Scopolamine Rapidly Increases Mammalian Target of Rapamycin Complex 1 Signaling, Synaptogenesis, and Antidepressant Behavioral Responses

Bhavya Voleti, Andrea Navarria, Rong-Jian Liu, Mounira Banasr, Nanxin Li, Rose Terwilliger, Gerard Sanacora, Tore Eid, George Aghajanian, and Ronald S. Duman

Background: Clinical studies report that scopolamine, an acetylcholine muscarinic receptor antagonist, produces rapid antidepressant effects in depressed patients, but the mechanisms underlying the therapeutic response have not been determined. The present study examines the role of the mammalian target of rapamycin complex 1 (mTORC1) and synaptogenesis, which have been implicated in the rapid actions of *N*-methyl-D-aspartate receptor antagonists.

Methods: The influence of scopolamine on mTORC1 signaling was determined by analysis of the phosphorylated and activated forms of mTORC1 signaling proteins in the prefrontal cortex (PFC). The numbers and function of spine synapses were analyzed by whole cell patch clamp recording and two-photon image analysis of PFC neurons. The actions of scopolamine were examined in the forced swim test in the absence or presence of selective mTORC1 and glutamate receptor inhibitors.

Results: The results demonstrate that a single, low dose of scopolamine rapidly increases mTORC1 signaling and the number and function of spine synapses in layer V pyramidal neurons in the PFC. Scopolamine administration also produces an antidepressant response in the forced swim test that is blocked by pretreatment with the mTORC1 inhibitor or by a glutamate alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor antagonist.

Conclusions: Taken together, the results demonstrate that the antidepressant actions of scopolamine require mTORC1 signaling and are associated with increased glutamate transmission, and synaptogenesis, similar to *N*-methyl-D-aspartate receptor antagonists. These findings provide novel targets for safer and more efficacious rapid-acting antidepressant agents.

Key Words: Acetylcholine, depression, GABA, glutamate, ketamine, synaptic plasticity

Depressive illness affects more than 15% of the population and results in enormous personal and socioeconomic consequences (1). Moreover, currently available medications that have been designed to block the reuptake or breakdown of monoamines have significant limitations, including low response rates (approximately one-third of patients achieve remission with the first prescribed antidepressant) and a therapeutic time lag of weeks to months (2). Clinical studies have identified two drug classes with rapid antidepressant actions in depressed patients: ketamine, a noncompetitive glutamate *N*-methyl-D-aspartate (NMDA) receptor antagonist (3–5), and scopolamine, a nonselective acetylcholine muscarinic receptor antagonist (6–9). The ability of these agents to produce a rapid response by mechanisms completely different from currently available agents represents a paradigm shift in the field of depression.

Recent work has begun to elucidate the molecular and cellular mechanisms underlying the actions of NMDA receptor antagonists. These studies demonstrate that ketamine rapidly stimulates the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and increases synaptogenesis in a rapamycin-sensitive manner in rat prefrontal cortex (PFC) (10,11). Similar effects have been reported for NMDA antagonists that are selective for the NMDA receptor subtype 2B (10) consistent with clinical reports (12). The mTORC1 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 (13). Conversely, a possible role for reduced mTORC1 signaling and synaptogenesis in the pathogenesis of depression is supported by postmortem studies demonstrating that levels of mTORC1 signaling and synaptic proteins are decreased in the PFC of depressed subjects (14). Stimulation of mTORC1-mediated synaptogenesis could represent a mechanism for rapid reversal of the behavioral and synaptic deficits that are caused by exposure to stress and have been implicated in depression (11).

The current study was undertaken to determine whether scopolamine rapidly increases mTORC1 signaling and synaptogenesis in the PFC and whether the behavioral actions of this muscarinic receptor antagonist require stimulation of mTORC1 signaling. The results demonstrate that scopolamine rapidly activates mTORC1 signaling, increases the number and function of spine synapses in the PFC, and produces mTORC1 dependent behavioral effects, similar to NMDA receptor antagonists.

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.

From the Laboratory of Molecular Psychiatry, Center for Genes and Behavior, Departments of Psychiatry, Neurobiology, and Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut.
Authors BV and AN contributed equally to this work.
Address correspondence to Ronald S. Duman, Ph.D., 34 Park Street, New Haven, CT 06520; E-mail: ronald.duman@yale.edu.
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Drug Administration and Surgical Procedure

Animals received a single acute injection of vehicle, scopolamine (intraperitoneal [IP]) or the preferential M1 selective antagonist telenzepine (subcutaneous). Tissue was collected from separate groups of animals for molecular or electrophysiologic studies, and separate cohorts were also used in behavioral paradigms or microdialysis experiments as described subsequently. For experiments involving central administration of rapamycin, rats were implanted with intracerebral ventricular (ICV) guide cannula under Nembutal anesthesia (IP 55 mg/kg) as previously reported (15,16). After recovery for 7 days, rapamycin (.2 nmol in 2 μL) or a vehicle (dimethyl sulfoxide) was delivered at the rate of .25 μL per minute 30 minutes before scopolamine injections. This dose of rapamycin is based on previous reports demonstrating effective and selective inhibition of the mTORC1 signaling (15,16).

