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Research report

A novel heterocyclic compound targeting the dopamine transporter improves performance in the radial arm maze and modulates dopamine receptors D1-D3

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HIGHLIGHTS

- CE-111 specifically targets DAT and inhibits dopamine reuptake.
- CE-111 improves spatial memory performance in radial arm maze.
- Blood-brain barrier in vitro model shows CE-111 permeates similar to modafinil.
- CE-111 modulates pre- and extra- synaptic but not postsynaptic dopamine receptors.

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ABSTRACT

A series of compounds targeting the dopamine transporter (DAT) haS been shown to improve memory performance most probably by re-uptake inhibition. Although specific DAT inhibitors are available, there is limited information about specificity, mechanism and in particular the effect on dopamine receptors. It was therefore the aim of the study to test the DAT inhibitor 4-(diphenyl-methanesulfinylmethyl)-2-methyl-thiazole (code: CE-111), synthetized in our laboratory for the specificity to target DAT, for the effects upon spatial memory and for induced dopamine receptor modulation. Re-uptake inhibition was tested for DAT (IC_{50} = 3.2 μM), serotonin transporter, SERT (IC_{50} = 272291 μM) and noradrenaline transporter, NET (IC_{50} = 174 μM). Spatial memory was studied in the radial arm maze (RAM) in male Sprague-Dawley rats that were intraperitoneally injected with CE-111 (1 or 10 mg/kg body weight). Performance in the RAM was improved using 1 and 10 mg/kg body weight of CE-111. Training and treatment effects on presynaptic, postsynaptic and extrasynaptic D1 and D2- receptors and dopamine receptor containing complexes as well as on activated DAT were observed. CE-111 was crossing the blood-brain barrier comparable to modafinil and was identified as effective to improve memory performance in the RAM. Dopamine re-uptake inhibition along with modulations in dopamine receptors are proposed as potential underlying mechanisms.

1. Introduction

Both D1-like and D2-like dopamine receptors play important roles in learning and memory, functions of the prefrontal cortex and hippocampus [1–3]. Dopamine autoreceptors that are located presynaptically regulate firing rate and neurotransmitter synthesis or release in response to extracellular dopamine levels [2].

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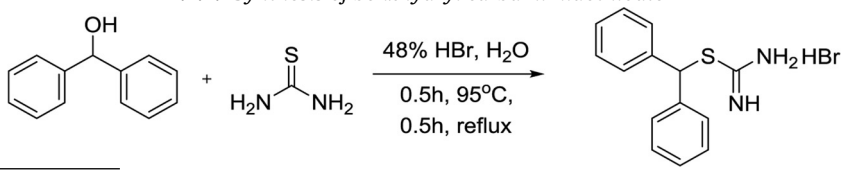
Presynaptic transduction and expression of D1R/D5R facilitates long-term potentiation (LTP) at the CA1 region of the hippocampus to subicular burst-spiking cell synapses through activation of the cAMP-PKA cascade [4]. Activation of D2-like autoreceptors (presynaptic receptors) leads to a decrease in midbrain dopamine neuron firing, dopamine synthesis and dopamine release, reduced locomotor activity and enhanced motivation for food-seeking behavior, whereas activation of postsynaptic D2-like receptors increases locomotor activity [5,6]. The presynaptic D2R plays an important role in hippocampal long-term synaptic plasticity and spatial learning [7]. D1R and D2R regulate synthesis and degradation of the dopamine transporter (DAT) which removes dopamine from the synaptic cleft to terminate dopamine-mediated signalling [8]. However, kinases like PKA or PKC phosphorylate DAT, that is either internalized to cease the transport or operate in reverse; as such, the phosphorylated DAT may release dopamine into the synapse [9]. The phosphorylation of DAT at the Thr^{53} site is important for amphetamine- induced DAT-mediated dopamine release [10]. Inhibition of DAT has been shown to enhance LTP in the hippocampal CA1- region mediated by the D3R [11]. In general, dopamine levels at the synaptic cleft or at the extrasynaptic region determine activity and function of dopamine receptors. Low dopamine concentrations (<500 nM) have been shown to enhance inhibitory postsynaptic currents (IPSCs) via D1 receptors whereas at high concentrations IPSCs are decreased via activation of D2R [12]. GABAergic inhibitory currents in CA1 pyramidal neurons are inhibited by activation of D3R and a D3-selective agonist, PD128907 enhances LTP in the CA1 stratum radiatum but not in stratum oriens [13].

Some psychostimulants inhibit DAT-mediated dopamine reuptake to elevate extracellular dopamine levels. As such, increased dopamine levels are associated with cognitive improvements [14]. Selective dopamine reuptake inhibitors such as modafinil and the benztropine analog AHN 2-005 improve both, hippocampal- and prefrontal cortex- dependent cognitive functions [15–18]. In search for highly selective DAT inhibitors, we synthesized a heterocyclic compound, CE-111 and tested its blocking efficacy on memory performance was assessed in a radial arm maze (RAM). Hippocampal dopamine receptors/transporter complexes were analysed by blue-native electrophoresis after CE-111 treatments in RAM training. Moreover, presynaptic, postsynaptic and extrasynaptic dopamine receptors/transporter were also studied to address the specific effects of memory training/CE-treatments on the hippocampal dopaminergic system.

2. Materials and methods

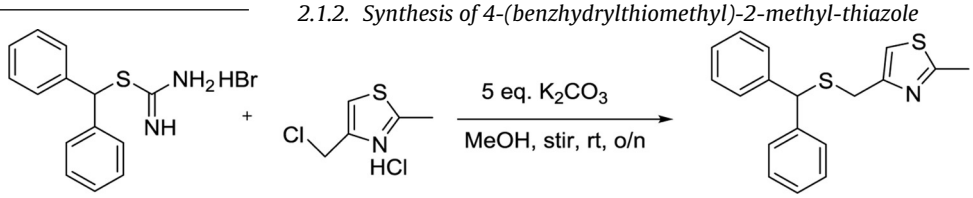
2.1. Synthesis of CE-111 (4-(benzhydrylsulfinylmethyl)-2-methyl-thiazole)

2.1.1. Synthesis of benzhydryl carbamimidothioate



Diphenylmethanol (13 g, 0.07 mol) and thiourea (6.5 g, 0.085 mol) are added in 0.5 l flask and 32.5 ml of water was added. The mixture is then heated to 95 °C (an emulsion is obtained) and 26 g of 48% HBr (0.322 mol, 4.6 equivalents) is then gradually added during 0.5 h. The mixture is heated under reflux (106–107 °C) for 0.5 h and cooled to 80–85 °C. The mixture is then cooled in ice and precipitated crystals formed. After filtration and washing with water, a colorless crystalline substance is obtained. The product is then dried in the high vacuum. About 9.62 g of the product was obtained as a white crystalline solid (yield: 74%).

2.1.2. Synthesis of 4-(benzhydrylthiomethyl)-2-methyl-thiazole

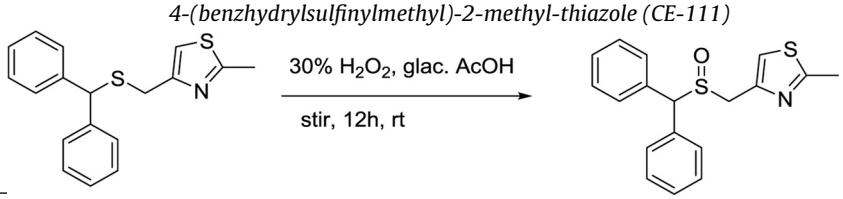


4-Chloromethyl-2-methyl-thiazole hydrochloride was obtained from Acros Organics (New Jersey, USA; Geel, Belgium).

