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The epileptogenic effect of seizures induced by hypoxia The role of NMDA and AMPA/KA antagonists

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Abstract

Hypoxia of the brain may alter further seizure susceptibility in a different way. In this study, we tried to answer the question how episode of convulsion induced by hypoxia (HS) changes further seizure susceptibility, and how *N*-methyl-D-aspartic acid (NMDA) and AMPA/KA receptor antagonists influence this process. Adult Albino Swiss mice exposed to hypoxia (5% O_2) developed clonic/tonic convulsions after about 340 s. Mice which underwent 10 s but not 5 s seizures episode subsequently exhibited significantly increased seizure susceptibility to low doses (equal ED_{16}) of bicuculline (BCC) and NMDA during a 3-week observation period. No morphological signs of brain tissue damage were seen in light microscope on the third day after a hypoxia-induced seizure (HS). Learning abilities assessed in passive avoidance test as well as spontaneous alternation were not disturbed after an HS episode. Pretreatment with AMPA/KA receptor antagonist NBQX effectively prolonged latency to HS and given immediately after seizure episode also attenuated subsequent convulsive susceptibility rise, however, NMDA receptor antagonist, MK-801, appeared to be ineffective. These results suggest that a seizure episode induced by hypoxia, depending on its duration, may play an epileptogenic role. The AMPA/KA receptor antagonist prolongs the latency to HS, and given after this episode, prevents the long-term epileptogenic effect.
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Keywords: Hypoxia; Seizures; MK-801; NBQX

1. Introduction

Episode of brain hypoxia/ischemia may alter seizure susceptibility in a different way. The detailed condition whether it confers protection or epileptogenic effect is still not strictly defined. Most of the models in which hypoxia/ischemia diminish further convulsive threshold are based on forebrain ischemia; global ischemia frequently has an opposite effect (Emerson et al., 1999; Heim et al., 1991; Towfighi et al., 1999). Therefore, it was suggested that an increase in seizure susceptibility depends on the changes in the brainstem. In both, forebrain ischemia model combined with hyperglycaemia (Siesjo et al., 1989; Uchino et al., 1996), and model of severe global hypoxia as seen in cardiac arrest in which neuronal necrosis occurs, the postischemic animals have increased susceptibility to convulsions (Kawai et al., 1995; Vanicky et al., 1997). So far it seems that the extent of hypoxia/ischemia, their duration and presence of morphologic damage, determines pro- or anticonvulsive influence of hypoxia/ischemia. Our previous experiments indicated that animals after a 5-min episode of hypoxia (5% oxygen, 95% nitrogen) showed decreased susceptibility to PTZ-seizures during the 7-day observation period (Rubaj et al., 2000). During those experiments, we observed that hypoxia of longer duration induced the generalized convulsions followed by death. The occurrence of those seizures seemed to abolish the development of protection (preconditioning) or even enhanced further convulsive susceptibility. In the clinic, seizures complicating hypoxia in adults are observed in several types of human pathology including: stroke (Kilpatrick et al., 1992; So et al., 1996), cardiopulmonary arrest (Snyder et al., 1980; Wijdicks and Young, 1994), bradyarrhythmias, vasovagal syncope, heart surgery, severe asthma pulmonary embolism, sleep apnea

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(Celesia et al., 1988; So et al., 1996). Some clinical studies have suggested that seizures complicating cerebral hypoxia are associated with increased risk of epilepsy in the future (Kilpatrick et al., 1992; Roach et al., 1996; Snyder et al., 1980; So et al., 1996). However, there is still no agreement whether such seizures should be regarded as the risk factor of epilepsy development in adults. In neonatal rats, seizures complicating hypoxia further increased seizure susceptibility (Jensen, 1995) and may accelerate neuronal death in the hippocampus (Dzhala et al., 2000). In the present work, we wished to examine the influence of hypoxia-induced seizure (HS) of different durations on further seizure susceptibility. By determining the changes in convulsive susceptibility to compounds with different mechanisms of action, we hope to better understand which of several possible pathways have been altered.

2. Materials and methods

The present study was conducted in compliance with the UK Animals Scientific Procedures Act 1986.

