Differential modulation of brain nicotinic acetylcholine receptor function by cytisine, varenicline, and two novel bispidine compounds: Emergent properties of a hybrid molecule

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Abbreviations: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; HBSS, Hank’s balanced saline solution

Structure-based names:
BPC, 2-(5-(3,7-diazabicyclo[3.3.1]nonan-3-yl)pyridin-3-yl)oxy)-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
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 oocytes and Human Embryonic Kidney 293 cells. Like varenicline and cytisine, BPC and BMSP are partial agonists of α4β2 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 α4* receptors that was not evident with BMSP. We evaluated the modulation of α4*- and α7-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. While 300 nM BPC strongly inhibited the ACh-evoked responses of LGN neurons, it did not inhibit the α7 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 α4β2* 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 since partial agonists can function as activity buffers, preventing over-activation 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 alpha subunits in vertebrates and seven non-alpha subunits (β1, β2, β3, β4, γ, δ, and ε), of which α2–α10 and β2–β4 are expressed in neurons and the others in muscle. Studies of knockout mice (Picciotto and Kenny, 2013) have implicated receptors containing α4 and β2 subunits in the reinforcing/addicting effects of nicotine. Receptors containing these subunits at varying ratios and sometimes also incorporating α5 subunits constitute the majority of the high-affinity nicotine-binding receptors in brain. Partial agonists at (α4β2*) nAChRs may be useful not only for managing nicotine reward and dependence, but also for managing the mood and depression symptoms that are co-morbid with smoking and smoking cessation (Mineur and Picciotto, 2010).

The most abundant types of α4 subunit-containing receptors (collectively designated as α4* receptors) include one configuration classified as a low sensitivity (LS) type with three α4 subunits and two β2 subunits (α4(3)β2(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 α4 subunits and three β2 subunits (α4(2)β2(3)), which responds to low concentrations of ACh and nicotine and with maximal currents limited at high concentrations. A third type of HS α4* receptor has two α4 subunits and two β2 subunits and an α5 subunit (α4(2)β2(2)α5). 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 α6, β2, and other subunits (such as α4β2α6β2β3). The α6 and β3 subunits are highly expressed in catecholaminergic neurons, particularly in the ventral tegmentum and substantia nigra. In ex vivo preparations, blocking these α6* 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 (α3β4*), and the homopentamers of α7 subunits. While muscle-type receptors are unequivocally off target, receptors containing α3, β4, and α5 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 α3-β4-α5 gene cluster have also been linked to smoking behavior. Activation of α7 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 α7 function have been implicated in schizophrenia, and schizophrenic individuals smoke at very high rates. These observations suggest that α7 and α3-containing receptors should not necessarily be considered off target but rather they may need to be targeted differently than α4* and α6* receptors. For people at risk for mental illness, it is likely that the function of α7 receptors should be especially spared.

The two drugs currently used as smoking cessation aids, cytisine and varenicline, have significant activity at α7 and α3* receptors, which may limit their utility and generate side effects. We report two novel compounds, BPC and BMSP (Figure 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 α3β4 subtypes or brain α7 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 (DHβE), methyllycaconitine citrate (MLA), (-)-nicotine, and cytisine were purchased from Sigma. Varenicline tartrate was purchased from

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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 (DMSO) 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 α1, β1, and δ clones used for receptor expression in *Xenopus 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 co-injected with α7 to improve the level and speed of α7 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 *Xenopus 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 min in 1.5 L frog-tank water containing 1 g of ethyl 3-aminobenzoate methanesulfonate (MS-222) buffered with sodium bicarbonate. The harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold NJ) for 2 h at room temperature in a calcium-free Barth’s solution (containing in mM: 88 NaCl, 1 KCl, 2.38 NaHCO3, 0.82 MgSO4, 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 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 CaCl₂, 10 HEPES, and 0.001 atropine, pH 7.2) at 2 ml/min for α7 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 co-applications 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 co-applied with ACh by the OpusXpress pipette delivery system for acute co-application 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 s in duration followed by a 181-s washout period for α7 receptors and 6 s with a 241-s 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 α1β1εδ, 60 μM for α7, 100 μM for α3β4, 10 μM for (α4)3(β2)2 (the HS form), 100 μM for (α4)2(β2)3 (the LS form), 10 μM for α4β2α5, and 30 μM for β3α4β2α6β2.

