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Effects of mGluR5 positive and negative allosteric modulators on brain damage evoked by hypoxia-ischemia in neonatal rats

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Abstract

In the present study, we examined the effects of negative and positive allosteric modulators of metabotropic gluta-tamate receptor 5 (mGluR5), fenobam and ADX47273, respectively, on brain damage induced by hypoxia-ischemia (H-I) in 7-day-old rats. The test drugs were administered intraperitoneally 10 min after H-I. Rectal body temperature was measured for 2.5 h after the insult. The number of apoptotic neurons in the immature rat brain was evaluated after 24 h. The wet weight of both hemispheres was determined 14 days after H-I, and its loss was used as an indi-cator of brain damage. In the vehicle-treated groups, H-I reduced the weight of the ipsilateral (ischemic) hemisphere by approximately 33% and sixfold increased the number of apoptotic cells in the cortex. Fenobam (10 mg/kg) and ADX47273 (5, 10, and 30 mg/kg) had no significant effect on brain damage, although application of fenobam at this dose significantly reduced the number of apoptotic cells. In contrast, fenobam (20 mg/kg) potentiated ischemic brain damage to 57.4% and had no effect on H-I-induced apoptosis. In all of the experimental groups, we detected no sig-nificant changes in the weight of the contralateral (control) hemisphere or the rectal temperature. In conclusion, in 7-day-old rats, the bidirectional modulation of mGluR5 by fenobam (10 mg/kg) and ADX47273 (all doses tested) did not result in significant changes in H-I-evoked brain damage, supporting our previous data indicating that also the antagonists of mGluR5 MPEP and MTEP, which reduce neuronal lesions in adult animals submitted to brain ischemia, were ineffective in 7-day-old rat pups.

Key words: *ischemia, neuroprotection, perinatal asphyxia, rat, metabotropic glutamate receptors, NMDA receptors.*

Introduction

Excitotoxicity mediated by ionotropic gluta-mate receptors, particularly *N*-methyl-*D*-aspartate receptors (NMDARs), is known to play a key role in brain damage caused by different forms of brain ischemia, including perinatal asphyxia [for review, see 11,14,38]. Preclinical studies have used various animal models of cerebral ischemia, demonstrating that NMDAR antagonists are neuroprotective [39]. However, because of the serious adverse effects of high-affinity NMDAR antagonists [34], their benefi-cial neuroprotective effects have not been directly confirmed in clinical tests [11]. Of particular concern is the developing brain, in which the pro-apoptotic

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effects of these compounds have been reported [12, 22,24].

Group I metabotropic glutamate receptors (mGluRs) comprising mGlu1 and mGlu5 subtypes, which potentiate the induction of calcium signals, may be an attractive therapeutic target because mGluR antagonism is relatively free of undesirable side effects, in which they only interfere with the modulatory control of excitatory neurotransmission instead of harming the tonic inhibition of excitation [3]. In addition it has been shown that mGlu5 receptors are positively coupled to NMDA receptors [6,29], and therefore they might also be involved in the NMDAR-mediated mechanisms associated with ischemic brain damage, and mGluR5 inhibition might be neuroprotective.

Many preclinical studies have demonstrated that mGluR1 antagonists were neuroprotective in brain ischemia in adult and developing animals [4,15, 19,23,35]. However, contradictory data have been reported concerning the neuroprotective effects of mGluR5 antagonists. Potent neuroprotection induced by the mGluR5 antagonists MPEP and MTEP in global brain ischemia in adult Mongolian gerbils [19,26] was not observed in another study [23]. Szydlowska et al. [35] reported that MTEP was highly neuroprotective in focal brain ischemia in adult rats, while in our previous study, this compound failed to provide neuroprotection against hypoxia-ischemia (H-I) in 7-day-old rats [19]. We tentatively attributed this difference in the effectiveness of mGluR5 antagonist in adult and developing rats to the distinctly specific properties of the developing brain, but this explanation requires verification by testing other pharmacological tools, including mGluR5 antagonists and mGluR5 positive allosteric modulators (PAMs), to determine how negative and positive mGluR5 modulation affects ischemic brain damage in immature rats.

