As a manuscript

Khalilov Ilgam Adegamovich

SECONDARY EPILEPTOGENESIS IN THE HIPPOCAMPUS OF NEWBORN RATS

Speciality 03.03.01 - «Physiology»

Synopsis

of the dissertation for a scientific degree

Doctor of biological sciences

Kazan 2019

The work was carried out in the Department of Human and Animal Physiology of the Institute of Fundamental Medicine and Biology of the Federal State Autonomous Educational Institution of Higher Education “Kazan (Volga Region) Federal University” (Kazan, Russia) and in the laboratory of early activity of the developing brain of the Mediterranean Institute of Neurobiology (Institut de Neurobiologie de la Mediterranée (Inmed) Inserm / AMU UMR1249 (Marseille, France).

Dissertation consultant: Khazipov Roustem Narimanovich, Doctor of medical sciences, leading scientist of the laboratory of Neurobiology, IFMaB of KFU.

Official opponents:
1. **Mukhina Irina Vasilievna**, Doctor of Biological Sciences, Professor, Department of Neurotechnology, Institute of Biology and Biotechnology, Federal State Autonomous Educational Institution of Higher Education “National Research Nizhny Novgorod State University named after N.I. Lobachevsky".
2. **Semyanov Alexey Vasilievich,** Doctor of Biological Sciences, Professor, Corresponding Member of the Russian Academy of Sciences, Head of the Department of Molecular Neurobiology and the Laboratory for Extrasynaptic Transfer of the Institute of Bioorganic Chemistry named after Academicians M.M. Shemyakin and Yu.A. Ovchinnikov Russian Academy of Sciences.
3. **Zaitsev Alexey Vasilievich**, Doctor of Biological Sciences, Professor, Head of the Laboratory of Molecular Mechanisms of Neural Interactions of the Institute of Evolutionary Physiology and Biochemistr. Sechenov Russian Academy of Sciences.

Leading organization: Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences

Planned date of defense of the dissertation: 11 march 2020 at 13:00 at the dissertation council of KFU 03.06 at the Kazan (Volga Region) Federal University. Address: Tatarstan Republic, Kazan, Karl Marx st., 76, Academic Council meeting room (208).

The dissertation text can be found in the Lobachevsky Scientific Library at the Kazan (Volga Region) Federal University

The synopsis was sent out «_____» _____2019.

Scientific secretary of the
Dissertation Council KFU 03.06.
Doct. of biol. sci., professor T.A. Anikina

GENERAL DESCRIPTION OF THE WORK

The relevance of the research and its degree of development

Epilepsy is one of the most common neurological diseases (according to various sources, from 0.5 to 1% of the population), characterized by repeated convulsive or non-convulsive seizures (epileptiform paroxysms (EP)) resulting from a spontaneous massive hypersynchronous electric discharge (“epileptic discharge”) a large group of neurons of the cerebral cortex. Epilepsy is characterized by spontaneous and repetitive EP. Non-epileptic paroxysms, which are observed in about 10% of the world's inhabitants, are even more common. Most often, EP is observed in newborns and children. Indeed, about 6% of children undergo at least one EP during their lifetime (Borusiak et al., 2010). In clinical practice, it is generally accepted that a single EP accompanied by an electro clinical epileptic seizure still does not allow diagnosing epilepsy, as well as a series of seizures if they are caused by provoking factors, for example, intoxication or high body temperature (febrile seizures).

Despite the significant successes of epileptology that have been achieved over the past decades and a large amount of data on cell-molecular and structural changes in the epileptic brain, the causes, basic conditions, and mechanisms of the formation of epilepsy (epileptogenesis) remain still not very effective. As a result, methods for slowing down or preventing epileptogenesis and treating epilepsy are still poorly understood. For example, in 30–40% of patients with temporal lobe epilepsy, epileptic seizures are resistant to treatment (non-curable) (Dalic and Cook, 2016; Engel, Jr., 2016). Moreover, participants in the International Congress on Epilepsy (Barcelona, Spain, 2017) concluded that, despite the introduction into clinical practice in recent years of more than ten new modern antiepileptic drugs (AED), the total number of patients with epilepsy and the number of patients with a form of disease resistant to treatment is not reduced. More and more scientists and clinicians are coming to the conclusion that in the fight against epilepsy, the foreground should be the development of ways to prevent the epileptogenic process, and not the development of new antiepileptic drugs.

Due to the highest prevalence of epilepsy among young children, as well as due to the age-related characteristics of the properties of neurons and neural networks, an important area of scientific research is the search for therapeutic ways to prevent the formation of an epileptic focus in children at an early age. Data from cohort studies indicate (Nunes et al., 2008; Pisani et al., 2012) that up to 50% of older children with a diagnosis of epilepsy suffered EP in the first months after birth, compared with 0.5% for a population of children in overall (Scher, 2006). It was shown that in newborns resistant to antiepileptic drugs, the risk of epilepsy in the future is 4 times higher than in children with normal development (Garfinkle et al., 2016; Toet et al., 2005). Population and clinical studies also confirm that the majority of adults with epilepsy are grown children with untreated epilepsy. Therefore, in our study, we

turned to the neonatal period, since it is known that it is at this age that epileptiform paroxysms are most often observed, which in the future can cause epilepsy (Holmes et al., 2002a; Holmes et al., 2002b). In most cases, the source of epileptiform activity in newborns is a damaged part of the brain, the so-called “epileptogenic focus,” which can be the result of birth trauma, asphyxiation, a tumor, vascular malformation, brain infection, etc. (Chapman et al., 2012). Epileptiform discharges from damaged areas of the immature brain easily extend to neighboring undamaged areas of the brain, including the contralateral (opposite) hemisphere, since excitation processes predominate in the developing brain of children (against the background of GABAergic inhibition) (Ben-Ari et al., 2007)

The hypothesis that epileptiform discharges during repeated repetition can contribute to the emergence of a new focus of epilepsy was formulated almost 140 years ago by the English scientist William Gower - "seizures beget seizures" (Gower, 1881), which means "convulsive discharges generate convulsive discharges" or " seizures give rise to seizures. " Later, in the sixties of the last century, based on his own experimental data on various types of adult animals and on the basis of clinical data, Dr. Frank Morrell developed the concept of “secondary epileptogenesis” (Morrell, 1960). The concept implies that repeatedly repeating epileptiform discharges that occur in an epileptogenic focus, spreading to neighboring intact brain regions and / or the contralateral hemisphere, can form a new epileptic focus - the “secondary epileptic focus”, which, despite the histological integrity of the tissue in this part of the brain capable of independently generating spontaneous epileptiform discharges. However, this concept has not been experimentally proven on an immature brain, mainly due to the lack of an adequate model that allows us to separate the background pathology from the actual epileptogenic effect of epileptiform discharges in undamaged parts of the brain.

To solve this problem, we developed a model of secondary *in vitro* epileptogenesis, which allows you to generate local epileptiform discharges and observe their epileptogenic effect in the intact tissue of the immature brain. In the process of creating the model, a preparation was developed consisting of two interconnected (commissural fibers) intact hippocampi of newborn rats or mice (7–9 day old, which, according to published data, approximately corresponds to the first year of a child’s life (Avishai-Eliner et al., 2002; Herlenius and Lagercrantz, 2004; Nehlig, 1997). A chamber specially designed for the preparation described above allows the left and right hippocampi and commissural fibers connecting them to three separate compartments and to perfuse them separately with different solutions, which in nd not mix chamber. We have developed a model of epileptogenesis secondary *in vitro* allows initiate epileptiform discharges in one of hippocampus (simulating "epileptogenic focus") application on his convulsive agent and study conduct and epileptogenic effect of these discharges on the contralateral hippocampus.

The search for specific markers of epileptogenicity of epileptiform discharges in an immature brain is currently the most urgent, since it is known that not in all cases epileptiform paroxysms lead to chronic epilepsy, and intensive treatment with

antiepileptic drugs of young children often causes serious and irreversible pathological changes in the developing brain. Therefore, for modern neurology, one of the urgent applied problems is the search for effective methods of preventing epileptogenesis and effective treatment of epilepsy in young children. It is important to note that existing antiepileptic drugs, which were developed mainly for the treatment of the mature brain, can stop seizures in the immature brain, but in many cases they do not prevent the long-term pathological changes in neuronal excitability that underlie epileptogenesis and epileptic discharges. Moreover, in newborns, some PEPs, in particular GABAergic drugs, such as barbiturates and benzodiazepines (first-choice drugs for newborns and young children), can even aggravate brain epileptiform activity in some cases. Therefore, currently there is an active search for antiepileptogenic drugs that delay or prevent the occurrence of epilepsy, which was also one of the objectives of this study. In particular, the proposed dissertation study assessed the possibility of using a model of secondary epileptogenesis to search for and test new and classic antiepileptic and antiepileptogenic drugs.

The aim and tasks of the research

The **aim** of this dissertation research was to identify the basic conditions and synaptic mechanisms underlying the induction and expression of epileptic activity in the developing hippocampus, including the study of the role of GABAergic neurotransmission and chloride cotransporters in the formation of the secondary epileptic focus. The second objective of the study was to identify potential therapeutic targets for preventing secondary epileptogenesis and for suppressing epileptic activity in the resulting secondary lesion.

In accordance with the goal, the following **tasks** were set:

1. To develop an experimental model of secondary epileptogenesis in the hippocampus of newborn rats *in vitro*.
2. Investigate the electrographic properties of epileptiform discharges and their distribution in the limbic system of rats in the postnatal period and determine the electrophysiological patterns of activity that are associated with secondary epileptogenesis.
3. Investigate the role of GABA (A), NMDA and AMPA receptors in ictogenesis and secondary epileptogenesis.
4. To study changes in chloride homeostasis and the role of chloride cotransporters in the generation of epileptiform discharges and in the formation of a secondary epileptic focus.
5. Investigate the antiepileptogenic and antiepileptic activity of the first choice anticonvulsants (phenobarbital and diazepam) and the NKCC1 bumetanide cotransporter blocker.

Scientific novelty, theoretical and scientific-practical significance of dissertation research

In the course of research work, new experimental models of intact and related structures of the limbic system of newborn rats *in vitro* were developed, including preparations of (1) the whole hippocampus, (2) the double hippocampus, which is a

complex of two (right and left) hippocampi connected to each other with other commissural fibers and (3) entorhinal-hippocampal-septal complex. Methods have been developed for microsurgical dissection of these preparations while preserving the integrity of both the structures themselves and the bonds between them, and the conditions of their maintenance and perfusion to ensure their viability. Experimental chambers with several compartments have also been developed to ensure separate perfusion of different structures, which is a prerequisite for the study of secondary epileptogenesis during the formation of MF. It was shown that the neurons and neuronal networks in the preparations developed by us retain viability and the ability to generate network discharges and oscillations, including epileptiform activity, in the limbic system of newborn rats *in vitro* until the end of the postnatal period (10th day after birth, P10). Our study showed that the use of these preparations can be extremely wide, from neurophysiological experiments to a detailed study of the morphology of neurons in normal and pathological conditions. Intact preparations, in contrast to thin slices, allow three-dimensional reconstruction of dendritic and axon branches of pyramidal cells and interneurons, as well as neuronal connections between the structures of the limbic system. The most significant advantage of intact preparations compared to thin slices is the preservation of the neural network both within the structures and the synaptic connections between them.

Electrophysiological experiments using the whole hippocampus preparation showed that: 1) an increase in epileptiform activity caused by the application of the glutamate AMPA / kainate (KA) agonist in pyramidal cells in the CA3 field of the hippocampus during the first postnatal week occurs in parallel with the strengthening of KA-induced postsynaptic currents; 2) the hippocampus is the primary structure that generates paroxysmal activity in the immature limbic system in response to the action of KA; 3) the spread of paroxysmal activity in other structures of the limbic system is age-dependent; 4) in the preparation of the whole hippocampus, ictal discharges can be caused at a much earlier age than in thin slices of this structure, which correlates with data obtained in in vivo experiments, in which it is shown that ictal epileptiform discharges can be caused already in three-day (P3) baby rats.

