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Differential Modulation of Brain Nicotinic Acetylcholine Receptor Function by Cytisine, Varenicline, and Two Novel Bispidine Compounds: Emergent Properties of a Hybrid Molecule[S]

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ABSTRACT

Partial agonist therapies for the treatment of nicotine addiction and dependence depend on both agonistic and antagonistic effects of the ligands, and side effects associated with other nAChRs greatly limit the efficacy of nicotinic partial agonists. We evaluated the in vitro pharmacological properties of four partial agonists, two current smoking cessation drugs, varenicline and cytisine, and two novel bispidine compounds, BPC and BMSP, by using defined nAChR subtypes expressed in *Xenopus laevis* oocytes and human embryonic kidney 293 cells. Similar to varenicline and cytisine, BPC and BMSP are partial agonists of $\alpha4\beta2$ nAChRs, although BMSP produced very little activation of these receptors. Unlike varenicline and cytisine, BPC and BMSP showed desired low activity. BPC produced mecamylamine-sensitive steady-state activation of $\alpha4^*$ receptors that was not evident with BMSP. We evaluated the modulation of $\alpha4^*$- and $\alpha7$-mediated responses in rat lateral geniculate nucleus (LGN) neurons and hippocampal stratum radiatum (SR) interneurons, respectively. The LGN neurons were sensitive to a very low concentration of varenicline, and the SR interneuron responses were also sensitive to varenicline at a submicromolar concentration. Although 300 nM BPC strongly inhibited the ACh-evoked responses of LGN neurons, it did not inhibit the $\alpha7$ currents of SR interneurons. Similar results were observed with 300 nM BMSP. Additionally, the bispidine compounds were efficacious in the mouse tail suspension test, demonstrating that they affect receptors in the brain when delivered systemically. Our data indicate that BPC and BMSP are promising $\alpha4\beta2^*$ partial agonists for pharmacotherapeutics.

Introduction

Partial agonist-based therapies are attractive for indications where there is a natural or induced imbalance in a neural regulatory system, because partial agonists can function as activity buffers, preventing overactivation by exogenous or endogenous agents and providing basal activity on their own in the absence of other stimuli. Both of these forms of action may be important for management of drug addiction, dependence, and mood symptoms associated with nicotine and cigarette smoking.

There is a rich variety of nicotinic acetylcholine receptor (nAChR) subtypes in the brain and periphery (for review, see Millar and Gotti, 2009). Receptors are pentamers, and there are ten α-subunits in vertebrates and seven non-alpha subunits ($\beta1$, $\beta2$, $\beta3$, $\beta4$, γ, δ, and ε), of which $\alpha2-\alpha10$ and $\beta2-\beta4$ are expressed in neurons and the others in muscle. Studies of knockout mice (Picciotto and Kenny, 2013) have implicated receptors containing $\alpha4$ and $\beta2$ subunits in the reinforcing/addicting effects of nicotine. Receptors containing these subunits at varying ratios and sometimes also incorporating $\alpha5$ subunits constitute the majority of the high-affinity nicotine-binding receptors in brain. Partial agonists at ($\alpha4\beta2^*$) nAChRs may be useful not only for managing nicotine reward and dependence but also for managing the mood and depression symptoms that are comorbid with smoking and smoking cessation (Mineur and Picciotto, 2010).

The most abundant types of $\alpha4$ subunit-containing receptors (collectively designated as $\alpha4^*$ receptors) include one configuration classified as a low sensitivity (LS) type with three $\alpha4$ subunits and two $\beta2$ subunits [$\alpha4(3)\beta2(2)$], which responds to ACh and nicotine with progressively larger responses across a relatively wide range of concentrations. A second configuration is classified as a high sensitivity (HS) subtype with two $\alpha4$ subunits and three $\beta2$ subunits [$\alpha4(2)\beta2(3)$],

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ABBREVIATIONS: ACh, acetylcholine; ACSF, artificial cerebral spinal fluid; BPC, 2-(5-(3,7-diazabicyclo[3.3.1]nonan-3-yl)pyridin-3-yloxy)-*N,N*-dimethylethanamine; BMSP, 1-(3,7-diazabicyclo[3.3.1]nonan-3-yl)-2-(4-(methylsulfonyl)phenyl)ethanone; mecamylamine, *N*-2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride; HS, high sensitivity; LGN, lateral geniculate nucleus; LS, low sensitivity; nAChR, nicotinic acetylcholine receptor; RIC-3, human resistance-to-cholinesterase 3; SD, Sprague-Dawley; SR, stratum radiatum.

which responds to low concentrations of ACh and nicotine and with maximal currents limited at high concentrations. A third type of HS $\alpha4^*$ receptor has two $\alpha4$ subunits and two $\beta2$ subunits and an $\alpha5$ subunit [$\alpha4(2)\beta2(2)\alpha5$]. The generation of nAChR subunit concatamers (Zhou et al., 2003; Kuryatov and Lindstrom, 2011; Stokes and Papke, 2012) has allowed for heterologous expression of receptors with defined subunit composition, an approach we have taken advantage of in this study.

Another class of receptors that has been associated with nicotine reward contains the $\alpha6$, $\beta2$, and other subunits (such as $\alpha4\beta2\alpha6\beta2\beta3$). The $\alpha6$ and $\beta3$ subunits are highly expressed in catecholaminergic neurons, particularly in the ventral tegmentum and substantia nigra. In ex vivo preparations, blocking these $\alpha6^*$ receptors with highly selective toxins decreases nicotine-evoked dopamine release (Grady et al., 2002), and blockade or knockout of these nAChRs in vivo decreases nicotine self-administration (Pons et al., 2008; Brunzell et al., 2010; Gotti et al., 2010).

In the pursuit of drugs to manage nicotine dependence, three classes of receptors may be hypothesized to be "off-target": receptors of the neuromuscular junction, receptors in autonomic ganglia ($\alpha3\beta4^*$), and the homopentamers of $\alpha7$ subunits. Although muscle-type receptors are unequivocally off target, receptors containing $\alpha3$, $\beta4$, and $\alpha5$ subunits are also found in limited areas of the brain, where they have been implicated in aversive effects of high nicotine dosages. Polymorphisms in the $\alpha3$-$\beta4$-$\alpha5$ gene cluster have also been linked to smoking behavior. Activation of $\alpha7$ receptors in the nucleus accumbens may decrease the motivation to self-administer nicotine (Brunzell and McIntosh, 2012) and has been implicated in the drive of people with schizophrenia to self-medicate by smoking (Leonard et al., 2007). Deficiencies in $\alpha7$ function have been implicated in schizophrenia, and individuals with schizophrenia smoke at very high rates. These observations suggest that $\alpha7$- and $\alpha3$-containing receptors should not necessarily be considered off target but rather they may need to be targeted differently than $\alpha4^*$ and $\alpha6^*$ receptors. For people at risk for mental illness, it is likely that the function of $\alpha7$ receptors should be especially spared.

The two drugs currently used as smoking cessation aids, cytisine and varenicline, have significant activity at $\alpha7$ and $\alpha3^*$ receptors, which may limit their utility and generate side effects. We report two novel compounds, BPC and BMSP (Fig. 1A), which modulate the brain nAChRs most strongly implicated in addiction, with reduced likelihood of perturbing the function of other nAChR such as the ganglionic $\alpha3\beta4$ subtypes or brain $\alpha7$ receptors. The extension of our studies with these agents will therefore be useful to ultimately evaluate which nAChR subtypes are the best targets for treating nicotine dependence. The development and characterization of such agents for smoking cessation aids may also have crossover value for other indications such as augmentation therapy for depression (Mineur and Picciotto, 2010).

Materials and Methods

Agents. Solvents and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Cell culture supplies were purchased from Life Technologies (Grand Island NY). Acetylcholine chloride (ACh), atropine, *N*-2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine hydrochloride (mecamylamine), dihydro-β-erythroidine hydrobromide, methyllycaconitine citrate, (−)-nicotine, and cytisine were purchased from Sigma-Aldrich. Varenicline tartrate was purchased from Tocris/R&D Systems, Inc. (Minneapolis, MN). BPC and BMSP were synthesized and provided by Dr. D. Guendisch. Fresh ACh stock solutions were made each day of experimentation. Stock solutions of BPC and BMSP were prepared in dimethyl sulfoxide and stored at −20°C. Working solutions were prepared freshly each day at the desired concentration from the stored stock.

Heterologous Expression of nAChRs in *Xenopus laevis* Oocytes. Mouse muscle nAChR $\alpha1$, $\beta1$, and δ clones used for receptor expression in *X. laevis* oocytes were obtained from Dr. J. Boulter (University of California, Los Angeles, CA), and the mouse ε clone was provided by Dr. P. Gardner (University of Massachusetts Medical School, Worcester, MA). Human nAChR clones and concatamers were obtained from Dr. J. Lindstrom (University of Pennsylvania, Philadelphia, PA). The human resistance-to-cholinesterase 3 (RIC-3) clone, obtained from Dr. M. Treinin (Hebrew University, Jerusalem, Israel), was coinjected with $\alpha7$ to improve the level and speed of $\alpha7$ receptor expression without affecting the pharmacological properties of the receptors (Halevi et al., 2003). Subsequent to linearization and purification of the plasmid cDNAs, cRNAs were prepared using the mMessage mMachine in vitro RNA transfection kits (Ambion/Life Technologies, Austin, TX).

