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SPECIAL ISSUE

Semax, an analogue of adrenocorticotropin (4–10), binds specifically and increases levels of brain-derived neurotrophic factor protein in rat basal forebrain

Oleg V. Dolotov,* Ekaterina A. Karpenko,* Tamara S. Seredenina,* Lyudmila S. Inozemtseva,* Natalia G. Levitskaya,* Yuriy A. Zolotarev,* Andrey A. Kamensky,*,† Igor A. Grivennikov,* Juergen Engele‡ and Nikolay F. Myasoedov*

**Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russian Federation*
†M. V. Lomonosov Moscow State University, Moscow, Russian Federation
‡University of Leipzig, Institute of Anatomy, Leipzig, Germany

Abstract

The heptapeptide Semax (Met-Glu-His-Phe-Pro-Gly-Pro) is an analogue of the N-terminal fragment (4–10) of adrenocorticotropic hormone which, after intranasal application, has profound effects on learning and memory formation in rodents and humans, and also exerts marked neuroprotective effects. A clue to the molecular mechanism underlying this neurotropic action was recently given by the observation that Semax stimulates the synthesis of brain-derived neurotrophic factor (BDNF), a potent modulator of synaptic plasticity, in astrocytes cultured from rat basal forebrain. In the present study, we investigated whether Semax affects BDNF levels in rat basal forebrain upon intranasal application of the peptide. In addition, we examined whether cell membranes isolated from this brain region contained binding sites for Semax. The binding of tritium-labelled Semax was found to be time dependent, specific and reversible. Specific Semax binding required calcium ions and was characterized by a mean ± SEM dissociation constant (K_D) of 2.4 ± 1.0 nM and a B_{MAX} value of 33.5 ± 7.9 fmol/mg protein. Sandwich immunoenzymatic analysis revealed that Semax applied intranasally at 50 and 250 μg/kg bodyweight resulted in a rapid increase in BDNF levels after 3 h in the basal forebrain, but not in the cerebellum. These results point to the presence of specific binding sites for Semax in the rat basal forebrain. In addition, these findings indicate that the cognitive effects exerted by Semax might be associated, at least in part, with increased BDNF protein levels in this brain region.

Keywords: adenocorticotropic hormone (4–10) analogue, basal forebrain, binding sites, brain-derived neurotrophic factor, cognitive effects.
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N-terminal fragments of adrenocorticotropic hormone (ACTH) – a member of the melanocortin family of peptides – are well known for their potent neuroregenerative and cognitive activities (Strand *et al*. 1993a,b). The heptapeptide Semax (Met-Glu-His-Phe-Pro-Gly-Pro) is an analogue of the ACTH(4–10) fragment (Met-Glu-His-Phe-Arg-Trp-Gly). Semax is completely devoid of any hormonal activity associated with the full-length ACTH molecule, which stimulates learning and memory formation in rodents and humans (Ponomareva-Stepnaya *et al*. 1984; Kaplan *et al*. 1996; Ashmarin *et al*. 1997). In addition, Semax profoundly interferes with several forebrain and hippocampal functions; it increases selective attention at the moment of information reception, improves memory consolidation, and promotes learning abilities (Ashmarin *et al*. 1995, 1997). Further major advantages of this peptide in comparison with the ACTH(4–10)

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Address correspondence and reprint requests to Igor A. Grivennikov, Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov square 2, 123182 Moscow, Russia. E-mail: igorag@img.ras.ru

Abbreviations used: ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor.

fragment are its greater resistance to enzymatic cleavage and thus a prolonged action *in vivo* (20 times) (Potaman *et al*. 1991). Semax is the active component of the novel drugs, 'SEMAX – 0.1% solution' and 'SEMAX −1.0% solution', which are applied intranasally. In Russia these drugs are used for treatment of brain hypoxia and ischaemia, stroke, cranial and brain trauma, to facilitate adaptive processes to extreme situations, and to improve learning abilities and memory formation (Ashmarin *et al*. 1997; Myasoedov *et al*. 1999).

