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TC-2559: A novel orally active ligand selective at neuronal acetylcholine receptors

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Abstract

TC-2559 [(*E*)-*N*-Methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine] is a novel nicotinic agonist markedly more selective than recently reported novel nicotinic receptor ligands (selectivity ratio for central nervous system (CNS) to peripheral nervous system (PNS) > 4000). TC-2559 competes effectively with [^{3}H]-nicotine binding (K_i = 5 nM) but not with [^{125}I]-bungarotoxin (> 50,000 nM). Dopamine release from striatal synaptosomes and ion flux from thalamic synaptosomes indicate that TC-2559 is potent and efficacious in the activation of CNS receptors and significantly reduced glutamate-induced neurotoxicity in vitro. TC-2559 has no detectable effects on muscle and ganglion-type nicotinic acetylcholine receptors at concentrations up to 1 mM. TC-2559 significantly attenuates scopolamine-induced cognitive deficits in a step-through passive avoidance task. Acute and repeated oral dosing of TC-2559 enhances performance in a radial arm maze task. In contrast to the effects of equimolar concentrations of (−) nicotine, TC-2559 does not induce hypothermia and locomotor activity is not enhanced following repeated daily administration of 14 days. TC-2559 has a markedly enhanced CNS-PNS selectivity ratio and an intra-CNS selectivity as evidenced by the improved cognition without increased locomotor activity. The in vitro and in vivo studies in the present study suggest that TC-2559 has the desired profile to be further evaluated as a potential therapeutic agent for neurodegenerative diseases.

Keywords: TC-2559; Nicotinic receptor; Cognition; Central nervous system

1. Introduction

The potential therapeutic benefit of nicotinic receptor ligands in a variety of neurodegenerative pathologies involving the central nervous system (CNS) has energized research efforts to develop nicotinic acetylcholine receptor subtype-selective ligands. In particular, there has been a concerted effort to develop nicotinic compounds with selectivity for CNS nicotinic acetylcholine receptors as potential pharmaceutical tools in the management of these disorders (e.g., ABT-418 from Abbott laboratories, Decker et al., 1994, and Arneric et al., 1995; TC-2403 from Targacept, Bencherif et al., 1996, and Lippiello et al., 1996; SIB-1508Y from SIBIA, Cosford et al., 1996).

The structural heterogeneity derived from the diversity of genes that encode for the various nicotinic acetylcholine receptor subunits is manifested as multi-dimensional heterogeneity of nicotinic cholinergic neurotransmission. Sixteen genes encoding for vertebrate nicotinic acetylcholine receptors subunits have been cloned, suggesting the possibility for considerable diversity in receptor subtype structure and function, and for potential therapeutic targets.

A successful approach to the identification of these nicotinic acetylcholine receptors involves neurotransmitter release studies in situ from brain synaptosomes or tissue. Nicotinic ligands have been shown to have a number of pharmacological effects, many of which may be related to effects upon neurotransmitter release. Release of acetylcholine, dopamine, serotonin, and norepinephrine have been reported (Summers and Giacobini, 1995; Summers et al., 1996). Confirmatory reports and additional studies have included the modulation in the CNS of glutamate, γ-amino butyric acid (GABA), takykinins and peptides (reviewed in Brioni et al., 1997). The distribution and function of nicotinic acetylcholine receptors is consistent with the view that nicotinic cholinergic signaling is involved in the regulation of the key neurochemicals in the brain and influences nicotine-sensitive neuronal processes

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involved in sensory processing and cognition. There is emerging evidence supporting the hypothesis that these neuromodulatory effects are provided by heterogeneous populations of nicotinic acetylcholine receptors that are distinguishable based on their anatomical, functional, pharmacological and molecular biological profiles (reviewed in Gotti et al., 1997).

Neurodegenerative diseases like Alzheimer's disease are characterized by neuronal degeneration and death. Agents capable of reducing or eliminating neuronal degeneration offer the potential to slow the progression or delay the onset of Alzheimer's disease and other neurodegenerative diseases. Evidence accumulated over more than a decade suggests that nicotinic acetylcholine receptor-selective ligands can offer neuroprotective effects in numerous models in vitro; (e.g., Akaike et al., 1994; Marin et al., 1994; Kaneko et al., 1997; Kihara et al., 1998) or in vivo (e.g., Janson et al., 1988; Janson and Moller, 1993; Sjak-Shie and Meyer, 1993). Other nicotinic receptor ligands have also been shown to be neuroprotective (ABT-418, Donnelly-Roberts et al., 1996; GTS-21, Nanri et al., 1997; RJR-2403, and RJR-1734, Lippiello et al., 1998; Bencherif et al., 1998a). In vitro models demonstrate neuroprotective effects of nicotinic receptor ligands on inhibition of neuronal death resulting from beta-amyloid toxicity, *N*-methyl-D-aspartate-mediated cytotoxicity or growth factor deprivation. In vivo models demonstrate rescue from neuronal death resulting from surgically induced neuronal loss (nucleus basalis lesions in young and aged animals, mesodiencephalic hemitransection) and chemically induced neurotoxicity (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) models, and systemic kainic acid-induced excitotoxic effects).

The reduction or elimination of undesirable effects at peripheral sites, particularly cardiovascular sites, and the development of nicotinic receptor ligands having improved selectivity for CNS nicotinic acetylcholine receptors subtypes provide an innovative approach for the development of therapies for Alzheimer's disease and a number of other CNS disorders. The compelling nature of the evidence sited above has motivated the development of several nicotinic-based therapeutics for the treatment of Alzheimer's disease (reviewed in Gotti et al., 1997). The present report summarizes the pharmacological and behavioral profiles of TC-2559. The results indicate that TC-2559 has a markedly enhanced selectivity for CNS receptors when compared with previously reported nicotinic receptor ligands (e.g., ABT-418, ABT-594).

