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A novel heterocyclic compound improves working memory in the radial arm maze and modulates the dopamine receptor D1R in frontal cortex of the Sprague-Dawley rat

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Highlights

- CE-125 is non-neurotoxic compound and specific reuptake inhibitor targeting the DAT and thus increasing dopamine levels in the synaptic cleft.
- CE-125 improves spatial memory performance and increases the WMI in a radial arm maze.
- CE-125 modulates the dopamine receptor D1R.

Abstract

A series of compounds have been shown to enhance cognitive function via the dopaminergic system and indeed the search for more active and less toxic compounds is continuing. It was therefore the aim of the study to synthetise and test a novel heterocyclic compound for cognitive enhancement in a paradigm for working memory. Specific and effective dopamine re-uptake

inhibition (DAT (IC50 = 4,1 ± 0,8 µM) made us test this compound in a radial arm maze (RAM) in the rat.

CE-125 (4-((benzhydrylsulfinyl)methyl)-2-cyclopropylthiazole), was tested for dopamine (DAT), serotonin and norepinephrine re-uptake inhibition by a well-established system. The working memory index (WMI) was evaluated in male Sprague Dawley rats that were intraperitoneally injected with CE-125 (1 or 10 mg/kg body weight). In order to evaluate basic neurotoxicity, the open field, elevated plus maze, rota rod studies and the forced swim test were carried out. Frontal cortex was taken at the last day of the RAM test and dopamine receptors D1R and D2R, DAT and phosphorylated DAT protein levels were determined.
On the 10th day both doses were increasing the WMI as compared to the vehicle-treated group. In both, trained and treated groups, D1R levels were significantly reduced while D2R levels were unchanged. DAT levels were comparable between all groups while phosphorylated DAT levels were increased in the trained group treated with 1 mg/kg body weight.
CE-125 as a probably non-neurotoxic compound and specific reuptake inhibitor was shown to increase performance (WMI) and modulation of the dopaminergic system is proposed as a possible mechanism of action.

Key words:

Dopamine reuptake inhibition

4-((benzhydrylsulfinyl)methyl)-2-cyclopropylthiazole

Radial arm maze

Sprague-Dawley rat

Working memory

1. Introduction

The dopaminergic system plays a key role for learning and memory [1] and a series of publications have been reporting enhancement of learning and memory via the dopaminergic system including D1 receptor agonists, DOPA or DOPAergics [2, 3, 4, 5, 6]. Dopamine re-uptake inhibition by DAT reuptake inhibitors is a useful pharmacological principle and a series of non-cocaine-non-metamphetamine-like DAT re-uptake inhibitors, compounds as e.g. modafinil were studied. And indeed, modafinil serves as a lead compound for synthesis of modafinil analogs and is well-studied in rodents and even in Humans.

Turner and coworkers [7], have shown cognitive enhancing effects of modafinil in healthy volunteers and Müller et al. [8] reported subtle effects on working memory in Humans. Minzenberg and Carter [9] reviewed neurochemical action and effects on cognition in rodents, healthy adults and across psychiatric disorders that are considered advantageous for cognitive processes. Rasetti and coworkers [10] have proposed that modafinil enhances the efficiency of prefrontal cortical cognitive information processing while dampening reactivity to threatening stimuli in the amygdala. Administration of modafinil improved working memory in metamphetamine-dependent individuals [11] and reliably enhanced task enjoyment and performance in several cognitive tests of planning and working memory [12]. Moreover, modafinil treatment along with cognitive training is associated with improved learning in healthy volunteers [13]. In rodents, modafinil restores memory performance in sleep deprived mice [14] and Shuman and coworkers [15] have demonstrated dose- and time dependent improvement of fear memory in the mouse. In the rat, modafinil was shown to enhance hippocampal-dependent but not frontal cortex-dependent spatial memory tasks -it even blocked LTP in frontal cortex [16]. A positive effect of modafinil on working memory was reported by Murphy and coworkers in the rat using a Morris Water Maze task [17]. Shanmugasundaram et al. [18] showed the task-dependent effect on memory in aversive learning paradigms and Karabacak et al. [19] revealed modafinil-mediated cognitive enhancement in a radial arm maze. Bezu and coworkers finally have shown that modafinil as well as levodopa reveal a drug-independent precise timing of spatial working memory modulation [20].

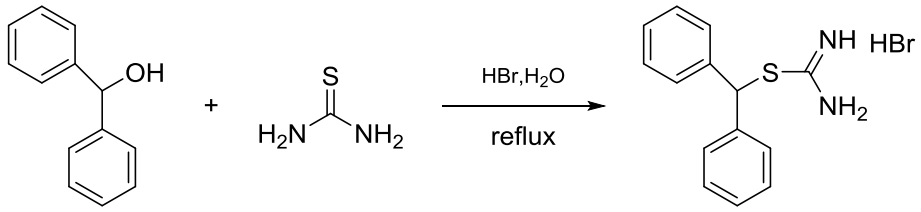
Given that modafinil and its analogs may have potential for enhancing certain forms of cognitive functions including working memory, it was the aim of the study to synthetise modafinil

analogs with high specificity and absence of neurotoxicity in order to evaluate their pharmacological properties in paradigms of working and reference memory.

