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THE COUMARIN SCOPOLETIN POTENTIATES ACETYLCHOLINE RELEASE FROM SYNAPTOSOMES, AMPLIFIES HIPPOCAMPAL LONG-TERM POTENTIATION AND AMELIORATES ANTICHOLINERGIC- AND AGE-IMPAIRED MEMORY

A. HORNICK,[a1] A. LIEB,[a1] N. P. VO,[b] J. M. ROLLINGER,[c] H. STUPPNER[c] AND H. PRAST[a]*

[a]*Institute of Pharmacy/Pharmacology and Toxicology, University of Innsbruck, Peter-Mayr-Str.1, A-6020 Innsbruck, Austria*

[b]*Pharmacology and Clinical Pharmacy Department, School of Pharmacy, Ho Chi Minh City University of Medicine and Pharmacy, 41 Dinh Tien Hoang Street, District 1, Ho Chi Minh City, Vietnam*

[c]*Institute of Pharmacy/Pharmacognosy and Center for Molecular Biosciences Innsbruck, University of Innsbruck, Innrain 52c, A-6020 Innsbruck, Austria*

Abstract—In a previous study the simple, naturally derived coumarin scopoletin (SCT) was identified as an inhibitor of acetylcholinesterase (AChE), using a pharmacophore-based virtual screening approach. In this study the potential of SCT as procholinergic and cognition-enhancing therapeutic was investigated in a more detailed way, using different experimental approaches like measuring newly synthesized acetylcholine (ACh) in synaptosomes, long-term potentiation (LTP) experiments in hippocampal slices, and behavior studies. SCT enhanced the K^+-stimulated release of ACh from rat frontal cortex synaptosomes, showing a bell-shaped dose effect curve (E_{max}: 4 μM). This effect was blocked by the nicotinic ACh receptor (nAChR) antagonists mecamylamine (MEC) and dihydro-β-erythroidine (DHE). The nAChR agonist (and AChE inhibitor) galantamine induced a similar increase in ACh release (E_{max}: 1 μM). SCT potentiated LTP in hippocampal slices of rat brain. The high-frequency stimulation (HFS)-induced, N-methyl-D-aspartate (NMDA) receptor dependent LTP of field excitatory postsynaptic potentials at CA3-CA1 synapses was greatly enhanced by pre-HFS application of SCT (4 μM for 4 min). This effect was mimicked by nicotine (2 μM) and abolished by MEC, suggesting an effect on nAChRs. SCT did not restore the total inhibition of LTP by NMDA receptor antagonist D, L-2-amino-5-phosphonopentanoic acid (AP-5). SCT (2 μg, i.c.v.) increased T-maze alternation and ameliorated novel object recognition of mice with scopolamine-induced cholinergic deficit. It also reduced age-associated deficits in object memory of 15–18-month-old mice (2 mg/kg sc). Our findings suggest that SCT possesses memory-improving properties, which are based on its direct nAChR agonistic activity. Therefore, SCT might be able to rescue impaired cholinergic functions by enhancing nAChR-mediated release of neurotransmitters and promoting neural plasticity in hippocampus. © 2011 IBRO. Published by Elsevier Ltd. Open access under CC BY-NC-ND license.

Key words: ACh release, long-term potentiation, hippocampus slice, nicotinic acetylcholine receptor, T-maze, object recognition.

In a previous study scopoletin (SCT) was identified as a putative inhibitor of acetylcholinesterase (AChE) based on a computer-aided screening approach. The coumarin was found to fit to a structure-based pharmacophore model constructed from interaction of galantamine to the enzyme (Rollinger et al., 2004; Stuppner et al., 2005). However, *in vitro* tests showed a rather high IC_{50} for AChE of 135 μM. Nevertheless SCT at low concentrations (2 μM) was able to enhance brain ACh in the rat brain (Rollinger et al., 2004) suggesting that the compound might possess additional properties. Among substances with coumarin scaffolds, several compounds can be found exerting memory-ameliorating properties. For example, nodakenin reduced the memory deficit induced by the anticholinergic scopolamine (SCOP) in mice (Kim et al., 2007). Ensaculin, a synthetically modified natural coumarin, also showed anti-dementia activity (Hoerr and Noeldner, 2002). Several coumarin derivatives were reported to have inhibitory activity at AChE, monoamino oxidase (MAO), or both (Shen et al., 2005; Chimenti et al., 2004; Brühlmann et al., 2001). Additionally, antioxidative properties were found for some compounds of this chemical class (Lin et al., 2008; Tiyagi et al., 2005).

SCT has previously been described as an anti-inflammatory (Muschietti et al., 2001; Calixto et al., 2003) and antiproliferative agent (Fujioka et al., 1999) that exhibits activities like the inhibition of inducible nitric oxide synthase (Kim et al., 1999; Kang et al., 1999) and prostaglandin synthase (Farah and Samuelsson, 1992). It also inhibits the MAO at moderate concentrations (Yun et al., 2001) and may act as an antioxidant (Shaw et al., 2003) and a radical scavenger (Toda, 2002). Several drugs that bind to AChE were shown to also interact with an allosteric binding site of nicotinic ACh receptors (nAChRs). This behavior has also been reported for galantamine, which was used as ligand template for the identification of SCT (Rollinger et al., 2004). Therefore, it can be speculated that SCT might possess an affinity to nAChRs.

[1] These authors contributed equally to this work.

*Corresponding author. Tel: +43-512-507-5618; fax: +43-512-507-2931.
E-mail address: helmut.prast@uibk.ac.at (H. Prast).

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer's dementia; AP-5, D,L-2-amino-5-phosphonopentanoic acid; CSF, cerebrospinal fluid; DHE, dihydro-β-erythroidine; DMSO, dimethyl sulfoxide; fEPSPs, field excitatory postsynaptic potentials; HFS, high-frequency stimulation; LTP, long-term potentiation; MAO, monoamino oxidase; MEC, mecamylamine; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; SCOP, scopolamine; SCT, scopoletin.

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A drug that enhances cholinergic neurotransmission and also exerts antioxidative and anti-inflammatory actions would be highly desirable as a therapeutic for neurodegenerative diseases such as Alzheimer's dementia (AD). Additionally, nAChR agonists are of high interest because they may also decrease the in excess synthesis and deposition of beta-amyloid peptide (Buckingham et al., 2009).

The aim of our study was to investigate whether SCT possesses cholinergic transmission-enhancing, synaptic plasticity-increasing, and cognition and memory-improving properties. For this purpose, we studied the effects of SCT on ACh release from brain cortex synaptosomes, on hippocampal long-term potentiation (LTP) and in behavioral tasks, which are sensitive to procholinergic drugs.

