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Cytisine-Based Nicotinic Partial Agonists as Novel Antidepressant Compounds

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ABSTRACT

Nicotine and other nicotinic agents are thought to regulate mood in human subjects and have antidepressant-like properties in animal models. Recent studies have demonstrated that blockade of nicotinic acetylcholine receptors (nAChRs) including those containing the β2 subunit (β2*), results in antidepressant-like effects. Previous studies have shown that cytisine, a partial agonist at α4/β2* nAChRs, and a full agonist at α3/β4* and α7 nAChRs, has antidepressant-like properties in several rodent models of antidepressant efficacy; however, it is not clear whether more selective partial agonists will also be effective in these models. We tested cytisine and two derivatives, 5-bromo-cytisine (5-Br-Cyt) and 3-(pyridin-3′-yl)-cytisine (3-pyr-Cyt) for their ability to act as a partial agonist of different nAChR subtypes and to show antidepressant-like activity in C57/BL6 mice in the tail suspension, the forced-swim, and the novelty-suppressed feeding tests. 3-pyr-Cyt was a partial agonist with very low efficacy at α4/β2* nAChRS but had no agonist effects at other nAChRs normally targeted by cytisine, and it was effective in mouse models of antidepressant efficacy. Animals showed dose-dependent antidepressant-like effects in all three behavioral paradigms. 5-Br-Cyt was not effective in behavioral tests when administered peripherally, probably because of its inability to penetrate the blood-brain barrier, because it efficiently reduced immobility in the tail suspension test when administered intraventricularly. These results suggest that novel nicotinic partial agonists may provide new possibilities for development of drugs to treat mood disorders.

Numerous studies have suggested that tobacco smoke can modulate depressive symptoms in human subjects and that nicotinic agents can have antidepressant-like effects in animal models (Picciotto et al., 2002). Individuals with a history of depression have an approximately 50% higher incidence of smoking than the general population (Glassman et al., 1990). In addition, nicotine [(*S*)-3-(1-methyl-2-pyrrolidinyl)pyridine] patch can reduce symptoms of depression in nonsmokers (Salin-Pascual et al., 1995), whereas smoking cessation can exacerbate symptoms of depression (Glassman et al., 1990). Animal studies have also shown that chronic nicotine administration can elicit antidepressant-like effects in rats, both in the learned helplessness (Semba et al., 1998) and the forced swim (Djurić et al., 1999; Tizabi et al., 1999) paradigms.

Nicotine exerts its effects by binding to, activating, and desensitizing nicotinic acetylcholine receptors (nAChRs) in the central nervous system and autonomic ganglia (Picciotto et al., 2008). α4/β2* receptors (where * indicates the possible inclusion of other nAChR subunits, such as α5, α6, or β3 along with α4 and β2) are the most widely expressed nAChRs in the central nervous system and also have the highest affinity for nicotine, whereas α7* nAChRs, which are also found at high levels in brain, form functional homomers and are highly expressed in the hippocampus and cortex but also found in most other brain regions (Zoli et al., 1998). In the peripheral nervous system, α3/β4* nAChRs are the main ganglionic subtype, but they are only expressed at relatively low levels in the brain (Gotti et al., 2006). Compared with acetylcholine (2-acetoxy-*N*,*N*,*N*-trimethylethanaminium), the endogenous ligand of nAChRs, nicotine acts much like a partial agonist (Papke et al., 2007).

It seems somewhat paradoxical that increased endogenous acetylcholine levels result in depression-like symptoms

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; HS, high sensitivity; LS, low sensitivity; 5-Br-Cyt, 5-bromo-cytisine; 3-pyr-Cyt, 3-(pyridin-3′-yl)-cytisine.

(Janowsky et al., 1972), whereas nicotine administration can decrease depressive symptoms (Salin-Pascual et al., 1995); however, chronic nicotine administration can desensitize nAChRs (Reitstetter et al., 1999), resulting in functional antagonism (for reviews, see (Quick and Lester, 2002; Picciotto et al., 2008). Thus, blockade (i.e., antagonism and/or receptor desensitization) rather than activation of nAChRs might have antidepressant effects in human subjects. This hypothesis is supported by the fact that mecamylamine [(1*R*,2*R*,4*S*)-*N*,2,3,3-tetramethylbicyclo[2.2.1]heptan-2-amine], a nonselective nAChR antagonist, decreases symptoms of depression in patients with Tourette's syndrome (Janowsky et al., 1972) and has antidepressant-like effects in mice (Caldarone et al., 2004; Rabenstein et al., 2006; Mineur et al., 2007c). Similar antidepressant-like effects have been observed in mice treated with cytisine [(1*R*,5*S*)-1,2,3,4,5,6-hexahydro-1,5-methano-8*H*-pyrido [1,2*a*][1,5]diazocin-8-one] (Mineur et al., 2007c), a quinolizidine alkaloid isolated from the seeds of *Cytisus* sp. and related members of Leguminosae (Fabaceae). Cytisine is a partial agonist at β2* nAChRs and a full agonist at β4* nAChRs (Papke and Heinemann, 1994; Picciotto et al., 1995). Taken together, these data indicate that blockade of β2* nAChRs may be critical for the antidepressant-like effects of nicotinic agents. Likewise, studies in knockout mice have demonstrated that the absence of β2* nAChRs throughout development can lead to antidepressant-like phenotypes (Caldarone et al., 2004), and these mice are insensitive to the antidepressant-like effects of the nicotinic antagonist mecamylamine (Rabenstein et al., 2006). Moreover, amitriptyline, a tricyclic antidepressant, has no effect in β2 subunit knockout mice (Caldarone et al., 2004), strongly suggesting that β2* nAChRs are 3-(10,11-dihydro-5*H*-dibenzo[[*a*,*d*]]cycloheptene-5-ylidene)-*N*,*N*-dimethyl-1-propanamine important for the function of classical antidepressants. Indeed, it has been proposed that the antidepressant effects of fluoxetine [*N*-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine] could involve the acceleration of nAChR desensitization (García-Colunga et al., 1997).

Mecamylamine has been successfully used to treat depression in combination with selective serotonin reuptake inhibitors in clinical trials to treat patients resistant to the SSRIs alone (George et al., 2008); however, mecamylamine can result in side effects, including dysregulation of the autonomic system, probably because of its ability to block ganglionic α3/β4* nAChRs. Likewise, cytisine can decrease the effects of endogenous ACh at β2* nAChRs because of its high affinity and low efficacy as a partial agonist; however, cytisine is a full agonist at α3/β4* nAChRs at low concentrations, which results in autonomic activation and potential toxic side effects (Etter, 2006). Based on these observations, we started to investigate cytisine derivatives to identify compounds with greater selectivity for β2* nAChRs but relatively low affinity and efficacy at α3/β4* nAChRs (Fitch et al., 2005). We hypothesized that compounds that were more selective partial agonists of β2* nAChRs would have antidepressant-like effects in rodent behavioral models, with higher efficacy (and potentially fewer side effects).

Materials and Methods

Synthesis

Compounds were derived as described previously (Imming et al., 2001; Fitch et al., 2005). In brief, cytisine was obtained by isolation from seeds and pods from *Laburnum anagyroides* and *Laburnum watereri* and was subsequently protected at the secondary nitrogen with *N*-*t*Boc-anhydride. Halogenation was performed with *N*-bromosuccinimide for introduction of a bromo substituent on the *N*-*t*Boc-cytisine. The resulting products were 3- and 5-bromo derivatives along with the 3,5-dibromo derivative, which were subsequently separated by column chromatography on silica.