2. Materials and Methods

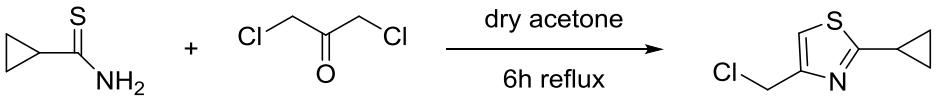
2.1. Synthesis of compound CE-125

2.1.1 Synthesis of [(diphenylmethyl)sulfanyl]methanimideamide



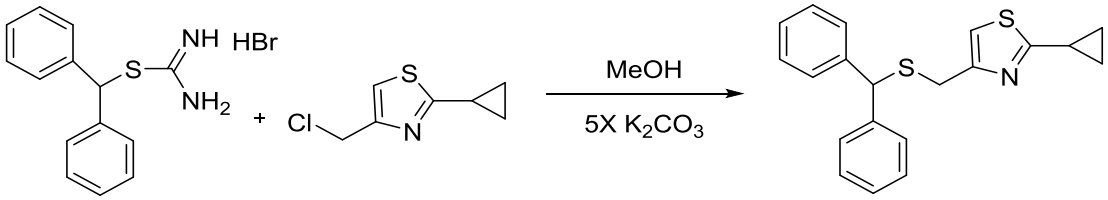
Diphenylmethanol (26 g, 0.14 mol) and thiourea (13.0 g, 0.17 mol) are added to a 1 L two-neck round bottom flask and 65 mL of water were added. The mixture is then heated to 95oC (an emulsion is obtained) and 52 g of 48% HBr (0.644 mol, 4.6 equivalents) is then gradually added during 30 min. The mixture is heated under reflux (106 – 107 ^{o}C) for 30 min and cooled to 80-85 ^{o}C. The mixture is then cooled in ice and a precipitate with crystals is formed. After filtration and washing with water a colorless crystalline substance is obtained. The product is then dried in vacuo. 19.24 g of the product were obtained as a white crystalline solid (yield: 74%).

2.1.2 Synthesis of 4-(chloromethyl)-2-cyclopropylthiazole



In a round bottom flask, 2 g (20 mmol) of cyclopropanecarbothioamide is dissolved in 60 mL of dry acetone and then 2.4 g (20 mmol) of 1,3-dichloropropan-2-one are added. The reaction mixture is then stirred under total reflux for 6 h and left to cool afterwards overnight at the room temperature. The product is then concentrated in vacuo and dried in high vacuum for one hour. Via this procedure 1.7 g of solid yellow product is obtained, that is subsequently used for further synthesis steps as such (yield: 49%).

2.1.3. Synthesis of 4-((benzhydrylthio)methyl)-2-cyclopropylthiazole

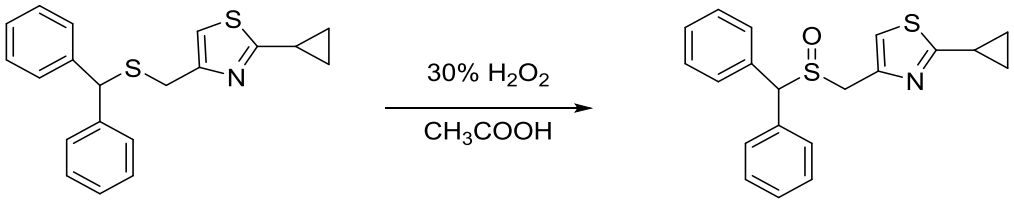


In a round bottom flask 3.23 g (10 mmol) of [(diphenylmethyl)sulfanyl]methanimideamide are dissolved in 100 mL of methanol. Subsequently, 1.7 g (9.8 mmol) of 4-(chloromethyl)-2-cyclopropylthiazole and 6.9 g (5 equivalents, 50 mmol) of potassium carbonate are added to the mixture. The mixture is left to stir for 2 days at room temperature.

Methanol is removed in vacuo and 150 mL of water are added. The reaction product is then extracted (3x) with 100 mL of ethylacetate. Organic phases are collected, combined, dried with Na_2SO_4, and filtered and the product is concentrated in vacuo.

The crude product is purified via flash column chromatography on silica gel. 5% methanol in dichloromethane is used as mobile phase. The separated product is concentrated via a rotary vacuum pump and cooled in a refrigerator overnight. By this procedure 2.45 g of a semi-solid product are obtained (yield: 72.6%).

