ORIGINAL ARTICLE

Ascorbic acid and alpha-tocopherol protect age-dependently from hypoxia-induced changes of cortical excitability in developing rats

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Abstract

OBJECTIVES: The effects of ascorbic acid and α-tocopherol pre-treatment on hypoxia induced changes in brain cortex excitability were tested in immature rats exposed chronically to simulated altitude of 7 000 m.

METHODS: Rat pups were kept together with their mothers for 8 hours a day in hypobaric chamber since the day of the birth till the postnatal day 11 or 17. Each day immediately before placing to hypobaric chamber pups were pretreated intraperitoneally either with ascorbic acid (100 mg/kg) or α-tocopherol (1 500 mg/kg). Cortical afterdischarges were elicited by repeated stimulation of the right sensorimotor cortex. The duration of evoked cortical afterdischarges was analyzed.

RESULTS: Duration of cortical afterdischarges progressively declines with age. Hypoxia prolonged the duration of afterdischarges in 12-, 18- and 25-day-old animals. Pretratment with ascorbic acid or α-tocopherol shorted afterdischarges duration in youngest experimental group when compared with animals exposed to hypoxia only.

CONCLUSION: Hypoxia significantly affects the brain cortex excitability by prolonging afterdischarges duration. This effect differs with age. Antioxidant pretreatment brought about shorter duration of cortical afterdischarges only in the youngest experimental group. The antioxidant effect is therefore age dependent.

Abbreviations:

CNS	- Central Nervous System
ADs	- Cortical afterdischarges
ECoG	- Electrocorticography
AA	- Ascorbic acid
TOC	- α-tocopherol
ROS	- reactive oxygen species
FRS	- free radical scavengers
PD	- postnatal day

INTRODUCTION

Impact of perinatal and postnatal hypoxia and its sequels were studied extensively in the past (Vanucci and Vanucci 2005, Trojan and Stastny 1988) and are of the great importance even nowadays. Perinatal hypoxic-ischemic encephalopathy is a major cause of acute mortality and chronic neurologic morbidity in children (Vannucci 2000; Volpe 2001) and hypoxia event is involved in the process of epileptogenesis (Towfighi *et al.* 1999). In the central nervous system hypoxia affects the neurons by triggering the excitotoxic pathways with final glutamate release. So called „glutamate loop" might overload the neuronal circuits and cause the cell death (Doble 1999). Secondly, "reperfusion or reoxygenation period" is significantly involved in hypoxic/ischemic brain damage via several factors, mainly the reactive oxygen species (ROS) and reactive nitrogen species that are responsible for tissue impairment and neuronal and glial cell loss (Fellman and Raivio 1997; Buonocore *et al.* 2001; Carloni *et al.* 2008). Noteworthy is the fact, that brain tissue has relative poor antioxidant capacities and antioxidant defense system, so hypoxia challenge can easily damage the fragile equilibrium of free radical homeostasis with many injurious consequences, mainly the generation, spreading and maintenance of convulsive episodes (Rauca *et al.* 1999; Kaur & Ling 2008; Warner *et al.* 2004). One of the possible ways how to defeat the deleterious effects of free radicals is to increase the brain tissue antioxidative capacity by delivering free radical scavengers (FRS). FRS role in epileptogenesis is not yet fully understood and possible anticonvulsant properties of FRS are dubious. We decided to test the influence of ascorbic acid (AA) and α-tocopherol (TOC) on hypoxia induced changes of sensorimotor cortex excitability, mainly because these two substances were repeatedly reported to influence seizure susceptibility in differently designed experiments. TOC was capable to attenuate seizure in pentylenetetrazol convulsion model (Bashkatova *et al.* 2003), TOC was showed to possess neuroprotective properties on ethanol-induced toxicity (Kozan *et al.* 2009), and some effect was observed on penicillin-induced seizure (Ayyildiz *et al.* 2006; 2007). On the other hand, nearly no effect of TOC on PTZ-induced seizures was described by Levy and coworkers (Levy *et al.* 1990; 1992). Yet it is not excluded, that TOC can influence the transduction pathways and processes of epileptogenesis independently on its antioxidant properties (Brigelius-Flohe & Traber 1999; Azzi & Stocker 2000). AA plays many roles in CNS, including neuronal microenviroment maintenance. It is an essential nutrient needed for many metabolic reactions (Rice 2000). AA exhibits also some anticonvulsant properties in, e.g. pilocarpine-induced convulsions (Xavier *et al.* 2007). The aim of the present study was to test the impact of AA and TOC pretreatment on hypoxia induced changes of brain cortex excitability. Experimental pattern of repeated electrical stimulation and analysis of afterdischarges (ADs) duration is a widely accepted model for testing pro/anti-convulsive properties of different substances and is used in our laboratory for many years. Four age groups were tested in our experiment (12-, 18-, 25- and 35-day-old) because of their relevance to hypoxia/ischemia event and secondly to enlighten the developmental differences (seizure-prone behavior is age dependent) related to possible benefits of AA and TOC cortex seizure susceptibility.

