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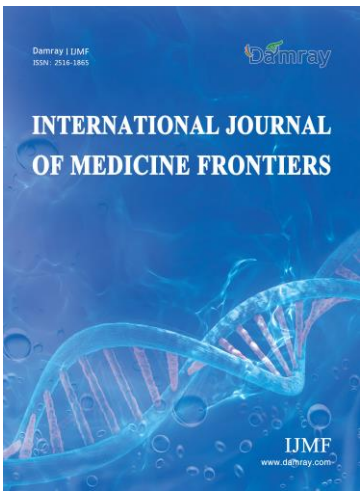

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The Dipeptide Mimetic of the Neurotrophin-3 Loop 4, Compound GTS-302, Exhibits Antidepressant-Like Activity in a Mouse Social Defeat Stress Model

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Abstract

Experimental and clinical data indicate the involvement of neurotrophin-3 (NT-3) in the pathogenesis of depression. A dimeric dipeptide mimetic of the NT-3 loop 4, bis-(N-gamma-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide (GTS-302), demonstrating antidepressant-like activity in the forced swimming test in mice (0.5-10 mg/kg, ip), was created at the Research Zakusov Institute of Pharmacology. The aim of this study was to investigate the antidepressant-like activity of the dipeptide GTS-302 in an experimental model of depression. Male C57Bl/6 mice were exposed to a 10-day social defeat stress. They received daily ip injections of the dipeptide GTS-302 at a dose of 1 mg/kg for the entire duration of the stress period. Following the stress, the sucrose preference test was conducted to assess anhedonia. Hippocampal tissue was then collected for the analysis of synaptic proteins (synaptophysin, PSD-95) and BDNF levels using Western blot analysis. In stressed mice, sucrose preference decreased by 16% compared to non-stressed controls, accompanied by an 18% reduction in hippocampal BDNF levels. Treatment with compound GTS-302 nearly completely counteracted these impairments. Therefore, the dipeptide mimetic of NT-3, GTS-302, holds promise as a potential antidepressant.

Keywords

NT-3, dimeric dipeptide mimetic, GTS-302, depression, social defeat stress, anhedonia, BDNF

1. Introduction

Depression is the most prevalent mental disorder worldwide. According to contemporary understanding, depression is associated with impaired neuroplasticity in corticolimbic brain areas, such as the prefrontal cortex, hippocampus, and amygdala, which are involved in mood regulation [1].

Literary data indicate the role of neurotrophin-3 (NT-3) in the neurobiological processes underlying the maintenance of emotional states. NT-3 and its specific TrkC receptors are widely distributed in the cortex and limbic

system, where they are involved in regulating synaptic plasticity [2-5]. NT-3 stimulates dopaminergic and serotonergic neurotransmission [6, 7], participates in the maintenance of hippocampal neurogenesis [4], and stimulates the synthesis and release of BDNF [8, 9]—another neurotrophin whose deficiency is associated with depression [10]. It is known that NT-3, in addition to TrkC receptors, binds with lower affinity to TrkB receptors specific to BDNF [11]. This interaction likely leads to the activation of molecular mechanisms underlying the antidepressant-like effects of BDNF.

In clinical studies, it has been established that the serum levels of NT-3 are reduced in depression, displaying a correlation with the disease's severity [12]. Postmortem examinations have unveiled decreased NT-3 levels in the brain cortex of untreated depressed patients in comparison to unaffected individuals [13]. Treatment with antidepressants resulted in the restoration of NT-3 levels [13]. Antidepressant-like activity of NT-3 has been observed in in vivo studies following its intracerebral administration [14].

Thus, NT-3, along with BDNF, possesses significant therapeutic potential for treating depression. The clinical application of full-size neurotrophins is limited by their low bioavailability. Addressing this issue is possible through the creation of pharmacologically suitable low-molecular-weight mimetics.

We have designed a dimeric dipeptide NT-3 loop 4 mimetic - bis-(N-gamma-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide (GTS-302) [15], based on the original hypothesis [16] on the crucial role of the most surface-exposed, often central, dipeptide fragments of β-turns within the loop-like structures of neurotrophins in receptor. Like other members of the neurotrophin family, NT-3 is a symmetrical homodimer with monomeric units containing seven β-strands forming three antiparallel β-sheets. The most exposed loop 4, specifically the fragment Ser^{91}–Glu^{92}–Asn^{93}–Asn^{94}–Lys^{95}, features turning regions strategically positioned for optimal receptor interaction.

