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Research report

A daily single dose of a novel modafinil analogue CE-123 improves memory acquisition and memory retrieval

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ABSTRACT

Dopamine reuptake inhibitors have been shown to improve cognitive parameters in various tasks and animal models. We recently reported a series of modafinil analogues, of which the most promising, 5-((benzhydrylsulfinyl)methyl) thiazole (CE-123), was selected for further development. The present study aims to characterize pharmacological properties of CE-123 and to investigate the potential to enhance memory performance in a rat model. *In vitro* transporter assays were performed in cells expressing human transporters. CE-123 blocked uptake of [3H] dopamine (IC50 = 4.606 μM) while effects on serotonin (SERT) and the norepinephrine transporter (NET) were negligible. Blood-brain barrier and pharmacokinetic studies showed that the compound reached the brain and lower elimination than R-modafinil. The Pro-cognitive effect was evaluated in a spatial hole-board task in male Sprague-Dawley rats and CE-123 enhances memory acquisition and memory retrieval, represented by significantly increased reference memory indices and shortened latency. Since DAT blockers can be considered as indirect dopamine receptor agonists, western blotting was used to quantify protein levels of dopamine receptors D1R, D2R and D5R and DAT in the synaptosomal fraction of hippocampal subregions CA1, CA3 and dentate gyrus (DG). CE-123 administration in rats increased total DAT levels and D1R protein levels were significantly increased in CA1 and CA3 in treated/trained groups. The increase of D5R was observed in DG only. Dopamine receptors, particularly D1R, seem to play a role in mediating CE-123-induced memory enhancement. Dopamine reuptake inhibition by CE-123 may represent a novel and improved stimulant therapeutic for impairments of cognitive functions.

1. Introduction

The DA transporter (DAT), which governs the spatial and temporal dynamics of dopamine neurotransmission by driving reuptake from the extracellular space into the presynaptic neurons, is a major target for several psychostimulant drugs including cocaine, amphetamine and methylphenidate, likely mediating their abuse-related effects [1–4]. Most classical psychostimulants exhibit a dose-dependent biphasic effect on cognitive functions, learning and memory [5,6]. However, their behavioral profile is also influenced by their action on and different affinity for multiple monoamine transporters [7].

Cocaine, unlike amphetamine, acts as a simple inhibitor that binds to the DAT and inhibits its transport activity without stimulating reverse transport activity [8]. Although a multitude of DAT inhibitors that share cocaine-like behavioral effects exists, a number of atypical DAT inhibitors (*e.g.*, analogs of benztropine, RTI-371, GBR12909, modafinil) exhibit different behavioral properties with a minimal abuse liability [9–14]. Moreover, benztropine and modafinil have been reported to antagonize the effect of cocaine [15,16], suggesting a unique therapeutic potential for treatment of stimulant dependence. Several of

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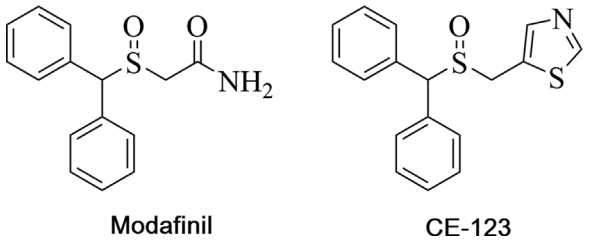


Fig. 1. Molecular formulae of CE-123 and parent compound modafinil.

them show pro-cognitive effects similar to those of low-dose psychostimulants. These agents, however, are only moderately selective inhibitors of DAT. The fact that a locomotor stimulant effect and addictiveness are not a common property of all DAT inhibitors, demonstrates that DAT may possess some of the ligand-specific pleiotropic attributes inherent to G-protein–coupled receptors [17,18].

A parent compound of CE-123, modafinil (Fig. 1) is a wake-promoting drug approved for the treatment of narcolepsy, sleep apnea and shift-work sleep disorders [19]. Modafinil has gained increasing interest for its cognitive enhancing properties in healthy individuals [20,21] and individuals with some cognitive dysfunctions, including attention deficit hyperactivity disorder (ADHD) [22] and schizophrenia [23]. Despite a weak binding potential for DAT, modafinil occupies more than 50% of DAT at therapeutic doses [24], that makes modafinil a candidate for off-prescription use for cognitive enhancement.

Although the mechanism of action of modafinil is not well understood, multiple neurotransmitter systems have been implicated in its activity [25]. A major molecular target of modafinil is the DAT, however, several studies reported significant occupancy of NET by modafinil [26]. *In vitro* (in cells expressing human transporters), modafinil inhibits DAT activity (IC50 6.4–13 μM) and with weaker potency NET (IC50 35.6–182 μM) [27–29]. Given the important role of dopamine for executive functions, attention and cognition, acting on dopaminergic system may be a key action that promotes pro-cognitive effects. Therefore, the design of improved selective dopamine reuptake inhibitors for cognitive enhancement represents the basic strategy for own modafinil analogue development.

We recently reported a series of modafinil analogues [30–33] displaying improved pharmacological properties compared to the parent compound with pro-cognition action. We have recently demonstrated that CE-123 enhances cognitive flexibility without producing impulsive responding [34] and therefore CE-123 was selected for further testing of its cognition enhancing properties.

In the present study, animals were tested in a hole-board task, a food-rewarded test of spatial learning and memory. The efficiency of DAT inhibitors is stimulus-dependent and is therefore limited by the tone of presynaptic activity [7]. Novelty or reward-related stimuli increase activity of DA neurons in the VTA [35,36] and stimulate DA release in hippocampus and prefrontal cortex [37,38]. The effect of DA receptor stimulation on learning and memory that is predominantly mediated by prefrontal cortex and hippocampus is well-documented. Activation of D1-like receptors enhance synaptic plasticity, in particular long-term potentiation [39,40], one of the cellular mechanisms of learning and memory [41]. D1-like receptors seem to be key mediators of downstream effects subsequent to DAT inhibition. However, effects of DAT inhibitors on the hippocampus were so far neglected.

To generate a profile of subregion-specific protein level changes of dopamine receptors and the DAT following drug treatment, three major hippocampal subregions were examined individually.

Herein we report a novel modafinil analogue CE-123 with superior pharmacological properties compared to the parent compound with respect to DAT, SERT and NET, specifically inhibits DAT *in vitro*, presumably penetrates the BBB, shows slower elimination from blood, CSF and brain and exerts no adverse effects and is significantly increasing performance in a spatial memory task.

2. Materials and methods

2.1. Synthesis of 5-((benzhydrylsulfinyl)methyl)thiazole (CE-123)

2.1.1. Synthesis of S-di-phenylmethyl-isothiuronium hydrobromide

Diphenylmethanol (26.0 g, 140 mmol) and thiourea (13.0 g, 170 mmol) were mixed in a 1 L two-neck round bottomed flask. Water (65 mL) was added and the mixture was heated to 95 °C (an emulsion was obtained) and 52 g of 48% HBr (644 mol, 4.6 equivalents) was then added dropwise during 0.5 h. The mixture was refluxed for additional 0.5 h and cooled to room temperature. The product was precipitated at 0 °C. Subsequently, products were filtered off and washed with cold water, dried under reduced pressure to yield 19.24 g of a white solid (yield: 74%).

2.1.2. Synthesis of 5-(chloromethyl)thiazole hydrochloride

In a round bottom flask 10 g (86.8 mmol) of 5-(hydroxymethyl) thiazole were dissolved in 100 mL of dichloromethane and cooled to 0 °C. Then 6.3 mL (1 equivalent, 86.8 mmol) of thionyl chloride was added dropwise to the reaction mixture and stirred overnight at room temperature. The product was then concentrated at reduced pressure and dried in high vacuum for one hour to yield 9.1 g of a solid orange material (yield: 78.5%).