In a round bottom flask 3.51 g (10.86 mmol) of benzhydryl carbamimidothioate is dissolved in 75 ml of methanol. Afterwards, 2 g (10.86 mmol) of 4-chloromethyl-2-methyl-1,3-thiazole and 7.5 g (5 equivalents) of potassium carbonate are added to the mixture. The mixture is left to stir overnight at room temperature. Methanol is evaporated and water is added. The solution is then extracted (3x) with 100 ml of ethylacetate. Organic phases are collected, combined, dried with Na_2SO_4 and then filtered. Ethylacetate is removed by rotary evaporation and then semi-solid product is obtained. Crude product is purified via the flash column chromatography on silica gel. Methanol of 5% in dichloromethane is used as the mobile phase. About 2.6 g of the crude solid product is obtained (yield: 59.07%).

H NMR (200 MHz, DMSO-d_6): 2.63 (3H, s, CH_3), 3.62 (2H, s, CH_2), 5.33 (1H, s, CH), 7.15 (1H, s, $CH_{thiazole}$), 7.21–7.46 (10H, m, $CH_{aromatic}$).

2.1.3. Synthesis of 4-(benzhydrylsulfinylmethyl)-2-methyl-thiazole (CE-111)



In a round bottom flask, 1.44 g (4.62 mmol) of 4-(benzhydrylthiomethyl)-2-methylthiazole is dissolved in 10 ml of glacial acetic acid (174.82 mmol). 0.55 ml (4.85 mmol) of 30% hydrogen peroxide is dropped into the solution and stirred for 12 h. Acid is neutralized with 5% sodium bicarbonate and ice.

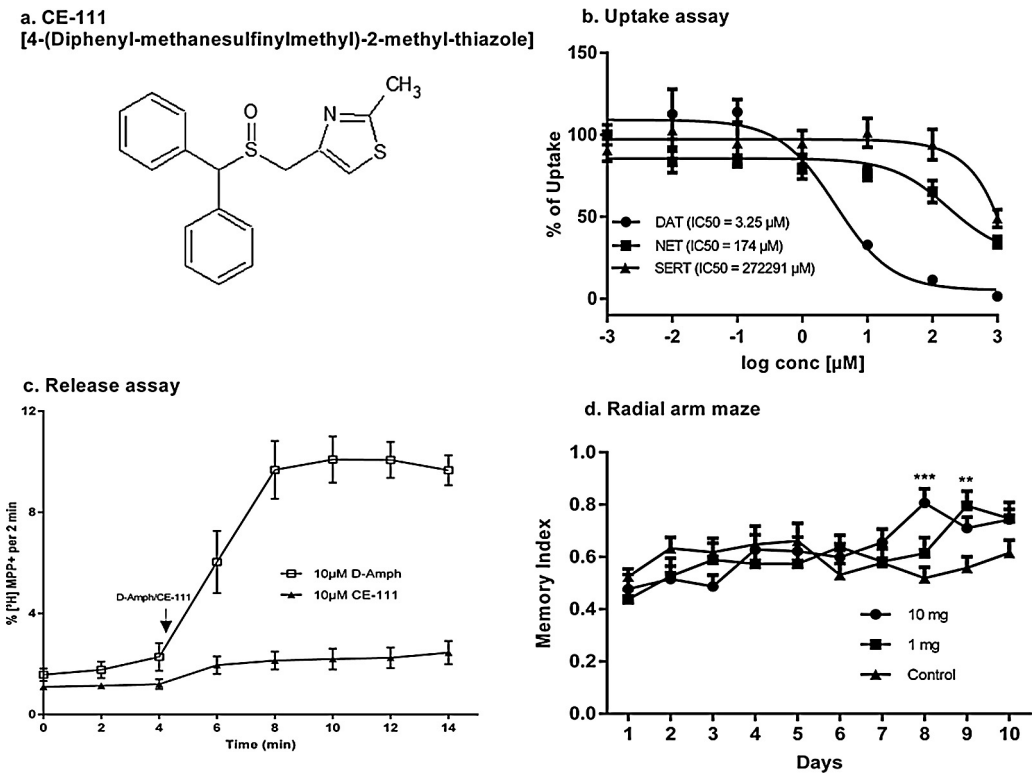


Fig. 1. (a) Structure of CE-111 (4-(diphenyl-methanesulfinylmethyl)-2-methyl-thiazole). (b) Evaluation of CE-111 activity on HEK293 cells stably expressing human isoforms of DAT, SERT and NET. It specifically inhibited DAT-mediated dopamine uptake (n = 3). (c) Substrate-efflux assay was performed to examine whether CE-111 has any ability to release the neurotransmitter from inside the cells, like amphetamine does. Figure depicts that CE-111 is not behaving as an amphetamine like compound. (d) Behavioural experiments on RAM show that both 10mg and 1mg groups improved memory performance on 8th and 9th day, respectively ($F(9, 269) = 4.660$, $P < 0.0001$). The data was analyzed using repeated measurements ANOVA with Bonferroni post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$. Values are expressed as mean ± s.e.m, n = 8–10.

Aqueous mixture is extracted (3x) with 50 ml of ethyl acetate. Organic phases are collected, combined, dried with Na_2SO_4 then filtered and ethyl acetate is concentrated on rotary evaporator. Semi solid product is purified via flash column chromatography on silica gel. 5% methanol in dichloromethane is used as a mobile phase. Product is dried in a high vacuum. By this procedure 1.12 g of the final pure product was obtained (yield: 73.98%) (Fig. 1a and Supplementary Fig. 1).

MS. $[M+H^+] = 328.0824$ (MW is 327.08), $[M+Na^+] = 350.0644$.

H NMR (200 MHz, DMSO-d_6): 2.65 (3H, s, CH_3), 3.73–3.99 (2H, dd, CH_2), 5.43 (1H, s, CH), 7.32–7.58 (11H, aromatic CH).

2.2. Uptake and release assays

Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from PAA Laboratories GmbH (Pasching, Austria). Fetal calf serum was purchased from Invitrogen. [3H]5-HT ([3H]5-hydroxytryptamine; [3H]serotonin; 28.3 μCi/mmol) and [3H]DA ([3H]dihydroxyphenylethylamine, [3H]dopamine; 46 μCi/mmol) were purchased from Perkin Elmer, Boston, MA. [3H]1-Methyl-4-phenylpyridinium ([3H]MPP+; 85 μCi/mmol) was supplied by American Radiolabeled Chemicals (St. Louis, MO). Paroxetine was purchased from Santa Cruz Biotechnology, USA, while mazindole and D-amphetamine were purchased from Sigma–Aldrich Co.