Oocytes were surgically removed from mature female *X. laevis* frogs (Nasco, Ft. Atkinson, WI) and injected with appropriate nAChR subunit cRNAs as described previously (Papke and Stokes, 2010). Frogs were maintained in the Animal Care Service facility of the University of Florida, and all procedures were approved by the University of Florida Institutional Animal Care and Use Committee. In brief, the frog was first anesthetized for 15–20 minutes in 1.5 liter frog-tank water containing 1 g of ethyl 3-aminobenzoate methanesulfonate buffered with sodium bicarbonate. The harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals,

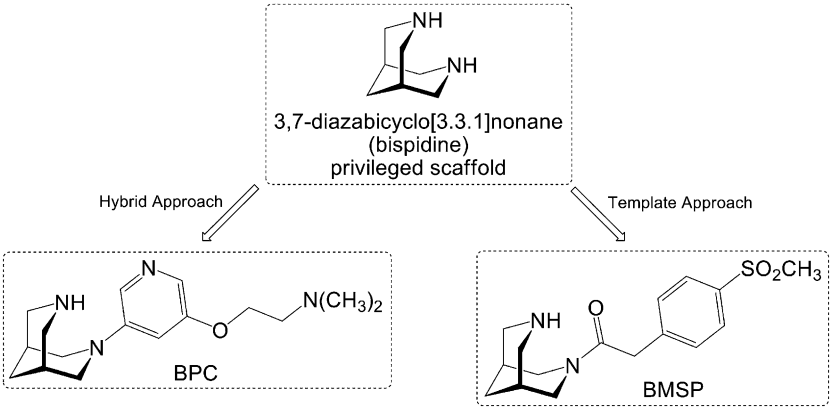

Fig. 1. Chemical structures of BPC and BMSP.

Freehold, NJ) for 2 hours at room temperature in a calcium-free Barth's solution (containing in mM: 88 NaCl, 1 KCl, 2.38 $NaHCO_3$, 0.82 $MgSO_4$, 15 HEPES, and 12 mg/l tetracycline, pH 7.6) to remove the follicular layers. Stage V oocytes were subsequently isolated and injected with 50 nl of 5–20 ng of nAChR subunit cRNA. Recordings were carried out 1–7 days after injection.

Two-Electrode Voltage Clamp Electrophysiology. Experiments were conducted using OpusXpress 6000A (Molecular Devices, Union City, CA) (Papke and Stokes, 2010). Both the voltage and current electrodes were filled with 3 M KCl. Oocytes were voltage-clamped at –60 mV. The oocytes were bath-perfused with Ringer's solution (containing in mM: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 HEPES, and 0.001 atropine, pH 7.2) at 2 ml/min for $\alpha7$ receptors and at 4 ml/min for other subtypes. To evaluate the effects of different partial agonists on ACh-evoked responses of various nAChR subtypes expressed in oocytes, baseline conditions were defined by two initial applications of ACh made before coapplications of experimental compounds with the control ACh. The agonist solutions were applied from a 96-well plate via disposable tips, and the test partial agonists were either coapplied with ACh by the OpusXpress pipette delivery system for acute coapplication experiments or introduced into the bath using the OpusXpress system to switch the running buffer for bath application experiments. For the concentration-response study, drug applications alternated between ACh controls and experimental compounds. Unless otherwise indicated, drug applications were 12 seconds in duration followed by a 181-second washout period for $\alpha7$ receptors and 6 seconds with a 241-second washout for other subtypes. A typical recording for each oocyte contained two initial control applications of ACh, an experimental compound application, and then a follow up control application of ACh to determine the desensitization or rundown of the receptors. The control ACh concentrations were 30 μM for $\alpha1\beta1\varepsilon\delta$, 60 μM for $\alpha7$, 100 μM for $\alpha3\beta4$, 10 μM for $(\alpha4)_2(\beta2)_3$ (the HS form), 100 μM for $(\alpha4)_3(\beta2)_2$ (the LS form), 10 μM for $\alpha4\beta2\alpha5$, and 30 μM for $\beta3\alpha4\beta2\alpha6\beta2$.

Data were collected at 50 Hz, filtered at 20 Hz, analyzed by Clampfit 9.2 (Molecular Devices) and Excel 2003 (Microsoft, Redmond, WA), and normalized to the averaged peak current or net charge response of the two initial ACh controls (Papke and Porter Papke, 2002). Data were expressed as means ± S.E.M. from at least four oocytes for each experiment and plotted by KaleidaGraph 3.6.2 (Synergy Software, Reading, PA).

Whole-Cell Patch-Clamp Electrophysiology. The A7R3HC10 cells stably expressing human $\alpha7$ and human RIC-3 were generated from low passage number human embryonic kidney 293 cells obtained from American Type Culture Collection (Manassas, VA) (Williams et al., 2012). The A7R3HC10 cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.45 mg/ml Geneticin selective antibiotic (G418 sulfate) and 0.015 mg/ml hygromycin at 37°C with 5% CO_2. For normal passaging, cells were dissociated with 1 mM ethylenediamine tetraacetic acid (EDTA) in calcium- and magnesium-free Hanks' balanced saline solution (Life Technologies) to avoid nonselective damage to the $\alpha7$ nAChRs expressed on the cell surface. Cells with 8–12 passages after stable transfection were used for whole-cell patch-clamp recordings.

The 12-mm glass coverslips (Thermo Fisher Scientific) were coated with 0.1 mg/ml poly-D-lysine (Sigma-Aldrich) at 37°C for 5 minutes. A7R3HC10 cells were plated onto the coverslips 1–4 days before recording. Whole-cell recordings were performed at room temperature using an Axopatch 200B amplifier (Molecular Devices). Briefly, cells were bathed in an external solution containing (in mM) 165 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 5 HEPES, and 0.001 atropine, pH 7.35. Patch pipettes (3–5 MΩ) were pulled from borosilicate glass (o.d. of 1.5 mm and i.d. of 0.86 mm; Sutter Instruments, Novato, CA) using a Flaming/Brown micropipette puller (Model P-97; Sutter Instruments) and filled with an internal solution containing (in mM) 120 CsCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES, and 5 MgATP, pH 7.35. Cells were voltage-clamped at −70 mV and perfused with external solution at a flow rate of 4 ml/min. Local application of 1 mM ACh was made every 60 seconds using single-barrel pipettes attached to a Picospritzer III pressure system

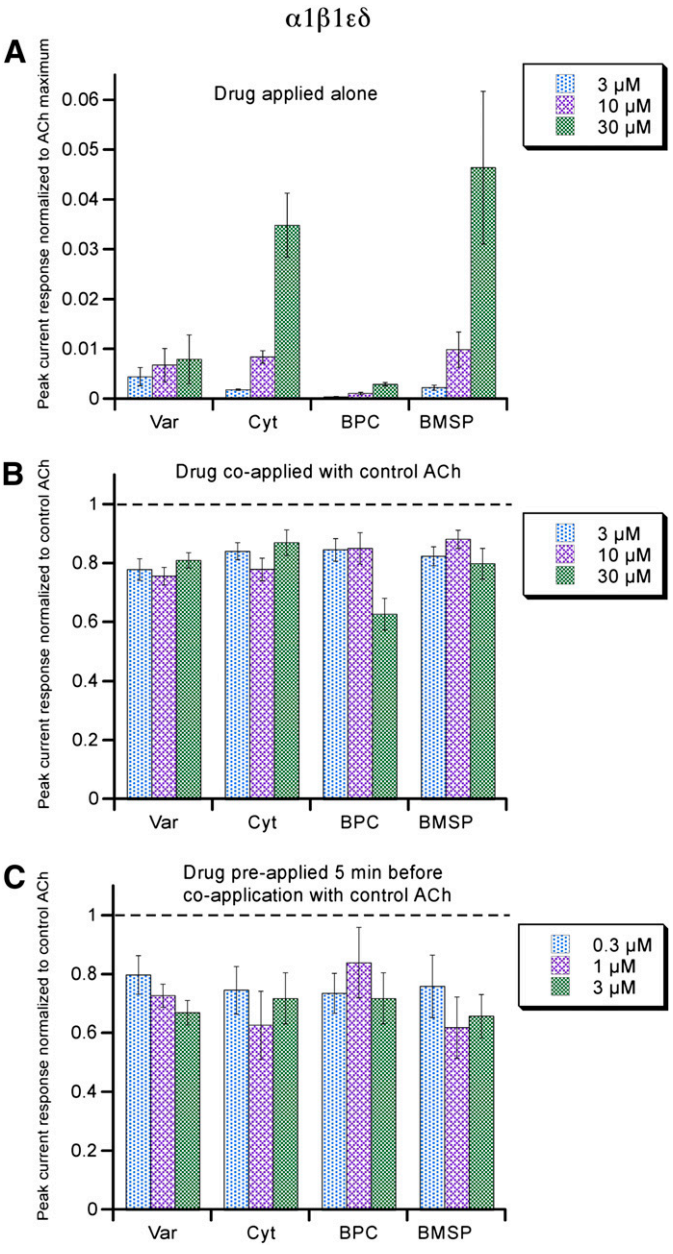

Fig. 2. Effects of various concentrations of varenicline (Var), cytisine (Cyt), BPC, and BMSP on mouse $\alpha1\beta1\varepsilon\delta$ nAChR expressed in *X. laevis* oocytes. (A) The agonist activity of Var, Cyt, BPC, and BMSP was characterized by comparing the peak current responses evoked by each compound to the maximum responses evoked by 30 μM ACh. (B) Varenicline, cytisine, BPC, or BMSP was coapplied with 30 μM ACh. The antagonist activity of each compound was characterized by normalizing the responses to the averaged control responses evoked by 30 μM ACh alone. (C) Varenicline, cytisine, BPC, or BMSP was preapplied for 5 minutes before the coapplication with 30 μM ACh. The antagonist activity of each compound was characterized by normalizing the responses to the averaged ACh control responses. Data represent the normalized averages (± S.E.M.) in terms of peak amplitude from 4 to 8 oocytes.

