Behavioural Pharmacology 1999; **10**:549–553

SHORT REPORT

Memory restoring and neuroprotective effects of the proline-containing dipeptide, GVS-111, in a photochemical stroke model

R.U. Ostrovskaya[a], G.A. Romanova[b], I.V. Barskov[c], E.V. Shanina[c], T.A. Gudasheva[a], I.V. Victorov[c], T.A. Voronina[a] and S.B. Seredenin[a]

[a]*Institute of Pharmacology,* [b]*Institute of General Pathology and Pathophysiology, and* [c]*Brain Research Institute, Russian Academy of Medical Sciences, Moscow, Russia*

Correspondence to Rita Ostrovskaya, Baltiyskaya Str. 8, Moscow 125315, Russia. E-mail: rita.o@postman.ru

Received 22 March 1999; accepted as revised 9 June 1999

Local thrombosis of the frontal cortex (Fr1 and Fr3 fields), caused by combination of the intravenous photosensitive dye Rose Bengal administration with focused high-intensity illumination of the frontal bone, was shown to provoke a pronounced deficit in step-through passive avoidance performance in rats without concomitant motor disturbances. *N*-Phenylacetyl-L-prolylglycine ethyl ester (GVS-111) administered intravenously at a dose of 0.5 mg/kg/day, for the first time 1 h after ischaemic lesion and then for 9 post-operative days, with the last administration 15 min before testing, attenuated the deficit. This treatment significantly diminished the volume of the infarcted area. Thus, post-ischaemic injection of GVS-111 demonstrated both cognition-restoring and neuroprotective properties. The cognition-restoring effect is probably based on an increase in neocortical and hippocampal neuronal plasticity. Neuroprotective effects of GVS-111 combine antioxidant activity with the ability to attenuate glutamate-provoked neurotoxicity and block voltage-gated ionic channels, i.e. the compound mitigates the main metabolic shifts involved in pathogenesis of brain ischaemia. © **1999 Lippincott Williams & Wilkins.**

Keywords: substituted prolyl-containing dipeptide, GVS-111, photochemically induced thrombosis, cognition, neuroprotection, rat

INTRODUCTION

Because of the high frequency and severity of stroke the development of substances that might directly or indirectly alleviate its consequences represents a clinical challenge of great importance. A cognitive deficit is a typical symptom of stroke, occurring in 35% of patients. The data on the effectiveness in stroke of the standard cognition enhancer, piracetam, are inconsistent and rather negative. Over the last decade our team has been developing a new approach to the design of cognition enhancers imitating the structure and conformation of piracetam with dipeptides, containing one of two pyrrolidine-containing amino acids, pyroglutamic acid or proline. This approach is based on an assumed peptidergic mechanism of activity of piracetam (Gudasheva *et al.*, 1996). A proline-containing dipeptide, GVS-111 (*N*-phenylacetyl-L-prolylglycine ethyl ester) was chosen from the series of acylproline-containing dipeptides because of its high cognition-restoring activity (Seredenin *et al.*, 1995): the range of GVS-111 effective doses are 0.1–1.2 mg/kg compared to those for piracetam of 200–800 mg/kg. The advantages of GVS-111 over longer peptides consists in its higher biological stability and effectiveness after systemic administration (see Ostrovskaya *et al.*, 1997 for details). The purpose of this study was to evaluate the effect of GVS-111 on general behaviour, passive avoidance performance and size of lesion in photochemically induced local cortical ischaemia, widely believed to be a model of stroke (Watson *et al.*, 1985; Hunter *et al.*, 1995; Rodgers and Hunter, 1997).

METHODS

Subjects

Experimentally naive male Wistar rats (180–220 g) were housed (10 animals per cage) under standard conditions with unlimited access to food and water on a 12 h light–12 h dark cycle. All animal housing and experimental procedures, including cortical ischaemia, were authorized and approved by the Ethical

Animal Use and Care Committee of the Institute of Pharmacology.

