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Excitatory Aminoacids and Epileptic Seizures in Immature Brain

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Summary

Data on convulsant and anticonvulsant action of drugs influencing excitatory amino acid receptors in developing rats are reviewed. Agonists of NMDA type of receptors NMDA and homocysteic acid, elicited an age-related seizure pattern – flexion, emprosthotonic seizures – in the first three postnatal weeks of rats. Generalized clonic-tonic seizures appeared only after a longer latency. Kainic acid administration resulted in epileptic automatisms and later in minimal, clonic seizures followed by generalized tonic-clonic seizures. A decrease of sensitivity to convulsant action with age is a general rule for all agonists tested. Different anticonvulsant action of NMDA and nonNMDA antagonists was demonstrated in a model of generalized tonic-clonic seizures induced by pentetrazol, whereas their action against epileptic afterdischarges elicited by electrical stimulation of cerebral cortex was similar. Again, higher efficacy in younger animals was a rule. As far as metabotropic glutamate receptors are concerned, agonists of groups II and III were shown to protect against convulsant action of homocysteic acid in immature rats and an antagonist of group I receptors MPEP suppressed the tonic phase of generalized tonic-clonic seizures induced by pentetrazol more efficiently in younger than in more mature rat pups. Unfortunately, a higher sensitivity to the action of antagonists of ionotropic glutamate receptors was demonstrated also for unwanted side effects (motor functions were compromized). In contrast, glutamate metabotropic receptor antagonist MPEP did not exhibit any serious side effects in rat pups.

Key words

Pharmacology • NMDA receptors • nonNMDA receptors • Glutamate metabotropic receptors • Convulsions • Ontogeny • Rat

Excitatory amino acid receptors and their role in epilepsy

Glutamate, as well as other excitatory amino acids, are acting via two types of receptors, ionotropic glutamate receptors (iGluR) which are ligand-gated cation specific ion channels and metabotropic glutamate receptors (mGluR) which are G-protein-coupled receptors. Ionotropic glutamate receptors are classified according to their prototypic agonists: NMDA (N-methyl-D-aspartate), kainate and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid). Kainate and AMPA receptors are sometimes put togeher as nonNMDA receptors. Various agonist and competitive as well as non-competitive antagonists and modulators are known since eighties (for review see Watkins 1991,

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Palfreyman and Baron 1991, Carter *et al.* 1991, Foster 1991, Honoré 1991). To date, eight mGluR subtypes (mGluR1 - mGluR8) together with splice variants have been cloned and are divided into three groups, defined on the basis of their amino acid sequence homology, signal transduction mechanism, and agonist selectivity (Nakanishi 1992, Pin and Duvoisin 1995). Group I receptors (mGluR1 and 5) are positively coupled to phosphoinositide hydrolysis, with consequent mobilization of intracelular Ca^{2+}; group II receptors (mGluR2 and 3) are negatively coupled to the adenylate cyclase; and group III (mGluR4, 6, 7, and 8) are also negatively linked to adenylate cyclase, but exhibit an agonist preference different than that of group II mGluRs (Pin and Duvoisin 1995, Conn and Pin 1997).