(General Valve Corp., Fairfield, NJ) with Teflon tubing (10–20 psi for 3 seconds). The drug pipette was positioned approximately 10–15 μm from the cell and loaded with 1.5 mM ACh because of the 1.5-fold dilution factor inherent in the picospritzer drug delivery (Williams et al., 2012). Five baseline responses induced by ACh alone were initially recorded, followed by a 20-minute bath application of specific agonist or partial agonist. To minimize the mixture of control solution (external solution alone) and drug solution (external solution containing specific

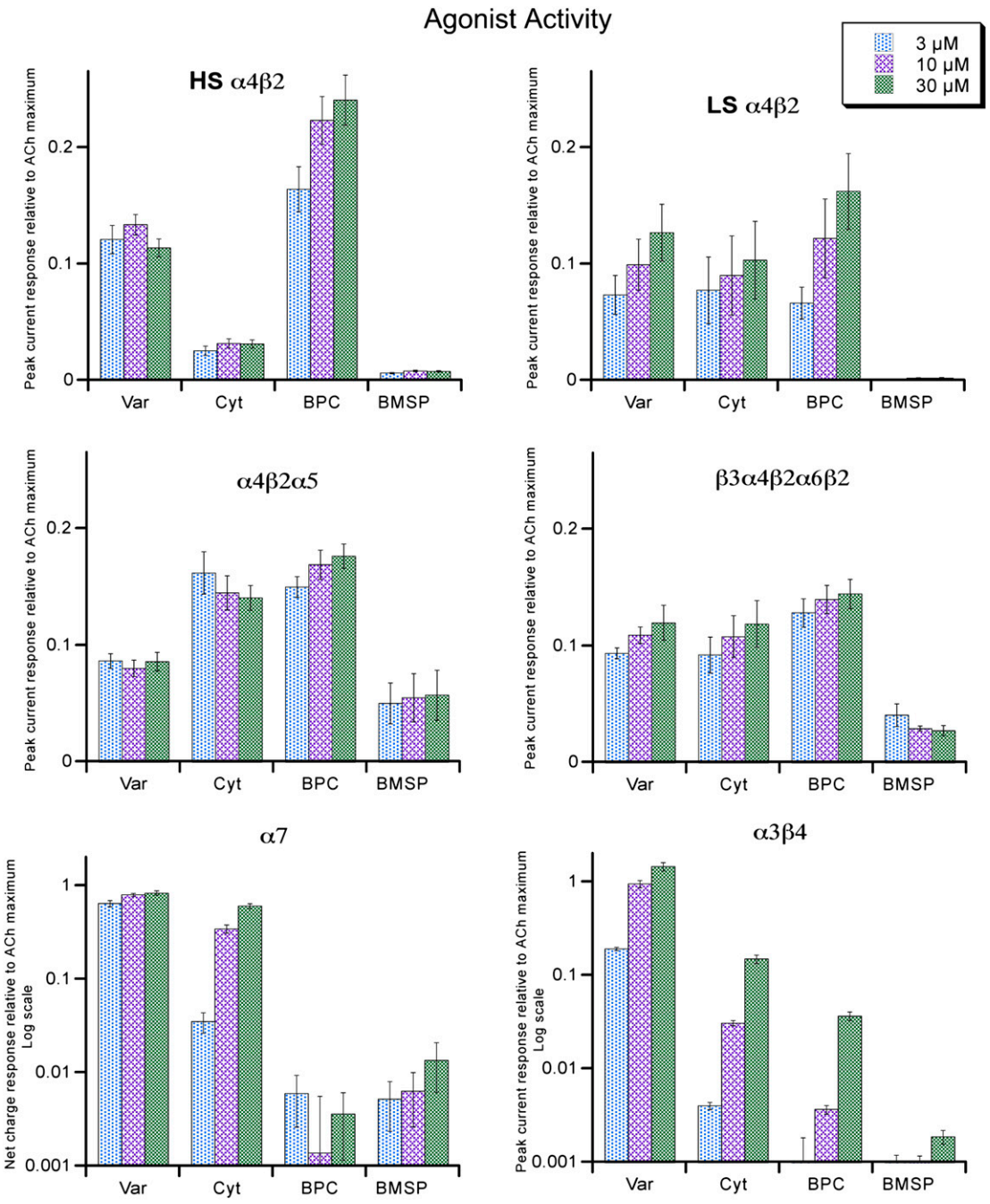

Fig. 3. Summary of the agonist activity of varenicline (Var), cytisine (Cyt), BPC, and BMSP obtained with human $(\alpha4)_2(\beta2)_3$ (HS $\alpha4\beta2$), $(\alpha4)_3(\beta2)_2$ (LS $\alpha4\beta2$), $\alpha4\beta2\alpha5$, $\beta3\alpha4\beta2\alpha6\beta2$, $\alpha7$, and $\alpha3\beta4$ nAChR expressed in *X. laevis* oocytes. Pure populations of HS $\alpha4\beta2$, LS $\alpha4\beta2$, and $\alpha4\beta2\alpha5$ nAChR were obtained by coexpressing the human $\beta2$-6-$\alpha4$ concatamer with monomeric $\alpha4$, $\beta2$, and $\alpha5$ (Zhou et al., 2003; Kuryatov et al., 2008; Papke et al., 2011), respectively. The acute application of these compounds activated small currents in oocytes expressing specific nAChR subtypes. The partial agonist activity of these compounds was characterized by comparing the responses (measured as peak current for non-$\alpha7$ and net charge for $\alpha7$) evoked by varying concentrations of varenicline, cytisine, BPC, and BMSP to the maximum responses evoked by ACh. The control ACh concentrations were: 10 μM for HS $\alpha4\beta2$, 100 μM for LS $\alpha4\beta2$, 10 μM for $\alpha4\beta2\alpha5$, 30 μM for $\beta3\alpha4\beta2\alpha6\beta2$, 60 μM for $\alpha7$, and 100 μM for $\alpha3\beta4$. Data are shown as the averaged normalized data ($\pm$ S.E.M.) from $n \geq 4$ oocytes at each condition.

agent), a Valve Driver II fluid control system (General Valve) was used to rapidly switch the running buffer. Recordings were filtered to 5 kHz and digitized at 20 kHz with a DigiData 1322A digitizer (Molecular Devices) using Clampex 9.2 (Molecular Devices). The access resistance, input resistance, and whole-cell capacitance were monitored throughout the experiment by a 10-millisecond/10-mV pulse before each response. Data were analyzed with Clampfit 10.3 (Molecular Devices) and Excel 2003. Cells with access resistance >40 MΩ or holding current >200 pA were excluded from analysis. Responses were measured as peak currents. Data were plotted by KaleidaGraph and represented as means ± S.E.M. of 4–7 cells.

Rat Brain Slice Recording. Preparation of rat brain slices and whole-cell patch-clamp recordings were carried out as described previously (López-Hernández et al., 2009). All procedures involving animals were approved by the University of Florida Institutional Animal Care and Use Committee and were in accord with the National

Institutes of Health Guide for the Care and Use of Laboratory Animals. Young male Sprague-Dawley (SD) rats were anesthetized with isoflurane (Patterson Veterinary Supply, Inc., Devons, MA) and swiftly decapitated. For whole-cell recordings of lateral geniculate nucleus (LGN) neurons, male SD rats of postnatal day 22–31 were used, while for interneurons of the stratum radiatum (SR), male SD rats of postnatal day 22–29 were used. Transverse (300 μm) whole-brain slices were prepared using a vibratome (Pelco, Redding, CA) and a high Mg^{2+}/low Ca^{2+} ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.5 $MgSO_4$, 1 $CaCl_2$, 10 D-glucose, and 25.9 $NaHCO_3$, saturated with 95% O_2–5% CO_2. Slices were incubated at 30°C for 30 minutes and then left at room temperature until they were transferred to a submerged chamber (Warner Instruments, Hamden, CT) for recording. During experiments, slices were perfused at a rate of 2 ml/min with normal ACSF containing (in mM) 126 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.5 $MgSO_4$, 2.4 $CaCl_2$, 11 D-glucose, 25.9 $NaHCO_3$, and 0.004 atropine, saturated with 95% O_2–5% CO_2 at 30°C. The LGN neurons and the SR interneurons were visualized with infrared differential interference contrast microscopy using an E600FN microscope (Nikon, Tokyo, Japan).

Patch-clamp recording pipettes and single-barrel drug application pipettes were pulled from borosilicate glass with an o.d. and i.d. of 1.5 and 0.86 mm, respectively (Sutter Instruments, Novato, CA). The recording pipettes were filled with an internal solution of (in mM) 125 K-gluconate, 1 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 MgATP, 0.3 Na_3GTP, and 10 HEPES, pH 7.35. Neurons were held at −70 mV, and a −10 mV/10 millisecond test pulse was applied before each response to determine access resistance, input resistance, and whole-cell capacitance. Local somatic applications of ACh (1 mM pipette concentration) were made every 30 seconds using single-barrel glass pipettes attached to a picospritzer (General Valve) with Teflon tubing (14–18 psi for 20 milliseconds). The single-barrel drug application pipettes were usually placed within 10–15 μm of the cell soma. In the coapplication experiments for each neuron, five baseline responses evoked by ACh were recorded, followed by responses evoked by applications of ACh in combination with bath application of the agonist, partial agonist, or antagonist. These agents were introduced into the ACSF using a syringe pump (KD Scientific, Holliston, MA) loaded with a 50-fold concentrated stock solution diluted to the final concentration in the perfusion line prior to entering the recording chamber (at a pump rate of 2.4 ml/h). Evoked responses were then recorded for 18–20 minutes. It should be noted that pressure application from a drug pipette containing 1 mM ACh delivered an effective concentration of approximately 30 μM to the soma of hippocampal SR interneurons in SD rat brain slices; thus a dilution factor of 30 would be expected for each ACh pulse pressure-ejected in our system (López-Hernández et al., 2007). Signals were recorded using a MultiClamp 700A amplifier (Molecular Devices), digitized using a Digidata 1322A (Molecular Devices), sampled at 20 kHz using Clampex 9.2, and filtered at 6 KHz. Data analysis was done with Clampfit 10.3 (Molecular Devices), Excel 2003, and Kaleidagraph 3.0.2. Cells with access resistances >60 MΩ or those requiring holding currents >300 pA for LGN neurons and >200 pA for SR interneurons were not included in the final analyses. Data are represented as means ± S.E.M. of 7–13 neurons.

Tail Suspension Test in Mice. Adult C57BL/6 male mice (20–30 g, 3 to 5 months of age) were used for the experiments. The animals were housed in a room with controlled temperature (24 ± 1°C) and a 12-hour light/12-hour dark cycle. All animals were acclimatized to the laboratory environment for at least 48 hours before the experiments. Food and water were available ad libitum. The experimental protocols were approved by the Animal Care and Use Committee at Yale University. On the day of testing, mice were transferred to the testing room at least 30 minutes prior to the first test. The tail suspension test was carried out as previously described (Mineur et al., 2007). BPC and BMSP were administered intraperitoneally 30 minutes before the test at doses of 2 and 5 mg/kg, respectively, dissolved in phosphate-buffered saline (pH 7.4) and injected at a volume of 10 ml/kg. Mice were gently suspended by a small paperclip taped to the tip of their tail, about 4/5 the distance from the base. The total time spent immobile during the 6-minute testing period was recorded. Mice were returned to the holding room after every animal was tested.

