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Vývojové změny vybraných podjednotek NMDA a AMPA receptorů a účinky jejich antagonistů ve fyziologických a epileptických dějích

Developmental changes in expression levels of the chosen subunits of NMDA and AMPA receptors and action of their antagonists on physiological and epileptic phenomena

Disertační práce

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Abbreviations:

- (3-MPA)–3-mercaptopropionic acid
- (AD)–Epileptic afterdischarge
- (AMPArs) 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid receptors
- (BBB)–Blood-brain-barrier
- (BDNF)–Brain-derived-neurotrophic-factor
- (CA) –Cornu ammonis
- (CaN)–Calcineurin
- (CNS)–Central nervous system
- (CONTROL)– control animals injected with saline
- (CRE)–cAMP response elements
- (CREB)–cAMP response element-binding protein
- (CREST)–Calcium-responsive transactivator protein
- (DG)–Dentate gyrus
- (DGNs)–Dentate gyrus neurons
- (DH)–Dorsal hippocampus
- (DPCPX)– 8-cyclopentyl-1,3-dipropylxanthine
- (E15)–Embryonic day 15
- (EPSCs)– Excitatory postsynaptic currents
- (EPSP)– Excitatory postsynaptic potential
- (ERK1/2)– Extracellular-signal-regulated kinases (ERKs) or classical MAP kinases
- (GABA)–  $\gamma$ -Aminobutyric acid
- (GABAARs)–  $\gamma$ -Aminobutyric acid type A receptors
- (GluA1, 2, 3, and 4)– AMPA receptor major subunits
- (GRIN2A)– gene encoding NR2A subunit of NMDA receptor
- (GRIN2A/GRIN2B)– ratio of relative mRNA level of both, GRIN2A and GRIN2B genes.
- (GRIN2B)–gene encoding NR2B subunit of NMDA receptor
- (HA)–Homocysteic acid
- (HCN) – Hyperpolarisation-activated cyclic nucleotide-gated member of potassium channel superfamily
- (HCN1)–Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1
- (HDAC)–Histone deacetylase
- (IMPase)–Inositol monophosphatase
- (INH)–Isonicotinehydrazide
- (IP3)– Inositol 1,4,5-trisphosphate
- (KA)–Kainic acid
- (KCC2)–Potassium-chloride cotransporter
- (LiCl/Para)– lithium chloride/paraldehyde controls
- (LiCl/Pilo-SE)–Lithium-pilocarpine model of status epilepticus
- (M1R)– Muscarinic acetylcholine receptor subtype 1

- (MD)–Mediodorsal nucleus of the thalamus
- (mRNA)–Messenger RNA
- (NADPH)– Nicotinamide adenine dinucleotide phosphate
- (NKCC2)– Sodium–potassium–chloride cotransporter
- (NMDA)– N–methyl–D–aspartate
- (NMDARs)– N–methyl–D–aspartate Receptors
- (NRF1)– Nuclear respiratory factor 1
- (NRSE/ RE1)– Neuron–restrictive silencer element.
- (P)– Postnatal day (P12; P15; P18; P25; P72)
- (PDS)–Paroxysmal depolarisation shift
- (PTZ)–Pentylene-tetrazol
- (Q)– Glutamine
- (R)–Arginine
- (REST)–Silencing Transcription factor also known as Neuron–Restrictive Silencer Factor (NRSF)
- (Ro–19–4603)–5,6–Dihydro–5–methyl–6–oxo–4H–imidazo[1,5–a]thieno[2,3–f][1,4]diazepine–3–carboxylic acid 1,1–dimethylethyl ester
- (RTN)–Reticular thalamic nucleus
- (SE)–Pilocarpine induced status epilepticus
- (SE)–Status epilepticus
- (Sp1)–Transcription factor specificity protein 1
- (TMIV) – 4 transmembrane domain model
- (VH)–Ventral hippocampus
- (VP)–Ventroposterior nucleus of the thalamus
- (VPL) –The ventral posterolateral nucleus
- (VPL)–Ventral posterior lateral nucleus
- (VPM)– Ventral posteromedial nucleus

## Introduction

Definitions of epileptic seizure and epilepsy were proposed by International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). An epileptic seizure is defined as a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain. Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition. The definition of epilepsy requires the occurrence of at least one epileptic seizure (Fisher et al., 2005).

Glutamate is the main excitatory neurotransmitter and excitatory neurotransmission is necessary for normal brain development. Elevated level of glutamate and excitation allows the postnatal brain to develop quickly, however it may also make it vulnerable towards seizures and epilepsy that can cause cognitive and neurological disabilities (Kolb and Wishaw, 1998; Baram, 2003; Haut et al., 2004, Jiang et al. 2010). Three types of ionotropic glutamate receptors (ligand-gated ion channels), were named according to their agonists: N-methyl-D-aspartate (NMDA) and 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl), propionic acid (AMPA) and kainate (KA). These receptors are composed from four subunits. During early stages of postnatal development, subunit composition of NMDA and AMPA receptors undergo intensive changes (Monyer et al., 1994; Matsuda et al. 2002; Wee et al., 2008; Pachernegg et al., 2012; Paoletti et al., 2013). Participation and role of these receptors in mechanisms of development of seizures and epilepsy became one of the main targets of intensive investigation (De Sarro et al., 2005; Rektor 2013; Di Maio et al., 2013).

### *Arrest of seizures as an active process*

In a seizure focus, neurons exhibit synchronized electrical response called paroxysmal depolarization shift (PDS) and it is followed by afterhyperpolarization. The afterhyperpolarization is an active process which is generated primarily by calcium and voltage-dependent  $K^+$  channels, as well as GABAA mediated chloride (release of  $\gamma$ -aminobutyric acid (GABA)–the major inhibitory neurotransmitter can activate GABAA receptors (GABAARs)) conductance. Increased  $Ca^{2+}$  entry during the PDS activates a  $Ca^{2+}$ -dependent membrane  $K^+$  conductance that allows  $K^+$  efflux, membrane hyperpolarization, and cessation of firing. Like  $Ca^{2+}$ ,  $Na^+$  entry may activate  $Na^+$ -dependent  $K^+$  current that reduces neuronal excitability by hyperpolarizing the membrane. The afterhyperpolarization is particularly dependent on feedback inhibition by surrounding GABA-ergic inhibitory

interneurons. Under normal circumstances, the afterhyperpolarization limits the duration and spread of PDS. During development of a focal seizure – the surround inhibition in individual neurons gradually disappears, the membrane hyperpolarization fails and high–frequency action potentials are generated continuously due to labile character of GABAergic transmission. This misbalance between excitation and inhibition is the most important factor in the onset of a seizure (Kandel et al., 2000).

### *Excitability of immature brain*

Immature brain is highly prone to develop seizures and epilepsy due to its high excitability and because the inhibitory system involved in seizures termination is not yet fully developed. In rats, GABA does not achieve maximal expression levels until the fourth postnatal week (Swann et al., 1989; Brooks–Kayal, et al., 2001). In addition, in the immature brain GABA ( $\gamma$ –amino butyric acid) can be both: depolarizing (excitatory) and hyperpolarizing (inhibitory) (Loturco et al., 1995). GABA<sub>A</sub> receptors (GABA<sub>A</sub>R) activation in immature neurons (when the intracellular chloride concentration is higher than the extracellular concentration) leads to an efflux of chloride and depolarization (Rakhade et al., 2009). This is caused by a low expression of the specific chloride exporter, potassium–chloride cotransporter KCC2 during early development and high expression level of the sodium–potassium–chloride cotransporter NKCC2, a chloride importer (Dzhala et al., 2005; Rakhade et al., 2009). It has been reported that activation of BDNF receptors by brain–derived–neurotrophic–factor (BDNF) – can induce seizures by reduction levels of KCC2 messenger RNA (mRNA) and protein in rat hippocampal slice (Rivera et al., 2002; Rakhade et al., 2009). Neuronal excitability is also affected by expression of voltage–gated ion channels, which is developmentally regulated (Rakhade et al., 2009). In neonatal rats, neurotransmitter release depends on both N–type and P/Q–type voltage–gated calcium channels but during maturation, exclusively the P/Q–type channels, which are formed by Cav2.1 subunits, take over this function. This interferes with the normal hyperpolarizing potassium ion current and promotes repetitive action potential firing (Pape, 1996; Iwasaki, 2000; Noebels, 2003). HCN channel is the hyperpolarization–activated cyclic nucleotide–gated member of potassium channel superfamily. These channels are responsible for maintenance of resting membrane potential and dendritic excitability (Pape et al., 1996; Rakhade et al., 2009). Expression of HCN1 channel isoform in immature neurons is low, but it is increasing with age and dendritic excitability can thus be reduced (Chen et al., 2001).

### ***Susceptibility of epileptic seizures and status epilepticus in developing brain***

Status epilepticus (SE) is a life-threatening, neurologic emergency. The most widely used definition for SE is "a seizure lasting more than 30 minutes or recurrent seizures for more than 30 minutes during which the patient does not regain consciousness". Operational definition: generalized, convulsive status epilepticus in adults and older children (>5 years old) refers to at least 5 min of continuous seizures or two or more discrete seizures between which there is incomplete recovery of consciousness. Mechanistic definition: generalized, convulsive status epilepticus refers to a condition in which there is a failure of the "normal" factors that serve to terminate a typical generalized tonic-clonic seizure (Lowenstein 1999). The incidence of SE is highest in patients during the first and after 60th year of life (Haut et al., 2004). High incidence of status epilepticus in infants and children might be due to immaturity of mechanisms arresting seizures, what was demonstrated in amygdala-induced afterdischarges (Moshé and Albala 1983), in hippocampal (Velíšek and Mareš 1991) as well as cortical afterdischarges (Mares et al., 2002) in rats. There is little information concerning effects of convulsive generalized SE on the immature brain due to a high mortality mainly because of respiratory failure after long lasting tonic-clonic seizures (Haut et al., 2004). Therefore, several animal models of SE with long-lasting clonic seizures and low mortality were developed (Ben-Ari, 1985; Turski et al., 1986; Pitkanen et al., 2002). A great variability of the results has been reported in animal models of SE induced by chemoconvulsants such as lithium chloride-pilocarpine model (LiCl/Pilo, or LiCl/Pilo-SE).

### ***Sensitivity to epileptogenic agents***

Experimental seizures can be elicited either pharmacologically or electrically. Both types of seizure induction may be used for quantification of sensitivity of developing brain. Pharmacological elicitation of epileptic seizures is based on a fact that nearly all epileptic seizures are due to a predominance of excitation over inhibition. Seizures can be evoked by drugs augmenting excitatory processes (mostly glutamatergic neurotransmission) or by drugs interfering with inhibitory processes (mainly with inhibition mediated by GABAA receptor complex). Excitatory amino acids and their agonists acting on (mostly ionotropic) glutamate receptors are used as convulsant drugs. N-methyl-D-aspartate (NMDA) receptors are the main target for this action. NMDA itself was studied during postnatal development of rats and it was demonstrated that it is able to elicit not only generalized clonic-tonic seizures but also flexion, emprosthotonic seizures. Generalized clonic-tonic seizures could be elicited throughout whole development and convulsant doses markedly increased with maturation demonstrating thus

high sensitivity NMDA receptors in the immature brain. Flexion seizures represent an age-bound type of seizures; they could be elicited in immature rats up to postnatal day 18 (P18) but not later (Mareš and Velíšek 1992). Similar data were obtained with an agonist of NMDA receptors homocysteic acid (Mareš et al. 1997). Convulsant action of AMPA receptor agonists was demonstrated with quisqualic acid but no quantification was made (Thurber et al. 1994). Convulsant action of kainic acid (KA), a prototypic agonist of the third type of ionotropic glutamate receptors, was studied in immature rats by four laboratories (Cherubini et al. 1983, Albala et al. 1984). Low doses of KA induce nonconvulsive seizures characterized by epileptic automatisms, higher doses led to secondary generalization and thus to convulsive seizures. Again, there is an increased sensitivity of immature rats but detailed quantification was done only in our laboratory. The youngest age group studied (P7) was the most sensitive but developmental changes were not as huge as in the case of NMDA (Velíšková et al. 1988).

There is an extensive literature concerning the opposite way of seizure elicitation – suppression of inhibition. GABA, glycine and adenosine served as targets for possible convulsant action of their antagonist. Various ways of suppression of GABAergic inhibition were used. Inhibition of GABA synthesizing enzyme glutamate decarboxylase with 3-mercaptopropionic acid (3-MPA) (Mareš et al. 1993), or isonicotinic acid hydrazide (INH) (Mareš and Trojan 1991), competitive antagonism at GABA<sub>A</sub> receptors with bicuculline (Zouhar et al. 1989), or antagonism at other binding sites of GABA<sub>A</sub> supramolecular receptor complex – benzodiazepine receptor inverse agonist Ro 19-4603 (Kubová and Mareš 1994), picrotoxin. Specific position among drugs blocking GABA<sub>A</sub> mediated inhibition has pentylenetetrazol (PTZ) (Mareš and Schickerová 1980, Pineau 1999). It probably acts as an antagonist at three binding sites of GABA<sub>A</sub> receptor complex. Convulsant drugs acting on GABA<sub>A</sub> receptors elicit in dependence on the dose three different seizure models – EEG spike and wave rhythm, minimal clonic seizures and generalized tonic-clonic seizures. All drugs with this mechanism of convulsant action exhibit highest effect in the third postnatal week of rats (Velíšek et al. 1992). No exact quantification is at disposal for drugs interfering with glycine inhibitory receptors (strychnine) but results are similar to the action of pentylenetetrazol, i.e. the highest sensitivity is in the third week of postnatal life (Kubová and Mareš 1995). Adenosine receptors could be antagonized non-specifically (aminophylline) but detailed quantification is missing (Mareš et al. 1994). Specific antagonism of inhibitory A<sub>1</sub> adenosine receptors by DPCPX resulted in more severe convulsant effects in P12 than in P25 rats (Mareš 2010). Rhythmic electrical stimulation of sensorimotor cortical region was used for quantification of seizure

susceptibility. Epileptic afterdischarge (AD) formed by spike-and-wave rhythm accompanied by clonic seizures demonstrated the highest sensitivity of P18 rats. If transition into the second, limbic type of afterdischarges was studied, threshold intensities necessary for elicitation of this type of seizures decreased with age but always were substantially higher than thresholds for spike-and-wave afterdischarges (Mareš et al. 2002). Combination of advantages of both ways of seizure induction is represented by local application of convulsant drugs. An antagonist of GABAA receptor bicuculline could be used in its water soluble form (bicuculline methiodide). The application on sensorimotor cortical region demonstrated that cortical epileptogenic focus could be elicited at very early developmental stage – in 5- and 7-day-old rats (Soukupová et al. 1993) but again, detailed quantification is missing. These results are in agreement with local application of penicillin (acting probably also on GABAA receptors) on the cerebral cortex (Mareš 1973) or into the amygdala (Kudo and Yamauchi 1988). Both studies have shown that epileptogenic focus could be formed since P3. On the other hand, acetylcholine applied locally on sensorimotor cortical region was found to elicit epileptogenic foci since P9 (Staudacherová et al. 1978), i.e. at the developmental stage when excitatory action of acetylcholine on cortical neurons develops. Status epilepticus can be induced by LiCl/Pilo administration in rats at P12, which (when compared to human development) is considered as an infant/toddler (Haut et al., 2004). Previous study shows that the LiCl/Pilo induced SE at early developmental stage (e.g, at P12) induced plastic changes as well as long lasting structural and functional consequences. Changes are mostly pronounced in such brain regions as: hippocampus (acute neurodegeneration, reduced volume at adulthood, mossy fiber sprouting, altered learning, memory and other cognitive functions), amygdala and perirhinal cortex (volume reduction of these structures was observed in adult animals) (Nairismagi et al., 2006), thalamus (neuronal injury, necrotic neuronal injury and activation of microglia). In general it was proposed that young animals are more sensitive to epileptogenic stimuli as well as to development of epilepsy, but more resistant to brain injury (Cavalheiro et al., 1987; Lado et al., 2002; Kubova et al., 2004).

#### *Lithium chloride–pilocarpine model of SE–mechanism*

In animals, the SE can be induced by systemic administration of pilocarpine. Since the administration of LiCl improve animal survival by decreasing the dose of pilocarpine by about 10-fold, many of investigators use pilocarpine in combination with LiCl pretreatment. Induction of SE by pilocarpine depends on activation of M1 muscarinic receptor. It leads to phospholipase C (PLC)–dependent generation of DAG and IP3 and subsequent release of

calcium from intracellular stores. High  $\text{Ca}^{2+}$  concentration promote release of glutamate (Smolders et al., 1997). Increased levels of glutamate acting through AMPARs and KA receptors allow the entrance on  $\text{Na}^+$  and  $\text{Ca}^{2+}$  into the cell. This leads to release of magnesium blockade from NMDARs and their activation. These events allow entrance of even more  $\text{Ca}^{2+}$  into the postsynaptic cell. Activation of ERK1/2 (MAP kinase–mitogen–activated protein kinases) also stimulates NMDA receptors.  $\text{IP}_3$ –mediated surge in intracellular  $\text{Ca}^{2+}$  and its increased influx through NMDARs leads to activation of NOX (NADPH oxidase) and generation of superoxide. It results in oxidative modification of cell surface NMDARs with impairment of receptor function. These events can trigger local activation of neuronal nitric oxide synthase (nNOS) in close association with the NMDA receptor, and NOX. Thus,  $\text{Ca}^{2+}$  influx through NMDARs channels leads to production of superoxide via NOX activation. Calcium uptake by mitochondria in combination with NO (nitric oxide) production triggers cell death (Di Maio et al. 2011). The persisting seizures induced by Pilo can be managed by means of paraldehyde administration. Paraldehyde has been used as an anti–convulsant for over 50 years. It is currently used when other anticonvulsants, including benzodiazepines or phenytoin and phenobarbital, have failed to stop an acute tonic–clonic convulsion (Rowland et al., 2009).

### ***AMPArs and their ontogeny***

Receptors mediating most of the fast excitatory synaptic transmission in the brain are ionotropic glutamate receptors of AMPA type. Their prolonged activation is highly neurotoxic and plays a critical role in generation and spread of cortical seizure activity. AMPA are heteromeric glutamate receptors consisting of four subunits: GluA1, 2, 3, and 4 in varying stoichiometries (Sommer et al. 1991; Hollmann and Heinemann 1994). Their diversity can be increased by alternative splicing or RNA editing leading to formation of the receptor channel with different biophysical properties (Lilliu et al. 2001; Kumar et al. 2002). Each subunit of AMPA receptor is encoded by separated gene. Their transcripts show unique area– and temporal–expression pattern and are independently regulated during development (Lilliu et al., 2001). If an AMPAR lacks a GluA2 subunit, then it will be permeable to sodium, potassium, and calcium, but presence of a GluA2 subunit will usually render the channel impermeable to calcium (Hsu et al., 2010). Additionally, in a process of RNA editing, an exonic glutamine (Q) codon in a pore–forming segment of GluA2 subunit protein, may be changed to an arginine (R) (Sommer et al., 1991). AMPARs containing the edited form GluA2 exhibit outwardly rectifying currents and little  $\text{Ca}^{2+}$  permeability, whereas those containing not edited GluA2 generate inwardly rectifying currents and are permeable to  $\text{Ca}^{2+}$  ions (Verdoorn et al., 1991;

Burnashev et al., 1995). These two forms of GluA2 have been called respectively flip and flop (Standley et al., 1995; Szczurowska and Mares 2013).

### *Ca<sup>2+</sup> permeability of AMPARs*

Calcium-permeable AMPARs have been reported to play an important role in various physiological (synaptic transmission, synaptic plasticity) and pathological (seizures, epilepsy) processes (Washburn et al., 1997; Cull-Candy et al., 2006; Kwak and Weiss, 2006). AMPARs are functionally and molecularly distinct in different cell types of the CNS (see table 2.). Rapidly gated calcium permeable AMPARs specific expression pattern in interneurons and relay neurons might be responsible for reduction of the time interval between individual EPSCs, the time shortening of EPSCs and initiation of the postsynaptic action potential (Jack and Redman, 1971). Termination of the EPSCs can be reached by activation of Ca<sup>2+</sup> dependent K<sup>+</sup> channels via AMPARs mediated Ca<sup>2+</sup> current (Geiger et al., 1995).

**Table.1. Functional properties and subunit composition of AMPARs in different cell types**

Cell Type	Deactivation time	Desensitization time	Calcium permeability	Relative Abundance			
				GluA1	GluA2	GluA3	GluA4
Neocortex	+	++++	*	*	*****	**	*
CA3 Pyramidal cells	++	+++++	*	*****	****	*	-
Bergman glial cells	+	+++	***	*****	*	**	***
DG granule cells	+	++++	*	*****	****	**	-
Interneurons DG basket cells	+	++++	**	*****	**	**	**

Relative deactivation and desensitization time: + Very fast, ++ fast, +++ moderate, ++++ slow, +++++ very slow. Relative expression level and calcium permeability: \* Very low, \*\* low, \*\*\* moderate, \*\*\*\* high, \*\*\*\*\* very high, - not detected (according to Geiger et al., 1995; Szczurowska and Mares 2013).

An AMPAR subunit expression profile differs in hippocampal principal neurons (CA3 pyramidal cells, hilar mossy cells) and interneurons (DG basket cells, hilar interneurons) (Amaral et al., 1990). The GluA2 and GluA4 transcripts in Bergmann glial cells (radial glia, which are present still during adulthood) and DG granule cells are almost completely edited at the R/Q site. The reason for more rapid desensitization of AMPARs in DG granule cells than those in CA3 pyramidal cells or hilar mossy cells is the low degree of R/Q site edited versions

in DG granule cells. This could be also due to the virtual absence of GluA2 and the high level of GluA4 in Bergmann glial cells (Geiger et al., 1995; Szczurowska and Mares 2013).

#### *AMPARs subunits expression: developmental changes*

Low levels of expression AMPARs GluA2 subunit at early stages of CNS development (hence a high  $\text{Ca}^{2+}$ -permeability) (Durand and Zukin, 1993; Monyer et al., 1994) might participate in the increased seizure susceptibility in the immature brain (Szczurowska and Mares 2013). Increased excitability seems to be caused by hyperexcitation conducted by  $\text{Ca}^{2+}$ -permeable AMPARs and subsequent excitotoxic death of inhibitory GABA-ergic interneurons (Moshe et al., 1983). However, AMPARs lacking GluA2 may still be formed even in the presence of GluA2 mRNA (Greger et al., 2003; Cull-Candy et al., 2006). Messenger RNAs of GluA1 and GluA4 subunit are expressed at higher levels in mesencephalon than in striatum, which develops later during prenatal development (E15–P0) (Lilliu et al., 2001). Immunoreactivity for GluA2/3 subunits is concentrated postsynaptically at corticothalamic synapses and their activation by cortical stimulation results in fast EPSCs and slower NMDA receptor-based EPSCs (Salt and Eaton, 1996). The great amount of GluA2 and GluA3 proteins directed to corticothalamic synapses underlines the role of corticothalamic input in enhancing or gating of sensory transmission through the thalamus (McCormick and Bal, 1994; Liu, 1997) and in setting up reciprocal thalamocortical oscillations (Liu, 1997). At early stages of development, GluA3 and GluA4 subunits are broadly expressed in hippocampus. Later in development GluA4 expression decrease, while GluA2 expression level increase in this structure. Thus, it becomes predominant AMPAR subunit in the hippocampus of adult rat brain. In rats cortical pyramidal neurons, synaptic AMPARs switch from GluA2-lacking to GluA2-containing, occur between P13 and P21 (Kumar et al., 2002). However, AMPARs on dendrites of the same neurons exhibit characteristics of those GluA2-containing AMPARs already at P6 until P40 (Brill and Huguenard, 2008; Hsu et al., 2010) therefore dendrites of cortical pyramidal neurons at early stages of postnatal development are probably  $\text{Ca}^{2+}$ -impermeable. The expression of the flop (not edited R/Q i.e.  $\text{Ca}^{2+}$  permeable variant) version of GluA subunits remain constant until P14, whereas the flip variants (edited R/Q i.e.  $\text{Ca}^{2+}$ -impermeable) are expressed at low levels at birth, and increase considerably between P8 and P14 (Standley et al., 1995). There are also some reports (Pellegrini-Giampietro et al., 1992) showing that most of GluA transcripts are transiently overexpressed, when compared to adult levels, with a peak expression at about P14 in hippocampus (Standley et al., 1995; Szczurowska and Mares 2013).

### ***Involvement of AMPARs in SE***

Seizures can cause rapid trafficking– and endocytosis–mediated alterations in AMPAR subunit composition and function. Rapidly after seizures, hyperexcitability of hippocampal networks may cause transition from normal to epileptic networks. Expression of GluA2 subunit and therefore the  $\text{Ca}^{2+}$  –permeability of AMPARs changes in various seizure models. With an increased potentiation of hippocampal epileptiform activity mediated by AMPARs, there is also an elevation of AMPA–mediated calcium permeability (Sanchez et al., 2001). This hyperexcitability is probably a consequence of a decrease in GABAergic inhibition induced by  $\text{Ca}^{2+}$  activated phosphatase, Calcineurin C (CaN).

It has been reported that the levels of GluA2 expression changes during two weeks following SE and prior to the development of spontaneous seizures (i.e. 1.5 months following SE) in this model (Roch et al., 2002; Raol et al., 2003). In the dentate gyrus, an increase of GluA3 mRNA level is induced 12 h after LiCl/Pilo treatment, while a clear decrease in GluA1 mRNA level and no significant change in GluA2 mRNA level can be observed (Condorelli et al., 1994). However, Grooms et al. (2000) and Hu et al. (2012) found a marked decrease in GluA2 and GluA1 expression 12–16h after SE. The reduction in GluA2 might serve as a “molecular switch”, leading to the arrangement of  $\text{Ca}^{2+}$  permeable AMPA receptors and enhancing the toxicity of endogenous glutamate following a neurological insult (Hu et al., 2012). Interestingly, 3 h after SE in the whole brain of immature animals the subunit composition of AMPARs changes from predominantly GluA3/4 subunits to predominantly GluA2/3 subunits (Hu et al., 2012). There is also an increase in GluA2 mRNA in mature dentate gyrus neurons (DGNs) after SE. (Liu et al., 2004; Porter et al., 2006). The seizure induced expression of  $\text{Ca}^{2+}$ –permeable AMPAR is developmentally regulated and contributes to the susceptibility of the immature brain to the epileptic activity (Cull–Candy et al., 2006).

### ***NMDARs and their ontogeny***

NMDARs are responsible for normal brain development; they are involved in numerous physiological (neuronal growth and migration, memory and plasticity) and pathological mechanisms (development of epilepsy, neurodegeneration associated with Parkinson, Alzheimer or Huntington diseases). NMDA receptors are ubiquitously distributed throughout the central nervous system (CNS). They are located mainly postsynaptically, but some of them are present on presynaptic membranes where they can play a role of auto– or heteroreceptors (Conti et al. 1997). These receptors can be also found on cortical astrocytes (Lee et al. 2010).

NMDARs are heteromeric complexes of four various subunits surrounding central ion channel permeable for  $\text{Ca}^{2+}$ . All subunits can be present in a form of different splice variants which exhibit diverse physiological and pharmacological properties. At the moment, seven different NMDA receptor subunits have been determined (NR1, NR2A–D, NR3A and NR3B). Functional NMDA receptors are assembled from at least one (more often two) constitutive, glycine-binding NR1 subunit (there are 8 known isoforms of NR1); one or two NR2A–D glutamate-binding subunits presence of which modulates ion channel functional properties (Monyer et al., 1994; Traynelis et al., 2010; Low and Wee, 2010). NR3A–B subunits do not form functional receptors alone but can additionally assemble with NR1–NR2 complexes and further increase NMDARs functional diversity (Sucher et al., 1995; Wong et al., 2002, Szczurowska 2013).

During first two postnatal weeks in rats (P1 –P14), brain is highly sensitive to the toxic effects of activation of NMDA receptors. Early in life, neurons of neocortex exhibit large, long-duration NMDAR-mediated excitatory postsynaptic currents (EPSCs) and these are characteristic for NR2B subunit containing NMDARs (Miyamoto et al., 2001). During the first postnatal week however, EPSCs of the NMDAR becomes shorter and faster (Barth and Malenka 2001; Lu et al., 2001; Liu et al., 2004). NR2B dominated currents probably cause greater  $\text{Ca}^{2+}$  entry through NMDAR central channel in developing synapses, assisting establishment of thalamocortical circuitry (Constantine–Paton et al., 1998; Cull–Candy et al., 2001). The level of NR2A subunit expression considerably increases during the first postnatal week in rodents, and become predominant, however the levels of NR2B do not change dramatically throughout development (Zhong et al., 1995; Stocca and Vicini, 1998). As a result, a change in ratio of the synaptic NR2B/NR2A is responsible for the developmental shortening of NMDARs mediated current (Lu et al., 2001, Szczurowska and Mares 2013).

### *NR1 subunit*

Formation of eight different NR1 splice variants is possible thanks to the insertion or deletion of three short exon cassettes, exon 5 in the N terminus (N1) and exons 21 and 22 in the C terminus (C1, C2) domains of the NR1 molecule (see Fig.1). Subunit NR1–1 is the full-length clone containing both C-terminal exons, NR1–2 lacks exon 21, NR1–3 lacks exon 22, and NR1–4 lacks both, letters (a) and (b) indicate a-absence and b-presence of exon 5 (Durand et al. 1993). These splice variants differ in their spatial and temporal expression patterns, and have different properties in interaction with protein kinases (Bradley et al., 2006, Szczurowska

and Mares 2013). NR1 subunits expression begins already at embryonic day 14 (E14), reaches the peak at third postnatal week and then slightly declines towards the adulthood (Laurie et al., 1994; Paupard et al., 1997). The NR1-b variant of NR1 (without the N-terminal N1 exon) is expressed mainly in neonatal sensorimotor cortex, caudate nucleus, and thalamus and CA3 layer in hippocampus, while NR1-a (which contains the N1 exon 5 cassette) is expressed abundantly throughout the brain and is found in all principal cells in hippocampus. Expression of NR1-1 variant is restricted to rostral parts (caudate, hippocampus, cortex) and NR1-4 to caudal parts (thalamus, cerebellum). Therefore, the composition and number of NMDAR signaling complexes may be dynamically regulated through the splicing of NR1 throughout development (Hoffmann et al., 2000; Hollmann., 1993; Heinemann, 1994; Laurie et al., 1994).

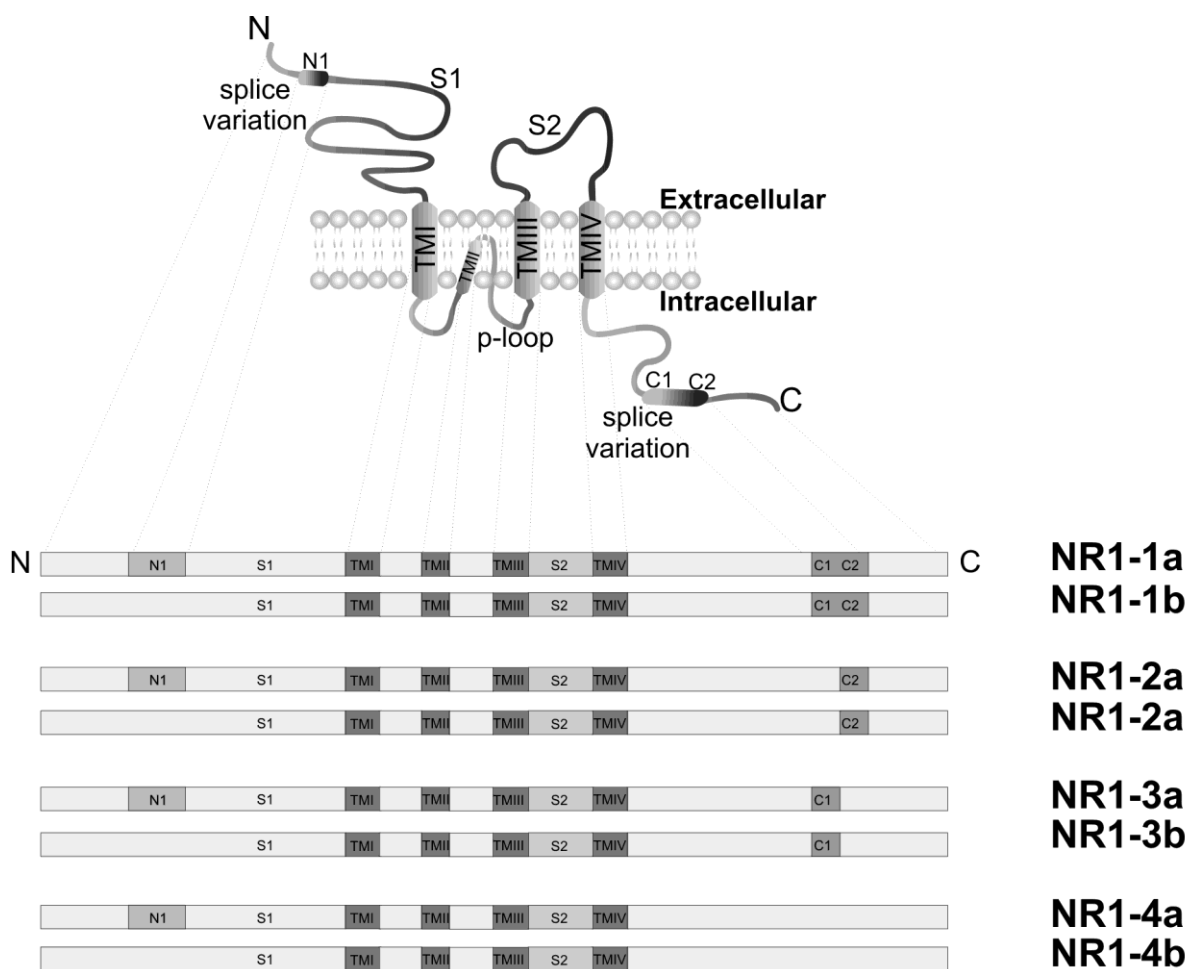


Fig.1. Schematic representation of NR1 subunit structure and its splice variants.