Cholinergic nerve endings in the frontal brain cortex express nicotinic autoreceptors, which enhance ACh release. Hence, synaptosomes from this brain area are well suited to investigate the mechanism of putative nAChR agonists (Tzavara et al., 2003; Zhang et al., 2002; Duffy et al., 2009; Roman et al., 2004; Raiteri et al., 1974; Marchi et al., 1999). The hippocampus is central to learning and memory, and it is known that nAChRs contribute to these functions (Jones et al., 1999). LTP is a form of synaptic plasticity, which is thought to underlie learning and memory processes (Bliss and Collingridge, 1993; Martin and Morris, 2002). Congruently, hippocampal LTP is impaired in aged animals, in cholinergic deficit, and in animal models of AD (Froc et al., 2003). Numerous agents including cholinergic enhancers and nAChR agonists show procognitive properties *in vivo* and are also effective in promoting neurotransmission and/or LTP in hippocampal slices (Lynch, 2002; Lagostena et al., 2008; Sliwinski et al., 2004). We investigated the effects of SCT in learning and memory tasks in aged mice and in mice rendered amnesic with the anticholinergic scopolamine. This pretreatment has been widely used for the detection of nootropic and procholinerigic activity of agents such as AChE inhibitors and nAChR agonists. The alternation rate in T-maze and the recognition of novel objects vs. familiar ones is sensitive to inhibition and stimulation of cholinergic neurotransmission (Kokkinidis and Anisman, 1976; Delatour and Gisquet-Verrier, 1996). The T-maze task measures spatial working memory function while the object recognition measures a nonspatial memory with the characteristics of episodic memory (Gerlai, 1998; Ennaceur and Delacour, 1988). Both forms of memory are impaired in the course of AD with episodic memory generally being the first and most severely affected one (Desgranges et al., 1996; Gainotti et al., 1998; Petersen et al., 2000; Remy et al., 2005).

EXPERIMENTAL PROCEDURES

Ethical statement

The experimental procedures and number of animals used in this study were approved by an ethical committee at the Federal Ministry of Science and Research of the Republic of Austria and conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The experiments were designed in such a way that the number of animals used and their suffering was minimized.

Drugs and chemicals

[^{3}H]-choline (0.03 μM, 17.5×10^6 Bq/g was purchased from New England Nuclear, Boston, MA, USA). D, L-2-amino-5-phosphonopentanoic acid (AP-5), galantamine hydrobromide, mecamylamine hydrochloride (MEC), dihydro-β-erythroidine (DHE), and scopolamine hydrobromide (SCOP) were from Tocris BioScience, Bristol, UK. Picrotoxin was bought from Research Biochemicals International, Natick MA, USA. Atropine sulfate, acetylcholine hydrochloride (ACh), (±)-nicotine, and SCT were purchased from Sigma, St. Louis, MO, USA.

Synaptosomes

Preparation. Synaptosomes were prepared from the brain frontal cortex of male Sprague–Dawley rats (4 months old, Research Institute for Laboratory Animal Breeding, University of Vienna, Himberg, Austria). The pellet between 1000 and 15000 g of a 0.32 M sucrose homogenate was collected and resuspended in basal medium (mM: NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, NaH_2PO_4 1.0, $NaHCO_3$ 22, and glucose 10), aerated with 95% O_2-5% CO_2 at 37 °C, pH 7.2–7.4 (Raiteri and Raiteri, 2000).

Superfusion protocol. The synaptosomal suspension was incubated with [^{3}H]-choline (0.03 μM, 17.5×10^6 Bq/g) for 15 min at 37 °C and then diluted aliquots were applied on filters in a superfusion system maintained at 37 °C (Raiteri et al., 1974). Basal and stimulation media (basal medium with 15 mM K^+) were supplemented with 1 μM ACh and 0.1 μM atropine. After a pre-superfusion period of 30 min, the synaptosomes showed a constant outflow of newly synthesized [^{3}H]-ACh (Marchi et al., 1983). The K^+-stimulated release of [^{3}H]-ACh was determined in two subsequently collected fractions of 6 min. Fraction 1 (control): 6 min of superfusion with basal medium, fraction 2 (stimulation): 1.5 min of superfusion with stimulation medium followed by 4.5 min basal medium. SCT and galantamine were diluted from stock solutions prepared with a minimum of dimethyl sulfoxide (DMSO). These drugs were present in the superfusion medium concomitantly with 15 mM K^+ (for 1.5 min). Antagonists MEC and DHE were present from 8 min before stimulation throughout the experiment.

Statistical analysis. The amount of [^{3}H]-ACh released in each sample was calculated as fraction of the total synaptosomal tritium at the start of the respective collection period (tritium in the sample/sum of tritium in the respective fractions and in the synaptosomes on the filter). K^+-evoked release was expressed as percentage of basal release rate. Each *n* represents the mean of an experiment with one synaptosomal preparation in at least three superfusion chambers. Statistical significance of group variances was calculated by one-way analysis of variance (ANOVA) followed by Bonferroni's test.

Hippocampal slices

Preparation. Coronal brain slices containing hippocampi (thickness, 400 μm) were prepared from Sprague–Dawley rats (65–104 g body weight). After decapitation the brains were removed and sectioned at 0 °C on a vibroslicer in artificial cerebrospinal fluid (CSF; composition in mM: NaCl 120, KCl 2.5, $MgSO_4$ 2.0, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 2, glucose 10) aerated with 95% O_2-5% CO_2 to a final pH of 7.35. Slices were then placed in a maintenance chamber and slowly warmed up from 16 °C to 30 °C (Capron et al., 2006).

Recording of evoked fEPSPs. After 1.5 to 3 h the slices were transferred to an interface-type chamber (BSC-2, Automate

Scientific) and superfused with 100 μM picrotoxin-containing CSF at 1 ml/min at 30 °C. The Schaffer collateral tract was electrically stimulated with a bipolar electrode. fEPSPs in the CA1 region were evoked by pulses at 30% of maximally effective strength at a frequency of 0.05 Hz. LTP was evoked by high-frequency stimulation (HFS) with one train of pulses at a frequency of 100 Hz for 1 s at 60% of maximal effective strength. fEPSPs were recorded with a glass electrode (1–2 MΩ resistance, filled with CSF), filtered between 2 and 5 kHz, and recorded with pClamp9 (Electrophysiology Data Acquisition and Analysis Software, Axon Instruments).

Drugs. Stock solutions of drugs were prepared as solutions in CSF (nicotine, MEC, AP-5) and DMSO (SCT, galantamine) as required and diluted immediately before use. Control experiments were carried out with solutions containing the same concentrations of DMSO as the respective drug solution.

Statistical analysis. fEPSP amplitude was calculated off-line and normalized to the mean of 10 min prior to HFS. Statistical analysis was carried out by ANOVA followed by Bonferroni post hoc test.

Memory and behavior

Animals. C56BL/6N male, 4–6-month-old and 16–18-month-old mice (Charles River Laboratories, Sulzfeld, Germany), were used in the behavioral studies. The mice were housed in groups of four in cages (40 (L)×25 (W)×15 (H) cm^3) at constant humidity (50–55%) and temperature (22±1 °C) on a 12:12 h light/dark cycle (7:00–19:00 h), with food and water *ad libitum*. Younger mice (4–6 months) were implanted with i.c.v. cannulas for application of SCOP and SCT. The aged mice were injected with SCT by the s.c. route.

Surgery. The 4–6-month-old mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.) and a guide cannula (gauge 25, o.d. 0.52 mm, i.d. 0.26 mm, and 7 mm long) was stereotaxically implanted into the brain area 0.5 mm higher than the lateral cerebral ventricle (−0.1 mm posterior, 0.8 mm lateral to bregma, −2.2 mm ventral to the dura) (Franklin and Paxinos, 1997). Each animal was allowed to recover for 5 days prior to experimentation.