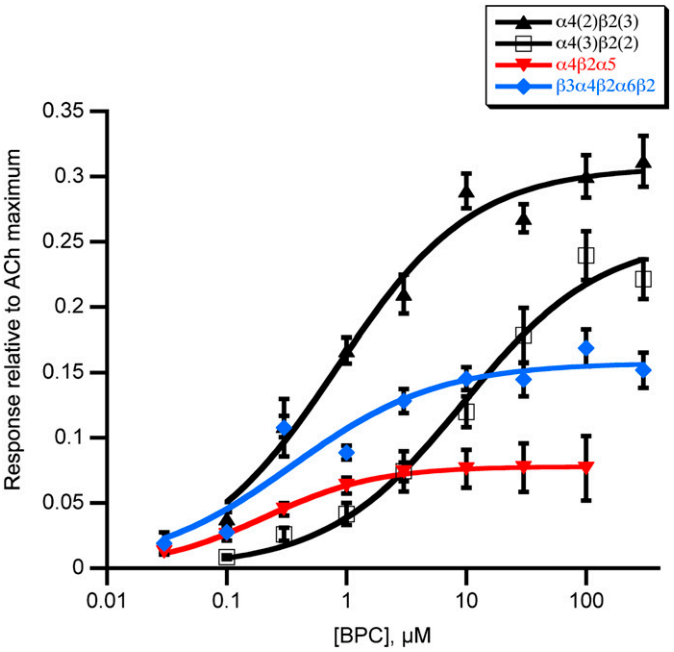

Fig. 4. Concentration-response curves for BPC on $\alpha4\beta2$* nAChRs. Data are peak current responses calculated relative to control ACh responses obtained from the same cells and subsequently normalized to the maximum ACh response determined in separate experiments (not shown). Control ACh concentrations were: 10 μM for HS $\alpha4\beta2$ [$(\alpha4)_2(\beta2)_3$], 100 μM for LS $\alpha4\beta2$ [$(\alpha4)_3(\beta2)_2$], 10 μM for $\alpha4\beta2\alpha5$, and 30 μM for $\beta3\alpha4\beta2\alpha6\beta2$. Values represent the averages (± S.E.M.) of at least four oocytes.

Results

Pharmacological Activity at nAChR Subtypes Expressed in *X. laevis* Oocytes. We evaluated the in vitro pharmacological properties of BPC and BMSP (Fig. 1) compared with the current smoking cessation drugs using concatamers of human nAChR subtypes along with an excess of single free subunits in *X. laevis* oocytes, allowing for expression of defined subunit compositions (Zhou et al., 2003). All four agents showed relatively little activity for mouse muscle nAChR, evoking no more than 5% of the ACh response when applied alone (Fig. 2A) and producing relatively little antagonism of ACh-evoked responses when either coapplied with ACh (Fig. 2B) or preincubated with the receptors prior to ACh application (Fig. 2C), although BPC did produce a small inhibition at the highest concentration tested.

TABLE 1
Concentration-response data for BPC

Receptor	I_{max}	EC_{50}
		μM
$\alpha4(2)\beta2(3)$	0.31 ± 0.01	0.8 ± 0.2
$\alpha4(3)\beta2(2)$	0.27 ± 0.02	9.4 ± 3.2
$\alpha4(2)\beta2(2)\alpha5$	0.10 ± 0.01	0.21 ± 0.01
$\beta3\alpha4\beta2\alpha6\beta2$	0.16 ± 0.01	0.35 ± 0.16

The agonist activity was evaluated for both HS and LS forms of human $\alpha4\beta2$ nAChR and other nAChR subtypes (Fig. 3). Like varenicline and cytisine, BPC is a partial agonist of $\alpha4\beta2$ receptors, especially of the HS subtype. However, BMSP produced very little activation of these $\alpha4\beta2$ receptors. BPC had a level of partial agonist activity for human $\alpha4\beta2\alpha5$ receptors that was comparable to cytisine, whereas BMSP was least efficacious among the four agents on this receptor as well as $\alpha4\beta2\alpha6\beta2\beta3$ receptors. Compared with the cytisine-based molecules, the two bispidine compounds showed the reduced activity for the two alternative nAChR subtypes, $\alpha7$ and $\alpha3\beta4$.

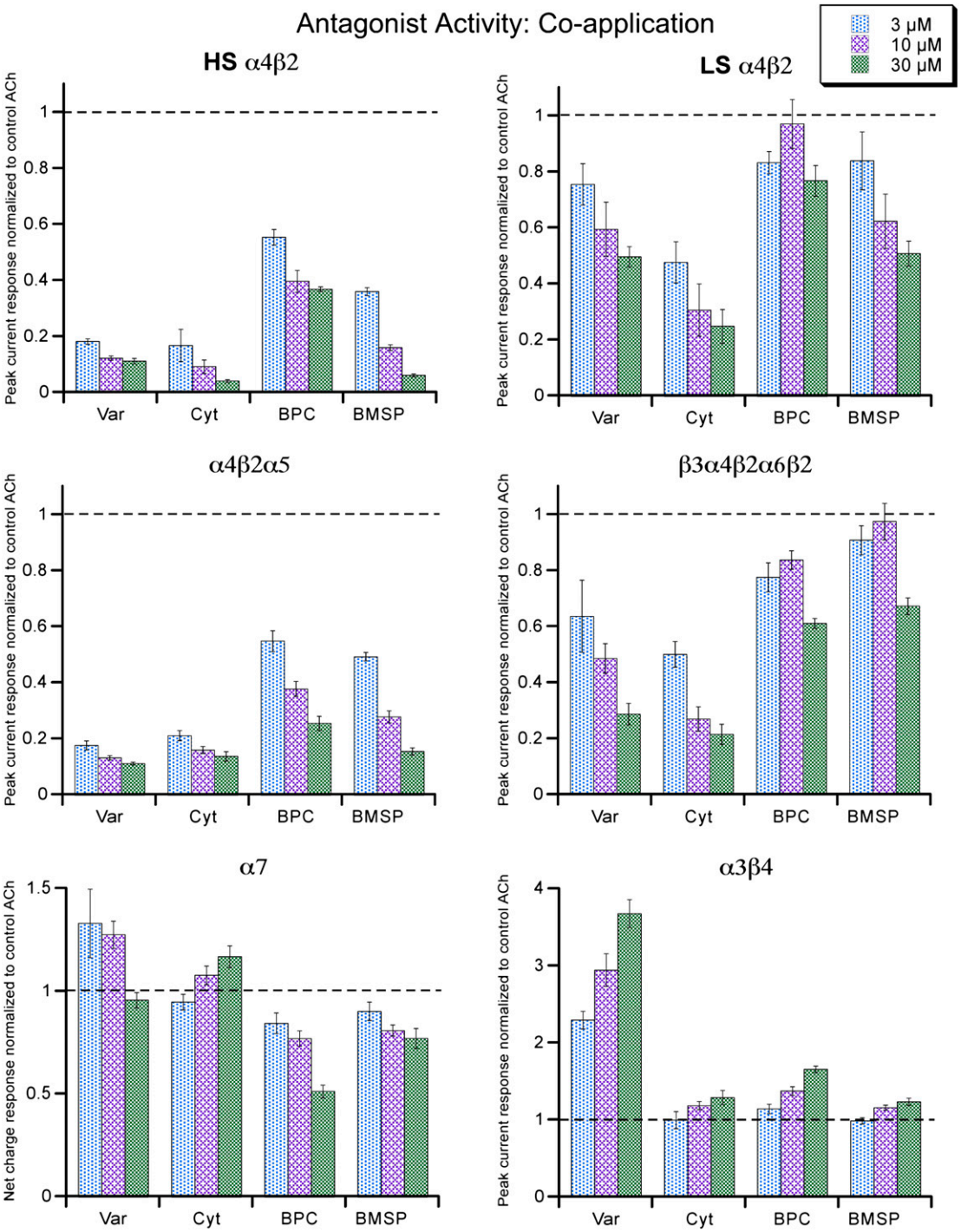

Fig. 5. Inhibition of ACh-evoked responses of various human nAChR subtypes expressed in *X. laevis* oocytes by acute coapplication of varenicline (Var), cytisine (Cyt), BPC, or BMSP. Each partial agonist was coapplied at increasing concentrations with control ACh. The antagonist activity of these compounds was characterized by normalizing the responses (measured as peak current for non-$\alpha7$ and net charge for $\alpha7$) to the averaged ACh controls. The control ACh concentrations used were: 10 μM for HS $\alpha4\beta2$, 100 μM for LS $\alpha4\beta2$, 10 μM for $\alpha4\beta2\alpha5$, 30 μM for $\beta3\alpha4\beta2\alpha6\beta2$, 60 μM for $\alpha7$, and 100 μM for $\alpha3\beta4$. Data are shown as the averaged normalized data ($\pm$ S.E.M.) from $n \geq 4$ oocytes at each condition.

Although the agonist activity of BMSP was too low to characterize effectively, BPC evoked significant currents when applied alone to $\alpha 4$* nAChR. Therefore we conducted full concentration-response studies, shown in Fig. 4. BPC was most efficacious for HS $\alpha 4\beta 2$ [$\alpha 4(2)\beta 2(3)$] receptors and, not surprisingly, least potent for LS $\alpha 4\beta 2$ [$\alpha 4(3)\beta 2(2)$] nAChRs (Table 1).

