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Low-Molecular Neurotrophin-3 Mimetics with Different Patterns of Postreceptor Signaling Activation Attenuate Differentially Morphine Withdrawal in Rats

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Abstract—The accumulated evidence suggests that varying levels of tyrosine kinase receptor signaling pathway activity may regulate opiate-associated neuroadaptation of noradrenergic system. Neurotrophin-3 (NT-3) interacts with tropomyosin receptor kinases (TRKs), binding mainly to TRKC receptors, which are expressed within noradrenergic neurons in the blue spot (*locus coeruleus*, LC). Considering the difficulties in delivering full-length neurotrophins to the CNS after systemic administration, low-molecular mimetics of loop 4 in NT-3, hexamethylenediamide bis-(N-monosuccinyl-L-asparaginyl-L-asparagine) (GTS-301), and hexamethylenediamide bis-(N-γ-oxybutyryl-L-glutamyl-L-asparagine) (GTS-302), activating TRKC and TRKB receptors, were synthesized. The aim of the study is comparative examination of the effects of NT-3 dipeptide mimetics on the signs of morphine withdrawal in outbred white rats with opiate dependence, as well as investigation of activation of postreceptor signaling pathways by the mimetics. Dipeptides GTS-301 and GTS-302 after acute administration at doses of 0.1, 1.0, and 10.0 mg/kg (i.p., intraperitoneal) had a dose-dependent effect on the specific morphine withdrawal symptoms with the most effective dose being 1.0 mg/kg. Maximum decrease in the total index of morphine withdrawal syndrome for GTS-301 was 31.3% and for GTS-302 – 41.4%. Unlike GTS-301, GTS-302 weakened mechanical allodynia induced by morphine withdrawal, reducing tactile sensitivity. When studying activation of the postreceptor signaling pathways by the NT-3 mimetics in the HT-22 hippocampal cell culture, a different pattern of postreceptor signaling was shown: GTS-302 (10^{-6} M), similar to NT-3, activates all three MAPK/ERK, PI3K/AKT/mTOR, and PLCγ1 pathways, while GTS-301 (10^{-6} M) triggers only MAPK/ERK and PLCγ1 pathways. Thus, the identified features of attenuation of the morphine withdrawal syndrome in the rats under GTS-301 and GTS-302 effects could be associated with different activation pattern of the postreceptor pathways.

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Keywords: neurotrophin-3 mimetics, withdrawal syndrome, morphine, rats, MAPK/ERK, PI3K/AKT, PLCγ1, HT-22

INTRODUCTION

Drug dependence is often associated with painful withdrawal symptoms experienced by the patients when they stop abruptly or reduce rapidly the dose of analgesics. Somatic component of drug dependence is assumed to be associated to a greater degree with mesolimbic areas of the brain, blue spot (*locus coeruleus*, LC)

Abbreviations: AKT, protein kinase B; ERKs, extracellular signal-regulated kinases; LC (Lat. *locus coeruleus*), blue spot; MAPK, mitogen-activated protein kinase; mTOR, rapamycin complex 1; NT-3, neurotrophin-3; PI3K, phosphatidylinositol 3-kinase; PLC, phospholipase C; TRKA, TRKB, TRKC, tropomyosin receptor protein kinases of A, B, and C types, respectively; WS, withdrawal syndrome.

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and periaqueductal gray area [1, 2]. Hyperactivity and disinhibition of brainstem noradrenergic neurons are considered to be the key mechanisms of aversive state and many somatic symptoms that occur during the acute opioid withdrawal [3].

