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Research Article

In search of new brain biomarkers of stress

Petr D. Shabanov[1], Aleksandra A. Blazhenko[1], Aleksandr S. Devyashin[1], Platon P. Khokhlov[1], Andrei A. Lebedev[1]

1 *Institute of Experimental Medicine, 12 Acad. Pavlov Str., St. Petersburg 197376, Russia*

Corresponding author: Petr D. Shabanov (pdshabanov@mail.ru)

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Abstract

The aim: of the study was to investigate the level of ghrelin in various brain structures during a stress response in Zebrafish to a predator, to evaluate this indicator as a potential biomarker of stress, and the effect of a benzodiazepine tranquilizer (phenazepam) on stress-induced changes

Materials and methods: The object of the study was Zebrafish, or *Danio rerio* wild type, which was subjected to stress by exposure to a predator *Hypsophrys nicaraguensis* from the cichlid family. In the tail tissue, the level of cortisol was determined, in the brain – the level of total (acylated and non-acylated) ghrelin by the method of enzyme-linked immunosorbent assay. The benzodiazepine anxiolytic phenazepam (1 mg/L), a ghrelin antagonist [D-Lys3]-GHRP-6 (0.333 mg/l) and corticotropin-releasing hormone (CRF; 0.4 mg/L) were used as the pharmacological agents.

Results and discussion: Exposure to a predator, just as administering CRF, more than doubled the level of cortisol in the tail tissue. [D-Lys3]-GHRP-6 and phenazepam prevented an increase in a tissue cortisol level. Simultaneously, in the medulla oblongata and cerebellum, the phylogenetically most ancient structures, rather than in the forebrain (telencephalon) or in the midbrain (corpora bigemia), the level of ghrelin was recorded about 500 pg/g of total protein. In response to exposure to a predator, the level of ghrelin increased in the forebrain and midbrain to nanogram concentrations and moderately decreased in the cerebellum. The effect was prevented by phenazepam and [D-Lys3]-GHRP-6.

Conclusion: Increases in ghrelin in the brain in response to stressful situations can be seen as a functional brain biomarker of stress, along with increased levels of tissue cortisol levels. Both of these effects are prevented by both the ghrelin antagonist and the benzodiazepine tranquilizer. The mechanism of action of the tranquilizer is a functional antagonism between the GABAergic system of the brain and the ghrelin system.

Keywords

Danio rerio, stress, cortisol, brain ghrelin, ghrelin antagonists, phenazepam, tranquilizers.

Introduction

Traditionally, the impact of stress factors on the body is assessed by the state of the hypothalamic-pituitary-adrenal system (Bovenkerk and Kaldewaij 2015, De Abreu et al. 2018). The increasing production of glucocorticoid hormones, the content of which is determined in the blood, has been considered as a common stress criterion since H. Selye's famous studies (Selye 1971). When simulating stress conditions in laboratory animals, mainly rodents (rats, mice), the main focus is on the concentration of corticosterone in the blood, which

dominates among the glucocorticoid hormones in such animals. In humans, the adrenal response to the action of any stress-producing agent is known to be an increased production of cortisol (Gorwood et al. 2016). Corticosterone and cortisol differ significantly in their functional characteristics and biological activity (Willner 1995, Griffiths et al. 2012). Therefore, when choosing a stress model, one is guided by such biological objects in which cortisol is an indicator of stress. Among laboratory animals, these include fish, in particular Zebrafish, or *Danio rerio*, a wild type found in shallow waters of Southeast Asia and actively reproduced as an aquarium animal (Menke et al. 2001, Griffiths et al. 2012, De Abreu et al. 2018). A feature of *Danio rerio* is also the non-isolation of individual genetic lines of the species, but the use of exclusively wild-type animals as a biological object for laboratory research.

The indicators of the state of the hypothalamic-pituitary-adrenal system in Zebrafish are currently well studied. At the same time, it was confirmed that a response to a stress-producing agent in Zebrafish is manifested as an increase in the level of cortisol in the blood (Griffiths et al. 2012). This fish species can be used to study the effect of pharmacological agents, evaluating both the integral behavior of animals and the biochemical characteristics of the brain, muscles, liver, and blood (Lebedev et al. 2019, Shabanov et al. 2020).

