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Semax, An ACTH(4-10) Analogue with Nootropic Properties, Activates Dopaminergic and Serotoninergic Brain Systems in Rodents*

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Corticotrophin (ACTH) and its analogues, particularly Semax (Met-Glu-His-Phe-Pro-Gly-Pro), demonstrate nootropic activity. Close functional and anatomical links have been established between melanocortinergic and monoaminergic brain systems. The aim of present work was to investigate the effects of Semax on neurochemical parameters of dopaminergic- and serotonergic systems in rodents. The tissue content of 5-hydroxyindoleacetic acid (5-HIAA) in the striatum was significantly increased (+25%) 2 h after Semax administration. The extracellular striatal level of 5-HIAA gradually increased up to 180% within 1–4 h after Semax (0.15 mg/kg, ip) administration. This peptide alone failed to alter the tissue and extracellular concentrations of dopamine and its metabolites. Semax injected 20 min prior D-amphetamine dramatically enhanced the effects of the latter on the extracellular level of dopamine and on the locomotor activity of animals. Our results reveal the positive modulatory effect of Semax on the striatal serotonergic system and the ability of Semax to enhance both the striatal release of dopamine and locomotor behavior elicited by D-amphetamine.

KEY WORDS: 3,4-Dihydroxyphenylacetic acid; 5-hydroxyindoleacetic acid; ACTH analogues; cognitive enhancers; D-amphetamine; dopamine; dopamine receptors; locomotor activity; melanocortin receptors; neurotrophic factors; Semax; serotonin.

INTRODUCTION

The search for drugs improving memory and enhancing cognitive functions in humans and the study of mechanisms of their action seem to be a challenging scientific problem. The investigations of de Wied initiated studies that demonstrated the role of corticotropin (ACTH) and its fragments in conditioned behavior (1). Further, it was shown that ACTH and its analogues display noortopic properties, improve memory and learning processes (2–4). The ACTH(4–7) fragment appeared to be the shortest sequence required to influence mammalian behavior, exhibiting the same potency as ACTH(4–10) (3). A number of ACTH analogues have been synthesized. Some of them are more stable and more potent than the natural N-terminal ACTH fragments (3,5). Among these analogues, ORG 2766 [H-Met(O2)-Glu-His-Phe-D-Lys-Phe-OH] has been investigated most intensively and became the prototype in studies on the

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influence of melanocortins on the central nervous system (3,6,7).

Another ACTH(4–10) analogue has been synthesized and named Semax. The peptide has the structure Met-Glu-His-Phe-Pro-Gly-Pro, which includes the minimal tetrapeptide sequence of ACTH(4–7). The Pro-Gly-Pro fragment of Semax is responsible for its metabolic stability and the relatively long duration of effects (8). Semax penetrates into the brain after an intravenous injection (9) or intranasal application (10,11) and demonstrates nootropic activity in animals and humans (8,10,12). Presently, Semax is used as a remedy for the treatment of cognitive disorders and ischemic strokes. However, the neurochemical mechanisms underlying the effects of Semax on the brain functions are still unclear.

Recently, the close functional and anatomical link has been established between the melanocortinergic and monoaminergic systems in the brain (13–15). Melanocortin (MC) receptors were found to be expressed in dopaminergic nuclei and the main dopaminergic projection areas. Also serotonergic and adrenergic nuclei express the melanocortin MC4 receptor, suggesting that melanocortins may modulate the brain monoaminergic systems (13,14). Interestingly, a few ACTH analogues, including Semax, display properties of the MC receptor antagonist (16). ACTH and its analogues are thus able to affect the dopaminergic (17–20) and serotonergic (21) brain systems. ACTH(1–24) and α-melanotropin (α-MSH), an MC receptor agonists, stimulate dopamine release in the caudate nucleus (19) and nucleus accumbens (20). On the other hand, the brain monoaminergic systems have been also shown to contribute to learning and memory processes (22–24), as well as being involved in depression and anxiety disorders. The aim of the present work was therefore to investigate the effects of Semax on the neurochemical parameters of the monoaminergic (dopaminergic and serotonergic) systems in the rodent brain.