Based on the double hippocampus preparation, we developed a model of secondary epileptogenesis, the use of which allowed us to demonstrate for the first time that repeated ictal discharges in one hippocampus can be carried out in the contralateral hippocampus and lead to the formation of a secondary (mirror) epileptic focus in it, and confirm the hypothesis of “seizures beget seizures” in an immature brain *in vitro*. It was experimentally demonstrated for the first time that the formation of a secondary epilepsy focus directly depends on the activation of both glutamate and GABA (A) receptors. In addition, we have established that this process depends on the presence of HF in the ictal discharges. It was found that in the pyramidal cells of the epileptic focus, the intracellular concentration of chloride ions increases, which leads to a positive shift in the potential for reversing currents through the ion channels of GABA (A) receptors and enhancing the exciting effect of GABA. It was shown for the first time that bumetanide, an antagonist of the cationic-chloride cotransporter NKCC1, does not prevent the formation of a secondary epileptic focus,

but blocks spontaneous epileptiform activity in the secondary focus. Moreover, we have shown that the genetic elimination of the NKCC1 cotransporter also does not prevent the formation of an epileptic focus.

The electrophysiological method we developed for evaluating the performance of chloride cotransporters allowed us to detect delayed elimination of chloride ions from MF neurons, an increased intracellular concentration of chloride ions, and to establish that the exciting effect of GABA in "epileptic" neurons is mainly due to down regulation of the KCC2 potassium-chloride cotransporter.

It was revealed that an increase in the intracellular concentration of chloride ions and an increase in the excitatory effect of GABA on neurons of the secondary focus is the main cause of the paradoxical aggravation of epileptic discharges in the immature brain, which is often observed with the use of first-choice antiepileptic drugs (phenobarbital and diazepam), which are positive allosteric G modulators (A modulators) receptors to prevent seizures in infants and young children. It was also shown for the first time that phenobarbital, in contrast to diazepam, inhibits AMPA / kainate-receptor-mediated postsynaptic currents and, in the initial stages of epileptogenesis, has an anticonvulsant and antiepileptogenic effect. However, in the already formed focus, both drugs aggravated epileptic activity. Finally, based on the above results, we proposed a strategy for the prevention of pharmacoinduced aggravation of epileptiform discharges with GABAergic antiepileptic drugs by the combined use of these drugs in combination with the NKCC1 cotransporter blocker bumetanide.

Thus, the model of secondary epileptogenesis that we developed opens up new possibilities for studying the pathogenesis and treatment of epilepsy in the developing brain, as well as for studying the mechanism of action of existing and new antiepileptic drugs both in the treatment of epilepsy, including both their anticonvulsant effect and in preventing secondary epileptogenesis in immature brain.

The dissertation is one of the fundamental scientific research and is of practical importance. The results of the thesis can be used in scientific research, in the process of training students of biological, medical and pharmaceutical universities, in the development of new antiepileptic and antiepileptogenic drugs for the treatment of epilepsy in newborns and young children.

Key Points:

1. Preparations of the whole and double hippocampus, as well as the entorhinal-hippocampal-septal complex of newborn rats *in vitro*, ensure the preservation of neurons and synaptic connections within and between these structures, are characterized by high viability and have several advantages compared with brain slices for studying the morphology of neurons and functions of neural networks in a developing limbic system. In particular, the developed preparations allow one to study the generation and distribution of epileptiform activity, as well as the processes of epileptogenesis in the structures of the limbic system for several days *in vitro*.

2. Multiple pharmacologically induced ictal discharges in the preparation of the whole hippocampus induce long-term epileptiform changes, which are manifested by spontaneous ictal and interictal discharges. Ictal discharges are also propagated through the commissural fibers into the contralateral hippocampus and are capable of causing the formation of a secondary (mirror) epileptic focus there. Activation of GABA (A) and NMDA receptors and the presence of high-frequency (80–140 Hz) oscillations (HFO) in the contralateral hippocampus are necessary conditions for the formation of a mirror epileptic focus.

3. The key mechanism of secondary epileptogenesis is a disturbance of chloride homeostasis due to dysfunction of the potassium-chloride cotransporter KCC2, which leads to a slowdown in the elimination of chloride ions and an increase in their intracellular concentration, as well as to an increase in the excitatory effect of GABA in neurons of the epileptic focus. Disturbance of chloride homeostasis in the epileptic focus can be corrected by blocking the cationic chloride cotransporter NKCC1 using a selective antagonist of bumetanide, which reduces the intracellular concentration of chloride ions and the excitatory action of GABA and leads to the suppression of paroxysmal activity in the epileptic focus.

4. Anticonvulsants of the first choice for newborns positive allosteric modulators of GABA (A) receptors phenobarbital and diazepam have a multidirectional effect on secondary epileptogenesis. Diazepam does not prevent the generation or propagation of KA-induced ictal discharges, nor the formation of a secondary epileptic focus. Phenobarbital also inhibits ictal discharges and prevents secondary epileptogenesis, which is due to the additional inhibitory effect of phenobarbital on AMPA / kainite receptor-mediated postsynaptic currents. In the already formed epileptic focus, both drugs, both phenobarbital and diazepam, enhance the excitatory action of GABA and aggravate epileptic activity.

Dissertant's personal contribution to the research

The author independently formulated the goal and objectives of the dissertation research. The scientific results presented in the thesis were obtained with the personal participation of the author at all stages of the work, including design, organization and conduct of experiments. The author is a leading developer of the innovative preparations “the whole hippocampus” and “double hippocampus”, on the basis of which a key model of the dissertation research is developed - a model of secondary *in vitro* epileptogenesis. Analysis and processing of experimental material and the preparation of research results for publication were also carried out by the author independently.

Reliability level and approbation of results

The main results of the dissertation research were presented at international symposia and conferences, in particular: the International Congress on Epilepsy (26th International Congress on Epilepsy, Paris, 2005), the European Congress on Neuroscience (FENS) (Geneva, 2009), the International Congress on Epilepsy (29th International Epilepsy Congress, Rome, Italy 2011), Society for Neurosciences Annual Forums of the USA (Society for Neurosciences): 26rd, 27th Annual Meeting, USA, New Orleans, 1997; 28th Annual Meeting, USA, Los Angeles, 1998; 35th

Annual Meeting, USA, Washington, 2005); 1st Mediterranean Neuroscience Conference, Montpellier, 1997; Human Frontier Hippocampal Conference, Paris, 1997; 3ème Colloque de la Société des Neurosciences, Bordeaux, France, 1997; European Congress on Epileptology (7th European Congress on Epileptology, Helsinki, Finland, 2006), European Congress on Epileptology (8th European Congress on Epileptology, Berlin, 2008), Eastern Mediterranean Congress on Epilepsy (2nd Eastern Mediterranean Epilepsy Congress, Dubai, 2010),

III International Congress dedicated to A.F. Samoilov "Fundamental and clinical electrophysiology. Actual issues of arrhythmology "(Kazan, Russia, 2019).

The reliability of the results obtained is also confirmed by the author's publications in peer-reviewed scientific journals, as well as by their citation index (> 4500) and the Hirsch index - 24 (according to WoS)

The structure and scope of the dissertation

The dissertation is presented on 257 pages of typewritten text and consists of an introduction, three chapters, a conclusion, findings and a list of references, including 411 titles. The work is illustrated by 64 figures and 1 table.

1. MATERIALS AND METHODS OF RESEARCH

All animal experiments were carried out in compliance with the principles of humanity and in compliance with the requirements for working with experimental animals in Russia (order of the Ministry of Health No. 755 of 08/12/1977) and in accordance with the European Convention for the Protection of Vertebrate Animals, with Directives 86/609 / EEC). Experiments were performed on hippocampal slices of newborn and adult animals and on intact hippocampi (in toto) of newborns. To prepare the preparations, laboratory Wistar rats and C57BL mice were used. The procedures for preparing slices of the hippocampus and intact hippocampi are described in detail in previous publications (Ben-Ari at al., 1989) and (Khalilov at al., 1997; Khalilov at al., 1999), respectively.

Electrophysiological methods of registration and data analysis

Patch-clamp registration in different configurations (in the configuration of the whole cell: "whole-cell") with current fixation or potential fixation: "current-clamp" and "voltage-clamp", respectively, or in the configuration on the "cell": "cell - attached " was performed using Axopatch 200A and Multi Clamp 700B amplifiers.

Extracellular recordings of local field potentials and population activity of neurons was performed using electrodes made of tungsten wire (diameter 50 μm, California Fine Wire, Grover Beach, CA). The amplification and digitization of the recorded signals was carried out using a DAM80A amplifier. Electrical stimulation was carried out using a bipolar tungsten electrode (wire diameter 50 μm). The spectral power of ictal discharges was analyzed using AutoSignal v1.7 software (SeaSolve Software Inc.).

Measurement of the dynamics of extracellular potassium and calcium

The ion-selective microelectrode was made of a double-barreled borosilicate glass tube (2GC150FS, Clark Electromedical, Pangbourne, Reading, UK), in which a filament pipette (filamentous processes) was used for extracellular registration of

field electric potentials. The microelectrode without filament was exposed to dimethyl-trimethyl-silylamine vapor. After drying at 200 ° C, the pipette without filament was filled with a solution containing the corresponding ion sensor. Fluka 60398 was used as a sensor for K^+ ions, and Fluka 21196 for Ca2 + ions.

Focal application of GABA and glutamate

For local application of GABA and glutamate, glass pipettes (1–2 Mom) were used, filled with ACSF solution with the addition of either GABA (100–200 μM) or glutamate (100 μM). The power of the picospritzer, the duration of the application, and the distance from the tip of the pipette to the body of the detected neuron were selected so that the signal amplitude in response to the application of the substance did not exceed 300 pA.

The obtained data were processed and analyzed using the following software: pClamp 10.0 (Molecular Devices), miniAnalysis (Synaptosoft) Origin 7.0 (Microcal Software) and AutoSignal (SeaSolve Software Inc.). Group data are presented as mean ± standard error. A statistical assessment of the existence of significant differences in the compared samples was evaluated using t-student test and analysis of variance with two factors (two-way ANOVA). The level of confidence $p < 0.05$ was taken as significantly significant.

2. MAIN RESULTS AND DISCUSSION

2.1. A new preparations to study the generation and propagation of network activity in the limbic system of the immature brain

Studies using the preparation of thin slices of different structures of animal brain and brain of people who underwent surgical intervention are of great importance for understanding the mechanisms of brain function in normal and pathological conditions. However, the preparation of thin slices has significant disadvantages. Since most of the interneuronal connections of brain structures are lost during the preparation of thin slices, the study of processes such as the generation and distribution of synchronized network activity becomes problematic and ineffective. To overcome the above disadvantages, we have developed preparations of the whole hippocampus and related structures of the limbic system of newborn rodents *in vitro*.

Morphological aspects of the whole hippocampus preparation

Light microscopy showed that the preparation of the whole hippocampus prepared from the brain of newborns (from P0 to P10) in rats that were *in vitro* for up to 10 hours, and even from the brain of older rats (P15, 4 hours *in vitro*), the main histological characteristics the hippocampus and the morphology of the vast majority of neurons, including interneurons, are well preserved. An electron microscopic examination of an intact hippocampus preparation, maintained *in vitro*, also confirmed the good morphological safety of neurons. Immunohistochemical studies of GAD-labeled neurons (GAD - glutamic acid decarboxylase (glutamate decarboxylase) also confirmed the good preservation of intact P0–15 hippocampi (Khalilov et al., 1997). The results of our study, in full accordance with previously

published data obtained on slices of rat hippocampus recorded in situ (Rozenberg at al., 1989; Dupuy and Houser, 1996) confirmed the presence of GAD-labeled neurons and their terminals in all layers of the hippocampus.

Electrophysiological properties of the intact hippocampus

To study the electrophysiological properties of the intact hippocampus *in vitro*, we used two methods of registration: 1) extracellular registration of field potentials and population activity of a group of neurons; 2) patch-clamp registration of the activity of single neurons in different configurations.

The average membrane potential of the studied neurons was -62 ± 4 mV (n = 74), and these cells in response to the depolarizing current step generated action potentials with overshoot. In addition, the characteristic giant depolarizing potentials (GDP) (Ben-Ari at al., 1989), which are the result of the synchronous discharge of hippocampal neurons (Khazipov at al., 1997; Leinekugel at al., 1997), were recorded both in interneurons and in the pyramidal cells.

Thus, our data indicate that the main morphological and physiological properties of the pyramidal cells and interneurons in the intact hippocampus *in vitro* are not altered and the new preparation can be used in pharmacological experiments.

Unique Properties and Capabilities of Intact Hippocampus

The intact hippocampus preparation developed by us can be extremely successfully applied, in particular, for a detailed study of the morphology of neurons and the processes of generation and distribution of synchronized network activity within the intact hippocampus and in complex preparations: septum - hippocampus, hippocampus - hippocampus, hippocampus - entorhinal cortex.

