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TC-5619: An alpha7 neuronal nicotinic receptor-selective agonist that demonstrates efficacy in animal models of the positive and negative symptoms and cognitive dysfunction of schizophrenia

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Abstract

A growing body of evidence suggests that the alpha7 neuronal nicotinic receptor (NNR) subtype is an important target for the development of novel therapies to treat schizophrenia, offering the possibility to address not only the positive but also the cognitive and negative symptoms associated with the disease. In order to probe the relationship of alpha7 function to relevant behavioral correlates we employed TC-5619, a novel selective agonist for the alpha7 NNR subtype. TC-5619 binds with very high affinity to the alpha7 subtype and is a potent full agonist. TC-5619 has little or no activity at other nicotinic receptors, including the α4β2, ganglionic (α3β4) and muscle subtypes. The transgenic *th*(*tk*−)/*th*(*tk*−) mouse model that reflects many of the developmental, anatomical, and multi-transmitter biochemical aspects of schizophrenia was used to assess the antipsychotic effects of TC-5619. In these mice TC-5619 acted both alone and synergistically with the antipsychotic clozapine to correct impaired pre-pulse inhibition (PPI) and social behavior which model positive and negative symptoms, respectively. Antipsychotic and cognitive effects of TC-5619 were also assessed in rats. Similar to the results in the transgenic mice, TC-5619 significantly reversed apomorphine-induced PPI deficits. In a novel object recognition paradigm in rats TC-5619 demonstrated long-lasting enhancement of memory over a wide dose range. These results suggest that alpha7-selective agonists such as TC-5619, either alone or in combination with antipsychotics, could offer a new approach to treating the constellation of symptoms associated with schizophrenia, including cognitive dysfunction.

Keywords

Nicotinic receptor; Alpha7; Acetylcholine; Cholinergic; Schizophrenia; Antipsychotic

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1. Introduction

Schizophrenia is a complex disorder, displaying dysfunction in three defined areas: positive symptoms (hallucinations, delusions, thought disorder), negative symptoms (social withdrawal, anhedonia and apathy) and cognition (attention, memory, executive function) [1]. With the introduction of “typical” antipsychotic drugs in the 1950s, control over many of the positive (psychotic) symptoms was achieved, but undesirable extrapyramidal side effects (EPS) such as akathisia, parkinsonism, and tardive dyskinesia, as well as cognitive blunting, social withdrawal, anhedonia and apathy led to poor compliance with treatment regimens [2]. Despite claims that second-generation antipsychotics produce fewer EPS compared with first-generation neuroleptics, some studies suggest that the incidence of treatment-emergent EPS and changes in EPS ratings are not significantly different [3]. Newer “atypical” antipsychotics have addressed some of the concerns over side effects, but they remain generally deficient in treating negative and cognitive symptoms [4]. In an attempt to overcome these treatment deficits alternative pharmacological strategies have begun to emerge.

Epidemiological data showing that a high proportion of schizophrenic patients are heavy smokers compared to the general population has prompted speculation that schizophrenics smoke to alleviate the symptoms of the disease. Adding credence to this hypothesis are data showing that nicotine or nicotinic agonists improve a number of symptoms, both positive and cognitive, associated with schizophrenia including smooth pursuit eye movement [5], attention deficits [6] and sensory gating [7] in humans. Moreover, nicotine administered via skin patch reverses some of the haloperidol-related cognitive impairments in tests assessing memory and reaction time [8].

Physiological evidence supporting a role for neuronal nicotinic receptors (NNRs) in schizophrenia derives from post-mortem studies showing decreases in the densities of both α4β2 and alpha7 NNR subtypes in schizophrenic patients [9,10]. These two NNR subtypes are the most abundant in the mammalian brain and have been shown to modulate multiple neuronal pathways involved in schizophrenia [11]. Familial linkage studies have associated regions on chromosome 15, which contains the alpha7 NNR gene, with schizophrenia and polymorphisms have been described in the promoter region of the alpha7 NNR gene further implicating alpha7 NNRs in schizophrenia [12].

Targeting alpha7 NNRs as a therapeutic strategy has led to the development of novel alpha7-selective compounds [13]. These compounds have shown varying effects on positive and cognitive symptoms in animal models, but none have demonstrated efficacy across all the three domains (positive, negative and cognitive). In the present studies we describe the effects of a novel alpha7 NNR-selective compound (TC-5619) in a transgenic mouse model previously shown to express a schizophrenia-like behavioral phenotype [14]. The *th*(*tk*−)/*th*(*tk*−) mouse model is based on expression of a dominant negative mutant of the fibroblast growth factor receptor-1 [FGFR1(tk−)] from the catecholaminergic, neuron-specific tyrosine hydroxylase (TH) gene promoter. Changes in FGF and its receptor FGFR1 have been described in the brains of schizophrenia patients suggesting that impaired FGF signaling could underlie abnormal brain development and function associated with this disorder [15]. In homozygous *th*(*tk*−)/*th*(*tk*−) mice there is a significant reduction in the size of TH-immunoreactive neurons in the substantia nigra and the ventral tegmental area and a reduced density of dopamine (DA) transporter in the striatum, demonstrating an impaired development of the nigro-striatal DA system. Paradoxically, *th*(*tk*−)/*th*(*tk*−) mice have increased levels of DA, homovanillic acid and 3-methoxytyramine in the striatum, indicative of excessive DA transmission. In contrast, there is a reduction of these measures present in the prefrontal cortex. These changes are consistent with human PET studies and evidence of

increased subcortical DA synthesis and lower prefrontal dopaminergic activity in schizophrenia [1,16]. As a result the *th*(*tk*−)/*th*(*tk*−) mice display impaired pre-pulse inhibition (PPI), a measure of impaired sensory gating similar to that seen in schizophrenia, where the degree to which PPI is affected correlates with symptom severity [17]. This inhibition is reversed by a DA receptor antagonist, the typical antipsychoticflupentixol [14] as well as by the atypical antipsychotic quetiapine [59], providing further validation of the model. We show that the alpha7 NNR-selective agonist TC-5619 can correct behaviors that stem from abnormal development of dopaminergic systems in the brains of these mice similar to those found in the human condition, including improvement of PPI and social interaction (a surrogate for negative symptoms). The effects of TC-5619 were also corroborated in rat models that showed an improvement in both sensory gating and cognitive function. Thus TC-5619 appears to produce effects that could impact all the three primary domains associated with the disease. We also describe enhancement of the effects of TC-5619 by the atypical antipsychotic clozapine.

2. Methods

2.1. Binding and ion flux assays

[^{3}H]-methyllycaconitine ([^{3}H]-MLA) binding was determined in hippocampal membranes and HEK/human alpha7/RIC3 membranes (cells obtained from Jon Lindstrom, Philadelphia, PA) using standard methods as described previously [18]. [^{3}H]-nicotine binding to α4β2 NNRs in rat cortical membrane preparations or in human SH-EP1 cell (Ron Lukas, Phoenix, AZ) membranes was assayed using standard methods adapted from published procedures [19]. The IC_{50} (concentration of the compound that produces 50% inhibition of binding) was determined by least squares non-linear regression using GraphPad Prism software (GraphPad, San Diego, CA). The clonal cell lines PC12 (Shooter) and SH-SY5Y were utilized to examine rat and human ganglion-type nicotinic receptors respectively, and the TE671/RD clonal line was used to examine human muscle-type nicotinic receptors. Functional activation of these receptors was determined by $^{86}Rb^+$ efflux assays according to published protocols [20]. Receptor selectivity testing was performed by Caliper Life Sciences (formerly NovaScreen) using a customized side effects profile screen that included over 65 receptor and enzyme targets. Activity at each target was determined using TC-5619 at 10 μM.

