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Selank Peptide Causes Changes in Gene Expression in the Hippocampus of Rats in the Early Hours after Acute Restraint Stress

I. B. Filippenkov[a,*], N. Yu. Glazova[a,b], E. A. Sebentsova[a,b], I. V. Mozgovoy[a], V. V. Stavchansky[a], N. F. Myasoedov[a], N. G. Levitskaya[a,b], S. A. Limborska[a], and L. V. Dergunova[a]

[a] *National Research Center "Kurchatov Institute", Moscow, Russia*
[b] *Faculty of Physics, Moscow State University, Moscow, Russia*
E-mail: filippenkov-ib.img@yandex.ru

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Stress is a risk factor for the development of anxiety-depressive disorders, cardiovascular diseases, and cognitive impairment. The peptide drug ThrLysProArgProGlyPro (Selank), an analogue of endogenous tuftsin, has entered clinical practice as an anxiolytic agent acting as a positive allosteric modulator. In a model of acute restraint stress, high-throughput RNA sequencing (RNA-Seq) is used to demonstrate that Selank is able to significantly alter gene expression in the rat hippocampus 2 h after stress exposure. Thus, the introduction of Selank (300 μg/kg) to rats 30 min before the start of immobilization lasting 1 h leads to a change in the expression of 549 genes (fold change >1.5 and $Padj < 0.05$), which are related to the systems of processing and presentation of antigens and transmission of nerve impulses. At the same time, when Selank is administered to rats in the absence of stress, no significant change in gene expression in the hippocampus is observed. Thus, Selank can regulate the processes caused by acute stress at the molecular-genetic level already in the early hours after acute stress, without affecting genomic activity in the absence of such an impact.

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INTRODUCTION

Numerous clinical and experimental data indicate that both acute and chronic stress cause physiological changes in the body of humans and animals that may underlie many pathological conditions [1]. Stress is a risk factor for the development of anxiety-depressive disorders, post-traumatic stress disorders, cardiovascular diseases, and cognitive impairment [2–4]. However, the neurobiological mechanisms mediating the acute and long-term effects of stress are not well understood [5]. The study of the mechanisms of negative consequences of acute stress and the search for means of their pharmacological correction are a pressing problem in modern neurobiology and medicine.

Currently, methods are being actively developed to reduce the scale of stress-related influence on the brain by protecting nerve cells with physiologically active substances. Thus, many physiological processes in the body are subject to direct or indirect regulation by substances of a peptide nature [6, 7]. One of the drugs created on the basis of natural regulatory peptides is ThrLysProArgProGlyPro (**Selank**), which is an analogue of the endogenous fragment of the heavy chain of immunoglobulin G, tuftsin, stabilized by the tripeptide ProGlyPro (**PGP**). Selank entered clinical practice as an anxiolytic agent with stimulating, nootropic and emotional normalizing effects with minimal side effects [8].

According to [9], Selank is capable of the positive allosteric modulation of GABA receptors. It is known that the effector action of peptides is often associated with the modulation of not just individual receptor systems, but with the influence on many types of cellular receptors. In turn, receptors can influence a number of signaling and metabolic pathways, including indirectly affecting gene expression in the nucleus [10, 11]. In response to stress, stress-response mediators (hormones, peptides, neurotransmitters) are released, which, by acting on the corresponding receptors in the central nervous system, can regulate gene expression in various areas of the brain [12–14]. Moreover, the ability of Selank to allosterically bind to receptors under conditions of the orthosteric binding of receptors to stress mediators can probably lead to significant changes in the signals transmitted to cells during stress. The use of genomic approaches, in particular those based on the study of changes in the level of mRNA of genes, may allow us to understand the genesis of the processes triggered by Selank under

stress, which underlie the mechanisms of action of the peptide. Thus, previously, using the PCR method, it was shown that Selank affects gene expression (*Gabra6*, *Gabrb1*, *Gabrb3*, *Gabre* etc.) associated with neurotransmission in the frontal cortex of rats [15]. These data suggest that Selank may modulate the gene-expression profile of the GABA system, as well as neurosignaling and neuroreception.

In this study, we use the high-throughput RNA sequencing method (**RNA-Seq**), to study the effect of Selank on the activity of the genome of rat brain cells in the first hours after acute restraint stress and to evaluate the signaling systems modulated by the action of Selank during stress. Changes in mRNA levels of genes in the hippocampus, a key brain structure involved in the control of anxiety-related behavior and the response to acute stress, have been studied [4]. As a result, it is revealed that Selank in the early hours after acute stress causes a change in the expression of hundreds of genes (fold change > 1.5 and $Padj < 0.05$), related to the systems of processing and presentation of antigens and transmission of nerve impulses in the rat hippocampus. Moreover, the peptide does not affect the genomic activity of hippocampal cells under normal, nonstressful conditions. Thus, Selank, against the background of the development of a stress reaction, can provide global regulation of the activity of hippocampal cells at the molecular genetic level.

EXPERIMENTAL

Animals

Experimental groups. The experiments were carried out on male Wistar rats weighing 200–230 g. The animals were kept in a vivarium with a 12-h light regimen with free access to water and standard laboratory food.

The heptapeptide ThrLysProArgProGlyPro (Selank) was synthesized in the Laboratory of Molecular Pharmacology of Peptides, National Research Centre "Kurchatov Institute." The peptide was administered in an aqueous solution at a dose of 300 μg/kg intraperitoneally in a volume of 1 mL/kg 30 min before the start of stress exposure. Control animals were administered an equivalent volume of distilled water at the appropriate times.

Four groups of rats were used: nonstressed rats, those given a vehicle (**NS-V**) or Selank (**NS-L**), as well as stressed rats that received a vehicle (**STR-V**) or Selank (**STR-L**). Rats were divided into groups randomly. The time, dosage, and route of administration of Selank were selected based on studies [16–18].

Acute-Stress Model

Stress factor. Immobilization in combination with acoustic exposure lasting 1 h was used as a stress factor. During the immobilization period, the rat was placed an individual restrainer for laboratory animals (manufactured by OOO Open Science, Russia). The sound of an electric bell was used as an acoustic stimulus (3 min, bell; then 1 min, pause).