For uptake experiments, the human isoforms of DAT, SERT and NET were expressed in HEK293 (HEK-DAT, HEK-SERT and HEK-NET) cells. CE-111-mediated monoamine transporter effects on substrate uptake were analysed as described previously [19,20]. In brief, cells were grown in poly-D-lysine (PDL) coated 96-well plates. CE-111 was dissolved in DMSO and subsequently diluted in Krebs–Ringer–HEPES buffer (KHB; 25 mM HEPES.NaOH, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgSO_4$ supplemented with 5 mM D-glucose). To determine unspecific uptake in HEK-DAT and HEK-NET 10 μM of mazindole were used while 10 μM of paroxetine were used for HEK-SERT. The tritiated substrates used for HEK-DAT, HEK-SERT and HEK-NET were 0.2 μM ^{3}H-Dop, 0.4 μM ^{3}H-5HT and 0.05 μM ^{3}H MPP$^+$, respectively. Cells were washed once with KHB buffer and incubated with compounds either 5 min for HEK-DAT and HEK-SERT cells or 8 min for HEK-NET cells. Subsequently, substrates were added and the reactions were stopped with ice-cold KHB buffer after either 1 min for HEK-DAT and HEK-SERT cells or 3 min for HEK-NET cells. Cells were lysed with 1% SDS and released radioactivity was measured by a liquid scintillation counter (Tri-carb-2300TR, Perkin Elmer).

The substrate/efflux experiments were performed as described before [21]. Briefly, HEK-DAT cells were grown in 5 mm diameter PDL-coated coverslips. Cells were incubated with 0.1 μM ^{3}H 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 buffer for 40 min (0.7 ml/min) at 25 °C to obtain stable baselines. The experiment was started with the collection of fractions (2 min). After collection of first three baseline fractions, either CE-111 or D-Amphetamine was added as depicted in Fig. 1C. Finally, the remaining radioactivity was collected by treating with 1% SDS.

2.3. Transport studies across blood-brain barrier in vitro model

The used Transwell model was based on mouse cell line cerebEND which was a kind gift from Prof. Carola Förster [22]. CerebEND cells form a tight barrier similar to primary mouse brain endothelial cells and have been characterized as blood-brain barrier *in vitro* model in a comprehensive manner previously [22,23]. CerebEND cells were cultivated on collagen IV coated Transwell inserts (1 μm pore size, BDL353103, Becton & Dickenson)

as described in Neuhaus et al. [23]. On day 13 after cell seeding transport studies were accomplished according to Novakova et al. [24]. In brief, 12-well plates prefilled with DMEM (Dulbecco's modified Eagle medium) and substance solutions were prewarmed at 37 °C. After medium exchange to pure DMEM, cell layers were equilibrated at 37 °C for 30 min. The transport study started after addition of compound solutions to the apical compartment, and inserts were transferred into a new, prewarmed and DMEM filled well in the incubator at 37 °C after five, 15, 30 and 60 min. Measurement of transendothelial electrical resistance (TEER) before and after the studies confirmed cell layer integrity throughout the total experiments. Stock solutions of Diazepam, CE-111 and Modafinil were 100 mM in DMSO, tested compound concentration was 100 μM in DMEM (test solution). Diazepam was added to CE-111 or Modafinil in DMEM as internal standard to account for cell layer's variability. Basolateral samples, apical solution after the transport study and test solutions were collected and prepared for following HPLC analysis by precipitation with acetonitril in a ratio of 1:1 at 4 °C for at least 30 min. After centrifugation supernatants were subjected to HPLC analysis which was accomplished as recently published [24] applying 50:50 CH_3CN:potassium phosphate buffer (10 mM, pH = 3) as elution buffer (retention time for Modafinil: 3.9 min; CE-111 8.7 min; Diazepam: 10.5 min) and UV-detection at 220/254 nm. Peak areas were used to calculate cleared volume versus (vs.) time curves and permeability coefficients following the clearance principle according to Novakova et al. [24]. PK_{all} accords to the permeability coefficient in μm/min across the cell layer and the membrane support. For PK_{cell} values [μm/min] the permeability across blank inserts without cells was subtracted resulting in the permeability of the compounds only across the cell layer.

2.4. Radial arm maze (RAM)

Twelve to fourteen weeks old male Sprague Dawley rats (n = 72) were used in all experiments. All procedures were carried out according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC) and 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. The maintenance of the animals, details about the apparatus and the performance of the 12 arms containing RAM experiment was described previously [19,20].

In brief; rats were handled for 30 min/day for 5 days to adapt to the experimenter and to the surrounding conditions. The amount of food was restricted prior to the actual experiment to reduce the body weight to ~80–85% for motivation. The food restriction was maintained during handling and subsequent training days. Water was provided *ad libitum* during the handling, habituation and training. Twelve arms RAM was used to train the animals and access the memory formation. Two days before the training period, rats were allowed to explore the maze (habituation) for 5 min and eat the food pellets which were scattered all over the maze. During the 10 training days (1 trial/day), eight arms were baited with food to assess working memory and four remained un-baited to access reference memory. The pattern of baited and un-baited arms was consistent throughout the training for each rat but differed among rats. CE-111 was freshly dissolved in 100% DMSO and injected intraperitoneally in doses of 1 and 10 mg/kg body weight, 1 ml/kg everyday 30 min before the start of the training sessions. Pure DMSO was used as vehicle.

Each trial began by placing the rat in the central platform, after 10 s the cylinder was slowly lifted. The session lasted for 8 min or until all the baited arms (n = 8) were entered-whatever occurred first. Arms were baited with a small pellet only once, 3 cm from the end. Re-entry into a baited arm was counted as a working memory error (WME), whereas any entry into an un-baited arm was recorded as a reference memory error (RME). Untrained animals (yoked) were also treated same as trained animals in terms of handling, habituation and food restriction and exposed to the maze for the same amount of time as their trained counterparts but without food rewards in the arms. Thus, they do not learn and form any memory. The training sessions were recorded with a computerized tracking video camcorder: 1/3 SSAM HR EX VIEW HAD. Six hours after the l of the tenth training session, the animals were deeply anaesthetized with CO_2 and killed by neck dislocation to study the protein synthesis-dependent dopamine receptors and transporter. Brain tissues were quickly removed and hippocampi were rapidly dissected on a cold plate set at 4–6 °C and stored at −80 °C.