In the design of compound GTS-302 (see Fig. 1), we preserved the - Glu^{92}-Asn^{93}- dipeptide fragment, substituting the preceding neighbor residue Ser^{91} with a bioisostere, namely a gamma-hydroxybutyric acid residue. The dimeric structure of neurotrophin was imitated using a hexamethylenediamine spacer at the C-terminus. The GTS-302 dipeptide was synthesized by classical peptide synthesis in solution using the strategy of Z/tBu protecting groups by the method of activated pentafluorophenyl and succinimide esters of L-amino acids [15].

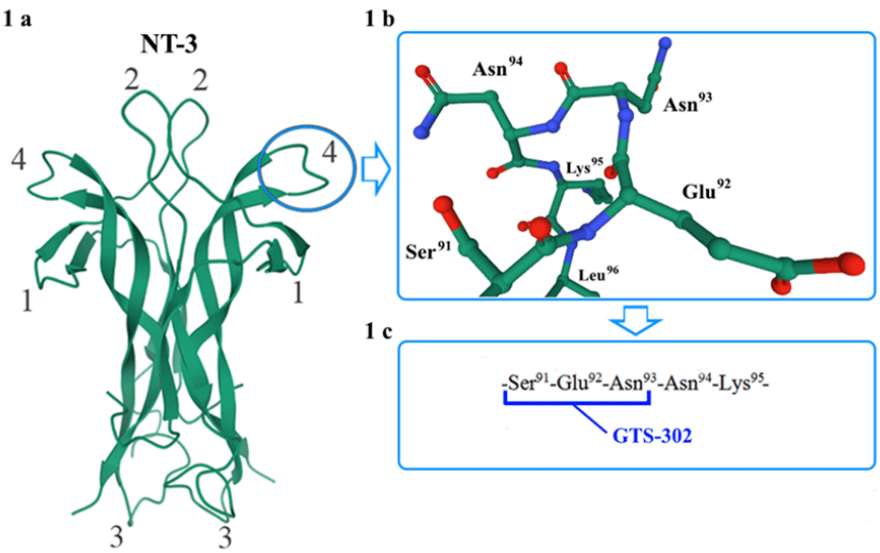

Figure 1. Design of NT-3 loop 4 dipeptide mimetic GTS-302. 1a - general view of NT-3 crystal structure (pdb ID: 1nt3), 1b – surface-exposed part of the loop 4, 1c - scheme of selection of the amino acids residues for design of GTS-302.

In vitro studies revealed that GTS-302, similar to full-length NT-3, activates TrkC and, to a lesser extent, TrkB receptors [15]. This dipeptide, administered intraperitoneally (i.p.) at doses ranging from 0.5 to 10 mg/kg, displayed significant antidepressant-like activity in the forced swimming test in mice, reducing immobility time by 20-35% [17]. Notably, the reference drug amitriptyline (at 10 mg/kg) reduced immobility time by 40% in the same test.

The aim of this study was to investigate the antidepressant-like activity of the dipeptide GTS-302 in a mouse

model of depression induced by chronic social defeat stress. Compared to the forced swimming test, commonly used for the preliminary screening of potential antidepressants, this model aligns more closely with human pathology, since stress of social etiology is one of the most common factors contributing to the development of depression. In this model, we investigated the impact of GTS-302 following chronic intraperitoneal administration on anhedonia, as well as on the levels of synaptic markers (synaptophysin, PSD-95), and BDNF in the hippocampus of stressed mice.

2. Materials and methods

Chemicals. Dipeptide GTS-302 (bis-(N-gamma-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide) was synthesized as described previously [15]. GTS-302 is a white crystalline substance. Mp 173-178°C (decomp); $[\alpha]_D^{23}$ –7.76° (c 1, DMSO). HRMS $[M + Na]^+$: calcd. for $C_{31}H_{54}N_8O_{14}Na$ 797.36, found 797.3647.

Anti-synaptophysin antibodies were obtained from BD Bioscience (USA); anti-BDNF and anti-PSD-95 antibodies were sourced from ThermoFisher (USA); and anti-β-actin antibodies were acquired from Abcam (UK). Secondary antibodies targeting rabbit or mouse IgG were purchased from ThermoFisher (USA).