3D reconstruction of neurons in an intact hippocampus preparation

The intact hippocampus preparation, unlike thin slices, allows you to completely reconstruct all dendritic and axon branches of the pyramidal cells (Fig. 1A) and interneurons (Fig. 1B) and obtain a three-dimensional (3D) image of these neurons (Fig. 1C).

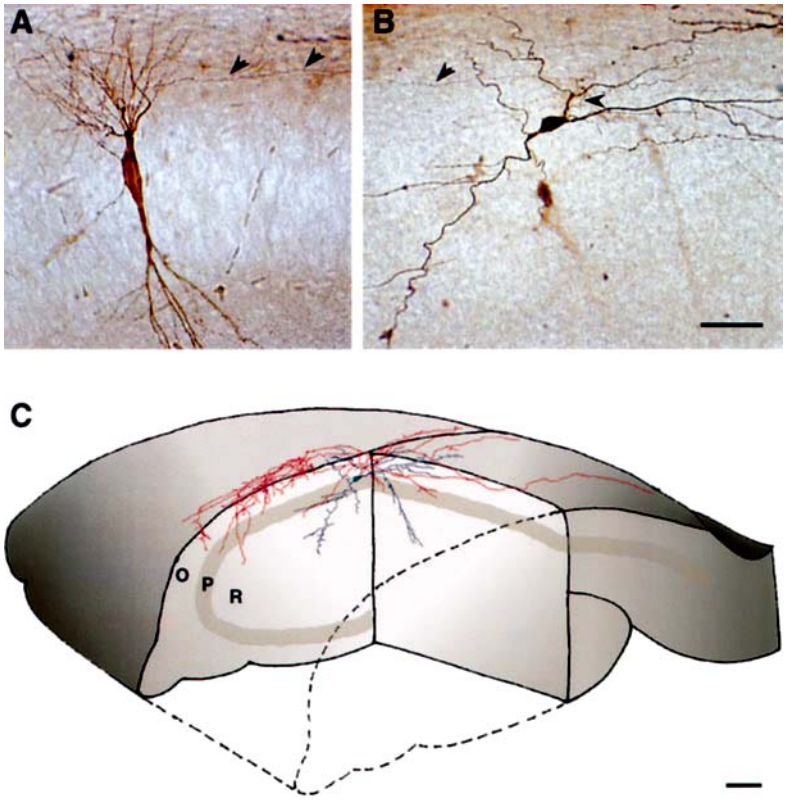

Figure 1. Morphological characteristics and 3D reconstruction of neurons in the preparation of the whole hippocampus *in vitro.*

For experiments, an intact P7 hippocampus was used, which was *in vitro* for 8 h. (**A**–**B**) Biocytin detection on 100 μm thick sections demonstrates the labeling of the soma, dendritic arborization, and axon (arrows) of a pyramidal neuron (**A**) and an interneuron (**B**) in the CA1 region. (**C**) 3-D reconstruction of the biocytin-filled interneuron illustrated in (**B**). This interneuron displays a fusiform cell body located at the limit between *stratum oriens* and *stratum pyramidale* and gives rise to three primary dendrites that arborize in the *stratum oriens*, *stratum pyramidale*, and *stratum radiatum* of the CA1 region. The dendritic tree is mainly oriented in the transverse plane. This interneuron exhibits an axonal arborization that extends over 700 μm in the rostrocaudal plane, mainly in stratum oriens. The axon length in the rostro-caudal direction is more than 1 mm. Scale bars, 100 μm..

The use of the intact hippocampus preparation allows reconstructing dendritic and axon branches of neurons in complex preparations: septum - hippocampus, hippocampus - hippocampus, hippocampus - entorhinal cortex.

Registration of network electrical activity of an intact hippocampus preparation

The use of the whole hippocampal preparation allows one to register and study the generation and propagation of network discharges, such as GDP and epileptiform activity. Thus, using the hippocampus preparation, we were able to record for the first time long (30–250 s) ictal discharges in the immature hippocampus, which were induced by bicucullin, kainate, increased extracellular concentration of potassium ions and high-frequency stimulation (kindling). In thin slices of the hippocampus, only short (<1 s) interictal discharges are generated in such experiments.

2.2. Generation and propagation of epileptiform discharges in the limbic system of newborn rats during development

The likelihood of generating epileptiform discharges and the formation of an epileptic focus in young children is much higher than in adults. However, animal experiments show that immature brain neurons are much more resistant than adult animal neurons to pathological changes, such as cell death in response to repeated epileptiform discharges. Despite this, in newborns, compared with adults, epileptiform discharges more often lead to the development of various neurological disorders, including epilepsy (Ben-Ari, 1985; Holmes et al., 1998; Nitecka et al., 1984). This fact indicates that the adverse effects of repeated ictal discharges or status epilepticus can be caused not only by the death of neurons, but also by a alteration of the mechanisms of development and formation of synaptic connections in neural networks (Goodman and Shatz, 1993). Understanding the principles of the functioning of these mechanisms in an immature brain is extremely important because it is believed that epileptiform paroxysms in young children do not require immediate aggressive treatment due to the absence of massive cell death in response to repeated epileptiform discharges and due to the adverse effects of antiepileptic drugs on developing brains (Holmes et al., 1997; Holmes and Ben-Ari, 1998).

Unlike adult rats, intraventricular injection of KA in newborn rats caused an ictal discharge only at the end of the third week of life (Albala et al., 1984; Cherubini et al., 1983; Stafstrom et al., 1992). Functional mapping using labeled 2 DG revealed that KA injection in younger rats generated epileptiform discharges only in the CA3 field of both hippocampi and in the lateral septum (Tremblay et al., 1984). These data obtained in *in vivo* experiments show that, although the immature hippocampus has a lower threshold for the epileptogenic effects of KA, the propagation of epileptiform discharges from the hippocampus to other limbic structures plays an important role in epileptogenesis. However, there are technical difficulties in registering epileptiform discharges in newborn rats and mice *in vivo*. That is why in the dissertation research we first used the new preparation that we developed.

Profile of age-related changes in kainate-induced epileptiform discharges

To determine the profile of age-related changes in epileptiform discharges caused by the application of KA in the washing solution, we performed simultaneous extracellular registration of electrical responses in the pyramidal layer of CA3 field and patch-clamp registration of CA3 of pyramidal cells. In the hippocampus of newborn rat pups (P0 – P1), under control conditions, the pattern of electrical activity was characterized by the presence of spontaneous GDPs, which were simultaneously recorded by both electrodes and generated at a frequency of 4.6 ± 1.3 min^{-1} (n = 5).

A typical response to KA application (250 nmol) was biphasic. During the first phase, there was a short-term increase in the frequency of GDPs to 1.0 ± 0.2 s^{-1}. Then followed the phase of desynchronization, accompanied by the suppression of GDPs. In the second phase, the frequency of GABAergic postsynaptic currents, as well as the frequency of spikes on the extracellular electrode, increased sharply. However, in response to KA application, even at high concentrations (1 μM), we did not observe

either population spikes on the extracellular electrode or polysynaptic responses. Thus, at this age, KA is not able to cause epileptiform activity.

In hippocampus of P2 – P3 rat pups, the response to the application of KA in 10 experiments out of 21 was similar to the response observed at an earlier age. In 11 experiments out of 21, short interictal epileptiform discharges generated during an increase in the frequency of GDPs. Only in one case did the application of the KA evoke responses similar to ictal discharges with characteristic tonic and clonic phases. Patch clamp registration allowed us to find that interictal discharges are associated with glutamate-mediated currents in CA3 pyramidal cells.

In hippocampi P4 – P7, rat pups generated classical ictal discharges in 23 experiments out of 41. KA-induced ictal discharge consists of 4 phases. Epileptiform activity begins with several high-amplitude (0.5–2 mV) interictal discharges (phase 1). The second phase, the so-called tonic phase, is characterized by rhythmic (6–20 Hz) oscillations, which tend to decrease towards the end of the tonic phase and towards the beginning of the third - clonic - phase. After a series of clonic discharges, the phase of postictal depression sets in (phase 4), during which spontaneous and induced network neuronal activity is completely absent. Thus, the epileptiform activity caused by KA in the hippocampus of newborn (P4) rats has a characteristic sequence of phases of ictal discharges previously recorded in experiments *in vivo* (Bragin et al., 1997).

Correlation between an increase in the predisposition of the hippocampus to the generation of epileptiform discharges and an increase in postsynaptic responses to kainate

In the mature hippocampus, KA-induced epileptiform activity is due to the activation of kainate receptors of the pyramidal cells in the CA3 field. Therefore, the following group of experiments was undertaken to identify a correlation between the phenomenon of a sharp increase in age of the hippocampus's predisposition to the generation of epileptiform discharges and an increase in the sensitivity of CA3 pyramidal cells to KA during the first postnatal week.

To determine age-dependent changes in the sensitivity of CA3 of pyramidal cells to KA, the substance was added to the washing solution in the presence of tetrodotoxin (TTX, 1 μM). In the hippocampi P0 – P1 rat, application of KA (250 nM) did not cause postsynaptic current in CA3 pyramidal cells (n = 9). Starting from two days of age, the application of the KA in all experiments was accompanied by the generation of inward currents, the amplitude of which progressively increased with age. KA-induced currents were also generated when NMDA, GABA (A) and AMPA receptor blockers were added to the perfusion solution: APV (50 μM), bicucullin (10 μM) and GYKI 56355 (30 μM), respectively, but were sensitive to short application of the non-selective AMPA / kainate receptor antagonist CNQX (10 μM), which indicates that these currents are the result of activation of high affinity kainate receptors.

Thus, during the first week of postnatal development, there is a gradual increase in postsynaptic currents in response to the application of KA, which

significantly correlates with the age profile of changes in the generation of KA-induced epileptiform discharges.

Age profile of epileptiform discharge of the limbic system

To study the age-related features of propagating epileptic discharges between the structures of the limbic system, we have developed new combined hippocampal preparations, including septum and / or entorhinal cortex. In newborn animals (P2), the KA generated a series of interictal discharges that were propagated to the septum with a delay of 54 ± 11 ms (n = 6). After transection of the connections between the hippocampus and septum, epileptiform activity was observed only in the hippocampus (n = 5). In rat P2, which included the hippocampus and entorhinal cortex, hippocampal interictal discharges were not generated in the entorhinal cortex (n = 4). In rat P6, KA-induced ictal discharges were generated in both the septum and entorhinal cortex. In all experiments, hippocampal discharges preceded the discharges generated in the septum (delay was 34 ± 15 ms, n = 4). Transection of neuronal connections between the hippocampus and septum demonstrated that in an isolated septum, KA is not able to generate epileptiform activity, as in the hippocampus of younger rats (P2 rats). However, unlike rat P2, the application of KA in rat P6 was accompanied by epileptiform activity in the entorhinal cortex (n = 6). Transection of the neuronal connections between the hippocampus and the entorhinal cortex showed that KA-induced epileptiform discharges originate in the hippocampus and are propagated to entorhinal cortex.

Thus, in accordance with the data obtained in *in vivo* experiments, the hippocampus is the primary structure generating paroxysmal activity in the immature limbic system in response to the action of KA, the generation and propagation of which in the limbic system are age-dependent.

3. Secondary epileptogenesis in immature brain *in vitro*

Clinical studies show that the likelihood of generating epileptiform discharges in children, especially in newborns, is much higher than in adults (Ben-Ari, 1985; Chen et al., 1999; Hauser, 1995; Holmes and Ben-Ari, 1998; Moshe et al., 1983; Moshe and Albala, 1983). This can be explained by the fact that for the immature brain, the predominance of excitation processes over inhibition processes is characteristic. In most cases, the source of epileptiform activity is a damaged part of the brain - the so-called primary epileptogenic focus, which may be the result of birth trauma, asphyxiation, etc. Epileptiform discharges in an immature brain can easily spread to neighboring undamaged parts of the brain, including the contralateral (opposite) hemisphere. The hypothesis that multiple epileptiform discharges may contribute to the emergence of a new focus of epilepsy was put forward almost 140 years ago by the English scientist William Gauver: “seizures beget seizures” (Gower, 1881), which means “convulsive discharges generate convulsive discharges”. The study of the mechanism of this phenomenon, called “secondary epileptogenesis”, is of great clinical importance, since patients with repeated and prolonged epileptic seizures can have a double pathology, which can significantly reduce the effectiveness of surgical intervention in pharmacologically resistant forms of epilepsy (Li et al., 1999). However, to date, the hypothesis of secondary epileptogenesis in an

immature brain has not been experimentally confirmed. In order to experimentally confirm this hypothesis, the author of the dissertation developed an *in vitro* model of secondary epileptogenesis, which allows one to artificially generate local repeating epileptiform discharges and observe their epileptogenic effect on the intact tissue of the immature brain.