EXPERIMENTAL PROCEDURE

Animals. Adult male C57/bl mice (18–24 g; Krjukovo, Moscow, Russia) were housed 10 per cage. Adult male Sprague–Dawley rats (250–300 g; Brain Research Center, University of Tampere, Finland) were housed two per cage. The animals received freely food and water and were kept at 12-h light/dark cycle. Behavioral studies were performed between 10:00 AM and 5:00 PM. All studies were approved by Russian Academy of Medical Sciences Guide for Care and Use of Laboratory Animals and by the Animal Experiment Committee of the University of Tampere.

Drugs. Semax (Met-Glu-His-Phe-Pro-Gly-Pro) was synthesized in the Institute of Molecular Genetics, Russian Academy of Sciences (Moscow, Russia) and D-amphetamine sulfate was purchased from Sigma (St. Louis, MO). Semax (0.15 and 0.6 mg/kg) and D-amphetamine sulfate (5 and 2 mg/kg, free base) were dissolved in saline (0.9% NaCl) and administered intraperitoneally (i.p.). All other chemicals used were obtained from common commercial sources and were of the highest purity available.

Determination of the Tissue Content of Monoamines and Their Metabolites in the Brain Structures of C57/bl Mice. C57/bl mice were sacrificed 0.5 or 2 h after Semax (0.15 mg/kg, i.p.) administration (8–10 animals per group). The hypothalamus and striatum were isolated on ice (+4°C) and stored in liquid nitrogen. The brain structures were homogenized in 0.1 M perchloric acid with 0.5 μM 3,4-dihydroxybenzoic acid as an internal standard and centrifuged (10,000 × *g*, 10 min, 4°C; K70D, Germany). The supernatants were analyzed with high performance liquid chromatography with electrochemical detection (HPLC/ED) (25). Dopamine (DA), its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) as well as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were detected with a glassy carbon electrode set at +0.8 V (BAS LC-4B, Bioanalytical Systems, West Lafayette, IN). The mobile phase contained 0.1 M citrate-phosphate buffer (pH 2.9), 1.85 mM 1-octanesulfonic acid, 0.27 mM ethylenediaminetetra-acetate (EDTA) and 8% acetonitrile. Monoamines and their metabolites were separated by the analytic reverse-phase column Phenomemex-C18, 4 μm, 150 × 4.6 mm. The flow rate was 1.0 ml/min.

Determination of the Extracellular Levels of Monoamines and Their Metabolites in Freely Moving Sprague–Dawley Rats. The animals were anesthetized with chloral hydrate (400 mg/kg, i.p.). The custom-made concentric dialysis probes (3 mm dialysis membrane, 0.5 mm outer diameter, 10 kDa cutoff, Hospal, Italy) were implanted into the right striatum (coordinates: AP +0.5, L+3, DV +6.5; relative to Bregma, according to (26). At 20–24 h after surgery, the dialysis probes were connected to a microperfusion system (CMA, Sweden) and perfused at 2 μl/min with artificial cerebrospinal fluid containing (in mM): Na^+ 155.0, K^+ 2.9, Ca^{2+} 1.1, Mg^{2+} 0.8, and Cl^- 133.0; pH 7.4 (27). After an equilibration period of 1.5 h, dialysate fractions were subsequently collected every 20 min. The location of the microdialysis probes was verified post-mortem with cryostat microtome sections of the brain.

At least four basal samples were taken before the first drug administration. Semax (0.15 or 0.6 mg/kg, i.p.) was administered either alone or in combination with the dopamine agonist D-amphetamine (D-AMPH) at the dose of 5 mg/kg (free base), i.p., 20 min after the Semax injection. Each group consisted of 6–7 rats.

The dialysate fractions were analyzed with HPLC/ED. The potentials of electrochemical detection electrodes (Microdialysis Cell 5014B, Coulochem II, ESA Inc., Chelsdora, USA) were set at −175 mV and +200 mV. The analytic column and the mobile phase were the same as described above.

