Received: 28 October 2022 | Revised: 16 January 2023 | Accepted: 13 February 2023

DOI: 10.1111/cbdd.14221

RESEARCH ARTICLE

# Synthetic corticotropins and the GABA-receptor system: Direct and delayed effects

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**Abstract**

The central effectors of the stress system are greatly interconnected and include, among others, a large group of peptides derived from proopiomelanocortin. In addition to natural corticotropins, a number of artificial molecules that contain some ACTH fragments in their structure are also referred to members of this family. Some of them possess a wide range of biological activity. The molecular mechanism underlying the biological activity of such peptides is partly based on allosteric modulation of various receptors. We analyzed the ability of some biologically active synthetic corticotropins (ACTH(4-7)PGP, ACTH(6-9)PGP, ACTH(7-10)PGP), and glyproline PGPL to affect the GABA-receptor system of rat brain. The effects of the peptides were studied in the isolated plasma membranes of brain cells, as well as after systemic peptide administration in the rat model of acute restraint stress. The delayed effect of stress or preadministration of each of the studied peptides on [$^3$H]GABA binding was different for its high- and low-affinity-specific sites. The studied peptides individually affected the binding of [$^3$H]GABA in their own way. Acute restraint stress caused a decrease in [$^3$H]GABA binding at its low-affine site and did not affected the high-affine site. Preliminary peptide administration did not influence this effect of stress.

**KEYWORDS**

ACTH, allosteric modulation, corticotropin, GABA, neuropeptides, peptides, receptor, Semax, stress

**Abbreviations:** ACTH, adrenocorticotropic hormone; ACTH(4-7)PGP (Semax), heptapeptide Met-Glu-His-Phe-Pro-Gly-Pro; ACTH(6-9)PGP, heptapeptide His-Phe-Arg-Trp-Pro-Gly-Pro; ACTH(7-10)PGP, heptapeptide Phe-Arg-Trp-Gly-Pro-Gly-Pro; ARS, acute restraint stress; Bmax, maximum number of specific binding sites; BSA, bovine serum albumin; DPM, disintegrations per minute, measure of the activity of the source of radioactivity; GABAR, GABA receptor; MSH, melanocyte stimulating hormone; PBS, phosphate-buffered saline; PGPL, tetrapeptide Pro-Gly-Pro-Leu; PMSF, phenylmethylsulfonyl fluoride, GABA, gamma-aminobutyric acid; POMC, proopiomelanocortin; Proglyprol, Kd, binding constant; Selank, heptapeptide Thr-Lys-Pro-Arg-Pro-Gly-Pro.

  

## 1 | INTRODUCTION

Exposure to unexpected and adverse forces (stress factors) actually triggers various reactions in the body that are necessary to enhance the probability of survival in new conditions (Maier & Watkins, 1998). The failure to cope with stress (the inability to form an adaptation) leads to the development of various physiological, neurological, cognitive and psychological disorders, including depression and anxiety (Sousa et al., 2018). The main peripheral effectors of the stress system are glucocorticoids and catechol amines (norepinephrine and adrenaline), which are regulated by the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system, respectively (Tsigos & Chrousos, 2002). The central effectors of the stress system are greatly interconnected and include: hypothalamic hormones, corticotropin-releasing hormone and proopiomelanocortin (POMC) derived peptides (Charmandari et al., 2005; Chrousos, 2009); other different neuropeptides (opioids, oxytocin, vasopressin, neuropeptide Y and etc.), gut-brain axis communication agents (Stengel & Tache, 2018), and regulatory peptides (Bali et al., 2014; Gyires & Feher, 2017; Wei et al., 2020). The latter have been studied to a lesser extent, but are of great interest. Short regulatory peptides (up to 20 amino acid long proteins) are known as universal natural regulators of metabolism and various physiological processes of the brain (Solcia et al., 1987; Track, 1983). They maintain homeostasis, contribute to the adaptation of cells to stress factors, participate in the mechanisms of neuroprotection, proliferation, etc. (Yu et al., 2020). In this issue, we focused on the POMC derived peptides family (Harno et al., 2018), in particular, on short synthetic peptides derived from adrenocorticotropic hormone (ACTH).

In response to stress, corticotrophin releasing hormone, stimulates release of ACTH from the pituitary cells, which, in turn, (by moving with the blood flow) stimulates secretion of glucocorticoid steroid hormones from adrenal cortex cells. But in the brain, ACTH undergoes farther processing and is cleaved into corticotropin-like intermediate peptide (CLIP) and alpha melanocyte stimulating hormone (α-MSH). The same process is found in the skin, but α-MSH plays distinct roles in these tissues (Stevens & White, 2009). Farther biodegradation of α-MSH in the brain leads to the formation of even shorter, but biologically active peptides, which do not evoke corticosteroid secretion, but affect various processes in the brain.

The POMC family includes a lot of hormones and neuropeptides (among them: α-, β-, γ-MSH, ACTH, β- and γ-lipotropins, [Met]enkephalin, β-endorphin, etc). (Bertagna, 1994; Lim & Khoo, 2020; Lowry, 2016). The processing of pro-peptide, as well as spectrum of derived peptides, is tissue-specific, and, depending on the case, we can obtain a various sets of peptides involved in completely diverse biological functions (Raffin-Sanson et al., 2003). For example, the peptides derived from ACTH(1-13) affect neural growth during development and regeneration (Strand, Lee, et al., 1993). ACTH(4-9) and ACTH(4-10) are capable of sustaining neurite outgrowth in the absence of nerve growth factor, accelerating and enhancing nerve regeneration and muscle reinnervation (Strand, Zuccarelli, et al., 1993). It has been shown that ACTH(1-24) and ACTH(4-10) affect the brainstem plasticity—accelerates vestibular compensation and can act directly on some vestibular neurons in vitro, (Darlington et al., 1991; Smith & Darlington, 1991).