Animals. The study was conducted on 60 male C57Bl/6 mice weighing 20-22 g, obtained from the Branch "Andreevka" of the Federal State Budgetary Institution of Science "Scientific Center for Biomedical Technologies" of the Federal Medical and Biological Agency (Moscow region, Russia). The animals were provided with ad libitum access to food and water and were maintained under natural light-dark cycles. The experiments involving animals were conducted in accordance with international regulations (Directive 2010/63/EU of the European Parliament and of the Council of the European Union of September 22, 2010, on the protection of animals used for scientific purposes). The conduct of experiments was approved by the Biomedical Ethics Committee of the V.V. Zakusov Research Institute of Pharmacology (protocol № 2, January 30, 2023).

Study design. The mice were randomly divided into 4 groups, each consisting of 10 animals. Two groups of mice ("Stress" and "Stress+GTS-302") were subjected to social defeat stress, where both aggressors and victims were male C57Bl/6 mice. Mice from the "Stress+GTS-302" group received GTS-302 ip at a dose of 1 mg/kg in the form of an aqueous solution daily for 10 days during the period of stress induction. The dose of GTS-302 was selected based on previous experiments as the most active one [17]. Mice from the "Stress" group received distilled water in the same regimen. The other two groups of mice ("Control" and "Control+GTS-302") were not subjected to stress. Mice in these groups also received either distilled water or GTS-302 at a dose of 1 mg/kg for 10 days. The ip administration volume was 10 mL per 1 kg of body weight. Twenty-four hours after the completion of the stress and the last administration of the compounds, the sucrose preference test was conducted to assess anhedonia. Subsequently, hippocampal tissue was collected for the further determination of synaptic proteins (synaptophysin, PSD-95) and BDNF content using Western blot analysis. The study design is presented in Figure 2.

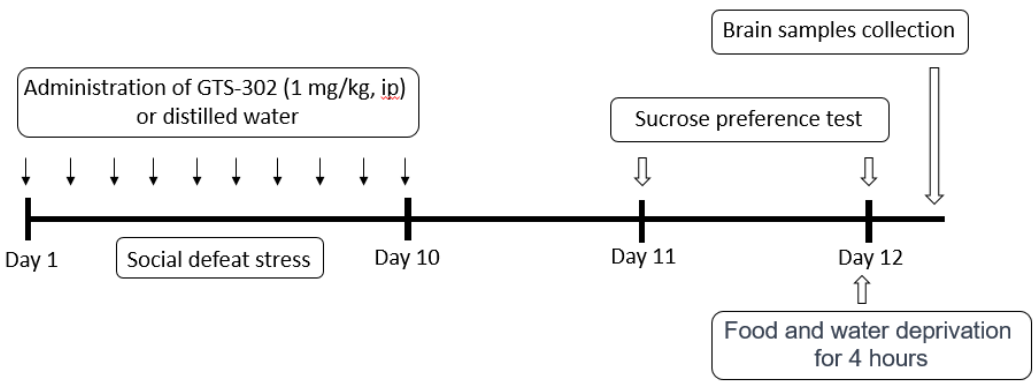

Figure 2. Study design.

Social defeat stress. Depressive-like state in mice was induced using chronic social defeat stress, caused by repeated experiences of defeat in daily confrontations between males, as described [18,19]. Animals were placed in pairs inside cages measuring 28x14x10 cm, separated by a perforated transparent plastic partition, allowing visual

and sensory contact while preventing physical interaction. From the third day onward and daily at the same time for 10 days, the partition was removed for 10 minutes, allowing physical contact between the animals. This resulted in the establishment of roles as "aggressor" and "victim" among the mice. The victim was identified as a defending, non-aggressive male, assuming a characteristic "submissive" posture, and it was marked for further identification purposes. Every 2-3 days, the "victims" were rotated by placing them in cages with unfamiliar "aggressors", thereby intensifying the impact of the stressor. In cases of excessive aggression towards the victim (persistent biting even after the "victim" displayed a submissive posture), the partition was restored to its place before the completion of the 10-minute interval, thus terminating the confrontation. Since not all pairs of mice engaged in active confrontations, not all "victim" mice were subjected to aggressor attacks daily, despite the rotation of the "victims." Only those "victims" that experienced daily social stress were included in the further study. As a result, there were 7 mice in each of the "Stress" and "Stress+GTS-302" groups.

