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Cortical epileptic afterdischarges in immature rats are differently influenced by NMDA receptor antagonists

Romana Šlamberová[a,b], Pavel Mareš[a,*]

[a]*Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic*
[b]*Charles University, 3rd Medical School, Prague, Czech Republic*

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Abstract

Epileptic afterdischarges elicited by stimulation of sensorimotor cortex were chosen to test anticonvulsant effects of NMDA receptor antagonists in developing rats (12, 18 and 25 days old) with implanted electrodes. Afterdischarges were elicited four times with 10-min intervals in the experiments with dizocilpine and 20 min with the other two drugs. Dizocilpine (0.5 or 1 mg/kg), CGP 40116 (0.1, 0.5 or 1 mg/kg) or 2-amino-7-phosphonoheptanoic acid (AP7, 30 or 60 mg/kg) was injected intraperitoneally between the first and second stimulation. Intensity of movements accompanying stimulation was diminished regularly only by CGP 40116. Duration of afterdischarges was reduced and intensity of clonic seizures was decreased by CGP 40116 in all age groups; dizocilpine exhibited similar action in 25- and 18-day-old rats, AP7 only in 25-day-old animals. Anticonvulsant action of the three NMDA antagonists exhibited different developmental profiles in our model; this difference might be due to developmental changes of NMDA receptors.

Keywords: Epileptic seizure; Cerebral cortex; NMDA receptor antagonist; Development; (Rat)

1. Introduction

Excitatory amino acids glutamate and aspartate represent the main excitatory neurotransmitters in mammalian brain. They exhibit their action by means of ionotropic and metabotropic receptors. Three types of ionotropic receptors for excitatory amino acids are classified according to their agonists: *N*-methyl-D-aspartate (NMDA), α-methyl-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate (Fagg and Massieu, 1991). The participation of excitatory amino acids in epileptogenesis was repeatedly demonstrated (for review see Schwarcz and Ben-Ari, 1986; Dingledine et al., 1990). Agonists of NMDA type of excitatory amino acid receptor are potent convulsants in adult (Czuczwar et al., 1985; Moreau et al., 1989) as well as immature animals (Schoepp et al., 1990; Mareš et al., 2004).

Antagonists of NMDA receptors have been known for more than 20 years (Davies et al., 1981; Wong et al., 1986) therefore studies of their anticonvulsant action started in the early eighties (Czuczwar and Meldrum, 1982; Croucher et al., 1982). They exhibit strong anticonvulsant action in many animal models of epileptic seizures (for review see Chapman, 1991).

Developmental studies demonstrated higher sensitivity of NMDA type of excitatory amino acid receptors in immature rats in comparison with adult animals (Hamon and Heinemann, 1988; McDonald et al., 1988) therefore we studied anticonvulsant actions of NMDA receptor antagonists in developing rats. Previously we have demonstrated a potent specific action of noncompetitive and competitive NMDA receptor antagonists against generalized tonic–clonic seizures elicited by systemic pentetrazol administration (Mareš et al., 2004). High efficacy of these drugs in the youngest age group studied (7-day-old rat pups) decreased with age. This decrease was more marked with noncompetitive than with competitive

* Corresponding author. Tel.: +420 2 4106 2549; fax: +420 24106 2488.
E-mail address: maresp@biomed.cas.cz (P. Mareš).

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antagonists. These data are in agreement with developmental changes of convulsant action of *N*-methyl-D-aspartate where the youngest rats were found to be the most sensitive ones (Schoepp et al., 1990; Mareš and Velepileptic afterdischarges induced by low-frequency cortical stimulation. Individual stimuli elicit jerks of head and contralateral forelimb muscles. With increasing stimulation intensity there appears an epileptic afterdischarge formed by spike-and-wave rhythm in the electroencephalogram and accompanied by clonic seizures (Mareš et al., 2002). There are at least three different phenomena to be evaluated in this model: direct activation of motor system by cortical stimulation, generation of spike-and-wave rhythm of thalamocortical origin (Avanzini et al., 1992) and activation of the motor system by epileptic activity. In addition, 12-day-old rat pups exhibit a progressive prolongation of afterdischarges with repeated stimulations (Kubová et al., 1993) similar to partial kindling described by Racine (1978). Ketamine exhibited marked anticonvulsant action in this model in 12- and 25-day-old rats but only moderate effects in 18-day-old animals (Kubová and Mareš, 1995). We are unable to explain this developmental irregularity; data on development of NMDA receptors (Insel et al., 1990) as well as our findings with ketamine action against pentetrazol-induced motor seizures in immature rats (Velíšek et al., 1989) clearly speak in favor of ketamine action even in 18-day-old rats. The aim of this study was to test if our previous results in the model of cortical afterdischarges (Kubová and Mareš, 1995) reflect a specific action of ketamine (which has other mechanisms of action in addition to antagonism at NMDA receptors—Smith et al., 1981; Lodge et al., 1982) or if it is a rule for all NMDA receptor antagonists. Therefore we compared the action of a noncompetitive NMDA receptor antagonist dizocilpine, and two competitive antagonists AP7 and CGP 40116 in the model of cortical epileptic afterdischarges. In addition, the results may delineate the anticonvulsant profile of these drugs in developing rats because cortical afterdischarges may represent a model of human myoclonic seizures (Kubová et al., 1993). The results of our study might also be used for comparison with the promising new NMDA receptor antagonists, specific for NR2B subunit (Nikam and Meltzer, 2002; Barton and White, 2004).