One of the important functions for partial agonists used as smoking cessation agents is through their ability to modulate the intrinsic responsiveness of the $\alpha 4\beta 2$* receptors to nicotine and ACh. Therefore we tested the ability of these agents to decrease agonist (ACh)-evoked responses. Note that similar effects can be obtained with nicotine as an agonist. However,

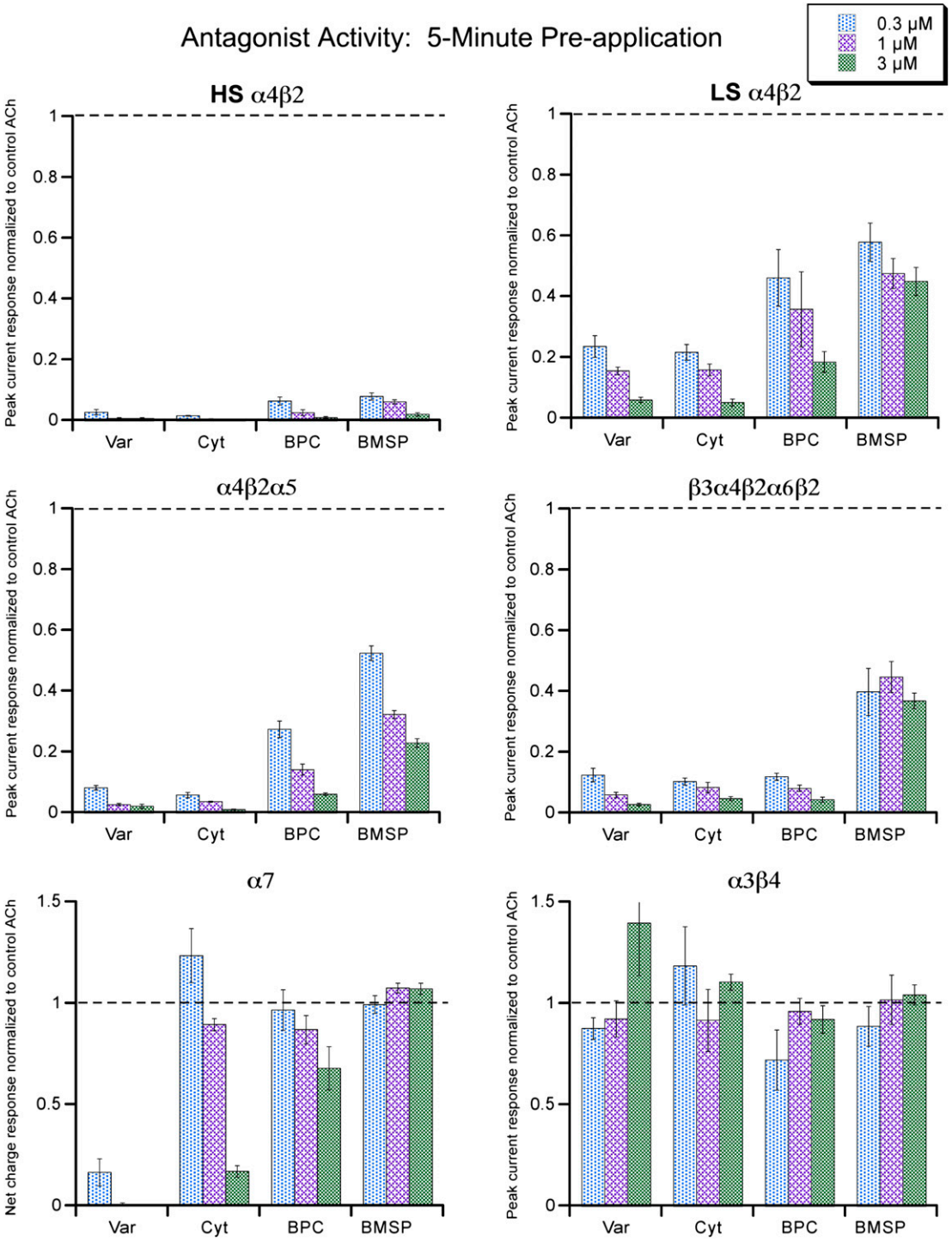

Fig. 6. Inhibition of ACh-evoked responses of various human nAChR subtypes expressed in *X. laevis* oocytes by bath application of varenicline (Var), cytisine (Cyt), BPC, or BMSP. A 5-minute preapplication of each partial agonist of a range of concentrations was made before the application of control ACh. The antagonist activity of these compounds was characterized by normalizing the responses (measured as peak current for non-$\alpha 7$ and net charge for $\alpha 7$) to the averaged ACh controls. The control ACh concentrations used were: 10 μM for HS $\alpha 4\beta 2$, 100 μM for LS $\alpha 4\beta 2$, 10 μM for $\alpha 4\beta 2\alpha 5$, 30 μM for $\beta 3\alpha 4\beta 2\alpha 6\beta 2$, 60 μM for $\alpha 7$, and 100 μM for $\alpha 3\beta 4$. Data are shown as the averaged normalized data ($\pm$ S.E.M.) from $n \geq 4$ oocytes at each condition.

because nicotine is sequestered and concentrated in the oocytes, we relied on the more readily reversible effects of ACh to probe for receptor function.

Of the neuronal nAChR subtypes tested, HS $\alpha4\beta2$ receptors were the most sensitive to all four agents as antagonists in acute coapplication experiments with ACh (Fig. 5), although they were least sensitive to BPC compared with the other three. All four agents inhibited $\alpha4\beta2\alpha5$ receptors, whereas $\alpha6^*$ receptors were most sensitive to the current smoking cessation drugs using this simple coapplication protocol. Because of their intrinsic agonist activity, cytisine and varenicline caused an additive activation, rather than inhibition of $\alpha3\beta4$ and $\alpha7$ receptors, especially in the case of varenicline on $\alpha3\beta4$ nAChR. These nAChR subtypes have been hypothesized to be off-target for smoking cessation therapies, and the bispidine compounds had relatively little effect on them. Note that because varenicline and cytisine differ significantly in their effects on human and rat $\alpha3\beta4$ receptors (Stokes and Papke, 2012), we also tested BPC and BMSP on rat $\alpha3\beta4$ receptors expressed in *X. laevis* oocytes. Neither drug produced significant inhibition of responses evoked by 100 μM ACh when coapplied at 10 μM, and likewise, neither compound produced significant responses when applied alone at 100 μM to cells expressing rat $\alpha3\beta4$ receptors (data not shown).

Acute coapplication of nicotinic agents is not a very good model for investigating drug-receptor interactions in vivo, because therapeutic agents will be present before and during the endogenous release of ACh or the rapid delivery of nicotine to the brain via cigarette smoking. Therefore, we also tested the modulation of ACh-evoked responses when the drugs were preincubated with the receptors for 5 minutes (Fig. 6). With this protocol, several types of $\alpha4^*$ receptors were sensitive to all four agents, with the greatest effects obtained on the HS $\alpha4\beta2$ receptors. There was also a rather selective inhibition of $\alpha7$-mediated ACh responses with varenicline.

Although preincubations better emulate in vivo drug interactions than do coapplications, a better model still is to use bath applications of the drugs at very low concentrations and observe the longer term perturbation of receptor function.

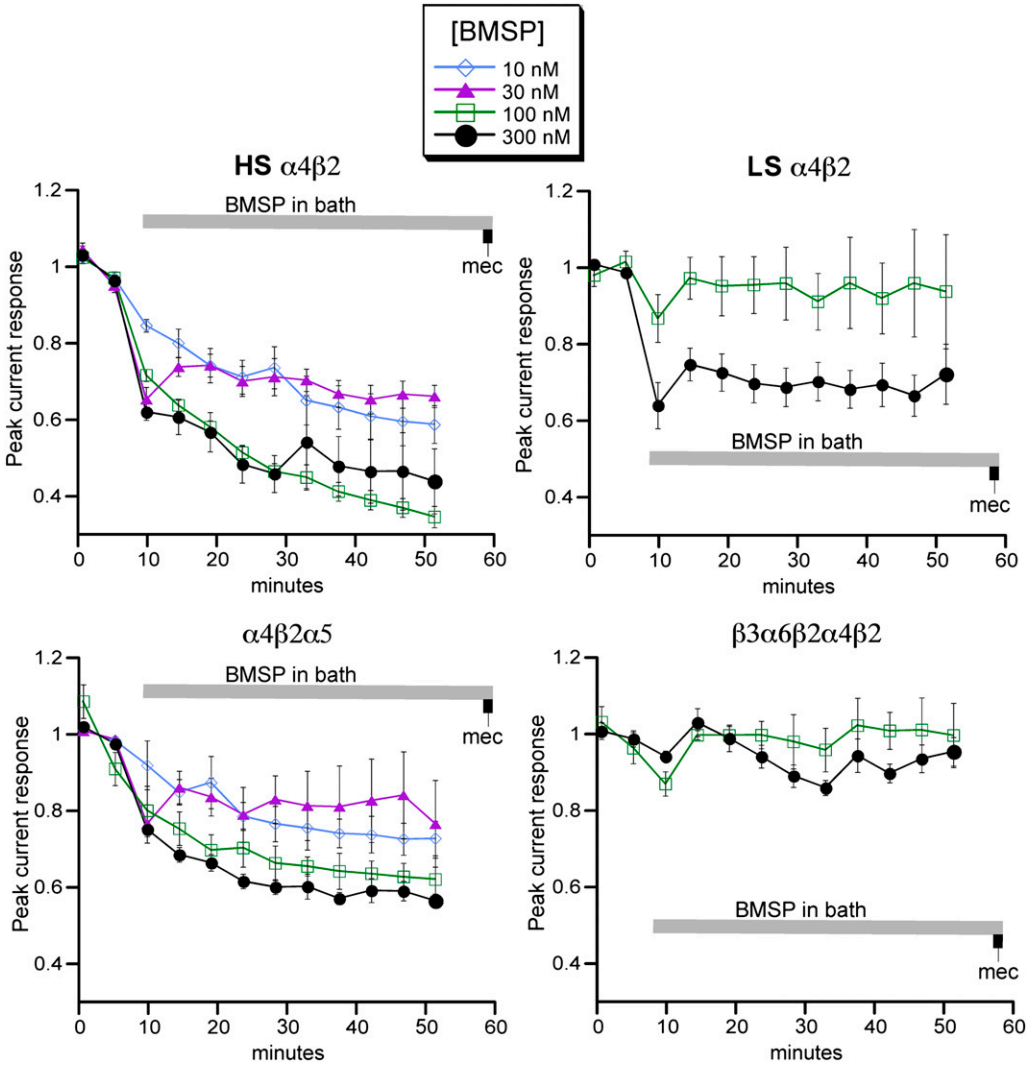

Fig. 7. Effects of bath-applied BMSP on the ACh-evoked responses of human $\alpha4\beta2$, $\alpha4\beta2\alpha5$, and $\beta3\alpha6\beta2\alpha4\beta2$ nAChR expressed in *X. laevis* oocytes. After measuring two baseline ACh-evoked responses, BMSP was added to the bath solution, and the cells were repeatedly probed for their ACh responses. The tested concentrations of BMSP were 10, 30, 100, and 300 nM for HS $\alpha4\beta2$ and $\alpha4\beta2\alpha5$ and 100 and 300 nM for LS $\alpha4\beta2$ and $\beta3\alpha6\beta2\alpha4\beta_2$. All data points represent an average of at least four oocytes (± S.E.M.) for each condition.

