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Effects of Peptides $ACTH_{6–9}$ PGP and $ACTH_{4–7}$-PGP on Anxiety Levels in Rats in Punished and Unpunished Behavior

S. A. Dodonova,[1] I. I. Bobyntsev,[1] A. E. Belykh,[1] I. A. Telegina,[1] Yu. A. Muzaleva,[1] L. A. Andreeva,[2] and N. F. Myasoedov[2]

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Melanocortins form a class of regulatory peptide currently under active study; these include such biologically active substances as adrenocorticotropic hormone (ACTH) and α- β-, and γ-melanocyte-stimulating hormone (MSH). ACTH fragments, like other peptides of the melanocortins family, have marked neurotropic effects, particularly stimulating learning, memory, and attention processes. The active center of ACTH required for activation of all types of melanocortin receptor, is the sequence His-Phe-Arg-Trp and corresponds to fragment $ACTH_{6–9}$. We report here studies of the synthetic peptide $ACTH_{6–9}$-PGP, whose structure contains the natural fragment $ACTH_{6–9}$ stabilized at the C terminal by attachment of the amino acid sequence prolyl-glycyl-proline (PGP) to increase resistance to the actions of carboxypeptidases. The effects of i.p. administration of $ACTH_{6–9}$-PGP at doses of 0.5, 5, 50, 150, and 450 μg/kg as single does 15 min before assessment of anxiety levels in rats in the Vogel conflict situation test (punished behavior) and the elevated plus maze (unpunished behavior) were studied. The effects of $ACTH_{6–9}$-PGP in these tests were compared with those of its structural analog $ACTH_{4–7}$-PGP at doses of 50, 150, and 450 μg/kg. The results showed that $ACTH_{6–9}$-PGP in the Vogel conflict situation test increased anxiety levels in rats, which was apparent as decreases in drinking time (at all doses) and reductions in the number of licks (at doses of 0.5 and 150 μg/kg) ($p < 0.01$). $ACTH_{6–9}$-PGP had no significant effect on the rats' behavior in the elevated plus maze. $ACTH_{4–7}$-PGP had no effects in the models of punished and unpunished behavior. Thus, $ACTH_{6–9}$-PGP, in contract to $ACTH_{4–7}$-PGP, was found to affect anxiety levels in rats, depending on the dose of peptide and the behavioral model used.

Keywords: regulatory peptides, ACTH, anxiety, Vogel test, elevated plus maze, rats.

Melanocortins are currently one of the most intensely studied classes of regulatory peptide. Their wide spectrum of physiological effects, the discovery of the family of melanocortin receptors (MC1R-NC5R) [1, 2] in a variety of brain structures, and the ability of short peptide sequences to cross the blood–brain barrier, allow adrenocorticotropic hormone (ACTH) fragments to be regarded as a potential model for further study and clinical use as medications [2–4].

N-terminal fragments of ACTH are known to have neurotrophic, analgesic, and nootropic effects [3]. Melanocortins have been shown to affect emotional status and body responses to stress [5]. The sequence His-Phe-Arg-Trp (HFRW), which corresponds to the fragment $ACTH_{6–9}$, is known to be the active center of the ACTH molecule and is required for activation of all types of MCR [6–9]. His-Phe-Arg-Trp has been shown to be the critical pharmacophore [10–12] for all endogenous MCR agonists, i.e., it behaves as the structure required for optimum supramolecular interactions with defined biological targets. The His-Phe-Arg-Trp sequence is absolutely necessary and sufficient for activation of all types of MCR except MC2R, whose activation is mediated by two binding domains: His-Phe-Arg-Trp and Lys-Lys-Arg-Arg ($ACTH_{15–18}$) [7, 9].

Modification of this fragment by attachment of the tripeptide Pro-Gly-Pro (PGP) to the C terminal with the aim of increasing resistance to the action of carboxypeptidases

[1] Kursk State Medical University, Kursk, Russia; e-mail: dodonovasveta@mail.ru.
[2] Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia.

also confers neurotropic activity [12]. The structurally close synthetic fragment $ACTH_{4–7}$-PGP (the active substance in the medicine Semax) has nootropic, anxiolytic, neurotrophic, and analgesic activities [13]. N-terminal ACTH fragments are known to lack hormone activity, which allows the properties of the initial peptide sequence to be discriminated from the effects of adrenal hormones [3]. An important question in studies of peptide compounds with neurotropic activity is their ability to cross the blood–brain barrier (BBB) – $ACTH_{4–7}$-PGP, like $ACTH_{6–9}$-PGP, has been shown to be able to cross the BBB and interact with brain structures [14, 15].