TMI–IV transmembrane domains; N1–exon 5 splice variation site; C1 and C2–exons 21 and 22 splice variation sites; S1 and S2–agonist binding domain (NR1–glycine–binding, NR2–glutamate–binding, and NR3–glycine–binding) (Szczurowska and Mares 2013)

#### *NR2A subunit*

Analysis of different rat and mice brain areas showed that from P0 to P2, levels of synthesized mRNA and protein of NR2A were very low in cerebral cortex and striatum, while in CA1 region of hippocampus expression of mRNA of NR2A subunit at P0 was easily detectable. Expression of NR2A between P5 to P10 increases significantly in the whole brain on both mRNA and protein levels (the increase was moderate in different cortical areas). It reaches a peak at P12 to P15 and remains at these levels (Wenzel et al., 1997; Liu et al., 2004). Expression of NR2A is highest in hippocampus, cerebral cortex, and thalamus. In hippocampus, NR2A mRNA signals are already strong at the time of birth but its protein levels remain low at least up to P10. This delay is caused by posttranscriptional mechanisms that regulate NR2A protein synthesis (Wood et al., 1996; Wenzel et al., 1997).

#### *NR2B subunit*

In cortex and hippocampus, NR2B mRNA subunit expression is already very strong at birth, while in the other structures expression is moderate or very low. Levels of NR2B mRNA do not dramatically change since P5, but its protein level increases notably in cerebral cortex and olfactory bulb (see table 1). From the P7 to P10, both levels of NR2B mRNA and protein increases in whole brain to reach a peak at P21 and then slightly decreases toward adulthood (Wenzel et al., 1997). In adult hippocampus, NR2B are present at the connections of the perforant path with CA3 cells, but are usually not detected at mossy fibers synapses (Paoletti et al., 2013). The NR2A and NR2B subunits are the predominant NR2 subunits in the adult hippocampus and neocortex (Monyer et al., 1994; Sheng et al., 1994) and new synapses rich in NR2A are added to those in which NR2B subunits predominate instead of switching subunits (Liu et al., 2004). There is general agreement that activity and visual experience increases the NR2A/NR2B ratio at the protein level, (Yashiro et al., 2008; Chen et al. 2006, Schurowska and Mares 2013).

#### *NR2C and NR2D subunits*

NMDA receptors containing NR2C or NR2D subunits are activated by glycine and glutamate with higher potency than receptors containing NR2A or NR2B. They are less sensitive to extracellular  $Mg^{2+}$  and exhibit lower maximal opening probability (Mosley et al., 2010).

NMDA receptors containing NR2D have been shown to exhibit remarkably slow deactivation following removal of glutamate (Carter et al., 1988; Monyer et al., 1994). NR2D subunits are co-assembled at the synapse with other NR2 subunits forming triheteromeric receptors (NR1/NR2B/NR2D) which may not exhibit the slow deactivation of pure NR1/NR2D receptors but may display other NR2D-like properties (Traynelis et al., 1998; Cull-Candy et al., 2001). NR2C and NR2D subunits also show localization patterns distinct from NR2A and NR2B, with prominent expression in the cerebellum, in the basal ganglia. (Monyer et al., 1994; Carter et al., 1988). NR2D and NR2C mRNAs are expressed in hippocampal and cortical interneurons but are barely expressed in principal cells (Monyer et al., 1994). NR2C mRNA subunit signals can be already detected at P0 in cerebellum, but no NR2C protein could be detected even at P10 (Monyer et al., 1994; Watanabe et al., 1994; Wenzel et al., 1997). It has been reported that the late appearance of the NR2C protein coincides with the termination of granule cell migration and the completion of the cerebellar circuitry. In the thalamus and olfactory bulb, on the other hand, the time course of the appearance of NR2C mRNA and its protein is very similar. Therefore, in the cerebellum a specific signal for the translation NR2C subunit protein is present. Levels of NR2C increase dramatically during third postnatal week when the mature circuitry is established (Wenzel et al., 1997). NR2D subunit is abundantly expressed in the caudal regions of the embryonic brain, its expression decrease rapidly during first two postnatal weeks. During adulthood, NR2D expression is low and restricted mostly to mesencephalon and diencephalon regions (Szczurowska and Mares 2013).

#### *NR3A and NR3B subunits*

NR3A subunits unlike NR2, form a glycine binding structure (Yao et al., 2008; Henson et al., 2010). The tightening of a ring of threonines that is present in the external vestibule of the channel of NR3A containing NMDARs exhibit reduction in  $\text{Ca}^{2+}$  permeability, ionic currents, and  $\text{Mg}^{2+}$  sensitivity (Wada et al., 2006). Compared to NR1/NR2 NMDARs, heterotrimeric complex of NR1, NR2 and NR3 subunits, forms an NMDAR with novel properties and attenuated currents (Henson et al., 2010). NR3A subunit plays an important role in preventing the premature synapse formation. Removal of NR3A allow for the insertion of mature NMDARs that in turn trigger AMPAR introduction (Sucher et al., 1995). NR3A subunits are expressed at high level at early stages of development. NR3A mRNA appears in the rat CNS by E16, peaks at second postnatal week and its expression gradually decreases during maturation (Henson et al., 2010; Paoletti et al., 2013). On the other hand, NR3B expression

demonstrates opposite developmental profile. Its levels are low at early life and progressively increase until adult life (Fukaya et al., 2005; Paoletti et al., 2013; Szczurowska and Mares 2013).

### ***Involvement of NMDARs in SE***

Most of the researches concerning developmental changes in NMDARs subunits expression pattern were focused on hippocampal formation (Haut et al., 2004). In hippocampal area, mRNA levels of NMDARs subunits change dynamically during development. In a model of LiCl/Pilo induced SE in immature brain (P20), the area of maximal neuronal injury is ventral hippocampus (VH) (Ekstrand et al., 2011).

In in vitro pilocarpine-induced epileptic activity, reactive oxygen species (ROS) production cause NMDA-mediated oxidative injury leading to apoptosis. In the LiCl/Pilo model of SE induction, IP3 synthesis is necessary for activation of two independent pathways, which together cause abnormal NMDAR subunit expression: a) Ca<sup>2+</sup>-dependent NADPH oxidase activation and b) ERK1/2 phosphorylation. Together these pathways are responsible for short-term up-regulation (overexpression) of the NR2B subunit in vitro and in vivo. Moreover, later after Pilo administration, NR2B subunits can regulate NR2A subunits expression level in two ways: a) by hyperactivation of NR2B induced via ERK1/2 activation or, b) independently, by oxidative stress-mediated NR2B overexpression. It was reported that NR2A but not NR2B subunit containing NMDARs activation was required for development of limbic epilepsy in kindling and pilocarpine models (Chen et al., 2007).

NR2B containing NMDARs are restored in adult brain in temporal lobe epilepsy (TLE), so neuronal hyperexcitability in epilepsy may be caused by mechanisms similar to those during early development (Di Maio et al., 2011). On the other hand, proteolysis of NR2B subunit by calpain was observed in hippocampus in animals after SE and it varies within neonatal period (Dong et al., 2006; Szczurowska and Mares 2013).

### ***NMDARs and AMPARs: general trafficking and signaling***

Production of functional NMDA receptors is limited, at least in part, by the availability of NR2 subunits (Prybylowski et al., 2004). NMDARs assemble in the endoplasmic reticulum and interact with membrane-associated guanylate kinases (MAGUKs) which are a family of proteins (including SAP102-synapse associated protein 102, SAP97 synapse associated protein 97, PSD-93- postsynaptic density protein 93kDa, and PSD-95- postsynaptic density protein 95kDa) play a role in scaffolding of the postsynaptic density (PSD). The NMDAR/MAGUK

interaction is mediated by the PDZ-binding domain of the NR2 subunit and Sec8, a protein of the exocyst complex. This interaction is necessary for the delivery of the NMDAR to the synapse. NMDARs are internalized by clathrin-mediated endocytosis (McGee et al., 2003; Sans et al., 2003). Synaptic and extrasynaptic receptors differ in their subunit composition and are also differentially regulated in response to phosphorylation changes (Li et al., 2002). The developmental subunit switch is dependent upon preferential MAGUK binding to either GluN2A or GluN2B subunits (Sans et al., 2003). NR2B-containing receptors delivery to the dendrite is possible thanks to binding of the motor protein KIF17 to a multiprotein complex containing the NR2B (Prybylowski et al., 2004). The extracellular matrix (ECM) protein Reelin modulates NR2B surface diffusion, and Reelin overexpression decreases synaptic NR2B expression and reduces NR1/NR2B-mediated synaptic currents (Gladding et al., 2011;Groc et al., 2007).

NMDARs interact with numerous different intracellular signaling pathways by cooperation with several cellular kinases including Protein Kinase A (PKA), Protein Kinase C (PKC), Extracellular signal-regulated kinase 1/2 (ERK1/2) or Calmodulin-dependent Protein Kinase II (CaM II kinase) which directly interact with C-terminal domain of NR2B subunit. The ERK1/2 (extracellular signal-regulated kinase 1/2) signaling pathway is responsible for activation of different transcription factors and plays an important role in synaptic plasticity and cell survival (Sweatt, 2004; Thomas and Huganir, 2004). In general, activation of ERK is coupled to cellular survival (Hetman et al., 2006). It is differentially regulated by calcium influx through NMDARs, depending on their subunit composition (Sava et al., 2012). An excessive NMDAR activation evokes neuronal degeneration by excitotoxic mechanism during early stage of neuronal development (Sava et al., 2012). On the other hand, different pools of NR2B-containing NMDA receptors are coupled to ERK in a different way: synaptic receptors are positively coupled, while extrasynaptic NR2B-containing receptors are negatively coupled to ERK. In developing hippocampal neurons, NR2B-containing NMDA receptors have been shown to mediate both pro-death and pro-survival signaling (Martel et al., 2009; Sava et al., 2012; Szczurowska and Mares 2013).

ERK1/2 pathway involves small GTPase- Ras, which is activated by specific guanine nucleotide exchange factors (GEFs) and inhibited by GTPase activating proteins (GAPs) (Thomas and Huganir, 2004). Both RasGEF and RasGAP can directly interact with NR2B subunit of NMDA receptor. Synaptic RasGAP (SynGAP) is present in high concentrations in postsynaptic densities and associate with NMDARs via PSD-95 proteins.

The other signaling molecule acting downstream of NMDA receptors is the tyrosine phosphatase: STRiatal-Enriched Phosphatase (STEP). Phosphorylation of the serine residue in STEP kinase-domain decreases STEP activity and reduce its affinity for substrates. Dephosphorylation of this same serine residue activates STEP. This activation can occur via calcineurin activity promoted by NMDA receptor-mediated  $\text{Ca}^{2+}$  influx. In basal conditions STEP activity is low, but when activated, STEP can down-regulate ERK2 by its dephosphorylation and influence excitotoxicity mediated via NMDARs (Paul et al., 2010). Both phosphorylation and dephosphorylation of ERK2 is  $\text{Ca}^{2+}$  influx-dependent. It was reported that induction of large, delayed  $\text{Ca}^{2+}$  influx (characteristic for NR2B subunit containing NMDAR) cause dephosphorylation and therefore STEP activation. As a result regulation of activation of the STEP by NR2B can explain NMDARs involvement in the inhibition of ERK activity and excitotoxicity (Paul et al., 2010).

AMPA receptors undergo kinesin and/or dynein mediated vesicular trafficking in dendrites. The  $\text{Ca}^{2+}$ -sensitive motor protein, Myosin Vb, is also involved in the dendritic vesicular trafficking of GluA1-containing AMPARs (Henley et al 2011). Influx of the  $\text{Ca}^{2+}$  via activated NMDARs stimulates CaMKII which in turn, phosphorylates GluA1 subunit of AMPARs. MyoVa is  $\text{Ca}^{2+}$  activated motor protein, which recognizes and couple phosphorylated GluA1 connected with Rab11 adaptor complex and then transport them along actin filaments. Activated by  $\text{Ca}^{2+}$  MyoVb on the other hand, transports AMPAR along the actin cytoskeleton to sites of exocytosis. Cytoskeletal adaptor protein 4.1N links AMPARs with actin filaments. When PKC phosphorylate, GluA1A at serine 816 and 818 its affinity for 4.1N increases and AMPA receptor can be inserted into membrane. On the other hand, phosphorylation of GluA1 at serine 845 by PKA, is responsible for insertion of AMPAR at extrasynaptic and perisynaptic sites where syntaxin 4 mediates membrane fusion events at the sites of exocytosis. Later these receptors are replaced by edited GluA2-containing AMPA receptors (Yang et al., 2008). PKC isoform protein kinase M zeta (PKMz) (Ling et al., 2002), maintains AMPARs at synapses by downregulating GluA2-containing receptor internalization. RNA editing of GluA2 has been shown to be important for exocytosis (Araki et al., 2010).

The signaling of Ras/ER pathway leads to AMPAR insertion into the dendritic membrane up away from the synaptic site of potentiation (Ling et al., 2002; Henley et al 2011; Hoogenraad et al, 2010; Patterson et al., 2010). In mature neurons NR2A-NMDARs promote Ras-ERK activation as well as surface expression of GluA1 subunit of AMPARs while NR2B-NMDARs inhibit both processes. On the other hand, changes in NR2A or NR2B expression

levels and signaling do not markedly influence GluA2 AMPA receptor expression subunit. Loss of GluA1/GluA2 subunits combination mediated by NR2B is rather balanced by the gain in GluA2/GluA3 therefore preventing calcium permeability of AMPARs (Kim et al., 2005; Szczurowska and Mares 2013).

***Neuronal circuits involved in brain damage after SE in immature rats.***

Probable mechanism of neuronal damage within hippocampus after LiCl/Pilo SE insult is neurotoxicity caused by overactivation of hippocampal excitatory circuits and subsequent damage of subpopulations of inhibitory interneurons. It has been proposed that the differences in severity of damage in different hippocampal subfields are due to differences in synaptic inputs (Druga et al., 2005). After SE, CA 1 and CA 3 hippocampal subfields show substantial interlaminar differences in number of degenerating neurons in stratum oriens and pyramidale (Pikkarainen et al., 1999). More damage can be seen in stratum oriens, which is supplied by collaterals of pyramidal neurons and receives more prominent excitatory (glutamatergic) inputs than the other layers (Somogyi and Klausberger, 2005). LiCl/Pilo-induced SE produces neuronal degeneration also in many thalamic nuclei in immature rats and it is highly related to age. Because all connections of dorsal thalamic nuclei with cortex are excitatory, the hyperexcitability within thalamocortical circuits caused by SE leads to massive glutamate release and subsequent excitotoxic damage of thalamic nuclei (Turski et al. 1989; Macchi, 1997; Druga et al., 2005). Immature brain exhibits high sensitivity to excitatory aminoacids, on the other hand SE-induced brain damage at early stages of development is less severe probably due to immaturity of neuronal afferents to thalamic nuclei at that age and their irregular maturation during 2nd and 3rd postnatal week (Mareš et al., 2004). The midline thalamic nuclei, receive different reciprocal connections from prelimbic and infralimbic cortical areas as well as from medial prefrontal cortex. These connections can be amplified by additional excitatory input from the amygdala and hippocampus (Ishikawa and Nakamura, 2003; Kubova et al., 2002). Thus it is possible that these thalamic nuclei participate in the synchronization of activity across limbic structures (Groenewegen and Berendse, 1994; Druga et al., 2005). Lateral thalamic nuclei have a number of projections to the cortices and additionally are interconnected with amygdala, thus amygdalar activity can spread to the thalamus (Doron and LeDoux, 1999; Shibata, 2000; Druga et al., 2005). Therefore damage seen in the lateral and caudal thalamic nuclei might be also related to hyperactivity in the thalamocortical circuits. Lateral amygdala receives strong projections from the dorsal part of medial geniculate nucleus, while the ventral part of this nucleus projects to primary auditory cortex. The amygdalar

epileptiform activity can be transferred firstly to the auditory cortex and further to medial geniculate nucleus (Doron and LeDoux, 1999; Pitkanen et al., 2000). Differences in the distribution of degenerating neurons in the lateral dorsal thalamic nucleus between young (in P12 and P15 rats in the basal part of this nucleus) and older animals (mainly in its dorsal part) suggest a role of gradual maturation of mutual connections between thalamus and cortex (Druga et al., 2005).

### ***Ictogenic and anticonvulsant action of drugs influencing NMDA and AMPA receptors***

Agonists of NMDA receptors (NMDA, homocysteic acid) elicit epileptic seizures. Their efficacy is high at early stages of development and decreases with age (Mareš and Velíšek, 1992, Mareš et al., 1997). Similar but not so marked attenuation of efficacy during maturation was observed with the anticonvulsant action of some antagonists of NMDA receptors and their anticonvulsant action (Velíšek et al., 1990, 1991; Mikolášová et al., 1994; Kubová and Mareš, 1995). Ifenprodil, a selective antagonist for NR2B subunit-containing NMDA receptors, exhibits anticonvulsant action only during the first three postnatal weeks in rats (Mareš and Mikulecká, 2009). Antagonists of AMPA receptors CNQX, DNQX and NBQX have only moderate anticonvulsant action against pentetrazol-induced seizures expressed during the first three postnatal weeks (Velíšek et al., 1995). NBQX but not noncompetitive antagonist of AMPA receptors GYKI52466 exhibited decreasing action against cortical epileptic afterdischarges during development (Mareš et al., 1997; Kubová et al., 1997; Szczurowska and Mares 2013).

## **Hypotheses**

- Selective blockade of NR2B subunit containing NMDARs at early stages of postnatal development can represent age-specific anticonvulsant treatment.
- Selective blockade of Ca<sup>2+</sup>-permeable AMPARs has an anticonvulsant effect in immature brain.
- The early-life LiCl/Pilocarpine-status epilepticus, may induce changes in expression levels of NR2A and NR2B (NMDARs) and GluA2 (AMPA) subunits, that are present not only during development, but also in adult brain.

## **Aims of the study**

- To analyze the influence of a specific antagonist of NMDARs containing NR2B subunit (Ro-25-6981) on physiological and pathological brain activity in immature rats.
- To determine the effect of administration of Ca<sup>2+</sup>-permeable AMPARs antagonist (IEM 1460) on epileptic phenomena in developing rats.
- To determine how normal ontogeny of NR2A and NR2B (NMDARs) as well as GluA2 (AMPA) subunits expression is affected in the brain after status epilepticus induced at early developmental stage, and if possible changes can persist in adulthood.

## Materials and Methods

### *Animals*

The experiments were carried out in male rat pups of albino Wistar strain (breeding of the Institute of Physiology Academy of Sciences, Prague). Animals were housed under standard conditions (food and water ad libitum, 12:12 h light: dark cycle, temperature  $22\pm 1^\circ\text{C}$ ). The day of birth was counted as zero and all litters were reduced to ten pups. Rat pups were taken from their mothers just before testing. The experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic to be in agreement with the Animal Protection Law of the Czech Republic (fully compatible with European Community Council directives 86/609/EEC).

### *Drugs*

An antagonist of calcium permeable AMPA receptors IEM1460 (1-trimethylammonio-5-(1-adamantane-methylammonio)pentane dibromide) purchased from Tocris, UK, was freshly dissolved in physiological saline in a concentration of 2 mg/ml. The intraperitoneally administered doses 3, 10 and 20 mg/kg used in both experiments were selected on the basis of data of Gmiro et al. (2008) and Borowicz and Banach (2007). Control animals were injected with saline in a volume corresponding to the highest dose of IEM1460.

PTZ (Pentylentetrazol, Metrazol) (purchased from Sigma-Aldrich) is a GABA<sub>A</sub> receptor antagonist that is commonly used as a convulsant agent in animal models to investigate the mechanisms of seizures.

Ro-25-6981-maleate ( $\alpha$ R, $\beta$ S)- $\alpha$ -(4-Hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1 piperidinepropanol maleate (purchased from Tocris bioscience) is a potent and selective activity-dependent blocker of NMDA receptors containing the NR2B subunit. Ro 25-6981 was freshly dissolved in saline (1 mg/ml) before beginning of each experiment. The drug was administered intraperitoneally in doses of 1 or 3 mg/kg. Control animals received saline (1 ml/kg). The doses were chosen according to previously published literature (Boyce et al., 1999, Kosowski and Liljequist, 2004, Higgins et al., 2005 and Kos et al., 2011).

## *Effects of IEM 1460 on different types of seizures induced in immature rats : Experimental procedures*

### *Convulsions induced by pentylenetetrazol*

Three age groups of male Wistar albino rats at postnatal day (P) P12, P18 and P25 were used (8 animals per each group). Rats were pretreated with IEM1460 and 30 min later (100mg/kg) PTZ (Sigma) was injected subcutaneously. Animals were then observed for 30 min in isolation. The two types of seizures (minimal clonic and generalized tonic-clonic) observed during the experiment were marked individually on a time scale and their incidence, pattern and latencies were evaluated. Severity of seizures was quantified using a modified five-point Racine scale (Pohl and Mareš, 1987 and Mareš, 2013).

### *Electrophysiology*

#### *Surgery*

In experiments with cortical afterdischarges, four age groups of male Wistar albino rats were used: P12, P18, P25, and an additional group of P80 adult animals. Animals were anesthetized with ether and flat silver electrodes were implanted epidurally. Two stimulation electrodes were placed over the right sensorimotor cortex (AP +1 and -1; L 2.5 mm). Recording electrodes were placed over left sensorimotor area (AP 0; L 2.5), left parietal as well as left and right occipital areas. The ground and indifferent electrodes were placed over the cerebellum. The entire assembly was fixed to the skull with a fast-curing dental acrylic and animals were allowed to recover for at least 1 h. Body temperature of 12- and 18-day-old rats was maintained during recovery and whole registration period by means of a pad heated to 34 °C (i.e. normal temperature in the nest).

#### *Cortical epileptic afterdischarges*

Sensorimotor cortical area was stimulated by 15-s series of biphasic pulses of 1-ms duration and 8-Hz frequency (3-5 mA intensity). EEG was amplified, digitalized at a frequency of 1 kHz using CED Power1401 system (Cambridge Electronic Design) and the data were saved on a computer (see an example of ADs recording in Fig. 2.). Behavior of the animals was coded directly into the recording. Stimulation was repeated six times at 20-min intervals. The first stimulation was always a control one; 10 min after the end of an afterdischarge IEM1460 was injected Spike 2 software (Cambridge Electronic Design) was used for evaluation of afterdischarge duration.

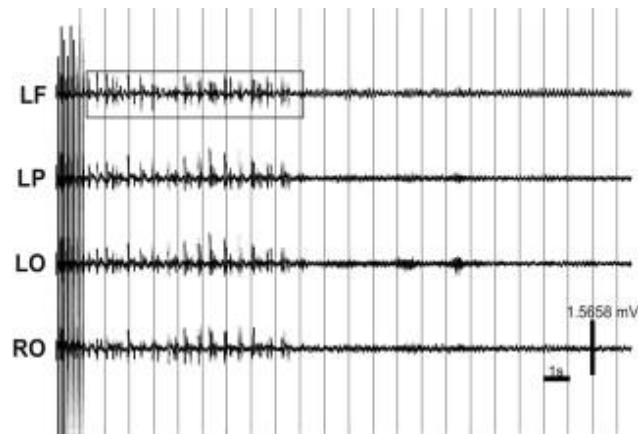


Fig. 2. Original recording of cortical epileptic afterdischarge (ADs) from P25 rat. Four traces from top to bottom: LF, left frontal; LP, left parietal; LO, left occipital; RO, right occipital leads always in a reference connection. Amplitude calibration 1.56 mV, time mark 1 s.

### *Statistics*

Incidence of minimal clonic and generalized tonic–clonic seizures in control and experimental animals was compared by means of Fisher exact test; latencies and severity of seizures were statistically evaluated using analysis of variance (ANOVA) with subsequent pairwise comparison by Holm–Sidak test (SigmaStat® SYSTAT).

Durations of afterdischarges measured off–line (absolute values) were statistically evaluated with ANOVA and subsequent Holm–Sidak test. Repeated measure (RM) ANOVA was used for comparison of the six afterdischarges in individual age and dose groups; one–way ANOVA for comparison of corresponding ADs in control and treated groups. Absolute values of durations were used for calculation. Relative duration of ADs (the first AD is always taken as 100%) is presented in figures for an easy comparison of various groups. Parametric ANOVA or nonparametric ANOVA on Range was used according to the initial testing of distribution of data (automatically performed by SigmaStat). Level of statistical significance was set at 5%.

## *Effects of a specific NR2B/NMDA antagonist Ro 25–6981 on cortical evoked potentials and epileptic afterdischarges in immature rats*

### *Animals and surgery*

Surgery was performed as described above, with an exception that only three age groups of male Wistar albino rats at postnatal day (P) P12, P18 and P25 were used for each experiment (8 animals per each group).

### *Electrophysiology*

#### *Stimulation and EEG recording*

For stimulation of sensorimotor cortex, isolated pulse stimulator (Model 2100 A–M Systems) with a constant current output was used. For recording of all electrophysiological signals, TDT Open Project Program (Tucker–Davis Technologies) was used. All obtained signals were amplified (RA16PA preamplifier and Pentusa Base Station, Tucker–Davis Technologies, FL, USA) and digitalized at 2 kHz for evoked potentials and at 1 kHz for epileptic afterdischarges.

#### *Single pulse evoked potentials*

Single 1–ms pulses with intensities increasing from 0.4 to 5.0 mA (0.4, 0.6, 0.8, 1.0, 1.4, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mA) were applied. First cycle of stimulations was a control one, then Ro 25–6981 or saline were injected and 20 min later the second stimulation series started. The software automatically averaged five subsequent responses (at each of 13 different current intensities used) and the amplitude was measured between peaks of N1 (first negative) and P2 (second positive) waves (Fig.3.a.). First positive wave could not be used because it was often distorted by stimulation artifact.

#### *Paired pulse evoked potentials*

The threshold stimulation intensity was found for each animal and double times this intensity was used to elicit paired responses with interpulse intervals from 50 to 1000 ms. Two cycles of stimulations were again performed: first before administration of the drug, and the second 20 min after Ro 25–6981 or saline administration. Amplitude of the first (A1) and second (A2) response was again measured between peaks of N1 (first negative) and P2 (second positive) waves (Fig.3.b.). The A2/A1 ratio was calculated for each interval.

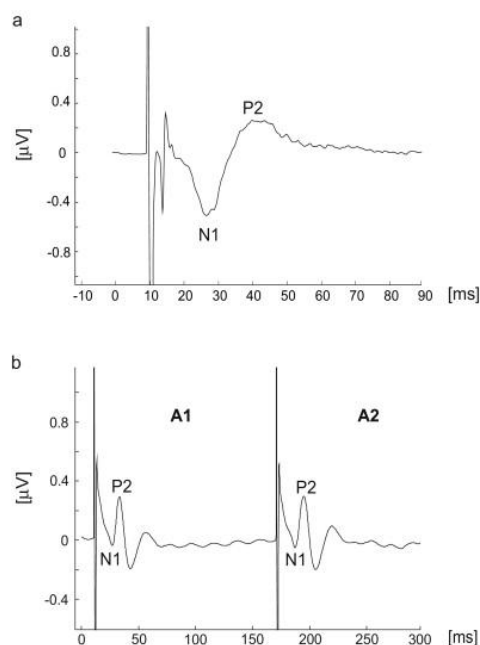


Fig.3. An example of single (a) and paired (b) pulse evoked potentials from P25 animal. N1, first negative wave. P2, second positive wave. N1–P2, measurement of amplitude difference. A1, amplitude of the first response. A2, amplitude of the second response. Y axis, amplitude (V). X axis, time (ms).

#### *Cortical afterdischarges (ADs)*

The same stimulation paradigm as for IEM1460 was used. Ro 25–6981 or saline were always injected 10 min after the first AD (i.e. after the predrug control stimulation). Additional series of 12–day–old rats with 30–min intervals were used to determine the duration of effects of Ro 25–6981. Duration of individual ADs was evaluated off–line by means of EEG Signal analyzer© software (Jakub Otahal 2004–2008 FGU ASCR, Prague, Czech Republic).

#### *Cortical afterdischarges elicited by stimulation with increasing intensity*

Cortical afterdischarges were elicited similarly as described above with an exception that stimulation was repeated at 10 min intervals and intensities increased stepwise from 0.2 to 15 mA (0.2, 0.4, 0.6, 0.8, 1, 1.4, 1.8, 2.2, 2.6, 3, 3.5, 4, 5, 6, 8, 10.5, 12, 15 mA). The Ro 25–6981 or saline were injected immediately after stimulation with 4 mA, i.e. after the 12th stimulation when the rats had a history of six ADs on the average. Changes in duration of ADs were again evaluated.

### *Statistics*

Statistical evaluation of all data was performed by means of One Way ANOVA or ANOVA on Ranks, followed by Holm–Sidak post hoc test. The SigmaStat® SYSTAT software tested the distribution of data and offered either parametric or nonparametric test. Additional statistical analysis was performed by means of Mann–Whitney Rank Sum Test. Statistics was calculated from original data (absolute values); in contrast, figures for ADs with six stimulations were prepared using relative durations (duration of the first, predrug AD was always taken as 100%). The level of significance was set at  $P < 0.05$ .

Analysis of developmental changes in expression of mRNA and protein level of GluA2 subunit of AMPAR (GRIA2A gene), as well as NR2A and NR2B subunits of NMRARs (GRIN2A and GRIN2B genes), in controls and in animals after pilocarpine–induced status epilepticus

### *Pilocarpine–induced status epilepticus*

Status epilepticus was elicited by administration of pilocarpine (40mg/kg i.p.) in 12–day–old animals pretreated with LiCl (3meq/kg ip.) 24h earlier, at P11. After 1.5h of seizures, paraldehyde in a dose of 0.07ml/kg was injected. After seizing has stopped, rat pups were returned to their mothers and their condition and weight were checked daily until the day of brain tissue isolation. The control animals received saline (1ml/kg) and an additional control group of animals received LiCl (3meq/kg ip.) at P11 and paraldehyde in a dose of 0.07ml/kg at P12.

### *TaqMan quantitative RT–PCR assays*

Five to six male rats per each age: P12, P15, P18, P25, P72; and treatment group (controls, LiCl/Para controls and SE animals) were killed by anesthesia overdose. The brains were immediately removed, pre–cooled in the ice–cold saline and its different regions i.e. cortex frontal (CXFR), cortex occipital (CXOC), parietal cortex (CXPAR), dorsal hippocampus (HD), ventral hippocampus (HV) and thalamus (TH) were dissected out on ice, snap–frozen in liquid nitrogen and total RNA was immediately isolated. Total RNA was isolated from all brain samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich, St. Louis, MO) according to the manufacturer’s instructions and RNA content was determined using a NanoDrop Spectrophotometer (NanoDrop Products, Wilmington, DE, USA). An aliquot (1µg) of total RNA from each sample was used for reverse transcription. Synthesis of the cDNA was performed on the day of RNA isolation according to the manufacturer’s protocol using High

Capacity Reverse Transcription Kit (Life Technologies) with 40 U RNaseOUT Inhibitor (Invitrogen La Jolla, CA), the cDNA was then stored at  $-20^{\circ}\text{C}$  until further analysis. The qPCR was performed in the ABI 7000 Sequence Detection System (Life Technologies). All primers used were developed as TaqMan Gene Expression Assays obtained from Applied Biosystems. All primers used were developed as TaqMan Gene Expression Assays obtained from Life Technologies. Target genes: GRIN2A (Rn00561341\_m1); GRIN2B (Rn00561352\_m1) and GRIA2 (Rn00568514\_m1) labeled with FAM/MBG were used in conjunction with Eukaryotic 18S rRNA Endogenous Control (4319413E) 18SrRNA (VIC/MGB Probe, Primer Limited) as a normalization factor. RT-PCR reactions were performed in 30 $\mu\text{l}$  aliquots on a 96-well optical reaction plate containing TaqMan Gene Expression Master Mix (Life Technologies). TaqMan probes and cDNA were processed using the following thermal cycles parameters: The amplification conditions consisted of uracil-DNA glycosylase (UDG) incubation at  $50^{\circ}\text{C}$  for 2 min and initial DNA polymerase enzyme activation at  $95^{\circ}\text{C}$  for 10 min, followed by 50 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 s and annealing/extension at  $60^{\circ}\text{C}$  for 1 min.