Neurotrophin-3 (NT-3) belongs to the family of neurotrophins and interacts with the tropomyosin-associated protein kinases of A, B, and C types (TRKA, TRKB, and TRKC, respectively), binding preferentially to the TRKC receptors, which are expressed in the noradrenergic LC neurons [4]. It is known that LC expresses high levels of NT-3 and TRKC [5, 6], and NT-3 also increases survival of the LC neurons *in vitro* [7]. Involvement of the TRKC signaling pathways in the opiate-induced adaptation of the catecholaminergic system has been shown in *in vitro* [7] and *in vivo* experiments [8]. Transgenic mice overexpressing TRKC receptors (TgNTRK3) showed an altered frequency of spontaneous excitation of the LC neurons and of the noradrenergic system response to chronic exposure to opiates, which is probably associated with the shifts in regulation of neurotrophins [9]. The accumulated evidence suggests that changing combinations and activity levels of the TRK receptor signaling pathways in the neuronal circuits, which are interconnected with noradrenergic neurons, could regulate and fine-tune the opiate-induced adaptations of noradrenergic system. Considering the difficulties in delivering full-length neurotrophins to the CNS during systemic administration, the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies of the Federal State Budgetary Scientific Institution is developing small-molecule NT-3 mimetics, which were designed using the proprietary technology for constructing dipeptide neurotrophin mimetics [10]. The most exposed part in the NT-3 structure is loop 4, a fragment of which (-Ser91-Glu92-Asn93-Asn94-Lys95-Leu96-), presumably occupies geometrically the most advantageous position for interaction with the receptor. When constructing the GTS-301 mimetic, the dipeptide fragment (-Asn93-Asn94-) of the beta-turn-like region was retained and the preceding Glu92 amino acid residue was replaced with the succinic acid residue. Dimeric structure of neurotrophin was reproduced using the hexamethylenediamine spacer at the C-terminus (hexamethylenediamide bis-(*N*-monosuccinyl-*L*-asparaginyl-*L*-asparagine) [11]. Another NT-3 mimetic, GTS-302, was constructed from the same fragment of the loop 4, but possessed one amino acid residue shifted to the left relative to (-Asn93-Asn94-), with the dipeptide region (-Glu92-Asn93-) retained and the preceding Ser91 amino acid residue replaced with gamma-hydroxybutyric acid; dimerization was also carried out with the hexamethylenediamine (hexamethylenediamide bis-(*N*-γ-oxybutyryl-*L*-glutamyl-*L*-asparagine)) spacer [12]. The aim of the study is comparative examination of the effects of NT-3 loop 4 mimetic on morphine withdrawal symptoms in the animals with formed opiate dependence, as well as investigation of activation of the postreceptor signaling pathways by mimetics.

MATERIALS AND METHODS

***In vivo* experiments.** The experiments were performed with outbred white male rats weighing 230-250 g (n = 112) (FSBSI, Scientific Center of Biomedical Technologies of the Federal Medical Biological Agency, Stolbovaya Branch). Animals were kept in groups of 8 individuals in a cage in the vivarium of the Federal Research Center for Innovator and Emerging Biomedical and Pharmaceutical Technologies (FSBSI) at temperature of 21-23°C and relative humidity of 40-60% with natural illumination and free access to water and briquetted feed for 10 days before testing.

Drugs. Morphine hydrochloride (Minmedbioprom, Chimkentbiopharm, substance) was dissolved in distilled water for injection and administered intraperitoneally (i.p.) at the rate of 0.1 ml/100 g of rat body weight. GTS-301 [melting point was 214-229°C (with decomposition); $[\alpha]_D^{22}$ – 20.2° (c = 1, DMSO)] and GTS-302 [melting point was 173-178°C (with decomposition); $[\alpha]_D^{23}$ – 7.76° (c = 1, DMSO)] [11] were synthesized in the chemistry department of the Federal Research Center of Original and Promising Biomedical and Pharmaceutical Technologies of the Federal State Budgetary Scientific Institution and administered i.p. as a suspension in 1% aqueous Tween 80 at a rate of 0.1 ml/100 g of animal weight.

Method of opiate dependence formation and assessment of somatic symptoms of morphine withdrawal syndrome. Morphine dependence was induced in rats and behavior was evaluated for specific signs of withdrawal syndrome (WS) according to the scheme described earlier [13]. To obtain morphine-dependent animals, the drug was administered to animals in increasing doses (10-20 mg/kg) 2 times a day with an interval of 8 h for 5 days: 1 day – 10 and 10 mg/kg; 2nd day – 10 and 20 mg/kg; 3rd day – 20 and 20 mg/kg; 4th day – 20 and 20 mg/kg; 5th day – 20 mg/kg. On the 5th day of the experiment, 5 h after the last morphine injection, GTS-301 and GTS-302 were administered at doses of 0.1, 1.0, and 10.0 mg/kg (i.p.), and control animals (groups 0.0) were injected with water containing 1% (*v/v*) Tween 80 in an equivalent volume 60 min before testing. Animals were tested for specific signs of morphine WS for 5 min in an open field (illuminated round arena with a diameter of 80 cm) 15 min after administration of naloxone, opiate receptor antagonist (Du Pont De Nemours Int. Switzerland) at a dose of 1.0 mg/kg (i.p.). Specific symptoms of morphine WS

Table 1. Specific symptoms of morphine withdrawal syndrome in rats

Withdrawal signs	Assessment	Withdrawal signs	Assessment
Diarrhea	points 1-5	spasms	+/–
Teeth chattering	number of episodes	stereotypy	+/–
"Wet-dog" shakes	number of episodes	vocalization	+/–
Escape attempts	+/–	chewing	+/–
Urination	+/–	nosebleed	+/–
Postural disturbance	+/–	rhinorrhea	+/–
Piloerection	+/–	head shakes	+/–
Dyspnea	+/–	paw shakes	+/–
Ptosis	+/–	convulsions	+/–

Note. The mean level of WS severity in the control group (group 0.0) was taken as 100%.

were recorded for all groups. Total index (TI) of the WS severity for each animal and average values for the experimental and control groups were calculated based on alternative symptoms (sign presence – 1 point, sign absence – 0 points), a total of 18 WS signs were recorded, each animal had its own set of signs (Table 1).

