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The role of main excitatory and inhibitory transmitters in epileptic seizures and the effect of antiepileptic drugs in the immature brain

Ph.D. Thesis

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GENERAL INTRODUCTION

Approximately one third of patients with epilepsy is resistant to pharmacotherapy, therefore studies on pathophysiology and pharmacology of epileptic seizures and epileptic syndromes are necessary *(Engel J. Jr. and Schwartzkroin A. P. 2006; Engel J. Jr. and Pedley T. A. 2008)*. Specific need for developmental studies is based on the fact that the number of newly diagnosed cases of epilepsy differs with age with the highest incidence during infancy and childhood. More than 75% of patients experience their first seizure before 18th year of life;, the first postnatal year is especially critical. Based on the epidemiology studies, seizures are the most frequently diagnosed symptoms in pediatric neurology *(Hauser W. A. and Kurland,L. T. 1975; Mathern G. W.2006)*.

1. Neurotransmiters and the receptors

Epilepsies are characterized by repeated appearance of epileptic seizures. Epileptic seizures are due to hypersynchronous activity of cerebral neurons and the pathogenetic basis of nearly all types of epileptic seizures is a marked predominance of excitation over inhibition. It is possible to elicit experimental epileptic seizures either by suppression of inhibition or by augmentation of excitation. Therefore main neurotransmitter systems – inhibitory using GABA and excitatory using glutamate as a transmitter – represent a common target in experimental epileptology.

Neurotransmitters are molecules of varied nature (quaternary amines, amino acids, catecholamines or peptides), which are released by neurons at chemical synapses and transmit a message from a neuron to another neuron, or to effector cell, or a message from a sensory cell to a neuron *(Hammond C.2001a)*. Since the year 1961, when Curtis and collaborators observed that glutamate has a depolarizing effect on neurons it is widely accepted as

excitatory amino acid transmitter *(Hammond C.2001c)*. Six years later Krnjevic and Schwartz described that γ-aminobutyric acid (GABA) mediates inhibitory synaptic transmission in adult vertebrate central nervous system (CNS) *(Hammond C.2001b)*.

1.1. GABA as an inhibitory transmitter

Majority of inhibitory synaptic transmission in the brain and spinal cord is generated by the small amino acid GABA. It acts on ionotropic $GABA_A$ (Fig.1) and $GABA_C$ receptors and metabotropic $GABA_B$ receptors. Activating the $GABA_A$ and $GABA_C$ receptor gates a Cl^- channel and increase Cl^- influx via the postsynaptic membrane that mediates fast synaptic inhibition. Both types of receptors are biochemically, pharmacologically a physiologically different *(Chebib M. and Johnston G. A. 1999)*. Binding of GABA on the $GABA_B$ receptor triggers a second messenger cascades which often activates K^+ channel *(Meldrum B. S.1989)*.

Role of $GABA_B$ receptor in epileptic seizures is a matter of controversy. Activation of these receptors results in mixed anti- and proconvulsant action (e.g. *Mares P. and Kubova H. 2008*). This type of GABA receptors was not studied in our experiments.

$GABA_A$ receptor, as well as $GABA_C$ receptor, belongs to the family of heteropentameric ligand-gated ion channels *(Ortells M. O. and Lunt G. G. 1995)*. This receptor is composed by extracellular N-terminal domain, four transmembrane components (TM1-TM4) and extracellular C-terminus. Large hydrophilic N-terminal domain is connected to TM1 and carries the neurotransmitter site and glycosylation sites. The hydrophilic loop separating TM3 and TM4 facing the cytoplasm contains phophorylation sites *(Luscher B. and Keller C. A. 2004)*.

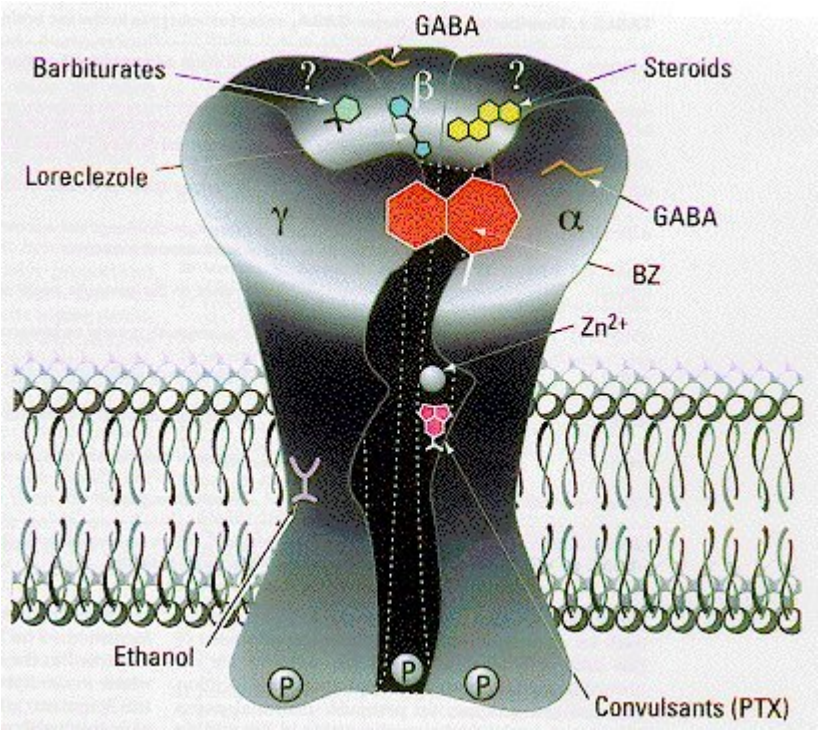

Fig. 1 $GABA_A$ receptor and its binding sites

Unique structural heterogeneity of $GABA_A$ receptor has been demonstrated and 19 different genes that encoded the subunits have been described. There are six α (α_1-α_6), three β (β_1-β_3), three γ (γ_1-γ_3), one δ, one ε, one θ, one π and three ρ (ρ_1-ρ_3) in mammalian CNS *(Sieghart W. et al. 1999; Whiting P. J. et al. 1997)*. The subunits stoichiometry of native $GABA_A$ receptors is unknown. Hypothetically, co-expression of 2α, 2 β and single γ_2 subunits composed pentameric channel receptor and modulate the properties, which are similar to those of native receptor *(Sieghart W. et al. 1999)*.

Theoretically, the subunit diversity allows numerous combinations and the subunit co-assembling can form homomeric or heteromeric $GABA_A$ receptors *(Costa E. et al. 2002)*. However, homomeric $GABA_A$ receptor has not been identified in the brain so far. Furthermore, each of the δ, ε, θ and π subunits appeared to replace the γ subunit.

The ρ-subunits form the $GABA_C$ receptor *(Chebib M. 2004)*. Main ligand-binding site of the $GABA_C$ receptor is located between two neighboring subunits in extracellular part. $GABA_C$ receptor is very distinct from the other two GABA receptors in terms of

pharmacological agonist and antagonist profiles (*Chebib M. and Johnston G. A. 1999*; *Chebib M. and Johnston G. A. 1999*; *Chebib M. 2004*; *Enz R. and Cutting G. R. 1998*; *Osolodkin D. I. et al. 2007*). To compare with $GABA_A$ receptors, the activation with GABA is characterized by longer mean-open times and smaller Cl^- conductance of $GABA_C$ receptors (*Feigenspan A. et al. 1993a*; *Feigenspan A. et al. 1993b*). Therefore, conformationally restricted analogues of GABA, such as selective agonists - (+)-cis-2-aminomethylcyclopropane carboxylic acid ((+)-CAMP) and cis-4-aminocrotonic acid (CACA) (*Chebib M. Johnston G.A. 1997*; *Chebib M. Johnston G.A. 1999*); or selective antagonist - (1,2,5,6-tetrahydropyridine-4-yl) methylphosphinic acid (TAMPA) (*Ragozzino D. et al. 1996*); have been studied to determine $GABA_C$ receptor profile (*Chebib M. and Johnston G.A. 1999*; *Chebib M. 2004*; *Enz R.and Cutting G.R. 1998*; *Osolodkin D. I. et al. 2007*).

The expression of the known subunits can modify different GABA sensitivity and the functional $GABA_A$ receptor channel properties. Moreover, the subunits localized at different parts of cellular surface (extrasynaptic, perisynaptic or postsynaptic sites) exhibit different subunit composition and properties. Extrasynapticaly and perisynapticaly localized $GABA_A$ receptors show high GABA sensitivity and slow inactivation. Those receptors, which exhibit lower GABA sensitivity and fast inactivation, are found postsynaptically (*Luscher B. and Keller C.A. 2004*).