All the peptides mentioned above are the short ACTH/α-MSH derivatives with closely located or overlapping amino acid sequences (if look at the primary structure of the native peptide). But such sequences do not always mean that derived peptides will have the same or very similar activity. In this study, we chose the ACTH(4-10) derived peptides with closely spaced and/or overlapping amino acid sequences: ACTH(4-7), ACTH(6-9), and ACTH(7-10). These bioactive peptides are promising for therapeutic application, but, as a rule, display a short blood plasma half-life due to proteolytic degradation by active proteases. It is known many attempts to increase the stability of biologically active peptides by modifying their structure, for example: conjugation to IG(Fc) fragments, fatty acids or polyethylene glycol, cyclization of peptides, introduction of D-isomers of amino acids, esterification, N-acylation, and etc. (Gentilucci et al., 2010; Vlieghe et al., 2010; Yao et al., 2018; Zhang et al., 2018). Numerous synthetic analogs of ACTH fragments were created by replacing the natural amino acids in the ACTH molecule with D-isomers: HOE 427 (Hock et al., 1988), ORG 2766 (Wolterink et al., 1991), and BIM-22015 (Attella et al., 1992). These analogs were more resistant to the action of proteases and more active than native peptides. Nevertheless, the presence of non-natural amino acids and radicals in the structure of these ACTH analogs yielded some new and at times negative biologic features; thus, no medical preparations based on the ACTH analogs described above have been produced.

One of the most interesting and biologically safe ways (in terms of future drugs) is to attach a stabilizing amino acid tail to the C-end of the peptide. For example, long serine-rich C-terminal sequence NSSSSGSSGAGQ of human γ3-MSH (Low et al., 2020), or short proline-rich C-terminal sequence PGP of collagen (Ashmarin et al., 2005). Based on the last of the above approaches, an analog of fragment ACTH(4-10) was synthesized. The peptide consists of the ACTH(4-7) fragment and the C-terminal tripeptide Pro-Gly-Pro. A number of studies have shown that ACTH(4-7)PGP (Semax) improves

learning and memory and exerts neuroprotective activity (Ashmarin et al., 2005). At present, Semax has been successfully used for the clinical treatment of stroke, cerebrovascular insufficiency and optic nerve atrophy (Kolomin et al., 2013; Tarasov et al., 2018). Subsequently, the C-terminal proline-enriched corticotropins ACTH(6-9)PGP and ACTH(7-10)PGP as well as tetrapeptide Pro-Gly-Pro-Leu (PGPL) were synthesized. It was shown that all of these proline-rich peptides possess high biological activity. For example, the peptides ACTH(6-9)PGP and ACTH(7-10)PGP showed neurotropic and anxiolytic activities (Glazova et al., 2011; Levitskaya et al., 2019). ACTH(6-9)PGP enhanced cell viability in the $H_2O_2$, tBH, and KCN cytotoxicity models (Akimov et al., 2021), significantly increased the number of survived cultured cortical neurons in the model of glutamate excitotoxicity (Bakaeva et al., 2020), showed an anxiolytic effect in chronic immobilization stress model (Vorvul et al., 2022). Tetrapeptide PGPL affected blood hemodynamics (Myasoedov et al., 2016; Obergan et al., 2011), reduced the activity of apoptosis (caused by "social" stress) and decreased the levels of caspase-3 and caspase-8 in the blood serum of white rats (Yasenyavskaya et al., 2021).

The molecular mechanisms underlying the biological activity of regulatory neuropeptides is largely related to their specific ligand-receptor interactions on brain target cells plasmatic membranes. Peptides can act as orthosteric ligands of their corresponding receptors (Vyunova et al., 2016), as well as affect the functional activity of various neuroreceptor systems as allosteric modulators (Bezuglov et al., 2015). One of the targets of the peptide action may be the Gamma-aminobutyric acid (GABA) system. GABA is the main inhibitory neurotransmitter of the CNS. The neurotransmitter plays a major role in the modulation of virtually cognitive and behavioral processes, including anxiety and stress responses (Hasler et al., 2010). It was shown earlier that anxiolytic effects of Selank (heptapeptide Thr-Lys-Pro-Arg-Pro-Gly-Pro) can be associated with subtype- and subunit-selective, concentration-dependent allosteric modulation of GABA receptors (GABAR) by the peptide. It is notable also Selank ability to block GABAR modulatory activity of benzodiazepines (Vyunova et al., 2018). The preliminary intranasal administration of Selank (2 h prior to the isolation of plasma membranes) also induces changes in the level of [$^3$H]GABA binding, but does not affect the affinity of the receptors (Vyunova et al., 2014). Study of the influence of ACTH-like peptides on the GABA system have shown that subchronic systemic administration of either ACTH(1-39) or ACTH(4-10) in rats causes an increase in midbrain GABA-receptor binding (Kendall et al., 1982). But in vitro studies have demonstrated that ACTH(1-24) and ACTH(4-10) inhibited the binding of GABA-receptor agonist in cerebral cortex cells (Ito et al., 1988). Our experiments also showed that ACTH(4-7)PGP is able to modulate [$^3$H]GABA-specific binding to corresponding receptors on rat brain cells plasmatic membranes (Vyunova et al., 2019). But the effects of short ACTH-like peptides on the GABA system have not been sufficiently investigated. In this issue we analyzed the ability of the biologically active synthetic corticotropins ACTH(6-9)PGP, ACTH(7-10)PGP and ACTH(4-7)PGP as well as tetrapeptide PGPL to affect the GABA-receptor system of rat brain cells. We have studied the direct influence of these peptides on [$^3$H]GABA binding in isolated plasma membranes and the effects of systemic peptide administration on GABA system in normal conditions and after the acute somatosensory restraint stress. The changes in GABA-receptor binding characteristics under stress conditions were of particular interest. In the acute restraint stress model, we analyzed the delayed effect of peptides on the GABA-receptor system, namely, after a sufficient time has elapsed for the peptide to reach its target in the brain, trigger cellular response processes and be destroyed by proteolytic enzymes. It was shown earlier that the peptides studied are able to entry into the brain after systemic administration, amount of the peptides in the brain reached its maximum in several minutes after injection, and then diminished at 1–2 h (Potaman et al., 1991; Shevchenko et al., 2014, 2015).