We suggested that, in addition to cortisol, the state of the ghrelin peptide system can be used as a stress marker (Lebedev et al. 2019). Ghrelin, or growth hormone secretagogue, is a hormone containing 28 amino acid residues, formed during the sequential proteolytic degradation of the protein precursor preproghrelin and proghrelin. Ghrelin is synthesized primarily in the stomach and secreted into the general bloodstream. In plasma, ghrelin exists in two forms: acetylated ghrelin and desacyl ghrelin. It is believed that post-translational acylation of ghrelin is necessary for its functional activity. Ghrelin is acylated at the 3rd serine residue by the enzyme ghrelin-O-acyltransferase (GOAT). Acylated ghrelin is characterized by a post-translational modification, which is unique for oligopeptides, namely, the addition of an octanoic acid residue to a serine amino acid residue by means of an ester bond. This presence of an acyl (alkyl) residue is necessary for the binding of ghrelin to the corresponding receptor in the central nervous system (CNS) – GHSR 1A (Goldstein et al. 2011, Tine et al. 2016). Ghrelin is involved in stress reactions, eating behavior, the formation of addiction to chemical (alcohol, opiates, psychostimulants) and non-chemical (gambling, eating disorders) factors (Silverman et al. 2010, Lebedev et al. 2019, Shabanov et al. 2020). Taking into account that the mechanisms of relapse in all types of addiction are mainly associated with stressful effects (Shabanov et al. 2020), this study provided for the consideration of the ghrelin system as one of the biomarkers of stress and an indispensable participant in the formation of obsessive-compulsive and impulsive addiction elements.

The genetic and peptide structure of ghrelin was determined in teleost fishes, including *Danio rerio* (Tine et al. 2016). H-terminal nuclei with acyl-modified sites are strictly conserved in all vertebrates. As in vertebrates, in *Danio rerio*, ghrelin mRNA is expressed mainly in the stomach, pancreas, and brain structures (Tine et al. 2016). Ghrelin in *Danio rerio* is a regulator of hormone production by the pituitary gland, food saturation, and intestinal function, which are similar functions in humans.

The aim of the present study was to study the level of ghrelin in various brain structures during a stress response in Zebrafish to a predator, to evaluate this indicator as a potential biomarker of stress, and the effect of a benzodiazepine tranquilizer (phenazepam) on stress-induced changes.

Materials and methods

Animals

We used adult fish *Danio rerio* (Cyprinidae, Teleostei) at the age of 6–8 months (young adult animals) from Aqua Piter company and raised at the Institute of Experimental Medicine, all *Danio rerio* being wild type. The intact animals (n = 96, both sexes) were used for testing. The volume of tanks for keeping the fish was 40 liters (3 tanks in total), each containing 20–30 animals. The animals were kept under standard light conditions (8.00–20.00) at a water temperature of 22 ± 2 °C, and were fed twice a day with standard food "Tetramin tropical flakes". The study was approved by the local ethics committee of the Federal State Budgetary Scientific Institution "Institute of Experimental Medicine".

Predator stress model

In the experiments with a predator, the test fish was first placed into a small glass tank with a volume of 200 ml with a dissolved pharmacological substance (or water blanks) for 5 min, then – into a tank with a predator *Hypsophrys nicaraguensis* (10 × 10 × 10 cm^3) for 5 min, and then it was decapitated in the cold (-10 °C).

Pharmacological substances

For a pharmacological analysis, corticotropin-releasing hormone (CRF) was used, which was dissolved in water at the concentration of 0.4 mg/L, ghrelin antagonist [D-Lys3]-GHRP-6 – 0.333 mg/L, phenazepam – 1 mg/L and exposed for 5 minutes. [D-Lys3]-GHRP-6 is a peptide fragment of substance P, exhibiting the properties of an antagonist of GHSR 1A receptors (Shabanov et al. 2020). Phenazepam belongs to the group of benzodiazepine anxiolytics (Shabanov et al. 2017, Lebedev et al. 2018). CRF and [D-Lys3]-GHRP-6 are received from Tocris (UK).

Determination of cortisol and ghrelin

Cortisol was assessed in the muscle tissue of the tail of fish, using a highly sensitive enzyme-linked immunosorbent assay (ELISA, AlcorBio, St. Petersburg, Russia) and was expressed in ng/mg protein, which was determined according to Bradford (Lebedev et al. 2019). Evaluation of a level of ghrelin in the brain of fish was also carried out using ELISA. The animals were removed from the tank, deeply anesthetized by placing them on a cooled surface (-10 °C), decapitated, and the brain was removed. The brain was surgically divided under an MBS-1 binocular magnifier into 3 anatomical parts (Figs 1, 2): the forebrain (anatomical telencephalon), the midbrain (corpora bigemia in fish), and the cerebellum with the medulla oblongata (Menke et al. 2001). Then the material for ELISA was prepared, using Ghrelin FISH, a MyBioSource ELISA kit. The ghrelin level was expressed as pg/mg total protein.

