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Title Page:

The novel α7 nicotinic acetylcholine receptor agonist N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide (ABBF) improves working and recognition memory in rodents

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Running Title Page:

Running Title: ABBF improves working and recognition memory

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List of nonstandard abbreviations:

ABBF: N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide, ANOVA: analysis of variance, HEPES: N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid), LSD: Fisher's Least Significant Difference, MLA: methyllycaconitine, nAChR: nicotinic acetylcholine receptor,

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Abstract:

The relative contribution of α4β2, α7 and other nicotinic acetylcholine receptor subtypes (nAChRs) to the memory enhancing versus the addictive effects of nicotine is the subject of ongoing debate. In the present study we characterized the pharmacological and behavioral properties of the α7 nAChR agonist N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide (ABBF). ABBF bound to α7 nAChR in rat brain membranes (K_i = 62 nM) and to recombinant human 5-HT_3 receptors (K_i = 60 nM). ABBF was a potent agonist at the recombinant rat and human α7 nAChR expressed in *Xenopus* oocytes, but did not show agonist activity at other nAChR subtypes. ABBF acted as an antagonist of the 5-HT_3 receptor and α3β4, α4β2 and muscle nAChRs (at higher concentrations). ABBF improved social recognition memory in rats (0.3–1 mg/kg, p.o.). This improvement was blocked by intracerebroventricular administration of the α7 nAChR antagonist methyllycaconitine (10 µg), indicating that it is mediated by α7 nAChR agonism. In addition, ABBF improved working memory of aged rats in a water maze repeated acquisition paradigm (1 mg/kg, p.o.) and object recognition memory in mice (0.3-1 mg/kg, p.o.). Rats trained to discriminate nicotine (0.4 mg/kg, s.c.) from vehicle did not generalize to ABBF (0.3-30 mg/kg, p.o.) suggesting that the nicotine cue is not mediated by the α7 nAChR and that selective α7 nAChR agonists may not share the abuse liability of nicotine. Our results support the hypothesis that α7 nAChR agonists may provide a novel therapeutic strategy for the treatment of cognitive deficits with low abuse potential.

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Introduction

Nicotine enhances cognitive functions, such as attention, learning, consolidation and retention, in both animals and humans, through activation of brain nicotinic acetylcholine receptors (nAChRs) (Levin et al., 1999; Levin et al., 2006). These ligand-gated ion channels are homopentamers formed by 5 identical subunits (α7 nAChR) or heteropentamers comprised of multiple α and β subunits. Various isoforms of these subunits have been identified (α2-α10; β2-β4; for a review, see Paterson and Nordberg, 2000; Gotti et al., 2006). The most common nAChRs found in the brain are the α7 subtype with a low affinity for nicotine and the α4β2 subtype with a high affinity for nicotine. Evidence from neuroanatomical, electrophysiological, and behavioral studies support a role for both of these receptor subtypes in processes of learning and memory.

Studies using [^{125}I]α-bungarotoxin and [^{3}H]cytisine to label α7 and α4β2 nAChRs, respectively, have identified high densities of these receptors in the hippocampus, a brain area which plays an important role in learning and memory (Paterson and Nordberg, 2000). The α4β2 nAChR agonist A-85380, the α7 nAChR agonist AR-R 17779, and the α7 nAChR agonist (and weak α4β2 antagonist) DMXB (GTS-21) modulated the induction of hippocampal long-term potentiation (LTP) in rats, indicating a role in neuronal plasticity (Gordon et al., 1998; Chen et al., 2000; Fujii et al., 2000; Hunter et al., 1994). Co-application of the α7 nAChR antagonist methyllycaconitine (MLA) inhibited the effects of AR-R 17779, whereas MLA alone had no effects on LTP (Chen et al., 2000).

Both α7 and α4β2 nAChR agonists have been shown to improve performance in learning and memory tasks (Levin et al., 2006). GTS-21 improved performance in long-delay trials of a delayed-matching-to-sample test in monkeys (Briggs et al., 1997), in the Lashley III maze and one-way active avoidance, and increased general learning and reference memory in the 17-arm radial maze in rats (Arendash et al., 1995). In clinical trials with healthy volunteers GTS-21 improved attention, working and episodic memory (Kitagawa et al., 2003). GTS-21 is a weak partial agonist of human α7 nAChRs and inhibits α4β2 nAChRs and 5-HT$_3$ receptors (Briggs et al., 1997; Kem et al., 2004). The more selective α7 nAChR agonist, AR-R 17779, improved long-term win-shift acquisition in the eight-arm radial maze (Levin et al., 1999). Infusion of MLA into the hippocampus impaired working memory in a similar radial arm maze task (Felix and Levin,

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1997). However, AR-R 17779 failed to improve performance in a 5-choice serial reaction time task (Grottick and Higgins, 2000). Recently, we described improvements in social recognition memory after treatment with AR-R 17779 (van Kampen et al., 2004). Together, these results indicate a role for α7 nAChRs in learning and memory, rather than in attention processes. The observation that the ß2-subunit is critical for the addictive effects of nicotine (Picciotto et al., 1998), and that α7 nAChRs are apparently not involved in the rewarding effects of nicotine (Grottick et al., 2000; Brioni et al., 1996), suggests that selective α7 nAChR agonists may have no, or only low, abuse potential. This property would add to the value of selective α7 nAChR agonists for the treatment of memory disorders.

We have examined the effects of the novel α7 nAChR agonist N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide (ABBF; Figure 1A) on the performance of normal adult rats in a social memory task and on object recognition in mice. In addition, we examined the effects of treatment with ABBF on the performance of aged (33 – 34 months old) rats in a working memory-specific version of the Morris water escape task. Previous studies have suggested that the discriminative stimulus effect of nicotine is closely related to its positive reinforcing stimulus effect and that a failure to generalize to the nicotine cue could be indicative for a lack of nicotine-like abuse potential (for discussion, see Merlo Pich et al., 1999; Stolerman et al., 1999). Therefore, we tested whether ABBF would generalize to the nicotine cue in rats.

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Methods

Materials

Ethanol absolute, 99.8%, was obtained from Riedel-de Haen (Seelze, Germany). Solutol^{TM} HS 15 (12-hydroxystearic-acid ethoxylate) was obtained from BASF (Ludwigshafen, Germany). Tylose^{TM} MH300P (methylhydroxyethyl-cellulose) was obtained from Hoechst AG, (Frankfurt, Germany). Methyllycaconitine (MLA) was obtained from Research Biochemicals International/Sigma-Aldrich (Deisenhofen, Germany). Acetylcholine and (-)-nicotine hydrogen tartrate were purchased from Sigma (St. Louis, MO, USA). N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamid (ABBF, Figure 1A) was synthesized by the Medical Chemistry Department of Bayer HealthCare (Wuppertal, Germany).

Membrane preparation and binding assays

Rats were decapitated, and the brains were rapidly removed and placed in ice cold homogenisation buffer [10 % w/v (0.32 M) sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride, 0.01 % (w/v) NaN_3, pH 7.4, 4°C]. Brains were homogenised at 600 rpm in a Potter glass teflon homogeniser. The resulting suspension was centrifuged (1000 x g, 4°C, 10 min) and the supernatant collected. The pellet was resuspended (20 % w/v) and the suspension was recentrifuged (1000 x g, 4°C, 10 min). Both supernatants were combined and centrifuged (15000 x g, 4°C, 30 min). The resulting pellet (P2 fraction) was resuspended in binding buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, pH 7.4) and centrifuged (15000 x g, 4°C, 30 min). The resuspension and centrifugation were repeated once.

The final pellet was resuspended in binding buffer and incubated in a final volume of 250 µl (0.2 mg membrane protein / assay) in the presence of 2 nM [^{3}H]methyllycaconitine, 0.1 % (w/v) bovine serum albumin and different concentrations of the test substance for 2.5 h (21°C). Nonspecific binding was determined in the presence of 10 µM MLA (Methyllycaconitine).

The incubation was terminated by the addition of 4 ml PBS (20 mM Na_2HPO_4, 5 mM KH_2PO_4, 150 mM NaCl, pH 7.4, 4°C) and rapid filtration using a Brandel cell harvester and type A/E glass fibre filters (Gelman Sciences), pretreated for 3 h with 0.3 % (v/v) polyethyleneimine. Filters were

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washed twice with 4 ml PBS (4°C) and bound [^{3}H]methyllycaconitine was determined by scintillation counting. All tests were performed in triplicate.