For 3-(pyridin-3′-yl)-cytisine, the 3-bromo derivative was further derivatized by introducing the pyridine-3-yl moiety from the corresponding pyridine-3-boronic acid via a Suzuki cross-coupling reaction under inert atmosphere in a mixture of 1,2-dimethoxyethane/water and $Pd(PPh_3)_4$ as a catalyst by means of microwave irradiation. The crude reaction mixture was liberated from the catalyst by extraction on an SPE C-18 column (Alltech Deutschland GmbH, Hamburg, Germany) with a methanol/water mixture and was purified by high-performance liquid chromatography on a reversed-phase column with a methanol/water gradient.

Both *N*-*t*Boc-protected derivatives were cleaved from the *t*Boc protection group by means of microwave irradiation in pure water. Subsequent lyophilization yielded the compounds at >99% purity (liquid chromatography/mass spectrometry). 5-Bromo-cytisine was selected because it showed an antagonist-like profile for α4/β2 nAChRs. In contrast, 3-bromo cytisine is an agonist. Therefore, we investigated whether a 3-substituted compound with a larger substituent might bias the functional properties toward partial agonism.

Cytisine and its derivatives were tested by radioligand binding assays for their abilities to compete for [^{3}H]epibatidine and [^{3}H]-methyllycaconitine binding sites in rat forebrain (α4/β2*, α7*), pig adrenals (α3/β4*) and *Torpedo californica* electroplax [(α1)2β1γδ] membrane fractions as has been described previously (Fig. 1) (Gündisch et al., 1999; Mukhin et al., 2000; Gohlke et al., 2002).

ACh Receptor Clones

Human nAChR receptor clones and α4/β2 concatamers were the generous gift of Dr. Jon Lindstrom (University of Pennsylvania, Philadelphia, PA).

Expression in *Xenopus laevis* Oocytes

Mature (>9 cm) female *X. laevis* African frogs (Nasco, Ft. Atkinson, WI) were used as a source of oocytes. Before surgery, the frogs were anesthetized by placing them in a 1.5 g/l solution of MS222 for 30 min. Oocytes were removed from an incision made in the abdomen.

Harvested oocytes were treated with 1.25 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) for 2 h at room temperature in calcium-free Barth's solution (88 mM NaCl, 1 mM KCl, 2.38 mM $NaHCO_3$, 0.82 mM $MgS0_4$, 15 mM HEPES, pH 7.6, and 12 mg/l tetracycline) to remove the follicular layer. Stage 5 oocytes were isolated and injected with 50 nl (5–20 ng) of each subunit cRNA (Papke and Heinemann, 1994; Papke and Porter Papke, 2002; Papke et al., 2007). Recordings were normally conducted 2 to 5 days after injection.

Electrophysiology

Experiments were conducted using OpusXpress6000A (Molecular Devices, Sunnyvale, CA). OpusXpress is an integrated system that provides automated impalement and voltage clamp of up to eight oocytes in parallel. Both the voltage and current electrodes were filled with 3 M KCl. The oocytes were clamped at a holding potential of −60 mV.

Data were collected at 50 Hz and filtered at 20 Hz for α7 and at 5 Hz for α4β2. The oocytes were bath-perfused with Ringer's solution. Agonist solutions were delivered from a 96-well plate using disposable tips. Flow rates were set at 2 ml/min for α7 and at 4 ml/min for α4/β2.

Experimental Protocols and Data Analysis

Responses of α7 nAChRs were calculated as net charge (Papke and Porter Papke, 2002), and responses of other nAChR subtypes are

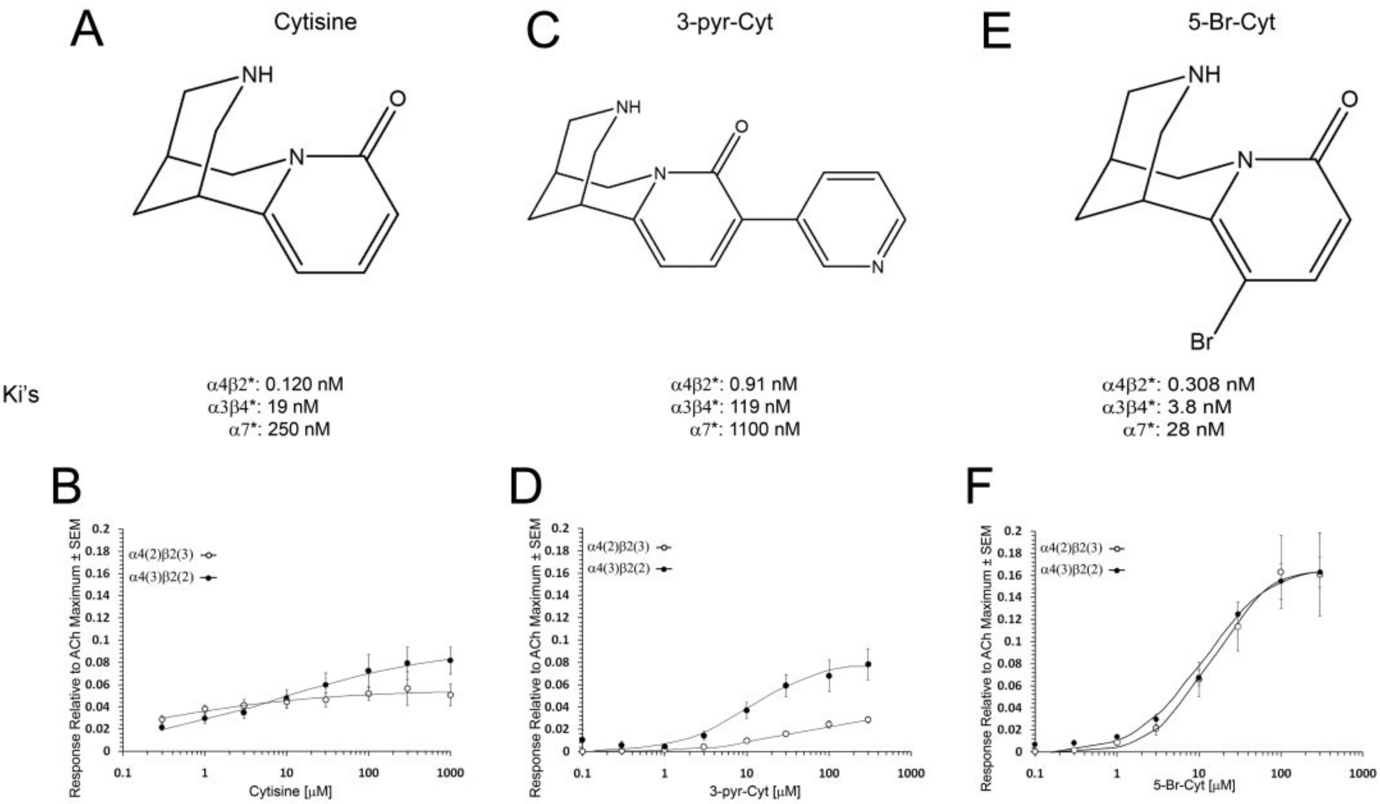

Fig. 1. Partial agonist properties of cytisine and derivatives at high- and low-sensitivity α4β4 nAChRs. Structure and activity of the compounds used in this study: cytisine (A and B), 3-pyr-Cyt (C and D), and 5-Br-Cyt (E and F). K_i values have been published previously and were determined by competition for [^{3}H]epibatidine and [^{3}H]methyllycaconitine binding sites using radioligand binding in rat forebrain (α4/β2*, α7*), pig adrenals (α3/β4*), and *T. californica* electroplax [$(\alpha1)_2\beta1\gamma\delta$] membrane fractions (Gündisch et al., 1999; Mukhin et al., 2000; Gohlke et al., 2002). The high-sensitivity form of α4β2 [α4(2)β2(3)], was generated by the coexpression of RNA coding the β2–6-α4 concatamer along with monomeric β2 (Zhou et al., 2003). The low-sensitivity form of α4β2 [α4(3)β2(2)] was generated by the coexpression of RNA coding the β2–6-α4 concatamer along with monomeric α4 (Zhou et al., 2003). The data plotted (B, D, and F) represent the average responses (±S.E.M.) from at least four oocytes at each concentration and have been normalized relative to the maximum ACh-evoked responses for each receptor subtype (see *Materials and Methods*).