The sucrose preference test [20] was used to assess anhedonia, one of the key symptoms of depressive-like state [21]. Mice were individually housed and provided with simultaneous free access to two bottles for 18 hours. One bottle contained a 1% sucrose solution, and the other contained water (Test 1). Subsequently, the mice underwent food and water deprivation for 4 hours, after which they were given access to two bottles again, with water and the sucrose solution, switching their positions (Test 2). Consumption of water and the sucrose solution was assessed by weighing the bottles before and after the test. Sucrose preference was calculated using the following formula: (M suc)/(M suc + M water)*100%, where M suc—consumed sucrose solution, M water—consumed water mass.

Sample collection. After completion of the behavioral testing, the mice were euthanized using cervical dislocation and decapitated. The brains were extracted, and the hippocampi were isolated at 0-4°C, then frozen in liquid nitrogen and stored at -80°C.

Western blotting. After thawing, brain tissue samples from animals of the same group were pooled into samples, ensuring that the number of samples was not less than three. The samples were then homogenized at 4°C using a glass homogenizer with a lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100), pH 7.5, containing protease inhibitors (pepstatin, bestatin, leupeptin, and aprotinin), at a tissue-to-buffer ratio of 1:5 (mass/volume). Then, the samples were incubated for 20 minutes at 4°C and centrifuged for 30 minutes at 14.000 rpm at the same temperature. The protein concentration in the supernatant was determined using the Folin-Lowry method [22]. The supernatant proteins were separated on a 12% polyacrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane by electroelution. After transferring the proteins from the gel to the membrane, it was cut according to the molecular weight of the studied proteins. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with 1% Tween 20 (TBST) for 1.5 hours. Subsequently, the membranes were incubated overnight at 4-5°C with antibodies against synaptophysin (diluted 1:500), PSD-95 (diluted 1:500), BDNF (diluted 1:1000), and β-actin (diluted 1:5000). Excess antibodies were washed off with TBST containing 0.1% (w/v) non-fat dry milk for 1.5 hours. Subsequently, the membranes were incubated for 2 hours at 4-5°C with secondary antibodies against rabbit IgG or mouse IgG conjugated with horseradish peroxidase. Protein detection was performed after washing off the secondary antibodies in TBST buffer using enhanced chemiluminescence reagents (ECL reagents) with the Alliance Q4 gel documentation system. Densitometry analysis of the obtained images was carried out using the GIMP2 software. Since the blots were part of the same membrane, they used the same internal control (β-actin).

Statistical analysis was performed using the GraphPad Prism 8.0 software (GraphPad Software, USA). Behavioral data were assessed for normality using the Shapiro-Wilk test. As the data distribution in the samples was found to be normal, intergroup comparisons were conducted using parametric two-way analysis of variance (two-way ANOVA) followed by multiple post hoc comparisons using the Sidak test. For biochemical data, intergroup comparisons were conducted using the Mann-Whitney U test. Differences were considered statistically significant at $p<0.05$. Data are presented as means and standard errors of the mean.

3. Results

3.1 The dipeptide GTS-302 prevents the development of anhedonia

No intergroup differences were observed in Test 1 of sucrose preference, conducted without prior food and water deprivation, (Table 1).

Table 1. Sucrose solution preference without prior food and water deprivation

Experimental group	Number of animals	Sucrose solution preference, %
Control	10	54.1±1.9
GTS-302	7	52.0±1.5
Stress	10	55.6±3.0
Stress + GTS-302	7	59.8±2.0

Data are presented as means and standard errors of the mean.

In Test 2, conducted after a 4-hour period of food and water deprivation, the consumption of sucrose solution by mice in the "Stress" group was statistically significantly reduced by 16% compared to the "Control" group (Table 2). This indicates that stress induced the development of anhedonia, which is one of the key indicators of depressive-like state [21].

In stressed mice receiving GTS-302, the preference for the sucrose solution was statistically significantly increased compared to the "Stress" group, reaching levels higher than in the control group (Table 2). Dipeptide GTS-302 also significantly increased sucrose solution consumption in animals not subjected to stress (Table 2).

Table 2. Sucrose solution preference after preceding food and water deprivation

Experimental group	Number of animals	Sucrose solution preference, %
Control	10	67.4±2.4
GTS-302	7	78.6±1.3**
Stress	10	56.9±5.0*
Stress + GTS-302	7	85.3±2.0####

Notes. The data are presented as means and standard errors of the mean. * - p<0.05, ** - p<0.01 compared to the "Control" group; #### - p<0.0001 compared to the "Stress" group (two-way ANOVA, Sidak's post-hoc test).

Therefore, GTS-302 completely counteracted the development of anhedonia induced by chronic social defeat stress.

