Behavioural Brain Research xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Synthetic enhancer compounds, besides acting on biogenic amine system, influence the glutamate transmission and stress response*

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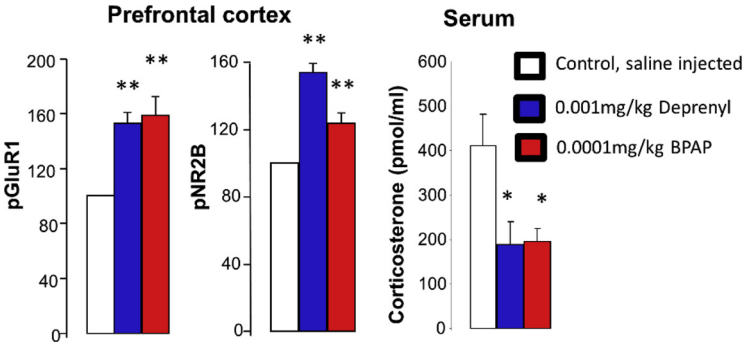
GRAPHICAL ABSTRACT



Three weeks treatment with specific doses of enhancer drugs increased the protein level of phosphorylated glutamatergic receptor subunits in the prefrontal cortex and reduced the resting corticosterone level during the active period in male rats. *p < 0.05, **p < 0.01 vs Control

ARTICLE INFO

Keywords:
Selegiline/(-)-deprenyl
(2*R*)-1-(1-benzofuran-2-yl)-N-propylpentane-2-amine (BPAP)
pGluR1 receptor
pGluN2B receptor subunit
Corticosterone
Stress triad

ABSTRACT

Pharmaceutically available enhancer selegiline/(-)-deprenyl (DEP) in the clinically used dose shows antidepressant effect, but nothing is known about this effect in enhancer dose, and its effect on co-morbid anxiety. Moreover, data about the antidepressant/antianxiety effects of the serotonin-influencing enhancer, (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine (BPAP) are also missing. The aim of the present paper is to establish the role of enhancer regulation in anxiety and follow the changes in the phosphorylation of glutamate subunits in prefrontal cortex as well as stress-related organ and hormonal changes as possible background mechanism. The effect of 3-week-treatment of rats with specific (0.001 mg/kg for DEP, 0.0001 mg/kg for BPAP) and non-specific (0.1 mg/kg for DEP, 0.05 mg/kg for BPAP) enhancer doses were evaluated on anxiety-like behavior in the elevated plus maze (EPM) and open-field (OF) tests. Phosphorylated glutamatergic GluR1 and GluN2B subunits were analyzed by Western blot. Changes in the stress-regulatory system were evaluated by measuring the organ weights and blood corticosterone concentrations. Non-specific enhancer doses had a tendency for anxiolysis on EPM, while only 0.1 mg/kg DEP elevated motility in OF. Specific enhancer doses significantly increased the expression of both glutamatergic receptor subunits; non-specific doses elevated only pGluR1. Treatments had no effects on stress-like organ weights; however, the specific enhancer doses significantly reduced the dark phase resting corticosterone levels. The study proved the enhancer-sensitivity of the glutamatergic transmitter system and suggested enhancer-induced stabilization of stress-hormone levels without major impact on non-stimulated anxiety-like behavior.

* This paper is dedicated to the memory of Joseph Knoll, who passed away in 2018.

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https://doi.org/10.1016/j.bbr.2019.112290
Received 16 May 2019; Received in revised form 29 August 2019; Accepted 7 October 2019

1. Introduction

Endogenous and exogenous enhancer substances facilitate the impulse propagation mediated release of monoamines in the brain opening a new perspective for neuronal regulations [1,2]. We demonstrated the enhancer-sensitivity of the catecholaminergic and serotonergic transmitter systems and identified β-phenylethylamine (PEA) as a natural catecholaminergic activity enhancer substance. Selegiline/(-)-deprenyl (DEP) was proved to be its artificially produced pair [3,4]. Tryptamine is the natural enhancer of the serotonergic neurons, and (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine (BPAP) was developed as its synthetic pair [5,6]. They exert their 'specific' enhancer effect in femto-/picomolar and their 'non-specific' enhancer effect about in a thousand orders higher concentration range [1,7].

In line with the monoamine hypothesis of depression [8], enhancer compounds may significantly improve the effectiveness of drug therapy in major depression and its combination with uptake inhibitors may substantially diminish the number of therapy resistant cases [9,10]. The role of enhancer effect in this respect remains to be clarified. Moreover, one of the limitations of the usage of DEP as an antidepressant is the missing information about its effect on comorbid psychiatric disorders e.g. stress-related anxiety [11].

Previous results using both non-specific [12] and specific enhancer concentrations [7] confirmed that DEP as well as BPAP significantly extend the lifespan of different mammalian species and reduce tumor manifestation [13]. However, these studies suggested that the enhancer regulation may not be exclusive to the biogenic amine system. Glutamate, being the main excitatory neurotransmitter in the brain, is a feasible target. Multiple lines of evidence suggest that glutamatergic neurotransmission plays fundamental role in the pathophysiology of stress-related disorders [14,15]. The medial prefrontal cortex (mPFC) seems to be an important area, where glutamatergic system may influence mood [16]. For example, maternal separation-induced depression led to an upregulation of the N-methyl-D-aspartate receptor 2B (GluN2B) subunit mRNA in the mPFC [17]. Moreover, manipulating the number of GluN2B subunits in the mPFC [18] or the phosphorylation of the subunit may contribute to antidepressant efficacy [19]. However, the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunit GluR1 content in the mPFC was also linked to inflammation-induced depression [20].