2. Methods

Three age groups of Wistar albino rats were used—12, 18 and 25 days old. The experiments were approved by Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences to be in agreement with Animal Protection Law of the Czech Republic (fully compatible with European Community Council directives 86/609/EEC). Electrode implantation was performed under ether anesthesia. Flat silver electrodes were implanted epidurally; two stimulation electrodes over the right sensorimotor, frontal cortical area (coordinates AP=−1 and +1, L=2 mm in relation to bregma), recording electrodes over left sensorimotor region (AP=0, L=2), right and left visual, occipital areas. The coordinates for occipital electrodes were calculated from the adult values of AP=6 and L=4 mm. The recalculation was based on the actual bregma–lambda distance, 8 mm was taken as a background value for adult rats. An indifferent electrode was placed into the nasal bone. All electrodes were cemented to the skull by fast curing dental acrylic. The surgical procedure took less than 15 min.

After the recovery from ether anesthesia (for at least 1 h) animals were neurologically examined (righting and placing reflexes), fed with a sucrose solution (suckling reflex thus examined) and only then stimulation started. Fifteen-second series of biphasic rectangular pulses of 1-ms duration and 8-Hz frequency were generated by a constant current stimulator. The intensity of electric stimulation ranged from 2.5 to 5 mA and was chosen to reliably evoke afterdischarges. When the suprathreshold intensity was found the drugs or saline was injected. The first afterdischarge was thus always a predrug one; it served as a background for measurements of effects in the subsequent stimulation series. The stimulation series were repeated three more times with the same intensity of stimulation current and with an interval between the end of afterdischarges and the beginning of the next stimulation lasting 10 min for dizocilpine and 20 min for AP7 and CGP 40116, respectively. Dizocilpine (Research Institute for Pharmacy and Biochemistry, Prague) and CGP 40116 (a generous gift of Novartis, Basel) were administered intraperitoneally as freshly prepared solutions in physiological saline–dizocilpine 5 min after the end of the first AD in doses of 0.5 or 1 mg/kg, and CGP 40116 at 10 min after the end of the first AD in doses of 0.1, 0.5 or 1 mg/kg. AP7 (Research Institute for Pharmacy and Biochemistry, Prague) dissolved in dimethylsulfoxide (DMSO) was administered intraperitoneally 10 min after the end of the first AD in doses of 30 or 60 mg/kg. The difference of the timing was based on pilot experiments demonstrating faster onset of action of dizocilpine in comparison with the other two drugs. Solutions were prepared with different concentrations of drugs to inject always the same volume

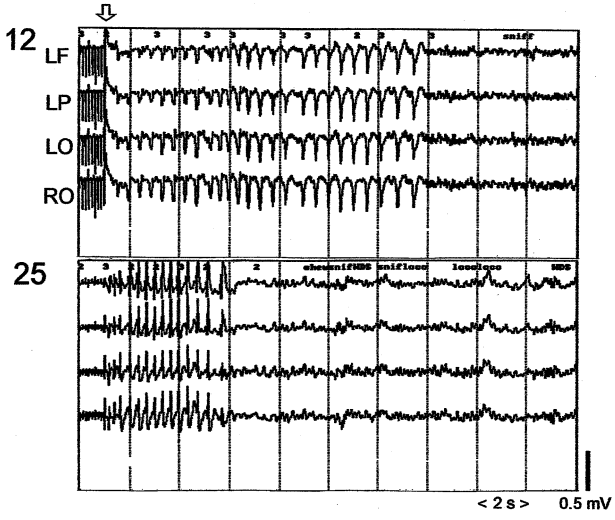

Fig. 1. Electroencephalographic recording of afterdischarges in 12- (upper part) and 25-day-old (lower part) rat. Individual leads from top to bottom: left frontal (LF), left parietal (LP), left occipital (LO) and right occipital (RO) in reference connection. An arrow marks the end of stimulation. Amplitude calibration=0.5 mV, time mark=2 s.