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 Papke, 2002). Data were expressed as means ± SEM 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 α7 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 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% CO₂. For normal passaging, cells were dissociated with 1 mM ethylenediamine tetraacetic acid (EDTA) in calcium- and magnesium-free Hank’s balanced saline solution (Life Technologies) to avoid non-selective damage to the α7 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 min. 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 CaCl₂, 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 MgCl₂, 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 s using single-barrel pipettes attached to a Picospritzer III pressure system (General Valve Corp., Fairfield NJ) with teflon tubing (10-20 psi for 3 s). The drug pipette was positioned approximately 10-15 μm from the cell and loaded with 1.5 mM ACh due to the 1.5-fold dilution factor inherent in the picospritzer drug delivery (Williams et al., 2012). Five baseline responses induced by ACh alone was initially recorded, followed by a 20-min 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 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-ms/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 ± SEM 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 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 Mg²⁺/low Ca²⁺ ice-cold artificial cerebral spinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 2.5 MgSO₄, 1 CaCl₂, 10 D-glucose, and 25.9 NaHCO₃, saturated with 95% O₂-5% CO₂. Slices were incubated at 30°C for 30 min 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 NaH₂PO₄, 1.5 MgSO₄, 2.4 CaCl₂, 11 D-glucose, 25.9 NaHCO₃, and 0.004 atropine, saturated with 95% O₂-5% CO₂ 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 mm 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 CaCl₂, 2 MgCl₂, 1 EGTA, 2 MgATP, 0.3 Na₃GTP, and 10 HEPES, pH 7.35. Neurons were held at -70 mV, and a -10 mV/10 ms 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 s using single-barrel glass pipettes attached to a picospritzer (General Valve) with teflon tubing (14-18 psi for 20 ms). The single-barrel drug application pipettes were usually placed within 10-15 μm of the cell soma. In the co-application 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 min. 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 322A (Molecular Devices), sampled at 20 kHz using Clampex 9.2, and filtered at 6 KHz. Data analysis was done with Clampfit.
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 h light/12 h dark cycle. All animals were acclimatized to the laboratory environment for at least 48 h before the experiments. Food and water were available ad libitum. The experimental protocols have been 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 min prior to the first test. The tail suspension test was carried out as previously described (Mineur et al., 2007). BPC and BMSP were administered i.p. 30 min before the test at doses of 2 mg/kg 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 min testing period was recorded. Mice were returned to the holding room after every animal was tested.

Results

Pharmacological activity at nAChR subtypes expressed in Xenopus oocytes

We evaluated the in vitro pharmacological properties of BPC and BMSP (Figure 1) compared to the current smoking cessation drugs using concatamers of human nAChR subtypes along with an excess of single free subunits in Xenopus 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 (Figure 2A) and producing relatively little antagonism of ACh-evoked responses when either co-applied with ACh (Figure 2B) or pre-incubated with the receptors prior to ACh application (Figure 2C), although BPC did produce a small inhibition at the highest concentration tested.

The agonist activity was evaluated for both HS and LS forms of human α4β2 nAChR and other nAChR subtypes (Figure 3). Like varenicline and cytisine, BPC is a partial agonist of α4β2 receptors, especially of the HS subtype. However, BMSP produced very little activation of these α4β2 receptors. BPC had a level of partial agonist activity for human α4β2α5 receptors that was comparable to cytisine, while BMSP was
least efficacious among the four agents on this receptor as well as \( \alpha 4 \beta 2 \alpha 6 \beta 2 \beta 3 \) receptors. Compared to the cytisine-based molecules, the two bispidine compounds showed the reduced activity for the two alternative nAChR subtypes, \( \alpha 7 \) and \( \alpha 3 \beta 4 \).