2.1.3. Synthesis of 5-((benzhydrylthio)methyl)thiazole

In a round bottomed flask 13.73 g (42 mmol) of *S*-di-phenylmethyl-isothiuronium hydrobromide was dissolved in 150 mL of methanol. Afterwards, 7.09 g (42 mmol) of 5-(chloromethyl)-thiazole hydrochloride and 29.31 g (5 equivalents, 210 mmol) of potassium carbonate were added to the mixture. The mixture was stirred for 2 days at room temperature. Methanol was removed under reduced pressure and 150 mL of water were added. The reaction products were extracted with ethylacetate, dried over Na_2SO_4, filtered off and the products were concentrated. The crude product was purified *via* flash column chromatography on silica gel (5% methanol in dichloromethane) to yield 17.9 g of white crystals (yield: 72.71%).

2.1.4. Synthesis of 5-((benzhydrylsulfinyl)methyl)thiazole

17.9 g (60.25 mmol) of 5-((benzhydrylthio)methyl)thiazole were dissolved in 50 mL of glacial acetic acid and 6.95 mL (60.25 mmol) of 30% H_2O_2 was dropped into the solution and stirred for 12 h. The reaction mixture was neutralized with cold 5% sodium bicarbonate solution. Reaction products were extracted with ethyl acetate, dried over Na_2SO_4, filtered off and ethyl acetate was removed under reduced pressure. The solid product (8.8 g) was purified by flash column chromatography on silica gel (5% methanol in dichloromethane), concentrated and dried in high vacuum to yield 6.6 g of the white crystalline product (yield: 50.2%) The C-18 HPLC - determined purity was 99.7% (Supplementary data). $[M+H^+] = 314.0667$, $[M+Na^+] = 336.0486$ (Supplementary data).

^{1}H NMR (500 MHz, CDCl3-d, 23 °C): $\delta = 8.86$ (s, 1H, CH, thiazole-2), 7.67 (s, 1H, CH, thiazol-4), 7.39 (2x CH-2,6, phenyls), 7.45 (CH-3,5, phenyl), 7.39 (CH-3,5, phenyl), 7.36 (2x CH-4, phenyls), 4.65 (s, 1H, CH), $\delta = 4.13$, 3.89 (AB, 2H, CH2) (Supplementary data).

^{13}C[42]NMR (125.75 MHz, CDCl3-d, 23 °C): $\delta = 46.64$ (CH2), 69.95 (CH), 128.54 (C-4, phenyl), 128.77 (C-4, phenyl), 129.33 (2x C-3,5, phenyl), 129.52 (2x C-3,5, phenyls), 128.66 (C-2,6, phenyl), 128.81 (C-2,6, phenyl), 134.05 (Cq-1, phenyl), 134.74 (Cq-1, phenyl), 124.89 (C-2, thiazole), 144.18 (C-4, thiazole), 154.97 (C-5, thiazole). (Supplementary data).

Details on formulae, separation and information on purity are given in Supplementary data.

2.2. Molecular docking

The protein homology model was kindly provided by the group of

Gerhard Ecker from the University of Vienna. The provided homology model was derived from the *drosophila* DAT structure as described in Saha et al. [43]. The DAT structure was in complex with nortriptyline [44].

Molecular docking was performed using Autodock Vina [45]. The ligands were prepared using the Maestro Modelling suite as shown in Fig. 3. The ligands were prepared with flexible bonds using the automatic detection of Autodock Vina. For the molecular docking run standard parameters were used, protein flexibility was not regarded and the exhaustiveness level was set to 16. Waters were removed and the binding site was defined as the center of mass of nortriptyline. The search space was restricted to the protein structure. Docking analysis was performed using compound R/S-CE-123 and cocaine.

The interaction pattern between the best docked poses and the amino acids in the binding pocket were visualized using the Maestro Modelling suite and the pharmacophore models were generated using LigandScout [46].

2.3. Reuptake inhibition assays

Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Handels GmbH (Austria). [^{3}H]5-HT (Hydroxytryptamine; 5-[1,2-^{3}H[N]]; 27,8 Ci/mmol), [^{3}H]DA (Dihydroxyphenylethylamine; 3,4-[ring-2,5,6-3[H]]-Dopamine; 36,6 Ci/mmol) and [^{3}H]MPP+ (Methyl-4-phenylpyridinium iodide; 1-[methyl-^{3}H]; 80 Ci/mmol) were purchased from Perkin Elmer, Boston, MA.

Effects of CE-123 on reuptake of their respective substrates were analyzed as described by [30,47]. Briefly, HEK293 cells stably expressing human isoforms of the dopamine transporter (DAT), the norepinephrine transporter (NET) and the serotonin transporter (SERT) were used for reuptake inhibition assays. All cell lines were seeded on 96-well plates pre-coated with poly-D-lysine (PDL) (5×10^4 cells/well) 24 h prior to the experiment. Each well was washed with 100 µL of Krebs-HEPES buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl2·2H2O, 2 mM MgCl2·6H2O, 5 mM D-(+)-Glucose monohydrate, pH 7.3). Cells were pre-incubated 5 min in KHB containing different dilutions (0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM, 0.1 mM and 1 mM) of CE-123. CE-123 was dissolved first in 99.9% dimethyl sulfoxide (DMSO) and subsequently diluted in KHB. Afterwards, cells were incubated in KHB containing the same dilutions of CE-123 with addition of 0.2 µM [^{3}H]-dopamine (for HEK-DAT), 0.05 µM [^{3}H]MPP+ (for HEK-NET) and 0.4 µM [^{3}H]5-HT (for HEK-SERT). Incubation times were 1 min for HEK-DAT and HEK-SERT and 3 min for HEK-NET. For determination of non-specific uptake in HEK-DAT and HEK-NET 10 µM mazindole was used and 10 µM paroxetine was used for HEK-SERT. After incubation at room temperature, reactions were stopped by the addition of 100 µL of ice-cold KHB. Finally, cells were lysed with 300 µL of 1% SDS and released radioactivity was measured by a liquid scintillation counter (Tri-carb-2300TR, Perkin Elmer).

2.4. DAT-release assay

Monensin sodium salt and the D-amphetamine hemisulfate salt were purchased from Sigma-Aldrich Co. The substrate/efflux experiments were performed as described before [47]. Briefly, HEK-DAT cells were grown in 5 mm diameter PDL-coated coverslips. Cells were incubated with 0.05 µM [3H]MPP+ at 37 °C for 20 min. The coverslips were transferred onto superfusion chambers (0.2 mL) and excess radioactivity was washed out with KHB for 40 min (0.7 mL/min) at 25 °C to obtain stable baselines. During the experiment the buffer was exchanged either to monensin or remained at control buffer after the collection of three baseline fractions for another four fractions. Subsequently, CE-123 or D-amphetamine was added for another five fractions as indicated in Fig. 1B. Finally, the remaining radioactivity was collected by treatment with 1% SDS.

2.5. Transport across blood-brain barrier in vitro model

The transport studies were accomplished with Transwell models using mouse cell line cerebEND which was a kind gift from Prof. Carola Förster [48]. CerebEND cells are able to form tight cell layers similar to primary mouse brain endothelial cells and have been characterized as blood-brain barrier *in vitro* model in a comprehensive manner previously [48,49]. Experiments were conducted on collagen IV coated Transwell inserts as described recently [31,49]. Compound solutions were added to the apical compartment, and inserts were transferred at 37 °C into DMEM (+0.2% DMSO) filled wells after five, 15, 30 and 60 min. Transendothelial electrical resistance (TEER) values before and after the studies confirmed that cell layer integrity was not compromised during experiments. Diazepam and CE-123 (100 mM) were dissolved in DMSO, compounds were diluted in DMEM to a test concentration of 100 µM (test solution). Diazepam was added to CE-123 in DMEM as internal standard to account for cell layer's variability. Samples of the test solution, the basolateral and apical compartments after the transport study were collected and prepared for HPLC analysis as recently described in detail [31,50]. Cleared volume *versus* (*vs.*) time curves and permeability coefficients were calculated using peak areas of HPLC analysis following the clearance principle according to Novakova et al. [50]. Permeability coefficient PCall [µm/min] describes the transport across the cell layer and the membrane support. PCcell values [µm/min] mean the permeability across only the cell layer (after substraction of transport results across blank inserts without cells).

