Neuroprotective Effect of (R, S)-4-phosphonophenylglycine Against Neuronal Damage Associated with Homocysteic Acid-induced Seizures in Immature Rats

Langmeier M.[1], J. Folbergrová J.[2], Haugvicová R.[3], Riljak V.[1]
[1]Institute of Physiology of the First Faculty of Medicine, Charles University in Prague, Czech Republic;
[2]Institute of Physiology of the Academy of Sciences of the Czech Republic, Prague, Czech Republic;
[3]Institute of Microbiology of the Academy of Sciences of the Czech Republic, Prague, Czech Republic

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Mailing Address: Professor Miloš Langmeier, MD., DSc., Institute of Physiology, First Faculty of Medicine, Albertov 5, 128 00 Prague 2, Czech Republic, Phone: +420 224 968 401, Fax: +420 224 918 816, e-mail: mlangm@lf1.cuni.cz

Abstract: Incidence of human epilepsy in infants and children is high and prolonged seizures in the early developmental period can cause brain damage and lead to serious consequences later in the life. The present study was aimed to investigate potential protective effect of (R, S)-4-phosphonophenylglycine ((R, S)-PPG), a potent and selective group III mGluR agonist, on brain damage associated with homocysteic acid-induced seizures in immature 12-day-old rats. This compound does not exhibit any proconvulsive effect. Moreover, (R, S)-PPG was shown to protect NMDA and quinolinic acid-induced lesions in rats. Seizures were induced by bilateral intracerebroventricular (i.c.v.) infusion of homocysteic acid (DL-HCA, 600 nmol/side). (R, S)-PPG was given by bilateral i.c.v. infusions (5 nmol/side) at 15- to 20-min time intervals prior to administration of DL-HCA. After 1 or 6 days of survival, animals in all experimental groups (13-day-old and 18-day-old) were perfused transcardially under deep ether anaesthesia with heparinized normal saline and subsequently with the fixation solution (4% paraformaldehyde in the phosphate buffer, pH 7.4, both solutions at room temperature). Two histological methods were used in our study. Fluoro-Jade B dye is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration and staining with bis-benzimide (Hoechst 33342) was used to detect apoptotic cells according nuclei with condensed and/or fragmented DNA. Animals perfused 1 day after the treatment (13-day-old): After only (R, S)-PPG application, no obvious pathological changes were found. After only DL-HCA application, distinct destruction of the hippocampal region both in the dorsal and ventral hippocampus was observed. Particularly affected were cells in the CA1 and CA3 regions. In addition, neurons with segmented or fragmented nuclei were found in the granule cell layer of the dentate gyrus. (R, S)-PPG + DL-HCA administration resulted in a lower number of Fluoro-Jade B positive cells. All areas of the hippocampus were protected by (R, S)-PPG pre-treatment. Animals perfused 6 days after the treatment (18-day-old): In the group where only (R, S)-PPG has been applied, no obvious pathological changes were found in the hippocampal area. After only DL-HCA administration almost complete destruction of the hippocampal region both in the dorsal and ventral hippocampus was observed. Particularly affected were the cells in the CA1 and CA3 regions, granule cells of the dentate gyrus and many interneurons in all hippocampal areas. (R, S)-PPG + DL-HCA administration resulted in lower number of Fluoro-Jade B positive cells. All areas of the hippocampus have been protected by (R, S)-PPG pre-treatment. In conclusion, the present data support the hypothesis that (R, S)-PPG can have a beneficial effect in those disorders where excitotoxicity is one of the dominant pathogenetic mechanisms.

Introduction

It is well established that glutamate is a major excitatory neurotransmitter in the central nervous system and the knowledge of the mechanism modulating its release and effects is highly important for the understanding of the pathogenesis of excitotoxicity, which plays a crucial role in various forms of neuronal damage and degeneration. Glutamate and its related agonists act on ionotropic glutamate receptors (iGluR) and it is well documented that excessive activation of these receptor subtypes is involved in wide range of animal models of epilepsy [1, 2].

