Available online at www.sciencedirect.com

ScienceDirect

Mendeleev Commun., 2023, **33**, 786–789

Mendeleev Communications

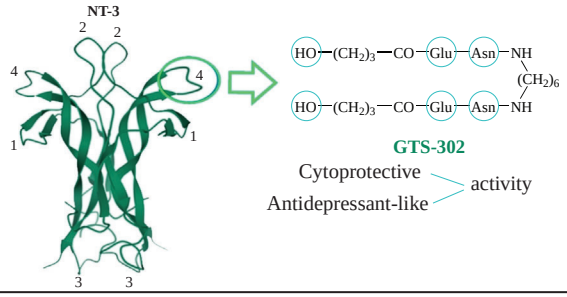
Design and synthesis of a novel dipeptide mimetic of the 4th loop of neurotrophin-3 and its pharmacological effects

Alexey V. Tarasiuk, Nellya M. Sazonova, Marina V. Melnikova, Sergey V. Pomogaybo, Ilya O. Logvinov, Sergey V. Nikolaev, Dmitry M. Nikiforov, Tatiana A. Antipova, Polina Yu. Povarnina, Yulia V. Vakhitova, Tatiana A. Gudasheva* and Sergey B. Seredenin

V. V. Zakusov Research Institute of Pharmacology, 125315 Moscow, Russian Federation.
E-mail: gudasheva@academpharm.ru

DOI: 10.1016/j.mencom.2023.10.016

A novel neurotrophin-3 loop 4 dipeptide mimetic, bis(*N*-γ-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide (GTS-302), was designed and synthesized. Compound GTS-302 activated the tyrosine kinase receptors TrkC and TrkB, exhibited cytoprotective activity (10^{-8}–10^{-5} M) in oxidative stress model in HT-22 cells and in 6-hydroxydopamine-induced SH-SY5Y cell damage. It also demonstrated an antidepressant-like activity in the forced swim test in mice (0.5–10.0 mg kg^{-1}, intraperitoneally).



Keywords: dipeptide mimetic, low-molecular mimetic, design, neurotrophin-3, cytoprotective activity, antidepressant-like activity.

Proteins of the neurotrophin family (NGF, BDNF, NT-3) play an important role in the development of the central nervous system and in the maintaining of neuronal homeostasis in the adult organism. Under pathological conditions, neurotrophins, generally, promote activation of endogenous protection and regeneration mechanisms.[1–3] Neurotrophins exert effects on neurons by signaling through neurotrophin tyrosine kinase (Trk) receptors. Unlike NGF and BDNF that selectively interact with TrkA and TrkB, respectively, NT-3 amenable to bind with all three subtypes of Trk receptors such as TrkA, TrkB, and TrkC, however, favoring the latter one.[4] Although all Trk receptors have been shown to widely express in the brain areas, TrkC is the most prevalent subtype in the locus coeruleus, a small brainstem nucleus,[1] that plays a significant role in the physiological response to stressful stimuli and is involved in the pathophysiology of affective and anxiety disorders, thus indicating the engagement of NT-3 in those conditions. Numerous animal studies have revealed neuroprotective, antidepressant-like, anxiolytic and other neuropsychotropic effects of NT-3 when administered intracerebrally.[5–7] To date, NT-3 is believed to have an antidepressant-like activities due to its ability to modulate monoamine synaptic transmission and involvement into mechanisms of hippocampal neuroplasticity.[8]

Taking into account the above mentioned, NT-3, as well as other neurotrophines, is considered as a starting point for the development of original therapeutics targeting the CNS disorders, such as depression, Parkinson's and Alzheimer's diseases. However, the major obstacles to clinical translation of exogenously administered full-sized neurotrophin are due to rapid peripheral enzymatic degradation, poor tissue distribution of this large molecule, including its crossing of blood–tissue barriers, instability in biological milieus and undesirable side effects because of pleiotropic activities of the native NT-3. To overcome these limitations, various strategies for the therapeutic application of neurotrophin have been proposed, including the development of small systemic available mimetics of NT-3.