Assessment of the emotional state of animals. Ninety minutes after the end of the stress exposure (3 h after the injection), the elevated plus maze (EPM) test was used to assess the emotional state of the rats. The experimental chamber of the labyrinth (OOO Open Science, Russia) consists of four arms diverging from the center. Two opposite arms are closed at the ends by walls and darkened, the other two are open and brightly lit. The rat was placed in the center of the maze and the time spent in various sections of the maze (central platform, and open and closed arms of the maze), the number of entries into the open and closed arms, and the number of hangings from the open arms were recorded for 5 min.

Rats were euthanized by decapitation 3.5 h after injection. Each comparison group included 10 rats.

Statistical processing of the results of assessing the emotional state of animals. For statistical processing of the obtained results, the Statistica software package, version 10, was used. When comparing the time spent in different compartments of the maze, a three-way ANOVA was used (factors COMPARTMENT, STRESS, and SELANK). Comparison of the number of entries into the arms and the number of hangings was performed using 2-way ANOVA (factors SELANK and STRESS). In case of the statistically significant effect of factors or their interaction, the Fisher LSD criterion was used for further assessment of the differences between groups in post-hoc analysis.

Transcriptome Analysis

Obtaining tissue. The hippocampal region was removed from the rat brain. Samples of this tissue were placed in RNAlater solution for 24 h at 0°C and then stored at –70°C.

Obtaining RNA. Total RNA was isolated from the obtained rat hippocampal samples using Trizol reagent (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. The isolated RNA was treated with DNase 1 (Thermo Fisher Scientific) in the presence of RiboLock RNase Inhibitor (Thermo Fisher Scientific) according to the manufacturer's recommendations. The quality and quantity of isolated total RNA were checked on a Bio-Analyser using the RNA 6000 Nano Kit (Agilent, USA). The RNA integrity number (RIN) was at least 9.

Table 1. Characterization of primers for real-time PCR

Gene	Primer (5'–3'): forward (F), reverse (R)	RefSeq	Product size (bp)	E ± SE
Pla2g3	F: CAAGTTCCACCTGCTCAACA, R: GTGCCTTTATCCCAGAAATG	NM_001106015	207	2.02 ± 0
Hif3a	F: GTGAGCACCATGACGAAACCCTCG, R: ATGCGCCTCACAATCAGCTACTTGC	NM_022528.2	134	1.96 ± 0
Cd74	F: GTGTCTCTGTCCTGGTGGCT, R: CGCATCAGCAAGGGGAGTAG	NM_013069.2	193	1.87 ± 0.18
Grm3	F: ACCCTCTGTCCCAACACCA, R: TACTTCCCACCTGTCTGCT	NM_001105712	216	2.02 ± 0.03
Adcy5	F: TGTCTTCGTGCTGGCTCTGT, R: TGGTAGTACAGTTCATCATTGC	NM_022600	210	1.94 ± 0
Cnot3	F: GCTCTACAGATAGTGAAGTCA, R: TGCTGGTTGCTGTGGACA	NM_001107471.2	70	1.82 ± 0.05
Cyr61	F: CTGTCTTTGGCACGGAACCT, R: ATTTCTTGGTCTTGCTGC	NM_031327.3	249	2.05 ± 0.04
Gapdh	F: ACTCTACCCACGGCAAGTTCAACG, R: GTAGACTCCACGACATACTCAGCAC	NM_017008.4	148	2.01 ± 0.03

E ± SE is the PCR efficiency ± standard error of the mean (SEM), RefSeq is the identifier of the reference nucleotide sequence of mRNA, and bp is base pairs of nucleotides.

Whole genome RNA sequencing (RNA-Seq). The polyA fraction of the RNA was obtained from the total RNA using Dynabeads® mRNA Purification Kit (Ambion, USA). Next, libraries were prepared from polyA-RNA for massively parallel sequencing using the NEBNext® mRNA Library Prep Reagent Set (NEB, USA). The concentration of libraries was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) on a Qbit 2.0 instrument. The distribution of library-fragment lengths was performed using the Agilent High Sensitivity DNA Kit (Agilent, USA). RNA-Seq was performed on a HiSeq1500 instrument (Illumina, USA) with the generation of at least 10 million short reads of 1 × 50 nucleotides in length. Each group NS-V, NS-L, STR-V, and STR-L included three animals.

Processing the RNA-Seq results. Tophat and Cuffflinks programs were used for mapping and read counting. The gene-expression levels were calculated as fragments per kilobase per million reads (FPKM) using the Cuffdiff program. Only those genes that changed their expression level by more than 1.5 times were considered to be differentially expressed genes (**DEG**). Differences that had probability value of p, adjusted with the Benjamini–Hochberg correction (***Padj***) < 0.05, were considered significant.

*Synthesis of complementary DNA (**cDNA**)* was performed in a 20-μL reaction mixture containing 5-μg RNA using the reagents of the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer's instructions. Oligo$(dT)_{18}$ primers were used for mRNA analysis.

Real-time polymerase chain reaction. The synthesized cDNA was used as a template for real-time PCR with the intercalating dye SYBR Green I. Primers were selected using the Oligo Analyzer Tool (https://www.idtdna.com/pages/tools/oligoanalyzer) and synthesized by ZAO Eurogen Ru (Table 1). cDNA amplification was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) as described in [18]. Each group included five animals.

Processing of the PCR results in real time. Each cDNA sample was analyzed in triplicate. From three repeated measurements, the average value of the cycle threshold (**Ct**) was calculated. The mRNA level of the gene glyceraldehyde-3-phosphate dehydrogenase *Gapdh* was used to normalize the PCR results. The calculations were performed using the Relative Expression Software Tool (REST) 2005 [19]. To estimate the level of the studied mRNAs relative to the mRNA of the comparison gene, the formula $E^{Ct(ref)}/E^{Ct(tar)}$ was used, where E is the efficiency of the PCR reaction, Ct(tar) is the average Ct value for the studied RNA and $E^{Ct(ref)}$, for mRNA of the comparison gene (*Gapdh*). The PCR efficiency was assessed using amplification of a series of standard dilutions of cDNA. The efficiency values for all PCR reactions were in the range of 1.82–2.05 (Table 1). When com-

paring data groups with each other, differences with a probability of $p < 0.05$ were considered significant. Additional data processing was performed using Microsoft Excel.

Bioinformatics Analysis

Analysis of functional annotations of genes. Software for annotation, visualization, and integrated discovery (**DAVID v2021**) [20] and for gene set enrichment analysis (**GSEA**) [21] were used to annotate the DEG functions. When comparing data groups, statistically significant differences were taken into account with a probability *Padj* < 0.05 (with the Benjamini–Hochberg correction in DAVID v2021 and FDR in GSEA).

