Neuroprotective effect of glutamate-substituted analog of gramicidin A is mediated by the uncoupling of mitochondria

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ABSTRACT

Background: Reactive oxygen species are grossly produced in the brain after cerebral ischemia and reperfusion causing neuronal cell death. Mitochondrial production of reactive oxygen species is nonlinearly related to the value of the mitochondrial membrane potential with significant increment at values exceeding 150 mV. Therefore, limited uncoupling of oxidative phosphorylation could be beneficial for cells exposed to deleterious oxidative stress-associated conditions by preventing excessive generation of reactive oxygen species.
Methods: Protonophoric and uncoupling activities of different peptides were measured using pyranine-loaded liposomes and isolated mitochondria. To evaluate the effect of glutamate-substituted analog of gramicidin A ([Glu1]gA) administration on the brain ischemic damage, we employed the in vitro model of neuronal hypoxia using primary neuronal cell cultures and the in vivo model of cerebral ischemia induced in rats by the middle cerebral artery occlusion.
Results: [Glu1]gA was the most effective in proton-transferring activity among several N-terminally substituted analogs of gramicidin A tested in liposomes and rat brain and liver mitochondria. The peptides were found to be protective against ischemia-induced neuronal cell death and they lowered mitochondrial membrane potential in cultured neurons and diminished reactive oxygen species production in isolated brain mitochondria. The intranasal administration of [Glu1]gA remarkably diminished the infarct size indicated in MR-images of a brain at day 1 after the middle cerebral artery occlusion. In [Glu1]gA-treated rats, the ischemia-induced brain swelling and behavioral dysfunction were significantly suppressed.
Conclusions: The glutamate-substituted analogs of gramicidin A displaying protonophoric and uncoupling activities protect neural cells and the brain from the injury caused by ischemia/reperfusion.
General significance: [Glu1]gA may be potentially used as a therapeutic agent to prevent neuron damage after stroke.

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1. Introduction

Stroke remains the number 1 cause of disability and the third leading cause of deaths. Worldwide, the burden of ischemic and hemorrhagic stroke increased significantly in the period between 1990 and 2010 in terms of the absolute number of people with these pathologies [1]. Despite the significant socioeconomic burden attributed to stroke, there are no therapeutic approaches currently available besides thrombolytic treatments, which have a narrow permissive time window after the stroke and limited availability [2]. Therefore, the development of novel neuroprotective drugs is a crucial issue for treatment of brain ischemia.

Many ischemia-induced neurological pathologies including stroke are associated with high oxidative stress [3]. The shift of mitochondrial redox balance in any direction may cause a wide spectrum of neurological disturbances from oxidative modification of vital components to selected cell death resulting in neurological deficit or, in extreme case, a death of the organism. An anti-oxidative strategy is needed with specific attention to mitochondria being both a source of, and a target for reactive oxygen species (ROS) accompanying oxidative stress associated with ischemia. Thus, the central role of mitochondria in launching and enhancing the pathological cascades caused by oxidative stress makes them possible target for pharmacological intervention against oxidative stress-related pathologies. It is generally accepted that mitochondrial production of reactive oxygen species is nonlinearly linked to the

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value of the mitochondrial membrane potential with significant increment of H_2O_2 generation at values exceeding 150 mV. Due to this, high values of the membrane potential may be deleterious, specifically under pathological conditions associated with oxidative stress [4,5]. Thus, it may be concluded that extrinsic chemical uncouplers reduce the membrane potential by shunting protons directly across the inner mitochondrial membrane and thereby reducing the production of mitochondrial ROS.

Cytotoxicity of uncouplers is generally attributed to an excessive increase in proton conductivity of the inner mitochondrial membrane leading to an uncontrolled decrease in ATP synthesis and resultant cell death [6]. Besides, all known uncouplers inhibit respiratory chain at high concentrations. In our previous publication we showed that a proton channel formed by the pentadecapeptide gramicidin A with glutamate substituted for valine at the N-terminus ([Glu1]gA), effectively decreased membrane potential of mitochondria in cells while exhibiting low cytotoxicity [7]. This observation was quite unexpected since the parent peptide gA exhibits very high cytotoxicity and can be used as a topical antibiotic only. It is generally accepted that in bilayer lipid membranes gA acquires a $\beta^{6.3}$-helical conformation and head-to-head association of two gA molecules via six hydrogen bonds that leads to the formation of a transmembrane channel [8–12]. N-terminal modifications of gA were shown to affect substantially its channel properties, leading even to complete inability of channel formation. Noteworthy, almost complete loss of potassium conductivity in case of desformylgramicidin was combined with much lesser reduction of proton conductivity with respect to parent gA [13,14], so that the desformylated peptide was able to uncouple oxidative phosphorylation [15]. It was suggested that low cytotoxicity of [Glu1]gA is a result of suppressed permeability for potassium and sodium ions while retaining permeability for protons [7]. Besides, low cytotoxicity could be attributed to the increased capability of [Glu1]gA to permeate through membranes and/or redistribute between different membranes in cells [7].

The present study dealt with a series of gA analogs that were N-terminally modified with protonatable amino acid residues — a glutamic acid and an aspartic acid. Based on the relationship between $\Delta\Psi_m$ and mitochondrial ROS production we hypothesized that administration of substituted analogs of gramicidin A as mitochondrial uncoupling agents might be an effective and safe approach for the treatment of acute ischemic stroke, while avoiding side effects and associated systemic toxicities that typically occur with classic uncoupling agents.

2. Materials and methods

2.1. Synthesis of gA analogs

Analogs of gramicidin A, $i.e.$ [Glu1]gA (HCO-EGALAVVVWLWLWLW-NH(CH$_2$)$_2$OH), [Glu3]gA (HCO-VGELAVVVWLWLWLW-NH(CH$_2$)$_2$OH), [Glu2]gA (HCO-VEALAVVVWLWLWLW-NH(CH$_2$)$_2$OH), [Glu5]gA (HCO-VGALEVVVWLWLWLW-NH(CH$_2$)$_2$OH), and [Asp1]gA (HCO-DGALAVVVWLWLWLW-NH(CH$_2$)$_2$OH), were prepared by standard solid-phase Nα-Fmoc strategy on 2-chloro-trytil chloride polystyrene resin using the diisopropylcarbodiimide/1-hydroxybenzotriazole coupling system and 4-methyl-piperidine for Fmoc-group deprotection. Fmoc-ethanolamine was attached to the resin in dry DCM with N-ethyldiisopropylamine. N-terminal formylation was carried out with 2-nitrophenyl formate and N-ethyldiisopropylamine. The peptides were cleaved off the resin and deprotected with trifluoroacetic acid-1,2-ethanedithiol–water (94:3:3) mixture and purified by RP-HPLC up to >95%. Synthesis conformity was checked by MALDI-TOF MS of the resultant peptides. Gramicidin A (gA) was from Fluka.