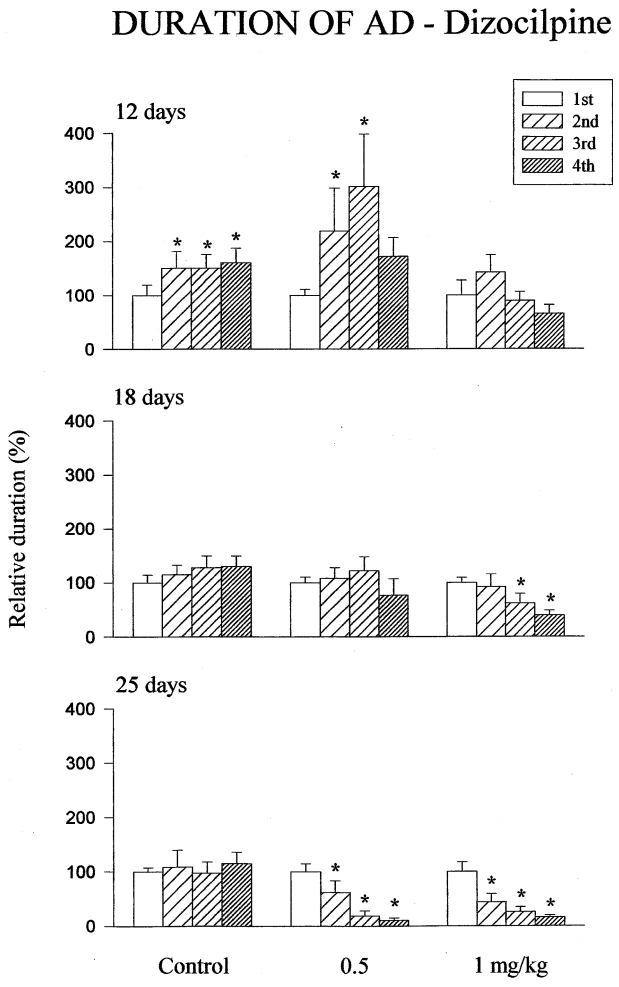

Fig. 2. Effects of dizocilpine on duration of afterdischarges (mean+S.E.M.) in rats 12, 18 and 25 days old (from top to bottom). The first afterdischarge was always a control one; an antagonist or saline was injected only at a time indicated in Methods section after this afterdischarge. Abscissa—the four afterdischarges (ADs) in control and two experimental (doses of 0.5 mg/kg and 1 mg/kg) groups. Ordinates—relative duration of the ADs, the firstpredrug AD was taken as 100% in each age and dose group. Significant differences in comparison with the first, predrug AD are marked by asterisks. Explanation of individual columns is on the right side of figure.

(1 ml/kg). Control groups were formed for each drug separately by siblings of experimental animals, i.e. each litter was divided into experimental and control rats. Controls for dizocilpine and CGP 40116 received physiological saline (different time schedules were used in these two control groups), those for AP7 were injected with DMSO. The injected volume of solvents was identical with that used for drugs (1 ml/kg). The solvents were administered at the same time intervals as the corresponding drugs. Each age and dose group consisted of eight to ten animals.

Motor phenomena accompanying stimulation and afterdischarges were recorded and quantified using a modified five-point scale of Racine (1972). The only change was in point 1; epileptic automatisms like intense orienting reaction in the known environment or wet dog shakes, i.e. elements of normal behavior but performed in inappropriate space and time were classified as this point. These activities were not synchronous with individual stimuli or sharp EEG graphoelements. The duration of afterdischarges was measured. Statistical evaluation of both afterdischarge duration and scores was performed by means of one-way repeated measure analysis of variance (RM ANOVA) with subsequent pairwise comparison according to Dunnett's method (SigmaStat® SPSS) in each age and dose group. Comparison of different doses of the same drug was realized similarly by means of two-way analysis of variance with factors number of afterdischarges and dose of the drug. Statistics was always calculated from absolute values; duration of afterdischarges is presented in figures as relative values for better comparison of data for individual age groups and drugs. The average of the first afterdischarge in the given group is taken as 100%. The level of statistical significance was set at $P<0.05$.