MATERIAL AND METHODS

Animals

All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and in agreement with the guidelines of the Animal Protection Law of the Czech Republic. Rat pups entered the experiment at postnatal day 0 (PD 0, day of birth counted as zero). There were at least 10 animals in each experimental group. They were keeping together with their mothers for 8 hours a day (except day 6, 7, 13 and 14) in hypobaric chamber till the PD17 (except the youngest experimental group – this group was exposed to hypoxia only till PD11). Each day immediately before placing to hypobaric chamber pups were pretreated intraperitoneally either with ascorbic acid (100 mg/kg) or α-tocopherol (1 500 mg/kg) or sham-treated in equal volume. The injection was followed by exposure to 41 kPa hypobaric hypoxia (simulated altitude 7 000 m), which was reached in 2 minutes (30 kPa/min) and lasted 8 hours. In-between hypoxia sessions animals were housed at a constant temperature (23±1 °C) and relative humidity (60%) with a fixed 12 h light/dark cycle (with lights on at 07:00), under ambient pressure conditions (app. 101 kPa) and fed (or their mothers) with food and water *ad libitum*.

Electrophysiology

Electrophysiological experiments took place on PD12 (24 hours after last hypoxia procedure), 18 (24 hours after last hypoxia procedure), 25 (8 days after last hypoxia procedure) and 35 (18 days after last hypoxia procedure). On that testing day animals were transported into the experimental room, weighed, marked and randomly assigned into particular experimental groups. All tests were performed between 9 AM and 5 PM. For monitoring ECoG and electrical stimulations six silver electrodes were implanted epidurally through the cranium under deep anaesthesia: two stimulation electrodes (right sensorimotor cortex), three registration electrodes (left sensorimotor cortex, left and right visual cortex) and reference electrode (placed into nasal bone). Recording and other experimental manipulations were carried out after the recovery of righting and suckling reflexes (i.e. approximately 15 min after the surgery), then the cortical afterdischarges were elicited by stimulation of the right sensorimotor cortex.

We used constant current stimulation (bipolar pulses – pulse period 1 ms; duration of stimulation 15 s; frequency 8 Hz; intensity 3–5 mA, which is sufficient for ADs eliciting). The basic stimulation intensity level was set at 3 mA. In case of no response, another stimulation of 4 mA was used 5 min after the first stimulation. The process was similarly repeated with 5 mA stimulation. Finally, if no epileptic graphoelements appeared after the 5 mA stimulation, the animal was excluded from the experiment. If a distinct response (epileptic graphoelements) was recorded, the stimulation was repeated five times at one-minute intervals (timed from the end of each seizure to the beginning of the next stimulation). The duration of evoked ADs and the shape of evoked graphoelements were recorded. Electrocorticograms were recorded 5 minutes before the very first stimulation and during whole stimulation process. The behaviour of rats was video-recorded.

Statistics

Differences in ADs duration between the experimental groups were compared with one-way ANOVA. For data not normally distributed, Kruskal-Wallis one-way ANOVA on Ranks and a Dunn's post hoc analysis was used.