2.2. Patch clamp electrophysiology

Patch clamp electrophysiology studies of the human alpha7 NNR using *Xenopus laevis* oocytes were performed in the laboratory of Roger Papke (University of Florida, Gainesville, FL) according to previously published procedures [21]. Experiments were conducted using OpusXpress 6000A (Axon Instruments, Union City, 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. Cells were voltage-clamped at a holding potential of −60 mV. Data were collected at 50 Hz and filtered at 20 Hz. Cells were bath-perfused with Ringer's solution, and agonist solutions were delivered from a 96-well plate via disposable tips, which eliminated any possibility of cross-contamination. Flow rates were set at 2 ml/min. Fresh acetylcholine stock solutions were made daily in Ringer's solution. Drug applications alternated between ACh controls and TC-5619. Applications were 12 s in duration followed by 181 s washout periods.

Responses were calculated as net charge for α7 receptors [22]. Each oocyte received an initial control application of ACh, then an experimental drug application, and then a follow-up control application of ACh (300 μM for α7 receptors and 30 μM for α4β2 receptors). Responses to experimental drug applications were calculated relative to the preceding ACh

control responses in order to normalize the data, compensating for the varying levels of channel expression among the oocytes. Since 300 μM ACh evoked maximal net charge responses from α7 receptors, normalization to the ACh controls effectively normalized the data to ACh maximum responses. Means and standard errors (SEM) were calculated from the normalized responses of at least four oocytes for each experimental concentration. Individual oocytes were used for not more than one dose–response study. For concentration–response relations, data derived from net charge analyses were plotted using Kaleidagraph 3.0.2 (Abelbeck Software; Reading, PA), and curves were generated from the Hill equation:

$$\text{Response}=\frac{I_{\max}[\text{agonist}]^n}{[\text{agonist}]^n+(\text{EC}_{50})^n}$$

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.

2.3. Behavioral studies in mice

2.3.1. Animals—The homozygous transgenic *th*(*tk*−)/*th*(*tk*−) mouse model has been previously described [14,59]. Briefly, these mice express FGFR1(TK−) fused to rat tyrosine hydroxylase (TH) gene promoter (4.5 kb). The progenies were screened for the presence of the transgene by PCR amplification of tail DNA. Homozygous transgenic and control mice (transgene free) lines were derived from crossing the same BCF1 (C57BL/10J/C3H/HeJ) parental mice. The lines showed stable behavioral differences in all the generations investigated [14]. Mice used in all specific experiments were from multiple litters. All mice used (males and females) were singly housed throughout behavioral testing and had been singly housed for at least four weeks before testing. Mice were housed on a light:dark cycle of 12:12 h with free access to food and water. All behavioral and anatomical procedures were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with approval from the University at Buffalo IACUC.

2.3.2. Pre-pulse inhibition in transgenic mice—Startle reactivity was measured using two chambers (SR-LAB; San Diego Instruments, San Diego, CA, USA). Each chamber consisted of a clear non-restrictive Plexiglas cylinder resting on a platform inside a ventilated box. A high-frequency loudspeaker inside the chamber produced both a continuous background noise of 68 dB and the 120-dB startle pulse. Vibrations of the Plexiglas cylinder caused by the whole-body startle response of the animal were transduced into analog signals by a piezoelectric unit attached to the platform. Pre-pulse inhibition (PPI) session: All PPI test sessions consisted of startle trials (pulse-alone), pre-pulse trials (pre-pulse + pulse), and no-stimulus trials. The pulse-alone trial consisted of a 40-ms 120-dB pulse of broad-band noise. PPI was measured by pre-pulse + pulse trials that consisted of a 20-ms noise pre-pulse, 100-ms delay, followed by a 40-ms 120-dB startle pulse (120-ms onset-to-onset interval). The acoustic pre-pulse intensities were 4, 8 and 16 dB above the 68-dB background noise (i.e. 72, 76 and 84 dB). The no-stimulus trial consisted of background noise only. The test session began and ended with five presentations of the pulse-alone trial; in between, each acoustic or no-stimulus trial type was presented 10 times in a pseudo-random order. There was an average of 15 s (range, 12–30 s) between trials. For the drug studies, the mice were placed into the startle chambers 30 min after each administration, and a 68-dB background noise level was presented for a 10-min acclimation period and continued throughout the test session. The level of PPI was calculated as a percentage score for each acoustic pre-pulse trial type as follows:

$$\%\text{PPI} = 100 - \frac{\text{startle response for pre-pulse+pulse}}{\text{startle response for pulse-alone}} \times 100$$

The magnitude of the acoustic startle response was calculated as the average response to all of the pulse-alone trials, excluding the first and last blocks of five pulse-alone trials presented.

Animals in one cohort of 8 control mice (5 male and 3 female) and 8 *th*(*tk*−)/*th*(*tk*−) mice (6 male and 2 female) were tested repeatedly between the ages of 6 and 8 months. Animals of both genotypes were given injections of vehicle and each drug dose. Our hypothesis was related to genotypic differences in behavior. Therefore, given the limited number of mice (preventing counterbalance design with meaningful number of mice per day/treatment) we chose to give all mice (control and transgenic) the same treatment in a given session. Mice were tested twice per week (at least 2 days apart) in one vehicle and one drug session. The order of the vehicle and drug injection days was changed each week. PPI and startle magnitude in vehicle test sessions were analyzed to determine if these measures remained stable with repeated testing. No effects of repeated testing over time were observed. Therefore, the results of all vehicle sessions were averaged. Injections of vehicle, TC-5619 (0.1 or 0.3 mg/kg) or clozapine (3.0 mg/kg) were administered i.p. in a volume of 175 μl/35 g of body weight. Drug doses were calculated as freebase.

For statistics, overall repeated measures ANOVAs were used at each drug dose with genotype, drug treatment and pre-pulse intensity level as the factors. For brevity, the main effects of pre-pulse intensity (which were always statistically significant) are not discussed. Separate repeated measures ANOVAs were conducted within each genotype using drug treatment as a factor. Post hoc LSD analyses were used to determine treatment effects at individual pre-pulse intensities only after a statistically significant main effect of genotype or drug treatment.

2.3.3. Social pair test in transgenic mice—Behaviors were measured in one cohort of 8 control and 8 *th*(*tk*−)/*th*(*tk*−) male mice. All subjects were singly housed on a 12:12 light:dark cycle. Food and water were available *ad libitum* in the home cage. The experiments began when the subjects were 5 months old. Our hypothesis was related to genotypic differences in behavior so we chose to give all mice (control and transgenic) the same treatment in a given session. To determine baseline, all the mice were tested with vehicle injections. Approximately two weeks later, all subjects were tested following administration of TC-5619 (0.3 mg/kg injected i.p.). The subjects were subsequently given a low dose of clozapine (3 mg/kg), a low dose of TC-5619 (0.1 mg/kg), and the low dose of clozapine plus the low dose of TC-5619. There was one week between drug treatments. Five weeks later, the same animals were again tested with vehicle injections to ensure that the baseline behavior of the animals did not change after treatment or with age. The performance of vehicle injected *th*(*tk*−)/*th*(*tk*−) mice and non-transgenic controls on all tests remained unchanged between 5 and 9 months of age (data not shown).

Each testing day followed the same time course. The animals were injected with saline or drug. Forty-five minutes later the animals were given two 3-min social behavior tests (one with a female and one with a male). Thirty minutes after the social behavior test, the animals were placed in the open-field for 10 min. Thirty minutes after the open-field test, the animals were given a 3-min trial on the elevated plus maze. Subjects were returned to their home cages after each test.

Social behavior was tested using a variant of the resident-intruder paradigm, in which a stimulus animal was introduced into the subject's home cage for 3 min. Prior to testing the subjects' home cages were not changed for at least 4 days to allow them to establish the cage as their territory. Each subject was tested with a different stimulus animal and each stimulus animal was used only once per testing day. Animals of both genotypes were given injections of vehicle and each drug dose. A stimulus female was placed into the cage of control and *th*(*tk*−)/*th*(*tk*−) subjects. After 3 min, the stimulus female was removed. Thirty minutes later a stimulus male was placed into the subject's home cage. After 3 min, the stimulus animal was removed. All testing occurred in the dark-phase (i.e. the active phase in the nocturnal mouse) of the light cycle under red light illumination. The interaction was videotaped from the side using the Nightshot feature on a Sony video camera (DRV-120, Sony Corporation). The behavior of the subject was quantified from the videotape using the Observer Mobile (Noldus Information Technologies, Sterling, VA). The number of bouts observed and the amount of time engaged in the following behaviors was measured: general social contact (contact with the stimulus animal), sniffing (both anogenital and non-anogenital contact), and non-social behavior (auto-grooming). The experimenter scoring the behavior was blind to the genotype and treatment of the subjects.