2. Materials and methods

2.1. Animals

Female (175–200 g) and male (200–350 g) Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC, USA) were used in the in vitro and in vivo studies, respectively. Rats had ad libitum access to drinking water and rat chow, except for rats assigned to radial-arm maze study. For these rats, body weights were maintained at approximately 85% of free-feeding levels. Studies were conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

2.2. In vitro studies

2.2.1. Synthesis and structure of TC-2559

(*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine (5-ethoxy-metanicotine; Fig. 1) was synthesized by the palladium-catalyzed coupling ($Pd(OAc)_2$, $P(o\text{-tol})_3$, Et_3N, CH_3CN) (Heck reaction) of 3-bromo-5-ethoxypyridine and *N*-methyl-*N*-(*tert*-butoxycarbonyl)-3-buten-1-amine, followed by treatment of the resulting intermediate, (*E*)-*N*-methyl-*N*-(*tert*-butoxycarbonyl)-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine with 6N HCl in tetrahydrofurane or trifluoroacetic acid in anisole (Fig. 1). The required 3-bromo-5-ethoxypyridine was prepared by reaction of 3,5-dibromopyridine (Lancaster Synthesis, Windham, NH) with sodium ethoxide in *N*,*N*-dimethyl formamide (DMF). The olefin, *N*-methyl-*N*-(*tert*-butoxycarbonyl)-3-buten-1-amine was prepared by reaction of 4-bromo-1-butene (Aldrich Chemical, Milwaukee, WI) with an excess of methylamine in DMF in the presence of K_2CO_3. Treatment of the resulting intermediate, *N*-methyl-3-buten-1-amine (Courtois et al., 1986; Wille and Goubeau, 1972) with di-*tert*-butyl-dicarbonate, afforded *N*-methyl-*N*-(*tert*-butoxycarbonyl)-3-buten-1-amine. For pharmacological evaluation, (*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine was converted to its sesquifumarate or hemigalactarate (TC-2559) salts (Fig. 1). For the sake of conciseness and clarity, both salts are referred to as TC-2559 in this report, but experiments were conducted with either salt based upon availability (MW of the free base = 206.282).

2.2.2. Model cell systems

2.2.2.1. Clonal cells. Cells of the TE671/RD human clone and the PC12 rat pheochromocytoma cell line were used according to routine protocols (Bencherif et al., 1996). Briefly, cells of the TE671/RD human clone and the PC12 rat pheochromocytoma were maintained in proliferative growth phase in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL) supplemented with 10% horse serum, 5% fetal calf serum (Hyclone), and antibiotics (penicillin/streptomycin).

2.2.2.2. Rat brain synaptosomes. Striatal or thalamic tissue was prepared as previously described (Bencherif et al., 1996). Briefly, female Sprague–Dawley rats (100–200 g) were killed by decapitation after anesthesia with 70% CO_2.

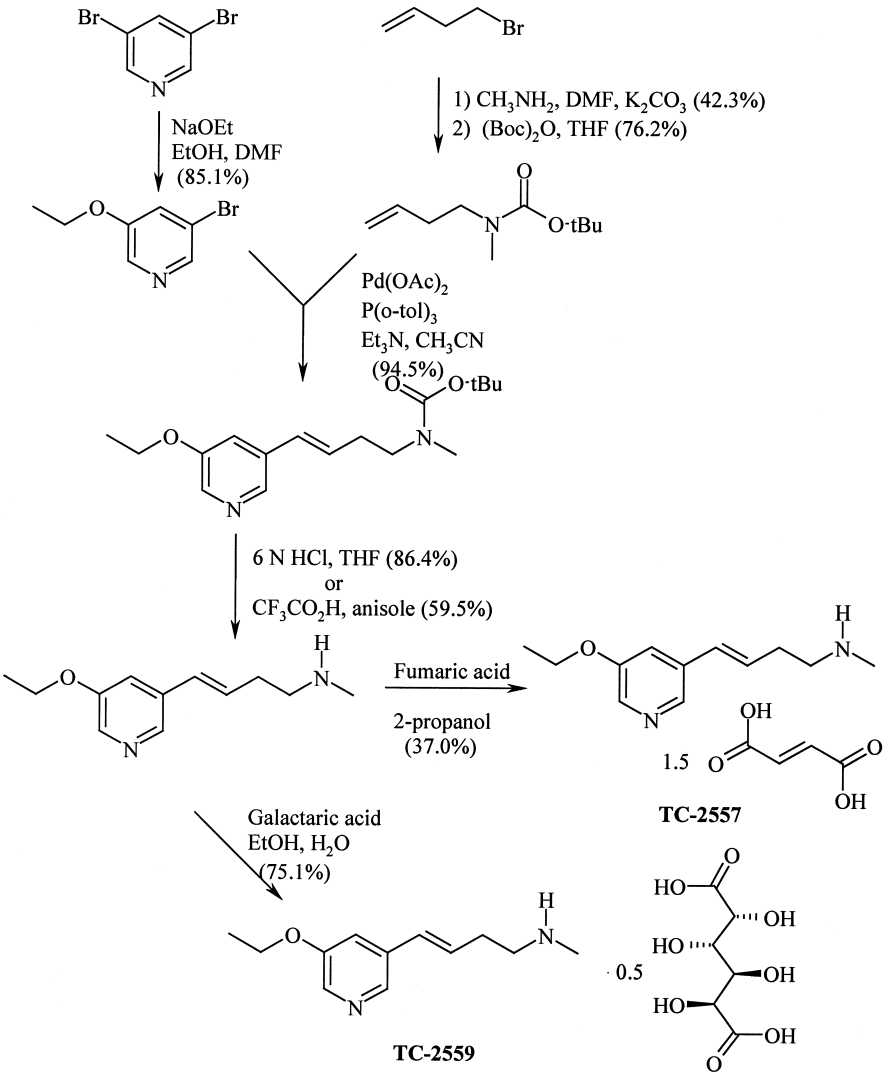

Fig. 1. Synthetic route and structure for TC-2559.

Striatal or thalamic tissue was rapidly dissected out and homogenized in 0.32 M sucrose containing 5 mM HEPES pH 7.4 (7.5 ml per striatum) using a glass/glass homogenizer. The tissue was then centrifuged for $1000 \times g$ for 10 min and the pellet discarded. The supernatant was centrifuged at $12{,}000 \times g$ for 20 min. The resultant pellet was re-suspended in perfusion buffer (128 mM NaCl, 1.2 mM KH_2PO_4, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, l mM ascorbic acid, 0.01 mM pargyline HCl and 10 mM glucose pH 7.4) and centrifuged for 15 min at $25{,}000 \times g$. The final pellet was resuspended in perfusion buffer, 1 ml per striatum. For binding experiments, tissue was frozen until required and then thawed, centrifuged at $48{,}000 \times g$ and the pellet resuspended in perfusion buffer.