Changes in tactile sensitivity in rats were assessed using a standard set of 20 nylon monofilaments (von Frey filaments set; Ugo Basile, Italy). During testing, a single filament was applied to the surface of the hind paws of the rat for 1-2 seconds through the mesh surface of the platform, withdrawal or displacement of a paw was considered as a positive reaction. Threshold level of the tactile stimulus was determined by its lowest response value in rats, according to the gram-based scale indicated on each filament. During the study, the testing procedure was carried out twice: on the day preceding the first day of morphine administration and 24 h after morphine withdrawal [13].

***In vitro* experiments.** *Cell cultivation*. Experiments were carried out with HT-22 mouse hippocampus cells (Cell Bank of Utrecht University, Holland). All experiments were performed under strictly sterile conditions. Cells were cultured at 37°C under 5% CO_2 in a DMEM medium (Dulbecco's modified Eagle's medium; HyClone, USA) [14] containing 5% FBS (fetal bovine serum; Gibco, USA) and 2 mM *L*-glutamine (ICN Pharmaceuticals, USA). The culture medium was changed 24 h after seeding and every 2-3 days after that. Reseeding into 75-cm^2 culture flasks (TPP, Switzerland) was performed 3 times a week.

Western blot analysis. Samples were lysed in a 50 mM Tris-HCl (pH 7.5) buffer containing 5 mM EDTA, 1 mM dithiothreitol, 1% (*w/v*) Triton X-100 at 5, 15, 30, 60, and 180 min after adding NT-3 (100 ng/mL) (Sigma, USA), or GTS-301 (L,L) (10^{-6} M), or GTS-302 (L,L) (10^{-6} M). NT-3 (100 ng/mL) was used as a positive control. Non-phosphorylated protein kinase B (AKT), extracellular signal-regulated kinases (ERK1/ERK2), and phospholipase C (PLCγ1) were used as loading controls. Protein level was examined in the cytosolic fraction. Protein concentration in the samples was measured using Lowry method [15]. 75 μg of protein was loaded into run in the gel. Proteins were separated using electrophoresis in a 10% polyacrylamide gel with a Mini-Protean Tetra Cell electrophoretic system (Bio-Rad, USA) [16]. The proteins were then transferred to a PVDF membrane (Santa Cruz, USA) in a Trans-Blot Turbo transfer system (Bio-Rad). All Western blots were preincubated in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl, 1% (*v/v*) Tween-20; pH = 7.5) containing 3% (*w/v*) BSA (Bio-Rad) for 1 h using primary anti-p-AKT1/2/3 antibodies (# PA5-104445; Invitrogen, Thermo Fisher Scientific, USA), anti-p-ERK1/2 (# PA5-37828; Invitrogen, Thermo Fisher Scientific), anti-p-PLCγ1 (#2821S; Cell Signaling Technology, USA) against AKT (# PA5-77855; Invitrogen, Thermo Fisher Scientific), ERK1/ERK2 (#61-7400; Invitrogen, Thermo Fisher Scientific), and PLCγ1 (#2822S; Cell Signaling Technology) at a dilution of 1 : 1000 overnight at 4°C. Next, after washing in a TBS-T buffer containing 0.05% (*w/v*) BSA, the membranes were incubated in the presence of secondary goat anti-rabbit IgG antibodies (# 31460; Thermo Fisher Scientific) conjugated with horseradish peroxidase (at 1 : 1000 dilution) for 1 h. Detection of proteins was performed after washing from secondary antibodies in a buffer with ECL reagents (# 32106; Pierce ECL WB Substrate (Thermo Fisher Scientific)) using Alliance 4.7 gel documentation system (UVITEC, UK). Densitometry of the images was performed using the GIMP2 software.

Statistical analysis of the results of *in vivo* experiments was performed by descriptive statistics, one-way analysis of variance (ANOVA), repeated measures ANOVA for related groups; the results of *in vitro* experiments were analyzed using Mann–Whitney U test and Statistica 10 software.

RESULTS

The obtained results show that GTS-301 and GTS-302 (Fig. 1) exhibit a dose-dependent effect on the specific signs of morphine withdrawal in rats.

