cm \times 76.5 cm \times 40 cm, Plexiglas) with a small amount of food in the center. Animals were allowed to explore the open field for 8 minutes. The latency to feed, specifically the time it took for the animal to approach and take the first bite of the food, was recorded by a stopwatch. Home cage food intake was measured right after the test as a control value.

Immunoblotting

Prefrontal cortex synaptosomes were prepared as previously reported (25) and sonicated in protein lysis buffer. Protein concentration was determined by bicinchoninic acid protein assay. For western blotting, equal amounts of protein (10–20 μ g) for each sample were loaded into 10% to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Polyvinylidene difluoride membranes (Bio-Rad, Hercules, California) with transferred proteins were blocked in phosphate buffered saline + .1% Tween 20 (PBST; Sigma) for 1 hour and kept with primary antibodies overnight at 4°C. The following primary antibodies were used: synapsin I (BD Biosciences, San Jose, California), postsynaptic density protein 95 (PSD95) (Invitrogen, Carlsbad, California), and glutamate receptor 1 (GluR1) (Abcam, Cambridge, Massachusetts). The next day, blots were washed three times in PBST and incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit secondary antibody (1:5000 to 1:10000; Vector Laboratories, Burlingame, California) for 1 hour. After three final washes with PBST, bands were

detected using enhanced chemiluminescence. The blots were then incubated in stripping buffer for 30 minutes at 37°C followed by three washes with PBST. The stripped blots were blocked for 1 hour and incubated with primary antibody directed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Advanced Immunochemical, Long Beach, California) for loading control. Densitometric analysis of immunoreactivity for each protein was conducted using National Institutes of Health ImageJ software (Bethesda, Maryland). Immunoreactivity was normalized to the control group for each protein.

Brain Slice Preparation and Electrophysiological Recordings

Brain slices were prepared as previously described (13,25). Briefly, 1 day after drug treatments, rats were anesthetized (chloral hydrate, 400 mg/kg IP) and brains removed and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) in which sucrose (252 mmol/L) was substituted for sodium chloride (sucrose-ACSF). A block of tissue containing prefrontal cortex and coronal slices (400 μ m) were cut in sucrose-ACSF with an oscillating-blade tissue slicer. Slices were placed in a submerged recording chamber; bath temperature was then raised slowly to 32°C. Known concentrations of drugs in ACSF were applied through a stopcock arrangement (~4 mL/min) to reach the slice within 7 to 10 seconds. The standard ACSF (pH 7.35), equilibrated with 95% oxygen/5% carbon dioxide, contained 128 mmol/L sodium chloride, 3 mmol/L potassium chloride, 2 mmol/L calcium chloride, 2 mmol/L magnesium sulfate, 24 mmol/L sodium bicarbonate, 1.25 mmol/L sodium phosphate, and 10 mmol/L d-glucose. There was recovery period of 1 to 2 hours before recording.

Pyramidal neurons in layer V were visualized by video microscopy using a microscope (60 \times infrared lens; Olympus, Center Valley Pennsylvania) with infrared differential interference contrast. Patch pipettes (3–5 M Ω) were pulled from glass tubing using a Flaming-Brown Horizontal Puller (Sutter, Novato, California). The pipette solution contained the following: 115 mmol/L potassium gluconate, 5 mmol/L potassium chloride, 2 mmol/L magnesium chloride, 2 mmol/L magnesium adenosine triphosphate, 2 mmol/L disodium adenosine 5'-triphosphate, 10 mmol/L sodium-phosphocreatine, 4 mmol/L disodium guanosine 5'-triphosphate, and 10 mmol/L HEPEs, pH 7.33 (American Bioanalytical, Natick, Massachusetts). Neurobiotin (.3%; Vector Laboratories) was added to the pipette solution to mark cells for later processing and imaging.

Whole-cell recordings were with an Axoclamp-2B amplifier (Molecular Devices, Sunnyvale, California). The output signal was low-pass filtered at 3 kHz and digitized at 15 kHz; data were acquired by pClamp 9.2/Digidata 1320 software (Molecular Devices). Series resistance, which was monitored throughout the experiment, was usually between 4 and 8 M Ω . To minimize series resistance errors, cells were discarded if series resistance rose above 10 Ω . Postsynaptic currents were studied in the continuous single-electrode voltage-clamp mode (3000 Hz low-pass filter) clamped near resting potential (75 mV \pm 5 mV).