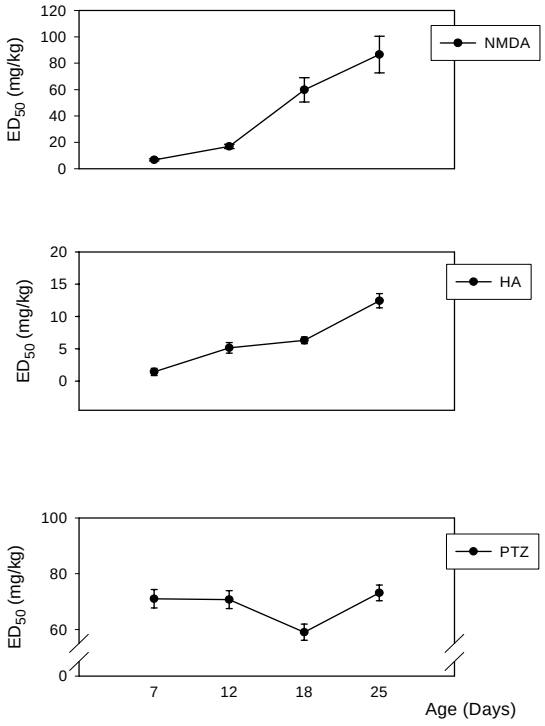

Fig. 1. Fifty percent convulsant doses of N-methyl-D-aspartate (NMDA, top), homocysteic acid (HA, middle) and pentetrazol (PTZ, bottom) in four age groups of immature rats. Both excitatory amino acid agonists demonstrate the highest efficacy in the youngest rats whereas pentetrazol, a convulsant drug influencing GABA-A receptors, exhibits different developmental profile. Abscissae: age of rats in days; ordinates: fifty percent convulsant doses in mg/kg (intraperitoneal injections).

As to the epilepsy and ionotropic glutmate receptors, there exist several reviews (e.g. Dingledine *et al.* 1990, Chapman 1991, Meldrum 1994). Practically all agonists are able to induce epileptic seizures and brain damage whereas antagonists have been shown to be anticonvulsant. The role played by metabotropic glutamate receptors depends on the type of receptors: activation of type I is proconvulsant and convulsant, whereas activation of type II and III is anticonvulsant (for review Moldrich *et al.* 2003). The existing data concern mostly adult experimental animals; human epilepsy starts in half of patients during infancy and childhood (Sander and Sillanpää 1997) and therefore studies of our laboratory have been concentrated on features of epileptic seizures (including the action of agonists and antagonists of excitatory amino acids) in developing brain.

Drugs acting on ionotropic glutamate receptors

Convulsant action of agonists of ionotropic glutamate receptors

Developmental studies appeared in eightees: Albala *et al.* (1984), Cavalheiro *et al.* (1983), Cherubini *et al.* (1983), Ben-Ari's group (Ben-Ari *et al.* 1984, Berger *et al.* 1984, Nitecka *et al.* 1984, Tremblay *et al.* 1984), our laboratory (Velíšková *et al.* 1988) and Babb *et al.* (1995) described seizures induced by kainic acid. Even 7-day-old rat pups do exhibit seizures after systemic administration of kainic acid and the sensitivity of this age group as well as of 12-day-old group is higher than that of adult animals (Velíšková *et al.* 1988). Kainic acid elicits epileptic automatisms as the first sign of its action (scratching as a predominant automatism in 7- and 12-day-old rat pups and wet dog shakes in older animals). Minimal clonic seizures restricted to forelimb and head muscles appear after a longer latency. This type of seizures is not common during the first two weeks of postnatal life but it is typical for older animals forming a pattern of kainic acid-induced status epilepticus. Very young animals exhibit an easy transition into generalized tonic-clonic seizures, this transition is uncommon in 25-day-old and adult rats (Velíšková *et al.* 1988). Schoepp *et al.* (1990) compared convulsant action of three agonists of excitatory amino acids in a group of 7- to 11-day-old rat pups and found that not only kainic acid but also N-methyl-D-aspartate (NMDA), AMPA and quisqualate elicit seizures in immature rats. These seizures may be prevented by administration of antagonists specific for individual types of excitatory amino acid receptors. We performed a study of seizures elicited by NMDA at different levels of postnatal development of rats (Mareš and Velíšek 1992). Initial immobility was followed by a locomotor hyperactivity