Majority of $GABA_A$ receptor is localized on somatodentritic compartments and axon initial segments of neuron. However, in the hippocampus, those receptors with α_2 subunits have been identified at mossy fiber terminals, which contain also other potential neurotransmitters (*Walker M. C. et al. 2002*). Hypothetically, $GABA_A$ receptors with $\alpha_{1,2,3}$ subunits mainly show synaptic localization in the cortex and hippocampus, while those containing α_5 subunits are frequently extrasynaptic and can be seen in the olfactory bulb and hippocampal pyramidal cells (*Costa E. et al. 2002*). In the hippocampus, they probably contribute to the synaptic plasticity by the short-lasting repetitive synchronous firing

(*Galarreta M. and Hestrin S. 1999*). The β and γ subunits are common part of $GABA_A$ receptors in whole brain.

The subunit combinations also follow expression pattern of regional distribution. The δ subunit shows high expression in cerebellar granule cells, thalamus and olfactory bulb (*Galarreta M. and Hestrin S. 1999*; *Laurie D. J. et al. 1992*). The ε subunit has very limited distribution and it has been found in hypothalamus and hillus of hippocampal dentate gyrus (*Galarreta M. and Hestrin S. 1999*; *Whiting P. J. 1999*). The π subunit, called "peripheral" one according to its expression in the peripheral organs such as uterus, while in the brain it shows very low intensity in the cortex and hippocampus (*Hedblom E. and Kirkness E. F. 1997*). The regional expression of the θ subunit is similar to β one, it is seen in substantia nigra and striatum. Furthermore, it is abundant in tissue with catecholamines (*Walker M. C. et al. 2002*).

The variety of subunits, which compose the $GABA_A$ receptor, can influence the binding affinity of different drugs and thus they can modify function of $GABA_A$ receptors. The endogenous (neurosteroids, GABA) as well as exogenous substances (benzodiazepines, barbiturates or general anesthetics) positively modulate the activity of the $GABA_A$ receptor-Cl^- channel complex. Their binding sites are localized in distinct part at the supramolecular complex and moreover, their action through the binding sites use different mechanisms and they can cause allosteric modification at other sites *(Mehta A. K. and Ticku M. K. 1999*; *Sieghart W. 1995*).

Two molecules of GABA are required for confirmation change of the $GABA_A$ receptor channel complex and opening of chloride channel. These two sites are localized between α and β subunits *(Hammond C.2001b)*.

Clinically frequently used drugs, benzodiazepines, act trough the binding site that is located at the interface between $\alpha_{1,2,3,5}$ and γ subunits. They increase the opening frequency of Cl^- channel without changing the mean burst duration or the mean number of opening per

burst of $GABA_A$ receptor *(Costa E. et al. 2002; Hammond C.2001b)*. According to pharmacological properties of benzodiazepines heteromeric $GABA_A$ receptors containing α_1 subunits produce the sedative, amnestic and anticonvulsant activity of diazepam (*Rudolph U. et al. 1999*) and those with α_2 subunits mediate anxiolytic effect (*Low K. et al. 2000*). Moreover, in the motor neurons and in the dorsal horn of spinal cord the α_5, α_2 or α_3 subunits composing the $GABA_A$ receptors may produce the relaxant activity (*Bohlhalter S. et al. 1996*).

Hypnotic and antiepileptic agents, barbiturates, affect the $GABA_A$ receptor via the distinct binding site with the mechanism, which increase the duration of single opening and burst, however the frequency of channel opening is not changed *(Hammond C.2001b)*.

Neurosteroids are formed *de novo* by neuronal tissue *(Baulieu E. E.1981)* or can be produced in the brain tissue from peripheral steroid hormones such as progesterone, deoxycorticosterone and testosterone, which can cross blood-brain barrier (*Majewska M. D. 1992*). Endogenous or synthetic neurosteroids such as epalons alter neuronal excitability by rapidly acting on the neuronal membrane (*Gee K. W. et al. 1995*). Epalons interact as positive allosteric modulators at a unique receptor site of the $GABA_A$ receptor-Cl^- channel complex in CNS (*Gee K. W. 1988*; *Gee K. W. et al. 1995*; *Harrison N. L. and Simmonds M.A. 1984*; *Majewska M. D. et al. 1986*). The neurosteroid mechanism is in increasing the channel open probability and together with GABA increase the duration of single and burst opening *(Mehta A. K. and Ticku M. K. 1999)*. Epalons provide anticonvulsant activity, which is separated from their hormonal effects (*Belelli D. et al. 1989*; *Gee K. W. et al. 1995*).

Reduction of the $GABA_A$ current can be mediated by the competitive antagonist, bicuculline by binding to the same receptor site as GABA and by channel blocker picrotoxin, which bind to the site localized in the ion channel. Inverse benzodiazepine agonists (such as β-carborbolines) reversibly decreased total $GABA_A$ current due to binding to benzodiazepine receptor *(Hammond C.2001b)*.

Majority of inhibitory action in adult CNS is induced by activation of resting $GABA_A$ receptors, where the Cl^- influx induces membrane hyperpolarisation. In contrast, in immature hippocampus the $GABA_A$ receptor activation leads to depolarization due to high intracellular Cl^- ions concentration and causes excitation. This changing leads to depolarization and may activate voltage-gated Ca^{2+} and NMDA-regulated channels in immature neurons (*Ganguly K. et al. 2001*).

1.2. Glutamate as main excitatory transmitter

Glutamate mediates most of the excitatory neurotransmission in the mammalian CNS. However, together with related excitatory amino acids it may be toxic to CNS (*Collingridge G. L. and Leseter R. A.1989*; *Ozawa S. et al. 1998*). Glutamate receptors are expressed mainly in the CNS and participate in physiological process such as synaptic plasticity, learning and memory, but also participate in the formation of neural networks during development (*Ozawa S. et al. 1998*). The glutamate neurotoxicity may be also involved in the genesis of various neurological disorders that include epilepsy, ischemic brain damage, head trauma, neuropathic pain and neurodegenerative disorders (Parkinson's disease, Alzheimer's disease, Huntington's chorea and amyotrophic lateral sclerosis) (*Dingledine R. et al. 1999*).

Glutamate physiological action is exerted through the activation of two types of receptors: ionotropic (iGluR) and metabotropic (mGluR). mGluRs are coupled to G proteins and control the activity of membrane enzymes and ion channels (*Bockaert J. et al. 1993*; *Loscher W. 1998b*). Action of glutamate on iGluRs is always excitatory but activation of mGluRs can result in both excitation or inhibition *(Kandel E. R. et al.2000)*. Traditionally, from the pharmacological point of view, iGluRs can be distinguished into three groups on the basis of their affinities and functional responses to the exogenous excitatory amino acids agonists N-methyl-D-aspartate (NMDA) (Fig. 2), α-amino-3-hydroxy-5-metyl-4-isoxazole (AMPA) and kainic acid (KA) (*Meldrum B. S. 1991*). In addition, another differentiation of

iGluRs can be made, namely to NMDA and non-NMDA receptors. The drug APV (2-amino-5-phosphonovaleric acid), which is the antagonist on the NMDA receptors selectively blocked NMDA but can not modify the AMPA or KA receptors, while both of them are blocked by quinoxallinediones (*Collingridge G. L. and Lesseter R. A. 1989*; *Hollmann M. and Heinemann S. 1994*; *Kandel E. R. et al.2000)*.

The transmembrane topology of iGluR is different from the other ligand-gated ion channels such as nicotinic acetylcholine (Ach) and $GABA_A$ receptors. According to the Hollmann's transmembrane topology model, these receptor subunits have in common a large extracellular N-terminus domain, four hydrophobic membrane segments (M1-4), three of them are transmembrane (M1, M2, M4) and one cytoplasm-facing re-entrant membrane loop (M2). The entire region between M3 and M4 is extracellular and the C-terminus is placed intracellularly (*Dingledine R. et al. 1999*).

The function of postsynaptic iGluRs is to mediate fast excitatory synaptic transmission by converting the binding of glutamate to a rapid and transient increase in conduction of both Na^+ and K^+, with nearly equal permeability. Some AMPA receptors and all NMDA receptors are also permeable to Ca^{2+} *(Kandel E. R. et al.2000)*.

AMPA receptors are homo- and hetero-tetramers consisting of subunits known as GluR1-GluR4, which can mediate fast excitatory synaptic signals in the brain. By recent evidence, these receptors are involved in synaptic plasticity (learning and memory) (*Dingledine R. et al. 1999*; *Ozawa S. et al. 1998*). Similarly, KA receptors are homo- and hetero-tetramers and they are also responsible for excitatory synaptic transmission. Furthermore, they are involved in modulation of neurotransmitter release from the presynaptic terminal. KA receptors are composed of the subunits GluR5-GluR7, KA1 and KA2. A third family of iGluRs are the NMDA receptors, which are formed as heterotetramers that contain both of NR1 and NR2 (NR2A-NRD) subunits, in some cases NR3 (NR3A, NR3B) subunits. The unique properties of NMDA receptor channels are implicated in physiological functions

such as learning, memory as well as formation of neural networks during development (*Dingledine R. et al. 1999*; *Madden D. R. 2002*).