Fenobam is a selective negative allosteric modulator of mGluR5, with anxiolytic and analgesic properties [13,25]. Recently mGluR5 PAMs emerged as a new group of drugs that may potentiate NMDAR-mediated currents by utilizing the functional coupling between mGluR5 and NMDARs [6]. Behavioral studies demonstrated that ADX47273, which is an mGluR5 PAM, enhances NMDA-dependent functions in vitro and in vivo in adult rodents and exerts antipsychotic-like and pro-cognitive effects [7,17,29,32,33]. To our knowledge, neither feno-

bam nor ADX47273 has been tested in studies of neuroprotection in brain ischemia, particularly in a rat model of perinatal asphyxia.

The aim of the present study was to assess the effects of mGluR5 modulation by fenobam and ADX47273 on ischemic brain damage in developing animals using a rat model of perinatal asphyxia. Weight deficit of the ipsilateral (ischemic) hemispheres served as an indicator of ischemic brain damage.

Material and methods

ADX47273 [S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone], fenobam [N-(3-chlorophenyl)-N'-(4,5-dihydro-1-methyl-4-oxo-1H-imidazole-2-yl)urea], and the vehicles dimethyl sulfoxide (DMSO) and Cremophor were obtained from Sigma-Aldrich (Poznan, Poland). ADX47273 was suspended in a mixture of DMSO and Cremophor (1 : 1, v/v), and the vehicle for fenobam was a mixture of DMSO and Cremophor (1 : 9, v/v).

Rat pups of both genders were obtained from the breeding animal house of the Mossakowski Medical Research Centre PAS in Warsaw. The animal experiments were approved by the Third Local Ethical Committee in Warsaw and performed in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and corresponding Polish government regulations concerning animal experimentation. All efforts were made to minimize animal suffering and the number of animals required.

A model of H-I in 7-day-old Wistar rat pups was used as previously described [27], with slight modifications [19,20]. Briefly, halothane-anesthetized rats were subjected to occlusion of the left common carotid artery. After 1 h, they were exposed to a gas mixture that contained 7.3% oxygen in nitrogen for 75 min at 36°C. The test drugs were administered intraperitoneally (i.p.) 10 min after the termination of hypoxia. The solutions were administered in a volume of 3.3 ml/kg (approximately 50 µl per animal, each weighing approximately 15 g). The concentration of the drugs in stock solutions was adjusted according to the final doses required (5, 10, and 30 mg/kg ADX47273 and 10 and 20 mg/kg fenobam). Control animals received corresponding volumes of the vehicles. The pups were then returned to their dams, kept for 2.5 h at 27°C, with control

of their rectal body temperature [19,20]. Then the dams were housed at an environmental temperature of 20°C under a 12 h/12 h light/dark cycle.

On postnatal day 21 (i.e., 14 days after the insult), the rats were anesthetized with halothane and decapitated. Both cerebral hemispheres were weighed. Brain damage was reflected by a deficit in the wet weight of the ipsilateral (left) ischemic hemisphere and is expressed as a percentage of the wet weight of the contralateral (right) control hemisphere [19,20]. This method of assessing the developing brain damage in a rat model of HI has recently been used by a number of other research groups [8,17,30]. Our previous control experiments demonstrated equal weight of the left and right hemisphere in naive and sham-operated animals and that H-I induction in 7-day-old rats does not interfere with the mass of the contralateral (reference) hemisphere. The obtained data are expressed as means ± SEM, with the number of repetitions (n) provided in parentheses. Statistical significance was tested using analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. Values of $p < 0.05$ were considered statistically significant.