Spine Density Analysis

After completion of recording, slices were transferred to 4% paraformaldehyde (.1 mol/L phosphate buffer) and stored overnight at 4°C. Slices were then processed with streptavidin conjugated to Alexa 594 (1:1000) for visualization of labeled cells. Labeled neurons within layer V of anterior cingulate and prelimbic medial prefrontal cortex were imaged with a dual-photon Ti:Sapphire laser scanning system (810 nanometers; Mai Tai, Spectra Physics, Mountain View, California) coupled to direct detection Radiance 2000 laser scanner (Zeiss Microimaging, Thornwood, New York) mounted on an

Olympus BX50WI microscope (Olympus), using a 60 \times (.9 numerical aperture) water-immersion objective. For spine density analysis, Z-stacks usually consisted of two to five scans at high zoom at 1- μ m steps in the z axis. Spine density was sampled in two zones: 1) tips of tuft branches as they approach the pial membrane and 2) proximal tuft dendrites just distal to the bifurcation of the apical shaft (previous studies had shown that, in contrast to basilar dendrites, the distal tuft in layer V pyramidal cells is especially sensitive to chronic stress [13]). Results were expressed in terms of spine density per 10 μ m.

Results

NMDA Receptor Antagonists Rapidly Reverse the Behavioral Deficits Caused by CUS: Requirement for mTOR Signaling

The development of depressive behaviors, notably anhedonia, with CUS exposure and requirement for chronic antidepressant treatment to reverse these effects, make CUS one of the most valid models of depression. Although stress and behavioral testing variables also make it one of the most difficult (12), we have successfully established a CUS paradigm in our laboratory (27,31). In the present study, CUS-exposed rats, when compared with unstressed control rats, exhibited a reduction in sucrose preference test (SPT), an indication of anhedonia behavior (Figure 1B). In addition, CUS exposure increased the latency to feed in a novel environment (NSFT), an indication of increased anxiety levels (Figure 1C). Administration of a single dose of ketamine rapidly reversed the CUS-induced behavioral deficits in both the SPT and NSFT (Figure 1B,C). Acute ketamine injection did not affect sucrose preference in nonstressed control rats (Figure S1 in Supplement 1). In addition, there was no difference in home cage feeding conducted immediately following the NSFT (data not shown), indicating that the effects of ketamine were not due to general increase in feeding. In contrast to the rapid actions of ketamine, chronic (21 days) administration of typical antidepressants is required to produce similar behavioral actions in these paradigms (11). The SPT was also conducted at additional time points, with continued exposure to CUS, to determine if the actions of ketamine are transient or sustained. The results demonstrate that a single dose of ketamine produces a long-lasting (up to 7 days) increase in sucrose preference relative to CUS-exposed animals (Figure 1F), a time frame comparable with that reported in clinical studies (4). The performance of CUS and control animals remained consistent across different time points (Table S1 in Supplement 1), indicating that the animals did not habituate to SPT after repeated testing.

We next determined if a selective glutamate NMDA receptor 2B (NR2B) antagonist, Ro 25-6981, produces similar effects in the CUS paradigm. Previous studies have reported that Ro 25-6981 has antidepressant actions in the forced swim and learned helplessness paradigms (7,25), and we have reported that the behavioral and synaptic actions of Ro 25-6981 are dependent on mTOR signaling. In the present study, we show that administration of Ro 25-6981 also rapidly reversed the behavior deficits caused by CUS exposure in both SPT and NSFT (Figure 1D,E). In addition, the actions of Ro 25-6981 in the SPT were long-lasting (up to 7 days) (Figure 1G), similar to the actions of ketamine. A previous study has reported that the behavioral actions of Ro 25-6981 in the FST do not persist as long as ketamine (7). In the present study, the actions of Ro 25-6981 were long-lasting, although there was some reduction at the 7-day time point.