## 2 | MATERIALS AND METHODS

### 2.1 | Reagents

$CaCl_2$ (Fluka); Tris (tris(hydroxymethyl)aminomethane) from BioRad Laboratories (Hercules, CA); bovine serum albumin (BSA), EDTA, benzamidine, Pepstatin A, bacitracin, Phenylmethylsulfonyl fluoride (PMSF), GABA were from Tocris (Bristol, UK); phosphate-buffered saline (PBS) was from PanEco (Moscow, Russia). All peptides used in the study (ACTH(4-7)PGP, ACTH(6-9)PGP, ACTH(7-10)PGP and PGPL), as well as tritium-labeled GABA ([$^3$H]GABA) were synthesized at the Institute of Molecular Genetics of National Research Centre "Kurchatov Institute", (Moscow, Russia).

### 2.2 | Buffers

Buffer A1 (10 mM Tris–HCl, pH 7.4 at 4°C, saccharose 0.32 M, 1 mM EDTA, 1 mM benzamidine, 0.1 mM PMSF); Buffer A2 (10 mM Tris–HCl, pH 7.4 at 4°C, 0.22 M saccharose); Buffer B (50 mM Tris–HCl, 1 mM $CaCl_2$, 0.003% BSA, pH 7.4 at 30°C for incubation or pH 7.4 at 4°C for plates washing).

## 2.3 | Animals

Male adult albino Wistar rats (mean body weight, 200–250 g) obtained from the Experimental Animal Centre "Stolbovaya" (Russia) were used in this study. The animals were housed in plastic cages under standard laboratory conditions, which included a controlled ambient temperature (22–25°C), a 12 h light/dark cycle, 60% ± 10% humidity, and free access to water and food. All rats were adapted (including daily handling) to the housing conditions for at least 1 week before experiment.

## 2.4 | Experimental design

In studying of the direct peptide effects on binding of [$^3$H]GABA to isolated plasma membranes intact rats were used. The rats were not subjected to any manipulation except for handling. For the plasma membrane isolation, the rats were rapidly decapitated, their brains were washed with cold PBS, and brain samples were isolated.

The effects of systemic peptide administration on the GABA-receptor system in rats were studied in the normal conditions and after acute stress exposure. Four groups of animals were used in the experiment with each peptide. The groups were as follows: "control", "peptide", "stress-control" and "stress-peptide" (10 rats per group). Apart from that, intact rats were used to assess the effects of experimental manipulations.

To reduce the number of animals used, the brain samples were obtained from rats in which the effects of stress and peptides on behavior were studied. The study of the peptide effects on behavior was carried out as follows. For four consecutive days, all rats, except intact ones, were trained in the food-motivated maze task. On the fifth day of the experiment, the rats were divided into four groups. The rats of the "stress-peptide" and "peptide" groups were administered with the corresponding peptide, while the rats of the "control" and "stress-control" groups received a vehicle injection. The peptides were injected intraperitoneally as an aqueous solution; ACTH(6-9)PGP and ACTH(7-10)PGP in doses of 0.1 mg/kg, PGPL–0.2 mg/kg body weight. The dosage of the peptides was chosen based on the previous studies demonstrating a positive peptide effects in rats (Filippenkov et al., 2021; Tarasov et al., 2018). Thirty minutes after peptide administration, rats of the "stress-control" and "stress-peptide" groups were exposed to restraint stress for 1 h. During this time, the animals of the "control" and "peptide" groups remained in their home cages. Thirty minutes after termination of stress exposure, the retention of the food-motivated maze task was examined. The rats were sacrificed 30 min after the testing procedure, their brains were rapidly extracted. The brain structures were separated, frozen in liquid nitrogen, stored at −80°C, and used in this study.

## 2.5 | Acute restraint stress

(ARS). Restraint stress model is widely used to study the effects of acute and chronic stress exposure in rats. In our study, a rat was submitted to restraint by placing it into a cylindrical plastic restrainer (165 × 55 × 55 mm; OpenScience, Russia). Acute stress was caused by the restraint combined with bright lighting (500 lx) and intermittent acoustic exposure (sounding of an electric bell, 80 dB) for 60 min. After the ARS procedure, rats were returned to their home cages.

## 2.6 | Isolation of plasma membranes

Rat brain cells plasmatic membranes were isolated at 4°C. The rats were decapitated; their brains were washed with cold PBS, brain structures (cortex and hippocampus) were isolated, and added to Buffer A1. The resulting samples were homogenized in 10 volumes of buffer using a Teflon-in-glass homogenizer, and then the homogenate was centrifuged at 1000 *g* for 20 min, the sediment removed, and the supernatant centrifuged at 40,000 *g* for 30 min. The dense brown mitochondria-rich sediment at the bottom of the tube was removed and the less-dense translucent sediment of membranes was resuspended in Buffer A, transferred to a clean tube, and centrifuged again at 40,000 *g* for 30 min. The sediment was resuspended in Buffer A2, divided into portions, frozen in liquid nitrogen, and stored no longer than 30 days at −70°C. Protein concentration in membrane samples was measured according to the Hartree–Lowry method.

