

RG3487, a Novel Nicotinic $\alpha 7$ Receptor Partial Agonist, Improves Cognition and Sensorimotor Gating in Rodents

Tanya L. Wallace, Patrick M. Callahan, Ashok Tehim, Daniel Bertrand, Geoffrey Tombaugh, Shaojie Wang, Walter Xie, Wayne B. Rowe, Voon Ong, Elizabeth Graham, Alvin V. Terry, Jr., Joshua S. Rodefer, Brian Herbert, Michael Murray, Richard Porter, Luca Santarelli, David A. Lowe

CNS Discovery Research, Roche Palo Alto LLC, 3431 Hillview Avenue, Palo Alto, CA (*T.L.W.*); Memory Pharmaceuticals Corp., 100 Philips Parkway, Montvale NJ (*P.M.C.*, *A.T.*, *G.T.*, *S.W.*, *W.X.*, *W.B.R.*, *V.O.*, *E.G.*, *B.H.*, *M.M.*, *D.A.L.*); HiQ Screening Sàrl, Geneva Switzerland (*D.B.*); Medical College of Georgia, Augusta GA (*A.T.*); University of Iowa, Iowa City IA (*J.S.R.*); CNS Clinical Research and Early Development F. Hoffmann LaRoche Ltd, Basel Switzerland (*R.P.*, *L.S.*).

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Corresponding Author:

Tanya L. Wallace, Ph.D.

SRI International

333 Ravenswood Avenue, M/S 100-69

Menlo Park, CA 94025

650-859-6041 tel

650-859-3153 fax

tanya.wallace@sri.com

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Abbreviations: NOR, Novel Object Recognition; MWM, Morris Water Maze; PPI, Prepulse Inhibition of Startle; MED, minimally effective dose; EC₅₀, 50% Effective Concentration; Serotonin 3, 5-HT₃; alpha 7 nicotinic acetylcholine receptor (α 7nAChR)

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Abstract

Neuronal nicotinic alpha 7 acetylcholine receptors ($\alpha 7$ nAChR) are expressed primarily in the brain and are implicated in modulating many cognitive functions (e.g., attention, working and episodic memory). Not surprisingly, much effort has been committed to the development of molecules acting at $\alpha 7$ nAChRs as potential therapies for a variety of CNS diseases (e.g., Alzheimer's). RG3487, N-[(3S)-1-azabicyclo[2.2.2]oct-3-yl]-1H-indazole-3-carboxamide hydrochloride ($C_{15}H_{19}ClN_4O$), binds potently to the human $\alpha 7$ nAChR ($K_i = 6$ nM), in which it acts as a partial agonist (63-69% of acetylcholine) as assessed by whole cell patch-clamp recordings in both oocytes and QM7 cell lines, respectively. RG3487 activates human $\alpha 7$ nAChRs with an $EC_{50} = 0.8$ μ M (oocytes) and 7.7 μ M (QM7 cells). RG3487 also exhibits antagonist properties at the serotonin 3 receptor (5-HT₃R; $IC_{50} = 2.8$ nM [oocytes], 32.7 nM [N1E-115 cells]). In vivo, RG3487 improved object recognition memory in rats following acute (minimally effective dose [MED]: 1.0 mg/kg, po) or repeated (10 day) administration at brain and plasma concentrations in the low nM range. Spatial learning deficits in age-impaired rats were reversed following RG3487 administration (MED: 0.03 mg/kg, ip) as evaluated in the Morris water-maze task. Using the prepulse inhibition (PPI) of startle model of sensorimotor gating, RG3487 improved apomorphine-induced deficits in PPI performance (MED: 0.03 mg/kg, ip), and reversed PCP-induced impairments in an attentional set shifting model of executive function (MED: ≤ 0.03 mg/kg, ip). Cumulative evidence from these studies indicates RG3487 is a novel and potent $\alpha 7$ nAChR partial agonist that improves cognitive performance and sensorimotor gating.

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Introduction

Nicotine is capable of improving cognitive processes, including attention, working memory and long-term memory, across a variety of species (Newhouse et al., 1997; Rezvani and Levin, 2001; Pichat et al., 2007). The $\alpha 7$ and $\alpha 4\beta 2$ nicotinic acetylcholine receptor (nAChR) subtypes are the most prominent in the central nervous system and are believed to mediate the pro-cognitive properties of nicotine. Moreover, nAChRs have been implicated in disease states characterized by cognitive impairments, including Alzheimer's, schizophrenia and attention deficit hyperactivity disorder, suggesting a potential role of the nicotinic system in disease pathology (Rezvani and Levin, 2001). Thus, much recent attention has been devoted to exploring the potential for novel therapies directed towards nAChRs (Bencherif and Schmitt, 2002).

Nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of ligand-gated receptors that include GABA_A, glycine, and serotonin-3 (5-HT₃) receptors (Gotti et al., 2006; Dani and Bertrand, 2007). The structural and functional diversity of the nAChRs derives from nine α and three β subunits that combine to form three main heteropentameric receptors ($\alpha 4\beta 2$, $\alpha 6\alpha 4\beta 2\beta 3$, $\alpha 6\beta 2\beta 3$), and one homopentameric receptor ($\alpha 7$). The $\alpha 7$ nAChR is the only known homopentameric receptor in the mammalian brain, with five identical ACh binding sites located at the interface between each subunit. In addition, recent evidence has demonstrated that the $\alpha 7$ subunit can combine with the $\beta 2$ subunit *in vitro* and *in vivo* (Liu et al., 2009).

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In particular, the $\alpha 7$ nAChR is an attractive target for cognitive impairing disorders in that this receptor subtype is highly expressed in key brain regions involved with cognitive processing (e.g., hippocampus, cortex), with limited peripheral expression (Levin and Rezvani, 2000; Gotti et al., 2006), and activation of the $\alpha 7$ nAChR has been shown to improve cognitive function. For example, RG3487 has been reported to improve sustained attention in rodents following acute administration (Rezvani et al., 2009). The $\alpha 7$ nAChR is primarily characterized by its high permeability to calcium ions and its rapid desensitization upon agonist binding (Seguela et al., 1993). Localization of the $\alpha 7$ nAChR is both pre- and post-synaptic and this receptor subtype is involved in numerous processes including modulation of neurotransmitter release, regulation of postsynaptic excitability, long-term potentiation and cognitive function. Nicotine has been shown to enhance excitatory synaptic neurotransmission through activation of presynaptic $\alpha 7$ nAChRs (McGehee et al., 1995) and these data have been corroborated more recently by the use of selective $\alpha 7$ nAChR agonists (e.g., SSR180711) (Pichat et al., 2007). In addition, activation of $\alpha 7$ nAChRs by A-582941 has been shown to enhance ERK_{1/2} and CREB phosphorylation, both important post-synaptic mediators of long-term memory (Bitner et al., 2007), which may contribute to the pro-cognitive properties of the $\alpha 7$ nAChR selective agonists.

The $\alpha 7$ nAChRs, rather than high-affinity nicotinic or muscarinic cholinergic receptors, are central to the function of auditory sensory gating, in which schizophrenic patients show marked deficits (Martin et al., 2004). Activation of $\alpha 7$ nAChRs has also been shown to increase GABAergic neurotransmission, which is hypothesized to restore sensory gating deficits

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associated with schizophrenia (Hajos et al., 2005). In addition, abnormal P50 suppression has been linked to genetic markers at the locus of the $\alpha 7$ nAChR subunit gene on chromosome 15q13-14, and the $\alpha 7$ nAChR expression is reduced in postmortem brain tissue from schizophrenic patients. In agreement with these hypotheses, administration of $\alpha 7$ nAChR agents (e.g., tropisetron) and the non-selective nicotinic agonist, nicotine, reverse the P50 auditory gating deficit observed in schizophrenic patients (Simosky et al., 2002). More recently, evidence has emerged that $\alpha 7$ nAChR agonists may be therapeutic for cognitive deficits in schizophrenia. Administration of the $\alpha 7$ nAChR partial agonist, GTS-21, to nonsmoking schizophrenic patients significantly improved cognitive performance in the Repeatable Battery for Assessment of Neuropsychological Status (RBANS) test, and it normalized the P50 auditory evoked potential (Olincy et al., 2006). However GTS-21, as well as RG3487, did not reverse cognitive impairments in schizophrenics using the MATRICS scale (Freedman et al., 2008); (Umbricht, 2009). Thus, it remains to be elucidated whether $\alpha 7$ nAChR agonists may improve cognitive deficits in these patients.

Overall, there is a large unmet medical need for safe and efficacious treatments for cognitive disorders (e.g., Alzheimer's). The studies in this paper describe the pharmacological characterization of RG3487 (formerly known as MEM3454), a novel partial agonist of the $\alpha 7$ nAChR with 5-HT₃ receptor antagonist properties currently being evaluated for the treatment of cognitive impairments in human disease.

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Methods

Receptor rat brain membrane preparations: Rat brains (Pel-Freez Biologicals, 56005-2) were placed into ice-cold 0.32 M sucrose containing protease inhibitor (Protease Inhibitor Cocktail Tablets, Roche Cat# 11697498001, one tablet per 50 ml). The tissue was homogenized and centrifuged for 10 min at 1,000 x g, 4°C. The supernatant was centrifuged at 20,000 x g at 4°C. The pellet was then homogenized in ice-cold water for 10 sec, centrifuged for 30 min at 39,000 x g at 4°C. The last two steps were repeated twice and the remaining pellet was re-suspended in binding buffer (200 mM TRIS-HCl, 20 mM HEPES, pH 7.5, 144 mM NaCl, 1.5 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 0.1% (w/v) BSA). The protein concentration was adjusted to approximately 2.5 mg/ml. The membrane preparation was stored at -80°C.