Gene-specific calibration curves were generated from serial dilutions of standard cDNA and the mRNA levels of all genes of interest were normalized to the reference gene (18SrRNA). In the saline control, LiCl and paraldehyde control, and experimental (after Status Epilepticus) groups all genes were quantified at postnatal days (P) 12 (results shown only for GRIN2A and GRIN2B and GRIA2A of control animals) 15, 18, 25 and 72 (young adult) animals.

### *Statistics*

Statistical analyses of differences in expression levels of genes GRIN2A, GRIN2B and GRIA2A were performed. Differences in the levels of expression of selected genes between age groups, between different brain regions and between corresponding treatments groups were calculated by means of One Way ANOVA analysis followed by Fisher-LSD test. The level of significance was set at  $P < 0.05$ . (SigmaStat® SYSTAT).

### *Western blotting (GluA2)*

Per each age (P12, P15, P18, P25, P72) and treatment group (controls, LiCl/Para controls and SE animals), male rats ( $n=3$ ) were killed by anesthesia overdose. The brains were immediately removed, pre-cooled in the ice-cold saline and its different regions i.e. frontal cortex (CXFR), occipital cortex (CXOC), parietal cortex (CXPAN), hippocampus dorsal (HD), hippocampus ventral (HV) and thalamus (TH) were dissected out on ice, snap-frozen in liquid nitrogen and

stored ( $-86^{\circ}\text{C}$ ) until the day of protein isolation. The tissue was homogenized in a RIPA Buffer (Sigma–Aldrich) with Protease Inhibitor Coctail (Sigma–Aldrich). The total protein concentration in each sample (triple measurement per each sample) was determined according to the manufacturer’s protocol using QuantiPro BCA Assay Kit (Sigma–Aldrich). The protein samples were mixed (1:1) with 2x Laemmli Sample Buffer (BIO–RAD) to reach the final concentration of  $1.66\mu\text{g}/\mu\text{l}$ . Samples were heated for 10 min at  $90^{\circ}\text{C}$  and loaded onto pre–cast Mini–PROTEAN TGX–Stain–Free 10% SDS–polyacrylamide gels (BIO–RAD) ( $25\mu\text{g}$  of total protein per line) and separated eletrophoretically. To determine the total protein content in each loaded sample, the stain free gels after electrophoresis were activated by exposure to UV light and imaged using ChemiDoc MP Imaging System (BIO–RAD). Total protein in individual lanes was quantified using Image Lab 5.1. (Gilda and Gomez, 2013) and used as an internal control for normalization. To transfer proteins onto nitrocellulose membrane, the Trans–Blot Turbo RTA transfer Kit (BIO–RAD) and Trans–Blot Turbo Blotting System (BIO–RAD) were used according to the manufacturer’s protocol. After protein transfer, membranes were imaged for Stain–Free staining and total protein was quantified using Image Lab 5.1. software. The blotting membrane was blocked with 3% non–fat milk for 2h and incubated overnight at  $4^{\circ}\text{C}$  with rabbit anti–Gria2 (Anti–AMPA Receptor 2 (GluA2) (extracellular) AGC–005, Alomone Labs Ltd, Jerusalem, Israel) (1:500). The proteins were detected with peroxidase conjugated monoclonal Mouse anti–rabbit secondary antibody (Jacson Immuno Research Laboratory) (1:10000), visualized with the chemiluminescence reagents Clarity Western ECL Substrate (BIO–RAD), captured using ChemiDoc MP Imaging System (BIO–RAD) and quantified (analyses of band intensity normalized to total protein content of corresponding sample in stain free gel ) using Image Lab 5.1. software. To enable comparison of the results between individual blots, an extra normalization factor was included. An additional sample, (the same in each single gel), was used a cross–reference between blots.

### *Statistics*

Differences in the levels of expression of selected GluA2 protein between age groups, between different brain regions and between corresponding treatment groups were calculated by means of One Way ANOVA analysis followed by Fisher–LSD test. The level of significance was set at  $P<0.05$ . (SigmaStat® SYSTAT).

## Results

### *Influence of a specific antagonist of Ca<sup>2+</sup>-permeable AMPARs, IEM1460 on different types of seizures in developing rats.*

#### *Convulsions induced by pentylenetetrazol*

Incidence of minimal seizures was not significantly affected in P12 and P25. The 20-mg/kg dose of IEM1460 tended to increase the incidence of these seizures in P12 rats where minimal seizures are not common under control conditions (Fig.4.). The minimal seizures were found only in four out of eight P25 control rats, the result probably due to an early appearance of generalized seizures. The incidence of minimal seizures tended to decrease in P18 rats but statistically significant difference was found only after the 10-mg/kg dose (Fig.4.). Latencies of minimal seizures were significantly prolonged by the 20-mg/kg dose of IEM1460 in all three age groups (Fig.5.). Generalized tonic-clonic seizures were observed in all control rats in all three age groups. There were no significant changes in the youngest group. The 20-mg/kg dose decreased the incidence of GTCS in P18 and the 10- and 20-mg/kg doses exhibited the same effect in P25 animals (Fig.4.). Latencies of GTCS were significantly prolonged by the 3- and 20-mg/kg doses in P12 rats. The latencies remained unchanged by IEM1460 in P18 animals; the P25 rats exhibited dose-dependent increase of these latencies with a significant change after the 20-mg/kg dose (Fig.5.). Seizure severity can be taken mainly as an expression of presence or absence of GTCS (Fig.4.). In agreement with decreased incidence of these seizures after the 20-mg/kg dose in P18 animals and after the two higher doses in P25 rats, seizure severity was significantly decreased by IEM1460 in these age groups.

## PTZ-induced seizures and IEM1460

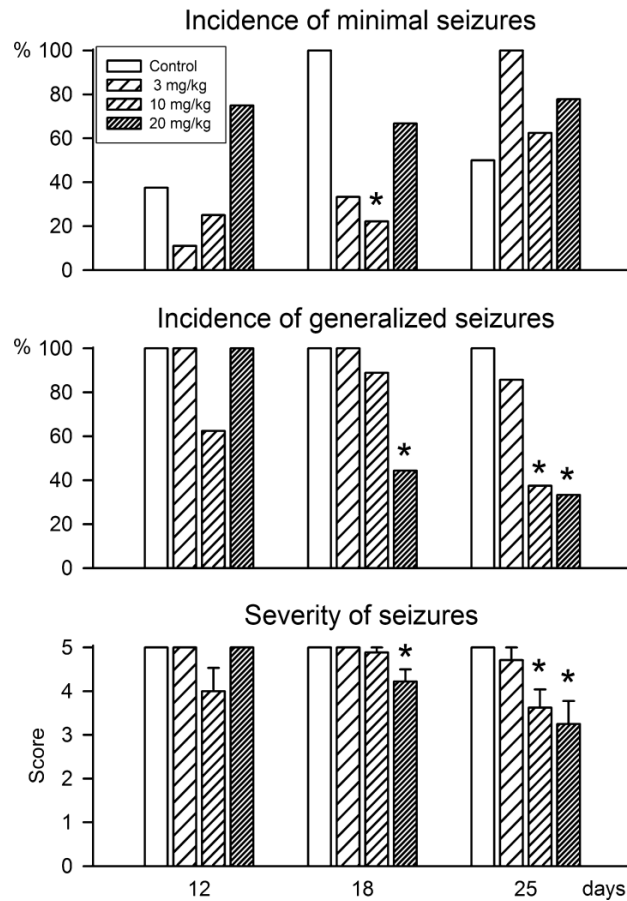


Fig.4. Effects of IEM1460 on PTZ-induced seizures in immature rats. From top to bottom: incidence of minimal clonic seizures; incidence of generalized tonic-clonic seizures; severity of seizures expressed by a score. Abscissae – three age groups (12-, 18- and 25-day-old rats); ordinates – for incidence: percentage of animals exhibiting seizures; for seizure severity: five-point scale (Pohl and Mareš, 1987). For doses of IEM1460, see inset. \*– denote significant difference from appropriate control groups.

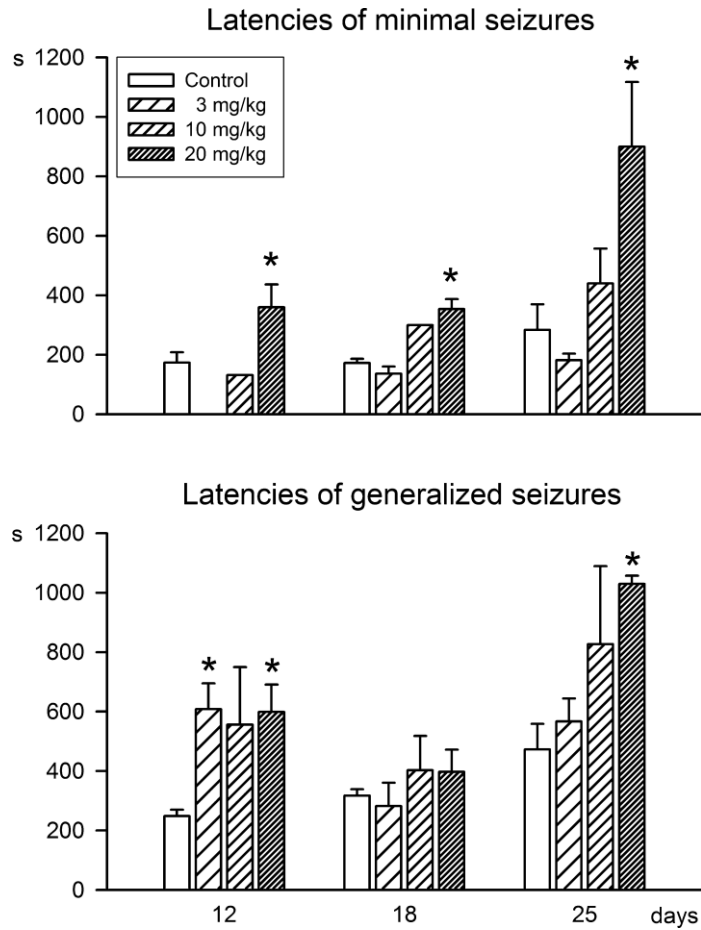


Fig.5. Effect of IEM1460 on latencies of minimal clonic seizures (upper part) and generalized tonic-clonic seizures (lower part). Abscissae – three age groups (P12, P15, P25); ordinates – for doses of IEM1460 see inset, t– latencies in seconds. \*– denote significant difference from appropriate control groups.

## Cortical afterdischarges

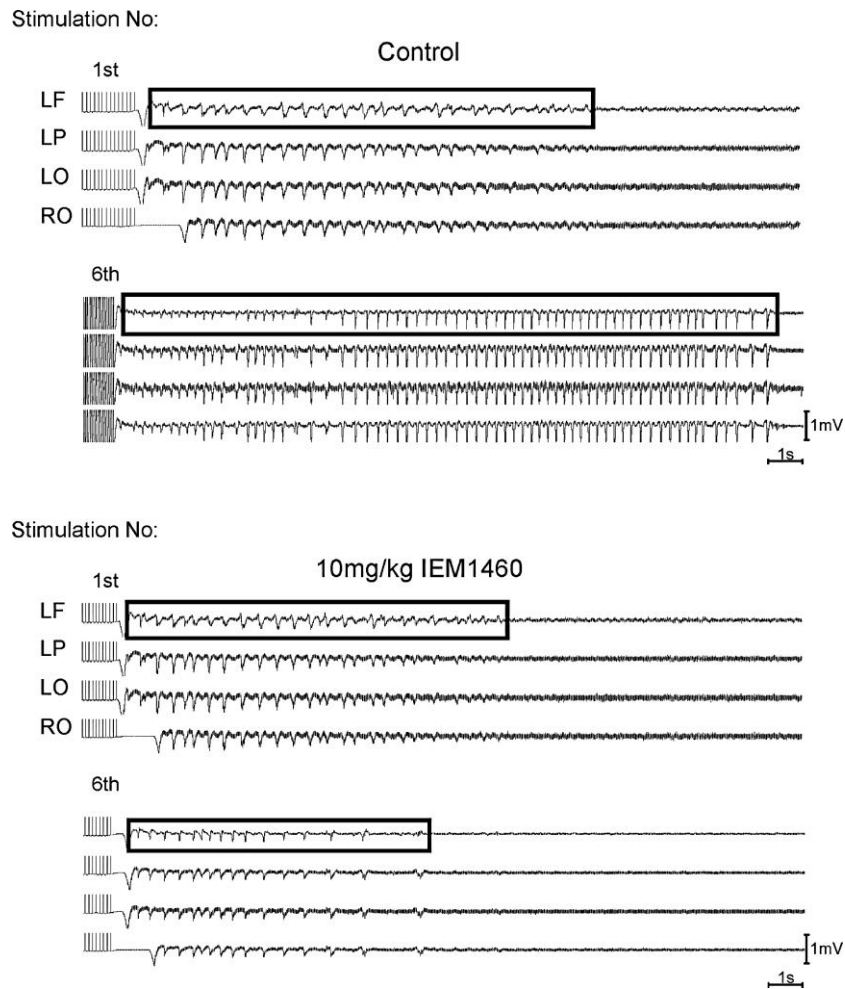


Fig.6. Original EEG recordings of cortical afterdischarges from P12 rats. Afterdischarges in the first lead are in the black rectangles. Upper part – control animal, lower part – rat injected with 10 mg/kg of IEM1460 immediately after the first AD. Each part presents the first (predrug) and the sixth AD. Individual leads: LF – left frontal, LP – left parietal, LO – left occipital, RO – right occipital cortical region in reference connection. Time mark 1 s, amplitude calibration 1 mV.

The control group of the youngest animals exhibited a marked progressive prolongation of ADs with repeated stimulations. This effect was untouched by the 3–mg/kg dose of IEM1460, slightly delayed by the 20–mg/kg dose and completely blocked by the middle (10–mg/kg) dose (Fig. 6. and Fig. 7.). The prolongation of ADs was much less expressed in control P18 rats and the level of statistical significance was not reached. In contrast, the 10–mg/kg dose of IEM1460 significantly prolonged ADs in this age group, especially the third to the sixth ADs were significantly longer than the first, predrug AD (Fig. 7.). The control P25 animals exhibited only the fifth AD longer than the first AD, effects of IEM1460 were similar but less expressed than in P18 rats. Again, the changes induced by the 10–mg/kg dose were more pronounced than effects of the other two doses. IEM1460 administration at either dose did not have any significant effect on the duration of cortical ADs in adult, P80 animals. No changes of motor phenomena during stimulation or during AD were seen in any age and dose group.

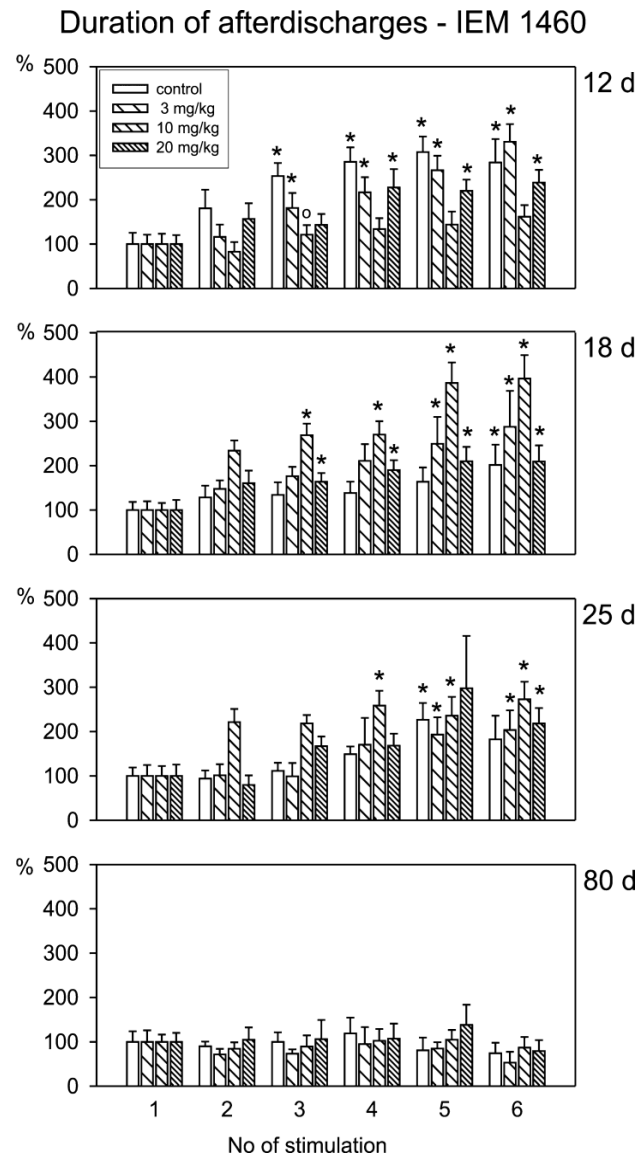


Fig.7. Effects of IEM1460 on relative duration of afterdischarges. Duration of the first, predrug AD is always taken as 100%. From top to bottom: rats 12, 18, 25 and 80 days old. Abscissae – six consecutive ADs; ordinates – duration in seconds. Individual doses – see inset. Asterisks denote significant differences in comparison with the appropriate first AD, open circles denote significant difference in comparison with corresponding control.

***Influence of Ro 25–6981, a specific antagonist of NMDARs containing NR2B subunit on evoked potentials and cortical afterdischarges in developing rats.***

*Single pulse evoked potentials*

All three age groups of control rats exhibited an increase in amplitude of responses with increasing stimulation intensity. The Ro 25–6981 at the dose of 3 mg/kg caused a significant decrease of N1–P2 amplitude at higher stimulation intensities in comparison to the controls in all three age groups. Effects in P12 animals where amplitude of responses did not augment with increasing intensities of stimuli were more marked than in older rats. It was demonstrated as a reduction of steepness of input–output curves (I/O), i.e. curves expressing changes of amplitude with intensity of stimulation (Fig. 8.). The effects of the 3–mg/kg dose in P12 animals were prominent at intensities of stimulation from 2.5 to 5.0 mA and in P18 animals from 3.5 to 5.0 mA. In P25 animals, similar effect of the 3–mg/kg dose was observed only at 3.0 and 5.0 mA stimulation intensities. Administration of the 1–mg/kg dose in P12 animals had significant effect on N1–P2 amplitude only at 4.5 mA stimulation intensity. In P18 animals, the 1–mg/kg dose caused that N1–P2 amplitude tended to increase, but the level of significance was not reached. Surprisingly, the 1–mg/kg dose of Ro 25–6981 caused a marked decrease of N1–P2 amplitude at 3.0–5.0–mA stimulation intensities in P25 animals in comparison to control group. Differences between the two doses were significant in all age groups.

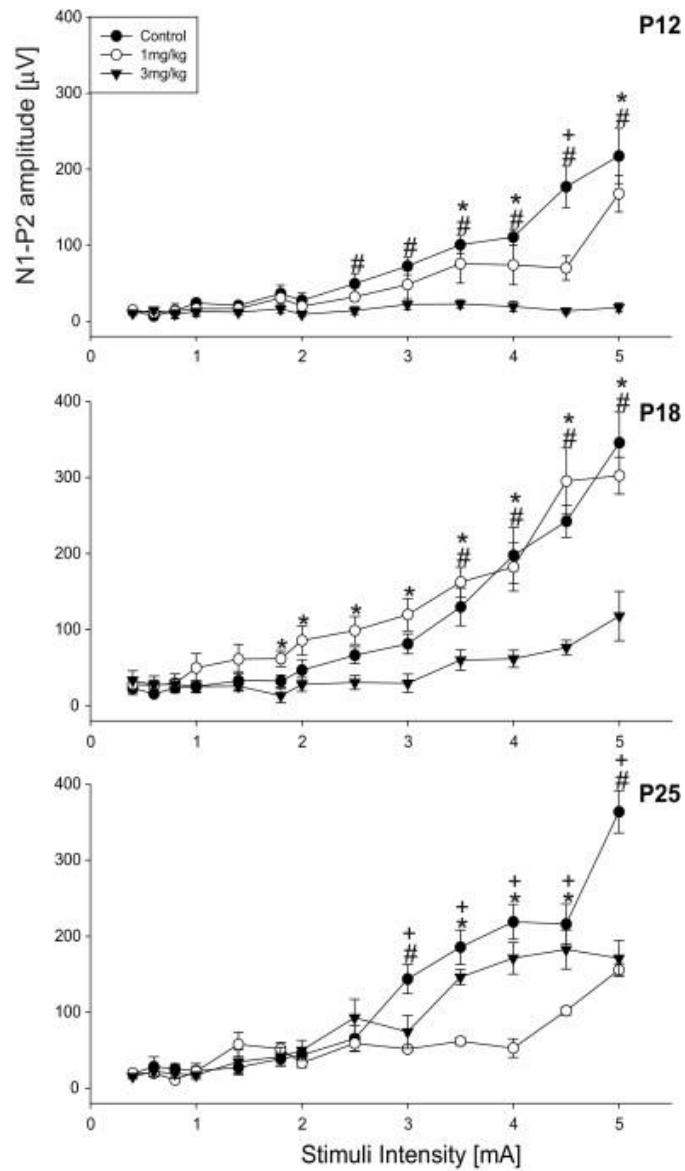


Fig. 8. Effect of Ro 25–6981 on input–output (I/O) curves of single pulse evoked potentials in P12, P18 and P25 (from top to bottom). I/O curves represent changes of (N1–P2) amplitude expressed as a mean  $\pm$  S.E.M. with increasing intensity of stimulation. X axis, intensity of stimulation pulses in mA; Y axis, amplitude in  $\mu\text{V}$ . Significant differences are marked as follows: (+) 1–mg/kg dose vs. control; (#) 3–mg/kg dose vs. control; (\*) 1–mg/kg dose vs. 3–mg/kg dose group.

### *Paired pulse evoked potentials*

Administration of Ro 25–6981 in either dose did not have any significant effect on paired pulse potentiation or depression (data not shown).

### *Cortical afterdischarges elicited with constant intensity of stimulation*

The duration of ADs elicited with 30–min intervals in control P12 animals significantly increased with repeated stimulation in comparison with the first (control) ADs (Fig. 9.). Administration of 1 mg/kg of Ro 25–6981 caused shortening of the following ADs, but it was not significant in comparison to the first ADs. The 3–mg/kg dose had similar effect, ADs shortening was more pronounced than after 1–mg/kg dose. Likewise, to the 1–mg/kg dose, ADs shortening with repeated stimulations was not statistically significant when compared to control ADs. On the other hand, the effects of 1–mg/kg and 3–mg/kg doses of Ro 25–6981 were especially marked after third ADs, i.e. 50 min after drug administration. After both doses of the drug, ADs duration was significantly shorter in comparison to corresponding control ADs only at that time point. Despite of lack of statistical significance in comparison to corresponding control group, the 3–mg/kg dose of Ro 25–6981 administration exhibited effect against ADs prolongation with repeated stimulation. This effect was observed up to 110 min after administration of the drug (Fig.9.).

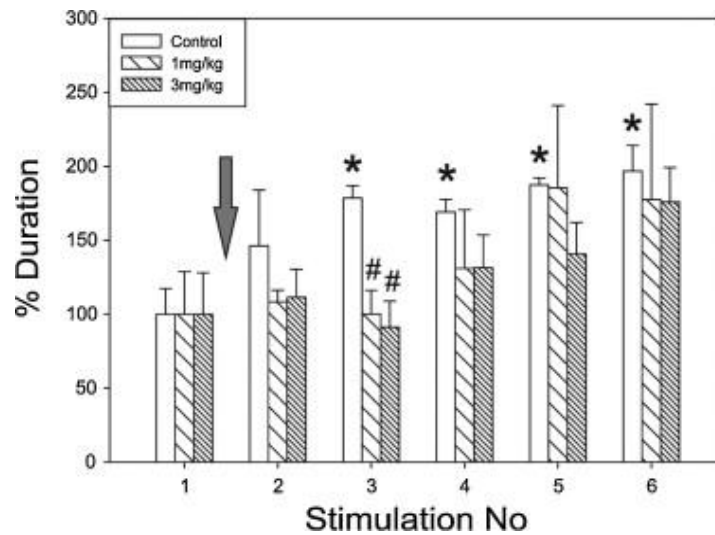


Fig.9. Effects of Ro 25–9681 on relative duration of ADs (mean  $\pm$  S.E.M.) in P12 animals. Abscissa: number of stimulation series. Ordinate, relative duration compared to the first AD taken as 100%. Stimulation series were repeated with 30–min intervals. Arrow indicates the time when the Ro 25–6981 was injected (10 min after first stimulation). (\*) indicates significant difference in comparison to the first (control) stimulation; (#) indicates significant difference in comparison to corresponding control ADs.

As for ADs elicited with 20–min intervals, control rats at P12 exhibited marked progressive increase of ADs duration with repeated stimulations (Fig. 10.). This effect was not so marked in the two older groups. Administration of Ro 25–6981 at the dose of 1 mg/kg did not affect ADs duration in the P12 animals. Significant prolongation of ADs after 5th and 6th stimulation was the same as in the control group. In contrast, the 3–mg/kg dose significantly affected ADs duration, progressive prolongation of post–injection ADs was blocked (Fig. 10). Duration of ADs in P18 and P25 rats did not exhibit any significant changes after either dose of Ro 25–6981.

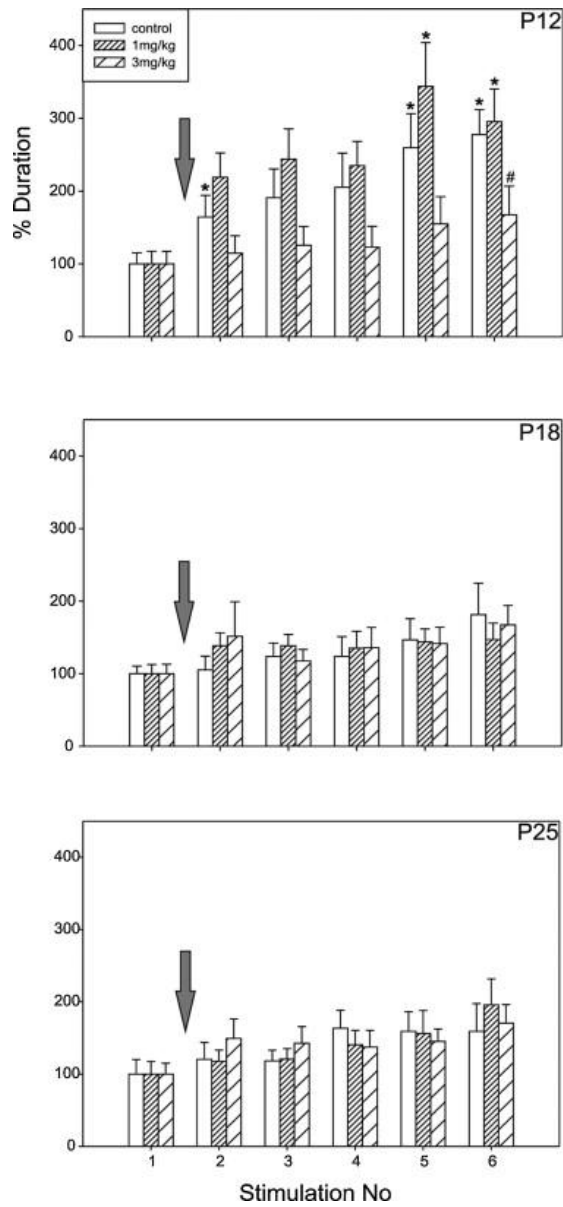


Fig.10. Effects of Ro 25-9681 administration on relative duration of ADs (mean  $\pm$  S.E.M.) in all three age groups of animals tested (from top to bottom P12, P18 and P25). Stimulation was repeated with 20-min intervals, the first AD was again taken as 100%. Details as in Fig.9.

*Afterdischarges elicited by increasing intensities of stimulation*

All age groups of control animals exhibited increasing duration of ADs with increasing intensities of stimulation. This effect was more pronounced in P12 than in older animals. If Ro 25-6981 was injected after stimulation with 4.0-mA current intensity the ADs duration ceased to increase (Fig.11.). The difference from controls was particularly marked after the lower dose of the drug (Fig.11.). In P18, administration of the 1-mg/kg dose resulted in a tendency to ADs shortening but the level of statistical significance was not reached. The same tendency was observed in P25 but after the 3-mg/kg dose, the increase of ADs duration was not as steep as in controls.

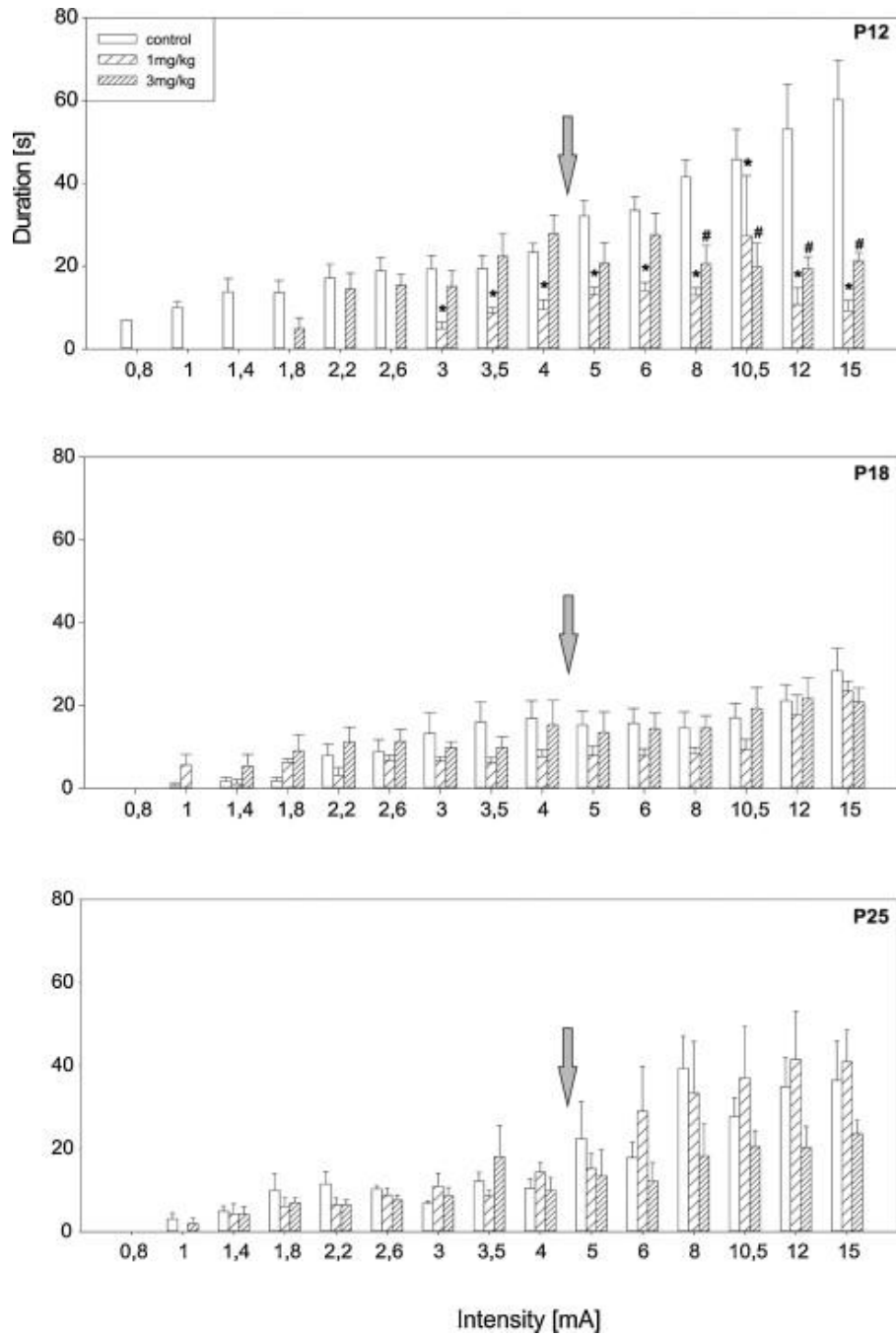


Fig.11. Effects of Ro-25-6981 on absolute duration of ADs (mean  $\pm$  S.E.M.) elicited by stimulation with increasing current intensities (mA) in animals at P12, P18, and P25. Stimulation was repeated at 10-min intervals. Abscissa, intensity of stimulation current; ordinate, duration of ADs in seconds. Significant differences in ADs duration were marked by: (\*) between 1.0-mg/kg dose and control animals; (#) between 3.0-mg/kg group and control animals. Arrows indicate the time when the Ro 25-6981 was injected (immediately after stimulation with 4 mA intensity).