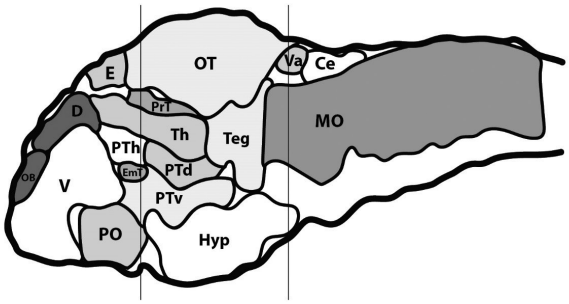

Figure 1. Layout of the *Danio rerio* brain for sampling. **Note:** Ce – cerebellar plate; D – dorsal telencephalon/pallium; E – epiphysis; EmT – eminentia thalami; Hyp – hypothalamus; MO – medulla oblongata; OB – olfactory bulb; OT – optic tectum; PO – preoptic area; PrT – pretectum; PTd – posterior tuberculum dorsal part; PTh – prethalamus; PTv – posterior tuberculum ventral part; Teg – tegmentum; Th – thalamus; V – ventral telencephalon/subpallium; Va – valvula cerebelli.

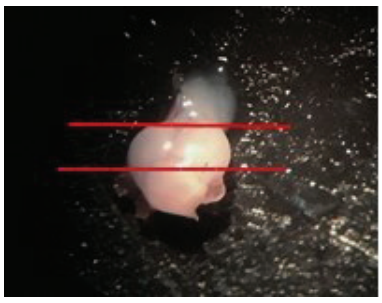

Figure 2. General view of the *Danio rerio* brain from the front, with boundaries of the anatomical sections. **Note:** The size of the fish brain averaged 2.7 mm, and the brain weight was 7.5 mg. Each isolated part did not exceed 1 mm in size and was no more than 3 mg in weight.

Statistical analysis

Statistical processing of the obtained data was carried out using Statistica v.6 and Origin v.7.5 packages. The normality of distributions was assessed using the Kolmogorov-Smirnov test, and the significance of differences between the groups was assessed using the Student's t-test or the Mann-Whitney U-test.

Results and discussion

Determination of cortisol in the muscle tissue of the rat tail showed that its basal level was 0.346 ± 0.035 ng/mg of protein. Exposure to a predator increased the cortisol level to 0.720 ± 0.052 ng/mg of protein, that is, more than 2 times. Exposure to CRF had a similar effect; in this case, the cortisol level increased to 0.896 ± 0.068 ng/mg of protein, that is, 2.6 times. The ghrelin antagonist [D-Lys3]-GHRP-6 and phenazepam did not significantly change the level of cortisol in the intact fish, but reduced the level of cortisol increased by exposure to a predator to practically the level of the intact animals (Table 1).

Table 1. Cortisol Levels (ng/mg of protein) in *Danio Rerio* Tail Muscle After Predator Exposure, CRF and Ghrelin Antagonist [D-Lys3]-GHRP-6 Administration

Animal group	Cortisol level, ng/mg of protein	Percentage of changes (%)	Nature of changes
Control (intact fish)	0.346 ± 0.035	0	0
Exposure to a predator	0.720 ± 0.052*	+208*	↑↑
CRF in intact fish	0.896 ± 0.068**	+258**	↑↑↑
[D-Lys3]-GHRP-6 in intact fish	0.356 ± 0.043	+2.9	0
[D-Lys3]-GHRP-6 + predator	0.357 ± 0.029#	-202#	0↓
Phenazepam in intact fish	0.363 ± 0.049	+5.4	0
Phenazepam + predator	0.384 ± 0.032#	-188#	0↓

Note: * – $p < 0.05$; ** – $p < 0.01$ in relation to the control group; # – $p < 0.05$ in relation to the group of fish exposed to a predator. ↑↑ – moderate activation; ↑↑↑ – pronounced activation; 0 – no changes; 0↓ – normalization (compensation of the effect of increased cortisol).