We previously applied this approach to characterize varenicline and cytisine (Papke et al., 2011). Figure 7 shows that bath application of BMSP at submicromolar concentrations effectively downregulated the responses of all $\alpha4^*$ receptors, other than those also including the $\alpha6$ subunit. All of the $\alpha4^*$ receptors were also functionally downregulated by bath application of 100 nM BPC (Fig. 8A).

At the end of bath application experiments, we routinely applied 100 μM mecamylamine to determine if there were steady-state currents that produced an apparent shift in baseline, as was previously reported for nicotine, varenicline, and cytisine (Papke et al., 2011). As shown in Fig. 8B, 100 nM BPC strongly decreased the size of the ACh-evoked responses and also generated a steady-state current revealed by mecamylamine application. This steady-state current was not observed with BMSP. For HS $\alpha4\beta2$, $\alpha4\beta2\alpha5$, and $\alpha6^*$ receptors, bath application of 100 nM BPC produced steady-state activation that was approximately 2–3% the size of the peak current responses to control applications of ACh.

Modulation of ACh-Evoked Responses in Cells Stably Expressing $\alpha7$ and RIC-3. We evaluated the effects of bath-applied nicotine and the $\alpha4^*$ partial agonists on the modulation of ACh-evoked responses of A7R3HC10 cells which stably express human $\alpha7$ nAChR (Williams et al., 2012). As shown in Fig. 9, 300 nM nicotine produced ~50% downregulation in the ACh-evoked responses, which was greater than the effects observed when BPC or BMSP were bath-applied at 300 nM. In contrast, as expected from the oocyte data, $\alpha7$-mediated responses were very sensitive to bath applications of varenicline, so that 50 nM varenicline produced ~70% reduction in the ACh-evoked responses. It required higher than 1 μM of cytisine in the bath to produce a comparable downregulation of ACh evoked responses (1.7 μM cytisine produced 78 $\pm$ 3% inhibition after 15 minutes, data not shown).

Modulation of ACh-Evoked Responses of Native nAChR Subtypes in Brain Slices. To investigate and compare these agents on native nAChR subtypes in the brain, we focused on two neuronal types: the primary neurons in the lateral geniculate nucleus (LGN), which we previously showed to express primarily $\alpha4^*$ receptors (Papke and Thinschmidt, 2009), and the $\alpha7$-expressing interneurons of the hippocampal stratum radiatum (SR) (López-Hernández et al., 2009). In the absence of modulation by bath-applied drugs, the ACh-evoked responses in LGN neurons were stable, or in the case of the SR interneurons, showed a small run-up (Supplemental Fig. 1A), as is sometimes seen in in vitro experiments (Papke et al., 2011). As expected, the responses of the LGN neurons were sensitive to bath application of dihydro-β-erythroidine and the SR interneurons to the $\alpha7$-selective antagonist methyllycaconitine (Supplemental Fig. 1B).

The responses of LGN neurons were sensitive to a very low concentration of nicotine, whereas the SR interneuron responses were insensitive to nicotine at a submicromolar concentration (Fig. 10A). In contrast to the relatively selective effects of nicotine on LGN neurons, LGN neurons and SR interneurons were both sensitive to varenicline at submicromolar concentrations (Fig. 10B). Both types of neurons were approximately 10-fold less sensitive to bath applications of cytisine (Fig. 10C).

Although 300 nM BPC strongly inhibited the ACh-evoked responses of the LGN neurons, it produced no effects on the $\alpha7$-mediated responses of SR interneurons (Fig. 11A). Similar results were obtained with BMSP, although as expected from the oocyte data the downregulation of $\alpha4\beta2$ responses by 300 nM BMSP was less than observed with 300 nM BPC (Fig. 11B).

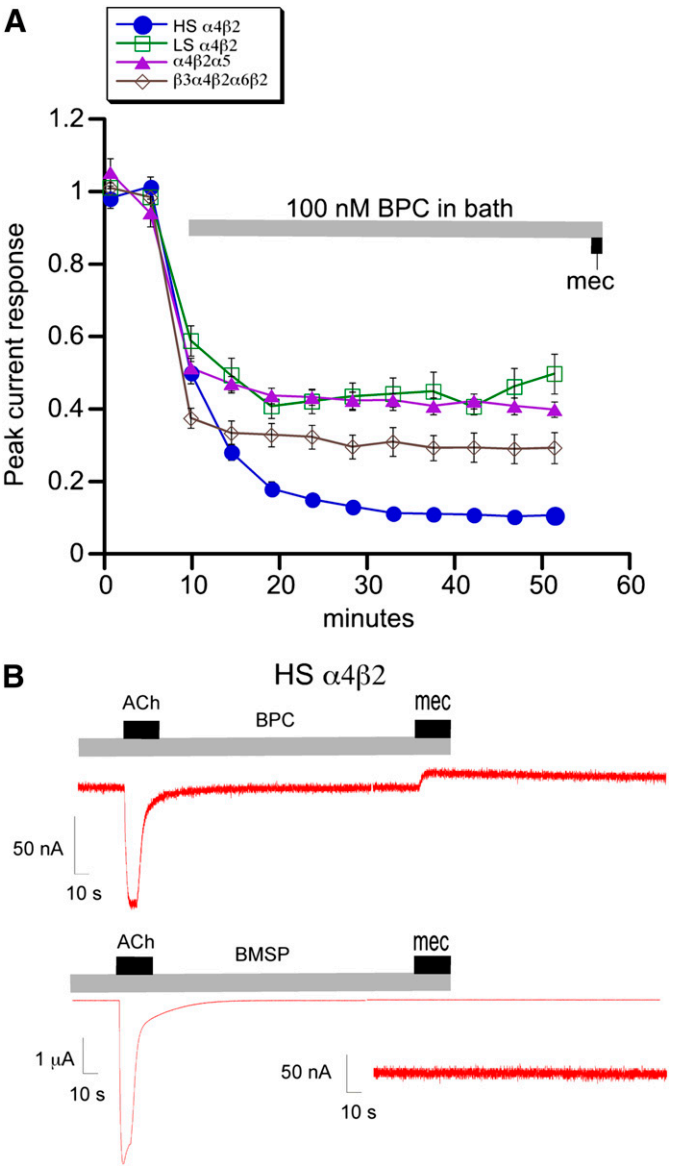

Fig. 8. (A) Inhibition of ACh-evoked responses of *X. laevis* oocytes expressing human HS $\alpha4\beta2$, LS $\alpha4\beta2$, $\alpha4\beta2\alpha5$, and $\beta3\alpha6\beta2\alpha4\beta2$ nAChR by bath application of 100 nM BPC. Before the addition of BPC to the bath, two applications of ACh were made to obtain the baseline responses. The cells were then repeatedly probed for the ACh responses at intervals of 277 seconds. (B) Effects of mecamylamine (mec) on the steady-state HS $\alpha4\beta2$ nAChR baseline current promoted by bath-applied BPC. With the presence of 100 nM BPC or 100 nM BMSP in the bath (gray bar), the application of 10 μM ACh (black bar) stimulated a transient current, as illustrated by the representative traces. With continuous BPC bath application, 100 μM mecamylamine was applied (open bar) to produce a sustained shift in baseline current. This baseline current remained at the same level in a bath solution without BPC, indicating that the steady-state activation of HS $\alpha4\beta2$ nAChR by bath-applied BPC can cause a baseline decline from the control level. However, such baseline shift was not observed with BMSP and mecamylamine coapplication. The 100 μM mecamylamine (open bar) did not reveal any baseline current with continuous BMSP bath application (gray bar).

In Vivo Activity. BPC and BMSP were evaluated for their efficacy in the tail suspension test in mice to determine whether they could alter behaviors associated with smoking

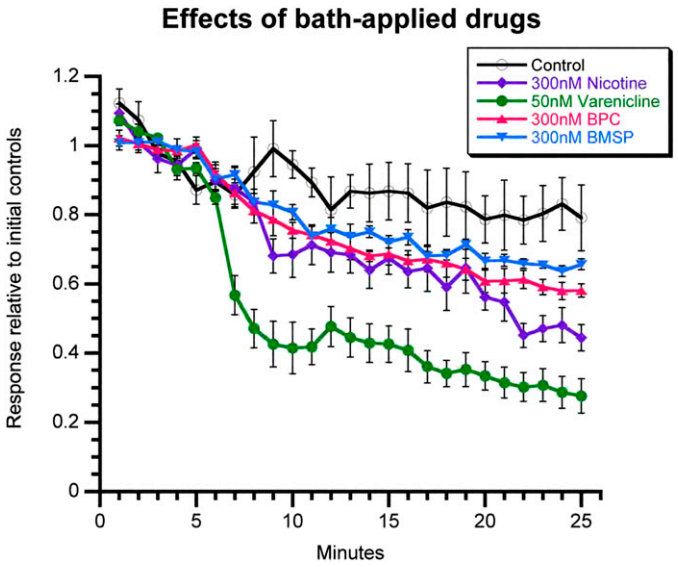

Fig. 9. Effects of bath-applied nicotine and partial agonists on ACh-evoked responses of $\alpha 7$ nAChR expressed in A7R3HC10 cells. Data were normalized to the averaged peak current of the five initial responses prior to the bath application of drug and represented as means ± S.E.M. of 4–7 cells.

cessation in vivo. This test is commonly used to evaluate potential antidepressant drugs, and cytisine was previously shown to reduce immobility in this assay (Mineur et al., 2007), whereas reports also suggest antidepressant-like effects of varenicline (Rollema et al., 2009). Both BPC and BMSP reduced immobility in this behavioral model at the concentrations tested (Fig. 12). The efficacy of the compounds in the mouse tail suspension test was similar to that of other nicotinic compounds tested previously and was equivalent to the effects of classic antidepressant medications on immobility time, although BPC and BMSP were somewhat less potent than cytisine or varenicline. The behavioral effects of BPC and BMSP in the tail suspension test also suggest that both agents are able to cross the blood-brain barrier and enter the brain.