All iGluRs are the cationic channels permeable to Na^+ and K^+. Some non-NMDA receptors and all NMDA receptors are also permeable to Ca^{2+}. NMDA and KA receptors have higher affinity for glutamate than AMPA receptors *(Hammond C.2001c)*.

During the normal excitatory postsynaptic potential (EPSP), non-NMDA receptors are those, which generate the early large phase of the EPSP with permeability to Na^+ and K^+, but not to the Ca^{2+} ions. The AMPA receptors mediate a fast-rising EPSP, while the KA-receptor-mediated EPSP is smaller and slower one, thus giving a slow-rising low-amplitude EPSP component [36]. Probably these variations in combination of the iGluRs and their subunits play important role in the synaptic plasticity during development (*Takai H. et al. 2003*).

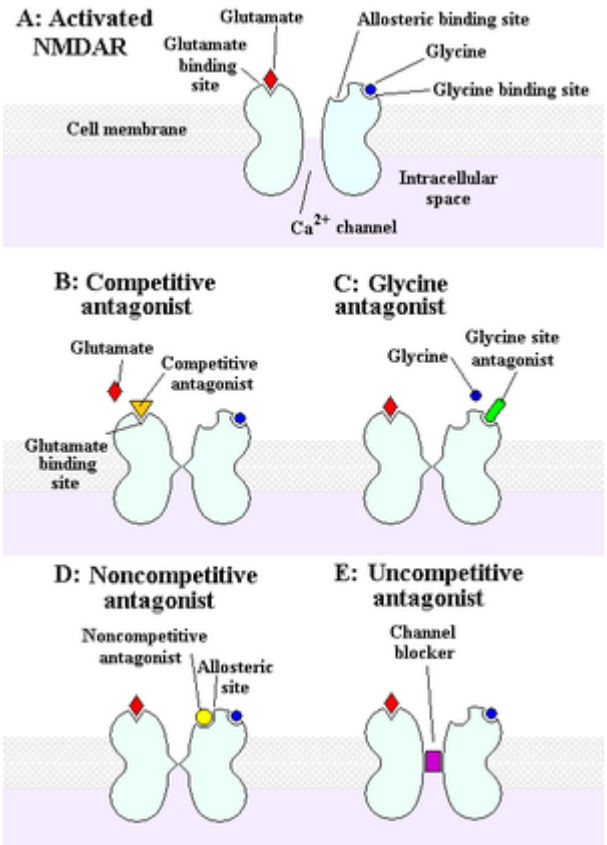

Fig. 2 N-methyl-D-aspartate receptor and its binding sites

The NMDA receptor complex is unique in comparison with the other ligand-gated ion channels, because for the efficient function, not only agonist but also cofactor binding and membrane depolarization are necessary. Thus, this receptor has a more complex role based on three characteristic properties. As mentioned above, NMDA receptor opens in response to glutamate more slowly than AMPA receptors. Requirement for glycine as another substance to be present together with the NMDA agonist in the extracellular space to allow opening of the channel complex introduces the glycine as a cofactor for proper functioning of NMDA receptor *(Hammond C.2001c; Kandel E. R. et al.2000)*. Furthermore, channel opening is also regulated by voltage-dependent Mg^{2+} block. At the resting membrane potential extracellular Mg^{2+} ion binds deeply into the open NMDA channel pore and blocks the activation of the receptor complex. Thus ions cannot enter the intracellular space and the NMDA receptor component of synaptic currents is blocked. This block can be removed when the membrane is sufficiently depolarized and allows the influx of ions (Na^{+} and Ca^{2+}) via the activated NMDA receptor. Therefore, glutamate first activates non-NMDA receptors *(Loscher W. 1998b)*. The resting membrane can be depolarized by single activation of the co-localized postsynaptic non-NMDA receptors (for example, glutamate binding to the AMPA receptors). The EPSP response is smaller comparing to the depolarization by repeated firing of the presynaptic neuron that summate the EPSP (long-duration EPSP) or generate much larger current carried by Ca^{2+} *(Dingledine R. et al. 1999; Kandel E. R. et al.2000)*. This Ca^{2+} influx can trigger the variety of intracellular signaling cascades, which can lead to changes of the certain neuronal functions such as long-lasting modification in the synapse and also have a close relationship to synaptic plasticity during CNS development *(Dingledine R. et al. 1999; Kandel E. R. et al.2000)*. In contrast, the excessive Ca^{2+} influx through the NMDA receptors can finally, via the calcium-dependent proteases and phospholipases, produce free radicals, which are toxic to the cells and induce neuronal death *(Kandel E. R. et al.2000; Takai H. et al. 2003)*.

Different studies examining subunits mRNA or protein distribution or using the animals with specific subunit genes deleted and comparing the functional properties of native and recombinant receptors have allowed to analyze different receptor channels properties. The GluR channel properties and their different sensitivities to the exogenous or endogenous substances, permeation and block by divalent ions, kinetic properties and interaction with intracellular proteins can be modified by subunit recombination (*Cull-Candy S. et al. 2001*; *Yamakura T. and Shimoji K. 1999*).

The distribution of different subunits of NMDA and non-NMDA receptors changed during the development of CNS *(Kandel E. R. et al.2000; Takai H. et al. 2003)*. In adult synapses both the NMDA and AMPA receptors can be present in the same postsynaptic membrane, while other synapses contain NMDA receptors channel complex only. In contrast to adult receptor combination, only NMDA receptors are displayed in the immature CNS *(Kandel E. R. et al.2000)*.

The functional characteristics of NMDA receptor channels depend on the expression and combination of the NR subunits during the brain maturation, which can probably explain the physiological and pathophysiological differences between the adult and immature CNS. By *in situ* hybridization studies at different stages of development, we can see the different regional distribution of the NMDA subunits in rodents *(Yamakura T. and Shimoji K. 1999)*. It has been showed that NR1 subtypes strongly influence NMDA properties. In the adults, the NR1s are spread ubiquitously throughout the brain. Furthermore, they are localized in the postsynaptic densities, mainly around vesicles and associated dendrites. In contrast, the NR2s display distinct regional pattern. The NR2A subunits are spread all over the brain, predominantly in the cerebral cortex, hippocampus and cerebellum. The NR2B subunits are expressed in the forebrain regions such as cerebral cortex, hippocampus, septum, caudate-putamen and olfactory bulb. The mRNA of NR2C displayed strong expression in cerebellum (the granule cell layer) and weak expression in the olfactory bulb and thalamus. NR2D

subunits are only moderately expressed in the thalamus, brain stem and the olfactory bulb. Both of NR2C or NR2D are probably mostly expressed at interneurons (*Ozawa S. et al. 1998*).

Recent studies showed that regional distribution of functional NMDA subunits differ during the brain development. During the embryonic time of CNS maturation, NR1 subunits are spread from the temporal region of the cerebral cortex at earlier stages to the later expression in the hippocampus. After birth, NR1s are distributed all over the neonatal brain mainly in the cerebral cortex and pyramidal cells in the hippocampus (*Ozawa S. et al. 1998*; *Takai H. et al. 2003*).

The NMDA-NR2 subunits displayed different pre- and postnatal expression pattern. At embryonic time, NR2Bs are abundantly expressed in cerebellum (the granule cells) and later they are replaced by the NR2C subunits around the birth. The NR2D are detected in diencephalon and brain stem in the fetal brain. The NR2As are widely spread, while the NR2C subunits are expressed mostly in cerebellum in the neonate brain (*Ozawa S. et al. 1998*; *Takai H. et al. 2003*).

The number of substances regulates the NMDA-receptor-channel efficiency in different ways, while at least two conditions are required for NMDA receptor activity such as the presence of ligand and the depolarization of the membrane (*MacDermott A. B. et al. 1986*; *Meldrum B. S. 1991*).