reported as peak currents. Each oocyte received initial control applications of ACh, and then experimental drug applications, and follow-up control applications of ACh. The optimal control ACh concentrations were empirically determined in separate experiments. For α7 nAChRs, the control ACh concentration was 300 μM; for α3β4 nAChRs, the ACh control was 100 μM; and for α4β2 nAChRs formed after the injection of α4 and β2 RNA at 1:1 ratio, the ACh control was 30 μM. Because the injection of RNAs at the 1:1 ratio typically produces a heterogeneous population of receptors, with two alternative subunit stoichiometries [α4(2)β2(3) and α4(3)β2(2)], we used a concatamer of the α4 and β2 subunits (Zhou et al., 2003) to force the formation either α4(2)β2(3) receptors, which have a high sensitivity to relatively low concentrations of ACh or nicotine (HS α4β2) or α4(3)β2(2) receptors that generate larger current but require relative high concentrations of ACh or nicotine for maximal activation ACh (LS α4β2). Specifically the β2–6-α4 concatamer was coexpressed with either monomeric α4 or β2 to generate either the low sensitivity (LS) or HS forms of the receptors, respectively. In these receptors, the agonist binding sites are within each of the two concatamers and the monomeric subunit forms the fifth structural subunit in the pentamer, although this has not been established for cytisine and its analogs. For the high- and low-sensitivity α4/β2 nAChRs formed with concatamers, the ACh controls were 10 and 100 μM, respectively. Responses to experimental drug applications were calculated relative to the preceding ACh control responses to normalize the data, compensating for the varying levels of channel expression among the oocytes. Mean values and S.E.M. were calculated from the normalized responses of at least four oocytes for each experimental concentration. Values measured relative to the ACh controls were expressed relative to ACh-evoked maximal responses based on separate ACh concentration-response studies (data not shown) that provided the ratio between the ACh control responses and the maximal ACh-evoked responses for each receptor subtype. For concentration-response relationships, data were plotted using Kaleidagraph 3.0.2 (Abelbeck/Synergy, Reading, PA), and curves were generated from the Hill equation: response = $I_{\max}$ [agonist]^n/[agonist]^n + $(\mathrm{EC}_{50})^n$, where $I_{\max}$ denotes the maximal response for a particular agonist/subunit combination, and n represents the Hill coefficient. $I_{\max}$, n, and the EC_{50} were all unconstrained for the fitting procedures.

Animals

Three-month-old C57BL/6J (B6) male mice (25–30 g) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were group-housed (5 mice/cage) under standard laboratory conditions (21 ± 2°C, 12:12-h light/dark cycle, lights on at 7:00 AM) with food and water available ad libitum, unless otherwise noted. At least 2 weeks of rest followed their arrival; during that time, mice were marked on their tail with a permanent marker for identification and were randomly assigned to one of the different treatment groups (n = 10/group unless otherwise stated). All procedures were approved by the Yale University Animal Care and Use Committee.

General Testing Conditions

Mice were habituated to the testing room at least 30 min before any behavioral evaluation. All tests took place between 12:00 PM and 5:00 PM The testing area was dimmed to limit stress or anxiety.

During behavioral evaluation, the experimenters were blinded to treatment.

Treatments: Experimental Design

The concentrations of the compounds used in this study were based on previous studies using cytisine (Mineur et al., 2007c), on the relative affinities of each compound for α4/β2 nAChRs, their molecular weight, and their availability. Pilot studies also helped refine the dose range used in behavioral assays. In addition, we found that using higher doses than those described here were not well tolerated by the mice and led to decreased locomotion, confounding the outcome of behavioral testing. Cytisine (0.3, 0.6, 1.0, and 1.5 mg/kg), 5-Br-Cyt (0.3, 0.6, 0.9, 1.2 mg/kg), 3-pyr-Cyt (0.3, 0.6, 0.9 mg/kg), nicotine (expressed as free base; 0.03, 0.09, 0.5, and 1.0 mg/kg), and fluoxetine (used as a positive control; 10 mg/kg) were diluted in phosphate-buffered saline (saline). Saline or drugs were injected intraperitoneally 30 min before the tail suspension test or the forced swim test. The two tests were separated by 48 h. After the latter test, saline or drug was injected daily (between 9:00 AM and 11:00 AM) for 15 days, and mice were subsequently tested in the novelty-suppressed feeding test. Testing for novelty-suppressed feeding was performed before daily injection to avoid acute effects of the drugs. Nicotine was not used in this test because it decreases appetite, confounding the motivation to seek food in the novelty-suppressed feeding test (Mineur et al., 2007c).

For the experiment with intraventricular infusion of 5-Br-Cyt, 20 naive mice (10 5-Br-Cyt-treated and 10 vehicle-treated) underwent cannulation surgery. A concentration of 50 ng of 5-Br-Cyt/1 μl/animal was used based on the molarity of the compound, its pharmacological properties, and comparisons with the effects of the other compounds. The solution was prepared in artificial cerebrospinal fluid and was infused slowly over ~30 s, 20 to 30 min before the beginning of the tail suspension test. The animals from the control group (artificial cerebrospinal fluid) received only artificial cerebrospinal fluid.

Surgery

Mice were anesthetized with a mixture of xylazine/ketamine diluted in saline (100 and 10 mg/kg, respectively) at 10 ml/kg. After complete anesthesia, mice were placed in a stereotaxic frame, and one guide canula (3-mm pedestal; designed to receive cannulas of gauge 33; Plastic Products, Roanoke, VA) was implanted per animal (from bregma, antero-posterior, +1.1 mm; lateral, 1 mm; ventral, −3.5 mm). After cannulation, mice were singly housed and allowed to recover for 1 week before testing.

After testing, mice were irreversibly anesthetized with chloral hydrate and canula placement was checked. After verification of placement, seven control-treated and eight 5-Br-Cyt-treated animals were used for subsequent analysis.

Behavioral Assays

Mice underwent three behavioral tests sequentially: the tail suspension test, the forced swim test (48 h later), and then the novelty-suppressed feeding test (15 days later). Although somewhat stressful, the tail suspension and forced swim tests were of short duration, and 48 h of rest was observed between each paradigm. Mice were not handled before testing. The novelty-suppressed feeding test took place 15 days after the tests sensitive to acute antidepressant treatment. C57BL/6J males are particularly resistant to stress, even chronic stressors (Mineur et al., 2006, 2007a). Previously published articles have used a similar design, and we have validated the performance in single versus repeated tests in C57BL/6 mice with several antidepressant-like compounds and mecamylamine (Rabenstein et al., 2006; Mineur et al., 2007b,c). This design allows us to reduce the number of animals used for these studies resulting in n = 10 for each dose. Each mouse was subjected to the same tests, from less stressful to more stressful as recommended in previously published work (Crawley, 2008). In previous experiments, we have never seen a difference in animals subjected to the forced swim test alone or after the tail suspension test (data not shown). Likewise, the novelty-suppressed feeding test was not affected by earlier testing in the forced swim test in our hands (data not shown).