The Assessment of Locomotor Activity of C57/bl Mice. The effects of Semax (0.6 mg/kg, i.p.), D-AMPH (2.0 mg/kg, i.p.) and their combination on the locomotor activity of C57/bl mice were also studied. The dose of D-AMPH was chosen to elicit a clear enhancement in the locomotor activity. Each group consisted of 10–12 mice. The locomotor activity was recorded in every mouse 3 times for 10-min periods using the Opto-Varimex apparatus. The first session (intact mice) was 0–10 min, followed by an injection of Semax or saline. The second session was 20–30 min. The animals received an injection of D-AMPH or saline at 30 min. The third

session 50–60 min began 20 min after D-AMPH administration. The scheme of drug administration in the microdialisys and behavioral experiments was the same.

Statistical Analysis. The tissue contents of monoamines and their metabolites were analyzed by Mann–Whitney *U* test using Statistica v. 5.0 software. In the microdialysis experiments, the mean basal levels were calculated for each rat as the mean values of four basal samples before drug or vehicle administration. The relative magnitudes of the effects of treatments are expressed as percentage changes from this baseline (100%). Comparison of different groups in the microdialysis studies was made using two-way repeated measures ANOVA and Duncan's post–hoc test (Statistica v. 5.0 software). In the behavioral studies, the locomotor activity in the third session that represents the effects of both drugs is expressed as percent of activity in the previous (second) session (100%). Comparison of different groups in the behavioral studies was made using one- way ANOVA. The data are presented as mean values ± SEM.

RESULTS

Effects of Semax on the Tissue and Extracellular Concentrations of 5-HT and its Metabolite

The tissue contents of 5-HT and its metabolite 5-HIAA in the hypothalamus of control C57/bl mice were 7.00 ± 0.29 and 1.88 ± 0.12 nmol/g wet tissue, respectively. The striatal tissue contents of 5-HT and 5-HIAA were 3.32 ± 0.12 and 1.75 ± 0.15 nmol/g wet tissue, respectively. Semax (0.15 mg/kg, i.p.) significantly increased ($P < 0.05$, *U*-test) the tissue concentrations of 5-HIAA in the hypothalamus at 0.5 h and in the hypothalamus and striatum at 2 h after the treatment (Fig. 1). This effect was more pronounced at 2 h. The peptide failed to alter the tissue content of 5-HT in either cerebral structure.

Similar results were obtained in the microdialysis experiments on freely moving Sprague–Dawley rats. The basal level of 5-HIAA in the dialysates from the striatum was 297 ± 7 pmol/ml. The extracellular level of 5-HIAA gradually increased after Semax administration (0.15 and 0.6 mg/kg, i.p.) during the experiment (Fig. 2). The significant changes ($P < 0.05$, ANOVA) were observed after 100 min. This effect was greater at the Semax dose of 0.15 mg/kg. The striatal level of 5-HT was below the detection level in our microdialysis experiments.

Effects of Semax on Tissue and the Extracellular Concentration of DA and its Metabolites

The tissue contents of DA and its metabolites DOPAC and HVA in the striatum of control C57/bl mice were 90.0 ± 2.8, 5.11 ± 0.16 and 9.43 ± 0.40 nmol/g wet tissue, respectively. The basal extracellular levels of DA, DOPAC and HVA in the striatal dialysates were 0.83 ± 0.12, 996 ± 25 and 761 ± 37 pmol/ml, respectively. Semax alone (0.15 and 0.6 mg/kg, i.p.) failed to alter these tissue and extracellular concentrations (data are not shown).