and much higher sensititvity of immature rats to convulsant action of NMDA (7-day-old rats exhibit generalized clonic-tonic seizures after the 7.5 mg/kg dose of NMDA, whereas 75 mg/kg has to be used in 25-day-old animals) was demonstrated in this study (Fig. 1). Minimal clonic seizures were never observed after NMDA administration. In contrast, an age-dependent type of seizures (flexion, emprosthotonic seizures) was observed during the first three postnatal weeks. A decrease of sensitivity during postnatal development as well as age-dependent flexion seizures were later demonstrated also for homocysteic acid, a sulphur containing amino acid acting primarily on NMDA receptors (Mareš *et al.* 1997a). There is a high mortality with systemic administration of both NMDA and homocysteic acid and therefore we started to use intracerebroventricular application of homocysteic acid to have a possibility to study long-lasting consequences of this insult. Homocysteic acid applied intracerebroventricularly in 12-day-old rats induced generalized clonic-tonic seizures, recurring frequently for several hours. The mortality with this seizure model, at least during the acute phase of seizures, was minimal (Folbergrová *et al.* 2000). Massive neuronal degeneration was observed in many brain regions following these seizures after one and/or 6 days of survival. In hippocampal formation, necrotic neurons were present in hippocampal CA1 and CA3 fields, whereas in the granule cell layer of dentate gyrus neurons with segmented or fragmented nuclei in various stages of degeneration were detected, displaying the features of apoptotic death (Langmeier *et al.* 2003). For illustration, neuronal degeneration in CA1 and CA3 hippocampal fields and dentate gyrus, evaluated by FluoroJade B staining, is shown in Figure 2.

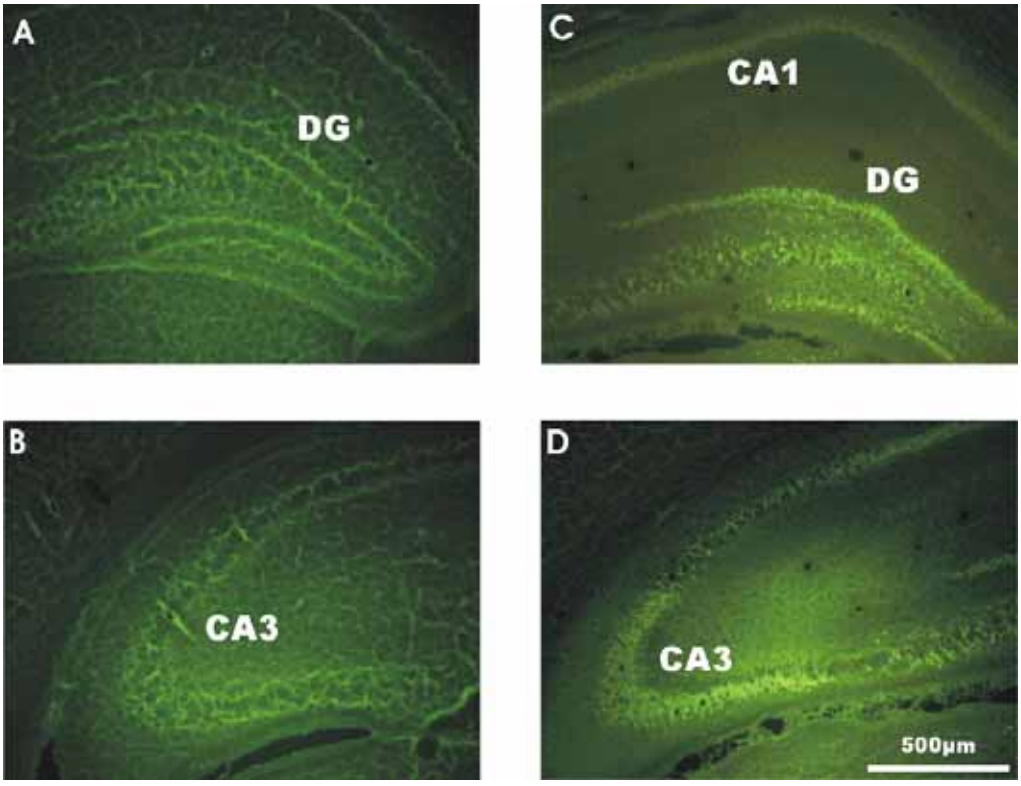

Fig. 2. Fluorescence photomicrographs of Fluoro Jade B (FJB) stained sections demonstrating neurodegeneration induced by homocysteic acid-induced seizures. A and B – control rat, C and D – rat 24 hours after intracerebroventricular homocysteic acid administration (on postnatal day 12). A and C – dentate gyrus (DG); B and D – CA3 field of dorsal hippocampus. Numerous FJB-positive neurons are present in both structures of the treated animal. Scale bar 500 μm.