The wide spectrum of neurotropic effects of melanocortins and their fragments and synthetic analogs, along with the potential for use in correcting a variety of pathological states, suggests value in studying the effects of $ACTH_{6–9}$-PGP, which includes the amino acid sequence His-Phe-Arg-Trp (the active center of the ACTH molecule) on behavioral reactions in animals, such as fear and anxiety.

The aim of the present work was to study the effects of $ACTH_{6–9}$-PGP on anxiety levels in rats in punished and unpunished behavior in comparison to the effects of $ACTH_{4–6}$-PGP.

Methods. Experiments were carried out on 180 male Wistar rats weighing 250–300 g. Animals were kept in cages of 10 individuals in standard animal-house conditions and had free access to standard granulated feed and water with a 12-h light regime (12 h light, 12 h dark) and a controlled temperature (22 ± 2°C). Stress responses to handling by the experimenter were minimized by daily handling. All procedures were conducted in compliance with EC directives for the protection of animals used for scientific purposes – EU Directive 2010/63/EU of September 22, 2010, the "Regulations for Laboratory Practice in the Russian Federation," approved by order of the Russian Federation Ministry of Health No. 708n of August 23, 2010, and with monitoring by the Ethics Committee of Kursk State Medical University, Russian Ministry of Health (protocol No. 3 of September 28, 2015).

Studies used physiological saline (0.9% sodium chloride) and peptides $ACTH_{6–9}$-PGP and $ACTH_{4–7}$-PGP, synthesized at the Institute of Molecular Genetics, Russian Academy of Sciences. Peptides were dissolved in physiological saline and administered to animals as single i.p. doses 15 min before experiments started. $ACTH_{6–9}$-PGP was used at the following doses in different experimental groups: 0.5, 5, 50, 150, and 450 μg/kg, while $ACTH_{4–7}$-PGP was used at doses of 50, 150, and 450 μg/kg. Doses were selected on the basis of published data on the effective dose range for the structurally similar molecule $ACTH_{4–7}$-PGP [12, 13]. Control animals received the same volume of physiological saline, i.e., 1 ml/kg.

Testing of animals in each series of experiments was performed on a number of sequential days, as per the study design. Day 1: group 1 ($ACTH_{6–9}$-PGP 0.5 μg/kg); group 2 ($ACTH_{6–9}$-PGP 50 μg/kg); group 3 ($ACTH_{6–9}$-PGP 450 μg/kg). Day 2: group 4 ($ACTH_{6–9}$-PGP 5 μg/kg); group 5 (controls given physiological saline); group 6 ($ACTH_{6–9}$-PGP 150 μg/kg). Day 3: group 7 ($ACTH_{4–7}$-PGP 50 μg/kg); group 8 ($ACTH_{4–7}$-PGP 150 μg/kg); group 9 ($ACTH_{4–7}$-PGP 450 μg/kg).

Anxiety levels were evaluated in two series of experiments: series 1 used the elevated plus maze (EPM) (one control and eight experimental groups each of 10 animals (n = 90)); series 2 used the Vogel conflict situation (also one control and eight experimental groups each of 10 rats (n = 90)) [16].

Vogel conflict situation test. The apparatus for the conflict situation consisted of three parts: an experimental chamber, a shocker, and a recording system (PanLab Harvard Apparatus, Spain). The experimental chamber was a cage of size 180 × 220 × 420 mm with a mesh floor and a bottle containing water. The floor of the experimental chamber and the nipple of the bottle were connected to the shocker. Experiments lasted three days. Animals were completely deprived of water during the first 24 h. On the next day, i.e., after 24-h deprivation, the skill of taking water from the bottle was developed: the animal was placed in the experimental chamber for 5 min. During this time, the rat explored the apparatus and found the bottle. After 5 min of training, the animal was placed in the cage with free access to water for 20 min. On experimental day 3, rats were again placed in the experimental apparatus for 5 min. During the experiments, after the first 20 free licks of the bottle nipple, rats received an electric shock of 0.3 mA for 5 sec. Rats were subsequently punished with these shocks after every 20 licks [17]. The following criteria were used for assessment of anxiety: the number of water sips taken from the bottle (the number of licks of the bottle during electrical stimulation), the number of electric shocks, and the duration of drinking.