Tail Suspension Test. Mice were suspended by the tail by gently taping the tail to a paper clip that was then attached to a length of string, and time spent immobile was recorded over a 6-min period (Mineur et al., 2006). After completion of the test, mice were returned to a holding cage until all cage-mates were tested. At the end of all experiments, mice were returned to their home cage and transferred back to the holding room.

Forced Swim Test. Mice were gently placed in a 4-liter beaker (18 cm in diameter) filled with 15 cm of water (~25–26°C) to prevent mice from touching the bottom of the beaker with their paws or tail. Time spent immobile during the 15-min testing period was recorded. The 15-min test was chosen based on our prior experience with C57BL/6J mice, because this strain shows very little immobility during the first 5 min of testing (Caldarone et al., 2003, 2004; Mineur et al., 2006), the time point often used in other strains of mice and in rats. After testing, each mouse was placed in a warm holding cage (30–35°C) with bedding covered by a paper towel. After each mouse was tested, animals were returned to the holding room.

Acute Locomotor Activity Measurements. Mice were placed in a clean Plexiglas cage (48 × 22 × 18 cm) for 20 min after injection of one of the nicotinic compounds. Locomotor activity was recorded using the OptiMax system (Columbus Instruments, Columbus, OH). All mice from the same cage were tested at the same time in separate locomotor boxes. Subjects were returned to their home cage at the end of the test.

Novelty-Suppressed Feeding Test. The protocol for novelty-suppressed feeding was based on previously published paradigms (for review, see Dulawa et al., 2005). After 15 days of drug treatment, mice were weighed, and food was removed from the cage. Twenty-four hours later, mice were transferred to the testing room, weighed again, placed in a clean holding cage, and allowed to habituate for at least 30 min. The testing apparatus consisted of a clear Plexiglas enclosure (40 × 40 × 17 cm) with a lid. The floor was covered with 2 cm of corncob bedding. A small piece of mouse chow was placed in the center of the arena on a piece of white circular filter paper (9.5 cm in diameter). At the start of the experiment, each mouse was placed in the corner of the testing area, and the time to the first feeding event was recorded. Immediately after the mouse began to eat, the subject was placed alone for 5 min in its original home cage with a pre-weighed piece of lab chow. At the end of the 5-min period, the amount of food consumed was determined. After all mice from a single cage were tested, mice were returned to their home cage.

Statistical Analyses

Data from the behavioral assays were evaluated with analyses of variance, with treatment and concentration as between subject factors. When relevant, post hoc analyses were performed by t tests with Bonferroni/Dunnett's correction for multiple comparisons. α was set at 5%.

Results

Electrophysiological Properties of 3-pyr-Cyt and 5-Br-Cyt

The electrophysiological properties of 3-pyr-Cyt and 5-Br-Cyt are summarized in Tables 1 and 2. Cytisine (Fig. 1A) was less potent but more efficacious at low sensitivity (LS) α4β2 receptors (concatamers containing three α4 and two β2 subunits; I_{max} 10% that of ACh; Fig. 1B) compared with HS α4β2 receptors (concatamers containing two α4 and three β2 subunits). In contrast, 3-pyr-Cyt (Fig. 1C) is a relatively weak partial agonist for both the LS and HS receptors (efficacy 8

TABLE 1
Maximal responses and potencies of the compounds used in this study with respect to activation of nAChRs expressed in oocytes
The parameters are those of the fits to the Hill equation (see *Materials and Methods*). I_{max} values are expressed relative to ACh I_{max}. Note that the responses of α4(2)β2(3) receptors were too small to obtain reliable curve fits (N.A.).

Agonist	Subunit	Activation		
		I_{max}	n	EC_{50}
Ach	hα4(3)β2(2)	1	0.76 ± 0.1	73 ± 12
Ach	hα4(2)β2(3)	1	0.9 ± 0.1	1.7 ± 0.3
5-Br-Cyt	hα4(3)β2(2)	0.17 ± 0.01	1.2 ± 0.1	15.1 ± 1.7
5-Br-Cyt	hα4(2)β2(3)	0.17 ± 0.01	1.0 ± 0.1	13.3 ± 1.7
5-Br-Cyt	Mixed α4β2	0.162 ± 0.005	1.4 ± 0.1	38 ± 3
5-Br-Cyt	hα3β4	0.66 ± 0.04	1.66 ± 0.16	124 ± 13
5-Br-Cyt	hα7	0.386 ± 0.015	2.4 ± 0.4	18.1 ± 1.8
3-pyr-Cyt	hα4(3)β2(2)	0.03 ± 0.001	0.81 ± 0.03	31 ± 3
3-pyr-Cyt	hα4(2)β2(3)	0.08 ± 0.01	1.0 ± 0.2	12 ± 4
3-pyr-Cyt	Mixed α4β2	0.023 ± 0.002	1.2 ± 0.2	33 ± 6
Cytisine	hα4(3)β2(2)	0.10 ± 0.02	0.35 ± 0.06	12.7 ± 8.7
Cytisine	hα4(2)β2(3)	≤0.05	N.A.	N.A.

N.A., not applicable.

and 3%, respectively), with similar potencies for both forms of α4β2 nAChRs (Fig. 1D). Another cytisine derivative, 5-Br-Cyt (Fig. 1E), showed no significant differences in its activity at LS and HS forms of α4/β2 nAChRs (Fig. 1F) and was a partial agonist with approximately 16% of the efficacy of ACh.

Consistent with what has been reported for the effects of cytisine (Sigma-Aldrich, St. Louis, MO) (Papke et al., 2007) on rat nAChRs expressed in oocytes, 5-Br-Cyt was relatively efficacious at α7 and α3β4 nAChRs (Fig. 2A). In contrast, 3-pyr-Cyt (Fig. 2B) produced very little activation (≤5% ACh maximum) of α3β4 or α7 receptors at concentrations ≤100 μM, making it a weak partial agonist for all of these neuronal nAChR subtypes. The partial agonist activity of 3-pyr-Cyt for α4β2 nAChRs was further studied in ACh coapplication experiments. As expected, coapplication of the partial agonist 3-pyr-Cyt with the full agonist ACh to cells expressing α4β2 nAChRs resulted in decreased responses compared with those produced by ACh alone, dependent on 3-pyr-Cyt concentration and limited by the intrinsic activity of 3-pyr-Cyt (Fig. 2C). There was no evidence for residual desensitization produced by the compounds used in the present study (data not shown). That is, ACh controls recorded 5 min after the application of the experimental compounds were not significantly different from those recorded before experimental drug application, throughout the concentration ranges tested. Although this experiment confirms that 3-pyr-Cyt can bind to α4β2 receptors and limit ACh responses, it should be noted that this coapplication protocol certainly underestimates the potency that 3-pyr-Cyt would have for similar effects in vivo. In a therapeutic context, the potency of 3-pyr-Cyt for modulating endogenous cholinergic signals after systemic delivery would most probably correspond to its very high affinity for inducing and maintaining α4β2 receptors in the desensitized state, as measured by binding studies.

TABLE 2
Maximal response and potency of 3-pyr-Cyt used in this study on inhibition of the response to 30 μM ACh

Drug	Subunit	Inhibition of 30 μM ACh-Evoked Response	
		n	IC_{50}
3-pyr-Cyt	Mixed α4β2	−0.8 ± 0.1	60 ± 12

Behavioral Effects of Nicotine, Cytisine, 3-pyr-Cyt, and 5-Br-Cyt

Tail Suspension Test. There was a dose-dependent effect of 3-pyr-Cyt and cytisine but not 5-Br-Cyt on immobility in the tail suspension test [$F(3,48) = 3.18$, $p = 0.032$; $F(3,47) = 4.46$, $p = 0.007$; and $F(4,44) = 0.58$, $p = 0.68$, respectively; Fig. 3, A–C].