Experimental design and drug treatment. Experiments were conducted between 8:00 and 16:00 h. Mice with i.c.v. cannulas were randomly divided into four experimental groups: vehicle; SCOP 20 μg; SCT 2 μg; and SCOP 20 μg plus SCT 2 μg. The drugs were applied in 1 μl of vehicle solution (SCOP: saline, SCT: 3 DMSO: 7 sterile water). I.c.v. injections were carried out 15 min before the start of the tests. Aged mice obtained SCT s.c. 30 min prior to object memory test (vehicle: 1 DMSO: 1 EtOH, diluted with olive oil as required).

T-maze continuous alternation experiments. Alternation behavior was tested under dimmed illumination (40 lx) in a T-maze equipped with guillotine doors at the start box and at the entrances of the T-arms as described by Gerlai (Gerlai, 1998).

Testing started with a forced-choice trial in which one of the goal arms was blocked by a guillotine door. A mouse was arrested in the start box. The door of the box was opened after 10 s, and the mouse was allowed to explore the open arm. After return the mouse was confined for 5 s by lowering the guillotine door. The subsequent 14 trials were "free-choice" trials in which the two doors of the goal arms were open. After a mouse entered one goal arm, the direct entrance into the other one was blocked by closing the door. After each return to the start box, the mouse was again confined for 5 s. A session was terminated as soon as 14 free-choice trials had been performed or when 30 min had elapsed.

The percentage of alternation for each mouse was calculated as the ratio of the actual number of alternation to the possible number (defined as the total number of arm entries minus one) multiplied by 100. The time required to finish the 14 trials (running time) was also registered.

Novel object recognition task. The novel object recognition task was performed as described by Ennaceur and Delacour (1988). Each mouse was habituated to the test box for 10 min in the absence of objects. During training session (T1) on the following day, two identical objects were placed in the back corners of the box, and behavior including exploration of the two objects was recorded for 10 min. After 18 h during testing trial (T2), a novel object replaced one of the objects now familiar to the mouse presented in T1, and the mouse was again allowed to explore for 10 min. An additional group of mice was tested after a forgetting interval of 3 h. The times spent by mice in exploring each object during experiment were analyzed on recorded videos. Exploration was defined as directing the nose toward the object at a distance of less than 1 cm or touching the object with its forepaw or nose.

Measures of retention in this task are object exploration time ratio (object exploration time with a novel object during testing divided by object exploration time with a familiar object during testing) and discrimination index (difference in exploration time expressed as a proportion of the total time spent exploring the two objects in T2). These data were calculated as previously described (Ennaceur and Delacour, 1988; Hornick et al., 2008).

Open-field test. Open-field behavior was investigated in a box of 41×41×41 cm^3 at illumination strength of 100 lx. Behaviors were recorded with an activity monitoring system based on infrared photobeams. The time of locomotor activity, the overall distance traveled by the mice, and the activity in the central zone (28×28 cm^2) were monitored for 10 min (Prut and Belzung, 2003).

RESULTS

Release study

A 1.5-min pulse of 15 mM K^+ at the start of the 6 min of the stimulation sample increased ACh release by 292.8± 30.0% of the basal release measured in the preceding sample (Fig. 1). This effect was half maximal at most since 30 mM K^+ increased ACh release by 629.4±73.3% (n=4) over basal release (not shown). SCT added to the stimu-

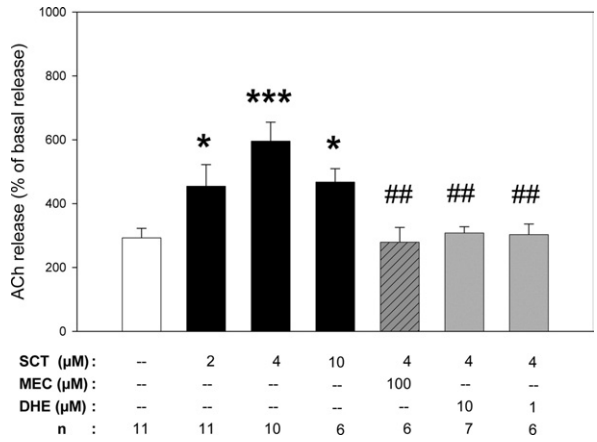

Fig. 1. Effect of scopoletin (SCT) on 15 mM K^+-induced release of [^{3}H]-ACh from prefrontal cortex synaptosomes and inhibition by the antagonists MEC and DHE. The medium contained 1 μM ACh and 0.1 μM atropine. Bars represent K^+-evoked release as percentage of basal release, mean±SEM. Each n represents number of experiments done in triplicate. ANOVA and Bonferroni post hoc test, * $P<0.05$, *** $P<0.001$ from control, ## $P<0.01$ from SCT 4 μM.

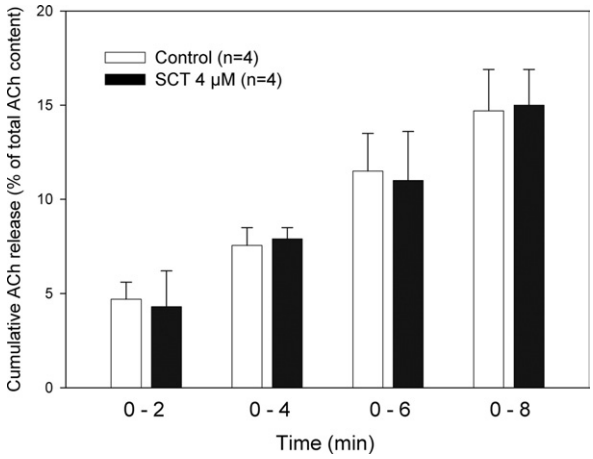

Fig. 2. Effect of scopoletin (SCT) on basal (non-stimulated) release of [^{3}H]-ACh from prefrontal cortex synaptosomes. The medium contained 1 μM ACh and 0.1 μM atropine. Bars represent cumulative release as % of total [^{3}H]-ACh present in the synaptosomes on the filter at the beginning of superfusion, mean±SEM. Number of experiments done in triplicate (*n*) was four.

lation medium at the concentrations 2, 4, and 10 μM enhanced the 15 mM K^+-evoked release showing a curve with maximum at 4 μM (595.6±59.6%). The presence of MEC (100 μM) and of DHE (10 and 1 μM) in the medium blocked the potentiation of ACh release induced by SCT (Fig. 1). The antagonists alone did not influence 15 mM K^+-induced release (not shown. MEC: 275.1±44.6%, n=4; DHE: 301.6±29.8%, n=3). SCT did not influence ACh release under the basal, nonstimulating K^+ concentration of 3 mM (Fig. 2). The potentiation of K^+-evoked ACh release was also found with galantamine, also showing a bell-shaped dose dependency. The maximal effect was observed at 1 μM (607.9±72.1%) and was also blocked by MEC (100 μM) (Fig. 3).

Electrophysiological study

Basal neurotransmission. The effect of SCT on basal neurotransmission and neuronal plasticity was investigated in rat hippocampal slices. fEPSP was evoked in the CA1 subregion by stimulation of Schaffer collaterals. SCT did not influence the basal fEPSP amplitude when applied at concentrations of 4 and 20 μM. Only the relatively high concentration of 200 μM SCT (not used in the LTP study) enhanced fEPSP amplitude under basal conditions (180.3±13.8% of control, n=4; not shown). Nicotine at concentrations of 2 and 10 μM and the antagonists MEC (10 μM) and AP-5 (100 μM) did not influence the magnitude of the basal fEPSP amplitude (not shown).