2.6. Plasma, cerebrospinal fluid (CSF) and brain levels of CE-123

Pharmacokinetic analysis was performed by Pharmacelsus (Saarbrücken, Germany). Sprague Dawley rats were treated with CE-123 or R-modafinil at a dose of 10 mg/kg *via* intraperitoneal administration. Blood, CSF and brains were collected from rats at 15 min, 1 and 7 h after dosing. The plasma was prepared within 45 min after sampling and was kept at −20 °C until being assayed. Rats were sacrificed by inhalation of an overdose of isoflurane for collection of CSF and brain. Rats were placed in a stereotaxic device and aliquots of 20 µL CSF were obtained by puncture of the cisterna magna. The sample was frozen on dry ice within 1–2 min of sampling. Subsequently, brains were dissected and frozen on dry ice within 1–2 min of sampling.

Plasma, CSF and brain levels of CE-123 were measured by HPLC–MS (Thermo TSQ Quantum Discovery Max, Thermo Fisher Scientific, USA) by standard validated protocols defined by the contractor (Pharmacelsus, Germany).

2.7. Animals

Forty-eight male Sprague Dawley rats, age between 12 and 14 weeks, divided in 4 groups were used for the experiments. They were bred and maintained in cages made of Makrolon filled with autoclaved woodchips in the Core Unit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. Food and water in bottles were available ad libitum and reared under normal animal facility conditions (temperature: 22 ± 2 °C; humidity: 55 ± 5%; 12 h artificial light/12 h dark cycle: light on at 7:00 a.m.). All procedures were carried out according to the guidelines of the Ethics committee, Medical University of Vienna, and were approved by Federal Ministry of Education, Science and Culture, Austria (BMWFW-66.009/0114-WF/II/3b/2014). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.8. Hole-board apparatus and procedure

Hippocampus-dependent spatial learning and memory was evaluated by a hole-board task as previously described with a small modification [51,52]. The hole-board setup consisted of a black plastic

board (1 m × 1 m) with 16 regularly arranged holes (7 cm in diameter and 7 cm deep) and was surrounded by plexiglass walls 30 cm in height (from BiObserve, St. Augustin, Germany). The walls were marked with different black and white cues at each side. Four out of 16 holes were baited in a fixed pattern with standard food pellets (dustless precision pellets, 45 mg, Bio-Serv, Frenchtown, NJ, USA). Beneath the hole-board there was a second board on which food pellets were scattered randomly to mask olfactory cues. The apparatus was kept at an elevation of 80 cm above the floor in a room with indirect illumination by floor positioned lamps directed to the ceiling providing equal light intensities in all corners. A camera fixed to the ceiling viewing the experimental area monitored trials and videos were stored digitally.

Rats were kept in their home cages within the testing room throughout the experiments. They were handled for 15 min each day for adaptation 3 consecutive days before habituation. The body weight of the animals was recorded from first day of handling throughout the experiment. The animals were food-deprived (receiving 5–6 g food (ssniff Spezialdiäten GmbH) per day and ad libitum water) to decrease the body weight to 80–85% of free feeding weight and maintained the same throughout the experiment.

Prior to the start of the training on the fourth and fifth day of food deprivation, rats were given two habituation sessions in order to familiarize them to the hole-board setup and to the reward system. On the first day of habituation, pellets were placed all over the board as well as in the holes and rats were allowed to explore the apparatus and eat the food for fifteen minutes, whereas on the second day, pellets were placed only in the holes and exploration/eating time was reduced to five minutes. After free exploration of the apparatus the animals were carefully picked up and taken back to the home cage.

During three days of training and testing (five trials on day 1, four trials on day 2, and the retention trial on day 3) a fixed pattern of baited holes (4 out of 16) with a 20 min inter-trial interval was maintained throughout the experiment for all rats to assess working memory, while the other arms were always left un-baited in order to test reference memory. The training began at 9:30 a.m. and the retention trial (day 3) began 24 h after the last trial on day 2 (10:30 a.m.). Each trial started by placing the rat at one of the corners of the hole-board and the start position changed subsequently in the following trials. A trial was stopped after 2 min or when an animal had found all 4 pellets. The board was cleaned with 1% Incidin® after each trial in order to remove any olfactory cues. The time needed to find all of the pellets (latency), the working-memory errors (i.e., visiting a hole that was baited but had already been visited, with the pellet picked up during a specific trial) and the reference-memory errors (i.e., visiting an unbaited hole) were counted. The memory index was calculated for working (first entry into baited holes/total visits of baited holes)(WMI) and reference memory (total visits of baited holes/total visits of all holes)(RMI). Thus a value of 1 represents no error reflecting best performance whereas zero errors combined with no hole visits indicate the index as zero. On the last day, one hour after the retention trial, animals were deeply anaesthetized with CO_2 and sacrificed by cervical dislocation. Brain tissues were quickly removed, hippocampi were rapidly dissected on a cold plate set at 4–6 °C and CA1, CA3 and DG were micro-dissected from left and right hippocampi under a stereoscope as described previously [53]. The tissues were stored separately at −80 °C for further biochemical analysis.

Untrained/yoked rats underwent similar handling, habituation and food restriction regimen and spent the same amount of time in the hole-board as their trained counterpart rats except there was no food during the trials, thus minimizing the likelihood of learning to associate the cues with the reward.

2.9. Drug administration

CE-123 was freshly dissolved in 100% DMSO and administered *via* intraperitoneal injection everyday 30 min before the start of the behavioral experiment throughout the training sessions. Rats received 1 mL/kg drug or vehicle (DMSO) administered intraperitoneally in doses of 1 and 10 mg/kg body weight. Drug treatment did not impair general health condition such as movement, gait, salivation, sedation, tremor, fur condition, convulsion, diarrhoea, etc. (Supplementary data).

2.10. Behavioral tests

Behavioral tests including Open Field [54], Elevated Plus Maze [54], Forced Swim Test [55,56] and Rota Rod [54,55] were used to determine potential effect of CE-123 on anxiety-, exploratory behavior, anti-depressant effect and motor function. A neurological observational battery [54] was applied to reveal defects in gait or posture, changes in muscle tone, grip strength, visual acuity and temperature. All procedures are described in details in Supplementary data.

2.11. Synaptosome isolation

Hippocampal subregions were homogenized with pipette tips in a Syn-PER synaptic protein extraction reagent (Thermo Scientific, Rockford, IL, USA) containing a Protease Inhibitor Cocktail (PIC, Roche Molecular Biochemicals) and a Phosphatase Inhibitor Cocktail (PhosStop, Roche Molecular Biochemicals). The homogenate was centrifuged at 1200 × *g* for 10 min to remove cell debris. The resulting supernatant was centrifuged at 15,000 × *g* for 30 min. The synaptosome pellets were resuspended in SDS-buffer (50 mM TrisHCl pH 8.0, 150 mM NaCl, 1% SDS, 1x PhosStop, 1x PIC). Protein concentration was estimated using a bicinchoninic (BCA) assay kit (Pierce, Rockford, IL, USA) according to manufacturer's instruction.

2.12. Immunoblotting

Protein levels of D1R, D2R, D5R, DAT, DATp were determined using a standard western blotting procedure [32]. Polyclonal rabbit anti-D1R (1:5000, GeneScript, USA), rabbit anti-D2R (1:5000, GeneScript), rabbit anti-D5R (1:3000, ab181623, Abcam), rabbit anti-DAT (1:3000, GeneScript) and rabbit anti-DATp (1:3000, ab183486, Abcam) antibodies were used. Specific peptide sequences were used for immunization and custom-production of the affinity-purified rabbit polyclonal antibodies D1R (-TSTMDEAGLPAERD-), D2R (-NWSRPFNGSEGKAD-) and DAT (-TNSTLINPPQTPVEAQERETW-) (GenScript, Piscataway, NJ, USA) and specificity of antibodies was determined [31]. Five to thirty micrograms of each sample were incubated at 37 °C for 30 min prior to separation on 10% SDS-PAGE. For immunoblotting, proteins were transferred to PVDF membranes (GE Healthcare), blocked for one hour at 22 °C with 5% non-fat milk in TBS containing 0.1% Tween-20 (0.1% TBST), and incubated with primary antibodies for 16 h at 4 °C. All antibodies were incubated in 5% non-fat dry milk in 0.1% TBST. Membranes were then washed six times for 10 min in 0.1% TBST. An HRP-conjugated anti-rabbit secondary antibody (1:10,000, ab191866, Abcam) was used and blots were visualised with ECL solution (#170-5061, BIO–RAD). Immunoreactive bands were imaged on films, digitized at a resolution of 600 d.p.i., and quantitated using ImageJ software. As loading control membranes were stained with PhastGel BlueR (GE Healthcare) according to the manufacturer's instruction. Immunoblot data were normalized to corresponding whole-lane densitometric volumes of total protein-stained membranes as previously described [57].