First of all, a preparation was developed consisting of two intact hippocampi connected (by commissural fibers) - 7–9 day old - rats or mice (Khalilov et al., 1997). The chamber, specially designed for the preparation described above, allows the left and right hippocampi and commissural fibers connecting them to three separate compartments and to perfuse them separately with different solutions that do not mix in the chamber (Khalilov et al., 2003). The developed model of secondary epileptogenesis allows one to induce ictal discharges in one of the hippocampi and to study the epileptogenic effect of discharges in the contralateral hippocampus.

Repeated ictal discharges and the formation of mirror focus

A short (2–3 min) application of KA (250 nM) to one of the hippocampi leads to the generation of an ictal discharge in this hippocampus. These discharges with a slight delay are propagated to the contralateral hippocampus. Each subsequent application of the KA (with an interval of 15 minutes) led, firstly, to a progressive increase in the duration of ictal discharges from 89.8 ± 4.6 s (for the first application) to 117.0 ± 6.2 s (for the fifteenth application) and secondly, to an increase in the generation of epileptiform clonic discharges originating in the contralateral hippocampus and propagating to the ipsilateral hippocampus (Fig. 2A, B, C). After 15 KA applications in 69% of experiments (n = 42), both hippocampi generated synchronous spontaneous ictal discharges (Fig. 2G).

The blockade of interhippocampal conduction by application of TTX into the middle compartment or the cutting of commissural fibers confirmed the assumption that the contralateral hippocampus was transformed into an epileptic focus, since the contralateral hippocampus was able to independently generate spontaneous and / or evoked epileptiform discharges (Fig. 2D).

Thus, it was experimentally shown for the first time that repeated ictal discharges in an immature hippocampus can cause the formation of a secondary epileptogenic mirror focus (MF).

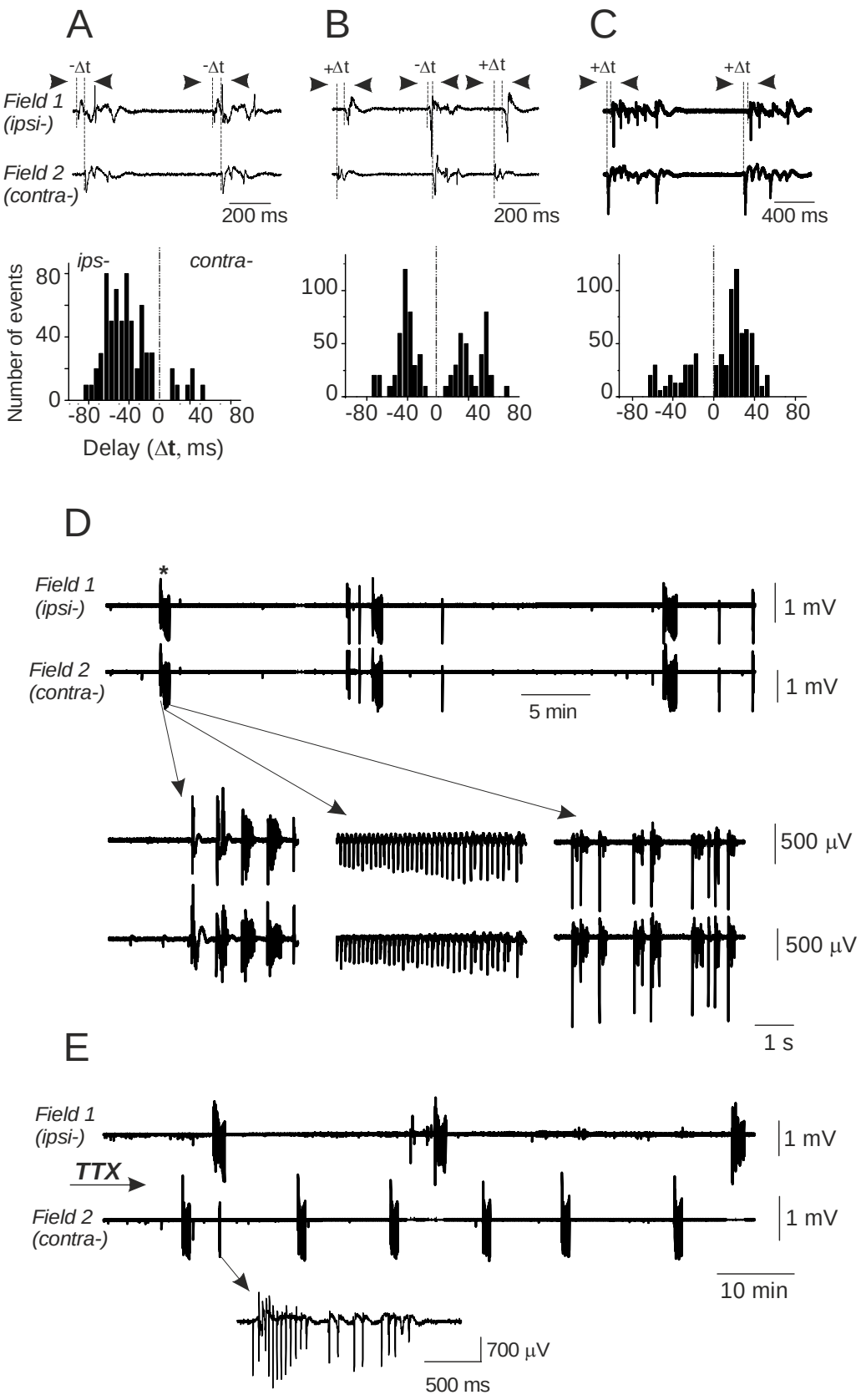

Figure 2. The formation of mirror focus. (**A, B, C**) Distribution histograms (lower row) of inter-hippocampal delays (**Δt**) of epileptiform events during the clonic phase of the ictal discharge (upper row) caused after the first (**A**) and seventh (**B**) applications of the KA. It is seen that during the first application of the KA, in comparison with the seventh, epileptiform discharges are mainly generated in the ipsilateral hippocampus and are propagated to the contralateral one. After the

formation of the MF, spontaneous discharges are noted mainly in the contralateral hippocampus (**C, D**). (**E**) Application of TTX into the central compartment of the camera. It is seen that spontaneous and induced (*) asynchronous ictal epileptiform discharges are generated both in the ipsilateral and contralateral hippocampi.

The results of our experiments indicate that repeated ictal discharges can transform the intact structure of an immature brain into a "chronic" epileptic focus (Fig. 3).

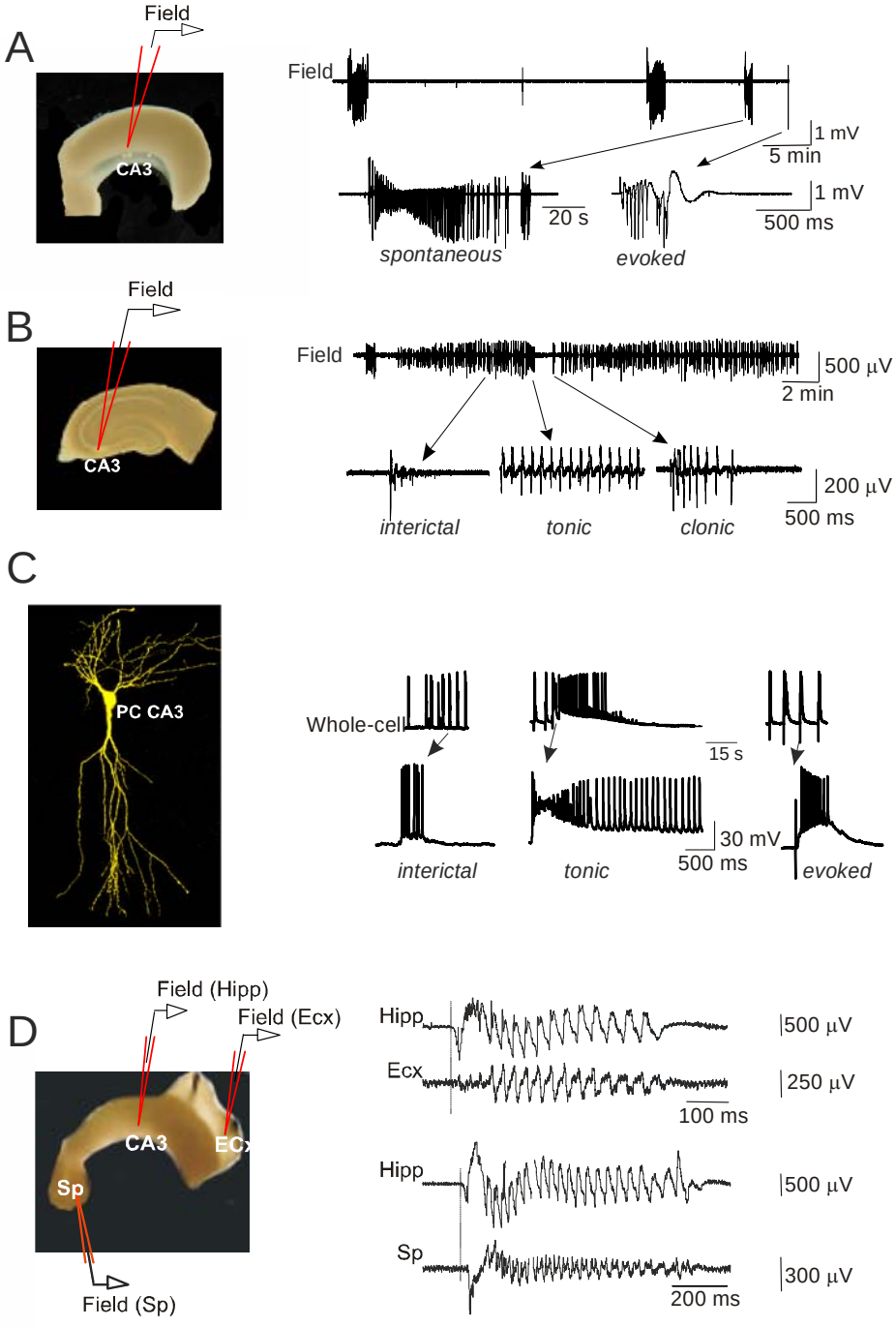

Figure 3. "Chronic" epileptogenesis in the secondary focus. (**A – D**) Registration of electrical activity in the contralateral hippocampus 24 hours after the onset of epileptic focus: (**A**) intact hippocampus (Hipp.), (**B**) a slice obtained from this intact hippocampus, (**C**) pyramidal cell stained with biocetin CA3, (**D**) intact hippocampus with septum (septum, **Sp**) and entorhinal cortex (**Ecx**). Left: photos of registered

preparations and CA3 pyramidal cells. Right: (**A**) Extracellular registration of spontaneous and evoked epileptiform activity in the whole hippocampus (lower on an extended time scale); (**B**) Extracellular registration in a hippocampal slice showing different phases of a spontaneous ictal discharge; (**B**) Spontaneous and induced discharges of action potentials in CA3 pyramidal cell in the slice prepared from the contralateral hippocampus after the formation of the MF (lower on an extended time scale); (**D**) Paired extracellular registration, demonstrating the generation and propagation of spontaneous epileptiform activity from the hippocampus to the septum and entorhinal cortex.

NMDA receptor activation needed for secondary focus formation

The model we created allowed us to demonstrate that multiple synaptic activation of neurons in the contralateral hippocampus by ictal discharges is necessary for the formation of MF. So, if during the experiment to block the synaptic activity of neurons of the contralateral hippocampus by adding CNQX or TTX to the perfusion solution, the ictal discharges are blocked and the formation of MF is completely prevented. If only NMDA receptors in the contralateral hippocampus are selectively blocked by adding APV (100 μM) to the perfusion solution, epileptic discharges continue to be generated in both hippocampi, but MF does not occur even after 25 KA-induced ictal discharges.

Excitatory action of GABA in the secondary epileptic focus

It is known that GABAergic synaptic transmission plays a major role in epileptogenesis and in the treatment of epilepsy. Therefore, we compared the action of the GABA(A) receptor blocker in the control tissue and in the slices obtained from the MF. In control slices, the addition of bicuculline to the perfusing solution led first to the blocking of GDP, and then to the generation of interictal epileptiform activity. In slices prepared from MF hippocampus, the effect was the opposite – bicuculline completely but reversibly blocked spontaneous epileptiform activity. In control slices, in contrast to "epileptic" slices, APV and CNQX completely blocked evoked action potentials (AP). AP that were not blocked in the presence of glutamate receptor blockers were completely blocked in both slices by the addition of bicuculline, indicating that they were caused by the excitatory action of GABA.