We hypothesized that synthetic enhancer drugs will influence anxiety through changes in the phosphorylation of glutamatergic receptors in the mPFC and stress-adaptation. We compared the effect of specific and non-specific peak doses of DEP and BPAP. As chronic treatment is necessary for the development of antidepressant effect in human beings, we were focusing on prolonged, three-week-treatment. The elevated plus maze (EPM) test was used for measuring anxiety and the prolonged observation (30 min) in the open-field aimed to filter any confounding locomotor effect. As a possible background mechanism, changes of phosphorylated AMPA and NMDA receptor subunits (pGluR1, pGluN2B, respectively) were measured by Western blot in the mPFC. As anxiety and depression are closely linked to stress, we also examined the chronic stress-like organ changes (body weight change, adrenal, thymus and spleen weights; stress triad [21]), as well as the resting corticosterone blood level at the end of the treatment among basal conditions.

2. Materials and methods

2.1. Animals

We wanted to check the enhancer activity after sexual maturation, when the endogenous enhancer activity starts to decline [2]. Therefore 2-month-old (around 200 g) male Wistar rats (Semmelweis University's breeding colony, Budapest, Hungary) were used. They were kept in standard cages (5/group) under 12 h light-dark cycles with lights on between 17.00 and 5.00 h, to be able examine the behavior during their active phase. The rats received tap water and rat chow ad libitum. Behavioral testing and sample collections were done during the early dark phase. The blood and brain samples were taken during rest, around 24 h after the last treatment. All manipulations of the animals were approved by the local committee for animal health and care and performed according to the European Communities Council Directive recommendations for the care and use of laboratory animals (2010/63/EU).

2.2. Drugs

We have obtained selegiline/(-)-deprenyl (DEP) from Sanofi-Chinoin (Budapest, Hungary) and (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine (BPAP) from FP Pharmaceutical Corporation (Osaka, Japan). Drugs were dissolved daily in physiological saline.

2.3. Experimental procedure

Rats were injected subcutaneously once daily for 21 days, during the early dark phase. The body weight of the animals was checked twice a week and the dose was adjusted to the actual body weight.

The following groups were formed (n = 8–9/group):

1 Control, saline injection 1 ml/kg
2 DEP 0.001 mg/kg, specific enhancer dose
3 DEP 0.1 mg/kg, non-specific enhancer dose
4 BPAP 0.0001 mg/kg, specific enhancer dose
5 BPAP 0.05 mg/kg, non-specific enhancer dose

Thirty minutes after the 21st treatment, the anxiety-like profile of the rats was tested for 5 min in the EPM and immediately after the general locomotion was measured. Rats were decapitated 24 h after the last treatment. The adrenal gland, thymus and spleen were dissected for assessment of chronic stress-like changes and trunk blood was collected for corticosterone determination by radioimmunoassay. Brains were immediately dissected, mPFC rapidly removed and frozen in liquid nitrogen and later analyzed for pGluR1 (AMPA receptor subunit) and pGluN2B (NMDA receptor subunit) by Western blot.

2.4. Behavioral tests

2.4.1. Elevated plus maze (EPM) test

The maze, consisting of two open arms (50 x 15 cm) and two closed arms (50 x 15 cm, with a 40 cm surrounding wall) extending from a central platform (15 x 15 cm) and opposing each other, was elevated to 50 cm in an isolated dark testing room. The arms of maze were illuminated (180 lux). Rats were placed on the central platform of the maze facing to the open arm. Observation started immediately and lasted for 5 min. Behavior of the rats was recorded by a video-camera, scored later by an experimenter blind to the treatment groups. The maze was cleaned between each animal by 10% ethanol solution.

Percent of time spent in the open arms and ratio of open/total (open plus closed) arm entries were calculated and used as measures of anxiety-like behavior. The number of closed arm entries was an estimate of general locomotor activity. An entry into a compartment was defined as having three paws of the rat in the defined compartment.

During the five-minute test, several ethological-oriented measures were also analyzed. Risk assessment behavior including stretch-attend posture (SAP, the trunk extends and then flexes back to original position) and head dipping (HD, the head flexes below the edge of the open arm) were determined. These behavioral patterns have been associated with detection and analysis of threats or threatening situations. In addition, we analyzed rearing, an exploratory behavioral posture and grooming (animals licks themselves using their forelimbs, mouth, and hind limbs, often in an orderly sequence). Anxiolytic drugs decrease the

number of risk assessment behaviors during the EPM test. These parameters are more sensitive for anxiety-related behavior than conventional measures obtained from the EPM test alone [22,23].

2.4.2. Open-field (OF) test

Locomotor activity was measured for 30 min by the "CONDUCTA System for behavioral and activity studies" (Experimetria Ltd, Budapest, Hungary). The apparatus consists of three black-painted testing boxes (40 × 50 x 50 cm, each) set in an isolated room. Conducta detects movements of the experimental animals by high-density arrays of infrared diodes. Variables recorded and presented are as follows:

Ambulation: horizontal locomotion (walking, running).

Local movements: sniffing, grooming, stereotyped head movements.

Rearing: vertical locomotion.

Upright locomotion: smaller changes in vertical locomotion.

Jumping: vertical escape movement.

Immobility: no apparent movement.

The total time as well as frequency of appearance of each variable was recorded. In case of ambulation the distance travelled was also calculated.