In order to evaluate the effects of fenobam in dosage of 10 or 20 mg/kg applied 10 min after hypoxia-ischemia on ischemia-induced apoptosis, the animals treated as described above were sacrificed 24 hours after hypoxia-ischemia. The animals were deeply anesthetized using Nembutal (80 mg/kg w. m. *i.p.*) and perfused through the ascending aorta with 0.9% NaCl in 0.01 M sodium-potassium phosphate buffer pH 7.4 (PBS), followed by *in situ* perfusion-fixation with ice-cold fixatives applied under gravity. Afterwards, the brains were saturated with sucrose through immersing in 10, 20 and 30% (w/v) sucrose solutions in PBS and cut into 40 µm-thick free-floating coronal sections using a cryostat. Morphology of cell nuclei was examined on brain sections mounted on microscope slides. Sections were washed in PBS and incubated in a 1 µg/ml solution of Hoechst stain (bisbenzimide dye in PBS) for 2 minutes, at room temperature. The stain was then drained off and cover slipped with PBS for visualization. The number of apoptotic cells at the last stage of apoptosis (formation of apoptotic bodies) was measured using a Zeiss fluorescent microscope with a 40× (NA 1.0) objective with additional 10× ocular zoom. Three to four animals from each experimental group were investigated. For each animal we

analyzed 6-8 sections. The dying cells were counted within 10 microscopic fields of observation located randomly within the somatosensory region of the cerebral cortex.

Results

The evaluation of brain damage 14 days after H-I in untreated animals revealed a 33.4% loss of weight of the ipsilateral brain hemisphere, indicating the same degree of brain damage induced by H-I (results not shown). The same level of brain damage was found in the vehicle-treated groups (Figs. 1 and 3). Compared to the corresponding vehicle control animals with a reduction of weight of the ischemic hemisphere at the level of 34.3 ± 2.19% ($n = 27$), brain damage of 24.6 ± 2.45% ($n = 35$) found in animals treated with the mGluR5 negative allosteric modulator fenobam (10 mg/kg) did not differ significantly ($p > 0.05$). In contrast, 20 mg/kg fenobam significantly ($p < 0.05$) potentiated brain damage to 57.4 ± 3.2% ($n = 18$) (Fig. 1).

In order to investigate the influence of fenobam on apoptosis in the rat brain, evoked by hypoxia-isch-
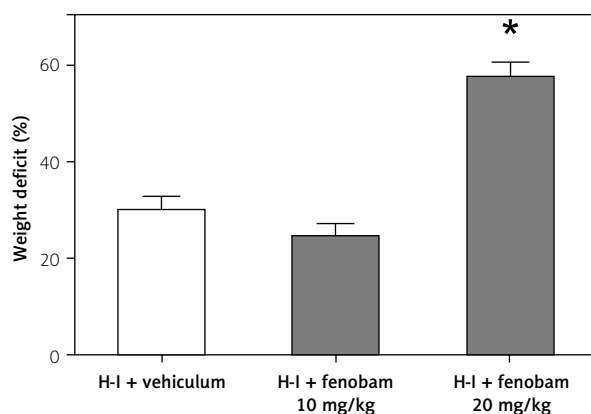
Fig. 1. Effects of fenobam on brain damage induced by hypoxia-ischemia in 7-day-old rats. Indicated doses of fenobam (Fen) or vehicle were administered *i.p.* 10 min after hypoxia-ischemia (H-I). Brain damage was evaluated by weighing the brain hemispheres 14 days after hypoxia-ischemia and is expressed as the weight deficit of the ipsilateral hemisphere as a percentage of the weight of the contralateral hemisphere. The bars represent mean ± SEM (group size: $n \geq 19$ rats per time point in each group). *$p < 0.05$, significant difference from vehicle-treated controls (H-I+veh; ANOVA followed by Tukey's multiple-comparison test).

emia, the animals were treated with fenobam in dosage of 10 or 20 mg/kg. Then, the number of apoptotic neurons was assessed 24 hours after the insult. The results presented in Figure 2A and B demonstrate that in the group of rats treated with fenobam in the dose of 20 mg/kg the mean number of apoptotic cells per field of observation was at the same level as observed in the H-I group (about 60 cells per field). Treatment with fenobam in the dose of 10 mg/kg significantly reduced the number of apoptotic cells by 36.7%.