Antagonists of these receptor subtypes (both of the NMDA and non-NMDA) have been demonstrated as effective anticonvulsants [3, 4, 5]. Unfortunately, it is known that application of the latter substances is accompanied by undesirable side effects such as sedation, disturbances of motor coordination and performance, ataxia and memory loss [6, 7]. As a result of these findings, an interest of researchers was focused on the subfamily of metabotropic glutamate receptors [8]. These receptors do not function as mediators, but rather as modulators of brain excitability via presynaptic, postsynaptic, and glial mechanisms [9]. Glutamate receptors of this group activate second messenger-mediated biochemical signalling cascades via GTP-binding proteins [10, 11]. Metabotropic glutamate receptors have been subdivided into three main classes on the basis of their function, sequence similarity or agonist preference: Group I receptors (comprising mGluR1 and mGluR5) are positively coupled to phosphoinositide hydrolysis, with consequent mobilisation of intracellular Ca^{2+}; group II (mGluR2 and mGluR3) are negatively coupled to adenylate cyclase while group III (mGluR4, 6, 7 and 8) are also negatively linked to adenylate cyclase, but show a different agonist preference than group II mGluRs. [12, 13]. Group III mGluRs, especially mGluR7, are widely distributed in the rat brain [14] and immunocytochemical studies have demonstrated that mGluR4 and 7 are localised presynaptically in the hippocampal formation [15]. It is in accordance with the assumption that these receptors act as inhibitory presynaptic autoreceptors and therefore can decrease synaptic transmission, most probably via a reduction of the Ca^{2+}-dependent release of glutamate [16], by inhibition of NMDA receptor [17] and by inhibition of cation influx through Ca^{2+}-permeable AMPA receptors [18]. All these facts indicate that agonists at group III mGluRs might have the potency to prevent pathological changes which are accompanied with excessive release of glutamate. This theory is also supported by the fact that vulnerable neurones respond to the increased extracellular glutamate concentration by an upregulation of mGluR4 mRNA levels [19].

Agonists for group III mGluRs such as L (+)-2-amino-4-phosphonobutyric acid (L-AP4) and L-serine-O-phosphate (L-SOP) exerted antiepileptogenic and anticonvulsant activity in kindled rats [20] and reduced anoxia-induced damage in primary hippocampal neurones [21]. At high doses, however, both L-AP4 and

L-SOP had proconvulsive effect [22] which might not be due to group III mGluR stimulation. (R, S)-4-phosphonophenylglycine was shown to be a potent and selective group III mGluR agonist, which did not exhibit any proconvulsive effect even at very high doses [22, 23]. Moreover, (R, S)-PPG protected against NMDA and quinolinic acid – induced lesions in rats [22].

Incidence of human epilepsy in infants and children is high and prolonged seizures in the early developmental period can cause brain damage and lead to serious consequences later in the life. Homocysteic acid DL-HCA is endogenous sulphur containing excitatory amino acid acting both at NMDA and non-NMDA receptors [25]. HCA applied intracerebroventricularly (i.c.v.) in 12-day-old rats induced generalized clonic-tonic seizures, recurring frequently for several hours [5]. The mortality in this seizure model is minimal, and in hippocampal formation, necrotic as well as apoptotic cells were observed after DL-HCA i.c.v. administration [26]. Recently we have demonstrated that (R, S)-PPG has a potent anticonvulsant effect against seizures induced in 12-day-old rats by DL-HCA [23].

In the present study, we were interested whether (R, S)-PPG could provide a protection of neuronal damage associated with these seizures. 12-day-old rats were chosen because of their level of brain maturation which is comparable to the early postnatal period in human infants [24].

Materials and methods

Animals

Immature, 12-day-old male Wistar albino rats were used for these experiments. The rat pups were removed from their mothers 1 hour before the experiments and placed into a plastic observation chamber on an electrically heated pad at 34 °C (i.e., the temperature of the nest). Before the operation animals were anesthetized by ether and fixed in a stereotaxic apparatus modified for rat pups [5]. Bilateral stainless-steel guide cannulas (26-gauge, 5 mm in length) were stereotaxically implanted 1mm above the lateral ventricles (AP, 0.2–0.3 mm caudally from the bregma; L, ±1.6 mm; V, 3.3 mm from the skull surface) the cannulas were secured with fast-curing dental acrylic and the pups were allowed a minimum of 90 min to recover from surgery. Seizures were induced by bilateral i.c.v. infusion of DL-HCA (600 nmol/side) using stainless-steel internal cannulas (33-gauge, 6 mm in length), each connected by a polyethylene tube to a 10-μl Hamilton syringe. Injections of DL-HCA were done in a volume of 0.5 μl at the rate of 0.17 μl/min using a SP200i infusion pump (WPI, USA).