Glutamate does not cross mature hemato-encephalic barrier, but convulsant action of high doses can be demonstrated in immature rats. Intraperitoneal administration of 4 g/kg dose of glutamate reliably induces minimal clonic as well as generalized tonic-clonic seizures in 7-, 12- and 18-day-old rats (Mareš *et al.* 2000); induction of seizures in older rats appears only exceptionally.

Another pattern of seizure activity has been demonstrated after administration of homocysteine (Kubová *et al.* 1995). The first signs of action are represented by moderate motor activities (e.g. tremor, shuffling of forepaws) in all age groups and by orienting activity since the age of 18 days. Flexion seizures are common in 7- and 12-day-old rat pups but in contrast to NMDA and homocysteic acid not in 18-day-old ones. Minimal clonic and generalized tonic-clonic seizures can be observed in all age groups. Doses necessary to elicit minimal seizures exhibit an U-shape when related to the age of rats whereas the possibility to induce generalized tonic-clonic seizures moderately increases with age.

Pattern of seizures is different according to the type of agonists. The very first signs induced by NMDA, homocysteic acid and homocysteine reflect action on the motor system (immobility, then hyperactivity), whereas kainic acid elicits epileptic automatisms. This difference reflects different target structures: agonists of NMDA receptors influence primarily motor system, kainic acid has its target in limbic structures (Ben-Ari 1985). As to the convulsions, all drugs used are able to elicit generalized seizures only the sequence of tonic and clonic phases is different. Typical NMDA agonists never induce minimal clonic seizures generated in basal forebrain structures (Browning and Nelson 1986) but they elicit age-bound emprosthotonic seizures. Unfortunately, we do not know the generator of these flexion seizures as well as reasons for their disappearance with maturation.

Seizures induced by agonists of ionotropic excitatory amino acid receptors are very difficult to be pharmacologically suppressed. Among kainic acid-induced epileptic phenomena in developing rats automatisms and tonic-clonic seizures are resistant to carbamazepine, valproate, phenobarbital and clonazepam, but all these drugs are active against minimal clonic seizures (Velíšek *et al.* 1992b). Automatisms may be suppressed by α_2 noradrenergic agonist clonidine but not by 5-HT_2 antagonist ritanserin (Velíšek *et al.* 1994). NMDA-induced seizures are also resistant to antiepileptics because clonazepam even at a high dose of 1 mg/kg i.p. is not able to suppress initial signs, flexion seizures as well as generalized clonic-tonic seizures in any age group (Velíšek and Mareš 1995).

Interaction of subconvulsant doses of excitatory amino acid agonists with another model of epileptic seizures – cortical afterdischarges – demonstrates very specific relationships (Mareš *et al.* 2002b). The afterdischarges are prolonged by kainic acid and homocysteine in all age groups studied whereas NMDA exhibits this action only in 12-day-old rats. Pattern of convulsions in 12-day-old rats is changed by NMDA and homocysteine from clonic seizures of forelimb and head muscles to flexion seizures. Homocysteine administration facilitates transition into the limbic type of afterdischarges (regularly seen only after very high intensities of stimulation) in 18- and 25-day-old rats as well as to long-lasting (tens of minutes) generalized tonic-clonic seizures in 25-day-old animals. The last pattern is never induced by cortical stimulation alone. Kainic acid did not change the pattern of afterdischarges but typical kainic acid-induced nonconvulsive seizures appear in the EEG during interstimulation intervals (Mareš *et al.* 2002a). Data for adult animals are similar, in addition the threshold for limbic type of afterdischarges is decreased (Koryntová *et al.* 1997, Koryntová and Mareš 1998). There is a mutual potentiation of action of the two epileptogenic agents used in these experiments – subconvulsant doses of excitatory amino acid agonists elicit motor seizures characteristic for higher doses and duration of EEG afterdischarges is markedly prolonged. Motor seizures accompanying cortical afterdischarges reflect the level of maturation of the central nervous system.

Anticonvulsant action of antagonists of ionotropic glutamate receptors

Antagonists for both NMDA and nonNMDA receptors are known as potent anticonvulsants (for review see Chapman 1991).

