Allosteric Modulation of Related Ligand-Gated Ion Channels Synergistically Induces Long-Term Potentiation in the Hippocampus and Enhances Cognition

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ABSTRACT

$\alpha 5$ Subunit-containing GABA_A receptors (GABA_ARs) and $\alpha 7$ neuronal nicotinic-acetylcholine receptors (nAChRs) are members of the Cys-loop family of ligand-gated ion channels (LGICs) that mediate cognitive and attentional processes in the hippocampus. $\alpha 5$ GABA_ARs alter network activity by tonic inhibition of CA1/CA3 pyramidal cells of the hippocampus. Postsynaptic $\alpha 7$ nAChRs in the hippocampus regulate inhibitory GABAergic interneuron activity required for synchronization of pyramidal neurons in the CA1, whereas presynaptic $\alpha 7$ nAChRs regulate glutamate release. Can simultaneous allosteric modulation of these LGICs produce synergistic effects on cognition? We show that combined transient application of two allosteric modulators that individually 1) inhibit $\alpha 5$ GABA_ARs and 2) enhance $\alpha 7$ nAChRs causes long-term potentiation (LTP) of

mossy fiber stimulation-induced excitatory postsynaptic currents (EPSC) from CA1 pyramidal neurons of rat hippocampal slices. The LTP effect evoked by two compounds is replicated by 3-(2,5-difluorophenyl)-6-(*N*-ethylindol-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine (522-054), a compound we designed to simultaneously inhibit $\alpha 5$ GABA_ARs and enhance $\alpha 7$ nAChRs. Selective antagonists for either receptor block sustained EPSC potentiation produced by 522-054. In vivo, 522-054 enhances performance in the radial arm maze and facilitates attentional states in the five-choice serial reaction time trial with similar receptor antagonist sensitivity. These observations may translate into therapeutic utility of dual action compounds in diseases of hippocampal-based cognitive impairment.

Introduction

The cognitive dysfunction that arises from Alzheimer's disease originates from multiple pathogenic mechanisms including genetic factors, β -amyloid deposition, tau pathology, apoptosis, neurotrophic deficit, neuronal loss, cerebrovascular impairment, and multiple neurotransmitter deficits. Diseases of cognitive impairment with polygenic origin, such as Alzheimer's disease or schizophrenia, may not respond ade-

quately to a drug therapy that is selective for a single pharmacological target. In particular, current treatments (donepezil, rivastigmine, and galantamine) that augment the cholinergic neurotransmitter deficits associated with cognitive deficits in Alzheimer's disease may have only marginal cost effectiveness (Green et al., 2005; Loveman et al., 2006; Takeda et al., 2006). Attempts to maximize cost effectiveness for treatment of elderly patients with Alzheimer's disease will be crucial because current treatments may have limited utility and are complicated by excessive pill burden, leading to reduced compliance and poor therapeutic outcomes. This situation is particularly acute given the large demographics about to enter the highest-risk age group for developing the disease. Moreover, polypharmacy, drug-drug interactions, adverse reactions, and noncompliance are substantial thera-

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ABBREVIATIONS: nAChR, neuronal nicotinic-acetylcholine receptor; GABA_AR, GABA_A receptor; EPSC, excitatory postsynaptic current; LGIC, ligand-gated ion channel; 5-CSRTT, five-choice serial reaction time trial; LTP, long-term potentiation; MLA, methyllycaconitine; NAM, negative allosteric modulator; PAM, positive allosteric modulator; RAM, radial arm maze; 522-054, 3-(2,5-difluorophenyl)-6-(*N*-ethylindol-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine; compound **6**, *N*-(4-chlorophenyl)- α -[[(4-chlorophenyl)amino]methylene]-3-methyl-5-isoxazoleacetamide; DMSO, dimethyl sulfoxide; ACSF, artificial cerebrospinal fluid; ITI, intertrial interval; LH, lateral hold; BZ, benzodiazepine.

peutic problems in the pharmacological management of elderly patients. A multifunctional drug targeting cognitive centers of the central nervous system could simultaneously address these issues for Alzheimer's or other diseases where impaired cognition is a central clinical component.

Two clinically validated pharmacological targets for cognition are $\alpha 7$ nAChRs and $\alpha 5$ GABA_ARs (Olinicy et al., 2006; Freedman et al., 2008; Attack, 2010). $\alpha 7$ nAChRs are rapidly desensitizing ligand-gated Ca²⁺ channels that are abundantly expressed in the hippocampus, a limbic structure intimately linked to cognition, attention processing, and memory formation (Gotti et al., 1997). In the hippocampus postsynaptic $\alpha 7$ nAChRs regulate inhibitory GABAergic interneuron activity required for synchronization of pyramidal neurons in the CA1, whereas presynaptic $\alpha 7$ nAChRs regulate glutamate release (Fabian-Fine et al., 2001; Buhler and Dunwhiddie, 2002).

