PhD THESIS

INVOLVEMENT OF GAP JUNCTIONS IN EPILEPTOGENESIS AND MANIFESTATION OF SEIZURES OF THE IMMATURE AND ADULT NEOCORTEX

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INTRODUCTION AND AIMS

Epilepsy is a functional disorder of the central nervous system, which can be characterized by excessive abnormal, synchronized rhythmic firing of large populations of neurons termed seizures. The hypothesis that cellular interconnection through electrical synapses, could be a mechanism underlying epileptiform discharges was introduced more than a decade ago and is supported by recent electrophysiological and anatomical data. The blockade of GJal communication has been shown to reduce seizures in various *in vitro* models of epileptiform discharges. Treatment that favors GJ channels opening has been found to promote seizure-like activity. Nevertheless, to date, very little evidence has been published showing an involvement of GJs in seizures under *in vivo* conditions.

Clinical experience and various experimental data indicate that the developing nervous system is more sensitive than the mature one to different convulsive effects. Although the physiological factors underlying this differential epileptogenicity have not been fully clarified, the higher susceptibility of the immature brain can be explained by certain characteristic neurobiological features. Intercellular communication via GJ channels is an important form of cell-to-cell communication in early brain development. It is believed that there is a possible correlation between the high seizure susceptibility of the immature brain and the elevated communication through the GJ channels. However, the role of GJal coupling in epilepsy in the developing nervous system is still not fully understood.

Recently, there has become an increasing awareness that glial cells are actually an integral part of the electrical circuitry of the brain. Spontaneous activity in astrocytes and neurons is organized in neuronal/astrocytic networks in which neuronal activity regulates the network properties of astrocytes. On the other hand astrocytes can release glutamate and other neuroactive substances providing feedback on the excitability of the adjacent neurons. Although, accumulating data support the idea that GJal communication plays a significant role in neuronal synchronisation during epileptiform activity, the function and involvement of different types of connexins in the initiation, maintenance and propagation of seizure is not obvious yet and need further elucidation.

Accordingly, the aims of our study were to investigate the following questions using the 4-aminopyridine (4-AP) *in vivo* epilepsy model:

- the functional involvement of neural and/or glial GJal communication of the adult neocortex in:
    - normal physiological activity of the cortex (basic cortical electric activity, evoked responses),
    - epileptogenesis and seizure susceptibility,
    - induction and maintenance of epileptiform activity,
    - control of the duration of seizures,

- the selective contribution of neuron specific GJs via Cx36 to epileptogenesis, ictogenesis and propagation of seizures in adult animals,

- the functional significance of neural and/or glial GJal communication in the epileptogenicity and seizure susceptibility of the immature mammalian brain,

- the basal mRNA expression levels for connexin (Cx) 26 (neuron specific, developing), Cx32 (oligodendrocyte specific), Cx36 (neuron specific, matured) and Cx43 (astrocyte specific) during the development,

- the changes induced by epileptiform activity in the mRNAs level for Cx26, 32, 36 and 43 both in the developing and adult animals.

MATERIALS AND METHODS

Animals

For electrophysiological and molecular biological measurements, P9-28 (18-50g) and adult (200-300g) Wistar rats of both genders were used. The animals were bred in our laboratory and housed under standard laboratory conditions, with food and water available ad libitum. All experimental procedures were conducted in accordance with the United States Public Health Service's *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Szeged.

Drugs

For electrophysiological and pharmacological experiments: 4-AP, carbenoxolone, quinine, octanol and trimethylamine (TMA) were purchased from Sigma, St.Louis, USA.

For molecular biological measurments: RNAzol B reagent was purchased from Tel-Test Inc., Friendswood, TX, USA. RNase-free DNaseI was obtained from Boehringer-Mannheim, Germany. dNTP was obtained from Roche, Indianapolis, IN, USA. M-MuLV reverse transcriptase, Taq polymerase, 1.5% agarose gel, β-actin and the primers of Cx26, 32, 36 and 43 were purchased from Sigma, St.Louis, USA.