Both, NMDA as a selective agonist and glutamate as non-selective agonist, act through the glutamate (glu) site of the NMDA receptor. The binding of glycine (cofactor) to a strychnine-intensive glycine (gly) site on the receptor increases the frequency of agonist-induced channel opening and it is absolutely required for NMDA channel activation (*Meldrum B. S. 1991*). The extracellular Mg^{2+} ions regulate the NMDA receptor channel via Mg^{2+} binding site located within the channel pore by blocking the current flow. This voltage-depended block is present at normal level of Mg^{2+} in the extracellular space at resting

membrane *(Kandel E. R. et al.2000; Mayer M. L. et al. 1984; Meldrum B. S. 1991)*. In addition, there are more regulatory sites on the NMDA receptor channel, which control NMDA-mediated activity further *(Loscher W. 1998b)*. The "phencyclidine (PCP) site" is present within the pore channel that is distinct from Mg^{2+} binding site. The "uncompetitive" NMDA receptor antagonists (NMDA receptor open-channel blockers) such as PCP, ketamine and MK 801 (dizocilpine) inhibit the channel opening of NMDA receptor by acting via this binding site *(Kandel E. R. et al.2000; Meldrum B. S. 1991; Woodruff G. N. et al. 1987)*.

The polyamine-binding site, when activated, modulates the NMDA current by increasing channel opening probability (which is due in part to an increase in glycine binding). Ifenprodil or eliprodil are the drugs which act as antagonist at the polyamine site and thereby only modulate NMDA receptor function *(Lipton S. A. 1993)*.

Another abundant metal (Zn^{2+}) in CNS potently inhibits NMDA receptor activation at the physiological extracellular concentration. It was challenged that Zn^{2+} act as non-competitive antagonist by binding to a site, which is distinct from the Mg^{2+} binding site and lies outside of the NMDA channel pore *(Huang E. P. 1997; Kandel E. R. et al.2000)*.

In addition, recent studies described many substances, which probably differently modulate the NMDA receptor, such as redox agents, nitric oxide (NO), proton (pH) and other compounds *(Lipton S. A. 1993)*.

From the pathophysiological point of view the NMDA and AMPA receptors contribute to seizure elaboration *(Dingledine R. et al. 1990)*, so pharmacological strategies inhibiting both iGluR types might be promising *(Loscher W. 1998a)*.

Most AMPA receptor channels display permeability of Na^{+} and K^{+}, but they do not conduct Ca^{2+}. However, it was identified that there are also the Ca^{2+}-permeable AMPA receptors in the CNS. The similarity of the NMDA and AMPA receptors by their permeability to Ca^{2+} ions through the channel complex is the function of the single-amino-acid (arginine (R) or glutamine (Q)) expressed at certain position in pore-forming M2 region of GluR2

subunit. When the glutamine is placed in Q/R site of the GluR2 and only Q-GluR2-subunits formed the AMPA receptor channel, the permeability to Ca^{2+} is present. While arginine replaced the glutamine-Q/R position just in one of the GluR2 subunits, the AMPA receptor channel is not permeable for Ca^{2+} *(Dingledine R. et al. 1999; Kandel E. R. et al.2000; Ozawa S. et al. 1998; Seeburg P. H. 1993)*.

Similarly, the subtype combinations determine the pharmacological diversity of NMDA receptors by changing binding affinity for the exogenous and endogenous substances and the influence on transmission of the receptor channel. The sensitivity of the NMDA receptors to glutamate is strongly supported by identification of the NR2s, while the binding site of cofactor glycine is placed at the NR1 subunits (*Ozawa S. et al. 1998*). There are differences in further kinetics such as deactivation time and excitatory postsynaptic current (EPSC) decay what is also the function of incorporation of the NR2s to NMDA receptors skeleton; NR2A shortens the time scale properties opposing to the NR2Ds (*Cull-Candy S. et al. 2001*; *Takai H. et al. 2003*). The gating characteristics of the receptor channel generating the "high-conductance" channel opening with high affinity to extracellular Mg^{2+} depend on NR2A or NR2B subunits. In contrast, receptors with NR2C and NR2D subunits give rise to low conductance with a low affinity to extracellular Mg^{2+} ions. Moreover this characteristic is also supported by evidence of "clear" NMDA receptor composition by the NR2C or NR2Ds in absence of NR3 subunits, which also give rise to low-conductance channel opening (*Takai H. et al. 2003*). The functional channel characteristics such as single channel properties are due to expression of the NR2s during development. The immature neurons mainly express the NR2Bs and NR2Ds, usually together with either NR2A or NR2C subunits. Furthermore, the NR2s expression developmental pattern depends on neuronal activity (*Ozawa S. et al. 1998*; *Takai H. et al. 2003*). The co-expression of NR1 and NR2 is essential for forming functional receptors (*Grimwood S. et al. 1995*).

2. Seizure models elicited by interaction with the main neurotransmitter systems

Three developmental models were used in these studies. Pentylenetetrazol was chosen as a drug antagonizing GABA-A receptors, N-methyl-D-aspartate as an agonist of the most important type of ionotropic glutamate receptors, and flurothyl as a drug without exactly known mechanism of action but with a high probability without marked action on the two main neurotransmitter systems.

2.1. PTZ-induced seizures as a developmental model

Excitatory effect of PTZ in the brain stem has been used in the past to stimulate circulatory and respiratory systems in patients (*Loscher W. 2009*). What is still in use are specific epileptic phenomena elicited by PTZ in preclinical testing of potential new antiepileptic drugs.

PTZ predominantly acts as an antagonist at ionotropic GABA-A receptor by binding to the picrotoxine site in the chloride channel (*Ramanjaneyulu R .and Ticku M. K. 1984*).

PTZ is well dissolved in normal saline and in water and can be administered systematically subcutaneously (SC), intraperitoneally (IP) and/or intravenously (IV) or locally into CNS (neocortex, hippocampus, amygdala, SN, area tempests). PTZ can be administrated once, repetitively or continuously (as intravenous infusion in mice or rats). Most common administration is subcutaneous, minimal clonic seizures induced by subcutaneous injection of PTZ are a part of obligatory preclinical testing of drugs.

The calculation of the time needed to produce convulsions (seizure latency) and the amount of PTZ is recalculated to 50% convulsive dose expressed in mg/kg. Different endpoints can be used in the PTZ model: incidence of seizures, seizure severity and duration, as well as mortality. IV infusion allows precise calculation of various doses of PTZ (threshold, 50% convulsive dose, 95% convulsive dose). Seizure activity is usually monitored

by behavioral observation; EEG recording brings more data but it is time and money consuming.

In the rats older than three weeks, PTZ in increasing doses can induce a nonspecific activation at the beginning and then four different epileptic phenomena. Nonspecific activation is represented by increased locomotor activity and orienting reaction. The first epileptic event is characterized by behavioral arrest (freezing) with episodes of spike-and-wave activity (sometimes in a spindle-like form) in the EEG. Duration of these episodes is usually between five and ten seconds. The second phenomenon is formed by isolated myoclonic twitches of whole body mostly with isolated sharp waves in EEG recording. Clonic seizures restricted to head and forelimb muscles (minimal seizures) represent the third event. They last some tens of seconds and are accompanied by spike-and-wave rhythm in the EEG. Finally, generalized seizures (major seizures) with tonic and clonic phases can be observed with whole spectrum of EEG changes (irregular high-amplitude spikes, slow waves forming spike-and-waves complexes and dysrhythmic activity). In addition, increasing doses shorten the latency to onset of seizures, augment the severity of convulsions and induce also an incidence of lethal outcome. Moreover, the observed initial stages can fail to appear if high doses are used (*de Casrilevitz M. et al. 1971*; *Mares P. and Schickerova R. 1980a*; *Mares P. et al. 1980b*; *Mares P. and Velisek L. 1983*; *Vernadakis A. and Woodbury D. M. 1969*).

In rodents, the convulsant activity changes with age (*de Casrilevitz M. et al. 1971*; *Mares P. and Schickerova R 1980a*; *Mares P. et al. 1980b*; *Mares P. and Velisek L. 1983*; *Vernadakis A. and Woodbury D. M.. 1969*). Animals in the first and second week of life are unable to generate spike-and-wave episodes with behavioral freezing as well as minimal clonic seizures. In contrast, single myoclonic jerks and generalized (major) seizures are not age-dependent and can be elicited at all developmental stages (*de Casrilevitz M. et al. 1971*; *Mares P. and Schickerova R. 1980a*; *Mares P. et al. 1980b*; *Mares P. and Velisek L. 1983*; *Vernadakis A. and Woodbury D. M. 1969*). Transition between the immature and mature set

of PTZ-induced phenomena is not abrupt. Rats in the third postnatal week can exhibit atypical episode of myoclonic seizures called "transitional group (*de Casrilevitz M. et al. 1971*), imperfect form of spike-and-wave rhythm may be recorded in a part of 15-day-old rats (*Schickerova R. et al. 1984*).