A dimeric dipeptide NT-3 loop 4 mimetic, namely, bis(*N*-monosuccinyl-L-asparaginyl-L-asparagine) hexamethylenediamide (GTS-301),[9] has previously been designed at the Zakusov Research Institute of Pharmacology, based on the original hypothesis on the key role of the most surface-exposed fragments of β-turns within the loop-like structures of neurotrophins for the interaction with the receptors.[10] Dipeptide GTS-301 was shown[9] to activate TrkC and TrkB receptors, displayed the neuroprotective activity *in vitro* at picomolar concentrations and demonstrated an antidepressant-like activity in the forced swim test in mice following subchronic intraperitoneal (ip) administration at doses of 10–40 mg kg^{-1}.

In this study, a novel NT-3 dipeptide mimetic, bis(*N*-γ-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide, GTS-302, has been designed and synthesized, that, as well as GTS-301, was based on the loop 4 of NT-3, but, with a shift by one amino acid residue to the N-terminus [Figure 1(*c*)]. It was assumed that this compound may differ quantitatively or qualitatively from GTS-301 in its pharmacological or biochemical properties. Design of the dipeptide GTS-302 was based on the crystal structure of NT-3 [Figure 1(*a*)].

Like other members of the neurotrophin family, NT-3 is a symmetrical homodimer whose monomeric units contain seven β-strands forming three antiparallel β-sheets. β-Strands are connected with each other by four outwardly exposed irregular structures called loops: 1st (residues 29–33), 2nd (42–47), 3rd (residues 58–75) and 4th (91–97) (see Figure 1**)**.[11] The most exposed is the loop 4 whose fragment Ser^{91}–Glu^{92}–Asn^{93}–Asn^{94}–Lys^{95} contains turning regions in its structure, presumably occupying the geometrically most favorable position for interaction with the receptor. When constructing compound GTS-302, we retained the –Glu^{92}–Asn^{93}– dipeptide fragment while the preceding neighbor

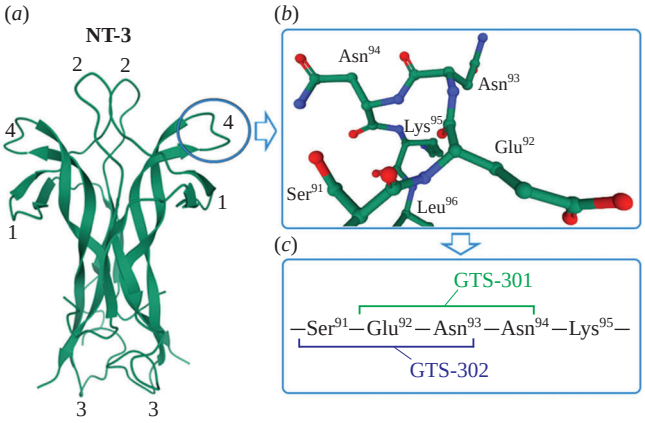
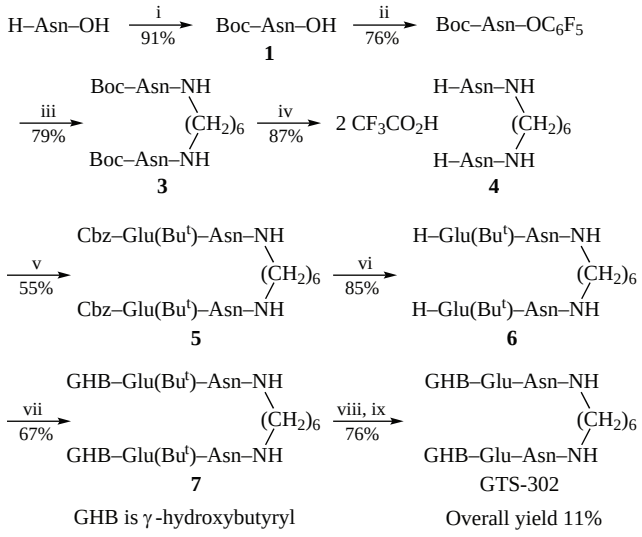