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 Figure 4. BPC was most efficacious for HS \( \alpha 4 \beta 2 \) \((\alpha 4(3)\beta 2(2))\) receptors and, not surprisingly, least potent for LS \( \alpha 4 \beta 2 \) \((\alpha 4(2)\beta 2(3))\) 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, since 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 \( \alpha 4 \beta 2 \) receptors were the most sensitive to all four agents as antagonists in acute co-application experiments with ACh (Figure 5), although they were least sensitive to BPC compared to the other three. All four agents inhibited \( \alpha 4 \beta 2 \alpha 5 \) receptors, while \( \alpha 6^* \) receptors were most sensitive to the current smoking cessation drugs using this simple co-application protocol. Due to their intrinsic agonist activity, cytisine and varenicline caused an additive activation, rather than inhibition of \( \alpha 3 \beta 4 \) and \( \alpha 7 \) receptors, especially in the case of varenicline on \( \alpha 3 \beta 4 \) 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 since varenicline and cytisine differ significantly in their effects on human and rat \( \alpha 3 \beta 4 \) receptors (Stokes and Papke, 2012), we also tested BPC and BMSP on rat \( \alpha 3 \beta 4 \) receptors expressed in *Xenopus* oocytes. Neither drug produced significant inhibition of responses evoked by 100 \( \mu \text{M} \) ACh when co-applied at 10 \( \mu \text{M} \), and likewise neither compound produced significant responses when applied alone at 100 \( \mu \text{M} \) to cells expressing rat \( \alpha 3 \beta 4 \) receptors (data not shown).

Acute co-application of nicotinic agents is not a very good model for investigating drug-receptor interactions in vivo, since 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 min (Figure 6). With this protocol, several types of \( \alpha 4^* \) receptors were sensitive to all four agents, with the greatest
effects obtained on the HS $\alpha_4\beta_2$ receptors. There was also a rather selective inhibition of $\alpha_7$-mediated ACh responses with varenicline.

Although preincubations better emulate in vivo drug interactions than do co-applications, 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. We have 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 down-regulated the responses of all $\alpha_4^*$ receptors, other than those also including the $\alpha_6$ subunit. All of the $\alpha_4^*$ receptors were also functionally down-regulated by bath application of 100 nM BPC (Figure 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 Figure 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 $\alpha_4\beta_2$, $\alpha_4\beta_2\alpha_5$, and $\alpha_6^*$ 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 $\alpha_7$ and RIC-3

We evaluated the effects of bath-applied nicotine and the $\alpha_4^*$ partial agonists on the modulation of ACh-evoked responses of A7R3HC10 cells which stably express human $\alpha_7$ nAChR (Williams et al., 2012). As shown in Figure 9, 300 nM nicotine produced ~50% down-regulation 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, $\alpha_7$-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 down-regulation of ACh evoked responses (1.7 µM cytisine produced 78 ± 3% inhibition after 15 min, data not shown).

Modulation of ACh-evoked responses of native nAChR subtypes in brain slices

In order 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 have previously shown express primarily $\alpha_4^*$ receptors (Papke
and Thinschmidt, 2009), and the α7-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 Figure 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 (DHβE) and the SR interneurons to the α7-selective antagonist methyllycaconitine (MLA) (Supplemental Figure 1B).

The responses of LGN neurons were sensitive to a very low concentration of nicotine, while the SR interneuron responses were insensitive to nicotine at a submicromolar concentration (Figure 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 (Figure 10B). Both types of neurons were approximately 10-fold less sensitive to bath applications of cytisine (Figure 10C).

While 300 nM BPC strongly inhibited the ACh-evoked responses of the LGN neurons, it produced no effects on the α7-mediated responses of SR interneurons (Figure 11A). Similar results were obtained with BMSP, although as expected from the oocyte data the down-regulation of α4β2 responses by 300 nM BMSP was less than observed with 300 nM BPC (Figure 11B).