2.2.3. Binding and functional studies

Standard procedures for ligand binding studies on membrane preparations were followed (Bencherif et al., 1996). Equilibrium binding assays were conducted by incubating membrane aliquots suspended in 300 μl assay buffer with 10 nM [^{3}H]nicotine (78.4 Ci/mmol; Dupont, New England Nuclear). Non-specific binding was determined in samples supplemented with 10 μM nicotine or 1 mM carbachol. Incubation was terminated by rapid filtration on a multimanifold tissue harvester (Brandel) using G/C filters presoaked in 0.33% polyethyleneimine (w/v). Unless specifically mentioned above, reagents were purchased from Sigma and were of the highest available grade. Radiolabeled ligands were purchased from New England Nuclear (NEN).

2.2.4. Functional studies

2.2.4.1. Ion flux in TE671 / RD and PC12 cells. Following $^{86}Rb^+$ radioisotope loading period (37°C), cells were washed twice with PBS and ligands were added to cells plated on micro-wells. The supernatant was collected following a 4-min exposure. Levels of non-specific ion flux were equivalent, whether defined using samples containing

agonist plus 100 μM D-tubocurarine or using blank samples that contained no agonist, and specific nicotinic acetylcholine receptor function was defined as total, experimentally determined ion flux in the presence of agonist +/− test compounds minus non-specific ion flux.

2.2.4.2. Dopamine uptake. The synaptosomal suspension was incubated for 10 min at 37°C in order to restore metabolic activity. [^{3}H]dopamine was added to a final concentration of 0.1 μM and the suspension incubated at 37°C for a further 10 min. Seventy-five-microliter aliquots of tissue were added to 96-well microtiter plate wells containing 250 μM perfusion buffer and then harvested onto Gelman AE filters (6 mm diameter) using an Inotech cell harvester while washing with 2 ml perfusion buffer.

2.2.4.3. Dopamine release and $^{86}RB^+$ release. Dopamine release and $^{86}Rb^+$ release were determined as previously described (Bencherif et al., 1998b). Release of dopamine or $^{86}Rb^+$ was determined in the presence of various ligands and was expressed as percentage of the maximal activation induced by nicotine (10 μM).

2.2.4.4. Residual inhibition. Experiments on residual inhibition were conducted by applying no drug, nicotine or TC-2559 (at the indicated concentrations) for 30 min in the superfusion buffer prior to exposure to 1 μM nicotine. The residual inhibition was determined as a fraction of remaining activation when compared to saline.

2.2.5. Neuroprotection studies

Neuroprotection studies were performed according to methods previously described (Akaike et al., 1994). Briefly, 2- to 3-week-old cultures of fetal rat brain prepared as described previously are exposed to test compound (10 μM) for 2 h prior to a 15-min incubation with 1 mM glutamate. Cells are assayed 24 h later for lactate dehydrogenase activities.

2.3. In vivo studies

2.3.1. Attenuation of scopolamine-induced amnesia in the step-through passive avoidance

Step-through latencies were recorded in passive avoidance chambers (Gemini Avoidance System, San Diego Instruments, San Diego, CA, USA). Attenuation of scopolamine-induced deficits was assayed with a one-trial step-through passive avoidance task using a paradigm previously described (Lippiello et al., 1996). During the acquisition day, rats received a subcutaneous injection of 0.5 μmol/kg scopolamine ($n = 10$) or saline ($n = 8$) 30 min prior to being placed in the chambers. Five minutes following scopolamine injection, each rat was administered a subcutaneous injection of either (−)-nicotine (0.3, 0.6, 1.8 or 3.0 μmol/kg; $n = 8–10$/group) or TC-2559 (0.6, 1, 3, 6 or 10 μmol/kg; $n = 8–10$/group). Thirty minutes following the scopolamine or vehicle injection, each rat was placed in the avoidance compartment. Immediately following the shock (0.5 mA for 2 s), the rats were removed from

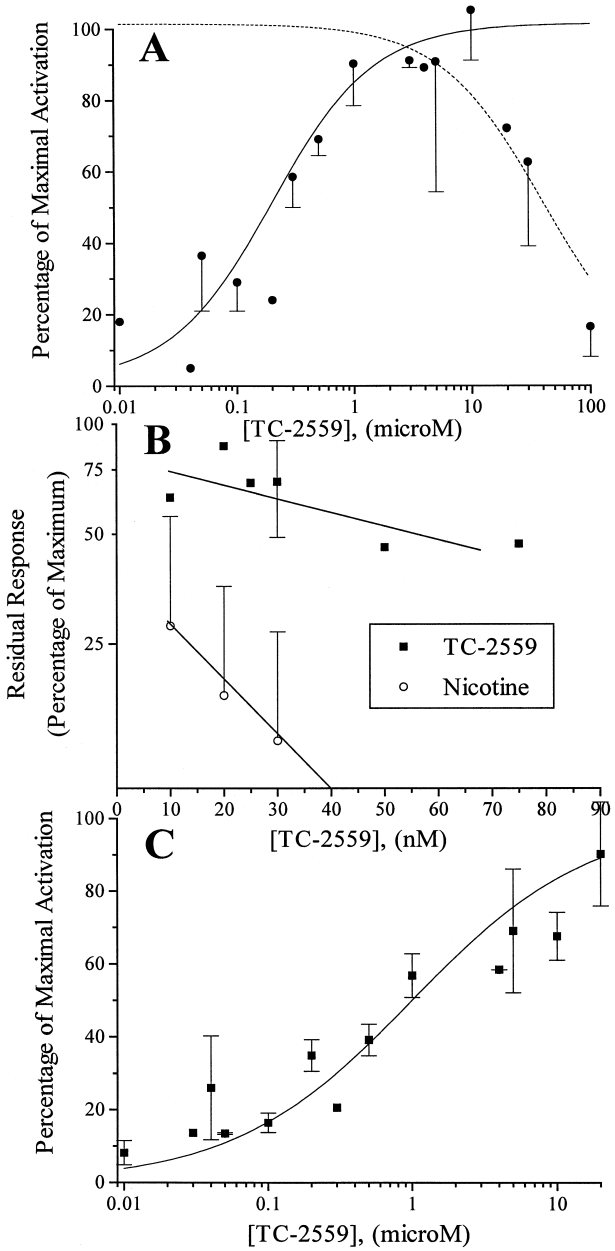

Fig. 2. (A) Effects of TC-2559 on dopamine release from rat striatal synaptosomes. All responses were normalized to the maximal response induced by nicotine (see Methods). Data points and bars represent mean ± S.E.M. of triplicate measurements from seven experiments. Activation curves (solid line) and inactivation curves (dashed line) are shown were fitted to the logistic equation described in Methods. (B) Residual activation of dopamine release from striatal synaptosomes following 20 min exposure to nicotine or TC-2559. The results indicate that TC-2559 induces less residual inhibition than does nicotine. Results are expressed in percentage of maximal response and are the mean ± S.E.M. (C) Effects of TC-2559 on $^{86}Rb^+$ efflux from rat thalamic synaptosomes. All responses were normalized to the maximal response induced by 10 μM nicotine (see Methods). Data points and bars represent mean ± S.E.M. of triplicate measurements of 10 experiments.