A

B
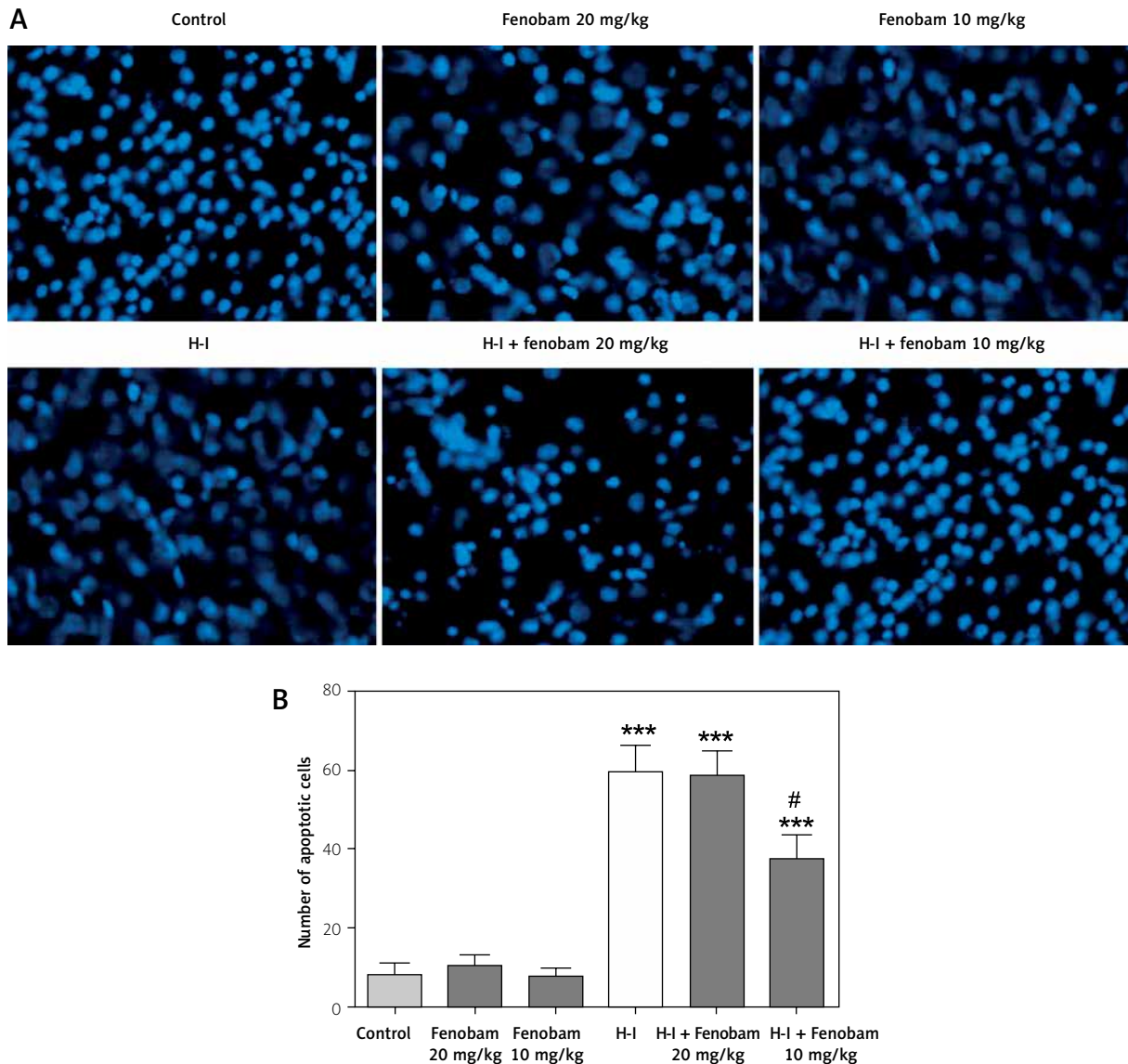
Fig. 2. The effect of fenobam on hypoxia-ischemia evoked induction of apoptosis in the cortex of immature rat brain assessed 24 hours after the insult. Morphology of cell nuclei in cerebral cortex visualized by Hoechst staining **(A)**. Note many apoptotic cells within the section subjected to H-I and H-I Fenobam + 20 mg/kg as well. Note the reduction of apoptotic cell number within the material of animals pretreated with fenobam 10 mg/kg. Quantitative evaluation of the number of apoptotic cells. Fluorescent images were taken using a Zeiss microscope equipped with a 40x (NA 1.0) objective with additional 10x ocular zoom. **B)** Bars represent means ± SEM (n = 4 animals for each experimental group). For each animal we analyzed 8-10 sections. Significant differences from the appropriate ischemic control (H-I) were tested by ANOVA followed by Tukey's multiple comparison test. *p < 0.001 vs. number of apoptotic cells assessed in the 24 h control group.