2.13. Statistical analysis

Data were analyzed by unpaired Student's *t*-test when comparing only two groups and with two-way ANOVA followed by Dunnett's or Bonferroni's *post hoc* test (as indicated in each figure legend), when comparing more than two groups (GraphPad Software, San Diego CA, USA). Non-linear regression analysis was carried out for reuptake and release assays. The probability level of $P < .05$ was considered as

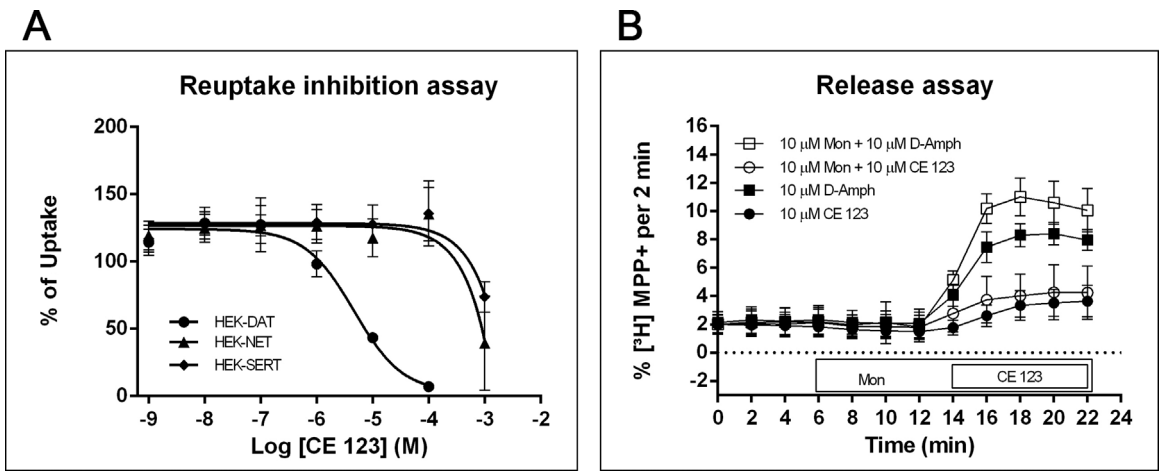


Fig. 2. (A) Reuptake inhibition of [3H]monoamines by CE-123. Inhibition of [^{3}H]DA, [^{3}H]MPP + and [^{3}H]5-HT reuptake by increasing concentrations of CE-123 in HEK293 cells stably expressing human isoforms of DAT, NET and SERT. Unspecific uptake was determined by using 10 µM mazindole for HEK-DAT and HEK-NET and 10 µM paroxetine for HEK-SERT. The percentage of maximum uptake was determined by using 1% DMSO in KHB. (B) DAT-release assay. The release assay was performed in HEK-DAT cells. Cells were grown on PDL-coated coverslips, treated with 0.05 µM [^{3}H]MPP + at 37 °C for 20 min and washed with KHB for 40 min in superfusion chambers. The first three (baseline) and next four fractions were without any compounds and with 10 µM monensin, respectively. Final five fractions were with either 10 µM CE-123 or 10 µM D-amphetamine. Non-linear regression analysis was carried out by using GraphPad Prism 5. Values are presented as mean ± SD.

statistically significant. Details are described in the corresponding sections.

3. Results

3.1. CE-123 strongly inhibits DAT-mediated dopamine reuptake without acting as a substrate

CE-123 was characterized in HEK293 cells expressing cloned human transporters. A reuptake inhibition assay was used to determine the efficacy of CE-123 to block the uptake of substrates by different monoamine transporters DAT, NET and SERT ([^{3}H]DA, [^{3}H]MPP$^+$ and [^{3}H]5-HT, respectively). CE-123 strongly inhibits DAT-mediated dopamine reuptake (IC_{50} = 4.606 µM ± 0.140) whilst inhibition of NET and SERT is negligible (Fig. S2A, Supplementary data).

The DA-release assay was performed to examine whether CE-123 acts as a substrate and induces release of [^{3}H]MPP + from HEK-DAT cells. 10 µM of CE-123 was used while 10 µM D-amphetamine serving as a positive control was assayed under the same conditions. Fig. 2B shows that CE-123 only negligibly induces DAT-mediated substrate efflux (Supplementary data). To facilitate reversal of normal transporter flux and unambiguously distinguish inhibitor from releaser [58,59], cells were pre-treated with 10 µM monensin. Monensin pretreatment did not increase CE-123-induced efflux of [^{3}H]MPP + , whereas efflux induced by DAT substrate D-amphetamine was significantly enhanced (Fig. 2B). The observation from HEK-DAT cells clearly indicates that CE-123 specifically blocks DAT without acting as a substrate.

3.2. Docking of CE-123 into DAT homology model

To characterize the CE-123 binding site in the DAT, we carried out a docking study of the two enantiomers with a DAT homology model derived from *drosophila* [43,60] (Fig. 3). A binding site of CE-123 enantiomers overlaps with the substrate-binding pocket in the center of DAT that is also the binding site for cocaine [44], modafinil [28] and benztropine analogues [61]. The highest scored ligand positions are shown in Fig. 3. The top-ranked binding poses revealed significant similarity between the binding modes of the enantiomers, although there are differences in the interacting amino acids: *e.g.* ASP476 is only present for S-CE-123 and ALA81 is only present as an interaction partner for R-CE-123. A closer look at the pharmacophore features revealed that the interactions are unspecific hydrophobic interactions. These differences are most likely due to small coordinate deviations between the two structures and do not reflect an independent and different binding mode. A common structural feature of CE-123 and modafinil is the lack of ionic interaction with negatively charged ASP79, an interaction demonstrated in cocaine binding. No direct differences are seen due to this interaction in the docked poses of S/R-CE-123 and cocaine, however.

3.3. CE-123 permeates across the blood-brain barrier in vitro

The cerebEND cell line possesses principal features of the blood-brain barrier (BBB) *in vivo*. In the present study cerebEND cell layers were used as an *in vitro* model to study the ability of CE-123 to cross the BBB. Transport studies across cerebEND cell layers revealed a permeability coefficient PCcell for CE-123 of 30.31 ± 6.94 µm/min (Fig. 4). Normalization of permeability data to internal control Diazepam resulted in a ratio of 0.87 ± 0.04 for CE-123. Thus, significant BBB permeability could be expected for CE-123, since Diazepam is known as a well permeating compound. Based on these findings following *in vivo* studies were accomplished.

3.4. Plasma, CSF and brain levels of CE-123

Next, we examined whether the data obtained in the *in vitro* model also holds true in an animal experiment; hence, we tested whether CE-123 can cross the BBB in the rat. We assessed in a pharmacokinetic study, plasma, CSF and brain concentrations of CE-123 or R-modafinil at 15 min, 1 and 7 h after the dose. Table 1 shows plasma, CSF and brain levels of drugs after a single intraperitoneal administration of CE-123 and R-modafinil at 10 mg/kg. For brain, the levels of CE-123 and R-modafinil were 4.4 ± 0.5 and 1.1 ± 0.3 µg/g, respectively, at 15 min, and 2.0 ± 0.4 and 0.1 ± 0.0 µg/g at 1 h; for CSF the respective values were 0.3 ± 0.1 and 0.5 ± 0.2, and 0.3 ± 0.04 and 0.1 ± 0.03 µg/mL; for plasma the respective values were 7.7 ± 1.1 and 2.3 ± 1.0, and 3.0 ± 0.9 and 0.2 ± 0.1 µg/mL. 7 h after the injection CE-123 was still detected in plasma, CSF and brain (Table 1). The brain level of 4.4 µg/g at 15 min indicates rapid brain entry for our compound. Moreover, the different plasma, CSF and brain levels of CE-123 and modafinil at the 15-min and 1-h time point demonstrates that CE-123 has a higher elimination rate constant than R-modafinil.