the chamber and returned to their home cage. The time to enter the dark chamber (i.e., step-through latency) was measured. Twenty-four hours later (retention trial), the latency to enter the dark compartment was again measured for each rat. If the rat did not enter the dark compartment within 300 s, the retention test was terminated and a ceiling score of 300 s was assigned.

2.3.2. Enhanced radial arm maze performance

Rats were habituated to the radial arm maze (Lafayette Instruments, Lafayette, IN, USA) with 45-mg Noyes® pellets scattered along each arm. Arm choices were recorded when the rat had placed all its paws beyond the threshold at the proximal end of the arm. The session ended when either the rat visited the eighth arm or 10 min elapsed. In subsequent training sessions, rats were trained to locate a food pellet in four of the eight arms and training continued for 10 sessions over a 2-week period. Prior to the start of the training, rats were divided into six groups ($n = 10$/group) and each group was paired with a distinct baiting pattern.

Maze testing was conducted over a six-day period. On days 1 and 6, rats were orally administered saline or one of four doses (0.6, 1, 3 and 6 μmol/kg) of TC-2559 and were placed on the maze 1-h post administration. Testing was completed when the last baited arm was visited or 10 min elapsed. On days 2–5, rats received daily administration of their appropriate solutions but were not tested on the maze. Two types of errors were scored: reference memory error was classified as when a rat entered a non-baited arm for the first time; and working memory error was classified as when a rat re-entered a baited arm from which food had already been retrieved on that trial. Subsequent re-entries into non-baited arms were scored as working memory errors. On days 1 and 6, rats were fed 1 h following maze testing. On days 2–5, each rat was fed 1 h after receiving its appropriate treatment.

2.3.3. Locomotor activity

Locomotor activity was assessed in photo-beam activity chambers (Med Associates, Georgia, VT, USA). Each chamber consisted of a clear, square Plexiglas box ($43.2 \times 43.2 \times 30.5$ cm) placed inside an activity monitor. Sixteen infrared photocell beams were located along axis of the floor of the chamber spaced 2.53 cm apart. Horizontal counts were sampled via Activity Monitor computer software (Med-Associates). Twenty-four hours prior to testing with TC-2559, each rat was habituated to an activity chamber for 180 min. During this habituation period only locomotor activity was recorded and no injections were administered. The following day, rats were placed in the activity boxes for 90 min, removed and injected subcutaneously with saline ($n = 11$), or TC-2559 at 1 μmol/kg ($n = 5$), 2 μmol/kg ($n = 5$), and 10 μmol/kg ($n = 4$). Immediately following dosing, each rat was returned to the locomotor activity testing apparatus for an additional 90 min and horizontal counts were sampled every 6 min. The effects of repeated dosing of TC-2559 and (−) nicotine on locomotor activity were evaluated in a separate group of rats ($n = 24$). Rats were habituated in locomotor activity chambers for 60 min, at 72, 48 and 24 h prior to testing. Beginning on day 4, rats ($n = 8$/group) received daily administration of saline, (−) nicotine (3.5 μmol/kg) or TC-2559 (3.5 μmol/kg). Immediately following the injections of the test compounds, animals were placed in the activity monitoring boxes for 60 min and horizontal counts were sampled every 5 min. The chronic dosing regiment of TC-2559 and (−) nicotine in combination with the 60-min locomotor activity session continued for 2 weeks.

2.3.4. Hypothermia

Body temperature readings were measured following implantation of telemetry devices (ER-4000 E-mitter, Mini-Mitter, Sunriver, OR, USA) and performed under general anesthesia using a combination of Ketamine (100

Table 1
Parameters for binding and activation of nicotinic acetylcholine receptors by nicotine, ABT-418, SIB-1508Y, and TC-2559. Values for nicotine and TC-2559 are mean ($n > 3$ for K_i's; $n > 7$ for Thalamic ion flux; $n > 5$ for striatal dopamine release and $n > 3$ for muscle and ganglion)

		Nicotine	ABT-418[a]	SIB-1508Y	TC-2559
Rat brain	K_i (nM)	3 ± 0.5	46 ± 9	4	5 ± 1
Striatum dopamine	EC_{50} nM	100 ± 28	1158 ± 178	3000	203 ± 52
	E_{max}	113 ± 17	81 ± 1	75	97 ± 12
Thalamic ion flux	EC_{50} nM	591 ± 120	620 ± 353	ND	367 ± 121
	E_{max}	87 ± 22	101 ± 27	50	93 ± 05
Muscle TE671/RD	EC_{50} μM	80	200	100[b]	> 1000
	E_{max}	100	100	33	< 20
Ganglionic PC12	EC_{50} μM	30	50	100[b]	> 1000
	E_{max}	100	100	< 20	< 20
Ratio 1[c]	Ggl/Str.	260	46	ND	> 5000
Ratio 2[c]	Ggl/Thl.	50	80	ND	> 4000

[a] Racemic mixture.
[b] 100 μM represent the highest dose tested; ND: not determined.
[c] Ratio of EC_{50}'s for ganglia and striatum (1); EC_{50}'s for ganglia and thalamus (2).