2.1. Animals and experimental conditions

Female Albino Swiss mice weighing 20–25 g were used in the study and were housed in a room maintained at 22 ± 1 °C with an alternating 12-h light–dark cycle with free access to food and water. Animals in the experimental groups were used only once.

2.2. Substances

The following substances were used in the experiments: dizocilpine maleate (MK-801, RBI, Natick, USA); (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(*f*) quinoxaline (NBQX, Tocris Cookson, Bristol, UK); bicuculline (BCC, Sigma, St. Louis, MO, USA); *N*-methyl-D-aspartic-acid (NMDA, Sigma). MK-801 and NMDA were dissolved in 0.9% NaCl. BCC was dissolved in saline acidified to pH 3 using 0.1 N HCl. NBQX was dissolved in a minimum quantity of 1 M NaOH, diluted with distillated water and adjusted to pH 8.0. Intracerebroventricular injections of NMDA were performed in constant volume of 2 μl/mouse according to the method of Haley and McCormick (1957). Control animals were injected with the same volume of vehicle applying the same procedure. MK-801, NBQX, BCC were dissolved in volume of 1 ml/kg b.w., and were injected subcutaneously.

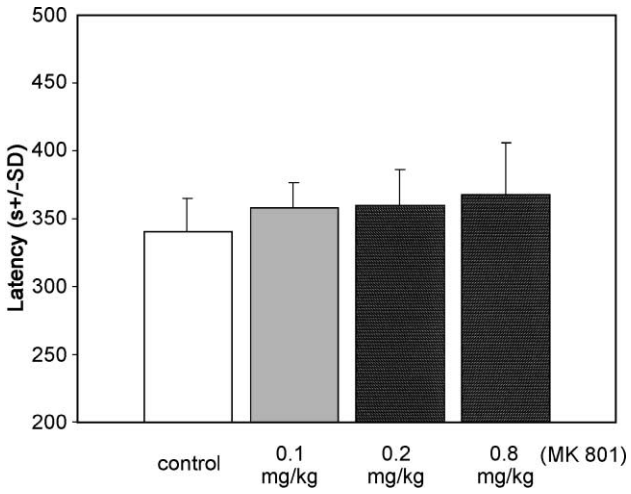

Fig. 1. The influence of MK-801 administered subcutaneously 20 min before exposure to hypoxia on latency to HS ($n = 15$). The differences are not statistically significant.

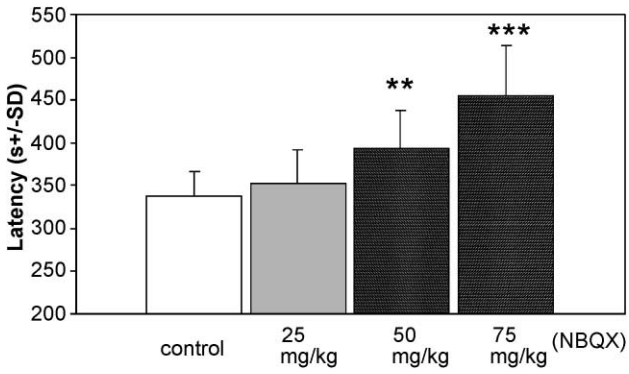

Fig. 2. The influence of NBQX administered subcutaneously 20 min before exposure to hypoxia on latency to HS ($n = 15$). $^{**}P < .01$ vs. control; $^{***}P < .001$ vs. control.

2.3. Hypoxia-induced seizures

Animals were placed in a plastic airtight chamber (volume 10 l) and were allowed to breathe spontaneously with normobaric gas mixture (5% of oxygen, 95% of nitrogen, flow 10 l/min). The latency to onset of seizure and death was measured. To examine the influence of glutamate antagonists on HS, animals received MK-801 (0.1–0.8 mg/kg), NBQX (25–75 mg/kg), or vehicle 20 min before hypoxia.