3.2 Dipeptide GTS-302 prevents the reduction of BDNF levels in the hippocampus

No intergroup differences were detected in the hippocampal levels of synaptic proteins synaptophysin and PSD-95 (Figs. 3, 4).

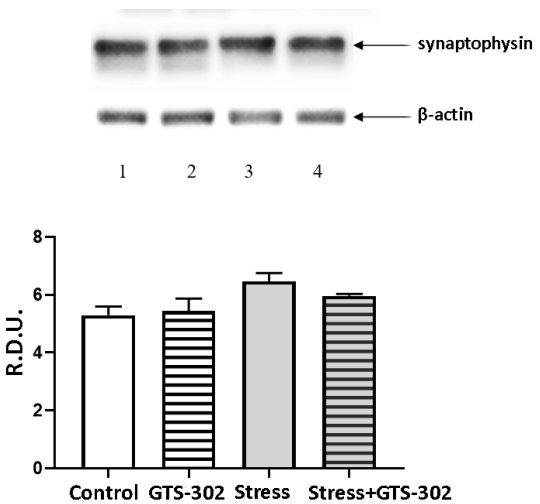

Figure 3. Synaptophysin levels in the hippocampus. Western blot analysis data. R.D.U—relative densitometric units. Lanes: 1—Control, 2—GTS-302, 3—Stress, 4—Stress+GTS-302. Data are presented as means ± standard errors of the mean.

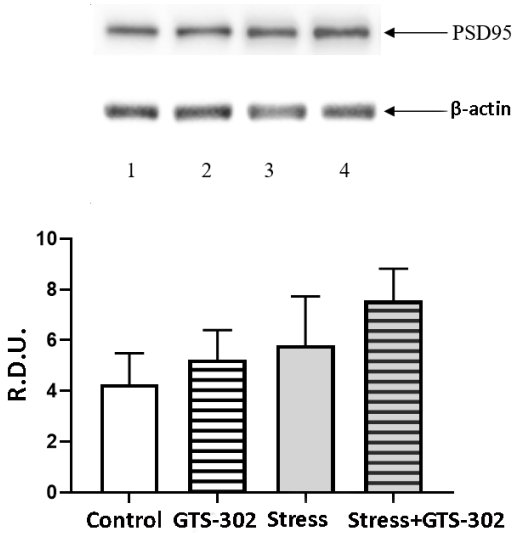

Figure 4. PSD-95 levels in the hippocampus. Western blot analysis data. R.D.U—relative densitometric units. Lanes: 1—Control, 2—GTS-302, 3—Stress, 4—Stress+GTS-302. Data are presented as means ± standard errors of the mean.

At the same time, the level of BDNF was significantly reduced in the hippocampus of stressed mice (R.D.U. = 5.4±0.1), showing an 18% decrease compared to the control group (R.D.U. = 6.6±0.4) (Fig. 5). In stressed animals treated with GTS-302, the BDNF content was significantly increased compared to the "Stress" group (R.D.U. = 6.4±0.4), nearly reaching the levels observed in control group (Fig. 5).

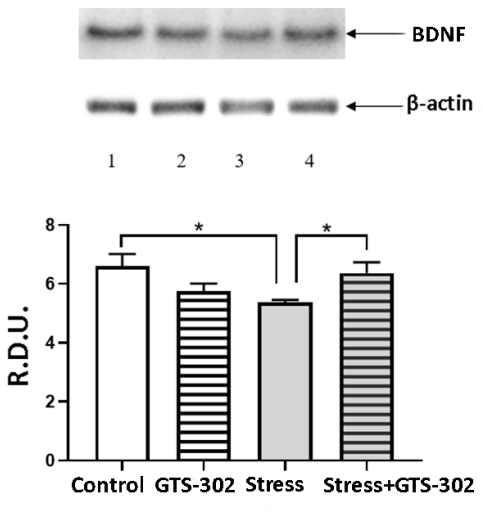

Figure 5. The dipeptide GTS-302 prevents the reduction of BDNF levels in the hippocampus of mice under conditions of chronic social defeat stress. Western blot analysis data. R.D.U—relative densitometric units. Lanes: 1—Control, 2—GTS-302, 3—Stress, 4—Stress+GTS-302. * - $p<0.05$ compared to the "Stress" group (Mann-Whitney U test).

Thus, GTS-302 almost completely counteracted the reduction of BDNF levels in the hippocampus under conditions of chronic social defeat stress.