$\alpha 5$ GABA_ARs are ligand-gated Cl⁻ channels selectively located on interneurons that control overall amplification of glutamatergic signaling in the hippocampus and control network activity by tonic inhibition of CA1/CA3 pyramidal cells of the hippocampus (Caraiscos et al., 2004; Glykys and Mody, 2006; Serwanski et al., 2006; Glykys et al., 2008; Pirker et al., 2008). GABA_ARs that contain $\alpha 5$ subunits show distinct immunocytochemical (Khrestchatsky et al., 1989; Fritschy and Mohler, 1995; Pirker et al., 2000), mRNA hybridization (Wisden et al., 1992), and selective radioligand binding (Sur et al., 1999; Howell et al., 2000; Li et al., 2001; Attack et al., 2005) patterns that are specific to hippocampal structures but not cortex or other areas of rat brain (McKernan et al., 1991). Furthermore, tonic inhibition of CA1 pyramidal cells in the hippocampus is mediated, in part, by $\alpha 5$ GABA_ARs (Caraiscos et al., 2004). Genetic alteration of $\alpha 5$ GABA_ARs causes behavioral phenotypes consistent with enhanced hippocampal-dependent learning and memory such as spatial (Collinson et al., 2002) and associative learning (Crestani et al., 2002). Hence multiple lines of evidence suggest that clinical evaluation of $\alpha 5$ GABA_ARs as a target for cognition enhancement should be explored.

The first evaluation in a clinical setting of the negative allosteric modulator (NAM) of $\alpha 5$ GABA_ARs, $\alpha 5$ IA, used an ethanol-induced deficit of word recall in normal young healthy volunteers (Nutt et al., 2007). The results of this study suggest that $\alpha 5$ IA can attenuate cognitive impairment caused by alcohol consumption. Moreover, the degree of improvement with $\alpha 5$ IA was proportional to the degree of ethanol-induced impairment (Nutt et al., 2007). These results are consistent with the observation that certain BZ site ligands can function as "alcohol antagonists" (Suzdak et al., 1986, 1988). On the other hand, $\alpha 5$ IA did not improve age-related memory deficits in healthy normal elderly volunteers relative to healthy young volunteers (Attack, 2010). Because the positive control (lorazepam) did not produce intended cognitive deficits in the elderly test subjects the overall results of this trial are equivocal. It remains to be seen whether modulation of $\alpha 5$ GABA_ARs can physiologically improve pathologically related memory deficits rather than just pharmacologically antagonize ethanol-induced cognitive deficits that, in part, target the same receptor system as $\alpha 5$ IA.

Cognition and attentional awareness rely on long-term potentiation (LTP), a form of synaptic plasticity required for memory formation. Although LTP generally requires a glu-

tamatergic signal for induction (tetanically or pharmacologically applied), direct activation of glutamate receptors is a problematic therapeutic strategy for Alzheimer's disease. An indirect method of generating LTP would be more attractive, especially if the spatial and temporal integrity of neurotransmission can be preserved. Activation of $\alpha 7$ nAChRs or inhibition of $\alpha 5$ GABA_ARs individually can augment LTP initiated by tetanic stimulation or glutamate receptor activation (Hunter et al., 1994; Gray et al., 1996; Martin et al., 2010). Because both receptor types are in close anatomical and functional apposition within LTP-generating circuits could simultaneous allosteric modulation of these LGICs induce LTP without direct glutamatergic activation? In the present studies, we provide compelling evidence that induction of LTP by this mechanism is not only possible but results in remarkable in vivo potency suggestive of functional synergism.

Materials and Methods

Synthesis of Compound 6 (*N*-(4-chlorophenyl)- α -[(4-chlorophenyl)amino]methylene]-3-methyl-5-isoxazoleacetamide). For *N*-(4-chlorophenyl)-3-methyl-5-isoxazoleacetamide, a suspension of 3-methyl-5-isoxazoleacetic acid (Sigma-Aldrich, St. Louis, MO; 3.72 g, 26.4 mmol) in 70 ml of CH₂Cl₂ was treated with neat oxalyl chloride (3 ml, 24.4 mmol). After stirring overnight at room temperature, the reaction was concentrated in vacuo. The residue was dissolved in benzene and concentrated to dryness. A solution of the acid chloride (2.57 g, 16.1 mmol) in 25 ml of CH₂Cl₂ was treated with 4-chloroaniline (2.06 g, 16.1 mmol). A solution of Et₃N (2.5 ml, 17.9 mmol) in 20 ml of CH₂Cl₂ was added dropwise. After the addition was complete, the reaction was extracted with a 1 M aqueous HCl solution. The organic layer was dried (Na₂SO₄), filtered, and concentrated in vacuo. The aqueous layer was made basic and extracted with EtOAc. The pooled EtOAc layers were washed with brine, dried (Na₂SO₄), filtered, and concentrated. The solids obtained were combined and washed with CH₂Cl₂, affording 1.8 g of the amide as a solid.

For *N*-(4-chlorophenyl)- α -[(4-chlorophenyl)amino]methylene]-3-methyl-5-isoxazoleacetamide, to a solution of *N*-(4-chlorophenyl)-3-methyl-5-isoxazoleacetamide (447 mg, 1.78 mmol) in neat *N,N*-dimethylformamide dimethylacetal (3 ml) was added to 2 ml of *N*-methyl-2-pyrrolidinone. The resulting solution was stirred at room temperature overnight and concentrated to dryness. The crude product was purified by column chromatography (2:1 EtOAc/hexanes) affording 394 mg (72%) of the intermediate dimethylaminoenaminone as a solid. A solution of this enaminone (128 mg, 0.42 mmol) in 5.5 ml of a 3 N aqueous HCl solution was treated with tetrahydrofuran (4 ml) and stirred at room temperature for 75 min. The reaction was partitioned between EtOAc and water. The EtOAc layer was washed with brine, dried (Na₂SO₄), filtered, and concentrated. The residue (124 mg) was dissolved in toluene (5 ml), and neat 4-chloroaniline (56.8 mg, 0.44 mmol) was added. After stirring overnight at room temperature, the reaction was concentrated in vacuo, and the residue was triturated with MeOH. The solid that formed was then recrystallized from MeOH, affording compound **6** as a light yellow solid. ¹H NMR (400 MHz, CDCl₃) δ 11.45 (d, 1H, *J* = 12.5 Hz), 8.25 (s, 1H), 7.67 (d, 1H, *J* = 12.5 Hz), 7.46 (d, 2H, *J* = 8.8 Hz), 7.28 (d, 4H, *J* = 8.8 Hz), 6.98 (d, 2H, *J* = 8.8 Hz), 5.98 (s, 1H), 2.31 (s, 3H) ppm; MS *m/z* 388 [MH⁺].