Despite these clinical benefits, the cellular and molecular mechanisms underlying the action of Semax in the brain are largely unknown. At the cellular level, Semax was shown to prevent the death of cultured rat basal forebrain cholinergic neurones, and to stimulate the activity of choline acetyltransferase (Grivennikov *et al*. 1999) and acetylcholinesterase (Alexidze *et al*. 1983). In addition, we recently found that Semax induced a rapid eight-fold and five-fold increase in brain-derived neurotrophic factor (BDNF) and nerve growth factor mRNA levels respectively, when applied to primary glial cultures of rat basal forebrain (Shadrina *et al*. 2001), suggesting that Semax might modulate brain functions by affecting neurotrophin synthesis. Another important issue is whether Semax affects cell function through cell membrane receptors. Previous attempts to demonstrate binding of tritium-labelled Semax to rat total brain plasma membranes were unsuccessful (Arefyeva *et al*. 1992). This might have been because levels of Semax-binding sites within whole brain cell membrane preparations are very low or, alternatively, might point to a non-receptor mechanism of action. In order to distinguish between these two possibilities, we investigated whether intranasal application of Semax modulates BDNF levels in the rat basal forebrain and, if so, whether Semax binding is detectable in this brain area.

Materials and methods

Peptide application and determination of BDNF protein levels

Six male Wistar rats (bodyweight 250–300 g) were housed under standard conditions with a 12-h illumination cycle (lights on from 09.00 to 21.00 hours) with food and water available *ad libitum*. All experiments were approved by the local animal ethics committee of the Biological Department of M. V. Lomonosov Moscow State University. For experiments, rats were randomly assigned to the control or Semax group ($n = 5$). Semax aqueous solution at concentrations indicated in the text was applied intranasally in a total volume of 100 μL/kg bodyweight. Equivalent volumes of distilled water were applied to control animals. The application started at 09.30 hours and continued for 30 min. After 3 and 24 h, rats were killed by decapitation, and brain tissue samples were quickly isolated, frozen in liquid nitrogen and stored at − 80°C. The samples were homogenized in 1 mL 100 mM Tris buffer (pH 7.7) per 100 mg wet-weight tissue at 4°C. This buffer contained 400 mM NaCl, 0.4% Triton X-100, 2% 'Block and Sample buffer' from the BDNF $E^{®}_{max}$ ImmunoAssay System (Promega, Madison, WI, USA), 1 mM phenylmethylsulphonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 1 mM benzamidine. The homogenates were centrifuged at 14 000 *g* for 15 min at 4°C and supernatants were stored at − 80°C until use. BDNF levels were determined using the BDNF $E^{®}_{max}$ ImmunoAssay System (Promega), according to the manufacturer's instructions. Acid treatment of samples was used initially according to the manufacturer's recommendations and led to results similar to those obtained with the non-acid extraction method. Total protein concentrations of samples were determined by a modified method of Lowry (Peterson 1977). The heptapeptide Semax was synthesized at the Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia. The purity of each batch was usually not less than 99% as assessed by HPLC analysis.

Cell membrane preparations

Cell membranes were prepared from rat brain according to Gray and Whittaker (1962) with some minor modifications. Adult Wistar rats (250–300 g) were killed by decapitation, and basal forebrains were removed and rinsed in ice-cold phosphate-buffered saline. Brain tissues were homogenized in 10 volumes of 10 mM Tris-HCl (pH 7.4) containing 0.32 M sucrose, 1 mM EDTA, 1 mM benzamidine and 0.1 mM phenylmethylsulphonyl fluoride (buffer 1) in a Teflon–glass homogenizer. Homogenization and all further procedures were carried out at 4°C. Homogenates were centrifuged for 20 min at 1000 *g,* and the supernatant was further centrifuged for an additional 30 min at 40 000 *g*. The resulting compact brown pellet, enriched in mitochondria, was discarded, whereas the less compact light-coloured pellet containing membranes was suspended in buffer 1 and washed twice with the same buffer. The pellet was resuspended in 10 mM Tris-HCl (pH 7.4) containing 0.22 M sucrose and 1 mg/mL bovine serum albumin, and stored in liquid nitrogen. Total protein concentrations were determined by a modified method of Lowry (Peterson 1977).