4. Discussion

In the present study, anhedonia (reduced ability to experience pleasure), one of the key symptoms of depression, was utilized as an indicator of depressive-like state in mice subjected to chronic social defeat stress. Anhedonia was

assessed in the sucrose preference test, where its criterion was considered as a preference for sucrose solution <65% [20].

The initial sucrose preference test was conducted without prior exposure of mice to the sucrose solution, resulting in low consumption rates across all groups (ranging from 50-60% of total fluid intake). This low consumption of sweet liquid may be attributed to neophobia, indicating the animals' fear of unfamiliar substances as documented in the literature [23]. Subsequently, we repeated the sucrose preference test, considering the initial trial as the mice's adaptation phase to the sucrose solution. To stimulate food motivation, a common practice involves food and water deprivation before such tests [24]. Hence, mice were deprived of food and water for 4 hours prior to measuring sucrose solution consumption. In this modified setup, sucrose solution preference was 67.4% in the non-stressed control group, whereas it significantly dropped to 56.9% in stressed mice. The administration of dipeptide GTS-302 significantly alleviated anhedonia, raising sucrose solution preference to 85.3%.

It is noteworthy that GTS-302 significantly increased sucrose solution consumption even in control mice not subjected to stress. According to literature data, the level of sucrose solution preference not only reflects the animals' ability to experience pleasure but also their memory of the location of the bottle with sweet water [24]. It has been shown that during prolonged access of mice to bottles with water and sucrose solution, the number of approaches to the bottle with sweet water increases due to a decrease in the number of "incorrect" choices [25]. Additionally, a separate study found that optogenetic inhibition of neurons in the medial prefrontal cortex led to reduced preference for sucrose solution in mice due to disruptions in learning [24]. Our previous research [17] demonstrated that GTS-302, following acute intraperitoneal administration, enhances memory in rats in the novel object recognition test. It can be assumed that the observed increase in sucrose solution preference in non-stressed mice treated with GTS-302 is linked to the mnemonic properties of the dipeptide.

Intriguingly, we previously found that the dimeric dipeptide mimetic of BDNF (GSB-106) completely reinstates diminished sucrose solution preference in a mouse model of chronic social defeat stress [19], however, it does not impact this parameter in non-stressed mice. Unlike GTS-302, GSB-106 did not demonstrate mnemotropic effects upon acute administration [26].

Furthermore, we assessed the levels of synaptic proteins synaptophysin and PSD-95, as well as the neurotrophin BDNF, in the hippocampus of mice using Western blot analysis. The hippocampus stands as a pivotal brain region implicated in the pathophysiology of depression, with a reduction in its volume being the most consistent finding in neuroimaging studies of individuals with depression [27]. Both experimental and clinical data emphasize that degenerative alterations in the hippocampus during depression are linked to a deficiency in BDNF, a factor that plays a fundamental role in regulating hippocampal neuroplasticity [28]. In the present study, no influence of stress on the hippocampal levels of synaptophysin and PSD-95 was detected. However, the BDNF content in stressed mice was significantly reduced by 18% compared to the control group. The decrease in BDNF level in the hippocampus is considered a relevant indicator of a depression-like state [29,30]. Therefore, our obtained biochemical data align with the observed anhedonic behavior in stressed mice. The absence of a decrease in the levels of synaptic markers may suggest a relatively mild degree of degenerative changes.

The NT-3 mimetic, GTS-302, nearly completely reinstated hippocampal BDNF levels in stressed mice, aligning with existing literature suggesting that NT-3 stimulates the synthesis and release of BDNF [8, 9]. The effect of GTS-302 could also be attributed to the dipeptide, similar to full-length NT-3 [4], which might stimulate hippocampal neurogenesis, thereby mitigating the decline in neuroplasticity. Uncovering the precise mechanisms underlying the antidepressant-like action of NT-3 requires more in-depth research.

5. Conclusion

Thus, the dimeric dipeptide mimetic of NT-3, compound GTS-302, administered chronically, completely prevents the development of depression-like states in mice induced by social defeat stress.

Author contributions

TAG: Supervision, Conceptualization, Writing—review and editing. DVL: Supervision, Writing—review and editing. PYuP: Investigation, Methodology, Writing-original draft. TAA: Conceptualization, Methodology, Writing-original draft. AVT, SNM: Investigation, Methodology, Validation, Formal analysis. DMN, IOL, SVN: Investigation, Formal analysis. All authors read and approved the submitted version.

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