Effect of nicotine and SCT on LTP. HFS of Schaffer collaterals (100 Hz for 1 s) under drug-free conditions induced a pronounced LTP. Immediately after HFS the fEPSP amplitude increased on an average to 197.9± 19.7% of the pretetanus value and stabilized at a level of 136.7±7.4% (Figs. 4A, B and 5A, B). HFS-induced LTP was N-methyl-D-aspartate (NMDA) receptor dependent since in presence of the antagonist AP-5 only a short-term potentiation was expressed (immediate peak amplitude of 176.6±17.2% followed by a rapid decline to nonpotentiated basal values within 10 min; n=4; not shown). Nicotine applied to the slice pre-HFS for 4 min at the concentration of 2 μM potently enhanced the HFS-induced LTP. The agonist induced an increase of the fEPSP amplitude during the immediate peak phase to 278.1±48.9% of control value. After the period of decline the stabilized amplitude was still at an elevated level (171.6±6.1%) (Fig. 4A, B).

Also SCT induced a potent facilitation of hippocampal LTP. 4 μM (4 min pre HFS) boosted fEPSP amplitude to 247.7±19.1% and also the stabilized maintenance level of fEPSP was substantially higher than that after drug-free induction (192.4±5.9%) (Fig. 5A, C). The nicotinic AChR antagonist MEC abolished this potentiating effect of SCT to values not significantly different from drug-free conditions (158.3±9.0%) (Fig. 5A, C). The above mentioned inhibition of HFS-induced LTP by AP-5 was not rescued by SCT. In presence of AP-5 and treatment of SCT (4 μM), the HFS induced just a short potentiation, in magnitude and fast decline not differing from the effect produced by AP-5 alone (maximal amplitude 183.3±20.1%, steady-state phase 100.7±8.7% of control) (Fig. 5).

Behavioral study

Effect of SCT i.c.v. on T-maze alternation. SCT and SCOP did not disrupt normal behavior. Cholinergic symptoms such as salivation, lacrimation, tremor, and diarrhea were not observed at drug concentrations used in this study.

Each mouse was subjected to one forced-choice trial followed by free-choice trials. Control mice selected the novel arm of the maze at a rate significantly above chance (59.3±3.3%, n=11, P<0.05, Wilcoxon test). The dose of SCT in behavioral testing was estimated in pilot experiments, revealing ineffectiveness of concentrations below

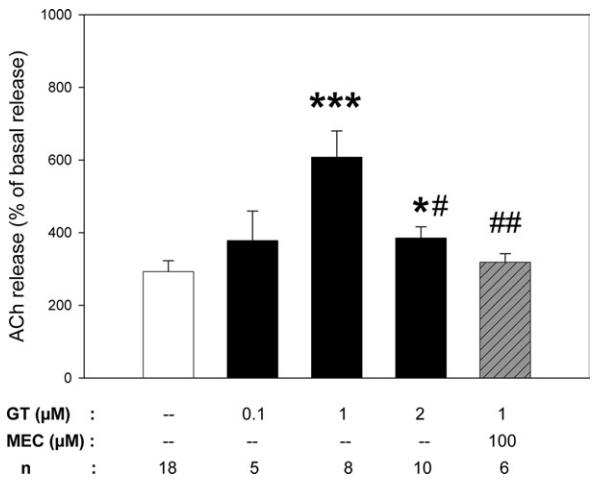

Fig. 3. Effect of galantamine (GT) on 15 mM K^+-induced release of [^{3}H]-ACh from prefrontal cortex synaptosomes and inhibition by MEC (100 μM). Bars represent K^+-evoked release as percentage of basal release, mean±SEM. Each *n* represents the number of experiments done in triplicate. ANOVA and Bonferroni post hoc test, * P<0.05, *** P<0.001 from control; # P<0.05, ## P<0.01 from GT 1 μM.

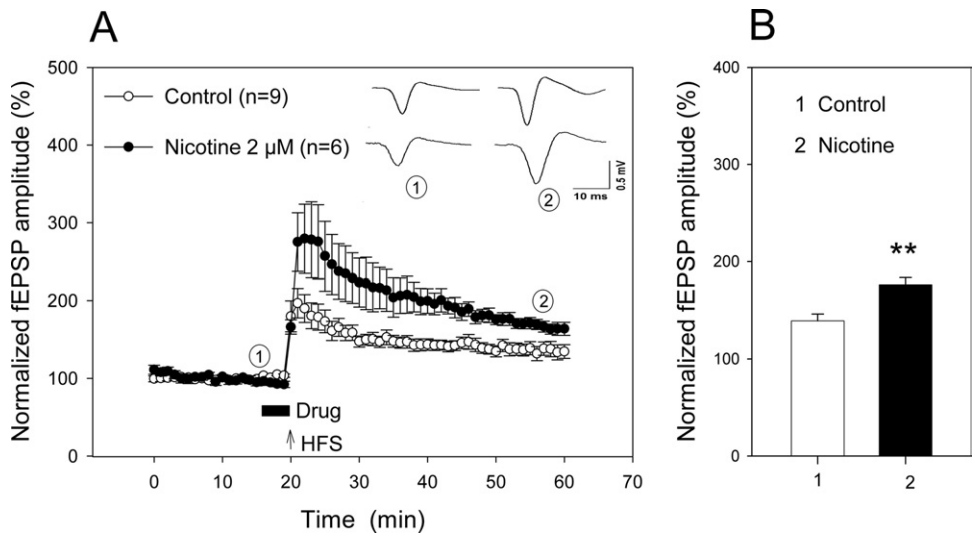

Fig. 4. Effect of nicotine on HFS-induced LTP. (A) Time course of fEPSP amplitudes. Percentage of change, means±SEM. Inserts show representative fEPSPs recorded at the indicated time (1) before HFS and (2) after HFS. (B) Percentage of change (means±SEM) in fEPSP amplitude measured 30–40 min after HFS. ANOVA and Bonferroni post hoc test. * $P<0.05$ vs. control.

0.4 µg ($n=5$, not shown). However, mice injected with 2 µg SCT showed an increased alternation rate of 71.3±2.5%, which indicates an enhanced preference for entering the unfamiliar maze arm (Fig. 6A). The running time was not influenced (Fig. 6B). In contrast to the coumarin, the anticholinergic drug SCOP induced a pronounced decrease in alternation rate to a value below chance level (37.0±2.4%) and an increase in running time (143.2±11.2%) (Fig. 6A, B). SCT reversed this SCOP-induced effect to values statistically not different from controls (alternation rate: 58.3±2.6; running time: 116.4±9.8%) (Fig. 6A, B).

Effect of SCT on object recognition. Following habituation to the arena, two identical objects were presented to the mouse during T1, and a novel and a familiar object during T2. Exploration times during T1 were not different between vehicle- and drug-treated groups, indicating that the treatments did not influence the inspection of objects (Figs. 7A and 8A).