Elevated plus maze (EPM). Studies of anxiety levels in rats in the EPM test were conducted using an experimental apparatus (PanLab Harvard Apparatus, Spain), which consisted four arms (two opposite arms without walls and two closed arms, with walls of height 300 mm) of length 500 mm and width 140 mm organized as a cross radiating from the central platform at right angles. The plus maze was elevated to a height of 500 mm from the floor. Experiments were run in conditions of artificial illumination: 300 lx for the open arms, 240 lx for the central platform, and 45 lx for the closed arms. In contrast to the Vogel conflict situation test, these conditions imposed low levels of stress on the animals. Immediately before experiments started, the rat was kept in a dark box for 5 min. The animal was then placed in the EPM, on the central platform, with the head facing an open arm, and the following indicators were monitored for 5 min: time spent in the open arms, time spent in the closed arms, time spent in the central platform, numbers of entries into the open and closed arms and the central platform,

TABLE. 1. Anxiety Indicators in the Vogel Test after i.p. Administration of $ACTH_{6–9}$-PGP and $ACTH_{4–7}$-PGP (n = 90, Me [Q1, Q3])

Indicator	Number of licks	Number of shocks	Duration of drinking, sec
Control (n = 10)	40 [39; 41]	2 [1; 2]	7.62 [4.82; 28.87]
$ACTH_{6–9}$-PGP			
0.5 μg/kg (n = 10)	3.0 [0.0; 20.0]*	0.0 [0.0; 1.0]*	2.1 [0.0; 4.9]*
5 μg/kg (n = 10)	20.0 [20.0; 23.0]	1.0 [1.0; 1.0]	2.9 [1.7; 5.8]*
50 μg/kg (n = 10)	40.0 [0.0; 60.0]	2.0 [0.0; 3.0]	3.5 [0.0; 5.1]*
150 μg/kg (n = 10)	15.0 [0.0; 20.0]*	1.0 [0.0; 1.0]*	1.9 [0.2; 3.6]*
450 μg/kg (n = 10)	40.0 [20.0; 40.0]	2.0 [1.0; 2.0]	3.2 [1.4; 10.1]*
$ACTH_{4–7}$-PGP			
50 μg/kg (n = 10)	40.0 [20.0; 44.0]	2.0 [1.0; 2.0]	4.1 [2.0; 8.8]
150 μg/kg (n = 10)	50.0 [15.0; 31.0]	2.0 [2.0; 3.0]	4.6 [3.3; 24.8]
450 μg/kg (n = 10	40.0 [40.0; 60.0]	2.0 [2.0; 3.0]	7.2 [3.3; 21.8]

*Significant differences ($p \le 0.05$), Mann–Whitney test with Benjamini–Hochberg correction, compared with the control group.

grooming, and head dips from the open arms. Indicator recording and analysis were with a SMART Video Tracking System (PanLab Harvard Apparatus Spain).

Statistical analysis was run in MS Excel 2016, Statistica 13.3, and the R computation environment. Distributions of indicators in statistical sets were determined using the Shapiro–Wilks test and the equality of dispersions was assessed using the Levene test. Significant differences were identified using nonparametric unifactorial analysis with the Kruskal–Wallis test and between-group differences were identified by post hoc use of the Mann–Whitney test (U test) with the Benjamini–Hochberg correction. Non-normality of distributions led to expression of results as medians (Me) with lower (25) and upper (75) percentiles (Q1 and Q3). Results were regarded as significant at $p \le 0.05$.

Results. *Vogel conflict situation test*. Table 1 shows that administration of $ACTH_{6–9}$-PGP had marked dose-dependent effects on indicators recorded in the Vogel test. All experimental groups showed significant decreases in the duration of drinking: by 72% at 0.5 μg/kg (p = 0.02), 62% at 5 μg/kg (p = 0.03), 54% at 50 μg/kg (p = 0.04), 75% at 150 μg/kg (p = 0.01), and 59% at 450 μg/kg (p = 0.03). These findings are evidence that the peptide has an anxiogenic effect.