Table 2. Effect of GTS-301 on some symptoms of morphine withdrawal

Behavioral signs	GTS-301, doses, mg/kg			
	0.0	0.1	1.0	10.0
Diarrhea	4.13 ± 0.30	2.50 ± 0.60*	2.50 ± 0.70*	1.87 ± 0.77*
Postural disturbance	0.87 ± 0.00	1.00 ± 0.00	0.75 ± 0.16	0.38 ± 0.18*
Ptosis	0.38 ± 0.18	0.00 ± 0.00*	0.25 ± 0.16	0.25 ± 0.16
Teeth chattering	1.13 ± 0.78	1.75 ± 0.88	0.13 ± 0.12*	0.50 ± 0.50
“Wet-dog” shakes	2.50 ± 1.12	1.00 ± 0.42	1.00 ± 0.53	0.75 ± 0.52
Vocalization	0.50 ± 0.19	0.62 ± 0.18	0.13 ± 0.12*	0.75 ± 0.16
Head shakes	0.50 ± 0.19	0.38 ± 0.18	0.25 ± 0.16	0.25 ± 0.16
Paw shakes	0.75 ± 0.16	0.25 ± 0.16*	0.38 ± 0.18	0.13 ± 0.13**
Rhinorrhea	1.00 ± 0.00	0.88 ± 0.12	0.38 ± 0.18**	0.38 ± 0.18**

Note. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ – significant difference from the control (group “0.0”); each group consists of 8 animals; the data are presented as a mean ± standard error of the mean (M ± SEM).

Table 3. Effect of GTS-302 on some symptoms of morphine withdrawal

Behavioral signs	GTS-302, doses, mg/kg			
	0.0	0.1	1.0	10.0
Diarrhea	3.10 ± 0.71	3.9 ± 0.22	1.60 ± 0.70	1.50 ± 0.59
Postural disturbance	1.00 ± 0.00	0.25 ± 0.16***	0.25 ± 0.16***	0.38 ± 0.18**
Ptosis	0.38 ± 0.18	0.37 ± 0.18	0.25 ± 0.16	0.13 ± 0.11
Teeth chattering	1.88 ± 0.81	0.25 ± 0.25*	0.50 ± 0.50	0.13 ± 0.13*
“Wet-dog” shakes	0.70 ± 0.15	0.25 ± 0.25	0.0 ± 0.0*	0.0 ± 0.0*
Vocalization	0.65 ± 0.17	0.63 ± 0.18	0.13 ± 0.11*	0.49 ± 0.17
Head shakes	0.75 ± 0.16	0.61 ± 0.18	0.13 ± 0.13**	0.50 ± 0.19
Paw shakes	0.75 ± 0.16	0.25 ± 0.16*	0.38 ± 0.18	0.25 ± 0.16*
Rhinorrhea	0.62 ± 0.18	0.38 ± 0.18	0.50 ± 0.19	0.88 ± 0.12

Note. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ – significant difference from the control (group “0.0”); each group consists of 8 animals; the data are presented as a mean ± standard error of the mean (M ± SEM).

Decreases in the WS total index (TI) in the group “GTS-301; 0.1 mg/kg” was 12% ($F_{(1.14)} = 4.66$; $p < 0.05$); in the group “GTS-301; 1.0 mg/kg” it amounted to 31.1% ($F_{(1.14)} = 9.14$; $p < 0.01$); in the group “GTS-301; 10.0 mg/kg” – 26.5% ($F_{(1.14)} = 21.44$; $p < 0.001$), compared to the 0.0 control group. WS TI drop in the group “GTS-302; 0.1 mg/kg” was 26.9% ($F_{(1.14)} = 10.19$; $p < 0.01$); in the group “GTS-302; 1.0 mg/kg” it amounted to 41.4% ($F_{(1.14)} = 12.49$; $p < 0.01$), in the group “GTS-302; 10.0 mg/kg” – 36.5% ($F_{(1.14)} = 25.64$; $p < 0.001$). The most pronounced effect of NT-3 mimetics was demonstrated at the dose of 1.0 mg/kg. When evaluating correction of the individual signs of morphine WS, it is necessary to note that reduction in the diarrhea symptoms, as well as elimination of the postural disturbance, ptosis, paw shaking,