The requirement for activation of mTOR signaling in the actions of ketamine was determined using rapamycin, a selective inhibitor of mTOR. Rapamycin was administered (intracerebroventricular in-

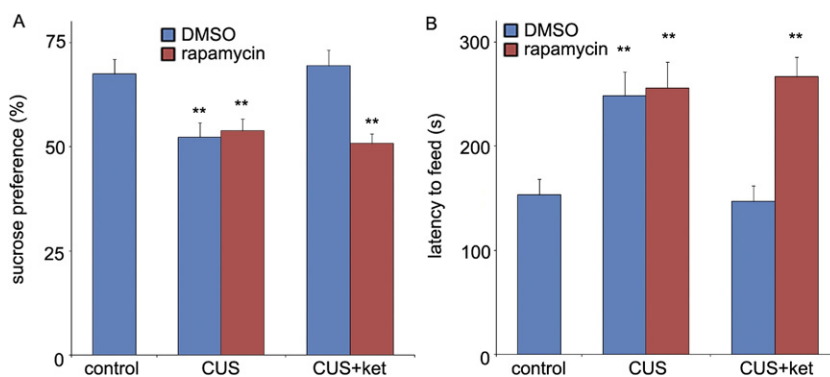


Figure 2. Rapid behavioral actions of ketamine in the chronic unpredictable stress model require mammalian target of rapamycin signaling. On day 21, rapamycin (.2 nmol, intracerebroventricular) or dimethyl sulfoxide was infused 30 minutes before ketamine (10 mg/kg, intraperitoneal) or vehicle treatment. The sucrose preference test was conducted 1 day (A) and the novelty-suppressed feeding test 2 days (B) after drug administration. Pretreatment with rapamycin completely abolished the behavioral actions of ketamine in both the sucrose preference (A) and novelty-suppressed feeding tests. (B). Values represent mean \pm SEM ($n = 6$ per group; $**p < .01$, analysis of variance). CUS, chronic unpredictable stress; DMSO, dimethyl sulfoxide; ket, ketamine.

fusion) 30 minutes before ketamine injection and behavioral testing was conducted the following day. Once again, ketamine completely reversed the behavioral deficits in the SPT and NSFT resulting from exposure to CUS (Figure 2A,B), and these effects were completely blocked by rapamycin pretreatment (Figure 2A,B). A single dose of rapamycin infusion alone, in the absence of ketamine and in stressed rats, had no effect in either test (Figure 2A,B).

Ketamine Rapidly Reverses the Deficit in Synaptic Proteins Resulting from CUS: Requirement for mTOR Signaling

Chronic stress paradigms have been demonstrated to profoundly alter brain structure and function in rodents, causing atrophy of pyramidal neurons in the PFC and the hippocampus (12,13,15–18,20,32). Studies were conducted to determine if our CUS paradigm results in alterations of synapse-associated proteins, as well as the number and function of spine synapses, and if ketamine can reverse these effects. Chronic unpredictable stress exposure (21 days) decreased levels of several well-characterized synaptic proteins in synaptoneurosome preparations of PFC (Figure 3). This included decreased levels of the presynaptic protein synapsin I and the postsynaptic proteins GluR1 and PSD95. Conversely, a single dose of ketamine administered to animals after 21 days of CUS exposure completely reversed the deficit of these synaptic proteins (Figure 3). Moreover, the ability of ketamine to reverse the deficit in synapsin I, GluR1, and PSD95 was blocked by infusion (intracerebroventricular) of rapamycin (Figure 3). Further analysis in another cohort shows that 7 days after drug treatments, ketamine still reversed CUS-induced deficits of synaptic protein expression (Figure S2 in Supplement 1), consistent with the behavioral actions of ketamine in the SPT (Figure 1).