The protocol of the experiments was approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic, to be in agreement with the Animal Protection Law of the Czech Republic, which is fully compatible with the guidelines of the European Community Council directives 86/609/EEC. The Institute possesses The

Statement of Compliance with Standards of Humane Care and Use of Laboratory Animals No. A5228-01 from the NIH. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Materials

For inducing seizures, DL-HCA (from Aldrich) was dissolved in saline and after adjusting the pH to ~ 7.0, only freshly prepared solutions were used. For evaluation of neuroprotective effect, the group III mGluR agonist (R, S)-PPG (from TOCRIS Cookson, Bristol, UK) was used. The drug was dissolved in saline containing a calculated amount of 5N NaOH to reach solubilisation and the pH was approximately 7.0. (R, S)-PPG was given by bilateral i.c.v. infusions at 15- to 20-min time intervals prior to administration of DL-HCA. The in vivo stability of the (R, S)-PPG is unknown. Therefore, it was infused i.c.v. at relatively short time interval before administration of DL-HCA to minimize its possible breakdown. Control animals received corresponding volume of saline. The selection of doses of (R, S)-PPG was based on our previous findings [23].

Two histological methods were used in our study. Fluoro-Jade B dye (FJB) (Histo-Chem Inc.) is an anionic fluorescein derivative useful for the histological staining of neurons undergoing degeneration [27]. Despite the fact that there is considerable information about the structure of the Fluoro-Jade dyes [28], much less is known about the chemical identity of the endogenous degeneration molecule to which it binds. Some authors speculated that the degeneration molecule is a polyamine [27]. It is well known that a number of polyamines are associated with cellular degeneration including putrescine, cadaverine, spermadine and histamine. However, there is no evidence that these isolated polyamines are unique to degenerating neurons, or that they would not evaporate or dissolve during histological processing. It is also not known if the endogenous degeneration molecule is produced via an anabolic or catabolic mechanism [29]. Despite all these facts it must be noted, that the neuronal labelling with Fluoro-Jade B is specific for degenerating neurons and that this relatively simple histological technique is relevant for the evaluation of neurodegeneration [27, 30, 31, 32, 33].

Second method, employing Hoechst 33342 (Sigma-Aldrich) staining, was used to identify apoptotic cells, according to their apoptotic nuclei with condensed and/or fragmented DNA.

Experimental groups

The animals were randomly divided into 4 groups. (a) Control group with saline, (b) animals given (R, S)-PPG alone (5 nmol/side), (c) seizure group with DL-HCA alone (600 nmol/side), (d) animals given (R, S)-PPG (5 nmol/side) 15–20 min prior to DL-HCA administration (600 nmol/side).

For our histological analysis totally 19 animals were used: group (a) 4 animals, group (b) 4 animals , group (c) 5 animals and group (d) 6 animals.

After 1 or 6 days of survival animals in all experimental groups (13-day-old and 18-day-old) were perfused transcardially under deep ether anaesthesia with heparinized normal saline and subsequently with the fixation solution (4% paraformaldehyde in the phosphate buffer, pH 7.4, both solutions at room temperature). The brains were removed from the skull, post fixed in the same solution for 24h in a refrigerator. Serial cryostat sections 40mm thick were stained with Fluoro-Jade B and DNA-specific dye bis-benzimide (Hoechst). The stained sections were dehydrated, cleared with xylene, and mounted in DPX (Fluka, Buchs).

Dorsal and ventral hippocampi were observed under the microscope Olympus Provis AX-70 with epifluorescence with blue (450–490 nm) excitation light. In addition, the localization of canullae was checked.

Semi-quantitative analysis

For the semi-quantitative analysis, Fluoro-Jade B positive cells were counted by two independent workers in all slices. Each area of the hippocampus in the slice was evaluated by the scale: (0) no FJB positive cells in the field of view, (+) sporadically present FJB cells in the field of view, (++) groups of FJB positive cells in the field of view and (+++) massive alteration, the study area is completely undergoing degeneration.