The hierarchical cluster analysis of DEG was performed using Heatmapper [22].

Gene networks. Cytoscape 3.8.2 software was used to visualize the regulatory network [23].

Other calculations and plots, including volcano plots, were constructed using Microsoft Excel (Microsoft Office 2010, Microsoft, USA).

Availability of Data and Materials

RNA-Seq data were deposited in the Sequence Read Archive database under the access code PRJNA1049747, https://www.ncbi.nlm.nih.gov/sra/PRJNA1049747).

RESEARCH RESULTS

Effect of Acute Restraint Stress and Selank Administration on the Behavior of Rats in the EPM Test

1.5 h after the end of stress exposure (3 h after administration of the peptide), the behavior of the rats was studied in the EPM test. The application of a three-factor ANOVA for time spent in different compartments of the maze showed a statistically significant effect of the COMPARTMENT factor ($F_{2,72} = 163.5$; $p < 0.0001$). In addition, a significant interaction between the STRESS and COMPARTMENT factors was observed ($F_{2,72} = 9.0$; $p < 0.001$), as well as the SELANK and COMPARTMENT factors ($F_{2,72} = 4.10$; $p = 0.02$). Further analysis showed that rats in all groups spent more time in the closed arms compared to both the open arms and the center of the maze ($p < 0.00001$). In the NS-V, NS-L, and STR-V groups, no significant differences were observed between the time in the open arms and in the center of the maze ($p > 0.45$). In the STR-L group, time spent in the open arms was statistically significantly greater than time spent in the center of the maze ($p = 0.04$). Between-group comparison showed a statistically significant reduction in time in the closed arms ($p = 0.003$), as well as an increase in time in the open arms at the trend level ($p = 0.07$) in the STR-V group relative to the NS-V group. In addition, a statistically significant increase in time in the open arms was recorded ($p = 0.0001$) and a decrease in time in the closed arms ($p < 0.0001$) in the STR-L group relative to the NS-V group. No significant differences were observed between the NS-L and NS-V groups ($p > 0.10$). The STR-L group showed a statistically significant increase in open-arm time ($p = 0.03$) compared with the STR-V group (Figs. 1a–1c).

The use of two-factor ANOVA analysis showed a statistically significant effect of the STRESS and SELANK factors on the number of entries into the open arms ($F_{1,36} = 14.38$; $p = 0.001$ and $F_{1,36} = 12.86$; $p = 0.001$) and hanging from the open arms of the labyrinth ($F_{1,36} = 12.67$; $p = 0.001$ and $F_{1,36} = 7.79$; $p = 0.01$). In addition, a significant influence of the SELANK factor on the number of entries into the closed arms was recorded ($F_{1,36} = 4.79$; $p = 0.035$). Significant interactions of the specified factors for these behavioral indicators in the EPM are noted there was no ($F_{1,36} < 1.8$; $p > 0.20$). Multiple comparison showed an increase in the number of open-arm entries at the trend level ($p < 0.10$) in the STR-V group relative to the NS-V group. In addition, a statistically significant increase in the number of entries into the open arms was recorded ($p < 0.00001$) and the number of hangings from the open arms ($p < 0.0001$) in the STR-L group relative to the NS-V group was recorded too. No significant differences were observed between the NS-L and NS-V groups ($p > 0.10$). In the STR-L group, there was a statistically significant increase in the number of open-arm entries ($p = 0.001$) and the number of hangings ($p = 0.01$) compared with the STR-V group. In addition, an increase in the number of entries into the closed arms was noted at the trend level ($p < 0.07$) in the STR-L group compared with the NS-V and STR-V groups (Figs. 1g–1e).

According to the results of the study, the administration of Selank to nonstressed animals did not lead to changes in behavior in the EPM test 3 hours after the injection. Rats that had experienced acute stress spent less time in the closed arms of the maze and more often entered the open arms compared to the nonstressed control, i.e., they demonstrated more active behavior. The nature of the changes in the behavior of animals that received an injection of Selank before stress exposure indicates a decrease in the level of anxiety and an increase in exploratory behavior, both in comparison with the control and with respect to stressed rats that were injected with the vehicle.

RNA-Seq Analysis of the Effects of Acute Stress on the Rat Hippocampal Transcriptome

RNA-Seq yielded changes in the mRNA level for 17 367 rat genes. The volcano plot illustrates the differences in mRNA expression between the STR-V and NS-V groups (Fig. 2a). 22 DEGs were detected (fold > 1.5; *Padj* < 0.05) under stress in the STR-V group rel-