2.1.4. Synthesis of 4-((benzhydrylsulfinyl)methyl)-2-cyclopropylthiazole (CE-125)



In a round bottom flask, 2.45 g (7.26 mmol) of 4-((benzhydrylthio)methyl)-2-cyclopropylthiazole is dissolved in 20 ml of glacial acetic acid. 0.75 ml (7.26 mmol) of 30% H_2O_2 is dropped into the solution and stirred for 12 hrs.

Acidity is neutralized with 5% sodium bicarbonate in ice. Reaction products are extracted (3x) with 100 mL of ethyl acetate. Organic phases are collected, combined, dried with Na_2SO_4, filtered and ethyl acetate is concentrated on a rotary evaporator.

A semi-solid brown product is purified via flash column chromatography on silica gel. 5% methanol in dichloromethane is used as a mobile phase. A crystalline product is obtained that is afterwards dried in a high vacuum. By this procedure 1.27 g of the solid white material was obtained (yield: 49.69%) The overall purity of the compound, determined by C18 analytical column-based HPLC method using reversed-phase conditions was 99.63% (Supplemental Figure 1).

$[M+H^+]$= 354.0981, $[M+Na^+]$= 376.0800 (Supplemental Figure 2).

1H NMR (500 MHz, CDCl3-d, 23°C): δ = 7.50 (C*H*-2,6, phenyl), 7.49 (C*H*-2,6, phenyl), 7.39 (2x C*H*-3,5, phenyl), 7.33 (2x C*H*-4, phenyl), 7.01 (s, 1H, C*H*, thiazole-5), 5.13 (s, 1H, C*H*), δ = 3.97, 3.75 (AB, 2H, CH_2), 2.32 (m, 1H, C*H*, cyclopropyl), 1.17 (d, 2H, CH_2 cyclopropyl), 1.09 (d, 2H, CH_2, cyclopropyl) (Supplemental Figure 3).

13C{1H}NMR (125.75 MHz, CDCl3-d, 23°C): δ = 173.98 (*C*-2, thiazole), 144.28 (*C*-4, thiazole), 116.90 (*C*-5, thiazole), 136.09 (*C*q-1, phenyl), 134.15 (*C*q-1, phenyl), 129.85 (*C*-2,6, phenyl), 129.07 (*C*-2,6, phenyl), 128.94 (*C*-3,5, phenyl), 128.60 (*C*-3,5, phenyl), 128.25 (*C*-4, phenyl), 128.17 (*C*-4, phenyl), 69.67 (*C*H), 51.33 (*C*H_2), 14.54 (*C*H, cyclopropyl), 11.32 (*C*H_2, cyclopropyl), 11.29 (*C*H_2, cyclopropyl) (Supplemental Figure 4).

2.1.5. Analytical Characterization (NMR, Mass Spectrometry, HPLC)

NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (UltraShield) using a 5 mm switchable probe (PA BBO 500SB BBF-H-D-05-Z, 1H, BB = 19F and 31P - 15N) with z axis gradients and automatic tuning and matching accessory (Bruker BioSpin). The resonance frequency for 1H NMR was 500.13 MHz and for 13C NMR 125.75 MHz. All measurements were performed for a solution in fully deuterated chloroform or methanol at 298 K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, NOESY, HSQC, and HMBC, were used as supplied by the manufacturer. Chemical shifts are referenced internally to the residual, non-deuterated solvent signal for chloroform 1H (δ 7.26 ppm) or methanol 1H (δ 3.31 ppm) and to the carbon signal of the solvent for chloroform 13C (δ 77.00 ppm) or methanol 13C (δ 49.00 ppm).

HRESIMS spectra were obtained on a maXis HD ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Samples were dissolved to 20 µg/mL in MeOH and directly infused

into the ESI source at a flow rate of 3 µL/min with a syringe pump. The ESI ion source was operated as follows: capillary voltage: 0.9 to 4.0 kV (individually optimized), nebulizer: 0.4 bar (N2), dry gas flow: 4 L/min (N2), and dry temperature: 200 °C. Mass spectra were recorded in the range of m/z 50 – 1550 in the positive-ion mode. The sum formulas were determined using Bruker Compass DataAnalysis 4.2 based on the mass accuracy ($\Delta m/z \leq 2$ ppm) and isotopic pattern matching (SmartFormula algorithm).

HPLC spectra were obtained on the LC-2010A HT Liquid Chromatograph device (Shimadzu Corporation, Tokyo, Japan). CE-125 was dissolved in MeOH at a concentration of 1 mg/mL. 10 µL of the sample were applied on Acclaim™ 120 C18 columns 2.1x150 mm (Thermo Scientific, Massachusetts, United States) under reversed-phase conditions (10-90% gradient of acetonitrile in water).