For 5-HT_3 receptor binding assays, membranes from HEK293 cells expressing human recombinant 5-HT_3 receptor (RB-HS3, Receptor Biology, Inc., MD, USA) were used. Membranes were diluted according to manufacturer's instructions in incubation buffer (50 mM Tris-Base, pH 7.4, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 % ascorbic acid, 10 µM Pargyline) and incubated in a volume of 200 µl (membrane protein concentration: 3 µg/assay) for 60 min (21°C) in the presence of 0.5 nM of the selective 5-HT_3 receptor radioligand [^{3}H]GR65630 (NEN Du Pont) and different concentrations of test compound. Nonspecific binding was determined in the presence of 100 µM 5-HT. The incubation was terminated by filtration through type A/E glass fibre filters (Gelman Sciences) or GF/B filters (Whatman), that were pretreated for at least 1 h with 0.3 % (v/v) polyethyleneimine. Filters were washed three times with 3 ml buffer (50 mM Tris-HCl 7,4; 4°C) and bound radioactivity was determined by scintillation counting. All tests were performed in triplicate.

The IC_{50}-values were determined from plots of binding activity versus log compound concentration using a sigmoidal curve fit (Prism Software Version 2.0, GraphPad Software Inc., San Diego, CA, USA). The dissociation constant K_i of test compounds was determined from their IC_{50}-values, the dissociation constant K_D and the concentration L of [^{3}H]methyllycaconitine or [^{3}H]GR65630 as appropriate, using the equation $K_i = IC_{50} / (1+L/K_D)$.

Electrophysiological assays

Preparation of *Xenopus* oocytes, injection of receptor cDNA and electrophysiological measurements of receptor activity were performed as described (Methfessel et al., 1986; Schnizler et al. 2003). Pieces of ovary were excised from anaesthesized adult female *Xenopus* laevis. The tissue was treated with collagenase (Sigma, Deisenhofen, Germany, 2 mg/ml) to release the oocytes from the follicle. Intact stage V oocytes were selected manually and placed into individual wells of 96-well plates (Greiner, Frickenhausen, Germany) filled with modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 5 mM TRIS/HCl, pH 7.4 with 50 µg/ml Gentamicin). Approximately 30 nl of cDNA solution,

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containing expression plasmids with inserts coding for the target receptors, were injected into the germinal vesicle of each oocyte using an automated system. Injected oocytes were incubated at 19°C for 3-8 days in modified Barth's solution before the measurements.

For electrophysiological recording, oocytes were impaled with two glass microelectrodes filled with pipette solution (1.5 M K-acetate and 0.1 M KCl). Voltage clamp was performed with a standard voltage clamp amplifier (Gene-Clamp 500 amplifier, Axon Instruments). For automated recording, the position of the cells and the recording headstage, the amplifier, the solution exchange, and the data acquisition were under full computer control using a software package developed by D. Bertrand (Geneva) as described by Schnizler et al. (2003). All the recordings reported here were obtained at a membrane potential of -80 mV. Oocytes were superfused with Normal Frog Ringer Solution (NFR:115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2). Solutions of test compounds in NFR were superfused through the recording chamber for 20 seconds while the voltage-clamp current was recorded. The wash-out time between applications of test solutions was 5 min.

Each measurement of a test solution was preceded by the application of a standard concentration of acetylcholine (for nAChRs) or serotonin (for 5-HT_3 receptors). Standard concentrations producing approximately 10 – 20% of the maximum response for each receptor were used as internal controls for differences in expression levels between different oocytes and changes of the response during an experiment. Current amplitudes evoked by the test solutions were normalized to that of the preceding acetylcholine- or serotonin application. The standard concentration of acetylcholine was different for each receptor subtype, depending on the subtype's sensitivity to acetylcholine. For rat and human α7 nAChRs, 50 µM acetylcholine were used as reference standard, the concentrations for rat α3β4, α4β2 and muscle nAChRs were 3.2 µM, 0.32 µM and 0.1 µM acetylcholine, respectively, and 0.5 µM serotonin was used as reference standard for the human 5-HT_{3A} receptor.

As initial assay for agonistic activity, 100 µM ABBF was applied to an oocyte expressing a high level of the corresponding receptor subtype. If an agonistic effect was noted, a full concentration-response curve was obtained. To test for antagonistic effects and potentiator activity of ABBF, various concentrations of the compound were applied together with the standard concentration of acetylcholine or 5-HT, and the resulting inward current was compared to the current level elicited

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by the standard concentration of agonist applied alone. EC_{50} values were calculated using GraphPad Prism.

Functional assays on muscarinic acetylcholine receptors (mAChRs):

Recombinant CHO cells stably expressing the human muscarinic acetylcholine receptor subtypes M1-M5 were used as cell based functional *in vitro* test. These cells stably expressed the calcium-sensititve photoprotein aequorin and the Gα16 protein thus allowing the coupling of all muscarinic receptors to phospholipase C and luminometric detection of the calcium release induced by agonist stimulation.

Cells were seeded two days before testing. The cell culture media (Dulbecco's modified Eagle's medium, 10% FCS, 2 mM glutamine, 10 mM HEPES) were changed on test day to tyrode solution (in mM: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 20 Glucose, 20 HEPES) containing coelenterazin (50 µM) for 4 h. Full dose response curves of test compounds were pre-pipetted in microtiter plates and transferred by a CyBio-pipetting robot to the cell plates. The light signals were detected immediately by a luminometer (lumibox) and the resulting dose response curves and EC_{50} values were calculated using GraphPad Prism.

Behavioral Experiments:

All animal experiments followed the principles of laboratory animal care and were in accordance with the guidelines given by European regulations and the German government and approved by the local authorities (Regierungspräsidium Düsseldorf, Germany).

Social recognition

Adult (4-5 months) male Wistar rats (HsdCpb:WU) and juveniles (4-5 weeks) were supplied by Harlan-Winkelmann (Borchen, Germany). The animals were housed in groups of three in type IV (adult rats) and type III (juvenile rats) Makrolon^{TM} cages, under a 12-h light-dark schedule (lights on at 7:00 a.m.). Food (ssniff, Soest, Germany) and water were available *ad libitum*, except

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during testing. Ambient room temperature (22°C) and relative humidity (55 ± 5%) were kept constant. The animals were randomly assigned to their respective treatment groups. Animals were adapted to laboratory housing conditions for one week prior to behavioral testing. One habituation session was performed under essentially similar conditions as the test session (see below). The social recognition test was performed as described by van Kampen et al. (2004).

The task consisted of two trials, separated by a 24-h retention interval. Adult animals were individually housed 30 min before testing. An enclosure (63 x 41 x 40 cm; aluminium side walls, Plexiglas front), was put over the cage 4 min prior to testing with the lid of the cage removed. During the first trial (T1), a juvenile was placed into the cage and the social investigation by the adult was measured cumulatively for 2 min by a trained observer. Sniffing and grooming of body parts, anogenital sniffing and close following were scored. ABBF (1 mg/kg, p.o.), dissolved in 10% ethanol / 20% Solutol / 70% distilled water in an application volume of 1 ml/kg was given 30 min, 1.5 h or 4 h before T1 to assess the duration of the effect after a single administration. After a retention interval of 24 h, social investigation time was measured in a second trial (T2) for 2 min, with the same juvenile being placed into the observation cage as in T1. A decreased duration of exploring the juvenile indicates social recognition. The difference was expressed as percentage reduction of the social investigation time during T2 in comparison with T1. This measure was subjected to analysis of variance (ANOVA) with the factor Treatment, supplemented with Fisher's Least Significant Difference (LSD) post-hoc comparisons between treatment groups.

To show that the effect is mediated by stimulation of α7 nAChRs, adult rats were treated with ABBF (1 mg/kg, p.o., 30 min before T1), and injected intracerebroventricularly (i.c.v.),with the α7 nAChR antagonist MLA [10 µg, 4 min before T1]. T2 was measured after a retention interval of 24 h, using the same juvenile as in T1.

A further experiment was carried out to reveal potential non-specific effects of ABBF, which may influence investigation of a conspecific, by placing a different (novel) juvenile into the observation cage at T2. Adult rats were injected p.o. with 0.3 mg/kg ABBF in saline (0.9% NaCl), 30 min before T1. T2 was conducted 24 h later, with either the *same* or a *different* juvenile as interaction partner. Non-specific effects may result in a shorter investigation time of the novel juvenile.

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Compounds with specific effects on cognition are expected to have no effect on the duration of investigating the novel juvenile.

Object recognition task:

Thirty (experiment 1) or sixty (experiment 2) male OF1 [(Ico:OF1(IOPS Caw)] mice, weighing 22 to 26 g, were supplied by IFFA CREDO (l'Arbresle, France). The mice were housed in groups of 10 in standard Makrolon™ type III cages and allowed to adapt to the laboratory for one week. During the course of behavioral testing, they were housed individually in standard Makrolon™ type II cages. The observation arena of the object recognition test consisted of a circular open field, 480 mm in diameter. The wall (height: 400 mm) and the floor consisted of transparent Makrolon™. Three different sets of objects, made of aluminium, were used.