Effect on SCOP-induced amnesia. Control mice spent an equal time exploring the two identical objects in T1 (not shown) but more time exploring the novel object than the familiar object after the 18-h interval in T2 (Fig. 7B). This group of mice achieved a mean discrimination index of 0.287±0.030 showing that they were able to discriminate well between familiar and novel objects (Fig. 7C). These mice did not yet reach the state of forgetting the objects since their performance was not significantly below that of mice tested after a 3-h interval (discrimination index: 0.314±0.048, $n=8$; not shown). SCOP (20 µg, i.c.v.) induced a drastic impairment of learning. The mice of this group did not discriminate at all between the novel object and the familiar object in the T2 (discrimination index: 0.029±0.019) (Fig. 7B, C). The exploratory activity was not impaired since the exploration time showed no significant difference to that of vehicle-treated control mice. SCT (2 µg, i.c.v.) did not improve the object recognition of normal mice (discrimination index: 0.304±0.034). However, it abolished the amnestic effect of SCOP. It potently increased the exploration ratio and discrimination index to values not significantly different from the control (0.219±0.039), indicating restoration of learning and memory abilities to a large extent (Fig. 7B, C).

Effect on age-induced impairment. Aged mice showed the required interest in the objects in T1 and T2 (6.93±0.73 and 6.66±0.55 s) (Fig. 8A). However, despite a short interval of 3 h, they did not show preferential exploration of the novel objects in T2 (exploration ratio: 0.935±0.106) (Fig. 8B). Apparently they were not able to discriminate novel objects from familiar ones (index: −0.024±0.050) (Fig. 8C). SCT (2 mg, s.c.) clearly restored the ability of these mice to recognize objects (ratio: 1.926±0.396, index: 0.270±0.053).

Effect of SCT on open-field locomotor behavior. The anticholinergic scopolamine enhanced locomotor activity in the open field as shown by the increase in the locomotion indexes, such as travel distance, travel time, and activity in the center area. Treatment with SCT exerted no significant effect on the locomotion indexes. SCT did also not influence the scopolamine-induced increase in travel distance and duration (Table 1).

DISCUSSION

SCT enhances ACh release from brain synaptosomes

In our study SCT potentiated the K^+-evoked release of ACh from superfused frontal cortex synaptosomes. This effect was abolished by the nAChR antagonist MEC and mimicked by galantamine. The nonstimulated, basal release was not influenced by drug concentrations used in this study. These findings suggest that the coumarin exerts an agonistic effect at nAChRs located at the synaptosomal

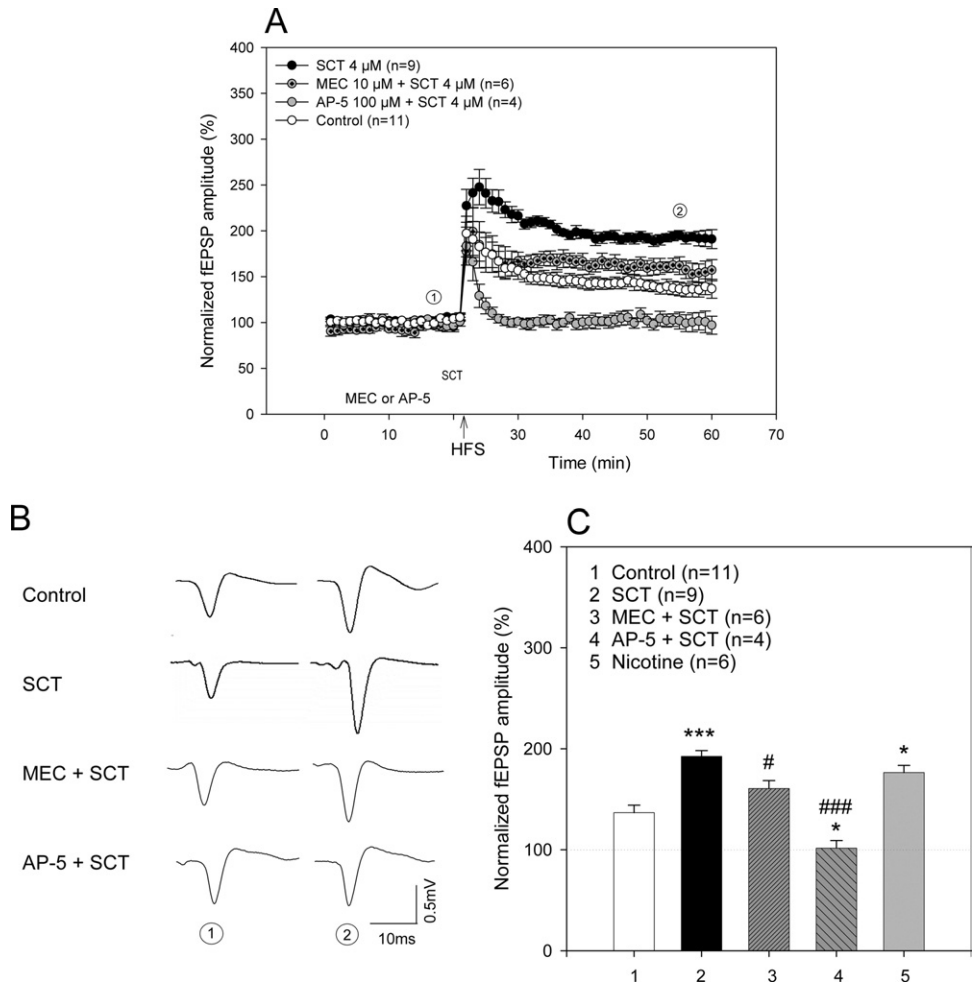

Fig. 5. Effect of scopoletin (SCT) and antagonists MEC and AP-5 on LTP. (A) Time course of fEPSP amplitudes. Percentage of change, means±SEM. (B) Representative fEPSPs recorded at the indicated time (1) before HFS and (2) after HFS. (C) Percentage of change (means±SEM) in fEPSP amplitude measured 30–40 min after delivery of HFS. Nicotine is shown for comparison. ANOVA and Bonferroni post hoc test. * $P<0.05$ vs. control, # $P<0.05$ vs. SCT.

membrane, which positively modulates ACh release. SCT is a very weak AChE inhibitor (EC_{50}: 100 μM), but AChE inhibition and muscarinic autoreceptors are not involved in this effect. Release-enhancing nAChRs and release-inhibiting muscarinic autoreceptors coexist on the cholinergic nerve terminals in cortex (Szerb et al., 1977; Marchi et al., 1983; Duffy et al., 2009). However, we blocked muscarinic receptors with atropine. Interaction of drugs with AChE does not alter ACh concentration in the superfusate because the released ACh is rapidly removed from the membrane surface (Garcia-Sanz et al., 2001).

Under basal, nonstimulated conditions SCT at concentrations below 10 μM did not affect the ACh release. It shares this property with galantamine. As mentioned in the introduction, SCT was found to fit to a pharmacophore model that had been generated from the binding of galantamine to the AChE (Rollinger et al., 2004). Galantamine and the structurally similar compounds physostigmine and codeine were shown to bind not only to AChE but also to an allosteric binding site of nAChRs (Storch et al., 1995; Samochocki et al., 2003). Hence, SCT and the three compounds mentioned above might share molecular domains which provide affinity to an allosteric site of nAChRs.