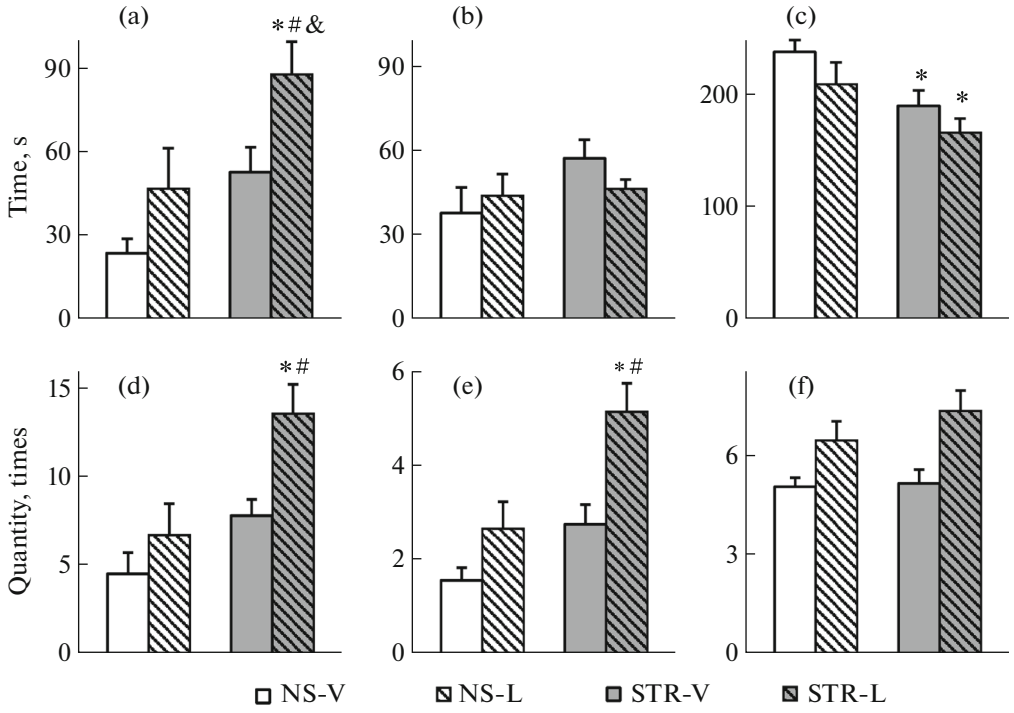

Fig. 1. Effect of acute stress and Selank on the behavior of rats in the EPM test. Selank was administered at a dose of 300 μg/kg 30 min before the start of stress exposure. Time spent in the open arms (a), in the center (b), and in the closed arms of the maze (c). The number of hangings from the open arms (d), entries into the open (e) and closed arms (f) of the maze. Data are presented as mean ± SEM; * is significant ($p < 0.01$) differences from the NS-V group; # is significant ($p < 0.03$) differences from the STR-V group; & is significant ($p < 0.05$) difference from the time in the center of the maze in the corresponding group.

ative to (vs.) NS-V. Of these, only three genes (*Alox15*, *Aox1*, *RT1-Da*) increased the expression level and 19 genes decreased it (*Gjb6*, *Gpd1*, *Gpr22*, *Met*, *Sep131*). The values of differential expression of the listed genes are presented in Fig. 2b.

Real-time PCR was used to study the expression of genes *Pla2g3*, *Cd74*, *Hif3a*, *Cnot3*, *Cyr61*, *Adcy5*, and *Grm3* in the comparison of STR-V vs. NS-V to validate the RNA-Seq results on an expanded sample set of animals (Fig. 2c). The real-time PCR results were in good agreement with the RNA-Seq results.

RNA-Seq Analysis of the Effect of Selank on the Hippocampal Transcriptome of Stressed and Control Rats

Comparison of the STR-L vs. STR-V groups, reflecting the effect of Selank on the rat hippocampal transcriptome under stress conditions, showed a significant change in the expression of a total of 549 genes. A volcano plot illustrating the differences in mRNA expression between the STR-L and STR-V groups is shown in Fig. 2d. The majority of DEGs in this comparison (353 DEGs) were upregulated, with genes *RT1-Ba*, *Cd74*, *RT1-Db1*, *RT1-Bb*, and *RT1-Da* by more than 25 times (Fig. 2d). There were also 196 DEGs (*Srpk3*, *Dqx1*, *Ascl2*, *Ercc2*), which reduced the level of their mRNA in comparison with STR-L vs. STR-V (Fig. 2d).

Importantly, no DEGs were identified that significantly changed mRNA levels in the NS-L vs. NS-V comparison, reflecting the effect of Selank on the rat hippocampal transcriptome under normal, non-stressed conditions (Fig. 2e).

Analysis of RNA-Seq Results in Different Comparison Groups

In the next step, a comparison was made of the spectra of DEGs that changed the mRNA level during acute stress exposure (STR-V vs. NS-V), as well as under the action of Selank under stress (STR-L vs. STR-V). Figure 3a shows in the form of a Venn diagram the 13 overlapping genes that significantly changed their expression level (>1.5-fold, $Padj < 0.05$) in the hippocampal region of rats both as a result of stress exposure and in response to the administration of Selank before stress relative to stressed animals that received the vehicle. The magnitudes of changes in the expression of these genes are shown in Fig. 3b. Only two genes *RT1-Da* and *Cd74*, encoding proteins of the major histocompatibility complex (MHC), co-directionally increased mRNA levels in both STR-V vs. NS-V and STR-L vs. STR-V. Another 11 genes