2.2. Reuptake inhibition assay by CE125

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

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 precoated with poly-D-lysine (PDL) (5x104 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, 3mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 2mM $MgCl_2 \cdot 6H_2O$, 5 mM D-(+)-glucose monohydrate, pH 7,3). Cells were preincubated 5 min in KHB containing different dilutions (0,1 µM, 1 µM, 10 µM, 0,1 mM and 1 mM) of CE 125. CE 125 was dissolved first in 99,9% dimethyl sulfoxide (DMSO) and subsequently diluted in KHB. Subsequently, cells were incubated in KHB containing the same dilutions of CE 125 with addition of 0,2 µM [3H]-dopamine (for HEK-DAT), 0,05 µM [3H]MPP+ (for HEK-NET) and 0,4 µM [3H]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 unspecific uptake in HEK-DAT and HEK-NET 10 µM mazindol were 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) [21, 22, 23].

2.3. Animals

60 male Sprague Dawley rats, aged between 12 and 14 weeks, were used in all experiments. They were bred and maintained in cages made of Makrolon and filled with autoclaved woodchips in the CoreUnit of Biomedical Research, Division of Laboratory Animal Science and Genetics, Medical University of Vienna. Food and water in bottles was available ad libitum. The room was illuminated with artificial light at an intensity of about 200 lx in 2 m from 5 a.m. to 7 p.m.. Experiments were carried out between 8 a.m. and 2 p.m. All procedures were carried out according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and 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.

2.4. Behavioural studies

To study the behavioural effects of CE-125 treatment, multiple experiments were carried out treating rats (10 per group) with an intraperitoneal dose of CE-125 10 mg/kg body weight for ten days and thereafter on the days of each test evaluation. The sequence of tests used was as follows: Open Field, Elevated plus Maze, Rota Rod, Neurological Observational Battery and Forced Swim Test. Simultaneously a group of 10 rats with DMSO treatment was studied as vehicle control.

2.4.1. Open field (OF)

Rats were observed by a video monitoring system consisting of a video camcorder coupled to the computational tracking system in an arena (100cm x 100cm long, with 40cm high walls) for 10 min. Each rat was placed in the center and following parameters were measured: a) pathlength, b) resting time, c) percent local movements, d) percent large movements, e) speed, f) times crossing the center and g) reversals [24].

2.4.2. Elevated plus maze (EPM)

Rats were observed for anxiety-like behavior. The EPM is made out of black PVC and consisted of 4 arms (each 50 cm long and 10 cm wide) arranged in the shape of a plus sign and elevated 70 cm above the floor. Two opposite arranged arms are open and the other two arms have 40 cm walls. All arms are interconnected by a 10 cm x 10 cm wide central area. Rats were observed with a video camcorder coupled to a computational tracking system in an arena. Rats were placed on the central area. Following parameters were recorded: a) the time spent in closed and open arms b) number of entries into the closed and open arms c) pathlength in closed and open arms. An entry was defined as having the rat placed all four legs into the box [24].

2.4.3. Rota rod

The Rota rod (Rota Rod "Economex", Columbus Instruments, Ohio USA) tests balance and coordination and is comprised of a rotating drum which accelerates from 4 to 40 rpm over the course of 5 min. The time at which each rat fell from the drum was recorded. Each rat received three pre-training trials. Subsequently, each rat completed three more consecutive trials and the longest time on the drum was used for analysis [25].

2.4.4. Neurological observational battery

The procedure follows the set up by Irwin [26]. A battery of tests was applied to reveal defects in gait or posture, changes in muscle tone, grip strength, visual acuity and temperature. To complete the assessment, vitally important reflexes were scored. In addition, during the manipulations, incidences of abnormal behavior, fear, irritability, aggression, excitability, salivation, lacrimation, were recorded [25].

2.4.5. Forced swim test (FST)

The test was performed as described previously [27]. The procedure consisted of two sessions, the pretest session and the test session, using the same apparatus and conditions (diameter 18 cm, height 40 cm, containing 23 cm of water maintained at 25 °C). During the pretest session, rats were forced to swim for 15 min; 24 h later, rats were placed in the same apparatus for 5 min, which is designated as test session. The duration of immobility during 5 min was recorded.

2.5. Radial arm maze (RAM)

2.5.1. Apparatus

The maze was made out of black plastic and kept at an elevation of 80 cm above the floor in a room with numerous visual cues. The central platform had a diameter of 50 cm with 12 arms (12 cm x 60 cm) projecting radially outwards. A plastic cylinder was used to restrict the movement of rats in the centre before the start of training. Lifting of the cylinder was controlled by a pulley system from the far end of the room.