Receptor saturation and competition binding studies: Saturation binding for $\alpha 7$ nAChR was carried out by incubating 200 μ g membrane protein with increasing concentrations of the selective $\alpha 7$ nAChR antagonist, [³H]-methyllycaconitine (MLA; 0.02-20 nM), in a final volume of 200 μ l binding buffer for 2 hours at room temperature. Competition binding assays were performed by incubating 200 μ g membrane proteins with 1 nM-10 μ M RG3487 dissolved in buffer (200 mM TRIS-HCl, 20 mM HEPES, pH 7.5, 144 mM NaCl, 1.5 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 0.1% (w/v) BSA) and 2 nM [³H]-MLA for 2 hours at room temperature. The nonspecific binding was defined by 10 μ M MLA.

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Saturation binding for the $\alpha 4\beta 2$ nAChR was carried out by incubating 300 μ g membrane protein with increasing concentration of the $\alpha 4\beta 2$ nAChR partial agonist, [3 H]-cytisine (0.02-30 nM), in a final volume of 1000 μ l binding buffer for 75 minutes at 4°C. Competition binding assays were performed by incubating 300 μ g membrane proteins with 1 nM-100 μ M RG3487 dissolved in buffer (50 mM TRIS-HCl, pH 7.4, 120 mM NaCl, 5.0 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂) and 1 nM [3 H]-cytisine for 75 min at at 4°C. The non-specific binding was defined by 1 mM nicotine.

Receptor binding for 5-HT₃ was assessed using human recombinant 5-HT₃ receptors expressed in HEK293 cells using standard transfection methods. RG3487 was tested to displace [3 H]-BRL-43694 (Granisetron; 2 nM), a 5-HT₃R selective antagonist, in rat brain cortical membranes for 60 minutes at room temperature. Competition binding assays were performed by incubating 2 μ g membrane protein with 0.001-100 μ M of RG3487 dissolved in buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA) and 2 nM [3 H]-BRL-43694 for 60 minutes at room temperature. MDL72222, a 5-HT₃R selective antagonist, was used to assess non-specific binding.

For all binding experiment, the binding reaction was terminated by vacuum filtration onto Filtermat A filter presoaked with 0.3% PEI using a Tomtec harvester. Following washing with buffer (50 mM Tris-HCl, pH 7.5) and drying in microwave oven, the radioactivity was counted using a Trilux Microbeta counter. All raw data were analyzed with Prism using the three parameter logistic equation.

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FLIPR: The neuroblastoma cells (SK-N-SH, ATCC, HTB-11) expressing $\alpha 7$ nAChR, $\alpha 3\beta 2$ nAChR, $\alpha 3\beta 4$ nAChR and other nAChRs were used in the FLIPR assay to test compound cross reactivity. Cells were seeded overnight in 100 μ l Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum. On the second day, the cells were incubated with 1x HBSS, 20 mM HEPES, 0.1% BSA, 2.5 mM probenecid, and 0.01% Pluronic acid F-127, pH 7.4 buffer in the presence of Fluo-3AM (Invitrogen (Molecular Probe); Carlsbad, CA; F-1241) at 37°C for 60 min. After washing three times to remove extracellular Fluo-3AM, the plates were filled with 100 μ l buffer and then placed onto a FLIPR (Molecular Devices; Sunnyvale, CA). Compound addition was performed by the FLIPR pipetting system. Fluorescence was monitored ($\lambda_{\text{ex}}=488$ nM, $\lambda_{\text{EM}}=540$ nM) for three minutes immediately after the compound addition. The relative fluorescence unit (RFU) was measured as peak fluorescence intensity minus basal fluorescence intensity.

For FLIPR studies with the human rhabdomyosarcoma muscle cells (ATCC No. CCL-136) expressing $\alpha 1\beta 1\gamma\delta$ nAChR the cells were seeded overnight in 100 μ l Dulbecco's modified Eagle's Medium, supplemented with 10% fetal bovine serum. On the second day, the cells were incubated with D-MEM+10%FBS, 20 mM HEPES, 0.1% BSA, 2.5 mM probenecid and 0.01% Pluronic F-127, pH 7.4 buffer in the presence of 4 μ M Fluo-3AM (Molecular Probe) at 37°C for 60 min in 50 ml VWR centrifuge tube. After washing three times with buffer (1x HBSS, 20 mM HEPES, 0.1% BSA, 2.5 mM probenecid Pluronic F-127, pH 7.4) to remove the Fluo-3AM, the cells were seeded into 96 well black wall and clear-bottom plates (Costar) at a density of 100,000

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cells per well in 100 μ l of the same buffer, and then placed onto the FLIPR (Molecular Devices). Compound additions were performed by FLIPR pipetting system. The fluorescence was monitored ($\lambda_{\text{ex}}=488$ nm, $\lambda_{\text{EM}}=540$ nm) for three minutes immediately after the compound addition. RFU was assessed as stated above.

RG3487 receptor binding studies conducted at neurotransmitter receptors, ion channels and enzymes were conducted at CEREP (Redmond, WA) using standard protocols and procedures described on their website (www.cerep.com).

Whole cell patch clamp recordings using Dynaflo: Whole-cell recordings were made at room temperature using fire polished borosilicate recording pipettes (~2–5 M Ω , World Precision Instruments; Sarasota, FL) filled with the following solution (in mM): CsCl (140), NaCl (4), HEPES (10), CaCl₂ (2), MgCl₂ (1), EGTA (5), pH = 7.3). Evoked currents were acquired using P-Clamp 9.0 software with an Axon Multiclamp 700A amplifier, low-pass filtered at 2 kHz and digitized with a Digidata 1322 at 5 kHz. The Dynaflo chip (Celectricon, Inc.; Gaithersburg, MD) was loaded with 80 μ L drug solution per well, grounded and the recording area was filled with ~2 mL extracellular solution (Ringers solution (in mM): NaCl (140), KCl (5), HEPES (10), CaCl₂ (2), MgCl₂ (1), glucose (10) and 1 μ M atropine; pH = 7.3). Cells were dissociated from the growth plate by gently washing the surface with 1mL extracellular solution and then transferred to the Dynaflo chip. Drug delivery was driven by pressure from a syringe pump (12 μ L/min) attached to the Dynaflo chip.

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For $\alpha 7$ nAChR recordings, a QM7 cell line stably expressing the human $\alpha 7$ nAChR was used. QM7 cells were cultured in medium 199, 10% FBS, 0.26% tryptose phosphate, 1% DMSO. During the recordings, cells were held at -70 mV and the holding potential was stepped to -90mV during drug application (1-sec) using the Dynaflo system. The responses were analyzed by evaluating both peak response and total net charge (i.e. integration of the area under the curve). RG3487 agonism was assessed by calculating evoked currents as a percentage of the cell's maximal response to acetylcholine (ACh) (1-3mM).

For 5HT₃R recordings, a N1E-115 cell line stably expressing human 5HT₃Rs was used to record native 5HT₃R currents. Inward 5HT₃R currents were evoked by applying 5-HT (10 μ M) for 1-2 seconds to the cell using the Dynaflo system. Drug antagonism was assessed by co-application with 5-HT. Drugs were prepared from frozen DMSO (\leq 0.1%) for RG3487 or diH₂O for ACh and 5-HT stock solutions immediately before use.

Oocyte procedures: All experiments were carried out on human nAChRs expressed in *Xenopus* oocytes using the method of cDNA expression. *Xenopus* oocytes were prepared and injected using standard procedures as described previously (Hogg et al., 2008). Briefly, ovaries were harvested from *Xenopus laevis* females that were deeply anesthetized. A small piece of ovary was isolated for immediate preparation while the remaining part was placed at 4 °C in a sterile Barth solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.82 mM

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MgSO₄·7H₂O, 0.33 mM Ca(NO₃)₂·4H₂O, 0.41 mM CaCl₂·6H₂O, at pH 7.4, and supplemented with 20 µg/ml of kanamycine, 100 unit/ml penicillin and 100 µg/ml streptomycin. All recordings were performed at 18 °C and cells superfused with OR2 medium containing: 82.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1.8 mM CaCl₂·2H₂O, 1mM MgCl₂·6H₂O, pH 7.4, and 1 µM atropine was added to prevent possible activation of endogenous muscarinic receptors.

Injections of cDNAs encoding for the human $\alpha 7$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\beta 2$ or 5HT_{3A, B}, were performed in at least one hundred oocytes using a proprietary automated injection device (Hogg et al., 2008) and receptor expression was examined at least two days later. Oocytes were penetrated with two electrodes and their membrane potential maintained at -80 mV throughout the experiment using standard voltage clamp procedures.

Oocyte recordings: Currents evoked by ACh or other agonists were recorded using an automated process equipped with standard two-electrode voltage-clamp configuration (TVEC). Unless indicated, cells were held at -80 mV. Data were captured and analyzed using a HiQScreen proprietary data acquisition and analysis software running under Matlab (Mathworks Inc., Natick MA).

ACh was prepared as a concentrated stock solution (10⁻¹ M) in water and then diluted in the recording medium to obtain the desired test concentration. Nicotine was diluted in water as stock solution (10⁻¹ M), kept frozen and diluted immediately prior to the experiment at the desired

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concentration. Compounds were prepared as stock solution (10^{-1} M) in DMSO and then diluted in the recording medium to obtain the desired test concentration. Residual DMSO did not exceed the concentration of 1%, a concentration that has no effect on *Xenopus* oocyte function.