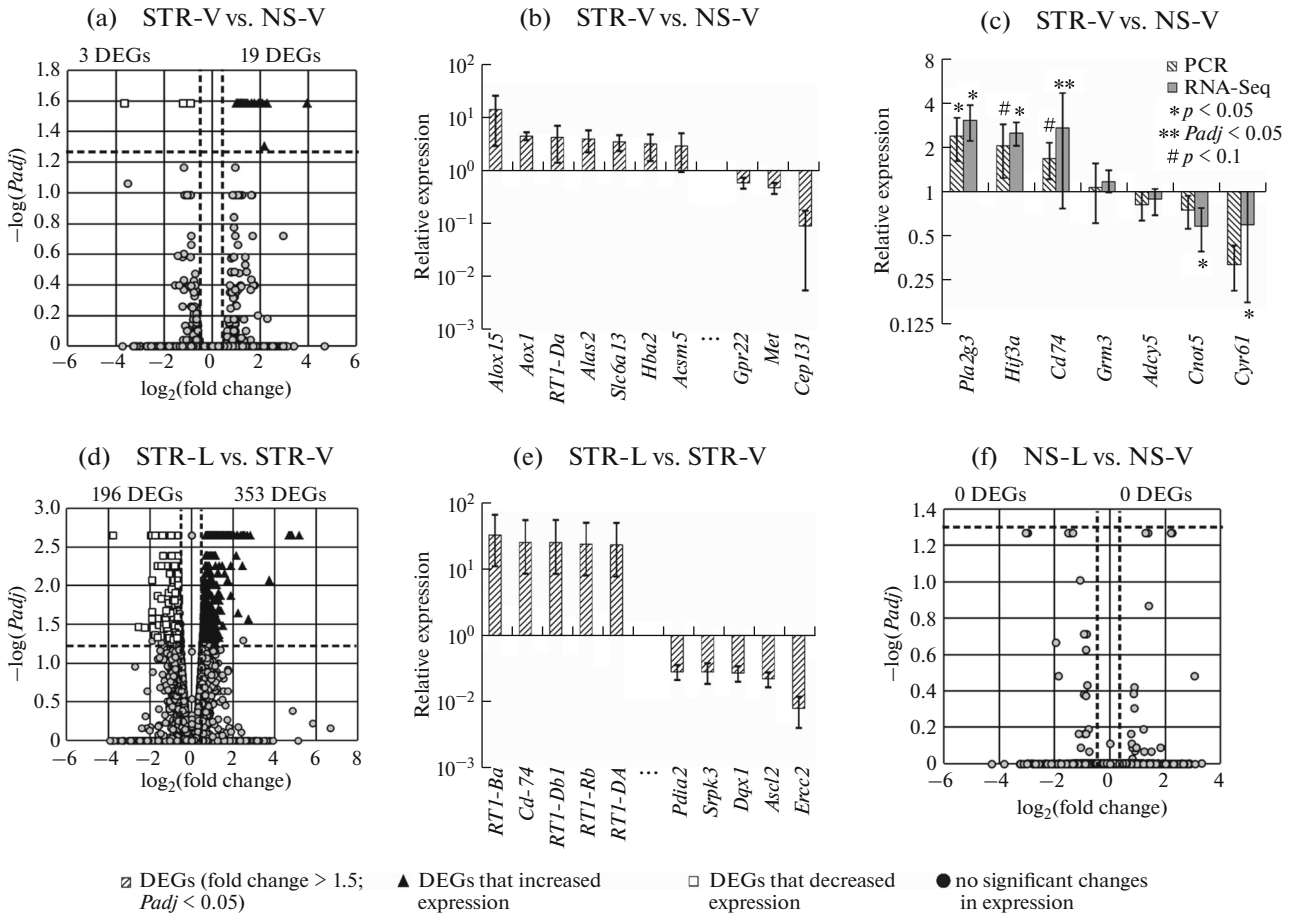

Fig. 2. RNA-Seq analysis of the effects of acute stress and Selank on the rat hippocampal transcriptome. Volcano plots illustrate the differences in mRNA expression between STR-V and NS-V (a), STR-L and STR-V (d), and NS-L and NS-V (f) groups. Genes that increased and decreased expression (fold >1.50 and *Padj* < 0.05) are marked with black triangles and white squares, respectively. Genes that did not change expression (fold ≤1.50 or *Padj* ≥0.05) are marked with gray circles. The 10 genes that showed the largest fold change in expression in the STR-V vs. NS-V (b) and STR-L vs. STR-V (e) comparisons are shown. Data are presented as mean ± SEM. The results of validation of the RNA-Seq results using real-time PCR are presented (c). Data are shown for the comparison of STR-V vs. NS-V.

(*Alox15*, *Aox1*, *Slc6a13*, *Acsm5*, *Igf2*, *Col3a1*, *Hbbl1* (*LOC100134871*), *Gpatch4*, *Ptgds*, *Gpr22,* and *Met*) changed expression level in the direction opposite to the effect of the stress itself when Selank was introduced.

Nine genes were identified (*Alas2*, *Hba2*, *Hba1*, *Col1a1*, *Rnase4*, *Mt2A*, *Gjb6*, *Gpd1*, and *Sep131*), which changed expression only under stress, predominantly (except *Sep131*) by raising it. Moreover, the largest number of DEGs (536) belonged to the category unique to the effects of Selank under stress. They are in the Venn diagram (Fig. 3a) in the STR-L vs. STR-V segment, but do not overlap with the DEGs in STR-V vs. NS-V. The top 10 genes from among them in terms of the fold change in expression are presented in Fig. 3c. Among them are genes *Itgal*, *C3*, *Ifi47*, *St14*, and *Tspo*, increased expression, and genes *Mapk15*, *Ttll9*, *Col27a1*, *Rtbdn*, and *Mroh7*, which had decreased expression in response to the introduction of Selank before stress relative to the stressed animals.

The results of the hierarchical cluster analysis of all genes that changed expression under stress (STR-V vs. NS-V), under the influence of Selank under stress (STR-L vs. STR-V), as well as the values of the fold changes in the expression of these genes upon the introduction of Selank under normal conditions (NS-L vs. NS-V) are illustrated in Fig. 3d. It is evident that the effect of Selank on gene expression is most pronounced, and the peptide causes compensation of the gene-expression profile disrupted by stress. The effect of Selank on gene expression is the least pronounced among the comparisons considered.