2.5.2. Procedure

RAM training was performed as described in Levin et al. [28] and Timofeeva et al. [29] with some modification. In brief, rats were handled for 5 days for adaptation (30 min/day/rat) and also to reduce the body weight to 85%. Water was provided ad libitum during the training. The amount of food (ssniff Spezialdiäten GmbH, Germany) was provided to maintain a lean, healthy body weight of approximately 85% of the free-feeding weight during training. Out of 12 arms, eight arms were baited with food during the training and four remained unbaited. Before the start of the training, rats were given two habituation sessions in which food was placed all over the maze and rats were allowed to explore the maze and eat the food for five minutes. During the training session, the same arms were baited for each rat once at the beginning of each session to assess working memory, while the other four arms were always left un-baited in order to test reference memory. The pattern of baited and un-baited arms was consistent throughout testing for each rat but differed among rats. Each trial started by placing the rat onto the central platform, after 10 s the cylinder was lifted slowly and the rat was allowed to enter any arm. The session lasted eight minutes or until all eight baited arms were entered-whatever occurred first.

The Working Memory Index (WMI) was calculated by dividing the number of first entries into the baited arms by total entries into the baited arms.

The rats were given 10 training sessions, one trial per day. Yoked group rats underwent similar handling, habituation and food restriction as trained group rats except there was no food during the trials so that they do not form any memory. The training sessions were recorded with a computerized tracking video camcorder: 1/3 SSAM HR EX VIEW HAD [23] Six hours after the end of the tenth training animals were deeply anaesthetized with CO2 and killed by neck dislocation.

Brain tissues were quickly removed and frontal cortices were rapidly dissected on a cold plate set at 4-6°C and stored at -80 °C for further biochemical analysis.

2.6. Preparation of synaptosomes

Synaptosomal preparation steps were performed on ice and all buffers contained protease (Sigma Aldrich Cat.# 11836145001) and phosphatase inhibitors (Sigma-Aldrich Cat.#4906837001).

Frontal Cortices of rats were homogenized in Syn-PER™ Synaptic Protein Extraction Reagent. (Thermo Scientific Cat.#87793).

The homogenate was centrifuged at 1200 x g for 10 min. The resulting supernatant was then centrifuged at 15000 x g for 25 min. After centrifugation, pellets were stored at -80° C until use. Synaptosome pellets were resuspended in extraction buffer (150mM NaCl, 50mM Tris, pH 8.0) containing SDS at 1.5% (w/v) final concentration. Samples were solubilized at room temperature for 30 min with vortexing every 10 min. Subsequently, the estimation of protein content was carried out by using the BCA protein assay kit (Pierce. Prod.#23225). Samples were then aliquoted and stored at -20° C for immunoblotting.

2.7. Western Blotting

Protein samples were mixed with sample buffer (Loading Sample Buffer, Bio-Rad, Cat.#161-0747) and incubated for 20 min at 37 °C. Various amounts of proteins were loaded onto SDS-PAGE gels (5 μg for D1 and D2 , 10 μg for phosphorylated DAT, 20 μg for DAT. SDS-PAGE was performed in the Peqlab electrophoresis system using 4 % stacking and 8 % or 10% resolving gel according to the molecular weight. The running buffer contained 25mM Tris, 192mM Glycine and 0,1% SDS (w/v). The voltage was set to 50V for 30 min, 100V for 30 min and finally to 150V for 1h.

Synaptosomal proteins were transferred from SDS-PAGE gels to PVDF membranes followed by blocking for 1h with 5 % (w/v) non-fat dry milk in TBS [12,95 mM Tris (pH7.5),154 mM NaCl]. Membranes were then incubated overnight at 4°C with diluted rabbit anti-rat primary antibodies, which included DRD1 (GenScript, 1:5000), DRD2 (GenScript, 1:5000) [30], DAT (GenScript,

1:5000) and pDAT (Abcam, 1:4000, ab183486). For subsequent detection HRP-conjugated anti-rabbit IgG was used (Abcam, 1:20000, ab6721). Membranes were developed with Clarity™Western ECL Substrate (Bio-Rad, Cat.#170-5061). Optical densities of immunoreactive bands were measured using software Image J (NIH). For normalisation of measured densitometric values, Coomassie blue R-350 stained membranes were used as loading control [31, 32].

2.8. Statistics

For assessment of IC50 values in the reuptake assay non-linear regression analysis was carried out. For Radial Arm Maze, the ANOVA test was used while for the other behavioral tests and comparison between the western blot results of CE-125 treated and control groups a non parametric t-test was applied. The level of significance was set to $P<0,05$. All calculations were performed using a GraphPad Prism version 6.00 for Windows, GraphPad Software2, San Diego, CA, USA.

3. Results

3.1. DAT reuptake inhibition

A reuptake inhibition assay was used to determine the efficacy of CE-125 to block the uptake of substrates [3H]DA, [3H]MPP+ and [3H]5-HT by their respective transporters DAT, NET and SERT. CE-125 displayed much higher potency for inhibiting uptake DAT (IC50 = 4,1 ± 0,8 µM; n = 3) as compared to NET (IC50 = 687,2 ± 152,8 µM; n = 3) and SERT (IC50 = 436,1 ± 129,0 µM; n = 3). Results are shown in Figure 1.