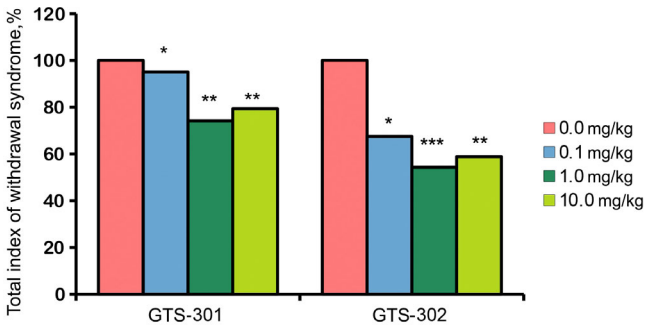

Fig. 1. Effect of the NT-3 loop 4 mimetics on the total index of naloxone-induced morphine withdrawal syndrome in the outbred male rats (as % of control). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ – significant difference from the control (group "0.0"); each group consists of 8 animals; the data are presented as a mean ± standard error of the mean (M ± SEM).

vocalization, and rhinorrhea were observed with GTS-301 administered at all doses examined (Table 2). For GTS-302, decrease in the severity or complete elimination of the signs, such as diarrhea, postural disturbance, teeth chattering, "wet-dog" shakes, vocalization, head, and paw shakes was recorded (Table 3).

In total, GTS-301 and GTS-302 attenuated 7 of the 18 analyzed signs of morphine WS, along with the greatest decrease in the total WS index (41.1%) observed in the GTS-302 group at a dose of 1.0 mg/kg (Table 3).

The main clinical symptoms of opioid withdrawal syndrome, in addition to vegetative disorders (yawning, rhinorrhea, watery eyes, diarrhea, pupillary dilation, piloerection, etc.), are often accompanied by the pain syndrome and hyperesthesia. The last ones are considered to be important diagnostic symptoms belonging to the category of disorders most severely experienced by patients. In additional series of experiments the effect of GTS-301 and GTS-302 on the thresholds of tactile sensitivity under conditions of morphine withdrawal in the dependent rats was examined. Pre-testing of the animals using von Frey filaments showed that the individual rats did not differ statistically significantly in the initial level of sensitivity to the mechanical tactile action: for GTS-301 it was $F_{(3.28)} = 0.957$, $p = 0.426$; for GTS-302 – $F_{(3.28)} = 1.159$, $p = 0.342$. During the morphine withdrawal modeling with GTS-301 a significant decrease in the sensitivity threshold was observed in the control and experimental groups compared to the initial values ($F_{(3.28)} = 4.078$; $p = 0.0002$), which is consistent with the data published previously [13]. However, GTS-301 at the studied single-administration doses did not affect mechanical allodynia formed during the morphine withdrawal. When evaluating efficacy of GTS-302, it was shown that, with the morphine withdrawal in the control and experimental groups, there was a significant decrease in the sensitivity threshold compared to the baseline level (pre-test) ($F_{(3.28)} = 5.125$; $p = 0.0059$). In contrast to GTS-301, GTS-302 increased dose-dependently the threshold of response to acute administration at all studied doses with the maximum effect observed for the dose of 1.0 mg/kg, at which there was a 3-fold increase in the recorded parameter compared to the control group ($F_{(1.14)} = 17.42$; $p = 0.009$). Nevertheless, in all experimental groups the level of tactile sensitivity remained lower than during the preliminary testing (Table 4).

Pharmacological advantages of GTS-302 over GTS-301 revealed by the *in vivo* modeling of opioid dependence may be due to peculiarities of activation of the postreceptor signaling pathways. Therefore, activation of the signaling ways of PI3K/AKT, MAPK/ERK, PLCγ1 by GTS-301 and GTS-302 mimetics in the HT-22 hippocampal cell culture was investigated. GTS-301 (10^{-6} M) and GTS-302 (10^{-6} M) were added to the culture medium as a sterile solution at effective concentrations established in the experiments on neuroprotective effect of these compounds on the HT-22 culture line of hippocampal cells [12, 17]. NT-3 (10^{-9} M) was used as a positive control. Cells were lysed at 5, 15, 30, 60, and 180 min after peptide adding.

Despite the fact that we have previously shown activation of the TRKB and TRKC receptors by both mimetics, a different pattern of postreceptor signaling in them was revealed: GTS-302 (10^{-6} M), similar to NT-3, activates all three MAPK/ERK, PI3K/AKT/mTOR, and PLCγ1 pathways, while GTS-301 (10^{-6} M) triggers only the MAPK/ERK and PLCγ1 signaling pathways. As can be seen from Figs. 2 and 3, a significant increase

Table 4. Effect of GTS-302 on thresholds of tactile sensitivity during the foot shock-induced stress in rats

Groups	Doses, mg/kg			
	0.0	0.1	1.0	10.0
Pre-test	1.35 ± 0.15	1.23 ± 0.14	1.05 ± 0.14	1.05 ± 0.09
Test	0.040 ± 0.005***	0.060 ± 0.005***,#	0.12 ± 0.02***,###	0.08 ± 0.01***,##

Note. Each group included 8 animals; *** $p < 0.001$ is a significant difference from the Pre-test group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ are significant differences from the control group. Data are presented in grams as a mean ± standard error of the mean (M ± SEM).