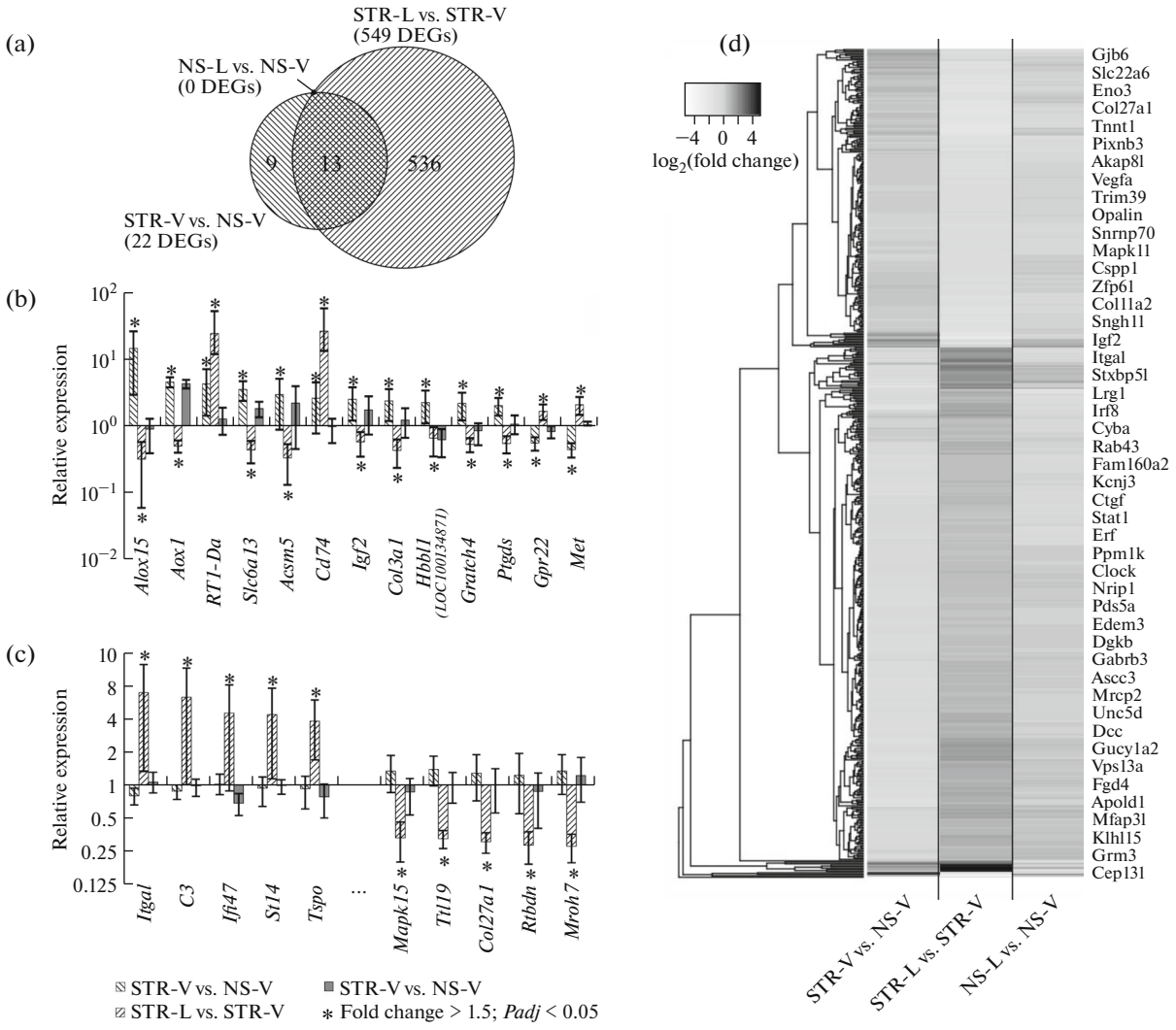

Fig. 3. Comparison of RNA-Seq results obtained in the hippocampus of stressed and nonstressed rats and after Selank administration. The Venn diagram illustrates the results obtained for the three pairwise comparisons STR-V vs. NS-V, STR-L vs. STR-V, and NS-L vs. NS-V (a). The numbers at the intersection of different gene sets in the Venn diagram indicate the number of DEGs. Relative expression values for DEGs that overlap between the two pairwise comparisons STR-V vs. NS-V and STR-L vs. STR-V (b). The 10 genes that showed the largest fold change in expression in the STR-L vs. STR-V comparison are shown (c). Data are presented as mean ± SEM. Hierarchical cluster analysis of all DEGs in three pairwise comparisons STR-V vs. NS-V, STR-L vs. STR-V, and NS-L vs. NS-V. Each column is a comparison group and each row is a gene, black bars are the maximum increase and white bars are the maximum decrease in relative expression (d).

Functional Annotations of DEGs Associated with the Effects of Acute Stress and Selank upon Stress

The DEG lists from the STR-V vs. NS-V and STR-L vs. STR-V comparison groups were processed using the DAVID v2021 functional enrichment analysis program. For the 22 DEGs identified upon stress, there were no significant associations with any signaling pathway (*Padj* < 0.05, where *Padj* is the probability of obtaining a false positive result with the Benjamini–Hochberg correction) in the comparison of STR-V vs. NS-V. Another GSEA tool that provides enrichment information using a more relaxed statistical approach (FDR $q < 0.05$), identified 22 pathways annotated in terms of the databases KEGG PATHWAYS (**KP**) and REACTOME PATHWAYS (**RP**), associated with DEGs for STR-V vs. NS-V. The most important pathways were ligand binding by scavenger receptors and signaling involving receptor tyrosine kinases.

DEGs identified as associated with the influence of Selank upon stress in the STR-L vs. STR-V comparison showed significant associations with 50 signaling pathways in DAVID v2021 data. These included pathways related to the immune system (phagosome, mitogen-activated protein kinase (MAPK) signaling pathway, cell-adhesion molecules, etc.) and neurosignal-