Using the method of perforated (gramicidin) patch-clamp registration, we compared the reversion potentials of GABA-mediated postsynaptic currents (E_{Cl}-) in slices obtained from control and" epileptic " hippocampi sustained for 24 hours under the same *in vitro* conditions. Experiments have shown that in MF neurons, in comparison with neurons of control slices, E_{Cl}- was significantly shifted towards positive values (control -69.8 ± 2.8 mV; MF -55.2 ± 1.9 mV, $P < 0.01$), and the membrane resting potentials of neurons had no statistically significant differences (control: -67.7 ± 0.5 mV, silces: -67.3 ± 0.6 mV, $P > 0.05$). Under the same conditions, patch-clamp registration in the current clamp mode showed that in MF neurons, in contrast to neurons in control slices, single electrical stimulation of synaptic inputs can cause AP discharges that were completely blocked when bicuculline was added. Thus, in MF, GABAergic synapses are excitatory.

Activation of GABA(A) receptors as a necessary condition for the formation of a secondary epileptic focus

To study the conditions for the formation of MF in these experiments, a GABA(A) receptor antagonist, bicuculline, was applied instead of KA. Bicuculline application resulted in the generation of epileptiform discharges that propagated to the contralateral hippocampus. Repeated application of antagonists, as in the case of KA, was accompanied by the formation of MF. However, it turned out that, unlike the contralateral one, the isolated ipsilateral hippocampus did not generate either spontaneous or induced discharges. Therefore, we can assume that in the immature hippocampus, GABA(A) receptor antagonists are not epileptogenic, but ictogenic agents. This observation points to a fundamental difference between ictal discharges during which GABA (A) receptors are inhibited or activated. Frequency-time analysis of ictal discharges showed that ictal discharges in the contralateral hippocampus, in contrast to the ipsilateral one, were accompanied by generation in the HFO. The average duration of the oscillations was 120 ± 4.3 ms (n = 63). Ictal discharges in the presence of GABA(A) receptor antagonists contained oscillations in a lower frequency range (19.4 ± 0.9 Hz, n = 63).

In similar experiments, we performed a time-frequency analysis of ictal discharges caused by the application of the KA. The analysis showed that in those experiments in which MF occurred, ictal discharges were accompanied by generation in the frequency range (80-120 Hz). In experiments in which ictal discharges in the contralateral hippocampus were accompanied by HFO, cutting of commissural fibers or application of TTX to the central compartment revealed the formation of MF. Other observations confirm the critical role of HFO in the formation of MF.

First, in 30 experiments (n = 37), in which ictal discharges were accompanied by HFO, the formation of MF occurred; in experiments in which HFO was absent (7 out of 37), the formation of MF did not occur.

Second, the time-frequency analysis of ictal discharges, in which APV, a blocker of NMDA receptors, prevented the formation of MF, also showed the absence of HFO (the average peak frequency is 9.6 ± 1.0 Hz; n = 4).

GABA(A) receptor antagonists have an ictogenic but antiepileptogenic effect

Based on data obtained in experiments with GABA(A) receptor blockers, we hypothesized that if GABA (A) receptors are involved in the formation of an epileptic focus, then their blockade can prevent the epileptogenic effect of other convulsants. We conducted experiments in which KA was applied to a single hippocampus, and the contralateral hippocampus was perfused with a solution containing bicuculline (20 μM). Under these conditions, HFO were generated only in the KA-applied hippocampus (81 ± 1 Hz), and in the contralateral hippocampus, the average peak frequency of oscillations was significantly lower – 19.4 ± 0.9 Hz (63 ictal events; $P < 0.01$; n = 5). Separation of the hippocampus revealed that only the ipsilateral hippocampus was epileptic (n = 5). In experiments in which KA was applied to one hippocampus in the presence of bicuculline, and the contralateral hippocampus was perfused with a control solution, similar results were obtained. HFO was generated only in the untreated bicuculline hippocampus.

Thus, blocking GABA (A) receptors causes epileptiform activity, but prevents epileptogenesis, which suggests a proepileptogenic effect of GABA (A) receptors in the immature hippocampus.

In mature neurons activation of GABAergic synapses is not a necessary condition for epileptogenesis

To confirm the possibility of projecting the results obtained in experiments on the immature hippocampus to the mature hippocampus, we simultaneously perfused slices of the mature and immature hippocampus in one common chamber. Application of bicuculline or gabazine (10 μM, 10 min) in slices of mature and immature hippocampus resulted in the generation of epileptiform activity. However, GABA (A) receptor blockers had an epileptogenic effect only in slices of the hippocampus of adult rats.

These experiments demonstrated that in the mature hippocampus, in contrast to the immature one, activation of GABA (A) receptors is not a necessary condition for epileptogenesis.

4. The role of chloride cotransporters in the generation of epileptiform discharges and the emergence of a secondary epileptic focus in the immature brain

Using simultaneous extracellular and perforated patch-clamp registrations, we first investigated the effect of bumetanide on the physiological network activity of immature hippocampal neurons. Experiments have shown that bumetanide (10 μM) completely blocked spontaneous GDPs and completely leveled the excitatory effect of GABA.

Bumetanide does not prevent the formation of MF, but blocks epileptiform activity in this focus

To study the effect of bumetanide on the formation of a secondary epilepsy focus, we also used the MF model that we developed. In these experiments, short applications of KA were performed against the background of continuous perfusion of the contralateral hippocampus with a solution containing bumetanide (10 μM). After 15 ictal discharges induced by KA in one hypocampus, both hippocampi started generating spontaneous synchronous ictal and interictal discharges, despite the presence of bumetanide in the washing solution of the contralateral hippocampus. Analysis of registrations showed that spontaneous ictal discharges always occurred first in the ipsilateral hippocampus, where bumetanide was absent. Separation of the hippocampus demonstrated that epileptiform activity is generated only in the ipsilateral hippocampus. However, after washing the bumetanide, the contralateral hippocampus was also able to generate spontaneous epileptiform discharges.

NKCC1 is only partially involved in the excitatory action of GABA in the neurons of the MF

We have experimentally confirmed that the excitatory action of GABA in MF neurons in the presence of bumetanide decreases, but does not completely disappear. These experiments show that in the presence of bumetanide, GABA continues to have a depolarizing action, which has an NKCC1-independent mechanism. To

investigate this effect, we measured and compared the membrane potentials (E_m) and driving forces (DF_{GABA}) in the CA3 pyramidal neurons of the control slices of the hippocampus and the MF slices. E_m in epileptic neurons was less than in control neurons, but the difference was not significant (P = 0.08). However, DF_{GABA} in "epileptic " neurons was significantly larger (13.2 ± 12.2 mV – in the control slices and 37.3 ± 8.1 mV – in "epileptic", P < 0.001), which is manifested in a positive shift of E_{GABA}. Bumetanide significantly reduced DF_{GABA} in MF neurons (from 37.3 ± 8.1 to 14.2 ± 7.6 mV, P < 0.001), although DF_{GABA} in MF neurons was greater than in control neurons (14.20 ± 7.6 mV versus 2.02 ± 4.8 mV in control neurons in the presence of bumetanide). Increasing the concentration of bumetanide (50 μM) or blocking epileptiform activity by adding CNQX + APV also did not lead to the disappearance of the increased positive value of $ДС_{GABA}$.

Thus, when NKCC1 activity was blocked by bumetanide, the depolarizing $ДС_{GABA}$ in “epileptic” neurons was 7 times higher than in control neurons.

5. The increase in $[Cl^-]_I$ and the excitatory action of GABA are the main cause of aggravation of epileptiform activity by phenobarbital

Despite more than 100 years of history, phenobarbital (PHB) is still the first choice drug for the treatment of convulsive conditions in children (Wheless et al., 2007; Bassan et al., 2008), and the second choice drug is diazepam (DZP). The mechanism of action of these drugs is mainly related to the potentiation of the action of GABA. However, these drugs are often ineffective, especially during generalized attacks (Painter et al., 1999), or may trigger an aggravation of seizures (Boylan et al., 2002; Guillet and Kwon, 2007). Therefore, understanding the causes and mechanisms of PHB alterations under repeated epileptiform discharges in young children is also important from a clinical point of view. Since the action of PHB is based on the potentiation of the action of GABA, the question arises whether the aggravation of epileptic seizures induced by PHB is due to the increased $[C^-]_I$ and excitatory action of GABA. The research results suggest that two main mechanisms can underlie the excitatory actions of GABA: increased activity of the chloride importer - NKCC1 cotransporter (Djala et al., 2005; Djala et al., 2008) and down regulation of the chloride cell exporter - KCC2 cotransporter (Jin et al., 2005; Pathak et al., 2007; Rivera et al., 2004; Viitanen et al., 2010).

Hypothetically, if the action of GABA is excitatory, then PHB should not block epileptiform discharges. To test the hypothesis of the study and the mechanisms underlying the permanently increased intracellular concentration of chloride ions, we used the MF model that we developed.

Phenobarbital suppresses initial ictal discharges, but aggravates epileptiform activity in MF

In order to test the effect of PHB when applying PHB (100 μM) throughout the experiment 15 minutes before the second application. The addition of PBS led to a decrease in the power of ictal discharges. After transection of commissural fibers

(after 15 applications of KA), epileptiform discharges were generated only in the ipsilateral hippocampus, which indicates the prevention of the formation of MF.

To test the effect of PHB on late and active changes, when GABA loses its inhibitory effect, PHB was applied to the contralateral hippocampus only after the 14^{th} application of KA on the ipsilateral hippocampus. It increased duration and amplitude, both evoked and spontaneous ictal discharges. In addition, the total power of ictal discharges increased by $24.2 \pm 7.3\%$.

Phenobarbital enhances the excitatory action of GABA and enhances the epileptiform activity in neurons MF.

Slices prepared from the isolated mirror focus hippocampi generate spontaneous interictal-like events in the 0.1–0.25 Hz range (Khalilov et al., 2003, 2005; Nardou et al., 2009). Phenobarbital augmented the number of interictal-like events (by $71 \pm 10\%$, $n = 20$, $P < 0.001$) and the number of spikes generated during and between interictal-like events ($65 \pm 11\%$, $n = 20$, $P < 0.001$). Therefore, phenobarbital aggravates epileptiform activities in the mirror focus Thus, PHB enhances the excitatory action of GABA in MF neurons, possibly due to the permanently high concentration of intracellular chloride as a result of impaired functioning of chloride cotransporters.

Are the pro-epileptic actions of phenobarbital mediated by the enhanced GABAergic excitation? We compared the effects of phenobarbital (100 μM) on control and mirror focus neurons using cell attached recordings. Focal GABA applications generated more action potentials in mirror focus neurons than in control ones (4.0 ± 0.3, $n = 6$ and 2.7 ± 0.2, $n = 7$, respectively, $P < 0.001$), suggesting a strengthening of GABA excitation in MF neurons. In contrast to control neurons, bath applications of phenobarbital increased the number of action potentials in MFones (from 4.0 ± 0.3 to 5.4 ± 0.4, $n = 6$, $P < 0.01$). Phenobarbital also increased significantly the perforated patch-clamp recorded post-synaptic currents generated by focal applications of GABA ($n = 4$): area from 177.5 ± 9.1 to 259.2 ± 7.4 nA/s ($P < 0.001$); amplitude: from 133.4 ± 3.8 to 162.9 ± 3.4 pA ($P < 0.001$); decay time: from 1953.7 ± 119.8 to 2359 ± 83.9 ms ($P < 0.001$) half-width: from 1346.7 ± 43.2 to 1557.2 ± 14.3 ms ($P < 0.001$). The rise time (10–90%) was not changed significantly (from 523 ± 18.6 to 562.8 ± 10.3 ms, $P = 0.53$). Therefore, phenobarbital augments GABA signals in mirror focus neurons possibly by a chronic increase of intracellular chloride. We next examined the roles of chloride cotransporters NKCC1 and KCC2 in these changes.