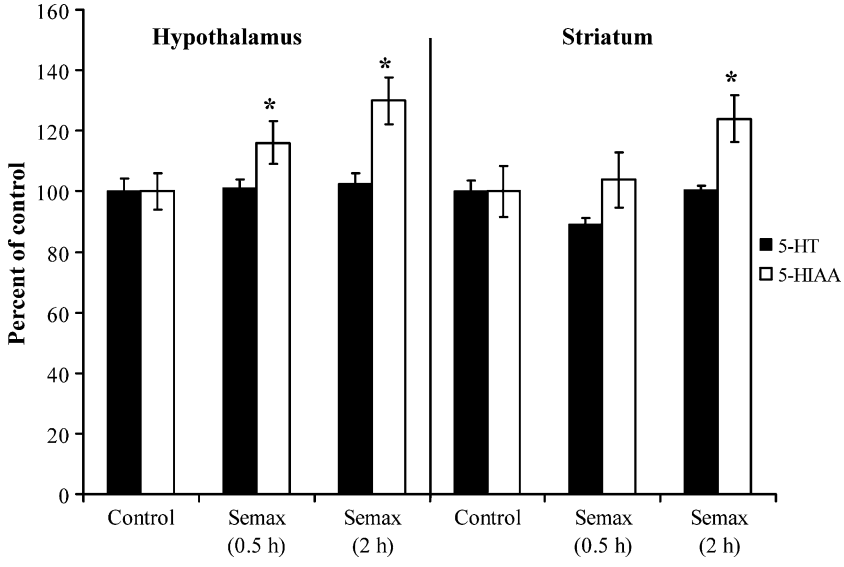

Fig. 1. Effects of Semax on the tissue concentrations of serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the hypothalamus and striatum of C57/bl mice 0.5 and 2 h after the treatment. Data are presented as percentages of the control group as mean values ± SEM ($n = 8$–10). *$P < 0.05$ versus control, as determined by *U*-test Mann–Whitney.

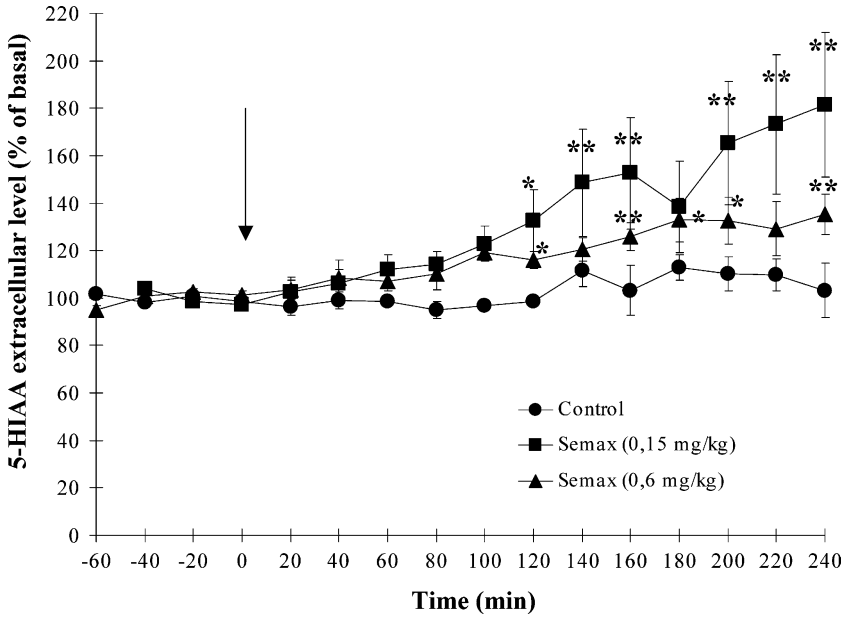

Fig. 2. .Effects of Semax on the extracellular level of 5-HIAA in the striatum of freely-moving Sprague–Dawley rats. Data are presented as percentages of the basal level as mean valus ± SEM (n = 6–7). Semax was administered i.p. at the moment indicated by the arrow at the dose of 0.15 mg/kg (squares) or 0.60 mg/kg (triangles). *P < 0.05; **P < 0.01 versus control, as determined by ANOVA followed by the post hoc Duncan's test.