Animals: Novel object recognition and locomotor activity tasks: male, Sprague-Dawley SD adult rats (Hilltop Labs, Scottsdale, PA); Morris water maze test: young adult (3-6 months) and aged (22-24 months), male Fischer F344 rats were obtained from Hilltop Labs (Scottsdale, PA). Attentional set shifting: Male, Long-Evans rats (Harlan Labs, Indianapolis, IN). Prepulse inhibition of startle: Male, albino, Wistar adult rats (Harlan Labs, Indianapolis, IN). All animals were maintained in temperature controlled rooms and all procedures were in compliance with local institutional animal care and use committee and AAALAC guidelines.

Drugs: RG3487 was dissolved in saline (0.9% sodium chloride) or de-ionized water (diH_2O) and was administered intraperitoneally 30-60 minutes before testing, except for the object recognition test, in which it was administered orally 60 minutes prior to training, or intraperitoneally immediately after training (consolidation experiment). Ondansetron was dissolved in diH_2O and administered orally 60 min prior to testing. Methyllycaconitine was dissolved in diH_2O and administered intraperitoneally 60 minutes before training. Apomorphine, dissolved in saline (0.9% sodium chloride), was administered subcutaneously 10 minutes before testing. Haloperidol was dissolved in saline and administered intraperitoneally 30 minutes before testing. Phencyclidine HCl (5.0 mg/ml) was prepared in saline and was administered

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twice daily (8:00 and 20:00) intraperitoneally for 7 days. The test doses and pretreatment times of the compounds were selected based on pharmacokinetic properties of the molecules, previously published scientific literature and/or personal experience of the investigators. All compounds were obtained from Sigma Aldrich (St. Louis MO) except for RG3487 which was synthesized in house (Roche / Memory).

Plasma Concentration Analysis: RG3487 plasma concentrations were determined using a validated assay. Briefly, RG3487 and the internal standard, [$^{13}\text{C}_6$]-RG3487 were extracted by protein precipitation into organic acetonitrile containing [$^{13}\text{C}_6$]-RG3487 using 0.20 mL of plasma. An aliquot of this extract was injected into a high performance liquid chromatography system coupled to a tandem mass spectrometer (LC/MS/MS). The analytes were separated by reverse-phase chromatography and detected using the selected reaction monitoring mode of MS/MS. Quantitation of plasma sample concentration was carried out by the comparison of the ratio of RG3487/[$^{13}\text{C}_6$]-RG3487 response against to form a calibration curve in the range of 0.250 to 512 ng/mL for RG3487 in plasma.

Novel Object Recognition: Prior to training, rats were acclimated to laboratory conditions (e.g., daily handling, weighing) and habituated to the test environment (e.g., placed in an opaque plastic training/testing chamber, 78.7 cm x 39.4 cm x 31.8 cm with bedding on the floor, inside dimly lit (4 lux) room for 10 minutes). During training, rats were placed in the chamber containing two identical objects (either a metal shaped tower [23 cm in height] or a metal conical

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cone with a chrome top [23 cm in height, 7.5 cm in diameter] for a 15 minute session. The objects were placed 14 cm from the sides of the two short walls and 18 cm from the sides of the long walls of the chamber; distance between the two objects was 25 cm. Following a retention delay interval of 48 hrs, animals were tested for recognition memory. In the test session, one object identical to that used in training (familiar) and one new (novel) object were placed in the chamber and the animal was allowed to explore for 5 minutes. The familiar and novel objects as well as chamber position of object were randomly assigned and objects were cleaned between sessions with a 50% ethanol solution to eliminate olfactory cues. Object exploration was scored when the animal directed its nose to the object at a distance of ≤ 2 cm and/or touched it with its nose; climbing or sitting on the object was not considered object exploration behavior. The primary behavioral measure was % time spent investigating the novel object time (seconds) (i.e., novel time / [novel + familiar time])*100.

Age-impaired Morris water maze: The Morris water maze test assesses spatial learning and memory. Briefly, animals were placed in a 1.5 meter diameter circular tank filled with 25 °C water and had 3 training sessions each day for 5 days using spatial cues around the room to locate a hidden escape platform (12 cm diameter) submerged 2 cm under the water. Maximum swim time was 120 seconds and the inter-trial interval was approximately 20 minutes. The start location for each trial was one of 7 different sites, each of which was used once per day. Based upon mean escape latency for all trials on days 3-5, aged rats (22-24 months old) were classified as either impaired (AI) or unimpaired (AU). The scores of AU rats were ≤ 0.5 standard

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deviations from the mean of the response from the young group, while the AI score was ≥ 2.0 standard deviations higher than the response from the young group.

On training day 6, drug or vehicle was administered to AI animals 30 minutes prior to the beginning of training. The same protocol was followed for days 7 and 8, with the last trial on day 8 being a probe trial. On training day 9, all aged rats trained in the visible-platform version of the task to assess visual acuity. In this paradigm the platform remained in the same position as during hidden platform training, but it was elevated 1 cm above the surface of the water and a black cylinder (10 cm high, 2.5 cm diameter) was placed in the center of the platform. The animals received 4 trials of 60 seconds duration, with a 30-second inter-trial interval. If an animal failed to swim to the platform on any trial, it was classified as sensory/motor impaired and was removed from subsequent analysis.

Attentional Set Shifting: The set-shifting task adapted for rodents uses olfactory and tactile stimuli, and experimental methods have been reported in detail previously (Rodefer et al., 2008). Briefly, rats were trained to dig in small bowls filled with sand to retrieve food rewards (Honey Nut Cheerios; General Mills, Minneapolis, MN) using sensory cues (i.e., odor, digging media).

Initially, a simple discrimination (SD) between either two odors or two digging media was presented, followed by a compound discrimination (CD) with the same positive stimulus as the initial SD. In the CD, a new dimension was introduced, but it was not a reliable predictor of the

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location of the food reward. An intra-dimensional shift (IDS) task was then presented; the IDS task was a compound discrimination in which the specific stimuli within both relevant and irrelevant dimensions were changed, but the relevant dimension (either odor or medium) remained the same. The IDS task was then reversed (IDSR), so that what was formerly the negative stimulus was changed to be the positive stimulus, with the irrelevant dimension still not predictive of the location of the reward. Then, the rats were presented with an extra-dimensional shift (EDS) task in which the formerly irrelevant dimension became the relevant one, whereas the originally relevant dimension no longer held predictive value. Finally, the EDS task was reversed (EDSR) such that the formerly negative stimulus became the positively reinforced stimulus. ED shift direction (i.e. odor to medium or medium to odor) was counterbalanced across subjects and was without effect on any of the behavioral variables ($p > 0.05$), so it was not considered in the presentation of results.

Following subchronic PCP injections, rats experienced a washout period of 10 days before beginning habituation and training to the set-shifting procedure. On the day of testing, rats were administered a dose of RG3487 or vehicle 30 min before the test session.

Prepulse inhibition of startle: The prepulse inhibition of startle paradigm has been described in detail previously (Hohnadel et al., 2007). Briefly, trials consisted of a prepulse (20 ms burst of white noise with intensities of 75, 80, or 85 dB) followed 100 ms later, by a startle stimulus (120 dB, 20 ms white noise). Animals were acclimated to test conditions for two days before the

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study began. On the day of drug testing, the rats were placed in the startle chamber (San Diego Instruments, San Diego, CA), and received 12 startle trials, 12 no-stimulus trials, and 12 trials of each of the prepulse/startle trials presented pseudo-randomly for a total of 60 trials. The inter-trial interval ranged from 10 to 30 s, and the total session lasted about 25-30 min. The startle trials consisted of single 120 dB white noise bursts lasting 20 ms. During the no-stimulus trial, no startle noise was presented, but the movement of the rat was scored, and represented a control trial for detecting differences in overall activity. Basal startle amplitude was determined as the mean amplitude of the 12 startle trials. Prepulse inhibition was calculated according to the formula $100 - 100\% \times (PP_x/P120)$, in which PP_x is the mean of the 12 prepulse inhibition trials (i.e., for each individual prepulse level), and $p120$ was the basal startle amplitude. The average level of PPI was also calculated (average of the responses to pp75, pp80, or pp85) and analyzed separately.

Locomotor Activity: Locomotor activity (LMA) was measured using activity chambers (42x42x30 cm; AccuScan Instruments Inc.; Ohio, USA) equipped with infrared photo sensors to measure horizontal and vertical activity. Twenty-four hours prior to testing, animals were brought to the laboratory and acclimated before being weighed, injected with saline (i.p.) and individually placed in the clear Plexiglas open field chamber located in a dimly lit (40 lux) room for 60 minutes of habituation. Approximately 24 hrs later, animals were brought back to the laboratory and acclimated before administration of vehicle or RG3487 (0.1 – 10 mg/kg, ip) in

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which LMA was recorded for 60 min. The primary behavioral measure was total distance traveled in the chamber.

Data Analysis: For most paradigms (NOR, PPI, MWM, LMA), data were analyzed using with a one-way (between group) ANOVA followed by post-hoc comparisons to identify individual differences. All comparisons were made with an experimental type I error rate (α) set at 0.05. The attentional set shifting data analyses were based on previously described methods (Rodefer et al., 2008). Briefly, set-shifting performance across all six phases of task was examined (SD, CD, IDS, IDS-reversal, EDS, and EDS-reversal) between animals treated with subchronic PCP or saline using a series of *a priori* planned contrasts using independent samples *t*-tests. All group variances were evaluated using Levene's Test for Equality of Variances. After performing the planned contrasts, performance in the EDS phase with an ANOVA, using corrected post hoc analyses to test mean differences.

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Results

RG3487 is the S-isomer of N-[(3S)-1-Azabicyclo[2.2.2]oct-3-yl]-1H-indazole-3-carboxamide hydrochloride (C₁₅H₁₉ClN₄O) with a molecular weight of 306.8 g/mol (as the HCl salt form). The chemical structure is shown in **Figure 1**.