Functional Preservation of NKCC1 and Down-Regulation of KCC2 in MF Neurons

We have shown that continuous perfusion of the contralateral hippocampus with the NKCC1 antagonist did not prevent the formation of MF (Nardou et al., 2009). In the experiments, we used the preparation of the whole hippocampus of knockout mice (P7 - P8) in which NKCC1 is genetically damaged ($NKCC1^{-/-}$). Both in hippocampi of control mice (wild type) and in hippocampi prepared from $NKCC1^{-/-}$ mice, the application of KA generated ictal discharges with HFO. Repeated application of the KA on one hippocampus was accompanied by the formation of the

MF (n = 5/5). Therefore, it can be assumed that the functional integrity of the NKCC1 cotransporter is not a necessary condition for the formation of a MF.

Since the classical patch-clamp method in immature cells, recording in the whole-cell configuration gives a significant error in measuring the resting potential (Tyzio et al., 2003), in the experiments we used the non-invasive patch-clamp method for detecting the currents of single ion channels of NMDA and GABA (A) receptors. The driving force of the GABA-mediated responses (DF_{GAMK}) was calculated from the current – voltage characteristics of the currents flowing through the GABA (A) channels (Tyzio et al., 2006).

The resting potential of neurons was determined by the current – voltage characteristics of currents flowing through single channels of NMDA receptors (Leinekugel et al., 1997).

In control neurons, the average DF_{GAMK} value was positive (13.24 ± 12.16 mV). Application of bumetanide (10 μM) led to an almost complete disappearance of the depolarizing DF_{GAMK} (2.56 ± 3.84 mV), which indicates the participation of NKCC1 in the depolarizing action of GABA(A) in control neurons. This is also directly evidenced by the value of DF_{GAMK} (–2.08 ± 6.27 mV) in neurons of NKCC1$^{-/-}$ mice. In MF neurons, the depolarizing DF_{GAMK} was significantly higher (37.28 ± 8.08 mV), and the value of E_m did not differ from the value of E_m of control neurons (75.2 ± 5.4 mV, n = 10 and 78.5 ± 2.3 mV, n = 14, respectively, P = 0.08), which indicates a permanent increase in $[Cl^-]_i$ in MF neurons. It should be noted that bumetanide (10 μM) significantly decreases DF_{GAMK} in MF neurons than in control neurons. This indicates that NKCC1 is fully functional and has increased activity in the neurons of MF. However, while the DF_{GAMK} values in the control neurons in the presence of bumetanide and in the NKCC1$^{-/-}$ mice neurons are about 0 mV, the depolarizing DF_{GAMK} in the presence of bumetanide in the same neurons of the MF remains significantly increased (14.19 ± 7.64 mV, n = 18 and 19.09 ± 2.71 mV, n = 26, respectively). Therefore, it is logical to assume that other mechanisms underlie the permanent positive shift of EGAMA in MF neurons.

Since KCC2 knockout mice (KCC2$^{-/-}$) die at birth, we used KCC2 antagonists to study the role of KCC2 in the regulation of $[Cl^-]_i$ in control neurons and in MF neurons. In control neurons, depolarizing DF_{GAMK} was practically absent in the presence of bumetanide. Blocking KCC2 by adding a higher concentration of bumetanide (100 μM) blocking both NKCC1 and KCC2 (Payne, 1997) or another KCC2 blocker, DIOA (10 μM) (Boulenguez et al., 2010) led to a pronounced positive shift in DF_{GAMK} (up to 27.73 ± 8.78 and 29.06 ± 4.36 mV, respectively.

Thus, the obtained data indicate the critical role of KCC2 in the regulation of $[Cl^-]_i$ and DSGAMK in control neurons.

In similar experiments with MF neurons, we obtained significantly different results. Blocking NKCC1 with a low concentration of bumetanide significantly reduced DF_{GAMK} in MF neurons (from ~ 37 mV to 15 mV). Approximately the same DF_{GAMK} value was in NKCC1$^{-/-}$ neurons. Therefore, it is obvious that NKCC1 plays a significant role in the regulation of $[Cl^-]$ and in the neurons of MF. However, the simultaneous blockade of NKCC1 and KCC2 did not lead to significant changes in

DF_{GAMK}, which indicates a reduced function of KCC2 in MF neurons. Therefore, in addition to the possible increased activity of NKCC1, a decrease in the activity of KCC2 can also be the reason for the increased depolarizing DF_{GAMK} in the neurons of MF.

Chloride removal is slowed down in mirror focus neurons due to downregulation of KCC2

To directly determine chloride removal from neurons, we used with some modifications a paradigm used by other authors (Zhu et al., 2005; Achilles et al., 2007; Brumback and Staley, 2008). In brief, neurons were recorded in the perforated patch-clamp mode to preserve intact chloride, in the presence of CNQX (10 μM) and APV (40 μM) to block ionotropic glutamate currents. The holding potential (VH) was adjusted to have no current flow i.e. $V_H = E_{GABA}$. GABA (200 μM) was then focally pressure applied for 50–100 ms through a patch pipette to the recording neurons every 20 s . Large voltage steps (to $V_H = 0$ mV, 1 min duration, three GABA pulses) were applied to depolarize neurons. Immediately after the end of the pulse, focal GABA generated inward currents suggesting that chloride has accumulated. This current was reduced progressively until it returned to pre-conditioning values ($V_H = E_{GABA}$). The latency of the time to recover (recuperation time) provides a good indication of the efficacy of KCC2 to remove chloride that has accumulated during the depolarization. Interestingly, after the return to control values, outward post-synaptic currents were recorded suggesting a possible over activity of KCC2.

In mirror focus neurons, recuperation time was considerably (5-fold) enhanced (from 68.4 ± 7.9 s in control to 306 ± 33.5 s in mirror focus neurons, $n = 18$ and $n = 6$, respectively, $P < 0.001$) suggesting a chronic deficiency in chloride removal. Interestingly, in contrast to naïve neurons, the transient increase of outward currents after recuperation was not observed in mirror focus neurons (not shown). Recuperation time is largely mediated by KCC2 as its specific antagonist DIOA (10 μM) or bumetanide (100 μM) dramatically enhanced the half-decay time [from 19.5 ± 1.9 s in control, $n = 18$ to 31.8 ± 4.3 s in DIOA and 30.9 ± 2.2 in bumetanide (100 μM, $n = 7$ and $n = 6$, respectively, $P < 0.001$). Recuperation time in these experiments was not quantifiable as there was no return to initial EGABA even after 10 min). Therefore, chloride removal is altered in M neurons following a downregulation of KCC2.

Internalization of KCC2 in MF neurons

We used a specific KCC2 antibody that does not label neurons in $KCC2^{-/-}$ to examine the cellular distribution of KCC2. In the rat and mice control hippocampi KCC2 was primarily located near the membrane of cell bodies and proximal processes of CA3 pyramidal neurons. The labelling was often observed in clusters. We observed, already at P4, clusters of KCC2 close to the cell membrane at the light and electron microscopy levels. In contrast, the labelling in CA3 pyramidal neurons was largely intra-cytoplasmic in mirror focus hippocampi with few clusters, suggesting an internalization of KCC2 after repeated ictal-like events. The differences between the cellular distribution of KCC2 in naïve and mirror focus

neurons was statistically significant with a sharp peak in control hippocampi around the membrane and a higher and spread out labelling over the cytoplasmic compartment in mirror focus neurons. Therefore, there is an internalization and loss of activity of KCC2 in mirror focus neurons.

Bumetanide prevents aggravation of epileptiform activity induced by phenobarbital in the MF

Since the NKCC1 antagonist bumetanide reduces chloride in epileptic neurons, we tested the possibility that it would also reduce the pro-epileptic actions of phenobarbital in mirror focus slices. When applied together, phenobarbital and bumetanide reduced/blocked interictal-like events generated spontaneously by mirror focus slices. However, the order of applications of the two agents revealed important differences. Applications of phenobarbital first aggravated interictal-like events that were then reduced by the additional application of bumetanide (n = 5). In contrast, when applied first, bumetanide strongly reduced interictal-like events that were replaced by giant depolarizing potential-like events. Addition of phenobarbital at that stage further reduced neuronal activity and giant depolarizing potential-like events (n = 5). Therefore, bumetanide ameliorates the effects of phenobarbital but it is preferable to apply bumetanide before phenobarbital to avoid its initial pro-epileptic actions and reinforcement of epileptiform activities**.**

6. Phenobarbital inhibits AMPA/kainite receptor-mediated currents and, unlike diazepam, has an inhibitory effect at the initial stages of epileptogenesis

Neonatal seizures are inherently different from adult seizures and can be refractory to anti epileptic drugs (AEDs). Benzodiazepines – notably diazepam (DZP) – and phenobarbital are the AEDs of first choice to treat neonatal seizures (Wheless et al., 2007; Bassan et al., 2008). In general, PB is first used in neonatal seizures whereas diazepam or lorazepam are preferred in febrile seizures. They are thought to reduce seizures by reinforcing the efficacy of GABAergic inhibition through a direct action on GABA(A) receptors mediated currents. However, there are several complicating factors with their use. In many conditions they fail to block seizures, produce an electro-clinical disconnection of EEG and clinical signs (Connell et al., 1989; Painter et al., 1999; Boylan et al., 2002; Yanay et al., 2004; Guillet and Kwon, 2007; Kaindl et al., 2008) and even aggravate them (Loscher and Honack, 1989; Guerrini et al., 1998; Perucca et al., 1998; Painter et al., 1999; Goodkin et al., 2008; Goodkin and Kapur, 2009). These aggravating actions are at least partly mediated by a persistent increase of $[Cl^-]_i$ leading to more seizures by enhancing the excitatory actions of GABA that heavily depend on this parameter (also see Cohen et al., 2002; Khalilov et al., 2003, 2005; Rivera et al., 2004; Glykys et al., 2009; Dzhala et al., 2010; Nardou et al., 2011). Experimental observations indicate that PHB efficiently blocks early seizures but aggravates established ones when first applied after many recurrent seizures. This is due to a persistent accumulation of chloride and excitatory actions of GABA that will be further enhanced by PHB (Glykys et al., 2009; Dzhala et al., 2010; Nardou et al., 2011). These effects are mediated by a persistent activation of the chloride importer NKCC1 and a down regulation and internalization of the chloride exporter KCC2 (Dzhala et al., 2010; Lee et al.,

2010; Nardou et al., 2011). Clearly, the regulation of $[Cl^-]_i$ constitutes a major source of problem to the use of these AEDs and stresses the importance of the history of seizures prior to treatment in determining the efficacy of the treatment.

Collectively however, these observations are based on the assumption that the actions of PHB, DZP, and other members of this family are solely mediated by GABA signaling. Yet, there are several indications that this may not be the case. Barbiturate anesthetics have been reported to antagonize glutamate evoked currents in spinal neurons (MacDonald and Barker, 1978a,b, 1982; Macdonald and Kelly, 1994) and to reduce recombinant AMPA-type glutamate receptor channels expressed in cell lines (Jin et al., 2010). Also, barbiturates are more efficacious than benzodiazepines to treat various brain disorders including neonatal seizures (Cheng et al., 2002; Soderpalm, 2002; Carmo and Barr, 2005; Bartha et al., 2007) raising the possibility that additional non-GABA mediated signaling may underlie a better efficacy to treat various neonatal seizures

At this stage of the study, using the MF model, we compared the effects of PHB and DZP on glutamate receptor-mediated currents in control neurons and in epileptic focus neurons.

Diazepam aggravates the propagating ictal-like discharges and does not prevent the formation of MF

We have shown that the application of PHB to the contralateral hippocampus was accompanied by a marked inhibition of the spreading ictal discharges and the prevention of the formation of MF. The application of diazepam in the same experiments, on the contrary, led to an increase in the spreading ictal discharges and did not prevent the formation of MF.

It is believed that HFO are an important factor determining the pathogenic action of ictal discharges. It has been shown that HFO (> 60 Hz), both in human epilepsy and in experimental animal epilepsy models, are one of the main signs of excessive pathogenicity of epileptic discharges (Bragin et al., 1999a; Bragin et al., 1999b; Jacobs et al., 2008a; Khalilov et al., 2005). As we have already experimentally demonstrated, in the preparation of the intact hippocampus, the presence of HFO during propagating ictal discharges is one of the determining conditions for the formation of MF. In these experiments, the PHB significantly reduced the frequency of HFO and the power of ictal discharges, while DZP, on the contrary, increased the range of HFO, the power and amplitude of the ictal discharges of the contralateral hippocampus by 19.2 ± 3.9% ($n = 7$, $P < 0.05$) and not prevented the formation of MF.

Thus, PHB and DZP in an immature hippocampus have opposite effects: PHB weakens, and DZP aggravates early ictal discharges.