Figure 1 Design of NT-3 loop 4 dipeptide mimetics: (*a*) general view of NT-3 crystal structure (pdb ID: 1nt3); (*b*) surface-exposed part of the loop 4; (*c*) scheme of selection of the amino acids residues for design of GTS-301 and GTS-302.

residue Ser^{91} was substituted with a homolog of its bioisostere, a γ-hydroxybutyric acid residue. The dimeric structure of neurotrophin was imitated using a hexamethylenediamine spacer at the C-terminus. Thus, a new dimeric dipeptide NT-3 loop 4 mimetic, bis(*N*-γ-hydroxybutyryl-L-glutamyl-L-asparagine) hexamethylenediamide, GTS-302, was constructed.

Dipeptide GTS-302 was synthesized by classical peptide synthesis in solution using the strategy of Cbz/Bu^t protecting groups by the method of activated pentafluorophenyl and succinimide esters of L-amino acids (Scheme 1). The peptide chain was extended from the C-terminus. At the first step, Boc-protected asparagine **1** was prepared by the reaction of asparagine sodium salt with Boc_2O in aqueous Pr^iOH.[12] Activated pentafluorophenyl ester of Boc-asparagine **2** was obtained using DCC and pentafluorophenol. Condensation of **2** with hexamethylenediamine in DMF afforded bis-asparagine **3** whose acidolysis with CF_3CO_2H/CH_2Cl_2 gave product **4**. Compound **4** was converted to the salt-free form with *N*,*N*-diisopropyl-*N*-ethylamine (DIPEA) and reacted with Cbz–Glu(Bu^t)–OSu (Su is *N*-hydroxysuccinimide residue) yielding the bis-dipeptide **5**. Removal of Cbz protection in **5** was performed by hydrogenolysis in methanol in the presence of 10% Pd/C. The thus deblocked bis-dipeptide **6** was treated with γ-butyrolactone at 60–70 °C for 4 h. The acylated product **7** was acidolyzed in the mixture of TFA and CH_2Cl_2 giving bis(hydrotrifluoroacetate) of the target product in good yield. The final product, GTS-302, was purified by preparative reverse-phase HPLC and then lyophilized (see Scheme 1). Peptide GTS-302 was chromatographically homogeneous, that was determined by TLC and reverse-phase HPLC assays. The structure of GTS-302 was confirmed by one-dimensional ^1H, ^{13}C and two-dimensional NMR spectroscopy, as well as electrospray ionization mass spectrometry (ESI-MS). Its diastereomeric purity was at least 98% according to NMR data.



Scheme 1 *Reagents and conditions*: i, Boc_2O, NaOH, Pr^iOH/H_2O, room temperature, 20 h, then HCl (pH 2.5); ii, C_6F_5OH, DCC, EtOAc, 10 °C, 7 h, then room temperature, 12 h; iii, $H_2N(CH_2)_6NH_2$, DMF, room temperature, 4 h; iv, CF_3CO_2H, CH_2Cl_2, room temperature, 2 h; v, Cbz-Glu(Bu^t)-OSu (Su is *N*-hydroxysuccinimide residue), DIPEA, DMF, room temperature, 12 h; vi, H_2, 10% Pd/C, MeOH; vii, γ-butyrolactone, DMF, 60–70 °C, 4 h; viii, CF_3CO_2H, CH_2Cl_2, room temperature, 2 h; ix, HPLC and lyophilization.

Further, we have evaluated the ability of GTS-302 to activate Trks receptors, its cytoprotective activity *in vitro*, and antidepressant-like activity *in vivo*. Western blot analysis with antibodies to Trk receptors phosphorylated at specific tyrosine residues was used to assay whether dipeptide GTS-302 was amenable to activate TrkC, TrkB, and TrkA receptors in mouse hippocampal HT-22 cells. Antibodies to total TrkC, TrkB, and TrkA were used as controls. It is well-known that phosphorylation of the specific tyrosine residues in Trks receptors promotes the activation of the receptors themselves and, in turn, the downstream signaling pathways. Activation of PI3K/AKT and MAPK/ERK signaling pathways is triggered following the phosphorylation of tyrosine residues at Tyr^{516}, Tyr^{515}, Tyr^{490} in TrkC, TrkB and TrkA receptors, respectively.[13,14]