$\alpha 5$ IA Synthesis. We used the method as described previously Sternfeld et al. (2004).

Synthesis of 522-054. For 5-(6-chloro-3-pyridazinyl)-1H-indole, to a solution of Na₂CO₃ (0.7 g, 6.6 mmol) in H₂O (5 ml) was added indole-5-boronic acid (1 g, 6.2 mmol) and EtOH (25 ml), stirred at room temperature for 30 min. 3,6-Dichloropyridazine (935 mg, 6.3 mmol), toluene (50 ml), and tetrakis(triphenylphosphine)palladium(0) (0.3 g) were added, then stirred at 90°C for 30 h, and evaporated. The residue

was treated with CH_2Cl_2 (100 ml) and filtered, and the filtrate was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by chromatography on silica gel, eluting with CH_2Cl_2 -MeOH (100:5) to give 5-(6-chloro-3-pyridazinyl)-1*H*-indole as a yellow solid (530 mg, 37%). ^1H NMR ($\text{DMSO}-d_6$) δ 6.52 (1H, s), 7.40 (1H, s), 7.50 (1H, d, 6.3 Hz), 7.86–7.90 (m, 2H), 8.27 (1H, d, 6.9 Hz), 8.32 (1H, s), 11.32 (1H, s) ppm; MS m/z 230 [MH $^+$].

For 2,5-difluorobenzoic hydrazide, to a solution of 2,5-difluorobenzoic acid (5 g, 31.6 mmol) in CH_2Cl_2 (60 ml) SOCl_2 (23 ml) was slowly added at room temperature. After this addition, the mixture was refluxed for 3 h, then evaporated and coevaporated with toluene. The residue was dissolved in CH_2Cl_2 (100 ml), anhydrous hydrazine (5 g) was added slowly, then refluxed for 4 h, and cooled to room temperature, then CH_2Cl_2 (100 ml) was added. The mixture was poured into a separatory funnel, washed with brine (3×100 ml), dried (Na_2SO_4), and evaporated. The residual solid was recrystallized from MeOH (15–20 ml). The colorless crystals were collected by filtration and dried to give 2,5-difluorobenzoic hydrazide (1.99 g, 56%). ^1H NMR ($\text{DMSO}-d_6$) δ 4.52 (2H, s), 7.29–7.33 (3H, m), 9.59 (1H, s) ppm; MS m/z 173 [MH $^+$].

For 3-(2,5-difluorophenyl)-6-(indol-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine, a mixture of (6-chloro-3-pyridazinyl)-1*H*-indole (850 mg, 3.7 mmol), 2,5-difluorobenzoic hydrazide (640 mg, 3.7 mmol), and $\text{Et}_3\text{N}\cdot\text{HCl}$ (0.5 g, 3.6 mmol) in xylene (20 ml) and dimethylformamide (2 ml) was stirred at 150°C for 3 days, then evaporated. The residue was treated with CH_2Cl_2 (100 ml), washed with brine (3×100 ml), dried (Na_2SO_4), and evaporated. The residue was purified by chromatography on silica gel, eluting with CH_2Cl_2 -MeOH (100:5) to give difluorophenyl)-6-(indol-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine as a yellowish solid (400 mg, 31%). ^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 6.53 (1H, s), 7.41 (1H, s), 7.50–7.58 (3H, m), 7.76 (1H, d, $J = 6.3$ Hz), 7.86 (1H, m), 8.08 (1H, d, $J = 7.2$ Hz), 8.28 (1H, s), 8.46 (1H, d, $J = 7.2$ Hz), 11.39 (1H, s) ppm; MS m/z 348 [MH $^+$].

For 3-(2,5-difluorophenyl)-6-(*N*-ethylindole-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine, difluorophenyl)-6-(indol-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine (370 mg, 1.00 mmol) and ground NaOH (60 mg, 1.5 mmol) in anhydrous dimethylformamide (15 ml) were stirred (25°C) for 30 min, then iodoethane (300 mg, 1.9 mmol) was added. The mixture was stirred (25°C) overnight and evaporated, and the residue was treated with CH_2Cl_2 (60 ml), washed with brine (3×100 ml), dried (Na_2SO_4), and evaporated. The residue was purified by chromatography on silica, eluting with CH_2Cl_2 -MeOH (100:5) to give 3-(2,5-difluorophenyl)-6-(*N*-ethylindole-5-yl)-1,2,4-triazolo[4,3-*b*]pyridazine as a pale white solid m/z (290 mg, 80%). ^1H NMR ($\text{DMSO}-d_6$) δ 1.33 (3H, t, $J = 5.4$ Hz), 4.21 (2H, q, $J = 5.4$ Hz), 6.55 (1H, d, $J = 2.1$ Hz), 7.47 (1H, d, $J = 2.1$ Hz), 7.54–7.5 (2H, m), 7.63 (1H, d, $J = 6.3$ Hz), 7.81 (1H, d, $J = 6.9$ Hz), 7.87–7.91 (1H, m), 8.93 (1H, d, $J = 7.2$ Hz), 8.28 (1H, s), 8.47 (1H, d, $J = 7.2$ Hz) ppm; MS m/z 376 [MH $^+$].