Diazepam enhances epileptiform activity in the mirror epileptic focus

In the preparation of the whole hippocampus after the formation of the MF, the application of DZP significantly increased the frequency, amplitude and duration of spontaneous ictal discharges and, accordingly, their total spectral power. The average spectral power of spontaneous ictal discharges after the application of DZP increased

by 80.0 ± 19.8% (n = 4, P <0.001). In slices prepared by MF, DZP also increased the frequency of spontaneous spikes and interictal discharges. As described above, in exactly the same experiments, PHB aggravated epileptiform activity in MF. Thus, the effects of PHB and DZP on epileptic activity depend on the stage of epileptogenesis and may even differ polarly at different stages of epileptogenesis. While primary discharges are blocked by PHB, but DZP are amplified, spontaneous discharges in an already formed secondary epileptic focus are amplified by both drugs. If the pro-epileptic effects of both drugs in the late stages of epileptogenesis are most likely due to a positive shift in E_{GABA} and an increase in GABAergic excitation, then their opposite effects in the early stages of epileptogenesis (when there are no epileptogenic shifts of E_{GABA} yet) are probably associated with effects not mediated by GABA(A) - receptors. Therefore, at the next stage of the study, we conducted a detailed pharmacological analysis of PHB and DZP, which included a study of their effects on physiological patterns of activity on hippocampal slices, as well as on synaptic responses mediated by glutamate receptors.

Diazepam enhances physiological neuronal activity of immature hippocampus

In control conditions, the physiological pattern of the immature hippocampal network activity is characterized by spontaneous network driven giant depolarizing potentials (GDPs; Ben-Ari et al., 1989) that occur spontaneously and can be evoked by electrical stimulation. GDPs are generated by the converging actions of GABA and glutamatergic synapses (Ben Ari et al., 2007). We tested the effects of DZP on the frequency of occurrence of extracellular recorded GDPs and MUAs. Diazepam, applied at a concentration of 2 μM, increased the frequency of GDPs by 215.1 ± 13.6% ($p < 0.001, n = 14$) and the frequency of spikes by 170 ± 14.8% ($p < 0.001, n = 14$). In similar experiments, bath application of PHB substantially reduced the amplitude of GDPs. PHB reduced also the frequency of spontaneous spikes

generated in CA3 pyramidal neurons by ~50% (see Nardou et al., 2011).

To determine the actions of DZP on GABA signaling, we tested the effects of DZP on responses generated by focal applications of GABA. GABA was focally applied (100 μM, 100 ms) in the CA3c region while simultaneous CA3c field and whole cell recordings (from pyramidal neurons in CA3a region) were performed. Focal GABA application generated GDPs. Application of DZP significantly increased the amplitude and duration of whole-cell recorded responses accompanied by a 17.2 ± 2.7% ($p < 0.05$) as well as the area and decay time constant of the currents increase in the amplitude of extracellular recorded GDPs and with a strong increase in the number of GDPs-associated spikes (98.1 ± 6.7%, $p < 0.001$, n = 5). Similar focal application of GABA generated 2–3 action potentials (spikes) in cell attached recordings (see Nardou et al., 2011). In contrast to DZP, bath applications of PHB (100 μM) significantly reduced the number of spikes (action potentials) from 2.7 ± 0.2 to 1.9 ± 0.2 (Nardou et al., 2011). Therefore, under control conditions PHB

reduces ongoing neuronal activity whereas DZP augments it. The observation that GABA acting PHB reduces and DZP augments ongoing neuronal activity raises the possibility of non-GABA mediated actions of these AEDs. We therefore compared their actions on glutamatergic signals that are instrumental in the generation of early network activity.

Phenobarbital but not DZP inhibits AMPA/kainate-receptor-mediated interictal-like discharges

To determine the effects of DZP and PHB on glutamatergic activity, we first studied their effects on field potentials recorded in control slices in the presence of bicuculline (10 μM), CGP (2 μM) and APV (40 μM), respectively, to block GABA(A), GABA(B), and NMDA receptors. In these conditions, all or none interictal-like discharges are generated spontaneously and by local electrical stimuli. Spontaneous interictal-like discharges are mediated by AMPA/kainate receptors as they were fully blocked by the selective antagonist CNQX (10 μM, n = 13). PHB fully blocked the evoked and spontaneous interictal-like discharges (n = 7). The frequency histograms reflect the powerful blockade of spikes and interictal-like discharges by PHB. As 10 μM bicuculline do not always fully block GABAergic signaling (Strata and Cherubini, 1994) we also repeated these experiments with 100 μM picrotoxin instead of bicuculline and observed similar actions of PHB (n = 3). In contrast, DZP (n = 6) failed to block or reduce these field potentials. Thus, DZP did not reduce the evoked or the spontaneous interictal-like discharges . Therefore, PHB but not DZP exerts a non-GABA mediated action on immature hippocampal neurons.

Phenobarbital but not DZP reduces AMPA/kainate receptor mediated postsynaptic currents

To examine the possible effects of PHB and DZP on AMPA/kainate receptors, we next tested their effects on the currents evoked in whole cell recordings by focal pressure applications of glutamate. In the presence of NMDA and GABA receptor antagonists, focal applications of glutamate (100 μM, 100 ms) from patch pipettes generated large currents mediated by AMPA/kainate receptors as they are completely blocked by CNQX (n = 9). PHB reduced significantly the amplitude of these currents (to 67.2 ± 8.1%, $p < 0.001$, n = 4). These effects fully recovered after wash out of PHB. In contrast, DZP had no effects on these currents (mean amplitude in DZP was 100.3 ± 4.9% of the control values, $p > 0.05$, n = 5). Next, in similar experimental conditions using fast agonist application techniques (Colquhoun et al., 1992) glutamate (1 mM, 2 ms) was applied to the nucleated patch clamp recorded CA3 pyramidal neurons. Phenobarbital significantly reduced the amplitude of glutamate induced currents (by 25.5 ± 0.5%, $p < 0.001$, n = 8). The decay time constant of these currents was not changed in the presence of PHB (3.6 ± 0.5 ms in control and 3.4 ± 0.4 ms in the presence of PHB, respectively, $p > 0.5$). In similar experiments DZP had no statistically significant effects on glutamate induced currents (mean current amplitude in DZP was 97.9 ± 3% of the control, $p > 0.3$, n = 4). In addition, we tested the effect of PHB and DZP on NMDA receptor mediated currents. In the presence of AMPA/kainate and GABAreceptor antagonists focal puff applications of glutamate (100 μM, 100 ms) generated (at +40 mV holding potential) large amplitude outward

currents. These currents are mediated by NMDA receptors as they were blocked by the NMDA receptor antagonist APV (40 μM, n = 11). Neither PHB nor DZP altered these currents. Therefore, PHB selectively reduces AMPA/kainate receptor mediated currents.

CONCLUSION

During the dissertation study, new *in vitro* preparations were developed to study the generation and propagation of network activity in the structures of the limbic system of the immature brain. Morphological studies have shown that these preparations can also be used extremely successfully for detailed study of the morphology of neurons, which, in contrast to thin slices, allow you to completely reconstruct all the dendritic and axon branches of neurons, get a three-dimensional reconstruction of these neurons and explore the neuronal connections between the structures of the limbic system.

Another advantage of the preparation of the whole hippocampus is that in the hippocampus already 3-4 – day-old rats, the application of KA causes long – term (1-2 min) classic tonic-clonic ictal discharges, characteristic of young children with generalized tonic-clonic seizures. In traditional thin slices of the hippocampus from P6-P7 rats, KA application causes only short (several hundred ms) interictal-like discharges, which, unlike ictal discharges, are accompanied by only a small increase in the extracellular concentration of potassium and calcium ions. The inability of hippocampal slices to generate ictal discharges can be explained by the fact that: 1) during their preparation, many interneuronal synaptic connections are lost and may disappear the minimum number of neurons required to generate an ictal discharge; 2) in thin slices, compared with the whole hippocampus, conditions for extra synaptic and field interactions, as well as for the accumulation of extracellular potassium ions, are worsened. Therefore, we can assume that in order to study the generation and propagation of physiological and epileptiform discharges in the immature brain, the preparations developed by us are more revalent than the slices. This is indicated by the fact that the whole hippocampus preparation is currently used in leading laboratories in Germany, Canada, Russia, United States, Finland, France and other countries.

Combined preparations of the hippocampus, including the septum and / or entorhinal cortex, allow us to study the features of epileptic discharges between different structures of the limbic system during the first postnatal week. Our experiments have shown that: 1) the increase in epileptiform activity in the CA3 pyramidal cells of the hippocampus with age occurs in parallel with the increase in KA-induced postsynaptic currents; 2) the hippocampus is the primary structure that generates paroxysmal activity in response to the action of KA and the propagation of this electrical activity to other structures of the limbic system is age-dependent.

The use of a preparation consisting of two hippocampus interconnected by commissural fibers and chamber with 3 compartments allowed us to develop a model of secondary epileptogenesis *in vitro*, which experimentally revealed the main mechanisms and conditions for the formation of a secondary epileptic focus in the immature hippocampus. First, our experiments show that ictal discharges caused in

one hippocampus are propagated to the contralateral hippocampus and such repeated ictal discharges (14 – 15 times, every 15 min) can transform the contralateral hippocampus into a secondary (mirror) epileptic focus (MF). Second, spontaneous or induced epileptiform discharges are also recorded in thin slices prepared from the contralateral hippocampus after the formation of MF. Third, epileptiform discharges that are generated in the MF are readily propagated to other structures of the limbic system, including the septum and entorhinal cortex, which indicates that the "kindling" effect is not limited only in the contralateral hippocampus. Fourth, one of the important conditions for the formation of MF is the activation of NMDA and GABA(A) receptors during ictal discharges. Fifth, ictal discharges without HFOs are also not accompanied by the appearance of MF. This indicates that there is a direct link between HFO during ictal discharges and formation of an epileptic focus. Therefore, it is possible to suggest the HFO during ictal discharges as a biomarker of the epileptogenicity of these discharges and the serious risk of secondary epileptic foci.

Based on the data obtained in the framework of the dissertation research, the concept of a "vicious circle of secondary epileptogenesis" was formulated, which is a cascade of pathophysiological changes in the developing hippocampus that occur as a result of repeated ictal-like discharges and lead to the formation of a “chronic” secondary epileptic focus (Fig.4).

1) In the initial conditions, the balance between excitation and inhibition in the hippocampus prevents the occurrence of super-synchronous high-amplitude activity, which is characteristic of epileptic discharges.

2) During propagation of epileptiform discharges from one hippocampus to the contralateral hippocampus start the process of induction of secondary epileptogenesis, which are based on co-activation of NMDA and GABA(A) receptors, which leads to increased intracellular concentration of chloride and calcium ions in the neurons of the epileptic focus. The precursor of long-term epileptiform changes is the presence of of HFO in the epileptiform discharges, the necessary condition for the generation of which is also the co-activation of NMDA and GABA (A) receptors.

3) The main reason for the increase in the intracellular concentration of chloride ions, as well as the decrease in the ability of neurons to remove chloride ions from neurons during epileptogenesis, is the dysfunction of the KCC2 chloride extruder as a result of its internalization through intracellular regulatory cascades not yet established.

4) Disruptions in intracellular chloride homeostasis due to a decrease in KCC2 activity lead to a positive shift in the potential for reversing postsynaptic responses mediated by GABA (A) receptors. As a result, a persistent excitatory GABA(A) is formed in the neurons of the secondary epileptic focus, a phenotype, which is the main mechanism for the expression of the epileptogenic process. At the same time, at the network level, the balance between the mechanisms of excitation and inhibition is shifted towards excitation, which results in the formation of a secondary epileptic

focus that can independently generate epileptiform activity and restart the next cycle of the “vicious circle” of epileptogenesis.

5) It is possible to break the “vicious circle” of secondary epileptogenesis at the level of induction by blocking the NMDA and GABA(A) receptors and thus preventing HFO. In the already formed focus, suppression of epileptiform activity is possible by correcting the intracellular homeostasis of chloride ions by blocking the NKCC1 chloride co-transporter with a selective antagonist bumetanide.