The anxiogenic effect of $ACTH_{6–9}$-PGP was also supported by decreases in the number of licks. Thus, peptide at 0.5 μg/kg decreased the number of punished water sips from the bottle by a factor of 6 ($p < 0.01$), while a dose of 150 μg/ml produced a 1.5-fold reduction ($p < 0.01$). In addition, there were significant reductions in the numbers of electric shocks received, by 200% (p = 0.02) and 50% (p = 0.03) at these doses. It should be noted that administration of $ACTH_{6–9}$-PGP at a dose of 5 μg/kg produced no statistically significant changes in the numbers of these behavioral acts as compared with the control group, though there were tendencies to decreases in the number of licks (by 50%, p = 0.09) and the number of electric shocks received (by 50%, p = 0.1).

As compared with the control group, $ACTH_{4–7}$-PGP at all doses produced no statistically significant effects on any of the indicators measured in the Vogel test.

Elevated plus maze. Administration of $ACTH_{6–9}$-PGP had no significant effect on the anxiety level of rats in the EPM test (Table 2), in contrast to the Vogel conflict situation test. Thus, only the dose of 150 μg/kg produced significant increases in the number of excursions into the open arms (p = 0.04), while doses of 50 and 450 μg/kg produced only tendencies to increases in this parameter (p = 0.09). $ACTH_{6–9}$-PHP at all test doses had no statistically significant influences on other test parameters (times spent by the animals in the open and closed arms and on the central platform, numbers of entries into the closed arms and central platform, grooming, and head dips from the open arms).

Similarly, administration of $ACTH_{4–7}$-PGP at all study doses also had no statistically significant effects on measures characterizing the behavior of the animal in the EPM as compared with the control group (Table 3).

Discussion. Studies of anxiety levels in the Vogel conflict situation test demonstrated imposition of significant levels of stress on the experimental animals by the prolonged (two days) deprivation of water, along with the emotional/pain factor. The actions of stress are known to affect the brain, with increases in the expression of a variety of melanocortin receptors, particularly MC4R mRNA, in the amygdala and hypothalamus [5, 18], these in turn playing an important role in forming fear and anxiety reactions [19]. Thus, agonists of this receptor can have anxiogenic effects [18], increasing cAMP expression in cells expressing MC4R

TABLE 2. Anxiety Indicators in the EPM Test after i.p. Administration of $ACTH_{6–9}$-PGP (n = 90, Me [Q1, Q3])

Indicator	Control (n = 10)	0.5 μg/kg (n = 10)	5 μg/kg (n = 10)	50 μg/kg (n = 10)	150 μg/kg (n = 10)	450 μg/kg (n = 10)
Time in open arms, sec	56.1 [41.7; 140.6]	57.6 [25.2; 77.0]	88.8 [53.9; 112.4]	28.3 [15.5; 51.12]	63.9 [39.9; 150.9]	67.5 [35.7; 113.1]
Time in closed arms, sec	173.2 [95.4; 246.2]	212.9 [189.6; 243.3]	138.4 [109.5; 203.1]	226.3 [205.0; 271.7]	151.7 [88.7; 232.1]	194.52 [139.6; 240.4]
Time in central platform, sec	30.61 [7.6; 60.6]	25.64 [18.6; 35.5]	53.8 [34.3; 75.6]	27.9 [23.0; 43.6]	46.1 [33.5; 64.8]	37.8 [19.1; 54.2]
Entries into open arms	3.0 [3.0; 5.0]	5.5 [4.0; 8.0]	7.0 [5.0; 9.0]	1.0 [0.0; 2.0]	7.0 [1.0; 10.0]*	9.0 [7.0; 15.0]
Entries into closed arms	6.0 [3.0; 12.0]	5.0 [4.0; 6.0]	5.0 [3.0; 10.0]	7.0 [3.0; 8.0]	5.0 [3.0; 18.0]	9.0 [5.0; 15.0]
Entries into central platform	9.5 [6.0; 16.0]	10.0 [5.0; 13.0]	13.0 [8.0; 18.0]	7.0 [5.0; 11.0]	13.0 [5.0; 25.0]	19.5 [9.0; 26.0]
Number of head dips	11.0 [6.0; 15.0]	8.50 [6.0; 11.0]	9.0 [9.0; 17.0]	6.0 [5.0; 8.0]	7.0 [6.0; 17.0]	11.5 [9.0; 13.0]
Grooming	0.5 [0; 1.0]	1.0 [0; 2.0]	1.0 [0; 1.0]	1.0 [0; 2.0]	0.0 [0; 1.0]	0.0 [0; 1.0]