Two-Electrode Voltage-Clamp Electrophysiology. Oocytes were obtained from *Xenopus laevis* frogs by using procedures approved and monitored by the University of California Irvine Institutional Animal Care and Use Committee. Individual *X. laevis* oocytes were injected with 0.005 to 50 ng of either $\alpha 7$ nAChR (Jon Lindstrom, University of Pennsylvania, Philadelphia, PA) or GABA $_A$ $\alpha 5\beta 3\gamma 2L$ (1:1:1; CoCensys Inc., Irvine, CA) subunit mRNA [transcription performed with the mMessage mMachine system (Ambion, Austin, TX) and diluted to 1 $\mu\text{g}/\mu\text{L}$]. Two-electrode voltage clamp recordings were made 3 to 14 days after mRNA injections at a holding voltage of -70 mV. The $\alpha 7$ nACh receptor recordings were performed in Ca^{2+} -free Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM BaCl_2 , 5 mM HEPES, pH 7.4) to limit Ca^{2+} -activated chloride currents. The GABA recordings were performed in standard Ringer's solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 5 mM HEPES, pH 7.4). Drug and wash solutions were applied with a microcapillary "linear array" for rapid (subsecond) application of agonists. Currents were recorded on a computer (PCLamp 9.0; Molecular Devices, Sunnyvale, CA). Concentration-effect data were fit to a four-parameter logistic equation (GraphPad Software Inc., San Diego, CA).

Hippocampal Slice Preparation and Whole-Cell Patch-Clamp Recordings. Horizontal hippocampal slices (310 μm thick) from Wistar rats aged 14 to 18 days were cut with a vibratome in icy artificial cerebrospinal fluid (ACSF) containing 122 mM NaCl, 3.5 mM KCl, 1.3 mM MgCl_2 , 2 mM CaCl_2 , 1.2 mM NaH_2PO_4 , 25 mM NaHCO_3 , and 10 mM glucose that was continuously bubbled with carboxygen (95% O_2 /5% CO_2) [see Tu et al. (2009)]. Slices were recovered in the continuously carboxygenated ACSF at room temperature for 1 h before use. Whole-cell patch-clamp studies were done in a submerged chamber perfused continuously with carboxygenated ACSF at a rate of 1 ml/min. Patch-clamp of neurons was performed under the guidance of IR-differential interference contrast optics using an Axopatch 200B patch amplifier (Molecular Devices) with a glass pipette filled with an internal solution containing 120 mM potassium gluconate, 2 mM NaCl, 5 mM MgATP, 0.3 mM Na_2GTP , 20 mM KCl, 10 mM HEPES, 1 mM EGTA, and 11.3 mM D-glucose, pH ~ 7.2 to 7.3 and osmolarity of ~ 270 to 280 mOsm. Series resistances ranging from 7 to 40 M Ω were not compensated for during recordings, but were monitored throughout the experiments. Recordings were discarded when a significant ($> 20\%$) change of series resistance was detected and was not the result of drug application.

Modulation of CA1 Synaptic Plasticity. The synaptic strength of CA1 pyramidal neurons was monitored by recording evoked EPSCs with whole-cell voltage patch-clamp held at -60 mV as described above. Evoked EPSCs were induced by electrically stimulating an electrode (FHC Inc., Bowdoinham, ME) placed in mossy fiber or Schaffer collateral pathways, respectively, as indicated in Fig. 1A. The stimulation pulse was generated by a Grass S88 stimulator (Grass Instruments, Quincy, MA) with a photoelectric stimulus isolation unit (Grass Instruments) that provides a constant current output. The stimulation intensity was adjusted to evoke a postsynaptic current of approximately 50 pA, and the intensity was usually approximately 0.1 to 0.5 mA for 0.1 ms for mossy fiber stimulation and 0.05 to 0.2 mA for Schaffer collateral stimulation. After 10-min stable baseline recording of EPSCs, compounds (receptor modulators) in ACSF were bath-applied for 20 min and then washed away with fresh ACSF. The amplitude of EPSCs was normalized with the 10-min average before modulator application. The effects of the modulators on the EPSC amplitude were analyzed at two time points, immediately before the wash and 30 min after the wash, to distinguish the transient effect in the presence of the modulators and the lasting effects after washout.

Five-Choice Serial Reaction Time Test. Adult male Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) weighing approximately 230 g on arrival were used for these experiments. Animals were group-housed with access to food and water ad libitum. All animals were maintained on a 12-h light/dark cycle, and all experiments were run during the light cycle. All experimental procedures followed guidelines approved by the University of California Irvine Institutional Animal Care and Use Committee and were consistent with federal guidelines. After being given 2 days to acclimate, the rats were placed on a food-restricted diet (~ 10 g chow/animal/day) until they reached $\sim 90\%$ of their free-feeding body weight. During this initial food restriction period, the rats were given 45-mg sucrose pellets (approximately five pellets/animal/day; TestDiet, Richmond, IN) in addition to their normal chow for acclimation. Once testing began, the rats were maintained at $\sim 90\%$ of their free-feeding weight for the duration of the experiment, but no longer received sucrose pellets in their home cages. The five-choice serial reaction time trial (5-CSRTT) box (Coulbourn Instruments, Allentown, PA) is a specialized operant chamber with five nose pokes on one wall and a feeding trough on the opposite wall. Each trial began with a delay interval [intertrial interval (ITI)]. After the ITI, a light was briefly illuminated (stimulus duration) in a single nose poke, and a correct response at that nose poke during the stimulus duration or a defined period of time after the cue [lateral hold (LH)] resulted in a single 45-mg sucrose pellet dropping in the trough and the feeding trough was briefly illuminated (5 s). Five seconds after a