Results

A detailed description of the character of seizures induced by i.c.v. infusion of DL-HCA to 12-day-old rats was given in our previous paper [5]. Briefly, already during the infusion of DL-HCA, majority of rats displayed high hyperactivity that led to convulsion in most of the pups within 3-5 min, in the remaining animals approximately within 10 min after infusion. The typical behavioural pattern usually began with a backward bending of the head and neck, accompanied by clonus of one or both forelimbs. The animals then usually fell to one side, but this was immediately corrected by the pups themselves. The intensity of motor phenomena gradually increased, usually leading to wild running, followed by falling to either side with clonic seizures of all extremities. This phase was followed by tonic extension or flexion of the forelimbs and tonic flexion of the hindlimbs. Most animals displayed repeated generalized clonic–tonic convulsions for 90–120 min, after which the frequency and intensity gradually diminished, nevertheless mild seizure activity could be observed even after 24 h. The mortality with this seizure model, at least during the acute phase of seizures, was minimal. As to the behaviour of animals after (R, S)-PPG administration, it was described in detail in our previous study [23] in which we tested various doses of this agonist. Pre-treatment with the present dose of (R, S)-PPG (5 nmol/each side) resulted in a highly pronounced anticonvulsant effect. No generalized clonic-tonic seizures appeared during the whole observation period (i.e. 2–3 h) and also

all initial forms of seizure activity (such as backward bending of the head etc.) were absent.

Histological evaluation

Slight dilatation of the ventricular system observed in the majority of rats (both control and experimental) most likely reflects reaction to the intraventricular administration of the solvent. This potential elevation of intraventricular pressure, however, did not affect the surrounding structures.

Animals perfused 1 day after the treatment (13-day-old, Table1)

In the group (a) (normal saline solution), neuropathological analysis after 1 day of survival did not detect any change in the hippocampus. The cytoarchitectonics of all analyzed brain regions corresponded to that expected in 13-day-old animals.

In the group (b) ((R, S)-PPG alone), also no obvious pathological changes were found in the hippocampal area of the animals. Only slight dilatation of the ventricular system was found in several animals. No Fluoro-Jade B positive cells have been detected; DNA specific staining Hoechst 33342 did not reveal any signs of apoptosis.

In the group (c) (DL-HCA alone), the destruction of the hippocampal region both in dorsal and ventral hippocampi was prominent. Particularly affected were the cells in the CA1-3 regions and granule cells of the dentate gyrus. The bilateral loss of CA3 pyramidal cells was observed in all experimental animals, this fact was confirmed by Fluoro-Jade B staining (Colour Figures 6A, 6B) with high number of FJB-positive cells. CA1 area of the hippocampus and hilus of the dentate gyrus were completely destroyed in most of the experimental animals, this fact was also confirmed by FJB dye. Numerous glial cells replaced the neuronal population

Table 1 – Animals perfused 1 day after the treatment (13-day-old)

	Exp. Group			
Area	Control	(R,S)-PPG	DL-HCA	(R,S)-PPG+DL-HCA
CA1	0	0	++	0
CA3	0	0	+++	0/+
Hilus	0	0	+++	0/+
VB DG	0	0	0	0
DB DG	0	0	0	0

Semi-quantitative analysis of Fluoro-Jade B positive cells in experimental groups: Control: group received the same volume of normal saline as experimental animals; (R, S)-PPG: rats given (R, S)-4-phosphonophenylglycine alone; DL-HCA: homocysteic acid treated rats; (R, S)-PPG + DL-HCA: rats pre-treated with (R, S)-4-phosphonophenylglycine 15-20 minutes prior to homocysteic acid administration. Each area of the hippocampus in the slice was evaluated by the scale: (0) no FJB positive cells in the field of view, (+) sporadically present FJB cells in the field of view, (++) groups of FJB positive cells in the field of view and (+++) massive alteration, the area studied is completely undergoing degeneration.

of this region. In addition, neurons with a segmented or fragmented nucleus were found in the granule cell layer of the dentate gyrus when DNA specific staining Hoechst was used (Colour Figure 8A). The cells, which were undergoing degeneration, were found at various stages of this process.

In the group (d) ((R, S)-PPG + DL-HCA) the number of Fluoro-Jade B positive cells was significantly lower. All areas of the hippocampus have been protected by (R, S)-PPG pre-treatment. Some Fluoro-Jade B positive cells have been found in CA3 of the hippocampus and in the hilus of the dentate gyrus. Hoechst staining did not detect any sign of apoptosis in any area of the hippocampus (Colour Figures 7A, 7B, 8B).

Animals perfused 6 days after the treatment (18-day-old, Table 2)

In the group (a) (normal saline solution), neuropathological analysis after 6 days of survival did not detect any change in the hippocampus. The cytoarchitectonics of all analyzed brain regions corresponded to that expected in 18-day-old animals.