Binding assay

All Semax binding experiments were carried out in 3.5-mL polystyrene tubes with uniformly tritium-labelled Semax (specific activity 60–120 Ci/mmol; prepared at the Institute of Molecular Genetics, Russian Academy of Sciences). The reaction mixture contained 10 nM [^{3}H]Semax and 1 mM $CaCl_2$ (except for Ca^{2+}, Mg^{2+} and Mn^{2+} ion dependency experiments) in 1 mL 50 mM Tris-HCl buffer (pH 7.4), without (total binding) or with 0.1 mM unlabelled Semax (non-specific binding). To assess the influence of Ca^{2+}, Mg^{2+} and Mn^{2+} ions on Semax binding, the reaction mixture was supplemented with various concentrations (100 nM to 1 mM) of $CaCl_2$, $MgCl_2$ and $MnSO_4$, and the amount of bound [^{3}H]Semax was determined after incubation for 45 min. The binding isotherm for Semax was obtained by determining [^{3}H]Semax binding in the presence of increasing concentrations (10 nM−10 μM) of unlabelled peptide. The reaction was started by adding membrane proteins (0.2 mg) to the reaction mixture. Reaction tubes were placed in a shaker and incubated at 30°C for 45 min. Samples were filtered through GF/B glass fibers (Whatman Maidstone, UK) presoaked with 0.3% polyethylenimine for 2 h at 4°C. Each tube was washed once with cold 50 mM Tris-HCl (pH 7.4) and filters were rinsed three times with 3 mL of this buffer. Air-dried filters were placed into scintillation vials containing 5 mL scintillation mixture [4 g 2,5-diphenyloxazole (PPO) and 0.2 g 1,4-bis (5-phenyl-2-oxazolyl) benzene (POPOP) per litre of toluene] and the