We always start developmental studies of possible anticonvulsant action with motor seizures induced by pentetrazol. The dose used in our laboratory allows to study models of two types of epileptic seizures in one experiment: minimal clonic seizures involving forelimb and head muscles and generalized tonic-clonic seizures (Velíšek *et al.* 1992a). All antagonists of NMDA type of receptors (competitive 2-amino-7-phosphonoheptanoic acid, CGP 39551 and CGP 40116; noncompetitive ketamine and dizocilpine [MK-801]; acting at modulatory sites – glutamic acid diethylester and kynurenic acid) exhibit powerfull anticonvulsant action restricted to generalized tonic-clonic seizures. Minimal clonic seizures were not influenced by these antagonists (Velíšek *et al.* 1989, 1990, 1991, 1995b, 1997, Velíšek and Mareš 1992, Haugvicová and Mareš 1998). Anticonvulsant action of antagonists of nonNMDA receptors in pentetrazol model is even more

specific than that of NMDA antagonists. Quinoxalinediones CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), DNQX (6,7-dinitro-quinoxaline-2,3-dione) and NBQX (2,3-dihydroxy-6-nitro-7-sulamoyl-benzo(F) quinoxaline) are active only against the tonic phase of generalized tonic-clonic seizures leaving both minimal and generalized clonic seizures unaffected (Velíšek *et al.* 1995a).