***Changes in developmental expression level of GRIA2A gene encoding GluA2 subunit of AMPARs in the brain of control animals and animals after LiCl/Pilo-SE induced at P12.***

The overall statistical analysis shows that the LiCl/Pilo-SE elicited at P12 of LiCl/Para administration at early stages of postnatal development had a significant influence on developmental expression profile of GRIA2A mRNA in all examined brain areas considered in this study (for detailed results of statistical analysis including F and P values, see Tables 2–7 below). As revealed by post hoc LSD-Fisher test, in animals subjected to LiCl/Pilo-SE at P12 there was a significant increase in GRIA2A mRNA level detected later in development. It was especially evident in CXOC (see Fig.14 and Table 4.); CXFR (see Fig.12 and Table 2.) and CXPAR (see Fig.13 and Table 3.). A marked increase in GRIA2A expression was also detected in TH at P25 (Fig.17 and Table 7.). In addition, early life LiCl/Pilo-SE caused that the GRIA2A mRNA level in CXPAR (see Fig.13 and Table 3.) and CXOC (see Fig.14 and Table 4.) was significantly decreased at P25 in relation to the control expression. There was also marked decrease in GRIA2A mRNA expression level following LiCl/Pilo-SE detected in the TH at P18 and P72. Furthermore, LiCl/Para administration at P11/P12 caused the GRIA2A mRNA expression level to be significantly increased in CXFR at P15 and P18, in CXPAR at P72, in CXOC at P18, in HD at P72 (see Fig.15 and Table 5.) and in the TH at P18 and P72. The marked decrease in GRIA2A mRNA expression after LiCl/Para injection was evident only in CXOC at P25. There were no significant changes in GRIA2A mRNA expression level detected in HV (see Fig.16 and Table 6.).

## Frontal cortex

The overall analysis revealed that the level of GRIA2A gene expression in CXFR (Fig.12a) of control animals, do not change significantly during development. On the other hand, there were marked changes detected in animals that were injected with LiCl/Para at P11/P12. Subsequent post hoc analysis showed (for details see Table 2), that the most marked decrease in the level of GRIA2A mRNA expression after LiCl/Para can be detected in P18 animals. Statistical analysis showed also that there was a main effect of SE on developmental GRIA2A mRNA expression levels in CXFR. Following tests revealed that there was a clear increase of GRIA2A mRNA level at day 6th after SE (P18), especially in relation to its level in animals 3 or 13 days after SE (P15 and P25 respectively) (Fig.12a). Analysis revealed also that injection of either LiCl/Para or Pilo had no main effect of LiCl/Para or SE on GRIA2A expression in P15 animals (3 days after LiCl/Para or Pilo) (Fig.12b). However, the elicitation of SE at P12, had strong influence on GRIA2A mRNA expression level 6 days later, i.e. at P18 and as demonstrated by subsequent post hoc test, the GRIA2A mRNA level in these animals was significantly higher when compared to P18 saline controls, or P18 LiCl/Para controls animals. There was no effect of LiCl/Para or SE on GRIA2A expression in animals 13 days after injection of LiCl/Para or Pilo (P25). On the other hand in P72 animals, (60 days after SE) the GRIA2A mRNA expression level was significantly higher in comparison to corresponding P72 control animals (Fig.12b).

**Table 2. Developmental changes in expression level of GRIA2A mRNA in the cortex frontal of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.12.a	FRONTAL CORTEX												
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 25)=0.286; P=0.884]					[F (3, 19)=5.951; P=0.005]				[F (3, 22 =6.880; P=0.002]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12		-	-	-	-								
P15	-		-	-	-		-	-	-		P<0.001 +	-	-
P18	-	-		-	-	P=0.001 ■		P=0.005 ■	-	-		-	-
P25	-	-	-		-	-	-		-	-	P<0.001 ▲		-
P72	-	-	-	-		-	-	-		-	P=0.013 ○	-	
Fig.12.b	FRONTAL CORTEX												
Age	P15			P18			P25		P72				
F values	[F (2, 15)=2.889; =0.087]			[F (2, 15)=46.084; P<0.001]			[F (2, 17)=0.615; P=0.552]		[F (2, 15)=6.293; P=0.010]				
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE			
Control	-	-	-	P<0.001 *	-	-	-	-	-	P=0.003 *			
LiCl/Para control		-		P<0.001 †		-		-		-			

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (†)- significant versus status epilepticus.

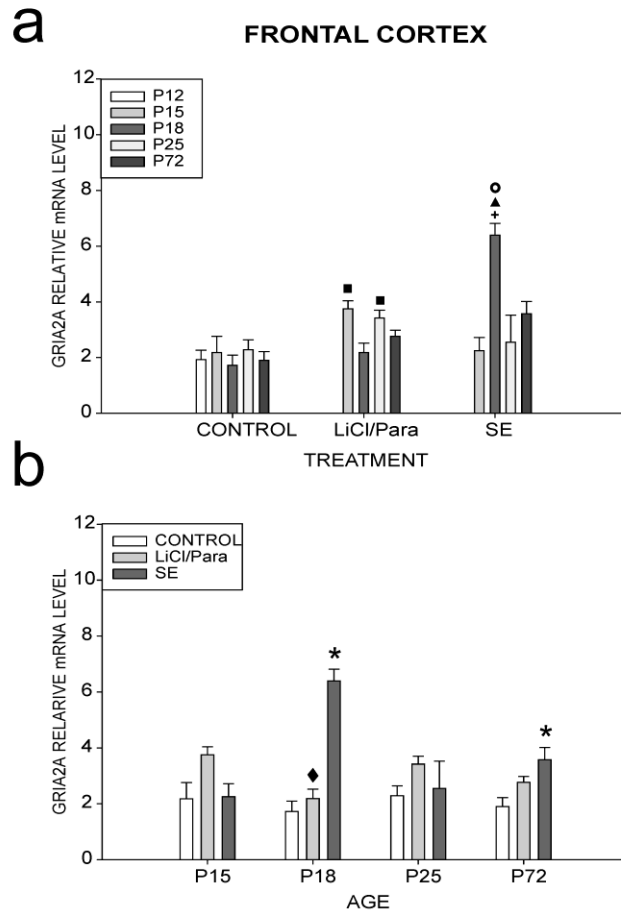


Fig.12. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the frontal cortex of control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #– significant versus P12, +– significant versus P15, ■– significant versus P18, ▲–significant versus P25, ○–significant versus P72. Part b) \*– significant versus group of saline controls; ◆– significant versus group of animals after status epilepticus.

## Parietal cortex

The overall statistical analysis showed that the GRIA2A mRNA expression level in the CXPAR of control animals is changing significantly during development. As revealed by subsequent post hoc test, it was markedly elevated at P18 in comparison to P12 or P15 (for details see Fig.13 and Table 3). The highest level of GRIA2A mRNA level was detected at P25, but later in development, at P72 it was slightly decreased (Fig.13a). There was no significant effect of LiCl/Para on GRIA2A mRNA expression level in the CXPAR of control animals. The LiCl/Pilo-SE induced at P12 also had a main effect of on developmental expression of GRIA2A mRNA in CXPAR. As revealed by following tests, its level detected at P18 was significantly higher than at any other age. Elicitation of LiCl/Pilo-SE at P12 had a main effect on GRIA2A expression 6 and 13 days after the insult, i.e. at P18 and P25. At P18, it was markedly higher when compared to its levels in saline and groups (Fig.13b), and at P25, it was significantly decreased in relation to LiCl/Para controls (Fig.13b). Additionally, a main effect of LiCl/Para administered at P11/P12 on GRIA2A expression in CXPAR was evident much later in development. A noticeable increase in GRIA2A mRNA was detected 60-days after the compounds were administered, and its level was significantly higher than in controls or LiCl/Pilo-SE group of animals (Fig. 13b).

**Table 3. Developmental changes in expression level of GRIA2A mRNA in the parietal cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.13.a	PARIETAL CORTEX												
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 26) = 7.431; P<0.001]					[F (3, 21) = 2.685; P=0.073]				[F (3, 20) = 8.149; P<0.001]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12		-	P=0.007 #	P<0.001 #	-								
P15	-		P<0.001 +	P<0.001 +	P=0.034 +		-	-	-		P<0.001 +	-	-
P18	-	-		-	-	-	-	-	-	-		-	-
P25	-	-	-	-	-	-	-	-	-	-	P<0.001 ▲		-
P72	-	-	-	P=0.027 ○		-	-	-		-	P=0.006 ○	-	
Fig.13.b	PARIETAL CORTEX												
Age	P15			P18			P25			P72			
F values	[F (2, 15) = 2.943; P=0.084]			[F (2, 16) = 22.891; P<0.001]			[F (2, 15) = 6.833; P=0.008]			[F (2, 15) = 4.886; P=0.023]			
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE			
Control	-	-	-	P<0.001 *	-	-	-	P=0.015 *	P=0.009 *	-			
LiCl/Para control		-		P<0.001 †			P=0.003 †			P=0.042 †			

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)- significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (†)- significant versus status epilepticus.

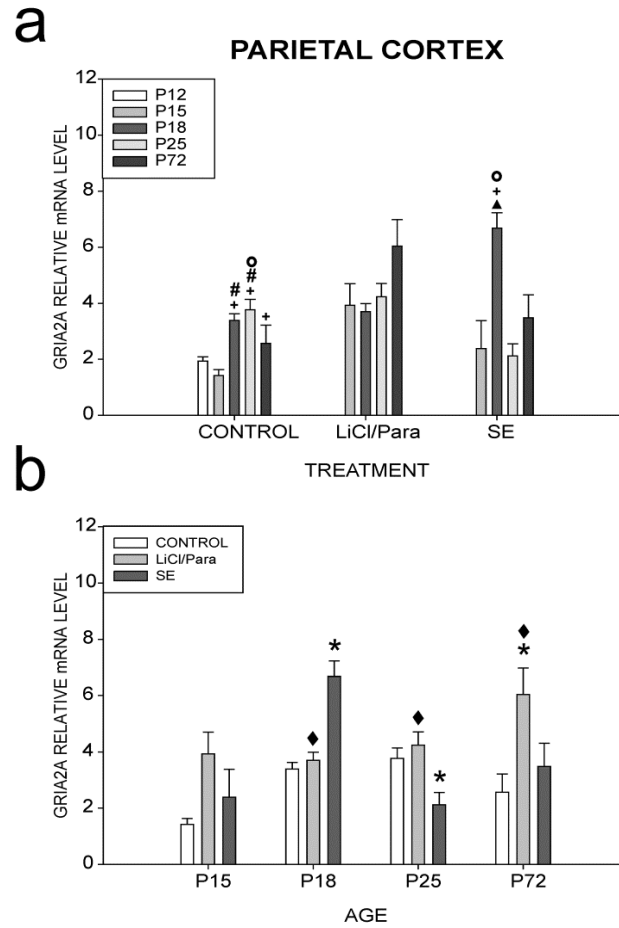


Fig.13. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the parietal cortex of control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #– significant versus P12, +– significant versus P15, ■– significant versus P18, ▲–significant versus P25, ○–significant versus P72. Part b) \*– significant versus group of saline controls; ◆– significant versus group of animals after status epilepticus.

## Occipital cortex

Analyses demonstrated that the level of GRIA2A mRNA expression in the CXOC significantly changes during development (for details see Table 4). The post hoc test revealed that it was markedly increased at P18, reaching the peak expression at P25, but slightly decreases towards adulthood (Fig.14a). The LiCl/Para administration at P11/P12 had also main effect on GRIA2A developmental expression in CXOC. As depicted in Fig.14a it was particularly elevated at P18 and it was significantly higher than at any other age of tested animals. Further post hoc analysis revealed that the GRIA2A level after LiCl/Para was also increased at P72, and it was significantly higher in relation to its level observed at P12 or P25. Elicitation of LiCl/Pilo-SE at P12 or administration of the LiCl/Para (P11/P12) caused GRIA2A mRNA expression level to be extremely increased in at P18 (Fig. 14b). At P25 on the other hand, the changes induced by LiCl/Para or LiCl/Pilo-SE administration early in life were opposite, and it caused significant decrease of GRIA2A mRNA expression (Fig. 14b).

**Table 4. Developmental changes in expression level of GRIA2A mRNA in the occipital cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.14.a	OCCIPITAL CORTEX												
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 30) = 7.012; P<0.001]					[F (3, 20) = 31.008; P<0.001]				[F (3, 21) = 56.314; P<0.001]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12		-	-	P=0.001 #	-								
P15	-		P=0.018 +	P<0.001 +			P<0.001 +	-	P=0.019 +		P<0.001 +	-	-
P18	-	-		-	-	-		-	-	-		-	-
P25	-	-	-		-	-	P<0.001 ▲		P=0.006 ▲	-	P<0.001 ▲		-
P72	-	-	-	P=0.001 ○		-	P<0.001 ○	-		-	P<0.001 ○	-	
Fig.14.b	OCCIPITAL CORTEX												
Age	P15		P18		P25		P72						
F values	[F (2, 15) = 2.579; P=0.109]		[F (2, 15) = 66.638; P<0.001]		[F (2, 16) = 8.594; P=0.003]		[F (2, 15) = 2.689; P=0.098]						
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE					
Control	-	-	P<0.001 *	P<0.001 *	P<0.001 *	P = 0.014 *	-	-					
LiCl/Para control		-		P=0.004 ♦		-		-					

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)- significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (♦)- significant versus status epilepticus.

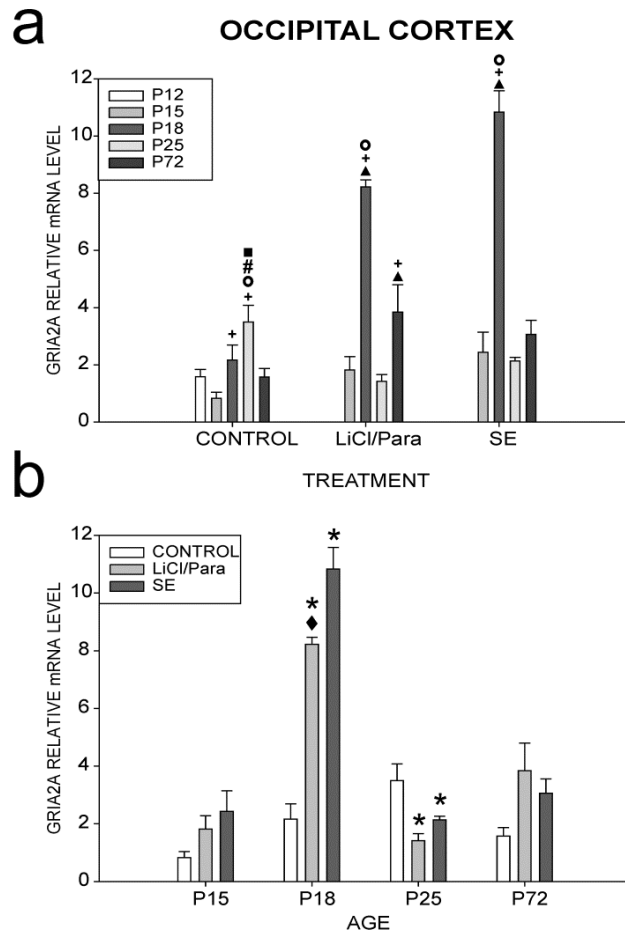


Fig.14. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the occipital cortex of control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #- significant versus P12, +- significant versus P15, ■- significant versus P18, ▲-significant versus P25, ○-significant versus P72. Part b) \*- significant versus group of saline controls; ◆- significant versus group of animals after status epilepticus.

## Dorsal hippocampus

There were no statistically significant changes of the GRIA2A mRNA expression levels in HD of control animals (for details see Table 5). Interestingly, the LiCl/Para and LiCl/Pilo-SE induced at P12 had its main significant effect early in adulthood, at P72, but no earlier during development (Fig.15). At P72, the GRIA2A mRNA level in the HD of animals subjected to LiCl/Pilo-SE was significantly higher than at any other tested stage of development (Fig. 15a) and in animals after LiCl/Para it was higher than its level detected in HD of saline controls (Fig.15b).

**Table 5. Developmental changes in expression level of GRIA2A mRNA in the dorsal hippocampus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.15.a													
DORSAL HIPPOCAMPUS													
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 24) = 0.945; P=0.455]					[F (3, 20) = 27.282; P<0.001]				[F (3, 21) = 3.543; P=0.032]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12		-	-	-	-								
P15	-		-	-			-	-	P<0.001 +		-	-	P=0.019+
P18	-	-		-	-	-		-	P<0.001 ■	-		-	P=0.008 ■
P25	-	-	-		-	-	-		P<0.001 ▲	-	-		P=0.0023 ▲
P72	-	-	-	-		-	-	-		-	-	-	
Fig.15.b													
DORSAL HIPPOCAMPUS													
Age	P15		P18		P25		P72						
F values	[F (2, 14) = 0.117; P=0.890]		[F (2, 15) = 0.508; P=0.611]		[F (2, 16) =1.922; P=0,179]		[F (2, 15) = 5.831; P=0.013]						
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE					
Control	-	-	-	-	-	-	P=0,004 *	-					
LiCl/Para control		-		-		-		-					

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)- significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

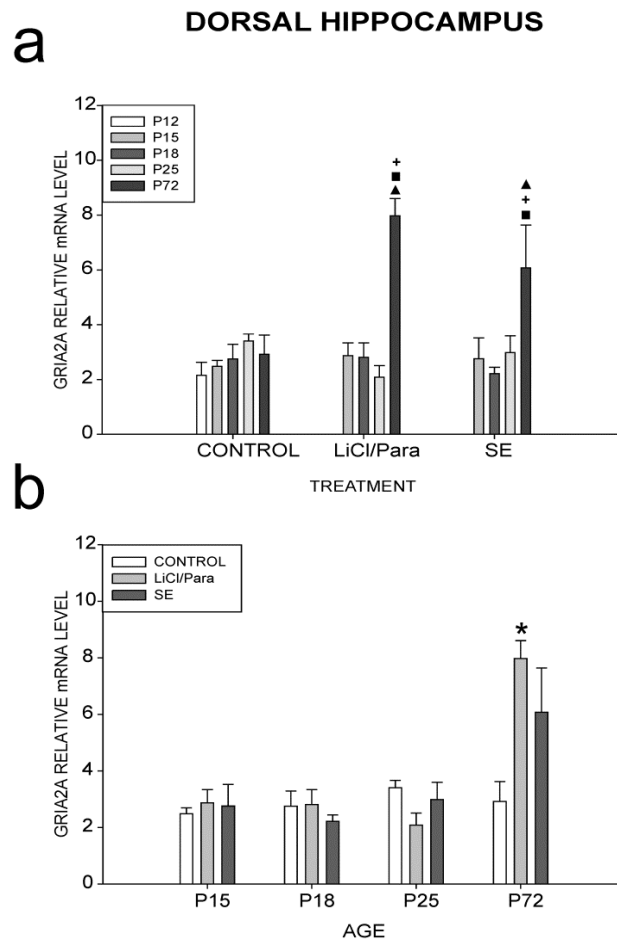


Fig.15. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the dorsal hippocampus of control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #– significant versus P12, +– significant versus P15, ■– significant versus P18, ▲–significant versus P25, ○–significant versus P72. Part b) \*– significant versus group of saline controls

## Ventral hippocampus

As revealed by One Way ANOVA, the level of GRIA2A mRNA expression in HV of (Fig.16a) control animals was significantly increasing with age (for details see Table 6). The following tests show that it was markedly higher at P25 and P72 than at P12 or P15. There were no statistically significant changes in developmental expression pattern of GRIA2A mRNA detected in HV of animals after administration of LiCl/Para or induction of LiCl/Pilo-SE (Fig.16a and b).

**Table 6. Developmental changes in expression level of GRIA2A mRNA in the ventral hippocampus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.16.a													
VENTRAL HIPPOCAMPUS													
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 25) = 2.819; P=0.047]					[F (3, 20) = 0.883; P=0.465]				[F (3, 21) = 1.714; P=0.195]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12		-	-	P=0.022 #	P=0.032 #								
P15	-		-	P=0.021 +	P=0.030 +		-	-	-		-	-	-
P18	-	-		-	-	-		-	-	-		-	-
P25	-	-	-	-	-	-	-		-	-	-		-
P72	-	-	-	-	-	-	-	-		-	-	-	
Fig.16.b													
VENTRAL HIPPOCAMPUS													
Age	P15			P18		P25		P72					
F values	[F (2, 15) =1.590; P=0, 236]			[F (2, 15) = 0.557; P=0.584]		[F (2, 15) =2.577; P=0.107]		[F (2,15) =0.938; P=0.413]					
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE					
Control	-	-	-	-	-	-	-	-					
LiCl/Para control		-		-		-		-					

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)- significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

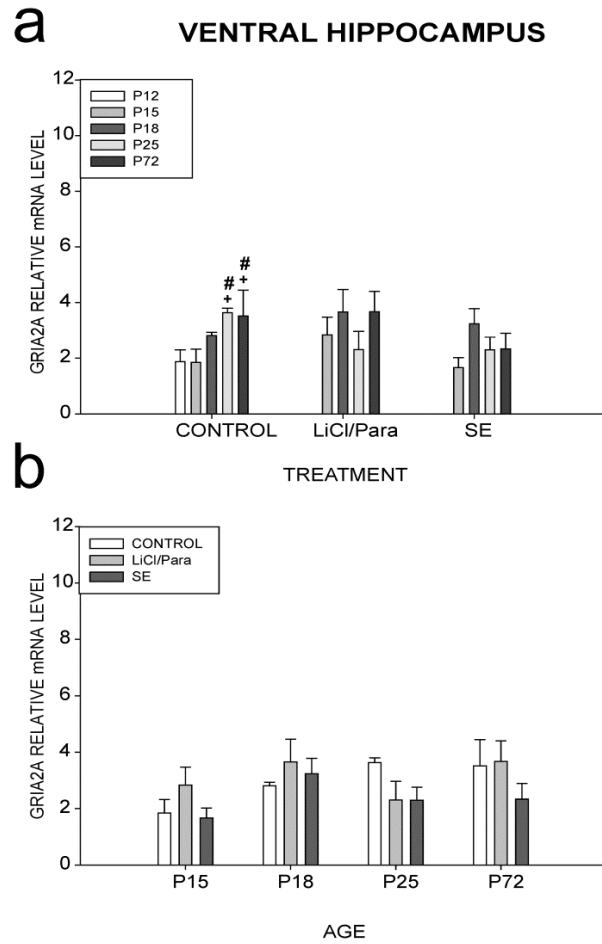


Fig.16. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the ventral hippocampus of control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #– significant versus P12, +– significant versus P15, ■– significant versus P18, ▲–significant versus P25, ○–significant versus P72. Part b) - no statistically significant differences were detected.

## Thalamus

As depicted in Fig.17, the GRIA2A mRNA expression levels in the TH of control animals was not changing significantly during development (for details see Table 7). Statistical analysis revealed that LiCl/Para or LiCl/Pilo-SE induced at P12 had a main effect on developmental GRIA2A expression level in the TH, and in both cases it was considerably increased at P25 (Fig.17a), and significantly higher than at P15 or P18. Additionally, the LiCl/Para administered at P12 had a main effect on GRIA2A mRNA level at P18, and it was higher than in saline controls or in animals after LiCl/Pilo-SE (Fig.17b). Similar tendency was detected also in P25 and P72 animals, but increase in GRIA2A level was not statistically significant (Fig.17b).

**Table 7. Developmental changes in expression level of GRIA2A mRNA in the thalamus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.**

Fig.17.a		THALAMUS											
Treatment	Control					LiCl/Para control				LiCl/Pilo-SE			
F values	[F (4, 26) = 1.332; P=0.285]					[F (3, 20) = 3.100; P=0.050]				[F (3, 20) = 6.333; P=0.003]			
Age	P12	P15	P18	P25	P72	P15	P18	P25	P72	P15	P18	P25	P72
P12	-	-	-	-	-	-	-	-	-	-	-	-	-
P15	-	-	-	-	-	-	-	P=0.019+	-	-	-	P=0.030+	-
P18	-	-	-	-	-	-	-	P=0.014■	-	-	-	P<0.001■	-
P25	-	-	-	-	-	-	-	-	-	-	-	-	-
P72	-	-	-	-	-	-	-	-	-	-	-	P=0.003 ○	-
Fig.17.b		THALAMUS											
Age	P15		P18		P25		P72						
F values	[F (2, 15) =0.929; P=0.417]		[F (2, 15) = 23.422; P<0.001]		[F (2, 15) =1.890; P=0.185]		[F (2, 15) = 2.108; P=0.148]						
Treatment	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE	LiCl/Para control	LiCl/Pilo-SE					
Control	-	-	P<0.001 *	-	-	-	-	-					
LiCl/Para control	-	-	-	P<0.001 ◆	-	-	-	-					

P- postnatal day; Expression level of GRIA2A mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)- significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

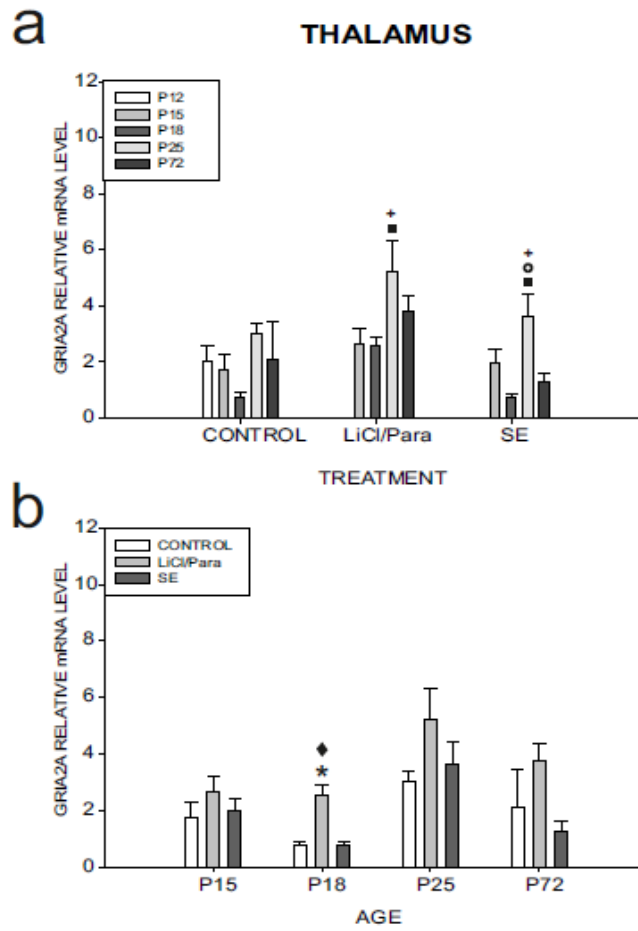


Fig.17. Developmental changes in expression level of GRIA2A mRNA (mean  $\pm$  S.E.M.) in the thalamus control animals (saline), in LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. Part a) #— significant versus P12, +— significant versus P15, ■—significant versus P18, ▲—significant versus P25, ○—significant versus P72. Part b) \*— significant versus group of saline controls; ◆— significant versus group of animals after status epilepticus.

***Developmental changes in expression of GluA2 subunit of AMPARs in different brain regions of control animals and animals after LiCl/Pilo–SE induced at P12.***

The developmental expression of GluA2 protein was changing greatly under control conditions and its profile differed among brain structures. The overall statistical analyses show that with an exception of CXP (Fig.18b), the GluA2 subunit level in the neocortex was gradually increasing and it reached its highest value in almost all tested neocortical regions at P18 (for details see Table 8). As revealed by subsequent statistical examination, the GluA2 level detected at P18 in the neocortex was significantly higher than at P15 and P25. The analyses also show that developmental changes of GluA2 protein levels in HD (Fig.18d), were quite similar to these detected in HV (Fig.18e). In both hippocampal parts, the level of GluA2 detected at P15 was significantly higher in comparison to its level at P25 and it was markedly increased at P72. The level of GluA2 in the TH of control animals (Fig.18f) was progressively decreasing with age from P12 up to P25. However, level of GluA2 detected in the TH at P72 was again increased (significant difference with P15, P18 and P25).

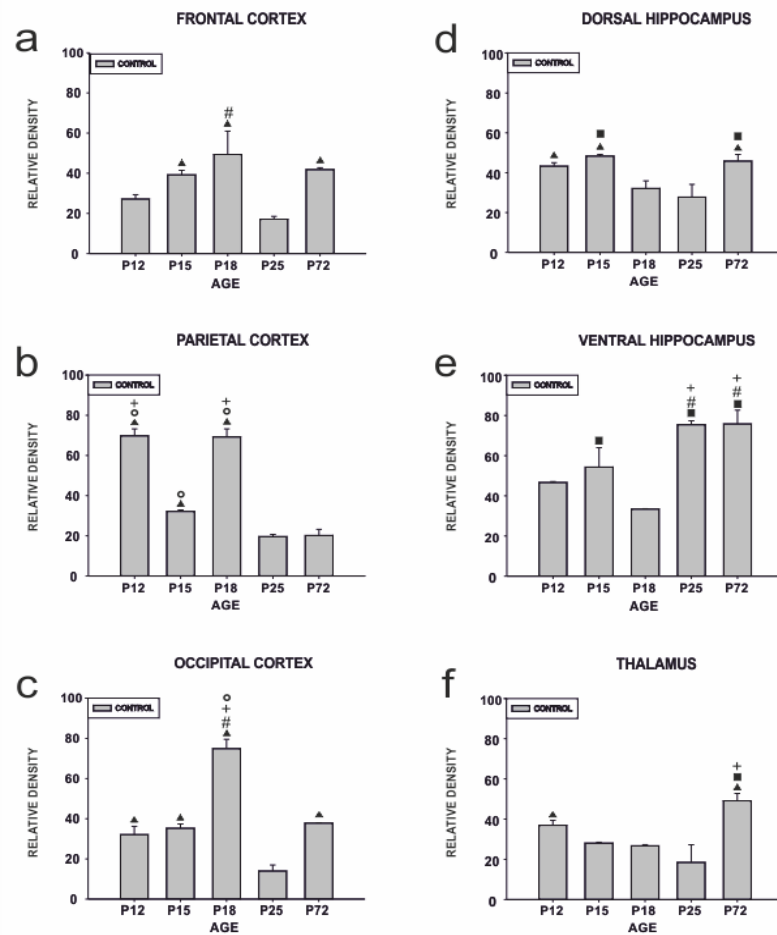


Fig.18. The developmental changes in expression level of GluA2 subunit protein of AMPARs in several brain areas of control animals measured by means of western blotting. Relative GluA2 signal density was depicted as a (mean  $\pm$  S.E.M.). a) cortex frontal, b) cortex parietal, c) cortex occipital, d) hippocampus dorsal, e) hippocampus ventral, f) thalamus. #– significant versus P12, +– significant versus P15, ■–significant versus P18, ▲–significant versus P25, ○– significant versus P72.

Table 8. Developmental changes in expression level of GluA2 subunit of AMPARs in different brain regions of control animals.

Fig.18.a.	FRONTAL CORTEX				
F value	[F (4, 10) = 5.495; P=0.013]				
Age	P12	P15	P18	P25	P72
P12	-	-	P=0.016 #	-	-
P15	-	-	-	-	-
P18	-	-	-	-	-
P25	-	P=0.017 ▲	P=0.002 ▲	-	P=0.009 ▲
P72	-	-	-	-	-
Fig.18.b.	PARIETAL CORTEX				
F value	[F (4, 10) = 80.058; P<0.001]				
Age	P12	P15	P18	P25	P72
P12	-	-	-	-	-
P15	P<0.001 +	-	P<0.001 +	-	-
P18	-	-	-	-	-
P25	P<0.001 ▲	P=0.011 ▲	P<0.001 ▲	-	-
P72	P<0.001 ○	P=0.014 ○	P<0.001 ○	-	-
Fig.18.c.	OCCIPITAL CORTEX				
F value	[F (4,10) = 46.680; P<0.001]				
Age	P12	P15	P18	P25	P72
P12	-	-	P<0.001 #	-	-
P15	-	-	P<0.001 +	-	-
P18	-	-	-	-	-
P25	P=0.003 ▲	P<0.001 ▲	P<0.001 ▲	-	P<0.001 ▲
P72	-	-	P<0.001 ○	-	-
Fig.18.d.	DORSAL HIPPOCAMPUS				
F value	[F (4, 10) = 5.784; P=0.011]				
Age	P12	P15	P18	P25	P72
P12	-	-	-	-	-
P15	-	-	-	-	-
P18	-	P=0.012 ■	-	-	P=0.027 ■
P25	P=0.015 ▲	P=0.003 ▲	-	-	P=0.007 ▲
P72	-	-	-	-	-
Fig.18.e.	VENTRAL HIPPOCAMPUS				
F value	[F (4, 10) = 11.861; P<0.001]				
Age	P12	P15	P18	P25	P72
P12	-	-	-	P=0.004 #	P=0.003 #
P15	-	-	-	P=0.019 +	P=0.018 +
P18	-	P=0.020 ■	-	P<0.001 ■	P<0.001 ■
P25	-	-	-	-	-
P72	-	-	-	-	-
Fig.18.f.	THALAMUS				
F value	[F (4, 10) = 7.045; P=0.006]				
Age	P12	P15	P18	P25	P72
P12	-	-	-	-	-
P15	-	-	-	-	P=0.007 +
P18	-	-	-	-	P=0.005 ■
P25	P=0.014 ▲	-	-	-	P<0.001 ▲
P72	-	-	-	-	-

P- postnatal day; Expression level of GluA2 protein was measured in animals under control conditions at 5 stages of postnatal development: P12, P15, P18, P25, P72; (3 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table shows all F and P values: (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)- significant versus P25; (○)-significant versus P72.