During two consecutive days, the mice were allowed to explore the empty apparatus twice for 5 min each day. The mice were pre-trained in pairs of two trials that were separated by a retention interval of 1 h. During the first trial (T1) the apparatus contained two identical objects. A mouse was taken from its home cage and placed into the apparatus, equidistant from the two objects, facing the wall in front of the experimenter. After T1 the mouse was transferred to its home cage. One hour later, the mouse was again placed into the apparatus for the second trial (T2). Now, the exploration arena contained two different objects, a familiar one from T1 and a novel object. The time spent exploring the two objects during T1 and T2 was recorded.

Exploration was scored whenever the mouse directed its nose to the object at a distance ≤ 20 mm and/or whenever it touched the object with its nose. Sitting on the object was not considered as exploratory behavior. In order to remove olfactory cues the objects were thoroughly cleaned after each trial. All combinations and locations of objects were used in a balanced manner. This reduces the effects of the individual preference of mice for particular locations or particular objects. The object recognition test provides measures for exploration and discrimination, i.e. non-cognitive effects of a drug can be distinguished from effects on memory performance (for details see: Prickaerts et al, 2005). The times spent exploring the familiar and new object during T2 were represented as 'a' and 'b', respectively. The discrimination index d2 was calculated as $d_2 = (b - a) / (a + b)$.

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As soon as the mice had reached a good discrimination performance (i.e. a discrimination index d2 ≥ 0.15; see below), pre-training continued with a retention interval of 24 h. Now, the retention performance stabilized around a d2 of 0 (= no discrimination). Each trial lasted 180 s. In order to habituate the mice to the p.o. administration of test compounds, they routinely received saline (10 ml/kg body weight) after the first trial (T1) of each pair. This phase of pre-training consisted of two trial pairs.

In the first experiment, the 6 mice with the lowest object exploration times were omitted from further testing. The remaining 24 animals were assigned to one of two groups, using a matched random assignment procedure. Matching was based on the inspection times in the last pre-training trial. Drug testing comprised two pairs of trials (T1, T2), separated by a 24-h retention interval. ABBF (1 mg/kg) or vehicle (0.5% tylose) was administered p.o., 30 min before T1, in an application volume of 5 ml/kg. During the first pair of trials, half of the mice were treated with vehicle, the other half received ABBF. During a second pair of trials after a 2 day washout period, the animals treated previously with vehicle received ABBF, whereas the animals previously treated with ABBF received vehicle only. Thus, each animal was treated once with vehicle and with ABBF. Consequently, results are based on 24 observations per treatment condition. Treatment effects were analyzed using a within-subjects (i.e. repeated measures) ANOVA.

In the second experiment, the 12 mice with the lowest object exploration times were omitted from further testing. The remaining 48 animals were assigned to one of four groups (n =12 per group), a vehicle control group (0.5% tylose) or a group treated with either 0.1, 0.3, or 1 mg/kg ABBF in 0.5% tylose, using a matched random assignment procedure. Matching was based on the means of the inspection times during T2 of the trial pairs with a 24-h retention interval. ABBF or vehicle was administered p.o., 30 min before T1. After the first pair of trials, all mice remained undisturbed for 17 days. Then, all mice were tested again in a second pair of trials. Thus, each animal was treated twice with vehicle or ABBF. The measures obtained during the two trial pairs were averaged per animal. These means were evaluated statistically by ANOVA with the factor Treatment (vehicle vs. 0.1, 0.3, or 1 mg/kg ABBF).

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Repeated Acquisition (working memory) performance of aged Fischer 344 X Brown-Norway rats in the water maze

Thirty-two aged male Fischer x Brown Norway (F344/NHsd x BN/RijHsd) F1 hybrid rats (FBNF1) were supplied by Harlan-Sprague Dawley (Indianapolis, Indiana, USA). They were housed in groups of four in standard Makrolon^{TM} type IV cages with sawdust bedding in an air conditioned room (22°C). The lights were on from 6:00 a.m. to 6:00 p.m.. Food (ssniff, Soest, Germany) and water were available *ad libitum*, except during testing. The rats were habituated to the local animal facilities for about 1.5 months before behavioral testing started. The Morris water escape training and the assessment of the effects of ABBF on spatial working memory were performed when the rats were 33 to 34 months old.

The rats were pre-trained in the standard Morris water escape task in five daily sessions, with 4 successive trials per session. For a detailed description of the standard task see van der Staay (2006). The 20 rats that were able to locate the escape platform were selected and allocated by matched random assignment on the escape latencies in the last acquisition session of the standard Morris water escape task to one of three treatment groups for the repeated acquisition task: a vehicle (0.5% tylose) control group (n=6), a group receiving 0.3 mg/kg ABBF (n=7), or a group receiving 1 mg/kg ABBF (n=7). Each day, ABBF was freshly suspended in 0.5% tylose. Vehicle or compound was administered p.o. in an application volume of 2 ml/kg, 30 min before each repeated acquisition session on four successive days. The animals received three pairs of trials in a session. Within a pair of trials, both trials were run in close succession. When a rat had completed a trial pair, it was gently dried with crêpe paper and returned to its home cage. The animals were kept warm under an infrared bulb (Original Hanau Solilux, 150 W) fixed about 60 cm above the floor of the cage. After an interval of approximately 15 min the next pair of trials was given. The start positions were at the virtual borders between quadrants at the rim of the pool. Out of the four alternative start positions, three were randomly selected per animal. For each trial pair within a session, a different start position was used. Within a daily session, the escape platform remained in the same position. Over the series of the four sessions, the platform was moved to a different position in each session.

For each rat, the travelled distance was averaged per session separately for the first and second trials of the pairs [average of first swims: ($trial_{1,1}$ + $trial_{2,1}$+ $trial_{3,1}$) / 3; average of second swims:

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($trial_{1,2}$ + $trial_{2,2}$ + $trial_{3,2}$) / 3; the first subscript represents the number of the trial pair within a session, the second subscript represents the trial within trial pairs]. The acquisition curve of the repeated acquisition task was analyzed further with a two-way ANOVA with the factors Treatment (vehicle vs. 0.3 or 1 mg/kg ABBF), and the repeated measures factors Sessions (sessions 1 to 4), and Trial Pairs (average of the first versus average of the second trials of the three trial pairs within a session).

Drug Discrimination:

Male Wistar rats (HsdCpb:WU) were purchased from Harlan-Winkelmann (Borchen, Germany). Body weight upon arrival at the laboratory was around 220 g, which gradually increased up to about 450 g during the course of the study. Rats were individually housed in Makrolon™ type III cages under a normal 12 h light period (light on at 7:00 a.m.). The animals had restricted access to food (approximately 13 g per day, standard pellets, ssniff, Soest, Germany) and were offered water *ad libitum*. Room temperature was maintained at 20-22 °C. Experiments were performed in sound- and light-attenuated standard operant chambers (Coulborn Instruments, Lehigh Valley, PA, USA). The chambers were equipped with two levers equidistant from a food tray between the levers. Food reinforcement (45 mg precision pellets, Bio-Serv, NJ, USA) was delivered by an automated food dispenser located outside of the chamber. Data collection and experimental contingencies were programmed using OPN software on a PC interfaced with the operant chamber. Ventilation and masking noise were provided by a fan mounted on the wall of the chamber. A white houselight was switched on during the sessions, which were conducted between 9:00 a.m. and 12:00 a.m..

In general, the procedure described by De Vry and Jentzsch (1998) was followed. After initial shaping to lever press for food reinforcement, the rats (*n*=16) were trained to discriminate 0.4 mg/kg nicotine hydrogen tartrate (s.c., *t*-15 min) from vehicle in a standard two-lever, fixed ratio:10 operant procedure. Daily sessions were conducted which were terminated either after the rat hat gained 50 reinforcers or when 10 min had elapsed, whichever event came first. For half of the animals, responses on the left lever were reinforced after nicotine, for the other half responses on this lever were reinforced after vehicle. The rats were injected with drug or vehicle

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according to the following sequence: D-D-V-D-V // V-D-V-V-D // D-V-D-V-V // D-D-V-D-V (D=drug, V=vehicle, //=no sessions during the weekends) with repetition. Discrimination criterion consisted of 10 consecutive sessions in which no more than 9 responses occurred on the non-reinforced lever before the first reinforcer was obtained. After reaching discrimination criterion, generalization tests were interspersed between the training sessions. During test sessions, responding on the selected lever, i.e., the lever on which 10 responses accumulated first, was reinforced for the remainder of the session. Generalization tests were separated by at least 3 training sessions in which vehicle and drug were correctly discriminated, i.e., less than 5 incorrect responses prior to the first reinforcer, and when at least 20 reinforcers were obtained per session. The animals were tested with different doses of nicotine hydrogen tartrate (0, 0.05, 0.1, 0.2, 0.4 and 0.8 mg/kg, s.c., 15 min before test) and ABBF (0, 0.3, 1, 10 and 30 mg/kg, p.o., 30 min before test).