Procedure

Each animal was first placed individually in a rectangular open field, inner dimensions 46 × 46 × 19 cm, and the number of squares crossed was registered automatically for 5 min. This procedure was followed by the passive avoidance procedure, performed according to the method of Bures *et al.* (1983). The equipment and training procedure have been described elsewhere (Ostrovskaya *et al.*, 1997). Having been placed in a large box, the rat explored it for a few seconds and then moved into a dark compartment. A sliding door was closed at once and the animal was left there for 5 min. After 1 h the procedure was repeated. On the next day the trials were repeated twice at 1-h intervals. During the second trial, immediately after entering the dark compartment, unavoidable electrical foot-shock (1.0 mA, 50 Hz, 4 s) was delivered through the grid floor. Twenty-four hours later, in order to assess the memory retention, the rat was placed in the lit compartment again and the latency of entering the dark compartment was observed for 300 s.

After testing in the passive avoidance paradigm, 33 rats were randomly divided into four groups: group I (n = 10) sham-operated rats treated intravenously for 9 days after the lesion with 0.9% NaCl solution (saline); group II (n = 7) subjected to the procedure of photochemically initiated cortical thrombosis and treated for 9 days after the lesion with the saline; group III (n = 7) subjected to the same procedure of cortical thrombosis and treated with 0.5 mg/kg/day GVS-111 intravenously first 1 h after the lesion, then daily for 9 days after the lesion with the last injection 15 min before the retention test; group IV (n = 9) sham-operated rats treated for 9 days with 0.5 mg/kg/day GVS-111. Freshly prepared GVS-111 solution in saline was used. The retention test was performed in all groups on the 9th day after operation.

Induction of photothrombosis

Focal photothrombotic cortical infarction was induced in frontal cortex (Fr1 and Fr3; Zilles and Wree, 1985) according to a modification of the method of Watson *et al.* (1985). The rats were anaesthetized with chloral-hydrate (300 mg/kg, i.p.). After intravenous injection of the photosensitive dye Rose Bengal (40 mg/kg, 3% solution in saline), each rat was placed in a stereotaxic frame and the scalp was incised. The skull was illuminated bilaterally with unfiltered cold white light (air-cooled 24 V, 250 W halogen bulb) directed by the fibre-optic light-guide 3 mm in diameter. The centres of the light beams were placed on each side of the skull 2.5 mm anterior to the bregma and 2.5 mm apart from the mid-line, corresponding to the frontal cortex (Paxinos and Watson, 1986). To prevent heat-mediated brain damage the irradiated skull surface was irrigated with the water (20°C). Sham-operated animals were subjected to the same procedure, except for Rose Bengal administration.

Morphometric studies

For histological studies and volumetric measurement of the infarcted cortical area, following behavioural testing (day 9), each rat was deeply anaesthetized with chloral-hydrate (300 mg/kg) and perfused transcardially with saline (100 ml), followed by 10% phosphate-buffered neutral formalin. After perfusion the animals were decapitated and heads were kept in a refrigerator (4°C); 20 h later the brains were removed. Serial coronal sections 100 μm thick were cut with a vibratome throughout the whole rostro-caudal extent of the infarcted area. The sections were stained with a 0.2% solution of methylene blue. The infarcted cortical area in each section was measured by digital planimetry, using the ASM Leitz analysing system. The total lesion volumes (V) were calculated using the formulae (Harman and Carpenter, 1950):

$$V = \frac{\sum Sn}{a^2} \times d$$

where a is the coefficient of the magnification of the ASM Leitz system, d is the thickness of each slice (in mm) and $\sum Sn$ is the total sum of the infarcted areas in all sections.

Data analysis

All data are expressed as mean ± SEM. Passive avoidance data are presented as the latency to enter the dark compartment on each of three trials: during the training, testing performed 24 h later and retention test, 9 days after the sham or real operation. Rats which did not acquire the avoidance response (i.e. show a latency shorter than 300 s before the operation) were discarded from the experiments. The initial latencies in the acquisition trial were first subjected to non-parametric ANOVA (Kruskal–Wallis one-way Analysis by Ranks). If this analysis of data for the training day revealed an absence of statistical differences between groups, a further analysis of the retention scores was performed, again using Kruskal–Wallis one-way Analysis by Ranks. If this analysis revealed a level of significance of 0.05 or

less, further pairwise comparisons were performed using the Mann–Whitney U-test. Differences between the pre-operative and post-operative tests within each group was analysed by means of the Wilcoxon *T*-test.