3.2. Open field

As shown in supplemental Figure 5 there were no differences between treated and untreated animals for parameters total distance travelled, resting time, local and large movements, average velocity, number of crossings of the center, frequency of spontaneous changes of direction and time spent at the margin.

3.3. Elevated plus maze

As shown in supplemental Figure 6 there were no significant differences between groups determining parameters time spent in the left open arm, left closed arm, total distance travelled,

distance travelled in open and closed arms, time spend in the open and closed arm and the resting time.

3.4. Rota rod

As given in supplemental Figure 7 there were no significant differences between times spent on the revolving rod.

3.5. Neurological observational battery

As given in supplemental table 1 there were no significant differences between groups.

3.6. Forced swim test

As shown in supplemental Figure 8 there were no significant differences between groups when immobility times were estimated.

3.7. Radial arm maze

Memory performance of rats from the 1 mg, 10 mg and control groups was progressively improving over the training days. The working memory index (WMI) was calculated by dividing the number of first entries into the baited arms by total entries into the baited arms. In the control group, the WMI was gradually increasing until the 6th day and remained stable during the further training days; however, continuous improvement was observed in 1 mg and 10 mg/kg body weight CE-125 treated groups (Figure 2). Thus, the significant memory improvement on the 10th day was observed in the 10 mg and 1 mg group compared to the control group ($F(9, 270) = 3.011$; $p < 0.01$). Significant difference in the latencies was also observed over the training days in the 10 mg group ($F(9,270) = 2.800$, $p < 0.001$) (supplemental Figure 9). Bonferroni post hoc analysis comparing the vehicle group with the CE-125 treated groups at different time points showed significant changes.

The WMI was assessed in the 12-arm radial maze over 10 days. Rats receiving CE-125 performed better as compared to vehicle. Both, 1mg/kg and 10mg/kg body weight trained rats showed an increase in WMI as days progressed ($F(9, 270) = 3.011$; $P< 0.01$). Data were analyzed using ANOVA and Bonferroni post hoc test. Data are represented as mean ± SEM. ** $p < 0.01$, vehicle vs. 1mg/kg; # $p < 0.05$, vehicle vs. 10mg/kg.

Latency was assessed in the 12-arm radial maze over 10 days. Rats receiving CE-125, 10mg/kg performed better as compared to vehicle and 1mg/kg dose ($F(9, 270) = 2.800$; $P< 0.001$).

Data were analyzed using ANOVA and Bonferroni post hoc test. Data are represented as mean ± SEM. ### $P < 0.001$, vehicle vs. 10mg/kg (Supplemental Figure 9).

3.8. Immunoblotting Results

3.8.1. D1R (Dopamine receptor D1)

Levels of D1R were significantly increased by training in the vehicle-treated group and the group treated with 10 mg/kg body weight is shown in Figures 3a,c CE-125 treatment by 1 mg/kg body weight did not modify D1 levels i.e. no significant difference was observed between trained and untrained animals (Figure 3b). 1 mg and 10mg/kg body weight of CE-125 reduced D1R levels in trained rats (Figures 3d,e).While 1 mg/kg body weight of CE-125 had no effect on D1R levels, 10mg/kg body weight significantly increased D1R levels in untrained animals (Figure 3f,g).

3.8.2. D2R

Levels of D2R were comparable in the trained and untrained vehicle-treated groups (Figure 4a). While D2R levels corresponding to the abovementioned groups were comparable, D2R levels in groups treated with 1 mg and 10 mg/kg body weight of CE-125 were significantly decreased in untrained animals (Figure 4b,c).

3.8.3. DAT

Neither in trained nor untrained, treated or untreated animals, there were no statistically significant changes of this transporter (supplemental Figure 10)

3.8.4. pDAT

Training significantly reduced the activated, phosphorylated DAT: pDAT levels as shown in Figure 5a. Treatment of trained animals with 1mg/kg body weight but not with 10 mg/kg body weight significantly increased pDT levels (Figure 5b,c). 10 mg/kg body weight of CE-125 significantly reduced pDAT in untrained animals (Figure 5 d).

4. Discussion

The major findings of the study are that both doses of CE-125 improved working memory performance.

It was shown that animals learned the task and that D1R and pDAT levels were modulated by training as well as by the compound.

Synthesis of CE-125 was shown to result into high purity and was characterized by NMR and small molecule mass spectrometry. Re-uptake inhibition revealed that the compound was inhibiting DAT re-uptake specifically as serotonin and norepinephrine transporters showed much lower reuptake inhibition at about two orders of magnitude.

Behavioural assays did not show any behavioural/toxic changes following daily intraperitoneal administrations of CE-125 at an intraperitoneal dose of 10mg/kg body weight.