Ketamine Rapidly Reverses the Deficit in Spine Density Resulting from CUS

A decrease in synapse-associated proteins indicates that CUS impairs synapse/spine formation and function and that ketamine reverses this deficit. To directly test this possibility, we examined the influence of CUS on spine number by two-photon laser imaging of the apical tuft of prelabeled layer V medial prefrontal cortex pyramidal neurons. The results demonstrate that CUS exposure significantly decreases spine density in both distal and proximal segments of the apical tuft 24 hours after the last stressor (Figure 4A). Administration of ketamine to CUS-exposed animals completely reversed the spine deficit on both the proximal and distal

apical tuft dendrites (Figure 4A). Further analysis of spine morphology revealed that CUS decreased the population of mature mushroom-like (large diameter, short length) spines, indicating a loss of

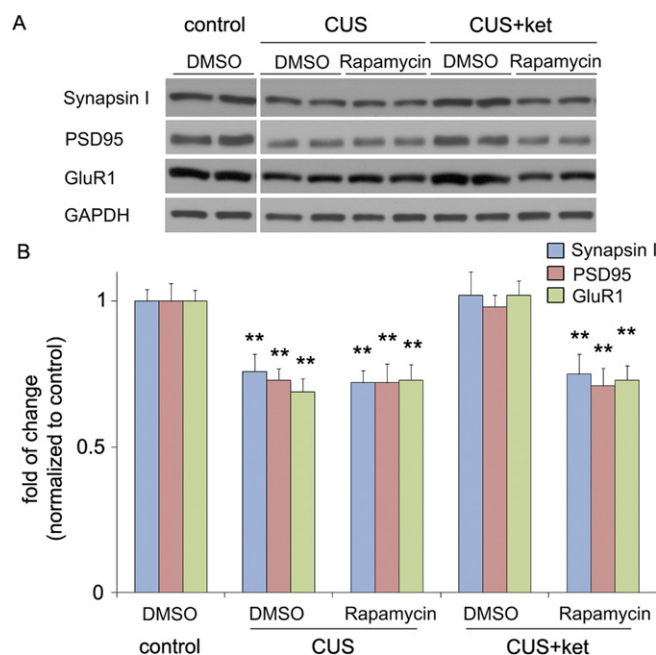


Figure 3. Chronic unpredictable stress exposure decreases synaptic proteins: rapid reversal by ketamine. Rats were exposed to chronic unpredictable stress and on day 21 were infused with vehicle or rapamycin (.2 nmol, intracerebroventricular) 30 minutes before ketamine (10 mg/kg, intraperitoneal) or vehicle treatment. Levels of synaptic proteins in prefrontal cortex synaptoneurosome were determined by western blot 2 days later. (A) Representative western blot images of synapsin I, glutamate receptor 1, and postsynaptic density protein 95 are shown, and (B) results were quantified and are the mean \pm SEM, percent of control ($n = 6$ animals; $**p < .01$, analysis of variance). Levels of glyceraldehyde-3-phosphate dehydrogenase were determined to control for differences in amounts of protein loading. Chronic unpredictable stress decreased expression of synapsin I, postsynaptic density protein 95, and glutamate receptor 1, and this deficit was completely reversed by a single dose of ketamine. Pretreatment with rapamycin completely abolished the effects of ketamine, but rapamycin infusion alone did not affect synaptic protein levels. CUS, chronic unpredictable stress; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GluR1, glutamate receptor 1; ket, ketamine; PSD95, postsynaptic density protein 95.