Therefore, it was confirmed that exposure to a predator (stressful situation) increases the level of cortisol in fish, which is abolished by the ghrelin antagonist [D-Lys3]-GHRP-6 and a benzodiazepine tranquilizer phenazepam. The experiment used the predator stress model, which is more commonly used in experiments on mammals. This experiment is an animal model of acute stress. In order to eliminate the additional stressing of animals, we used deep anesthesia of *Danio rerio* (by cooling) immediately after the experiment and obtaining the material (brain tissue) within the first minutes, which made it possible to more objectively evaluate the results obtained.

In intact *Danio rerio* specimens, ghrelin is detected only in the cerebellum (with the medulla oblongata), the most ancient and developed structure of the fish brain (Fig. 3A). When *Danio rerio* is exposed to a predator, the ghrelin level increases in the forebrain (telencephalon) and in the midbrain (corpora bigemia) and decreases in the cerebellum (Fig. 3B). Phenazepam 1 mg/L completely eliminates the increased level of ghrelin in all areas studied, both in the intact individuals (Fig. 3C) and in the animals exposed to a predator (Fig. 3D).

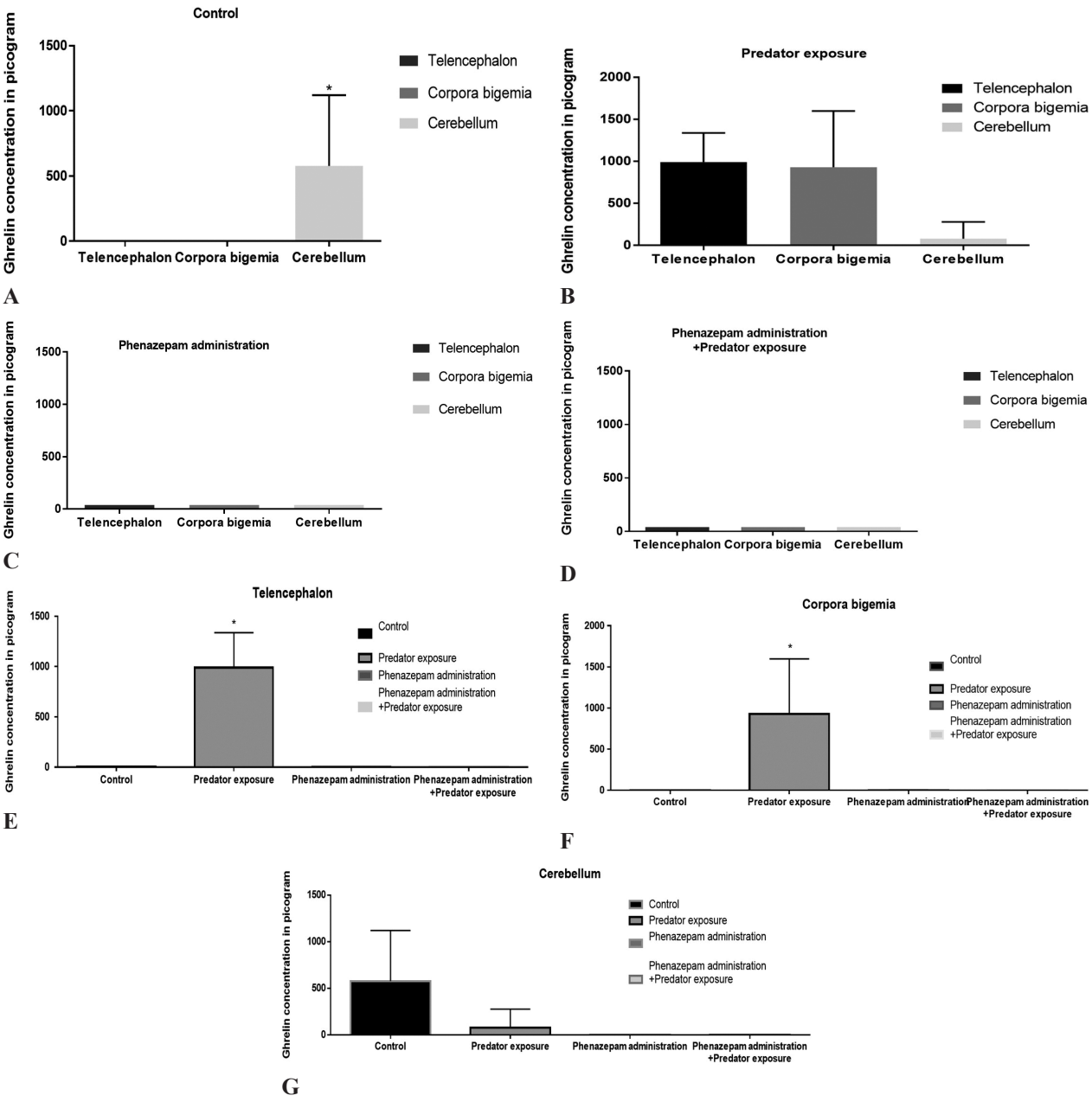

Figure 3. Effect of acute stress and phenazepam on a ghrelin content (pg/mg of total protein) in different parts of the *Danio rerio* brain. **Note: A** distribution of ghrelin levels in different parts of the *Danio rerio* brain; **B** the effect of exposure to a predator on the ghrelin level; **C** the effect of phenazepam on the level of ghrelin in control individuals; **D** the effect of phenazepam on the ghrelin level in individuals after exposure to a predator; **E** content of ghrelin in the forebrain; **F** content of ghrelin in the midbrain; **G** content of ghrelin in the cerebellum.

If we consider the effects of exposure to a predator on the level of ghrelin in the studied structures, conspicuous is the maximum reaction of the forebrain, when the level of ghrelin increases to nanogram concentrations (Fig. 3E), and, though to a lesser extent, but significantly increases the level of ghrelin in the midbrain (Fig. 3F). In the cerebellum, the level of ghrelin, on the contrary, decreases relative to the basal level increased to 500 pg/mg of total