2.4. Testing of further seizure susceptibility in BCC- and NMDA-induced seizures

Our preliminary, unpublished data indicate that HS increase further seizure susceptibility and also that the difference between the control group and the HS group is dependent on the dose of the convulsant. If lower doses of the convulsant are used, the difference is more significant, so the authors decided to use doses of convulsants equal to ED_{16}. The chemoconvulsive agents—BCC and NMDA at the doses: 2.8 mg/kg and 0.8 nmol, respectively, were applied at 1st, 3rd, 7th, 10th, 21st day after HS. After the injection of the chemoconvulsant, the animals ($n = 20$) were observed for 30 min in order to assess the occurrence of

Table 1
Subsequent convulsive susceptibility to BCC and NMDA at ED_{16} in animals after HS compared with control group ($n=20$)

Days after HS	1		3		7		10		21	
Experimental group	Control	HS	Control	HS	Control	HS	Control	HS	Control	HS
Susceptibility to BCC at ED_{16} (%)	20	75	15	80	15	75	20	75	15	70
Fisher's Exact Test	$P<.05$		$P<.01$		$P<.05$		$P<.05$		$P<.05$	
Susceptibility to NMDA at ED_{16} (%)	15	80	20	80	15	75	15	70	15	80
Fisher's Exact Test	$P<.05$		$P<.01$		$P<.05$		$P<.05$		$P<.05$	

clonic seizures. The percentage of animals with clonic convulsions was noted. To examine the influence of glutamate antagonists, animals received MK-801 (0.1–0.8 mg/kg), NBQX (25–75 mg/kg), or vehicle directly after the HS. Susceptibility to BCC and NMDA (at ED_{16}) was investigated on the third day after HS.

2.5. Learning abilities in passive avoidance task

The animals ($n=20$) were placed in an illuminated box ($10 \times 13 \times 15$ cm), connected to a dark box ($10 \times 13 \times 15$ cm), which was equipped with an electric grid floor. Entrance into the dark box was punished by an electric foot-shock. (0.6 mA for 1 s) The mice that did not enter the dark box within 60 s were excluded from the experiment. Directly after a single episode of HS elicited in conditions described above, the training phase was performed. Twenty-four hours later the same animals were put into the illuminated chamber and observed up to 180 s. Retention of memory was expressed as percentage of animals that did not enter the dark box during observation time.

2.6. Spontaneous alternation in Y-maze

Spontaneous alternation was assessed using the Y-maze test (procedure being modified from Sarter et al., 1988). The maze consisted of three equally spaced arms 15-cm long, 15-cm wide, with walls 15-cm high. The animal was placed at the end of one of the arms and allowed to traverse the apparatus freely for 8 min while the number of arm entries was recorded by photocells connected to the PC. The total number of arm entries was considered to reflect locomotor activity. An alternation was defined as entry into each of three arms on consecutive entries. The alternation score for each mouse was calculated as the ratio of the actual number of alternation to the possible number (defined as the total number of arm entries minus two).

Table 2
Subsequent convulsive susceptibility to BCC and NMDA at ED_{16} in animals after HS depending on duration of the episode ($n=20$)

	Days after HS									
	1		3		7		10		21	
Duration of HS episode (s)	5	10	5	10	5	10	5	10	5	10
Susceptibility to BCC at ED_{16} (%)	20	75	20	80	20	75	20	75	20	70
Fisher's Exact Test	$P<.05$		$P<.05$		$P<.05$		$P<.05$		$P<.05$	
Susceptibility to NMDA at ED_{16} (%)	20	80	20	85	20	75	20	70	15	75
Fisher's Exact Test	$P<.05$		$P<.01$		$P<.05$		$P<.05$		$P<.05$	

2.7. Determination of brain GABA content

On estimated (1, 3, 7, 10, 21) days after HS, animals were killed by decapitation. The heads were immediately placed in liquid nitrogen for 6 s. The brains were rapidly removed and GABA content was determined according to the method described by Lowe et al. (1953), with the modification of Sutton and Simmonds (1974). The GABA accumulation was expressed as micrograms of GABA per gram of the fresh tissue. In order to assess the influence of MK-801 and NBQX, the substances were administered directly after HS.