3. Results

3.1. Control animals

The first, preinjection stimulation always elicited an epileptic afterdischarge formed by spike-and-wave rhythm with approximately 3-Hz frequency in 18- and 25-day-old rats (Fig. 1) and by rhythmic sharp waves (with markedly lower frequency—1–2 Hz)

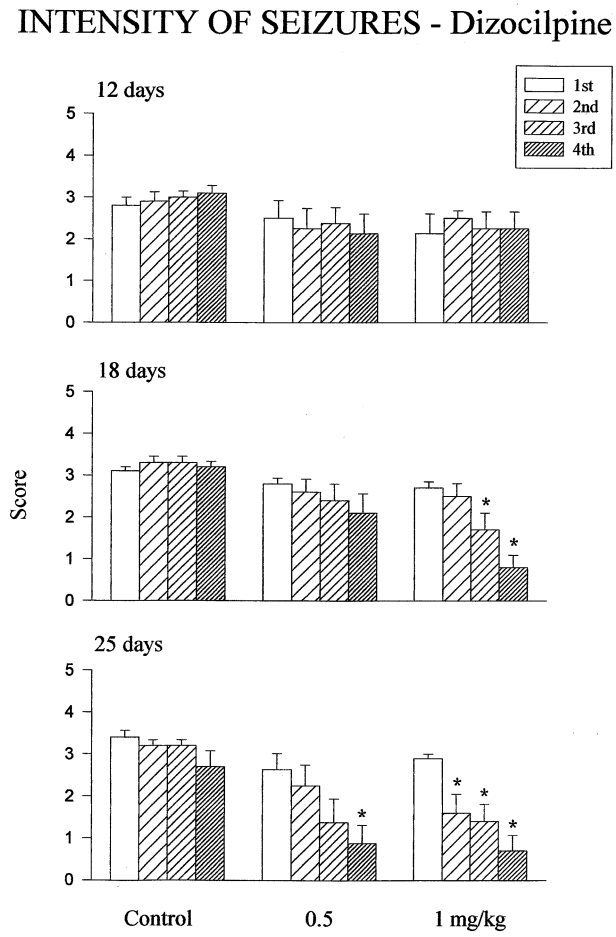

Fig. 3. Effects of dizocilpine on clonic seizures accompanying afterdischarges (mean intensity+S.E.M.). Details as in Fig. 2, only ordinates—five-point scale of intensity of motor phenomena according to modified Racine's scale.

in 12-day-old rat pups. Stimulation of the sensorimotor cortical area elicited clonic movements of head and forelimbs synchronous with individual stimuli. Sharp elements of the afterdischarges were accompanied by similar movements (clonic seizures); their frequency was thus lower. Duration of afterdischarges was longer in 12-day-old rat pups (average duration of the first afterdischarges from all groups of these rats was 12.1±2.3 s, mean±S.E.M.) than in the 18-day-old animals (8.4±1.2 s) the difference between 12- and 25-day-old rats (12.1±2.3 vs. 9.5±1.9 s) did not reach the level of significance. Repeated stimulations led to a progressive increase in duration of afterdischarges in 12-day-old control groups injected with physiological saline; similar tendency in DMSO-treated group did not reach a level of statistical significance. Only a tendency to progressive prolongation was observed in other age groups; statistically significant difference was found only for the fourth afterdischarge in comparison with the first one in the DMSO-treated group. Intensity of movements elicited by electrical stimulation as well as of clonic seizures related to afterdischarges did not significantly change with repeated stimulations. The results in animals injected with physiological saline and with dimethylsulfoxide did not markedly differ, i.e. DMSO did not significantly influence phenomena measured in our model.

3.2. Dizocilpine

Dizocilpine blocked the prolongation of afterdischarges in 12-day-old rats (Fig. 2). This action was seen only in the fourth afterdischarge after the lower dose and in the third and fourth afterdischarges after the higher dose. Duration of these afterdischarges did not significantly differ from the first predrug one. The higher dose of dizocilpine was found to progressively shorten the duration of the third and fourth afterdischarges in 18-day-old rats. In 25-day-old rats the duration of all three postdrug afterdischarges was significantly decreased by both doses of dizocilpine.

Suppression of the intensity of movements accompanying the electrical stimulation (i.e. significantly lower score) was observed only in the 18-day-old animals in the third and fourth stimulation series (data not shown). The intensity of clonic seizures accompanying the third and fourth afterdischarges was decreased by higher dose of dizocilpine in 18- and 25-day-old animals. The lower dose was effective only in the fourth afterdischarge in the 25-day-old rats (Fig. 3).