2.5. Western blot

The isolated mPFC was lysed with lysis buffer (containing: 20 mM Tris pH = 7.5, 150 mM NaCl, 2 mM EDTA, 0,5% Triton X-100, 0.5% Proteinase Inhibitor Cocktail (Sigma), 2 mM Na_3VO_4 and 10 mM NaF). Protein concentrations were measured by Bradford method. 30 µg total proteins were mixed with loading buffer containing β-mercaptoethanol and denatured at 99 °C for 5 min. Denatured samples were loaded onto a 10% SDS-polyacrylamide gel and separated for 40 min with 200 V. Proteins were transferred to a PVDF membrane with overnight blotting at 4 °C at a constant 75 mA current. The successful blotting procedure was verified with Ponceau staining. Non-specific binding sites were blocked with 5% non-fat dry milk dissolved in TBS (Tris-buffered saline). pGluR1 (at Ser845) (Cat. no.: #8084, Cell Signaling Technologies, Danver, MA) or pGluN2B (at Tyr1472) (Cat. no.: #4208, Cell Signaling Technologies) antibodies in a dilution of 1:500 were applied as primary antibodies. β-Actin (Cat. no.: A2228, Sigma-Aldrich, St. Louis, MO, USA) immunoreaction served as loading control. For negative control, 1% non-fat dry milk dissolved in TBS without primary antibody was applied. The membranes were incubated with the primary antibodies at 4 °C with gentle shaking overnight. Secondary antibodies were horseradish peroxidase-conjugated antibodies (HRP) from DakoCytomation, Glostrup, Denmark. Anti-rabbit immunoglobulins/HRP (Cat. no.: P0448, dilution: 1:2000) for pGluR1 and pGluN2B receptors and anti-mouse immunoglobulins/HRP (Cat.: no.: P0447 dilution: 1:2000) for β-actin were dissolved in 1% non-fat dry milk (TBS) applied for 1 h at room temperature with gentle shaking.

For washing procedures, TBS + 0.05% Tween20 was used for 5 x 5 min between each steps. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used for visualization and was detected by Kodak Image Station 4000 MM. Detected signals were measured with the ImageJ software. The signal intensity was normalized to control values and expressed in percentage.

2.6. Hormone assay

Trunk blood was centrifuged at 2100 rcf for 20 min at 4 °C (Janetzki K26D) and the serum was stored at −20 °C until analyzed. All samples were always analyzed in the same radioimmunoassay. Concentrations of serum corticosterone were measured in 10 µl unextracted serum by RIA as described earlier [24]. The intraassay coefficient of variation was 12.3%.

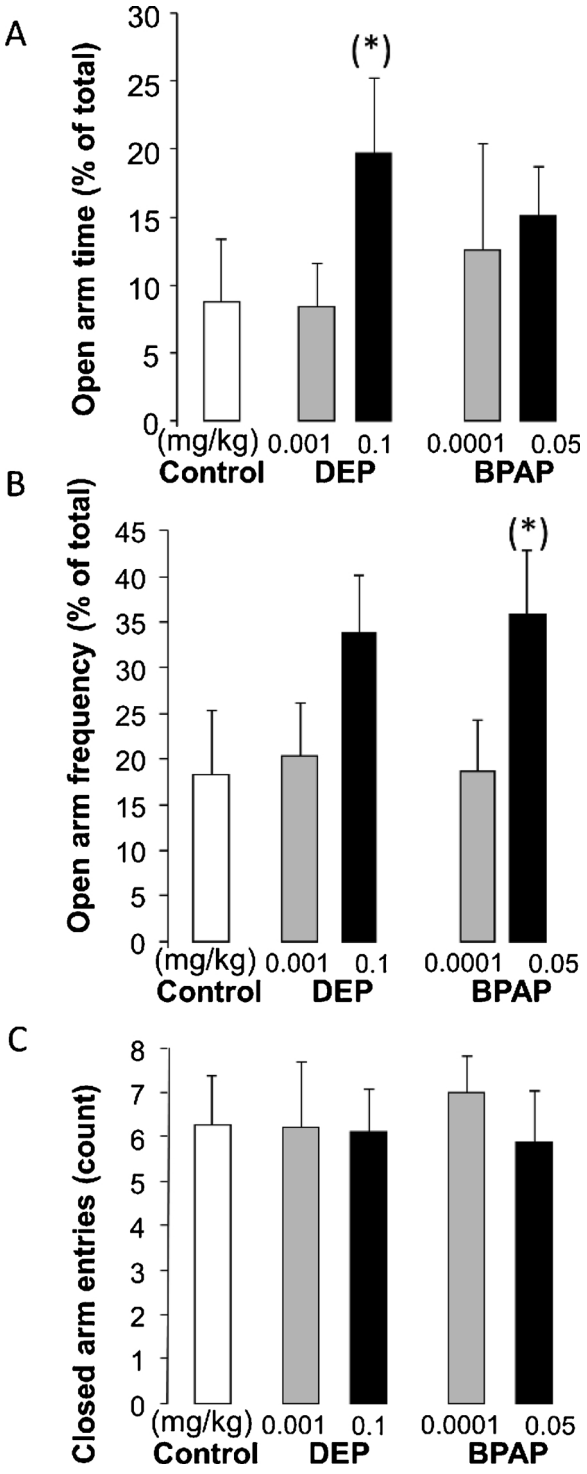

Fig. 1. Behavior in the elevated plus maze. (A) Open arm time reflects the anxiety state. (B) Open arm entries resemble the locomotion independent anxiety. (C) Closed arm entries are a measure of locomotion. N = 8–9/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine; (*) $0.05 < p \leq 0.1$ vs saline-treated group.