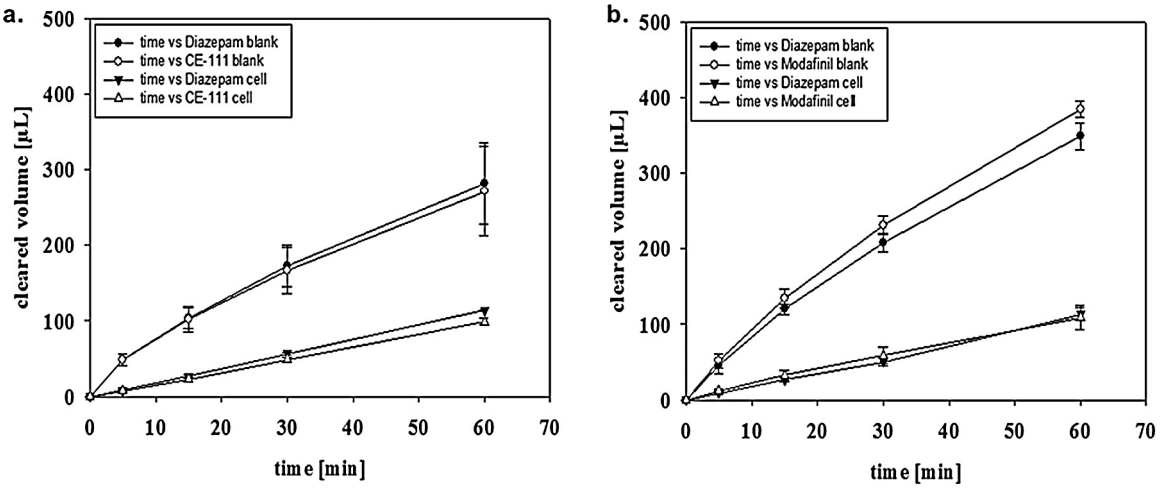
2.5. Sample preparation

Hippocampal total membrane fraction was prepared as described previously [25]. Briefly, homogenized hippocampi in ice-cold homogenization buffer [10 mM HEPES, pH 7.5, 300 mM sucrose, one complete protease inhibitor tablet (Roche Molecular Biochemicals) per 50 ml] were centrifuged for 10 min at 1000*g* and the nuclei pellet was discarded. The supernatant was centrifuged at 50, 000*g* for 30 min. The resulting pellet was resuspended in wash buffer (homogenization buffer without sucrose), incubated on ice for 30 min and centrifuged at 50, 000*g* for 30 min to obtain a membrane fraction. Membrane protein was extracted with extraction buffer (1.5 M 6-aminocaproic acid, 300 mM Bis–Tris, pH 7.0, 1% n-Dodecyl b-D-maltoside) and the protein content was measured by using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Presynaptic and postsynaptic membrane proteins were prepared as described in [26] with some modifications. All the steps were carried out at 4 °C unless it is indicated. Hippocampi were homogenized (0.5 mM $CaCl_2$, 10 mM $MgCl_2$, 320 mM sucrose and protease inhibitor) and brought to a final sucrose concentration of 1.25 M. The homogenate containing 1.25 M of sucrose was overlaid by 1 M sucrose and centrifuged at 100, 000*g* for 3 h. Interfaced synaptosomal fraction was collected, mixed with 0.1 mM $CaCl_2$ and centrifuged at 15, 000*g* for 30 min to obtain a synaptosomal pellet. The pellet was resuspended with 20 mM Tris buffer pH-6 containing 0.25 mM $CaCl_2$ and 1% Triton X-100, mixed/incubated for 30 min and then centrifuged at 50, 000*g* for 30 min. The supernatant containing the extrasynaptic membrane proteins was collected and the pellet was treated with 20 mM Tris buffer pH-8 containing 0.25 mM $CaCl_2$ and 1% Triton X-100 and after mixed/incubated for 1 h, the mixture was centrifuged at 50, 000*g* for 30 min. The supernatant containing presynaptic proteins was collected; the pellet containing PSD proteins was extracted with 0.25 mM tris buffer pH-7.4 containing 3% of SDS. Presynaptic and extrasynaptic proteins were precipitated overnight with 3 fold of acetone at −20 °C. Precipitated proteins were solubilized with 0.25 mM Tris buffer (pH-7.4) containing 3% of SDS.

2.6. Electrophoresis and western blotting

Blue-native electrophoresis and immunoblotting were closely performed as described before [25]. Specific peptide sequences were used to produce D1R (-TSTMDEAGLPAERD-), D2R (-NWSRPFNGSEGKAD-), D3R (-LRHPSLEGGAGMPS-), DAT (-TNSTLINPPQTPVEAQERETW-) and DATph (-TNSTLINPPQpTPVEAQERETW-) antibodies from GenScript. Antibodies were tested in SDS-PAGE and BN-PAGE (Supplementary Figs. 2 and 3).



	CE-111	Diazepam
PK_{all} [µm/min]	18.49 ± 0.81	21.47 ± 0.29
PK_{cel} [µm/min]	34.97 ± 6.43	45.20 ± 11.74
Ratio diazepam	0.79±0.07	1

	Modafinil	Diazepam
PK_{all} [µm/min]	17.19 ± 2.56	20.97 ± 1.58
PK_{cel} [µm/min]	23.49 ± 4.95	32.25 ± 3.94
Ratio diazepam	0.73±0.10	1

Fig. 2. Permeability of CE-111 (a) and modafinil (b) across the 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 PK_{all} [µm/min] revealed almost no difference between the transport of CE-111 and modafinil. Subtraction of the blank values led to permeability coefficient PK_{cell} [µm/min] values reflecting the permeability of the compounds only across the cell layer. Normalization to internal control diazepam showed almost no difference between the permeability of CE-111 and modafinil. The data are presented as mean ± SD, n = 3–5.

2.7. Immunohistochemistry

Rats were anesthetized and transcardially perfused with PBS and then with 4% paraformaldehyde. Excised brains were post-fixed with 4% paraformaldehyde for 1 h and 100 µm thick sections were made by using Vibratome. Finely powdered DIL dye was randomly placed on hippocampal sub regions followed by overnight incubation in PBS at the room temperature. Following 30 min of blocking with 10% donkey serum, either D1R or D2R antibody (1:200) was added and incubated for 24 h at 4 °C. Tissue was then washed with PBS for 30 min and incubated with secondary antibody (anti-rabbit IgG Alexa Fluor 647, 1:1000; Cell Signaling) for 1 h. After washing with PBS for 1hr, tissue was counterstained with DAPI (3 µg/ml). Then again the tissue was briefly washed with PBS and the tissue slide was mounted (DAKO; fluorescence mounting medium). The tissue was imaged with Zeiss LSM 700 using 63X oil immersion objective at 1 Air Unit and the 3D-image was constructed by using imageJ.

2.8. Statistics

Non-linear regression analysis was carried out to determine the IC_{50} values of CE-111 on DAT, NET and SERT. For behavioural data analysis, a repeated measurement of two way ANOVA with the factors, CE-111 and the time were used. A pairwise multiple comparison was done using the Bonferroni post hoc test. The densitometry values of western blot quantification were compared using two-way ANOVA with Bonferroni post hoc test for multiple comparisons to reveal the differences among groups. The probability level of $p < 0.05$ was considered as statistically significant. Statistics was done using GraphPad Prism 6 (version 6.00, GraphPad Software, San Diego CA, USA).

3. Results

3.1. CE-111 specifically blocked dopamine uptake and is not a substrate of DAT

The effect of CE-111 on monoamine transporters, DAT-, NET- and SERT- mediated respective substrate uptake (^{3}H-DA, ^{3}H-5HT and ^{3}H MPP+) was assessed using HEK293 cells which expressed the corresponding transporters. CE-111 strongly blocks DAT-mediated substrate uptake (IC_{50} = 3.25 ± 0.23SD µM) while weakly blocks NET (IC_{50} = 174 ± 15.5SD µM) and negligible effects on SERT (IC_{50} = 272291 ± 3453SD µM) (Fig. 1b). We then sought whether CE-111 specifically blocks DAT or it also behaves like a substrates of DAT to promote DAT-mediated release to increase the extracellular dopamine levels. We therefore performed a release assay to determine if CE-111 acts as a substrate to induce release of tritiated MPP^+ from HEK-DAT cells. CE-111 treatment did not induce DAT-mediated release while D-Amphetamine as a positive control did (Fig. 1c). The observations clearly indicate that CE-111 specifically blocks DAT without acting as a substrate of DAT to increase the extracellular dopamine levels.