It was hypothesized that the effects of Semax could be revealed during the hypo- or hyperactivity state of the brain dopaminergic systems. D-AMPH (5.0 mg/kg, i.p.), an indirect dopamine agonist, produced a sharp increase in the extracellular level of DA (up to 20 pmol/ml) in 20–40 min after injection (Fig. 3). The magnitude of the increase was about 20-fold in comparison with the basal values. After

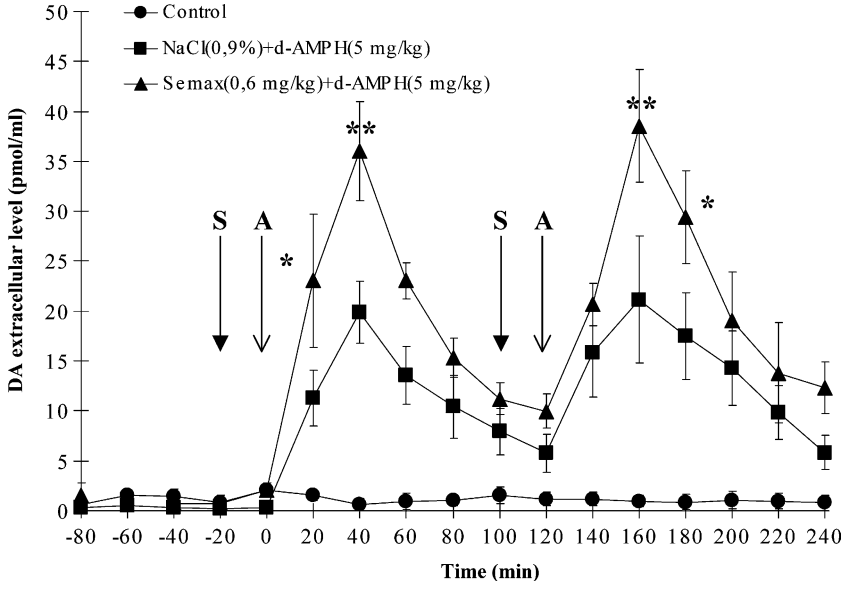

Fig. 3. Effects of Semax and D-amphetamine (D-AMPH) on the extracellular level of dopamine (DA) in the striatum of freely moving Sprague–Dawley rats. Data (pmol/ml) are presented as mean values ± SEM (n = 6–7). Semax and D-AMPH were administered i.p. at the doses of 0.6 and 5.0 mg/kg, respectively. The arrows indicate the moment of Semax (S) and D-AMPH (A) administration. *P < 0.05; **P < 0.01 versus D-AMPH alone, as determined by ANOVA followed by the post hoc Duncan's test. The D-AMPH group significantly (P < 0.01) differences from the control at all time points after the psychostimulant treatment (20–240 min).

this sharp initial increase the increase in DA was attenuated to 500–800% of the basal level at 120 min after the injection. The second injection of D-AMPH produced the same changes in the extracellular level of DA. D-AMPH reduced the extracellular level of DOPAC to 30% of the basal during 4 h (data are not shown).

Pretreatment with Semax (0.6 mg/kg, i.p., 20 min prior D-AMPH) markedly enhanced ($P < 0.05$, ANOVA) the D-AMPH-stimulated striatal DA level (Fig. 3) and accentuated to 20% of the basal ($P < 0.01$, ANOVA) the D-AMPH-induced decrease in the striatal DOPAC level (data are not shown). The peak DA level was markedly increased up to 35–40-fold in comparison with the basal. The same results were obtained after the second injection of D-AMPH in the Semax-pretreated animals (Fig. 3, 120–240 min).

Effects of Semax and D-AMPH on the Locomotor Activity of C57/bl Mice

To verify the modulating effects of Semax on the dopaminergic systems activated by D-AMPH, the locomotor activity of animals was also assessed. The effects of Semax and D-AMPH are shown in Fig. 4. The horizontal activity of control C57/bl mice was reduced during the course of experiments. Semax alone had no effect on this parameter (data are not shown). D-AMPH (2.0 mg/kg, i.p.) significantly increased ($P < 0.01$, ANOVA) the horizontal activity of mice up to 182 ± 18% of the activity in the preceding session. Pretreatment with Semax (0.6 mg/kg, i.p., 20 min prior D-AMPH) significantly enhanced up to 261 ± 30% ($P < 0.05$, ANOVA) the locomotor activity of mice enhanced by D-AMPH.