2.7. Statistical analysis

Values are presented as mean ± S.E.M. Data were analyzed by analysis of variance using one way or repeated measures ANOVA of the STATISTICA 13.0 software package (StatSoft Inc. Tulsa, Okla, US). Levene's test was not significant in all cases. Multiple pairwise comparisons were made by the Tukey HSD method. Correlations were made

by the Pearson method.

3. Results

3.1. Elevated plus maze (EPM) test

The control animals spent approximately 10% of the time in the open arm, which – theoretically – allows treatment-induced changes into both directions. Nevertheless, as Fig.1A shows, none of the treatments induced significant alterations ($F(4,38) = 0.87$, $p = 0.49$), despite an anxiolytic trend with the higher dose, 0.1 mg/kg, of DEP ($p = 0.10$ vs control). In addition, the DEP group treated with 0.1 mg/kg spent significantly lower time in the closed arm (Control: 64.9 ± 5.8% vs. DEP 0.1 mg/kg: 42.0 ± 6.1%; $p = 0.03$, data not presented in the figure). No change in the means of locomotion during this short, 5 min observation period, reflected by the closed arm entries was observed ($F(4,38) = 0.12$, $p = 0.97$) (Fig.1C). There might be alterations within the individual animals; therefore, we calculated also the locomotion independent measure of anxiety in the open arm entries (Fig.1B). The general analysis did not reveal any significant difference ($F(4,38) = 1.82$, $p = 0.14$), but the higher dose of BPAP (0.05 mg/kg) induced trends for anxiolysis ($p = 0.10$ vs. control). Moreover, in controls 3 out of 8 animals stayed in the closed arm during the entire 5-min observation period, while in DEP (0.1 mg/kg) and BPAP (0.05 mg/kg) groups only 1/9.

The ethological measures of anxiety (SAP ($F(4,39) = 0.87$, $p = 0.49$) and HD ($F(4,39) = 0.44$, $p = 0.78$)) as well as rearing ($F(4,39) = 0.59$, $p = 0.67$), and grooming ($F(4,39) = 0.59$, $p = 0.67$) did not show any alterations between the groups.

3.2. Open-field (OF) test

In the case of the distance travelled, the main effect of treatment was not significant ($F(4,39) = 0.81$, $p = 0.53$) (Table 1. 1st row), but the animals moved less in line with time (effect of time: $F(2,78) = 198.9$, $p < 0.01$) (Fig.2A). The difference between first and second/third 10 min was highly significant in each case ($p < 0.01$), while in the case of saline and 0.001 mg/kg DEP we could not detect further decrease during the last 10 min compared to second 10-min period. In all other cases (DEP 0.1 mg/kg, BPAP 0.0001 mg/kg, BPAP 0.05 mg/kg), there was a further decline ($p < 0.01$). The treatment influenced locomotion in a time-dependent fashion (treatment x time interaction: $F(8,78) = 2.69$, $p = 0.01$). During the first 10 min, 0.1 mg/kg-DEP-treated rats were more active compared both to saline and to 0.001 mg/kg-DEP-treated groups.

The time spent with ambulation was not affected by the treatment alone ($F(4,39) = 0.82$, $p = 0.52$) (Table 1. 2nd row) with significant time effect ($F(2,78) = 206.2$, $p < 0.01$) and treatment x time interaction ($F(8,78) = 2.36$, $p < 0.05$) (Fig. 2B). The post hoc analysis revealed similar differences between groups as was presented for distance travelled.

The 30 min velocity of movement was not influenced by treatment (Table 1. 3rd row), but a significant decrease was observed in the last 10-min period, compared to the first 10 min (data not shown) (time: $F(2,78) = 5.02$, $p < 0.01$).

Rearing frequency reduced with time ($F(2,78) = 31.3$, $p < 0.01$) (Table 1. 6th row). More specifically, except 0.001 mg/kg DEP group in all other cases there was a decline already during the second 10-min period, while further decline was detectable only in 0.0001 mg/kg BPAP group. In case of time spent rearing (Table 1. 7th row; Fig. 2C), the 0.1 mg/kg DEP group showed enhanced levels during the second and third 10-min observation periods compared to saline-treated group ($p < 0.05$).

Considering the frequency and duration of local movement (Table 1. 4th, 5th row), smaller changes in vertical locomotion (upright frequency, Table 1. 8th row and the time spent in upright position, Table 1. 9th row) as well as frequency of jumping (Table 1. 10th row) did not show treatment-related alterations.

Rats became more immobile with time ($F(2,78) = 27.6$, $p < 0.01$) (Table 1. 11th row; Fig.2D). Specifically, only in saline-treated animals the immobility time during the second 10 min was higher than during the first 10 min, while except 0.001 mg/kg DEP, in all other cases rats spent significantly longer immobile time during the third 10 min section than during the first 10 min.

3.3. Glutamate receptors

The amount of the phosphorylated AMPA subunit, pGluR1, was significantly enhanced in comparison to saline-treated controls by the treatment with both the specific and non-specific doses of DEP and BPAP ($F(4,39) = 9.78$, $p < 0.01$, Fig.3A).

The amount of the phosphorylated NMDA subunit, pGluN2B, was significantly increased by the treatment with the specific enhancer doses (0.001 mg/kg DEP and 0.0001 mg/kg BPAP) ($F(4,39) = 70.7$, $p < 0.01$, Fig.3B). In contrast, the quantity of the subunit was not influenced by the non-specific enhancer dose of DEP (0.1 mg/kg). Moreover, in the case of BPAP the non-specific enhancer dose (0.05 mg/kg) induced even a significant reduction compared to the control ($p < 0.01$).