2.3.4. Open-field activity in transgenic mice—The subjects were removed from their home cage and placed alone into a clean Plexiglas open-field testing arena (45 cm × 45 cm × 25 cm) for a 10 min testing session, after which they were returned to their home cage. The test was videotaped from above using a Sony TRV-350 Handycam video camera using the Nightshot feature. Movement was analyzed in detail using the Clever Sys. Inc. system.

2.3.5. Elevated plus maze in th(tk−)/th(tk−) transgenic mice—The subjects were placed in the center of a mouse elevated plus maze (San Diego Instruments, San Diego, CA). The test was videotaped from above using a Sony TRV-350 Handycam video camera using the Nightshot feature. The behavior of the subject was quantified from the videotape using the Observer Mobile (Noldus Information Technologies, Sterling, VA) by an observer unaware of the treatment or genotype of the subjects.

2.4. Behavioral studies in rats

2.4.1. Animals—For *in vivo* studies, adult male 200–350 g Sprague–Dawley rats (Charles River Laboratories, Raleigh, NC) were used. Animals had *ad libitum* access to drinking water and rodent chow. 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.

2.4.2. Pre-pulse inhibition (PPI) in rats—Startle activity was measured using Plexiglas animal enclosure boxes (17.7 cm × 9 cm × 11.2 cm) resting on a Plexiglas frame enclosed in a lighted, ventilated, sound-attenuating cabinet measuring 29.5 cm × 17.7 cm × 29 cm (inside of box dimensions). A high-frequency loudspeaker inside the chamber produced both a continuous background noise of 65 dB and the 118-dB startle pulse. Startle responses, reflecting the motion of animals following acoustic stimuli, were detected by a piezoelectric transducer mounted below the frame. Stabilimeter readings were rectified and recorded by a microcomputer and interface ensemble (Hamilton-Kinder, Poway, CA), and reported in Newton (N) units. All PPI testing was conducted as described above (Section 2.3.2). Rats were injected with TC-5619 (0.1, 0.3 or 1 mg/kg s.c. or vehicle) and then 10 min later injected with apomorphine (1.0 mg/kg s.c.). The animals were placed in the startle chamber 5 min after apomorphine administration and a 65-dB background noise level was presented for a 5-min acclimation period and continued throughout the test session. A two-way repeated measures (RM) ANOVA, with pre-pulse dB (RM) and treatment as the dependent

variables was performed to determine significant differences among treatment groups and post hoc analyses were performed where differences were noted. A p-value < 0.05 was considered statistically significant.

2.4.3. Novel object recognition in rats—A novel object recognition (NOR) task was used to determine the duration of cognitive-enhancing effects of TC-5619. The object recognition model is based on a rodent's spontaneous tendency to explore novel aspects of their environment and this exploratory activity can be an index of memory function [23]. The NOR test measures the capacity to recognize an object presented on two occasions, some time apart. The test arena consisted of a 17.5 in. × 17.5 in. clear Plexiglas™ with walls 12 in. in height. The arena was enclosed in an opaque, sound-attenuating chamber and the doors (opening to the front side) remained open. Firstly, a dose–response study was conducted to determine a minimum effective dose of TC-5619 by administering TC-5619 by oral gavage once daily 30 min prior to three NOR trials (i.e. habituation, acquisition and recall NOR trials). Inter-trial intervals of 24 h were imposed between the three trials. In the dose–response study, the minimum effective dose to produce cognitive-enhancing activity was determined to be 0.3 mg/kg (data not shown). Based on this outcome, TC-5619 (0.3 mg/kg) was administered by oral gavage once daily to separate groups of animals in order to determine the duration of effect at this dose level. On the first 2 days of this q.d. dosing paradigm, administrations were followed 30 min later by an exploratory (habituation) trial of 6 min duration on Day 1 (no objects) and an object recognition acquisition trial of 3 min duration on Day 2 (2 of the same objects). Inter-trial intervals of 24 h were imposed between trials on Day 1 and Day 2. On the third day the final OR trial, or recall trial (3 min; one familiar, one novel object) was started at either 30 min, 2 h, 6 h, 18 h or 24 h after compound administration. For the recall trial, a video camera was positioned approximately 36 in. from the unshielded side of the arena for videotaping of the animals' behaviors. These behaviors were subsequently hand-scored by a blinded observer assessing the time spent exploring a novel (object B) versus a familiar (object A) object during this recall exposure trial. Absolute exploration time for each object was recorded, and a recognition index (RI), expressed as a percentage, was calculated as follows:

$$\%\mathrm{RI}=\frac{\text{time investigating novel object}}{\text{total time investigating both novel+familiar objects}}\times 100$$

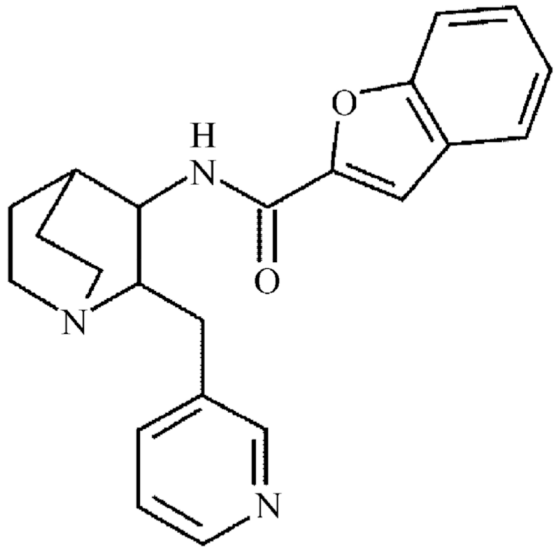
Student's t-tests were performed for each treatment group to determine statistically significant differences between exploration time for the familiar versus novel object and one-way ANOVAs (or comparable Kruskall–Wallace ANOVAs for non-parametrically distributed data) were performed to assess statistically significant differences among groups for %RI. Where statistically significant overall effects were found, post hoc analyses were performed. $p < 0.05$ was considered statistically significant.

2.5. Drugs and chemicals

TC-5619 was provided by Targacept, Inc. (Winston-Salem, NC). Haloperidol, (−)-apomorphine and clozapine were obtained from Sigma–Aldrich (St. Louis, MO). TC-5619 and haloperidol were dissolved in either 0.9% saline for s.c. administration or de-ionized H_2O for oral administration. Clozapine was dissolved in 0.9% saline for i.p. administration. (−)-Apomorphine was dissolved in saline containing 0.1% (w/v) ascorbic acid (Sigma) and refrigerated in the dark to protect against oxidative degradation.

3. Results

3.1. Structure and synthesis of TC-5619



TC-5619, *N*-[2-(pyridin-3-ylmethyl)-1-azabicyclo[2.2.2]oct-3-yl]-1-benzofuran-2-carboxamide, was prepared from commercially available quinuclidin-3-one by aldol condensation with 3-pyridinecarboxaldehyde to afford 2-(pyridin-3-yl)methylene quinuclidin-3-one followed by catalytic hydrogenation. The carbonyl moiety of the resulted 2-(pyridin-3-yl)methylquinuclidin-3-one was converted into an amino group by reductive amination. Final coupling of 3-amino-2-(pyridin-3-yl)methyl-1-azabicyclo[2.2.2] octane with benzo[b]furan-2-carboxylic acid provided *N*-[2-(pyridin-3-ylmethyl)-1-azabicyclo[2.2.2]oct-3-yl]-1-benzofuran-2-carboxamide [24].