(-)-Nicotine hydrogen tartrate was dissolved in 0.9% NaCl (saline) for s.c. administration. ABBF was suspended in 0.5% tylose and distilled water for p.o. administration. Compounds were administered in an application volume of 2 ml/kg body weight for p.o. and 1 ml/kg for s.c. administration.

Test results were expressed as the percentage of rats that selected the drug lever (% Drug Lever Selections). Generalization was considered to be complete if at least 80% drug lever selections was obtained. In addition, the percentage of animals that selected a lever (either drug or vehicle lever) was determined as an index of behavioral disruption (i.e., % Lever Selections). Least-square linear regression analysis was used to estimate ED_{50} values (and their 95% confidence limits) after log-probit conversion of the data.

Results:

In vitro pharmacology

ABBF had high affinity (K_i = 62 ± 20 nM, mean ± SEM, n = 4; Figure 1B) for α7 nAChR in rat brain membranes labelled with the α7 nAChR radioligand [^{3}H]methyllycaconitine. The compound was

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approximately 50 fold more potent than the natural agonist acetylcholine (K_i = 3 μM) and tenfold more potent than nicotine (K_i = 770 nM) measured using the same conditions.

ABBF inhibited binding of the 5-HT_3 receptor selective radioligand [^{3}H]GR65630 to the human recombinant 5-HT_3 receptor expressed in HEK293 cells with similar affinity (K_i = 60 ± 10 nM, Figure 1F).

The application of ABBF to *Xenopus* oocytes expressing recombinant rat or human α7 nAChRs produced a strong and reversible, rapidly desensitizing inward current, which is typical for a nicotinic agonist. The concentration-response curves for ABBF and acetylcholine (Figure 1C) suggested that ABBF was a full agonist of the rat and the human α7 nAChRs with an EC_{50} value of 3 μM (pEC_{50} ± SEM = 5.47 ± 0.20 and 5.51 ± 0.06, respectively), > 50 fold more potent than the activity of acetylcholine in this assay format (EC_{50} = 170 μM) (data for the human α7 nAChRs are shown in Figure 1C). Co-application of ABBF with a reference concentration of acetylcholine (50 μM) increased the acetylcholine response, shifting the concentration-response curve for ABBF to the left (EC_{50} = 0.5 μM, pEC_{50} ± SEM = 6.29 ± 0.09, Figure 1D). The most sensitive assay for the functional activity of ABBF was a brief (1 min) preincubation with ABBF that desensitizes the receptor and thus reduces subsequent responses to acetylcholine. Using this assay format, ABBF showed an IC_{50} value of 0.1 μM (pIC_{50} ± SEM = 7.01 ± 0.37, Figure 1E).

ABBF (tested at concentrations of up to 100 μM) had no agonist activity at recombinant α4β2, α3β4, and muscle nAChRs or 5-HT_3 receptors. Co-application of different concentrations of ABBF with constant reference concentrations of acetylcholine producing approximately 10 – 20% of the maximum response for each receptor showed that high concentrations of ABBF had an inhibitory effect on α3β4 (IC_{50} = 1.5 μM, pIC_{50} ± SEM = 5.83 ± 0.08, acetylcholine reference concentration = 3.2 μM), α4β2 (IC_{50} = 7.6 μM, pIC_{50} ± SEM = 5.12 ± 0.08, acetylcholine reference concentration = 0.32 μM), and muscle nAChRs (IC_{50} = 6.4 μM, pIC_{50} ± SEM = 5.19 ± 0.42, acetylcholine reference concentration = 0.1 μM).

ABBF was selective versus the muscarinic AChRs (EC_{50} and IC_{50} > 10 μM at M1-M5 mAChR).

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Behavioral Experiments

Social Recognition:

ABBF did not significantly change social investigation time during the first encounter (T1). After a 24h delay, vehicle treated adult rats did no longer show a reduced social investigation time at the second encounter (T2) with the same juvenile rat that they had inspected at T1, indicating that social recognition memory was lost after 24 h. ABBF significantly improved the social recognition performance of adult rats. To assess the duration of the effect, ABBF was administered at different times before T1. Treatment of adult rats with 1 mg/kg ABBF (p.o.) between 30 min and 4 h before T1 improved the recognition performance ($F_{3,28}$ = 4.99, $p < 0.01$). Post hoc LSD comparisons confirmed that at all intervals between drug administration and T1 (0.5 , 1.5 and 4 h), ABBF reduced the percent social investigation time at T2 (Figure 2A).

To exclude possible non-cognitive effects of ABBF on the adult rat that might change its investigation of the familiar juvenile at T2, the social investigation time was measured in parallel groups with a familiar or a novel juvenile. A two-way ANOVA followed by post-hoc LSD comparisons showed that only the group that was treated with 0.3 mg/kg ABBF (p.o.) and was confronted with the familiar juvenile at T2 had a significant reduction of the percent social investigation time at T2 ($p < 0.05$, Figure 2B).

In order to confirm that the improvement of recognition memory by ABBF was due to stimulation of α7 nAChR receptors, the effect of intracerebroventricular (i.c.v.) administration of the α7 nAChR antagonist MLA (10 µg) was tested. A two-way ANOVA revealed a significant effect of ABBF ($F_{1,23}$ = 4.67; $p < 0.05$), MLA ($F_{1,23}$ = 5.93, $p < 0.05$) and an interaction between the treatment with ABBF and MLA ($F_{1,23}$ = 7.92, $p < 0.01$). Post-hoc t-tests confirmed that treatment with 1 mg/kg ABBF improved social recognition performance; this effect was antagonised by i.c.v. administration of 10 µg MLA (Figure 2C).

Object recognition:

In both experiments, mice displayed a similar level of exploration of the objects during T1 after vehicle or drug treatment ($F_{1,46}$ = 0.73, n.s. and $F_{3,44}$ = 1.23, n.s, data not shown). During T2,

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vehicle treated mice spent as much time exploring a novel object as exploring a familiar object that they had been exposed to 24 h previously, resulting in a discrimination index of 0 (= no object recognition memory). Treatment with 1 mg/kg ABBF improved the object discrimination performance of the OF1 mice at T2 (d2: $F_{1,46}$ =15.89, $p < 0.01$; see Figure 3A). The exploration times at the novel object and the familiar one at T2 are depicted in Figure 3B.

In a second experiment, treatment with 0.3 and 1 mg/kg ABBF improved the object discrimination performance of the OF1 mice at T2 (d2: $F_{3,44} = 3.02$, $p < 0.05$; see Figure 3C), but not treatment with 0.1 mg/kg ABBF or vehicle. The exploration times at the novel object and the familiar one at T2 are depicted in Figure 3D.

Repeated Acquisition (working memory) performance of aged Fischer 344 X Brown-Norway rats in the water maze

Swimming speed was not affected in any of the sessions, i.e. the treatment did not affect sensorimotor performance or motivation of the rats. Treatment with ABBF did not affect the distance swum to reach the platform, averaged over the four repeated acquisition sessions (General mean: $F_{2,17} = 0.73$, n.s., see Figure 4). All groups learned to decrease the distance travelled across training sessions (Sessions: $F_{3,51} = 10.65$, $p < 0.01$). This improvement was similar for the three groups of rats (Sessions by Treatment interaction: $F_{6,51} = 0.54$, n.s.).

There was an overall effect of trial pairs, i.e. the average performance in the first trials differed from that in the second trials ($F_{1,17} = 7.30$, $p < 0.05$.). Treatment with ABBF affected the change in performance from the first to the second trials of pairs (Trial Pairs by Treatment interaction: $F_{2,17} = 8.02$, $p < 0.01$). There were no effects of sessions on this effect (Sessions by Trial Pairs interaction: $F_{3,51} = 0.55$, n.s.; Sessions by Trial Pairs by Treatment interaction: $F_{6,51} = 0.56$, n.s.).