## 2.7 | Radioligand binding

The tritium-labeled GABA ([$^3$H]GABA) with a specific radioactivity of 52 Ci/mmol and a radiochemical purity of over 95% synthesized at the Institute of Molecular Genetics of National Research Centre "Kurchatov Institute", according to (Shevchenko et al., 2013). The radioligand-receptor assay was performed on a special unit and we used MultiScreenHTS 96-Well Filter Plates (MultiScreen System, EMD Millipore, Darmstadt, Germany).

The incubation of the reaction mixture was performed directly in the wells of the standard 96-well

plates with GF/B filters (Millipore). The reaction mixture (final volume, 200 µL) contained 50 µL of the radioactively labeled ligand in solution and 50 µL of buffer (containing either unlabeled ligand or the compound under study, depending on the experiment point). The reaction was initiated by addition of 100 µL of membrane protein solution (whose final concentration in the incubation mixture was 0.2 mg/mL) dissolved in Buffer B with an added cocktail of inhibitors (100 µM PMSF +10 µM Bacitracin +5 µM Pepstatin A). The plates were kept at 30°C with continuous shaking for 20 min. After incubation, samples were passed straight through the filters at the bottom of the plates, which were then washed with three 200-µL portions of cold Buffer B. The plates were air-dried; filters were detached and transferred into scintillation vials each containing 4 mL of liquid scintillator (Unisolve 100; Koch-Light, Haverhill, UK), and radioactivity was measured using a Tri-Carb 2100R liquid scintillation counter (Packard BioScience, USA).

## 2.8 | Statistical analysis

Data from the radioligand-receptor binding assay were determined using nonlinear regression analysis (five parameter logistic curve) included into Pharmacology module of the SigmaPlot 10 software suite (Systat Software Inc, San Jose, CA). Intergroup comparisons of data were made by the analysis of variance (ANOVA) module of the SigmaPlot 10. Values on the graphs represent mean ± SE of three independent experiments. Each experiment was conducted on isolated plasmatic membranes of different groups of rats; each mean in experiment was obtained as average from six separated experimental volumes (wells of the standard 96-well plates with GF/B filters (Millipore)).

# 3 | RESULTS

## 3.1 | The radioligand-specific binding

[$^3$H]GABA was tested for the stability of its chemical structure during the incubation period (and more, up to 120 min), using the HPLC analysis method. Two sites of [$^3$H]GABA-specific binding to rat brain cells plasmatic membranes were found in all the tested brain structures (hippocampus, cortex). The main characteristics of [$^3$H]GABA-specific binding completely correlate with the data obtained in our previous study (Vyunova et al., 2019). The above allowed us to compare new and previously obtained results with a high degree of accuracy.

## 3.2 | The influence of peptides (within an ultra-wide range of concentration) on [$^3$H]GABA binding

In this part of the study we used a high affinity site of [$^3$H]GABA-specific binding corresponding to Kd = 18 nM and Bmax = 112 pmol/mg of membrane protein. The influence of studied peptides on [$^3$H]GABA binding to corresponding ($GABA_A$) receptors, located on rat cortical neurons plasmatic membranes, has been analyzed within an ultra-wide range of peptide concentration (femto- to micro-M) (Figure 1). The ACTH(7-10)PGP peptide showed the existence of a dose-dependent activity corridor that begins at a peptide concentration of 500 pM and retains the effect of 20% blocking of [$^3$H]GABA binding up to 500 nM (Figure 1). In this concentration range, there was a significant decrease in [$^3$H]GABA-specific binding compared to [$^3$H]GABA binding measured in the presence of unlabeled GABA (250 µm) ($p < .05$). The ACTH(4-7)PGP also decrease the [$^3$H]GABA-specific binding. A significant effect

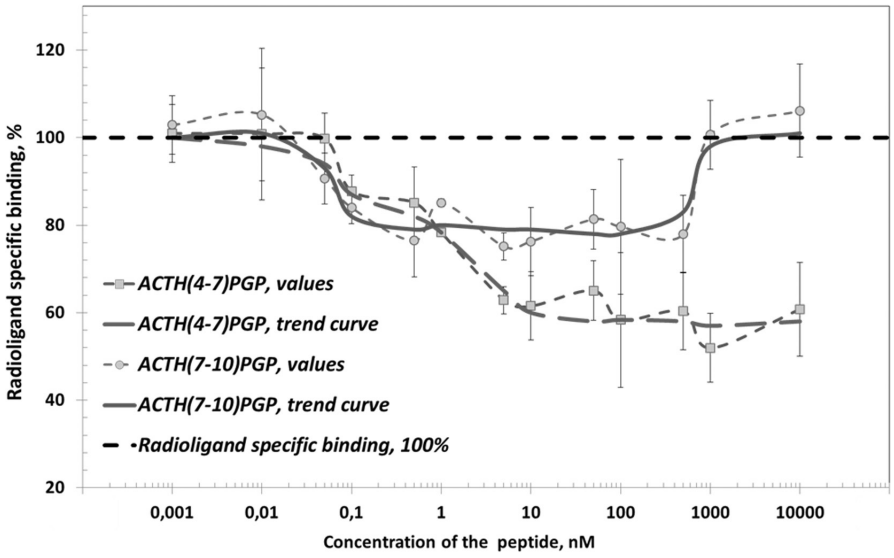


**FIGURE 1** Specific binding of [$^3$H]GABA in the presence of different concentrations of peptides to corresponding receptors on rat cerebral cortex neurons plasmatic membranes. The abscissa (logarithmic) represents the concentration of peptide in incubation mixture, nmol per liter. The ordinate represents the proportion of [$^3$H]GABA (20 nM) specific binding, %. A value of 100% corresponds to [$^3$H]GABA-specific binding, determined in the presence of unlabeled GABA (250 µM).

of the peptide was found at concentrations from 500 pM to 10 mcM ($0.01 < p < .05$). The ACTH(6-9)PGP and PGPL peptides were inactive in their effect on [$^3$H]GABA binding over the entire concentration range.