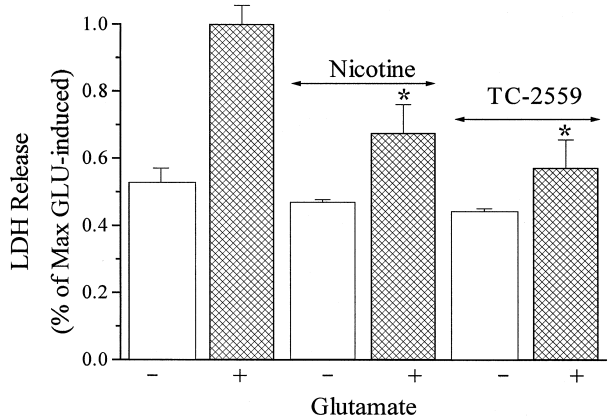

Fig. 3. Magnitude of neuroprotection of fetal rat brain cells following exposure to 1 mM glutamate. TC-2559 resulted in significant reversal of cell death as measured by lactate dehydrogenase activity. $^{*}P < 0.05$.

mg kg^{-1}) and Xylazine (20 mg kg^{-1}). The frequencies emitted from the transmitters were received by ER-4000 Energizer/Receivers and were converted to body temperature, recorded, and time/date stamped using *Vital View* computer software (Mini-Mitter).

Following the completion of the baseline pretreatment measurements (four baseline measurements were taken for each animal over a 2-week period), the rats were dosed with saline, (−) nicotine (0.3, 1 or 3 μmol/kg) and TC-2559 (0.3, 1 and 3 μmol/kg). Body temperature measurements were recorded continuously and plotted every 30 s for the next 180 min.

2.4. Statistics

Data obtained from the passive avoidance failed to meet the assumptions necessary for parametric analysis, therefore, Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks followed by Dunn's test were used to analyze step-through latencies from the retention trials. For the radial arm maze, statistical differences in working and reference memory errors following acute and chronic administration were assessed by ANOVA followed by Dunnett's test. $P < 0.05$ was considered significant. ANOVA followed by Dunnett's test for post-hoc comparisons were employed to analyze results from the locomotor and hypothermia studies.

2.5. Compounds

Scopolamine hydrobromide (Research Biochemicals International (RBI), Natick, MA), (−) nicotine (RBI) and TC-2559 (synthesized by the Medicinal Chemistry Dept. of Targacept) were dissolved in 0.9% sterile saline. Doses are expressed as μmol/kg and are expressed in terms of the free base concentration.

3. Results

3.1. In vitro studies

These studies were performed to evaluate the effects of the novel nicotinic agonist TC-2559 on CNS nicotinic acetylcholine receptor subtypes. Binding affinity of TC-2559 to CNS receptors was conducted in membrane preparations from rat brain cortex ($K_i = 5.5 \pm 1.6$ nM; $n = 4$). To assess the selectivity of TC-2559 for CNS nicotinic acetylcholine receptors, we evaluated the ability of TC-2559 to release dopamine from rat striatal synaptosomes. Our results indicate that TC-2559 stimulates dopamine release from striatal synaptosomes with potency and efficacy similar to that of nicotine ($EC_{50} = 203 \pm 52$ nM vs. 100 nM, respectively; $E_{max} = 97 \pm 12\%$ vs. nicotine; $n =$

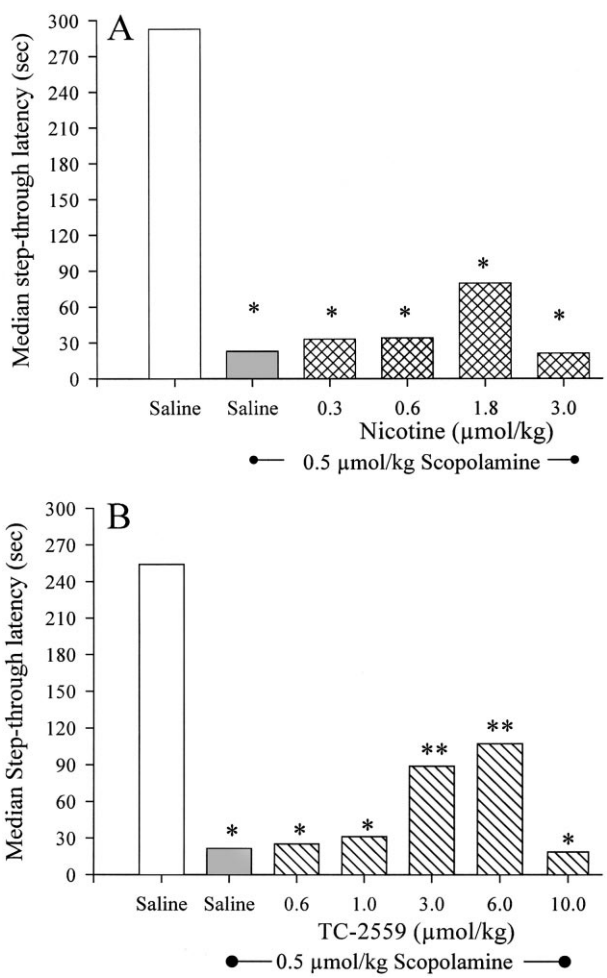

Fig. 4. Step-through latencies for Sprague–Dawley rats given saline (1 mg/kg), scopolamine (0.5 μmol/kg) alone, or scopolamine with different doses of (−) nicotine (0.3, 0.6, 1.8 and 3.0 μmol/kg) or TC-2559 (0.6, 1, 3, 6 or 10 μmol/kg) 24 h earlier. Results are expressed as median latencies. Kruskal–Wallis analysis of variance on ranks followed by Dunn's test: $^{*}p < 0.05$ vs. saline/saline, $^{**}P < 0.05$ from scopolamine/saline.