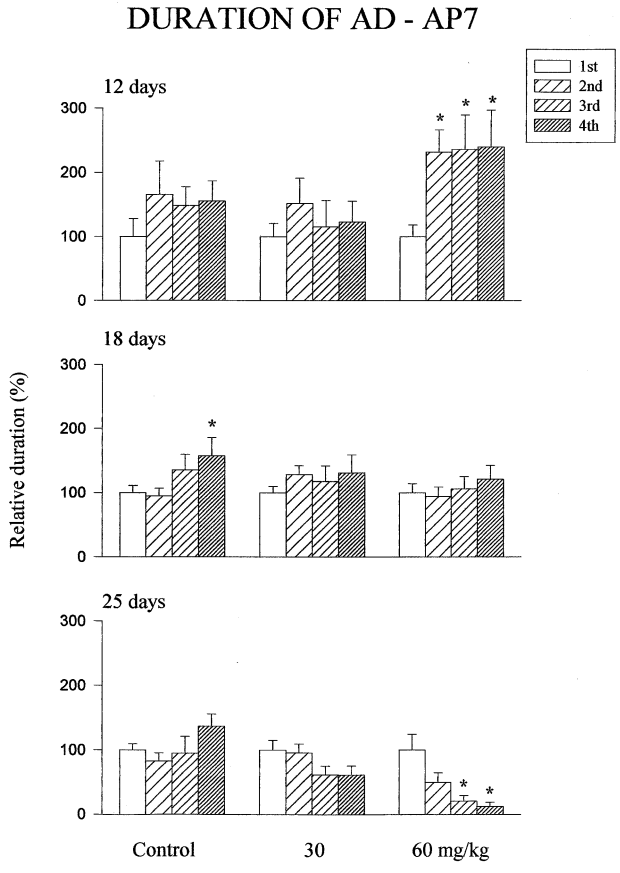

Fig. 4. Effects of AP7 on duration of afterdischarges (mean+S.E.M.). Details as in Fig. 2, only the doses were 30 mg/kg and 60 mg/kg.

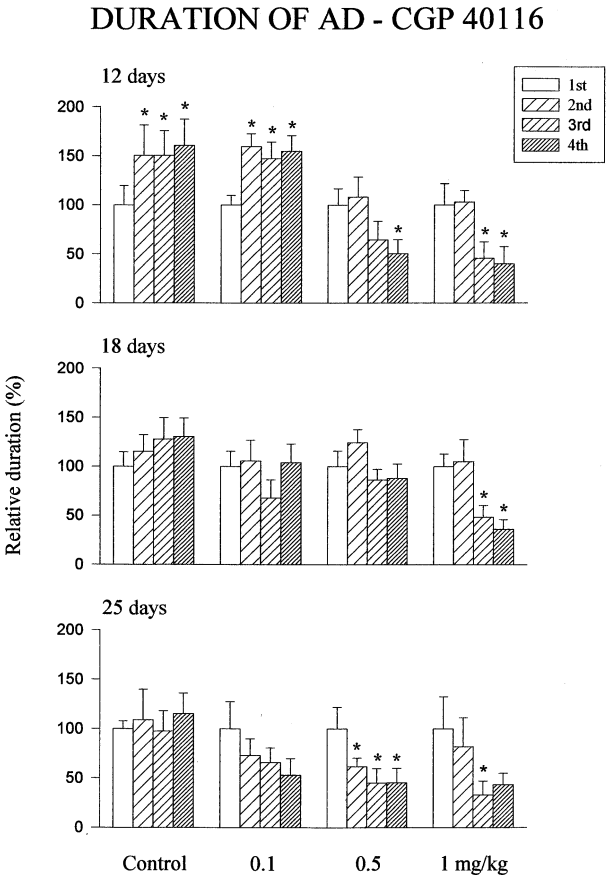

Fig. 5. Effects of CGP 40116 on duration of afterdischarges (mean+S.E.M.). Details as in Fig. 5, only ordinates—relative duration of the ADs, the first AD was always taken as 100%.

3.3. 2-Amino-7-phosphonoheptanoic acid (AP7)

The lower dose of AP7 resulted in a significant increase in afterdischarge duration in 12-day-old rats (Fig. 4). On the contrary, the significant prolongation of the fourth afterdischarge induced by dimethylsulfoxide in the control group did not appear after either dose of AP7 in 18-day-old rats. The higher dose of AP7 resulted in shortening of the third and fourth afterdischarges in 25-day-old animals.