The enhancer compounds both in their specific and non-specific dose failed to induce any significant changes on the expression of the non-phosphorylated GluR1 (data not shown).

Table 1
Open field behavior.

	Control	DEP		BPAP		F(4,39)	p
		0.001 mg/kg	0.1 mg/kg	0.0001 mg/kg	0.05 mg/kg		
1. Distance travelled (cm)	2227.2 ± 262.9	2546.8 ± 177.1	2881.0 ± 374.7	2639.0 ± 206.5	2759.0 ± 282.1	0.81	0.53
2. Ambulation time (sec)	208.0 ± 21.1	241.6 ± 16.3	260.4 ± 28.3	245.0 ± 16.2	251.4 ± 22.5	0.82	0.52
3. Velocity (cm/sec)	10.6 ± 0.20	10.6 ± 0.42	10.9 ± 0.25	10.7 ± 0.21	10.8 ± 0.24	0.23	0.92
4. Local movement count	245.0 ± 5.2	246.4 ± 6.0	236.0 ± 4.3	246.4 ± 5.0	252.1 ± 6.4	1.38	0.34
5. Local movement time (sec)	1146.9 ± 37.1	1114.7 ± 36.6	1085.7 ± 32.6	1140.8 ± 26.0	1068.9 ± 35.5	1.00	0.41
6. Rearing count	55.6 ± 5.1	68.8 ± 9.9	79.9 ± 8.4	76.1 ± 6.3	69.2 ± 9.4	1.23	0.31
7. Rearing time (sec)	158.1 ± 19.3	191.6 ± 38.9	253.8 ± 24.9	207.2 ± 21.6	201.8 ± 28.6	1.49	0.23
8. Upright count	58.5 ± 8.2	60.3 ± 8.3	78.1 ± 6.9	71.4 ± 3.7	59.0 ± 8.5	1.46	0.23
9. Upright time (sec)	80.9 ± 12.6	82.1 ± 12.5	107.7 ± 10.6	99.1 ± 7.4	78.6 ± 12.9	1.31	0.28
10. Jumping count	0.63 ± 0.26	0.00 ± 0.00	0.33 ± 0.24	0.44 ± 0.34	0.11 ± 0.11	1.25	0.31
11. Immobility time (sec)	288.0 ± 31.7	253.2 ± 56.0	201.1 ± 25.7	208.0 ± 31.7	278.9 ± 47.9	0.95	0.44

The detailed analysis of the open-field during the entire 30 min did not reveal any significant difference between the groups. N = 8–9/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine.