There is not only qualitative but also quantitative development of PTZ-induced epileptic phenomena. CD50 values of PTZ for motor seizures differ in rats from postnatal day 7 to adulthood: the lowest CD50 for generalized seizures was found in 18-day-old rats with higher values for both younger and older animals. There are comparable doses for adults and pups (7- and 12-day old), even the higher doses (CD50 = 66.6 mg/kg in 7-day-old, 58.9 mg/kg in 12-day-old and 57.6 mg/kg in 90-day-old rats after sc administration) (*Velisek L. et al. 1992*). Similar finding was published by Vernadakis; she found the 16-day-old rats to be most sensitive to convulsant action of PTZ. Eighteen-day-old rats were not studied in her experiments (*Vernadakis A. and Woodbury D. M. 1969*). In contrast, CD50 for minimal seizures decreases from postnatal day 18 up to adulthood (*Velisek L. et al. 1992*).

It is always necessary to take into account strain (and probably also breeding) differences if outcome of individual studies is compared (*de Casrilevitz M. et al. 1971*; *Mares P. et al. 1980a*; *Mares P. et al. 1980b*; *Mares P. et al. 1983*; *Vernadakis A. and Woodbury D. M. 1969*).

There are some important features of different application routes. Timed intravenous PTZ infusion into tail vein substantially decreased number of animals, which are necessary to have statistically valid data. If more than one endpoint is used in this test, this way of administration yields very detailed data *(Loscher W. 2009)*. On the other hand, this type of application is much more time consuming and therefore it is not used by pharmaceutical companies. An additional problem is in intravenous application in very young rodents (*Loscher W. 2009*).

2.2. NMDA-induced seizures as a model of age-dependent seizures

Seizures induced by methylated aspartate do not represent a common model. They are used only in a few laboratories.

NMDA (methylated aspartate) or racemic mixture N-methyl-DL-aspartate (NMDLA) is well dissolved in normal saline and to elicit motor seizures it can be administered intracerebrally (*Lees G. J. 1995*), intracerebroventricularlly (ICV) (*Swinyard E. A. et al. 1991*) or more often systematically (IP) by a single bolus injection (*Singh L. et al. 1991*) or by intravenous infusion (*Singh L. et al. 1991*).

Description of seizures elicited by systemically administered NMDLA in adult mice was published by Singh (1991). Clonic-tonic generalized seizures described by Singh could be elicited also after focal microinjection into brain tissue such as unilateral amygdala (*Hirayasu Y. and Wada J. A. 1992*), massa intermedia (in the middle thalamus) (*Ishimoto T. et al. 2000*) or hippocampus (*Lees G. J. 1995*).

Systemic administration of NMDA induced in adult rodents hyperlocomotor activity, (running typically in "eight-shaped" trajectory) and extreme agitation as the first sign. Intensity of this locomotor activity increases with the dose of NMDA. The hyperactivity is later replaced by ataxia and then by automatisms (tail flicking and/or biting of the tail or paws). These phenomena probably represent the first specific stage of NMDA-induced epileptic activities. Next step is formed by generalized clonic-tonic seizures. If the animals progress up to this stage, mortality is practically 100%.

All these phenomena could be observed also in immature rats but the sensitivity is extremely high in 7-day-old rats and decreases with age (*Mares P. and Velisek L. 1992*). What is different is the intensity of individual events, especially of hyperlocomotion. It is highly expressed in 25-day-old rats, not so markedly in younger animals, probably due to the not yet fully mature motor abilities. A special pattern of seizures appears during the first three postnatal weeks. This type of seizures – flexion - is specific for convulsant drugs acting on

NMDA receptors, it cannot be elicited by agonists of AMPA or kainate receptors. The hyperflexion of head, body and tail with a loss of righting ability and with or without sound effects called emprosthotonus ("ball" position). Eighteen-day-old rats exhibit flexion seizure (emprosthotonus) after lower doses of NMDA; if high doses are used they can be masked by generalized seizures which appear with a short latency (*Mares P. and Velisek L. 1992*).

EEG correlate in cortical and hippocampal recording is not specific. Generally, amplitude suppression occurs almost immediately after NMDA administration in young as well as adult animals. It can be combined with "serrated" waves (consisting of fast low-voltage activity superimposed on huge slow waves) (our data) accompanying the behavioral arrest. Almost isoelectric lines mixed with slow hippocampal waves are recorded during the flexion seizures (our data).

2.3. Flurothyl-induced seizures in immature rats

Flurothyl [bis (2,2,2-trifluoroethyl) ether] when inhaled is a potent CNS stimulant and a rapidly acting volatile convulsant (*Krantz J. C., Jr. et al. 1957*; *Truitt E. B., Jr. and Ebersberger E. M. 1960*). The mechanism of the flurothylinduced convulsant effect is unknown. Flurothyl may affect synaptic transmission either presynaptically, postsynaptically, or both (*Richter J. et al. 1977*; *Schuck S. L. and Shulman A. 1971*).

The behavioral pattern of flurothyl induced seizures is age dependent *(Sperber E. F. and Moshe S. L. 1988)*. In young adult rats (postnatal days [PN] 60), flurothyl initially induces clonic seizures consisting of head and forelimb clonus with preservation of the righting reflex. Several episodes of clonic seizures can occur. With increasing amounts of flurothyl, tonic or tonic-clonic seizures appear characterized by wild running and loss of the righting reflex. Thirty-day-old rats (PN 30) display "adult-like'' behavioral seizure patterns *(Veliskova J. 1999)*. In rat pups (PN 9 and PN 15), a clonic seizure is followed almost

immediately by a tonic-clonic seizure. The latency to seizure onset is also age dependent (*Sperber E. F.and Moshe S. L. 1988*; *Velisek L. et al. 1995a*).

3. Anticonvulsant drugs with mechanism of action connected with GABAergic inhibition

3.1. Ganaxolone

Neurosteroids are steroid hormones that can be formed de novo in the central nervous system (CNS) *(Baulieu E. E.1981)*. Neurosteroids can also be produced in brain tissues from the conversion of peripheral steroid hormones such as progesterone, deoxycorticosterone, and testosterone, which can cross the blood-brain barrier (*Majewska M. D. 1992*). Endogenous neurosteroids as well as synthetic neuroactive steroids alter neuronal excitability rapidly (within seconds to minutes) acting on the neuronal membrane (*Gee K. W. et al. 1995*; *Paul S. M. and Purdy R. H. 1992*). The endogenous metabolites of progesterone and their synthetic analogue alphaxalone are the best described neurosteroids (*Gee K. W. et al. 1995*; *Phillipps G. H. 1975*). There is a class of neuroactive steroids called "epalons" that are devoid of hormonal effects with high specific activity for the $GABA_A$ receptor complex *(Baulieu E. E.1981; Belelli D. et al. 1989; Gee K. W. et al. 1995)*. Epalons interact as positive allosteric modulators at a unique receptor site of the $GABA_A$ receptor C1- channel complex in the CNS *(Gee K. W. 1988; Gee K. W. et al. 1995; Harrison N. L. and Simmonds M. A. 1984; Majewska M. D. et al. 1986; Majewska M. D. 1992; Paul S. M. and Purdy R. H. 1992)*. As positive modulators of $GABA_A$ receptor complex, epalons have anticonvulsant effects (*Majewska M. D. 1992*). Ganaxolone (hydroxy-3P-methyl-pregnan-20- one; GNX) is a newly developed 3P-methylated synthetic epalon derived from the endogenous neuroactive steroid 3-hydroxy-5-pregnan-20-one (3ά,5ά-P) (*Carter R. B. et al. 1997*; *Monaghan E. P. et al. 1997*). GNX was developped to avoid rapid catabolism characteristic for natural metabolites of progesterone.

In vitro studies show that GNX has a positive allosteric modulatory effect at the GABA, receptor-Cl^- channel complex (*Carter R. B. et al. 1997*). Data from in vivo studies demonstrate that GNX is a potent anticonvulsant against clonic pentylenetetrazol (PTZ)-, bicuculline-, t-butylbicyclophosphorothionate-, cocaine-, and aminophylline-induced seizures after systemic administration in adult rats or mice (*Carter R. B. et al. 1997*; *Gasior M. et al. 1997*). Systemic GNX administration exhibits a protective effect against stage 5 kindled convulsions produced by corneal kindling in adult rats (*Carter R. B. et al. 1997*). The effective doses of GNX in this study are below those that induce ataxia in rotorod test. Tonic convulsions induced by maximal electroshock are also effectively suppressed by GNX, but at doses that cause ataxia. This anticonvulsant effect is more pronounced in adult mice than in rats (*Carter R. B. et al. 1997*). GNX has no influence on NMDA-induced seizures but decreases the acute lethality of NMDA in adult mice (*Laurie D. J. et al. 1992*). GNX treatment also does not influence strychnine-induced seizures in mice (*Carter R. B. et al. 1997*). It should be noted that in adult mice, the same or lower doses of GNX that produce anticonvulsant effects have also anxiolytic effects as tested on PTZ-induced behavioral changes (*Hauser W. A. 1994*).