3.2. CE-111 permeated across the blood-brain barrier in vitro similar to modafinil

Before conducting the *in vivo* experiments, the ability of CE-111 to cross the blood-brain barrier was assessed *in vitro*. Transport studies across cerebEND cell layers revealed that CE-111 permeated in a similar manner compared to modafinil (Fig. 2). Normalization of permeability data to internal control diazepam resulted in ratios of 0.79 ± 0.07 for CE-111 and 0.73 ± 0.10 for Modafinil, respectively. Thus, data suggested significant blood-brain barrier permeability of CE-111 since modafinil is known as a well permeating compound. Based on these findings following *in vivo* studies were accomplished.

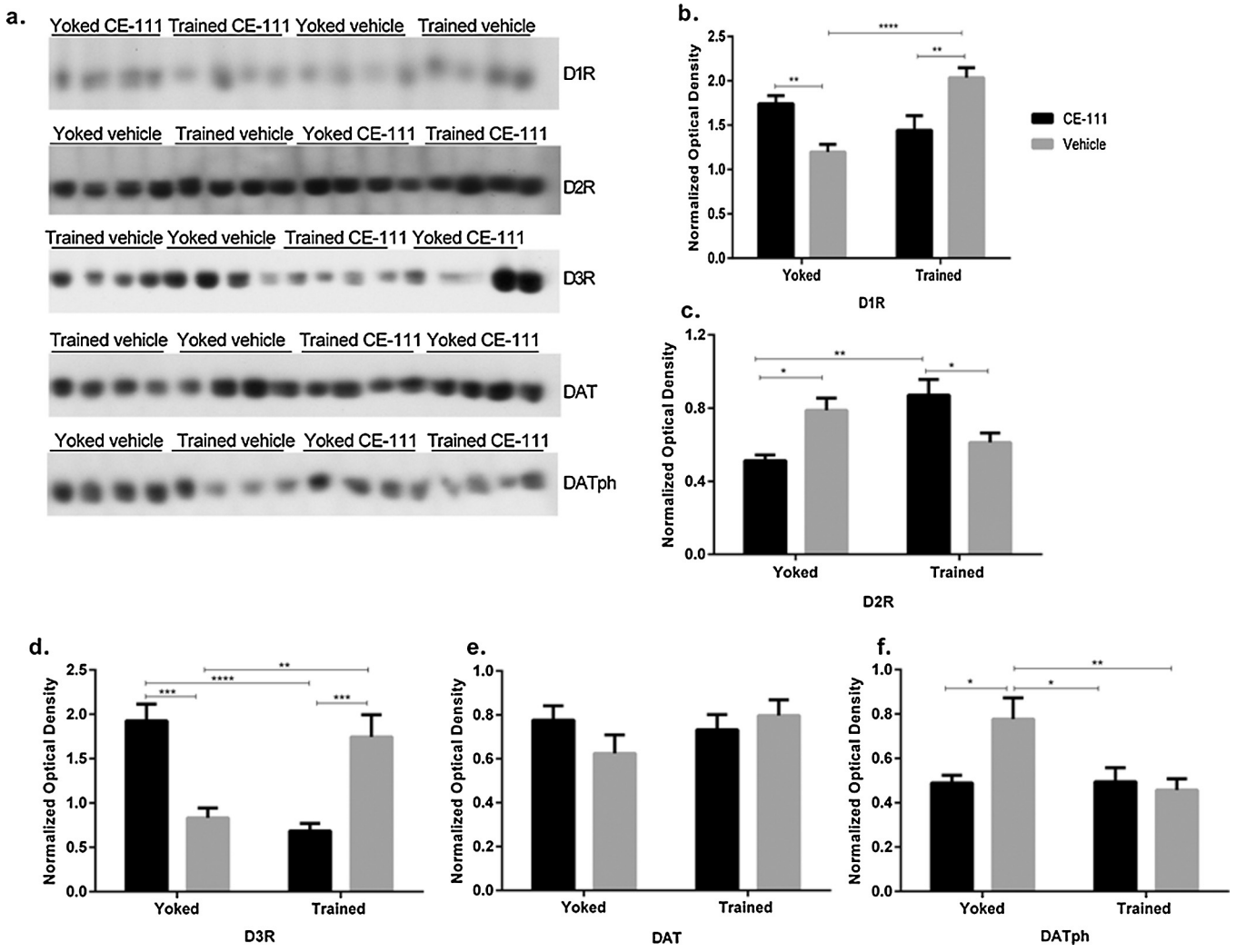


Fig. 3. BN-PAGE western blot of 10 mg/kg body weight group. (a) Hippocampal membrane proteins (native) were separated by blue-native electrophoresis and immunoblotted with indicated antibodies (n = 8–10). Molecular weight range was between 720 kD and 480 kD. (b–f) Quantified results of D1R ($F(1,28) = 24.87$, $P < 0.0001$), D2R ($F(1,29) = 18.76$, $P = 0.0002$), D3R ($F(1,27) = 44.33$, $P < 0.0001$), DAT ($F(1,29) = 2.229$, $P = 0.1462$) and DAT^{ph} ($DAT\,Thr^{53}$) ($F(1,31) = 6.709$, $P = 0.0145$). The data was analyzed by two-way ANOVA with Bonferroni post hoc test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Values are expressed as mean ± s.e.m, n = 8–10.

3.3. CE-111 improved memory performance

Memory performance of rats from the 1 mg, 10 mg and control groups was progressively improved over the training days. The memory index (MI) was calculated by dividing the number of first entries into the baited arms by total entries into the baited arms. In the control group, memory index was gradually increased until 6th day and remained stable during the further training days; however, continuous improvement was observed in 1 mg and 10 mg CE-111 treated groups. Thus, the significant memory improvement at the 8th and 9th day was respectively observed in the 10 mg and 1 mg group compared to the control group ($F(9, 269) = 4.660$, $P < 0.0001$) (Fig. 1d).

3.4. Memory training and CE-111 treatments modulated dopamine receptors and transporter complexes

Hippocampal dopamine receptors and transporter complexes were separated by BN-PAGE and subsequently immunoblotted with specified antibodies as mentioned earlier. The Coomassie R-350 stained PVDF membrane was used as the loading control [27] (Supplementary Fig. 4). Fig. 3a shows blue native-western blot images of dopamine receptors and transporters from 10 mg/kg group. As seen in Fig. 3b, the D1R complex levels were reduced in CE-111 trained and vehicle yoked groups compared to vehicle trained group. Furthermore, they were also reduced in vehicle yoked compared to CE-111 yoked group ($F(1, 28) = 24.87$, $P < 0.0001$). Whereas, the D2R complex was reduced in CE-111 yoked group compared to CE-111 trained and vehicle yoked groups and in vehicle trained group compared to CE-111 trained group ($F(1, 29) = 18.76$, $P = 0.0002$) (Fig. 3c). The D3R complex levels were reduced in CE-111 trained and vehicle yoked groups compared to vehicle trained group while it was significantly increased in CE-111 yoked group compared to vehicle yoked group ($F(1, 27) = 44.33$, $P < 0.0001$) (Fig. 3d). No significant changes were observed in DAT-containing complexes ($F(1, 29) = 2.229$, $P = 0.1462$) (Fig. 3e). However, DAT^{ph} (Thr^{53})-containing complexes were significantly reduced in CE-111 yoked, CE-111 trained and vehicle trained groups compared to vehicle yoked group ($F(1, 31) = 6.709$, $P = 0.0145$) (Fig. 3f). Like 10 mg/kg group, significant changes of dopamine receptors or transporter were observed in the 1 mg/kg body weight CE-111 treated group (Supplementary Fig. 5).