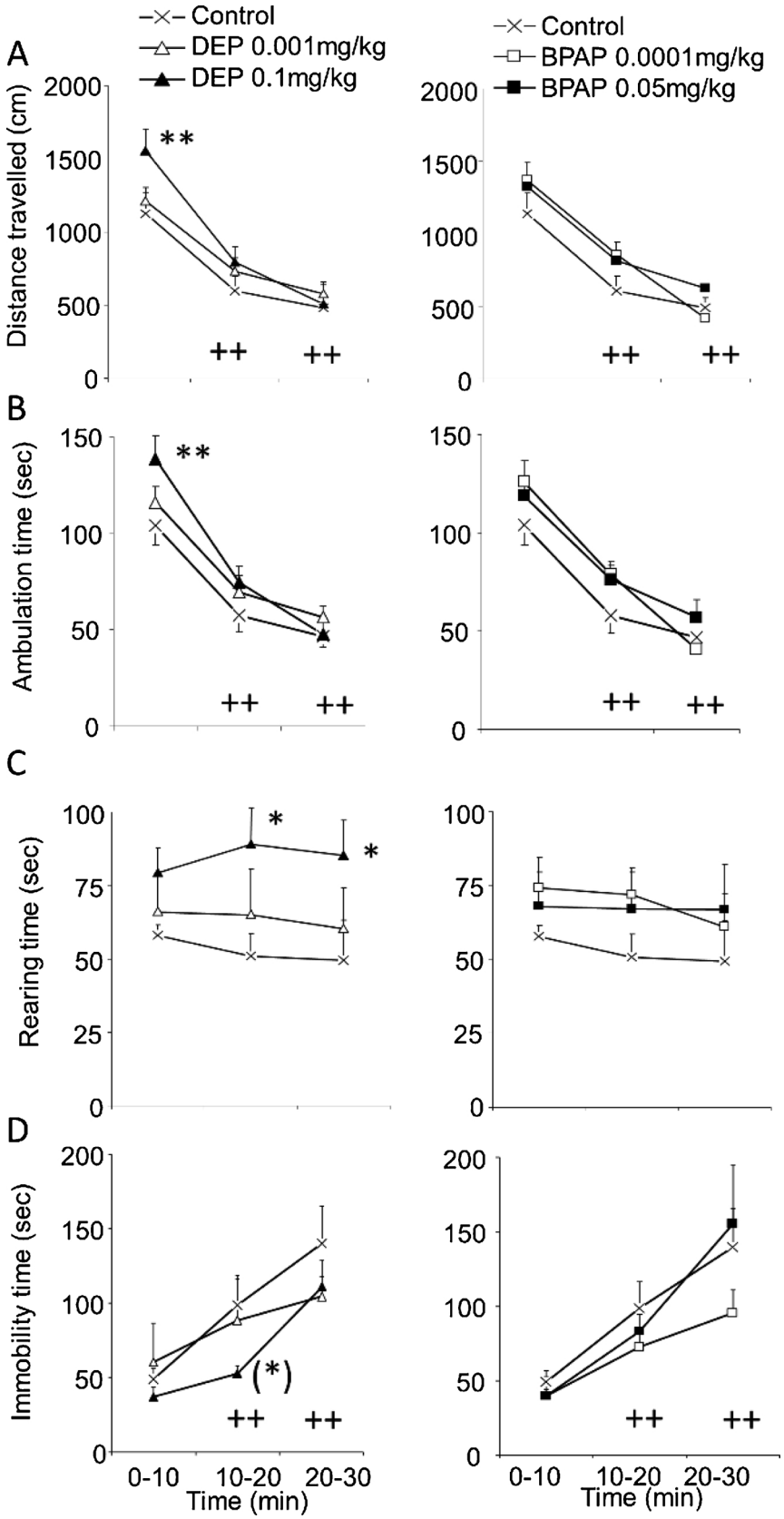

Fig. 2. Behavior on the open-field during 30 min in 10 min sequences. (A) Distance travelled and (B) ambulation time were significantly enhanced by higher doses of DEP during the first 10 min observation period. (C) The same treatment elevated the rearing activity during the last 20 min, (D) but none of the treatment had an effect on immobility. We presented the 5 groups separately only for better visibility. The controls were the same on both sides. N = 8–9/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine; (*)$0.05 < p \leq 0.1$, *$p < 0.05$, **$p < 0.01$ vs saline-treated group; + +$p < 0.01$ vs the first 10 min.

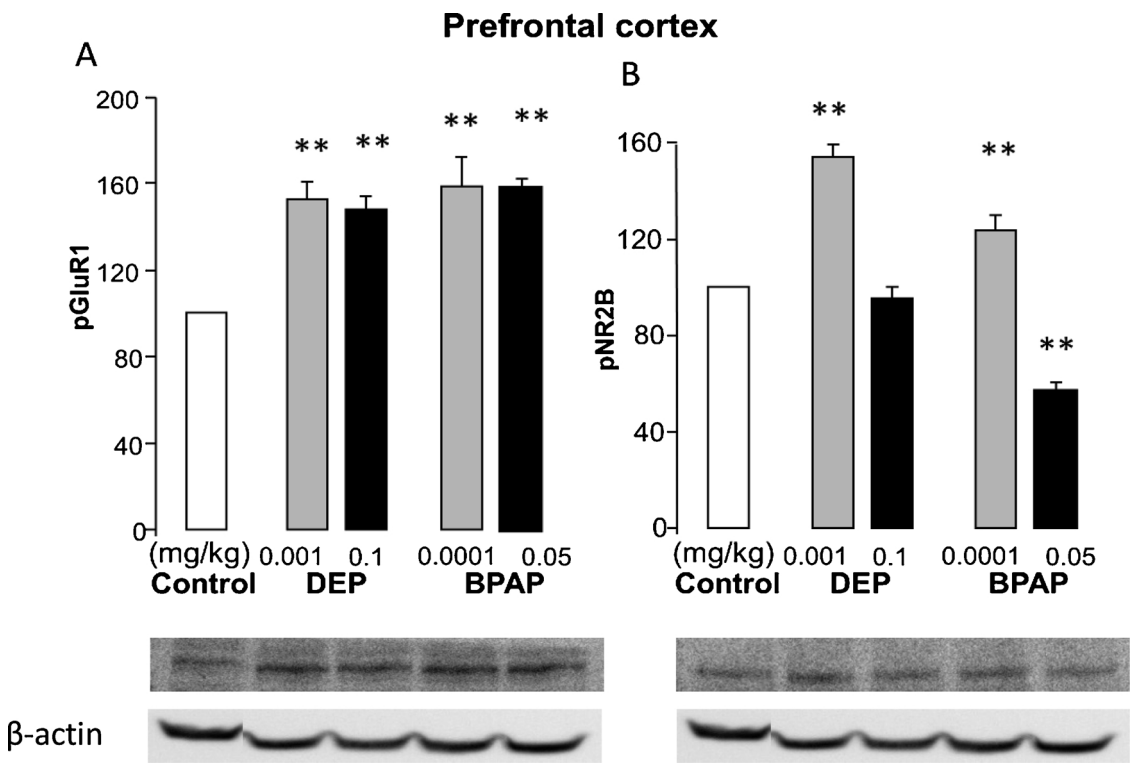

Fig. 3. Changes in the protein amount of phosphorylated glutamatergic receptor subunits measured by Western blot in the mPFC. (A) The level of phosphorylated AMPA receptor subunit (pGluR1) (B) The level of phosphorylated NMDA receptor subunit (pGluN2B) in animals treated with specific and non-specific enhancer doses of DEP and BPAP. n = 3/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine; **p < 0.01 vs saline treated group.

3.4. Changes in the stress-adaptation

There was no significant alteration between the groups in any measured body and organ weight parameters (Table 2.).

On the contrary to the somatic parameters, serum corticosterone levels were influenced by the treatment ($F(4,39) = 3.84$, $p = 0.01$, Fig.4) with lower detectable level in animals treated with the lower, specific enhancer doses measured under rest during the active, dark phase ($p < 0.05$).