Previous work in primates and rodents proposed that D1R is linked to WM: Brozoski et al.[33] showed that selective depletion of dopamine in the dorsolateral PFC of rhesus monkeys is reversed by administration of levodopa or apomorphine, a combined D1/2 agonist and indeed, blockade of hippocampal D1-class receptors during learning impairs one-trial place memory [34]. The DR antagonist SCH23390 is known to inhibit spatial and non-spatial memory in the rat [35] and results from several behavioral studies showed that D1R is critical for memory performance in various working memory paradigms [36,38,38,39,40]. D1R agonism was suggested long ago to play a major role in PFC for WM processes [41,42] and the ability to use previously acquired spatial information in the RAM requires D1R activation in PFC and D1R modulation of hippocampal inputs in turn pointing to a key role of D1R in WM [43]. In the current study D1R levels in frontal cortex were increased by training but not by training and CE-125 which is, however not contradicting previous work as reports on D1R in literature did not measure frontal cortex levels of this receptor.

pDAT i.e. activated levels were decreased in trained untreated rats of the current study in frontal cortex and in previous work in hippocampus [24], but in the current study administration of 1mg/kg body weight of CE-125 was increasing pDAT levels and the interpretation of these results remains elusive. CE-104, an analog of CE-125, however, failed to show statistically different pDAT levels in frontal cortex of trained and treated animals[23] Binding of CEs to DAT with subsequent inhibition of dopamine reuptake may not be associated with changes of DAT or pDAT levels.

Taken together, CE-125 is an active and specific DAT reuptake inhibitor, significantly improving WM in a radial arm maze in the absence of adverse behavioral changes. It will be

challenging to follow up this compound in further pharmacological and pharmaceutical investigations.

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Figure legends

Figure 1

Evaluation of CE-125 activity on HEK293 cells stably expressing human DAT, SERT and NET. It specifically inhibited DAT-mediated dopamine uptake (n = 3).

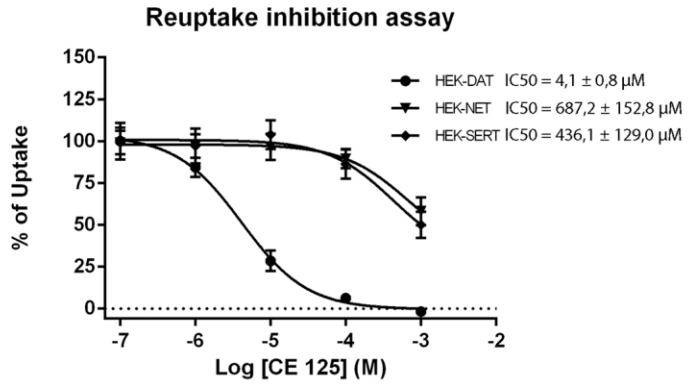

Figure 2

Working memory index (WMI), assessment of CE-125 in the 12-arm radial maze over 10 days. Rats receiving CE-125 performed better as compared to vehicle. Both, 1mg/kg and 10mg/kg CE-125 trained rats showed an increase in WMI as days progressed ($F(9, 270) = 3.011$; $P < 0.01$). Data were analyzed using ANOVA and Bonferroni post hoc tests. Data are represented as mean ± SEM. ** $p < 0.01$, vehicle vs. 1mg/kg; # $p < 0.05$, vehicle vs. 10mg/kg.