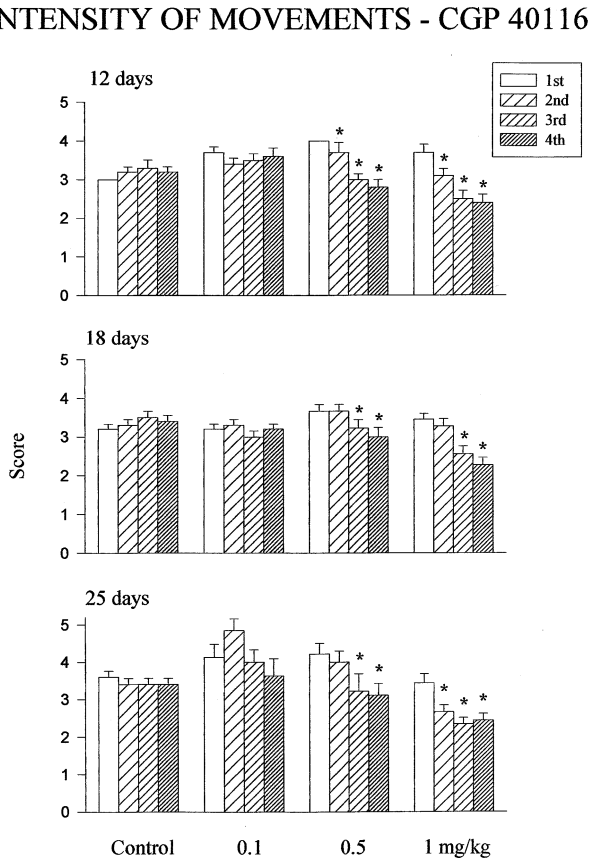

Fig. 6. Effects of CGP 40116 on movements accompanying stimulations (mean intensity+S.E.M.) in rats 12, 18 and 25 days old (from top to bottom). Abscissa—first to fourth stimulations in a control and three experimental groups (doses of 0.1 mg/kg, 0.5 mg/kg and 1 mg/kg), ordinates—five-point scale of intensity of motor phenomena. Description of individual columns is on the right. Asterisks again denote the significant difference in comparison with the first, predrug stimulation.

The intensity of movements accompanying the cortical stimulation was influenced rather exceptionally (in 25-day-old rats only). The intensity of clonic seizures was diminished after the higher dose in the third and fourth afterdischarges again in the oldest group (data not shown).

3.4. CGP 40116

The lowest dose of CGP 40116 was unable to block the progressive prolongation of afterdischarges in 12-day-old rat pups whereas the 0.5- and especially the 1-mg/kg dose suppressed the duration of afterdischarges. The only significant change found in 18-day-old rats was a shortening of the third and fourth afterdischarges after the highest dose of CGP 40116. Twenty-five-day-old animals exhibited a decrease in afterdischarge duration in all three afterdischarges after 0.5 mg/kg, the 1-mg/kg dose led only to a significant shortening of the third afterdischarge due to high variability of results (Fig. 5).

The intensity of movements accompanying stimulation was significantly diminished after the two higher doses in all age groups (Fig. 6). The intensity of clonic seizures accompanying afterdischarges was decreased by the two higher doses in all age groups, too (Fig. 7).

4. Discussion

The differences between 12-day-old rat pups and the two older groups reflect maturation of the cerebral cortex. Longer duration and lower frequency of epileptic graphoelements is due to imperfect synchronization of activities of cortical neurons (Prince and Gutnick, 1972) as well as to an absence of bursting neurons (Franceschetti et al., 1993). Rhythmic thalamocortical activities including spike-and-wave rhythm in the EEG appear in rats only during the third postnatal week (Mareš et al., 1982) therefore we cannot expect the same EEG pattern at various stages of postnatal development.

The effects found may be divided into two categories: a direct effect on the motor system reflected in suppression of movements accompanying stimulation and an anticonvulsant action expressed as a block of progressive prolongation, shortening and/or complete suppression of afterdischarges, and attenuation of motor seizures. The effects of the three antagonists against these phenomena markedly differ.

Movements elicited directly by stimulation of sensorimotor cortical area may be compared to localized seizure activity as described by Voskuyl et al. (1992) and Krupp and Löscher (1998). This phenomenon was more resistant to