In the developing brain the anticonvulsant activity of GNX was demonstrated by our data and Mareš et al., (*Mares P. and Stehlikova M. 2009b*) in rats. Tonic-clonic seizures are more sensitive to GNX treatment than clonic seizures in PTZ-induced convulsions (*Mares P. and Stehlikova M. 2009b*). Interestingly, PTZ-induced minimal clonic seizures are not influenced by GNX (*Mares P. and Stehlikova M. 2009b*). However, minimal clonic seizures can be elicited only after the third postnatal week of rats following PTZ administration (*Mares P. and Schickerova R. 1980a*; *Mares P. and Velisek L.. 1983*). GNX was active in the model of epileptic afterdischarges (ADs) induced by electrical stimulation of sensorimotor cortical region in developing rats (*Mares P. 2005*; *Mares P. and Stehlikova M. 2009b*).

The best anticonvulsant effects of GNX were prominent in PN 12 rats (*Mares P. et al. 2009a*) in PTZ model, as well as in cortical epileptic ADs.

The effects are dose dependent (our data) and lasted for at least 60 min. Similar data were obtained with anticonvulsant action of THDOS conjugate (triethylammonium 3-hydroxy-20-oxo-5 pregnan-21yl hydrogrnsuccinate) and HOHP (3alpha-hydroxy-21xi,22-oxido-21-homo-5alpha-pregnan-20-on) as demonstrated by Mareš at all in PN12 rats (*Mares P. 2005*; *Mares P. et al. 2006*). The effects of GNX in the flurothyl model are most prominent when the drug is administered 10 min before testing. This finding is in agreement with the time-course data of a previous study in adult mice and rats that demonstrated the best effects of GNX shortly (10 min) after GNX administration *(Carter R. B. et al. 1997)*.

Preclinical and clinical studies described side effects of GNX (*Laxer K. et al. 2000*; *Mares P. and Stehlikova M. 2009b*; *Pieribone V. A. et al. 2007*). GNX treatment produced ataxia when tested on the rotorod tested [Carter RB, 1997] or motor toxicity at high doses ($TD5_{,,}$ = 24.8 mg/kg in mice) as measured by inverted-screen toxicity (*Gasior M. et al. 1997*)]. In cortical ADs model, GNX did not compromise motor phenomena in young animals (*Mares P. and Stehlikova M. 2009b*). However, the most effective dose of GNX (10mg/kg) affected motor behavior. The side effects are collectively described as "moderate" because the motor activity of these animals was not fully suppressed and rats used all four limbs to pull themselves forward. The sedation and somnolence were the most common adverse event reported by patient in clinical trials (*Laxer K. et al. 2000*; *Pieribone V. A. et al. 2007*).

3.2. Pyridoxine

Vitamin B6, as a cofactor, catalyzes metabolism of various amino acids in human body. In the brain, its main biologically active form, pyridoxal-5'-phosphate (PLP), is involved in transformations of the enzymes, which play crucial roles in metabolic pathways of amino acids such as glutamate and GABA (*Chung S. H. and Cox R. A.. 1983*). Pyridoxine is

used as a therapeutic agent mostly is clinical neurology. Epilepsy, behavioral problems and other neuropsychological conditions required chronic, long-term administration of pyridoxine for a whole lifetime with the risk of side effects (*Bernstein A. L. 1990*).

In adult patients, high vitamin B6 intake may have toxic effects in the peripheral nervous system (PNS) - sensory (*Schaumburg H. et al. 1983*; *Windebank A. J. et al. 1985*) as well as mild motor neuropathy (*Morra M. et al. 1993*). It is sometimes described as 'megavitamine' syndrome (*Dordain G. and Deffond D. 1994*). Similar effects may occur after a chronic abuse of relatively low doses of B6 (*Morra M. et al. 1993*), and even modest doses of B6 cannot be regarded as innocent in the PNS (*Bender D. A. 1989*) while opposite is true for the central nervous system which could be protected by vitamine B6 (*Snodgrass S. R. 1992*).

In pediatric neurology, high-dose of vitamine B6 is used in treatment of rare intractable seizures - pyridoxine-dependent epilepsy, which is probably due to abnormal binding of pyridoxal phosphate to glutamic acid decarboxylase leading to a decrease of gamma-aminobutyric acid. Mutations in the gene encoding this enzyme may also induce convulsions (*Bass N. E. et al. 1996*; *Gospe S. M., Jr. 2006*; *Leonard J. V. 2007*; *Pearl P. L. and Gibson K. M. 2004*; *Pearl P. L. et al. 2007*; *Pietz J. et al. 1993*). Moreover, pyridoxine is potential antiepileptic drug, recommended in those cases where the conventional therapy (ACTH, oral corticosteroids or vigabatrin) of infatile spasms failed (*Tsao C. Y. 2009*). Recently another clinical disorder of GABA metabolism, the pyridoxal 5'-phosphate dependent seizures, has been described in infants (*Gospe S. M., Jr. 2006*; *Leonard J. V. 2007*; *Pearl P. L. 2009*). Developing CNS appears to be more sensitive to the toxic effects of B6. In fatal overdose with Bendectine (composed of 1:1 mixture of doxylamine succinate and pyridoxine HCl (*Tyl R. W. et al. 1988*) in an infant, tonic-clonic seizures developed as a result of either doxylamine or pyridoxine toxicity (*Bayley M. et al. 1975*). Moreover, there is a

report of a paradoxical rise of seizure frequency with EEG correlations developed following B6 administration in newborn with intractable epileptic seizures (*Hammen A. et al. 1998*).

In adult patients, pyridoxine is infused in large doses as a specific antidote for isoniazid-induced seizures (*Maw G. and Aitken P. 2003*; *Tai W. P. et al. 2008*; *Tajender V. and Saluja J. 2006*).

In adult rats, B6 has specific proconvulsant activity. Intravenous or intracerebroventricular administration of B6 induces tonic-clonic seizures with frequent lethal outcome (*Schaeffer M. C. 1993*). Therefore, the results indicate that high doses of B6 may have proconvulsant effects, especially in the immature brain (*Ebadi M. et al. 1982*; *Ebadi M. et al. 1985*).

Multiple mechanisms of action of B6 in the brain could represent an alternative explanation. While low doses of B6 have anticonvulsant potency possibly due to the indirect support of GABA synthesis (*Ebadi M. et al. 1982*; *Ebadi M. et al. 1985*), high doses of B6 have indeed proconvulsant effects due to unknown mechanisms (*Ebadi M. et al. 1982*; *Ebadi M. et al. 1985*). Studies suggest a direct interaction of high doses of B6 with the GABAA receptor (*Ishioka N. et al. 1995*) or decreasing GABA content (*Waymire K. G. et al. 1995*). Although the i.c.v. administration of B6 metabolite pyridoxal phosphate induces almost instant seizures (depending on the dose), it is not clear from which structure these seizures arise and what is the cellular mechanism of the pyridoxal seizure induction (*Kouyoumdjian J. C. and Ebadi M. 1981; Ebadi M. et al. 1982*; *Ebadi M. et al. 1985*). The principal metabolite of B6—pyridoxal phosphate—acts as a coenzyme in many metabolic reactions *(Ebadi M. et al. 1982; Ebadi M. et al. 1985; Kouyoumdjian J. C. and Ebadi M. 1981)*. Therefore, one could speculate about other modes of B6 action (possibly involving additional metabolic reactions utilizing even higher than proconvulsant doses of B6) that could supersede or mask proconvulsant effects of B6.

4. Anticonvulsant drugs with mechanism of action connected with glutamatergic excitation

4.1. MK-801 (dizocilpine)

The anticonvulsant, central sympathomimetic and anxiolytic properties of compound MK-801 [(+)- 10,11-dihydro-5-methyl-5H-dibenzo (a,d) cycloheptene-5,10 imine] were described (*Clineschmidt B. V. et al. 1982a*; *Clineschmidt B. V. et al. 1982b*; *Clineschmidt B. V. et al. 1982c*). MK801 is a selective, non-competitive antagonist of excitatory amino acid transmitters at the NMDA receptor site (*Woodruff G. N. et al. 1987*). High effectiveness of MK-801 against epileptic seizures induced in vivo (*Clineschmidt B. V. et al. 1982a*) also led to the use of this anticonvulsant in a clinical study (*Troupin A. S. et al.1986*). In clinical trials MK-801 in high doses is effective against epileptic seizures, but it induced psychomimetic side effects. In lower doses, there is limited efficacy in treatment of epilepsy (*Fisher R. and Blum D. 1995*; *Porter R. J. 1989*). However, trials using MK-801 were not completely stopped; NMDA receptor system plays an important role in different human pathological states such as ischemic cerebrovascular disease (*Caccamo D. et al. 2004*; *Giacoia G. P. 1993*; *Ginsberg M. D. 1993*; *Scatton B. 1994*), learning and memory processes (*Ciamei A. et al. 2001*; *Rogawski M. A. and Wenk G. L. 2003*; *Tzschentke T. M. and Schmidt W.J. 2000*) or parkinsonism (*Johnston T. H. et al. 2005*; *Levin E. D. and Rezvani A. H. 2006*; *Ossowska K. et al. 1996*; *Sharp F. R. and Hendren R. L. 2007*).