The treatment effect on trial pairs was further evaluated by an analysis that compared the difference scores between the mean of all first trials and the mean of all second trials over the four repeated acquisition sessions. The overall difference score between first and second trials was affected by the drug treatment ($F_{2,17} = 8.02$, $p < 0.01$). Post-hoc comparisons confirmed that the group treated with 1 mg/kg ABBF reduced the distance swum to reach the platform during the second trials compared with the first trials. This was not the case for the vehicle-treated control

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group and the group treated with 0.3 mg/kg ABBF. Additional t-tests confirmed that the difference score of the group treated with 1 mg/kg (but not vehicle or 0.3 mg/kg) exceeded zero, i.e. the second trials had shorter escape latencies than the first trials (vehicle control: $t_5 = 1.49$, n.s.; 0.3 mg/kg ABBF: $t_6 = -1.80$, n.s.; 1 mg/kg ABBF: $t_6 = 3.34$, $p < 0.05$).

Drug Discrimination:

Fourteen out of 16 rats learned to discriminate nicotine (0.4 mg/kg, s.c.) from vehicle, the median number of sessions to reach criterion being 41 (range: 26-89 sessions). As assessed at the 15 min injection-test interval used during training, the generalization obtained with nicotine was dose-dependent [ED_{50} value (95% confidence limits): 0.11 (0.06-0.20) mg/kg, s.c.], and complete (≥ 80%) at 0.2, 0.4 and 0.8 mg/kg (Figure 5). Generalization occurred in the absence of behavioral disruption (100% lever selections at each dose tested). ABBF did not cause any behavioral disruption and only reached a maximal level of 33 % drug lever selection, i.e. did not generalize to the nicotine cue in the dose range tested (0.3 – 30 mg/kg, Figure 5).

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Discussion:

The present study characterizes ABBF as a potent α7 nAChR agonist that improves recognition and working memory in rodents and does not generalize to a nicotine cue.

ABBF competed with [^{3}H]methyllycaconitine for binding to α7 nAChRs in rat brain membranes with a K_i of 60 nM, which is approximately 50-fold more potent than the natural agonist acetylcholine (K_i = 3 μM) and tenfold more potent than nicotine (K_i = 770 nM).

The functional studies showed that ABBF is a selective agonist specific for the α7 nAChR subtype relative to the other nAChRs tested ($\alpha3\beta4$, $\alpha4\beta2$ and muscle nAChR). The nAChR subtypes used for the selectivity tests were chosen as representative of the major classes of heteropentameric nicotinic receptors: αβγδ for the muscle nAChR, $\alpha3\beta4$ to represent the ganglionic nAChR family, and $\alpha4\beta2$ as the major variety of nAChR in the CNS. ABBF also showed high affinity for the 5-HT_3 receptor in binding assays and antagonist activity in electrophysiological experiments. 5-HT_3 receptor antagonists are in clinical use as anti-emetics and are generally well-tolerated (Sorbe, 1996). Therefore, 5-HT_3 receptor antagonist activity would not preclude potential clinical application of a combined $\alpha7$ nAChR agonist / 5-HT_3 receptor antagonist such as ABBF. GTS-21, a weak partial $\alpha7$ nAChR agonist that has cognitive enhancing effects in healthy volunteers, is also an antagonist at 5-HT_3 receptor and inhibits $\alpha4\beta2$ nAChRs (Kitagawa et al., 2003; Kem et al., 2004). The observation that several ligand classes show high affinity to both $\alpha7$ nAChRs and 5-HT_3 receptors may be due to the relatively high degree of sequence similarity.

The activity of ABBF at the rat and human $\alpha7$ nAChR is very similar, in contrast to several other ligands that show species differences in their potency and efficacy due to four amino acids changes in the ligand binding site of the rat compared to the human $\alpha7$ nAChR (Stokes et al., 2004). On co-application with acetylcholine, the concentration-response curve of ABBF was shifted to the left, suggesting that low concentrations of ABBF can potentiate the responses to acetylcholine. The most sensitive assay for the functional activity of ABBF was preincubation that desensitized the $\alpha7$ nAChR in the oocyte assay and reduced subsequent responses to acetylcholine. This assay format, which is probably the most relevant for the effects observed after application *in vivo* (where low and constant levels of ABBF are expected to occur at the

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synapses) resulted in an IC_{50} value of 100 nM, in good agreement with the affinity measured in binding assays with the α7 nAChR radioligand [^{3}H]MLA (60 nM).

In order to further elucidate the role of α7 nAChR in learning and memory we examined the effects of ABBF in the social recognition test. This test measures the difference of the investigation time an adult animal displays during the first and the second encounter with a juvenile animal. Rodents have an innate interest in their conspecifics, and their olfactory discrimination capabilities result in recognition of a juvenile they have previously examined. This recognition leads to a shorter investigation time during the second encounter. In a previous study we demonstrated that the selective α7 nAChR agonist, AR-R 17779 improves the performance of rats in the social recognition test (van Kampen et al., 2004). Normal rats do not remember a previously inspected juvenile if the second encounter takes place after 24h – the investigation time is similar in both encounters. However, if rats are treated with the α7 nAChR agonist AR-R 17779 or the acetylcholinesterase inhibitor metrifonate before the first encounter, the social investigation time is significantly reduced during the second encounter.

In the present study we have demonstrated that another α7 nAChR agonist, ABBF, can also improve performance of rats in the social recognition test, thus confirming and extending the initial observations. Improved memory performance was observed after administration of ABBF for up to 4 h prior to T1 suggesting that no rapid desensitization of the effect after agonist application is taking place. In contrast to the in vitro experiments with recombinant receptor, desensitization may occur to a lower extent in vivo or the desensitized receptor conformation may still activate signal transduction pathways. The behavioral changes observed are memory specific, because treatment with ABBF did not reduce the investigation time after 24h, if a novel juvenile was presented instead of the same as in the first inspection period. The α7 nAChR antagonist MLA blocked the improved memory performance induced by ABBF, confirming that it is mediated by stimulation of α7 nAChRs, rather than functional antagonism caused by desensitization of the α7 nAChR. Moreover, it suggests that 5-HT_3 receptors are not involved in the cognitive effect of ABBF.

As a second test to assess the effects of ABBF on learning and memory we used the object recognition task, which was first described for rats (Ennaceur and Delacour, 1988) and

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subsequently adapted to mice (Dodart, Mathis and Ungerer, 1997). It is based on the spontaneous behavior of rodents to explore a novel object more than a familiar one, that they have already explored several min or h before. After a 24h retention interval, the animals will no longer discriminate between a known and an unfamiliar object. Performance in this task is improved by phosphodiesterase (PDE) 5 inhibitors (Prickaerts et al., 2005), PDE2 inhibitors (Boess et al., 2004) and impaired by scopolamine (Dodart, Mathis and Ungerer, 1997). The α7 nAChR agonist ABBF (0.3 and 1 mg/kg, p.o.) significantly increased exploration of the novel object, consistent with an improved memory of the familiar object.

In order to examine the effects of ABBF on working memory in the Morris water maze, we used a modification of the repeated acquisition paradigm described by Whishaw (1985). In previous studies we had found that aged (26-month-old) FBNF1 rats showed profound performance deficits in the Morris water escape task, when compared with adult counterparts, and that the age-related decline was even more profound in rats aged 33-34 months (van der Staay, 2006). In fact, 12 of the 32 aged rats in the present study were unable to acquire the standard task. These rats were excluded from training in the repeated acquisition task. We found that 1 mg/kg ABBF improved working memory in the very old FBNF1 rats, as evidenced by the decrease in distance travelled from the first to the second trials in trial pairs. This test can be considered a working memory variant of the Morris water escape task because within a session, the animal must use the information gathered in the first trial from a particular start position to the submerged platform in order to improve performance in the second. We have previously investigated in detail whether the improvement within a repeated acquisition session is a consequence of an overall improvement from trial to trial and found that there is true within trial-pairs improvement. The latency from the second trial in the pair to the first trial in the *next* pair increased in adult rats, indicating that a new start position represents a new problem (van der Staay and de Jonge, 1993). While α7 nAChR knock-out mice did not show impairments in the basic Morris water maze (Paylor et al., 1998), a deficit was reported in a delayed matching-to-place modification, pointing to a role of α7 nAChRs in working memory, in agreement with our observations (Fernandes et al., 2006).

While we have shown that the α7 nAChR antagonist MLA prevents the effect of ABBF in the social recognition test, we cannot exclude a contribution of 5-HT_3 receptor inhibition to the

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efficacy of ABBF in the other behavioral tests. However, the observations with other α7 nAChR agonists that cause varying degrees of 5-HT_3 receptor inhibition suggest that α7 nAChR activation is also responsible for the improvements in the object recognition and water maze tests. During the preparation of this manuscript, two other groups have reported on the activity of α7 nAChR agonists in models of learning and memory. SSR180711 enhanced performance in the object recognition task and restored MK-801 induced deficits in the water maze (Pichat et al., 2006). PHA-543,613 improved object recognition (Wishka et al., 2006). These studies support our present results and confirm the earlier observations with GTS-21 and AR-R 17779 (Arendash et al., 1995; Briggs et al., 1997; van Kampen et al., 2004).