RESULTS

Stimulation of sensorimotor cortical area brought about forelimbs movements in the rhythm of stimulation. The ADs in 12-day-old animals were represented by rhythmic sharp waves only, while ADs in older group formed spike-and wave rhythm. Clonic movements that accompanied elicited ADs were synchronous with sharp ECoG graphoelements.

Control animals

Results obtained from control groups (sham treated) confirmed previously described fact that the length of ADs progressively declines with age. First ADs length in 12-day-old animals is longer (18.74±12.02) compared to 18-day-old animals (8.47±5.82, $p<0.05$), 25-day-old animals (6.03±3.46, $p<0.05$) and 35-day-old animals (4.5±3.21, $p<0.05$). Secondly, in 12- and 18-day-old animals the duration of subsequent ADs (when compared with first ADs length) is unchanged or longer. Thirdly 25- and 35-day-old rats exhibited the post-ictal depression phenomenon characterized by the shortening of ADs elicited by subsequent electrical stimulation (mutual comparison of first ADs duration that served as baseline with the following ones).

Effects of hypoxia on ADs length

12-, 18-, and 25-day-old animals exposed to hypoxia exhibited the prolongation of ADs in our pattern of repeated stimulation when compared to animals not exposed to hypoxia (Figures 1 and 2). Analysis of ADs duration in oldest experimental group brought about increase in ADs length after the very first stimulation, while ADs after subsequent stimulations remained unaffected by hypoxia (except the sixth one).

Effect of ascorbic acid pretreatment on hypoxia-induce changes of cortex excitability

Pretreatment with ascorbic acid significantly influenced the ADs duration in 12-day-old rats: the 3rd, 4th, 5th and 6th ADs duration were significantly shorter when compared with those obtained from animals exposed to hypoxia and treated with saline only. No effect was observed in 18- and 35-day old animals. Certain tendency for ADs decrease was pronounced in 25-day-old animals but it didn´t reach the level of statistical significance (Figure 1).

Effect of pretreatment α-tocopherol on hypoxia-induce changes of cortex excitability

α-tocopherol significantly shorted the ADs duration in the youngest experimental group. ADs duration was significantly shorter after the 3rd, 4th, 5th and 6th stimulation, when compared with animals exposed to hypoxia and not treated with TOC. No effect on ADs length was observed in 18-, 25- and 35-day old animals (Figure 2).

DISCUSSION

Prenatal/perinatal hypoxic episode is an important medical issue nowadays, particularly because its role in process of further epileptogenesis. We decided to test changes of CNS susceptibility after the repeated hypobaric hypoxia and tried to influence the sequels of hypoxia by pretreatment with AA or TOC. Method of evoked cortical ADs is very efficient because it tests and senses the susceptibility and excitability of brain tissue and reflects changes of the brain homeostasis (Kalinčík & Marešová 2005; Vannuci & Vannuci 2005; Golan & Huleihel 2006). This seizures-prone behavior is maintained by mutual influence and disbalance of excitatory and inhibitory neuronal tissue mechanisms. The inhibitory mechanisms are developing during the period of early ontogenesis (reflecting the development of neurotransmitters systems) and so 12- and 18-day-old animal are yet unable to prevent prolongation of ADs after the repeated stimulation (Moshe 1987; Langmeier & Marešová 2005; Riljak *et al.* 2010). The inhibition of ADs prolongation could be observed in 25- and 35-day-old animals – the mentioned systems are probably sufficiently developed. This is manifested as post-ictal depression phenomenon: electrical stimulation following the very first afterdischarge brought about shorter duration of the following ADs. In our experimental pattern the long-term hypoxia prolonged ADs in two younger groups, while the 25- and 35-day-old animals remained unaffected (except the first elicited ADs). This result reflects the sensitivity of immature brain to hypoxia event and their ability to terminate seizures