protein in the control animals (Fig. 3G). Phenazepam neutralizes all the levels of ghrelin in the studied areas of the brain increased by exposure to a predator, which means that the study revealed that the evolutionarily younger structures, and first of all, the forebrain, react to the influence of a predator. The cerebellum, as the most evolutionarily ancient and most developed structure in fish, is more inert to the effects of a stress factor. And finally, tranquilizer phenazepam eliminates all the changes that occur under the influence of a predator.

The results of an enzyme-linked immunosorbent assay showed that in the muscle tissue of the tail of intact fish in all the individuals, the level of cortisol is determined, which is 0.346 ± 0.035 ng/mg of total protein. Exposure to a predator, as well as administration of CRF, more than doubles the level of cortisol, which indicates the involvement of the hypothalamic-pituitary-adrenal system in the response to a stress-inducing factor. The stress response,

accompanied by an increased level of cortisol in the tissues, is eliminated by both the anxiolytic phenazepam and the peptide antagonist ghrelin [D-Lys3]-GHRP-6. The data obtained confirm the involvement of the ghrelin system in the anxiolytic action of benzodiazepines, mainly due to functional antagonism between the ghrelin system and GABAergic mechanisms mediating the effect of benzodiazepines (Guo et al. 2012, Shabanov et al. 2017).

The enzyme-linked immunosorbent assay showed that in the control (intact) group of fish, ghrelin was detected only in the cerebellum and medulla oblongata, the most phylogenetically ancient structures of the brain, while the detection rate of ghrelin in the cerebellum was only 57.1 ± 2.8%. Stress-inducing exposure to a predator increases the level of ghrelin in the evolutionarily younger structures – the forebrain and midbrain, but decreases it in the cerebellum. At least several questions arise from the data presented. First, how adequate is the *Danio rerio* model of acute stress for studying the molecular mechanisms of stress? Secondly, is it possible to consider the change in the level of ghrelin due to stressful effects as a kind of biomarker of stress? And, thirdly, what are the mechanisms of action of phenazepam on the ghrelin peptide system?