**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 cessation in vivo. This test is commonly used to evaluate potential antidepressant drugs, and both cytisine has previously been shown to reduce immobility in this assay (Mineur et al., 2007) while 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 (Figure 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 classical 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 α4β2* 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 α4β2* (Ki = 600 nM) and α4β2α6β2β3 nAChR subtypes and is an agonist at α7 nAChRs with negligible effects on α3β4* 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 α4* 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 α4* agonist. In contrast to the hybrid BPC, BMSP, with very low α4* 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. While cytisine and varenicline have similar activity for human and rat α4* and α7 receptors (Papke and Heinemann, 1994; Papke and Papke, 2002; Mihalak et al., 2006, Papke et al., 2011), we have previously shown that they differ in their activity for human and rat ganglionic-type α3β4 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 α3β4 receptors.

Varenicline and cytisine are sometimes referred to as α4β2-selective partial agonists; however, we confirm that both compounds have significant efficacy at α3β4 and α7. The high levels of α7 modulation shown by varenicline may be of particular concern since this agent has been associated with adverse neuropsychiatric events (McClure et al., 2009; Moore et al., 2011). Cardiovascular events which have occurred with varenicline (Ware et al., 2013) might be due to effects on ganglionic α3β4 receptors. Our data show that BPC and BMSP come much closer to selectively regulating the activity of α4*. BPC is especially effective at targeting the HS form of α4* receptors, which may be important in smokers (Lester et al., 2009).

While α7 and α3β4* nAChR subtypes should not be dismissed as non-relevant 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 β2* 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 an off-target receptors for the treatment of nicotine dependence. While numerous lines of evidence support α4* and α6* 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 α3β4 receptors in the brain, most highly concentrated in the medial habenula, and some co-assembled with α5. The α5-containing receptors have been implicated in establishing aversive effects of high nicotine doses (Frahm et al., 2011), and, independent of α5, α3β4 receptors have also been implicated in nicotine reward and withdrawal (Jackson et al., 2013). Likewise, although homomeric α7 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 α7 and α5-containing receptors should be targeted, but whether they should be targeted differently from α4* and α6* receptors. The ideal drug might down-regulate the function of α4* and α6* receptors and enhance the function of α5-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 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 β2* 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, since in vivo effects are limited by other properties, including brain penetration and clearance. For instance, while 5-bromo-cytisine has high affinity and is a weak partial agonist of α4β2 nAChRs, it is not effective in behavioral models of antidepressant efficacy when injected peripherally, but was 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 α4 partial agonism from activity at α3β4 and α7, and to a large degree our data support the strength of that approach.

While 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.

While decreasing activity of β2* 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 α6* 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 α4β2α6β2β3 nAChRs than do the bispidine compounds, but it is not known whether this is important for their therapeutic efficacy. Since blockade of nAChRs containing the α4 subunit is sufficient to block nicotine reward (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 US, 30% of all smokers suffer from mental illness (Mackowick et al., 2012). While 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 have 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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Participated in research design: Peng, Mineur, Picciotto, and Papke
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Provided experimental materials: Guendisch, Eibl, Tomassoli
Wrote or contributed to the writing of the manuscript: Peng, Stokes, Guendisch, Picciotto, and Papke
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Footnote
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Table 1
Concentration-response data for BPC

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<th>Receptor</th>
<th>$I_{\text{max}}$</th>
<th>EC$_{50}$</th>
</tr>
</thead>
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<tr>
<td>$\alpha_4(2)\beta_2(3)$</td>
<td>0.31 ± 0.01</td>
<td>0.8 ± 0.2 µM</td>
</tr>
<tr>
<td>$\alpha_4(3)\beta_2(2)$</td>
<td>0.27 ± 0.02</td>
<td>9.4 ± 3.2 µM</td>
</tr>
<tr>
<td>$\alpha_4(2)\beta_2(2)\alpha_5$</td>
<td>0.10 ± 0.01</td>
<td>0.21 ± 0.01 µM</td>
</tr>
<tr>
<td>$\beta_3\alpha_4\beta_2\alpha_6\beta_2$</td>
<td>0.16 ± 0.01</td>
<td>0.35 ± 0.16 µM</td>
</tr>
</tbody>
</table>
Figure legends