Fig. 1. Simultaneous activation of $\alpha 7$ nACh and inhibition of $GABA_A \alpha 5$ receptors enhances hippocampal CA1 synaptic plasticity. **A**, recording (R) and stimulating (S) electrode placement in CA1, CA3, and dentate gyrus (DG) areas of hippocampus. EPSC traces were recorded in CA1 neurons after electrical stimulation of mossy fiber or Schaffer collateral pathways. **B**, structures of compound **6** (left) and $\alpha 5IA$ (right). **C** to **F**, effects of compound **6** ($1 \mu M$) (**C**), $\alpha 5IA$ ($1 \mu M$) (**D**), or coapplication of compound **6** ($0.5 \mu M$) and $\alpha 5IA$ ($0.5 \mu M$) (**E**) on the amplitude of EPSCs recorded in CA1 neurons after stimulation of the mossy fiber pathway before ($\circ$), during ($\bullet$), or after ($\circ$) application of drugs. **F**, effects of compound **6** and $\alpha 5IA$ on the amplitude of EPSCs before wash (filled bars) or 30 min after wash (open bars) ($n = 4-5$ slices). *, $P = 0.0165$, **, $P = 0.0049$ before wash; **, $P = 0.008$, after wash, Student's t test comparison to baseline before drug application. **G**, effect of compound **6** (0.1 mg/kg i.p.) and $\alpha 5IA$ (0.03 mg/kg i.p.) on scopolamine (S; 1.25 mg/kg i.p.) disruption of performance in the rat 5-CSRTT model ($n = 5-6$ animals per group). One-way ANOVA, *, $P = 0.032$, S + vehicle, *, $P = 0.021$, S + $\alpha 5IA$, **, $P = 0.008$, S + compound **6**, Dunnett's post hoc test comparison with vehicle control. Data are mean $\pm$ S.E.M.

correct response, the next trial ITI began. A response during the ITI was a premature response and resulted in a 5-s timeout, which was followed by a new trial ITI. An incorrect response during the stimulus duration or LH resulted in a 5-s timeout, which was followed by a new trial ITI. No response by the end of the LH resulted in an omission and a 5-s timeout, which was followed by a new trial ITI. An additional response at any nose poke within 5 s of a correct response was a perseverative response and resulted in a 5-s timeout, which was followed by a new trial ITI. Animals were tested once per day, and each session lasted for 100 stimulus trials (correct + incorrect + omission) or 30 min, whichever came first. Percentage of correct performance was calculated by taking the number of correct responses divided by the total number of stimulus trials and transformed to a percentage by multiplying by 100. Premature responses and perseverative responses were recorded separately and did not affect the number of stimulus trials. Naive animals were started at a protocol that used a 30-s stimulus duration and 30-s lateral hold. In this experiment, the intertrial interval for all protocols was 5 s. Once an individual animal achieved 65% correct performance at any protocol, it was moved onto the next, more difficult protocol. The next protocol used a 10-s stimulus duration with a 20-s lateral hold, the following used a 3-s stimulus duration and a 10-s lateral hold, and the final protocol used a 1-s stimulus duration and a 5-s lateral hold. Once the animals reached a stable baseline percentage of correct performance on the final protocol, test drugs were administered. A stable baseline was defined as 2 consecutive days with performances within 10% of each other. Drugs were administered intraperitoneally at a volume of 1 ml/kg. The vehicle used in all cases was 2% DMSO, 8% solutol, and 90% saline. The animals were randomized into five treatment groups: vehicle, 1 mg/kg scopolamine, 1 mg/kg scopolamine with 0.1 mg/kg 4-083, 1 mg/kg scopolamine with 0.03 mg/kg $\alpha 5IA$, and 1 mg/kg scopolamine with 0.1 mg/kg 4-083 and 0.03 mg/kg $\alpha 5IA$. In some cases, animals that received vehicle treatment and maintained their baseline were retested with a different, randomized drug group. All other animals received only a single treatment.

Eight-Arm Radial Maze. We used the method as described previously (Ng et al., 2007). Adult male Sprague-Dawley rats ($n = 6$ /group) were treated daily for 7 days for the effects of 522-054 on memory performance. On each of these 7 days, the animals were injected intraperitoneally with either vehicle or 522-054 at 0.03, 0.1, or 0.3 mg/kg. Thirty minutes after dosing, animals were placed in the eight-arm radial maze and evaluated in this individual "trial," during which two types of errors were scored: 1) working memory errors (revisits to arms that had been previously baited on the same training trial) and 2) reference memory errors (visits to any of the four arms that had never been baited). A percentage of correct performance measure was then calculated by the following formula: per-

centage of correct performance = number of correct entries/(maximum number of correct choices + number of errors) $\times$ 100. In addition, the choice accuracy measure was the number of baited arm entries until a working memory error was made (entries to repeat).