The overall statistical analysis revealed that the early life LiCl/Pilo-SE and LiCl/Para administration had a significant influence on the developmental expression of GluA2 subunit protein in majority of brain regions considered in this study (for details of statistical analysis: F and P values, see Table 9). As following post hoc tests show, the GluA2 level was significantly increased later in development in SE rats. It was especially evident at P18 in CXFR (Fig.19a) and in HD where it was higher than in corresponding saline controls (Fig.19d). Additionally, the GluA2 level was significantly increased at P72 in HD and in CXOC (Fig.19c) in relation to its control levels. In some brain areas of animals after LiCl/Pilo-SE, the GluA2 protein level was markedly decreased, and it was especially significant in the TH at P15 (Fig.19f), at P18 in CXPAR (Fig.19b), and at P72 in CXFR (Fig.19a). Analysis show that in the HV of SE animals (Fig.19e), the GluA2 protein level was significantly decreased in relation to GluA2 expression in corresponding controls at almost all stages of postnatal development (with an exception of P18 animals). The developmental level of GluA2 subunit expression was also highly affected by administration of LiCl/Para at early stages of development P11/P12. The expression of GluA2 protein after LiCl/Para administration was significantly increased at P15 in CXPAR (Fig.19b), P18 in TH (Fig.19f), and especially at P72 in CXOC (Fig.19c) and HD (Fig.19d). In HV (Fig.19e) of animals after LiCl/Para, the GluA2 level was significantly decreased throughout the development. Significant decrease in GluA2 subunit expression was also detected in TH of P15 animals (Fig.19f).

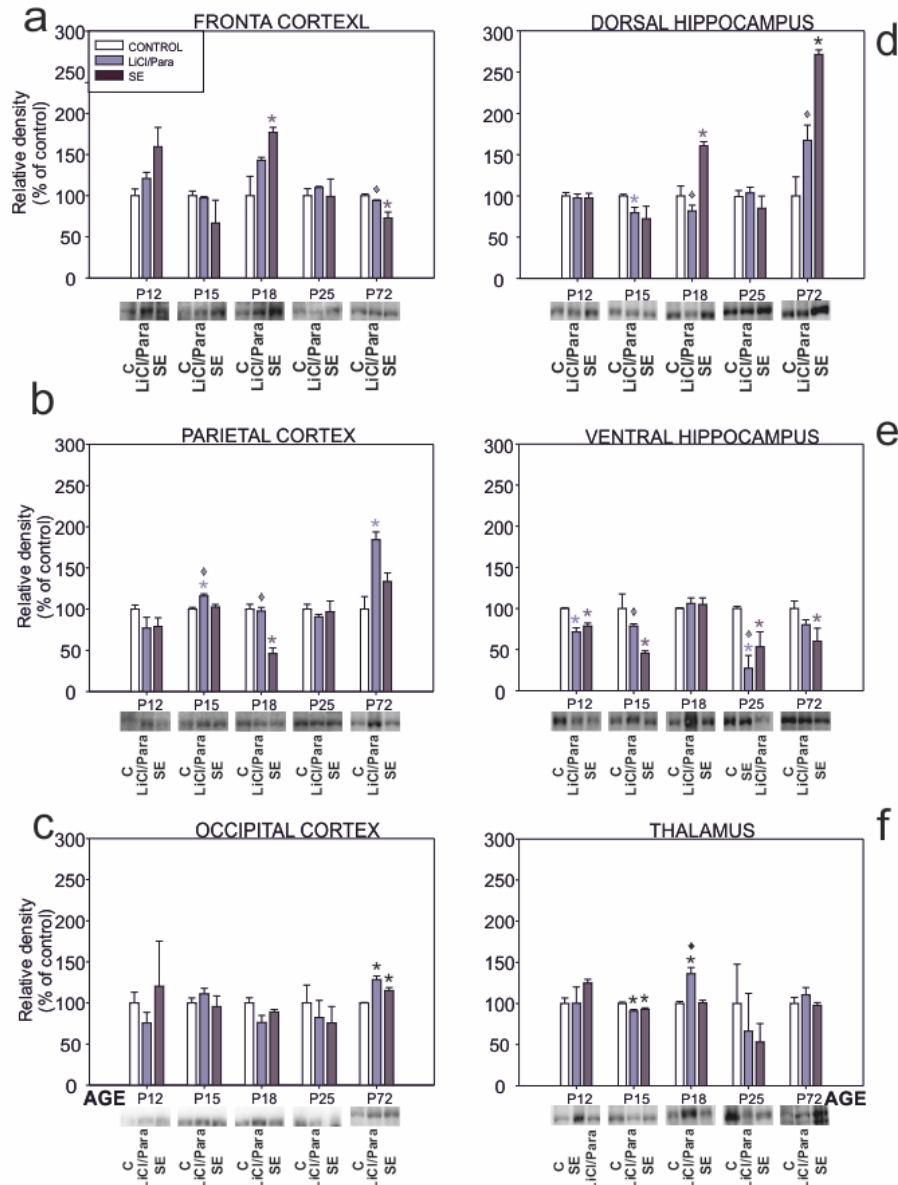


Fig.19. Developmental changes of GluA2 protein in the several brain areas of control animals (saline) (C), in LiCl/Para control animals (LiCl/Para) and animals after LiCl/Pilo-SE (SE) induced at P12. GluA2 protein expression level was measured in a) frontal cortex b) parietal cortex, c) occipital cortex, d) dorsal hippocampus, e) ventral hippocampus, f) thalamus and presented as a relative density (percent of 100% control density) (mean  $\pm$  S.E.M.). The representative blots (each sample was prepared from three individual samples from corresponding age and treatment group) were presented below corresponding graph. \*— significant versus group of saline controls; ♦— significant versus group of animals after status epilepticus.

**Table 9. Developmental changes in expression of GluA2 subunit of AMPARs in different brain regions of LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12.**

Fig.19.a. FRONTAL CORTEX										
Age	P12		P15		P18		P25		P72	
F values	[F (2, 6) = 1.776; P=0.248]		[F (2, 6) = 2.660; P=0.149]		[F (2, 6) = 6.440; P=0.032]		[F (2, 6) = 0.206; P=0.820]		[F (2, 6) = 18.970; P = 0.003]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	-	-	-	P=0.012 *	-	-	-	P=0.001 *
LiCl/Para	-	-	-	-	-	-	-	-	-	P=0.004 ♦
Fig.19.b. PARIETAL CORTEX										
Age	P12		P15		P18		P25		P72	
F values	[F (2, 6) = 2.501; P=0.162]		[F (2, 6) = 8.414; P=0.018]		[F (2, 6) = 43.053; P<0.001]		[F (2, 6) = 2.721; P=0.155]		[F (2, 6) = 7.402; P=0.024]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	P = 0.009 *	-	-	P<0.001 *	-	-	P=0.009 *	-
LiCl/Para	-	-	-	P=0.018 ♦	-	P<0.001 ♦	-	-	-	-
Fig.19.c. OCCIPITAL CORTEX										
Age	P12		P15		P18		P25		P72	
F values	[F (2, 6) =0.328; P=0.733]		[F (2, 6) = 0.779; P=0.500]		[F (2, 6) = 4.791; P=0.057]		[F (2, 6) =0.478; P=0.642]		[F (2, 6) = 12.026; P=0.008]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	-	-	-	-	-	-	P=0.003 *	P=0.041 *
LiCl/Para	-	-	-	-	-	-	-	-	-	-
Fig.19.d. DORSAL HIPPOCAMPUS										
Age	P12		P15		P18		P25		P72	
F values	[F (2, 6) = 0.0996; P=0.907]				[F (2, 6) = 21.915; P=0.002]		[F (2, 6) = 1.049; P=0.407]		[F (2, 6) = 13.060; P=0.007]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	t-test (4)= 3.657; P=0.022*	-	-	P=0.003 *	-	-	-	P=0.002 *
LiCl/Para	-	-	-	-	-	P<0.001 ♦	-	-	-	P=0.022 ♦
Fig.19.e. VENTRAL HIPPOCAMPUS										
Age	P12		P15		P18		P25		P72	
F values	[F (2, 6) = 30.577; P<0.001]		[F (2, 6) =7.064; P=0.026]		[F (2, 6) = 0.261; P=0.779]		[F (2, 6) = 33.953; P<0.001]		[F (2, 6) =6.109; P=0.036]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P<0.001 *	P=0.001 *	-	P=0.010 *	-	-	P<0.001 *	P=0.002 *	-	P=0.013 *
LiCl/Para	-	-	t-test (4) =13.309; P<0.001♦	-	-	-	-	P=0.027♦	-	-
Fig.19.f. THALAMUS										
Age	P12		P15		P18		P25		P72	
F values			[F (2, 6) = 8.081; P=0.020]		[F (2, 6) = 10.341; P=0.011]		[F (2, 6) = 0.526; P=0.616]		[F (2, 6) = 0836; P=0478]	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	t-test (4)= 2.822; P=0.048 *	P=0.008 *	P=0.028 *	P=0.007 *	-	-	-	-	-
LiCl/Para	-	-	-	-	-	P=0.008♦	-	-	-	-

P- postnatal day; Expression level of GluA2 protein was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12, P15, P18, P25, P72; (3 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Additional statistical analysis were performed by means of paired T-test. Table shows all F and P values; (-) not significant; (\*)- significant versus control; (♦)- significant versus status epilepticus.

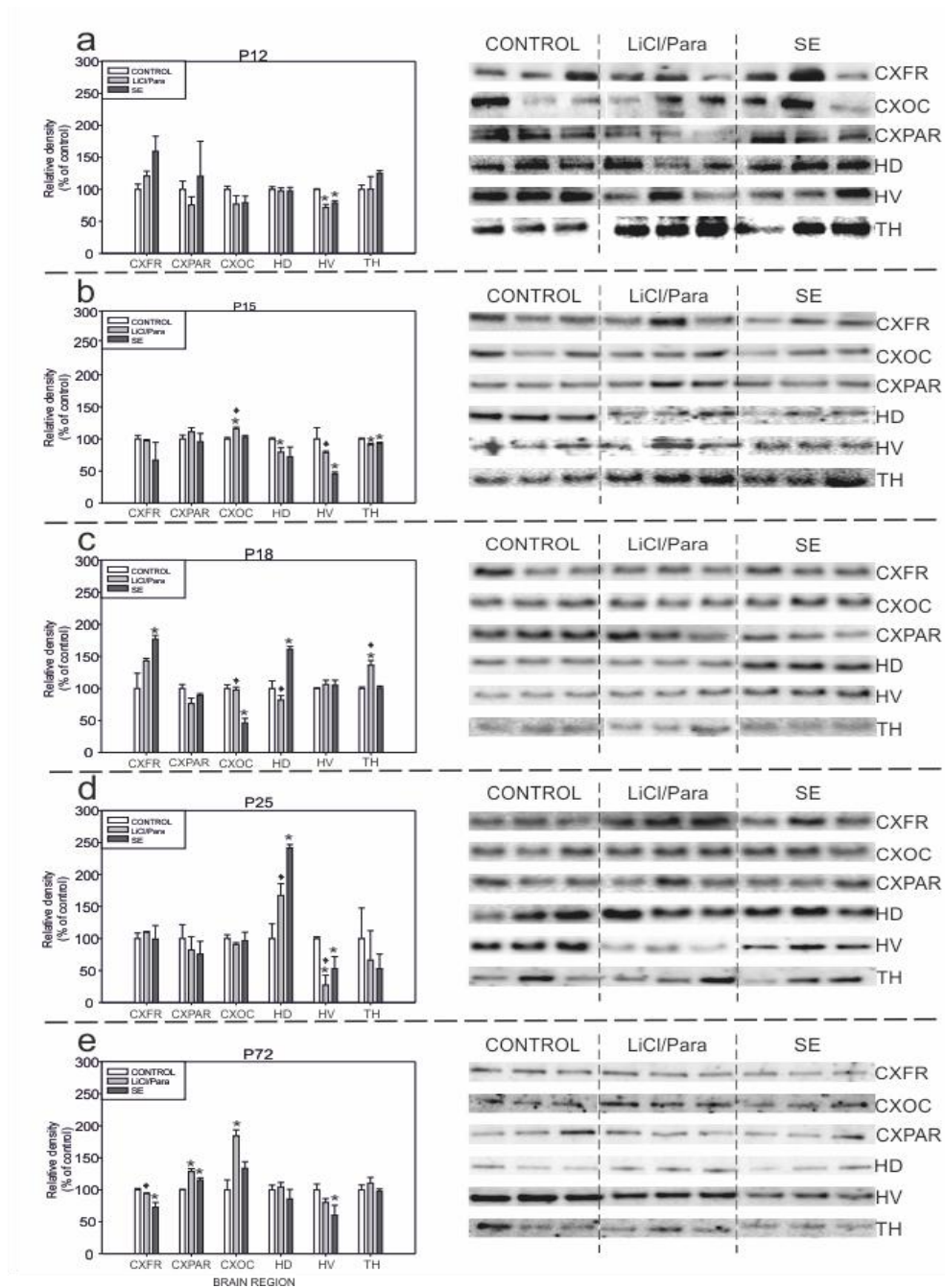


Fig.20. Changes in GluA2 subunit protein level at different stages of postnatal development in different brain areas of control animals, LiCl/Para control animals and animals after LiCl/Pilo-SE induced at P12. GluA2 protein expression level was measured at postnatal days (P): a) P12, b) P15, c) P18, d) P25, e) P72 in CXFR– frontal cortex, CXPAR– parietal cortex, CXOC– occipital cortex, HD– dorsal hippocampus, HV– ventral hippocampus and in TH– thalamus. Data were presented as a relative density (percent of signal density in controls 100%) (mean  $\pm$  S.E.M.). The original blots (three samples per each age and treatment group were presented on the left side of the panel). Significant differences marked as in Fig.19.

***Developmental changes in levels of expression of GRIN2A and GRIN2B genes encoding NR2A and NR2B subunits of NMDARs detected in control animals and animals subjected to LiCl/Pilo-SE at P12.***

Statistical analysis revealed that under control conditions the mRNA of GRIN2A, GRIN2B and their expression ratio in different brain structures change dynamically throughout the development (for details of statistical analysis: F and P values, see Tables 10–15 below). Analysis show that GRIN2A mRNA expression levels in CXFR (Fig.21a and Table 10), CXPAR (Fig.22a and Table 11), CXOC (Fig.23a and Table 12) and HD (Fig.24a and Table 13) detected at P18 and P72 were significantly increased especially when compared to its levels at P12, P15 and in some cases at P25. Additional tests revealed that the GRIN2A mRNA expression in the HV (Fig.25a and Table 14) and TH (Fig.26a and Table 15) was gradually increasing during development and its level at P25 and P72 was markedly higher than at any other tested age. Analysis displayed that the GRIN2B mRNA expression pattern in CXFR, CXPAR and HD of control animals detected at P25 was significantly higher than at any other tested age. In the CXPAR, in addition to elevated GRIN2B mRNA level at P25, it was also significantly increased at P12 and P18. The GRIN2B expression in the TH was increasing with age and its highest level detected at P72 was significantly higher than at earlier developmental stages. There were no statistically significant changes of GRIN2B mRNA expression level found in CXOC and HV of control animals. The overall analysis show that GRIN2A/GRIN2B mRNA expression ratio in the majority of the brain areas (CXFR, CXPAR, CXOC, HD) in control animals was significantly higher at P18 and P72 than at P12, P15 or P25. In the HV, the GRIN2A/GRIN2B ratio was increasing gradually with age reaching its highest values at P25 and P72 that were significantly higher than at P12 and P15. There were no significant changes in developmental GRIN2A/GRIN2B ratio detected in the TH. The overall statistical analyses show that both LiCl/Pilo-SE and LiCl/Para administration at early stages of postnatal development had a significant influence on GRIN2A and GRIN2B mRNA expression level later in development (for detailed results of statistical analysis including F and P values, see Tables 10–15). Additionally, the effect of LiCl/Pilo-SE and LiCl/Para on these mRNAs expression levels were very similar. Following, post hoc analysis showed that both, LiCl/Pilo-SE and LiCl/Para caused significant decrease in GRIN2A mRNA level in relation to its control expression. It was especially prominent at P15, P18 and P72 in the brain structures such as CXPAR (Fig.22b and Table 11), CXOC (Fig.23b and Table 12) and HD (Fig.24b and Table 13). Furthermore, analysis revealed that in the CXFR (Fig.21b and Table 10) and TH (Fig.26b

and Table 15) the significant decrease in GRIN2A mRNA level in animals subjected to LiCl/Pilo-SE, and LiCl/Para at P12, was detected only at P72. The tendencies in GRIN2A mRNA expression levels detected in HV of developing animals after LiCl/Pilo-SE or LiCl/Para were not statistically significant. The GRIN2B mRNA expression levels in developing brain were similarly affected (significantly increased in the majority of the brain structures) by both LiCl/Pilo-SE as well as LiCl/Para. In CXFR (Fig.21c and Table 10), CXPAR (Fig.22c and Table 11), CXOC (Fig.23c and Table 12) and HV (Fig.25c and Table 14), the GRIN2B mRNA levels were markedly higher in comparison to its control mRNA values especially at P15 and P18. Additionally, its level was also markedly increased at P25 and P72 in CXOC and HV. In HD of animals after LiCl/Pilo-SE and LiCl/Para, the GRIN2B mRNA was significantly increased only at P18 (Fig.24c and Table 13). After early-life SE the mRNA expression of this subunit was also significantly increased in TH at P25 (Fig.26c and Table 15), but there was no significant effect of LiCl/Para on GRIN2B mRNA expression detected in this structure. In general, statistical analysis revealed that the GRIN2A/GRIN2B mRNA expression ratio was significantly decreased after in SE and LiCl/Para animals in comparison with its control levels in all considered brain structures and at all tested stages of postnatal development (Fig.21–26 see part d).

## Frontal cortex

Overall analysis revealed that mRNA expression level of GRIN2A gene in the CXFR of control animals was changing significantly during development (Fig.21a). There was a significant increase in GRIN2A mRNA level in CXFR of P18 and in P72 animals (for details see Table 10). The expression of GRIN2A mRNA at P72 was also considerably higher when compared to its level detected at P25 and P12. The GRIN2B mRNA expression level during development was also changing greatly with age. Subsequent analysis demonstrated the significantly higher GRIN2B mRNA level in P25 animals when compared to P15, P18 or P72. As for GRIN2A/GRIN2B mRNA expression ratio, it was markedly increased at P18 and P72 when compared to the mRNA level at P25. The GRIN2A/GRIN2B ratio at P72 was also significantly higher than at P12. As for GRIN2A mRNA expression level in both groups of animals (LiCl/Para controls and SE rats - Fig.21b) was significantly lower only in P72 animals than in corresponding control animals (Table 10.). The GRIN2B mRNA expression level (Fig.21c) significantly increased in LiCl/Para and SE animals in comparison to controls at P15 and P18. The overall analysis revealed that GRIN2A/GRIN2B mRNA level ratio (Fig.21d after LiCl/Para or SE) was much lower than in controls what was especially marked in P15 and P18. In young adult SE animals (P72), this change was less pronounced but still significant (for details see Table 10).

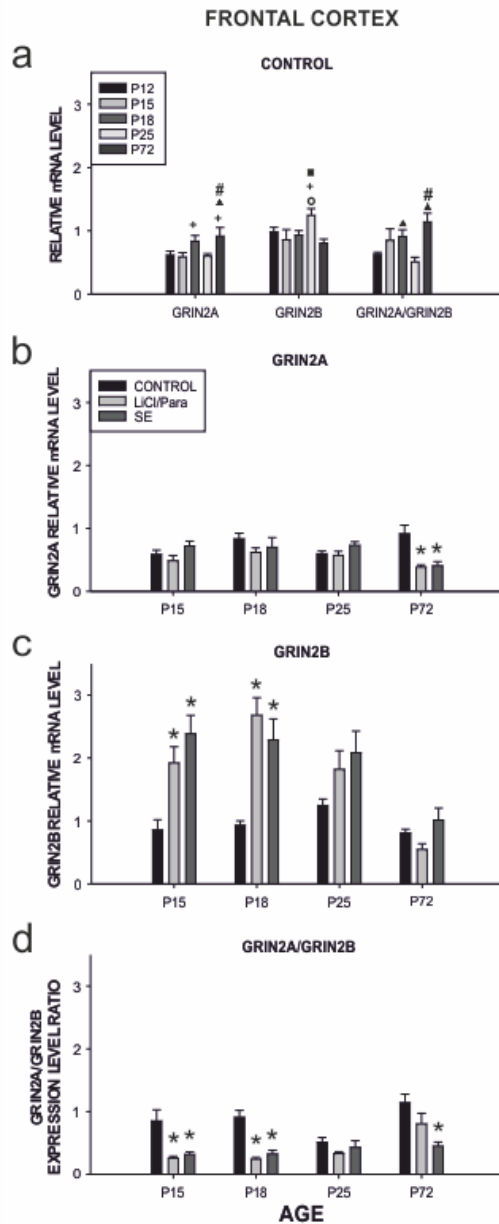


Fig.21. Developmental changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the cortex frontal. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Significant difference between individual groups in figure part a): #– significant versus P12; +- significant versus P15; ■–significant versus P18; ▲– significant versus P25; ○–significant versus P72. Significant difference between individual groups in parts b), c), and d): \*– significant versus group of saline controls; ◆– significant versus group of animals after status epilepticus.

Table 10. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the frontal cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

FRONTAL CORTEX																
CONTROL																
Fig.21.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B					
F values	[F (4, 26) = 3.097; P= 0.033]					[F (4, 26) =2.816; P=0.046]					[F (4, 26) =4.346; P =0.008]					
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	
P12	-	-	-	-	P=0.024 #	-	-	-	-	-	-	-	-	-	P= 0.005 #	
P15	-	-	P=0.050 +	-	P=0.014 +	-	-	-	P=0.012 +	-	-	-	-	-	-	
P18	-	-	-	-	-	-	-	-	P=0.032 ■	-	-	-	-	-	-	
P25	-	-	-	-	P=0.017 ▲	-	-	-	-	-	-	-	P=0.021 ▲	-	P<0.001 ▲	
P72	-	-	-	-	-	-	-	-	P=0.005 ○	-	-	-	-	-	-	
EXPERIMENTAL																
Fig.21.b.	GRIN2A															
Age	P15		P18		P25		P72									
F values	-		-		-		-		[F (2, 15) =10.868; P=0.001]							
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P<0.001 *	P=0.001 *
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.21.c.	GRIN2B															
Age	P15		P18		P25		P72									
F values	[F (2, 15) =10.181; P = 0.002]		[F (2, 19) =13.025; P<0.001]		-		-									
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.008 *	P<0.001 *	P<0.001 *	P=0.002 *	-	-	-	-	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.21.d.	GRIN2A/GRIN2B															
Age	P15		P18		P25		P72									
F values	[F (2, 15) =9.276; P= 0.002]		[F (2, 19) =27.927; P<0.001]		-		-		[F (2, 15) = 6.968; P=0.007]							
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.001 *	P= 0.003 *	P<0.001 *	P<0.001 *	-	-	-	-	-	-	-	-	-	-	P=0.002 *	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; at 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

Table 10. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the frontal cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

FRONTAL CORTEX																
CONTROL																
Fig.21.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B					
F values	[F (4, 26) = 3.097; P= 0.033]					[F (4, 26) =2.816; P=0.046]					[F (4, 26) =4.346; P =0.008]					
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	
P12	-	-	-	-	P=0.024 #	-	-	-	-	-	-	-	-	-	P= 0.005 #	
P15	-	-	P=0.050 +	-	P=0.014 +	-	-	-	P=0.012 +	-	-	-	-	-	-	
P18	-	-	-	-	-	-	-	-	P=0.032 ■	-	-	-	-	-	-	
P25	-	-	-	-	P=0.017 ▲	-	-	-	-	-	-	-	P=0.021 ▲	-	P<0.001 ▲	
P72	-	-	-	-	-	-	-	-	P=0.005 ○	-	-	-	-	-	-	
EXPERIMENTAL																
Fig.21.b.	GRIN2A															
Age	P15		P18		P25		P72									
F values	-		-		-		-		[F (2, 15) =10.868; P=0.001]							
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P<0.001 *	P=0.001 *
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.21.c.	GRIN2B															
Age	P15		P18		P25		P72									
F values	[F (2, 15) =10.181; P = 0.002]		[F (2, 19) =13.025; P<0.001]		-		-									
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.008 *	P<0.001 *	P<0.001 *	P=0.002 *	-	-	-	-	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.21.d.	GRIN2A/GRIN2B															
Age	P15		P18		P25		P72									
F values	[F (2, 15) =9.276; P= 0.002]		[F (2, 19) =27.927; P<0.001]		-		-		[F (2, 15) = 6.968; P=0.007]							
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.001 *	P= 0.003 *	P<0.001 *	P<0.001 *	-	-	-	-	-	-	-	-	-	-	P=0.002 *	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 5-7 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

## Parietal cortex

As overall statistical analysis revealed that, there were significant changes in expression levels of GRIN2A and GRIN2B genes as well as in GRIN2A/GRIN2B ratio in the CXPAR from control animals (Fig.22a). Subsequent analysis displayed that there was a significant elevation of GRIN2A mRNA expression at P18 and P72 (for details see Table 11). The GRIN2A mRNA level detected at P25 was significantly higher in comparison to its level at P15. As for the level of GRIN2B in CXPAR of control animals, it was significantly increased at P12 and P18, and its peak expression was detected at P25. As for GRIN2A/GRIN2B mRNA expression ratio, it was markedly increased only at P18 and P72. Performed statistical analysis revealed also that LiCl/Para administration and pilocarpine induced SE had a main effect of on GRIN2A and GRIN2B mRNA expression in the CXPAR (Fig. 22b and c). Subsequent analysis shows that in animals after LiCl/Pilo-SE, the GRIN2A mRNA level was markedly decreased at P18, and in LiCl/Para controls, it was significantly decreased at P25 when compared its levels detected in the control animals of the same age (see Fig.22b and Table 11). On the other hand, both LiCl/Para and SE markedly affected the GRIN2B mRNA in CXPAR, and at all tested ages with an exception of P72 the level of its expression was significantly increased in comparison to control values. As for P25, LiCl/Para caused marked decrease in GRIN2B mRNA when compared to its level after SE (Fig.22.c). Additionally, statistical analysis demonstrated that LiCl/Para and SE significantly influenced GRIN2A/GRIN2B mRNA ratio (Fig.22d) - its levels in saline controls were markedly decreased at all tested developmental stages.

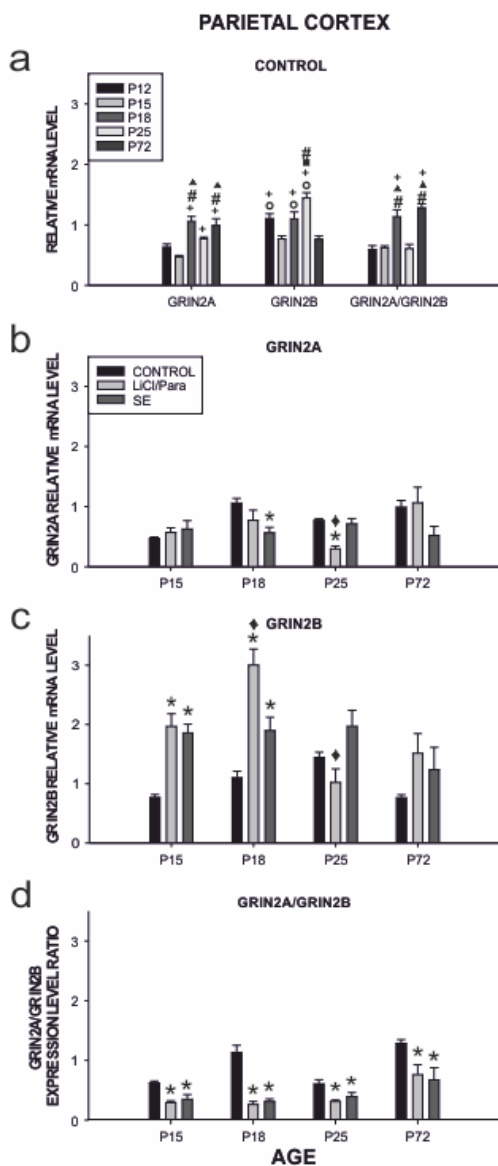


Fig.22. Changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the parietal cortex. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Statistical significance marked as in Fig.21.

Table 11. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the parietal cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

PARIETAL CORTEX															
CONTROL															
Fig.22.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B				
F values	[F (4, 28) =12.417; P<0.001]					[F (4, 28) = 10.646; P<0.001]					[F (4, 28) =15.930; P<0.001]				
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72
P12	-	-	P<0.001 #	-	P=0.002 #	-	-	-	P=0.010 #	-	-	-	P<0.001 #	-	P<0.001 #
P15	-	-	P<0.001 +	P=0.006 +	P<0.001 +	P=0.014 +	-	P=0.010 +	P<0.001 +	-	-	-	P<0.001 +	-	P<0.001 +
P18	-	-	-	-	-	-	-	-	P=0.006 ■	-	-	-	-	-	-
P25	-	-	P=0.004 ▲	-	P=0.033 ▲	-	-	-	-	-	-	-	P<0.001 ▲	-	P<0.001 ▲
P72	-	-	-	-	-	P=0.013 ○	-	P=0.009 ○	P<0.001 ○	-	-	-	-	-	-
EXPERIMENTAL															
Fig.22.b.	GRIN2A														
Age	P15		P18		P25		P72								
F values	-		[F (2, 17) = 4,953; P=0,020]		[F (2, 16) =18,550; P<0,001]		-								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	-	-	-	P=0.006 *	-	P<0.001 *	-	-							
LiCl/Para control	-	-	-	-	-	P<0.001♦	-	-							
Fig.22.c.	GRIN2B														
Age	P15		P18		P25		P72								
F values	[F (2, 16) = 18,720; P<0,001]		[F (2, 17) = 23,647; P<0,001]		[F (2, 16) =5,261; P=0,018]		-								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	P<0.001 *	P<0.001 *	P<0.001 *	P<0.001 *	-	-	-	-							
LiCl/Para control	-	-	-	P=0.002 †	-	P=0.005 †	-	-							
Fig.22.d.	GRIN2A/GRIN2B														
Age	P15		P18		P25		P72								
F values	[F (2, 16) =8,836; P=0,003]		[F (2, 17) =32,290; P<0,001]		P25 [F (2, 16) =6,875; P=0,007]		[F (2, 15) =4,551; P=0,029]								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	P=0.001 *	P=0,004 *	P<0,001 *	P<0,001 *	P=0,003 *	P=0,021 *	P= 0,014 *	P=0,030*							
LiCl/Para control	-	-	-	-	-	-	-	-							

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraaldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; at 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (♦)- significant versus status epilepticus.

## Occipital cortex

The overall statistical analysis showed that there were significant developmental changes in GRIN2A relative mRNA expression levels as well as in GRIN2A/GRIN2B mRNA ratio occurring in the CXOC of the control animals (Fig.23a). However, there were no marked changes detected in developmental expression levels of GRIN2B mRNA. The post hoc tests revealed there was a significant increase in GRIN2A mRNA levels in control animals at P18 and P72 (for details see Table 12). The age-related expression level of GRIN2B mRNA in CXOC was relatively stable while the GRIN2A/GRIN2B mRNA level ratio was significantly increased at P18 and P72. Overall analysis revealed marked influence of LiCl/Para and SE on GRIN2A mRNA expression level in CXOC at P15 and P18, while at P72 it was affected only in LiCl/Para controls (Fig.23b). With an exception of P25 animals, in each age group of tested animals the GRIN2A mRNA levels after LiCl/Para and LiCl/Pilo-SE were markedly lower than its control expression. Preformed analysis showed also the major effect of LiCl/Para and SE on the expression level of GRIN2B mRNA in occipital cortex in animals at P15, P18 and P25 but not at P72 (Fig.23c). The relative mRNA expression of this gene was strongly elevated after both LiCl/Para as well as SE in comparison to control group, with particularly high mRNA level in P18 animals after LiCl/Para. The LiCl/Para and SE had also main effect on developmental levels of GRIN2A/GRIN2B mRNA ratio. As revealed by overall analysis, the GRIN2A/GRIN2B level all tested ages was significantly decreased in LiCl/Para and SE rats when compared to the control values (Fig.23d).