Application of the mGluR5 PAM ADX47273 tended to increase brain damage (Fig. 3), but this effect did not reach statistical significance. To illustrate this, statistical analysis of data for ADX47273 10 mg/kg, which are also representative for other ADX47273 doses, demonstrated that the level of reduction of weight of the ischemic hemisphere in the ADX47273 treated group by 42.3 ± 3.25% ($n = 24$) did not differ significantly ($p > 0.05$) from the corresponding vehicle control data (34 ± 2.2%) ($n = 27$) (Fig. 3).

To evaluate the possible negative effects of fenobam and ADX47273 on brain development, and effects of the test substances on post-ischemic body temperature, the mass of the contralateral hemispheres and rectal body temperature were also measured. The vehicles for fenobam and ADX47273 as well as these test substances regardless of the drug dose administered had no effect on the mass of the contralateral hemispheres and did not interfere with rectal body temperature during the 2.5-h period after H-I (results not shown).

Discussion

Based on the well-established role of NMDARs in ischemic brain damage and the functional coupling of mGluR5 and NMDARs, one might expect that the negative modulation of mGluR5 provides neuroprotection, whereas the enhancement of mGluR5 activity exacerbates brain damage in animal models of brain ischemia. However, the results of the present study using a rat model of perinatal asphyxia and evaluating brain damage based on the ipsilateral hemisphere's mass deficit 14 days after the insult indicate that the mGluR5 negative and positive modulators fenobam and ADX47273 at typically used doses failed to influence brain damage in 7-day-old rats subjected to H-I, but fenobam at a high dose (20 mg/kg) enhanced it. Examination of the number of apoptotic cells in the brain 24 h after H-I showed a significant reduction in the number of apoptotic cells in the animals treated with the lower dose of fenobam, and no effect at its higher dose.

Our negative data are consistent with previously published results indicating that the other mGluR5 antagonist MTEP provided neuroprotection in brain ischemia in adult but not in developing rats [19,35]. Collectively these data appear to support the hypothesis that mGluR5 activity and consequently its functional coupling to NMDARs, which may determine
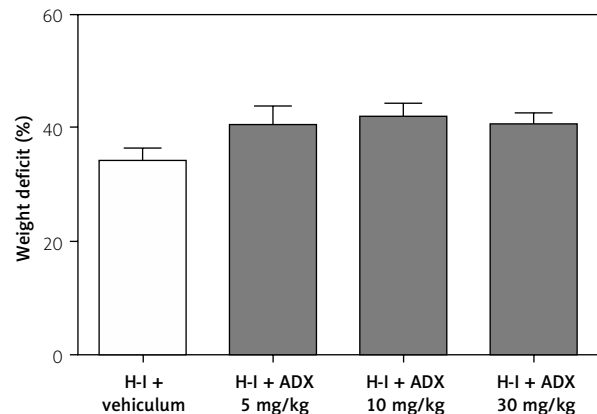
Fig. 3. Effects of ADX47273 on brain damage induced by hypoxia-ischemia in 7-day-old rats. Indicated doses of ADX47273 (ADX) or vehicle administered *i.p.* 10 min after hypoxia-ischemia (H-I). For other explanations see legends to Fig. 1.