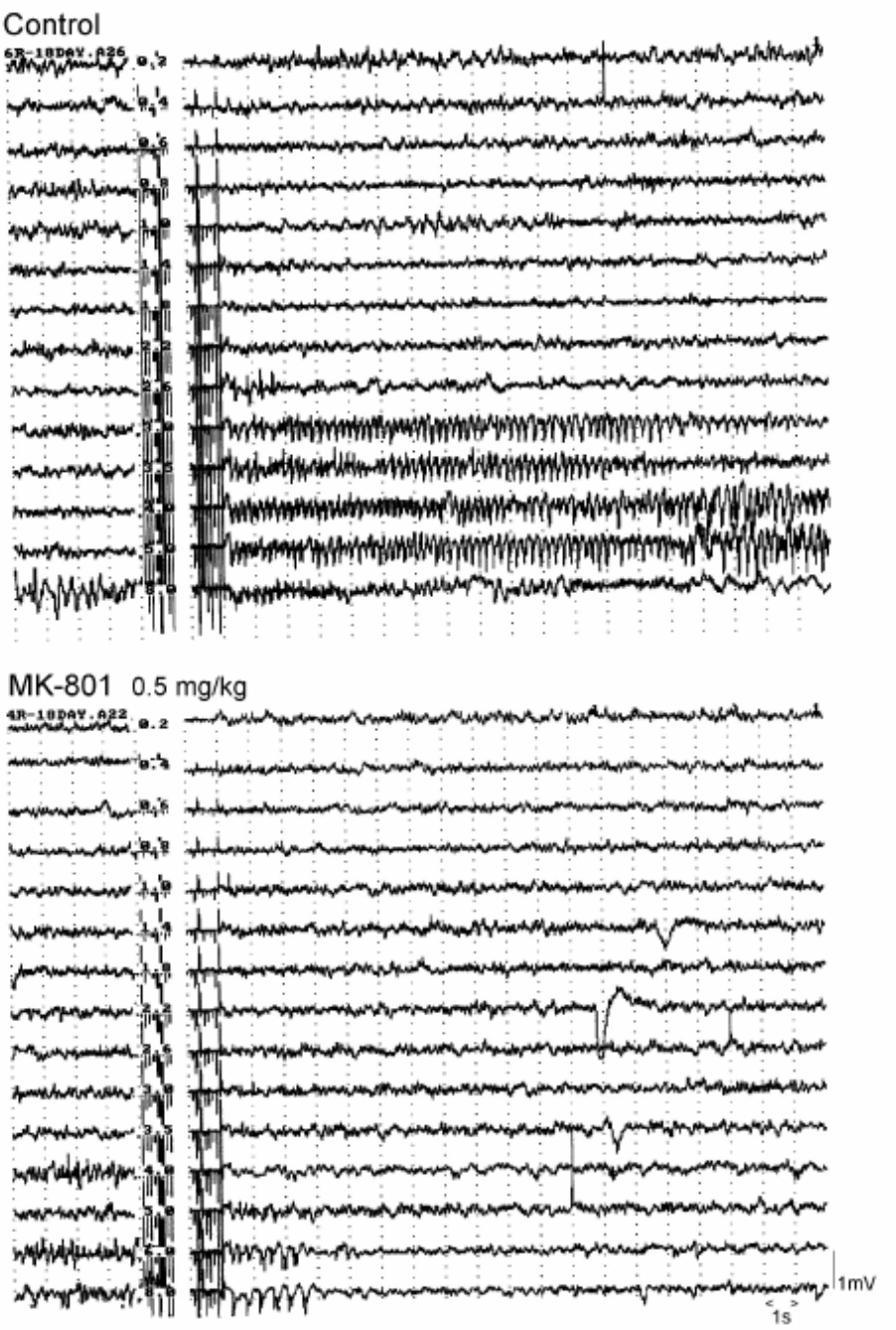

Fig. 3. Effects of rhythmic electrical stimulation of sensorimotor cortical area in two 18-day-old rats. Upper part – control animal, lower part – rat pretreated with MK-801 (dizocilpine, 0.5 mg/kg i.p.). Individual rows represent stimulations with increasing current intensities, left part – EEG immediately before stimulation, interruption during stimulation and right part – the last second of stimulation and 19 s after stimulation. Threshold intensity for elicitation of an epileptic afterdischarge was 2.6 mA in control rat and 5.0 mA in rat pretreated with MK-801. Time and amplitude calibration in the lower right corner.

As far as the seizures induced by homocysteine are concerned, the pretreatment with the selected antagonists of NMDA and nonNMDA receptors has revealed that there is apparently an age-dependent change in the anticonvulsant efficacy of nonNMDA receptor antagonists. Thus, only antagonists of NMDA receptors

(AP7 and MK-801) were anticonvulsant in 7-day-old rat pups whereas both NMDA (AP7, CGP 40116, MK-801) and nonNMDA (NBQX) receptor antagonists protect 18-day-old rats against homocysteine-induced seizures and their metabolic consequences (Folbergrová 1994, 1997). We have hypothetized that the observed difference in the protective efficacy of AMPA receptor antagonists might reflect some subtle change in AMPA receptor subunits composition, occurring during early postnatal development (Folbergrová 1997).