The finding that ABBF failed to induce a significant level of generalization to the nicotine cue (i.e., maximal level of 33 % generalization) is in accordance with previous findings obtained with another α7 nAChR agonist (AR-R 17779) in a similar drug discrimination assay (Kaiser et al., 1998). While we cannot completely rule out a contribution of the different routes of administration (s.c. for nicotine versus p.o. for ABBF), the fact that ABBF does not generalize to the nicotine cue at doses that are active in the memory tasks supports the suggestion that the discriminative effect of nicotine is not (primarily) mediated by the α7 nAChR. It was previously reported that the nicotine cue is blocked by antagonists of the β2-subunit of the nAChR (Shoaib et al., 2000), but not by selective α7 nAChR antagonists (Brioni et al., 1996). In addition, transgenic mice which lack the β2-subunit of the nAChR fail, or hardly learn to detect the nicotine cue (Shoaib et al., 2002), while α7 nAChR knock-out mice acquire discrimination of nicotine at a rate similar to wild-type mice (Stolerman et al., 2004). It has been argued that the discriminative stimulus effect of nicotine is closely related to its positive reinforcing stimulus effect (for discussion, see Merlo Pich et al., 1999; Stolerman et al., 1999), and that testing the extent of generalization to the nicotine cue offers an opportunity to assess whether such a compound has nicotine-like abuse potential. Interestingly, self-administration studies with nicotine have already indicated that the α7 nAChR most likely does not play a (major) role in the positive reinforcing stimulus of nicotine (Epping-Jordan et al., 1999; Grottick et al., 2000; Merlo-Pich et al., 1999). Therefore, it is suggested that selective α7 nAChR agonists do not share the abuse potential characteristics of nicotine.

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Taken together, the present study showed that the α7 nAChR agonist ABBF can improve performance in several learning and memory tests in both rats and mice without producing nicotine-like discriminative stimulus effects. Therefore α7 nAChR agonists may provide a novel therapeutic strategy for the treatment of cognitive deficits in patients suffering from memory disorders such as Alzheimer's disease and other dementias.

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Legends for Figures

Figure 1: Structure and *in vitro* pharmacological properties of ABBF

A) Structure of N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-7-[2-(methoxy)phenyl]-1-benzofuran-2-carboxamide (ABBF). B) ABBF (K_i = 62 nM) inhibited binding of the radioligand [^{3}H]MLA to α7 nAChR in rat brain membranes with > ten fold higher affinity than nicotine (K_i = 770 nM) and 50 fold higher affinity than acetylcholine (K_i = 3000 nM). C) Concentration response curves of ABBF (EC_{50} = 3 µM) and acetylcholine (EC_{50} = 170 µM) at the human α7 nAChR expressed in *Xenopus* oocytes. D) Potentiation of the response to 50 µM acetylcholine by different concentrations of ABBF (EC_{50} = 0.5 µM) on co-application with acetylcholine. E) Pre-application of ABBF reduced the subsequent response to 50 µM acetylcholine (IC_{50} = 100 nM). F) ABBF inhibited the binding of [^{3}H]GR65630 to the human 5-HT_{3A}R expressed in HEK293 cells. Data shown are means ± standard error of the mean (SEM) from triplicate (B, F) or 3 – 6 separate determinations (C, D, E).

Figure 2: ABBF improves social recognition memory in rats.

A) Vehicle treated adult rats showed no difference in their social investigation behavior during the second encounter (trial 2) with a juvenile rat they had explored 24 h previously (trial 1) indicating that social recognition memory is lost after 24 h. ABBF (1 mg/kg, p.o. administration 30 min or 1.5 h or 4 h before trial 1) reduced the time adult rats spent investigating the juvenile rat in trial 2 (n = 8 per group), indicating an improvement in social recognition memory. *: $p < 0.01$, significantly different from vehicle treated group. B) ABBF (0.3 mg/kg, p.o., 30 min before trial 1) reduced the time adult rats spent investigating the familiar juvenile but not a novel juvenile in trial 2 compared with trial 1 (n = 7-10 per group). *: $p < 0.05$, significantly different from vehicle treated group. C) ABBF (1 mg/kg, p.o., 30 min before T1) reduced the time adult rats spent investigating the juvenile in trial 2 compared with trial 1 (n = 6-7 per group). The reduction was prevented by administration of MLA (10 µg, i.c.v., 4 min before T1). #: $p < 0.05$, significantly different from group treated with 1 mg/kg ABBF. Data shown are means ± SEM.

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Figure 3: ABBF improves retention in the mouse object recognition task.

A) The discrimination index d2 of vehicle treated mice was 0, indicating no difference between the time spent exploring a novel object and a familiar object encountered 24 h previously. Treatment with 1 mg/kg ABBF significantly increased the discrimination index d2 compared to vehicle (0.5 % tylose), *: $p < 0.01$, suggesting an improvement in object recognition memory. B**)** Time spent exploring the familiar and novel object (same experiment as A). ABBF increases exploration of the novel object. C) Treatment with 0.3 or 1 mg/kg ABBF (but not 0.1 mg/kg) significantly increased the discrimination index d2 compared to vehicle (0.5 % tylose), *: $p < 0.05$. D**)** Time spent exploring the familiar and novel object (same experiment as C). Results are shown as means ± SEM.

Figure 4: ABBF improves performance of aged rats in a repeated acquisition task

Mean distance (cm) ± SEM travelled by 33 - 34 months old FBNF1 rats in the water maze before reaching the platform after treatment with A) vehicle (0.5% tylose), B) ABBF (0.3 mg/kg), C) ABBF (1 mg/kg). A-C: Data are shown as mean distance in first trials (filled circles) or second trials (open squares) of three trial pairs each in four different sessions. D) Mean distance travelled in all first trials combined and mean distance travelled in all second trials combined over the four repeated acquisition sessions of the three groups of rats are shown.**: $p < 0.05$ that difference score (mean of first versus mean of second trials) > 0

Figure 5: Drug discrimination: ABBF does not generalize to the nicotine cue

Generalization test results obtained with nicotine (s.c., *t*-15 min, *n* = 6-8 per dose) and ABBF (p.o., *t*-30 min, *n*=5 per dose) in rats trained to discriminate nicotine (0.4 mg/kg, s.c., *t*-15 min) from vehicle. Vehicle induced 0% generalization after s.c. or p.o. administration. Data points with a generalization level exceeding 80% were considered to reflect complete generalization. All rats selected a lever at each dose tested.

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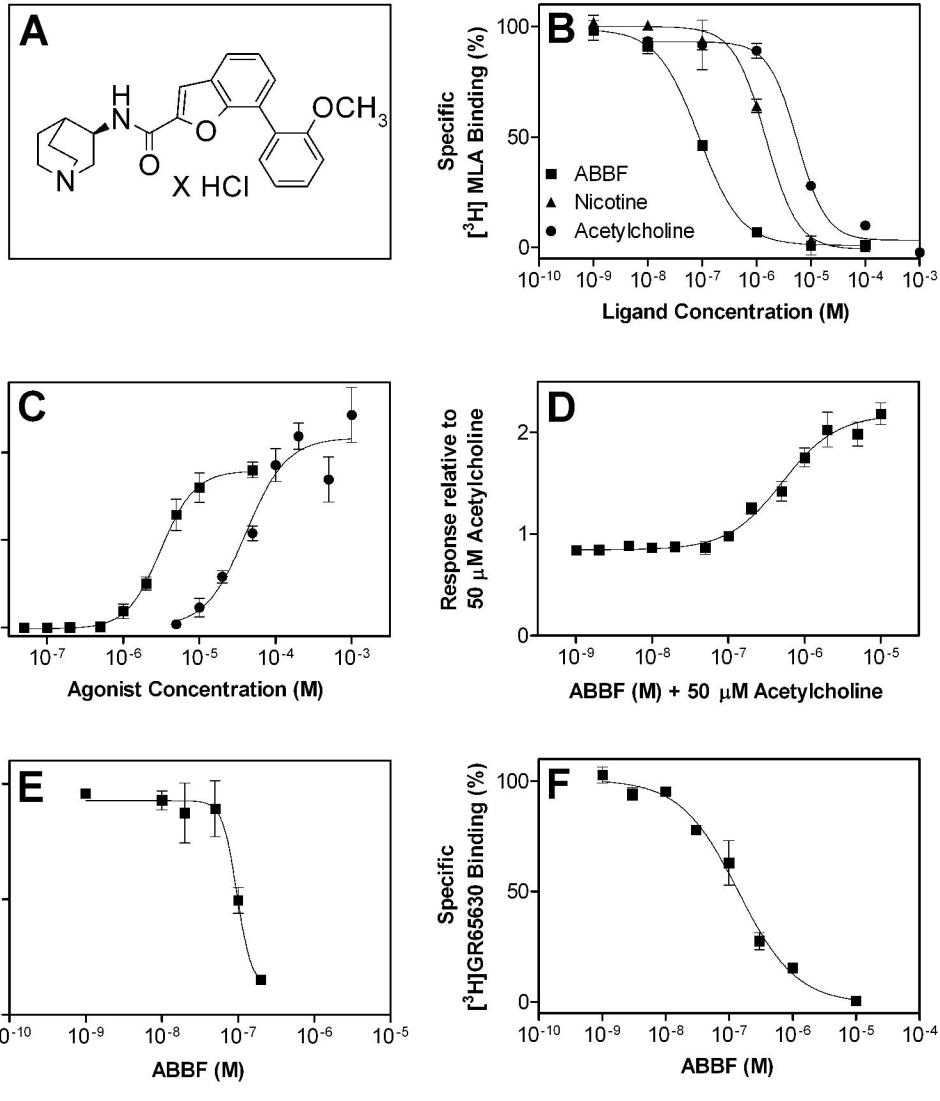
Figure 1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Ligand Concentration (M)
C
Response relative to
50 μM Acetylcholine
2
1
0
10-7 10-6 10-5 10-4 10-3
Agonist Concentration (M)
D
Response relative to
50 μM Acetylcholine
2
1
0
10-9 10-8 10-7 10-6 10-5
ABBF (M) + 50 μM Acetylcholine
E
Response relative to
50 μM Acetylcholine
1.0
0.5
0.0
10-10 10-9 10-8 10-7 10-6 10-5
ABBF (M)
F
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ABBF (M)