RESULTS

Behavioural testing

The animals which were subjected to the sham operation (Group I) or to the photochemically induced thrombosis (Group II) recovered from the narcosis and operation at the same time (around 2–3 h). Animals of group II demonstrated no changes in general behaviour, posture or gait, they reacted adequately to external stimuli (visual, acoustic, approaches of the experimenter's hand, touching); their locomotor activity also did not differ substantially from that of group I (data not presented). GVS-111, administered for 9 days, did not change general behaviour, reactivity to the external stimuli either in lesioned (group III) or sham-operated rats (group IV). GVS-111 treatment did not change locomotor activity. These data confirm our previous findings that, like standard cognition enhancers. GVS-111 neither stimulates nor depresses spontaneous locomotion (Seredenin *et al.*, 1995).

The latencies to enter the dark compartment on the acquistion trial of the passive avoidance test did not show significant difference between groups (4.9 ± 1.3; 6.8 ± 1.7; 4.8 ± 1.6; 5.7 ± 1.6). However, repetitive testing of the passive avoidance performance on post-operative day 9 revealed great differences between groups (Figure 1). In the control group the latency to enter the dark compartment tended to be shorter than before the lesion, but this difference was not statistically significant ($P > 0.05$), thus demonstrating only a tendency to retention loss over 9 days. Cortical ischaemia interfered with retention ($P < 0.001$). Pairwise comparison of the data from groups I and II showed that the dark compartment latency in saline-treated animals subjected to ischaemia was much shorter than that in sham-operated animals ($P < 0.05$). Rats subjected to the ischaemic lesion and treated post-operatively with GVS-111 demonstrated a statistically significant increase in latency, compared to ischaemic damaged saline-treated rats ($P < 0.01$ for the difference between groups III and II). Passive avoidance performance on day 9 in the group of GVS-111-treated animals subjected to ischaemic damage did not differ significantly from that of sham-operated rats ($P > 0.5$ for the differences in latency in groups III and I). In sham-operated animals, GVS-111 did not significantly affect passive avoidance retention, which did not differ from that in the control group ($P > 0.5$ for groups I and IV).

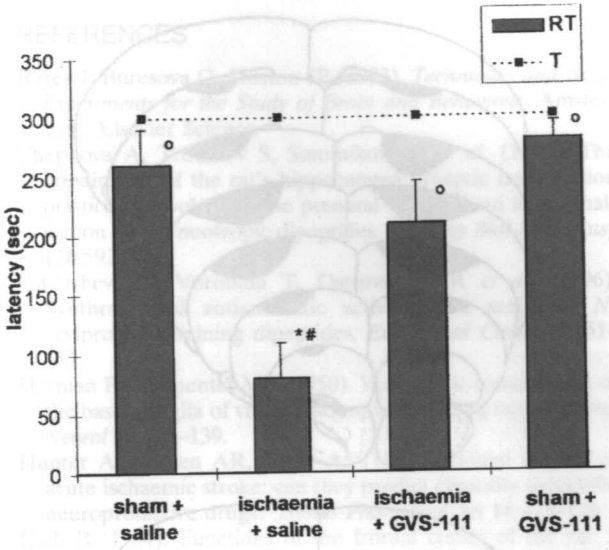

FIGURE 1. Latency to enter the dark compartment in a passive avoidance retention test, following photothrombotic ischaemia of the frontal cortex and treatment for 9 days with GVS-111. T – latency to enter during the first test (before the lesion), 300 s in all groups; RT – latency to enter in the retention testing (9 days after ischaemic lesion). *Significantly different from the sham-operated animals, subjected to ischaemic lesion, $P < 0.01$ (Mann–Whitney U-test); °significantly different from the saline-treated animals, $P < 0.01$ (Mann–Whitney U-test); #significantly different from data in the same group before the operation, $P < 0.001$ (Wilcoxon *T*-test).