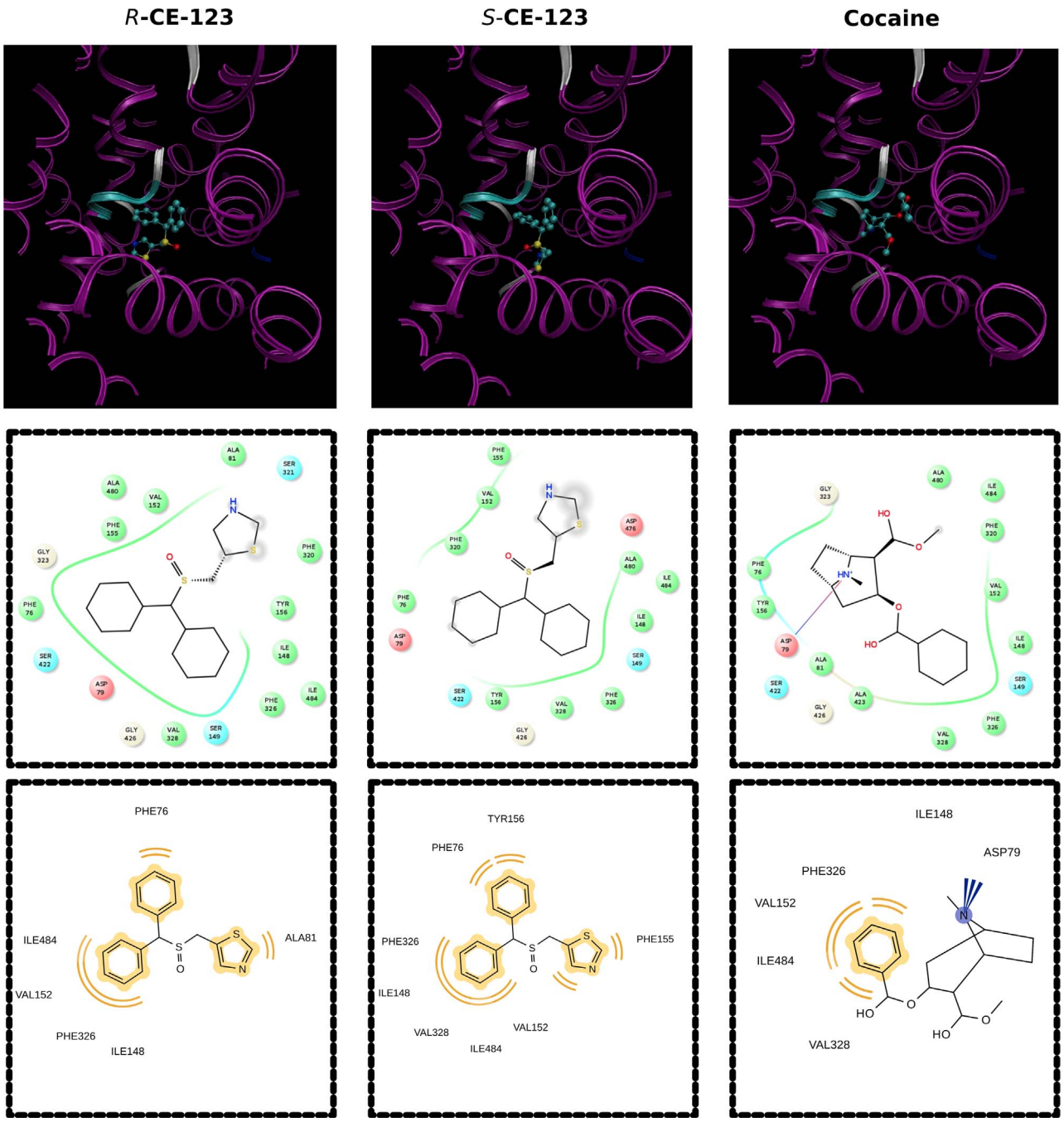
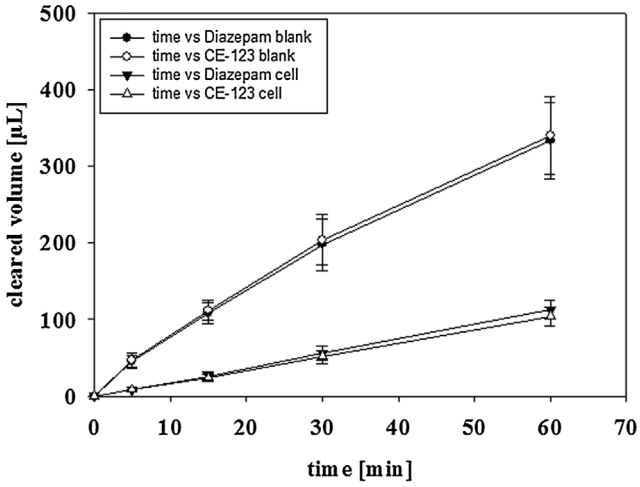


Fig. 3. Docking of R/S-CE-123 and cocaine into DAT homology model. The highest scored pose of R-CE-123, S-CE-123 and cocaine are shown in the upper panels as well as the ligand interaction diagram showing the interactions between the ligands and the surrounding amino acids in the middle panels. In the ligand interaction diagram the residues are represented as colored spheres (green = hydrophobic, cyan = polar, red = negatively charged, purple = positively charged) labeled with the residue name and number. Pharmacophore features are shown in the bottom panels indicating that – with the exception of an ionic interaction (shown in blue on cocaine) – only hydrophobic interactions (shown as yellow regions) are present. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Spatial hole-board training

The effect of CE-123 on spatial learning and memory was evaluated using a hole-board procedure with intraperitoneal administration of drug (1 mg/kg and 10 mg/kg) or vehicle. Working memory indices (WMI) and reference memory indices (RMI) were calculated for all training trials and used for analysis. The analysis of RMI and latency was performed for each day separately as they represent different phases of training [52]. A two-way ANOVA for repeated measures on RMI revealed a significant trial effect ($F_{4,48} = 17.88$, $P < .0001$) and trial x treatment effect ($F_{8,96} = 2.915$, $P < .0059$), but no significant treatment effect ($F_{2,24} = 2.177$, $P > .05$) at day 1. At day 2 a significant trial effect ($F_{3,36} = 10.35$, $P < .0001$) and treatment effect ($F_{2,24} = 4.683$, $P = .0192$), but no significant trial x treatment effect ($F_{6,72} = 1.765$, $P > .05$) was observed. The Dunnett's *post hoc* test revealed a significant difference between the CE-123 at 1 mg/kg and vehicle treated groups at day 2 ($P = .0294$). A one-way ANOVA at day 3 (consolidation phase) resulted in a significant treatment effect ($F_{2,36} = 12.38$, $p < .0001$). Trial-by-trial analysis revealed that reference memory indices were significantly higher on day 1, trial 3 at 10 mg/kg ($P = .0386$), trial 4 ($P = .0068$ at 10 mg/kg and $P = .0003$ at 1 mg/kg), at the end of the acquisition phase (day 2, trial 7, $P = .0147$,



	CE-123	Diazepam
PC_{all} [µm/min]	19.48 ± 2.22	21.24 ± 2.42
PC_{cell} [µm/min]	30.31 ± 6.94	34.91 ± 7.61
Ratio Diazepam	0.87±0.04	1

Fig. 4. Permeability of CE-123 across a blood-brain barrier *in vitro* model. Differences of cleared volume *versus* (*vs.*) time curves between the transport across the blank inserts and inserts with cultivated cerebEND cells confirmed the significant barrier formed by the blood-brain barrier *in vitro* model. Resulting permeability coefficients PCall [µm/min] revealed similar transport properties of CE-123 in comparison to Diazepam. Substraction of the blank values led to permeability coefficient PCcell [µm/min] values reflecting the permeability of the compounds only across the cell layer. The data are presented as mean ± SD, n = 5.

trial 9, P = .021 at 1 mg/kg) and during retrieval (day 3, trial 10, P < .0001 at 1mg/kg) in CE-123 treated groups compared to vehicle treated group (Fig. 5A).