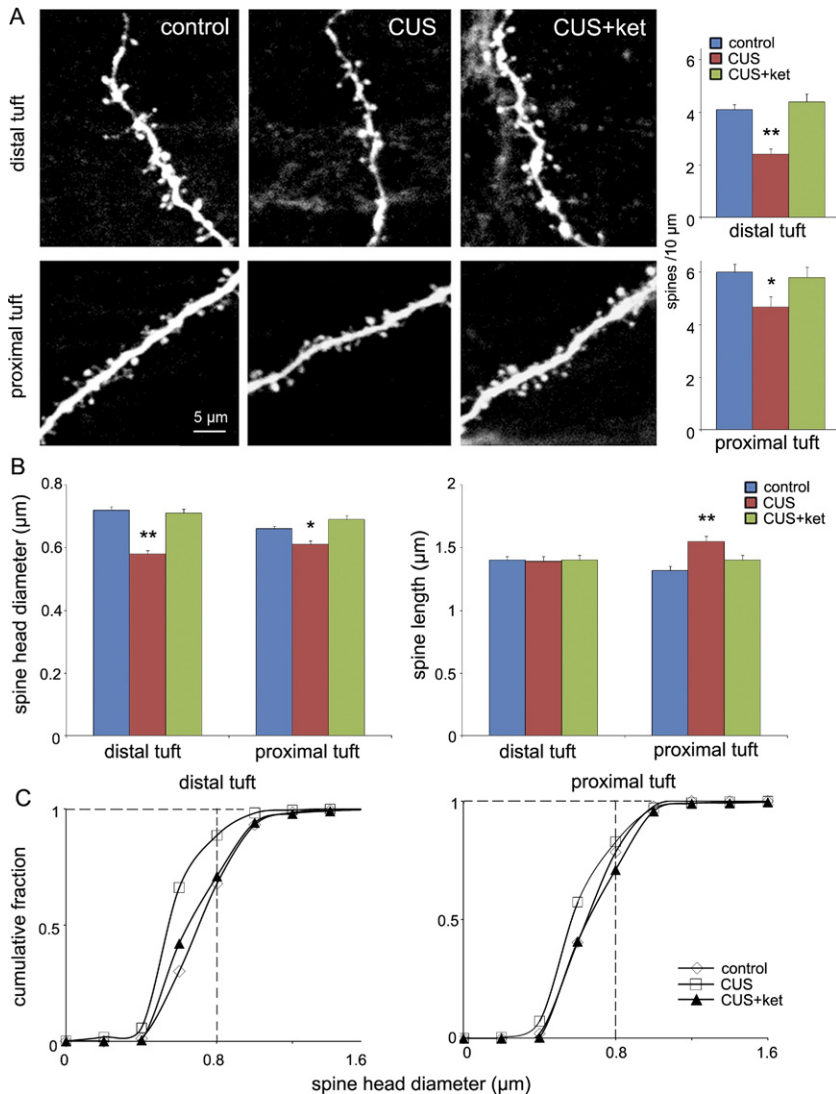


Figure 4. Chronic unpredictable stress (CUS) exposure decreases spine density in prefrontal cortex layer V pyramidal cells: rapid reversal by ketamine. Animals were exposed to CUS for 21 days and then received ketamine injections (10 mg/kg, intraperitoneal). Twenty-four hours later, slices of prefrontal cortex were prepared for whole-cell recordings followed by neurobiotin labeling and post hoc two-photon microscopy image of the neurobiotin-labeled layer V pyramidal cells. **(A)** Representative images are shown of high magnification Z-stack projections of distal and proximal segments of the layer V pyramidal cell apical tuft dendrites (scale: 5 μm). The density of spines was analyzed using Neurolucida Explorer (version 9; MBF Bioscience, Williston, Vermont) and the results are the mean ± SEM (~12 cells from four rats in each group; * $p < .05$; ** $p < .01$, analysis of variance). Chronic unpredictable stress decreased spine density of both distal and proximal segments of the apical tuft, and this deficit was completely reversed by ketamine treatment (bar graphs to right of images). **(B)** Quantification of distal and proximal spine head diameter and spine length. **(C)** Cumulative fraction curves for distal and proximal tuft spine diameter; note large decrease in population of large diameter, mushroom spines (> .8 μm; vertical dashed line) in the CUS group compared with other groups in the distal tuft; stress-induced changes are less pronounced in the proximal tuft, consistent with previous studies (13). ket, ketamine.

mature spines and synaptic connections. All of these deficits in spine density and morphology resulting from 21 days of CUS exposure were reversed rapidly and completely by a single dose of ketamine (Figure 4B,C).

Ketamine Rapidly Reverses the Deficit in Synaptic Functions Resulting from CUS

Electrophysiological analysis of the same cells demonstrates that the frequency/amplitude of excitatory postsynaptic currents (EPSCs) induced by serotonin (5-HT) and/or hypocretin/orexin are decreased by CUS (Figure 5). These functional deficits are consistent with the drastic reduction in large diameter (> .8 μm) mushroom spines caused by CUS exposure. Once again, a single dose of ketamine completely reversed the deficit of both the 5-HT- and hypocretin-induced EPSCs caused by 21 days of CUS exposure (Figure 5).