Immunoblotting

For analysis of mTORC1 signaling synaptoneurosomes were prepared and Western blotting for the phosphorylated forms of mTORC1 signaling proteins, as well as upstream kinases was conducted as previously described (16). The primary antibodies used for both phosphorylated and total proteins were phospho-mTORC1 (Ser2448), mTORC1, total p70 S6 kinase (S6K) (Thr389), phospho-S6K, total extracellular-signal regulated kinase (ERK), phospho-ERK (Thr202/Tyr204), total protein kinase B (PKB or Akt), phospho-Akt (all from Cell Signaling, Boston, Massachusetts), GluR1 (Abcam, Cambridge, Massachusetts), and glyceraldehyde-3-phosphate dehydrogenase (Advanced Immunochemical, Long Beach, California). Levels of immunoreactive bands were quantified by densitometry using National Institutes of Health Image J software and normalized to the control group for each protein.

Brain Slice Preparation and Electrophysiologic Recordings

Brain slices were prepared as previously described (16,17). Briefly, 1 day after scopolamine treatment, rats were anesthetized (chloral hydrate, 400 mg/kg, IP), and brains were removed. Coronal slices 400 μm thick were cut from a block of tissue containing the medial PFC, placed in a submerged recording chamber at 32°C in standard artificial cerebrospinal fluid (pH 7.35). There was recovery period of 1 to 2 hours before recording.

Pyramidal neurons in layer V were patched under visual control using a microscope (60× IR lens; Olympus, Center Valley, Pennsylvania) with infrared differential interference contrast microscopy. The pipette solution contained the following: 115 mmol/mL K gluconate, 5 mmol/mL KCl, 2 mmol/mL $MgCl_2$, 2 mmol/mL Mg-adenosine-5′-triphosphate, 2 mmol/mL Na_2adenosine-5′-triphosphate, 10 mmol/mL Na_2-phosphocreatine, .4 mmol/mL Na_2GTP, and 10 mmol/mL 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.33. Neurobiotin (.3%) was added to the pipette solution to mark cells for later processing and imaging.

Whole-cell recordings were made 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 MΩ. To minimize series resistance errors, cells were discarded if series resistance rose above 10 MΩ. Postsynaptic currents were studied in the continuous single-electrode voltage-clamp mode (3000 Hz low-pass filter) clamped near resting potential (75 ± 5 mV). Known concentrations of drugs in artificial cerebrospinal fluid were applied through a stopcock arrangement (~4 mL/min) to reach the slice within 7 to 10 seconds.

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 the fluorophore Alexa 594 (1:1000) for visualization of labeled cells; this procedure enabled visualization of even the most distant spines in the apical tuft. Labeled neurons within layer V of anterior cingulate and prelimbic medial PFC were imaged with a two-photon Ti:sapphire laser scanning system (810 nanometers; Mai Tai, Spectra Physics, Mountain View, California) coupled to direct detection Radiance 2000 BioRad laser scanner (Zeiss Micromaging, Thornwood, New York) mounted on a Olympus BX50WI microscope, using a 60× (.9 numerical aperture) water-immersion objective. For spine density analysis, Z-stacks usually consisted of two to five scans at high zoom at 1-μm 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 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 (18). Spine density and diameter were analyzed by automated Neuroleucida software (Autoneuron/Autospine; MBF Bioscience, Williston Vermont). Density results were expressed in terms of spine density per 10 μm.

Behavioral Analysis: Forced Swim Test

Behavioral responses in the forced swim test (FST) were conducted as previously described (16). Twenty-four hours before the treatment with scopolamine, rats were placed for 15 minutes in a clear cylinder with water (24 ± 1°C, 45 cm deep). Rats were administered scopolamine and 24 hours later were tested for immobilization in the FST. In the blocking studies, rapamycin was administered (.2 nmol in 2 μL, ICV) or 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) was administered (10 mg/kg, IP) 30 minutes before scopolamine. Video-recorded sessions were scored for total immobility time during the first 5 minutes by a blinded experimenter. Brains were collected and cannula placement was determined by histology; rats with incorrect placement were excluded. Immobility values were analyzed using a one-way analysis of variance with least significant difference post hoc tests as appropriate. Significance was determined at $p < .05$, and data were plotted as total seconds immobile.

Microdialysis and Glutamate Measurement

Rats were anaesthetized with pentobarbital (55 mg/kg, IP) and cannula guides CMA/11 (Harvard Apparatus, Holliston, Massachusetts) were stereotaxically implanted in the prefrontal cortex (anteroposterior +3.5 mm and lateral +.7 mm from bregma; dorsoventral 2 mm from dura). After handling daily for 7 days to reduce variability and decrease injection stress, the influence of scopolamine and ketamine administration on glutamate levels in freely moving rats was determined. Microdialysis probes (CMA/11, Harvard Apparatus; 2 mm length of the microdialysis membrane; molecular weight cutoff, 20 kDa) were lowered through the guide cannula so that the tip of the dialyzing membrane reached dorsoventral 4 mm from dura). Perfusion solution (Harvard Apparatus) was applied at a constant flow rate (1 μL/min) using a micropump (Harvard Apparatus). Effluents were collected every 10 minutes and were immediately frozen at −80°C. Sample collection started 90 minutes after the onset of perfusion to achieve stable conditions and was then collected every 10 minutes for 60 minutes before the injection of either ketamine (10 mg/kg, IP) or scopolamine (25 μg/kg, IP). Sampling was carried