Figure 2

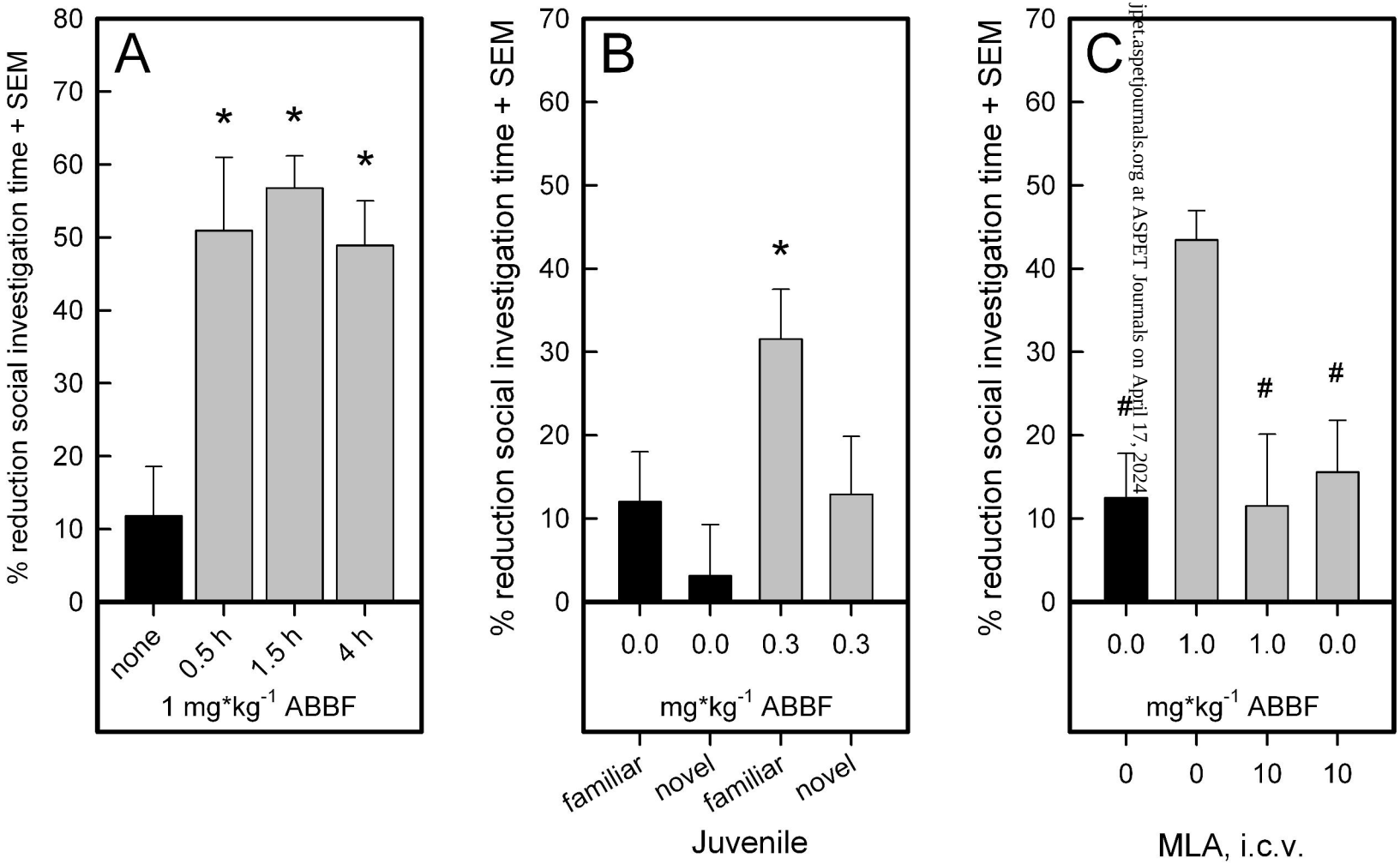

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Figure 3

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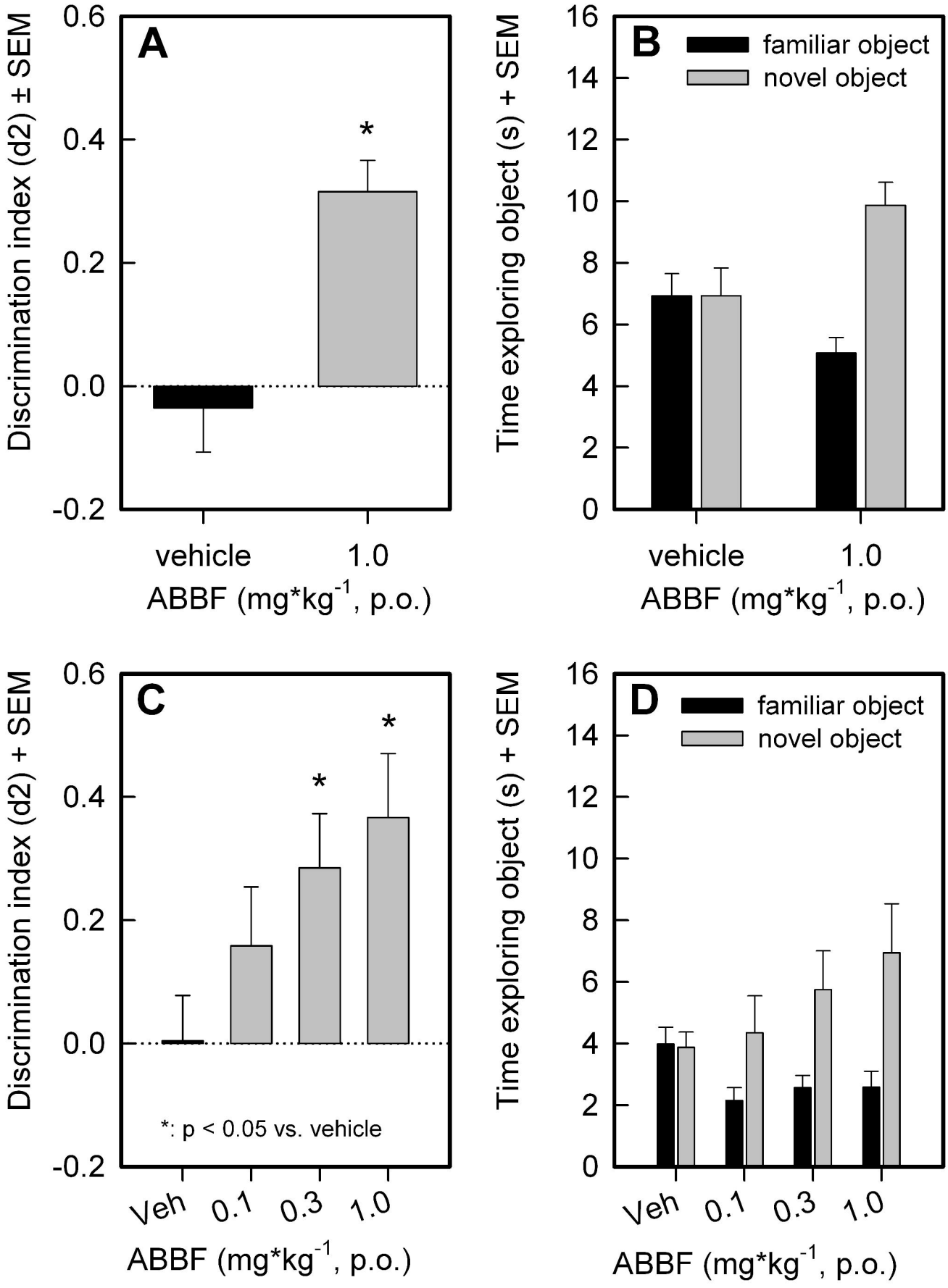