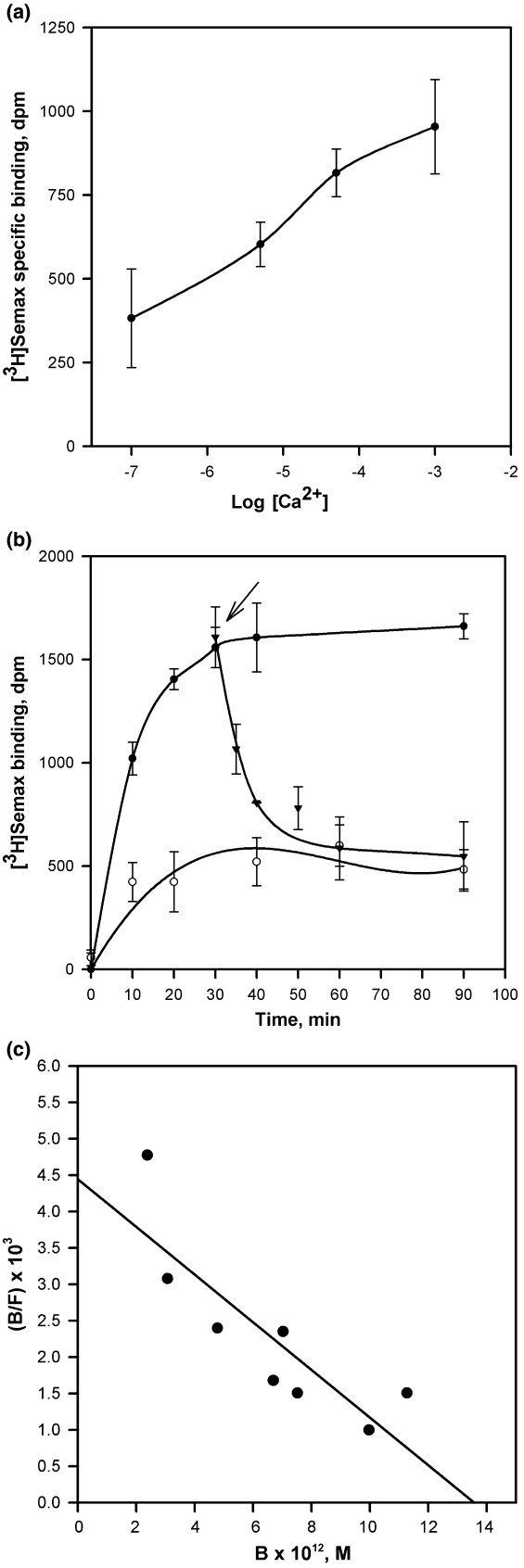

Fig. 1 [^{3}H]Semax binding to rat basal forebrain cell membranes. (a) Calcium dependence of [^{3}H]Semax binding to rat basal forebrain cell membranes. The incubation medium contained $CaCl_2$ at the indicated concentrations. Values are mean ± SEM ($n = 3$) from two independent experiments. (b) Time course of [^{3}H]Semax binding to rat basal forebrain cell membranes. Arrow indicates the addition of unlabelled Semax at a final concentration of 10 μM. Values are mean ± SEM ($n = 3$) from two independent experiments. ●, Total binding; ○, non-specific binding; ▼, binding after addition of unlabelled Semax. (c) Scatchard plot analysis of [^{3}H]Semax binding to rat basal forebrain cell membranes. B and F represent concentrations (mol/L) of the bound and free ligand, respectively.

radioactivity associated with the filters was determined on a Mark-3 scintillation counter (Nuclear Chicago, Des Plaines, IL, USA) with 30% efficiency. The reversibility of Semax binding was assessed by displacing bound [^{3}H]Semax with an excess of unlabelled Semax. To this end, the reaction mixture was additionally supplemented with 10 μM unlabelled peptide after a 30-min incubation period, and the amount of bound [^{3}H]Semax was determined at the indicated time intervals. All the samples were run in triplicate.

Results and discussion

We first studied the characteristics of [^{3}H]Semax binding to rat basal forebrain cell membranes. This analysis revealed that [^{3}H]Semax binding depended on the concentration of Ca^{2+} in the incubation medium (Fig. 1a), whereas it was not affected by Mg^{2+} (up to 1 mM). Mn^{2+} (1 mM) completely blocked specific binding of the peptide (data not shown). In addition, Semax binding was time dependent and reversible (Fig. 1b). The isotherm of Semax binding in Scatchard's plot was linear (Fig. 1c), and fitted best with the one-site binding model, exhibiting a mean ± SEM dissociation constant (K_D) of 2.4 ± 1.0 nM and a B_{MAX} value of 33.5 ± 7.9 fmol/mg protein. There was no specific binding of [^{3}H]Semax to membranes prepared from the rat cerebellum or cortex. Together these results indicate that Semax binding to rat basal forebrain cell membranes meets the fundamental criteria ascribed to receptor binding. To date, five subtypes of G protein-coupled melanocortin receptors have been characterized, and three of them (MC3-R, MC4-R and MC5-R) are expressed in mammalian brain (Hol *et al*. 1995). It was shown that ligand binding to these receptors is modulated by extracellular calcium (Zohar and Salomon 1993). Whether the observed calcium dependency of Semax binding to rat basal forebrain cell membranes reflects the involvement of one of the known melanocortin receptors requires further elucidation.

In the next series of experiments, we tested the ability of Semax to affect rat basal forebrain BDNF levels upon intranasal application. In our previous experiments, Semax resulted in a rapid (40 min) increase in BDNF mRNA levels in rat basal forebrain glial cell cultures (Shadrina *et al*. 2001), so in the present study we determined BDNF protein levels at 3 and 24 h after intranasal administration of Semax. Both a