Galantamine has no intrinsic activity per se, but it enhances the effect of ACh on nAChRs (Samochocki et al., 2003). Hence, the effect of SCT and galantamine on 15 mM K^+-evoked ACh release was investigated in presence of 1 μM extrasynaptosomal ACh. This concentration provides a low degree of activation of nAChRs, which does not lead to a significant desensitization (Roman et al., 2004). SCT induced a pronounced increase in the K^+-evoked release, and this effect was abolished by MEC and DHE. At the concentrations used in this study, DHE has selectivity for α and β subunits containing nAChRs and does not influence α_7 homomeric nAChRs (Papke et al., 2008). Hence, $\alpha_n\beta_n$-nAChRs might be responsible for the potentiating effect of SCT on ACh release. The effect of SCT resembles that of galantamine. Both agents stimulated K^+-evoked release with an apparently bell-shaped concentration-effect curve, and the maximum enhancing concentration of SCT (4 μM) was not far from that of galantamine (1 μM). At higher concentrations the release-

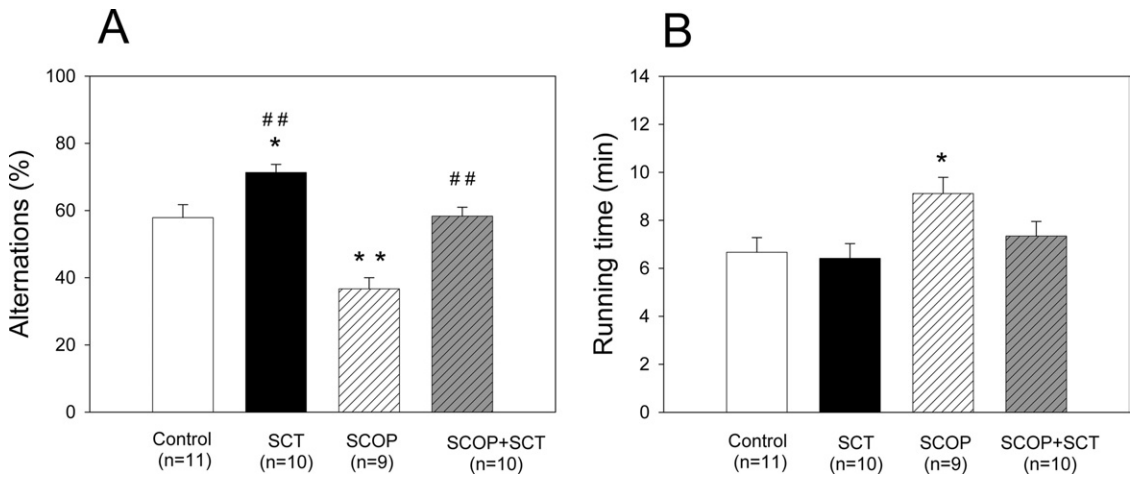

Fig. 6. Effect of scopoletin on T-maze alternation in scopolamine-amnestic mice. (A) Alternation rate, (B) running time. Scopoletin (SCT; 2 μg i.c.v.); Scopolamine (SCOP; 20 μg i.c.v.). Data are shown as means±SEM, analysis of variance (ANOVA) followed by multiple comparisons Tukey–Kramer test. * $P<0.05$, ** $P<0.01$ vs. control group; ## $P<0.01$ vs. SCOP group.

potentiating effect showed attenuation which might be attributed to desensitization of nAChRs. This has been demonstrated with galantamine in electrophysiological studies (Maelicke et al., 2000; Samochocki et al., 2003).

SCT amplifies hippocampal LTP

In our experiments HFS stimulation of Schaffer collaterals induced an LTP of evoked fEPSPs in the CA1 region. LTP showed NMDA glutamate receptor-dependent characteristics since in presence of the antagonist the initially potentiated fEPSPs decreased rapidly to the basal level.

It has been reported that this form of hippocampal LTP is amplified by nicotine (1 to 5 μM) and other nAChR agonists (Nakauchi et al., 2007; Fujii et al., 2000). Also under our experimental conditions the HFS-induced, NMDA-dependent LTP was strongly enhanced by nicotine when it was present during HFS. Nicotine increased the magnitude of LTP to a new maximum, which was not attained by stimulation alone. Other investigations found nicotine boosts short-term potentiation to LTP (Ge and Dani, 2005; Rosato-Siri et al., 2006) and enables LTP in slices from aged animals with impaired synaptic plasticity (Fujii and Sumikawa, 2001).

After showing the effect of nicotine on LTP, we tested whether SCT exerts similar properties. Findings with galantamine suggested that the potency of nAChR agonists to enhance synaptosomal ACh release parallels the potency to amplify hippocampal LTP. In our study with synaptosomes, 1 μM galantamine induced the maximum of ACh release, and furthermore, this concentration was recently reported to amplify hippocampal LTP (Moriguchi et al., 2009). Hence, we investigated the effect of SCT on LTP at 4 μM, the $E_{max.}$ of K^+-induced ACh release from cortex synaptosomes found in this study.

In our experiments SCT showed a strong effect on stimulation-evoked synaptic plasticity. It induced a pronounced amplification of LTP very similar to the effect of nicotine. This amplification was abolished by MEC, and SCT failed to rescue the inhibition of LTP by AP-5. These key findings suggest that SCT enhances NMDA-dependent LTP through agonistic action at nAChRs. Several other nAChR agonists, which were shown to improve memory, also potentiated LTP in CA1 or dentate gyrus (Newhouse et al., 2001; Lagostena et al., 2008; Timmermann et al., 2007).

The action of nAChR agonists depends largely on affected subtypes of nicotinic receptors, their location on the different neurons, and the mutual interaction between these cells. The hippocampus is endowed with nAChRs predominantly of the homomeric α_7 and the heteromeric $\alpha_4\beta_2$ subtype (Alkondon and Albuquerque, 1993, 2004). The nicotinic potentiation of LTP in CA1 was found to be mediated mainly by the α_7 subtype (Mann and Greenfield, 2003). However, it was also reported that both subtypes contribute to the facilitating effect suggesting that cognition-enhancing agents can act through each one of these nAChRs (Lagostena et al., 2008; Matsuyama and Matsumoto, 2003; Nakauchi et al., 2007). Recently, Moriguchi et al. showed under experimental conditions very similar to ours that galantamine potentiates NMDA-dependent LTP in CA1 of hippocampal slices via the α_7 subtype (Moriguchi et al., 2009). The underlying intracellular mechanism seems to consist in a potentiation of NMDA-induced currents in a PKC-dependent manner. The experiments with synaptosomes show that SCT activates β subunit containing DHE-sensitive subtypes. Whether SCT is selective for these subtypes or influences also α_7 nAChRs remains to be investigated.