Electrophysiological experiments

In vivo experiments were carried out on pentobarbital-anaesthetized rats; ictal epileptiform activity was induced by local application of 4-AP to the cortical surface. Four silver ball electrodes were used to record the ECoG from the primary focus, from the secondarily induced mirror focus, and from two other points, in order to detect the propagation of epileptiform discharges. The electrophysiological data were recorded continuously by means of an 8-channel electroencephalograph (with a low-frequency filter of 0.1 Hz and a high frequency-filter of 70 Hz) and stored in a computer memory for off-line analyses.

Pharmacological manipulation of GJal communication

Two types of experimental protocols were used in order to investigate the possible contribution of GJal communication either to epileptogenesis and seizure susceptibility in normal brain tissue (*pretreatment*) or to the induction, manifestation and propagation of seizures at already established epileptic foci (*treatment*).

In pretreatment and treatment experiments, carbenoxolone and octanol were used as broad-spectrum GJ blockers to uncouple both neuronal and glial GJs. Quinine an antimalarial drug was applied for selective blocking of neuron specific Cx36. TMA, an intracellular alkalinizing agent was used for opening of the GJs. In developing animals, GJs were manipulated with carbenoxolone or TMA.

Molecular biological measurements

Semiquantitative RT-PCR amplification was used to measure the levels of different connexin mRNAs at the untreated cortex or epileptic foci of developing and adult neocortex.

In adult animals samples were taken from the areas of the Pf and Mf, one hour after the onset of the first seizure. In developing animals due to the generalized appearance of epileptiform activity the cortical tissues of the Pf area were isolated. For control values, the identical cortical areas of animals without induced epileptic activity were used.

Statistical analysis

In *electrophysiological experiments*, the effects of the drugs were assessed by measuring the latency of the first ictal period, the number and duration of seizures, and the summated ictal activity (determined by multiplying the number of individual ictal periods by their durations measured during 20 min period) by analyzing the pattern (frequency and amplitude) of the seizure discharges both in the Pf and Mf and by recording the spread of seizure activity to other field of the cortex.

Data were analyzed with the aid of Digidata 1200B (BD, BNC, Axon Instruments, Inc.) after the experiments. Student's t-test was used to assess significant differences between the control and experimental groups of data. The level of statistical significance was set at $P \leq 0.05$.

In molecular biological measurements statistical analysis of the data was done by a two-way ANOVA. When significant differences were found with the overall analysis, the Tukey HSD test was used for post hoc comparisons between groups (significance criterion: $P \leq 0.05$).

RESULTS

Observations in adult animals:

(1) Manipulation of the functional state of the GJs in a normal physiological state of the adult brain (*pretreatment*) apparently did not influence the basic cortical electric activity and slightly modified the induction and expression of seizure discharges. These observations suggest that GJs have weak involvement in epileptogenesis and seizure susceptibility in the adult neocortex (*Szente et al, Neuroscience; 115/4:1067-1078, 2002*).

(2) In contrast, more expressive changes were observed in the seizure activity when the GJs were manipulated after 25-30 seizures (*treatment*), in comparison with the pretreatment situation. Blockade of the GJs with carbenoxolone or octanol exerted a strong anticonvulsive effect: shortened the duration of seizures, decreased the amplitude of the seizure discharges and depressed the propagation of epileptiform activity. On the other hand, opening of the GJs with trimethylamine had a robust proconvulsive effect and increased significantly the parameters of epileptiform activity mentioned before. In addition, the expression levels of Cx32, Cx43 and Cx36 mRNAs increased significantly in the active epileptic foci. These observations indicate that plastic modifications in the Cx32, 36 and 43 expression during an acute episode of repeated seizures might significantly amplify the synchronous interconnections among the cells and underlie the pathophysiological mechanism involved in ictogenesis and the propagation of seizures. In addition, dynamic changes in the opened or closed state of the GJs contribute to the control of duration of seizures (*Szente et al, Neuroscience; 115/4:1067-1078, 2002; Gajda et al., Epilepsia; 44/12:1610-1615, 2003*).