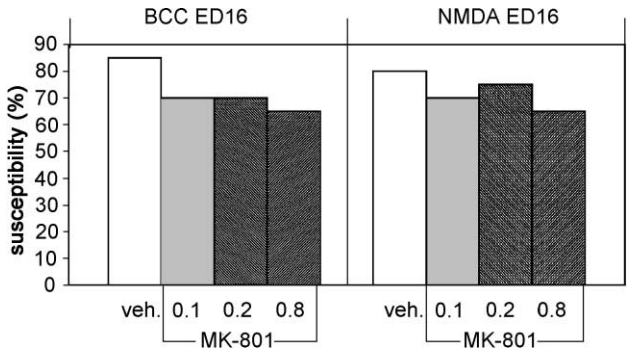

Fig. 3. The influence of MK-801 given subcutaneously immediately after HS on subsequent convulsive susceptibility to BCC and NMDA at ED_{16}, examined 3 days after the episode, compared with vehicle-treated control group ($n=20$). Differences are not significant.

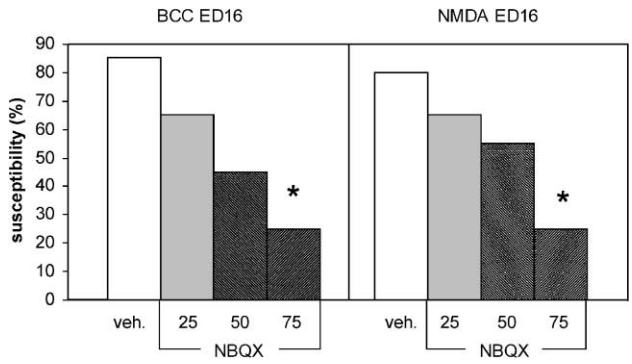

Fig. 4. The influence of NBQX given subcutaneous immediately after HS on subsequent convulsive susceptibility to BCC and NMDA at ED_{16}, examined 3 days after the episode, compared with vehicle-treated control group ($n=20$). $*P<.05$ vs. vehicle-treated control (veh).

2.8. Histological analysis

Three experimental and three control animals were randomly selected. Animals were killed 3 days after the HS. Brains were removed and postfixed with Baker solution (1% $CaCl_2$ in 10% buffered formaline) for 2 weeks. The brains then were dehydrated, embedded in paraffin and sectioned at 6 μm thickness for staining with hematoxylin and eosin or cresyl violet for light microscopic analysis.

2.9. Statistics

The differences in the latency to convulsions induced by hypoxia, spontaneous alternation, locomotor activity in Y-maze, as well as GABA content in the control and treated groups, were determined by a two-way repeated-measures ANOVA and corrected Bonferroni *t* test. The frequency of clonic activity induced by BCC and NMDA in animals after hypoxic seizures episode and learning abilities in passive avoidance task was compared in each treated group with those of vehicle controls by Fisher's Exact Test.

3. Results

3.1. Acute response to hypoxia

Ninety percent of animals in hypoxic conditions (5% O_2 and 95% N_2) revealed increased respiratory rate, hyperactivity, tremor, and generalized convulsions followed by death. Mean latency to onset of seizures was 340 ± 20 s. After 10 ± 1 s duration of seizures episode, apnea and death were observed. Ten percent of animals in these conditions died without convulsions. MK-801 (0.1–0.8 mg/kg) did not prolong the latency to onset of hypoxia-induced convulsions and death (Fig. 1).

NBQX (50 and 75 mg/kg) significantly prolonged the latency to seizures and death compared with vehicle-treated controls (Fig. 2).

3.2. Subsequent seizure susceptibility

No spontaneous seizures were observed in any animal during the observation period when continuous observation was possible. No sudden changes in neurological status, which may be indicative of an unwitnessed seizure occurring during the unmonitored portion of the day, were seen. Survival during a 3-week observation period was not significantly different between groups, there were no deaths in each of the group. Further epileptiform susceptibility was dependent on duration of HS. As compared with control not previously exposed to hypoxia, subjects that underwent 10 ± 1 s episode of HS had significantly greater susceptibility to seizures induced by BCC (2.8 mg/kg) and NMDA (0.8 nmol) (Table 1).

In contrast, animals that had exhibited only 5 ± 1 s episode of clonic seizures during hypoxia did not have further seizure susceptibility differing from those of the normoxic control animals (Table 2).

As compared with the vehicle-treated control group, there was no significant decrease of seizure susceptibility tested on the third day after hypoxia in subjects receiving MK-801 (Fig. 3).

Treatment with NBQX at the dose of 75 mg/kg prevented the increase of seizure susceptibility in both BCC- and NMDA-induced seizures (Fig. 4).