Morphological analysis

Cortical ischaemic lesion was clearly visible on the methylene blue stained brain sections. At its maximal extent the lesion went through the whole thickness of the cortex and was separated from the surrounding undamaged brain tissue by a well-defined border (Figure 2). Zones of ischaemic lesion were localized in areas Fr1 and Fr3 of the frontal lobes (Zilles and Wree, 1985; Paxinos and Watson, 1986) corresponding to the pre-frontal cortex according to the Krettek and Price classification (Krettek and Price, 1977; Kolb, 1984).

In saline-treated rats, sacrificed on day 9 after the intervention (group II), the ischaemic lesion included a central core of pan-necrosis often with round cavities in the peri-infarction zone. In these rats the mean total volume of the focal ischaemic lesion was 6.2 ± 1.14 mm^3. In the rats of group III treated with GVS-111 at doses of 0.5 mg/kg/day i.v. for 9 days after photothrombotic lesion the volume of the lesioned cortical area diminished significantly ($P < 0.001$) to

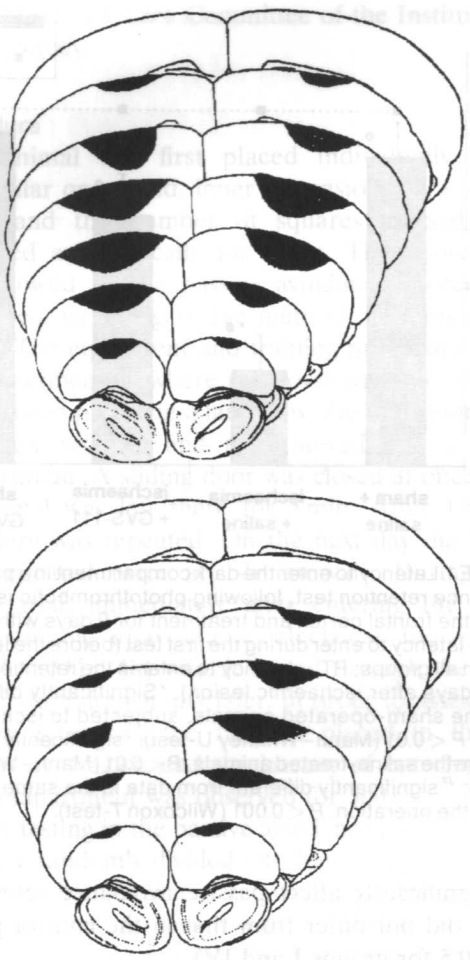

FIGURE 2. Infarcted areas in coronal sections of the frontal lobes (Fr1 and Fr3) in saline-treated (top) and GVS-III-treated (bottom) rats. Rats were sacrificed 9 days after photothrombotic infarction and subchronic (9 days) post-ischaemic treatment, either with saline or with GVS-III (0.5 mg/kg/day i.v.). Drawings have been made from serial coronal sections on the levels corresponding to the planes bregma +5.2 mm to bregma −1.8 mm (Paxinos and Watson, 1986) stained with 0.2% methylene blue. The thickness of each section is 0.1 mm, the distance between sections is 1.0 mm.

3.03 ± 0.5 mm^3. The damaged area did not usually contain cavities in the peri-infarction zone.

DISCUSSION

Taking into account the high variability of the clinical forms of brain ischaemia, many experimental models have been developed. Hunter *et al.* (1995) report that the photochemically induced stroke model is a relatively non-invasive model (no craniotomy is required because the skull is transparent to the light), which gives an opportunity to change the size and the location of the lesion. Selection of the prefrontal cortex as the place of thrombotic lesion was based on the key role of this cortical area in cognition. Judging by the severely impaired retention in the passive avoidance task, without deficiencies in movement coordination, muscle tone and locomotion, we obtained a purely cognitive deficit. These data are consistent with previous reports, according to which rats subjected to local ischaemia of the frontal cortex demonstrated a selective deficit in spatial learning (Rodgers and Hunter, 1997).