HT-22 cells were treated with NT-3 [100 ng ml^{-1}, ~10^{-9} M (positive control)[15]] or GTS-302 (10^{-6} M) for 5, 15, 30, 60, and 180 min and, thereafter, were lysed and prepared for immunoblotting. Concentration of GTS-302 (10^{-6} M) was chosen as the most active based on the data obtained in the cytoprotection assays (Table 1). The time intervals were chosen based on the results of experiments on Trk phosphorylation in the presence of dipeptide mimetics NGF and BDNF[16], as well as by NT-3 mimetic GTS-302.[9]

It was found that GTS-302, like the full-size NT-3, activates TrkC receptors 15 min after treatment and this activation maintains for, at least, 180 min (Figure 2).

Activation of TrkB receptors upon GTS-302 or NT-3 treatment was detected in 30 min, and was sustained for 180 min following GTS-302 administration, although for NT-3, values of tyrosine phosphorylation at Tyr^{515} were not statistically significant at this time point (Figure 3). As shown in Figure 4, GTS-302 did not activate TrkA receptors, whilst NT-3 evoked TrkA receptor phosphorylation only at 180 min. Thus, the pattern of activation of TrkC and TrkB receptors by small NT-3

Table 1 Cytoprotective activity of GTS-302 in the model of oxidative stress in HT-22 cells (MTT test).[a]

Experimental groups	Concentration/M	Optical density[b]	Activity (%)[c]
Control	0	0.182 ± 0.010	100
H_2O_2 (1.5 mM)	1.5×10^{-3}	0.087 ± 0.007[d]	0
NT-3 (100 ng ml^{-1})	3.4×10^{-9}	0.135 ± 0.006[e]	**51**
GTS-302	10^{-5}	0.106 ± 0.008[e]	**20**
	10^{-6}	0.109 ± 0.006[e]	**23**
	10^{-7}	0.104 ± 0.005[e]	**18**
	10^{-8}	0.103 ± 0.006[e]	**17**
	10^{-9}	0.095 ± 0.010	8

[a] Data are presented as means and standard deviations (M ± SD). [b] In optical density units. [c] Percent of the maximum possible effect calculated by the formula A (%) = $(D_{exp} - D_{ac})/(D_{pc} - D_{ac}) \times 100\%$, where D_{exp} is the optical absorption of the solution with GTS-302 or NT-3, D_{ac} is the optical absorption of the active control (with damage), D_{pc} is the optical absorption in passive control (no damage). [d] With $p \leqslant 0.05$ compared to passive control. [e] With $p \leqslant 0.05$ compared with active control (H_2O_2, Kruskal–Wallis test followed by Dunn's test).

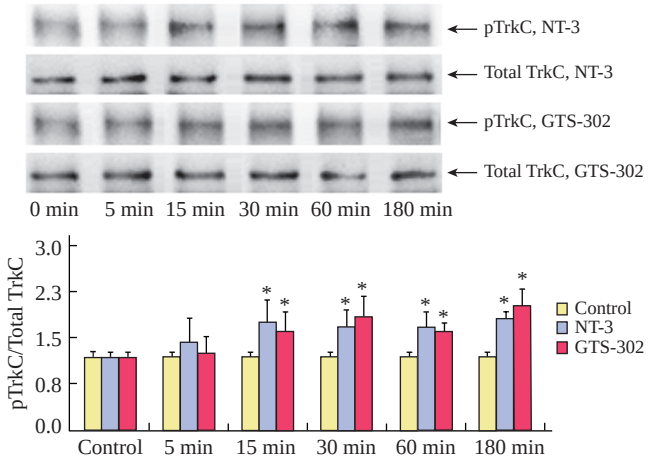


Figure 2 Phosphorylation level of the TrkC receptor following treatment with NT-3 (3.4×10^{-9} M) or GTS-302 (10^{-6} M) in HT-22 mice hippocampal cells. The original Western blots and the results of their densitometry are presented. Data are given as mean and standard deviations (M ± SD). The data are averages from three independent experiments. Significance of differences * $p \leqslant 0.05$ – from control (Mann–Whitney U test).