2.2. Use of animals

Experimental procedures were conducted in accordance with the European Community Council directives 2010/63/EU and the study was approved by the local institutional animal ethics committee. Experiments were performed on outbred white male rats (320–350 g). The animals had unlimited access to food and water and were kept in cages with a temperature controlled environment (20 $\pm$ 1 °C) with lights on from 9 AM to 9 PM. For all surgical procedures rats are anesthetized with i/p injection of 300 mg/kg (12%) chloral hydrate. A feedback-controlled heating pad maintained core temperature (37.0 $\pm$ 0.5 °C) during ischemia supplemented with an infrared lamp until awake.

2.3. Isolation of rat liver and brain mitochondria

Experiments were performed on outbred white male rats (180–200 g) fed $ad libitum$. Animal protocols were approved by the Institutional Review Boards. Rat liver mitochondria as a conventional and easy model of isolated mitochondria of a high purity were prepared by differential centrifugation [16] in a medium containing 250 mM sucrose, 10 mM MOPS, 1 mM EGTA, and bovine serum albumin (0.1 mg/ml), pH 7.4. The final washing was performed in the medium of the same composition. Protein concentration was determined using bicinchoninic acid as described in [17]. Rat brain mitochondria were isolated as previously described by Sims [18]. In brief, the brains were rapidly removed and placed in the ice-cold isolation buffer containing 0.32 M sucrose, 1 mM K$_2$EDTA, and 10 mM Tris base (pH 7.1) then brain fragments were broken with 10 strokes in a glass homogenizer. The homogenate was spinned at 1330 $\times g$ for 5 min. The supernatant was collected and recentrifuged at 21,200 $\times g$ for 10 min. Final pellet was collected and resuspended in 10 ml of isolation buffer containing 0.02% digitonin. After 10 min of keeping on ice the suspension was spinned at 6900 $\times g$ for 10 min. The fluffy white layer surrounding the mitochondria pellet (brown) was removed and mitochondria were resuspended in 12% Percoll solution with 0.32 M sucrose, 1 mM K$_2$EDTA, 10 mM Tris base (pH 7.1) and centrifuged at 6900 $\times g$ for 10 min. The supernatant was removed and the pellet was resuspended in isolation buffer with further centrifugation at 6900 $\times g$ for 10 min.

2.4. Mitochondrial respiration

Respiration of isolated mitochondria was measured using a standard polarographic technique with a Clark-type oxygen electrode ("Oroboros", Austria) at 25 °C using DATLAB software. The incubation medium contained 300 mM sucrose, 20 mM MOPS, 0.5 mM EDTA, 5.5 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, and 0.5 mg/ml BSA, pH 7.4.

2.5. Measurement of hydrogen peroxide

Hydrogen peroxide production was measured fluorimetrically employing the dye Amplex Red (Molecular Probes, Eugene, OR, USA) in combination with horseradish peroxidase [19]. In these experiments, the incubation medium was supplemented with 1 μM Amplex Red, and 5 U/ml horseradish peroxidase. The detection of H$_2$O$_2$ in mitochondrial suspensions was recorded as an increase in fluorescence of the dye at 585 nm with the excitation wavelength set at 550 nm. The dye response was calibrated by sequential additions of known amounts of hydrogen peroxide solution.

2.6. Proton transport in pyranine-loaded liposomes

The lumenal pH of the liposomes was assayed with pyranine [20] as described in [21] by a slightly modified procedure of [22]. To prepare pyranine-loaded liposomes, lipids (5 mg palmityloleoyl phosphatidylcholine (POPC) and 1 mg cholesterol) in a chloroform suspension were dried in a round-bottom flask under a stream of nitrogen. The lipids were then resuspended in buffer (100 mM KCl, 20 mM MES, 20 mM MOPS, 20 mM Tricine titrated with KOH to pH 6.0) containing 0.5 mM pyranine. The suspension was vortexed and then freeze–thawed three times. Unilamellar liposomes were prepared by extrusion

through 0.1-μm-pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound pyranine was then removed by passage through a Sephadex G-50 coarse column equilibrated with the same buffer solution. To measure the rate of pH dissipation in liposomes with lumenal pH 6.0, the liposomes were diluted in a solution buffered to pH 8 and supplemented with 10 mM p-xylene-bis-pyridinium bromide to suppress the fluorescence of leaked pyranine. The pH was estimated from the ratio F_{455}/F_{410} of the intensities of fluorescence measured at 505 nm upon excitation at 455 nm (F_{455}) and 410 nm (F_{410}), respectively [22], as monitored with the Panorama Fluorat 02 spectrofluorimeter. At the end of each recording, 1 μM nigericin was added to dissipate the remaining pH gradient.

2.7. Middle cerebral artery occlusion model of focal ischemia

Middle cerebral artery occlusion (MCAO) surgery or sham operation was performed as previously described [23]. Briefly, the right common carotid artery was exposed through a midline cervical incision. A heparinized intraluminal silicon-coated monofilament with Ø 0.25 mm was introduced *via* the external carotid artery into the internal carotid artery to occlude blood supply to the middle cerebral artery. After 60 min of occlusion, the filament was gently pulled out and the external carotid artery was permanently closed by cauterization. In sham-operated rats, the right common carotid artery was exposed and the external carotid artery was electrocoagulated without introducing the filament into the internal carotid artery. The rats were treated intranasal (i/n) by [Glu1]gA administered immediately after reperfusion. Saline (20 μl) containing [Glu1]gA at doses of 50, 5 and 0.5 μg/kg was administered intranasally as drops with a small pipette every 5 min into both sides of the nasal cavity, followed by 5 μl for the last dose (for a total of 20 min). Rats were randomly divided into the following groups: (1) Sham + Saline (n = 6), (2) MCAO + Saline (n = 8), (3) MCAO + [Glu1]gA 0.5 μg/kg (n = 7), (4) MCAO + [Glu1]gA 5 μg/kg (n = 7), and (5) MCAO + [Glu1]gA 50 μg/kg (n = 5). Infarct volume was quantified by analyzing brain MR-images obtained 24 h after the MCAO as described previously [24]. Brain swelling was also measured in MR-images and calculated using a formula: swelling (edema) = (the volume of the right hemisphere − the volume of the left hemisphere) / the volume of the left hemisphere [25]. Ischemic damage volume for each group was normalized to the mean for the group MCAO + Saline.