GVS-111, administered intravenously at doses of 0.5 mg/kg/day for 9 post-operative days, promoted the restoration of memory retention, damaged by the cortical infarction. As to possible reasons for this effect, it should be underlined that GVS-111, like piracetam, increases the amplitude of transcallosal responses (Ostrovskaya *et al.*, 1994) and restores hippocampal long-term potentiation damaged by pre-natal hypoxia (Chepkova *et al.*, 1995). These effects may contribute to the compound's cognition-restoring effect. The present experiments provide evidence that the action of GVS-111 is not confined to these purely functional effects. Judging by the morphological assessment, post-operative treatment with this substance causes a statistically significant diminution of the volume of the lesioned cortical tissues, showing a neuroprotective effect.

Systemic administration of the photosensitive dye Rose Bengal, known as an efficient photodynamic generator of singlet molecular oxygen, followed by exposure to high-intensity light produces free-radical-mediated endothelial damage. This causes a marked platelet aggregation in brain parenchymal vessels that results in cortical microvascular stasis and thrombotic lesion (Watson *et al.*, 1985). Ischaemia also initiates the cascade of events triggering disturbances of ionic homeostasis and glutamate-mediated toxicity, leading to a loss of neuronal cells. The destabilization of ionic homeostasis, mainly intracellular calcium and potassium accumulation, in the post-synaptic region is an important pathogenic mechanism of ischaemia. Blood supply insufficiency, increased glutamate release and decreased uptake causes a pronounced rise of glutamate content in the brain (Siesjo and Siesjo, 1996).

Experiments of Seredenin and Badyshtov (unpublished data) demonstrated that GVS-111 (0.5 mg/kg) decreases the amount of thiobarbituric acid reactive products (TBAR) in the brain of BALB/c mice exposed to an open field. Rats immobilized for 24 h show a dramatic increase in the content of TBAR in brain and blood, and GVS-111 prevented the accumulation of these lipid peroxidation products (Lysenko *et al.*, 1997).

In experiments on isolated neurones of *Helix pomatia* Solntseva and colleagues (1997) showed that GVS-111 diminishes the amplitude of high-threshold voltage-gated Ca^{2+} currents (threshold concentration of GVS-111 = 10^{-8} M). High-threshold Ca^{2+}-dependent K^+ current appeared to be even more sensitive to GVS-111 (threshold concentration = 10^{-9} M). The blockade of Ca^{2+} channels and of K^+ channels of Ca^{2+}-dependent type can overcome the intracellular calcium excess. The neurone-sparing effect of GVS-111 was demonstrated in experiments on cerebellar cells. GVS-111 (10^{-6} M) decreased the amount of damaged neurones in a culture subjected to a toxic concentration of glutamate (50 μM) from 79.8% to 43.2% (Ostrovskaya *et al.*, 1998).

The results suggest that GVS-111 possesses both cognition-restoring and neuroprotective properties. The cognition-restoring effect is probably based on an increase of neocortical and hippocampal neuronal plasticity. The neuroprotective effect of GVS-111 is of the 'cocktail' type: the substance combines antioxidant activity with the ability to attenuate glutamate-provoked neurotoxicity and block voltage-gated ionic channels, i.e. it mitigates the main metabolic shifts involved in the pathogenesis of brain ischaemia.

GVS-111 is a well-tolerated substance: while its effective doses are 0.1–1.2 mg/kg, its acute toxicity is shown to be 5000 mg/kg. One-month long GVS-111 administration at doses of 0.5 and 5.0 mg/kg did not cause either morphological or biochemical disturbances, embryotoxicity or teratogenity.

An essential factor to bear in mind when considering the treatment of ischaemic stroke is that a potential therapeutic agent must be efficacious when given after the ischaemic attack, because patients are treated after they have had a stroke. In our experiments GVS-111 demonstrated its effect when administered 1 h after thrombotic lesion and then repeatedly throughout the post-operative period. An important feature of GVS-111 is its effectiveness after intravenous administration – the most realistic way to treat stroke patients in critical conditions. Therefore, it appears reasonable to conclude that GVS-111 fits the main requirements put forward for neuroprotective substances potentially effective for stroke treatment (Hunter *et al.*, 1995).