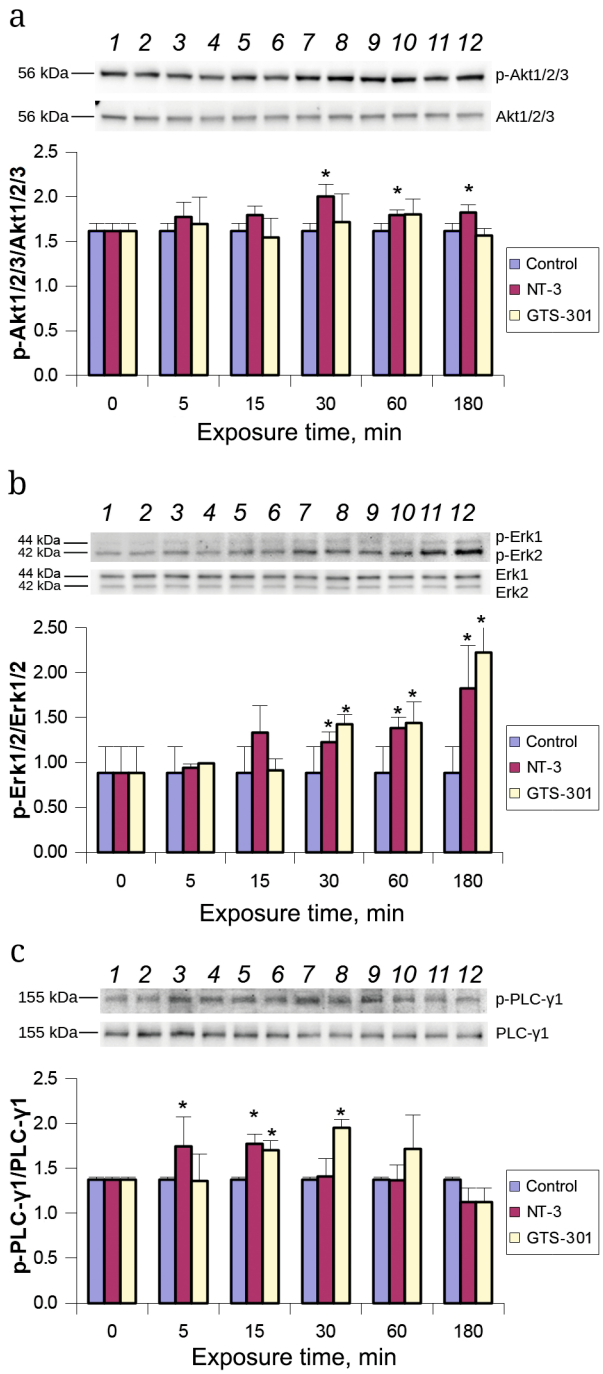

Fig. 2. Phosphorylation of AKT1/2/3 (a), ERK1/2 (b), and PLC-γ1 (c) after NT-3 and GTS-301 (10^{-6} M) adding of the HT-22 mouse hippocampal cell culture (original Western blots and densitometry results). Lanes: *1*, *6*) Control; *2*) GTS-301, 5 min; *3*) NT-3, 5 min; *4*) GTS-301, 15 min; *5*) NT-3, 15 min; *7*) GTS-301, 30 min; *8*) NT-3, 30 min; *9*) GTS-301, 60 min; *10*) NT-3, 60 min; *11*) GTS-301, 180 min; *12*) NT-3, 180 min. Data are presented as a mean ± standard deviation (M ± SD) of three independent experiments; * $p < 0.05$ is a significant difference from the control.