4. Correlations

There was a significant negative correlation between pGluN2B receptor protein amount and the time spent in SAP in EPM ($r = -0.56$, $p < 0.05$), as well as ambulation time ($r = -0.65$, $p = 0.01$) and distance travelled ($r = -0.61$, $p < 0.05$), rearing time ($r = -0.54$, $p = 0.05$) and rearing count ($r = -0.60$, $p < 0.05$) and the same receptor protein and absolute ($r = -0.60$, $p < 0.05$) and relative spleen weight ($r = -0.77$, $p < 0.01$). Moreover, negative correlation was detected between pGluN2B subunit and corticosterone levels, too ($r = -0.55$, $p < 0.05$).

5. Discussion

The present paper demonstrated the enhancer-sensitivity of the glutamatergic and the stress-regulatory systems without significant effects on locomotion and anxiety, measured on OF and EPM, respectively. Specifically, the phosphorylation of the glutamatergic AMPA receptor subunit GluR1 was sensitive (enhanced) toward both the specific and the non-specific doses of the synthetic enhancers (Fig. 3A). In contrast, the pGluN2B and corticosterone levels were significantly influenced (elevated or diminished, respectively) only by specific enhancer doses of DEP and BPAP (Figs. 3B, 4).

At present DEP is the only synthetic enhancer used in therapy, however, it is used in non-specific enhancer dose. A recent review summarized 50-year history of DEP, published in thousands of papers, registered already in more than 70 countries, marketed under more

Table 2
Somatic parameters.

	Control	DEP		BPAP		F(4,39)	p
		0.001 mg/kg	0.1 mg/kg	0.0001 mg/kg	0.05 mg/kg		
Weight (g)	348.5 ± 7.4	351.8 ± 7.4	344.6 ± 5.6	350.0 ± 5.1	343.6 ± 5.7	0.32	0.86
Body weight change during 3 weeks (g)	138.8 ± 3.2	139.1 ± 4.4	134.2 ± 3.6	147.3 ± 4.6	134.4 ± 5.6	1.48	0.23
Adrenal weight (mg)	62.2 ± 3.7	57.8 ± 2.5	60.7 ± 4.6	59.4 ± 1.6	65.3 ± 3.2	0.78	0.55
Relative weight of the adrenal gland (mg/kg)	178.4 ± 10.0	164.0 ± 5.4	176.0 ± 12.8	170.3 ± 6.3	189.8 ± 8.3	1.19	0.33
Thymus weight (mg)	648.4 ± 43.4	656.7 ± 43.7	643.0 ± 43.3	712.3 ± 42.0	637.3 ± 39.3	0.52	0.72
Relative weight of the thymus (mg/kg)	1871.6 ± 137.5	1869.4 ± 124.4	1863.0 ± 117.0	2033.1 ± 113.4	1857.6 ± 116.2	0.39	0.81
Spleen weight (mg)	877.5 ± 37.2	833.2 ± 37.9	974.2 ± 60.8	905.8 ± 31.7	879.1 ± 36.7	1.53	0.21
Relative weight of the spleen (mg/kg)	2520.8 ± 105.5	2367.2 ± 90.1	2816.4 ± 141.5	2587.9 ± 82.2	2559.4 ± 100.4	2.37	0.06

Chronic stress-like changes were not detected after 3 weeks treatment with either specific or non-specific enhancer doses of DEP and BPAP. N = 8–9/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine.

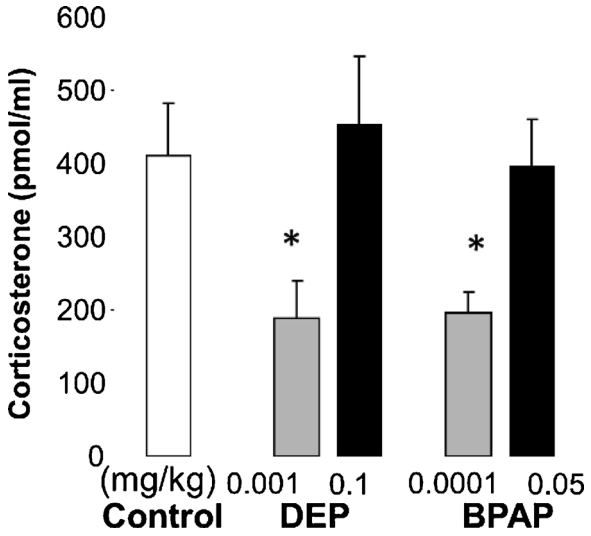

Fig. 4. Serum corticosterone levels (pmol/ml) at rest measured at the early dark phase 24 h after the last injection following 3 weeks treatment. Specific enhancer doses significantly reduced the activity-induced changes. n = 8–9/group; DEP: (-)-deprenyl; BPAP: (2*R*)-1-(1-benzofuran-2-yl)-*N*-propylpentane-2-amine; *p < 0.05 vs saline treated group.

than 100 trade names and is still increasingly used to improve life-quality in the latter decades [25]. The first step in clarifying its pharmacological spectrum was the realization that DEP is a selective MAO-B inhibitor [26], which was followed by the discovery that DEP is a unique MAO inhibitor free of cheese effect [27]. The final step in DEP history was the discovery of the enhancer-sensitive brain regulations and the demonstration that DEP is a synthetic enhancer compound [1,2].

Originally DEP was developed in 1964/1965 as a compound for treatment of depression [28], however, when its selectivity towards MAO-B was discovered it was introduced in the clinical practice as a potent and safe new compound for treatment of Parkinson's disease. Whereas many studies demonstrated the antidepressant effect of DEP [29,30], it was finally registered with antidepressant indication only in 2006, in the USA in its non-specific enhancer dose (around 5 mg/kg) and the transdermal patch. Administration of DEP was published to be suitable for long term treatment of major depressive disorder [31,32].