7; Fig. 2A). Similar results were obtained on $^{86}Rb^{+}$ release from rat thalamic synaptosomes ($EC_{50} = 367 \pm 121$ nM vs. 591 ± 120 for nicotine; $E_{max} = 107\%$ vs. nicotine; $n = 10$; Fig. 2B). Constant exposure to TC-2559 resulted in less residual inhibition of dopamine release from striatal synaptosomes as observed with nicotine. Pre-exposure to TC-2559 (20 nM for 30 min) resulted in a 13% loss of function (compared to vehicle) whereas nicotine under the same conditions resulted in complete desensitization (Fig. 2C). At the highest concentration tested (75 nM which is the maximal concentration that can be used without resulting in significant activation), TC-2559 resulted in 50% residual inhibition. To evaluate interaction of TC-2559 with nicotinic receptors expressed in peripheral tissue, we assessed the effects of nicotine and TC-2559 on the human muscle nicotinic acetylcholine receptor subtype in TE671/RD cells. Nicotine resulted in the partial activation of muscle receptors ($EC_{50} = 60$ μM and E_{max} of 60% compared to acetylcholine), whereas TC-2559 resulted in no significant activation at a concentration of up to 1 mM (at 100 μM $9 \pm 5\%$ compared to nicotine; $n = 4$, Table 1; $5 \pm 5\%$ at 300 μM and $26 \pm 13\%$ at 1 mM). The ability of TC-2559 to interact with ganglionic receptors was determined in PC12 cells (which express α3, α5, β2, β4, and α7) and a pharmacologically identified α3β4 nicotinic acetylcholine receptor subtype. Nicotine resulted in the activation of nicotinic acetylcholine receptors in PC12 cells ($EC_{50} = 20$ μM and E_{max} of 65% when compared to the endogenous neurotransmitter acetylcholine). At concentrations ranging from 100 μM to 1 mM, TC-2559 did not result in any detectable activation of ganglion-type receptors (0% compared to nicotine; $n = 3$; Table 1). Neuroprotective effects of TC-2559 were evaluated in fetal rat brain cells in cultures exposed to 1 mM glutamate with or without prior exposure to TC-2559. Cell death determined by lactate dehydrogenase activity in cultures indicates that nicotine (10 μM) and TC-2559 (10 μM) significantly inhibit the cytotoxic effects of glutamate (Table 1; Fig. 3).

3.2. In vivo studies

3.2.1. Attenuation of scopolamine-induced amnesia

The avoidance latencies from the retention session for the (−) nicotine treated animals indicate that the median step-through latencies (292.5 s) of the saline-treated rats were significantly different ($H = 16.016$; $df = 5$; $P < 0.007$) from the avoidance latencies (22.5 s) of the scopolamine-treated group. However, (−) nicotine, at all doses tested, failed to attenuate scopolamine-induced avoidance latencies (Fig. 4A). Conversely, TC-2559, at doses of 3 and 6 μmol/kg, significantly ($H = 23.80$; $df = 6$; $P < 0.001$) attenuated scopolamine-induced decrease of avoidance latencies of 21.5 s (Fig. 4B). The median step-through latencies for 3 and 6 μmol/kg were 88.7 and 105.7 s, respectively. Furthermore, the median step-through latencies of rats treated with 3 and 6 μmol/kg were not significantly different from the avoidance latencies (254.1 s) of the saline-treated animals (Fig. 4B).

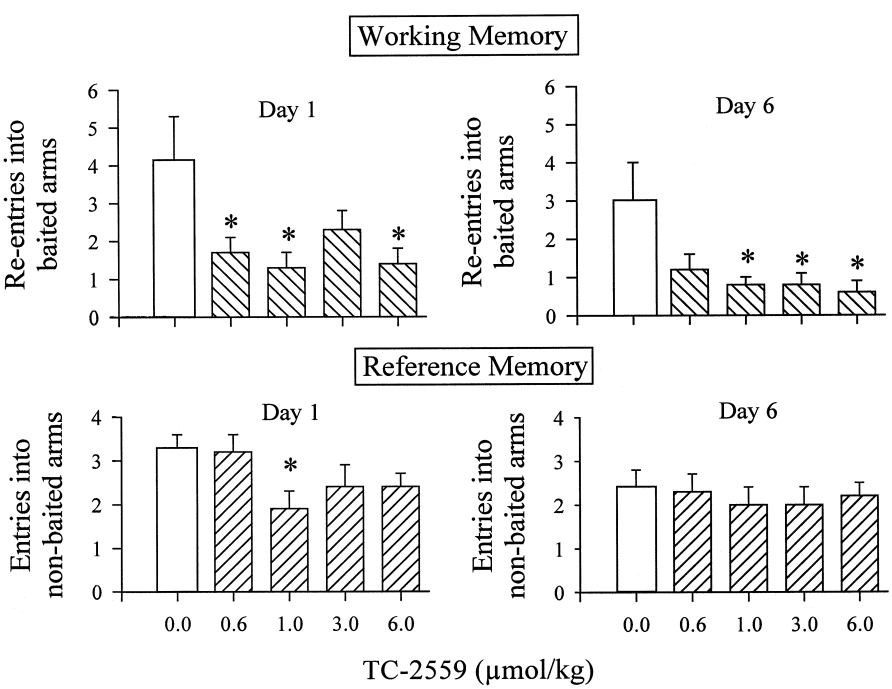

Fig. 5. Effect of oral TC-2559 administration on total number of re-entries into baited arms (top panel) and total number of entries into non-baited arms (bottom panel) after acute (day 1) and repeated (day 6) administrations as assessed in the radial arm maze task. Results are expressed as mean (±S.E.M.). One-way analysis of variance followed by Dunnett's test: $^{*}P < 0.05$ compared to saline control group.

3.2.2. Enhanced radial-arm maze performance

An additional cognitive study was conducted using a radial arm maze task. The radial arm maze consisted of baiting four of eight arms to evaluate the effects of TC-2559 on working and reference memories in rats (Fig. 5). Administration of TC-2559 significantly reduced the number of working memory errors (Fig. 5, upper-left panel) as assessed by re-entries into a previously baited arm at 3 and 10 μmol/kg [$F(4,48) = 2.939$, $P = 0.031$] when compared to errors in saline-treated rats. Overall, TC-2559 was less effective [$F(4,48) = 2.605$, $P = 0.049$] in reducing reference memory errors following acute oral dosing (Fig. 5, lower-left panel). However, following the administration of 1 μmol/kg, the reference memory errors were significantly decreased when compared to the saline-treated rats. In addition to decreases in working memory errors following acute administration, there were further significant reductions in working memory errors following 6 days of repeated administration for the rats that received 1, 3, and 6 μmol/kg doses of TC-2559 [$F(4,48) = 3.459$, $P = 0.0157$] when compared with saline-treated rats (Fig. 5, upper-right panel). Again, reference memory errors were not significantly altered following chronic treatment with TC-2559 (Fig. 5, lower-right panel).