(3) Selective blockade of interneuronal communication via Cx36 by quinine after repeated seizures resulted in the appearance of a new discharge pattern with frequencies above 15Hz at the initiation of seizures and increased the number of seizures. However, the summated ictal activity decreased, because of the significant reduction in the duration of the seizures. The amplitudes of the seizure discharges of all the patterns decreased, with the exception of those with frequencies of 11-12 Hz. Our observations indicate that specific blockade of neuronal Cx36 at the already active epileptic focus had an anticonvulsive effect and characteristically modified the manifestation of seizure discharges. It seems that GJ communication is differently involved in the induction and maintenance of seizure discharges. We suppose that

Cx36 GJ communication keeps control the activity of neuronal networks that are involved in the induction of seizures (*Gajda et al., Epilepsia; 46(12):1998-2004, 2005*).

(4) The selective and/or global blockade of neuronal and glial GJs resulted to some extent in different modifications in ictogenezis. Global blockade of the connexins following the blockade of Cx36 further decreased the summated ical activity and the amplitude of seizure discharges of all frequencies and further shortened the duration of seizures. Indeed, significant increases were detected in the levels of expression of the major astroglial Cx43 and oligodendroglial Cx32 mRNAs at the already active epileptic foci. Based on these observations we suggest that both neuronal and glial GJ communication contribute to the manifestation and propagation of seizures in the adult rat neocortex, although probably with different ways and degrees (*Gajda et al., Epilepsia; 46(12):1998-2004, 2005*).

Observations on developing animals:

(5) In developing animals, the basic ECoG and the 4-AP-induced epileptiform activity exhibited progressive changes. On maturation, new, faster components appeared with higher frequencies in the ECoG activity. The seizures became focalized and periodic; the discharges became faster with increasing frequency and the amplitudes of the discharges differentiated depending on the frequency. A transient decrease (P13-14) and then increase (P15-16) were detected in seizure susceptibility (*Gajda et al., Epilepsia; 47(6):1009-1022, 2006*).

(6) The basic expressions and the inducibility by epileptiform activity of Cx36, 43, 32 and 26 mRNAs displayed subtype-specific changes at the various developmental time points. Cx mRNA expressions were significantly upregulated around P16 (except for Cx26). The Cx26, 36 and 43 gene inducibility was highest around P16 and then declined significantly (*Gajda et al., Epilepsia; 47(6):1009-1022, 2006*).

(7) The GJ opener induced rhythmic synchronous cortical activity resembling seizure discharges at P9-12. In contrary to adults, manipulation of the GJs more efficiently modified both the epileptogenezis and the maintenance of seizure discharges in the young animals. Based on our results of electrophysiological, pharmacological and molecular biological experiments we suggest that the characteristic quantitative and qualitative composition of the GJ pool may be responsible part for the elevated epileptogenicity and seizure susceptibility of the developing brain (*Gajda et al., Epilepsia; 47(6):1009-1022, 2006*).

PRACTICAL IMPORTANCE

Epilepsy is one of the most common neurological disorder affecting about 4% individuals over their lifetimes. In up to 70-80% of people, seizures can be controlled with modern medicines and surgical techniques. However, up to 20-30% of people with epilepsy will continue to experience seizures even with the best available treatment. In addition, people with epilepsy suffer profound social, physical and psychological consequences.

The significant number of patents that experience poorly controlled seizures suggests that some clinically relevant proepileptic mechanisms are not targeted by any of the current available antiepileptic drugs. Based on our observations, we are convinced that drugs developed for modification of the functional state and/or the expression level of the GJs open new therapeutic approaches for epilepsy treatment.

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