Results

LTP in the Hippocampus Is Generated by Coapplication of an $\alpha 7$ nAChR-Positive Allosteric Modulator and $GABA_A \alpha 5$ -Negative Allosteric Modulator. Mossy fiber-stimulated hippocampal EPSCs recorded from the CA1 (Fig. 1A) are not enhanced in the presence of $1 \mu M$ compound **6**, a selective $\alpha 7$ nAChR positive allosteric modulator (PAM) (Ng et al., 2007) (Fig. 1, B and C). Application of $1 \mu M$ $\alpha 5IA$ (an $\alpha 5$ $GABA_A$ NAM; Attack, 2010) (Fig. 1B) caused an enhancement of EPSCs during drug exposure, but not after washout (Fig. 1, D and F), indicative of a lack of LTP generation. When both compounds were applied together at subthreshold concentrations ($0.5 \mu M$ each), a significant enhancement of EPSCs occurred that was maintained for at least 30 min after washout (Fig. 1, E and F). These results suggest that simultaneous inhibition of $\alpha 5$ $GABA_A$ Rs and enhancing $\alpha 7$ nAChRs causes a synergistic modification in the hippocampus such that an LTP of mossy fiber to CA1 EPSCs is produced. LTP is not observed after Schaffer collateral stimulation under similar treatment conditions.

Coapplication of an $\alpha 7$ nAChR PAM and $\alpha 5$ $GABA_A$ NAM Reverses Pharmacologically Induced Cognitive Deficits. The synergism between $\alpha 7$ nAChRs and $\alpha 5$ $GABA_A$ Rs is also observed in vivo. Negative modulation of $\alpha 5$ $GABA_A$ Rs or positive modulation of $\alpha 7$ nAChRs individually can elicit proattentive effects, but the interaction between both of these receptors on attention is unknown (Young et al., 2004; Attack, 2010). The 5-CSRTT model measures the attentional vigilance of an animal subject to obtain a food reward. In rats a subthreshold dose of either $\alpha 5IA$ (0.03 mg/kg i.p.) or compound **6** (0.1 mg/kg i.p.) alone did not reverse scopolamine-induced attentional impairment in 5-CSRTT (Fig. 1G) (Sternfield et al., 2004; Young et al., 2004; Ng et al., 2007). However, when both drugs were administered simultaneously, the scopolamine-induced deficit was reversed such that the response was not significantly different from vehicle control (Fig. 1G). Consistent with the hippocampal slice data,

the in vivo results suggest that a synergistic effect on hippocampal-mediated behavior can be produced by simultaneously inhibiting $\alpha 5$ GABA_AR and enhancing $\alpha 7$ nAChR-mediated activities.

$\alpha 7$ nAChR PAM and GABA_A $\alpha 5$ NAM Embodied in a Single Molecule. We designed a compound that has “dual activity” to confirm that synergism can be produced by the simultaneous inhibition of $\alpha 5$ GABA_A and enhancement $\alpha 7$ nAChR-mediated activities. In individual frog oocytes expressing either of these two LGICs, 522-054 (Fig. 2A, inset) is an $\alpha 5$ GABA_AR NAM and a selective $\alpha 7$ nAChR PAM (Fig. 2A). The compound simultaneously enhances and inhibits $\alpha 7$ nACh and $\alpha 5$ GABA_A receptor-mediated actions, respectively, in a concentration-dependent manner. 522-054 enhanced mossy fiber-stimulated EPSCs in the CA1 at 0.3 and 1.0 μ M but not at 0.1 μ M (Figs. 2, B, C, D, and G). Preapplication of flumazenil (a BZ site antagonist) blocked 522-054 elicited enhancement of EPSCs after mossy fiber stimulation (Fig. 2, E and G), indicating that 522-054 effects on $\alpha 5$ GABA_ARs are mediated through the BZ site. Likewise, methyllycaconitine (MLA), a selective $\alpha 7$ nAChR antagonist, blocked the 522-054 effect on EPSCs (Fig. 2, F and G).

522-054 Is Nootropic. 522-054 was evaluated for proattentive and procognitive effects in the rat 5-CSRTT and radial arm maze (RAM), respectively. In 5-CSRTT, scopol-

amine (1.25 mg/kg i.p.) caused a significant reduction in performance compared with vehicle control (Fig. 2H). 522-054 dose-dependently changed the percentage of baseline performance relative to scopolamine-treated animals in an inverted U-shaped dose response typical of nicotinic agonists (Fig. 2H) (Picciotto, 2003). Peak activity of 522-054 occurred at 0.003 mg/kg, an exceptionally low dose given the in vitro potency of the compound to modulate either $\alpha 5$ GABA_A or $\alpha 7$ nACh receptors (Fig. 2A). MLA or flumazenil blocked the effect of 522-054 on scopolamine-induced attentional deficits (Fig. 2H), suggesting that the potent effect of 522-054 on 5-CSRTT depends on $\alpha 7$ nACh or $\alpha 5$ GABA_A receptor modulation, respectively.