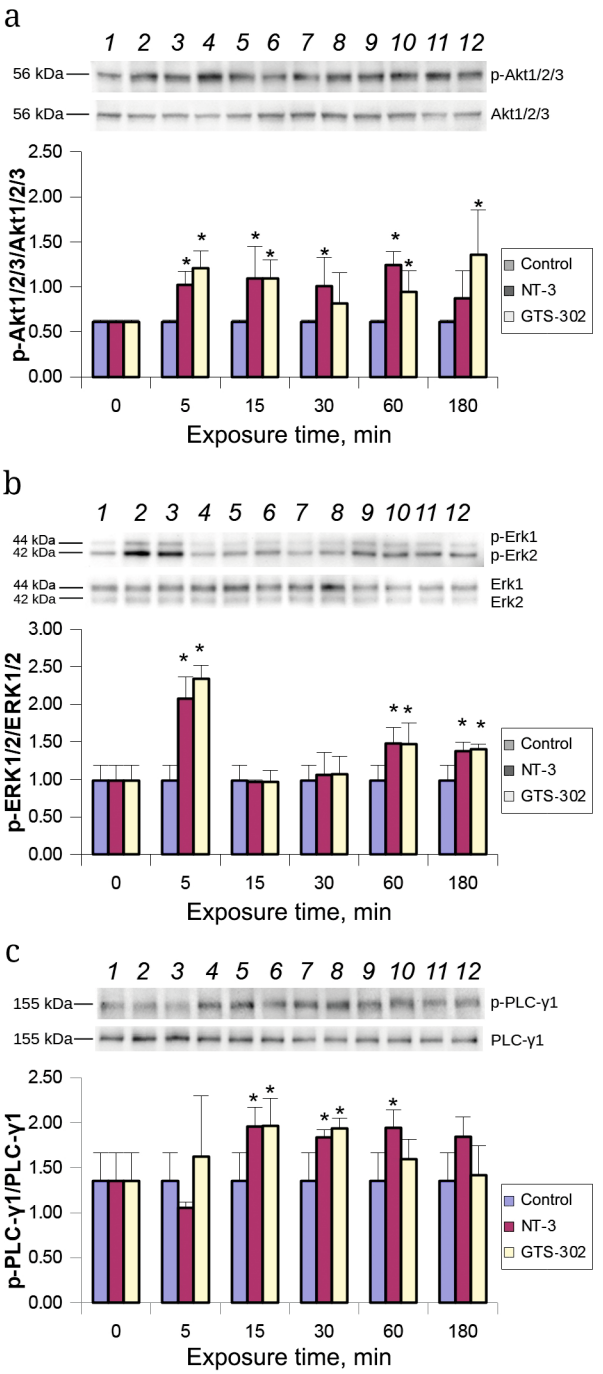

Fig. 3. Phosphorylation of AKT1/2/3 (a), ERK1/2 (b), PLC-γ1 (c) after NT-3 and GTS-302 (10^{-6} M) adding of the HT-22 mouse hippocampal cell culture (original Western blots and densitometry results). Lanes: *1*, *6*) Control; *2*) GTS-302, 5 min; *3*) NT-3, 5 min; *4*) GTS-302, 15 min; *5*) NT-3, 15 min; *7*) GTS-302, 30 min; *8*) NT-3, 30 min; *9*) GTS-302, 60 min; *10*) NT-3, 60 min; *11*) GTS-302, 180 min; *12*) NT-3, 180 min. Data are presented as a mean ± standard deviation (M ± SD) of three independent experiments; * $p < 0.05$ is a significant difference from the control.

in phosphorylation of AKT kinases was observed at 5, 15, 60, and 180 min after the GTS-302 (10^{-6} M) adding, as well as at 5, 15, 30, and 60 min after NT-3 adding; a significant increase in phosphorylation of Erk kinases was observed 5, 60, and 180 min after the GTS-302 adding; and 30, 60, and 180 min after the GTS-301

adding. The PLCγ1 signaling pathway was activated 15 and 30 min after adding of both GTS-302 and GTS-301.

DISCUSSION

When modeling morphine dependence in rats, naloxone at low doses triggers general negative affective state (motivational component), while at high doses naloxone induces somatic signs of withdrawal [18], and that was demonstrated in our work: development of the somatic-vegetative disturbances typical to opioid abstinence syndrome was observed in the rats 15 min after naloxone administration at a dose of 1 mg/kg. Previously, the *in vitro* experiments showed that treatment of the LC neurons with morphine led to 20%-decrease in the norepinephrine uptake and a 12%-decrease in the number of tyrosine hydroxylase-immunoreactive (TH+) cells. At the same time, increase in the norepinephrine uptake and number of the TH+ cells was noted in the cell culture supplemented with NT-3 [7]. In the *ex vivo* experiments, infusion of NT-3 directly into the ventral tegmental area in the rats prevented biochemical changes in the mesolimbic dopaminergic system that occur during prolonged action of morphine [19]. Despite the change in the NT-3 and TRKC mRNA levels in several brain areas during opiate withdrawal, there is very little evidence for the role of NT-3 in the development of opioid withdrawal *in vivo*. In mice with conditional ablation of NT-3 in the brain, somatic symptoms of opiate withdrawal were attenuated, which was accompanied with the reduced expression of tyrosine hydroxylase in the LC [20]. Opiate withdrawal led to the significant decrease in the levels of NT-3 mRNA in the LC 20 and 70 h after morphine withdrawal, while the TRKC mRNA levels were also below the control values [4]. It cannot be ruled out that the NT-3 mimetics could have certain advantages, when used as agents for rapid pharmacotherapy of the opioid withdrawal syndrome.