the effects of mGluR5 modulators on H-I-evoked brain damage, depend on the level of brain development, and that mGluR5 may not be fully operational in 7-day-old rats. We propose that the mechanism of unexpected potentiation of the ischemic brain damage evoked by fenobam 20 mg/kg may result from unspecific interactions of its metabolites.

In the present study, we used H-I in 7-day-old rats, which is an established model of perinatal asphyxia [27, for review see 37]. We used this model previously to test the neuroprotective potential of other mGluR ligands [19,20]. The involvement of NMDARs in the mechanisms of ischemic brain damage in this experimental model is clear [9,10,11,14,38,39], but less clear is the role of particular group ImGluRs subtypes. mGluR1 and mGluR5 have significantly different developmental profiles in various brain regions and in the subcellular localization [5,18]. Immunocytochemical studies have shown that mGluR5 predominates in the immature rat brain [28], whereas mGluR1 appears in the rat brain only in limited amounts at the end of the first postnatal week [18]. However, the results of functional studies demonstrated that mGluR1 rather than mGluR5 mediates transient calcium currents in rat brain neurons during early postnatal days [36]. Thus, a model of H-I in 7-day-old rats appears to be suitable for studies that address developmental aspects of the effects of mGluR5 modulation on ischemic brain damage. As pharmacological tools we used fenobam and ADX47273, which are selective

mGluR5 negative and positive allosteric modulators, respectively [16,25]. Oral fenobam administration in adult rats (10-30 mg/kg) exerted analgesic and anxiolytic-like effects and impaired learning [13], whereas the behavioral effects of ADX47273 were observed in rats treated with 10 and 30 mg/kg *i.p.* [16]. The doses of the test substances used in our study were based on the above data from the literature.

As mentioned above, based on theoretical assumptions, the negative modulation of mGluR5 should lead to a decrease in the activity of NMDARs and consequently neuroprotection in brain ischemia. However, the present study failed to demonstrate such effects of fenobam in the H-I model in 7-day-old rats. These results fit in with the divergent literature data on the neuroprotective effects of mGluR5 antagonists in different models of brain ischemia. Although Meli *et al.* [23] did not find a neuroprotective effect of the mGluR5 antagonist MPEP on global brain ischemia in adult Mongolian gerbils, other authors reported potent neuroprotection induced by the mGluR5 antagonists MPEP and MTEP in the same gerbil model and a model of focal brain ischemia in adult rats [2,19,26,35]. In contrast to the results of studies in adult animals, our previous study demonstrated a lack of neuroprotection provided by MTEP in 7-day-old rats subjected to H-I [19]. The present data are consistent with these earlier findings.

The aforementioned differences in the subcellular localization of mGluR1 and mGluR5 and minimal functional role of mGluR5 in calcium signaling during the first postnatal week [18,36] may suggest that mGluR5 may not be functional during this early developmental period with corresponding consequences for the operation of coupling between mGluR5 and NMDARs. It has been demonstrated that the antagonism of NMDARs in the developing brain increases constitutive apoptosis [12,24] and decreases brain weight 2 weeks after a single MK-801 injection [21]. Moreover, NMDAR antagonists interfere with thermoregulation and induce hypothermia in neonatal rats [9]. Our present data showed the absence of a decrease in the weight of the contralateral brain and of hypothermia in rats treated with fenobam, which is consistent with lack of interference with NMDAR activity.