DISCUSSION

It is generally accepted that the concentration of 5-HIAA, a metabolite of 5-HT, rises with an increase in the 5-HT, which may reflect an activation of the brain serotonergic system. Interestingly, the time course of the 5-HIAA accumulation in the striatum of C57/bl mice (Fig. 1) is closely follows the time course of the changes in the 5-HIAA extracellular level in the striatum in freely moving Sprague–Dawley rats (Fig. 2).

Presently, there are only a few studies on the effects of ACTH/MSH-like peptides on the brain serotonergic systems. MTII, a potent MC4 receptor agonist, has been reported to increase the firing rate of serotonergic neurons in the dorsal raphe nucleus (21). It is also suggested that ORG 2766, an ACTH analogue, is able to affect the serotonergic brain systems (28).

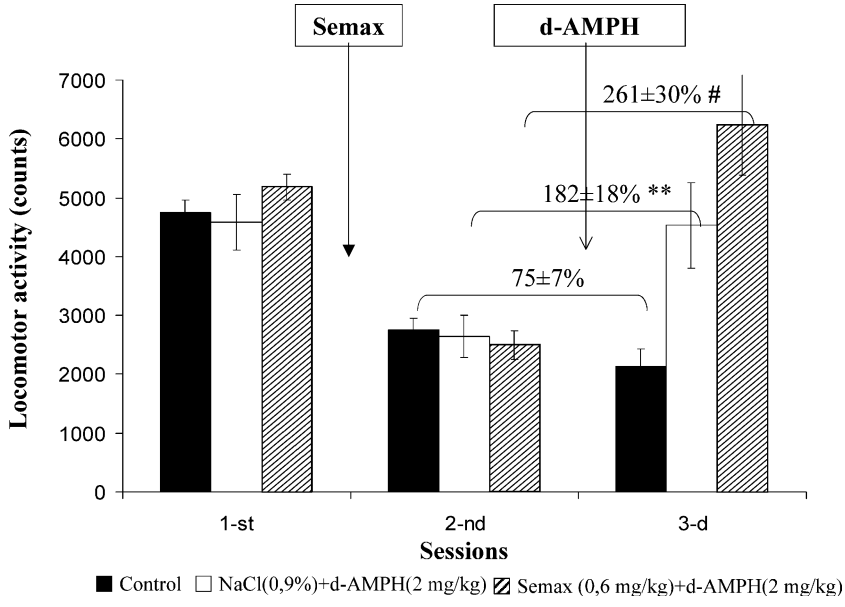

Fig. 4. Effects of Semax (0.6 mg/kg, i.p.) and D-AMPH (2.0 mg/kg, i.p.) on the locomotor activity of C57/bl mice. Data are presented as mean values ± SEM ($n = 10–12$). Semax and D-AMPH were administered i.p. at the doses of 0.6 and 2.0 mg/kg, respectively, at the moments indicated by the arrows. $^{**}P < 0.01$ versus control; $^{\#}P < 0.05$ versus D-AMPH, as determined by one way ANOVA.

The mechanisms underlying the effects of Semax on the serotonergic brain systems are unknown. It could be assumed that Semax is able to modulate the serotonergic systems by affecting the melanocortinergic system. In fact, the serotonergic nuclei express melanocortin MC4 receptors (13,15). Semax has been reported to antagonize the α-MSH-induced cAMP accumulation in HEK cells, expression of MC4 receptors *in vitro* and α-MSH-induced excessive grooming behavior in rats *in vivo* (16). The effects of the non-peptide compound MCL0129, an MC4 receptor antagonist with anxiolytic- and antidepressant-like activities in behavioral tests, are similar to those of the drugs that potentiate serotonergic transmission (29). In the light of this hypothesis, it is likely that the effects of Semax on the serotonergic brain systems could be mediated via antagonism with melanocortin MC4 receptors. However, other neurotransmitter systems could be also involved.