2.8. Limb-placing test

The modified version of the limb-placing test consisting of seven tasks was used to assess forelimb and hindlimb responses to tactile and proprioceptive stimulation [26]. The rats were habituated for handling and tested before operation and after the reperfusion for 24 h. For each task, the following scores were used: 2 points, normal response; 1 point, delayed and/or incomplete response; 0 point, no response. The integral score was evaluated over seven tasks.

2.9. Primary neuronal cultures of cerebral cortex

Primary neuronal cultures of cerebral cortex were obtained from embryos (16–18 days) of outbred white rats. Cultures were prepared according to Brewer [27] with the following modifications: cerebral cortex was dissected, meninges were removed and tissue was incubated for 15 min in trypsin/EDTA (0.05/0.02% wt./vol. in PBS) at 37 °C; the cultures were rinsed twice with PBS and once with dissociation medium (modified Eagle's medium with 10% fetal calf serum, 10 mM HEPES, 100 U penicillin–streptomycin/ml, 2 mM L-glutamine); dissociated by Pasteur pipette in dissociation medium, pelleted by centrifugation (210 g for 2 min at 21 °C) and redissociated in Neurobasal medium (NBM) with supplemental B27 (Invitrogen, Paisley, UK), 100 U penicillin–streptomycin/ml, 0.5 mM L-glutamine. Cell suspension was applied

to poly-L-lysine-coated 96-well plates. Cultures were kept at 37 °C and 5% CO_2 and after 4 days *in vitro* twice a week one-half of the medium was replaced by a basal medium. The cultures were maintained up to day *in vitro* (DIV) 7 and then used for experiments. The mitochondrial membrane potential in cultured neurons was evaluated by the fluorescent probe JC-1 forming J-aggregates (red fluorescence) at high values of the potential and displaying green fluorescence at low values of the membrane potential [28].

2.10. Oxygen–glucose deprivation

To achieve oxygen–glucose deprivation (OGD), a technique similar to that described by [29] was used. Primary rat cortical neurons were used for OGD experiments after a cultivation period of 7–8 DIV. For OGD the cultures were washed twice in glucose-free basic salt solution of the following content (in mM): NaCl 116, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 0.8, NaH_2PO_4 1; pH 7.3 and incubated in this solution in a humidified chamber filled with nitrogen (37 °C) for 5 h. Control cultures were incubated in NBM with supplemental B27 minus antioxidants under normoxic conditions (in a CO_2-incubator). Immediately after OGD and in control cultures, medium was removed and replaced with 100 μl per well of NBM-B-27 minus antioxidants. When treated, neurons received either [Glu3]gA or [Glu1]gA or gA at a dose of 2.5 μg/ml during the OGD. Cell injury was assessed using phase contrast microscopy and by measuring lactate dehydrogenase release (LDH) in the supernatant 24 h after OGD. Each value was derived from a minimum of eight cultures from three independent experiments.

2.11. Determination of lactate dehydrogenase activity

Lactate dehydrogenase activity in the cultivation medium reflected cell viability [30] in experiments examining the effects of OGD. Lactate dehydrogenase released from damaged cells was measured by standard kinetic assay for pyruvate (Hoffmann-La Roche Ltd., Basel, Switzerland). Briefly, culture medium was collected from each well and centrifuged at 10,000 g for 10 min. Supernatant was analyzed using A25 BioSystems biochemistry analyzer (BioSystems S.A., Spain) with the manufacturer's protocols for LDH activity.

2.12. Statistical analysis

Statistical analyses were performed using STATISTICA 7.0 for Windows (StatSoft, Inc.). Values given as mean ± standard error of means (SEM) apart from the neurological deficit scores are expressed as median ± interquartile ranges; the 25th to 75th percentiles are shown in the parentheses. Variance homogeneity was assessed with Levene's test. Statistical differences between groups in the data of infarct volume, brain swelling and LDH-release were analyzed using one-way ANOVA with Tukey's post hoc test. Statistical differences in limb-placing tests between groups were analyzed using Kruskal–Wallis test with the Mann–Whitney *u*-test (the Bonferroni's post hoc correction was applied). Differences were considered significant at P ≤ 0.05.

3. Results

3.1. Protonophoric activity of gramicidin A and its analogs

According to [20,31–33] liposomes loaded with the pH-sensitive probe pyranine can serve as a useful system to study the action of protonophores. Fig. 1 shows the kinetics of dissipation of a pre-formed pH gradient on membranes of liposomes after the addition of gramicidin A and its analogs. The effect of the peptides on the pH gradient developed within minutes and the induction of the proton permeability decreased in the series: gA > [Glu1]gA > [Glu3]gA > [Glu2]gA > [Glu5]gA,[Asp1]gA (Fig. 1). Noteworthy, the protonophoric assay with pyranine-loaded liposomes is usually carried out in the presence of
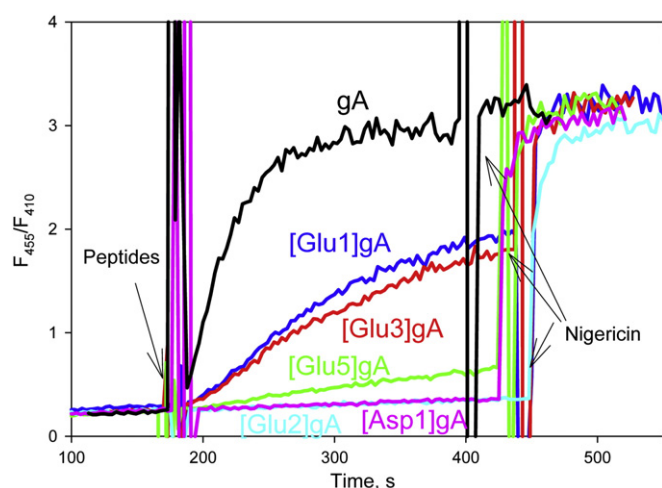
Fig. 1. Dissipation of pH gradient by different peptides (concentration 0.2 μg/ml) on membranes of pyranine-loaded liposomes formed from POPC/cholesterol. Inner liposome pH was estimated from the ratio (F455/F410) of pyranine fluorescence intensities measured at 505 nm upon excitation at 455 nm and 410 nm, respectively. 1 μM nigericin was added at 400–450 s to equilibrate the pH. Other conditions: see Materials and methods. Lipid concentration was 20 μg/ml.
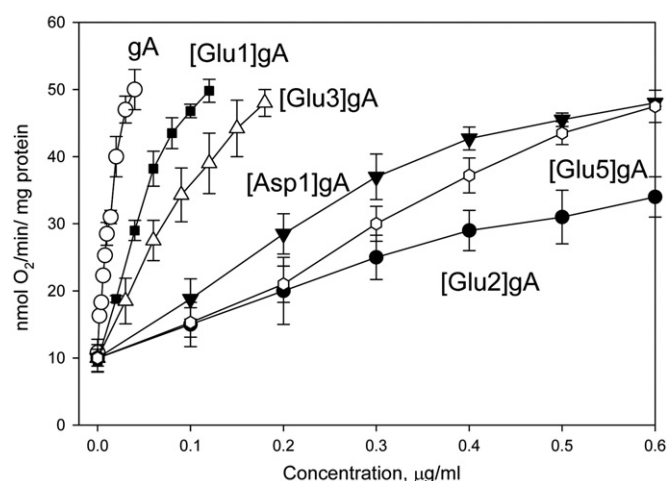