3.2.3. Locomotor activity: acute administration

The acute effects of TC-2559 on horizontal counts are shown as cumulative counts over the 90-min locomotor session (Fig. 6A). The 1 and 10 μmol/kg doses of TC-2559 resulted in significant [$F(4,21) = 3.626$, $P = 0.016$] reductions in horizontal counts at the 30-min time point when compared to the vehicle control group. Overall, TC-2559 resulted in a dose-dependent monophasic change in locomotor behavior with a sustained hypolocomotion at 60 min for the 1.0 μmol/kg dose of TC-2559 [$F(4,21) = 4.287$, $P = 0.008$] that lasted for the 90 min of monitoring when compared to the saline-treated group (Fig. 6A).

3.2.4. Locomotor activity: repeated administration

Following acute effects of TC-2559 on locomotor activity, experiments were conducted to determine whether sensitization (i.e., increases in locomotor responses) would develop in rats following repeated exposure to either (−) nicotine or TC-2559 (Fig. 6B). Rats received equimolar concentration (3.5 μmol/kg) of either (−) nicotine or TC-2559 once daily for 14 consecutive days. Horizontal counts were collected for each 60-min locomotor session. A within-group comparison (one-way repeated measures analysis of variance followed by Dunnett's test for post-hoc comparisons) indicated that horizontal counts were significantly [$F(7,14) = 11.06$, $P < 0.001$] increased by the third day of (−) nicotine administration when compared to similar counts from days 0 (baseline), 1 and 2 for the (−) nicotine-treated group (Fig. 6B). Over the course of 14 days, locomotor activity after chronic administration of (−) nicotine was nearly 3.5-fold higher than that after the first (−) nicotine session (day 1). Conversely, no apparent sensitization developed following repeated administration of TC-2559 ($P = 0.413$). In fact, locomotor activity following chronic administration of TC-2559 mirrored that of the saline-treated group (Fig. 6B).

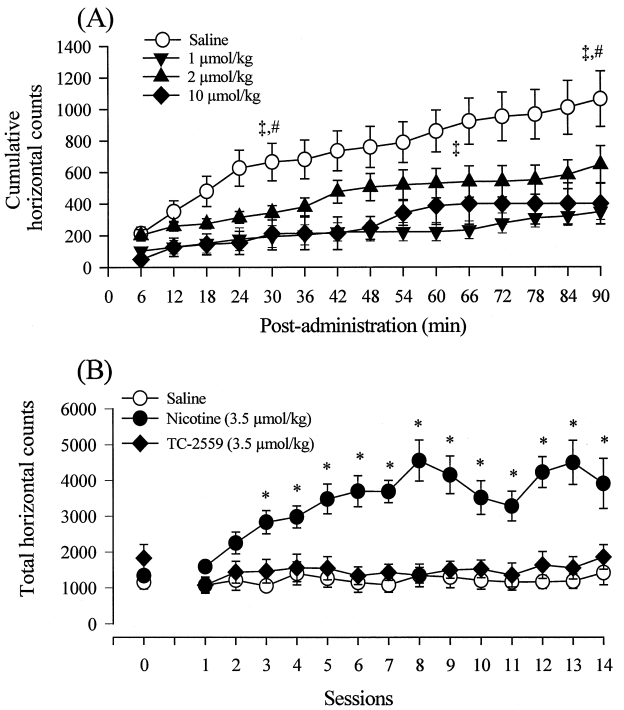

Fig. 6. Effect of subcutaneous TC-2559 administration following acute (A) and repeated dosing (B) on locomotor activity. For comparison, the effect of subcutaneous (−)-nicotine administration following repeated dosing was also examined. Results are presented either as cumulative horizontal counts over a 90-min period following acute dosing (A) or as total horizontal counts over a 60-min period following repeated dosing (B) (mean ± S.E.M.). One-way analysis of variance followed by Dunnett's test at the 30-, 60-, and 90-min time-points (A): ‡ $P < 0.05$, 2 μmol/kg treated group vs. saline; # $P < 0.05$, 10 μmol/kg treated group vs. saline, Dunnett's test. One-way analysis of variance followed by Dunnett's test (B). * $P < 0.05$ vs. saline-control.

3.2.5. Hypothermia

Prior to drug treatment, all rats had basal core body temperature of 37°C to 37.5°C and body temperature readings were not affected following subcutaneous administration of saline. (−) Nicotine dose-dependently induced hypothermia [$F(4,22) = 4.515$, $P < 0.08$] over the course of 40 min (20-60 min) following subcutaneous administration. Body temperature readings following the administration of 1 and 3 μmol/kg of (−) nicotine were 36.6°C and 36.5°C, respectively. The peak hypothermic effect induced by (−) nicotine was short-lived, occurring from 20–30 min post administration. Doses up to 10 μmol/kg of TC-2559 failed to produce any hypothermic effects.

4. Discussion

The novel nicotinic compound TC-2559 (*E*)-*N*-methyl-4-[3-(5-ethoxypyridin)yl]-3-buten-1-amine (5-ethoxymetanicotine) exhibits potency and selectivity for a subset of CNS nicotinic acetylcholine receptors and is impotent at ganglion and muscle subtype nicotinic acetylcholine receptors. The selectivity ratio as measured by the relative potency at ganglionic-type nicotinic acetylcholine receptor and CNS subtypes indicates that this compound is markedly more selective than some recently described novel nicotinic receptor ligands (e.g., ABT-418, ABT-594; see Table 1).

The selectivity and potency are demonstrated by the findings that TC-2559 binds with high affinity to nicotinic acetylcholine receptor receptors in rat brain, releases dopamine and induces $^{86}Rb^{+}$ efflux in synaptosomes and is ineffective in activating ganglionic-type receptors in PC12 cells and muscle-type receptors in human TE671/RD cells. The lack of interaction with ganglion-type receptors is consistent with the reduced ability of TC-2559 to induce undesirable cardiovascular side effects (changes in blood pressure and heart rate; data not shown).

TC-2559 is neuroprotective in vitro, and consistent with a recently published report on a structurally related compound, TC-2403 (Papke et al., 2000), TC-2559 resulted in significantly less residual inhibition than did (−) nicotine. This salient property of TC-2559 and analogues may be particularly useful for unraveling the biophysical events resulting in desensitization and in dissecting the contribution of agonism and desensitization processes in the mediation of nicotinic behavioral effects.