In vitro

RG3487 receptor binding studies

RG3487 displaced binding of the α 7nAChR antagonist [³H]-methyllycaconitine (MLA) (0.02 – 20 nM), with a K_i value of 6 nM (n = 3) in rat membrane preparations. In addition, the selectivity of RG3487 was investigated at a concentration of 10 μ M at binding sites for 60 different membrane and soluble receptors, ion channels and monoamine transporters (**Table 1**). RG3487 inhibited binding by less than 50% in all cases, except at the 5-HT₃R. In a binding assay using human recombinant 5-HT₃Rs expressed in HEK293 cells, RG3487 was tested to displace [³H]-BRL-43694, a 5-HT₃R selective antagonist. In this study, RG3487 showed high affinity to 5-HT₃Rs with a K_i value of 1.2 nM.

The effects of RG3487 in various *in vitro* cell biology assays were also investigated. RG3487 was profiled at a concentration of 10 μ M against a broad panel of enzymes (30 enzymes). The results showed less than 20% inhibition for all enzymes tested (**Table 1**).

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RG3487 acts as a partial agonist at $\alpha 7$ nACh receptors

The ability of RG3487 to activate $\alpha 7$ nAChRs was evaluated using the whole-cell patch clamp electrophysiological recordings in oocytes stably expressing the human $\alpha 7$ nAChR. In this study, RG3487 (0.1 – 300 μ M) evoked a maximum peak current (I_{\max}) that was 63 % of the current evoked by ACh (1280 μ M), suggesting that RG3487 is a partial agonist at the $\alpha 7$ nAChR (**Figure 2a**). The EC_{50} of RG3487 at the human $\alpha 7$ nAChR expressed in oocytes was $0.8 \pm 0.15 \mu$ M with a Hill coefficient of 1.4 ± 0.08 (n = 4). Using net charge analysis, the EC_{50} of RG3487 was $0.18 \pm 0.018 \mu$ M with a Hill coefficient of 1.5 ± 0.32 (n=3; not shown). The evoked current decreased at concentrations above 10 μ M RG3487.

To confirm that RG3487 acts directly as an agonist of the $\alpha 7$ nAChRs, a competition experiment was performed with the specific antagonist MLA. Pre-incubation (5 minutes) with 10 nM MLA fully inhibited the RG3487-evoked currents confirming that these responses arise from the activation of $\alpha 7$ nAChRs (not shown).

In addition to the activation of the $\alpha 7$ nAChRs expressed in oocytes, we also investigated the agonist activity of this compound using whole cell patch clamp recordings of human $\alpha 7$ nAChR stably expressed in QM7 cell lines. A range of RG3487 (0.1-100 μ M) concentrations was tested, and the I_{\max} was 69% of the maximal response to ACh (3 mM) (**Figure 2b**). The EC_{50} of RG3487 in QM7 cells was $7.7 \pm 1.7 \mu$ M (peak current, n = 5) as compared to ACh which

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exhibited an $EC_{50} = 268 \pm 22 \mu\text{M}$ (peak current, $n = 6$) with a Hill coefficient of 1.24 ± 0.09 .

When measured by net charge, the EC_{50} of RG3487 was $0.152 \pm 0.041 \mu\text{M}$ ($n = 5$) with a Hill coefficient of 1.6 ± 0.67 and the I_{max} 53% of ACh. For ACh, the net charge $EC_{50} = 22 \pm 1.4 \mu\text{M}$ ($n = 6$) and the Hill coefficient was 2.5 ± 0.34 .

Selectivity of RG3487 for $\alpha 7\text{nAChRs}$ versus other nAChR subtypes as assessed in oocytes

To evaluate the properties of RG3487 at non- $\alpha 7\text{nAChR}$ subtypes, oocytes expressing the desired receptor combination were first challenged with a reference ACh ($30 \mu\text{M}$) test pulse to assess evoked current amplitude, and then were challenged with increasing concentrations of RG3487. In oocytes expressing the human $\alpha 4$ - and $\beta 2\text{nACh}$ subunits, RG3487 ($0.1 - 1000 \mu\text{M}$) did not evoke any measurable currents. Similarly, RG3487 tested at 10 and 100 μM evoked no detectable currents at the following nACh receptors: $\alpha 4\beta 2\alpha 5$, $\alpha 4\beta 2\alpha 6\beta 3$, $\alpha 4\beta 2\alpha 6$, $\alpha 3\beta 2$ and $\alpha 2\beta 2$ (not shown).

In addition, RG3487 was assessed for potential cross-reactivity using a FLIPR functional assay at $\alpha 3\beta 2\text{nAChR}$ and $\alpha 3\beta 4\text{nAChR}$ expressed in SK-N-SH cell lines and $\alpha 1\beta 1\gamma\delta\text{nAChR}$ expressed in human rhabdomyosarcoma muscle cell lines. Application of RG3487, at concentrations of up to 100 μM , did not trigger any calcium mobilization signals, indicating that RG3487 does not have cross activity at these nAChRs subtypes as expressed in these cells.

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RG3487 is a potent antagonist at 5-HT₃R

Using *Xenopus* oocytes stably expressing the human 5-HT_{3A,B} receptor, the functional effects of RG3487 were assessed using whole-cell patch clamp electrophysiology. Currents evoked by 10 μ M 5-HT were measured after incubation with RG3487 (0.1-300 nM). RG3487 dose dependently inhibited 5-HT-evoked current when assessed in this system with an IC₅₀ = 2.84 \pm 0.81 nM and a Hill coefficient of 0.83 \pm 0.06 nM (n = 4) (**Figure 3a**). The current responses were normalized to the 5-HT I_{max}.

Further *in vitro* investigation of the interaction of RG3487 with 5-HT₃R_s was conducted in N1E-115 cell lines using whole-cell patch clamp recordings in the Dynaflo system. 5-HT (10 μ M) reliably evoked currents from N1E-115 cells with an EC₅₀ of 5.5 \pm 0.18 μ M and a Hill coefficient of 2.68 \pm 0.14 (**Figure 3b**) that were abolished with the co-application of the selective 5-HT₃R antagonist, ondansetron (100 nM; data not shown). When co-applied with 5-HT (10 μ M), RG3487 inhibited the 5-HT₃R-mediated current with an IC₅₀ of 32.7 \pm 0.9 nM, and a Hill slope equal to 1.3 \pm 0.4. RG3487 (0.001-1 μ M) did not exhibit any agonist properties when applied alone (data not shown).

Desensitization properties of RG3487 at α 7nAChR and α 4 β 2nAChR

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One of the key features of nAChRs is their strong desensitization upon exposure to an agonist.

To explore the effects of RG3487 on desensitization, *Xenopus* oocytes expressing the $\alpha 7$ nAChR were exposed to sustained (45 seconds) increasing concentrations of RG3487 (0.003-1 μ M) and then stimulated with a standard pulse of ACh (100 μ M). In this study, RG3487 desensitized the $\alpha 7$ nAChRs in a dose dependent manner ($IC_{50} = 40 \pm 4.1$ nM; Hill coefficient 2.7 ± 0.08) (**Figure 4**). Note that for the low concentration of the RG3487, an increase of the peak current is observed and that a reduction of the current becomes apparent for concentrations greater than 10 nM.

Using a similar protocol, investigation into the desensitization effects of RG3487 on heteromeric $\alpha 4\beta 2$ nAChRs was also conducted at 100 nM. Two control responses evoked by a brief ACh (30 μ M) test pulse were recorded and three responses were successively recorded in the presence of RG3487. In addition, a control response was measured at the end to evaluate receptor recovery. The results from this experiment revealed that at concentrations up to 100 nM, RG3487 did not alter the $\alpha 4\beta 2$ nAChR responses (data not shown).

In vivo

Plasma concentrations of RG3487 in rats

Following oral administration of RG3487 (0.3 -10 mg/kg) in fasted male SD rats, the mean C_{max} was typically observed between 0.5-4 hours (**Table 2**). In addition, to assess brain penetration of

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the compound, RG3487 was administered orally at 10 mg/kg to rats and animals were sacrificed at 1 hour post dose. Brains were removed and homogenized. Concentrations of RG3487 measured 1 hour after administration were 43.8 ± 11.8 ng/ml. Dividing by the plasma concentration at 1 hour, brain to plasma ratios were 0.13 ± 0.01 .

RG3487 Selectively Enhances Object Recognition Memory

To investigate the effects of RG3487 on episodic memory, rats were tested in the novel object recognition test. Administration of RG3487 (0.1-10 mg/kg, po) prior to training significantly increased object recognition memory at the 48-hour retention interval in a dose-related manner ($F_{(5, 37)} = 9.667$; $p < 0.05$) (MED = 1.0 mg/kg, po) (**Figure 5a**). Similar effects were observed following ip administration of RG3487 (0.01 – 1.0 mg/kg) ($F_{(3, 22)} = 4.634$; $p < 0.05$) (data not shown).

Pre-training administration of RG3487 suggests that its pro-cognitive effects may reflect improvements in the acquisition and/or consolidation of the recognition memory. To investigate the effect on consolidation specifically, administration of RG3487 directly after object recognition training was explored. In this paradigm, RG3487 (0.1 and 1.0 mg/kg, i.p.) significantly enhanced the percentage of time exploring the novel object when tested at the 48-hour retention delay interval as compared to vehicle treated controls ($F_{(3,25)} = 7.404$; $p < 0.05$) (**Figure 5b**).