The RAM behavioral paradigm evaluates both the attentional and cognitive elements required for learning and memory (Rezvani and Levin, 2001). In this widely used test for hippocampal-based cognitive performance animals rely on spatial navigation cues to find food rewards at the end of the maze arms. Animal studies have demonstrated that nicotine and other more selective $\alpha 7$ nAChR agonists can improve performance, whereas nicotinic antagonists impair performance in the RAM (Levin et al., 2002). We examined the effects of 522-054 on pharmacological disruption of acquired memory in the eight-arm RAM using a partial baiting paradigm (four baited arms selected randomly for each rat) (Ng et

Fig. 2. LGIC-mediated synergy induced by 522-054. A, effect of 522-054 on nicotine EC₅₀ and GABA EC₂₀ currents in human $\alpha 7$ nAChRs and GABA_A $\alpha 5\beta 3\gamma 2$ receptors, respectively. B to F, EPSC amplitude recorded in CA1 neurons after electrical stimulation of the mossy fiber pathway before (○), during (●), or after (○) application of 522-054 at 0.1 (B), 0.3 (C), or 1.0 μ M (D) with flumazenil (0.1 μ M) (E) or MLA (10 nM) (F) pretreatment. G, EPSC amplitude before wash (filled bars) and 30 min after washout (open bars) ($n = 3-4$ slices). *, $P = 0.014$, 0.3 μ M before wash; *, $P = 0.036$, MLA + 1 μ M; **, $P = 0.008$, 0.3 μ M after wash; **, $P = 0.004$, 1 μ M before wash; **, $P = 0.006$, 1 μ M after wash, Student's t test comparison with baseline before drug application. H, rat 5-CSRTT model performance, vehicle (V), scopolamine (S; 1.25 mg/kg i.p.), flumazenil (F; 10 mg/kg i.p.), and MLA (M; 3 mg/kg i.p.) ($n = 6-15$ animals per group). One-way ANOVA, **, $P = 0.00445$, Bonferroni's post hoc test comparison to scopolamine. I, scopolamine (S)-disrupted (1 mg/kg i.p.; filled bars) measures of memory in eight-arm radial maze compared with vehicle controls (open bars) ($n = 5-6$ animals per group). One-way ANOVA, **, $P = 0.0002$, entries to repeat; **, $P = 0.0002$, percentage of performance; **, $P = 0.0006$, total time, Dunnett's post hoc test comparison with vehicle + scopolamine. Data are mean $\pm$ S.E.M.

al., 2007) previously used to assess animal performance on this task (Levin, 2002; Ortega-Alvaro et al., 2006). We treated rats with 522-054 or vehicle during the first six trials and determined whether pharmacological disruption of acquired memory by scopolamine on the seventh trial day was reversible by 522-054. Scopolamine (1 mg/kg i.p.)-treated rats given 522-054 (0.03, 0.1, and 0.3 mg/kg i.p.) showed significant improvement in choice accuracy (entries to repeat; Fig. 2I) compared with scopolamine- and vehicle-treated rats (Fig. 2I). Rats treated with 0.03 mg/kg 522-054 also showed a nonsignificant trend toward reduction of short-term memory impairment (working memory errors; Fig. 2I) and preservation of long-term memory (reference memory errors; Fig. 2I) caused by scopolamine. At the same dose, a significant increase in performance (Fig. 2I) and reduced total time to maze completion (Fig. 2I) were observed. Collectively, these results suggest that 522-054 is remarkably potent at restoring learning, memory, and attention acutely disrupted by scopolamine.

Discussion

The physiological synergy between $\alpha 5$ GABA_A and $\alpha 7$ nACh receptors observed in the in vitro slice preparations and in the learning and memory paradigms can be explained by several observations related to each type of LGIC. The chemical-induced LTP observed by coapplication of an $\alpha 5$ GABA_AR NAM and $\alpha 7$ nAChR PAM or by application of a single "dual modulator" of both receptor types probably occurs by a lowering of the excitatory drive threshold caused by $\alpha 5$ GABA_AR blockade and enhanced glutamatergic signaling mediated by presynaptic and postsynaptic $\alpha 7$ nAChRs in the recurrent synapses of CA3 pyramidal cells. Inhibition of $\alpha 5$ GABA_ARs causes reduced shunting inhibition without significantly changing membrane potential. This change alters the input-output relationship of neurons and reduces the excitatory drive required to generate an action potential (Bonin et al., 2007; Martin et al., 2010). Complete loss of $\alpha 5$ GABA_AR-mediated tonic inhibition results in spontaneous gamma oscillations in the CA3 pyramidal cell layer, presumably because of the recurrent connections within the CA3 (Glykys et al., 2008). Hippocampal gamma frequency oscillations originating in the CA3 and CA1 regulate network activity that promotes the encoding of spatial information and formation of episodic memories (Whittington et al., 1995; Traub et al., 1996; Towers et al., 2004). $\alpha 7$ nAChRs in the CA3 are known to be located presynaptically and enhance glutamate release onto postsynaptic (RS)- α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and *N*-methyl-D-aspartic acid receptors because application of either 6,7-dinitroquinoxaline-2,3-dione or D(2)-2-amino-5-phosphonopentanoate blocks excitation of CA3 neurons by selective or nonselective agonists of $\alpha 7$ nAChRs (Huang et al., 2010). Moreover, the activation of $\alpha 7$ nAChRs in the CA3 can augment tetanically stimulated LTP because MLA routinely blocks the effect (Langostena et al., 2008; Huang et al., 2010).

An electrical stimulation can produce short-term potentiation in the presence of an $\alpha 5$ GABA_AR NAM alone because $\alpha 5$ GABA_AR inhibition reduces the threshold for action potential (Fig. 1D). The probability of LTP is increased in the additional presence of an $\alpha 7$ nAChR PAM, because properly timed presynaptic and postsynaptic nAChR activity coincides

with postsynaptic glutamate depolarization. Thus PAMs are optimally suited to enhance $\alpha 7$ nAChR activity, because they intrinsically preserve the temporal integrity of neuronal transmission. Postsynaptic depolarization mediated by $\alpha 7$ nAChRs amplifies postsynaptic glutamatergic currents, converting short-term potentiation to LTP, by multiple mechanisms including relieving the Mg²⁺ block of *N*-methyl-D-aspartic acid receptors and direct or indirect production of calcium signals that contribute to LTP (Ge and Dani, 2005).