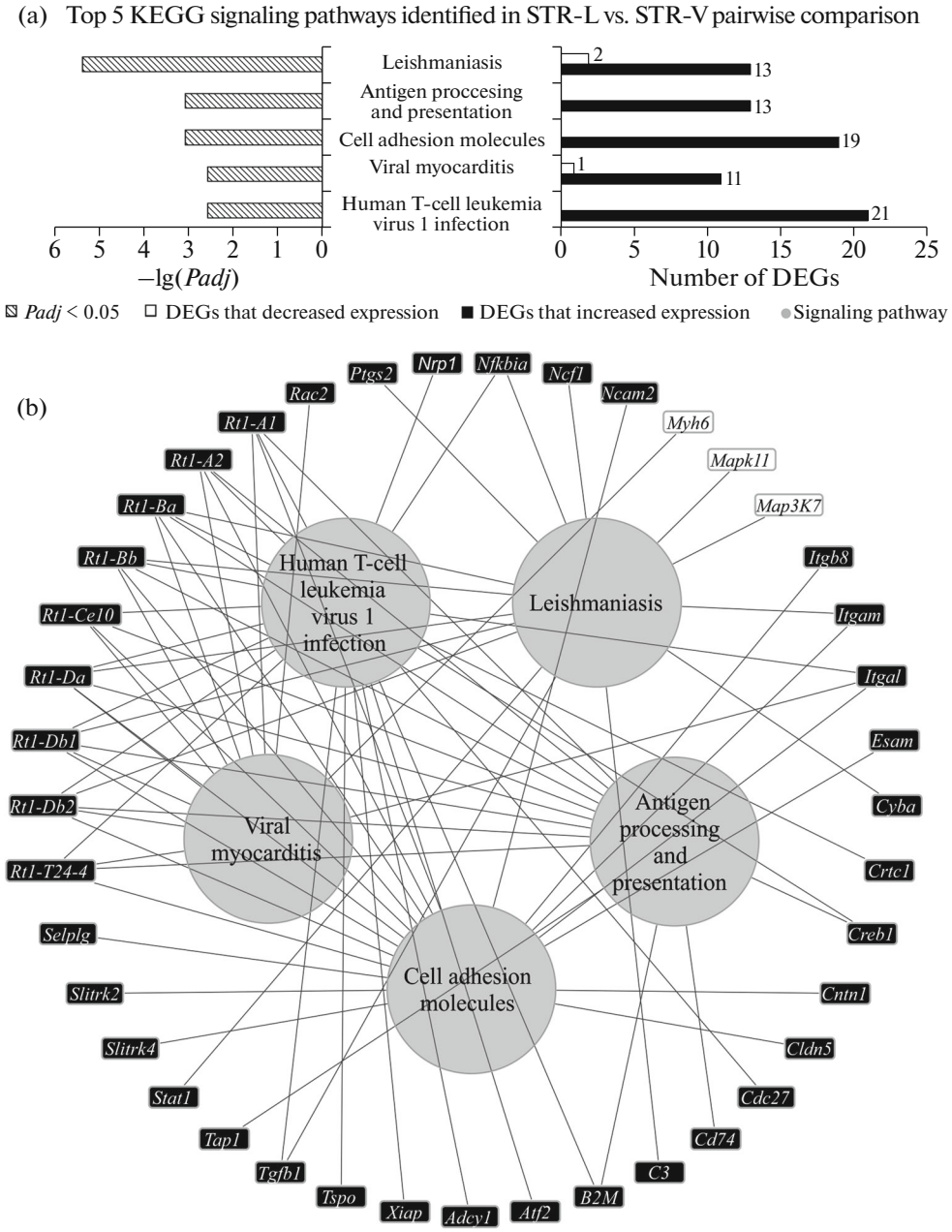

Fig. 4. Functional annotation analysis of DEGs associated with the effects of acute stress and stress-induced Selank. The top five significant KEGG signaling pathways associated with DEGs are shown in the comparison of STR-L vs. STR-V (a). For each signaling pathway, the number of genes that increased and decreased expression is shown, and the corresponding *Padj* values are given. A functional network of gene association with signaling pathways from among the five most significant ones and presented in Fig. a is presented (b). Only those DEGs that changed mRNA levels in the STR-L vs. STR-V comparison and those pathways that were significantly associated with these DEGs were selected for analysis. The nodes represent genes. Each line connecting the nodes indicates the involvement of the protein product of the corresponding gene in the functioning of the signaling pathway.

ing (neuronal system, glutamatergic synapse, dopaminergic synapse, calcium signaling pathway).

The top five pathways in terms of significance level in the STR-L vs. STR-V comparison are shown in Fig. 4a. These included leishmaniasis, cell-adhesion molecules, antigen processing and presentation, human T-cell leukemia virus 1 infection, and viral myocarditis, annotated in KP terms and primarily related to the immune and inflammatory response of cells. Notably, most of the DEGs associated with the listed pathways were upregulated by Selank under stress conditions.

Figure 4b shows the gene network characterizing the effect of Selank on the rat hippocampal transcrip-

tome under stress conditions. It shows genes whose protein products are involved in the functioning of signaling pathways related to the five most significant for the comparison STR-L vs. STR-V. There are 41 DEGs participating in the network, 38 of which (*RT1-Ba*, *Cd74*, *RT1-Db1*, and others) increased and only three (*Mapk11*, *Map3k7,* and *Myh6*) reduced mRNA levels in STR-L vs. STR-V. The largest number of genes are associated with the functioning of the pathways: human T-cell leukemia virus 1 infection and cell-adhesion molecules, 21 and 19 DEGs, respectively. The genes *RT1-Ba*, *RT1-Db1*, *RT1-Bb*, *RT1-Da*, and *RT1-Db2*, related to MHC activity, are involved in the functioning of all five pathways presented, while 26 genes are associated with only one pathway: the genes *Tspo*, *Xiap*, *Cdc27*, *Adcy1*, *Atf2*, *Crtc1*, and *Nrp1* are associated with human T-cell leukemia virus 1 infection; the genes *C3*, *Ptgs2*, *Cyba*, *Ncf1*, *Stat1*, *Mapk11*, and *Map3k7*, with Leishmaniasis; the genes *Itgb8*, *Ncam2*, *Slitrk4*, *Slitrk2*, *Esam*, *Selplg*, *Cntn1*, and *Cldn5*, with cell-adhesion molecules; the genes *Cd74* and *Tap1*, with antigen processing and presentation; and the genes *Rac2* and *Myh6*, with viral myocarditis.

DISCUSSION

Acute stress is known to cause both short-term and long-term behavioral, neurochemical, and structural changes, which may be based on various mechanisms [24, 25]. The duration of changes caused by acute stress ranges from several hours to several days [26, 27]. It is believed that stress-induced behavioral disturbances involve plastic changes in the central nervous system, which may take some time to develop [2].

In the presented study, changes in the behavior of rats in the EPM test were assessed 1.5 h after the end of stress exposure. The test is based on the innate avoidance of open illuminated spaces by rodents, which leads to a decrease in exploration of this area when a darkened and less dangerous area is available to them [28]. In the experiments conducted, rats of all groups spent more time in the closed arms of the maze, avoiding the open sections (open arms and the center of the maze). The stressor used resulted in a reduction in the time spent in the closed compartment, while no statistically significant change was recorded in the generally accepted indicators of anxiety levels, i.e., the number of entries and time in the open arms. There was also no change in the exploratory activity indicator: the number of hangings from the open arms of the maze. In studies [18], no changes in anxiety indices were also found in rats that had undergone similar exposure 4 h after the end of the stress. The nature of stress-induced changes in rat behavior both 1.5 and 4 h after restraint stress indicated increased avoidance motivation and hyperactivity in the animals [29].

The administration of Selank to animals not exposed to stress did not result in behavioral changes in the EPM test 3 h after injection. In the group of rats that received an injection of Selank before stress exposure, an increase in the time spent in the open arms and the number of entries and hangings from the open arms of the maze was observed, both in comparison with nonstressed animals and in comparison with rats that had undergone stress exposure. The nature of the observed changes indicates that the introduction of Selank leads to a decrease in anxiety and an increase in exploratory activity in rats that have undergone acute restraint stress but does not affect the normal behavior of animals.

In this study, RNA-Seq was used to obtain the spectra of DEGs associated with the effects of acute restraint stress and the Selank peptide in the rat hippocampus. The real-time PCR method made it possible to assess changes in the expression level of individual genes in an expanded sample of animals and obtain results consistent with RNA-Seq. Acute stress was shown to induce changes in the mRNA levels of 22 genes in the rat hippocampus 2 h after stress. The results of transcriptome analysis were compared with those obtained previously under conditions of a similar stress model, when changes in the mRNA level of genes were studied after a longer period of time after stress: after 4.5 h [18]. The results varied markedly. After 4.5 h, a change in the expression of a significantly larger number (1359) of genes associated with the regulation of RNA and protein biogenesis and metabolism, as well as cell neurotransmitter systems, was recorded [18]. The 22 DEGs identified 2 h after stress were primarily associated with the modulation of scavenger receptor activity and receptor tyrosine kinase signaling. The gene *Pla2g3*, encoding group-III phospholipase A2, was detected as DEGs by RNA-Seq and real-time PCR both at 2 h post-stress in this study and at 4.5 h post-stress in [18]. In [30] it was shown that *Pla2g3* gene copy number variations are associated with natural and artificial selection in adaptation to extreme climates. A change in *Pla2g3* gene expression was also reported when studying the effects of elevated glucocorticoid levels on the mouse hippocampal transcriptome. The expression of this gene changed both upon the administration of exogenous corticosterone and in response to the action of a stress factor [31]. According to our data, *Pla2g3* expression steadily increases both 2 and 4.5 h after stress and may characterize the general features of stress reactions in the rat hippocampus.