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To confirm that the RG3487-induced improvement in recognition memory was mediated through the $\alpha 7$ nAChR, animals were treated with the selective $\alpha 7$ nAChR antagonist MLA (0.3125, 1.25 and 5 mg/kg, ip) in combination with 3.0 mg/kg, po of RG3487. MLA (1.25 and 5 mg/kg) significantly antagonized the pro-cognitive effects of RG3487 ($F_{(4, 28)} = 6.241$; $p < 0.05$) (**Figure 5c**). Because RG3487 is also a potent antagonist at the 5-HT₃ receptor, we investigated the effects of the selective 5-HT₃ antagonist, ondansetron (0.1, 1 and 10 mg/kg, po) in the novel object recognition model. Ondansetron did not show any improvement in recognition memory at the 48 hour retention delay ($F_{(3, 21)} = 0.168$; $p > 0.05$) (**Figure 5d**), suggesting that the cognitive enhancing effect of RG3487 in the object recognition model was due to activation of the $\alpha 7$ nAChR.

It was of interest to determine whether the pro-cognitive effects of RG3487 could be observed following repeated administration. To this end, RG3487 (3 mg/kg, ip) was administered once daily for 10 days to determine its effects on object recognition memory. Repeated administration of RG3487 significantly enhanced the percentage of time animals spent investigating the novel object at the 48-hour delay interval ($t_{(17)} = -5.680$; $p < 0.05$) (**Figure 5e**) similar to the results from the acute administration study, suggesting that pharmacological tolerance to repeated daily dosing did not occur with RG3487.

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Improvement in spatial memory by RG3487 in age-impaired animals

In the Morris water maze test, age-impaired animals were identified and administered either RG3487 (0.01-10 mg/kg, ip) or vehicle prior to each of three additional training days. RG3487 treatment in rats significantly improved the aged-impaired performance deficits compared to vehicle-treated age-impaired animals ($F_{(6,473)} = 5.977$; $p < 0.05$) (**Figure 6**). Post hoc analyses revealed a significant reduction in swim latencies at 0.03-0.3 mg/kg doses of RG3487, whereas higher doses (1 and 10 mg/kg) were inactive. In age-impaired rats, the RG3487-mediated improvements in spatial navigation were reduced as the dose of the compound increased (rats: ≥ 1 mg/kg), yielding a U-shaped dose response curve. There were no statistically significant differences in swim speeds or visual acuity in age-impaired animals treated with RG3487 versus vehicle for either species (data not shown). These results demonstrate that RG3487 effectively attenuates age-related impairments in spatial learning and memory rats.

RG3487 improves executive function following sub-chronic PCP administration

In this study, subchronic PCP treatment produced a significant impairment in number of trials to criterion only in the extradimensional shift (EDS) phase ($t_{(16)} = 3.79$; $p < 0.05$). There were no suggestions of PCP-induced impairment on trials to criterion in SD, CD, IDS, IDS reversal or EDS reversal (all $p > 0.05$) consistent with previous data (Rodefer et al., 2008). Animals that received an acute injection of RG3487 (0.03-1 mg/kg, ip) prior to testing in the set shifting paradigm exhibited a treatment-dependent improvement in performance in the EDS ($F_{(4,40)} =$

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11.59, $p < 0.05$). Dunnett's post hoc analyses indicated that significant effects of RG3487 were observed at all doses tested ($p < 0.05$) when compared to the saline-treated PCP rats. Thus, acute administration of RG3487 attenuated the PCP-induced deficit on EDS performance in the set-shifting task (**Figure 7**).

RG3487 reverses apomorphine-induced deficits in sensorimotor gating

In this study, the prepulse stimuli utilized (75, 80 and 85 dB) clearly inhibited the startle response to a 120 dB auditory stimulus in a fashion that was dependent on the prepulse intensity (i.e., the greater the decibel level of the prepulse, the greater the inhibition of the startle response). Moreover, apomorphine (0.5 mg/kg, sc) significantly diminished PPI, which was significantly antagonized by the positive control, haloperidol (0.3 mg/kg, ip). Administration of RG3487 (0.01-1.0 mg/kg, ip) attenuated the apomorphine-induced deficit in PPI in young male rats in a dose dependent manner ($F_{(6, 90)} = 6.71$; $p < 0.05$) (**Figure 8**).

RG3487 does not alter locomotor activity

RG3487 was also assessed for effects on locomotor activity and rearing behaviour in SD rats in a 60 min session. At the doses tested, RG3487 (0.1-10 mg/kg, ip) did not significantly alter total distance travelled ($F_{(3, 14)} = 1.029$; $p > 0.05$) or rearing behavior ($F_{(3, 14)} = 0.237$; $p > 0.05$) (not shown).

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Discussion

Enhancing cognitive performance via activation of the $\alpha 7$ nAChR represents a promising new approach to treating diseases such as Alzheimer's. The present studies were conducted to characterize the *in vitro* and *in vivo* pharmacological properties of the novel compound, RG3487. In particular, RG3487 selectively activated the $\alpha 7$ nAChR without affecting other nicotinic receptors (i.e., >100-fold selectivity versus other nicotinic subtypes). In addition, RG3487 did not show any appreciable binding affinity for >90 other GPCRs, ion channels, monoamine transporters or enzymes, except for the 5-HT₃R at which it acted as an antagonist. When tested *in vivo*, RG3487 demonstrated consistent pro-cognitive properties following acute and repeated administration in healthy and impaired animal models. RG3487 also improved sensorimotor gating deficits in rats. These data suggest RG3487 has a pharmacological profile ideal for investigating the role of the $\alpha 7$ nAChR in cognitive disorders.

RG3487 exhibited low nanomolar binding affinity at the $\alpha 7$ nAChR in which it acted as a partial agonist when compared ACh in both oocytes and QM7 cells. Moreover, RG3487-mediated current activation in oocytes could be abolished by the selective $\alpha 7$ nAChR antagonist, MLA, confirming its agonist activity at the $\alpha 7$ nAChR. A difference in peak current EC₅₀ values was noted between the oocytes and the QM7 cells, which may be due to differing experimental conditions and/or differences in the physiological and pharmacological properties of the $\alpha 7$ nAChR expressed, a finding that has been reported previously with other $\alpha 7$ nAChR agonists

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(Biton et al., 2007). In addition, it is interesting to highlight the differences between peak current-derived EC_{50} values that exhibited μ M potencies as compared to the left-shifted EC_{50} values derived from net charge analysis in the nM range between the oocyte and cell line systems. It has been shown that EC_{50} values for $\alpha 7$ nAChR agonists derived from peak current amplitudes may underestimate agonist potency, owing to the rapid desensitizing properties of the receptor (Papke and Porter Papke, 2002). Thus, presumed differences in the rate of drug addition in our two recording systems may have introduced variability, contributing to the difference between the peak-derived EC_{50} values in oocytes and cell lines.

One of the key characteristics of the $\alpha 7$ nAChR is rapid desensitization following agonist binding (Revah et al., 1991; Seguela et al., 1993). Sustained exposure of *Xenopus* oocytes to increasing concentrations of RG3487 progressively prevented ACh from activating $\alpha 7$ nAChR, but not $\alpha 4\beta 2$ nAChR-mediated currents, consistent with the idea that RG3487 desensitized $\alpha 7$ nAChRs in a concentration dependent manner. Blockade of ACh-evoked currents following sustained exposure of RG3487 occurred at concentrations approximately 1000-fold less than those necessary to activate the receptor in oocytes. Critically, this low concentration range of RG3487 approximates the efficacious brain and plasma levels of compound identified in rat following oral administration. Notably, a pulse of ACh applied following sustained exposure of 3 and 10 nM RG3487 potentiated the ACh-evoked current which may reflect enhanced co-operativity of

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agonist binding at these low concentrations, as has been reported previously for the $\alpha 4\beta 2$ nAChR (Smulders et al., 2005).

The 5-HT₃R belongs to the superfamily of ligand-gated ion channels that includes the nAChRs (Maricq et al., 1991). Significant sequence homology exists between 5-HT₃Rs and $\alpha 7$ nAChRs including the ligand binding domain, and cross-reactivity of certain compounds (e.g., tropisetron) has been reported previously (Macor et al., 2001). Subsequently, we determined that RG3487 acts as a potent 5-HT₃R antagonist, as it inhibited 5-HT-induced current in both *Xenopus* oocytes and N1E-115 cell lines expressing 5-HT₃Rs and did not evoke currents when applied alone.

In an effort to expand on the *in vitro* characterization of RG3487, the compound was further assessed in multiple *in vivo* models of cognition. RG3487 produced dose-related improvements in episodic memory function in both young and age-impaired animal models as measured in the novel object recognition (NOR) and Morris water maze tasks, respectively. The observed pro-cognitive effects of RG3487 were mediated by the $\alpha 7$ nAChR in that the improvement in recognition memory could be blocked by administration of MLA. These data suggest that acute activation of the $\alpha 7$ nAChR is sufficient to produce pro-cognitive effects and is similar to previous reports with other selective $\alpha 7$ nAChR agonists (Bitner et al., 2007; Pichat et al., 2007). In addition, the RG3487-mediated improvement in episodic memory is in alignment with

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previously published data showing that this compound improves sustained attention following acute administration (Rezvani et al., 2009).

We also investigated the potential pro-cognitive properties of 5-HT₃R inhibition using the NOR model, because of the high affinity binding and functional antagonist properties of RG3487 at this receptor. Antagonism of the 5-HT₃R has been shown to improve cognitive performance in some animal paradigms (e.g., pharmacologically-induced hypocholinergic models) (Hodges et al., 1996), but not in all *in vivo* experimental systems tested (Pitsikas and Borsini, 1997). Using the selective, competitive 5-HT₃R antagonist, ondansetron, no improvement in recognition memory was observed in the present study. These data combined with MLA blockade of the cognitive enhancing effects of RG3487, as well as the reported pro-cognitive properties of selective α 7nAChR agonists themselves (e.g., A-583941, SSR-180711) (Bitner et al., 2007; Pichat et al., 2007), (Bitner et al., 2007; Pichat et al., 2007) suggest that α 7nAChR activation and not 5-HT₃R antagonism mediates the pro-cognitive properties of RG3487.