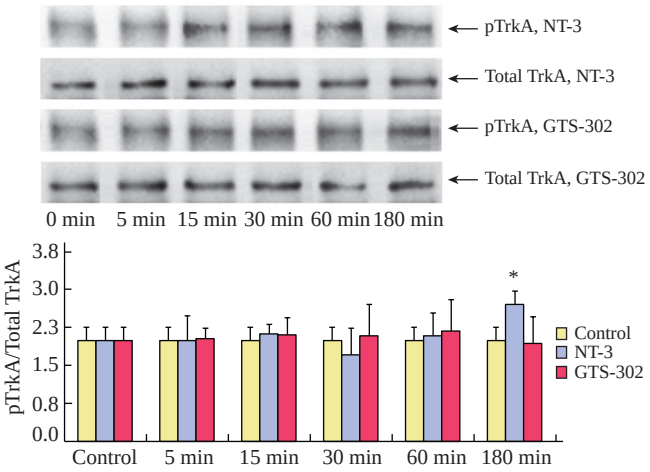


Figure 4 Phosphorylation level of the TrkA receptor after the addition of NT-3 (100 ng ml^{-1}) and GTS-302 (10^{-6} M) to mice hippocampal HT-22 cells. The original Western blots and the results of their densitometry are presented. Data are given as mean and standard deviations (M ± SD). The data are averages from three independent experiments. Significance of differences * $p \leqslant 0.05$ – from control (Mann–Whitney U test).

mimetic GTS-302 is similar to that caused by the full-length NT-3, with no effect on TrkA receptors phosphorylation.

The cytoprotective effects of GTS-302 were studied in a model of oxidative stress in HT-22 mouse hippocampal cells and in a model of 6-hydroxydopamine-induced toxicity in SH-SY5Y cells (a cellular model of Parkinson's disease). Cells were treated with GTS-302 at a range of concentrations of 10^{-9}–10^{-5} M 24 h before application of the damaging agents. NT-3 (100 ng ml^{-1}, ~10^{-9} M) was used as a positive control. It was found that GTS-302 under oxidative stress conditions prevented the decrease of cells viability in the concentration range of 10^{-8}–10^{-5} M, with no effect at a concentration of 10^{-9} M (see Table 1).

In a cellular model of Parkinson's disease, dipeptide GTS-302 exhibited cytoprotective activity at concentrations of 10^{-7}–10^{-5} M, and this effect was comparable with the activity of full-length NT-3. At a concentration of 10^{-8} M, effect of GTS-302 has not been detected (Table 2). Thus, the dipeptide mimetic of the 4th loop of NT-3, GTS-302, demonstrates cytoprotective activity *in vitro* in both models, similar to NT-3.

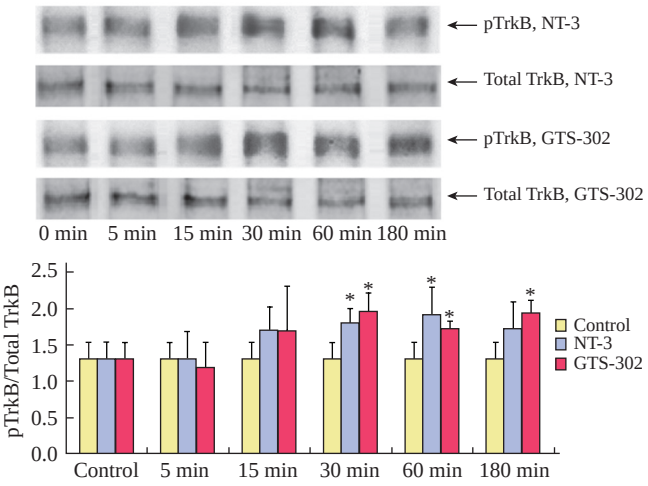


Figure 3 Phosphorylation level of the TrkB receptor after the addition of NT-3 (3.4×10^{-9} M) and GTS-302 (10^{-6} M) to mice hippocampal HT-22 cells. The original Western blots and the results of their densitometry are presented. Data are given as mean and standard deviations (M ± SD). The data are averages from three independent experiments. Significance of differences * $p \leqslant 0.05$ – from control (Mann–Whitney U test).