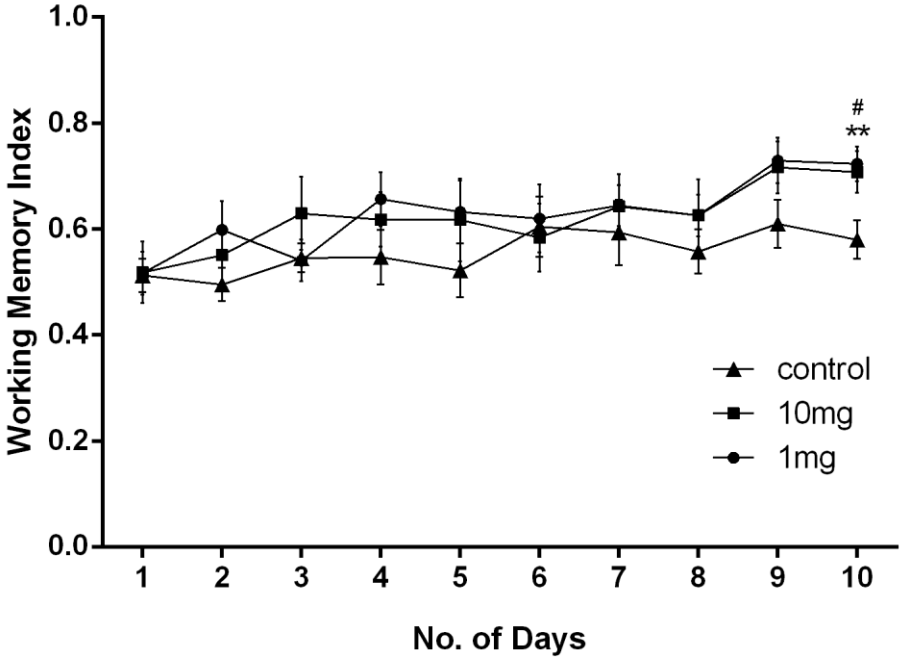

Figure 3

Representative western blot images of D1R in frontal cortex synaptosomal proteins comparing (a) vehicle-treated group and non-treated (Yoked). (b) Trained and untrained (Yoked) with CE-125 1mg/kg body weight. (c) Treated trained group with 10 mg/kg body weight and non-trained treated (Yoked) with 10 mg/kg body weight. (d,e) 1mg and 10mg/kg body weight of CE-125 trained rats against vehicle treated and trained (DMSO). (f,g) 1 and10 mg/kg body weight of CE-125 untrained group(Yoked) against vehicle (DMSO). Synaptosomes were prepared and run on SDS-PAGE and blotted onto PVDF membranes. Immunoreactivity of proteins was observed with a D1R antibody. The apparent molecular weight was 115 kDa (n =10). Densitometry was performed by ImageJ software and normalized with the total protein staining of the PVDF membrane by Coomassie R-350. Histograms showing the densitometric quantitative analysis of receptor and transporter levels (a-g). The data was analyzed by T-test and Statistics using GraphPad Prism. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Graphs are shown as mean± SD.

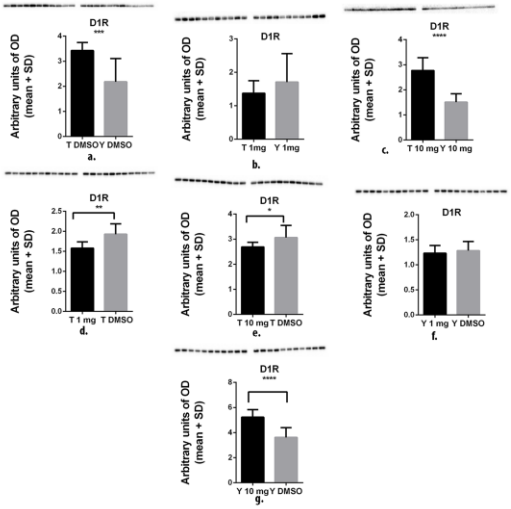

Figure 4

SDS western blot of D2R of (a) trained and untrained vehicle-treated groups (b,c) untrained animals (Yoked) treated with 1 mg and 10 mg/kg body weight of CE-125. The D2R antibody detected a band at the apparent molecular weight of 50 kDa. *P < 0.05, ****P < 0.0001. Values are expressed as mean ± SD, n = 10.

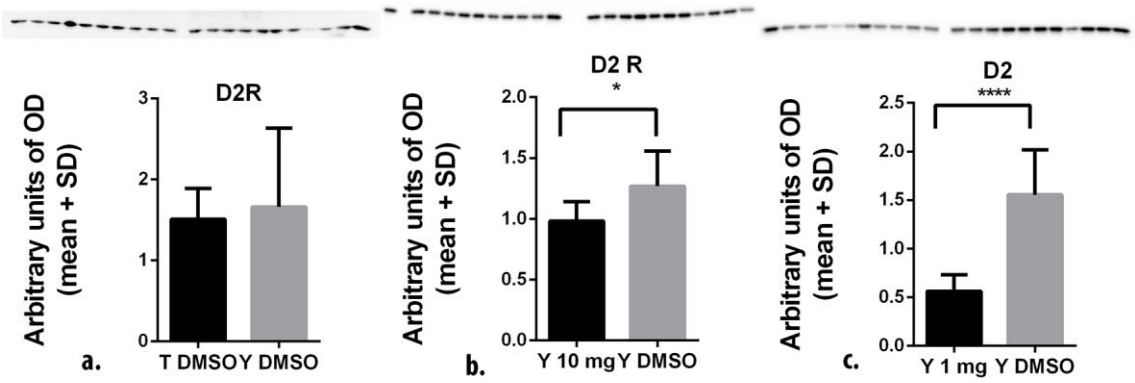

Figure 5

SDS western blot of pDAT of (a) trained treated vehicle group (DMSO) vs. untrained (Yoked) animals. (b,c). Treated trained groups with 1 mg and 10 mg/kg body weight of CE-125 against vehicle-treated group. (d) 10 mg/kg body weight of CE-125 untrained group (Yoked) against untrained vehicle group. The pDAT antibody recognized a band at the apparent molecular weight of 68 kDa. *P < 0.05, **P < 0.01. Values are expressed as mean ± SD, n = 10.