Discussion

Glutamatergic NMDA receptor antagonists, notably ketamine, have emerged as promising candidates for next-generation fast-acting antidepressants (33). The results of the present study demonstrate that a single dose of ketamine completely reverses the behavioral deficits caused by long-term exposure to CUS. Similar effects were observed with a selective NR2B antagonist, Ro 25-6981, which also

rapidly reversed the anhedonic and anxiogenic behaviors resulting from CUS. This is consistent with a recent report that administration of a selective NR2B receptor antagonist, CP-101606, produces rapid antidepressant effects in depressed patients (34). Reversal of the CUS effects by ketamine and Ro 25-6981 are sustained for up to 7 days, similar to the time course for the therapeutic actions of ketamine (3,35,36). Although previous studies have reported antidepressant actions of ketamine in models that are responsive to acute or subchronic administration of typical antidepressant agents (7,8,10), the current study provides a rigorous test of the rapid actions of NMDA antagonists in a CUS paradigm that requires chronic (21 days) antidepressant treatment (11,37). These findings differ from a recent negative CUS study (38), although the latter measured consumption instead of preference, which is generally considered a better measure of anhedonic behavior.

We recently reported that ketamine increases synaptic protein levels and produces antidepressant behavioral actions in non-stressed naive animals and that these effects are blocked by rapamycin, a selective inhibitor of mTOR (25). Similarly, we show here that ketamine reversal of the behavioral deficits caused by CUS is blocked by rapamycin, indicating that the effects of ketamine are mTOR-dependent alterations in spine/synapse. In support of this hypothesis, we found that CUS exposure decreased levels of synapsin I, GluR1, and PSD95, effects that were rapidly reversed by ket-

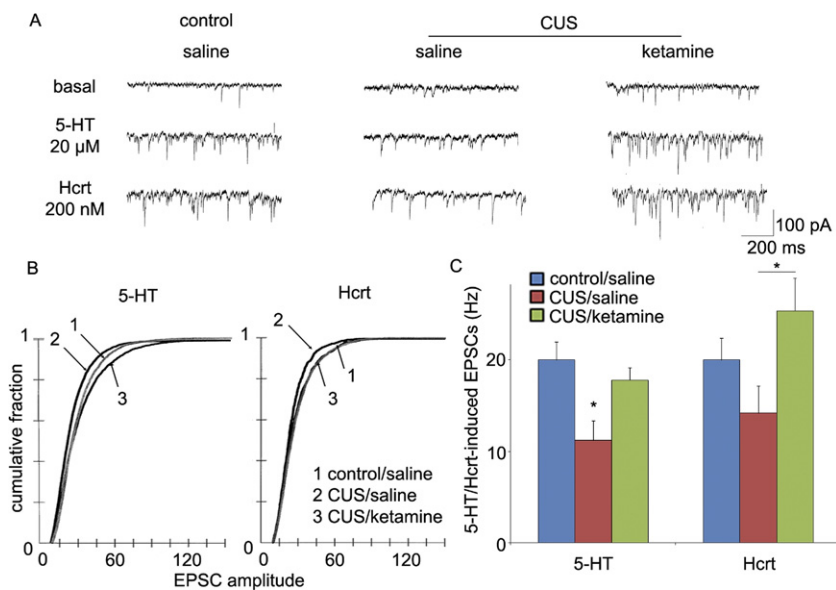


Figure 5. Chronic unpredictable stress (CUS) exposure decreases excitatory postsynaptic current (EPSC) responses in prefrontal cortex layer V pyramidal cells: rapid reversal by ketamine. **(A)** Sample whole-cell voltage-clamp traces of serotonin (5-HT) and hypocretin-induced EPSCs in slices from nonstressed control, CUS, or CUS + ketamine rats are shown (24 hours post drug treatment). **(B)** Frequency of 5-HT- and hypocretin-induced EPSCs and **(C)** cumulative probability distributions showing that CUS exposure causes decreases in amplitude ($p < .0001$ for 5-HT, Kolmogorov-Smirnov test- z value = 3.89; $p < .05$ for hypocretin, Kolmogorov-Smirnov test- z value = 1.92) ($n = 12$ neurons/group; * $p < .05$, analysis of variance). These deficits were completely reversed by a single dose of ketamine **(B, C)**. Hcrt, hypocretin.

amine in a rapamycin-dependent manner. The requirement for mTOR signaling is consistent with our recent findings that ketamine directly activates several components of the mTOR pathway (25). Another study has reported that rapamycin paradoxically produces an antidepressant response (39), although rapamycin was administered systemically, which could influence mTOR signaling and other kinases in peripheral tissues that could indirectly impact behavior. In addition, the latter article only used the forced swim and tail suspension, which are typically used as rapid drug screens and have limited validity as measures of depressive behavior.