Table 2 Cytoprotective activity of GTS-302 in the model of 6-hydroxydopamine-induced toxicity in SH-SY5Y cells (MTT test).[a]

Experimental groups	Concentration/M	Optical density[b]	Activity (%)[c]
Control	–	0.187 ± 0.010	100
6-OHDA (100 mM)	10^{-4}	0.163 ± 0.014[d]	0
NT-3 (100 ng ml^{-1})	3.4×10^{-9}	0.183 ± 0.006[e]	**83**
GTS-302	10^{-5}	0.179 ± 0.005[e]	**67**
	10^{-6}	0.182 ± 0.010[e]	**79**
	10^{-7}	0.180 ± 0.004[e]	**71**
	10^{-8}	0.179 ± 0.008	67

[a] Data are presented as means and standard deviations (M ± SD). [b] In optical density units. [c] Percent of the maximum possible effect calculated by the formula A (%) = $(D_{exp} - D_{ac})/(D_{pc} - D_{ac}) \times 100\%$, where D_{exp} is the optical absorption of the solution with GTS-302 or NT-3, D_{ac} is the optical absorption of the active control (with damage), D_{pc} is the optical absorption in passive control (no damage). [d] With $p \leqslant 0.05$ compared to passive control. [e] With $p \leqslant 0.05$ compared with active control (6-OHDA, Kruskal–Wallis test followed by Dunn's test).

Antidepressant-like effects of GTS-302 were studied in the forced swim test in BALB/c mice. It was found that the acute administration of the dipeptide at doses from 0.5 to 10.0 mg kg^{-1} (i.p.) significantly reduces the immobility time (Table 3). At a lower (0.1 mg kg^{-1}) and at a higher dose (20 and 50 mg kg^{-1}), GTS-302 demonstrated no activity in this animal test. Importantly, the degree of the antidepressant-like activity of GTS-302 is comparable with that of the classic antidepressant amitriptyline (see Table 3). The data obtained displayed a bell-shaped dose–effect dependence of GTS-302, which, as known, is a feature of regulatory peptides.[17]

In summary, GTS-302, an original dimeric dipeptide NT-3 loop 4 mimetic, like a full-sized neurotrophin, activates TrkC and TrkB receptors, exhibits cytoprotective activity *in vitro*, and has an antidepressant-like effect *in vivo*. Compared to the previously obtained dipeptide mimetic GTS-301, novel compound GTS-302 exhibits antidepressant-like activity at lower doses and even after acute administration. The threshold doses of GTS-301 and GTS-302 are 10 and 0.5 mg kg^{-1} (i.p.), respectively. At the same time, GTS-302 showed less pronounced neuroprotective activity compared to GTS-301. Dimeric dipeptide mimetics of NT-3 can be the basis for development of pharmacological agents, prospective for the treatment of diseases whose pathogenesis is associated with the alteration or dysfunction of native neurotrophin and related intracellular pathways.

Table 3 Antidepressant-like activity of the NT-3 mimetic GTS-302 upon its acute administration in the forced swim test in BALB/c mice.[a]

Group	Immobility time/s	Immobility time (% of control)	Activity (%)[b]
	Experiment 1		
Control	226.7 ± 7.5	100	0
GTS-302 (1 mg kg^{-1})	**183.6 ± 6.8**[c]	**81**	**19**
GTS-302 (5 mg kg^{-1})	**188.9 ± 10.2**[d]	**83**	**17**
GTS-302 (10 mg kg^{-1})	**177.8 ± 10.3**[e]	**78**	**22**
	Experiment 2		
Control	164.2 ± 7.5	100	0
GTS-302 (0.1 mg kg^{-1})	143.5 ± 15.2	87	13
GTS-302 (0.5 mg kg^{-1})	**113.5 ± 11.2**[d]	**69**	**31**
GTS-302 (20 mg kg^{-1})	125.2 ± 12.6	76	24
GTS-302 (50 mg kg^{-1})	132.1 ± 13.9	80	20
Amitriptyline (10 mg kg^{-1})	**119.7 ± 7.4**[d]	**73**	**27**