The answers to the first two questions are no doubt affirmative. *Danio rerio* can be used as an object for studying stress, since fish completely repeats the patterns of response to stress factors, like mammals, in particular, rodents, most often involved in studis of stress reactions (Pollak et al. 2010, Bovenkerk and Kaldewaij 2015, Tine et al. 2016). Moreover, in fish, the cortisol is the main stress hormone, as in humans, while in rats it is corticosterone, which is present in humans only in trace amounts (Lux and Kendler 2010, Silverman et al. 2010). This brings both objects of study closer together. The ghrelin system, consisting of at least three structural components (acylated ghrelin, deacylated ghrelin, and obestatin) and the corresponding receptors GHSR-1A and GHSR-1B in different structures of the body, including the central nervous system, is quite sensitive in terms of reactivity to stressful impact (Goldstein et al. 2011, Tine et al. 2016). For example, earlier in rats we showed a change in the level of ghrelin in stressed animals, which decreased under the influence of ghrelin antagonist [D-Lys3]-GHRP-6 (Shabanov et al. 2020). In the present study, total brain ghrelin was determined, which includes acylated (about 10% of the total amount) and desacyl ghrelin (about 85% of the total amount of ghrelin). Another question arising from these results is whether an increase in ghrelin in response to a stress factor is due to the *de novo* synthesis of ghrelin(s) in the brain or due to an increase in the transport of desacyl ghrelin in the central nervous system. Although these results do not directly answer this question, they support the assumption that an increase in ghrelin occurs due to the synthesis of peptides in the brain due to derepression of the ghrelin gene, which controls the synthesis of both acylated and desacyl ghrelin (Shabanov et al. 2020).

Finally, regarding the mechanisms of a possible effect of phenazepam on the ghrelin peptide system, benzodiazepines, including phenazepam, which is one of the most powerful anxiolytics, exhibit the properties of indirect agonists of the inhibitory system of γ-aminobutyric acid (GABA), interacting with two of the five $GABA_A$ receptor subunits sensitive to them. GABA is involved in the regulation of eating behavior, stress reactions, mediates addictive behavior in mammals (Reynolds and Berridge 2001), and from a molecular point of view interacts with many peptide systems of the brain, for example, the system of corticoliberin, orexins, and ghrelin (Guo et al. 2012, Shabanov et al. 2020). As a rule, GABA and the ghrelin system are in antagonistic functional relationships, hence the results obtained on a decrease in the level of ghrelin under the influence of phenazepam, which exhibits the properties of an indirect GABA agonist, are understandable. This phenomenon seems to be based on the suppression of ghrelin gene expression in response to stress by phenazepam, which is manifested by a decrease in the peptide level in the studied brain areas of *Danio rerio* fish.

Conclusion

1. The use of a highly sensitive enzyme-linked immunosorbent assay (ELISA) allows identifying the ghrelin peptide in various parts of the brain of *Danio rerio*.
2. The state of psycho-emotional impact (exposure of fish to a predator) significantly increases the level of cortisol in muscle tissue and the content of ghrelin, mainly in the forebrain and midbrain, but not in the cerebellum.
3. Anxiolytic phenazepam and ghrelin antagonist [D-Lys3]-GHRP-6 eliminate the consequences of a predator-induced stress on cortisol levels and the ghrelin system in *Danio rerio*.

Conflict of interests

The authors declare neither competing financial interests, nor conflict of interests.

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Author contributions

- **Petr D. Shabanov,** Dr. Habil. Med. Sci. (Pharmacology), Professor and Head, S.V. Anichkov Department of Neuropharmacology, e-mail: pdshabanov@mail.ru, **ORCID ID** https://orcid.org/0000-0003-1464-1127 (main idea, general supervision of the experiment, writing the article, proofreading).

- **Aleksandra A. Blazhenko,** post-graduate student, S.V. Anichkov Department of Neuropharmacology, e-mail: alexandrablazhenko@gmail.com, **ORCID ID** https://orcid.org/000-0002-8079-0991 (experimental work, obtaining the results, statistical analysis).

- **Aleksandr S. Devyashin,** post-graduate student, S.V. Anichkov Department of Neuropharmacology, e-mail: alexsanta93@mail.ru, **ORCID ID** https://orcid.org/0000-0002-1794-4230 (experimental work, obtaining the results, statistical analysis).

- **Platon P. Khokhlov,** PhD (Biochemistry), senior researcher, S.V. Anichkov Department of Neuropharmacology, e-mail: platonkh@list.ru, **ORCID ID** https://orcid.org/0000-0001-6553-9267 (experimental work, obtaining the results, statistical analysis).

- **Andrei A. Lebedev,** Dr. Habil. Biol. Sci. (Pharmacology), Professor, Head of the Laboratory of General Pharmacology, S.V. Anichkov Department of Neuropharmacology, e-mail: aalebedev-iem@rambler.ru, **ORCID ID** https://orcid.org/0000-0003-0297-0425 (organization of the experimental study, writing the article).