Post hoc t test analyses revealed that mice injected with 3-pyr-Cyt spent significantly less time immobile at 0.6 mg/kg ($p = 0.012$), whereas the effects of cytisine only reached significance at 1 mg/kg ($p = 0.004$). At a higher dose, cytisine (1.5 mg/kg) still showed significant effects in the tail suspension test ($p = 0.016$), whereas higher doses of 3-pyr-Cyt (0.9 mg/kg) showed a trend that did not reach significance ($p = 0.14$). In comparison, there was a significant main effect of nicotine [$F(4, 55) = 4.86$, $p = 0.002$]; however, post hoc analyses revealed that this interaction was mainly because of a significant difference between 0.03 and 1 mg/kg ($p < 0.0001$), whereas no significance was found between saline treatment and any of the doses of nicotine used in this study (Fig. 3D). Fluoxetine induced a significant reduction of immobility at 10 mg/kg compared with saline [$F(1, 19) = 6.05$, $p = 0.026$; Fig. 3D].

Forced Swim Test. Mice treated with cytisine or 3-pyr-Cyt, but not 5-Br-Cyt, showed a dose-dependent decrease in immobility in the forced swim test [$F(3,44) = 11.58$, $p < 0.0001$; $F(3,40) = 10.51$, $p < 0.0001$; and $F(4,40) = 1.80$, $p = 0.14$, respectively; Fig. 4, A–C]. Post hoc t tests indicated that mice were significantly less immobile when they were treated with 0.3, 0.6, or 0.9 mg/kg 3-pyr-Cyt ($p = 0.015$, $p < 0.0001$, and $p = 0.0014$, respectively). Mice treated with cytisine were significantly less immobile at doses of 0.75 and 1 mg/kg ($p = 0.014$ and $p < 0.0001$, respectively), but this effect was not seen at 1.5 mg/kg. Acute nicotine treatment had no significant main effect in the forced swim test [$F(4,55) = 1.17$, $p = 0.33$; Fig. 4D]; however, fluoxetine resulted in a significant reduction of immobility at 10 mg/kg compared with saline-treated animals [$F(1,16) = 5.34$, $p = 0.034$; Fig. 4D].

Locomotor Activity. No overall treatment effect of any of the compounds tested was detected on acute locomotor activity [$F(4,45) = 1.58$, $p = 0.33$; Fig. 5].

Novelty-Suppressed Feeding. As has been reported previously (Mineur et al., 2007c), chronic (15 days), but not

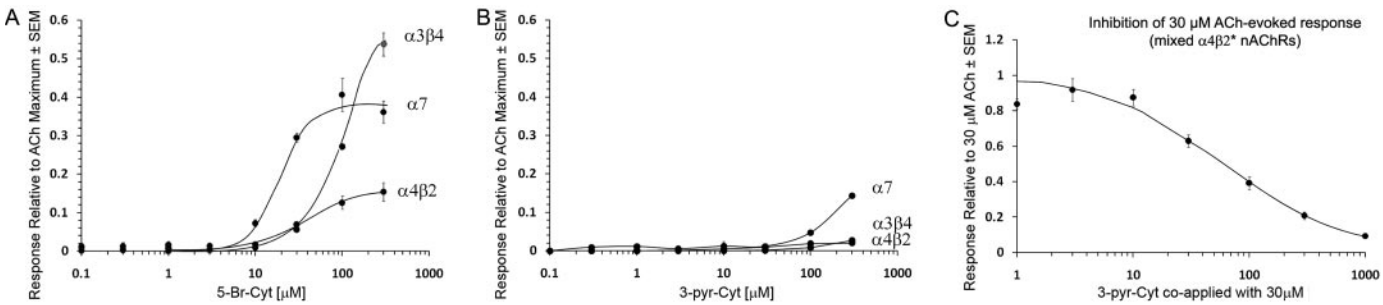

Fig. 2. Effects of 5-Br-Cyt and 3-pyr-Cyt on α4β2, α3β4, and α7 nAChR subtypes. 5-Br-Cyt (A) and 3-pyr-Cyt (B) were tested for their ability to activate α3β4 and α7 type nAChRs expressed in *X. laevis* oocytes and compared with activity at α4β2 nAChRs (Papke and Heinemann, 1994; Papke and Porter Papke, 2002; Papke et al., 2007). The data plotted represent the average responses (±S.E.M.) from at least four oocytes at each concentration and have been normalized relative to the ACh maximal responses for each receptor subtype (see *Materials and Methods*). C, effects of 3-pyr-Cyt on α4β2 receptors were also studied in coapplication experiments with ACh. Oocytes were tested for their responses to applications of 30 μM ACh, and these responses were compared with the responses evoked by 30 μM ACh coapplied with increasing concentrations of 3-pyr-Cyt. The coapplication responses of at least four oocytes (±S.E.M.) are plotted, normalized to the responses of the same oocytes to ACh alone. Note that in these experiments (A–C) the α4β2 receptors were formed from the coexpression of α4 and β2 monomers and so represent a mixed population of the high- and low-sensitivity subtypes.

acute, cytisine treatment decreased the latency to initiate feeding in the novelty-suppressed feeding test compared with saline treatment [$F(2, 32) = 2.42$, $p = 0.01$; Fig. 6A], but post hoc *t* test analyses show that this effect only reached significance at a dose of 1 mg/kg ($p = 0.0001$). A significant effect of 3-pyr-Cyt was also observed [$F(2, 33) = 3.39$, $p = 0.04$; Fig. 6B], and post hoc *t* test analyses revealed that this effect was only significant at 0.3 mg/kg 3-pyr-Cyt ($p = 0.023$) but not at 0.6 mg/kg ($p = 0.52$). No significant effect of 5-Br-Cyt was seen in the novelty-suppressed feeding test [$F(3, 36) = 0.10$, $p = 0.95$; Fig. 6C]. In comparison, fluoxetine induced a significant decrease in the time to first feed at 10 mg/kg com-