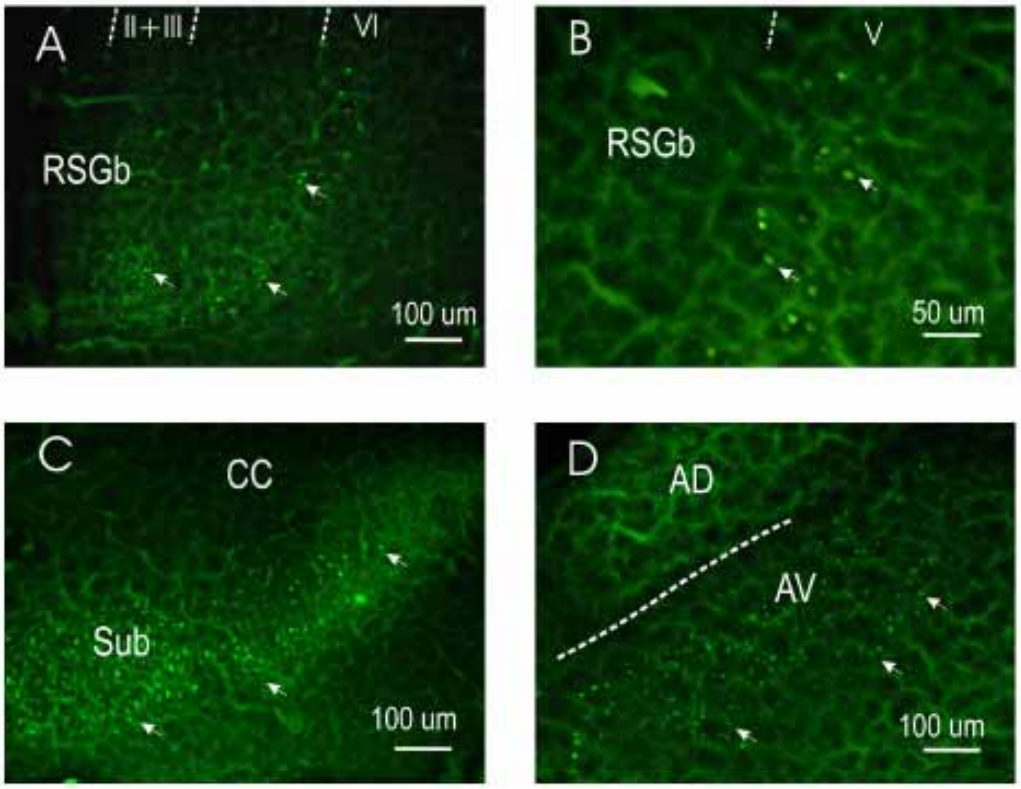

Fig. 4. Fluorescence photomicrographs of Fluoro Jade B (FJB) stained sections demonstrating neurodegeneration induced by single injection of NMDA antagonist dizocilpine (MK 801; 1 mg/kg i.p.) in P12 rats. To identify the cytoarchitectonic boundaries, adjacent sections stained with cresyl violet were used. The brain was partitioned into individual structures according to Paxinos and Watson (1997). Numerous FJB-positive neurons (white arrows) are present rostrally in the retrosplenial granular b cortex in layers II+III and VI (panel A) and more caudaly mostly in layer V (panel B). The large number of labeled neurons occurred also in subiculum (panel C). In the thalamus, many degenerating neurons were observed predominantly in the anteroventral nucleus (panel D). Abbreviations: AD – anterodorsal nucleus of the thalamus; AV - anteroventral nucleus of the thalamus; CC corpus callosum; RSG – the retrosplenial granular b cortex; Sub – subiculum. Scale bar A,C,D 100 µm, B 50 µm.

Seizures induced by intracerebroventricular administration of homocysteic acid could be prevented by both NMDA (AP7, MK-801) and nonNMDA (NBQX) receptor antagonists, as evaluated not only from the suppression of behavioral manifestations of seizures, but also in terms of selected indicators of brain energy metabolism (Folbergrová *et al.* 2000). In addition, we have demonstrated that the pronounced anticonvulsant effect against seizures induced both by homocysteine (Folbergrová 1997) and homocysteic acid (Folbergrová *et al.* 2000) could be achieved by the combined treatment with low subthreshold doses of NMDA and nonNMDA receptor antagonists.

Electrocortical recordings in rat pups with implanted electrodes allow to compare effects on EEG with effects on motor phenomena. Model of afterdischarges induced by electrical stimulation of sensorimotor cortical area enables to measure four different phenomena (Mareš *et al.* 2002b): movements induced by cortical stimulation (i.e. direct activation of the motor system); epileptic afterdischarges characterized by spike-and-wave rhythm (probably of thalamocortical origin); clonic seizures accompanying spike-and-wave afterdischarges (i.e. spread of epileptic thalamocortical activity into the motor system); and transition into the second, limbic type of afterdischarges characterized by fast spikes and/or delta waves combined with low amplitude spikes accompanied by behavioral automatisms (i.e. spread of epileptic acitivity into the limbic system). NMDA noncompetitive (and rather

nonspecific) antagonist ketamine exhibits marked action against cortical afterdischarges in 12- and 25-day-old rats but its effect in 18-day-old rats is weak (Kubová and Mareš 1995). More specific antagonists CGP 40116, 2-amino-7-phosphonoheptanoic acid and MK-801 exhibit very good anticonvulsant action in all three age groups studied (12, 18 and 25 days old – Šlamberová and Mareš - submitted). NonNMDA antagonists NBQX and GYKI 52366 are also reliably active in all three age groups (Kubová *et al.* 1997, Mareš *et al.* 1997b). Modification of the stimulation paradigm allowing to measure threshold intensities for individual phenomena demonstrated effect of both MK-801 and NBQX on thresholds for cortically-induced movements, spike-and-wave afterdischarges (Fig. 3) and clonic seizures but not on the transition into the limbic type of afterdischarges. In addition, in doses equipotent in pentetrazol-induced seizures MK-801 is more efficient than NBQX (Mareš – submitted).