Acknowledgement

This work was partly supported by RFBR grants 98-04-48587 and 98-04-49523.

REFERENCES

Bures J, Buresova O, Huston JP (1983). *Techniques and Basic Experiments for the Study of Brain and Behaviour*. Amsterdam: Elsevier Science.

Chepkova A, Trofimov S, Smolnikova N *et al.* (1995). The impairment of the rat's hippocampal synaptic transmission plasticity provoked by the prenatal hypoxia and its normalisation by the nootropic dipeptides. *Bull Exp Biol Med (Rus)* **120**:592–595.

Gudasheva T, Voronina T, Ostrovskaya R *et al.* (1996). Synthesis and antiamnestic activity of a series of *N*-acylprolyl-containing dipeptides. *Eur J Med Chem* **31**:151–157.

Harman PJ, Carpenter MV (1950). Volumetric comparisons of the basal ganglia of various primates including man. *J Comp Neurol* **93**:125–139.

Hunter AJ, Green AR, Cross AJ (1995). Animal models of acute ischaemic stroke: can they predict clinically successful neuroprotective drugs? *Trends Pharmacol Sci* **16**:123–128.

Kolb B (1984). Functions of the frontal cortex of the rat: a comparative review. *Brain Res Rev* **8**:65–68.

Krettek J, Price J (1977). Projections from the amygdaloid complex to the cerebral cortex and thalamus in the rat and cat. *J Comp Neurol* **172**:687–722.

Lysenko A, Uskova N, Ostrovskaya R *et al.* (1997). Antioxidant effect of dipeptidergic nootrop GVS-111. *Exp Clin Pharmacol* **60**:15–18.

Ostrovskaya RU, Gudasheva TA, Trofimov SS *et al.* (1994). GVS-111 an acyl-prolyl-containing dipeptide with nootropic properties. In: *Biological Basis of Individual Sensitivity to Psychotropic Drugs.* Seredenin SB, Longo G, Gaviragi G (editors). Edinburgh: Graffham Press, pp. 79–91.

Ostrovskaya R, Romanova G, Gudasheva T *et al.* (1997). Novel substituted acylprolyne containing dipeptide, GVS-111, promotes the restoration of the learning and memory damaged by the bilateral frontal lobectomy in rats. *Behav Pharmacol* **8**:261–268.

Ostrovskaya R, Gudasheva T, Andreeva N, Victorov I (1998). Neurometabolic effects of prolyl-containing cognition enhancer GVS-111 suggests its effectiveness in Alzheimer disease. *Neurosci Lett* Suppl. 51:S31.

Paxinos G, Watson Ch (1986). *The Rat Brain* (2nd edn). London: Academic Press.

Rodgers DC, Hunter AJ (1997). Photothrombotic lesions of the rat cortex impair acquisition of the water maze. *Pharmacol Biochem Behav* **56**:747–754.

Seredenin SB, Voronina TA, Gudasheva TA *et al.* (1995). Biologically active *N*-acylprolyldipeptides having antiamnestic, antihypoxic effects. *United States Patent,* number 5,439,930.

Siesjo BK, Siesjo P (1996). Mechanisms of secondary brain injury. *Eur J Anaesthesiol* **13**:247–268.

Solntseva E, Bukanova J, Ostrovskaya R *et al.* (1997). The effects of piracetam and its novel peptide analogue GVS-111 on neuronal voltage-gated calcium and potassium channels. *Gen Pharmacol* **29**:85–89.

Watson BD, Dietrich WD, Busto R *et al.* (1985). Induction of reproducible brain infarction by photochemically initiated thrombosis. *Ann Neurol* **17**:497–504.

Zilles K, Wree A (1985). Cortex: area and laminar structure. In: *The Rat Nervous System*. Paxinos G. (editor). Sydney: Academic Press, pp. 375–416.