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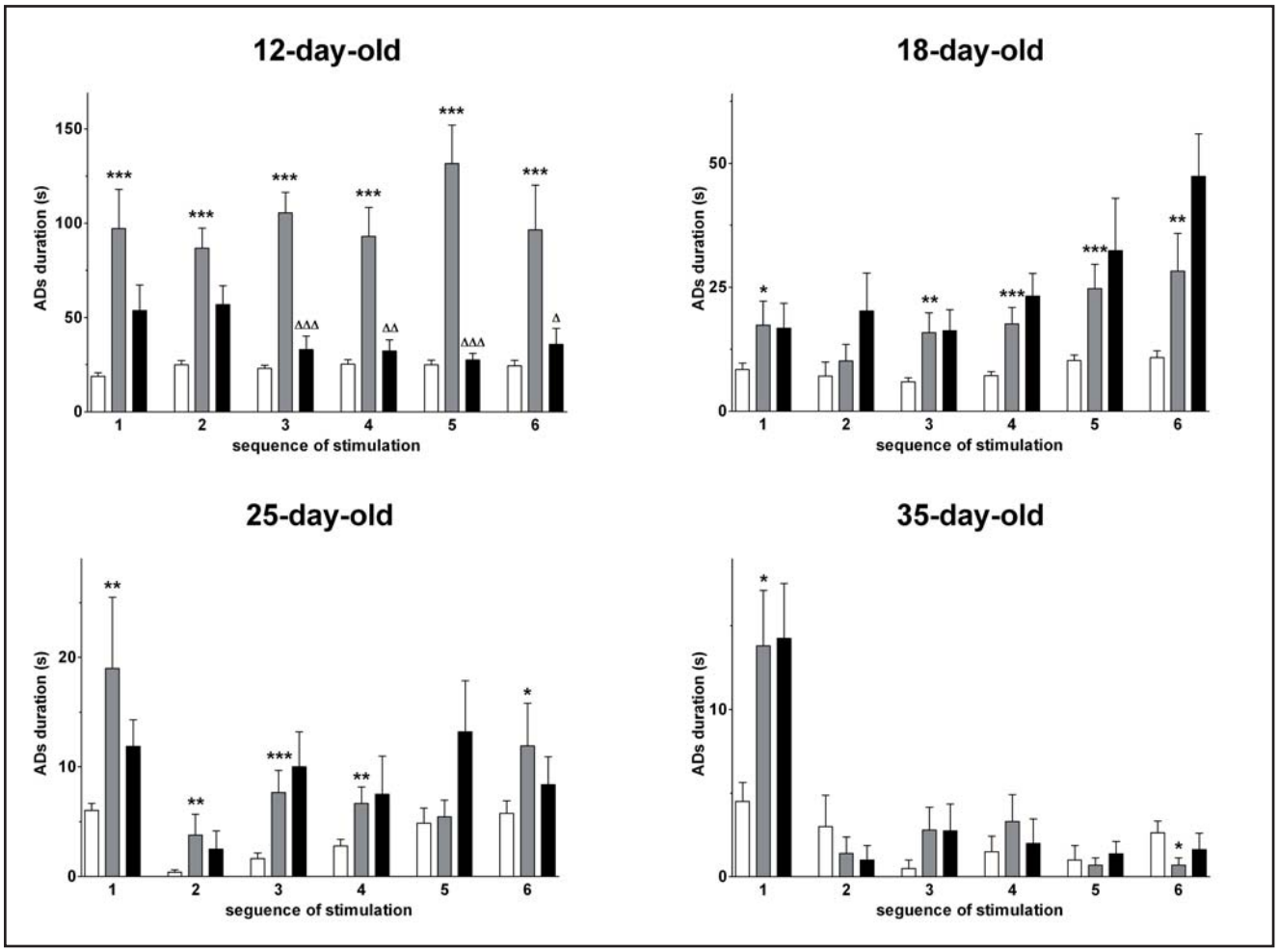

Fig. 1. Duration of ADs in 12, 18, 25 and 35-day-old rats. White columns – sham-treated animals (controls), grey columns – animals exposed to hypoxia, black columns – animals pretreated with ascorbic acid and exposed to hypoxia. 1-6 sequence of stimulation. Y axis represent the duration of ADs (seconds), * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, mutual comparison between controls and hypoxia exposed animals. $^{\Delta}$ indicates results significant at $p<0.05$, $^{\Delta\Delta}$ indicates results significant at $p<0.01$, $^{\Delta\Delta\Delta}$ indicates results significant at $p<0.001$, mutual comparison between hypoxia exposed animals and animals treated with ascorbic acid before each hypoxia exposition.