In contrast to (−) nicotine, TC-2559 failed to induce hypothermia in rats. Furthermore, repeated administration of (−) nicotine, but not TC-2559, resulted in behavioral sensitization as reflected in its ability to increase locomotor activity. These results support the hypothesis that TC-2559 exhibits a lower potency and efficacy than (−) nicotine for peripheral nicotinic acetylcholine receptor subtypes at muscle and ganglionic sites while maintaining binding affinity and activation of CNS nicotinic acetylcholine receptor subtype. Cognitive studies using the radial arm maze indicate that TC-2559 is effective following acute and repeated oral administration in the rat. These data suggest that TC-2559 exhibits the desired profile for potential use as a therapeutic agent.

The phenotypes of the nicotinic acetylcholine receptors mediating dopamine release from striatal structures and that present in the ventral tegmental area have been discussed (Marks et al., 1993; Le Novere et al, 1996; Goldner et al., 1997; Gerzanich et al., 1997). A complex phenotype has been proposed based on pharmacological, molecular biological and protein chemical studies. The putative subunit composition of the nicotinic acetylcholine receptor expressed in striatal structures may include a combination of $\alpha 3$, $\alpha 4$, $\beta 2$ and/or $\beta 3$ subunits. It has been proposed that sensitization of motoric effects is mediated through upregulation of dopaminergic neurotransmission in the ventral tegmental area whereas hypolocomotion may include a GABAergic stimulation in the nucleus accumbens. Recent studies have suggested an $\alpha 6$ containing nicotinic acetylcholine receptor as mediating nicotine responses in the ventral tegmental area based initially on the high expression of $\alpha 6$ mRNAs in dopaminergic neurons of the mesencephalic nuclei (Le Novere et al, 1996), the immunohistochemical localization of the $\alpha 6$ nicotinic acetylcholine receptor subunit in the ventral tegmental area (Goldner et al., 1997), and the functionality of $\alpha 6$-containing receptors (Gerzanich et al., 1997). Involvement of an $\alpha 6$ nicotinic acetylcholine receptor subunit in nicotine-sensitization is supported by recent results using in vivo antisense oligonucleotide (Le Novere et al., 1999). Our observations that TC-2559 is a potent full agonist in striatal preparations and is ineffective in inducing sensitization of locomotor activity following chronic administration also suggest heterogeneity in the subtypes mediating these two effects (i.e., dopamine release in striatum and putative activation of nicotinic acetylcholine receptor in the ventral tegmental area). Another possibility is that TC-2559 does not reach the relevant sites in the ventral tegmental area. This eventuality is minimized by the observations that TC-2559 is able to induce depressive effects on locomotor activity following acute dosing as well as being effective in improving cognitive functions. Results from the present animal studies clearly demonstrate that TC-2559 significantly improves performances of rats in two different cognitive tasks. In one paradigm, TC-2559 significantly attenuated scopolamine-induced deficits in the passive avoidance task and demonstrating oral efficacy in radial arm maze paradigm. A large body of evidence indicates that nicotine treatment improves cognitive function in animals and humans (Jones et al., 1992; Sahakian et al., 1989; Wilson et al., 1995; Arneric et al., 1995; Lippiello et al., 1996; Brioni et al., 1997). In addition, animals treated with the nicotinic receptor ligands TC-2403 or TC-1734 showed a marked long-lasting improvement of cognitive function (Lippiello et al., 1996, 1998). Animals with chemically and surgically induced amnesia showed improved cognitive function when treated with TC-2403 (Lippiello et al., 1996). Nicotine treatment via subcutaneous injection or transdermal patch improves attention and learning in humans, including Alzheimer's patients. Administration of ABT-418 to Alzheimer's patients resulted in improvement of cognitive function (Potter et al., 1999). In addition, these effects are maintained following repeated administration (6 days with once a day dosing) suggesting a lack of tolerance to the cognitive improvement.

Overwhelming evidence supports the lack of tolerance to the memory effects to nicotinic receptor ligands in rats, primates and humans. No tolerance to cognitive improvement with nicotine (Levin and Rose, 1990; Levin et al.,

1990), and GTS-21 (Arendash et al., 1995a,b) was observed and no decline in effectiveness in the delayed matching-to-sample task was observed in primates treated with ABT-418 (Arneric et al., 1995). No tolerance to the memory effects of TC-1734 was observed following chronic exposure (Lippiello et al., 1998). Similarly, no tolerance to the analgesic effects of ABT-594 or TC-2403 was seen following chronic administration (Bannon et al., 1998). The lack of tolerance to CNS-mediated effects of TC-2559 reported in this work is consistent with previous reports.

TC-2559 is neuroprotective in vitro. A number of studies have demonstrated a neuroprotective effect of nicotine and nicotinic receptor ligands both in vitro and in vivo. These include effects in in vitro models demonstrating neuroprotective effects of nicotinic receptor ligands on inhibition of neuronal death resulting from beta-amyloid toxicity (Salomon et al., 1996; Kihara et al., 1998), NMDA-mediated cytotoxicity (Akaike et al., 1994; Marin et al., 1994; Donnelly-Roberts et al., 1996; Kaneko et al., 1997), or growth factor deprivation (Yamashita and Nakamura, 1996). In vivo models demonstrating nicotine's ability to rescue neurones from death resulting from surgically induced nucleus basalis lesions in young and aged animals (Janson et al., 1988; Janson and Moller, 1993; Sjak-Shie and Meyer, 1993; Nanri et al., 1997), chemically induced neurotoxicity in MPTP models (Janson et al, 1988) and systemic kainic acid-induced excitotoxic effects (Borlongan et al., 1995) have been reported. The neuroprotective effects of nicotinic receptor ligands may provide additional chronic benefit in conditions where cognitive enhancement is sought.

Several recent reviews and study reports have presented perspectives on how nicotinic acetylcholine receptor-targeted compounds having some of the positive effects of nicotine (e.g., anxiolysis, anti-depressant, cognitive enhancement, neuroprotection) with reduced side effects might be developed. Thus, intense effort has been directed toward the development of nicotinic agonists that are potent and have selectivity for CNS nicotinic acetylcholine receptor subtypes. The recent discovery of nicotinic receptor ligands having selectivity for neuronal nicotinic acetylcholine receptors, such as TC-2403 and TC-2559, suggests that the successful clinical development of potent nicotinic-based therapeutics is imminent and may expand the therapeutic arsenal available for treatment of a variety of CNS disorders.

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