3.3. Passive avoidance task

Subjects, which had undergone HS, did not display impairment in learning abilities in this test 1, 3, 7, 10, and 21 days later (Fig. 5).

3.4. Y-maze

Spontaneous alternation as well as horizontal locomotor activity of animals 1, 3, 7, 10, 21 days after the HS did not differ from the control group (Fig. 6).

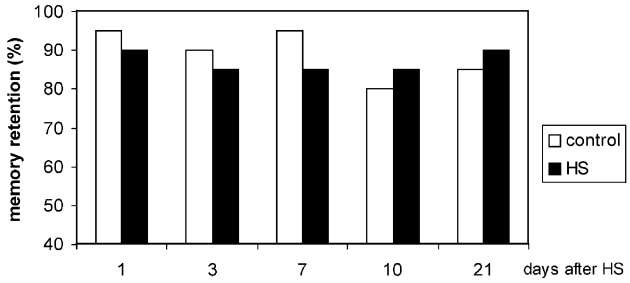

Fig. 5. The influence of HS on learning abilities in passive avoidance task, examined after the episode, compared with vehicle-treated control group ($n=20$). Differences are not significant.

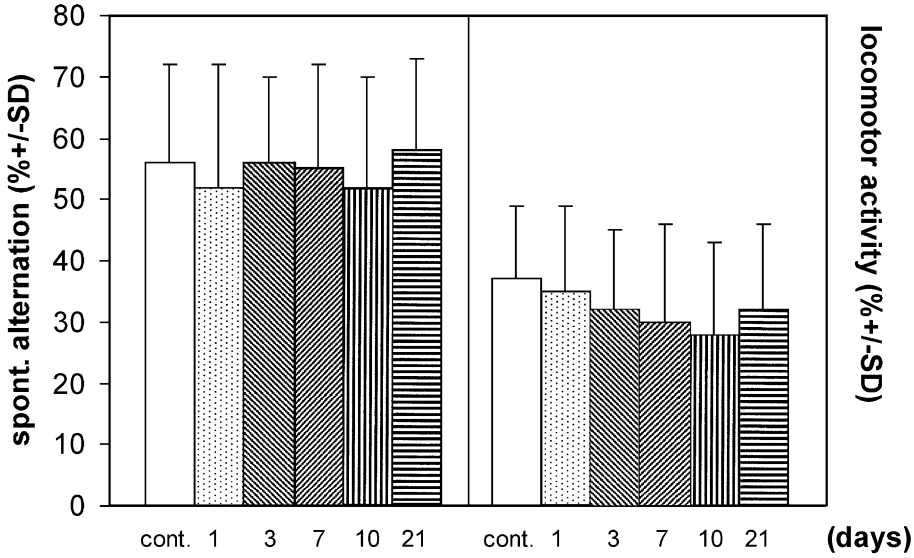

Fig. 6. The influence of HS on spontaneous alternation and locomotor activity in Y-maze, examined after the episode, compared with vehicle-treated control group ($n=20$). Differences are not significant.

3.5. Brain GABA content

As shown in Fig. 7, a significant decrease of brain GABA content in subjects after the episode of HS was noted (Fig. 7).

Administration of MK-801 as well as NBQX (Fig. 8) directly after seizures protected against this effect when examined 3 days after HS.

3.6. Histological analysis

Microscopic examination of the brain from animals after the HS showed no atrophic areas. Hematoxylin and eosin sections as well as cresyl violet showed no morphological signs of damage when assessed in light microscope (500 ×). The hippocampus, believed to be most vulnerable to hypoxic and seizures related injury, appeared to be intact.