Epileptic afterdischarges elicited by electrical stimulation of dorsal hippocampus are used only exceptionally in our laboratory. Both ketamine (Mikolášová *et al.* 1994) and MK-801 (Marešová and Mareš 1999) exhibit excellent anticonvulsant action in this model.

Unfortunately, the marked anticonvulsant action of antagonists of ionotropic glutamate receptors is compromised by their serious side effects. Both NMDA (MK-801 and CGP 40116 – Mikulecká and Mareš 2002) and nonNMDA (NBQX – Mareš *et al.* 1997b; GYKI 52366 – Kubová *et al.* 1997) antagonists induce ataxia demonstrated by various tests of motor skills of developing rats. In addition, even drugs not used for detailed testing of motor performance induce signs of ataxia. In addition, MK-801 induced neuronal degeneration in many brain regions when administered to 7-day-old rats (Ikonomidou *et al.* 1999). Our experiments in 12-day-old rat pups demonstrated degenerating neurons in cingular and retrosplenial cortex, subiculum and anteroventral thalamic nucleus (Fig. 4, data on file). Therefore clinical use of antagonists listed above is nearly impossible.

Drugs acting on metabotropic glutamate receptors

There are only a few data published on the role of metabotropic glutamate receptors in immature brain as concerns epileptic seizures and epilepsies. Research in this field is difficult: majority of drugs at disposal do not cross blood-brain barrier and therefore intracerebroventricular (icv) administration is necessary. The very first paper published by McDonald *et al.* (1993) demonstrated a possibility to elicit seizures by systemic administration of 1S,3R-ACPD in 7-day-old rat pups.

As to the potential anticonvulsant action of drugs acting on metabotropic glutamate receptors, seizures induced by icv administration of homocysteic acid have been used as a model in our laboratory (Folbergrová *et al.* 2001, 2003). Partial protection against these seizures could be induced by a group II agonist (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG IV) but this drug is convulsant at high doses. Marked protective action has been achieved by selective group II agonist (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (2R,4R-APCD), group II agonist and group I antagonist (S)-4-carboxy-3-hydroxyphenylglycine ((S)-4-C3HPG, Folbergrová *et al.* 2001) and group III agonists (RS)-1-amino-3-(phosphonoethylene)cyclobutanecarboxylic acid and (R,S)-4-phosphonophenylglycine ((R,S)-PPG, Folbergrová *et al.* 2001, 2003). Anticonvulsant action of all these drugs has been demonstrated not only by suppression of motor seizures but also by means of electroencephalographic recordings and by prevention of energy metabolite changes accompanying these seizures.

Some agonists and antagonists active after systemic administration were introduced recently. An antagonist of group I metabotropic receptors 2-methyl-phenylethynyl-propionic acid (MPEP, specific for mGlu5 subtype) is able to suppress specifically the tonic phase of generalized tonic-clonic seizures induced by pentetrazol in 12-, 18- and 25-day-old rat pups. A little higher doses are active also against minimal clonic seizures in those age groups where pentetrazol induces this type of seizures (18- and 25-day-old rats). In addition, it does not seriously compromise motor skills of developing rats (Mareš and Mikulecká – submitted). These data are promising but there is stil a long way to a possible clinical use.

Conclusions

Agonists of all types of ionotropic glutamate receptors are convulsants whereas antagonists at these receptors possess very strong anticonvulsant action in different models of epileptic seizures even in developing rodents. Unfortunately, competitive as well as noncompetitive antagonists available at present also exhibit very strong unwanted side effects. It is possible

that some new drugs specific only for a certain subtypes of these receptors (according to the subunit composition) will appear as useful for clinical practice. As far as metabotropic glutamate receptors are concerned, the existing data suggest that they may be considered a promising target for drug therapy in epilepsy (treatments with antagonists of group I and agonists of group II and III). However, the present knowledge is far from being sufficient for clinical studies.

Acknowledgements

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