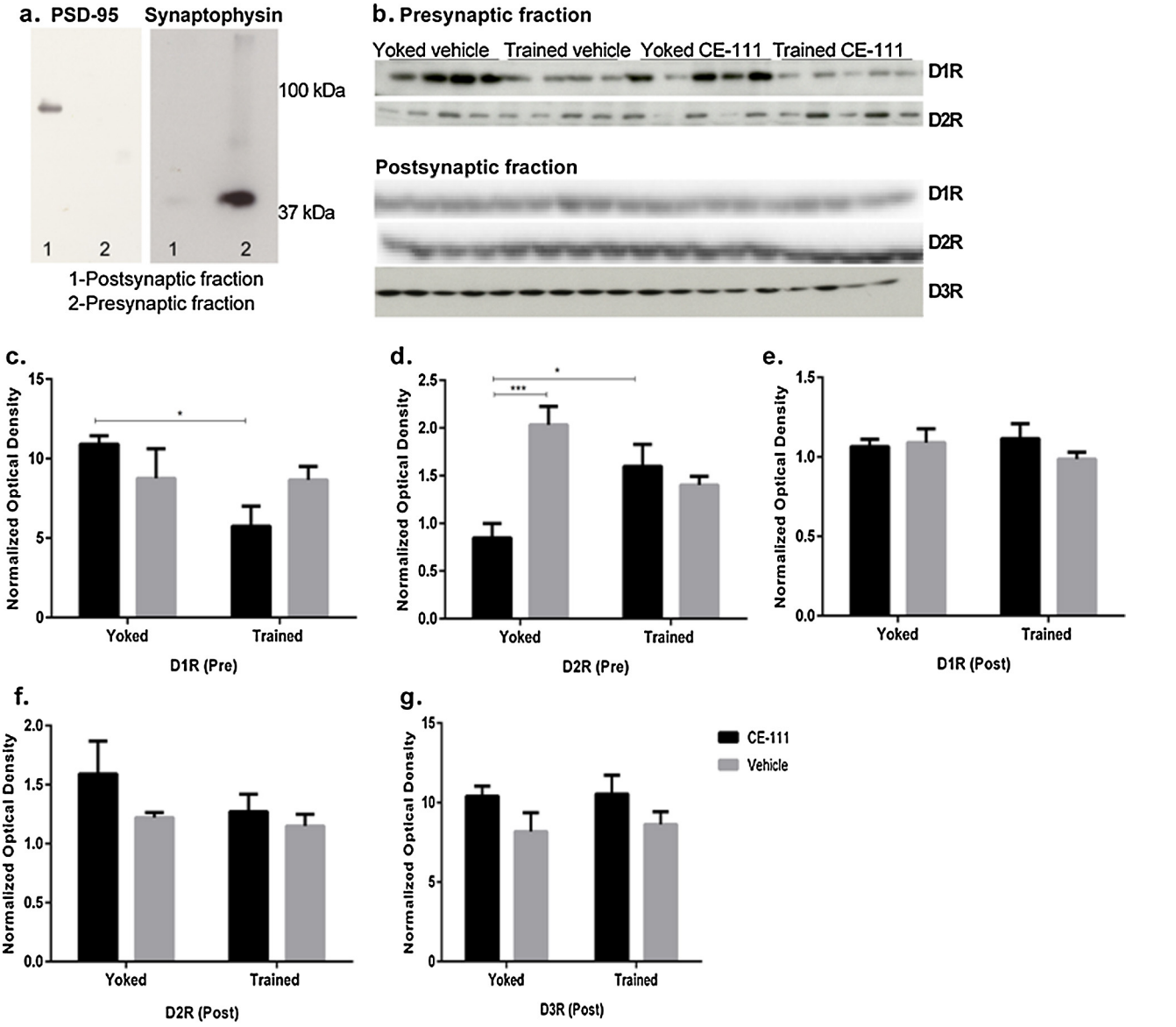


Fig. 4. Presynaptic and postsynaptic dopaminergic system of 1 mg/kg body weight group. (a) Postsynaptic marker PSD-95 was enriched in postsynaptic fraction, while presynaptic marker synaptophysin was enriched in presynaptic fraction, shows the quality of our separation methods. (b) SDS-PAGE western blotting of presynaptic and postsynaptic fraction with indicated antibodies. (c–g) Quantified results of D1R ($F(1, 30) = 4.372$, $P = 0.0451$) and D2 R ($F(1, 30) = 14.29$, $P = 0.0007$) from presynaptic fraction and D1R ($F(1, 32) = 1.081$, $P = 0.3064$), D2R ($F(1, 31) = 0.4490$, $P = 0.5078$) and D3R ($F(1, 31) = 0.02589$, $P = 0.8732$) from postsynaptic fraction. The data was analyzed by two way-ANOVA with Bonferroni post hoc test. $^{*}P < 0.05$, $^{***}P < 0.001$. Values are expressed as mean ± s.e.m, n = 8–9.

3.5. CE-111 modulated presynaptic and extrasynaptic dopamine receptors and transporter

We then sought to study the presynaptic, postsynaptic and extrasynaptic dopamine receptors as well as transporter, as it is unknown how their distribution is affected either during the memory formation or in treatment with the psychostimulants. The presynaptic, postsynaptic and extrasynaptic proteins were prepared as described in Section 2. The postsynaptic marker, PSD-95 and the presynaptic marker, synaptophysin, respectively observed at PSD and presynaptic fractions demonstrate the efficiency of sample preparation method (Fig. 4a). Western blot images from 1 mg/kg are shown in Fig. 4b. The presynaptic D1R complex levels were significantly decreased from CE-111 yoked to CE-111 trained group ($F(1, 30) = 4.372$, $P = 0.0451$) (Fig. 4c). The presynaptic D2R complex levels were significantly reduced in CE-111 yoked compared to CE-111 trained and vehicle yoked groups ($F(1, 30) = 14.29$, $P = 0.0007$) (Fig. 4d). Surprisingly, no significant changes in D1R, D2R and D3R complex levels were observed in the PSD fractions (D1R ($F(1, 32) = 1.081$, $P = 0.3064$), D2R ($F(1, 31) = 0.4490$, $P = 0.5078$) and D3R ($F(1, 31) = 0.02589$, $P = 0.8732$)) (Fig. 4e–g). However, the extrasynaptic D1R levels were significantly increased in CE-111 trained group compared to CE-111 yoked and vehicle trained groups ($F(1, 28) = 24.86$, $P < 0.0001$) (Fig. 5b). The D2R complex levels were significantly increased in CE-111 trained group when compared to CE-111 yoked group ($F(1, 31) = 9.794$, $P = 0.0038$) (Fig. 5c). Although the extrasynaptic DAT was comparable between the groups ($F(1, 31) = 1.884$, $P = 0.1798$), DAT Thr^{53} was significantly reduced in vehicle yoked group compared to CE-111 yoked and vehicle trained groups ($F(1, 32) = 5.559$, $P = 0.0247$) (Fig. 5d and e).