4. Discussion

Hypoxia can affect the further seizure susceptibility in a different manner, but the borderline whether it enhanced or diminished further seizure susceptibility is not yet established. In this study, we tried to evaluate the effect of convulsive episode induced by hypoxia on further convulsive susceptibility induced by commonly used chemical convulsants. We chose NMDA and BCC because of their specificity for NMDA receptor and GABA-A receptor, respectively. Because different convulsants produced different patterns of convulsive activity, we defined seizure

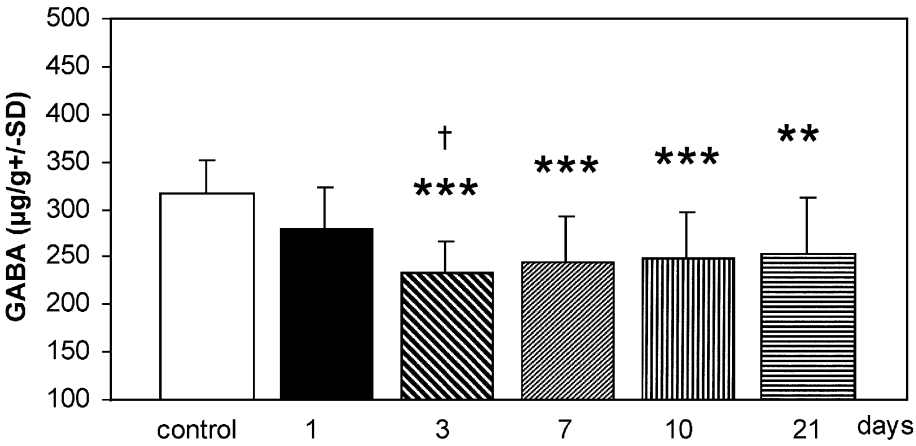

Fig. 7. The influence of single episode of HS on brain GABA content ($n=8$). $^{**}P<.01$ vs. control; $^{***}P<.001$ vs. control; $^{\dagger}P<.05$ vs. 1 (ANOVA).

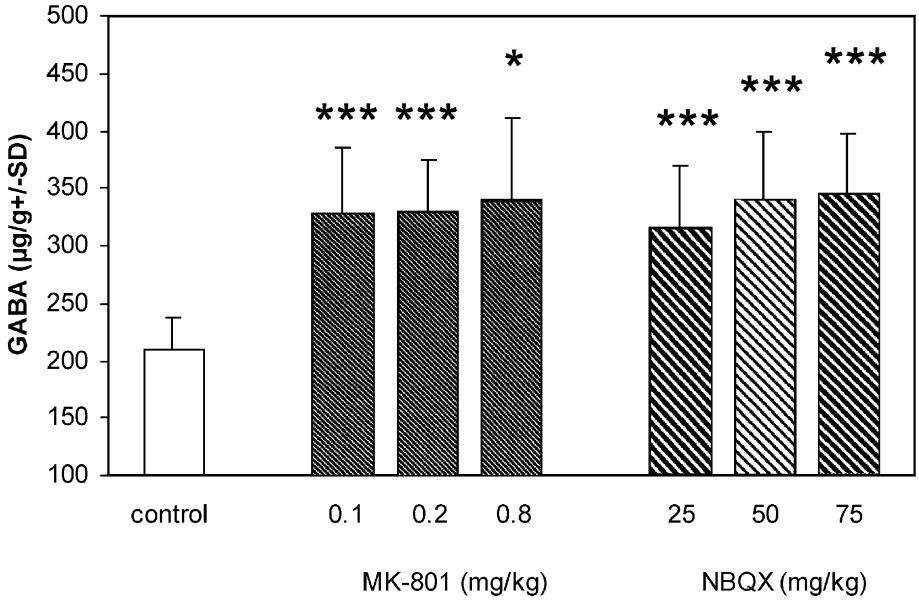

Fig. 8. The influence of MK-801 and NBQX administered immediately after single episode of HS on brain GABA content examined 3 days later ($n = 8$). * $P < .05$ vs. vehicle; * * * $P < .001$ vs. vehicle (ANOVA).

activity based on the most unambiguous characteristic endpoint clonic activity. Since hypoxia is a dynamic process, we determined seizures susceptibility at different times: on 1, 3, 10, 21 days after HS. Because it was similarly enhanced during the observation period, we decided to study the influence of glutamate antagonist on further susceptibility to seizures on the third day after insult. Exposure of adult mice to hypoxia, obtained by decreasing oxygen in the breathing mixture to 5%, results in the development of generalized clonic seizures, after about 340 s, followed by tonus, apnea, and death. A single 10-s (sublethal) episode of HS leads to increased long-term (3 weeks of observation) susceptibility to convulsions induced by BCC and NMDA while a 5-s episode does not. In a pilot study it appeared that the proconvulsive effect of HS diminished when higher doses of convulsants were used. Thus, the authors decided to use the doses equal to their ED_{16} in further experiments. It can be concluded that the duration of seizure aside from seizure occurrence itself during hypoxia is essential for further epileptogenesis. This effect is dependent on convulsant dose, and is greater when low doses are used.