### 3.3 | The influence of preliminary administration of the peptides on [$^3$H]GABA binding in norm and after the stress exposure

The detailed description of an acute restraint stress model and animal groups used ("control", "peptide", "stress-control", and "stress-peptide") is given in Methods Chapter. Previously, we showed that characteristics of [$^3$H]GABA-specific binding to its high- and low-affinity sites, localized on hippocampal cells plasmatic membranes of intact rats and of rats from the "control" group, are almost identical. Therefore, the experimental manipulations used had no effect on the hippocampal [$^3$H]GABA binding, which allowed us to consider the group ("control") as a base standard of comparison.

The effect of stress or preadministration of each of the studied peptides on [$^3$H]GABA binding was different for its two specific sites (high- and low-affinity). The ARS exposure does not affect the basic characteristics (Kd and Bmax) of [$^3$H]GABA binding to its high-affine-specific site (Figures 2–4, the area of low concentrations, up to 50 nM). (The observed for this site decrease of 10%–15% (of [$^3$H]GABA binding) is not statistically significant, $p > .5$). All the changes (if any) in [$^3$H]GABA binding after the stress and/or peptide exposure were observed only at its low-affine site (Figure 2–4, the area of high concentrations, 50–300 nM). In all the cases, the affinity remained the same, but the number of binding places changed. In all experiments, the "stress-control" group showed a decrease in [$^3$H]GABA binding by about 30% compared to the "control" group ($p < .05$) (Figures 2–4).

A comparison (for each of the peptides) between the groups: "peptide" versus "control", "stress-peptide" versus "control", "peptide" versus "stress-peptide", and "stress-peptide" versus "stress", revealed a significant variety of effects also only at low-affine site (Figures 2–4, the area of high concentrations, from 50 to 300 nM). The effects of the ACTH(7-10)PGP peptide are shown in Figure 2. In unstressed rats, the peptide administration led to significant decrease in hippocampal [$^3$H]GABA binding at concentration range from 100 to 300 nM [3H]GABA in comparison to "control" group ($p < .001$). Furthermore, the specific [$^3$H]GABA binding in the "ACTH(7-10)PGP" group was significantly decreased compared to "stress-control" (at concentrations 120–300 nM; $p < .05$) and "stress-ACTH(7-10)PGP" (at concentrations 100–300 nM; $p < .05$).

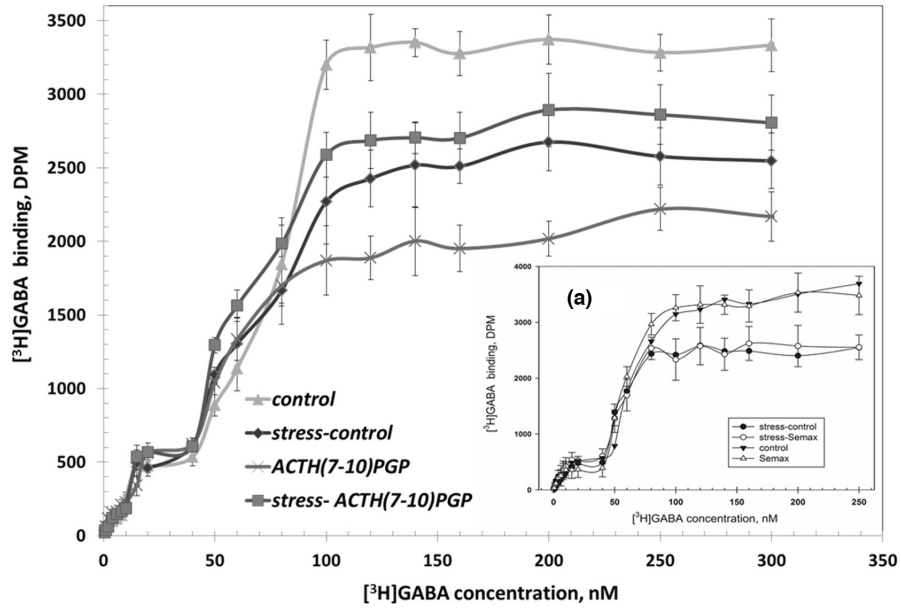


**FIGURE 2** Effects of the ACTH(7-10)PGP on specific binding of [$^3$H]GABA to rat hippocampal cells plasmatic membranes. The abscissa represents the concentration of radioligand ([$^3$H]GABA), nM. The ordinate represents the specific binding of [$^3$H]GABA, DPM. Values on the graphs represent mean ± SE of three independent experiments. Figure 2a shows (as a small thumbnail) the effects of ACTH(4-7)PGP, which are described in more detail in (Vyunova et al., 2019). (Rats of the "stress-control" and "stress-ACTH(7-10)PGP" groups were exposed to 1-hour restraint stress. During this time, animals of the "control" and "ACTH(7-10)PGP" groups stayed in their home cages. Thirty minutes before stress exposure, "stress-ACTH(7-10)PGP" and "ACTH(7-10)PGP" groups were administered the peptide, whereas rats of the "control" and "stress-control" groups were injected with a solvent.)