A two-way ANOVA for repeated measures on latency revealed a significant trial effect ($F_{4,48}$ = 5.333, P = .0012) and treatment effect ($F_{2,24}$ = 4.009, P = .0315), but no significant trial × treatment effect ($F_{8,96}$ = 1.147, P > .05) at day 1. At day 2 a significant trial effect ($F_{3,36}$ = 16.35, P < .0001) and treatment effect ($F_{2,24}$ = 4.131, P = .0287), but no significant trial x treatment effect ($F_{6,72}$ = 0.7827, P > .05) was observed. The Dunnett's *post hoc* test revealed significant differences between vehicle and CE-123 for both 1 mg/kg (P = .0402) and 10 mg/kg (P = .0477) doses at day 1, and between vehicle and CE-123 at 1 mg/kg (P = .0193) at day 2 (Fig. 5B). A one-way ANOVA at day 3 resulted in a significant treatment effect ($F_{2,36}$ = 8.707, P = .0008). The time to find all pellets was significantly decreased on day 1 (trial 2; trial 3; trial 4 and trial 5), on day 2 (trial 7 and trial 9) and during retrieval (day 3, trial 10, P = .0006) when compared drug-treated groups to the vehicle-treated group.

A two-way ANOVA analysis on WMI did not show significant differences between CE-123 treated groups and vehicle treated group (Fig. 5C). However, this may be due to a ceiling effect.

3.6. CE-123 does not affect coordinative function and locomotion

Potential neurotoxic effects of the CE-123 were examined in the open field (OF), the elevated plus maze (EPM), the forced swim test (FST) and the rota rod test. After 3 days of handling, animals were administrated CE-123 at a dose of 10 mg/kg or vehicle for the following 10 days. From the 5th day of drug administration behavioral tests were conducted in the following order: OF, EPM, observation battery, rota rod and FST. The motor coordinative function was evaluated through the rota rod test. Administration of CE-123 at 10 mg/kg dose did not produce any significant effect on motor coordination and locomotion (OF) (Supplementary data). Behavioral tests assessing anxiety-like behavior (OF and EPM) and a test used for evaluation of anti-depressant effects (FST) did not show significant changes between the drug- and vehicle-treated groups (Supplementary data).

3.7. Training and drug administration modulates synaptic dopamine receptor levels in hippocampal subregions

Because DA receptors are important regulators of DAT function, as well as downstream processes involved in memory function, we decided to examine dopamine receptor level changes in synaptosomal fractions of three major hippocampal subregions after treatment with the selective DAT inhibitor CE-123 (1 mg/kg). D1R protein levels were significantly increased in CA1 and CA3 hippocampal subregions following CE-123 treatment and training in the hole-board task. A simple administration of drug (no training) led to significant increase of D1R protein levels in CA1 and DG, whereas D1R protein levels in CA3 were significantly decreased (Fig. 6).

D2R protein levels were significantly decreased in CA1 in the trained group (P = .0232), whereas no significant changes in D2R protein levels were observed in other hippocampal subregions.

In DG D5R protein levels were significantly increased (P = .0375 for trained groups, P = .0325 for yoked groups) in the drug-treated group compared to the vehicle-treated group, neither in CA1, nor in CA3 significant changes in D5R protein levels were shown.

3.8. CE-123 modulates synaptic dopamine transporter levels in hippocampal subregions

We compared total DAT protein levels and phosphorylation of DAT at Thr53 (DATp) in the hippocampal subregions of drug-treated and vehicle-treated animals. Results have revealed altered dopamine transporter levels in hippocampal subregions following drug administration.

DA transporter reuptake inhibition by CE-123 in CA1 leads to increased phosphorylation of DAT at Thr53 in the trained group, whereas total DAT protein levels were not changed (Fig. 7; trained groups, P = .0002; yoked groups, P = .976).

In DG of trained animals total DAT protein levels were significantly increased in the drug-treated group as compared to vehicle-treated group (P < .0001).

In CA3 DATp protein levels were significantly decreased in the drug-treated group compared to vehicle-treated group in the yoked groups

Table 1
Plasma, CSF and brain levels of CE-123 and R-modafinil in Sprague Dawley rats after a single intraperitoneal administration of 10 mg/kg.

Time (min)	Plasma (ng/ml)				CSF (ng/ml)				Brain (ng/g)			
	CE-123		R-modafinil		CE-123		R-modafinil		CE-123		R-modafinil	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
15	7701.9	1097.4	2277.7	1030.8	606.9	52.6	524.5	197.0	4398.6	506.9	1088.8	259.1
60	2972.6	859.3	199.5	86.8	279.2	35.6	113.5	32.4	1987.7	354.3	117.7	4.9
420	64.1	43.4	0.0	0.0	4.9	3.0	1.2	2.0	20.9	21.7	0.0	0.0

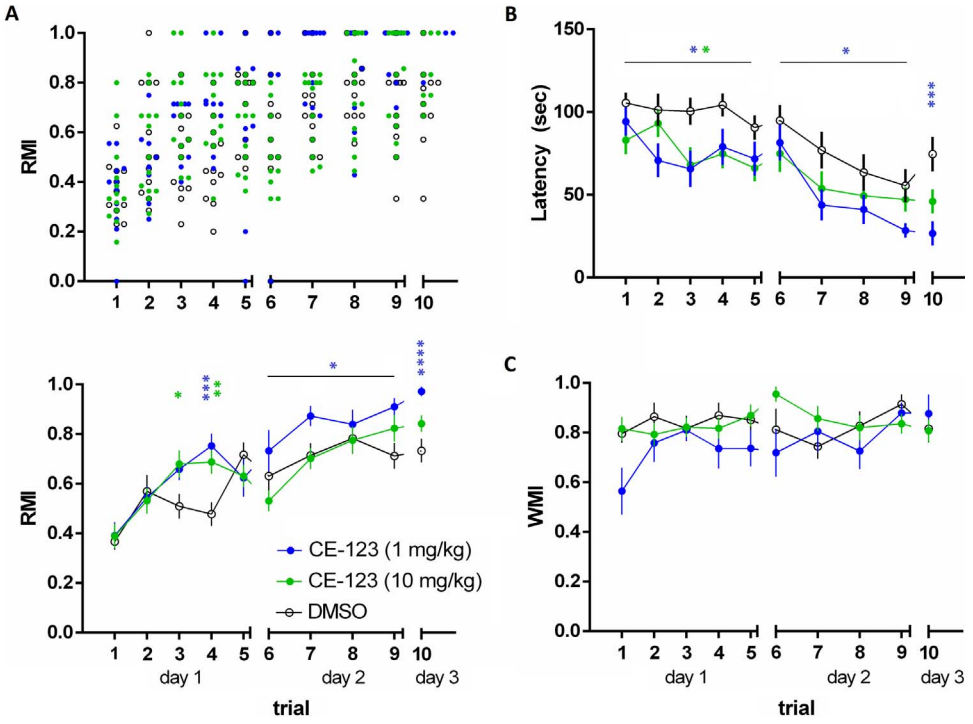


Fig. 5. (A) The performance was significantly better with improved RMI in the drug treated groups compared to vehicle during acquisition phase on day 1 and day 2 and during retrieval on day 3 of the hole-board testing (B). Latency to complete the task was significantly decreased in the drug treated groups compared to vehicle treated group during acquisition phase on day 1 at both doses and on day 2 and day 3 at dose of 1 mg/kg. (C) Working memory indices. Mean and SEM are shown in the graph for every trial. The data were analyzed using two-way ANOVA with Bonferroni's or Dunnett's *post hoc* analysis for day 1 and 2 and one-way ANOVA for day 3. (*P < .05; **P < .01; ***P < .001; ****P < .0001; n = 13).