In the group (b) ((R, S)-PPG alone), no obvious pathological changes were found in the hippocampal area of these animals. Similarly to the control rats, slight dilatation of the ventricular system, namely of the lateral ventricles was found in several animals. No Fluoro-Jade B positive cells have been detected; DNA specific staining Hoechst 33342 did not detect any signs of apoptosis (data not shown).

In the group (c) (DL-HCA alone), almost complete destruction of the hippocampal region both in dorsal and ventral hippocampi was found. Particularly affected were cells in the CA1-3 regions, granule cells of the dentate gyrus and many of the interneurons in all hippocampal fields. A heavy loss of CA3 pyramidal cells was observed in all experimental animals. Numerous glial cells replaced the neuronal population of this region (data not shown).

The glial response was also prominent in other hippocampal regions, being most intense in the hilus (Colour Figure 9A). In addition, neurons with the

Table 2 – Animals perfused 6 days after treatment (18-day-old)

	Exp. Group			
Area	Control	(R,S)-PPG	DL-HCA	(R,S)-PPG+DL-HCA
CA1	0	0	+	0
CA3	0	0	++	0
Hilus	0	0	++	0
VB DG	0	0	0	0
DB DG	0	0	0	0

Semi-quantitative analysis of Fluoro-Jade B positive cells in experimental groups: Control: group received the same volume of normal saline as experimental animals; (R, S)-PPG: rats given (R, S)-4-phosphonophenylglycine alone; DL-HCA: homocysteic acid treated rats, (R, S)-PPG + DL-HCA: rats pre-treated with (R, S)-4-phosphonophenylglycine 15–20 minutes prior to homocysteic acid administration. For evaluation see Table 1

segmented or fragmented nucleus were found in the granule cell layer of the dentate gyrus when DNA specific staining Hoechst was used. Massive neuronal degeneration was observed using Fluoro-Jade B dye (Colour Figure 9B). The cells, which were undergoing degeneration, were found at various stages of this process.

In the group (d) ((R, S)-PPG + DL-HCA), the number of Fluoro-Jade B positive cells was significantly lower. All areas of the hippocampus have been protected by (R, S)-PPG pre-treatment. Hoechst staining did not detect any signs of apoptosis in any area of the hippocampus (microphotograph not shown).

Discussion

Our results demonstrate that in accordance with our recent study [33], long-lasting seizure activity induced by the i.c.v. infusion of DL-homocysteic acid in 12-day-old rat pups leads to pronounced morphological changes in the hippocampus as it was revealed 1 and 6 days after the insult. The hippocampal formation is one of the most affected regions by various insults [25, 26, 30, 31, 33]. It was shown recently, that group III mGluR agonists might be a valuable class of drugs that can prevent early glutamate-mediated cell damage [34]. It was proposed that these agonists can avert convulsion and excitotoxic lesions in vivo [22, 23]. Our present experiments with excitotoxic lesions induced by DL-HCA i.c.v. administration confirm these results – when animals were pre-treated with a selective agonist for group III metabotropic glutamate receptors (R, S)-PPG prior to DL-HCA application, pathological changes observed in the hippocampal formation were significantly lower.

It has been shown that activation of the presynaptic group III mGluRs (that serve as autoreceptors), can reduce glutamate release and transmission at several glutamatergic synapses in the CNS [9, 10, 11]. Unfortunately it remains unclear if the latter one is the main mechanism of the anticonvulsant activity, or whether other factors also contribute, such as release of neuroprotective or neurotrophic factors. This must be elucidated in future experiments. It should be mentioned that FJB staining does not allow differentiating the type of nerve cell death (necrosis vs. apoptosis). Our findings support the possibility that the DL-HCA-induced cell loss is most probably apoptotic. However, the occurrence of necrosis can not be excluded [26]. The nuclear fragmentation demonstrated with DNA staining (bis-benzimide – Hoechst 33342) appears to speak in favour of apoptosis. This hypothesis is fully supported by the results of electron microscopic analysis in our preliminary experiments and it corresponds with findings of other authors [26, 35].

In conclusion, the present data support the hypothesis that (R, S)-PPG can have beneficial effects in diseases where excitotoxicity is one of the dominant pathogenetic mechanisms. This could apply, for example, to diseases like epilepsy.

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