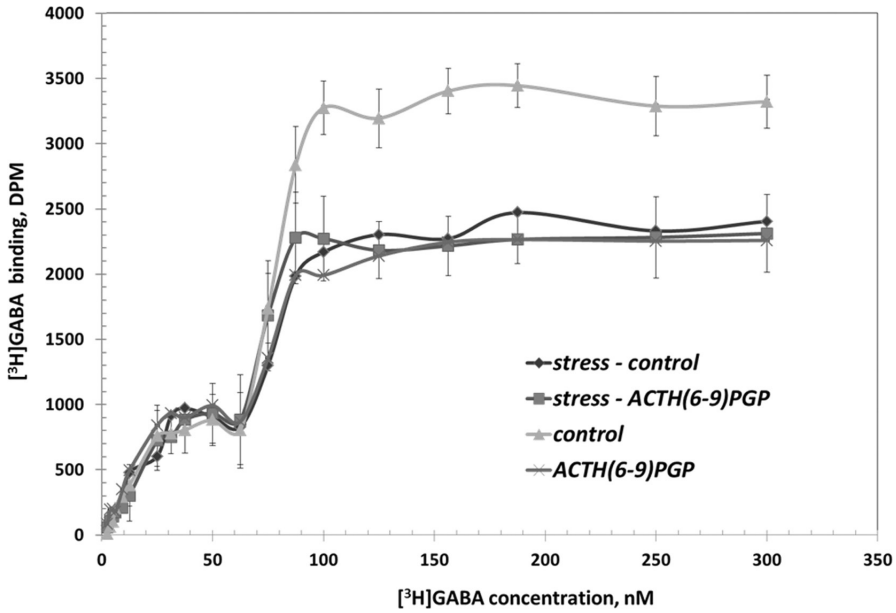


**FIGURE 3** Effects of the ACTH(6-9)PGP on specific binding of [$^3$H]GABA to rat hippocampal cells plasmatic membranes. The abscissa represents the concentration of radioligand ([$^3$H]GABA), nM. The ordinate represents the specific binding of [$^3$H]GABA, DPM. Values on the graphs represent mean ± SE of three independent experiments. (Rats of the "stress-control" and "stress-ACTH(6-9)PGP" groups were exposed to 1-h restraint stress. During this time, animals of the "control" and "ACTH(6-9)PGP" groups stayed in their home cages. Thirty minutes before stress exposure, "stress-ACTH(6-9)PGP" and "ACTH(6-9)PGP" groups were administered the peptide, whereas rats of the "control" and "stress-control" groups were injected with a solvent.)

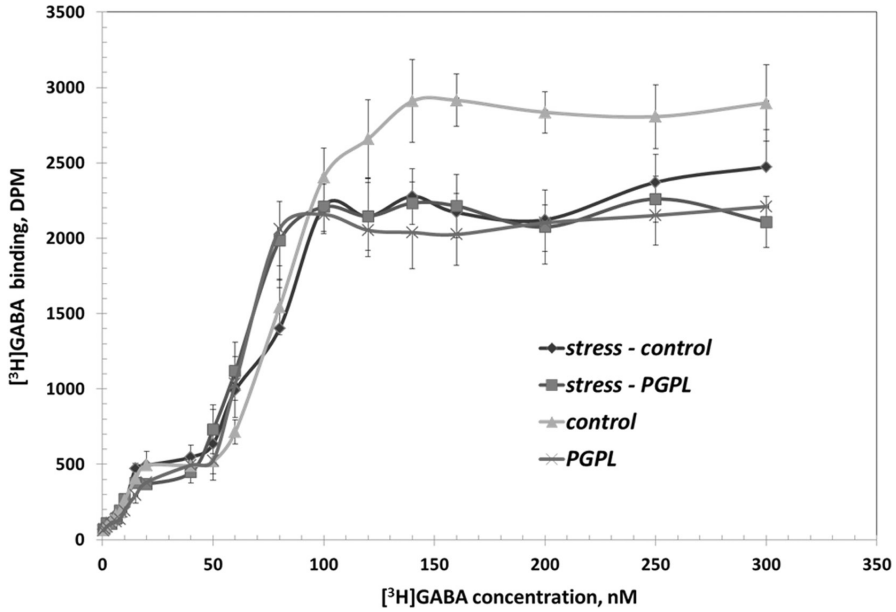


**FIGURE 4** Effects of the PGPL on specific binding of [$^3$H]GABA to rat hippocampal cells plasmatic membranes. The abscissa represents the concentration of radioligand ([$^3$H]GABA), nM. The ordinate represents the specific binding of [$^3$H]GABA, DPM. Values on the graphs represent mean ± SE of three independent experiments. (Rats of the "stress-control" and "stress-PGPL" groups were exposed to 1-h restraint stress. During this time, animals of the "control" and "PGPL" groups stayed in their home cages. Thirty minutes before stress exposure, "stress-PGPL" and "PGPL" groups were administered the peptide, whereas rats of the "control" and "stress-control" groups were injected with a solvent.)

In "stress-ACTH(7-10)PGP" group the [$^3$H]GABA binding was lower than in "control" one (100–300 nM; $p < .05$). We found no statistical difference between "stress-ACTH(7-10)PGP" and "stress-control" groups ($p > .05$). Figure 2a shows (as a small thumbnail) the effects of ACTH(4-7)PGP, which are described in more detail in Vyunova et al. (2019).

The effects of ACTH(6-9)PGP and PGPL peptides are shown in Figures 3 and 4, respectively. For both peptides, there was a significant difference in [$^3$H]GABA binding between the "control" and "peptide" groups, as well as "control" and "stress-peptide" groups at [$^3$H]GABA concentrations of 100–300 nM ($p < .001$). No significant differences were found between the groups "peptide", "stress-control", and "stress-peptide" ($p > .05$).