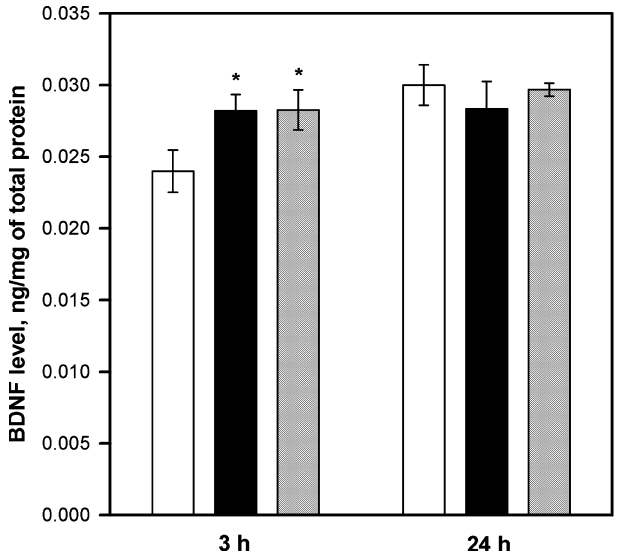

Fig. 2 Effect of Semax on BDNF protein levels in rat basal forebrain 3 and 24 h after intranasal application. Semax was applied intranasally at doses of 50 μg (black bars) or 250 μg (grey bars) per kg bodyweight. Equivalent volumes of distilled water were applied intranasally to control animals (white bars). BDNF levels were evaluated by ELISA. Mean BDNF protein levels in controls were 0.024 and 0.030 ng/mg total protein after 3 and 24 h respectively. Values are mean ± SEM ($n = 10$ rats) from one experiment. Similar results were observed in two additional independent experiments. *$p < 0.05$ versus controls (one-way ANOVA).

behaviourally active dose (50 μg/kg bodyweight; Ashmarin *et al*. 1995) and a higher dose (250 μg/kg) were tested. The selectivity of the effect was assessed by additionally determining BDNF protein levels in rat cerebellum. In the basal forebrain, Semax at both doses produced a statistically significant increase in BDNF levels after 3 h, whereas after 24 h BDNF levels were indistinguishable from those of controls (Fig. 2). In accordance with previous reports (Bova *et al*. 1998; Schaaf *et al*. 2000; Pollock *et al*. 2001), ongoing experiments in our laboratory revealed a fluctuation (possibly circadian modulation) of basal forebrain BDNF levels in control animals, characterized by a decline in BDNF levels during the light phase. In this respect, the observation that the Semax-induced increase in BDNF levels seen after 3 h was similar to the BDNF levels determined in control and Semax-treated animals after 24 h suggests that Semax modulates basal forebrain BDNF levels within its physiological (possibly circadian) range. In contrast to the basal forebrain, Semax failed to affect BDNF levels in the cerebellum after either 3 or 24 h (data not shown). It is noteworthy that cholinergic neurones of the basal forebrain are intimately related to learning and memory. BDNF stimulates the development of this neuronal population (Knusel *et al*. 1991) and thus also affects their function. Moreover, in addition to these neurotrophic effects, BDNF potently modulates short-term synaptic transmission and synaptic plasticity, e.g. long-term potentiation, by activating tyrosine kinase trkB receptors (Hall *et al*. 2000; Mizuno *et al*. 2000). The present demonstration of a transient increase in BDNF levels in the basal forebrain upon Semax application provides evidence for the involvement of BDNF in some cognitive effects of Semax.

In conclusion, our findings indicate that rat basal forebrain cell membranes contain binding sites for Semax and that the cognitive effects exerted by Semax might at least in part be associated with changes in BDNF protein levels in this brain region. Target regions of basal forebrain cholinergic neurones are hippocampus and cortex. As these brain areas are also involved in cognitive functions, future studies should investigate whether Semax really increases cortical BDNF expression or whether the redistribution or transport of BDNF from other brain areas, such as hippocampus, is affected. Preliminary data on the influence of Semax on BDNF mRNA and protein levels in hippocampus have been published (Dolotov *et al*. 2003). It should be noted that molecular mechanisms of cognitive effects of short melanocortins [ACTH(4–10) and its analogues] and the receptors involved are unknown (Adan *et al*. 1994). Thus our data are initial and preliminary, but represent an important step towards clarifying these mechanisms. Identification of nuclear targets and signal transduction pathways for Semax and endogenous melanocortins, as well as the confirmation of the present data by independent methods (western blot, immunocytochemistry), are required and are in progress in our laboratory.

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