[a]Data are presented as means and standard errors of the means (M ± SEM). [b]Activity was calculated by formula A (%) = $(T_c - T_{exp})/T_c \times 100\%$, where T_c is the immobility time in control, T_{exp} is the immobility time in the GTS-302 or Amitriptyline group. [c]With $p < 0.001$ compared with the control group (one-way analysis of variance, Dunnett's test). [d]With $p < 0.05$. [e]With $p < 0.01$.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.10.016.

References

1 E. Castrén, *Handb. Exp. Pharmacol*., 2014, **220**, 461.
2 M. Fujitani, Y. Otani and H. Miyajima, *Biomolecules*, 2021, **11**, 1730.
3 A. M. Weissmiller and C. Wu, *Transl. Neurodegener*., 2012, **1**, 14.
4 M. Bothwell, *Handb. Exp. Pharmacol*., 2014, **220**, 3.
5 J. Zhang, Q. Shi, P. Yang, X. Xu, X. Chen, C. Qi, J. Zhang, H. Lu, B. Zhao, P. Zheng, P. Zhang and Y. Liu, *Neuroscience*, 2012, **222**, 1.
6 Y. Shirayama, A. C.-H. Chen, S. Nakagawa, D. S. Russell and R. S. Duman, *J. Neurosci.*, 2002, **22**, 3251.
7 D. D'Amico, T. Gener, M. M. de Lagrán, M. V. Sanchez-Vives, M. Santos and M. Dierssen, *Neuropsychopharmacology*, 2017, **42**, 462.
8 A. S. de Miranda, J. L. V. M. de Barros and A. L. Teixeira, *Expert Opin. Ther. Targets*, 2020, **24**, 1225.
9 T. A. Gudasheva, N. M. Sazonova, A. V. Tarasiuk, I. O. Logvinov, T. A. Antipova, D. M. Nikiforov, P. Yu. Povarnina and S. B. Seredenin, *Dokl. Biochem. Biophys*., 2022, **505**, 160 (*Dokl. Ross. Akad. Nauk. Nauki Zhizni*, 2022, **505**, 303).
10 T. A. Gudasheva, T. A. Antipova and S. B. Seredenin, *Dokl. Biochem. Biophys.*, 2010, **434**, 262 (*Dokl. Akad. Nauk*, 2010, **434**, 549).
11 M. J. Butte, P. K. Hwang, W. C. Mobley and R. J. Fletterick, *Biochemistry*, 1998, **37**, 16846.
12 V. F. Pozdnev, *Chem. Nat. Compd*., 1974, **10**, 782 (*Khim. Prir. Soedin*., 1974, 764).
13 L. F. Reichardt, *Philos. Trans. R. Soc., B*, 2006, **361**, 1545.
14 T. Yang, S. M. Massa, K. C. Tran, D. A. Simmons, J. Rajadas, A. Y. Zeng, T. Jang, S. Carsanaro and F. M. Longo, *Neuropharmacology*, 2016, **110**, 343.
15 E. J. Huang, G. A. Wilkinson, I. Fariñas, C. Backus, K. Zang, S. L. Wong and L. F. Reichardt, *Development*, 1999, **126**, 2191.
16 T. A. Gudasheva, P. Yu. Povarnina, T. A. Antipova, Yu. N. Firsova, M. A. Konstantinopolsky and S. B. Seredenin, *J. Biomed. Sci*., 2015, **22**, 106.
17 A. J. Kastin and W. Pan, *Crit. Rev. Toxicol*., 2008, **38**, 629.

Received: 11th April 2023; Com. 23/7148