(P = .0205); whereas DATp protein levels in trained groups were comparable.

4. Discussion

In comparison to other psychostimulants including the lead compound modafinil, CE-123 with respect to NET, DAT and SERT is an apparently very selective DAT inhibitor with negligible binding to NET and SERT. The very selective inhibition of the DAT may underlie the fact that no neurological adverse side effects as *e.g.* coordination disturbances were observed after drug treatment in contrast to modafinil (racemate), which displayed impairment in motor coordination on the rota rod (data not shown). These effects are usually determined by kinetic characteristics and target specificity of the compound; higher doses or multiple targets usually exert adverse drug reactions [62].

The lack of cocaine-like locomotor stimulation could be also attributed to a DAT binding mode that is distinct from that shown by cocaine and cocaine-like compounds. Examples are modafinil and benztropine-like ligands, known to interact with an inward-facing conformational state [28,63–65]. Moreover, several atypical DAT inhibitors have been reported to attenuate cocaine-induced behavioural effects *in vivo* [66–69], suggesting a potential application of our compound as an anti-relapse medication. The docking experiments from the current study revealed that CE-123 lacks ionic interaction with negatively charged ASP79, an interaction demonstrated in cocaine binding. We could not show binding to inward-facing DAT conformation, however.

In order to reach its target in the CNS, CE-123 has to overcome the blood-brain barrier. Before *in vivo* studies were conducted, results of *in vitro* transport studies suggested that CE-123 could permeate in a manner similar to diazepam and in comparison to a previous study also similar to modafinil [31].

The structural modification in the design of CE-123 includes substitution of carboxyl-amide moiety of modafinil with a thiazole attached on position 5 *via* a methylene bridge to the sulfoxide moiety (Fig. 1). A substitution of the carboxyl-amide group with the heterocycle (thiazole) could result in the increased metabolic stability of the compound. Pharmacokinetic analysis indicated rapid brain uptake with brain levels higher than for R-modafinil, moreover, CE-123 was detected in the brain at 1 h post-administration in levels almost 20 times higher than those achieved following administration of R-modafinil. These data along with a memory enhancing effect observed in the hole-board test indicate that CE-123 presumably permeates across the blood-brain barrier and approaches its sites of action *in vivo*.

Over the years modafinil has gained popularity for its reported pro-cognitive effects, which were extensively evaluated in healthy individuals and psychiatric disorders [20,21]. Studies involving animals reported variable effects on different neurocognitive domains including attention, executive functions, learning and memory. Working memory-enhancing effect of modafinil has been demonstrated by employing distinct spatial memory tasks [29,70,71]. Effects of modafinil on long-term memory after chronic or subacute administration has been reported as well [52,72,73].

The memory-enhancing potential of CE-123 was evaluated in a spatial hole-board task in male Sprague-Dawley rats. Animals learned the hole-board task as shown by the gradually decreasing number of errors and latency during training trials. CE-123 shows a unique enhancing effect on memory acquisition on day 1 after single administration prior to training at both drug concentrations. A second dose on day 2 prior to training shows an enhancing effect only at 1 mg/kg, same as for the retrieval phase. A dose-dependent effect on cognition, learning and memory consolidation was reported previously for classical psychostimulants [6,74], as well as for modafinil [71,73].

Moreover, significantly decreased latency in a treated group may not only refer to enhanced cognitive performance, but may also indicate increased motivation to perform a given task [75], consistent with previous reports on motivation-enhancing effect of DAT inhibitors [76,77]. However, the biological relevance of this behavioral result has to be proven in further studies.

It has to be mentioned that the observed memory enhancing effect can be also attributed to a rescuing effect. DMSO, a commonly used drug delivery vehicle, has demonstrable pharmacological and pathological effects on the central nervous system [78,79]. We have recently reported that DMSO reduced spatial memory performance and LTP magnitude, when compared to saline control. R-modafinil rescued spatial memory and dentate gyrus synaptic plasticity from impairing

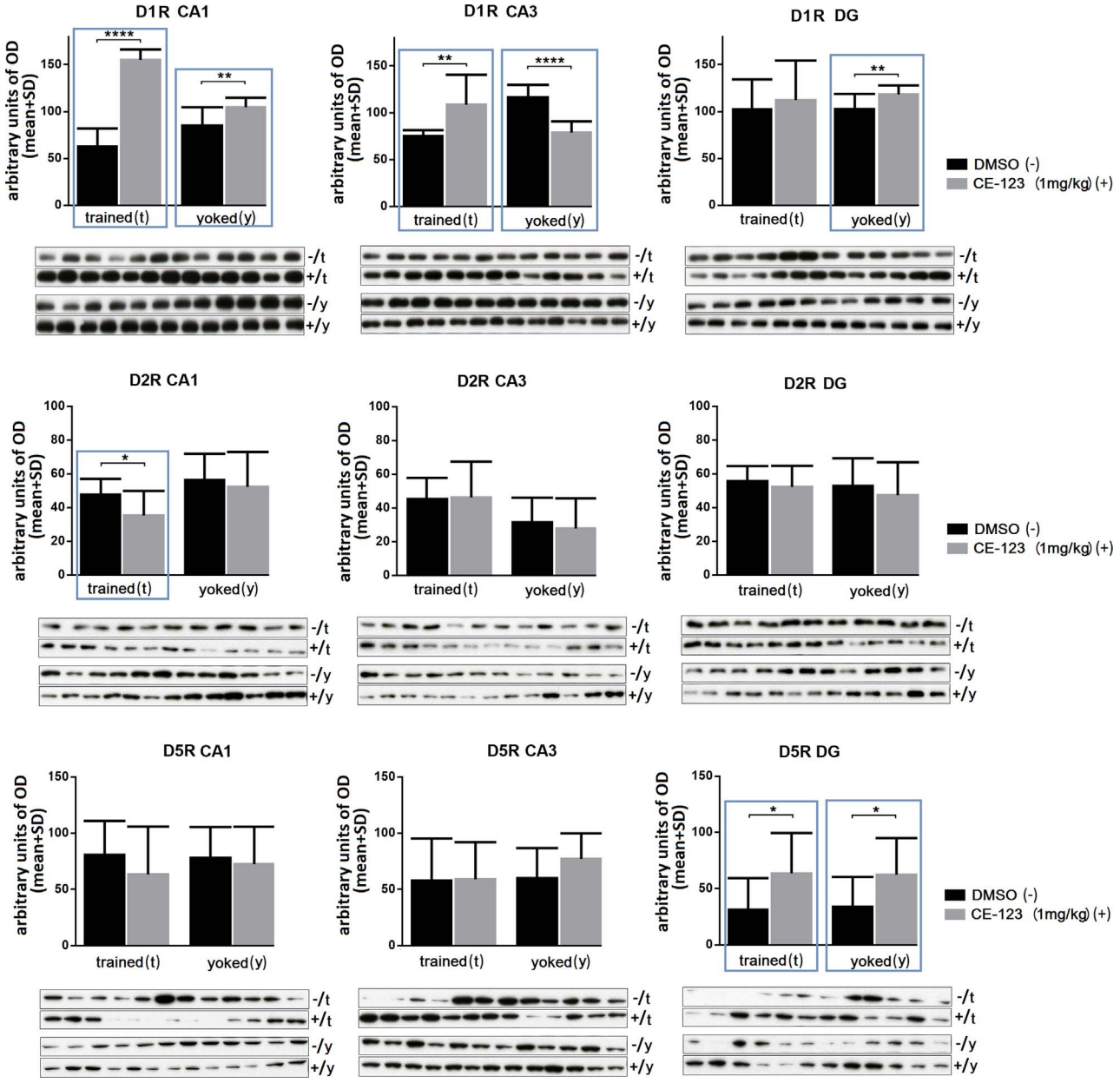


Fig. 6. CE-123-induced synaptosomal changes in dopamine receptor protein levels in hippocampal subregions CA1, CA3 and DG. Hippocampal synaptosomal proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. Apparent molecular weights were about 100 kDa for D1R, 50 kDa for D2R and for D5R. The data were analyzed by unpaired Student's *t*-test. *P < .05, **P < .01, ****P < .0001. Values are expressed as mean ± SD; n = 11–13.

effects of DMSO [52].