Figure 4

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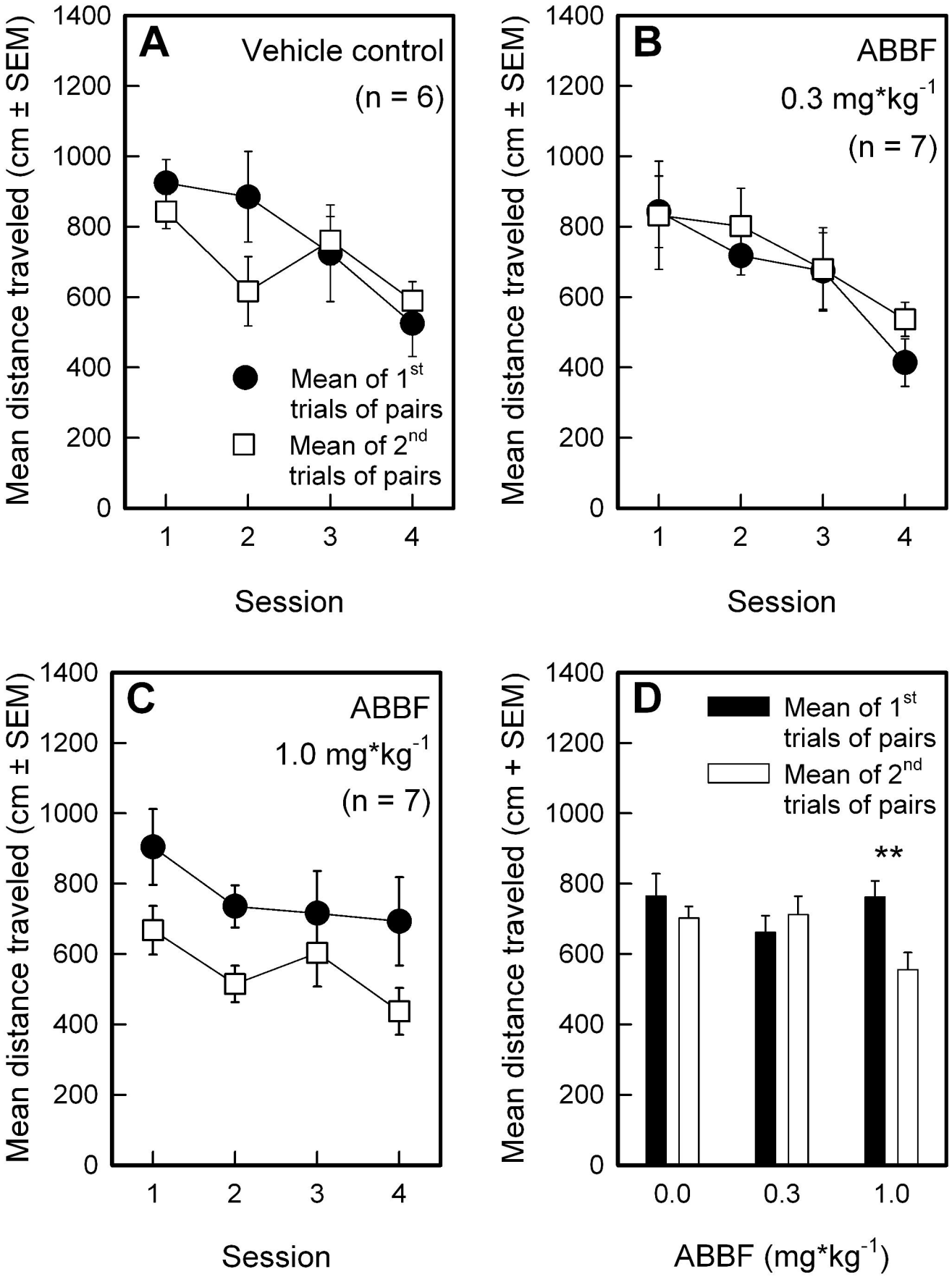

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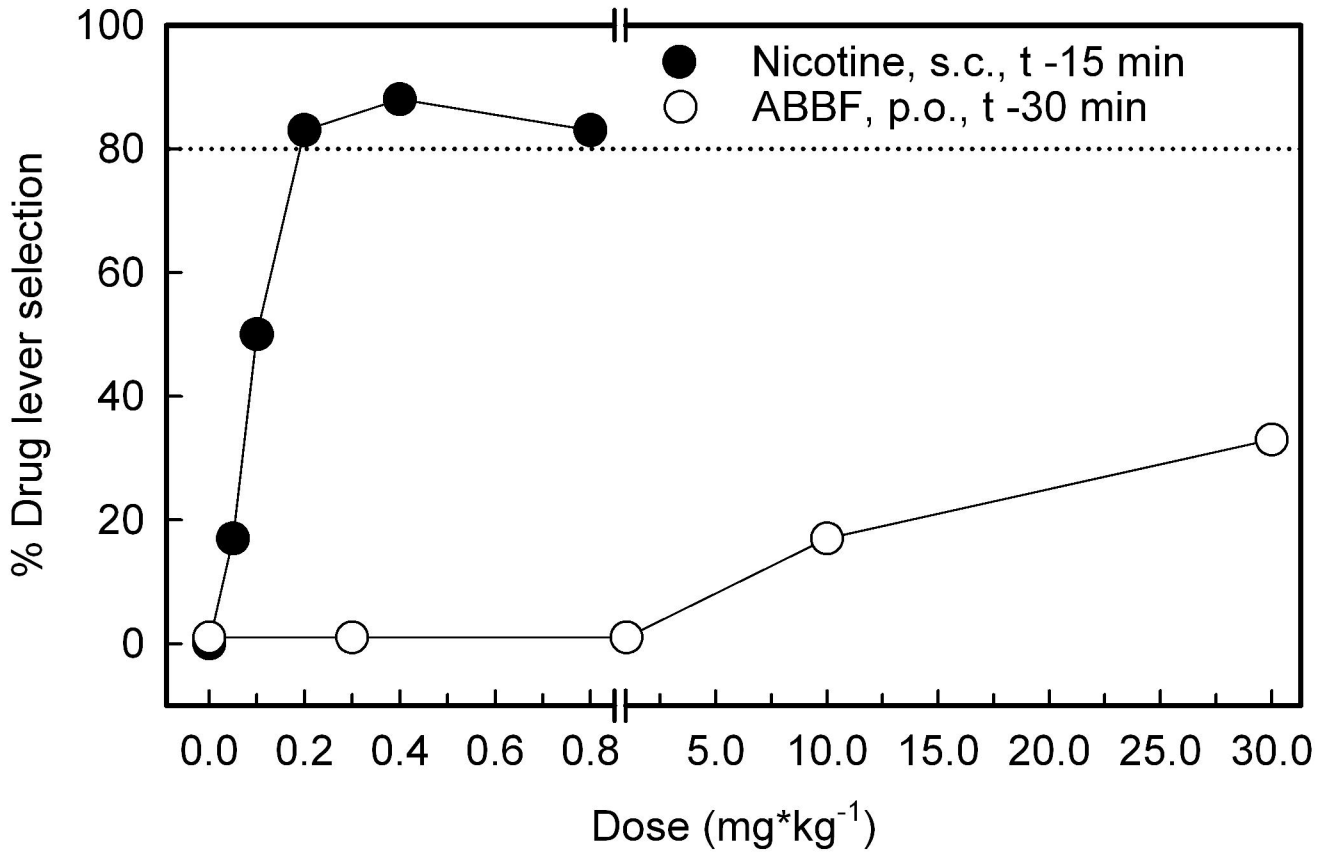
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