## 4 | DISCUSSION

The aim of the presented study was to evaluate the ability of the biologically active synthetic corticotropins ACTH(6-9)PGP, ACTH(7-10)PGP and ACTH(4-7)PGP as well as tetrapeptide PGPL to affect the GABA-receptor system of rat brain cells. In vitro experiment have shown that ACTH(7-10)PGP and ACTH(4-7)PGP inhibited the [$^3$H]GABA binding in isolated plasma membranes of brain cells, while peptides ACTH(6-9)PGP and PGPL were inactive. After systemic administration, peptides ACTH(6-9)PGP, ACTH(7-10)PGP and PGPL caused a decrease in GABA-receptor binding in hippocampus in unstressed rats. Acute restraint stress led to a decrease in [$^3$H]GABA binding at its low-affine site and did not affected the high-affine site. Preliminary peptide administration did not influence this effect of the stress.

The GABA system is directly or indirectly involved in many neuronal processes in the mammalian body, especially in processes related to learning and stress (as a system, whose main role is to regulate the excitability of neurons throughout the nervous system) (Hasler et al., 2010). In modern pharmacology, we can find a lot of drugs, which act as positive or negative modulators of the GABA-receptor system. Some of these drugs act on proteins involved in synthesis, degradation, and membrane transport of GABA, other—target the GABA receptors functional activity as allosteric modulators (Olsen, 2018). It is known a lot of peptides that are involved in the allosteric modulation of GABA receptors (Farzampour et al., 2015; Michaeli et al., 2020; Tonon et al., 2020). The modulatory effect of such peptides may change in the presence of another allosteric modulator (Kasian et al., 2017) or after exposure to stress (Chigr et al., 2001). For example, heptapeptide Selank affect the [$^3$H]GABA binding as a positive allosteric modulator, and the joint action of Selank and some of benzodiazepines also regulates activity of [$^3$H]GABA binding in specific manner, which is not cumulative and differs from either substance individually (Vyunova et al., 2018).

Numerous studies showed that the N-terminal region of the ACTH molecule is the principal region responsible for nootropic and neuroprotective activity of the hormone and that the ACTH(4-10) peptide is the minimal fragment retaining the behavioral effect of the full-length ACTH molecule, with complete loss of its hormonal activity (Ito et al., 1988). Data accumulated to date allow to conclude that the ACTH(4-10) sequence includes several regions that may cause different physiological effects (McDaniel, 1993). The importance of the ACTH(6-9) sequence for neurotrophic activity of ACTH-like peptides was indicated (Ericson et al., 2017). ACTH(4-7) is the shortest ACTH fragment retaining nootropic activity. The ACTH(7-10) fragment has a weak activity, but elongation of the molecule to ACTH(7-16) increasing the effect to the level comparable to those of ACTH(4-7) (de Wied, 1990). The corticotropins studied in the present work comprise the natural tetrapeptide ACTH(4-7), ACTH(6-9) or ACTH(7-10) and the fragment PGP in its structure. We had previously shown that all of these peptides have neurotropic activity, but the duration and magnitude of their effects were different (Glazova et al., 2011; Levitskaya et al., 2019). It was shown earlier that preadministration of the ACTH(4-7)PGP reduces memory impairments caused by acute stress (Glazova et al., 2018). But peptides ACTH(6-9)PGP, ACTH(7-10)PGP and PGPL do not have such an effect. The difference in effects suggests different mechanisms of action of peptides on the central nervous system.

In this study, we used several biologically active corticotropins with anxiolytic effects that may be associated with the GABAR system. The effect of some peptides on gene transcription in nerve cells in norm and under pathological conditions has been shown (Denisova et al., 2018; Dergunova et al., 2021; Filippenkov et al., 2020), including the effect on GABAR system genes (Filatova et al., 2017). It has previously been demonstrated that peptides ACTH(4-7)PGP and ACTH(6-9)PGP attenuated behavioral alteration induced by acute restraint stress (Filippenkov et al., 2021; Glazova et al., 2018). The antistress action of the peptides was associated with a correction of the hippocampal transcriptome profile disrupted by stress (Filippenkov et al., 2021). In the present study, we analyzed two aspects of the possible mechanism of the peptides activity: their direct influence on [$^3$H]GABA binding, and their ability to change the GABAR system functioning in vivo in normal conditions and after the acute stress exposure.

Recently, the heptapeptide ACTH(4-7)PGP has been shown to block about 40% of [$^3$H]GABA binding sites in dose-dependent manner (Vyunova et al., 2019). The effect started at peptide concentration of 100 pM and reached saturation up to 10 nM. In the same conditions, the ACTH(7-10)PGP peptide showed a 20% blocking effect within a dose-dependent activity corridor from

500 pM to 500 nM (Figure 1). The data obtained suggest that some ACTH-like peptides may act on the GABA-receptor complexes as allosteric modulators. We also obtained no effect of the ACTH(6-9)PGP and PGPL peptides on [$^3$H]GABA binding to its high affinity site. So, the peptides with overlapping amino acid sequences showed rather different modulatory activity. In the body, such a difference in regulatory activity can mean a difference in the resulting biological effects. The effects observed after peptide administration may be the result of the action of the pool of peptides—the initial peptide and the products of its proteolytic degradation (synacton) (Vyunova et al., 2016). As a result of proteolysis of the peptides studied, different derivatives are formed, which may cause differences in their effects (Shevchenko et al., 2015; Zolotarev et al., 2006).