Fig. 2. Effect of gramicidin A and its analogs on rat liver mitochondria respiration. Dose dependence of stimulation of respiration by gA, [Glu1]gA, [Glu3]gA, [Glu2]gA, [Glu5]gA, and [Asp1]gA. Points and error bars represent mean ± S.E. with at least three independent experiments. Substrate, succinate (5 mM) in the presence of 2 μM rotenone. FCCP-mediated maximal respiration was 61 ± 3 nmol O_2/min/mg protein.

valinomycin, the powerful K^+-ionophore. No pH changes could be observed with FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone) in the absence of valinomycin. According to [20], this effect is associated with Δψ formation on liposome membranes, which blocks proton transport without valinomycin. However, the peptide-mediated pH changes were insensitive to the presence of valinomycin in the system (data not shown). Apparently, this result can be attributed to the ability of the peptides to induce to some extent the potassium ion permeability as well as proton fluxes. The addition of nigericin at the end of each pH trace caused the dissipation of the remaining pH gradient on the membrane of liposomes showing the upper limit of the effect.

The series of amino acid-substituted peptides shows high specificity to carboxyl-containing amino acids and to their position. [Glu1]gA exhibited substantially higher proton conducting activity compared to [Asp1]gA, although glutamic acid differs from aspartic acid by one methylene group only. It can be assumed that the position of the carboxylic group with respect to proton conducting pathway plays a crucial role and in the case of [Asp1]gA it blocks the channel. Interestingly, glutamic acid in position 1 suppressed the gA proton conducting pathway to a lesser extent compared to other positions studied (Fig. 1).

3.2. Uncoupling of isolated mitochondria by gramicidin analogs and induced changes in mitochondrial ROS production

Gramicidin A is known to stimulate respiration and decrease membrane potential of isolated mitochondria [34] which is related to a phenomenon of respiratory control, *i.e.* the limitation placed on electron transport by the chemiosmotic gradient of proton ions on the inner mitochondrial membrane [35,36]. Uncouplers diminish the proton gradient and its control of the electron flow through the respiratory chain thus making electron transport/respiration run freely. It has been shown in our previous work that [Glu1]gA decreases the membrane potential in a way similar to parent gA [7]. In the present work we compared the stimulation of respiration of rat liver mitochondria by gA and different glutamate- and aspartate-substituted analogs. Fig. 2 shows concentration dependences of the stimulation of respiration of mitochondria with succinate as a substrate. The C_{50} values for gA, [Glu1]gA, [Glu3]gA, [Asp1]gA, [Glu5]gA, and [Glu2]gA were about 0.015 μg/ml, 0.04 μg/ml, 0.1 μg/ml, 0.2 μg/ml, 0.3 μg/ml, and 0.4 μg/ml, respectively. Therefore, the order of uncoupling activity of the glutamate-substituted peptides was similar to that of protonophoric

activity on liposomes. This result is in line with the general view of the close relationship between the protonophoric action of a compound and its ability to decrease mitochondrial membrane potential and to stimulate respiratory proton pumps [35,36]. However, the action of [Asp1]gA on mitochondria was substantially stronger than of [Glu5]gA (Fig. 2), while the reverse situation was observed on liposomes (Fig. 1). This effect can be attributed to the lipid-dependence of the action of the peptides, specifically to different dependence of the channels of different structures on the lipid environment. In fact, the ion channel activity of gramicidin A and its analogs is lipid dependent, especially exhibiting dependence on the thickness of the membrane [37] which can be different in liposomes and mitochondria. Besides, the uncoupling action of gramicidin analogs can proceed "locally", *i.e.* due to a cycling of protons within a proton domain operationally located at or near the proton pump as suggested in [38].
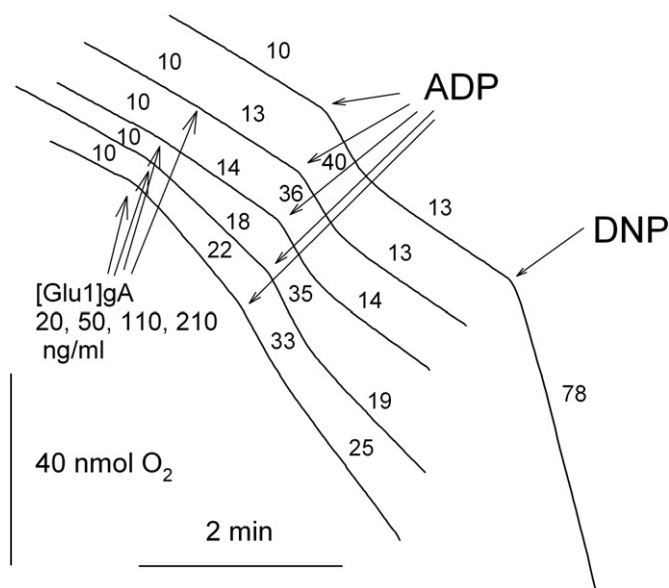

Fig. 3. Effect of different concentrations of [Glu1]gA on the basal respiration rate and respiration rate in states 3 and 4 of isolated rat liver mitochondria. Numbers at different parts of each record are respiration rates in relative units. Maximal respiration rate for control mitochondrial suspension could be reached by the supplement of 40 μM 2,4 dinitrophenol (DNP).