Discussion

For the purpose of developing $\alpha 4\beta 2^*$ ligands with increased selectivity, cytisine was structurally simplified down to its bispidine (3,7-diazabicyclo[3.3.1]nonane) skeleton. The bispidine scaffold, which can form cation-pi/HB interactions with nAChRs, displays weak inhibition of $\alpha 4\beta 2^*$ (K_i = 600 nM) and $\alpha 4\beta 2\alpha 6\beta 2\beta 3$ nAChR subtypes and is an agonist at $\alpha 7$ nAChRs with negligible effects on $\alpha 3\beta 4^*$ and muscle nAChRs (Tomassoli et al., 2011). It served as a synthetic starting point for new compound libraries, applying a hybrid (Meunier, 2008) and a template approach, where a second pharmacophoric element, a hydrogen bond acceptor motif, was introduced. These strategies led to compounds with improved $\alpha 4^*$ selectivity. The hybrid approach design, where two active pyridine-based nAChR ligands were overlapped at a hydrogen bond acceptor motif, generated BPC, which is a relatively efficacious $\alpha 4^*$ agonist. In contrast to the hybrid BPC, BMSP, with very low $\alpha 4^*$ efficacy, displays a carbonyl oxygen as its hydrogen bond acceptor functionality.

Our utilization of human nAChR subtypes makes our data relevant to therapeutics. By extending our studies to the native receptor subtypes in rodent brain, we both confirm the in vitro pharmacology and validate the drugs for further study in animal models. Although cytisine and varenicline have similar activity for human and rat $\alpha 4^*$ and $\alpha 7$ receptors (Papke and Heinemann, 1994; Papke and Porter Papke, 2002; Mihalak et al., 2006, Papke et al., 2011), we previously showed that they differ in their activity for human and rat ganglionic-type $\alpha 3\beta 4$ receptors (Stokes and Papke, 2012), for which they produce significant activation but with differences in potency and efficacy, indicating a significant likelihood for side effects in humans, especially for varenicline. In contrast, our data show that BPC and BMSP have minimal activity for human and rat $\alpha 3\beta 4$ receptors.

Varenicline and cytisine are sometimes referred to as $\alpha 4\beta 2$-selective partial agonists; however, we confirm that both compounds have significant efficacy at $\alpha 3\beta 4$ and $\alpha 7$. The high levels of $\alpha 7$ modulation shown by varenicline may be of particular concern because this agent has been associated with adverse neuropsychiatric events (McClure et al., 2009; Moore et al., 2011). Cardiovascular events that have occurred with varenicline (Ware et al., 2013) might be attributable to effects on ganglionic $\alpha 3\beta 4$ receptors. Our data show that BPC and BMSP come much closer to selectively regulating the activity of $\alpha 4^*$. BPC is especially effective at targeting the HS form of $\alpha 4^*$ receptors, which may be important in smokers (Lester et al., 2009).

Although $\alpha 7$ and $\alpha 3\beta 4^*$ nAChR subtypes should not be dismissed as nonrelevant targets for the management of smoking cessation, the fact that these receptors can induce both positive and negative effects related to addictive behavior make them problematic targets for smoking cessation strategy. Conversely, the demonstrated ability of $\beta 2^*$ nAChR blockade to blunt nicotine addiction, prevent relapse, and induce positive mood effects makes our focus highly relevant. It may not be possible to make a clear separation between on-target and off-target receptors for the treatment of nicotine dependence. Although numerous lines of evidence support $\alpha 4^*$ and $\alpha 6^*$ receptors as underlying the reinforcing effects of nicotine, special considerations must be given to other nAChR subtypes as well. Although primarily found in autonomic ganglia, there are $\alpha 3\beta 4$ receptors in the brain, most highly concentrated in the medial habenula, and some coassembled with $\alpha 5$. The $\alpha 5$-containing receptors have been implicated in establishing aversive effects of high nicotine doses (Frahm et al., 2011), and, independent of $\alpha 5$, $\alpha 3\beta 4$ receptors have also been implicated in nicotine reward and withdrawal (Jackson et al., 2013). Likewise, although homomeric $\alpha 7$ receptors in the brain are generally considered off-target for smoking therapies, these receptors contribute to circuits associated with reward (Mansvelder et al., 2002; Brunzell and McIntosh, 2012). The question then is, not only whether subtypes such as $\alpha 7$- and $\alpha 5$-containing receptors should be targeted but whether they should be targeted differently from $\alpha 4^*$ and $\alpha 6^*$ receptors. The ideal drug might downregulate the function of $\alpha 4^*$ and $\alpha 6^*$ receptors and enhance the function of $\alpha 5$-containing receptors and thereby increase the aversive effects of nicotine. Unfortunately, no such ideal drug exists, so at this point we must build up from the limited efficacy of the existing therapeutic drugs. Other preclinical drug development programs continue to work up from the cytisine scaffold (Mineur et al., 2009; Tasso et al., 2009; Sala et al., 2013). In some assays, the cytisine dimer (Sala et al., 2013) shows a similar progression in selectivity as

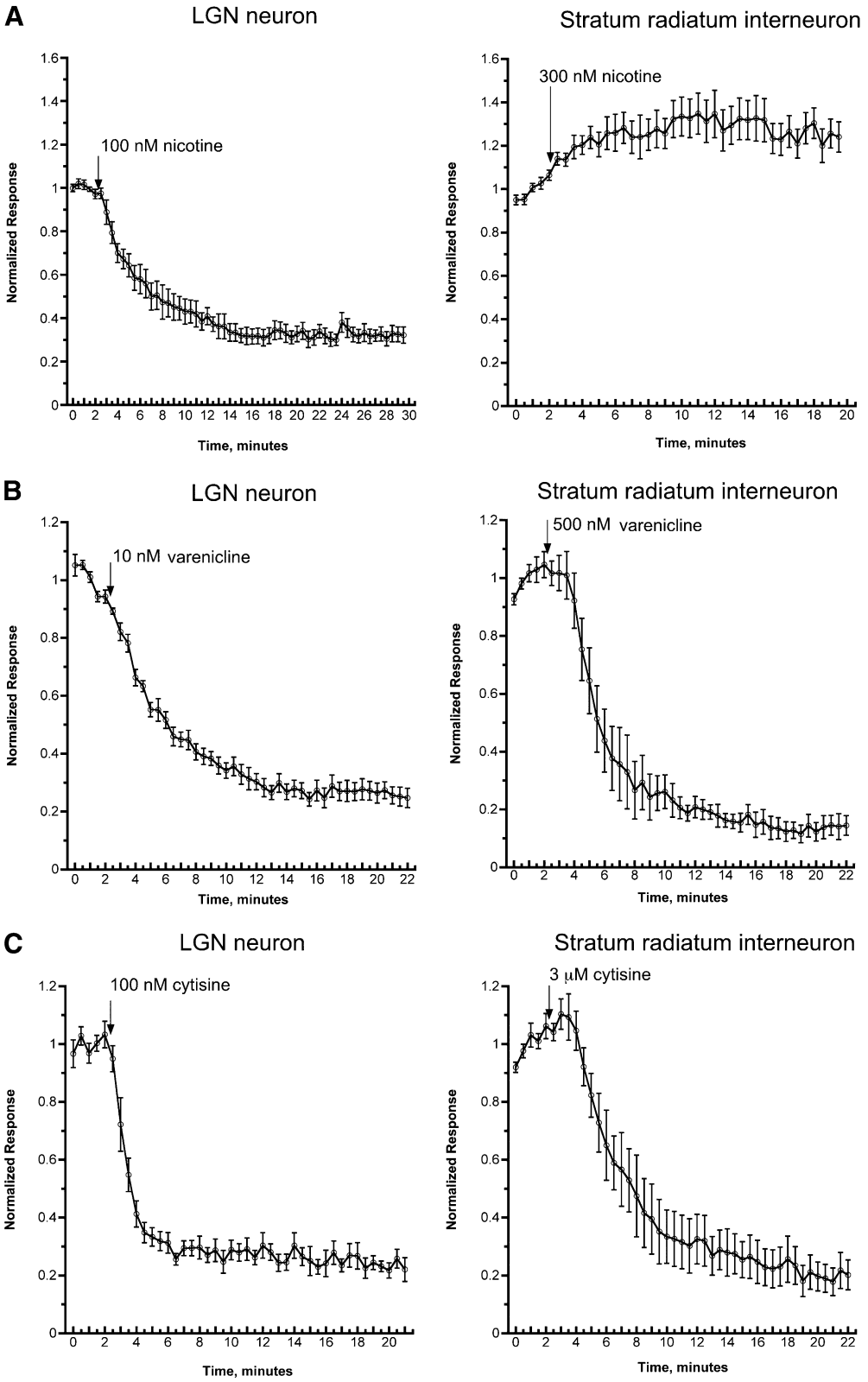

Fig. 10. Modulation of ACh-evoked currents of LGN neurons and SR interneurons by nicotine, varenicline, and cytisine. Left panels: Time courses for the 100 nM nicotine (A)-, 10 nM varenicline (B)-, and 100 nM cytisine (C)-induced peak current inhibition of ACh-evoked responses in LGN neurons. Right panels: Time courses for the modulation of ACh-evoked peak current responses by 300 nM nicotine (A), 500 nM varenicline (B), and 3 μM cytisine (C) in SR interneurons. ACh was applied using single-barrel pressure application pipettes containing 1 mM ACh with an interstimulus interval of 30 seconds. Five baseline responses were recorded followed by evoked responses in the presence of the test agonist or partial agonist. Data were normalized to the average of the first five ACh-evoked responses prior to the bath application of the test agent. Black arrows indicate the time of running the syringe pump to perfuse with ACSF containing the test agent. Data represent the averages of 7–13 neurons.

the bispidine compounds we report, but the cytisine dimer has significant intrinsic activity for stimulating dopamine release, which may not be ideal.