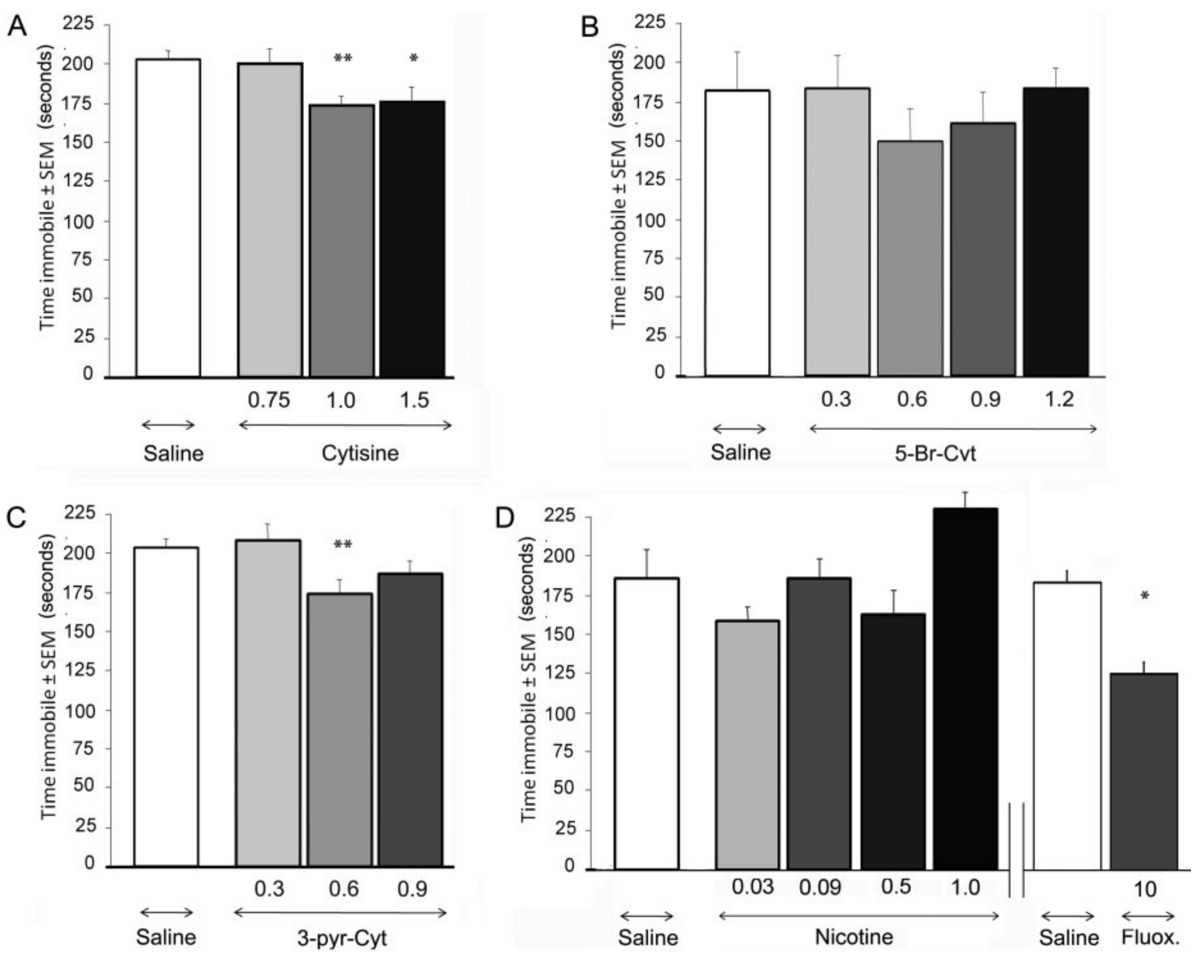

Fig. 3. Effects of cytisine, 3-pyr-Cyt, 5-Br-Cyt, nicotine, and fluoxetine in the tail suspension test. Total time spent immobile in the tail suspension test by C57BL/6J male mice treated with various doses of cytisine (A), 3-pyr-Cyt (B), 5-Br-Cyt (C), and nicotine and fluoxetine (D). $n = 10$/treatment group. The *x*-axis values indicate the dose injected in milligrams per kilogram. Error bars represent S.E.M.. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

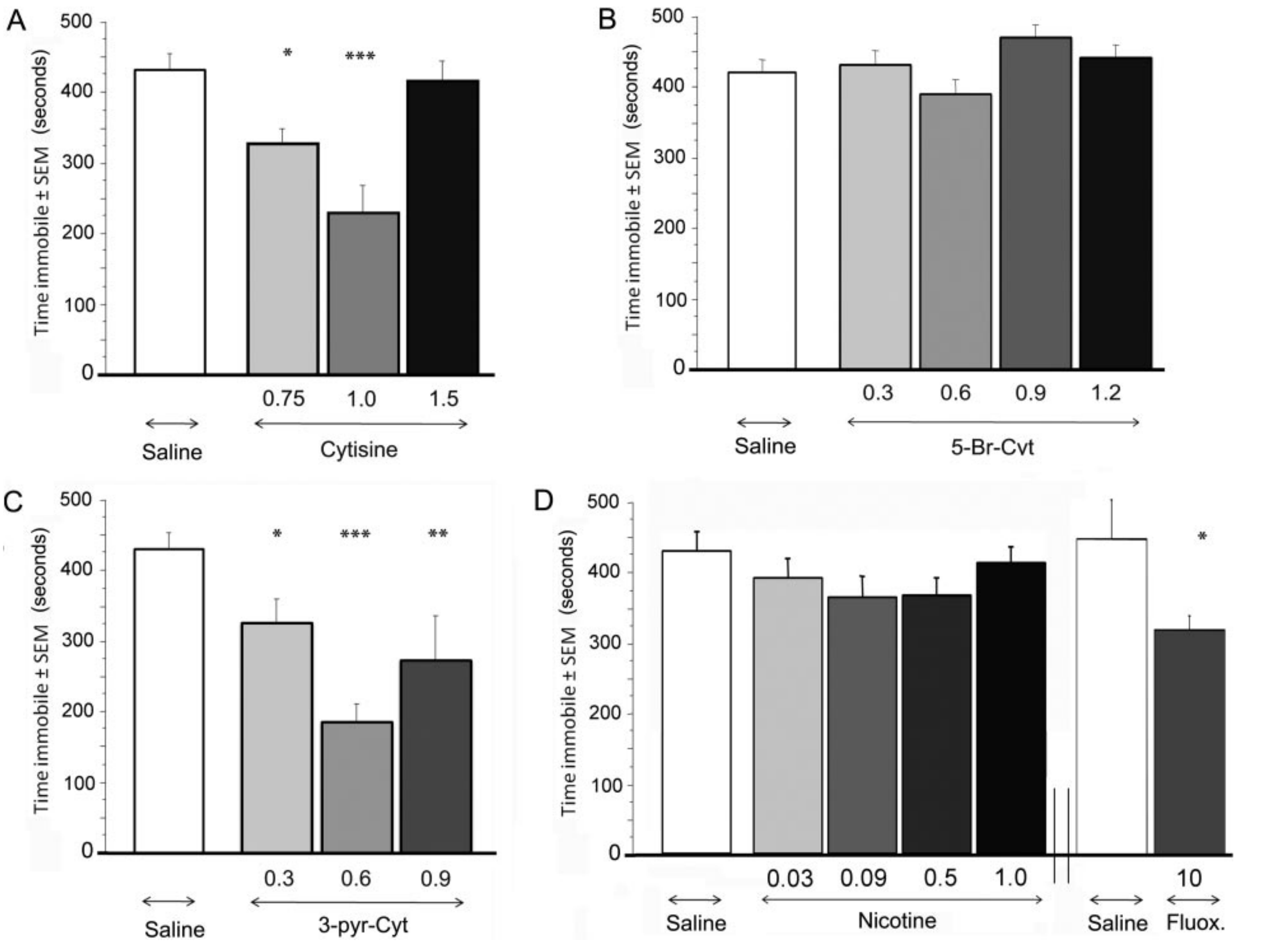

Fig. 4. Effects of cytisine, 3-pyr-Cyt, and 5-Br-Cyt in the forced swim test. Total time spent immobile in the forced swim test by C57BL/6J male mice treated with various doses of cytisine (A), 3-pyr-Cyt (B), 5-Br-Cyt (C), and nicotine and fluoxetine (D). n = 10/treatment group. The x-axis values indicate the dose injected in milligrams per kilogram. Error bars represent S.E.M.. $*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$.

pared with saline [$F(1, 13) = 9.09$, $p = 0.009$; Fig. 6D]. No effects on home-cage food consumption (5 min in home cage) or body weight were observed at any doses of cytisine, 3-pyr-Cyt, 5-Br-Cyt, or fluoxetine compared with saline-treated animals (data not shown).

Behavioral Effects of Centrally Administered 5-Br-Cyt

To determine whether the absence of effect of 5-Br-Cyt in these tests of antidepressant action was because of a lack of efficacy or a lack of penetration across the blood brain barrier, 5-Br-Cyt was infused into the ventricle. Centrally administered 5-Br-Cyt induced a significant antidepressant-like effect in the tail suspension test [$F(1,14) = 6.14$, $p = 0.026$; Fig. 7], suggesting that the lack of effect after peripheral administration was due to low penetration into the brain. It should be noted that the difference in baseline observed between controls of the intraperitoneal and intracerebroventricular 5-Br-Cyt experiments is probably due to the effects of canula implantation, more intensive handling of animals with central drug administration and single housing of cannulated mice.