Effects of SCT on memory and behavior

nAChR agonists and the AChE inhibitors dose dependently increase alternation in the T-maze, ameliorate object recognition, and decrease locomotor activity in the open field (Bontempi et al., 2003; Spowart-Manning and van der Staay, 2004; Sambeth et al., 2007; Pichat et al., 2007; Syenkiewicz-Jarosz et al., 2000). A useful dose of SCT for the behavioral investigations was estimated in

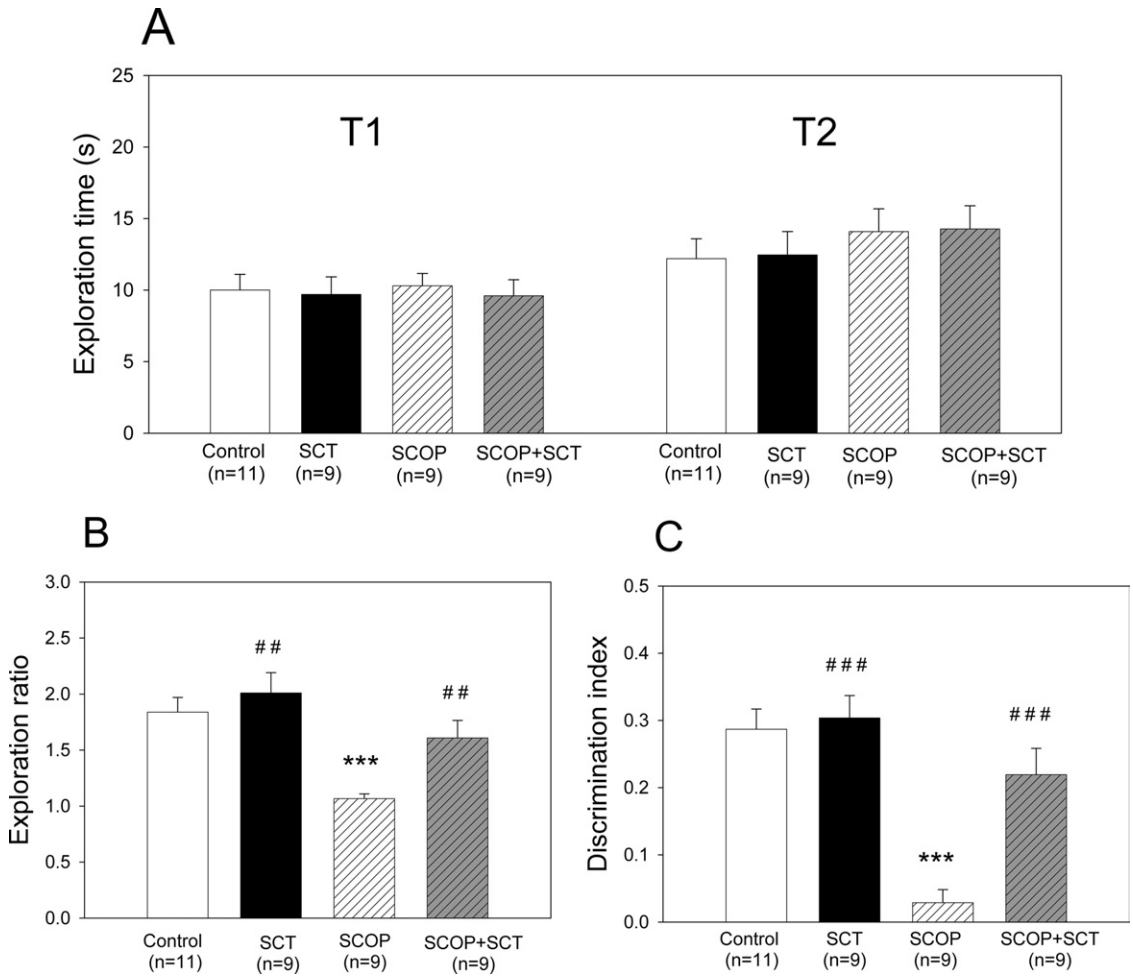

Fig. 7. Effect of scopoletin on object recognition in scopolamine-amnestic mice. Scopoletin (SCT) 2 μg i.c.v., scopolamine (SCOP) 20 μg i.c.v. (A) Exploration time, (B) Exploration ratio, and (C) Discrimination index. Data are shown as means±SEM, analysis of variance (ANOVA) followed by multiple comparisons Tukey–Kramer test. ** $P<0.01$, *** $P<0.001$ vs. control group; ## $P<0.01$, ### $P<0.001$ vs. SCOP group.

pilot experiments by testing increasing doses of SCT in the T-maze continuous alternation task (Bontempi et al., 2003).

T-maze alternation. This one trial test is well suited to study the effects of drugs on the cholinergic system. When placed in a T-maze, rats possess a strong tendency to

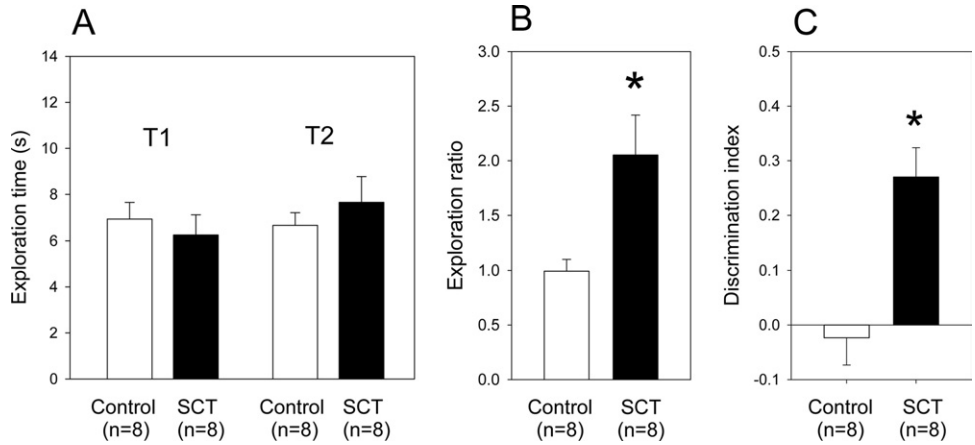

Fig. 8. Effect of scopoletin on object recognition in aged mice. Scopoletin (SCT) 2 mg/kg s.c. (A) Exploration time, (B) Exploration ratio, and (C) Discrimination index. Data are shown as means±SEM. Statistical analysis by *t*-test. * $P<0.05$.

Table 1. Effect of scopoletin (SCT) on locomotor indexes in the open-field paradigm. Scopoletin (SCT) 2 μg i.c.v.; 2 mg/kg s.c., scopolamine (SCOP) 20 μg i.c.v., n=8–12 animals per group

Mice	Treatment	Move distance (cm)	Move time (s)	Center distance (%)	Center time (%)
Age of 4–6 mon, SCT and SCOP i.c.v.	Control	3240±221	472.5±12.9	15.04±3.41	18.02±6.71
	SCT	2987±176	453.5±13.7	17.99±2.68	20.42±4.19
	SCOP	4039±201*	497.8±9.6	13.65±3.27	16.86±5.17
	SCT+SCOP	4416±146*	496.7±15.1	16.03±4.53	19.30±6.75
Age of 15–18 mon, SCT s.c.	Control	2018±189	388.7±25.6	20.37±5.82	21.11±3.17
	SCT	2286±325	409.2±34.8	23.18±4.77	22.57±2.73

Data are shown as means±SEM, analysis of variance (ANOVA) followed by multiple comparisons Tukey–Kramer test.
* $P<0.05$ vs. control group.