One argue which may explain even now the underutilization of DEP as an antidepressant is the missing information about its effect on co-morbid psychiatric disorders e.g. stress-related anxiety [11]. While there is little evidence of anxiolytic effect of non-specific enhancer doses of DEP so far (0.25 mg/kg in rats and 5–10 mg in human beings), no data are known at all about the anxiolytic effect of specific enhancer doses.

As generally chronic treatment is necessary for the development of antidepressant effect, we were focusing on prolonged, three-week-treatment. Our present data seem not to support a characteristic anxiolytic effect. In connection with this, however, we have to mention two important comments. First, that despite their wide use, the relevance of EPM and OF in measurement of pathological anxiety is questionable [33]. Avoidance of open places is an ethologically relevant behavior of the animals, thus, the ineffectiveness of our treatment on this 'normal' behavior is not a disadvantage. Secondly, we have to emphasize that enhancer compounds increase the stimulus induced release of catecholamines and serotonin and not the basal one [34]. The *in vitro* data are in good correlation with the *in vivo* results, which demonstrated the learning performance improving effect of DEP on low performing but the lack of effect on high performing rats. Previous studies with 0.25 mg/kg DEP found also only small antidepressant and anxiolytic effect in non-stimulated tests [35]. Moreover, recently published results on antidepressant effect of the non-specific dose of DEP during the forced-swim test, as well as its efficacy preventing the impairment of the hippocampal CA1 long-term potential provoked by low frequency stimulation before high frequency one, also support our hypothesis [36]. On the basis of these, we assume that anxiolytic effect of enhancers will be detected only in highly anxious rats (e.g. EPM or OF conducted after stress).

The precise mechanism behind the enhancer activity is not yet clarified. Recent data demonstrated, that antidepressant effect of DEP, or its action on the hippocampal long-term potentiation is independent from the MAO-B inhibition, as rasagiline, a potent MAO-B inhibitor was lack of these effects [36]. Interaction with distinct sites of vesicular monoamine-transporter2 might be the main mechanism of action of the enhancer-sensitive regulations which clarifies the bi-modal, bell-shaped concentration- effect curves of DEP and BPAP [1].

In the Western blot experiment, we have shown for the first time that enhancer drugs may influence the sensitivity of prefrontal cortical neurons to the glutamate as well beside the well-known effects on catecholaminergic and serotonergic systems. There was no difference in the effect of specific and non-specific enhancer doses in the case of AMPA receptor, suggesting that changes in this system might not be a specific enhancer effect. On the contrary, phosphorylation of the GluN2B receptor subunit seems to be specifically involved in the specific enhancer regulation. Though the mechanism behind the difference between the specific and non-specific doses of enhancer compounds is not clear, there are data in the literature supporting our observation. Previous results showing ineffectiveness of non-specific enhancer doses of DEP (0.05, 0.25 mg/kg) on the GluN2B level in the mPFC support this observation [37]. In addition, the non-specific dose BPAP showed a potent inhibitory effect on both p-Akt Ser473 as well as on Erk 1 and 2, while the specific dose provoked activation of both signaling pathways [38]. Actually, in our experiments the drug induced pGluN2B elevation correlated with some anxiety-like measures, like reduced SAP and rearing. Antagonizing the GluN2B subunit was shown to decrease anxiety [39] and anxiety-like behavior was accompanied by changes in mPFC GluN2B levels [40]. As there was no clear anxiolytic effect of the synthetic enhancer substances on EPM, we might assume that the glutamatergic system will contribute more to a pathological anxiety state.

Additionally, multiple lines of evidence suggest that glutamatergic neurotransmission plays fundamental role in the pathophysiology of stress-related disorders (see also introduction). Changes in GluN2B subunit in the mPFC might have contributed also to changes in stress-regulation as its elevation occured parallel with reduced corticosterone levels. Additionally, enhanced pGluN2B level was accompanied by reduced spleen weight (negative correlation), the latter reflecting an overall reduced corticosterone supply. Indeed, the cortical GluN2B plays a major role in modulating adaptive responses to stress [41].

According to the general view, chronic stressors enhance resting corticosterone levels [42]. Thus, our treatment did not induce any chronic stress-like changes, neither in hormone levels, nor in the stress-triad. However, in traditional stress research the blood samples are taken during the nadir of the hormone levels, at the beginning of the light phase, when the animals are inactive. In the present experiment, the behavior of the animals was examined during their active phase, thus, corticosterone was also taken at this time-point. Despite no overall effect on general locomotion, the enhancer drugs, in specific enhancer concentrations significantly reduced the activity-induced elevation in the main stress hormone. Considering the beneficial effects of the enhancer drugs (prolongation of lifespan [7], suppression of tumor manifestation [13]), we might assume that this smaller circadian elevation of corticosterone might be one of the mechanisms contributing to the beneficial effects of prolonged enhancer treatment.

6. Conclusion

Changes in the phosphorylated GluN2B subunit expression in the

prefrontal cortical neurons, as well as smaller resting corticosterone elevation during the dark phase of the day may contribute to the previously published beneficial effects of prolonged treatment with the enhancer substances on aged animals and on the tumor manifestation. These changes were specific to the enhancer regulation as were not detected after treatment with non-specific doses, but were not accompanied by changes in “normal”, ethologically relevant anxiety.

Funding

The authors thank for the financial support of our work by the FP Pharmaceutical Corporation (Osaka, Japan).

Conflict of interest statement

The authors declare no potential conflicts of interest.

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