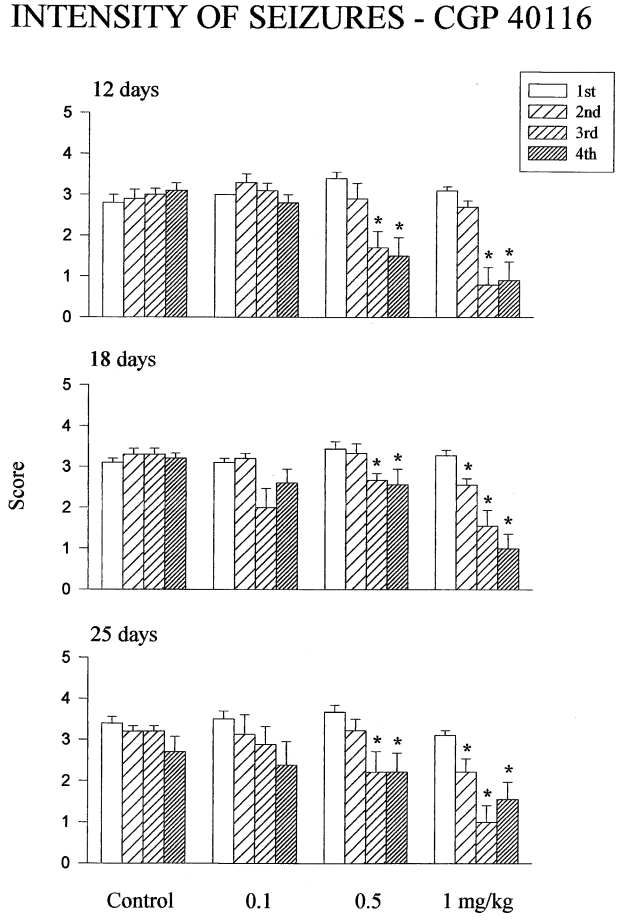

Fig. 7. Effects of CGP 40116 on severity of clonic seizures accompanying afterdischarges (mean intensity+S.E.M.). Details as in Fig. 5.

classical (Krupp and Löscher, 1998) as well as new (Della Paschoa et al., 2000) antiepileptic drugs than generalized seizure activity. In agreement with these data attenuation of these movements was regularly seen only after the administration of CGP 40116 in present experiments. Dizocilpine and AP7 did not exhibit a systematic effect in spite of the fact that impairment of the motor system was described for NMDA receptor antagonists in adult animals (Parsons et al., 1995) as well as in rat pups (Mikulecká and Mareš, 2002). Site of action of NMDA receptor antagonists on motor system is thus probably localized in subcortical structures.

Three epileptic phenomena were evaluated in our experiments: generation of cortical epileptic afterdischarges measured as their shortening; progressive prolongation of afterdischarges observed in 12-day-old animals; and clonic seizures accompanying afterdischarges. All three antagonists of NMDA receptors exhibit an anticonvulsant action but their efficacy varied according to the phenomenon measured and to the age.

Cortical epileptic afterdischarges characterized by spike-and-wave rhythm represent probably a thalamocortical phenomenon similarly as spike-and-wave rhythms elicited by other agents (Steriade and Deschenes, 1984). The rhythmic thalamocortical phenomena can be recorded since the third postnatal week in rats (Mareš et al., 1982; Avanzini et al., 1992); that is in agreement with EEG pattern of cortical afterdischarges (Mareš et al., 2002; present study). Thalamocortical system of 12-day-old rats cannot generate EEG spike-and-wave rhythm, but rhythmic sharp waves registered as an afterdischarge in this age group have probably the same significance. Mechanism of generation of the spike-and-wave rhythm is mainly GABAergic (Snead, 1995) but there are some results speaking in favor of the role of excitatory amino acids in the generation of this rhythm (Peeters et al., 1994; Koerner et al., 1996). Our data support a possible participation of excitatory mechanisms.

Progressive prolongation of afterdischarges is significant in 12-day-old control rat pups receiving saline but not in the control group injected with dimethylsulfoxide. This prolongation is due to a lack of postictal depression in this age group (Mareš et al., 1992) and an immaturity of inhibitory systems participating in this depression. A possible interaction of dimethylsulfoxide with these systems remains to be studied. In contrast to immaturity of inhibitory systems, NMDA receptors are present at this age (Insel et al., 1990) and the convulsant action of systemically administered NMDA is stronger than in older animals (Schoepp et al., 1990; Mareš and Velíšek, 1992). Marked action of dizocilpine and CGP 40116 against this "partial kindling" was thus predictable. The difference between the two doses of dizocilpine might be due to pharmacokinetic factors—lower dose exhibited the action only on the fourth afterdischarge whereas higher dose reversed the effect of repeated stimulations at the third and fourth stimulations. A hypothetical explanation might be in a different time necessary to reach the critical concentration of dizocilpine in brain. The potentiating effect of the higher dose of AP7 cannot be explained at present.

Motor seizures are due to a spread of epileptic activity into the motor structures up to the spinal cord level. At least part of pyramidal cells of the motor cortex uses excitatory amino acids as transmitter (as was demonstrated for cortico-striate pathway by Headley and Grillner, 1990) and therefore the effect of NMDA receptor antagonists was not surprising. Again AP7 was active only in 25-day-old rats and its inefficacy in younger animals remains to be analyzed.