A

ADP/O=2.0　　RC=3.6
ADP/O=1.35　RC=2.0
ADP/O=1.2　　RC=1.6

[Glu1]gA
10 ng/ml

[Glu1]gA
20 ng/ml

2 min

20 nmol O$_2$

B

[Glu1]gA
10 ng/ml

[Glu1]gA
20 ng/ml

20 nmol O$_2$

[Glu1]gA
30 ng/ml

[Glu1]gA
40 μg/ml

[Glu1]gA
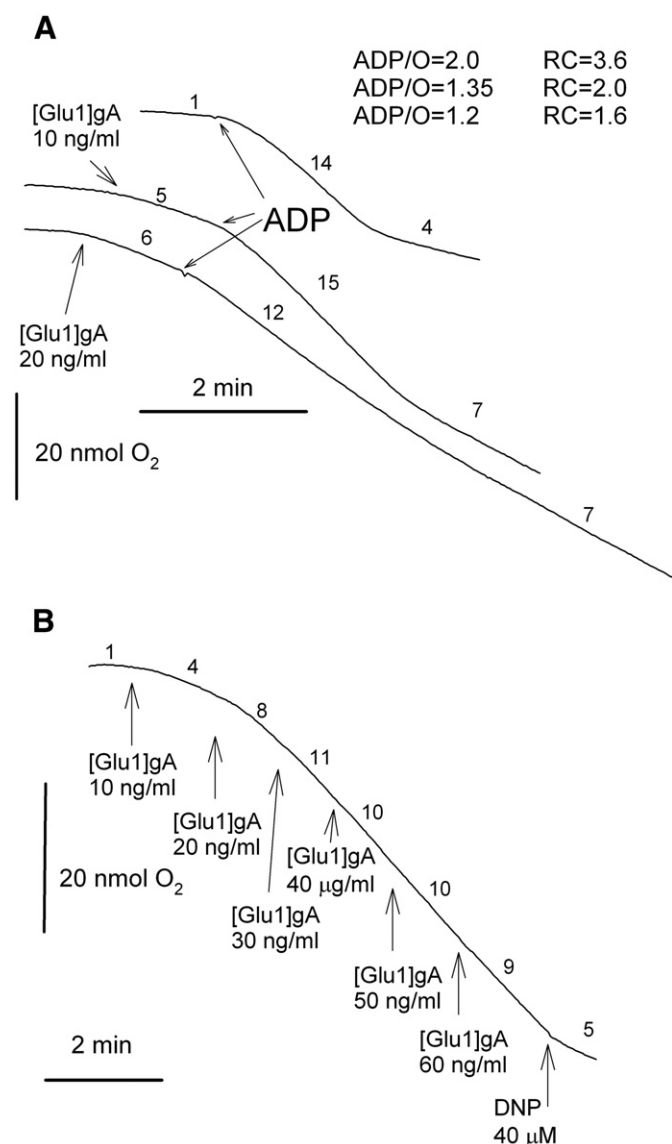50 ng/ml

[Glu1]gA
60 ng/ml

DNP
40 μM

2 min

Fig. 4. Effect of different concentrations of [Glu1]gA on the respiration of isolated rat brain mitochondria. A, The effect on the basal respiration rates and rates in states 3 and 4; top right, the values of ADP/O and respiratory control (RC) for control sample (top line) and of those at 10 and 20 ng/ml [Glu1]gA; B, Titration of the respiration rates with different concentrations of [Glu1]gA. DNP, 40 μM 2,4 dinitrophenol; numbers at different parts of each record are respiration rates in relative units.

Fig. 3 gives an indicative example of uncoupling activity of different concentrations of [Glu1]gA in liver mitochondria upon the addition of certain amount of ADP. As expected, uncoupling activity of gramicidin A analogs in liver mitochondria was concentration-dependent with increased basal respiration rate and respiration in state 4 going in parallel with increased concentration of gramicidin derivatives.

Isolated mitochondria from rat brain demonstrate even higher sensitivity to uncoupling potency of [Glu1]gA (Fig. 4) with maximal effect reached at 50 ng/ml (Fig. 4B). Note that in brain mitochondria [Glu1]gA concentrations higher than 10 ng/ml in concert with mild uncoupling inhibit respiratory chain (seen by a respiration rate at state 3, Fig. 4A) while this inhibition in rat liver mitochondria could be observed at 210 ng/ml (Fig. 3). As in liver mitochondria [Glu1]gA in brain mitochondria decrease ADP/O and respiratory control (Fig. 4A).

Since increased electron flux along mitochondrial respiratory chain associated with uncoupling potentially results in lower ROS production [36] we evaluated the rate of hydrogen peroxide production in isolated mitochondria measured using Amplex Red. H$_2$O$_2$ production supported