3.2. Selectivity of TC-5619 for the alpha7 NNR

TC-5619 is a potent inhibitor of [^{3}H]-MLA binding to the alpha7 receptor from rat brain, with a Ki of 1 nM in rat hippocampal membranes (Table 1). A similar binding affinity of 1 nM was obtained in a HEK293 cell line co-expressing human alpha7 and RIC3 cDNAs. TC-5619 has a lower affinity for the α4β2 receptor subtype. In competition binding studies with [^{3}H]-(S)-nicotine, TC-5619 displayed a Ki of 2800 nM at α4β2 receptors expressed in SH-EP1 cellular membranes and a Ki of 2100 nM at α4β2 receptors expressed in rat cortical membranes.

We also tested TC-5619 in a broad receptor selectivity battery (NovaScreen) and found minimal interactions with other non-nicotinic receptor classes, as defined by inhibition of receptor-selective ligand binding >50% at 10 μM. Based on this criterion, TC-5619 showed positive interactions with a non-selective opioid receptor assay (58% inhibition) and with the sodium site 2 (79% inhibition). Dose–response assessments of these interactions showed that the Ki values for the opioid site and for sodium site 2 were both 13 μM, providing a greater than 1000-fold separation from the binding affinity at alpha7. Due to the close sequence and structural homology between alpha7 and $5HT_3$ receptors, and previously reported interactions of some nicotinic ligands with both receptors, we examined the affinity of TC-5619 for $5HT_3$ receptors. Binding of TC-5619 (10 μM) to $5HT_3$ receptors displayed 59% inhibition of radioligand binding at the mouse receptor and 25% inhibition at the

human receptor. Investigation of functional activation at the human $5HT_3$ receptor suggested minimal to no activation; a maximal response of 15% was obtained at 100 μM TC-5619.

3.3. Functional activation of NNRs by TC-5619

Using patch clamp electrophysiological techniques, we examined the functional activity of TC-5619 at neuronal nicotinic receptors transiently expressed in *Xenopus* oocytes. At human alpha7 receptors, TC-5619 displayed an EC_{50} of 33 nM and an E_{max} of 100% relative to ACh (Fig. 1 and Table 1). In contrast to previously described alpha7 full agonists [25], residual inhibition of subsequent applications of ACh over the dose–response range for receptor activation was minimal. There was no detectable activation when TC-5619 was applied to oocytes expressing the human α4β2 subtype and no significant decreases in subsequent control responses to ACh, indicating that TC-5619 is neither an agonist nor antagonist at α4β2 (results not shown). TC-5619 produced very little functional activation of peripheral nicotinic acetylcholine receptors expressed in appropriate rat and human cell lines (Table 1). At 10 and 100 μM, TC-5619 produced no, or very low, activation of human muscle (5% and 12% of nicotine's E_{max}, respectively), rat ganglion (11% and 20% of nicotine's E_{max}, respectively) or human ganglion (6% and 11% of nicotine's E_{max}, respectively) receptors. The lack of interaction with muscle and ganglionic-type receptors suggests low potential for nicotinic side effects with TC-5619.

3.4. Effectiveness of TC-5619 in a preclinical model of positive symptoms of schizophrenia in mice

3.4.1. Attenuation of pre-pulse inhibition and startle response in transgenic mice—Inhibition of a motor (startle) response elicited by a weak sensory event, termed pre-pulse inhibition (PPI), provides an operational measure of sensorimotor gating, a system in the brain that is deficient in schizophrenia [26]. Reduction in PPI stemming from abnormal subcortical neurotransmission, has been used as a model of the positive symptoms. In the present study PPI was tested in one cohort of 8 *th*(*tk*−)/*th*(*tk*−) and 8 non-transgenic control mice. Consistent with previous findings in *th*(*tk*−)/*th*(*tk*−) mice, PPI was reduced (Fig. 2B; $p = 0.033$) relative to controls with vehicle-only injections (Fig. 2A). Control mice showed no statistically significant changes in PPI with administration of TC-5619 at 0.1 or 0.3 mg/kg (Fig. 2A), while *th*(*tk*−)/*th*(*tk*−) mice displayed significant improvements in PPI only at 0.3 mg/kg ($p = 0.011$) (Fig. 2B). At the 0.3 mg/kg dose, there was a statistically significant drug × genotype interaction effect ($p = 0.022$) demonstrating the ability of TC-5619 to normalize PPI in *th*(*tk*−)/*th*(*tk*−) mice.

The startle response of *th*(*tk*−)/*th*(*tk*−) mice was elevated relative to controls ($p = 0.019$) when injected with vehicle (Fig. 3B). TC-5619 reduced startle response in *th*(*tk*−)/*th*(*tk*−) mice (Fig. 3B; $p < 0.001$) and increased startle response ($p = 0.037$) in control mice at the 0.3 mg/kg dose (Fig. 3A). There was no statistically significant effect of drug on startle response at 0.1 mg/kg in either genotype. At both doses tested there was a significant drug × genotype interaction effect ($p = 0.017$ at 0.1 mg/kg, $p < 0.001$ at 0.3 mg/kg) indicating normalization of the startle response in *th*(*tk*−)/*th*(*tk*−) mice. These data suggest that TC-5619 may ameliorate the gating deficits associated with schizophrenia.

3.4.2. Enhancement of effects of TC-5619 on pre-pulse inhibition and startle response by antipsychotics in transgenic mice—The atypical antipsychotic drug clozapine was investigated for potential interactions with TC-5619. Administration of clozapine alone at 3.0 mg/kg had no effect on PPI in control mice (Fig. 2A). The PPI in *th*(*tk*−)/*th*(*tk*−) mice was slightly increased by clozapine but this effect did not reach statistical significance (Fig. 2B). When combined with TC-5619 at 0.1 mg/kg, a dose that did not improve PPI independently in either genotype, there was an improvement in PPI in *th*(*tk*−)/

th(*tk*−) mice (Fig. 2B; $p < 0.01$) and no effect in control mice (Fig. 2A). There was a statistically significant interaction effect of drug (clozapine 3.0 mg/kg and TC-5619 0.1 mg/kg combined) × genotype ($p = 0.039$) indicating an additive ability of both drugs at moderate doses to normalize PPI in *th*(*tk*−)/*th*(*tk*−) mice relative to control mice.

Clozapine at 3.0 mg/kg or TC-5619 at 0.1 mg/kg had no effect on startle response in either genotype; however when administered in combination, startle response was decreased in *th*(*tk*−)/*th*(*tk*−) mice (Fig. 3B; $p = 0.021$) and no effect was observed in control mice (Fig. 3A). There was a statistically significant drug × genotype (clozapine, 3.0 mg/kg and TC-5619, 0.1 mg/kg combined) interaction ($p = 0.031$).

3.5. Effects of TC-5619 in a preclinical model of negative symptoms of schizophrenia in mice

In addition to impaired sensory gating and hallucinations, which are associated with increased subcortical dopamine transmission, schizophrenic patients manifest negative symptoms such as social withdrawal and flattened affect. The social interaction model in rodents has been well established as a paradigm for testing antipsychotics with respect to their potential effects on negative symptoms in schizophrenia [27]. The *th*(*tk*−)/*th*(*tk*−) mouse models negative symptoms by showing diminished response to social cues. When exposed to a male or female stimulus, socially experienced male *th*(*tk*−)/*th*(*tk*−) mice engage significantly less in stimulus investigation than controls (Klejbor, unpublished observation). Other measures of sociability (e.g. anogenital investigation, contact time) were also shown to have diminished in these animals. In the present study social investigation of a female or male stimulus transgenic animal is less than that of controls (Fig. 4). Treatment with 0.3 mg/kg TC-5619 (high dose) increased investigation time of a female stimulus animal in both control and *th*(*tk*−)/*th*(*tk*−) mice. With a male stimulus animal, this dose of TC-5619 increased investigation time in *th*(*tk*−)/*th*(*tk*−) but not in control mice. Although clozapine or 0.1 mg/kg TC-5619 (low dose) had no effect on investigation time in any group, treatment with TC-5619 (low dose) and clozapine together increased the investigation time of a female stimulus in control and *th*(*tk*−)/*th*(*tk*−) mice. Treatment with TC-5619 (low dose) and clozapine together increased investigation time of a male stimulus animal in *th*(*tk*−)/*th*(*tk*−) but not in control mice. There were main effects of genotype ($F(1,78) = 35.11$, $p < 0.001$) and treatment ($F(5,78) = 25.62$, $p < 0.001$) but no interaction ($F(5,78) = 0.54$, $p = 0.75$) between them on time spent investigating a female stimulus animal (Fig. 4).With a male stimulus animal, there were main effects of genotype ($F(1,78) = 55.15$, $p < 0.001$) and treatment ($F(5,78) = 27.84$, $p < 0.001$) and an interaction ($F(5,78) = 5.70$, $p < 0.001$) between them on time spent investigating a male stimulus animal (Fig. 4).