In addition to effects on behaviors related to drug reward, decreasing activity of $\beta 2^*$ nAChRs has repeatedly been shown to be antidepressant-like. The bispidines tested, like various cytisine derivatives, have positive effects in tests of antidepressant efficacy. However, comparisons of in vitro properties and in vivo behavioral effects of novel nicotinic compounds must be made carefully, because in vivo effects are limited by

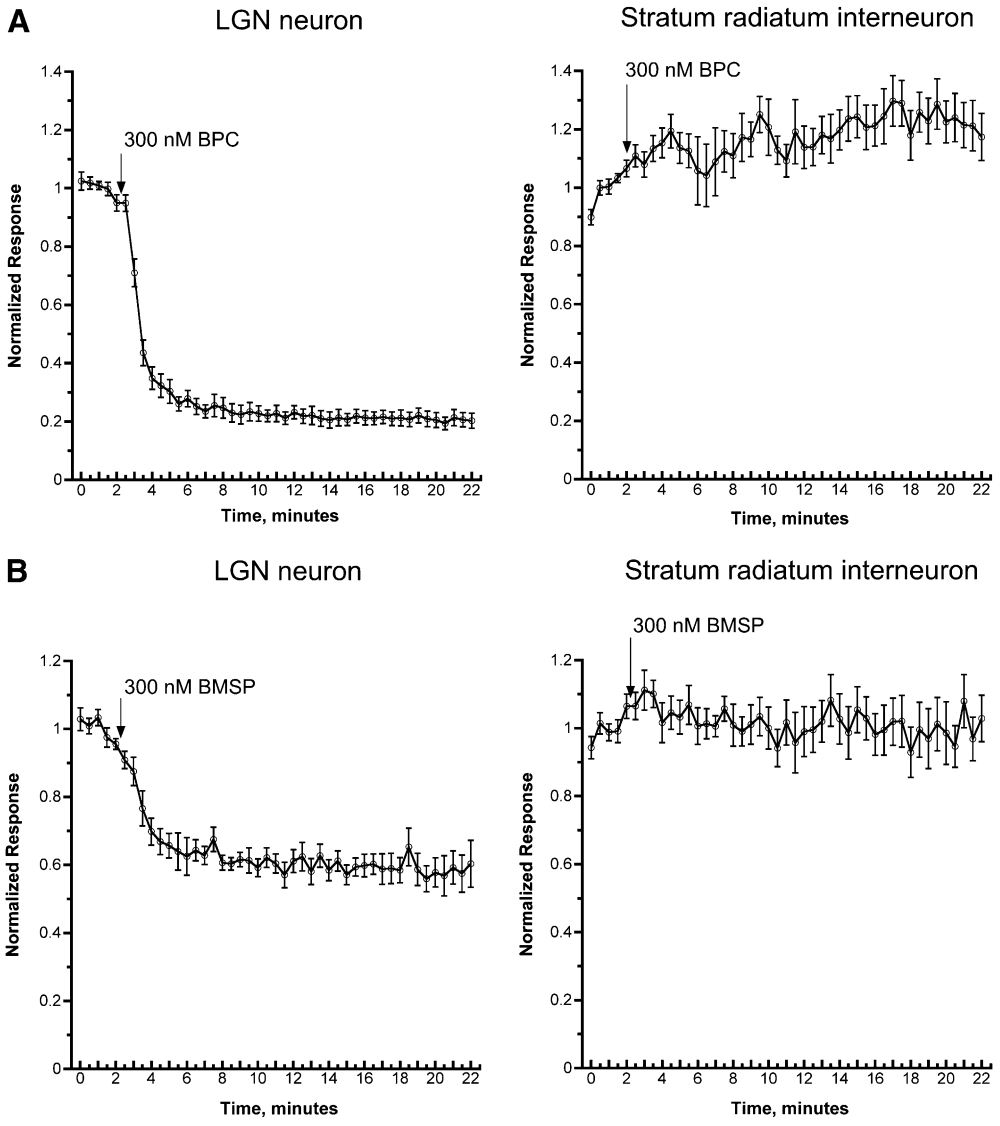

Fig. 11. Specific inhibition of ACh-evoked peak current responses in LGN neurons and SR interneurons by bath application of BPC (A) and BMSP (B). ACh was applied from a single-barrel pressure applicator, and after five baseline responses, either 300 nM BPC or 300 nM BMSP was added to the bath. Bath application of BPC (n = 10) and BMSP (n = 9) produced a respective 77 and 40% inhibition of the transient responses to acute applications of ACh in LGN neurons (A and B, left panels), whereas the bath application of BPC (n = 12) or BMSP (n = 9) had no significant effect on the ACh-evoked responses in SR interneurons (A and B, right panels).

other properties, including brain penetration and clearance. For instance, although 5-bromo-cytisine has high affinity and is a weak partial agonist of $\alpha4\beta2$ nAChRs, it is not effective in behavioral models of antidepressant efficacy when injected peripherally, but is effective when infused directly into the ventricles. Overall, the advancements toward the profile of an "ideal drug" based on the cytisine scaffold have been relatively small and incremental. Our basic starting point was the simpler bispidine scaffold; we were hoping that we could achieve a cleaner separation of $\alpha4$ partial agonism from activity at $\alpha3\beta4$ and $\alpha7$, and to a large degree our data support the strength of that approach.

Although both BPC and BMSP have improved selectivity profiles over varenicline and cytisine, there are interesting differences between the two agents. Specifically, BPC has much greater intrinsic agonist activity than does BMSP, so comparison of the two agents may indicate whether symptoms such as dysphoria during withdrawal can be managed best with an agent that provides a baseline of stimulation in the absence of the drug. Such activity may contribute to the ability of a medication to maintain abstinence following smoking cessation.

Although decreasing activity of $\beta2$* nAChRs has repeatedly been shown to produce antidepressant-like effects in mice, several clinical trials have suggested that blockade of nAChRs in conjunction with administration of antiserotonergic antidepressants may augment the efficacy of antidepressant therapies in humans (Philip et al., 2009). However, a large clinical trial of a mecamylamine isomer was not successful, suggesting that the use of truly selective partial agonists with some intrinsic efficacy at specific nAChR subtypes, such as BPC, may be a more successful strategy than complete nicotinic blockade. Such agents may have better compliance, fewer side effects, and may be particularly effective for treating the mood symptoms that occur during smoking cessation (Moore et al., 2011).

A number of studies have shown that $\alpha6$* nAChRs are involved in nicotine self-administration in rodents (Pons et al., 2008; Brunzell et al., 2010; Gotti et al., 2010). The current smoking cessation drugs cytisine and varenicline both have higher efficacy at $\alpha4\beta2\alpha6\beta2\beta3$ nAChRs than do the bispidine compounds, but it is not known whether this is important for their therapeutic efficacy. Because blockade of nAChRs containing the $\alpha4$ subunit is sufficient to block nicotine reward

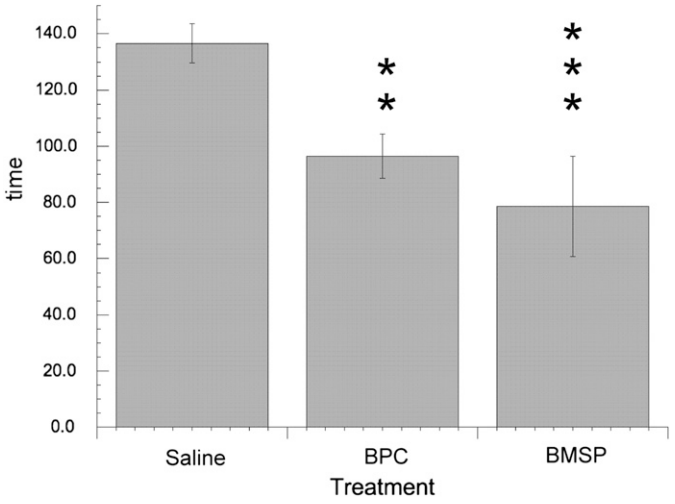

Fig. 12. The effects of BPC and BMSP on immobility time in the tail suspension test in mice (2 and 5 mg/kg, respectively). Results are represented as means ± S.E.M. with $n = 10$ in each group. Values are significant at $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group, based on a two-tailed t test with Bonferonni corrections.

(McGranahan et al., 2011), it is not clear whether activity at both α4* and α6* nAChRs will be necessary for an effective medication or whether only one of the two is essential. The identification of BMSP, which is highly specific for α4* nAChRs, will be extremely useful for answering this question.

Just as sparing α7 receptor function may be an important consideration in a smoking cessation therapy, equal consideration should be given to the self-medication needs of smokers suffering from, or at risk of, mental illness. The incidence of smoking is twice as high in the mentally ill as in the normal population and four times higher than normal in schizophrenia. In the United States, 30% of all smokers suffer from mental illness (Mackowick et al., 2012). Although varenicline may be especially bad for such individuals because of its potent suppression of α7 activation, a bispidine-type drug lacking any α7 effects may not fulfill the secondary drive for nicotine self-delivery that is not simply involved with dopamine-mediated reward.

In conclusion, we characterized two novel agents that offer potentially important alternative approaches for the experimental investigation and management of nicotine dependence and withdrawal. In vitro experiments with nAChR receptors and these agents indicate that they have a higher degree of selectivity for α4* and α6* nAChR than existing therapeutic agents and therefore will be useful in testing whether modulating the activity of these receptors will be sufficient to manage nicotine dependence with potentially fewer side effects. Their in vitro activity profiles correctly predicted their activity on native nAChR in brain slices, and, importantly, they were effective at sites in the mouse brain when given systemically. Moreover, the efficacy of these agents in the tail suspension test promotes their potential utility for managing the mood symptoms that occur during smoking cessation and nicotine withdrawal.

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Authorship Contributions

Participated in research design: Peng, Mineur, Picciotto, Papke.

Conducted experiments: Peng, Tian, Mineur.

Contributed new reagents or analytic tools: Guendisch, Eibl, Tomassoli.

Performed data analysis: Peng, Stokes, Mineur, Papke.

Wrote or contributed to the writing of the manuscript: Peng, Stokes, Guendisch, Picciotto, Papke.

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