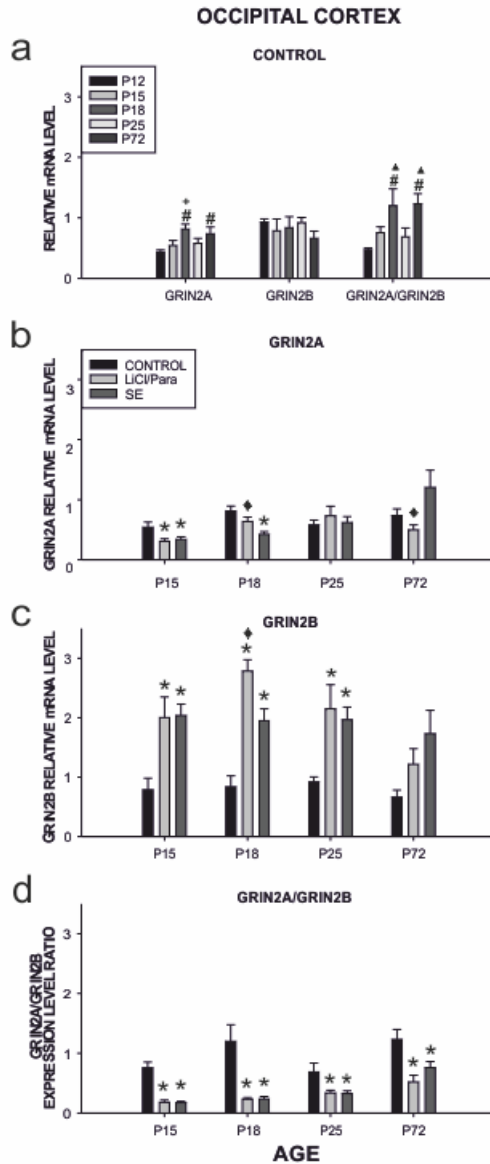


Fig.23. Changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the occipital cortex. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Statistical significance marked as in Fig.21.

Table 12. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the occipital cortex of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

OCCIPITAL CORTEX																
CONTROL																
Fig.23.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B					
F values	[F (4, 25) =3.363; P=0.025]					-					[F (4, 25) = 4.065; P=0.011]					
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	
P12	-	-	P=0.004 #	-	P=0.017 #	-	-	-	-	-	-	-	P=0.005 #	-	P=0.003 #	
P15	-	-	P=0.029 +	-	-	-	-	-	-	-	-	-	-	-	-	
P18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
P25	-	-	-	-	-	-	-	-	-	-	-	-	P=0.038 ▲	-	P=0.028 ▲	
P72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
EXPERIMENTAL																
Fig.23.b.	GRIN2A								GRIN2B							
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	[F (2, 15) = 4.498; P=0.029]		[F (2, 16) = 8.701; P=0.003]		-		[F (2, 15) = 3.831; P=0.045]		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.015 *	P=0.030 *	-	P<0.001 *	-	-	-	-	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	P=0.037 †	-	-	-	-	-	-	-	-	-	-	-	P=0.016 †
Fig.23.c.	GRIN2B								GRIN2A/GRIN2B							
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	[F (2, 15) = 7.829; P=0.005]		[F (2, 16) = 24.312; P<0.001]		[F (2, 17) =5.965; P=0.011]		-		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.004 *	P=0.003 *	P<0.001 *	P<0.001 *	P=0.006 *	P=0.011 *	-	-	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	P=0.007 †	-	-	-	-	-	-	-	-	-	-	-	-
Fig.23.d.	GRIN2A/GRIN2B								GRIN2A/GRIN2B							
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	[F (2, 15) = 20.517; P<0.001]		[F (2, 17) = 12.716; P<0.001]		[F (2, 17) = 5.495; P=0.014]		[F (2, 15) = 7.729; P=0.005]		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P<0.001 *	P<0.001 *	P<0.001 *	P<0.001 *	P=0.014 *	P=0.007 *	P=0.002 *	P=0.022 *	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; at 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (†)- significant versus status epilepticus.

## Dorsal hippocampus

Relative mRNA expression level of GRIN2A and GRIN2B genes in the HD of control animals was changing significantly during development (Fig.24a). The GRIN2A mRNA level was markedly increased at P18 and P72 when compared to its levels at P12 and P15 (for details see Table 13). As for GRIN2B mRNA expression level, it was varying greatly throughout the development with its peak expression at P25 and the lowest expression level detected at P72. The GRIN2A/GRIN2B expression ratio was also significantly changing with age - its levels at P18 and P72 were significantly higher than at P12 and P15. Statistical analysis displayed that LiCl/Para and LiCl/Pilo-SE had a marked influence on GRIN2A mRNA the expression level in HD. It was significantly decreased at P18 and P25 when compared to the levels in corresponding saline controls (Fig.24b). The LiCl/Pilo-SE and LiCl/Para had also main effect of on developmental GRIN2B expression level in HD (Fig. 24c., and it was highly increased 6 days after the insult, i.e. at P18. Moreover, SE as well as LiCl/Para administration had a main effect on the ratio of GRIN2A/GRIN2B mRNA expression in HD (Fig.24d). It was significantly decreased in almost all age groups of tested animals in comparison to the control levels.

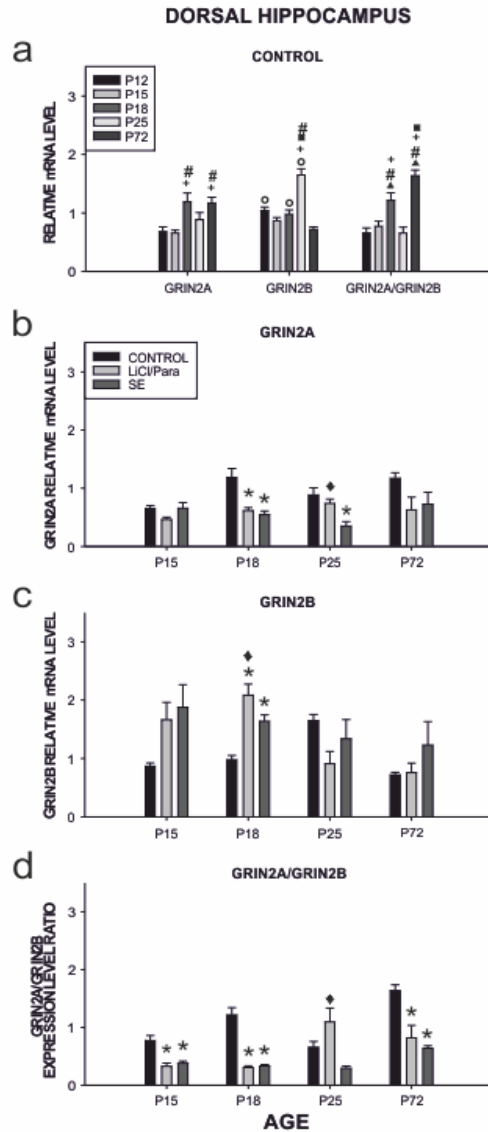


Fig.24. Changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the dorsal hippocampus. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Statistical significance marked as in Fig.21.

Table 13. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the dorsal hippocampus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

DORSAL HIPPOCAMPUS															
CONTROL															
Fig.24.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B				
F values	[F (4, 26) = 5.448; P=0.003]					[F (4, 26) = 24.122; P<0.001]					[F (4, 26) = 17.549; P<0.001]				
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72
P12	-	-	P=0.003 #	-	P=0.004 #	-	-	-	P<0.001 #	-	-	-	P<0.001 #	-	P<0.001 #
P15	-	-	P=0.002 +	-	P=0.003 +	-	-	-	P<0.001 +	-	-	-	P=0.006 +	-	P<0.001 +
P18	-	-	-	-	-	-	-	-	P<0.001 ■	-	-	-	-	-	P=0.008 ■
P25	-	-	-	-	-	-	-	-	-	-	-	-	P<0.001 ▲	-	P<0.001 ▲
P72	-	-	-	-	-	P=0.006 ○	-	P=0.024 ○	P<0.001 ○	-	-	-	-	-	-
EXPERIMENTAL															
Fig.24.b.	GRIN2A														
Age	P15		P18		P25		P72								
F values	-		[F (2, 18) = 14.068; P<0.001]		[F (2, 18) = 8.720; P=0.002]		[F (2, 18) = 6.851; P=0.006]								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	-	-	P<0.001 *	P<0.001 *	-	P<0.001 *	-	-							
LiCl/Para control	-	-	-	-	-	P=0.009	-	-							
Fig.24.c.	GRIN2B														
Age	P15		P18		P25		P72								
F values	-		[F (2, 18) = 15.510; P<0.001]		-		-								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	-	-	P<0.001 *	P=0.003 *	-	-	-	-							
LiCl/Para control	-	-	-	P=0.026 †	-	-	-	-							
Fig.24.d.	GRIN2A/GRIN2B														
Age	P15		P18		P25		P72								
F values	[F (2, 15) = 14.446; P<0.001]		[F (2, 18) = 54.870; P<0.001]		[F (2, 18) = 6.851; P=0.006]		[F (2, 17) = 10.014; P=0.001]								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE							
Control	P<0.001 *	P<0.001 *	P<0.001 *	P<0.001 *	-	-	-	-							
LiCl/Para control	-	-	-	-	-	P=0.002 †	-	-							

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (†)- significant versus status epilepticus.

## Ventral hippocampus

Statistical analysis revealed that the GRIN2A mRNA expression levels in HV of control animals (Fig.25a) was significantly increasing with age reaching its highest values at P25 and P72, while the GRIN2B mRNA expression remained relatively stable during development. As a result, the GRIN2A/GRIN2B mRNA expression ratio was significantly increased at P25 and P72 (for details see Table 14). The overall analysis displayed that there was no main effect of LiCl/Para or LiCl/Pilo-SE on GRIN2A mRNA expression level at any of tested ages and there was no significant difference when compared with expression in control animals (Fig.25b). As for the GRIN2B mRNA expression level in HV (Fig.25c), LiCl/Para as well as LiCl/Pilo-SE affected it markedly at P15, P18 and P72. With an exception of animals at P25, the GRIN2B mRNA level was significantly increased at all postnatal days mentioned above. The developmental profile of GRIN2A/GRIN2B mRNA expression ratio was markedly changed after LiCl/Para as well as LiCl/Pilo-SE. When compared to its control levels it was significantly decreased in animals at all postnatal days tested (Fig.25d).

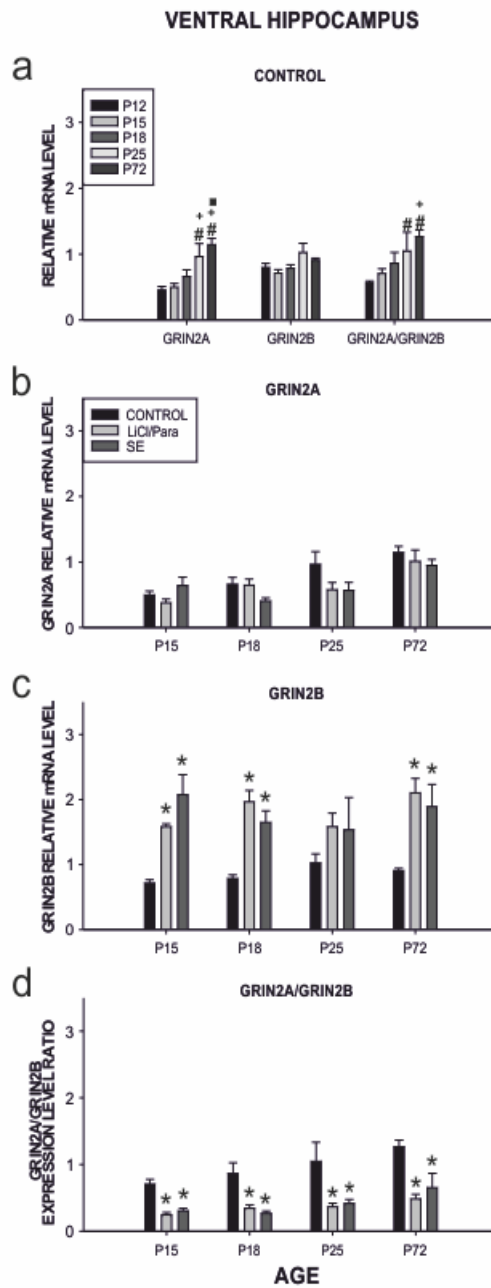


Fig.25. Changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the ventral hippocampus. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Statistical significance marked as in Fig.21.

Table 14. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the ventral hippocampus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

VENTRAL HIPPOCAMPUS																
CONTROL																
Fig.25.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B					
F values	[F (4, 26) = 7.263; P<0.001]					-					[F (4, 26) =3.203; P=0.029]					
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	
P12	-	-	-	P=0.004 #	P<0.001 #	-	-	-	-	-	-	-	-	P=0.038 #	P=0.004 #	
P15	-	-	-	P=0.006 +	P<0.001 +	-	-	-	-	-	-	-	-	-	P=0.014 +	
P18	-	-	-	-	P=0.005 ■	-	-	-	-	-	-	-	-	-	-	
P25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
P72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
EXPERIMENTAL																
Fig.25.b.	GRIN2A															
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	-		-		-		-		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.25.c.	GRIN2B															
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	[F (2, 17) = 13.434; P<0.001]		[F (2, 18) = 13.492; P<0.001]		-		[F (2, 16) =7.269; P=0.006]		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P=0.006 *	P<0.001 *	P<0.001 *	P=0.001 *	-	-	-	-	P=0.002 *	P=0.011 *	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fig.25.d.	GRIN2A/GRIN2B															
Age	P15		P18		P25		P72		P15		P18		P25		P72	
F values	[F (2, 17) = 23.331; P<0.001]		[F (2, 18) = 12.579; P<0.001]		[F (2, 17) = 5.771; P=0.012]		[F (2, 16) = 8.915; P=0.003]		-		-		-		-	
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE
Control	P<0.001 *	P<0.001 *	P<0.001 *	P<0.001 *	P=0.006 *	P=0.014 *	P<0.001 *	P=0.007 *	-	-	-	-	-	-	-	-
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-6 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (◆)- significant versus status epilepticus.

## Thalamus

In the TH of control animals, the expression of GRIN2A and GRIN2B mRNA was markedly changing with age (Fig.26a). The expression of both genes was increasing significantly during development, and as the post hoc test revealed, the GRIN2A mRNA level detected at P72 was significantly higher than at any other age of tested animals (for details see Table 15). The profile of GRIN2A/GRIN2A mRNA expression ratio in the TH remained stable throughout the development. As for GRIN2A mRNA level in the TH (Fig.26b), the overall analysis showed that it was significantly lower in LiCl/Para and SE animals when compared to controls only in adult P72 animals. As the subsequent analysis revealed, the level of GRIN2B mRNA was significantly higher in TH of SE rats than in control naïve animals as well as LiCl/Para controls. It was marked only 13 days after the insult, i.e. at P25 (Fig.26c). Due to mentioned changes in GRIN2A and GRIN2B mRNA expression profiles in the TH of SE or LiCl/Para animals, the level GRIN2A/GRIN2B mRNA expression ratio was significantly decreased in all tested age groups with an exception of animals at P25 (Fig.26d).

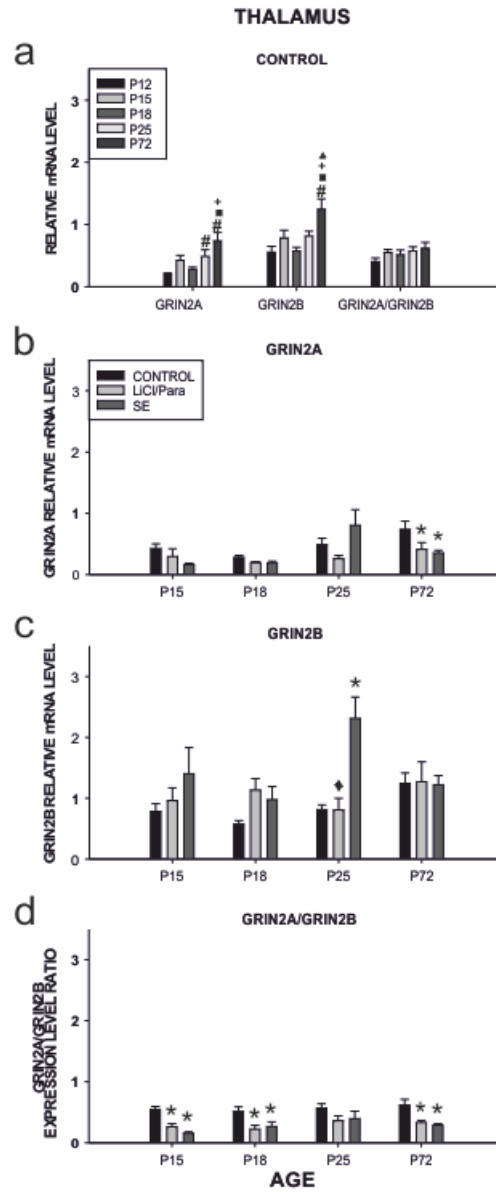


Fig.26. Changes in relative mRNA expression level of GRIN2A, GRIN2B and GRIN2A/GRIN2B ratio in the ventral hippocampus. (a) Levels of GRIN2A, GRIN2B and GRIN2A/GRIN2B mRNA ratio detected in control animals. Influence of LiCl/Paraldehyde and pilocarpine-induced status epilepticus on relative mRNA level of (b) GRIN2A, (c) GRIN2B (d) GRIN2A/GRIN2B ratio at different stages of postnatal development. All data were presented as mean  $\pm$  SEM values. Statistical significance marked as in Fig.21.

Table 15. Developmental changes in expression level of GRIN2A and GRIN2B mRNA in the thalamus of control animals, LiCl/Para control animals and animals after Pilo-SE induced at postnatal day 12.

THALAMUS															
CONTROL															
Fig.26.a. GENE	GRIN2A					GRIN2B					GRIN2A/GRIN2B				
F values	[F (4, 26) = 5.568; P=0.002]					[F (4, 26) = 6.042; P=0.001]					-				
Age	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72	P12	P15	P18	P25	P72
P12	-	-	-	P=0.038 #	P<0.001 #	-	-	-	-	P<0.001 #	-	-	-	-	-
P15	-	-	-	-	P=0.020 +	-	-	-	-	P=0.008 +	-	-	-	-	-
P18	-	-	-	-	P<0.001 ■	-	-	-	-	P<0.001 ■	-	-	-	-	-
P25	-	-	-	-	P=0.001 ▲	-	-	-	-	P=0.013 ▲	-	-	-	-	-
P72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EXPERIMENTAL															
Fig.26.b.	GRIN2A														
Age	P15		P18		P25		P72								
F values	-		-		-		-		[F (2, 16) = 3.839; P=0.043]						
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	
Control	-	-	-	-	-	-	-	-	-	-	-	-	P=0.048 *	P=0.022 *	
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Fig.26.c.	GRIN2B														
Age	P15		P18		P25		P72								
F values	-		-		[F (2, 15) = 23.256; P<0.001]		-								
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	
Control	-	-	-	-	-	-	-	P<0.001 *	-	-	-	-	-	-	
LiCl/Para control	-	-	-	-	-	-	-	P<0.001♦	-	-	-	-	-	-	
Fig.26.d.	GRIN2A/GRIN2B														
Age	P15		P18		P25		P72								
F values	[F (2, 17) = 24.204; P<0.001]		[F (2, 16) = 4.512; P=0.028]		-		-		[F (2, 16) = 7.524; P=0.005]						
Treatment	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	LiCl/Para	LiCl/Pilo-SE	
Control	P<0.001 *	P<0.001 *	P=0.012 *	P=0.014 *	-	-	-	-	P=0.007 *	P=0.003 *	-	-	-	-	
LiCl/Para control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

P- postnatal day; Expression level of GRIN2A and GRIN2B mRNA was measured in: controls- group of animals injected with saline, LiCl/Para controls-group of animals that received LiCl and paraaldehyde, LiCl/Pilo-SE- group of animals after P12 status epilepticus induced by pilocarpine; At 4-5 stages of postnatal development (time intervals following LiCl/Pilo-SE): P12 (only control group), P15, P18, P25, P72; (5-7 animals per each group). Statistical analysis: One-Way ANOVA followed by Fisher's least significant difference post hoc test. Level of significance: P<0.05. Table show F and P values only when significance was found; (-) not significant; (#)- significant versus P12; (+)- significant versus P15; (■)-significant versus P18; (▲)-significant versus P25; (○)-significant versus P72; (\*)- significant versus control; (♦)- significant versus status epilepticus.

## Discussion

### *Influence of a specific antagonist of Ca<sup>2+</sup>-permeable AMPARs, IEM1460 on different types of seizures in developing rats.*

The models of seizures used in this study have different sites of origin in the brain. The sites of origins were previously determined in the basal forebrain area and cerebral cortex for minimal clonic seizures and in the brainstem with subsequent involvement of anterior thalamus, retrosplenial cortex and dentate gyrus for generalized tonic-clonic seizures. The involvement of different brain structures in these seizures was determined by means of various techniques: electrical stimulation of different brain areas and brainstem transections (Browning and Nelson, 1985), a density of c-fos protein immunoreactivity as an indicator of cellular activation (André et al., 1998), single photon emission computed tomography to image cerebral blood flow changes (Blumenfeld et al., 2009), functional magnetic resonance imaging of the blood oxygen level-dependent signal activity of neurons (Brevard et al., 2006, Blumenfeld et al., 2009 and Zhang et al., 2014). During cortical ADs, a slow spike and wave rhythm (3–4 Hz) characteristic for cortico-thalamo-cortical oscillations can be observed in EEG (Pohl et al., 1986 and Avanzini et al., 1992), cortical epileptic afterdischarges appear as a consequence of local stimulation of somatosensory cortex, therefore, their site of origin is in the cerebral cortex.

Present study shows that the onset of PTZ-induced minimal clonic seizures in all three age groups of tested animals was significantly delayed (Fig.4.). This suggests that the blockade of the Ca<sup>2+</sup>-permeable AMPARs by IEM 1460 was sufficient to make the spread of the epileptic activity into the motor system more difficult. As it was described in the present and previous studies, the expression of GluA2 mRNA and protein varies in different parts of telencephalon, and in adults, its immunoreactivity is high in the majority of the structures (Sato et al., 1993 and Petralia et al., 1997). However, the lack or presence of mRNA of the GluA2 subunit alone does not necessarily determines the calcium permeability of AMPARs. It was reported that the calcium permeability of the AMPARs is determined by the Q/R site edition of the GluA2 subunit mRNA. Additionally, the Q/R site mRNA edition is responsible for the retention of this subunit in endoplasmic reticulum (ER), on the other hand, an unedited GluA2 is competent to exit the ER efficiently (Greger et al., 2003 and Greger et al., 2006). It should be kept in mind that AMPA receptors lacking GluA2 subunit may still be formed even in the presence of GluR2 mRNA (Greger et al., 2003, Cull-Candy et al., 2006; Szczurowska and Mareš, 2013). In addition, the formation of subunit assembly of AMPARs is regulated by

numerous factors such as N-terminal regions, phosphorylation, or their binding proteins (Leuschner and Hoch, 1999; Song and Huganir, 2002; Vandenberghe et al., 2005; Buldakova et al., 2007). It has been demonstrated by means of *in vitro* electrophysiological measurements (Kumar et al., 2002), kainate-stimulated  $\text{Co}^{2+}$ -labeling assay (Hsu et al., 2010) that there is a high level of  $\text{Ca}^{2+}$ -permeable AMPARs present in developing cerebral cortex. Our results are in agreement with these reports, as well as with the results of our analysis of GluA2 subunit developmental expression levels. As for the generalized tonic-clonic seizures (Fig.5.), the decreased incidence as well as prolonged latency of seizures after IEM1460 was marked only in 25-day-old rats. This suggests that IEM1460 administration brought adequate block of  $\text{Ca}^{2+}$ -permeable AMPARs to the brain areas involved in generation of the generalized tonic-clonic seizures in animals at P25, but not in the younger ones.

Originally, the site of generation of generalized tonic-clonic seizures has been localized into the brainstem (Browning and Nelson, 1985). In addition, spinal cord isolated from supraspinal structures was found to be able to generate generalized tonic-clonic seizures. Since, at least in adult animals, the brainstem exhibits low to moderate levels of expression of GluA2 subunit protein (Sato et al., 1993; Petralia et al., 1997), it can explain the effects of IEM1460 against generalized tonic-clonic seizures in older, P25 animals. However, the more recent studies (Brevard et al., 2006; Blumenfeld et al., 2009; Zhang et al., 2014) described that there is an increased activity in the anterior thalamus, retrosplenial cortex, dentate gyrus, cerebral cortex, basal ganglia or cerebellum just before or during the generalized seizure onset. This suggests that these brain areas may also contribute to the initiation and maintenance of generalized tonic-clonic seizure. Because in rats the process of maturation of the brain connectivity takes place mainly postnatally, our results can be explained by immaturity of the connections between individual brain structures important for generalized tonic-clonic seizures in animals at P12 and P18. On the other hand, the developmental profile of GluA2 expression differs from one brain structure involved in generation of the tonic clonic seizures to the other (Pellegrini-Giampietro et al., 1991; Pellegrini-Giampietro et al., 1997; Ho et al., 2007). These data demonstrating that the transmission between the mossy fibers and CA3 pyramidal cells uses a mixed population of  $\text{Ca}^{2+}$ -permeable and  $\text{Ca}^{2+}$ -impermeable AMPARs within the first 2–3 postnatal weeks (Ho et al., 2007) might suggest the explanation for our results. In addition, GABAergic interneurons of neocortex and hippocampus or dentate gyrus basket cells were described as ones exhibiting the high level of  $\text{Ca}^{2+}$ -permeability and low content of GluA2 subunit of AMPARs (Jonas et al., 1994; Geiger et al., 1995; Racca et al.,

1996; Pellegrini–Giampietro et al., 1997; Buldakova et al., 2007). There is a possibility that in our experiment, the IEM1460 had influence on  $\text{Ca}^{2+}$ -permeable AMPARs not only on excitatory neurons, but also on the GABAergic interneurons and basket cells leading to a decrease in inhibition. Consequently, we can imply that the lack of satisfactory effect of IEM1460 against generalized tonic–clonic seizures in P12 and P18 animals may be caused by the combination of several factors. Except from above–mentioned immaturity of the connections, the insufficient expression of the  $\text{Ca}^{2+}$ -permeable AMPARs and/or expression of these receptors on inhibitory neurons may play an important role.

The different development of  $\text{Ca}^{2+}$ -permeable and  $\text{Ca}^{2+}$ -impermeable AMPA receptor gated channels was demonstrated not only in individual brain parts (Kumar et al., 2002; Eybalin et al., 2004; Ho et al., 2007) but also in soma and dendrites of cortical pyramidal neurons (Kumar et al., 2002). These data might explain the irregular development of anticonvulsant action. As demonstrated in Kumar's study (Kumar et al., 2002), developmental “switch” in the somata of cortical pyramids takes place between postnatal days 13 and 21. Additionally, it was described in our study concerning evaluation of developmental expression profile of GluA2 subunit and study by Hsu et al., (2010), there is a significant increase in the proportion of neurons expressing  $\text{Ca}^{2+}$ -permeable AMPARs in the somatosensory cortex of rats between P 5 and P13. It is in agreement with findings of our electrophysiological experiments, showing that anticonvulsant effect against cortical afterdischarges is marked in rat pups aged P12 (Fig.6. and Fig.7.). Surprisingly, we have observed that IEM1460 administration had proconvulsant effect on ADs elicited in animals at their P18 and P25. Since ADs are of thalamocortical origin, it can be explained by the fact that the inputs from thalamocortical afferents activate inhibitory neurons more strongly than excitatory neurons triggering powerful feed–forward inhibition (Cruikshank et al., 2007). This thalamic activation of inhibitory interneurons occurs mainly via GluA2–lacking AMPA receptors (Hull et al., 2009). Therefore, we can suggest that selective blockade of calcium permeable AMPA receptors by IEM1460 at thalamocortical synapses can also decrease the level of activation of inhibitory neurons. Consequently, it can cause overall disinhibition, allowing a spread of excitation within thalamocortical circuits and leading to prolongation of ADs. However, the GluA2 mRNA within the thalamus is undetectable in P14 and P21 and in the adult animals (Pellegrini–Giampietro et al., 1991) suggesting that the  $\text{Ca}^{2+}$ -permeable AMPARs might be highly involved in the thalamocortical circuitry even in late phases of development.

Still, it stands in contrast to our recent results, showing that a noticeable level of both, mRNA as well as GluA2 protein is present in the thalamus of developing animals between P12 and P25. Additionally, the pyramidal cells of neocortex and hippocampus as well as granule cells of dentate gyrus exhibit low  $\text{Ca}^{2+}$ -permeability and more abundant mRNA of GluA2 subunit than GABAergic interneurons of neocortex and hippocampus or dentate gyrus basket cells (Jonas et al., 1994; Geiger et al., 1995; Racca et al., 1996; Pellegrini-Giampietro et al., 1997; He et al., 1998; Buldakova et al., 2007).

Glutamatergic synapses on inhibitory cells preferably use AMPARs, and these features can be found in different types of interneurons, i.e. in fast-spiking cells and low threshold spiking cells (Markram et al., 2004). Moreover, in the rat's neocortex, the currents mediated by GluR2-lacking AMPARs were not detected in the pyramidal-shaped neurons, but in some fast spiking interneurons already at P14 and P15 (Brill and Huguenard, 2008). So far, it was reported that IEM1460 is not able to affect inhibitory currents in pyramidal neurons of CA1 region of hippocampus. In this case, only the late components of EPSCs (excitatory postsynaptic currents) were strongly inhibited by this compound, suggesting the involvement of  $\text{Ca}^{2+}$ -permeable AMPARs in multi-synaptic excitatory pathways (Buldakova et al., 2007). Since the IEM1460 can also selectively block fast spiking interneurons (Gittis et al., 2011), it is probable that in our experiment this compound was able to influence cortical inhibitory neurons endowed with  $\text{Ca}^{2+}$ -permeable AMPAR leading to augmentation of cortical epileptic afterdischarges in animals at postnatal days 18 and 25. In original studies an intraperitoneal administration of IEM1460 in a doses of 1 and 3 mg/kg were used. Irregular relation between dose of IEM1460 and effect might be due to the fact, that the 20-mg/kg dose is too high and other effects of the drug may take place. Effect of IEM1460 against nicotine-induced seizures was explained by its action on cholinergic receptors (Gmiro et al., 2008) and this must be taken into account. Action on NMDA receptors described in vitro experiments is not probable because IEM1460 is one hundred times less active on these receptors than on AMPA receptors lacking GluA2 subunit (Bolshakov et al., 2005) and developmental profile of anticonvulsant action of IEM1460 substantially differs from that of antagonists of NMDA receptors (Mareš and Mikulecká, 2009). Anticonvulsant action demonstrated in the present experiments, markedly differ from effects of nonspecific AMPA receptor antagonists described previously in our laboratory – both NBQX and GYKI 52466 were active against cortical ADs in all three age groups of immature rats (Kubová et al., 1997, Mareš et al., 1997).

Our results indicate that the effects of IEM1460 on various types of seizures depend on their sites of origin in the brain, their developmental stage, and GluA2 subunit expression profile. To clarify the action of IEM1460 in developing brain, further studies are necessary.

***Influence of Ro 25–6981, a specific antagonist of NMDARs containing NR2B subunit on evoked potentials and cortical afterdischarges in developing rats.***

The results of this study indicate that at early stages of postnatal development (P12) Ro 25–6981 can significantly diminish physiological and pathological cortical excitability. In animals at P18 and P25, Ro 25–6981 significantly affected only the physiological interhemispheric responses evoked by single-pulse stimulation (but not the epileptic afterdischarges). This antagonist of NR2B/NMDARs (NMDA receptors containing NR2B subunit) exhibited clear anticonvulsant action against ADs in P12 animals. When the drug was administered after series of ADs, the activation-dependent anticonvulsant effect present in P12 animals was only outlined in the two older groups. For the purpose of our study, the drug was tested in order to determine how long its effects last in vivo. The test show, that in animals at P12, a single dose of Ro 25–6981 (3–mg/kg) is sufficient to affect the prolongation of ADs up to 110–min after drug administration (Fig.9.).

For our study, the Ro 25–6981 was chosen over another antagonist of NR2B/NMDARs ifenprodil, due to its higher affinity as well as activation-dependent action (Fischer et al., 1997; Burket et al., 2010; Di Maio et al., 2011; Lima–Ojeda et al., 2013). We decided to use 3–mg/kg dose, which was repeatedly shown not to impair cognitive or motor functions and improve task performance in behavioral experiments in adult animals (Boyce et al., 1999; Kosowski and Liljequist, 2004; Higgins et al., 2005; Kos et al., 2011). The lower, 1–mg/kg dose was chosen because of predominance of NR2B subunits in immature brain (Sheng et al., 1994; Wenzel et al., 1997).