Discussion

We investigated the electrophysiological and behavioral properties of two cytisine derivatives, developed with the goal of identifying α4β2 nAChR-selective partial agonists with lower affinity for other nAChR subtypes, including α3β4. Two compounds were selected that displayed selectivity as partial agonists of α4β2 nAChRs: 3-pyr-Cyt (a pyridine-3-yl derivative) and 5-Br-Cyt. Based on the observation that nicotinic antagonists are antidepressant-like (Rabenstein et al., 2006) and that acute administration of a nicotinic agonist is not consistently antidepressant-like (Figs. 3D and 4D), along with the observation that β2 knockout mice have similar phenotypes to wild-type mice treated with mecamylamine or classical antidepressants (Rabenstein et al., 2006), we propose that inactivation/inhibition of α4/β2* nAChRs is a plausible mechanism underlying the effects of cytisine derivatives in these behavioral models.

Compared with cytisine and nicotine, efficacious agonists at α3β4 and α7 receptors (Papke et al., 1994, 2007; Picciotto et al., 1995; Papke and Porter Papke, 2002), 3-pyr-Cyt showed little effect at α3β4 or α7 nAChRs; however, 3-pyr-Cyt was a high-affinity, low-efficacy partial agonist of α4β2 nAChRS (<10% activity compared with ACh). Both cytisine and 3-pyr-Cyt had differential effects on high- and low-sensitivity α4β2 nAChRs; however, whereas cytisine was a more efficacious partial agonist at LS nAChRs (10% ACh) and a low-efficacy partial agonist at HS nAChRs (less than 5%) as has been shown previously (Bermudez and Moroni, 2006), 3-pyr-Cyt was a low-efficacy partial agonist at both HS and

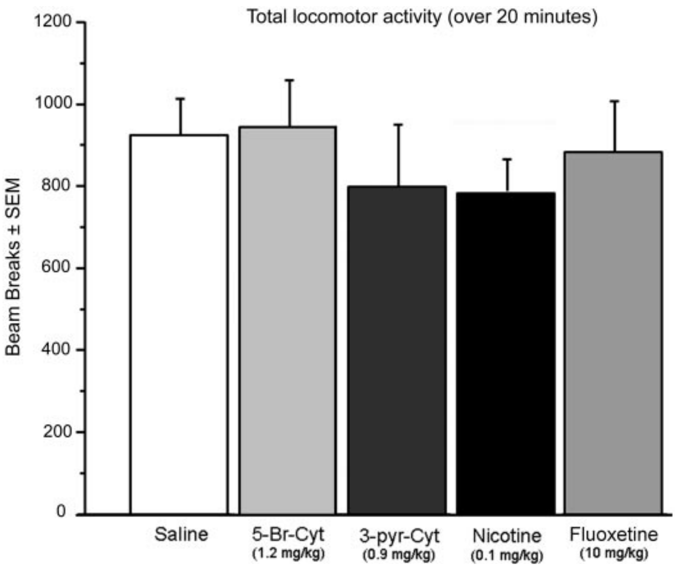

Fig. 5. Effects of acute cytisine, nicotine, 3-pyr-Cyt, and 5-Br-Cyt on locomotor activity. Horizontal activity was measured as total number of beam breaks in C57BL/6J male mice 20 min after cytisine, 3-pyr-Cyt, 5-Br-Cyt, or nicotine. $n = 10$/treatment group. Error bars represent S.E.M.

LS nAChRs (8 and 3% of ACh, respectively). Whether the small differences in efficacy at these receptors have measurable functional consequences in vivo remain unclear. Taken together, these data indicate that 3-pyr-Cyt is a very weak partial nicotinic agonist at α4β2 nAChRs with much less activity at α3β4 or α7 nAChRs than cytisine. In contrast, 5-Br-Cyt did not show differential effects at LS and HS nAChRs and had an average efficacy of ~18%, at least double that of 3-pyr-Cyt, at both receptor subtypes. 5-Br-Cyt also showed greater efficacy at α7 and α3β4 nAChRs than 3-pyr-Cyt.

Both cytisine and 3-pyr-Cyt showed antidepressant-like effects in mice across three different models of antidepressant efficacy. The cytisine used in this study was synthesized in house rather than obtained commercially as in previous studies (Mineur et al., 2007c) and was potentially more purified and therefore active at somewhat lower concentrations than in the previous study.

In the tail suspension test, 3-pyr-Cyt induced an antidepressant-like effect at lower concentrations than cytisine, whereas acute nicotine treatment had no significant effects at any of the doses tested. This provides further evidence that blockade rather than activation of β2* nAChRs is responsible for the antidepressant-like effects of nicotinic agents (Caldarone et al., 2004; Rabenstein et al., 2006; Mineur et al., 2007c) because 3-pyr-Cyt had very low efficacy but high potency at α4β2 nAChRs and low potency at both α3β4 and α7 nAChRs. 3-pyr-Cyt may be more effective in tests of antidepressant action than cytisine because it is a low-efficacy partial agonist at both LS and HS α4β2 nAChRs, whereas cytisine is a more effective agonist at LS nAChRs. We hypothesize that in vivo, 3-pyr-Cyt competes with endogenous ACh, limiting α4β2*-dependent cholinergic neurotransmission, similar to a competitive antagonist. The inverted U-shaped behavioral response to cytisine and 3-pyr-Cyt in the