To our knowledge the impact that fenobam has on induction of apoptosis in brain neurons by ischemia, hypoxia and similar pathological situations has not previously been investigated extensively. Ahn *et al.* [1] demonstrated in *in vitro* that fenobam given alone only marginally reduced activation by H_2O_2 of Akt and p38 in C6 cells or protected them against DNA damage. These authors revealed that fenobam significantly potentiated cytoprotective effects of the exogenous peptide construct PEP-1-FK506BP both in C6 cells submitted to oxidative stress and in the hippocampus of Mongolian gerbils after global forebrain ischemia. Our present results showed that application of fenobam at a dose of 10 mg/kg significantly reduced the number of apoptotic neurons in H-I challenged immature rat brains, which is a new and interesting finding. This finding may indicate that although mGluR5 participates in the H-I-evoked triggering of apoptosis in the rat pups, its role may still be rather weak. Indeed, this significant fenobam-evoked protection against neuronal apoptosis observed 24 hours after HI (Fig. 2) contrasts with only an insignificant tendency to neuroprotection as assessed after 14 days on the basis of the brain mass deficit (Fig. 1).

The justification for the use of ADX47273 was the same as for fenobam (i.e., NMDARs play an instrumental role in ischemic brain damage, and mGlu5 receptors are functionally coupled to NMDARs and potentiate glutamate-induced Ca^{2+} signaling). Although based on these assumptions the expected result for ADX47273 was exacerbation of damage, previously published results did not demonstrate an increase of ischemic brain injury by activation of mGluR5 in different experimental models. On the contrary, Bao *et al.* [2] used an adult rat model of focal brain ischemia and reported neuroprotection provided by the selective mGluR5 agonist CHPG. Meli *et al.* [23] reported that CHPG failed to exacerbate neuronal damage in an *in vitro* model of rat organotypic hippocampal slices challenged with oxygen-glucose deprivation (OGD), while the activation of group I mGluRs by DHPG was shown to induce tolerance of OGD in the same model [31,40]. Consistent with these data, in our present study we observed only a nonsignificant trend toward an exacerbation of ischemia-evoked brain damage induced by ADX47273.

Our results unexpectedly revealed that fenobam 20 mg/kg significantly increased damage in the ipsilateral hemisphere which was assessed based on brain mass deficit 14 days after H-I, and had no protective effect on the number of apoptotic cells evaluated one day after the insult. Considering the mechanisms of this phenomenon, one should take into account selectivity. The brain concentration of

30 mg/kg fenobam 40 min after oral administration in adult rats reaches 600 nM, whereas its IC_{50} for mGluR5 is only 58 nM [25]. Fenobam is also very rapidly metabolized in rats *in vivo* [41]. Therefore, the exacerbation of brain injury by i.p. administration of fenobam (20 mg/kg) may result from fenobam and/or its metabolite interactions with as-yet unidentified receptors other than mGluR5.

In summary, the present study evaluated the effects of negative and positive allosteric modulators of mGluR5, fenobam and ADX47273, respectively, on brain damage evoked by H-I in 7-day-old rats. We found no significant effects of such treatment. Only a weak tendency toward a neuroprotective effect of 10 mg/kg fenobam and equally nonsignificant potentiation of brain damage by the mGluR5 PAM ADX47273 were observed in this animal model. Moreover, aggravation of brain damage by fenobam at a dose of 20 mg/kg was noted. Our results are inconsistent with the theoretically predicted pattern of changes in ischemic brain damage evoked by the modulation of mGluR5 activity, based on the hypothesis that mGluR5 and its positive coupling to NMDARs may participate in these phenomena. Most likely functions of mGluR5 depend on brain development and may be inadequate in 7-day-old rat pups. Further studies using models of brain ischemia in adult animals are needed to verify whether the present findings are specific to juvenile animals and result from developmental changes in mGluR5 functions.

Acknowledgments

Disclosure

Authors report no conflict of interest.

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