Anticonvulsant action of Ro 25–6981 demonstrated in the present study is in full agreement with our earlier reports, presenting that ifenprodil display similar age-dependent effect against cortical epileptic afterdischarges (Mareš, 2014) as well as against pentetrazol-induced convulsive seizures (Mareš and Mikulecká, 2009) in immature rats. In our experiment, pretreatment with Ro 25–6981 resulted in markedly shortened ADs in P12 animals (Fig.10.), while ifenprodil was particularly effective in P15 animals (an age group not studied in the present experiment). In contrast to our present results, P18 and P25 animals pretreated with

ifenprodil expressed marked ADs prolongation during the repeated stimulations with the same, suprathreshold intensity. The outcomes of our experiment in which ADs were elicited by increasing intensities of the stimulation and the Ro 25–6981 was injected after several ADs (Fig.10.), are also similar to these received with ifenprodil. In the current study, Ro 25–6981 was significantly effective against ADs prolongation only in the youngest group of animals (P12), and its administration resulted in tendency to shorten ADs duration in the two older groups of animals. On the other hand, ifenprodil exhibited marked anticonvulsant action in P12, P15 and in P18 animals, but no such effects were observed in P25 animals. Properties of ifenprodil in P18 rat pups might be due to its less specific effect on NR2B/NMDARs and thus its influence on another target.

Since the cortical afterdischarges are of cortico–thalamo–cortical origin, the decrease or loss of the anticonvulsant action of both NR2B/NMDARs antagonists – ifenprodil (Mareš and Mikulecká, 2009; Mareš, 2014) and Ro 25–6981 (present results) – after the second postnatal week in rats fits with the developmental “switch” in synaptic NR2B/NMDARs to NR2A/NMDARs at thalamocortical synapses (Wenzel et al., 1997; Cull–Candy et al., 2001; Liu et al., 2004; Szczurowska and Mareš, 2013). Low efficacy of this compound to prevent ADs prolongation in older groups of tested rats is also in agreement with its failure to antagonize maximal electroshock seizures in adult mice even at very high doses (10 and 32 mg/kg, the 100–mg/kg dose afforded only minimal protection – Burket et al., 2010). Our results, indicating the anticonvulsant effect of Ro 25–6981 against ADs induced by repeated stimulation with increasing intensity (Fig.11.), is consistent with the *in vitro* ability of this compound to exhibit increased efficiency and faster onset of block of NMDARs at high concentration of agonist (Fischer et al., 1997).

As the GABAergic interneurons are responsible for the fine–tuning of cortical excitation and inhibition (Bacci et al., 2005; Suzuki et al., 2014), it is of great importance to mention that some types of GABAergic interneurons (neurogliaform (NG) cells and fast spiking (FS) cells) exhibit persistent firing. Persistent firing is an ability of interneurons to integrate excitatory inputs to the point that they can persistently fire even in the absence of the original stimulus (Suzuki et al., 2014). This phenomenon was observed not only in cell cultures, but also *in vivo* (especially in NG cells) and these interneurons may function as a activity–dependent “buffer” that can prevent the spread of neuronal hyperexcitability in the neocortex and hippocampus (Sheffield et al., 2011; Sheffield et al., 2013; Suzuki et al., 2014). The lack of significant prolongation of subsequent ADs in control P18 and P25 animals might

be explained by the phenomenon of persistent firing of GABAergic cortical interneurons. It fits with developmental profile of GABAergic interneurons properties, as these change dynamically throughout the development (Quilichini et al., 2012) and the process of interneuron maturation is very slow (it is incomplete even at P30 in mice) (Zhang, 2004; Le Magueresse and Monyer, 2013). Therefore, we can imply that marked ADs prolongation observed in control animals only at P12 is due to immaturity of GABAergic interneurons, and their ability of persistent firing.

The age specific effect of Ro 25–6981 was not observed in single pulse evoked potentials where all age groups were affected by the drug (Fig.8.). The effect of Ro 25–6981 in P25 group is confusing since only the 1–mg/kg dose (but not the 3–mg/kg dose) was able to reduce the amplitude of these responses. At present, we cannot fully explain this phenomenon. Nevertheless, the answer might be found in the NMDARs subunit composition and localization at the sites of transcallosal inputs in rat neocortex. It was reported that in pyramidal neurons of neocortical layer V, the excitatory postsynaptic currents arising from callosal inputs are carried out by NR2A/NMDARs and those arising from intracortical synaptic inputs are mediated by NR2B/NMDARs (Kumar and Huguenard, 2003; Köhr, 2006). Since the first waves of interhemispheric responses evoked by single pulse stimulation are monosynaptic transcallosal potentials (Tchekalarova et al., 2011), the significant influence of Ro 25–6981 on their amplitude in all age groups of tested animals was unexpected. One possible explanation might be in involvement of the second positive wave into measurement of amplitude – this wave is probably of intracortical origin. Another factor may be that postsynaptic sites at transcallosal inputs can carry both di- and triheteromeric NMDARs (containing NR2B subunit) and these can also be expressed extrasynaptically (Köhr, 2006).

Since the presynaptic NR2B/NMDARs and GABAergic cortical interneurons tightly cooperate in physiological modulation of excitation and inhibition, we need to acknowledge that they have an influence on amplitude of evoked potentials. The amplitude of evoked potentials in P18 and P25 control animals increase with increasing stimulation intensities; it suggests that persistent firing of GABAergic interneurons mentioned before do not contribute to this process, and another possibility has to be considered. It was demonstrated that NMDARs could be expressed at presynaptic sites of not only excitatory, but also of inhibitory neurons, where they can modulate presynaptic release and significantly contribute to inhibitory neuronal activity in hippocampus and neocortex (Conti et al., 1997; Corlew et al., 2007; Mathew and Hablitz, 2011). Presynaptic NMDA receptors are present on neocortical pyramidal

cells as well as on GABAergic nerve terminals in cerebellum and neocortex (DeBiasi et al., 1996; Paquet and Smith, 2000) where they modulate GABA release (Mathew and Hablitz, 2011). NMDA autoreceptor that are present on excitatory presynaptic terminals are likely to contain NR2B subunit and can facilitate glutamate release in rat entorhinal cortex (Berretta and Jones, 1996; Woodhall et al., 2001).

In the naïve brain, inhibitory synaptic transmission can be tonically modulated by activation of presynaptic NMDARs. These effects are developmentally regulated, and are particularly expressed at P12–15 but absent at P21–25 (Mathew and Hablitz, 2011). There is also developmental shift of the NR2B/NMDARs from the synaptic to extrasynaptic sites (Liu et al., 2004) and autoreceptor function seems to be reduced with development but not in the epileptic adults (Yang et al., 2006). Additionally, the loss of presynaptic NMDARs later in development might be related to switch in receptor subunits, to ones with a higher EC50 for glycine, or changes in glycine levels (Mathew and Hablitz, 2011). It is in agreement with the finding that saturating concentration of glycine for NR2A/NMDARs is higher than for NR2B/NMDARs (Li et al., 2008). It seems possible, that after stimulation with increasing intensities the autoreceptor function of presynaptic NR2B/NMDARs in P25 animals can be restored, as it was observed in epileptic adults (Yang et al., 2006). Hence, modulated by the NR2B/NMDARs, GABA release activity, which probably was brought back by high intensity stimulation, might not be blocked completely by the lower (1-mg/kg) dose of Ro 25–6981; therefore, it is possible that it caused the decrease in amplitude of single evoked potentials. Moreover, as in adult animals NR2B subunit is primarily expressed in the forebrain (Loftis and Janowsky, 2003), it is also likely that purely cortical activity can be affected by Ro 25–6981 even in the third and fourth postnatal week in rats.

Unfortunately, at the moment we have no explanation on the complete lack of effects of Ro 25–6981 on cortical potentiation or depression induced by paired pulse stimulation. The reason might be in relatively moderate intensity of stimulation (twofold threshold); in contrast, the effects on single-pulse responses were observed mostly at high stimulation intensities.

In conclusion, our results show that Ro 25–6981, a selective antagonist of NR2B/NMDARs, exhibits clear activation-dependent anticonvulsant action against epileptic afterdischarges (model of myoclonic seizures) only during the second postnatal week in rats. Consequently, we can imply that in older animals, receptors containing NR2B subunit do not contribute to generation of seizures in this model. Thus, Ro 25–6981 or other NR2B antagonists may represent a useful tool in pharmacotherapy of epileptic activity in immature

brain. This drug also significantly reduces the level of physiological excitability induced by single pulse stimulation of sensorimotor cortex in an age-independent manner and did not affect cortical excitability evoked by paired pulse stimulation. At the moment we cannot fully explain this in vivo action of Ro 25–6981 and further tests will be necessary. However, the level of activation of NMDARs containing NR2B subunit and their localization may play a role in the effects of Ro 25–6981 on cortical excitability.

***Developmental changes in expression level of the chosen subunits of NMDA and AMPA receptors in different brain regions of control animals.***

The results of the present study show that the gene and protein expression levels of GluA2 subunit of AMPARs as well as levels of expression of GRIN2A and GRIN2B genes encoding NR2A and NR2B subunits of NMDARs are changing dynamically throughout the development. These changes were detected not only in the in the different regions of neocortex but also in the deep brain structures, such as hippocampus and thalamus.

As for developmental changes in the levels of expression of GRIA2A gene and GluA2 protein, present results show that under control conditions, the age-related changes of GluA2 protein and its mRNA levels detected in different brain structures, seemed to take place independently from each other. In the whole neocortex (with an exception of CXFR), the expression of GRIA2A mRNA was generally increasing with age reaching its highest level at P18 and P25 (Fig.12–14, part a). Similar GRIA2A mRNA expression pattern was observed in HV (Fig.16a), HD (Fig.15a) and TH (Fig.17a), but its level was significantly increased later in development. As for GluA2 protein expression, while its highest levels in the CXFR (Fig.12a), CXPAR (Fig.13a) and CXOC (Fig.14a) were detected at P18, its expression in HV and TH was elevated mostly at P72 (Fig.18). With an exception of HV, the pattern of GluA2 protein expression in the above-mentioned structures at different postnatal ages was not similar to its mRNA levels. Present results stand in line with reports confirming that the overall expression level of GluA2 subunit is markedly increasing with age (Kumar et al., 2002) and this significant change occur during 2<sup>nd</sup> and 3<sup>rd</sup> week of postnatal life in rat cortical and hippocampal pyramidal neurons (Ho et al., 2007; Isaac et al., 2007). Additionally, our data support previous findings, showing that developmental switch from AMPARs lacking GluA2 to GluA2-containing AMPARs does not occur simultaneously in all parts of the central nervous system (Pellegrini-Giampietro et al., 1992, Tanaka et al., 2000; Eybalin et al., 2004; Talos et al., 2006; Szczurowska and Mareš, 2013; 2015). Additionally, since the AMPARs

present on the dendrites of cortical pyramidal neurons can exhibit characteristics of these GluA2-containing already at P6 (Brill and Huguenard, 2008; Hsu et al., 2010), it can explain different levels of the GluA2 protein expression detected in different regions of neocortex at the early stages of postnatal development. Similarly to results presented by the Pellegrini-Giampietro et al., 1992, displaying that most of the GluA transcripts are transiently overexpressed with a peak expression at about P14 in hippocampus (Standley et al., 1995), the results of the present study show that in both, HD and HV, the GluA2 protein level was markedly elevated at P12 and P15. As it has been detected (by means of immunocytochemistry), the expression of GluA2 and GluA3 AMPARs subunits in the TH of control animals is moderate with no major developmental changes (Spreafico et al., 1994). However, our data show that the GluA2 expression detected in the TH at P12 was relatively high and interestingly, mRNA and protein level of this subunit were significantly increased at P72. The developmental changes in the AMPARs subunit composition in the sensory relay synapses in the TH have a great influence on excitatory synaptic transmission as well as on strengthening and elimination of redundant inputs (Arsenault and Zhang, 2006). Therefore, our results showing that the GluA2 subunit expression level in immature and in adult TH is relatively high can indicate decreased calcium permeability of AMPARs. It is possible that this increased GluA2 subunit level is partially responsible for gating of sensory transmission through the thalamus (McCormick and Bal 1994, Liu 1997). Marked differences between expression profiles of GluA2 protein and GRIA2A mRNA may be explained by the fact that AMPARs lacking GluR2 may still be formed even in the presence of GluR2 mRNA (Greger et al., 2003; Cull-Candy et al., 2006).

As for the NMDARs, present results show that the expression levels of GRIN2A and GRIN2B genes encoding NR2A and NR2B subunits of NMDARs, changes greatly during development and their profiles differ among brain regions. Under control conditions, there was a general age-dependent increase in GRIN2A mRNA level and less pronounced developmental increase of GRIN2B mRNA levels detected in the majority of brain structures.

The GRIN2A mRNA expression levels detected in each neocortical region considered in this study were significantly increased especially at P18 and P72, similar expression pattern was observed in the HD (Fig.24a). Furthermore, the levels of expression of GRIN2A mRNA detected in the HV (Fig.25a) and TH (Fig.26a) of control animals were gradually increasing with age, with peak expression at P25 and P72. As for the GRIN2B mRNA, its expression pattern was similar in CXFR (Fig.21a), CXPAN (Fig.22a) and HD with its highest level

detected at P25, whereas in cortices isolated from adult rats, the GRIN2B mRNA level was lower than at other tested postnatal days. Interestingly, the mRNA expression of GRIN2B gene in the HV (Fig.25a) and CXOC (Fig.23a) was relatively stable throughout the development without any significant changes detected. The GRIN2B expression in the TH (Fig.26a) was increasing with its highest level revealed at P72, i.e. early in adulthood. With an exception of TH, developmental profiles of GRIN2A/GRIN2B mRNA ratio were very similar in the majority of the brain areas, and its highest values were detected at P18 and P72. The GRIN2A/GRIN2B mRNA levels detected in HV were gradually increasing with age reaching its highest values in early adulthood. Similar pattern was observed in HD but with additional increase in GRIN2A/GRIN2B ratio detected at P18.

These results stand in line with outcomes of the previous studies describing that at early stages of postnatal development there is little NR2A expression and NR2B predominates, but with cerebral maturation levels of NR2A increases more rapidly than these of NR2B (Monyer et al., 1994; Sheng et al., 1994). Regarding levels of the GRIN2A mRNA expression in the neocortex, these were significantly increasing with age reaching its highest expression level at P15 and at P25, what is consistent with data describing that NR2A expression reaches the peak at 2<sup>nd</sup> to 3<sup>rd</sup> postnatal week (Wenzel et al., 1997, Liu et al., 2004).

Developmental increase in GRIN2A/GRIN2B mRNA ratio revealed in our study, supports previously published data and indicates that preferential surge in GRIN2A expression may result in an age-related increase in the relative ratio of NR2A/NR2B (Cui et al., 2013). As reported by Quinlan et al. (1999) and Fox et al. (1999), the change in the NR2A/NR2B subunit ratio in the visual cortex of rats is altered by visual experience because it rapidly increases NR2A subunit protein level (Fox et al., 1999). Present results confirm it, and show that the GRIN2A mRNA level detected in the CXOC of control animals was increasing between P12 to P18, when rats open their eyes and start to explore their environment. It has been reported that timing of the developmental “switch” between NR2A and NR2B subunits of NMDARs observed in hippocampus, differs from other brain structures and occurs at least 7 days later than in thalamus or cortex (Guilarte and McGlothan, 1998). According to our data there were only minor differences between timing of the increase in GRIN2A mRNA level. It was evident only in HV and TH, where the highest GRIN2A levels were detected only at P25 and P72, while in all other structures high levels were found at P18. Similar developmental profile of GRIN2A mRNA levels detected in CXFR and TH can be explained by the fact that the somatosensory cortex and the thalamus are highly interconnected (Pandis et. al., 2006).

According to our data, the GRIN2A/GRIN2B mRNA ratio in TH in contrast to neocortex does not change significantly during development. Our results are similar to those of Wenzel et al. (1997) stating that GRIN2B mRNA levels increase in whole brain to reach a peak at P21 and then slightly decrease towards adulthood. Additionally, comparable expression profiles in the cortex and HD detected in the present study can be explained by the fact that gene expression in the HD correlates with cortical regions involved in information processing, whereas genes expressed in the HV correlate with amygdala and hypothalamus i.e. regions involved in emotion and stress (Fanselow and Dong, 2010).

By means of measured duration of NMDA-mediated currents, it was established that an increased NR2A/NR2B ratio is associated with a change in NMDAR properties (Flint et al., 1997). As it was recently described by Pandis et al. (2006), the lower NR2A/NR2B ratio would endow the NMDARs present in VH with different functional characteristics than these present in the DH, i.e. there is longer decay time of the NMDAR-mediated EPSPs in VH. It is consistent with our current results showing that at least in later stages of postnatal development the GRIN2A/GRIN2B ratio in HD is higher than that detected in HV. Yet, there is evidence that the signals of GRIN2A mRNA (in situ hybridization) in hippocampus are strong at the time of birth but its protein levels remain low at least up to P10 and it can be caused by posttranscriptional mechanisms that regulate NR2A protein synthesis (Wood et al., 1996; Wenzel et al., 1997; Szczurowska and Mares, 2013). Therefore, it is important to keep in mind that there is a certain delay in NR2A protein expression in relation to its mRNA levels. Furthermore, as it was shown by Yashiro et al. (2008), Chen et al. (2007), under conditions of low activity, NMDARs are able to stimulate local translation of NR2B subunits what can explain frequently reported differences between mRNA and protein levels of NR2B subunit.

### ***Changes in GRIA2A mRNA and GluA2 protein expression level in animals after LiCl/Pilo-SE induced at P12.***

The expression of genes and proteins encoding different subunits of AMPARs, change dramatically throughout the development and their normal ontogeny may be affected by pathological events such as severe seizures (Condorelli et al., 1994). The study of Koh and Jensen (2001) support the role of the changes in AMPARs subunit composition in epileptogenesis following early-life seizures. Since controlling and regulating the AMPARs subunits expression can potentially serve as a new manner of protection of the brain against damage induced by SE (Hu et al., 2012) it would be beneficial to determine the developmental profile of GluA2 subunit expression in distinct brain regions following early life SE.

To date, the majority of the research concerning modifications in gene and protein expression level occurring after seizures was focused on changes induced by LiCl/Pilo–SE itself and preferentially on hippocampus. Therefore, there is not enough information on LiCl/Pilo–SE induced changes in GRIA2A mRNA and GluA2 expression occurring in the brain structures other than hippocampus. In addition, there are almost no data considering influence of LiCl and paraldehyde that are commonly used in this animal model of status epilepticus on GRIN2A gene and GluA2 protein expression.

As the period of maximal seizure susceptibility in rats coincides with the 2<sup>nd</sup> and 3<sup>rd</sup> week of postnatal life (Sperber et al., 1999), present study was focused on the alteration in ontogeny of GluA2 protein and mRNA expression in different brain areas of rats subjected to LiCl/Pilo–SE or LiCl and paraldehyde at P12. Our results indicate that the LiCl/Pilo–SE induced at early stages of postnatal development as well as LiCl/Para, have strong influence on the mRNA and GluA2 protein expression later in life and these effects are brain region–specific.

The outcomes of this study show that, the level of the GRIA2A mRNA expression following LiCl/Pilo was changing independently to the level of GluA2 protein detected in the same brain areas (with an exception of HV). Additionally, the expression levels of mRNA and the GluA2 protein were highly affected not only in animals after LiCl/Pilo–SE induced at P12, but also in control group of animals that received injections of LiCl and paraldehyde. Present study show that in the majority of brain structures there is general tendency of GluA2 protein expression level to be decreased after both LiCl/Para and LiCl/Pilo–SE.

In the HV, the GluA2 protein expression was significantly decreased at almost all tested stages of postnatal development (Fig.19e; Fig.20), and there was a similar tendency for GRIA2A mRNA expression (Fig.16). Concerning the early changes in GluA2 subunit expression in the HV, our data stand in line with results of the previous studies by Grooms et al. (2000) and Hu et al. (2012) confirming that there is a marked decrease in GluA2 expression detected in the hippocampus after SE. It is also consistent with data stating that a general decrease in mRNA level of the (GluA–1–3) AMPARs subunits can be observed in the hippocampal layers after SE (Condorelli et al., 1994).

A substantial decrease in GluA2 protein level after LiCl/Pilo–SE was observed also in different brain regions tested in this study, but at various stages of postnatal development. Therefore, to some extent, presented data are similar to these describing that in the brains of the patients with refractory epilepsy, the level of GluA1/GluA2 AMPARs subunits ratio was

significantly increased (Talos et al., 2008; Sánchez Fernández and Loddenkemper, 2014, Loddenkemper et al., 2014). It was also observed in the cortex of rats following hypoxic–ischemic injury (Talos et al., 2006). Nevertheless, the animal data on SE–induced changes in GluA2 subunit expression in neonatal brain are controversial. While some of these are comparable to human data (Grooms et al., 2000; Hu et al., 2012), other reports indicate an increase in GluA2 subunit expression level (Porter et al., 2006).

Present results also show similar variability and the developmental expression levels of GRIA2A mRNA and its protein may be significantly higher following LiCl/Pilo–SE induced at P12 than its control values in some brain regions. In general, with an exception of CXFR (Fig.12) and HD (Fig.15), a significant increase in GRIA2A gene expression detected after LiCl/Pilo–SE was not reflected by changes in GluA2 subunit protein expression. The GRIA2A mRNA level was significantly increased in each of tested neocortical regions, but only at P18, i.e. 6 days after the LiCl/Pilo–SE (Fig. 12–14), while in the HD, the increase in GRIA2A mRNA expression was evident only at P72, i.e. early in adulthood (Fig.15). As for GluA2 protein expression in the neocortex, its increased level was detected only in the CXFR at P18, however, at the same postnatal day its expression was also markedly increased in HD. In addition, both, the GRIA2A mRNA and GluA2 protein level detected in HD were markedly increased even early in adulthood, at P72 (Fig.19a,d). As for the changes detected in the HD, our data confirm that the GRIA2A mRNA expression in the hippocampus changes during two weeks following SE prior to the development of spontaneous seizures (Roch et al., 2002; Raol et al., 2003). An increased GluA2 level was also reported in the hippocampus of young and adult animals (Condorelli et al., 1994; Porter et al., 2006; Hu et al., 2012) and in the post-mortem hippocampus samples from epileptic patients (Suzuki et al., 2000; Russo et al., 2013). Simultaneous changes in GluA2 subunit expression occurring in the CXFR and HD detected in the present study can be explained by the fact that convulsive seizures involve both hippocampus and neocortex (Sankar et al., 1998; Kubová et al., 2004).

Since a decreased expression of GluA2 subunit is thought to occur just before neuronal degeneration (Hanada, 2014), it may explain the fluctuations in developmental levels of GluA2 protein expression detected after P12 LiCl/Pilo–SE. The reduction in GluA2 level might serve as a “molecular switch”, leading to the arrangement of Ca<sup>2+</sup>–permeable AMPA receptors and enhancing the toxicity of endogenous glutamate following a neurological insult (Hu et al., 2012). Present results show that significant decrease in the GluA2 subunit expression in HV may be detected already 3h after the SE. As the GluA2 level in HV remained decreased

throughout the development, it suggests that this structure is highly prone to increased excitability. It is highly probable since the anterior hippocampus in humans (that corresponds to the HV in rodents) appears to be the most common site of seizure generation (McDonald et al., 1991). In addition, it is important to mention that the mRNA and protein expression profile of GluA2 subunit presented in this study differs substantially between HV and HD, and as it was previously reported, these marked dissimilarities between these two hippocampal poles confirm their different function and connectivity (Pandis et al., 2006).

An explanation for an increased level of GluA2 protein in the HD can be that the hippocampus, especially during immature stages, has the ability to activate the endogenous anti-epileptic mechanism to maintain the balance between excitation and inhibition after SE, and consequently reduce brain damage (Porter et al., 2006). It is possible that the high levels of GluA2 subunit expression in HD not only at P18 (i.e. 6 days after LiCl/Pilo-SE was induced) but also much later at P72 (60 days after LiCl/Pilo-SE) is related to elevated excitability that triggers mechanisms decreasing AMPARs calcium permeability via upregulation of GluA2 protein expression. Decrease in  $\text{Ca}^{2+}$ -permeability of AMPARs, can inhibit excessive  $\text{Ca}^{2+}$  influx and protect neurons from excitotoxic injury (Liu et al., 2004; Porter et al., 2006).

It has been reported that AMPARs containing GluA2 subunit are highly expressed at cortico-thalamic synapses (Salt and Eaton, 2006), but under control conditions their expression does not change dramatically throughout the development (Spreafico et al., 1994). The results of the present study confirms that the GRIA2A mRNA level in the TH of control animals, does not change significantly throughout the development (Fig.17a) however, there were some minor developmental changes detected in GluA2 protein levels (Fig.18f). On the other hand, there was a significant increase in GRIA2A mRNA expression after the LiCl/Pilo-SE detected in TH of P25 animals (Fig.17a) and decrease in GluA2 protein level in the TH at P15 (Fig.19f). As demonstrated by Druga et al. (2005), the severity of damage in majority of involved thalamic nuclei reach a peak at 12–24 h after LiCl/Pilo-SE. In this study, slightly elevated (but not significant) GluA2 protein level detected in the TH already 3h after the insult could be explained by the fact that increased GluA2 subunit expression is an immediate response to increased excitability (Porter et al., 2006). Present data also show that there was a significant decrease in GluA2 protein level detected in TH at P15 (3 days after LiCl/Pilo-SE). It stands in line with report stating that after LiCl/Pilo-SE in P12 animals, the degree of thalamic injury was markedly increased at P15 (Druga et al., 2005). Interestingly, at the same postnatal day, similar but still not significant changes were detected in the CXFR, while a

marked decrease in GluA2 level was detected in CXPAN and CXOC at P18, i.e. 6 days after LiCl/Pilo-SE.

The thalamus is involved in the regulation of the seizure threshold, pathogenesis of absence seizures, synchronization of seizure activity between limbic structures and the cortex as well as in the amplification and distribution of limbic seizures (Bertram et al., 1998; Druga et al., 2005). The neocortex and thalamus are highly and reciprocally interconnected and maturation of these connections plays a major role in establishing gating of sensory transmission through the thalamus (Liu, 1997; Price et al., 2006; Takeuchi et al., 2012). It has been reported that all of the dorsal thalamic nuclei interconnection with cerebral cortex is based on excitatory neurotransmission (Macchi and Jones, 1997) and therefore, LiCl/Pilo-SE induction can trigger a massive excitotoxic damage of this structure (Costa et al., 2004). According to our data the developmental changes in GluA2 protein levels detected in the TH are very similar to these in CXFR, it is therefore possible that excitotoxicity may spread from the thalamus to cortex and vice versa, causing the fluctuations in the GluA2 protein expression level.

The expression of GluA2 subunit is strongly influenced at the levels of transcription as well as translation. The GRIA2A transcription is regulated by positive and negative elements in the 5' proximal region of the promoter, while the mRNA translation efficiency can be regulated by structural motifs residing in the 5' or 3' untranslated regions (UTRs). Therefore, the differences observed between GluA2 subunit mRNAs and its protein expression might be clarified by the alterations in transcriptional and translational regulation, indicating a possible widespread uncoupling between the two functions (Tebaldi et al., 2012; Russo et al., 2013). The molecular mechanism explaining changes in GluA2 expression after neuronal insult has been described, and it involves positive regulatory elements such as NRF-1 (Nuclear respiratory factor 1) that is transcription factor important for regulating genes of cytochrome oxidase, and Sp1 (specificity protein 1) transcription factor located to 5' proximal region of the promoter. The promoter silencer element, RE1/NRSE (RE1-Silencing Transcription factor (REST)) that represses transcription by binding a DNA sequence element called the neuron-restrictive silencer element (NRSE) is also involved (Traynelis et al., 2010). The NRF-1 transcription factor binds to the GRIA2A promoter that is associated with acetylated H3 and H4 histones what is a critical step for GRIA2A transcription in neurons (Myers et al., 1998). Suppression of GluA2 expression occurs by binding of the GRIA2A RE1/NRSE element to the REST/NRSF repressor, which in turn recruits histone deacetylase (HDAC) complexes to the

GRIA2A promoter, resulting in chromatin remodeling and decreased expression (Traynelis et al., 2010). As for the translation of GluA2 mRNA, it is repressed at the initiation step, by the long forms of 5'UTRs and 3'UTRs. The transcripts of GRIA2A mRNA bearing the short 3'UTR are mostly associated with actively translating ribosomes, although the mRNAs of GluA2 that contain the long 3'UTR are generally retained in translationally inactive complexes of messenger ribonucleoprotein (mRNP) (Irier et al., 2009 a, b).

It has been reported that LiCl/Pilo-SE as well as kainate (Jia et al., 2006) can act in a manner of REST binding to RE1 element, causing histone deacetylation which in turn results in GRIA2A and GluA2 downregulation (Huang et al., 2002). Similarly, the ischemia episode can trigger the expression of the REST repressor and consequently reduces GluA2 expression in CA1 neurons that are destined to die (Calderone et al., 2003). Moreover, as mentioned above, decreased expression of GluA2 subunit is thought to occur just before neuronal degeneration (Hanada, 2014). These mechanisms can serve as an additional explanation for a marked decrease of GRIA2A mRNA (CXOC and CXPARG) and GluA2 protein levels (CXPARG, HV, TH) detected in this study after LiCl/Pilo-SE.

On the other hand, ischemic preconditioning can prevent the downregulation of GRIA2A in neurons, and it is thought to be caused by inhibition of REST expression in these same neurons (Calderone et al., 2003). In addition, some recent reports indicate that increased excitability is temporally associated with a rapid increase in GluA1 and GluA2 expression after seizures (Rakhade et al., 2008), and the translation of GluA2 mRNAs with the long 3'UTRs in rat hippocampus is relieved shortly after seizures (Irier et al., 2009 a, b).

It is possible, that similarly to ischemic preconditioning, early-life LiCl/Pilo-induced SE can trigger analogous mechanisms resulting in increased GRIA2A expression that can be detected at different stages of postnatal development. These mechanisms may serve as an explanation of a significant increase in the expression of GRIA2A detected in the neocortex of P18 rats. Moreover, because LiCl/Pilo-SE leads to generation of spontaneous seizures later in life, it can also explain a marked increase in GRIA2A mRNA level and GluA2 protein detected in HD, 60 days after LiCl/Pilo-SE.

Present study show also that the expression levels of mRNA and the GluA2 subunit protein of AMPAR were highly affected not only by LiCl/Pilo-SE, but also by an administration of LiCl at P11 followed by injection of paraaldehyde at P12. In general, the changes in expression of GRIA2A gene induced by the LiCl/Para administration were identified as a significant increase in GRIA2A level when compared to saline controls. There

was only one significant example of the opposite effect of LiCl/Para injection (compared to SE) on GRIA2A mRNA expression level in CXOC at P25 (Fig.14). As for the overall influence of LiCl/Para on developmental GluA2 subunit protein level, it was quite variable, and the detected significant changes were due to both, marked decrease as well as increase in this protein expression.

Lithium is broadly used as a treatment of manic–depressive disorder but at early stages of postnatal development, it has severe adverse effects on morphogenesis (Vezzani, 2009). In the LiCl/Pilo model of status epilepticus, activation of M1 muscarinic receptor by pilocarpine leads to hydrolysis of phosphatidylinositol bisphosphate (PIP<sub>2</sub>), generation of DAG and IP<sub>3</sub> and subsequent release of calcium from intracellular stores. As a result, a high intracellular Ca<sup>2+</sup> concentration promote release of glutamate (Smolders et al., 1997). The LiCl mechanism of action is thought to depend on the depletion of an endogenous source of inositol via inhibition of inositol monophosphatase (IMPase) that dephosphorylates the isomers of inositol monophosphate to produce inositol (Klein and Melton, 1996). Since the IMPase is recycling IP<sub>3</sub> to produce PIP<sub>2</sub>, LiCl administration can increase IP<sub>3</sub> concentration and therefore increase calcium release. It is probably why it has been demonstrated that the dose of pilocarpine used to induce SE can be decreased by about 10–fold and substantially improve survival of laboratory animals if the LiCl was used prior to pilocarpine injection. It is also known, that LiCl facilitates Pilo–induced seizures by activation of mononuclear cells and T–lymphocytes. Pilocarpine it thought to be poorly permeable through the blood–brain–barrier (BBB), however after the injection of 3mEq/kg of LiCl alone, the changes in peripheral markers of inflammation were detected, similar to these observed after full convulsant dose (350 mg/kg) of pilocarpine. Additionally, profound changes in peripheral immune cells were found 3 and 20 hours after LiCl injection and these accompanied by an increase in 4–5Hz EEG activity and a pronounced spike amplitude (Marchi et al., 2007). Early after pilocarpine or LiCl alone, the elevated levels of IL–1 are observed in the serum what is considered consistent with BBB breakdown. This allows pilocarpine to leak through BBB in order to stimulate muscarinic receptors in the brain (Vezzani et al., 2009).