Our results indicating that HS produce increase in further seizure susceptibility are consistent with those obtained on neonatal rat model (Applegate et al., 1996; Jensen, 1995, 1999; Jensen et al., 1992). The reason for the proconvulsive action of HS is still not clear. Seizure activity is an energy-consuming process and its occurrence during hypoxia may exacerbate the ATP depletion (Younkin et al., 1986) and hypoxia severity. However, microdialysis study in newborn pig brain during hypoxia did not show the difference in lactate/pyruvate concentration ratio in white and gray matter, between those animals having seizures during hypoxia and those which did not have them (Thoresen et al., 1998). In the light of these results, the hypothesis that increased energy consumption during hypoxia is responsible for their proconvusive action is still in doubt (however, only small groups of animals were used in this study). Hypoxia-preceded seizures may nonetheless contribute to neuronal injury by producing enhanced rise in excitatory amino acids in the extracellular fluid during subsequent seizure episodes (Young et al., 1992).

The convulsive threshold depends mainly on the balance between GABAergic and glutamatergic transmission. Thus, the observed increase in convulsive susceptibility after HS may be due to decrease in inhibitory tone—GABAergic system, excessive activities in excitatory glutamatergic circuitry, or both. Specific alternations in neurotransmitter concentrations and receptor densities and sensitivities, which underline the changes in seizure susceptibility, remain unknown. However, since the susceptibility to NMDA-induced convulsions is increased, glutamate-mediated pathways activity is enhanced. It is known that hypoxia/ischemia may increase NMDA binding and its mediated activity (Dalkara et al., 1996; Wei et al., 1997), although detailed changes are still extensively studied. Increased sensitivity to BCC-induced seizures observed during a 3-week observation period may imply that HS causes a long-lasting hypofunction of the GABAergic system. Cerebral hypoxia/ischemia as well as convulsions result in an increased extracellular GABA concentration in the brain (Bowdler and Green, 1982; Green et al., 1987; Iadarola and Gale, 1981) with a concomitant inhibition of GABA synthesis and release (Green et al., 1987, 1992)