alternate arm choices on successive trials. Testing of mice is not trivial since several strains are fearful in the novel situation, which may interfere with performance (Bertholet and Crusio, 1991; Dember, 1990; Spowart-Manning and van der Staay, 2004). In our experiments, the C57BL/6N mice showed alternating choices of right and left maze arms at a rate significantly above chance, indicating low anxiety and a clear expression of spatial memory in their behavior. The spontaneous alternation test is sensitive to the consequences of normal and pathological aging (Stone et al., 1992; Willig et al., 1987). Neurochemical pathways using ACh and dopamine in the septum and hippocampus have been implicated in the exploration of novel maze arms (Kokkinidis and Anisman, 1976; McFarland, 1989; Einat and Szechtman, 1995). Spontaneous alternation rates were often shown to be decreased by drugs that impair cholinergic transmission (Meyers and Domino, 1964; Kokkinidis and Anisman, 1976). In contrast, nAChR agonists including nicotine enhance alternation rate of aged and scopolamine-amnestic mice (Bontempi et al., 2003). Furthermore, AChE inhibitors increase alternation rate in untreated and pirenzepine-impaired animals (Delatour and Gisquet-Verrier, 1996; Egger et al., 1973; Ukai et al., 1995). Consistent with these reported effects of nAChR agonists and AChE inhibitors, SCT enhanced the alternation rate in untreated mice and reversed the scopolamine-induced decrease to control levels. The procholinergic effect might be interpreted as facilitation of spatial working memory, required to recognize the previously entered arm of the maze. However, the effect of scopolamine to reduce the alternation rate below chance level suggests that a modulation of neophobic behavior might also be involved in the effects of the anticholinergic and of SCT.

Novel object recognition. In our study, we also used the novel object recognition task. This paradigm reveals the ability of the animals to recognize the objects they have seen only once during the training trial. Normal mice have a natural propensity to explore novel objects, and when this bias is observed it is inferred that mice remember the familiar object. This kind of object recognition is based on a nonspatial memory with the characteristics of episodic memory (Ennaceur and Delacour, 1988), a form of memory that is primarily affected in senile dementia, age-associated impairment, and the Alzheimer's type of dementia (Flicker et al., 1985; Lee et al., 2003). Also in mice and rats, object discrimination appears to depend on the integrity of the cholinergic system (Bartolini et al., 1996; Dodart et al., 1997) since AChE inhibitors and nAChR agonists ameliorate memory in this task. Galantamine improved impairments of object recognition induced by beta-amyloid or lesion of magnocellular nucleus basalis (Wang et al., 2007; Rispoli et al., 2006). Nicotine, the α_7 nAChR agonist SEN12333 and donepezil improved object memory in scopolamine-treated rats (Sambeth et al., 2007; Roncarati et al., 2009).

Effectiveness of SCT in states of cholinergic deficit was investigated with the widely used scopolamine paradigm and in 15–18-month-old mice. Numerous studies have shown that scopolamine injected i.p. or s.c. disrupts object recognition. Also our results indicate that 20 μg scopolamine injected i.c.v. practically abolishes object memory in C57BL/6N mice. We show that even this total disruption of memory by the anticholinergic is almost restored by 2 μg SCT, suggesting a potent ameliorating action of SCT on the impaired cholinergic transmission.

In some studies nAChR agonists caused improvements of object recognition in normal, unimpaired mice; however, this was not the case in our study (Boess et al., 2007; Puma et al., 1999; Pichat et al., 2007; Wishka et al., 2006). The 4–6-month-old C57 BL6 mice used in our study may not have reached the state of forgetting the familiar objects within the delay interval of 18 h. In contrast, the 16–18-month-old mice were not able to retain the object memory for the interval of 3 h, a finding which indicates a severe age-associated impairment. Nevertheless, SCT applied s.c. caused a pronounced improvement of object recognition, showing its potential as memory enhancer and its property to reach the brain when administered systemically.

Open-field behavior. In the open-field paradigm SCT had neither a significant effect on move distance and time nor on the anxiety state-related locomotion in the center area. Also the locomotor indexes of the object memory test and of the alternation test were not significantly influenced by the drug, suggesting that the outcome depended on task-specific performance and learning abilities and not on unspecific influences. The amnesic agent SCOP enhanced locomotor activity, although we tried to keep the dose as low as possible. It has been shown that manipulations of the cholinergic brain systems affect open-field behavior. Anticholinergic drugs at higher doses increase

locomotor activity and cause hyperlocomotion in the open field. In contrast, nAChR agonists and AChE inhibitors have been reported to reduce spontaneous locomotor activity and counteract the stimulatory effect of antagonists such as scopolamine (Bontempi et al., 2003; Carlsson and Carlsson, 1989; Capone et al., 1999). In our study, SCT did not reduce scopolamine-evoked activity. Presumably the dose was too low to exert the antiscopolamine effect. The mechanism implicated in scopolamine-induced hyperactivity is different from the mechanism implicated in the scopolamine-induced memory deficit and probably related to a dopaminergic system interaction (Fernando et al., 1986; Itoh et al., 1993). Hence, the locomotor activation may be more resistant to nAChR-mediated enhancement of cholinergic transmission than the memory deficit.

To summarize, the pharmacological properties of SCT found in this study suggest that this molecule might be useful in the treatment of brain disturbances associated with dementia, such as AD. The main features of AD are progressive memory deterioration and disordered cognitive function resulting from loss of cholinergic transmission. In a significant proportion of AD patients, there is also a slowing of motor activity and extrapyramidal dysfunction resembling those seen in Parkinson's disease and behavioral abnormalities. Depressive symptoms occur in a large proportion of subjects with AD and other types of dementia, and this may be related to degeneration of noradrenaline and serotonin neurons innervating the limbic system (Newman, 1999; Palmer et al., 1988). The MAO inhibitory property of SCT might exert a beneficial effect against these catecholaminergic impairments. Yet AChEs including also the nAChR agonistic galantamine and the NMDA antagonist memantine are still the only drugs that produce statistically significant improvements in cognitive performance and in the activity of daily living in large trials with AD patients and in other types of dementia (Scott and Goa, 2000; Lilienfeld and Parys, 2000; Blesa et al., 2003; Onor et al., 2007; Schoenmakers et al., 2009). Accumulating evidence suggests that nAChR agonists may be superior to AChE inhibitors in the treatment of AD. It has been reported that the α_7 nAChR is involved in the beta-amyloidogenic pathway and plays a neuroprotective role in connection with the pathogenesis of AD (Liu et al., 2007; Qi et al., 2007; Woodruff-Pak and Gould, 2002). Hence, the effectiveness of SCT at the different subtypes is of high interest. Other beneficial properties of this agent are its antioxidant, radical scavenger, and anti-inflammatory activities (Shaw et al., 2003; Toda, 2002; Muschietti et al., 2001; Calixto et al., 2003). Oxidative stress, increased formation of oxygen radicals, and enhanced lipid peroxidation are induced by i.c.v. injection of beta-amyloid and are early hallmarks in AD and in its transgenic animal models (Sonnen et al., 2008, 2009). Several studies suggest that appropriate antioxidants can prevent neurodegeneration, as reviewed by Sonnen et al. (2008) and Zhao (2009).

CONCLUSIONS

In conclusion, we showed that the coumarin SCT amplifies presynaptic activity-dependent ACh release, enhances LTP in the CA1 area of the hippocampus, and exerts cognition-improving properties in cholinergically impaired and in age-impaired mice. We assume SCT acts as an agonist at nAChRs and enhances NMDA-dependent LTP. We suggest that this is the main mechanism of action by which SCT ameliorates memory.

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