In our work for the first time it was shown *in vivo* that the NT-3 dipeptide mimetics after acute systemic administration attenuate dramatically somatic signs of the naloxone-induced morphine withdrawal in rats, which is consistent with the idea of participation of neurotrophins in the mechanisms associated with formation of opiate dependence [21]. It was first shown in 1995 by *in situ* hybridization that the individual CNS neurons can express mRNA for high-affinity NT-3, TRKB, and TRKC receptors [22]. The ability of GTS-301 and GTS-302 to activate, same as full-length neurotrophin, two types of receptors (TRKB and TRKC) in the HT-22 hippocampal cells during oxidative stress modeling [17, 12] suggests high level of regulatory activity of the small-molecule NT-3 mimetics under pathological conditions that occur during withdrawal of psychoactive substances, including analgesics. GTS-301 and GTS-302 compounds attenuated significantly 7 of 18 signs of the morphine withdrawal. However, with acute systemic administration, GTS-302 caused not only the greatest decrease in the total index of withdrawal syndrome, but also weakened in a dose-dependent manner mechanical allodynia in the rats with drug dependence, which does not rule out existence of the structure-property relationship.

In this work, for the first time, differences in the activation patterns of postreceptor pathways by the NT-3 mimetics during interaction with TRK receptors have been demonstrated. One of the peculiarities of the GTS-302 action, similar to NT-3, is the ability to activate three intracellular cascades, while GTS-301 did not show the ability to activate the PI3K/AKT/mTOR signaling pathway. It is known that PI3K/AKT/mTOR inhibits the rapamycin complex 1 (mTOR), change in functioning of which has been established in substance abuse [23]. In the work of Zhu et al. [24] published in 2021, a possible role of the PI3K/AKT/mTOR signaling pathway in heroin addiction was revealed, since the authors showed involvement of the PI3K/AKT/mTOR phosphorylation in the nucleus accumbens in formation of the heroin-seeking behavior in rats. Gene expression analysis demonstrated statistically significant increase in the mTOR expression with the decreased levels of AKT as well as PI3K (phosphatidylinositol 3-kinase) expressions among the heroin-dependent patients compared to the control group ($p < 0.001$), with insufficient levels of PI3K and AKT phosphorylation and, in some cases, overexpression of mTOR ($p < 0.001$) [25].

It should be noted that neuroprotective properties aimed at attenuation of mechanical allodynia in hyperglycemia can be performed, among other things, through phosphorylation of AKT, as well as of its downstream target factor mTOR, that is, increase in viability of the Schwann cells seems to be mediated through the PI3K/AKT/mTOR signaling pathway [26]. However, in other work, the nociceptin opioid peptide receptor (NOP) antagonist, also known as FQ nociceptin/orphanin receptor (N/OFQ), alleviated mechanical allodynia associated with the chemotherapy-induced neuropathic pain by inhibiting paclitaxel-enhanced expression of PI3K and phospho-AKT in the dorsal root ganglion [27] Overall, the role of PI3K, in formation of individual symptoms of opiate dependence manifested as central sensitization after exposure to harmful stimuli remains completely unclear. However, the ability of GTS-302 to not only stop main manifestations of the morphine withdrawal syndrome, but also to weaken noticeably mechanical allodynia in the rats with formed drug dependence suggests that these effects of NT-3 mimetic may be due to involvement of the PI3K/AKT/mTOR signaling

pathway activation in the processes, including in the processes of central sensitization. This fact requires further investigation.

CONCLUSION

For the first time, the ability of small-molecule neurotrophin-3 mimetics to reduce morphine withdrawal syndrome in the opiate-dependent rats was experimentally proven, while the pharmacologically more significant effect of mimetic was observed that involves activation of all three main postreceptor signaling cascades (PI3K/AKT, MAPK/ERK, and PLCγ1) same as in the case of full-length neurotrophin.

Contributions. L.G.K. supervised the study, discussed the results of the study, prepared the manuscript; M.A.K. conducted the *in vivo* experiments, performed statistical analysis of the data; S.V.N. conducted the *in vitro* experiments, edited the manuscript and images; I.O.L. conducted the *in vitro* experiments, biochemical analysis; T.A.A. performed statistical analysis of the data and discussed the obtained results, prepared the manuscript; T.A.G. developed the concept and discussed results of the study.

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Ethics declarations. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed and were approved by the Biomedical Ethics Committee of the V. V. Zakusov Research Institute of Pharmacology of FSBSI (Protocol No. 01 of February 2, 2023). The authors of this work declare that they have no conflict of interests.

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