In contrast to the significant effect of TC-5619 on social interaction, the TC-5619 treatment had no effect on motor activity in the open-field test in either control or *th*(*tk*−)/*th*(*tk*−) mice (Fig. 5A, no main effect of treatment, $p > 0.05$). As previously reported, *th*(*tk*−)/*th*(*tk*−) spent more time in the center zone of the open-field and moved a greater distance than control mice (Fig. 5A, statistically significant main effect of genotype, $p < 0.05$). Also, there was no effect of TC-5619 on behavior in the elevated plus maze (Fig. 5B, no main effect of treatment, $p > 0.05$). The *th*(*tk*−)/*th*(*tk*−) mice spent significantly more time in the open arm, and consequently less time in the closed arms, than did controls (Fig. 5B, statistically significant main effect of genotype, $p < 0.05$).

3.6. Effects of TC-5619 in a preclinical model of positive symptoms of schizophrenia in rats

The psychostimulant apomorphine has been shown to impair PPI, and this effect can be reversed by administration of antipsychotic drugs. In this study, TC-5619 (0.3 mg/kg s.c.) reversed PPI deficits induced by administration of apomorphine (Fig. 6). In an overall

comparison among drug treatment factors alone, administration of saline plus 1.0 mg/kg (−)-apomorphine significantly reduced %PPI compared with saline + vehicle-treated animals ($p < 0.001$). TC-5619 significantly reversed ($p < 0.001$) apomorphine-induced PPI deficits following 0.3 mg/kg TC-5619 + 1.0 mg/kg apomorphine when compared to saline plus apomorphine. The typical antipsychotic haloperidol significantly reversed ($p < 0.001$) the PPI deficits induced by apomorphine following 0.3 mg/kg haloperidol + 1.0 mg/kg apomorphine when compared to saline plus apomorphine. These data provide additional evidence that TC-5619 may ameliorate the gating deficits associated with schizophrenia.

3.7. Effects of TC-5619 on novel object recognition in rats

In a duration of effect assessment of TC-5619 (0.3 mg/kg p.o.) in the novel object recognition paradigm, the average time spent exploring object A versus object B during the recall trial by the vehicle-treated group at 30 min, 6 h, or 24 h after the third vehicle administration was not significantly different ($p = 0.17$, $p = 0.35$, and $p = 0.12$, respectively). By comparison, at 30 min, 2 h, 6 h, and 18 h after the final sub-acute (q.d. × 3 days) administration of TC-5619 (0.3 mg/kg p.o.), subjects spent significantly more time investigating the novel object than the familiar object (Fig. 7A). Moreover, at 2 h (75%) and 6 h (71%), the recognition index (RI) was significantly increased in animals treated with 0.3 mg/kg TC-5619 compared with the RI (54%) of the vehicle-treated group at 30 min after final administration (Fig. 7B). The results demonstrate that TC-5619 facilitates memory in young rats up to 18 h after a third sub-acute daily administration.

4. Discussion

The present findings suggest that alpha7 NNR-selective agonists such as TC-5619 may have the potential to positively impact all the three primary domains of schizophrenia, including cognitive dysfunction as well as the positive and negative symptoms. Previous findings support the notion that alpha7 NNRs play a broad role in the neurobiological mechanisms that underlie the spectrum of deficits present in schizophrenia. Alpha7 NNRs are located in a number of brain structures relevant to schizophrenia, such as the hippocampus, lateral and medial geniculate nuclei, and reticular nucleus of the thalamus [11,28]. In animal studies, blockade of alpha7 NNRs induces gating deficits similar to those in schizophrenia [29] and alpha7 NNRs are directly involved in processes that mediate memory consolidation such as long-term potentiation (LTP) and sensory processing [30,31]. The well-known modulation by alpha7 NNRs of several key neurotransmitters including glutamate [32], GABA [33], and dopamine [34] is particularly relevant. It has been suggested that the GABAergic neurons in the reticular thalamic nucleus are crucially involved in the regulation of random background activity in specific thalamic nuclei and that dysfunction of this structure may play a central role in the predisposition to hallucinations and acute psychosis [35]. These neurons are characterized by high concentrations of alpha7 NNRs [36]. Activation of nicotinic receptors on terminals from reticular thalamic neurons normally increases the release of GABA onto thalamic relay cells, which improves the signal-to-noise ratio of neural activity in specific thalamic nuclei during arousal [37]. Thus cholinergic activation of alpha7 NNRs on GABAergic terminals from the reticular thalamic nucleus may be responsible for stimulus-specific inhibition of thalamic relay cells necessary for signal discrimination and sensory gating, making it likely that the improvements in PPI and startle response by TC-5619 in the present studies involve similar mechanisms.

The transgenic *th*(*tk*−)/*th*(*tk*−) mouse model has also been reported to express dopaminergic dysfunction similar to that in schizophrenia [14]. It has been suggested that in acute phases of schizophrenia episodic dopaminergic hyperactivity may complement the neurobiological deficits (e.g., alpha7 NNR hypo-functionality) that underlie lack of sensory gating [38]. In particular, dopaminergic hyperactivity and NNR abnormalities (the latter being related to

abnormal sensory gating) may be complementary in reducing the release of GABA onto thalamic relay cells and augmenting random background activity in thalamic relay nuclei as a result. Based on these observations, modulation of dopaminergic tone by activation of alpha7 NNRs would also be consistent with the present results. In addition, interaction of alpha7 activity with glutamatergic pathways cannot be ruled out as a contributing factor. For example, cortical hyper-excitation and psychotic symptom formation in schizophrenia have been linked to hypo-functional NMDA receptors on thalamic reticular neurons [39]. NMDA receptors are expressed on GABAergic neurons of the reticular thalamic nucleus, as are alpha7 NNRs, and their blockade leads to inhibition of reticular thalamic neurons with a reduction in the release of GABA onto thalamocortical cells. This provides yet another potential mechanistic link between alpha7 NNRs and schizophrenia.

Multiple genetic links have been reported for schizophrenia, including gene products that influence alpha7 neuronal nicotinic receptors. More recently, the integrative nuclear FGF receptor1 (FGFR1) signaling, or INFS, has been shown to affect several signaling mechanisms for which schizophrenia-linked mutations have been reported [40,41]. Changes in FGF and its receptor FGFR1 have also been described in the brains of schizophrenia and bipolar patients suggesting that impaired FGF signaling could underlie abnormal brain development and function associated with these disorders [15]. The transgenic *th*(*tk*−)/*th*(*tk*−) mouse model used in the present studies is characterized by disruption of FGF receptor signaling as well as developmental, anatomical, and biochemical changes similar to those found in schizophrenia [14,59]. With respect to the effects of the alpha7 agonist TC-5619 seen in the present studies, it is possible that they are in part associated with alpha7 NNR activation linked to modulation of FGF signaling pathways. For example, there is evidence that the fibroblast growth factor (FGF-2) gene is a target for nicotinic cholinergic signaling and that FGF-2 could be involved, in view of its neurotrophic functions, in NNR mechanisms mediating neuronal survival, trophism and plasticity [42]. Studies involving acute intermittent nicotine treatment have shown enhancement of neuronal precursor cell proliferation in the adult rat brain and that pre-treatment with mecamylamine, a non-selective NNR antagonist, blocks the enhanced precursor proliferation by nicotine [43]. These changes were accompanied by up-regulation of fibroblast growth factor-2 (FGF-2) mRNA and the expression of its receptor FGFR1 in cells with a precursor cell profile. Further, the effects of nicotine on neuronal precursor proliferation were mediated by FGF-2 via FGFR1 activation. Neuronal nicotinic receptors have in fact been shown to play a role in modulating and enhancing the activity of FGF signaling pathways [42], but the exact identities of the NNR subtypes involved have not been characterized.