At the next stage, we analyzed the system response to stress and/or the peptides administration. In particular, the time-delayed effects of the peptides on GABA-receptor system functioning in normal conditions and after the acute stress exposure. The stress model we used is consisted of 1-h animal restraint combined with 1-h acoustic exposure (Glazova et al., 2018). The procedure of restraint does not cause pain in animals, but includes aspects of both emotional and physical stress, as well as causes an unconditioned neuroendocrine response. At the same time, the acute scream sound was shown to increase the level of corticosterone, affects the levels of neurotransmitters in the striatum, hypothalamus and hippocampus of stressed rats (Hu et al., 2014).

In the present study, we have shown that acute restraint stress itself caused a 30% decrease in [$^3$H]GABA binding at its low-affine site (corresponding to the [$^3$H]GABA concentration from 50 to 300 nM) and did not affected the high-affine site (corresponding to the region of [$^3$H]GABA concentrations up to 50 nM). The Kd (binding constant) of both sites, as well as the thermodynamic characteristics of the receptor-ligand interactions remained unchanged. The literature data indicate that acute stress induces alterations in GABA-receptor system. Studies measuring [$^3$H]GABA binding suggest that the availability of low-affinity binding sites is rapidly affected following stress while the affinity is not affected (Skilbeck et al., 2010). Stress exposure can alter the GABAergic function via changes in GABA release and/or the expression of specific GABA-receptor subunits (Fan et al., 2019). The direction of the changes of [$^3$H]GABA binding vary depending on brain structure and the stress-paradigm used (Maguire, 2014; Skilbeck et al., 2010). Therefore, our results are consistent with the literature data.

In unstressed rats, the studied peptides affected the [$^3$H]GABA-specific binding in the same manner. The observed changes in binding were the result of a decrease in the number of available binding sites of [$^3$H]GABA. The ACTH(6-9)PGP and PGPL peptides themselves affected the [$^3$H]GABA binding at its low-affine site in the same way as stress—caused a 30% decrease (Figures 3 and 4). The ACTH(7-10)PGP peptide showed a slightly greater effect—about 40% of [$^3$H]GABA binding places were blocked (Figure 2). It was shown earlier that ACTH(4-7)PGP in the same experimental conditions was inactive (Figure 2a). Thus, in unstressed rats, the peptides ACTH(6-9)PGP, ACTH(7-10)PGP and PGPL, but not ACTH(4-7)PGP, cause the decrease in GABA binding as well as stress does. It has been suggested that disinhibition of the hippocampus caused by the dysfunction of the GABA system may be implicated in memory deficiency induced by stress. Such stress-induced attenuation of hippocampal GABAergic signaling alters glutamatergic synaptic plasticity and contributes to the learning and memory impairments associated with stress (Fan et al., 2019). As mentioned above, ACTH(4-7)PGP reduces memory impairments caused by acute stress (Glazova et al., 2018), but other peptides studied do not have such an effect. One can assume that different influences on the GABA system may determine the different behavioral effects of these peptides.

We have previously shown that peptides ACTH(4-7)PGP, ACTH(6-9)PGP, and ACTH(7-10)PGP have an anxiolytic effect when administered 15 min before testing (Glazova et al., 2011; Levitskaya et al., 2019). However, 4.5 h after administration, the effect of peptides on the level of anxiety was not registered (Filippenkov et al., 2021). In this study, rat brain samples were obtained 2.5 h after peptide administration, which was determined by the task of studying the effects of stress. The duration of the anxiolytic effects of peptides is probably less than 2.5 h.

The investigation of the influence of the peptides studied on stress-induced alteration in the GABAergic system did not reveal significant effects of the peptides. Probably, the behavioral effects of the studied peptides are not related to the hippocampal GABA system.

The direct and indirect peptide influence on the GABA system look like two fundamentally different, but working together mechanisms underlying the biological activity of regulatory peptides: one of them (direct) is fast and is based on temporary changes in the characteristics of specific ligand-receptor binding (allosteric modulation). The other is more complex, requires some time and causes a systemic response, the magnitude of which is determined by the structure of peptide and it's synactone. This both mechanisms work together and influence each other. The “indirect” response may be the result of a complex effect of the peptide on several receptor systems at once (Bezuglov et al., 2015; Vyunova et al., 2019).

## 5 | CONCLUSION

In the present study we have shown that peptides ACTH(6-9)PGP and ACTH(7-10)PGP but not ACTH(4-7)PGP caused a decrease in GABA-receptor binding in hippocampus after systemic administration. The finding that ACTH(6-9)PGP and ACTH(7-10)PGP can modify the binding characteristics of the hippocampal GABA-receptor recognition site suggests that this action may be responsible in part for some of the effect of these peptides. It was also shown that acute restraint stress induced a decrease in [3H]GABA binding in hippocampus. The dysfunction of the hippocampal GABA system may be implicated in the stress-induced memory deficit which we registered earlier in the ARS model (Glazova et al., 2018). The influence of the peptides studied on the stress-induced alterations in GABA system was not found. It should be noted that in this work, the effects of systemic peptide administration on GABA system were studied on the hippocampal cells plasmatic membranes. But ACTH-like peptides may differently regulate the GABA receptors in specific brain structures (Kendall et al., 1982). It cannot be ruled out that the GABAergic system in other brain regions can be affected by the peptides studied. So, further studies will be necessary to establish the mechanisms of central action of ACTH analogs.

### ACKNOWLEDGMENTS

All authors are thankful to their respective Institutes and Universities.

### FUNDING INFORMATION

No funding declared.

### CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**How to cite this article:** Vyunova, T. V., Andreeva, L. A., Shevchenko, K. V., Glazova, N. Y., Sebentsova, E. A., Levitskaya, N. G., & Myasoedov, N. F. (2023). Synthetic corticotropins and the GABA-receptor system: Direct and delayed effects. *Chemical Biology & Drug Design*, *00*, 1–13. https://doi.org/10.1111/cbdd.14221