Abnormal behavioral changes were observed following MK-801 IP administration. Hyperlocomotion induced by MK-801 was described in rats (*Frantz K. and Van Hartesveldt C. 1999*; *Koek W. et al. 1989*) , guinea pigs (*Jerram A. H. et al. 1996*), mice (*Tricklebank M. D. et al. 1989*), pigeons (*Koek W. et al. 1988*; *Koek W. et al. 1989*) or rhesus monkeys (*Koek W. et al. 1988*). However, this motor syndrome can be facilitated by local microinjection of MK-801 into the posterior parts of nucleus accumbens (ACC) not into caudate-putamen (CP) (*al-Khatib I. et al. 1995*). MK-801 is the most potent noncompetitive antagonist (compared to:

PCP, thienylcyclohexylpiperidine (TCP), (+)-N-allylnormetazocine [+)-NANM and ketamine) inducing a characteristic motor syndrome in mice (*Tricklebank M. D. et al. 1989*), rats, pigeons or rhesus monkeys (*Koek W. et al. 1988*).

This behavioral, motor syndrome (MK-801-induced motor syndrome) was intentionally studied (*Frantz K. and Van Hartesveldt C. 1999*; *Hiramatsu M. et al. 1989*; *Scorza M. C. et al. 2008*; *Valentine J. L. et al. 1996*) or mentioned as motor changes in different studies of anticonvulsant action (*Katsumori H. et al. 1998*; *Stafstrom C. E. et al. 1993*). These behavioral "side" effects may be used in studies of animal models of schizophrenia (*Ossowska K. et al. 2000*) or parkinsonism (*Johnston T. H. et al. 2005*; *Levin E. D. and Rezvani A. H. 2006*; *Ossowska K. et al. 1994*; *Ossowska K. et al. 1996*; *Sharp F. R. and Hendren R. L. 2007*).

In addition, MK-801 can elicit ataxia, but it is not effective as anestethic drug (measured by loss righting reflex) in rats. MK-801 or PCP elicit ataxia and MK-801 is probably toxic to Purkinje cells in cerebellum (*Nakki R. et al. 1996*).

Some data indicate that behavioral changes common for all NMDA antagonists must not be due only to NMDA-receptor blockade. Catecholaminergic systems and serotonergic neurotransmission may be significantly involved in the mechanisms by which MK-801 alters behavior in rats (*Loscher W. and Honack D. 1992*).

Effects of MK-801 on seizures

There are many studies documenting the effect of MK-801 on reactive seizure in mature CNS.

MK-801 prolonged latencies of PTZ-induced generalized tonic-clonic seizures in a dose-dependent manner. In contrast, the minimal clonic seizures remain unaffected. These different effects support the existence of two different generators for minimal clonic and generalized tonic-clonic seizures (*Browning R. A. 1985*; *Browning R. A. and Nelson D. K.*

1986; *Gale K. 1988*). According to these studies the generator of minimal seizures is considered to involve the structures of the forebrain ("forebrain seizures") whereas the brain stem structures are responsible for major (tonic-clonic) seizures. Moreover, it appears that NMDA receptor mediated transmission plays a dominant role in the genesis of generalized tonic-clonic seizures, whereas the NMDA receptor activity during clonic seizures appears to be less important. Pretreatment with MK-801 resulted in a dose-dependent anticonvulsant action in strychnine-induced generalized seizures. This action is specific - MK-801 suppressed the tonic, but not the clonic phase of seizures in adult rats (*Kubova H. and Mares P. 1994*).

MK-801 produced an effective and dose-dependent anticonvulsant action in the lithium-pilocarpine model but not in rats treated with a high dose pilocarpine alone (*Ormandy G. C. et al. 1989*). In contrast, MK-801 was found to exhibit proconvulsant action in the mouse pilocarpine model of complex partial seizures (*Starr M. S. and Starr B. S. 1993*). In the kainate model of limbic seizures in rats, MK-801 markedly reduced number of wet dog shakes as well as seizure severity elicited by kainate (*Loscher W. et al. 1994*). MK-801 also reduced cocaine- and methamphetamine-induced seizures in rats (*Derlet R. W. et al. 1990*). If MK-801 was administered intracerebroventricularly together with NMDA it antagonized NMDA-induced generalized seizures (*Chiamulera C. et al. 1990*).

MK-801 blocked motor seizures induced by electrical stimulation of hippocampal or amygdala kindled foci, but was more effective in reducing seizure severity and AD duration resulting from stimulation of the hippocampal focus (*Gilbert M. E. 1988*). MK-801 prevented an increase of the duration of maximal dentate activation; in addition, higher doses of MK-801 shortened the duration of maximal dentate activation (*Stringer J. L. and Lothman E. W. 1990*). MK-801 was found to be potent against the spread of seizures but less effective against partial seizures (*Williamson J. M. and Lothamn E. W. 1989*). The effect of single administration of MK-801 on the induction of audiogenic seizure susceptibility by noise in

immature rats was examined. Treatments with this non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist resulted in increases in noise exposure-dependent susceptibility (*Pierson M. and Swann J. 1991*).

MK-801 paradoxically enhanced electrographic seizures that preceded SE suppression in a model of limbic SE induced by 90 min of 'continuous' electrical stimulation of the hippocampus in rats (*Bertram E. H. and Lothman E. W. 1990*).

The data from the preclinical studies, where the action of MK-801 is tested in different models of epileptic seizures and the behavioral "side effects" models during ontogenesis in animals, suggested that MK-801 might affect not only NMDA receptor system.

4.2. 2-amino-7-phosphonoheptanoic acid (AP7)

One of ω-phosphonic amino acids, 2-amino-7-phosphonoheptanoic acid (AP7) was shown to be a potent competitive antagonist of N-methyl-D-aspartate (NMDA) receptors (*Evans R. H. et al. 1982*). This antgonism is a background for its anticonvulsant action (*Chapman A. G. et al. 1991*).

Anticonvulsant effect of AP7 has been studied in several types of experimental seizures in adult animals. Seizures produced by N-methyl-D-aspartic acid (NMDA) (*Meldrum B. S. et al. 1983*) were antagonized by pretreatment of AP7 and the same was demonstrated for clonic seizures induced by IP, SC or IV administration of N-methyl-DL-aspartic acid (NMDLA) (*Czuczwar S. J. and Meldrum B. 1982*; *Czuczwar S. J. et al. 1985*).

Similar anticonvulsant effect was demonstrated against seizures elicited by kainic acid (KA), 3-mercaptopropionic acid (3MPA), thiosemicarbazide (TSC), quinolinic acid, bicuculline, picrotoxin (*Czuczwar S. J. and Meldrum B. 1982*; *Nevins M. E. and Arnolde S. M. 1989*) and methyl-6,7-dimethoxy-4-ethyl-beta-carboline-3-carboxylate (DMCM) (*Czuczwar S. J. and Meldrum B. 1982*), or PTZ (*Croucher M. J. et al. 1982*; *Nevins M. E. and Arnolde S. M. 1989*). Pretreatment of DBA/2 mice (a strain of mice genetically susceptible to

sound-induced seizures) with AP7 blocked myoclonus or convulsions induced by trans-2,3-piperidine dicarboxylic acid (*Croucher M. J. et al. 1984*). Also, intracerebroventricular administration of AP7 acts against NMDA-induced clonic seizures (*Meldrum B. S. et al. 1983*) and 4-aminopyridine (4-AP)-induced generalized tonic-clonic seizures (*Fragoso-Veloz J. and Tapia R. 1992*). Moreover, AP7 protects against tonic extension seizures in submaximal and maximal electroconvulsive shock (ECS) models (*Nevins M. E. and Arnolde S. M. 1989*) or blocks the full motor seizures (stage 5) in electrical stimulation of the ventral hippocampus (*Peterson D. W. et al. 1984*) or prevented the development of seizure activity in amygdala kindling (*Croucher M. J. et al. 1995*).

However, AP7 is less potent than noncompetitive NMDA antagonist (MK-801, PCP) (*Fragoso-Veloz J. and Tapia R. 1992*; *Nevins M. E. and Arnolde S. M. 1989*).