A

20 nM H$_2$O$_2$

Succinate

CCCP

[Glu1]gA

200 s

B

20 nM H$_2$O$_2$

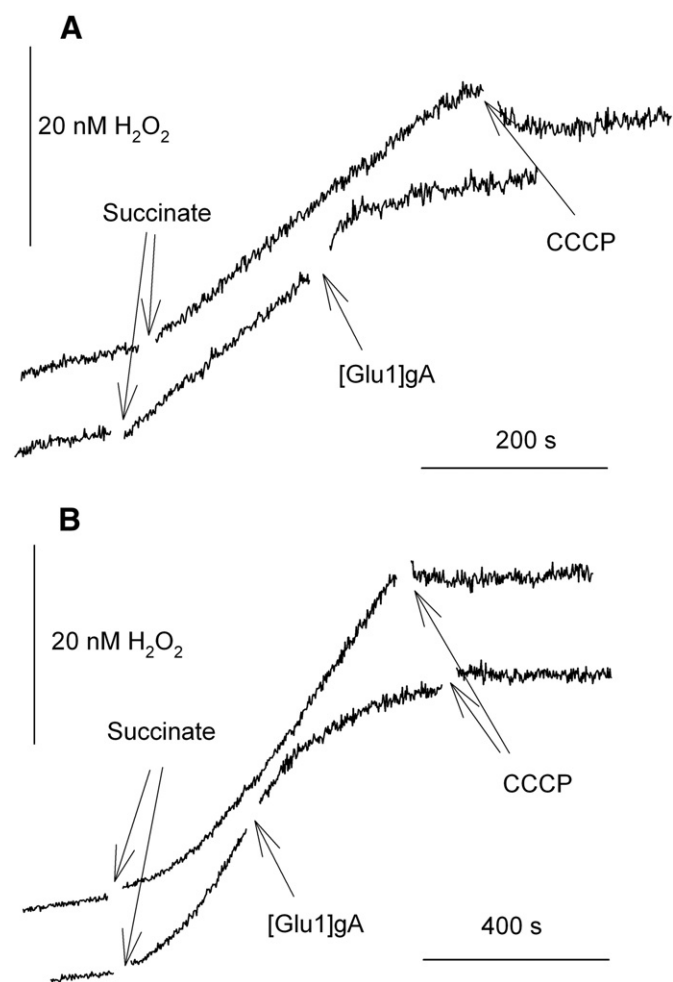Succinate

CCCP

[Glu1]gA

400 s

Fig. 5. Effect of [Glu1]gA on H$_2$O$_2$ production in rat liver (A) and brain (B) mitochondria. Incubation medium: 250 mM sucrose, 20 mM MOPS, 5 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 1 mM EGTA, 1 μM Amplex Red, and 5 U/ml horseradish peroxidase, pH 7.4. 3 mM succinate, 20 ng/ml [Glu1]gA and 0.5 μM CCCP (carbonyl cyanide m-chloro phenyl hydrazone) were added as shown. Each sample contained 0.5 mg/ml of mitochondrial protein.

by succinate (in the absence of rotenone) was rather high in both liver (Fig. 5A) and brain (Fig. 5B) mitochondria. Uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) as well as [Glu1]gA remarkably diminished H$_2$O$_2$ production in both mitochondrial suspensions. Potentially, lower ROS production especially under conditions of unwanted oxidative stress may be beneficial for organs including the brain.

3.3. Neuroprotective effect of substituted analogs of gramicidin A in OGD model

Although elevated mitochondrial respiration rate in many cases is an index of uncoupling of oxidation and phosphorylation, actual proof of the uncoupling process is an indication of the drop of the mitochondrial transmembrane potential. Fig. 6A demonstrates the effect of [Glu1]gA on the mitochondrial ΔΨ values in rat neuronal cultural cells estimated by a fluorescent membrane potential probe JC-1. The loss of mitochondrial red fluorescence and prevailing green fluorescence after administration of [Glu1]gA were reported on partial mitochondrial uncoupling.

We revealed that [Glu1]gA and [Glu3]gA were the most effective uncouplers, based on data obtained from the mitochondrial respiration and protonophoric activity measured in liposomes. The model of OGD on cultural neurons was used to estimate the capability of these peptides to protect neurons from ischemia-induced cell death. Neurons were exposed to 5 h OGD on 7–8 DIV and LDH-release was measured after 24 h of reoxygenation. Some cultures were treated with [Glu3]gA
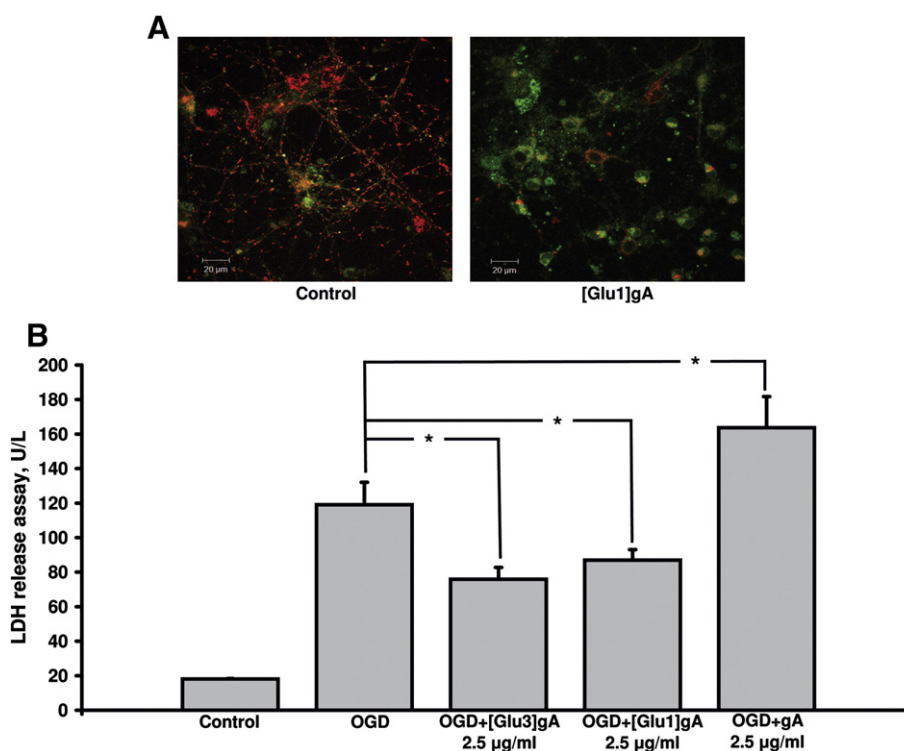
Fig. 6. Protective effect of substituted analogs of gramicidin A on OGD-induced damage of primary cultures of neurons isolated from cerebral cortex. (A) The representative fluorescent photomicrographs of control cortical neuronal cells and of those exposed to 2.5 μg/ml [Glu1]gA treated with 100 nM JC-1. While control cells contain numerous mitochondria carrying red fluorescence (high membrane potential), in cells treated with [Glu1]gA high potential mitochondria occupy much lesser areas. (B) Quantification of the viability of cortical neurons measured by LDH-release. OGD induced increase of LHD release in cultural medium while treatment with [Glu3]gA and [Glu1]gA but not gA alleviated it. * denotes significance from the OGD group (P < 0.05); number of cultures for each experiment = 8.

or [Glu1]gA or gA at a dose of 2.5 μg/ml during OGD. As shown in Fig. 6B, neurons exposed to OGD and 24 h reoxygenation underwent a significant cell death as assessed by LDH release. Neuronal cell death in this model was characterized by rounding and swelling of the cells apparently demonstrating that the majority of degenerating neurons die by a necrotic pathway (Suppl. 1). These observations of consistent cell death produced by our OGD-reperfusion model *in vitro* in parallel with *in vivo* experiments demonstrate that reperfusion causes a massive brain damage and severely impairs neurological functions when blood supply returns to the lesion area after a period of ischemia [39,40]. Substantial neuroprotection was observed in cells treated by [Glu1]gA and [Glu3]gA (Fig. 6B) where LDH-activity in culture medium drops from 119 ± 13.1 (untreated cells after OGD) to 87 ± 6.8 and 76 ± 6.1 U/l, respectively (P < 0.05). Neuronal cell death in [Glu1]gA-treated cell cultures is characterized by initial cell shrinkage with minimal cell swelling (Suppl. 1). Under identical conditions, gA did not possess any protective effect and even aggravated neuronal damage at the same concentration.