CE-123 had no effect on working memory performance, even though it was expected, probably due to a ceiling effect. To determine a full working memory-enhancing effects of CE-123, a more complex task would be required.

The hippocampus is a functionally complex brain region that plays a role in spatial navigation and memory. Each hippocampal subregion represents a distinct unit at the cellular, molecular and functional level and contributes differently to hippocampal information processing [80,81]. Dopamine neurotransmission affects hippocampal synaptic plasticity [82–84] and influences hippocampal-associated functions [85,86].

To study a possible mechanism of action, western blot analysis was used to quantify changes at the protein levels of DAT and dopamine receptors (D1R, D2R and D5R) in the synaptosomal fraction of the three major hippocampal subregions. Based on current knowledge, it is evident that D1-like receptors play a causal role in the establishment of information encoding and storage by gating hippocampal long term plasticity and may drive the qualitative processing of information being stored by the hippocampus [87]. Moreover, D1-like receptors seem to be key mediators of downstream effects subsequent to DAT inhibition. Several reports support this hypothesis. It has been shown that D1R is required for the majority of modafinil-induced effects on exploration [1], which is supported by the observation that D1/D5 antagonists attenuate modafinil-induced increases in activity and exploratory behaviour [1]. Modafinil and GBR-12909 induce an increase in motivation in a D1R expression-dependent manner [76]. With respect to spatial memory, D1/D5 antagonism impairs long-term spatial memory in rats [88] whereas D1/D5 agonism enhances spatial memory [88].

The current study revealed that CE-123 administration induces region- and training-specific changes in DA receptors protein levels. Drug treatment led to increase of D1R protein levels in CA1 and DG, however, in CA3 D1R protein level changes seems to be dependent on training and not simply reflect drug administration. In the DG drug

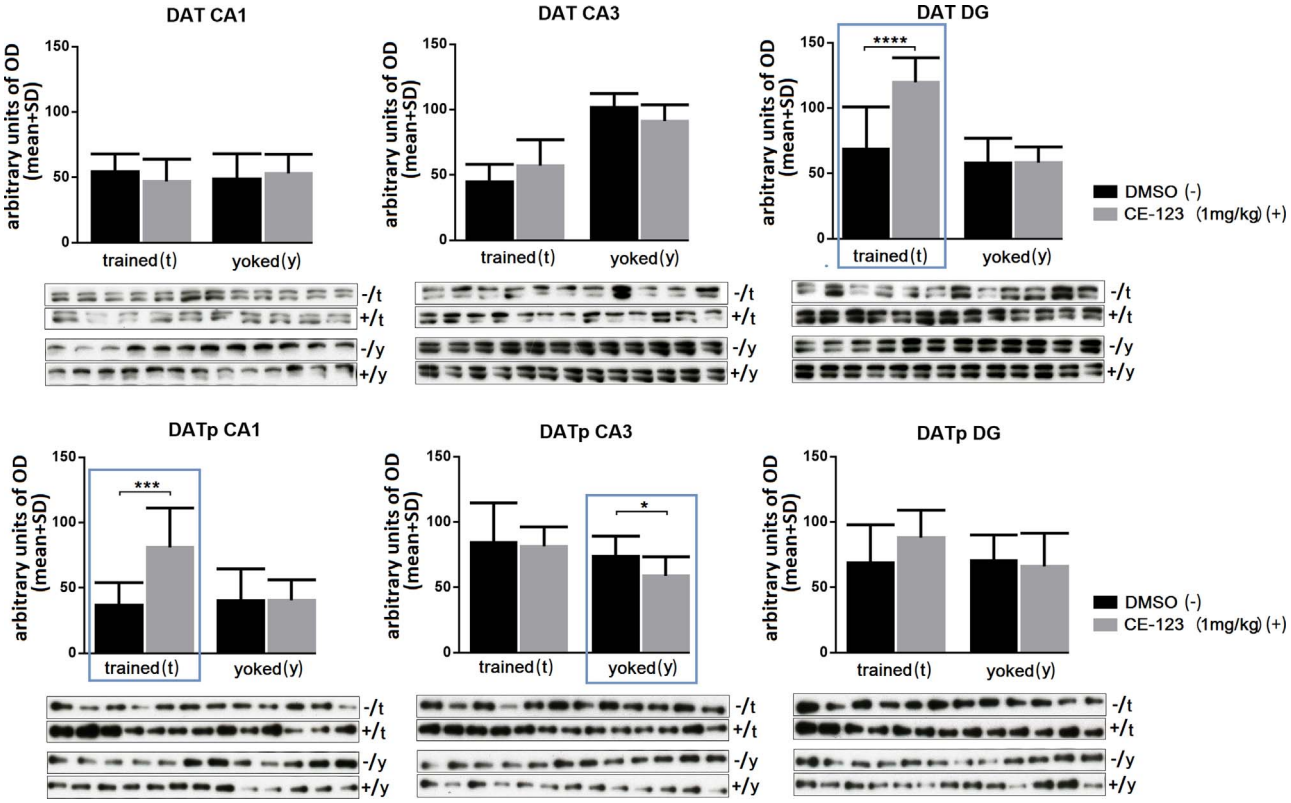


Fig. 7. CE-123-induced synaptosomal changes in DAT and DATp (Thr53) protein levels in hippocampal subregions CA1, CA3 and DG. Hippocampal synaptosomal proteins were separated by SDS-PAGE and immunoblotted with indicated antibodies. The apparent molecular weights were about 115 kDa for DAT and 75 kDa for DATp. Data were analysed by unpaired Student's *t*-test. *P < .05, ****P < .0001. Values are expressed as mean ± SD; n = 10–13.

administration leads to a significant increase of D5R protein levels that was training-independent. D1 and D5 receptors show differences in structural features, regional, cellular and subcellular distribution, probably predicting differences in receptor signalling as well as biological function [89]. Therefore, also different hippocampal subregions exerting distinct functions in information processing may be differently modulated by the activation of D1 and/or D5 receptors. We can only speculate that the cognition enhancing effect of DAT inhibitors, such as CE-123, may be due to drug-induced adaptations in the cellular mechanisms involving hippocampal D1/D5 receptor activation that in turn underlie the formation of a persistent memory trace in the hippocampus [90].

As already shown above, CE-123 directly modulates DAT function, however, dynamic regulation of surface density representing transport capacity may be involved as well [91]. Many psychostimulants influence DAT trafficking. Amphetamine increases internalization of DAT leading to a decrease of surface density [92]; in contrast, cocaine and methylphenidate increase DAT surface density [93,94]. Regulation of DAT surface expression is driven by ERK-dependent processes [95,96], which involve ERK-mediated phosphorylation of Thr53 regulating DAT insertion into the cell membrane. The membrane-proximal residue Thr53 which precedes Pro54 as the DAT ERK phosphorylation site [97] is the site playing a major role in the transport kinetic mechanism. DAT inhibitors such as nomifensine, modafinil and its analogues, appear to behave similarly in this mechanism as cocaine. We observed an increase of surface-associated functional DAT in CA1 in the treated/trained group. The findings indicate the presence of a rapid feedback mechanism associated with the phosphorylation state that regulates DA clearance by modulating DAT surface density. Therefore our compound may not only simply inhibit dopamine reuptake, but also prevent internalization of this transporter itself.

Previous studies have suggested that activation of the short D2R variant on presynaptic site initiates a signalling cascade to upregulate cell-surface DAT and enhance DAT function [95,98]. Own results show that CE-123-induced down-regulation of total D2R protein levels is training-dependent and occurs only in CA1. However, differentiation between the short and long D2R splicing variant would be required as they seem to have different neuronal distributions and functions.

Future studies with CE-123 need to address further behavioral, pharmacokinetic and molecular questions in several brain regions, including examination of interaction with a large array of different receptors and transporters, to unveil the specific and detailed molecular and cellular mechanism of action. Selectivity and the pharmacological profile may be improved by enantiomeric separation as follow up studies.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.01.032.

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