**Figure 1.** Chemical structures of BPC and BMSP.

**Figure 2.** Effects of various concentrations of varenicline (Var), cytisine (Cyt), BPC, and BMSP on mouse α1β1εδ nAChR expressed in *Xenopus* 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 co-applied 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 pre-applied for 5 minutes before the co-application 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 (± SEM) in terms of peak amplitude from 4-8 oocytes.

**Figure 3.** Summary of the agonist activity of varenicline (Var), cytisine (Cyt), BPC, and BMSP obtained with human (α4)2β2 (HS α4β2), (α4)2β2 (LS α4β2), α4β2α5, β3α4β2α6β2, α7, and α3β4 nAChR expressed in *Xenopus* oocytes. Pure populations of HS α4β2, LS α4β2, and α4β2α5 nAChR were obtained by co-expressing the human β2-6-α4 concatamer with monomeric α4, β2, and α5 (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-α7 and net charge for α7) 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 α4β2, 100 µM for LS α4β2, 10 µM for α4β2α5, 30 µM for β3α4β2α6β2, 60 µM for α7, and 100 µM for α3β4. Data are shown as the averaged normalized data (± SEM) from n ≥4 oocytes at each condition.

**Figure 4.** Concentration-response curves for BPC on α4β2* 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 α4β2...
\((\alpha_4)_2(\beta_2)_3\), 100 \(\mu\)M for LS \(\alpha_4\beta_2\) \(((\alpha_4)_3(\beta_2)_2)\), 10 \(\mu\)M for \(\alpha_4\beta_2\alpha_5\), and 30 \(\mu\)M for \(\beta_3\alpha_4\beta_2\alpha_6\beta_2\). Values represent the averages (± SEM) of at least four oocytes.

**Figure 5.** Inhibition of ACh-evoked responses of various human nAChR subtypes expressed in *Xenopus* oocytes by acute co-application of varenicline (Var), cytisine (Cyt), BPC, or BMSP. Each partial agonist was co-applied at increasing concentrations with 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 \(\mu\)M for HS \(\alpha_4\beta_2\), 100 \(\mu\)M for LS \(\alpha_4\beta_2\), 10 \(\mu\)M for \(\alpha_4\beta_2\alpha_5\), 30 \(\mu\)M for \(\beta_3\alpha_4\beta_2\alpha_6\beta_2\), 60 \(\mu\)M for \(\alpha_7\), and 100 \(\mu\)M for \(\alpha_3\beta_4\). Data are shown as the averaged normalized data (± SEM) from \(n \geq 4\) oocytes at each condition.

**Figure 6.** Inhibition of ACh-evoked responses of various human nAChR subtypes expressed in *Xenopus* oocytes by bath application of varenicline (Var), cytisine (Cyt), BPC, or BMSP. A five-minute pre-application 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 \(\mu\)M for HS \(\alpha_4\beta_2\), 100 \(\mu\)M for LS \(\alpha_4\beta_2\), 10 \(\mu\)M for \(\alpha_4\beta_2\alpha_5\), 30 \(\mu\)M for \(\beta_3\alpha_4\beta_2\alpha_6\beta_2\), 60 \(\mu\)M for \(\alpha_7\), and 100 \(\mu\)M for \(\alpha_3\beta_4\). Data are shown as the averaged normalized data (± SEM) from \(n \geq 4\) oocytes at each condition.

**Figure 7.** Effects of bath-applied BMSP on the ACh-evoked responses of human \(\alpha_4\beta_2\), \(\alpha_4\beta_2\alpha_5\), and \(\beta_3\alpha_6\beta_2\alpha_4\beta_2\) nAChR expressed in *Xenopus* 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 nM, 30 nM, 100 nM, and 300 nM for HS \(\alpha_4\beta_2\) and \(\alpha_4\beta_2\alpha_5\), and 100 nM and 300 nM for LS \(\alpha_4\beta_2\) and \(\beta_3\alpha_6\beta_2\alpha_4\beta_2\). All data points represent an average of at least four oocytes (± SEM) for each condition.