Paraldehyde is used in the clinic for treatment of acute tonic–clonic convulsion, but only when other anticonvulsants (benzodiazepines, phenytoin, and phenobarbital) have failed to stop seizures (Rowland et al., 2009). In human paraldehyde exhibit low toxicity when used at appropriate dose (0.3 ml/kg) and it can terminate over 60% of episodes of prolonged seizures within 10 min, however an exact mechanism of paraldehyde action remains unknown

(Rowland et al., 2009). Paraldehyde was used in present study in order to efficiently arrest behavioral seizures induced by Pilo-injection. However previous records show that the electrographic seizures in animals subjected to LiCl/Pilo-SE can be suppressed only transiently (Druga et al., 2005), and short ictal episodes in EEG could be recorded for up to 10–16 h after the beginning of SE (Kubová et al., 2004).

As the known LiCl mechanism of action directly involves intracellular signaling, it is highly probable that it can have also marked influence on gene and protein expression. Additionally, in our study LiCl was administered 24h before paraldehyde was injected, therefore, it is possible that the changes in GluA2 mRNA and protein expression level observed in LiCl/Para treated control animals were primarily caused by LiCl and combination of both compounds probably strengthen this effect.

Because the GRIA2A mRNA and GluA2 protein level were significantly affected, it suggests that LiCl/Para treatment had strong influence on both the transcriptional and translational mechanisms. Additionally, if LiCl/Para administration can cause marked increase in GRIA2A mRNA levels, there might be some tendency to activate neuroprotective mechanism that could decrease AMPARs  $Ca^{2+}$ -permeability via increasing GluA2 protein transcription. Since there is only little information on LiCl and Para mechanisms of action, it is almost impossible to compare our data with previous publications.

In conclusion, present study show that the LiCl/Pilo-SE induced at P12 may result in dramatic changes of GRIA2A mRNA and GluA2 protein expression that can persist throughout the development and may be detected even in adulthood. Based on the previously published data, increased GluA2 subunit expression is an immediate response to increased excitability and can serve as an early neuroprotective mechanism decreasing  $Ca^{2+}$ -permeability (Porter et al., 2006). Therefore, an increased levels of GluA2 subunit detected at P18 in CXFR and HD indicate both, increased excitability and that these structures are able to activate endogenous antiepileptic mechanism to maintain the balance between excitation and inhibition after SE. In addition, marked increase in this subunit expression observed in HD even at P72 indicates that this structure exhibits this ability even later in life. On the other hand, since a decreased expression of GluA2 subunit is thought to occur just before neuronal degeneration (Hanada, 2014), a general decrease in GluA2 expression level detected in the HV following LiCl/Pilo-SE might suggest that neurons of this structure are disposed to excitotoxic neuronal death not only during development but also in adulthood. In addition, present study show that LiCl and

paraldehyde administration had a strong influence on GRIA2A gene, and GluA2 protein developmental expression levels.

Still, the developmental alterations of GRIA2A mRNA expression after LiCl/Pilo–SE are irregular and differ from GluA2 protein expression profile. The differences in changes of GRIN2A mRNA and GluA2 protein level occurring in the different brain structures are quite controversial and to some extent might be explained by the fact that the GluA2 protein synthesis can be downregulated even if the GRIA2A mRNA is present (Greger et al., 2003; Cull–Candy et al., 2006). The lack of statistically significant changes in GluA2 subunit mRNA expression observed in different brain regions is possibly due to the cellular heterogeneity of the tissue samples (Sloviter et al., 2003; Porter et al., 2006). Furthermore, it has been also reported that in immature rats, even after a single episode of kainate–induced seizures results in a loss of total GluA2 subunit in the rat hippocampus and these changes may be undetectable when studying global tissue levels of expression (Cornejo et al., 2007).

***Developmental changes in the levels of expression of GRIN2A and GRIN2B genes encoding NR2A and NR2B subunits of NMDARs in animals after LiCl/Pilo–SE induced at P12***

It is well documented that the seizures that occur early in life may cause serious alterations in the expression levels of these NMDARs subunits. Since the manipulation with the genes and proteins expression of different NMDARs subunits can serve as a tool in preventing increased excitotoxicity and epileptogenesis, it is important to determine the developmental changes in NR2A and NR2B subunits expression occurring after early–life seizures. The majority of up to date reports concerning seizure–induced alterations of NMDARs subunits expression were focused on changes in hippocampus and limbic structures (Haut et al., 2004; Ekstrand et al., 2011; Toyoda et al., 2013). There is also growing evidence about changes induced by SE in the neocortex (Silva et al., 2005; Gashi et al., 2007; Fernandez and Loddenkemper, 2014). However, most of the researches concerning developmental changes in NR2A and NR2B subunits expression after LiCl/Pilo–SE were performed considering the neocortex as a whole structure without distinguishing between separate cortical areas.

In our study, we present analysis of developmental changes in expression levels of the genes encoding NR2A and NR2B subunits GRIN2A and GRIN2B in six different brain regions including: CXFR, CXPAN, CXOC, HD, HV and TH that were detected 3, 6, 13, and 60 days after LiCl/Pilo–SE was induced at P12. An additional control group of animals that received LiCl and paraldehyde at P11 and P12 (respectively) was also included. These data may provide

comprehensive information on developmental changes in expression levels of GRIN2A and GRIN2B genes caused by P12 LiCl/Pilo–SE and describe influence of early administration of LiCl and paraldehyde on expression of these genes throughout the brain.

The results of the present study are in agreement with data indicating that the developmental expression level of the GRIN2A and GRIN2B genes encoding proteins of NR2A and NR2B subunits are highly affected by LiCl/Pilo–SE induced at early stages of postnatal development. Interestingly, our data show that the expressions of genes of interest are also highly affected by administration of LiCl and paraldehyde, and these effects are very similar to those caused by LiCl/Pilo–SE.

The LiCl/Pilo–SE induced early in life as well as injection of LiCl/Para resulted in a decrease of developmental levels of GRIN2A mRNA expression that was significant in almost all tested brain regions. A substantial decrease in GRIN2A mRNA level occurring in CXPAR, CXOC and HD was detected especially 3–6 days after SE, while the marked decrease in CXFR and TH was evident later in life, i.e. at P72. In the brain of animals subjected to LiCl/Pilo–SE, the expression profile of GRIN2B gene was increased throughout the development, and similar tendency was observed in LiCl/Para control animals. This effect of LiCl/Pilo–SE on GRIN2B mRNA expression was observed in all tested brain areas except the TH (Fig.26c). In the majority of analyzed brain areas, an increase in GRIN2B mRNA level was especially significant 3 to 6 days (P18) after both, the LiCl/Pilo–SE or LiCl/Para injections. Due to these changes induced by LiCl/Pilo–SE, the GRIN2A/GRIN2B mRNA ratio was significantly decreased in all brain structures and at all postnatal days considered in this study.

Present data are similar to these showing that there is a marked reduction in expression of NR2A subunit occurring in the hippocampus of P10 rats after tetanus toxin–induced seizures, as well as in the whole brain of P9–P13 mice after flurothyl–induced seizures (Swann and Le, 2007; Fernandez and Loddenkemper, 2014). It has been described that the age–dependent increase in NR2A level can accelerate EPSCs in visual cortex (Roberts and Ramoa, 1999), parietal cortex (Kew et al., 1998), hippocampus (Gottmann et al., 1997) and other brain structures (Giza et al., 2006). Since developmental levels of the NR2A subunit expression correlates with changes of NMDARs biophysical properties in multiple brain structures. The decreased levels of GRIN2A mRNA indicate that P12 LiCl/Pilo–SE may markedly change NMDARs properties in different brain structures, and thus probably slow down normal brain development.

It has been demonstrated that the levels of GRIN2B mRNA were markedly increased in the pyramidal neurons of hippocampus from epileptic patients (Casillas–Espinosa et al., 2012) and the NR2B/NMDARs were restored in the adult brain of patients with TLE (Di Maio et al., 2013). The results of the present study supports these previously published data, since after a single episode of LiCl/Pilo–SE the developmental levels of GRIN2B mRNA expression were highly increased.

The LiCl/Pilo–SE that cause NMDARs hyperactivation can subsequently activate NADPH oxidase (NOX) and NMDAR–independent ERK1/2 phosphorylation, that may lead to early upregulation of NR2B subunit (Di Maio et al., 2011). The mechanism of GRIN2B mRNA transcription depends also on association of GRIN2B promoter with numerous regulatory elements such as Sp1 and CREB (cAMP response element–binding protein), a transcription factor capable of binding CRE (cAMP response elements) and the calcium–responsive transactivator (CREST), protein that regulate gene expression (Traynelis et al., 2010). It has been demonstrated that the GRIN2B gene expression level can rapidly increase in response to the blockade of GABA<sub>A</sub> receptors and stimulation of NMDARs induced by bicuculline (Qiu and Ghosh, 2008). It is probably due to an increasing activity of CRE binding protein, decreased HDAC activity, and increased association of the GRIN2B promoter with acetylated histones (Traynelis et al., 2010). Based on this information, it is possible that early–life LiCl/Pilo–SE induced hyperactivation of NMDARs and subsequent activation of NOX and ERK1/2 phosphorylation caused a rapid increase in GRIN2B mRNA expression. Marked increase in GRIN2B mRNA level detected in this study at P18–P25, may indicate that the LiCl/Pilo–SE induced hyperactivation of NMDARs endure preferentially 6–13 days following seizures.

In addition, since increased NR2B subunit expression is characteristic for developing neurons and its increased levels are observed even in adult epileptic brain, it is possible that recurrent seizures observed in adult epileptic patients with history of early–life SE, could be caused by mechanisms similar to those characteristic for immature brain (Holmes et al., 2002). Our data can confirm it, as an increased GRIN2B expression was detected even in the HV of adult animals subjected to LiCl/Pilo–SE at P12. Since an increased expression of GRIN2B mRNA could be detected in HV even in adulthood, it suggests that this hippocampal pole is particularly affected by LiCl/Pilo–SE.

The GRIN2A/GRIN2B mRNA expression ratio was significantly decreased following P12 LiCl /Pilo–SE. It was detected at all stages of postnatal development and in all brain

regions considered in this study. Present data support findings of Gashi et al. (2007) showing that in adult animals with a history of early life flurothyl- or pilocarpine-induced seizures, the GRIN2A/GRIN2B ratio in cortical areas was markedly decreased, yet our data indicate that this ratio was significantly decreased not only in neocortex but also in the HV, HD and TH. The distinct functions of HD (cognitive functions) and HV (emotion and affection) are determined by different connectivity of these hippocampal parts (Fanselow and Dong, 2010). It has been demonstrated that after seizures the ratio of NR2A/NR2B subunits in VH was lower than in HD suggesting different NMDAR function in these two hippocampal parts (Papatheodoropoulos et al., 2005; Pandis et al., 2006). However, in the present study, the GRIN2A/GRIN2B mRNA ratio after P12 LiCl/Pilo-SE was similarly decreased in both parts of the hippocampus. Furthermore, present results are consistent with data obtained from the patients after SE, showing that GRIN2B gene expression was markedly increased, resulting in significantly decreased ratio of GRIN2A/GRIN2B mRNA (Fernandez and Loddenkemper, 2014). In general, the NR2A/NR2B expression ratio can determine the level of neuronal excitability, and the lower GRIN2A mRNA levels in comparison to GRIN2B can influence the NMDARs-EPSCs and decrease threshold for seizures (Papatheodoropoulos et al., 2005). Since in the developmental profile of GRIN2A/GRIN2B mRNA ratio following early life- seizures was markedly decreased in all tested brain areas, we can imply that it can reflect increased excitability that can persists until adulthood.

The results of this study support previously reported data showing that the early-life seizures can partially reverse normally occurring age-dependent changes in NMDARs subunits expression not only in the hippocampus (Toyoda et al., 2013), but also in the cortex and thalamus (Fernandez and Loddenkemper, 2014). Consequently, we can imply that our data provide confirmation for previously described developmental changes in GRIN2A and GRIN2B expression induced by LiCl/Pilo-SE, and show that these changes occurred at similar stages of postnatal development especially in neocortex. In addition, since an increased level of GRIN2B is associated with higher activity of NMDARs, its significantly increased level detected in HV of P72 SE animals might indicate that early life seizures can highly affect this structure in terms of increasing its level of excitability, and it may last up to adulthood.

The developmental expression of GRIN2B mRNA described in our study was highly affected also in the brain of LiCl/Para-control animals similarly as after Pilo-SE. As it was mentioned above, it is possible that the process of transcription can be highly affected by combination of LiCl and paraldehyde, and it may explain mentioned alterations in GRIN2A

and GRIN2B mRNA expression levels. As the effects of both LiCl/Pilo–SE and LiCl/Para on the GRIN2A and GRIN2B genes expression levels were very similar, it is possible that LiCl/Para administration alone has comparable or even stronger influence on neuronal excitability than LiCl/Pilo–SE.

It seems likely that the single episode of early–life LiCl/Pilo–SE can markedly affect normal brain ontogeny by increasing expression of GRIN2B and decreasing expression of GRIN2A gene encoding even at later stages of postnatal development. Nevertheless, it is important to mention that at least NR2B subunit protein can be selectively removed from the synapses, for example by proteolysis (Ehlers 2003; Jurd et al., 2008), therefore it is needed to keep in mind that the expression of NR2A and NR2B subunits proteins might differ substantially from these of GRIN2A and GRIN2B genes.

## **Conclusions**

Since it is well established that NR2B/NMDARs are dominant in the highly excitable developing brain, it was hypothesized that blockade of NR2B subunit containing NMDARs at early stages of postnatal development by selective antagonists might represent age–specific anticonvulsant treatment. One of the aims of the present study was to analyze the influence of one of specific antagonists these receptors, (Ro 25–6981) on physiological and pathological brain activity in immature rats.

The results of this study indicate that the Ro 25–6981 can significantly diminish physiological and pathological cortical excitability at early stages of rat postnatal development. Analysis shows that this drug significantly reduces the level of physiological excitability induced by single pulse stimulation of sensorimotor cortex in an age–independent manner but did not affect cortical excitability evoked by paired pulse stimulation. We cannot fully explain this in vivo action of Ro 25–6981 and further tests will be necessary. On the other hand, this antagonist exhibits activation–dependent anticonvulsant action against cortical epileptic afterdischarges (model of myoclonic seizures) which can be detected only during the second postnatal week in rats. Consequently, we can imply that in older animals, receptors containing NR2B subunit do not contribute to generation of seizures in this model. Therefore, Ro 25–6981 or other NR2B antagonists may represent a useful tool in pharmacotherapy of epileptic activity in immature brain.

Another hypothesis raised in the present study was that  $\text{Ca}^{2+}$ –permeable AMPARs (lacking GluA2 subunit) highly represented in immature brain, could serve as another target for age–specific anticonvulsant treatment. Consequently, the aim of this part of the research was to

determine the effect of IEM 1460 (an antagonist of  $\text{Ca}^{2+}$ -permeable AMPARs) on epileptiform activity induced in developing brain using two models of seizures with different sites of origin in the brain. The IEM1460 was tested in seizures induced by pentetrazol as well as in cortical epileptic afterdischarges.

The IEM1460 significantly delayed the onset of PTZ-induced minimal clonic seizures (with site of origin in basal forebrain area and cerebral cortex) at all tested stages of postnatal development. As for generalized tonic-clonic seizures (site of origin is a brainstem with subsequent involvement of other brain structures), the IEM1460 administration suppressed the generalized tonic-clonic seizures, but only in 18- and 25-day-old animals. On the other hand, the anticonvulsant effect of IEM1460 against cortical afterdischarges was marked only in P12 rat pups, but its effect was clearly proconvulsant in P18 animals. It does not have any effect on ADs in adult animals. Presented data indicate that the effects of IEM1460 on various types of seizures greatly depend on its dose, age of animals, site of the brain where the seizures are generated and on expression profile of GluA2 subunit. In addition, this compound might block  $\text{Ca}^{2+}$ -permeable AMPARs located to cortical interneurons what can lead to profound disinhibition and consequently cause augmentation of seizures. To clarify the action of IEM1460 at different stages of brain development, further studies are necessary.

AMPA and NMDARs that have a great influence on normal neuronal excitability and their subunit composition change dynamically during development together with their properties. Severe seizures, such as status epilepticus, can greatly affect developmental expression of these subunits (NR2A and NR2B subunits of NMDARs and GluA2 subunit of AMPARs), and therefore change neuronal excitability and influence normal brain ontogeny. One more of raised hypothesis was, that the LiCl/Pilocarpine-status epilepticus induced in immature brain, bring change not only to developmental expression levels of NR2A and NR2B (NMDARs) and GluA2 (AMPARs) subunits, but these changes can also persist in adulthood. Therefore it was important to determine how normal ontogeny of NR2A and NR2B (NMDARs) as well as GluA2 (AMPARs containing GluA2 subunit are in general  $\text{Ca}^{2+}$  -impermeable) subunits expression is affected in the brain after early onset (P12) of status epilepticus induced by LiCl/Pilocarpine, and if these changes can be detected later in life. In order to reveal these changes, the qPCR and Western Blotting techniques were used to study the levels of expression of the chosen subunits in six selected brain regions at five time intervals following P12 LiCl/Pilo-SE. Moreover, to determine the effects of LiCl and

paraldehyde that are commonly used in this SE model, an additional control group of animals that received only LiCl/Para, was included.

Present study show that the LiCl/Pilo–SE induced at P12 may result in dramatic changes of GRIA2A mRNA and GluA2 protein expression that can persist throughout the development and may be detected even in adulthood. Based on the previously published data, increased GluA2 subunit expression is an immediate response to increased excitability and can serve as an early neuroprotective mechanism decreasing  $Ca^{2+}$ -permeability (Porter et al., 2006).

In conclusion, it is possible to state that an increased levels of GluA2 subunit detected at P18 in the frontal cortex and the dorsal hippocampus indicate increased excitability and that these structures are able to activate endogenous anti–epileptic mechanism to maintain the balance between excitation and inhibition after SE. In addition, marked increase in this subunit expression observed in the dorsal hippocampus even at P72, designate that this structure exhibit this ability even later in life. On the other hand, since a decreased expression of GluA2 subunit is thought to occur just before neuronal degeneration (Hanada, 2014), a general decrease in GluA2 expression level detected in the ventral hippocampus following LiCl/Pilo–SE may suggest that neurons of this structure are prone to excitotoxic neuronal death not only during development but also in adulthood. In addition, present study provides evidence that LiCl and paraldehyde administration to control rats had a strong influence on GRIA2A gene, and Glu2A protein developmental expression levels. Based on the results of the present study, we can confirm that the developmental expression level of GRIN2A and GRIN2B genes that encode NR2A and NR2B subunits of NMDARs are highly affected by LiCl/Pilo–SE induced at early stages of postnatal development. Present results stand in line with data showing that after LiCl/Pilo–SE induced at P12, an expression of GRIN2A gene is decreased, while expression of GRIN2B gene is markedly increased in the majority of the brain structures.

In conclusion, LiCl/Pilo–SE induced early in life caused decrease of the GRIN2A mRNA level in all tested brain regions, but it was significant only at different stages of postnatal development. It is possible that this decrease in expression of GRIN2A mRNA will be responsible for diminished levels of NR2A subunit protein, which may result in major alterations of the NMDARs kinetics in terms of affecting NMDA–dependent EPSCs. As for NR2B subunits, it seems that the single episode of P12 LiCl/Pilo–SE can markedly affect neuronal maturation by increasing developmental levels of the GRIN2B gene, encoding NR2B subunit throughout the brain. Present results are also suggesting that hyperactivation of

NMDARs by the Pilo–SE induced early in life, can cause a rapid and significant increase of the GRIN2B gene and consequently NR2B protein expression especially 3 to 13 days after the seizures. Since in HV this increased expression of GRIN2B was detected even in adulthood, it suggest that a single episode of the early-life SE might have long lasting consequences in terms of delaying normal brain maturation and that the ventral hippocampus is particularly affected. In addition, administration of LiCl and paraldehyde at early stages of postnatal development has similar influence on normal developmental profile of GRIN2A and GRIN2B expression as Pilo–SE. Interestingly, LiCl/Para caused even more robust changes in expression of GRIN2A (decrease) and GRIN2B (increase) than status epilepticus induced by LiCl/Pilo. It is possible that LiCl in combination with paraldehyde can strongly alter normal brain ontogeny in terms of changing properties of NMDARs.

Concerning the GRIN2A/GRIN2B mRNA expression ratio, it was significantly decreased immediately after SE induced at P12 by LiCl/Pilo, as well as after LiCl/Para administration. This change might be found throughout the postnatal development in all brain regions considered in this study. Since the low NR2A/NR2B ratio can indicate increased excitability (Papatheodoropoulos et al., 2005), present results designate that single episode of early–life SE may increase excitability for the rest of the development in the whole brain and it can sustain even during adulthood. Moreover, the LiCl/Para can exhibit similar effects.

The effects of the selective antagonists blocking different types of NMDA and AMPA receptors that are highly involved in seizure generation can reveal comprehensive information on involvement of GluA2–containing AMPARs and NR2A/NR2B–containing NMDARs in different types of seizures elicited at early stages of postnatal development. Additionally, investigation of the seizure–induced changes in these receptors subunits expression, provide relevant information on developmental expression changes of Ca<sup>2+</sup>–permeable AMPARs and NR2A or NR2B–containing NMDARs. These data may provide an important reference for further studies concerning changes in neuronal excitability induced by early–life seizures.

## Summary

During early stages of postnatal development, glutamate receptors of NMDA and AMPA type, undergo intensive functional changes due to modifications of their subunit composition (Pachernegg et al., 2012; Paoletti et al., 2013). The NR2B-containing NMDARs (NR2B/NMDARs) and GluA2-lacking AMPARs (Ca<sup>2+</sup>-permeable) that are highly expressed in immature brain, are implicated in increased excitability, seizures generation, excitotoxicity, and neuronal death (Vizi et al., 2013).

The IEM1460 as a specific antagonist of Ca<sup>2+</sup>-permeable AMPARs (lacking GluA2 subunit) can potentially exhibit anticonvulsant action at early stages of rat postnatal development. It was assessed in a two models of epileptic seizures induced in animals at P12, P18 and P25: pentylenetetrazol (PTZ)-induced convulsions and cortical afterdischarges (ADs) that are generated at different sites of origin in the brain (ADs were induced also in animals at P80). Our results indicate that the effects of IEM1460 on a various types of seizures depend on their sites of origin, developmental stage, and GluA2 subunit expression profile. To clarify the action of IEM1460 in immature CNS, further studies are necessary.

The Ro 25-6981 maleate is a highly selective and activity-dependent antagonist of NR2B/NMDARs. The aim of our study was to investigate the influence of Ro 25-6981 administration on physiological (single and paired pulse cortical interhemispheric evoked potentials) and epileptic brain activity (cortical afterdischarges (ADs) in animals during first 3 weeks of life. Our results designate that the Ro 25-6981 exhibits age- and activation-dependent anticonvulsant action at early postnatal development. In contrast, the influence of Ro 25-6981 on physiological excitability induced by single pulse stimulation of sensorimotor cortex does not depend on age. Since its action against ADs prolongation can be observed even 110min after the single administration of the drug, this compound may thus represent a useful agent in pharmacotherapy of epileptic seizures in immature brain.

Detailed developmental expression profile of NR2B, NR2A (NMDARs) GluA2 (AMPARs) subunits (tested in electrophysiological experiments) in different brain regions, can provide an important information on the potential targets for development of further age-specific antiepileptic drugs. Moreover, controlling and regulating the expression of AMPARs and NMDARs subunits may serve as a new essential tool for neuroprotection after seizures. In the present study, these analysis of the changes in the genes (GRIN2A,

GRIN2B and GRIA2A) and GluA2 protein levels were assessed in the six different regions of rat brain - frontal cortex, parietal cortex, occipital cortex, dorsal hippocampus, ventral hippocampus and thalamus, at five different developmental stages including young adults.

Present results confirm that in the rat brain, the mRNA and protein levels of GluA2 subunit are increasing with age., in the neocortex its expression reached its highest level during the third postnatal week (with the exception of frontal cortex), while in the deep brain structures an increase in the GluA2 expression level occurred later in life. Present study confirms previously published data that in the majority of the brain regions the GRIN2A mRNA expression was significantly increasing with age, while the level of GRIN2B mRNA was slightly decreased at later stages of postnatal development. In the the tested brain structures, the GRIN2A/GRIN2B mRNA expression ratio was increasing during development.

Lithium chloride and pilocarpine status epilepticus (LiCl/Pilo-SE) induced in rodents at early stages of postnatal development represents a model of severe seizures resulting in progressive signs and symptoms of temporal lobe epilepsy (TLE). Since early-life seizures, can markedly change the gene and protein expression of a specific subunits of NMDARs and AMPARs leading to alterations in their properties, there are numerous publications concerning developmental expression profile of these subunits. Nevertheless, the majority of previously published studies were focused on hippocampus and neocortex, taken as whole structures. The aim of this part of the study was to determine the influence of LiCl/Pilo-SE induced early in life on the developmental expression profile of the genes encoding NR2A and NR2B subunits of NMDARs and GluA2 subunit of AMPARs in six different brain regions at five stages of development. A control group was included using animals that received only LiCl and paraldehyde.

Present data confirm previously published results showing that developmental changes in the levels of selected NMDA and AMPARs subunits expression are highly affected by LiCl/Pilo-SE. The outcomes of this study show, that the LiCl/Pilo-SE induced at P12 may result in dramatic changes of GRIA2A mRNA and GluA2 protein expression that can persist throughout the development and may be detected even in adulthood. An increased expression of GluA2 AMPARs subunit is an immediate response to elevated excitability and can serve as an early neuroprotective mechanism decreasing  $Ca^{2+}$ -permeability (Porter et al., 2006). A surge in GRIA2A mRNA and GluA2 protein

expression that can be detected especially in dorsal hippocampus not only during development but also later in life, indicates that this hippocampal part is able to activate early endogenous anti-epileptic mechanisms to maintain the balance between excitation and inhibition after SE. Since a decreased expression of GluA2 subunit is thought to occur just before excitotoxic neuronal degeneration (Hanada, 2014), a marked decrease in the age-dependent expression of GluA2 level detected in the ventral hippocampus after P12 LiCl/Pilo-SE suggests that this structure is highly prone to excitotoxicity not only during development but even in adulthood.

The expression of GRIN2A and GRIN2B genes encoding NR2A and NR2B subunits were highly affected by early-life LiCl/Pilo-SE. Present results support data published formerly, displaying that there is general significant decrease in the GRIN2A mRNA and an early marked increase in GRIN2B mRNA developmental expression level. In general, changes in these genes expression may result in diminished levels of NR2A and increased level of NR2B subunit protein, which can cause alterations of the normal brain ontogeny in terms of changing NMDARs kinetics and thus the level of excitability. Our data indicate that the ventral hippocampus is particularly affected by LiCl/Pilo-SE, since increased expression of GRIN2B was there detected even in the adulthood. As for the GRIN2A/GRIN2B expression ratio, as its low level may indicate increased excitability (Papatheodoropoulos et al., 2005), present results designate that single episode of early-life SE may increase age-related excitability in the whole brain, and it can persist up to adulthood. Moreover, LiCl/Para can exhibit similar effects, and present data provide evidence that the early-life administration of LiCl and paraldehyde have a marked influence on developmental expression levels of Glu2A gene and protein, as well as GRIN2A and GRIN2B gene expression. It is possible that LiCl in combination with paraldehyde can strongly alter normal brain ontogeny in terms of changing NMDARs and AMPARs properties.

## Souhrn

Glutamátové receptory NMDA a AMPA typu procházejí během časných stadií postnatálního vývoje intenzivními funkčními změnami danými modifikací jejich podjednotkového složení (Pachernegg et al., 2012; Paoletti et al., 2013). Velké zastoupení NMDA receptorů obsahujících NR2B podjednotku a malá přítomnost AMPA receptorů obsahujících GluA2 podjednotku v nezralém mozku (neprostupných pro  $\text{Ca}^{2+}$ ) se podílejí na vysoké excitabilitě, vzniku záchvatů, excitotoxicitě a zániku neuronů (Vizi et al., 2013).

IEM1460 jako specifický antagonist AMPA receptorů prostupných pro  $\text{Ca}^{2+}$  (kde chybí GluA2 podjednotka) může vykazovat antikonvulsivní působení v časných stadiích vývoje mozku potkana. Naše výsledky ukázaly, že účinek IEM1460 na různé typy záchvatů závisí na jejich místu vzniku, stadiu vývoje a expresi GluA2 podjednotky.

Ro 25-6981 maleát je vysoce selektivní antagonist NMDA receptorů obsahujících podjednotku NR2B. Tato látka vykazuje antikonvulsivní aktivitu závislou na věku (v časném postnatálním období) a aktivaci NMDA receptorů. Antikonvulsivní efekt u 12-denních potkanů trval minimálně 110 minut, to znamená, že tato látka by mohla být užitečná ve farmakoterapii epileptických záchvatů v nezralém mozku.

Zajímali jsme se o podjednotky glutamátových receptorů, které jsme ovlivňovali v elektrofyziologických pokusech. Detailní vývojový profil exprese NR2B, NR2A a GluA2 podjednotek v různých částech mozku může přinést důležité informace o potenciálních cílech věkově specifické antiepileptické léčby. Stejně tak se tyto poznatky můžou uplatnit jako neuroprotektiva. V naší studii jsme stanovovali změny v genech GRIN2A, GRIN2B a GRIA2A a proteinu GluA2 v šesti oblastech mozku potkanů – frontální, parietální a okcipitální kůře, dorsálním a ventrálním hipokampu a v talamu – na pěti vývojových stadiích (až po mladé dospělé potkany).

Naše výsledky potvrdily, že v mozku potkana vzrůstá s věkem mRNA a množství proteinu GluA2 podjednotky a taky literární údaje o vývojovém profilu expresí genů GRIN2A a GRIN2B (kódujících podjednotky NR2A a NR2B NMDA receptorů).

Ve většině oblastí mozku exprese GRIN2A mRNA významně vzrůstá s věkem, zatímco množství GRIN2B mRNA lehce pokleslo v pozdějších stadiích vývoje. Ve většině mozkových oblastí se během vývoje poměr GRIN2A/GRIN2B mRNA exprese zvyšuje.

LiCl/pilokarpinový status epilepticus (SE) vyvolaný u hlodavců v časných stádiích ontogeneze představuje model těžkých záchvatů vedoucí progresivně k symptomům temporální epilepsie. Tyto záchvaty mohou změnit vývojový profil exprese genů a proteinů specifických podjednotek NMDA a AMPA receptorů. Existuje řada studií popisující vývojové změny těchto podjednotek, většinou v hipokampu, méně často v neokortexu, ale vždy jako celku. Cílem této části naší studie bylo zjistit vliv SE vyvolaného v časně ontogeneze na vývoj genů kódujících NR2A a NR2B podjednotky NMDA receptorů a GluA2 podjednotky AMPA receptorů v šesti oblastech mozku v čtyřech až pěti vývojových stádiích. Dodatečná kontrolní skupina byla tvořena zvířaty, která dostala jen LiCl a paraldehyd. Potvrdili jsme publikovaná data ukazující, že vývoj vybraných podjednotek NMDA a AMPA receptorů je silně ovlivněn SE. Naše výsledky prokázaly, že SE vyvolaný 12 den po narození vede k dramatickým změnám exprese GRIA2A mRNA a GluA2 proteinu během vývoje až do dospělosti. Vyšší exprese GluA2 podjednotky je bezprostřední odpovědí na zvýšenou excitabilitu a může představovat neuroprotektivní mechanismus snižující prostupnost pro  $Ca^{2+}$  (Porter et al., 2006). Zvýšená exprese GRIA2A genu a GluA2 proteinu v dorsálním hipokampu přetrvávají až do dospělosti. Znamená to, že tato část hipokampu je schopna aktivovat endogenní antiepileptické mechanismy, které udržují rovnováhu excitace a inhibice po SE. Snížená exprese GluA2 podjednotky, kterou jsme našli ve ventrálním hipokampu po SE, která se objevuje ještě před excitotoxickou degenerací neuronů (Hanada, 2014), může znamenat, že tato struktura je zranitelná excitotoxicitou nejenom během vývoje, ale i v dospělosti.

Expresí genů GRIN2A a GRIN2B kódujících NR2A a NR2B podjednotky byla výrazně ovlivněna SE vyvolaném v časném vývoji. Naše výsledky potvrdily literární data prokazující pokles GRIN2A mRNA a časně zvýšení úrovně GRIN2B mRNA. Tyto změny mohou vést ke sníženému výskytu proteinů NR2A a vyššímu výskytu NR2B, což mění normální vývoj kinetiky NMDA receptorů a tím úroveň excitability. Naše data ukazují, že ventrální hipokampus je obzvlášť ovlivněn SE, zvýšená exprese GRIN2B byla prokázána i v dospělosti. Nižší poměr GRIN2A/GRIN2B exprese může znamenat zvýšenou excitabilitu (Papatheodoropoulos et al., 2005). Naše výsledky demonstrují, že jedna epizoda SE v časném vývojovém období může zvyšovat excitabilitu v řadě částí mozku a tato změna může přetrvávat do dospělosti. Problémem je, že LiCl/paraldehydové kontroly vykazují podobné změny jak v expresi GluA2 genu a proteinu, tak i v expresi GRIN2A a GRIN2B. Kombinace LiCl a paraldehydu zřejmě silně mění normální vývoj glutamátových receptorů.

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