efficiently (Mares & Trojan 1990; Maresova *et al.* 2001). The early onset of oxygen deficiency is related to many events, such as neuron depolarization (Balestrino 1995), followed by (according to some sources is preceded) the secretion of glutamate (Choi 1988; Doble 1999). This glutamate overload sustains the depolarization-prone microenviroment and finally leads to excitotoxic damage of neuronal tissue (Doble 1999). Mentioned excitotoxic damage could be trigger by blocking oxidative phosphorylation, decreasing intracellular pH, or by mitochondria damage and depletion of energetic reserves (Vanucci & Vannuci 2005; Groennendaal *et al.* 1999; Maulik *et al.* 2001). It is well known, that excessive generation of free radicals is involved in excitability and seizure-related brain disturbances (Mori *et al.* 1990; Waldbaum *et al.* 2010). For such reason the free radicals scavengers were introduced and widely tested for their possibility to protect the neuronal tissue from excitotoxic damage, with special focus on immature brain, that is relatively poor on antioxidant defense systems (Buonacore 2007; Sies 1997; Wu *et al.* 2011). AA is water soluble substance, relatively abundant in the brain, capable to antagonize N-methyl-D-aspartate receptors (Miura 2006) and protecting lipoprotein from peroxidative damage by peroxyl radicals by scavenging the free radicals (Woods *et al.* 2001). TOC, the second tested substance in our experiment, is lipid soluble and could suppress the intracellular calcium increase and acts against membrane lipid peroxidation (Mattson *et al.* 1995; Zaidi *et al.* 2003). In our experimental pattern the youngest experimental group profited most from the prophylactic administration of AA and TOC. Surprisingly no effect of AA or TOC on ADs duration was observed in 18-day-old rats. It is noteworthy that the process of epileptogenesis is nonlinear with many "developmental windows" of relatively higher and lower resistance to epileptogenic stimuli