*Significant differences ($p \le 0.05$), Mann–Whitney test with Benjamini–Hochberg correction, compared with the control group.

[5, 18]. Given that $ACTH_{6–9}$-PGP is the minimum necessary sequence for endogenous melanocortin receptor agonists to have biological activity [6–10], it can be suggested that increases in anxiety levels in conditions of punished behavior and i.p. administration of study heptapeptide (the decrease in the total duration of drinking seen at all doses in rats in the Vogel test) reflect the interaction of the sequence His-Phe-Arg-Trp-Pro-Gly-Pro with MC4R and resultant action of these receptors [20].

Conversely, i.p. administration of $ACTH_{6–9}$-PGP in conditions of unpunished behavior had no effect on behavioral reactions reflecting anxiety levels in the experimental animals. This is probably linked with the minimal level of stress imposed by the EPM test, in contrast to the Vogel conflict situation. This leads to the suggestion that the level of stress-induced expression of melanocortin receptors in the corresponding brain structures was insufficient for expression of the anxiogenic effects of $ACTH_{6–9}$-PGP seen in our studies in conditions of punished behavior [20–22].

Given the complexity and multiplicity of mechanisms both involved in forming individual behavioral acts and being triggered during stress reactions, it can be suggested that the different effects of $ACTH_{6–9}$-PGP in punished and unpunished behavior may potentially also be linked with allosteric interactions between peptide and the receptors of other neurotransmitter systems, for example the GABAergic, serotoninergic, and dopaminergic systems, as has been demonstrated for $ACTH_{4–7}$-PGP. This latter is known to induce and change signals from a wide spectrum of receptors, inducing conformational changes and functional reconstruction [3]. In addition, peptides have their own binding sites where interactions allow for fine tuning of information signals [3] in situations of adaptation to external conditions, whose mechanisms are particularly clearly activated in the Vogel test, as compared with the EPM test. It can be suggested that as animals in the EPM test were not subjected to intense stress, they correspondingly had no requirement for activation of the adaptive processes accompanying activation of various transmitter systems.

The nonlinear dose-dependent nature of the effects noted above (for example, the absence of any effect of $ACTH_{6–9}$-PGP on the number of licks at doses of 5, 50, and 450 μg/kg vs. the presence of such an effect at doses of 0.5 and 150 μg/kg) may be linked with the characteristic biological features of the realization of the effects of regulatory peptides. The kinetic characteristics of chemical and biological processes occurring as a result of the actions of this class of physiologically active substances often have bell-shaped or U-shaped types of dose-effect curves. These features indicate marked effects at very low peptide concentrations and minor or distorted effects at higher or intermediate doses [23]. The directions of the effects of regulatory peptides at one dose or another largely depend on activation of one or another intracellular second messenger system. Thus, high ACTH concentrations activate cAMP synthesis on transmission of the cellular signal, while low concentrations activate the diacylglycerol-inositol phosphate pathway; this can subsequently determine the type of effect obtained [23].

At the same time, it should be noted that reports by other authors have previously described anxiolytic effects with $ACTH_{6–9}$-PGP in the EPM test [12], which were not confirmed in our studies. One reason for this difference may be the route of administration of test substances. In our studies, animals received peptide i.p., while the anxiolytic effect was obtained after intranasal administration of $ACTH_{6–9}$-PGP. The route of administration determines delivery of peptide and the mechanisms of its tissue distribution, metabolism, and bioavailability, which significantly influence

TABLE 3. Anxiety Indicators in the EPM Test after i.p. Administration of $ACTH_{4–7}$-PGP (n = 90, Me [Q1, Q3])