Protective effect of focal microinjections of AP7 into different CNS structures was demonstrated in model of limbic seizures. Bilateral application of AP7 into the entopeduncular nucleus (EP) (*Patel S. et al. 1986*), into the prepiriform cortex (PC) (*Millan M. H. et al. 1986*) or into the substantia nigra (SNR) pars reticulata (*Turski L. et al. 1986*) prevented pilocarine-induced seizures (*Millan M. H. et al. 1986*; *Patel S. et al. 1986*; *Turski L. et al. 1986*); bilateral application into substantia nigra (SNR) pars reticulata suppressed PTZ-induced tonic seizures (*Xie X. H. et al. 1991*) and unilateral injection into deep prepiriform cortex (DPC) prevented seizures induced by the intravenous administration of bicuculline in the rat (*Piredda S. and Gale K. 1986*).

Interestingly, the focal bilateral infusion of AP7 into (SNR) pars reticulata increased duration of electrographic KA-induced seizure discharges (*Tanaka K. et al. 1994*) or prolonged seizure duration in KA-induced status (*Tanaka K. et al. 1996*). These authors speculate that differences in seizure models may account for these contradictory findings. Recently, difference between two parts of SNR anterior and posterior was demonstrated to exhibit site-specific effects on seizures (*Fan X. D. et al. 1997*; *Moshe S. L. et al. 1994*;

Thompson K. et al. 2000; *Veliskova J. and Moshe S. L. 2001*). Bilateral microinfusions of GABAA agonists in the SNRanterior have anticonvulsant effects, while they have proconvulsant effects in the SNRposterior (*Thompson K. et al. 2000*; *Veliskova J. et al. 1996*). The same site-specificity was demonstrated for AP7 in the adult male rats. Anticonvulsant effects of AP7 infusions were demonstrated in the SNRanterior in agreement with data for other seizure models (*De Sarro G. et al. 1984*; *Maggio R. and Gale K. 1989*; *Turski L. et al. 1986*; *Wurpel J. N. et al. 1992*). The proconvulsant effect of AP7 infusions in the SNRposterior is consistent with the findings of Tanaka et al. (*Tanaka K. et al. 1994*). Blockade of NMDA receptors in both SNR subregions produced effects similar to augmentation of GABAergic neurotransmission (using intranigral infusions of GABAA receptor agonists) (*Moshe S. L. et al. 1994*; *Thompson K. et al. 2000*; *Veliskova J. et al. 1996*). Infusions of acidic solution in the SNRposterior have proconvulsant effects (*Velisek L. et al. 1998*). A possible mechanism may be the suppressive effect of acidosis on NMDA receptors leading to a decrease in neuronal excitability (*Tang C. M. et al. 1990*; *Traynelis S. F. and Cull-Candy S. G. 1990*), similar to the effect of AP7 infusions. The protons may also produce agonist-like effects on GABAA receptors (*Kaila K. 1994*); the proconvulsant effects of local pH changes in the SNRposterior are similar also to the effects of GABAA agonists in this region (*Moshe S. L. et al. 1994*; *Velisek L. et al. 1998*). These data show that the effects of the specific NMDA receptor antagonist AP7 are region specific. The substantia nigra pars reticulata (SNR) plays an important age- and sex-specific role in control of clonic seizures. Its involvement in control of tonic-clonic seizures is contradictory (*Velisek L. et al. 2006*).

NMDA receptor sensitivity changes during ontogenesis, higher efficacy was demonstrated in young animals (*Baudry M. et al. 1983*; *Tsumoto T. et al. 1987*; *Velisek L. et al. 1990*; *Wurpel J. N. et al. 1992*). Developmental studies of anticonvulsant action of AP7 are restricted to PTZ-induced generalized tonic-clonic seizures (*Velisek L. et al. 1990*), homocystein (*Folbergrova J. 1997*) and homocysteic acid (*Folbergrova J. et al. 2000*). The

marked anticonvulsant effect of this competitive NMDA antagonist - AP7 during development has been demonstrated however the strong unwanted side effects make some limitations (*Mares P. et al. 2004*).

AIMS OF STUDY

1. To study anticonvulsant action of drugs influencing inhibitory GABAergic system against seizures elicited by convulsants with different mechanisms of action during development.

2. To verify age-specific model of flexion seizures in immature rats by means of pharmacological intervention with drugs used in pediatric epileptology (with a focus on GABAergic system).

3. To study anticonvulsant action of drugs influencing excitatory glutamatergic system at different levels of brain maturation.

4. To analyze possible site of anticonvulsant action of antagonists of glutamate receptors of the NMDA type.

5. The general aim was to test the hypothesis that correction of the balance between inhibitory and excitatory systems is a possible way to efficient treatment of epileptic seizures at different stages of postnatal development.

GENERAL DISCUSSION

The main question posed at the beginning of this dissertation – if the disbalance between excitatory and inhibitory systems in the brain which form a background of nearly all types of epileptic seizures – may be answered positively. We were able to suppress epileptic seizures elicited by an insult targeted on one of these systems by a therapeutic intervention focused on the opposite system at all developmental stages studied. This question is important due to increasing amount of data demonstrating that the main inhibitory neurotransmitter in the mature brain, GABA, serves as an excitatory factor at early stages of development in relation to intracellular activity of chloride anions *(Ben-Ari Y., 2002; Dzhala V. I. et al. 2005)*. Our data clearly show that this excitatory action is restricted to the very first postnatal days (in hippocampus, data for other brain structures are sparse) and that rats in the second week of life exhibit normal, i.e. inhibitory action of GABA.

Our results demonstrated that it is possible to induce experimentally a model of some features of age-specific seizures and affect them pharmacologically similarly to clinical experience with epileptic syndromes in pediatric epileptology. NMDA-elicited flexion seizures model a type of seizures typical for Lennox-Gastaut syndrome in infancy and were recently used as an important part of a model of this severe age-dependent epileptic syndrome *(Velisek L. et al. 2007)*. An elaboration of this model is based on description of these specific seizures by Mareš and Velíšek (1992) and pharmacological verification presented in these theses.

In addition to special discussions in all papers included in these theses two points deserve special attention: 1) proconvulsant and convulsant action of pyridoxine and 2) site-specific anti- or proconvulsant action of NMDA receptor antagonist (AP7) in substantia nigra.

Ad 1) Pyridoxine is a coenzyme of many enzymes mostly those involved in metabolism of amino acids. Among these enzymes is glutamate decarboxylase, an enzyme

which produces GABA from glutamic acid, as well as GABA-aminotransferase, an enzyme playing a key role in catabolism of GABA *(Amadasi A. et al. 2007)*. Low doses exhibited expected anticonvulsant action probably by potentiation of glutamate decarboxylase activity but high doses (250 mg/kg) induced seizures. These seizures are nonconvulsive, they are hardly detectable without EEG recording. Mechanism of generation of these seizures is not known but increased activity of GABA-aminotransferase should be taken into account and changes of activity of other enzymes cannot be excluded. In spite of the fact that the 250-mg/kg dose is extremely high, this finding may be of clinical significance.

Ad 2) Different role of GABA receptors in anterior and posterior parts of substantia nigra in epileptic seizures was described. Effects of drugs potentiating GABAergic inhibition (muscimol, vigabatrin) or suppressing it (bicuculline) can be anticonvulsant or proconvulsant according to the site of microinjection *(Moshe S. L. and Garant D. S. 1996, Veliskova J. et al. 1996)*. Our data demonstrated that this region-specific effect is not bound to GABAergic system only and that antagonists of NMDA receptors exhibit the same site specificity as GABAergic drugs. An injection of AP7 into anterior part of substantia nigra resulted in an anticonvulsant action whereas injection into posterior part had a proconvulsant action. These parallels between effects of GABAergic and glutamatergic drugs demonstrated again a significance of balance between the two main neurotransmitter systems for generation as well as suppression of epileptic seizures.

Our results clearly shown that different types of epileptic seizures can be elicited if excitation prevails over inhibition and that this is valid for all developmental stages studied. None of our studies included rat pups in the first week of postnatal life where the role of GABA may be excitatory rather than inhibitory. Older data from our laboratory demonstrated that the inhibitory function of GABA expressed as an anticonvulsant action of drugs potentiating GABAergic inhibition is present even in 7-day-old rats *(Kubova H. and Mares P. 1991)*. Local application of bicuculline, a competitive antagonist of GABA, on the cerebral

cortex, induces an epileptiform focus even in 5-day-old rats *(Velískova J. et al. 1991)*. The balance between excitation and inhibition can be compromised as well as repaired by intervention in either system. This general principle is valid at all developmental stages studied in presented experiments.

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