The molecular genetic effect of Selank was studied by administering the peptide to rats both in the absence of stress and by subjecting them to acute restraint stress 30 min after injection. In rats without stress, no significant change in gene expression in the hippocampus was observed 3.5 h after Selank injection compared to the vehicle. However, 549 genes, primarily those associated with antigen processing and pre-

sentation systems and nerve-impulse transmission, significantly changed their expression when exposed to Selank under stress conditions. Importantly, 13 of the 22 genes identified as DEGs 2 h after acute restraint stress also changed in expression when exposed to Selank under stress, with the peptide predominantly reducing the expression of those genes that increased in expression under stress (*Gpatch4*, *Aox1*, *Alox15*, *LOC100134871* (*Hbb*), *Acsm5*, *Slc6a13*, *Igf2*, *Col3a1*, and *Ptgds*). Thus, 2 h after stress, Selank can indeed regulate stress-induced processes at the molecular genetic level, but at the same time has significant activity in relation to 536 genes, the expression level of which is not affected by the influence of stress. The functional annotations of these genes are related to the immune and neurosignaling systems. It was previously shown that Selank has pronounced immunotropic activity and is capable of inducing the secretion of interferons and exerting an antiviral effect [32]. When examining the most significant functional annotations of genes, signaling pathways of cell adhesion, T-cell function, and antigen processing and presentation were identified. The genes encoding proteins involved in all of these pathways were *RT1-Ba*, *RT1-Db1*, *RT1-Bb*, *RT1-Da*, and *RT1-Db2*, related to MHC activity. The expression level of these genes was increased in response to the administration of Selank upon stress 3.5 h after injection. It should be noted that no effect of stress on the expression of these genes 4.5 h after stress has been previously recorded [18]. At the same time, the gene *RT1-Bb* was identified as a DEG in Sprague–Dawley rats in response to heat stress [33]; the gene *RT1-EC12*, also belonging to the MHC class-II gene family, was associated with individual differences in anxiety in genetically heterogeneous NIH-HS rats [34]. The differential expression of MHC class-II family genes was previously identified [35] under conditions of ischemic damage to brain tissue and in response to the administration of peptides (derivatives of adrenocorticotropic hormone (**ACTH**)) 4.5 h after transient occlusion of the right middle cerebral artery in rats. These peptides, like Selank, include a fragment of PGP, which is characterized by high stability in the animal's body. It is likely that this fragment is capable of partially determining the activity of the peptides containing it. This is confirmed by the results obtained in [35, 36] in comparing the effects of ACTH(4–7)PGP and ACTH(6–9)PGP, as well as ACTH(4–7)PGP and PGP on gene expression in the rat brain under ischemic conditions. Comparison of the effect of Selank at 2 h and the effects of ACTH(4–7)PGP and ACTH(6–9)PGP 4.5 h after stress revealed 79 genes that were altered in expression by both Selank and at least one of the ACTH derivatives. Among the DEGs were *Zdhhc21*, *Uhmk1*, *Rpl36a*, *Rpl39*, and *Rpl22l1*, related to the regulation of transcription and translation, which were co-directionally increased in expression by all peptides under stress. At the same time, hundreds of genes that are not overlapping can characterize the individual properties of peptides and reflect the structural features of each of the compounds. In particular, the effect of Selank on *RT1-Ba*, *RT1-Db1*, *RT1-Bb*, *RT1-Da*, and *RT1-Db2* gene expression, which was not detected for ACTH derivatives under stress, may be associated with the activity of another fragment of Selank, tuftsin, its natural analogue, a regulatory peptide derived from the heavy chain of immunoglobulin G and possessing immunomodulatory properties.

Previously, a model was proposed that describes the effect of peptides on the transcriptome of brain cells that are simultaneously in a state of stress response. It is based on the fact that the signal coming from the receptor, with which mediators or stress hormones bind orthosterically, is modified due to the additional allosteric binding of the peptide to membrane receptors. The resulting signal elicits a corresponding transcriptome response, in part identical to that under the same stress conditions and in part specific to the peptide [18]. The detected differential expression of multiple genes, induced by Selank but not associated with the stress response itself, somewhat violates the linearity of the idea that the peptide can modulate only those signals that the cell receives from orthosteric interactions with effectors (mediators of stress reactions). An explanation may be the presence of the peptide's own orthosteric binding sites with receptors, as well as the greater complexity in regulating the transcriptome response. It should be noted that in this study, the transcriptome response after Selank upon stress was examined, assessing changes in the level of protein-coding mRNA only. However, it is known that many different types of RNA function in the cell, providing processes of regulation of gene expression. These may include short noncoding RNAs (microRNAs), which are capable of forming a microRNA–mRNA duplex that directs the degradation of mRNA or the repression of its translation [37]. Much attention is also paid to a new class of RNA of a circular nature [38, 39]. These RNAs have increased stability and tissue-specific expression, which makes them particularly interesting objects, including for applied and translational research [40–42]. Functionally circular RNAs (**circRNA**) are capable of forming a microRNA–circRNA duplex on par with mRNA, which allows them to influence microRNA-mediated mRNA repression [43–45]. Given the diversity of regulatory properties of different types of RNA, the mechanism of Selank's effect on the transcriptome of nerve cells may be extremely nonlinear and requires further study using functional genomics methods.

CONCLUSIONS

The study showed that Selank in the early hours after acute stress can regulate stress-induced processes at the molecular genetic level without affecting genomic activity under nonstress conditions. How-

ever, the presence of a specific peptide response that is not associated with the stress response itself indicates a more complex mechanism of its action. We expect that further studies of the role of noncoding RNAs in the regulation of gene expression will allow us to develop a deeper understanding of the nature and spatiotemporal regulation of peptide activity in the brain under normal conditions and stress.

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CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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