Indicator	Control (n = 10)	50 μg/kg (n = 10)	150 μg/kg (n = 10)	450 μg/kg (n = 10)
Time in open arms, sec	56.1 [41.7; 140.6]	61.6 [18.3; 119.2]	36.24 [32.6; 103.4]	29.8 [13.4; 100.7]
Time in closed arms, sec	173.2 [95.4; 246.2]	220.7 [108.6; 250.4]	228.7 [164.3; 259.8]	252.9 [181.9; 268.9]
Time in central platform, sec	30.6 [7.6; 60.6]	21.4 [16.8; 59.9]	24.4 [9.6; 39.1]	16.2 [4.1; 25.2]
Entries into open arms	3.0 [3.0; 5.0]	4.0 [2.0; 7.0]	4.0 [2.0; 6.0]	4.5 [1.0; 6.0]
Entries into closed arms	6.0 [3.0; 12.0]	6.0 [2.0; 11.0]	4.0 [3.0; 11.0]	4.0 [2.0; 9.0]
Entries into central platform	9.5 [6.0; 16.0]	8.0 [4.0; 18.0]	5.0 [4.0; 18.0]	7.0 [3.0; 11.0]
Number of head dips	11.0 [6.0; 15.0]	8.0 [4.0; 13.0]	7.0 [4.0; 18.0]	6.0 [4.0; 13.0]
Grooming	0.5 [0; 1.0]	1.0 [0.0; 1.0]	1.0 [1.0; 1.0]	1.0 [0.0; 1.0]

*Significant differences ($p \le 0.05$), Mann–Whitney test with Benjamini–Hochberg correction, compared with the control group.

the final peptide concentration in brain structures [24, 25]. For example, intracerebroventricular administration of the peptide formulations Cortexin and Cerebrolysin had moderate anxiolytic effects, while systemic administration produces an anxiogenic effect [26]. Neurotrophic effects of different extents and directions determined by different routes of administration have also been demonstrated for the structurally close $ACTH_{6–9}$-PGP analog $ACTH_{4–7}$-PGP [3, 13].

Considering the dose-dependent nature of the effects of neuropeptides, our use of the i.p. route of administration provides the most accurate doses of test agents, as intranasal administration can involve losses of drug due to inadequate administration technique, incorrect selection of buffer, and reflex activation of swallowing and sneezing [27]. This fact is quite critical because changes in the concentrations of ACTH and its fragments determine the signal transmission pathway, which in turn influences the direction and extent of the effects [23].

The lack of effect of $ACTH_{4–7}$-PGP on anxiety levels in animals in both of the tests used in the present study are consistent with published data. For example, single doses of $ACTH_{4–7}$-PGP given 15 min before tests had no effect on anxiety levels in animals [5]. Despite the structural closeness of the heptapeptides studied here, differences in the amino acid sequences, i.e., the absence of the active ACTH center from $ACTH_{4–7}$-PGP (unlike the situation with $ACTH_{6–9}$-PGP), have the result that Semax lacks the anxiogenic activity typical of melanocortins. Furthermore, $ACTH_{4–7}$-PGP can display the property of an MC4R antagonist, inducing anxiolytic and antidepressant effects [3, 5]. These properties are not seen in normal conditions and become apparent on the background of increased anxiety and depressivity levels due to the effects of $ACTH_{4–7}$-PGP on the functional activity of the serotoninergic system [5]. It is important to note that the peptide has a "restorative" action in conditions of elevated anxiety.

The similar characteristics of the regulatory influences, apparent as differential realization of effects depending on the level of tension of the adaptive systems of the body, bring $ACTH_{4–7}$-PGP and $ACTH_{6–9}$-PGP closer together. It is interesting to note that these properties are also characteristic of other regulatory peptides whose effects increase with increases in the intensity of the actions of stimuli on the body, producing greater changes in various indicators [28].

Thus, the extent and direction of the effects of the N-terminal ACTH fragments on anxiety in rats in punished and unpunished behavior depend on the peptide dose used and the behavioral model employed. The effects of $ACTH_{6–9}$-PGP are anxiogenic in nature in the Vogel conflict situation test and absent in the EPM test, while $ACTH_{4–7}$-PGP produced no effects on anxiety levels in either test.

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