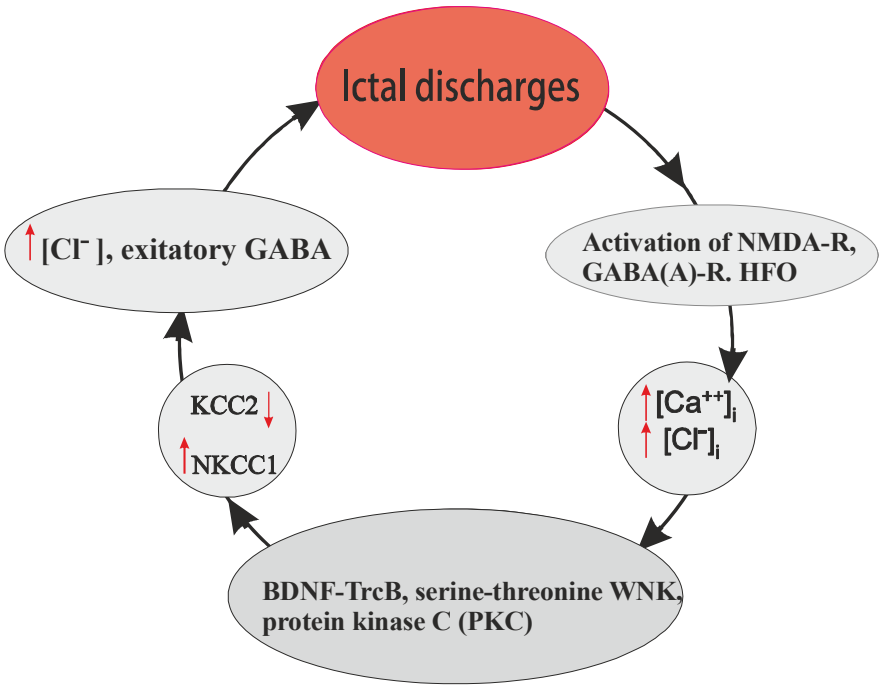

Figure 4. The "vicious circle" of secondary epileptogenesis.

FINDINGS

1. Preparations of the whole and double hippocampus, as well as the entorhinal-hippocampal-septal complex of newborn rats *in vitro*, developed by us in the course of the dissertation research, have several advantages over thin sections, as they allow: a) to completely reconstruct dendritic and axon branches of pyramidal cells and interneurons, get a three-dimensional reconstruction of these neurons; b) to study the inter structural connections in the limbic system and the processes of generation and propagation of synchronized network activity within and between the structures of the limbic system of newborn rats and mice.

2. Studies of age-related changes in the generation and distribution of epileptiform discharges caused by KA have led to a number of important observations: a) age-dependent increase in epileptiform activity in the hippocampus during the first postnatal week occurs in parallel with an increase in KA-induced postsynaptic currents in CA3 pyramidal neurons; b) the hippocampus is the primary structure generating paroxysmal discharges in the limbic system of newborn rats in response to the action of KA; the distribution of these discharges into other structures of the limbic system is age-dependent; c) in the preparation of the whole

hippocampus, ictal discharges can be caused at a much earlier age than in thin sections.

3. Repeated ictal discharges induced by KA in the preparation of the whole hippocampus can provoke a long-term epileptiform transformation in the activity of the neural network of the hippocampus, which generates itself as spontaneous paroxysmal discharges that persist for several days. Ictal discharges are propagated through the commissural fibers into the contralateral hippocampus, where they can also induce the formation of MF.

4. A prerequisite for long-term epileptiform changes and the formation of MF in the hippocampus in newborn rats is the activation of NMDA and GABA(A) receptors and the presence of HFO in the ictal discharges of the contralateral hippocampus. The presence of HFO in ictal discharges is a reliable biomarker of the epileptogenic process. GABA(A) receptor antagonists have an ictogenic effect, but prevent the formation of MF. In slices of the hippocampus of adult rats, unlike newborns, blocking GABA(A) receptors does not prevent epileptogenesis.

5. The formation of MF in the hippocampus in newborn rats is accompanied by an increase in the excitatory effect of GABA on CA3 hippocampal pyramidal cells, which is due to a positive shifts of the GABA(A) receptor reversal potential. An increase in the intracellular concentration of chloride ions and a decrease in the rate of removal of chloride ions from MF neurons are due to dysfunction of the KCC2 cotransporter as a result of its internalization.

6. Recovery of chloride homeostasis and a decrease in the excitatory effect of GABA on MF neurons using the selective blocker of the cationic chlorine cotransporter NKCC1 bumetanide do not prevent the formation of MF, but bumetanide blocks epileptiform activity in the already formed MF.

7. The genetic elimination of NKCC1 also does not prevents the formation of MF, but, unlike the pharmacological blocking of the NKCC1 cotransporter by bumetanide, it does not have an antiepileptic effect in MF.

8. Allosteric modulators of GABA(A) -receptors PHB and DZP differently affect secondary epileptogenesis. DZP prevents neither the generation, nor the propagation of KA-induced ictal discharges, nor the formation of a secondary epileptic focus. PHB suppresses ictal discharge and prevents secondary epileptogenesis, which is due to the additional inhibitory effect of PHB on AMPA / kainate receptor-mediated postsynaptic currents. In the already formed MF, both drugs enhance the excitatory effect of GABA and aggravate epileptiform activity. The blockade of the NKCC1 cotransporter by bumetanide prevents the aggravation of epileptiform activity generated by PHB in MF neurons.

LIST OF MAIN PUBLICATIONS ON THE TOPIC OF THE DISSERTATION

1. **Khalilov I.**, Khazipov R., Esclapez M. & Ben-Ari Y. (1997) Bicuculline induces ictal seizures in the intact hippocampus recorded in vitro // ***European Journal of Pharmacology***. -V.319(2-3). -P. R5-R6.

2. **Khalilov I._** , Esclapez M., Medina I., Aggoun J., Lamsa K., Leinkugel X., Khazipov R. & Ben-Ari Y. (1997) A novel in Vitro preparation: the intact Hippocampal Formation // ***Neuron*.** –V.19(4). –P. 743-749.
3. Khazipov R., Leinekugel X., **Khalilov I.** , Gaiarsa J.L. & Ben-Ari Y. (1997) Synchronization of GABAergic interneuronal network in CA3 subfield of neonatal rat hippocampal slices // ***Journal of Physiology*** (London). –V.498. –N.3. –P. 763-772.
4. Leinekugel X., Medina I., **Khalilov I.**, Ben-Ari Y. & Khazipov R. (1997) Ca2+ oscillations mediated by the synergistic excitatory actions of $GABA_A$ and NMDA receptors in the neonatal hippocampus // ***Neuron***. –V.18. –P. 243-255.
5. Leinekugel X., **Khalilov I. A**., Ben-Ari, Y Khazipov R.N. (1998) Giant Depolarizing Potentials: the Septal Pole of the Hippocampus Paces the Activity of the Developing Intact Septohippocampal Complex In Vitro//***Journal of Neuroscience***. –V.18. –N.16. -P.6349-6357.
6. **Khalilov I.**, Dzhala V., Medina I., Leinekugel X., Melyan Z., Lamsa K. , Khazipov R. & Ben-Ari Y.(1999) Maturation of kainate-induced epileptiform activities in interconnected intact neonatal limbic structures in vitro // ***European Journal of Neuroscience***. –V.11. –P. 3468-3480.
7. **Khalilov I.**, Dzhala V., Ben-Ari Y. and Khazipov R. (1999) Dual role of GABA in the neonatal rat hippocampus // ***Developmental Neuroscience*.** –V.21. –P. 310-319.
8. Leinekugel X., **Khalilov I.**, McLean H., Caillard O., Gaiarsa J.-L., Ben-Ari Y., Khazipov R. (1999) GABA is the principal fast-acting excitatory transmitter in the neonatal brain // ***Advances in neurology.*** *–V.*79. –P. 189-201.
9. **Khalilov I.,** Morozova E. and Gozlan H. (2002) Status epilepticus induced by propagating seizures in the neonatal rat hippocampus *in vitro* // ***Epilepsies***. –V.14. –N.3. –P. 195-201.
10. **Khalilov I.,** Holmes GL and Ben-Ari Y. (2003) *In vitro* formation of a secondary epileptogenic mirror focus by interhippocampal propagation of seizures ***// Nature Neuroscience.*** -V.6. –P. 1079-1085.
11. Khazipov R., **Khalilov I.,** Tyzio R., Morozova E., Ben-Ari Y. and Holmes GL. (2004) Developmental Changes in GABAergic Actions and Seizure Susceptibility in the Rat Hippocampus // ***European Journal of Neuroscience***. **–V.**19. –N.3. –P. 590-600.
12. **Khalilov I**, Le Van Quyen M, Gozlan H, Ben-Ari Y. **(**2005) Epileptogenic actions of GABA and fast oscillations in the developing hippocampus // ***Neuron*.** –V.48. –N.5. –P. 787-796.
13. MHFOel Le Van Quyen, **Ilgam Khalilov ,** Yehezkel Ben-Ari. (2006)The dark side of high-frequency oscillations in the developing brain // ***Trends in Neuroscience.*** V.29. –N.7. –P. 419-427.
14. Romain Nardou, Yehezkel Ben-Ari and **Ilgam Khalilov.** (2009) Bumetanide, an NKCC1 Antagonist, Does Not Prevent Formation of Epileptogenic Focus but Blocks Epileptic Focus Seizures in Immature Rat Hippocampus // ***Journal of Neurophysiology.*** –V.101. –N.6. –P. 2878-2888.

15. Tyzio R., **Khalilov I.,** Represa A., Crepel V., Zilberter Y., Rheims S., Aniksztejn L., Cossart R., Nardou R., Mukhtarov M., Minlebaev M., Epsztein J., Milh M., Becq H., Jorquera I., Bulteau C., Fohlen M., Oliver V., Dulac O., Dorfmüller G., Delalande O., Ben-Ari Y., Khazipov R. (2009) Inhibitory actions of the gamma-aminobutyric acid in pediatric Sturge-Weber syndrome // ***Annals of Neurology.*** –V.66. –N.2. –P. 209-218.
16. Ben-Ari Y and **Khalilov I.** Oscillatory activity: Seizures Beget Seizures in the Developing and Adult Brains: Central Role of GABA and High Frequency Oscillations. (2009) // In: Philip A. Schwartzkroin, editor Encyclopedia of ***Basic Epilepsy Research.*** -Vol 2. Oxford: Academic Press; -P. 1019-1023.
17. Nardou R., Yamamoto S., Chazal G., Bhar A., Ferrand N., Dulac O., Ben-Ari Y., **Khalilov I.** (2011) Neuronal chloride accumulation and excitatory GABA underlie aggravation of neonatal epileptiform activities by phenobarbital // ***Brain.*** –P.134(Pt 4). –P. 987-1002.
18. Nardou R., Yamamoto S., Bhar A., Burnashev N., Ben-Ari Y., **Khalilov I.** (2011) Phenobarbital but Not Diazepam Reduces AMPA/kainate Receptor Mediated Currents and Exerts Opposite Actions on Initial Seizures in the Neonatal Rat Hippocampus // ***Frontiers in Cellular Neuroscience.*** –V. 5. –A.16.
19. **Khalilov I.**, Chazal G., Chudotvorova I., Pellegrino C., Corby S., Ferrand N., Gubkina O., Nardou R., Tyzio R., Yamamoto S., Jentsch T.J., Hübner C.A., Gaiarsa J.L., Ben-Ari Y., Medina I. (2011) Enhanced Synaptic Activity and Epileptiform Events in the Embryonic KCC2 Deficient Hippocampus. // ***Frontiers in Cellular Neuroscience.*** –V.5. –A.23.
20. Ben-Ari Y, **Khalilov I**, Kahle KT, Cherubini E. (2012) The GABA Excitatory/Inhibitory Shift in Brain Maturation and Neurological Disorders**.** ***The Neuroscientist.*** –V.18(5)-P. 467-486.
21. **И. А. Халилов**, Г.Ф. Ситдикова, Р.Н. Хазипов, А.Л Зефиров. (2014) Вторичный эпилептогенез в незрелом мозге: роль ГАМК // ***Журнал неврологии и психиатрии им. С.С. Корсакова.*** 114(4): 41-51.
22. Khazipov R, Valeeva G, **Khalilov I.** (2015) Depolarizing GABA and developmental epilepsies // ***CNS Neuroscience & Therapeutics*** **. – V.**21. -N2. –P. 83-91.

Author's e-mail: ilgam.khalilov@gmail.com

Address for reviews of the synopsis: 420008, Kazan, Kremlyovskaya st, 18, Kazan (Volga Region) Federal University, отдел аттестации научно-педагогических кадров, to Scientific secretary of the Dissertation Council KFU 03.06. Anikina Tatyana Andreevna, tel: (843)238-76-01, e-mail: tania57vgl@rambler.ru