In addition to the acute nootropic effects of RG3487, no evidence of tachyphylaxis was observed following chronic (i.e., 10 day) administration of this compound as tested in the NOR model. Although rapid desensitization of the α 7nChR is a key characteristic of this ligand-gated ion channel (Revah et al., 1991; Seguela et al., 1993), it appears that a balance between receptor activation and desensitization may occur with RG3487 that results in a net pro-cognitive effect

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following repeated dosing. This effect may be due to pharmacokinetic properties of RG3487, as well as the low concentrations of this molecule that are sufficient to activate a subset of $\alpha 7$ nChRs at any given moment allowing time for other previously activated receptors to recover from desensitization. Overall, these data make it tempting to speculate that transient inactivation of the $\alpha 7$ nAChR following RG3487 binding does not translate into a functional limitation for this drug target.

The administration of RG3487 either prior to training or immediately after training improved long-term memory in the NOR model, indicating that RG3487 can influence both acquisition and consolidation phases of memory formation. One mechanism underlying this effect may be due to enhanced neurotransmission through activation of $\alpha 7$ nAChRs localized presynaptically (Radcliffe and Dani, 1998). To this end, selective activation of $\alpha 7$ nAChRs in primary neuronal culture systems and *in vivo* has been shown to enhance extracellular concentrations of neurotransmitters with recognized involvement in cognitive processes (e.g., glutamate, acetylcholine, dopamine) (Radcliffe and Dani, 1998; Biton et al., 2007; Pichat et al., 2007).

The pro-cognitive effects of RG3487 described may also be mediated by postsynaptic activation of $\alpha 7$ nAChRs. Not only have $\alpha 7$ nAChRs been identified post-synaptically, but their high Ca^{2+} permeability suggests that metabotropic Ca^{2+} -mediated second messenger signaling could underlie its cognitive enhancing properties (Berg and Conroy, 2002). In support of this concept,

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activation of $\alpha 7$ nAChRs can trigger Ca^{2+} -induced Ca^{2+} release from internal stores (Dickinson et al., 2007), which has been linked to transcriptional regulation and synaptic plasticity (Berg and Conroy). To this end, Bitner et al. (2007) report activation of the ERK_{1/2} pathway and subsequent downstream phosphorylation of cAMP response element binding (CREB) protein, a critical mediator of synaptic plasticity following administration of the selective $\alpha 7$ nAChR agonist A-58294.

Both Alzheimer's and schizophrenic patients exhibit alterations in the expression of the $\alpha 7$ nAChR (Freedman et al., 1995; Court et al., 1999), which may contribute to the cognitive impairments observed in these diseases. In addition, genetic polymorphisms in the promoter region of the $\alpha 7$ nAChR gene on chromosome 15 have been linked to sensory gating deficits in schizophrenic patients (Leonard et al., 1996). When tested using the pre-pulse inhibition (PPI) of startle model of sensorimotor gating, RG3487 improved the ability of the animal to inhibit responding following apomorphine-induced impairments. These data compare favorably with standard antipsychotic agents (e.g., risperidone, haloperidol) in the PPI model, and are in agreement with some, but not all, results reported for other $\alpha 7$ nAChR agonists in this model (Stevens et al., 1998; Schreiber et al., 2002).

In some of the *in vivo* assays we used to test RG3487 (i.e., PPI, MWM), a U-shaped dose response was observed, which has been reported previously for nAChR agonists (Picciotto,

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2003; Feuerbach et al., 2009). Notably in the oocyte system, a decrease in RG3487-evoked currents was observed at concentrations above 10 μ M, which may be due to rapid receptor desensitization, a well recognized property of the α 7nAChR (Revah et al., 1991; Seguela et al., 1993), or to open channel blockade (Colquhoun et al., 1979). These *in vitro* findings may provide a mechanistic explanation for the inverted U-shaped dose responses that were observed *in vivo*.

In addition to improving sensorimotor gating deficits, RG3487 also improved executive function deficits induced by sub-chronic PCP administration using an attentional set-shifting paradigm, which is considered an analogous task to the Wisconsin Card Sort Test in humans (Rodefer et al., 2008). Schizophrenic patients, as well as animals that receive a subchronic regimen of PCP, show a specific disruption in the ability to acquire the extra-dimensional set-shift discrimination that may be attributable to NMDA receptor hypofunction. Typical and atypical antipsychotic medications, which are useful in treating the positive symptoms of schizophrenia, do little to improve cognitive dysfunction in this patient population and in the extra-dimensional set shifting (Rodefer et al., 2008). It remains to be determined whether agonists directed at the α 7nAChR will improve executive function in schizophrenic patients, as the clinical data to date have shown moderate to no effect on cognitive parameters in this patient population (Olincy et al., 2006; Freedman et al., 2008; Umbricht, 2009).

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The development of $\alpha 7$ nAChR agonists as potential therapeutic agents for cognitive impairment in diseases such as Alzheimer's is supported by data showing improvement in attention, learning and memory following activation of this receptor subtype. RG3487 is a selective $\alpha 7$ nAChR partial agonist that exhibits pro-cognitive effects in healthy and age-impaired animals across multiple cognitive domains. In addition, RG3487 effectively reverses pharmacologically-induced sensorimotor and executive function impairments, two paradigms postulated to model aspects of schizophrenia. These preclinical findings suggest that RG3487 may be a promising therapeutic agent for treating cognitive deficits in human disease.

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Authorship contributions:

Participated in research design: Wallace, Callahan, Tehim, Bertrand, Tombaugh, Wang, Xie, Rowe, Terry, Rodefer, Herbert, Murray, Santarelli, Lowe

Conducted experiments: Callahan, Bertrand, Tombaugh, Wang, Xie, Rowe, Ong, Graham, Terry, Rodefer, Herbert, Murray

Contributed new reagents or analytic tools: Wang, Xie, Ong, Murray

Performed data analysis: Wallace, Callahan, Bertrand, Tombaugh, Wang, Xie, Rowe, Terry, Rodefer, Murray, Lowe

Wrote or contributed to the writing of the manuscript: Wallace, Callahan, Bertrand, Tombaugh, Wang, Rowe, Terry, Rodefer, Porter, Lowe

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Figure Legends

Figure 1: *Molecular structure of RG3487*

Figure 2: *Electrophysiological recordings of RG3487-mediated current activation at the human $\alpha 7$ nAChR.* Whole cell patch-clamp recordings in **a)** *Xenopus* oocytes expressing h $\alpha 7$ nAChR in response to a series of RG3487 concentrations (0.1-300 μ M). RG3487 maximum peak current (n = 4) was 63% of ACh (1280 μ M) response, with an EC₅₀ of 0.8 μ M. **b)** QM7 cell line expressing h $\alpha 7$ nAChR exposed to RG3487 (0.1-100 μ M). RG3487 maximum peak current (n = 5) was 69% of ACh (3000 μ M) with EC₅₀ = 7.7 μ M. Activation currents were normalized to ACh. Results are presented as mean \pm SEM.

Figure 3: *Patch clamp recordings of RG3487-mediated current inhibition at 5-HT₃ receptors.* **a)** RG3487 (0.0001-0.3 μ M) inhibited 5-HT (10 μ M)-induced current activation mediated through the human 5-HT₃R with an IC₅₀ = 2.8 nM (n = 4) in *Xenopus* oocytes; **b)** RG3487 (0.001-1 μ M) inhibited 5-HT (10 μ M)-induced current activation mediated through the 5-HT₃R with an IC₅₀ = 30 nM (n = 5) in N1E-115 cell lines expressing this receptor subtype. Results are presented as mean \pm SEM.

Figure 4: *Electrophysiological recordings of current inhibition following sustained exposure to RG3487.* Plot of the peak current as a function of the logarithm of RG3487 (0.003-1 μ M) and

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normalized to ACh (100 μ M) response yielded the concentration inhibition curve, $IC_{50} = 40$ nM ($n = 4$) in *Xenopus* oocytes expressing human $\alpha 7$ nAChR. Results are presented as mean \pm SEM.

Figure 5: Effects of RG3487 administration on novel object recognition. **a)** RG3487 (0.1-10 mg/kg, po) administered 1 hour before training significantly increased % time investigating the novel object when rats were tested 48-hours later. Similar effects were shown following intraperitoneal administration of RG3487 (0.01-1.0 mg/kg) (not shown). **b)** Administration of RG3487 (0.01-1.0 mg/kg, ip) immediately after training, also enhanced the % time animals spent investigating the novel object during the test. **c)** RG3487 (3.0 mg/kg, po)-mediated improvement in recognition memory was dose dependently blocked by the $\alpha 7$ nAChR selective antagonist MLA (0.3125, 1.25 and 5.0 mg/kg, ip) when both compounds were co-administered prior to training. **d)** The selective 5-HT₃ receptor antagonist, ondansetron (0.1-10 mg/kg, po), administered 1 hour before training did not alter the % time spent investigating the novel object as compared to vehicle treated animals. **e)** RG3487 (3 mg/kg, ip) improved recognition memory following 10 days of once daily administration with the last dose being given 1 hour prior to training. Results are presented as mean \pm SEM. * $p < 0.05$ versus vehicle. # $p < 0.05$ versus RG3487 alone. N = 6-8 / group.