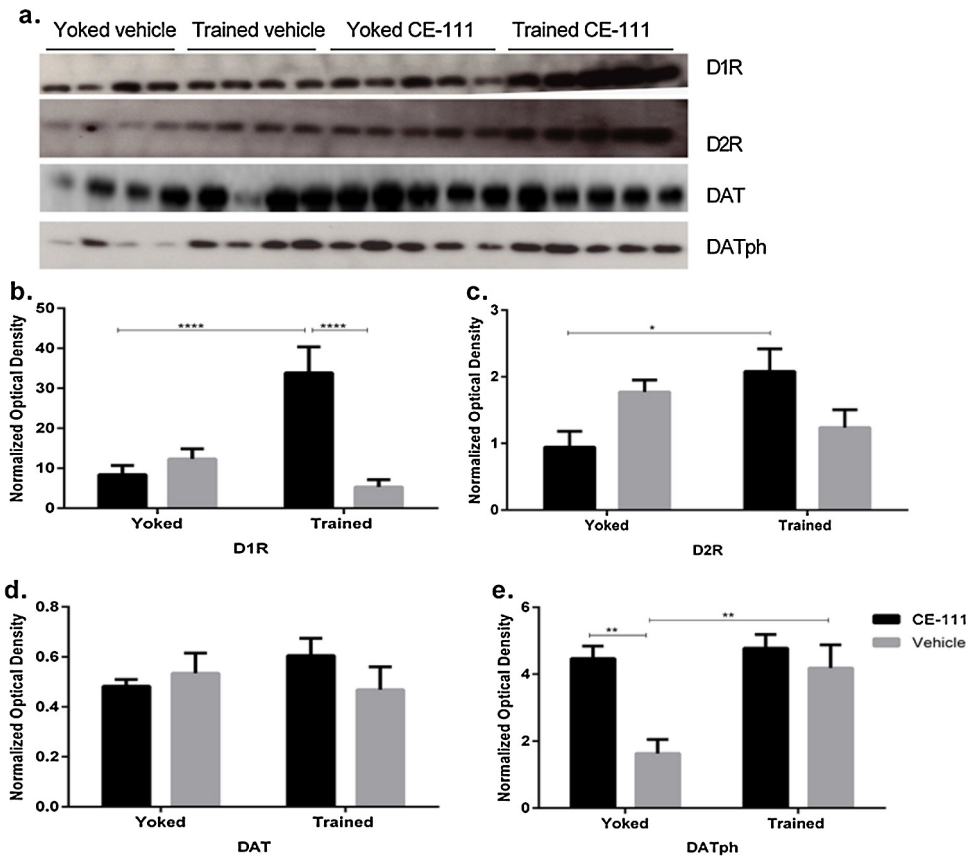


Fig. 5. Extrasynaptic dopaminergic system of 1 mg/kg body weight group. (a) SDS-PAGE western blotting of extrasynaptic fraction with indicated antibodies. (b–e) Quantified results of D1R ($F(1, 28) = 24.86$, $P < 0.0001$), D2R ($F(1, 31) = 9.794$, $P = 0.0038$), DAT ($F(1, 31) = 1.884$, $P = 0.1798$) and DAT^{ph} ($DAT\ Thr^{53}$) ($F(1, 32) = 5.559$, $P = 0.0247$). The data was analyzed by two-way ANOVA with Bonferroni post hoc test. **$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. Values are expressed as mean ± s.e.m, n = 8–9.

3.6. Distribution of D1R and D2R

In order to elucidate the distribution of D1R and D2R on the hippocampal pyramidal cells, we stained neurons with DIL dye and co-localized the dopamine receptors. D1R is mainly expressed around the soma but also expressed on dendritic spines and axonal segments of CA1 pyramidal cells (Fig. 6a). Like D1R, D2Rs are expressed both, around the soma and dendritic spines (Fig. 6b). This observation suggests that both D1R and D2R may have different functional properties on neuronal plasticity based on their cellular distribution.

4. Discussion

4.1. CE-111 is a specific DAT inhibitor and improves memory performance

Psychostimulants can induce diverse behavioral effects and might lead to either cognitive improvements or deficits and behavioral adverse effects [28]. These effects are usually determined by the dosages or target specificity of the compound; higher doses or multiple targets usually exerts adverse side effects [29]. Cocaine inhibits NET, DAT and SERT, as an example [30]. Here, CE-111 selectively inhibited DAT-mediated rather than NET- or SERT-mediated uptake suggesting that it might serve as a potential specific psychostimulant. Prior to *in vivo* studies, *in vitro* transport across the blood-brain barrier *in vitro* model showed that CE-111 permeated similar such as modafinil. In contrast to the benztropine analog, AHN 2-005 that induced improvement in cognitive performance only at the 10 mg/kg dose, but not at the 1 mg/kg concentration [16], we observed a continuous memory improvement using CE-111 during the last three days of training at both, 10 mg/kg and 1 mg/kg dosages. Moreover, modafinil is effective at a comparable dose of approximately 1–3 mg/kg body weight in medical use but it is less specifically acting at the dopamine transporter.

4.2. Effect of CE-111 on D1R-, D2R-, D3R-, DAT- and DAT^{ph}-containing complexes

The D1R are exclusively excitatory, while D2R are inhibitory, and D3R either inhibitory or excitatory. D1R is important for hippocampal spatial long-term memory formation [31,32], while mice lacking D2R and D3R showed impaired spatial working memory [33]. D1R complex levels were reduced in the vehicle yoked group compared to the CE-111 yoked group suggesting an influence of CE-111 on D1R-containing complexes, which were abundant in the vehicle trained group compared to both vehicle yoked groups and the CE-111 trained group. However, the level of D1R-complex was similar between CE-111 yoked and CE-111 trained groups. This result suggests that CE-111 has the same effect on D1R in trained and control groups while D1R was differentially modulated during training in vehicle groups. Interestingly, we observed completely opposite effects on D2R-containing complexes. The D2R complex level was reduced in CE-111 yoked group compared to vehicle yoked group, but increased in CE-111 trained group compared to vehicle trained group. It suggests that CE-111 modulates D2R during memory training while control groups had no effect on D2R. Though D1R and D2R belong to different subfamilies of