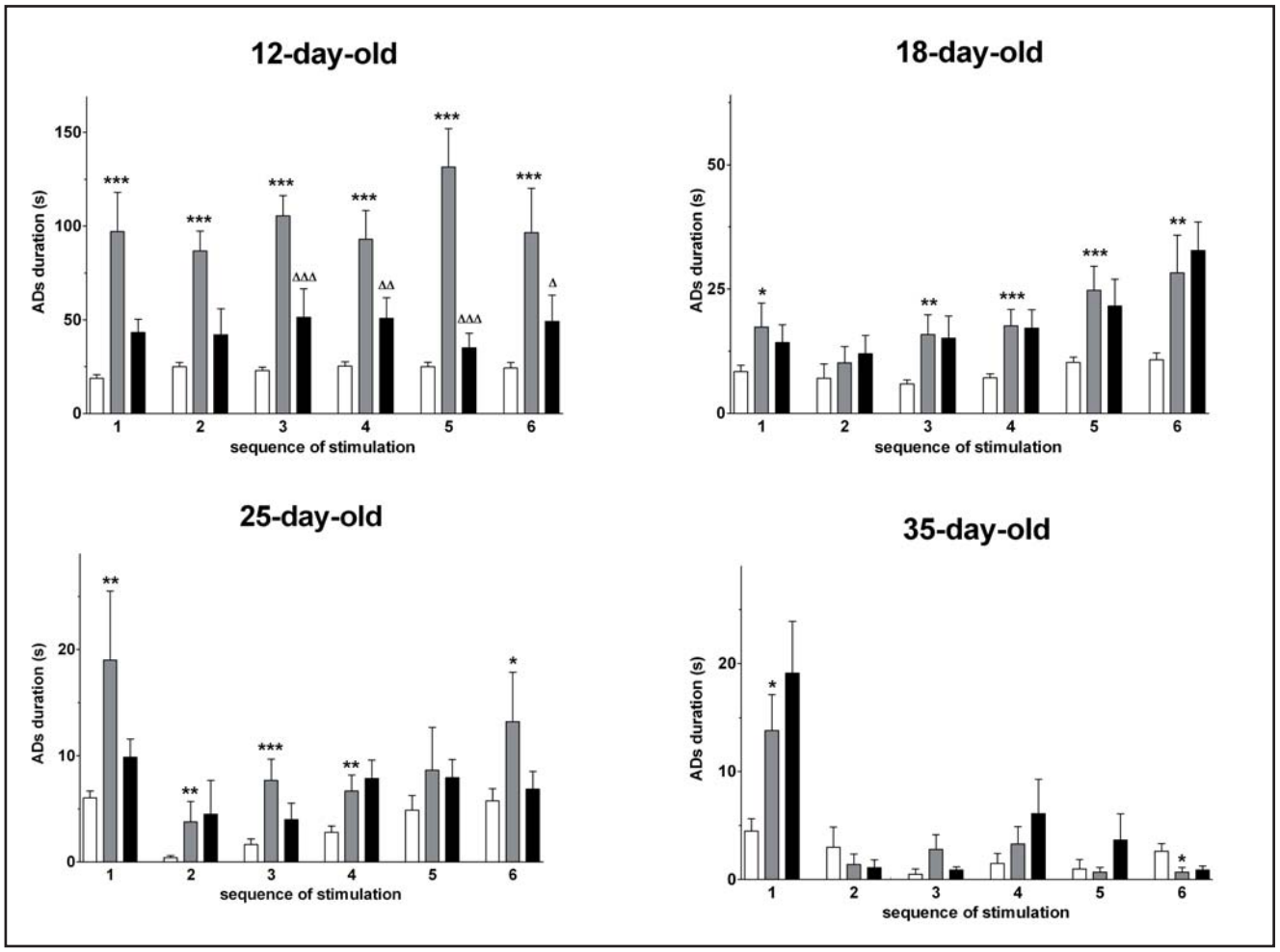

Fig. 2. Duration of ADs in 12, 18, 25 and 35-day-old rats. White columns – sham-treated animals (controls), grey columns – animals exposed to hypoxia, black columns – animals pretreated with α-tocopherol and exposed to hypoxia. 1-6 sequence of stimulation. Y axis represent the duration of ADs (seconds), * indicates results significant at $p<0.05$, ** indicates results significant at $p<0.01$, *** indicates results significant at $p<0.001$, mutual comparison between controls and hypoxia exposed animals. $^{\Delta}$ indicates results significant at $p<0.05$, $^{\Delta\Delta}$ indicates results significant at $p<0.01$, $^{\Delta\Delta\Delta}$ indicates results significant at $p<0.001$, mutual comparison between hypoxia exposed animals and animals treated with α-tocopherol before each hypoxia exposition.

(Schwartzkroin 1984) and probably at this developmental stage antioxidants used in our experiment were not capable to enhance the seizure arrest and the level of cortical excitability influenced by hypoxia challenges.

Interestingly, 25-day-old rats challenged to hypobaric hypoxia exhibited an increase in ADs duration compared to age matched controls, however neither AA nor TOC brought about decrease in the length of epileptic seizure elicited by repeated electrical stimulation. We exclude the possible exhaustion of the system generating ADs, because even after the last stimulation the observed ADs was longer compared to ADs elicited after the first stimulation. In the oldest experimental group exposed to hypoxia only the first ADs was longer when compared with controls. Explanation of such differences includes not only developmental aspects, but also the length of period in-between the hypoxia exposition and electrophysiological experiments ("resting" period between last hypoxia exposition and ADs elicitation was 18 days in 35-day-old animals, while only 24-hours in two youngest groups). Future experiments (hypoxia exposition prolonged till the 34th day) could clarify such age-related differences. As mentioned above, the hypoxia-related damage is caused by several factors and generation of free ROS is only one of them. Supplementation with ROS scavengers is obviously insufficient in this (25- and 35-day-old) developmental brain stage. We can speculate that seizure arresting mechanisms were impaired by other factors (that remained unaffected by our treatment) rather than by excessive ROS generation.

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