Figure 6: Effects of RG3487 on spatial learning in age-impaired rats. Aged rats (22-24 months old) were trained for 5 days for spatial learning and based upon mean escape latency for all trials on days 3-5, aged animals were classified as either impaired (AI) or unimpaired (AU).

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On days 6-8, AI animals were administered vehicle (V) or RG3487 (0.01 – 10 mg/kg, ip) 30 min before testing and latency to reach the escape platform was measured. RG3487 (0.03-0.3 mg/kg) reduced latency to reach the platform, without altering swim speeds. Results are presented as mean \pm SEM. * $p < 0.05$ versus vehicle. N = 7-13 / group.

Figure 7: *Effects of RG3487 on attentional set shifting in rats.* Subchronic phencyclidine (PCP) administration (5 mg/kg, ip, bid for 7 days) to rats followed by a 10 day washout period impaired performance on the extradimensional shift (i.e., increased trials to completion) versus vehicle treated animals. Acute administration of RG3487 (0.03 – 1 mg/kg, ip) significantly reversed the PCP-induced deficit. Results are presented as mean \pm SEM. * $p < 0.05$ versus vehicle. # $p < 0.05$ versus PCP treated rats. N = 9 / group.

Figure 8: *Effects of RG3487 on prepulse inhibition of startle response.* Acute administration of Apomorphine (Apo; 0.5 mg/kg, sc) impaired PPI in rats that was reversed by haloperidol (Hal; 0.3 mg/kg, ip) co-administration. RG3487 (0.03 – 0.3 mg/kg, ip) also reversed apo-induced impairments following acute administration as compared to vehicle (Veh). Results are presented as mean \pm SEM. * $p < 0.05$ versus vehicle. # $p < 0.05$ versus apomorphine. N = 10-20 / group.

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Table 1: In vitro pharmacology of RG3487: Receptor binding, enzyme and cell-based assays

Target	% Control (10 μ M)	Target	% Control (10 μ M)	Target	% Control (10 μ M)
A1 (<i>h</i>)	97.5	CCR1 (<i>h</i>)	90.7	Phospholipase A2	108
A2A (<i>h</i>)	94.5	H1 (<i>h</i>)	81.9	COX1 (<i>h</i>)	115
A3 (<i>h</i>)	95	H2 (<i>h</i>)	75.5	COX2 (<i>h</i>)	95.8
α 1 (non-selective)	83	MC4 (<i>h</i>)	104	5-LO	94.5
α 2 (non-selective)	100	ML1 (<i>h</i>)	95.9	Constitutive NOS (<i>h</i>)	120
β 1 (<i>h</i>)	77.3	M1 (<i>h</i>)	77.8	Phosphodiesterase 1	99.8
β 2 (<i>h</i>)	73	M2 (<i>h</i>)	87	Phosphodiesterase 2 (<i>h</i>)	99
AT1 (<i>h</i>)	76.3	M3 (<i>h</i>)	83.6	Phosphodiesterase 3 (<i>h</i>)	99.5
AT2 (<i>h</i>)	98.4	M4 (<i>h</i>)	90.4	Phosphodiesterase 5 (<i>h</i>)	108
BZD (central)	85.5	M5 (<i>h</i>)	101	ACE (<i>h</i>)	96.2
BZD (peripheral)	88.2	P2X	108	ECE-1 (<i>h</i>)	99.3
BB (non-selective)	103	P2Y	102	Elastase (<i>h</i>)	99.2
B2 (<i>h</i>)	91.9	5-HT1A (<i>h</i>)	95.5	Caspase-8 (<i>h</i>)	107
CGRP (<i>h</i>)	101	5-HT1B	88.3	Cathepsin L (<i>h</i>)	90.3
CB1 (<i>h</i>)	77.5	5-HT2A (<i>h</i>)	51.4	Neutral endopeptidase (<i>h</i>)	88.3
CCK1 (CCKA) (<i>h</i>)	108	5-HT2C (<i>h</i>)	73.1	MMP-9 (<i>h</i>)	96.4
CCK2 (CCKB) (<i>h</i>)	99.1	5-HT3 (<i>h</i>)	0.1	Tryptase (<i>h</i>)	98.3
D1 (<i>h</i>)	94.3	5-HT5A (<i>h</i>)	73.5	Ca ²⁺ -ATPase pump	107
D2S (<i>h</i>)	106	5-HT6 (<i>h</i>)	77.6	Phosphatase IB (<i>h</i>)	99.2
D3 (<i>h</i>)	96.5	5-HT7 (<i>h</i>)	84.7	CAM kinase II	105
D4.4 (<i>h</i>)	115	σ (non-selective)	70	EGFR-tyrosine kinase (<i>h</i>)	100
D5 (<i>h</i>)	86.2	sst (non-selective)	103	ERK ₂ (P42 ^{mapk})	99.3
ETA (<i>h</i>)	88.4	VPAC1 (VIP1) (<i>h</i>)	95.2	PKA (<i>h</i>)	109
ETB (<i>h</i>)	106	V1a (<i>h</i>)	86.3	PKC	98.8
GABA (non-selective)	74.8	Ca ²⁺ chan (L, verap site)	82.7	Acetylcholinesterase (<i>h</i>)	101
GAL1 (<i>h</i>)	107	K ⁺ V chan	89.8	MAO-A (<i>h</i>)	99.6
GAL2 (<i>h</i>)	82.5	SK ⁺ Ca chan	94.5	MAO-B (<i>h</i>)	103
PDGF	104	Na ⁺ chan (site 2)	52.7	ATPase (Na ⁺ /K ⁺)	102
CXCR2 (IL-8B) (<i>h</i>)	109	Cl ⁻ chan	110	AcetylCoA synthetase	95.6
TNF- α (<i>h</i>)	94	NE transporter (<i>h</i>)	88.4	Carbonic anhydrase II (<i>h</i>)	88.3

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Table 2: RG3487 plasma concentrations following oral administration in male, Sprague Dawley rats.

RG3487 plasma concentration (ng/ml) following oral administration (mean ± SEM)								
<u>Dose (mg/kg)</u>	<u>0.5 h</u>	<u>1 h</u>	<u>2 h</u>	<u>4 h</u>	<u>6 h</u>	<u>8 h</u>	<u>24 h</u>	<u>AUC_{0-8h} (ng.hr/ml)</u>
0.3	4.1 ± 0.2	2.6 ± 0.1	3.8 ± 0.7	4.4 ± 1.6	1.3 ± 0.6	0.4 ± 0.02	BQL	16.7 ± 2.0
1	21.5 ± 2.3	22.2 ± 3.0	24.2 ± 2.2	10.8 ± 1.9	3.7 ± 0.6	1.5 ± 0.1	BQL	87.2 ± 9.7
3	38.8 ± 3.6	45.9 ± 7.2	54.1 ± 5.0	64.2 ± 30.0	47.0 ± 25.0	26 ± 16.0	BQL	290 ± 27.6
10	591 ± 137	343 ± 55.4	262 ± 53	211 ± 25.6	92.6 ± 8.5	33 ± 4.8	BQL	1531 ± 239.7
	n = 3	n = 6	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3