Because schizophrenia encompasses multiple neural domains it would be desirable to develop novel therapies that address all aspects of the disorder, including both positive and negative symptoms as well as cognitive dysfunction. Alpha7 agonists with varying pharmacologies have been described, providing evidence for the potential treatment of multiple domains in schizophrenia [44]. Many have demonstrated efficacy in animal models of sensory gating, such as PNU-282987 [45] and PHA-709829 [46]. Others have shown promise in addressing cognitive dysfunction, such as ABBF [47] and SSR-180711 [48]. A-582941 has been reported to affect both cognition and sensory gating [49]. Similarly, DMXB (or GTS-21), one of the first alpha7 NNR-selective ligands described, was shown to reverse auditory gating deficits in DBA/2 mice [50], suggesting the potential to ameliorate positive symptoms. In addition, GTS-21 was found to enhance attention, working memory, and episodic secondary memory in human volunteers [51]. By comparison, the selective alpha7 agonist AR-R17779 appears to improve learning and memory [52] and social recognition [53] but is relatively ineffective in restoring pre-pulse inhibition [54]. Allosteric modulators of alpha7 function have appeared, some that affect sensory gating such as PNU-120596 [55] and others that enhance cognitive function such as NS1738 [56]. Despite

the many beneficial effects demonstrated in animal models, many alpha7 ligands have also shown a propensity for 5HT3 or hERG interactions, which could lead to unwanted side effects [46,49]. The development of several compounds showing promise in preclinical studies has been limited due to nausea, cardiovascular arrythmias or CNS-related side effects [46,49,60]. Another challenge to the development of alpha7 ligands as therapeutics is that their positive effects could potentially be reduced by concomitant medications used in schizophrenia such as antipsychotics, some of which have been shown to inhibit the activity of alpha7 NNRs [58,61]. The inclusion of patients taking clozapine may have obscured the effects of DMXB-A in an initial phase 2 trial in schizophrenia [57].

With respect to cognitive effects, TC-5619 demonstrated significant enhancement of short-term working memory in the novel object recognition paradigm. Moreover, effects on memory were seen up to 18 h following oral administration, suggesting a positive effect on long-term memory consolidation as well. Any compound of utility for the cognitive dysfunction of schizophrenia must not diminish the effects of classical or atypical antipsychotics against the positive symptoms of schizophrenia. Thus, it is interesting that in addition to the cognitive-enhancing properties TC-5619 also reversed apomorphine-induced pre-pulse inhibition both in mice and rats. This characteristic of TC-5619 may provide an additional benefit against the positive symptoms associated with schizophrenia. The efficacy of TC-5619 in the social withdrawal model in mice suggests that the compound also has the potential to target negative symptoms of the disease.

Another unique feature of TC-5619 is that its effects appear to be additive or possibly synergistic with those of antipsychotics, further supporting the therapeutic potential of alpha7-selective compounds not only as monotherapy but also as add-on therapies in combination with existing drugs. Future studies will be necessary to explain the mechanism(s) by which this effect occurs. In conclusion, the present studies provide proof of concept that alpha7 NNR-selective compounds may provide a novel therapeutic approach for treating symptomatic domains of schizophrenia possibly beyond cognitive dysfunction.

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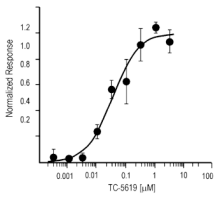

Fig. 1.

Effects of TC-5619 on alpha7 NNRs expressed in oocytes. Dose–response for TC-5619-evoked currents in human alpha7 receptors expressed in *Xenopus* oocytes. Data were normalized to the net charge of control 300 μM ACh responses obtained 5 min before the experimental agonist-evoked responses. Each point represents the Mean ± SEM of the normalized responses of at least four oocytes.

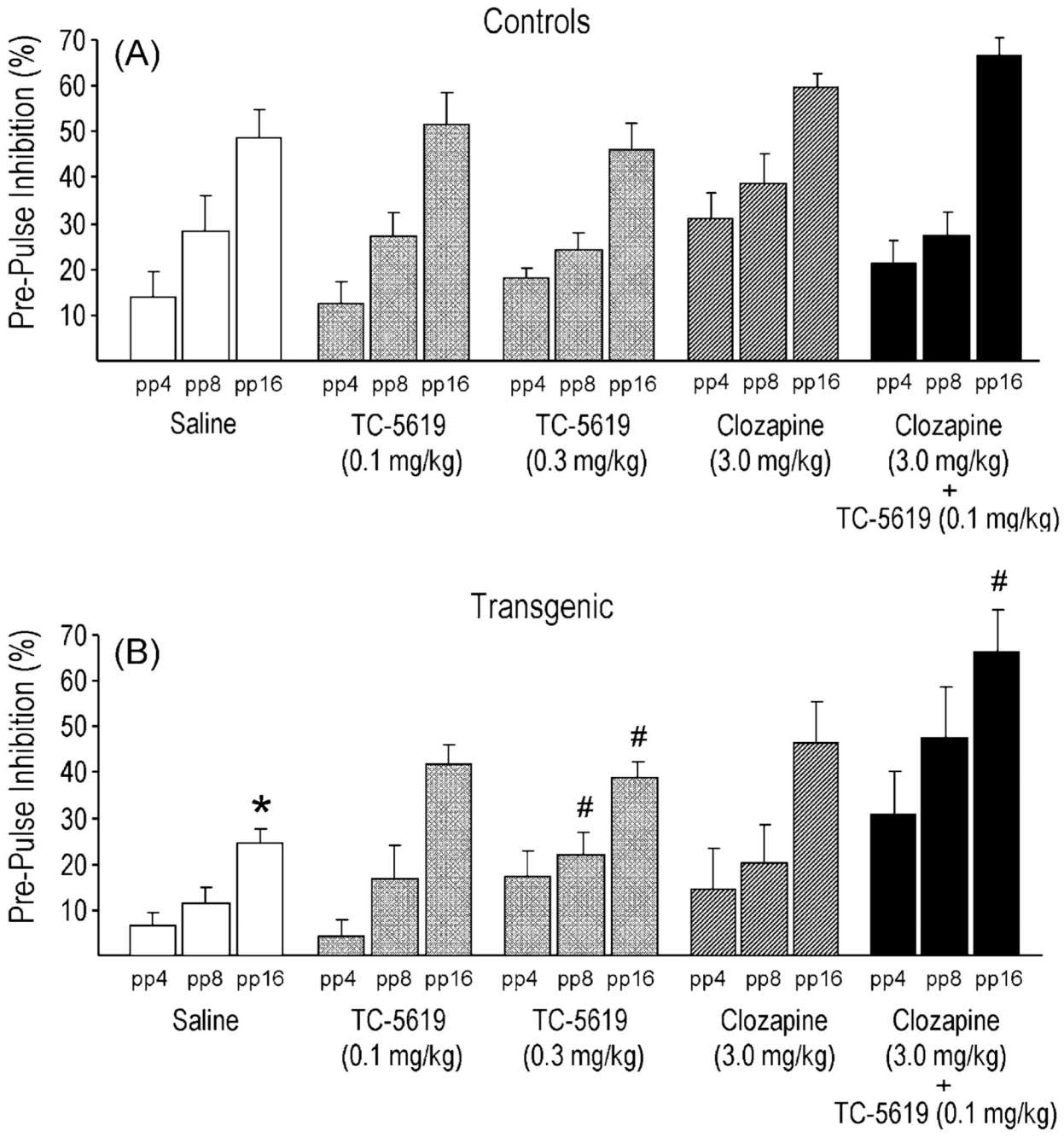

Fig. 2.