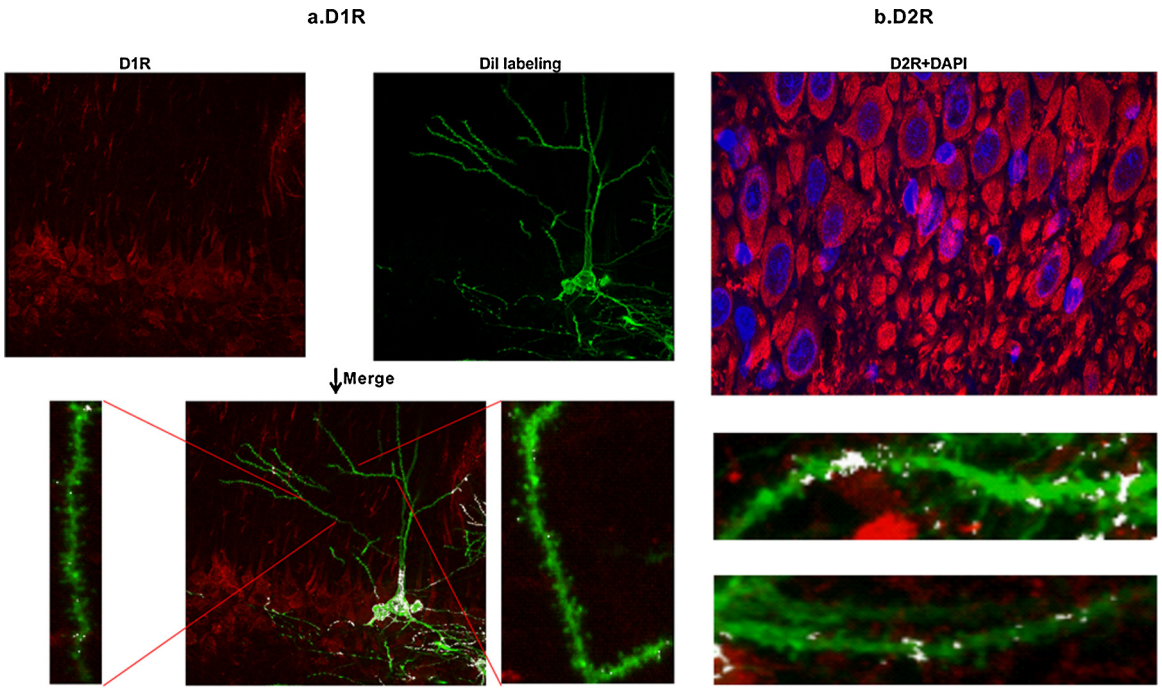


Fig. 6. Distribution of hippocampal D1R and D2R. (a) Immunohistochemistry with D1R and DIL labelling. White spots on the soma and dendritic spines are co-localized points. (b) Upper panel shows D2R around the soma with DAPI in blue. Lower panel with DIL and D2R are shown white co-localized points.

dopamine receptors, either co-expression or co-activation of D1R-D2R elevates intracellular calcium levels, suggesting that D1R and D2R could also show similar functional properties [34]. Unlike to either D1R or D2R, both, training and CE-treatments differentially modulated D3R-containing complexes. D3R levels were higher in the CE-111 yoked group compared to the vehicle yoked group and the CE-111 trained group. This suggests that CE-111 facilitates the expression of plasma membrane-associated D3R levels, but the memory training reduces to the level of yoked control. On the other hand, D3R levels were abundant in the vehicle trained group compared to CE-111 trained and vehicle yoked groups, suggesting that training without CE-111 promotes more D3R-contaning complexes on the plasma membrane. Taken together, CE-111 and memory training modulate D1R-, D2R- and D3R-contaning complexes, but in a different manner. Interestingly, the total level of DAT-containing complexes was not altered while DAT^{ph} (Thr53) was increased in the vehicle yoked group compared to the other three groups. It suggests that phosphorylation at threonine 53 is an important target site to regulate either trafficking or re-uptake/efflux property of DAT during drug treatments and memory formation.

4.3. CE-111 and presynaptic and extrasynaptic dopamine receptors

GABAergic neurons in globus pallidus express presynaptic D2R which inhibits GABA release. The presynaptic D2R synapse becomes super-sensitized after dopamine loss. These supersensitive D2Rs may compensate for the loss of dopamine in Parkinson's disease to induce a strong disinhibition of globus pallidus neuron activity that may contribute to the motor-stimulating effects [35]. Dopamine release facilitates motor functions through the activation of postsynaptic D2R. However, presynaptic D2R is activated or recruited at high levels of dopamine release through a feedback mechanism in order to suppress the motor functions [36]. In other words, postsynaptic D2R activates multiple signalling pathways while presynaptic D2R controls dopamine synthesis and release. D2R knockout mice (D2KO) showed impaired hippocampus-dependent LTP, LTD and spatial memory. However, when the presynaptic D2R ($D2^{DATcre}$) was selectively deleted, the hippocampal LTD was impaired and LTP remained intact in the CA1, whereas spatial memory was equally impaired as in D2KO mice [7]. These studies suggest that presynaptic D2R but not postsynaptic D2R plays a vital role in hippocampus-dependent spatial memory and LTD which is as well associated with spatial memory consolidation and novelty detection [37,38]. In addition, the stimulation of inhibitory D2R lowers extracellular dopamine levels in striatum and prefrontal cortex [39]. Here, the presynaptic D2R was significantly reduced in the CE-111 yoked group compared to the vehicle yoked group. Furthermore, in the CE-111 trained group D2R levels were comparable with those of the vehicle trained group, but significantly higher when compared to the CE-111 yoked group. Interestingly, the same pattern of increased D2R was observed from presynaptic fraction and native protein studies (total membrane fraction). Herein, these results strongly support the importance of our studies on the presynaptic dopaminergic system.

Dopamine reduces the hippocampal and the entorhinal excitatory input to subicular neurons by decreasing the glutamate release following activation of the presynaptic D1R [40]. It also inhibits NMDAR-mediated excitatory postsynaptic potentials through presynaptic D1R [41]. The postsynaptic D1R physically interacts with the GluN1 subunit of the NMDAR to avoid ligand-induced internalization in order to facilitate both, NMDAR and D1R-mediated signalling [42]. These statements are in line with the view that NMDAR-mediated synaptic plasticity is respectively facilitated and inhibited by postsynaptic D1R and presynaptic D1R. Our results indicate that presynaptic D1R was significantly decreased in the CE-111 trained group compared to the CE-111 vehicle group; thus our results suggests that CE-111 may probably facilitate NMDAR-mediated signalling to enhance memory formation. It is noteworthy to mention here that the extrasynaptic D1R might be from extrasynaptic regions of both presynapse and postsynapse. On the whole, we speculate that the presence of CE-111 might have exerted a modulatory action of pre- and

extrasynaptic D1R; whereas it has no effect on postsynaptic D1R as the postsynaptic D1R remained comparable between groups. Future studies should address the functional role of presynaptic and postsynaptic D1R in memory formation and in the treatment of psychostimulants in more detail. Similar to the postsynaptic D1R and D2R levels, the postsynaptic D3R levels are not different between groups. Because we could not detect D3R in presynaptic and extrasynaptic fractions, we conclude that D3R is mainly expressed in hippocampal postsynaptic regions. The activation of hippocampal postsynaptic D3R reduces GABAergic transmission in the CA1 region by inducing PKA-dependent endocytosis of GABA receptors [13]. Cocaine-induced synaptic plasticity of mesencephalic dopaminergic neurons is mediated by presynaptic D3R [43].

Both DAT and DAT Thr^{53} were mainly detected in the extrasynaptic and not in presynaptic fraction, proposing that DAT is primarily accumulated at the extrasynaptic sites. Similar to DAT-containing complexes, the extrasynaptic DAT levels were also comparable between the groups. However, DAT Thr^{53} was significantly reduced in the vehicle yoked group compared to the remaining groups. Even though the functional properties of DAT Thr^{53} are not known yet, an in vitro study proposed that DAT Thr^{53} site plays an important role in DAT-mediated reuptake [10]. Our IHC studies showed the presence of D1R and D2R at the somatic sites and dendritic spines, showing that dopaminergic neurons target both spines and soma. However, the physiological and functional roles of dendritic and somatic dopaminergic receptors are unknown. Future studies should address its distinguished properties during drug treatments, learning and memory.

Herein, we propose that the novel compound CE-111 specifically inhibits DAT-mediated dopamine uptake as well as improves memory performance of rats in the RAM. At the molecular level, the presence of CE-111 modulates D1R, D2R, D3R and DAT^{ph}- containing receptor complexes. Most importantly CE-111 modulates the presynaptic and extrasynaptic dopamine receptors without affecting postsynaptic dopamine receptors.

Conflict of interest

The authors declare that there is no conflict of interest

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.06.011.

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