Marked differences in efficacy of individual NMDA receptor antagonists are not surprising. They were demonstrated in a model of status epilepticus in adult rats (Yen et al., 2004). Comparing the three antagonists used in our experiments, the most potent action was exhibited by CGP 40116; this drug was effective against all three epileptic phenomena studied in all age groups. This result is in sharp contradiction with data of Della Paschoa et al. (2000). They demonstrated only a moderate action of a very high dose of CGP 40116 (5 mg/kg i.v.) on both threshold values measured in a model of cortical stimulation with continuously increasing intensity. This contradiction may be explained by a substantial difference in the two cortical stimulation models: repeated suprathreshold stimulations in our experiments vs. a progressive increase of stimulation intensity in the other model (Voskuyl et al., 1989). There are additional factors—placement of stimulation electrodes and, last but not least, age of animals. Dizocilpine did not differ from CGP 40116 in the 18- and 25-day-old rats but the youngest group was less influenced by this drug. This finding differs from our older data on the action of dizocilpine on generalized tonic–clonic seizures – younger animals were more sensitive to the anticonvulsant action of dizocilpine than 18- and 25-day-old ones (Mareš et al., 2004) – as well as from results of the study of motor skills under the influence of dizocilpine and CGP 40116 (Mikulecká and Mareš, 2002). Due to these results the pharmacokinetic reasons may be excluded. Pharmacodynamic reasons (different composition of NMDA receptor subunits in immature brain, different relation between dizocilpine binding site and cationic channel) might be discussed only hypothetically because there are not sufficient data in this field. The only background might be formed by findings of Morin et al. (1989) that dizocilpine binding is overexpressed in the brainstem of immature rats (brainstem is the crucial structure for generation of generalized tonic–clonic seizures—Browning and Nelson, 1986) whereas the amount of these sites is between 55% (10-day-old rats) and 80% (15-day-old rats) in the cerebral cortex.

As AP7 is concerned the results were surprising. Marked efficacy in 25-day-old rats in contrast to nearly nil or paradoxical proconvulsant effect in younger pups speaks against the poor penetration into the brain. Were the blood–brain barrier a reason of developmental changes quite opposite effect has to be seen (Saunders, 1992). In addition, AP7 penetrates regularly at least into cerebrospinal fluid of adult rats (Compton et al., 1988). Again, explanation of our

findings may be only hypothetical, probably in the field of composition of subunits of NMDA receptors in structures important for generation of cortical afterdischarges. This hypothetical explanation is based on published data on differential development of NMDA receptor subunits NR1, NR2A–D at the level of mRNAs as well as proteins (Sheng et al., 1994; Portera-Cailliau et al., 1996; Wenzel et al., 1997). At present, attention is focused on subtype specific NMDA receptor antagonists namely drugs acting on receptors with NR2B subunit (Nikam and Meltzer, 2002). There are promising data about their anticonvulsant action in adult rodents (Armstrong et al., 1998, Barton and White, 2004) but developmental studies remain to be performed.

Our results demonstrated that developmental changes of the anticonvulsant action of NMDA receptor antagonists depend on the drug as well as model used. Anticonvulsant effects of different NMDA receptor antagonists against generalized tonic–clonic seizures induced by pentetrazol exhibit identical developmental tendency — a decrease of efficacy with brain maturation (Mareš et al., 2004). The action of NMDA receptor antagonists in a model of cortical epileptic afterdischarges does not exhibit such a simple developmental change. At present we cannot explain low efficacy of AP7 in 12- and 18-day-old rats. The developmental irregularity of the action of ketamine (lower efficacy in 18-than in both 12- and 25-day-old rats—Kubová and Mareš, 1995) might be due to a low specificity of ketamine as an NMDA receptor antagonist, i.e. to its other mechanisms of action. This explanation is supported by the present data on dizocilpine: this more specific noncompetitive antagonist exhibited a marked effect in 18-day-old rats. Anticonvulsant action of CGP 40116 and dizocilpine on cortical epileptic afterdischarges in immature rats might predict clinical efficacy of NMDA antagonists against myoclonic seizures in pediatric patients. On the other hand, both drugs exhibit serious side effects in immature rats (Mikulecká and Mareš, 2002) which make their clinical application impossible. It is therefore necessary to focus attention either on low-affinity or subunit-specific NMDA receptor antagonists (Nikam and Meltzer, 2002; Barton and White, 2004).

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