Measurement of the appropriate concentration responses for each receptor type to uncover the minimum level of each drug required to produce LTP in the hippocampal slices can be complicated. The simplest interaction between two targets is to show that two ineffective concentrations, when combined, produce a response that neither concentration alone does. The intent of our studies was to show that using half the inactive concentration of each compound combined could produce an effect where neither alone could. Concentration responses could be conducted to "titrate" the level at which, in the presence of a fixed concentration of the other drug, the synergy of the combination is revealed. Alternatively, the absolute minimum combination levels of both compounds required for the synergistic effect can be determined. Future studies can be performed to precisely quantitate what we have already shown qualitatively. Complications also extend to the behavioral evaluation of two compounds in the 5-CSRTT model and the potential synergy to reverse scopolamine-induced cognitive deficits. The in vivo models to assess this dose-response relationship could potentially be confounded by pharmacokinetic interactions whereby one compound influences the rate of absorption, distribution, metabolism, and excretion of the other compound to cause apparent synergism. However, such a possibility is reduced by the observations with 522-054 that recapitulates the actions of simultaneous exposure to its component mechanisms observed with the two individual compounds.

We hypothesize that the synergistic effect of 522-054 on hippocampal EPSCs originates in the CA3 because under similar recording and bath procedures nontetanic stimuli delivered to the Schaffer collaterals do not produce LTP. The role of $\alpha 7$ nAChRs on GABAergic interneurons in the CA1 may explain why nontetanic Schaffer collateral stimulation is not potentiated by 522-054 or the combination of a $\alpha 7$ nAChR PAM and a GABA_A $\alpha 5$ NAM. Postsynaptic $\alpha 7$ nAChRs in the CA1 promote GABA release from interneurons to inhibit pyramidal cell firing rate (Ji et al., 2001). The postsynaptic $\alpha 7$ nAChRs that enhance GABA interneuron activity are opposed by presynaptic $\alpha 7$ nAChRs in the CA1 that promote glutamate release directly onto pyramidal cells. This counterbalance is not apparent in the CA3, which suggests why $\alpha 7$ nAChRs can synergize with $\alpha 5$ GABA_ARs in this particular hippocampal region.

Synergism between two related LGICs can manifest as a chemical induction of LTP in the hippocampus. This mechanism is reflected behaviorally in the RAM and 5-CSRTT paradigms by the microgram/kilogram potencies of 522-054, because it is an allosteric modulator of $\alpha 5$ GABA_ARs and $\alpha 7$ nAChRs. We speculate that this apparent manifestation of behavior synergy depends on the preservation of the spatial and temporal integrity signaling that only an allosteric modulatory mechanism can provide. We show that 522-054 selectively reverses the general decline in cognitive perfor-

mance caused by scopolamine because of potential synergy induced by simultaneous modulation of $\alpha 7$ nACh and GABA $_A$ $\alpha 5$ receptors. It is important to also consider the use of scopolamine as the single agent to disrupt cognitive function in this interpretation. For example, scopolamine disruption of cognitive performance in rats could be caused by nonspecific effects such as altered locomotor activity that could confound the interpretation of whether scopolamine is an appropriate drug to induce cognitive impairment. For the 5-CSRTT model locomotor activity is not likely an issue because the doses used do not impair locomotor activity (Ortega-Alvaro et al., 2006). Scopolamine's utility as a drug to disrupt cognition revolves around whether an agent is intended to be used to selectively or nonselectively disrupt cognition. The pitfalls of the use of scopolamine in this regard have been highlighted (Klinkenberg and Blokland, 2010). Although it is suggested that a more selective cognitive impairment could be produced by selective muscarinic M1 receptor antagonism, an M1-selective muscarinic-mediated deficit may not fully recapitulate the diffuse cognitive deficit (e.g., on working memory, attention, strategy) observed in human diseases such as Alzheimer's. Moreover M1-selective antagonists have not yet been fully validated in this role. Nevertheless, the use of a nonselective drug, such as scopolamine, would probably be a more stringent assessment of the overall efficacy of a cognitive enhancer because physiological antagonism of the cognitive deficit is being measured rather than a pharmacological antagonism of a highly selective agent that disrupts cognition.

522-054 demonstrates the benefits of dual allosteric modulation of different receptors mediating similar outcomes (i.e., improved learning and memory) to produce synergies that result in enhanced pharmacological potency. This synergistic strategy can be translated clinically into optimal therapeutic efficacy, reduced dosage and adverse effects, mitigated dependence on polypharmacy, and the concomitant side effects that are problematic in the therapeutic management of Alzheimer's disease or other diseases of cognitive impairment (Buccafusco, 2009).

Authorship Contributions

Participated in research design: Johnstone, Gu, Yoshimura, Villegier, Hogenkamp, Whittemore, Belluzzi, Yakel, and Gee.

Conducted experiments: Johnstone, Gu, Yoshimura, Villegier, Hogenkamp, Whittemore, Huang, and Tran.

Contributed new reagents or analytic tools: Johnstone and Hogenkamp.

Performed data analysis: Johnstone, Gu, Yoshimura, Villegier, Whittemore, Belluzzi, Yakel, and Gee.

Wrote or contributed to the writing of the manuscript: Johnstone, Gu, Yoshimura, Hogenkamp, Whittemore, and Gee.

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