Effects of TC-5619 or TC-5619 and clozapine together on pre-pulse inhibition in mice. TC-5619 alone (0.1 and 0.3 mg/kg), clozapine alone (3.0 mg/kg) or both drugs together were administered i.p. PPI was measured in age-matched controls (Panel A) and in transgenic *th*(*tk*−)/*th*(*tk*−) mice (Panel B) as described in Section 2. Results are expressed as Mean ± SEM. *Significant difference from control group receiving same treatment at individual stimulus intensity (post hoc; $p < 0.05$, after significant main effect of genotype F value; ANOVA). #Significant difference within genotype from vehicle group at individual stimulus intensity (post hoc LSD; $p < 0.05$, after significant main effect of drug F value; ANOVA).

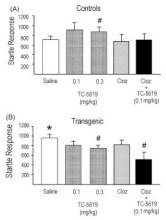

Fig. 3.

Effects of TC-5619 or TC-5619 and clozapine together on startle response in mice. TC-5619 alone (0.1 and 0.3 mg/kg), clozapine alone (3.0 mg/kg) or both drugs together were administered i.p. Startle response was measured in age-matched controls (Panel A) and *th*(*tk*−)/*th*(*tk*−) mice (Panel B) as described in Section 2. Results are expressed as Mean ± SEM. *Significant difference from control group receiving same treatment ($p < 0.05$, significant main effect of genotype F value; ANOVA). #Significant difference within genotype from vehicle group ($p < 0.05$, significant main effect of drug F value; ANOVA).

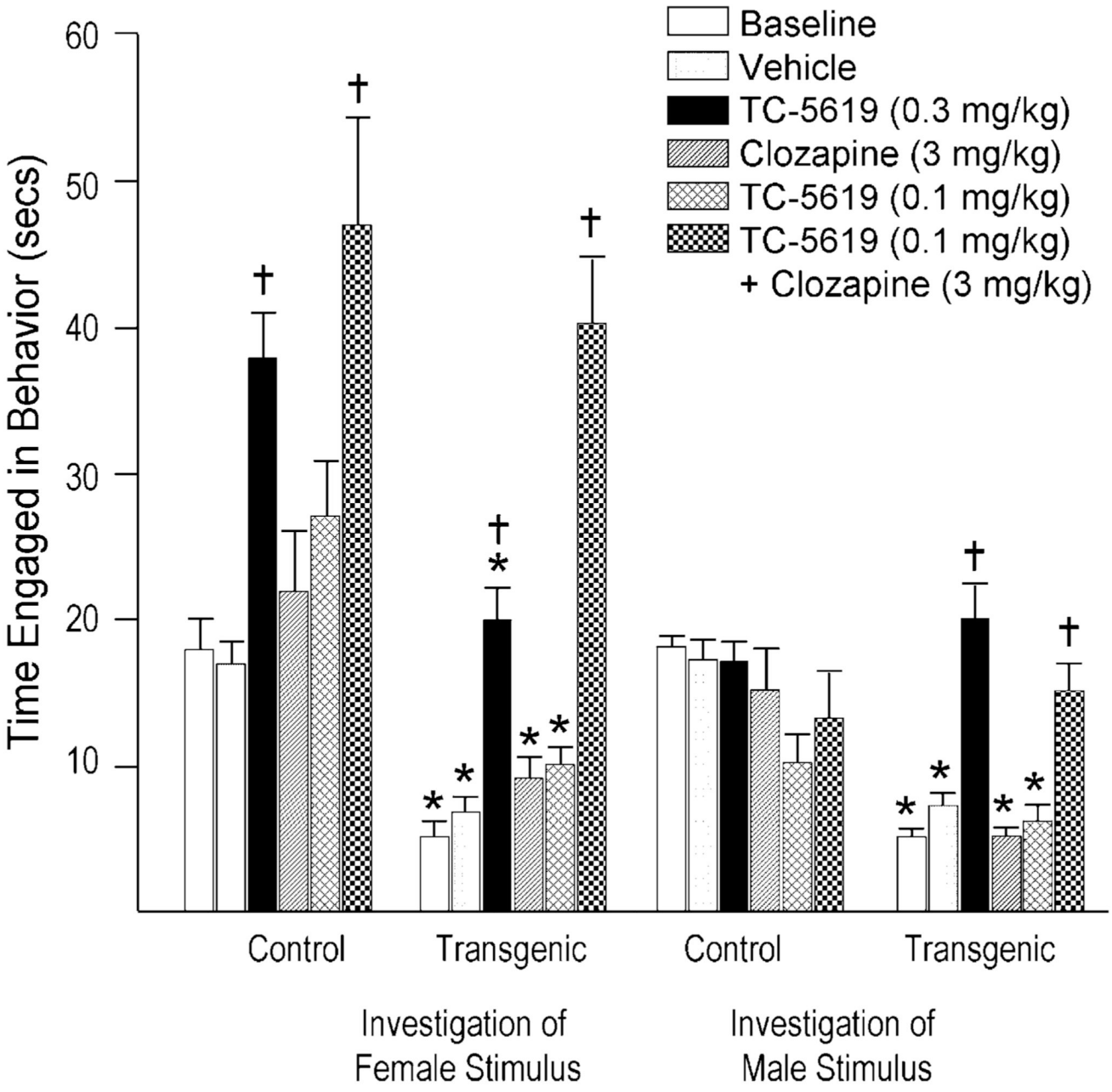

Fig. 4.

Effects of TC-5619 or TC-5619 and clozapine together on social investigation in mice. In the absence of drug (baseline and vehicle groups), control mice spend more time investigating stimulus animals than transgenic *th*(*tk*−)/*th*(*tk*−) mice. Treatment with TC-5619 (0.3 mg/kg) increased the time subjects of both genotypes spent investigating the stimulus animal. There was no difference in investigation time between drug-treated control mice and drug-treated *th*(*tk*−)/*th*(*tk*−) mice. Low doses of clozapine (3.0 mg/kg) or TC-5619 (0.1 mg/kg) alone had no effect on investigation time of either a female or male stimulus animal. These behaviorally inactive doses of TC-5619 and clozapine administered together increase social investigation of female stimulus animals in both genotypes. With a male

stimulus animal, however, treatment with clozapine and TC-5619 (low dose) increased investigation time in *th*(*tk*−)/*th*(*tk*−), but not control mice. †Significantly different from other groups of the same genotype ($p < 0.05$). *Significantly different from control group of the same treatment ($p < 0.05$).

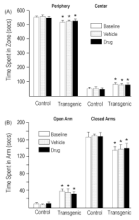

Fig. 5.

Effects of TC-5619 on locomotor activity in the open-field test and elevated plus maze in mice. (Panel A) The time control or *th*(*tk*−)/*th*(*tk*−) mice spent in the periphery or center zone of the open-field. Although *th*(*tk*−)/*th*(*tk*−) mice spent significantly more time in the center (and consequently significantly less time in the periphery) than controls, there was no effect of TC-5619 (0.3 mg/kg). *Significantly different from controls ($p < 0.05$). (Panel B) TC-5619 had no effect on time spent in the open and the closed arms of the elevated plus maze. As expected, subjects of both genotypes spent significantly more time in the closed arms than in the open arms. The *th*(*tk*−)/*th*(*tk*−) mice spent significantly more time in the open arms than controls. There was no effect of drug. *Significantly different from controls ($p < 0.05$).