3.4. [Glu1]gA protects against ischemic brain injury

Our findings in the *in vitro* OGD model provided strong rationale to examine whether [Glu1]gA is able to protect brain tissue against ischemia-induced damage *in vivo*. To address this hypothesis, we used MCAO focal ischemia model that was found to be optimal in lowering infarct size variability [41]. After MCAO, we observed extensive cortical and striatal damage and also variable partial damage to the hypothalamus and amygdale outside the vascular territory of the middle cerebral artery. When compared with the vehicle group, the treatment with [Glu1]gA which was initiated immediately after reperfusion at doses of 0.5 and 5 μg/kg significantly reduced infarct volume by 23% and 30%, respectively (P < 0.05) (Fig. 7B). Moreover, the treatment with 5 μg/kg [Glu1]gA significantly reduced brain swelling (P < 0.05) (Fig. 7C). There was no significant effect on infarct volume and brain

swelling after treatment with 50 μg/kg [Glu1]gA (Fig. 7C). The neurological score after 24 h of reperfusion demonstrates that [Glu1]gA significantly decreases neurological deficit of the ischemic animals. While the intact rats before the induction of ischemia scored 14 (14–14) in limbplacing test, and sham-operated animals scored 13.75 (13.13–14), rats after ischemia demonstrated only 2 (2–2.25) (P < 0.05). Treatment with [Glu1]gA at doses of 0.5 and 5 μg/kg reduced the neurological deficits to 5 (4–5) and 8 (6–8) scores, respectively (P < 0.05) (Fig. 7D). Dose of 50 μg/kg [Glu1]gA was not protective. This finding, in line with *in vitro* results, strongly supports the idea about [Glu1]gA's neuroprotective ability in ischemia-induced stroke.

4. Discussion

Blockade of blood flow in major blood vessels leads to a state of ischemia associated with oxidative stress where mitochondria play an essential role as a source of ROS and, simultaneously, their main target for ROS. ROS in small amounts are necessary for cellular metabolism, however, their increased levels in pathologies is a threat to the existence of the cell and organ. Oxidative stress is one of the most important factors that exacerbate brain damage after cerebral ischemia and reperfusion [42]. It is believed, that mitochondria play a critical role in ischemia-induced brain pathologies by producing excessive amounts of ROS and reactive nitrogen species [43,44]. The burst of these aggressive species is involved in direct cytotoxic effects, including protein and lipid peroxidation, oxidative DNA damage, and post-ischemic inflammatory injury. This occurs through redox-mediated signaling pathways resulting in pathological modification of cellular compartments including mitochondrial respiratory chain and ATP synthase finally yielding a substantial cell death [45–47]. Therefore, the control of ROS production is important for achieving neuroprotection against ischemia/reperfusion injury.
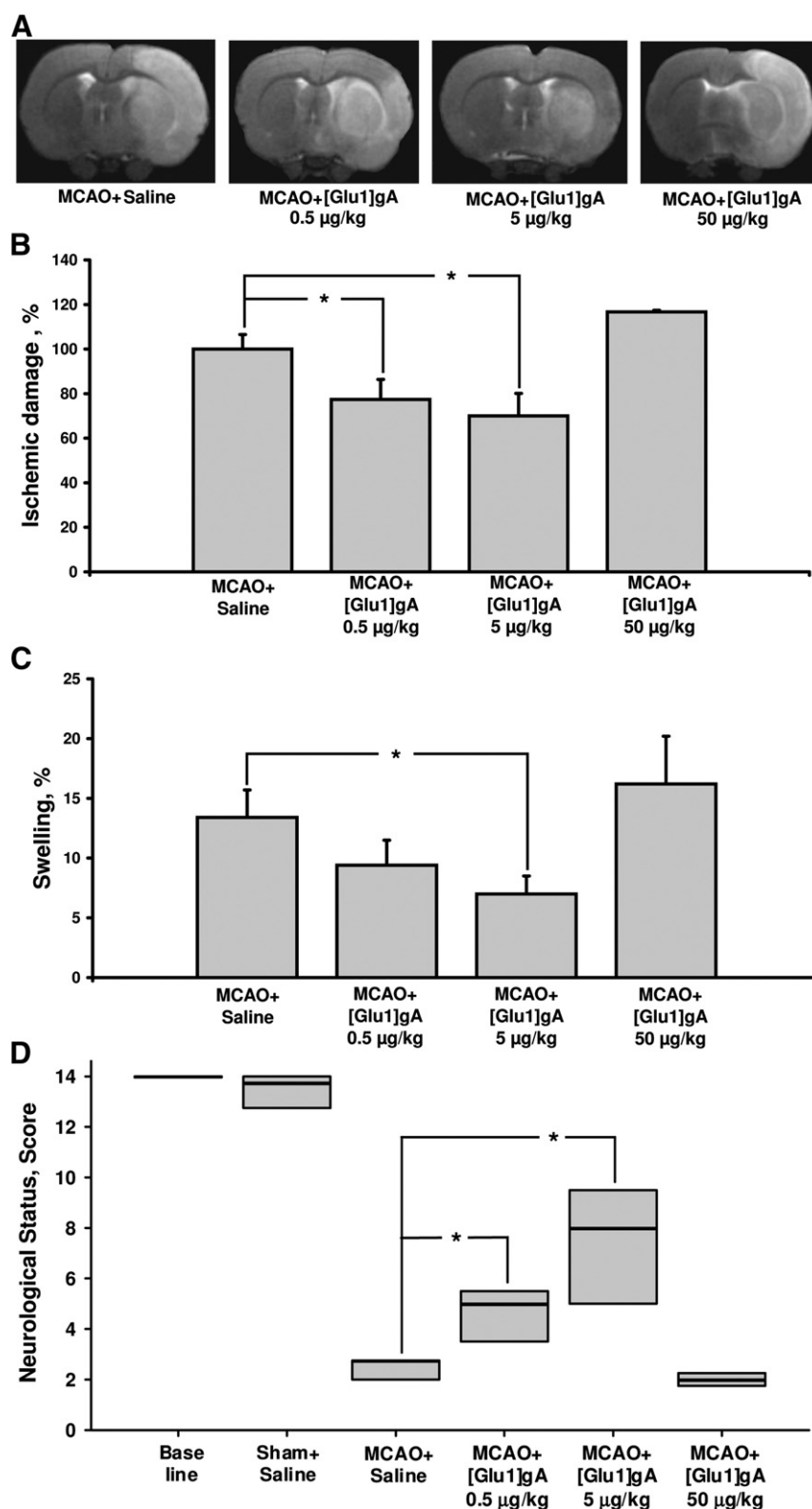
Fig. 7. Post-insult [Glu1]gA treatment reduces ischemia/reperfusion-induced brain damage. The animals were subjected to ischemia for 1 h followed by reperfusion for 24 h. [Glu1]gA was instilled i/n immediately after reperfusion at doses of 0.5, 5 and 50 μg/kg. (A) Representative T2-weighted MR-images were obtained 24 h after reperfusion (each image covered 0.8 mm thick brain section). Hyperintense regions refer to ischemic areas. (B) Infarct volume and (C) brain edema (swelling) evaluated by using MRI with analysis of T2-weighted images. (D) Neurological status estimated using limb-placing test. The box indicates the interquartile ranges, and the solid bar indicates the median. * denotes significant difference from the MCAO + Saline group ($P < 0.05$) (one-way ANOVA, followed by Tukey's post hoc analysis for (B) and (C); Kruskal–Wallis test with the Mann–Whitney u-test for (D)).