We examined the possibility that mTOR inhibition underlies the decrease in synaptic proteins caused by CUS but found no significant differences in levels of phosphorylated/activated mTOR, 4E-Binding Protein 1, and p70S6 kinase (Figure S3 in Supplement 1). It is possible that there is a complex time course for stress regulation of mTOR signaling (e.g., S6 kinase is upregulated by exposure to acute restraint stress) (40). Alternatively, the decrease in synaptic proteins could be mediated by other mechanisms, including increased degradation or via other signaling pathways upstream of mTOR (e.g., neurotrophic factor stimulated kinases are decreased by exposure to repeated stress or corticosterone) (41–45). Further studies of these and other pathways are currently being conducted to characterize the mechanisms underlying the neuronal atrophy caused by chronic stress exposure.

Decreased levels of synaptic proteins resulting from CUS is consistent with previous studies demonstrating that chronic stress causes dendritic atrophy of neurons in limbic brain regions, including the PFC (14–19). This possibility is confirmed in the present study, which shows that CUS decreases the density of apical spines in layer V pyramidal neurons of the PFC. Notably, CUS caused a marked reduction in the population of large-diameter mushroom spines, which correlates with a significant reduction in the amplitude as well as frequency of both 5-HT- and hypocretin-induced EPSCs. These transmitter-induced synaptic currents involve both cortical-cortical (5-HT) and thalamocortical (hypocretin) synaptic inputs (13), indicating that diverse pathways are affected. Administration of ketamine rapidly and completely reversed these deficits in spine density and function, indicating a causal relationship between the morphological and physiological responses. However, it is possible that the 5-HT- and hypocretin-induced excitatory postsynaptic potentials reflect only a functional change in synaptic

efficacy and further studies of mini excitatory postsynaptic potentials, measured under depolarization blockade, may be useful to directly test this causal interaction.

Dendritic atrophy of PFC neurons in response to chronic CUS is consistent with previous reports with other paradigms, such as chronic restraint stress (15–19), and provides a cellular mechanism that could explain the decreased PFC volume reported in depressed patients (20–23). Neuronal atrophy and loss of spines/synapses in the PFC could underlie the behavioral deficits observed in depressed patients (46), including reduced cognitive functions and loss of inhibitory control of emotions, perhaps mediated by amygdala circuits (47,48).

The ability of ketamine to increase synaptogenesis represents a fundamental, conceptual frame shift in our understanding of synaptic plasticity and the treatment of depression. First, the results demonstrate a basic characteristic of brain plasticity, the ability to rapidly stimulate the formation and function of new spines/synapses. Second, the findings show that the dendritic atrophy resulting from long-term stress exposure is reversible. This also implies that the structural deficits caused by and implicated in the pathophysiology of mood disorders, including treatment-resistant depression, are reversible and that the rapid actions of ketamine are mediated by increased synaptogenesis. Brain imaging studies that directly or indirectly measure synaptogenesis and/or glutamate transmission will be required to directly test this hypothesis in depressed patients.

In summary, the results provide direct evidence that NMDA receptor antagonists rapidly reverse the behavioral, morphological, and physiological deficits resulting from chronic stress via a rapamycin-sensitive, mTOR pathway. These findings, together with earlier studies, provide promising targets for development of next-generation fast-acting antidepressant medications that are safer than ketamine. This possibility is supported by the finding that an NR2B selective antagonist also produces rapid antidepressant actions in the CUS model (present study), as well as other paradigms (7,25), and increases mTOR-dependent synaptogenesis (25). Studies are underway to test additional targets in the glutamate-mTOR signaling pathway that could also be developed as rapid-acting antidepressants.

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Supplementary material cited in this article is available online.

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