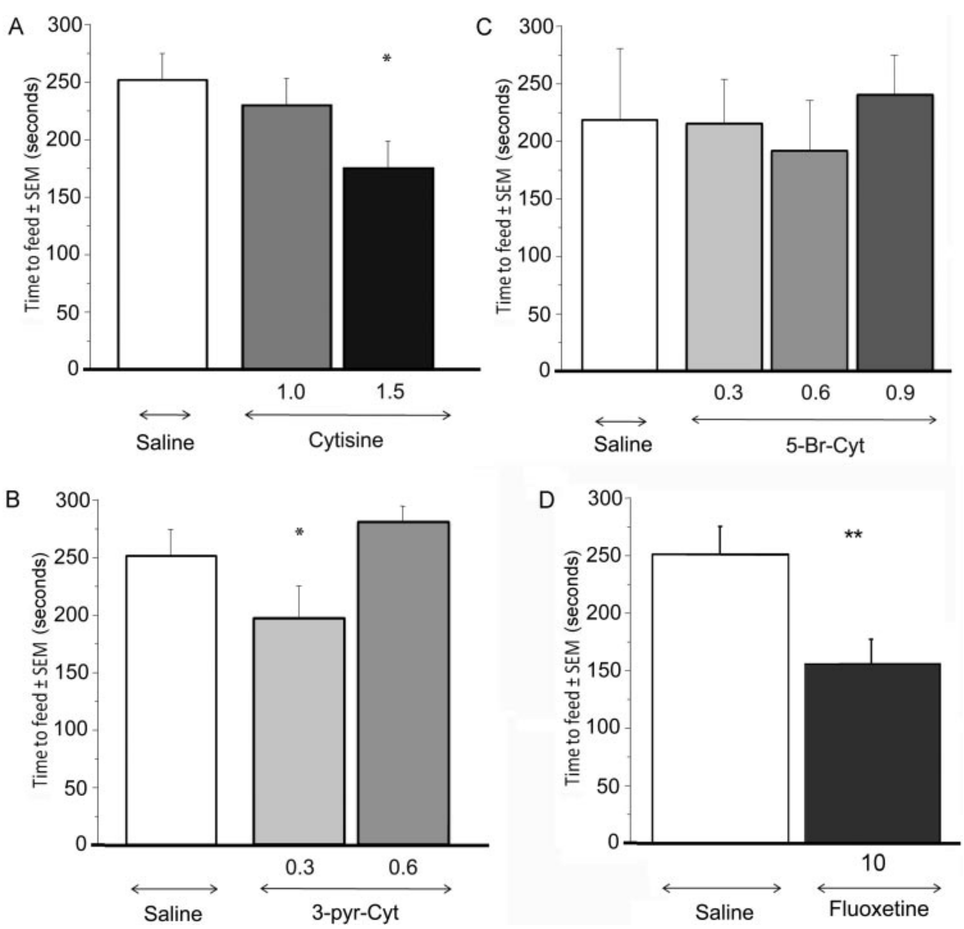

Fig. 6. Effects of cytisine, 3-pyr-Cyt, and 5-Br-Cyt in the novelty suppressed feeding test. Time required for C57BL/6J male mice to initiate a feeding episode in the novelty suppressed feeding test after chronic injection (15 days) of various doses of cytisine (A), 3-pyr-Cyt (B), and 5-Br-Cyt (C). $n = 10$/treatment group. The x-axis values indicate the dose injected in milligrams per kilogram. Error bars represent S.E.M. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

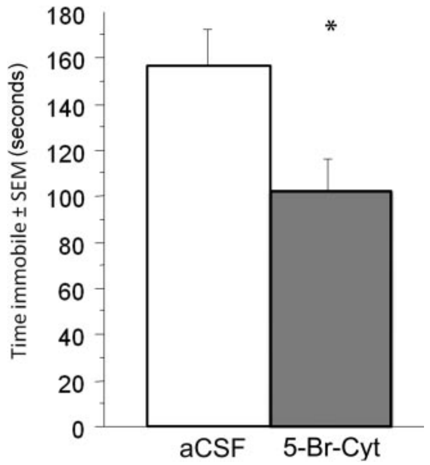

Fig. 7. Effects of centrally administered 5-Br-Cyt in the tail suspension test. Total time spent immobile in the tail suspension test by C57BL/6J male mice after 50 ng i.c.v. 5-Br-Cyt. $n = 8$/treatment group. Error bars represent S.E.M. $*$, $p < 0.05$.

forced swim test is most likely because of nonspecific deleterious effects because higher doses of nicotinic agents tend to be aversive and hypothermic, two consequences that would increase immobility. Thus, a limited reduction of (α4β2) nAChR activity seems to be necessary to result in antidepressant-like effects without affecting other critical pathways. In addition, a fine balance between agonism and antagonism of nAChRs may be required for efficacy in these behavioral tests (Picciotto et al., 2008). α7 nAChRs may also play a role in the response to antidepressants under specific conditions in C57BL/6J mice, because the effects of the nicotinic antagonist mecamylamine are abolished in α7 knockout mice (Rabenstein et al., 2006); however, efficacy of the nicotinic compounds tested here does not seem to depend on their intrinsic activity at α7 nAChRs. It is also possible that 3-pyr-Cyt has increased bioavailability in the brain compared with cytisine. Indeed, cytisine has poor blood-brain barrier penetration, limiting its efficacy in the central nervous system (Coe et al., 2005). Likewise, the lack of behavioral effects observed with 5-Br-Cyt may be because of greatly reduced brain penetration.

In support of this hypothesis, 5-Br-Cyt, which had a greater affinity than 3-pyr-Cyt for nAChR subtypes in binding assays, and was a high-affinity, low-efficacy partial agonist at α4β2 nAChRs, unexpectedly had no effect in the different paradigms used to assess antidepressant-like efficacy when administered peripherally. However, local infusion directly into the ventricle induced antidepressant-like effects in the tail suspension test. Thus, the most likely explanation for the lack of behavioral effect of systemic 5-Br-Cyt is that this compound is likely to have low brain penetration. One possibility could be that peripherally administered 5-Br-Cyt forms an epoxide (at the double bond in 3–4 position of the pyridone), resulting in a hydroxyl group or a glutathione conjugate, both of which are very hydrophilic and might have altered effects at nAChRs. The lack of effect on feeding and body weight of 5-Br-Cyt suggests that the lack of behavioral effects resulting from peripheral administration is not confined to tests of antidepressant action. In addition, we are confident these results do not reflect a failed study with 5-Br-cyt because we repeated the tests on three independent groups of animals and never saw a response.

Both cytisine and 3-pyr-Cyt were effective in the forced swim and tail suspension tests, although both compounds were less potent in the tail suspension test. Although the tail suspension and forced swim tests are both tests of antidepressant efficacy that are based on a similar principle (immobility in response to a stressor), the two tests are dependent on somewhat different behavioral variables. For example, differential sensitivity to temperature could alter the results of the forced swim test but would probably have little effect on the tail suspension test. Nicotinic agents and cholinergic modulation are known to affect body temperature, likely through effects on the autonomic system such as vasoconstriction (Tritto et al., 2004). In addition, the tail suspension test is shorter in duration than the forced swim test, which may provide more time for the experimental compounds to alter behavior in the swim test, thus increasing its sensitivity. Despite the dose difference between the tests, 3-pyr-Cyt was more efficient than cytisine across paradigms. It is not known whether these compounds are fully metabolized in 24 h; thus, there could be some carryover from one test to another, potentially explaining a stronger effect in the forced swim test compared with the tail suspension test. However, given the very short half-life of cytisine, these compounds might be rapidly metabolized.

The novelty-suppressed feeding test is only sensitive to chronic, and not acute, treatment with classical antidepressants (Dulawa and Hen, 2005), and it is thus thought to be sensitive to the neuronal adaptations that result in antidepressant effects in human depressed patients, because antidepressants must be administered for several weeks in patients to be effective. Chronic treatment with either 3-pyr-Cyt or cytisine resulted in a reduction in the time to first feed in the novelty-suppressed feeding test. This effect of 3-pyr-Cyt was only observed at one of the doses used, and the size of the effect was relatively small. Contrary to cytisine that showed an antidepressant-like effect in this test at concentration similar to those observed in both the tail suspension and the forced swim tests, 3-pyr-Cyt was only effective at a lower dose than those active in the two other tests. Many nicotinic agents can decrease feeding, which can be an important confound in this paradigm. For example, both nicotine (Jo et al., 2002) and cytisine (Mineur et al., 2007c) can decrease feeding and body weight. In the current study, 3-pyr-Cyt modulated neither feeding nor body weight (data not shown). Anxiety-like behavior could also alter performance in this test, and cytisine seems to be somewhat anxiogenic (Mineur et al., 2007c), potentially blunting the antidepressant like-effects measured in this paradigm. Interestingly, β4 null-mutant mice show reduced anxiety-like behavior in several paradigms (Salas et al., 2003) and activation of β4* nAChRs may be responsible for the ability of cytisine to increase anxiety-like behaviors. 3-pyr-Cyt should not activate α3β4 nAChRs to the same extent as cytisine, thus if the anxiogenic effects of nicotinic agents involve this nAChR subtype, 3-pyr-Cyt may not increase anxiety-like behavior to the same extent as cytisine.

In summary, we have evaluated the electrophysiological and antidepressant-like effects of two new cytisine derivatives that are weak nicotinic partial agonists with greater selectivity and affinity for β2* nAChRs. One compound, 3-pyr-Cyt, showed greater efficacy than cytisine in three tests of antidepressant efficacy. Taken together, these data suggest that fine tuning the pharmacology of nicotinic partial agonist may result in novel therapeutic compounds to treat mood disorders.

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