BQL = Below Quantitation Limit

Figure 1

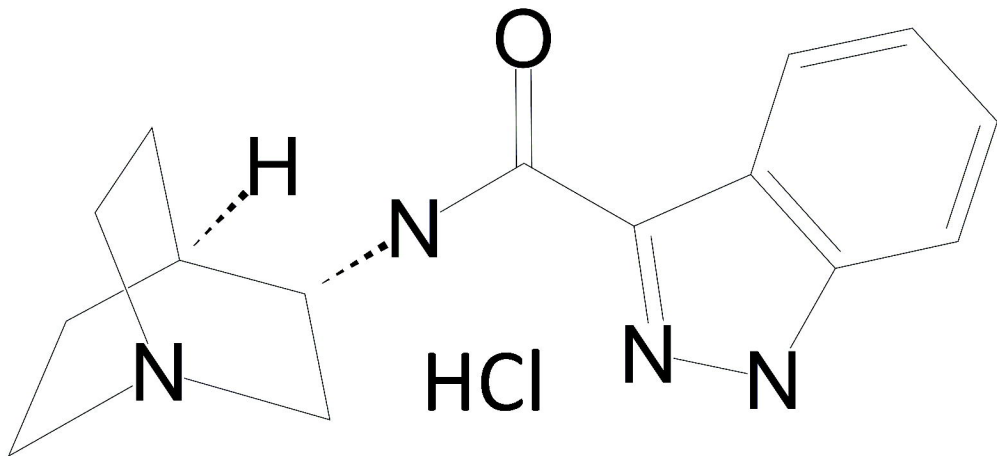


Figure 2a

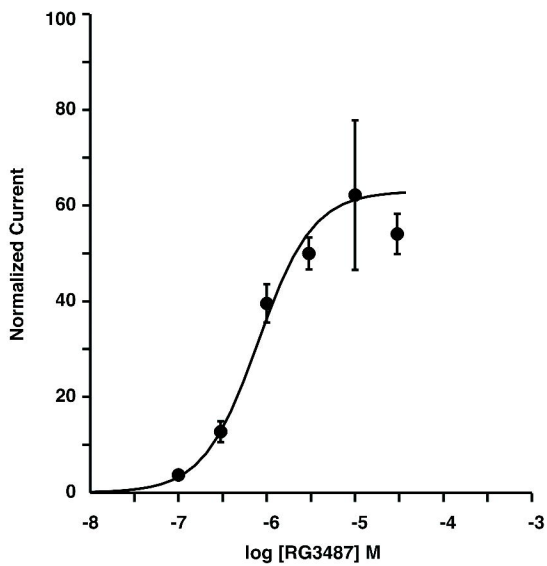


Figure 2b

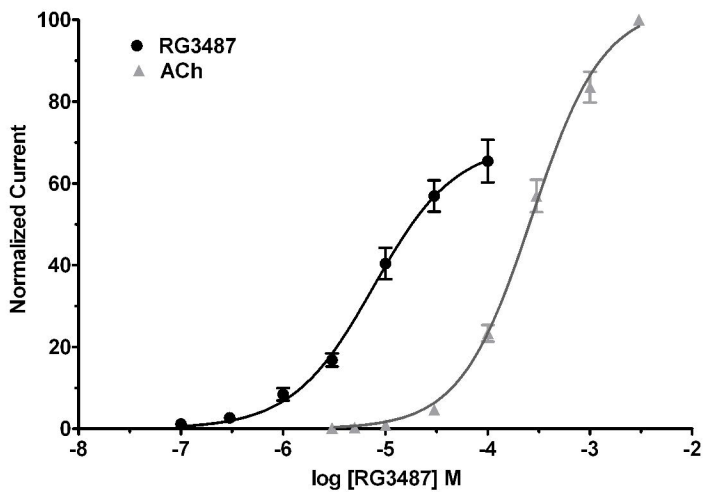


Figure 3a

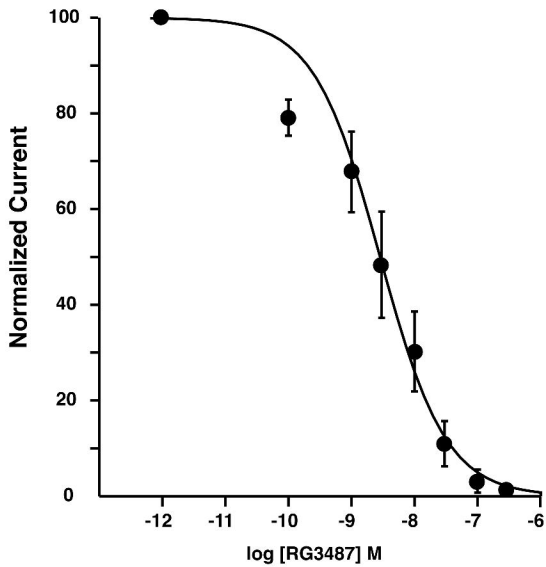


Figure 3b

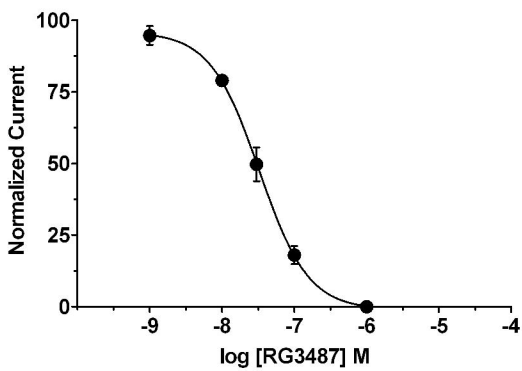


Figure 4

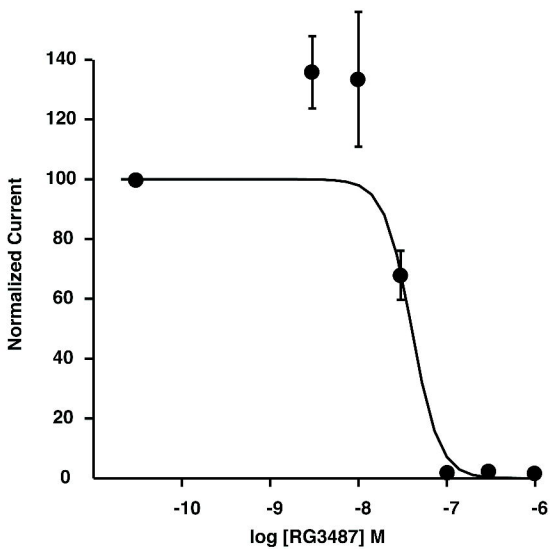


Figure 5a

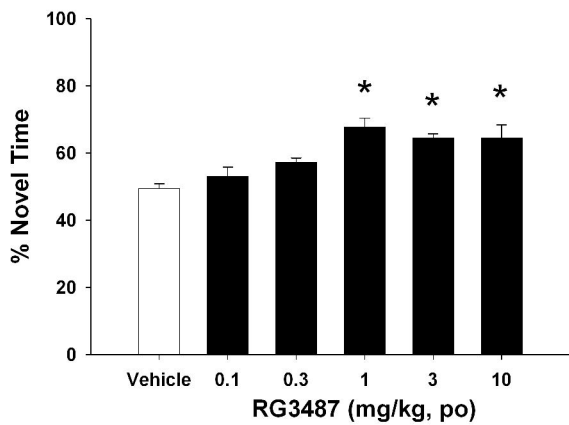


Figure 5b

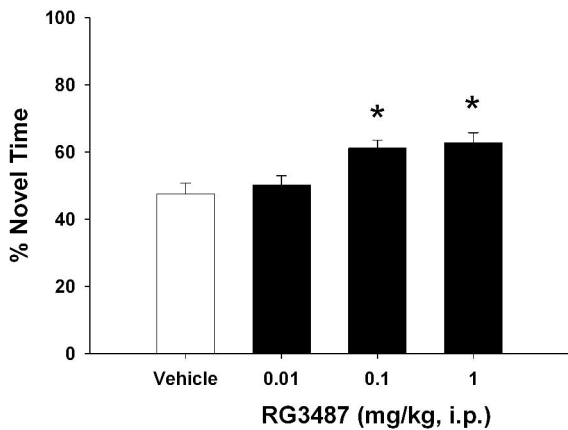


Figure 5c

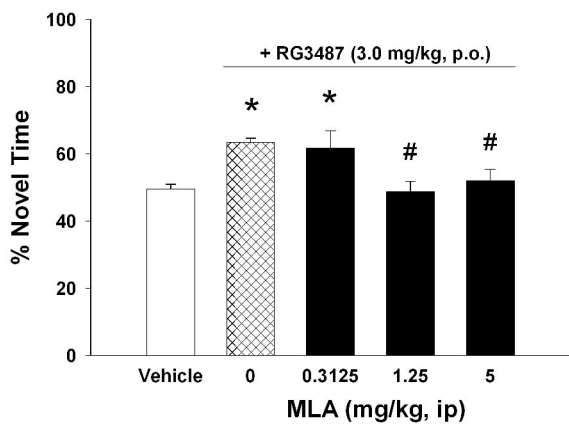


Figure 5d

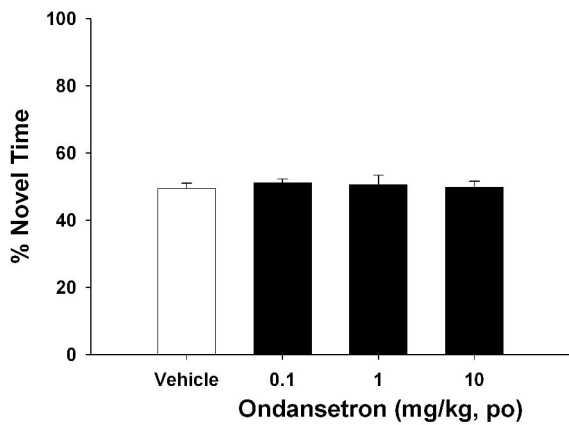


Figure 5e

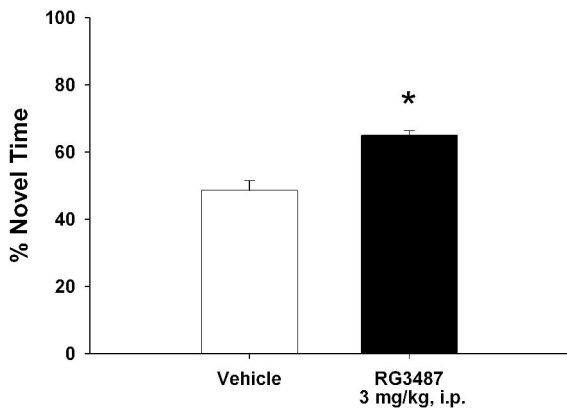


Figure 6

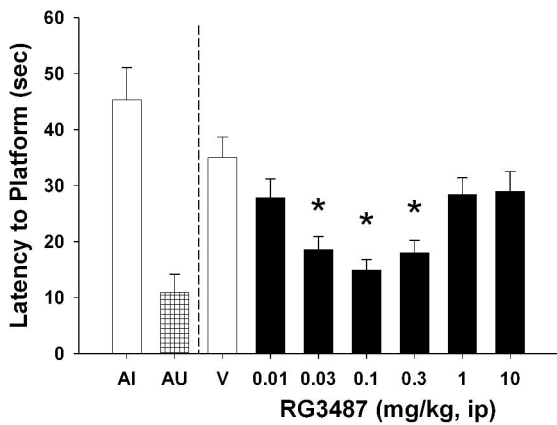


Figure 7

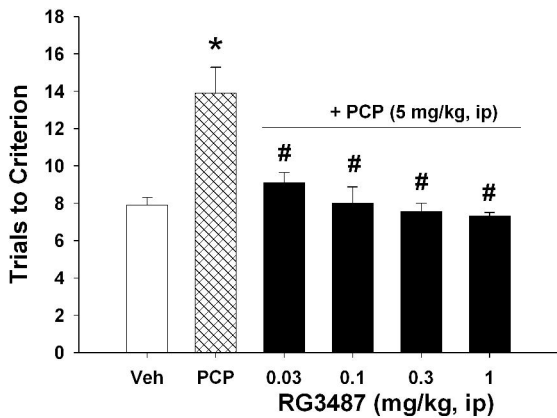


Figure 8