It is known that partial uncoupling of oxidative phosphorylation and respiration can decrease the generation of ROS by mitochondria [4]. Thus, one of the approaches to protect ischemic brain from damage is the treatment with chemical uncouplers [48–50]. Controlled uncoupling that prevents undesirable consequences of excessive mito-chondrial membrane potential should be one of the major goals of

neuropharmacology. Gramicidin A conventionally behaving as an uncoupler in isolated mitochondria and chloroplasts could be considered as a candidate for mitochondrial uncoupling in tissues [7,15]. However, gA is characterized by high toxicity, in particular, it perturbs ion balance across plasma membrane even at low concentrations [51]. Therefore, to avoid the gA toxicity, one could try to alter its ionic selectivity by modifying the amino acid sequence or attaching certain groups to its N-termini.

In this study we examined a series of gA analogs that were N-terminally modified with protonatable amino acid residues — glutamic acid and aspartic acid. We were searching for the most effective uncoupler with the lowest toxic effects on cells. The neuroprotective effects of substituted analogs of gramicidin A on neurons cell culture and animal model of brain ischemia and their activity as uncouplers *in vitro* were comparatively analyzed in this work. Comparison of various glutamate-substituted analogs of gramicidin A showed that the most effective uncouplers were [Glu1]gA and [Glu3]gA. Further investigation of the effect of these compounds on neuronal cultures showed their high potency in preventing cell death caused by OGD, as an *in vitro* model of stroke. At the same time, the native gA was toxic in these experiments. Previously, we have shown that the toxicity of [Glu1]gA was observed at concentrations by an order of magnitude higher than those of gA and did not appear at concentrations that efficiently reduce mitochondrial membrane potential in cells [7]. Based on these data, we selected [Glu1]gA for studying its neuroprotective properties in a model of stroke *in vivo*. We used the intranasal administration of the peptide due to several reasons. First of all, intranasal administration provides many advantages for clinical use, in practice it is non-invasive and easy to apply for the fastest in direct delivering of therapeutic agents to the brain. Intranasally administered drugs penetrate the brain parenchyma and cerebrospinal fluid within minutes using an extracellular route through perineural and/or perivascular spaces through the unique anatomical connection provided by the olfactory and trigeminal nerves [52]. In addition, this approach has been already applied for peptide/protein drug delivery because of: 1) bypassing the blood–brain barrier, 2) avoidance of hepatic first-pass drug metabolism, and 3) elimination of the need for systemic delivery, thereby reducing systemic adverse effects. A number of studies demonstrated advantages of intranasal peptide administration for treatment of stroke [53–55].

In this study we showed that [Glu1]gA administered intranasally effectively protects the brain from ischemic injury in doses 1000 times smaller than the classical uncouplers FCCP and DNP (2,4 dinitrophenol), for which the therapeutic dose for experimental stroke is about 5 mg/kg [49,50]. Moreover, it is well known that DNP has very narrow therapeutic window since in rodents the toxic concentrations of DNP exceeded therapeutic concentration only 2–3 fold [56,57]. DNP was widely used in clinical practice for weight loss at the beginning of the 20th century and was banned by the FDA due to multiple deaths caused by side-effects associated with overdosing and severe uncoupling of mitochondria [58–61]. Thus, the classical and well studied uncouplers, DNP and FCCP, seem not to have further perspectives to be used in clinics as neuroprotectors, and there is a need for developing new ones for effective stroke treatment. As it was shown previously, [Glu1]gA in wide range of uncoupling concentrations (0.01–10 μg/ml) did not alter cell morphology and was non-toxic for cell cultures [7]. In the experiments *in vivo* [Glu1]gA was neuroprotective at doses of 0.5–5 μg/kg, whereas the toxic dose was 50 μg/kg only. Thus, the therapeutic permissive window was 10 to 100 fold which makes them significantly more harmless for potential clinical use in comparison with DNP.

Our results highlight the importance of early adverse mitochondrial events in brain damage during ischemia/reperfusion and support the concept that mitochondrial uncoupling may be an important neuroprotective strategy for the treatment of stroke and other brain pathologies associated with cellular oxidative stress. Our results show that [Glu1]gA has a significant neuroprotective action that could be explained by its uncoupling action on mitochondria. Thus, mitochondrial uncoupling by substituted analogs of gramicidin A might be a clinically relevant therapeutic strategy for treatment of brain ischemic pathologies, especially, stroke.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.09.002.

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