thus producing hypofunction of the GABAergic system. Elevated glutamatergic activity, as observed during hypoxia, may additionally produce depression of the GABA-A receptor function (Marty and Llano, 1995). On the other hand, there is good evidence that GABA exerts an inhibitory tone on glutamate-mediated neuronal activity (Kanter et al., 1996) and blocks NMDA-stimulated calcium influx in the rat brain's cortical slice (Riveros and Orrego, 1999). Thus, the mutual interaction between GABA and the glutamatergic system may set the new balance directed toward excitation. Usually following global ischemia/hypoxia, hippocampal CA1 cells degenerate over 2–4 days, a process called delayed neuronal death (Kirino, 1982). In our study, no significant histological damage occurs in the model, suggesting that only functional alterations take place in neurons when exposed to HS insult. It is essential that functional as well as histological end points be used to evaluate neuronal injury since after ischemia CA1 cells may be functionally impaired while maintaining normal morphology (Hori and Carpenter, 1994a,b). To determine hippocampal function, memory tests (Volpe et al., 1992; Wang and Huang, 1990), passive avoidance, and spontaneous alternation in Y-maze were performed. Since mice after the HS episode did not have a disturbed memory function during a 3-week observation in both tests, which indicates an undisturbed hippocampal function, it can be concluded that long-term changes in seizure susceptibility are dissociated from neurobehavioral consequences of HS. Several lines of research in experimental animals have implicated a role for the excitatory amino acid glutamate in the production of hypoxic/ischemic brain damage in the immature and adult brain (Andine et al., 1991, 1992; Siesjo et al., 1989). Experimental studies have shown that glutamate antagonists are capable of reducing the severity of hypoxic/ischemic brain damage and possess anticonvulsant properties. It would be rational to test their effectiveness in HS. Pretreatment with NBQX, AMPA/KA receptor antagonist prolonged the latency to HS while MK-801, NMDA receptors antagonist did not. In the next step of the experiment, we tested the effectiveness of MK-801 and NBQX given immediately after the convulsive episode induced by hypoxia against the epileptogenic effect of HS. It was found that only NBQX at the highest dose is partially able to prevent the epileptogenic effect of hypoxia. To minimize the nonspecific effect of MK-801, we decided to test the doses up to 0.8 mg/kg ip since higher doses of MK 801 (>0.5 mg/kg) have been reported to increase serotoninergic (Hiramatsu et al., 1989; Loscher and Honack, 1992; Whitton et al., 1992) and dopaminergic neurotransmission (Hiramatsu et al., 1989; Verma and Moghaddam, 1996) in the brain. Experimental and clinical data suggest that activation of both systems may have protective effects in the pathophysiology of hypoxic/ischemic brain damage (Prehn et al., 1993; Ishige et al., 2001) as well as in epilepsy (Jobe et al., 1999; Watanabe et al., 2000; Kanner and Balabanov, 2002). Our results can indicate that in adult mice, similarly to neonatal rats (Jensen et al., 1995), activation of AMPA/KA but not NMDA receptors may partially mediate the acute and chronic epileptogenic effect of hypoxia. Obtained results indicate that blockade of AMPA/KA receptor seems to be superior over blockade of NMDA receptors in HS. In fact, NMDA receptor antagonists are highly neuroprotective in animal models of focal brain ischemia, although not in transient global ischemia (Buchan, 1990; Buchan et al., 1991a,b). Contributing factor of this reason may be extracellular acidity due to accumulation of lactic acid during global ischemia, an event less prominent in the penumbra of focal ischemia where perfusion is partially maintained. That acidic shift selectively down-regulates NMDA receptors and NMDA receptor-mediated excitotoxicity but enhances AMPA receptor-mediated excitotoxicity (Lee et al., 1999), which could be responsible for NMDA receptor antagonist ineffectiveness in global brain hypoxia/ischemia. The observed lack of protection may additionally result from antagonist-induced damage masking a protective effect. There are reports indicating that NMDA antagonists can be damaging. MK-801 actually causes neuronal damage when administered during normoxia (Allen and Iversen, 1990; Emerson et al., 1999; Olney et al., 1989) and enhances neuronal damage in CA1 during global ischemia (Buchan et al., 1991a,b; Li and Buchan, 1993), at the doses that are used for protection. After transient global ischemia, there is excess glutamate during the early phase of oxygen reperfusion (Araki et al., 1993; Baker et al., 1991; Benveniste et al., 1984). This is important because glutamate itself is far more toxic in the presence of oxygen than in anoxia (Dubinsky et al., 1995). Since the elevation of glutamate may be observed several hours after a hypoxic episode (Andine et al., 1991), we thus hoped that NMDA and AMPA/KA receptor antagonists would be protective when added after the insult. Furthermore, in a clinical situation, hypoxic injury cannot usually be predicted that is why administration of glutamate receptor antagonist after injury would be more clinically relevant. In accordance with our results, most groups have found no protection by early postischemic application of NMDA antagonists in global ischemia (Fleischer et al., 1989; Nellgard and Wieloch, 1992; Sheardown et al., 1993). Contrary, AMPA/KA receptor antagonist NBQX was reported to attenuate neuronal damage when added at different times up to 12 h after two- and four-vessel occlusion ischemia (Buchan, 1990; Buchan et al., 1991a,b; Nellgard and Wieloch, 1992; Volterra et al., 1994), suggesting that delayed activation of AMPA/KA receptors was damaging. Because the temperature in our experiments was not strictly monitored, we cannot exclude that hypothermia is responsible for observed protective effect of NBQX as suggested in gerbils (Nurse and Corbett, 1996).

In conclusion, obtained results indicate that seizures complicating hypoxia act as an epileptogenic factor. This

effect depends on the duration of seizure induced by hypoxia and doses of convulsant used for further seizure susceptibility assessment. Its epileptogenic effect can be blocked by glutamate AMPA/KA antagonist NBQX. The effect of NMDA receptor antagonist MK 801 at the tested doses was not significant.

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