**Figure 8.** A. Inhibition of ACh-evoked responses of *Xenopus* oocytes expressing human HS \(\alpha_4\beta_2\), LS \(\alpha_4\beta_2\), \(\alpha_4\beta_2\alpha_5\), and \(\beta_3\alpha_6\beta_2\alpha_4\beta_2\) 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 s. B. Effects of mecamylamine (mec) on the steady-state
HS α4β2 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 α4β2 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).

**Figure 9.** Effects of bath-applied nicotine and partial agonists on ACh-evoked responses of α7 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 ± SEM of 4-7 cells.

**Figure 10.** Modulation of ACh-evoked currents of LGN neurons and SR interneurons by nicotine, varenicline, and cytisine. Left panels: Time courses for the (A) 100 nM nicotine, (B) 10 nM varenicline, and (C) 100 nM cytisine-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 (A) 300 nM nicotine, (B) 500 nM varenicline, and (C) 3 μM cytisine in SR interneurons. ACh was applied using single-barrel pressure application pipettes containing 1 mM ACh with an interstimulus interval of 30 s. 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.

**Figure 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&B, left panels), whereas the bath
The application of BPC (n=12) or BMSP (n=9) had no significant effect on the ACh-evoked responses in SR interneurons (A&B, right panels).

**Figure 12.** The effects of BPC and BMSP on immobility time in the tail suspension test in mice (2 mg/kg and 5 mg/kg, respectively). Results are represented as mean ± SEM with n=10 in each group. Values are significant at ** P <0.01, *** P <0.001 when compared with the control group, based on a two-tailed t-test with Bonferroni corrections.
Figure 1

3,7-diazabicyclo[3.3.1]nonane (bispidine) privileged scaffold

Hybrid Approach

BPC

Template Approach

BMSP

SO₂CH₃
Figure 2

Graph A: Drug applied alone
- Peak current response normalized to ACh maximum
- Var, Cyt, BPC, BMSP
- Concentrations: 3 μM, 10 μM, 30 μM

Graph B: Drug co-applied with control ACh
- Peak current response normalized to control ACh
- Var, Cyt, BPC, BMSP
- Concentrations: 3 μM, 10 μM, 30 μM

Graph C: Drug pre-applied 5 min before co-application with control ACh
- Peak current response normalized to control ACh
- Var, Cyt, BPC, BMSP
- Concentrations: 0.3 μM, 1 μM, 3 μM
Figure 3

Agonist Activity

HS α4β2

LS α4β2

α4β2α5

β3α4β2α6β2

α7

α3β4

Log scale
Figure 4

The graph shows the response relative to ACh maximum as a function of [BPC] in μM for different isoforms of the α4β2 and α4β2α5 receptors. The curves represent different isoforms:

- α4(2)β2(3) (black triangles)
- α4(3)β2(2) (black squares)
- α4β2α5 (red triangles)
- β3α4β2α6β2 (blue diamonds)

The data points are indicated with error bars, showing the variability in the response at different concentrations of BPC.
Antagonist Activity: 5-Minute Pre-application

**HS α4β2**

<table>
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<tr>
<th>Var</th>
<th>Cyt</th>
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<th>BMSP</th>
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**LS α4β2**

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**α4β2α5**

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**β3α4β2α6β2**

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**α7**

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**α3β4**

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</table>
Figure 7
Figure 10

A. LGN neuron vs. Stratum radiatum interneuron with 100 nM nicotine.

B. LGN neuron vs. Stratum radiatum interneuron with 10 nM vorenicline.

C. LGN neuron vs. Stratum radiatum interneuron with 100 nM cytisine.

Figure 10 continues...
Figure 11