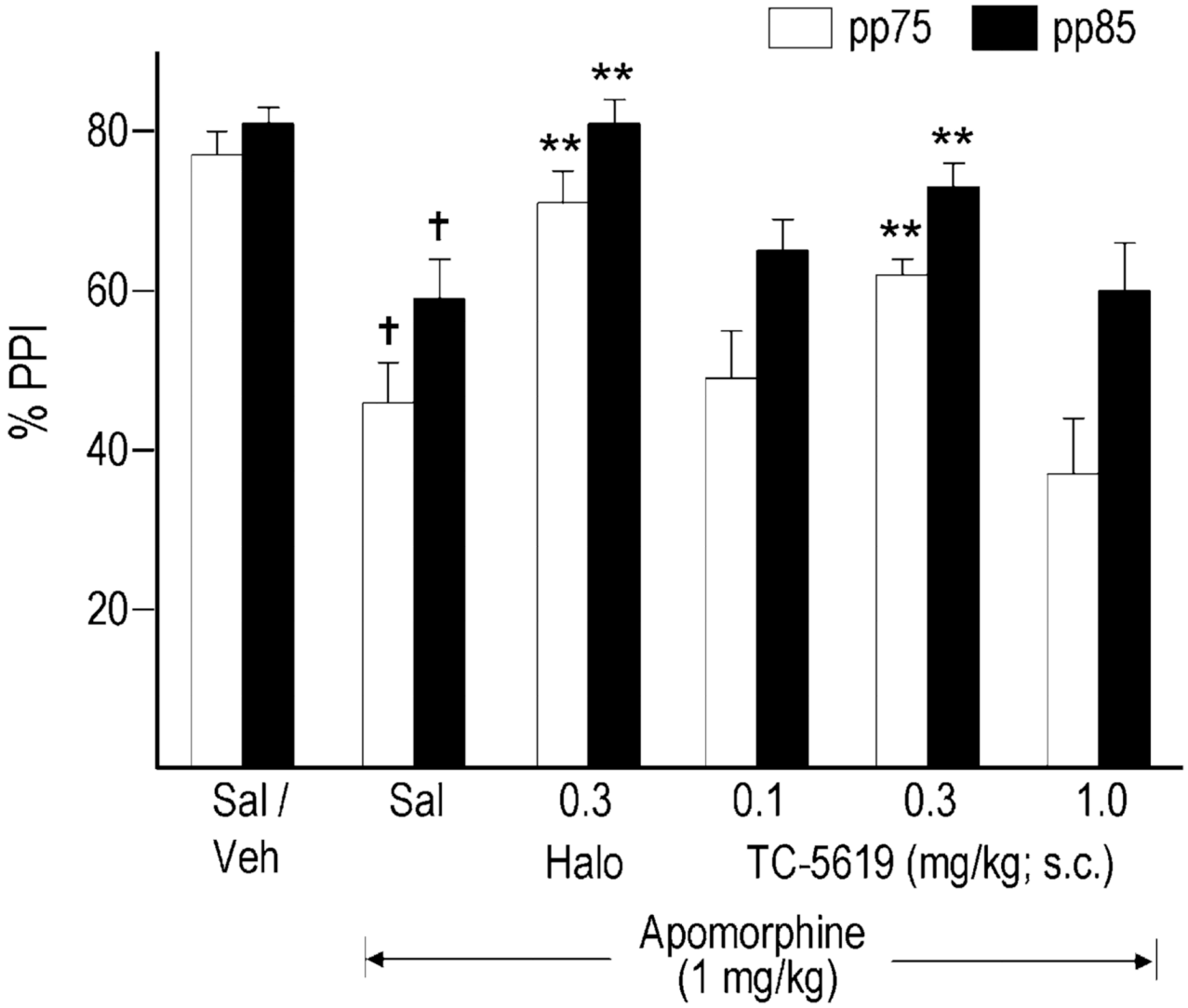

Fig. 6.

Effects of TC-5619 on apomorphine-induced impairment of pre-pulse inhibition in rats. To examine pre-pulse (PP) inhibition in rats, the PP trials involved either a pre-pulse of 75 dB (=10 dB over background) or 85 dB (=20 dB over background) of 20 ms duration with onset 100 ms prior to a 120-dB pulse of 40 ms duration. The average inter-trial interval was set to 40 s with a range of 20–60 s, and the inter-trial interval length was randomized. The startle response was measured for 100 ms from the onset of the 120-dB pulse presentation. The magnitude of the "flinch" of a startled rat was measured. The level of PPI was calculated as a percentage score for each acoustic pre-pulse trial type as follows: %PPI = 100 − {[(startle response for pre-pulse + pulse)/(startle response for pulse-alone)] × 100}. Data are expressed as Mean ± SEM. $^{\dagger}p < 0.001$ vs. saline/vehicle; $^{**}p < 0.001$ vs. saline plus apomorphine.

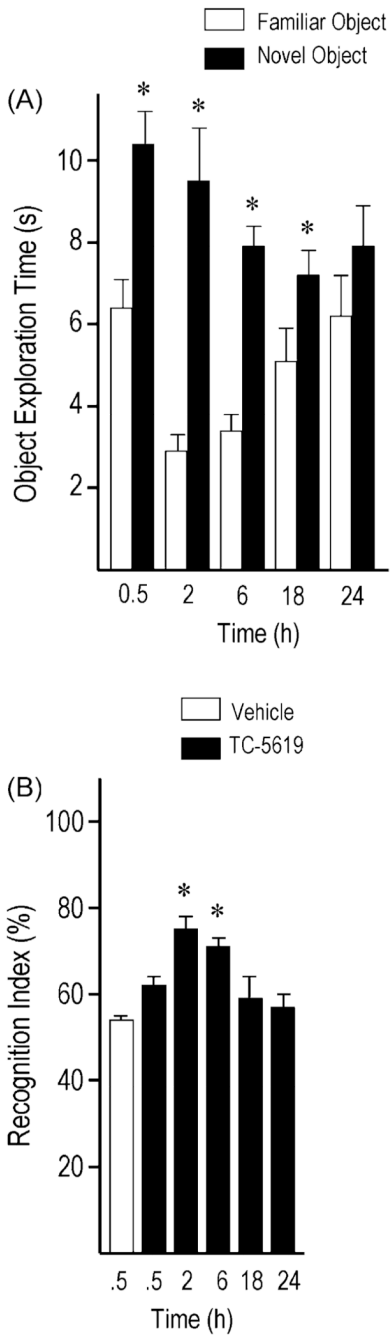

Fig. 7.

Effects of TC-5619 on cognition in a novel object recognition paradigm in rats. (Panel A) TC-5619 was administered p.o. (0.3 mg/kg) and the exploration times were determined at 0.5, 2, 6, 18 and 24 h post-administration as described in Section 2. Data are expressed as Mean ± SEM. $^{*}p < 0.05$ vs. vehicle controls. (Panel B) A % recognition index was calculated (%RI = [(time investigating novel object)/(total time investigating both novel + familiar objects)] ×100) at various times following administration of TC-5619 (0.3 mg/kg p.o.). Results represent the recognition index (RI) as a function of time following administration of TC-5619 and are expressed as Mean ± SEM. $^{*}p < 0.05$ vs. vehicle controls.

Table 1

TC-5619 binding and function parameters at nicotinic receptor subtypes.

NNR subtype/parameter	Source	Parameter	Parameter value (Mean ± SE)
Alpha7 binding	Rat hippocampus	Ki (nM)	1.00 ± 0.50
	Human HEK alpha7/RIC3 cells		1.00 ± 0.04
Alpha7 function (voltage clamp)	*Xenopus laevis* oocytes	EC50 (nM)	33 ± 10
		E_{max} (%ACh)	100 ± 7
α4β2 binding	Rat cortex	Ki (nM)	2100 ± 400
	Human SH-EP1 cells		2800 ± 1300
Muscle function (Rb^+ flux)	Human TE-671 cells	% nicotine @ 10 μM	5 ± 2
		% nicotine @ 100 μM	12 ± 7
Ganglion function (Rb^+ flux)	Rat PC-12 Shooter cells	% nicotine @ 10 μM	11 ± 6
		% nicotine @ 100 μM	20 ± 8
	Human SH-SY5Y cells	% nicotine @ 10 μM	6 ± 2
		% nicotine @ 100 μM	11 ± 1