Our results did not reveal any significant effect of Semax alone on the tissue and extracellular levels of DA and its metabolites. We hypothesized that effects of Semax could be revealed during the hypo- or hyperactivity state of the dopaminergic brain systems and therefore the indirect dopamine agonist D-AMPH was used to enhance dopaminergic neurotransmission. Indeed, D-AMPH (5 mg/kg, i.p) induced a sharp increase in the DA extracellular level in the rat striatum. Our results show that pretreatment of animals with Semax potentiates the effects of D-AMPH on the extracellular levels of DA and DOPAC in the striatum of Sprague–Dawley rats. It is previously reported that ACTH/MSH peptides concentration-dependently antagonize the inhibition of DA release induced by the DA receptor agonist TL-99 in striatal slices *in vitro* (17). The effects of ACTH/MSH peptides are similar to that of sulpiride, a dopamine D2 antagonist. Florijn et al. (18) showed an involvement of dopamine D2 autoreceptors in the dopaminergic effects of ACTH-like peptides. ACTH(1–24) was likewise reported to decrease the binding of an dopamine D2 agonist *N-n*-propyl[^{3}H]norapomorphine to the dopamine D2 receptors in rat striatal membranes *in vitro* in a concentration-dependent manner with an IC50 of 10^{-6} M. The dopaminergic effect of ACTH/MSH peptides is apparently due to the competitive inhibitory interaction between melanocortins and dopamine D2 autoreceptors. The extracellular DA level has significantly increased (up to 150% to basal) in the caudate nucleus in freely moving rats after icv administration of 1 μg ACTH(1–24) (19). It could be also assumed that Semax may interact with dopaminergic autoreceptors as do the ACTH-like peptides.

The potentiating effect of Semax on dopaminergic neurotransmission in the brain was confirmed by using the behavioral model in mice. It is well known that D-AMPH induces a sharp increase in the striatal DA level and locomotor hyperactivity in animals. As shown in Fig. 4, pretreatment with Semax significantly enhances locomotor hyperactivity produced by D-AMPH at the dose of 2.0 mg/kg, i.p. Both neurochemical and behavioral results evidence modulating effects of Semax on striatal dopaminergic neurotransmission.

It has been previously reported that Semax increases expression of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in glial cell cultures *in vitro* (30) as well as the BDNF level in the basal forebrain ganglia and hippocampus of Wistar rats *in vivo* (11). Recently, it was also found that BDNF stimulates dopaminergic neurotransmission in the brain. It enhanced KCl-stimulated dopamine release in a dose-dependent manner *in vitro* (31–33). This potentiation was shown to be mediated via TrkB receptors and required activation of the MEK (mitogen-activated/extracellular-signal regulated kinase) and PI3K (phosphatidylinositol-3 kinase) pathways (33). Furthermore, both the stimulation of dopamine release and the induction of dopamine-related behaviors by methamphetamine are significantly suppressed by pretreatment with an injection into the nucleus accumbens of either BDNF or TrkB antibody (34). In view of the data described above, the BDNF/TrkB pathway could be also involved in the effects of Semax on dopaminergic neurotransmission in the brain.

The monoaminergic brain systems are known to play an important role in the pathogenesis depression and anxiety. Drugs effective in the treatment of depression enhance primarily serotonergic and noradrenergic transmission in the brain. Recently, it was found that the BDNF/TrkB pathway may be involved in the effects of antidepressant drugs (35–37). Basic research in rodents has demonstrated that exposure to stress decreases the levels of BDNF in the brain regions associated with depression. The clinical studies have also suggested that a decreased level of BDNF may be involved in the pathogenesis of the major depressive disorder (38,39). In contrast, antidepressant treatment produces the opposite effect (36). In addition, BDNF by itself produces antidepressant effects in behavioral models of depression,

providing further support for the hypothesis that BDNF contributes to the therapeutic action of antidepressant treatment (40).

The enhancement of monoaminergic neurotransmission, revealed in the present study, as well as the increase of BDNF expression in the brain by Semax led us to suggest that the peptide could also show efficacy in the treatment of dopamine and/or serotonin deficiency states, for example, in mental depressions.

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