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

PhD Thesis

Zita Gajda

Supervisor:
Dr. Magdolna Szente, D.Sc.
Professor of University of Szeged

Department of Comparative Physiology
University of Szeged

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1. Abbreviations

4-AP	4-aminopyridine
Cx	connexin
GJ	gap junction
Pf	primary focus
Mf	mirror focus
RT-PCR	reverse-transcriptase polymerase chain reaction
i.p.	intraperitoneally
TMA	trimethylamine
P	postnatal day

2. Introduction

Epilepsy is a chronic, functional disorder of the central nervous system, which is characterized by spontaneous repetitive seizures. The cellular background of the seizure discharges is the sudden, excessive, abnormal synchronized rhythmic firing of large populations of neurons. Epilepsy affects about 4% of individuals over their lifetimes in the developed countries. Despite progress in understanding the pathogenesis of experimental seizures and epilepsy, the cellular basis of human epilepsy remains, for the large part, a mystery. In the absence of understanding of epileptogenesis, drug therapy of epilepsy is directed at the control of symptoms, i.e. the suppression of seizures by chronic administration of antiepileptic drugs. Nonetheless, seizures remain uncontrolled in about 25% of all epilepsies despite antiepileptic drug therapy. Surgery may help a small number of people whose seizures do not respond to medication. The significant number of patients that suffers poorly controlled seizures suggests that some clinically relevant proepileptic mechanisms are not targeted by any of the current available antiepileptic drugs. The reason for this could be that the currently available anticonvulsants were identified by using the same classical epilepsy models, which mainly involve the same actions, without a consideration of the variations in the pathophysiological mechanisms that result in epilepsy.

There is growing evidence that, besides the chemical synapses, direct coupling via gap junction (GJ) channels provides a second major pathway, contributing to normal and abnormal brain functions both during development and in the adult brain (Dermietzel and Spary, 1993; Jefferys, 1995; Connors and Long, 2004; Nakase and Naus, 2004). The GJs are specialized membrane regions composed of aggregates of transmembrane channels, which directly connect the cytoplasm of adjacent cells (Fig.1). GJs play an important role in forming a functional network or syncytium of cells by allowing the transfer of small molecules or the conduction of electrical activation (Nicholson, 2003; Benett and Zukin, 2004). Each intercellular channel is formed by the conjunction of two hemichannels, or connexons, formed by the hexameric assembly of subunits proteins called connexins (Cxs). Eleven Cx subtypes are found in the central nervous system (Jefferys, 1995; Nakase and Naus, 2004). The subset and the expression of Cxs vary depending on the type of cell and the stage of development (see Table I for details of cellular expression of Cxs).

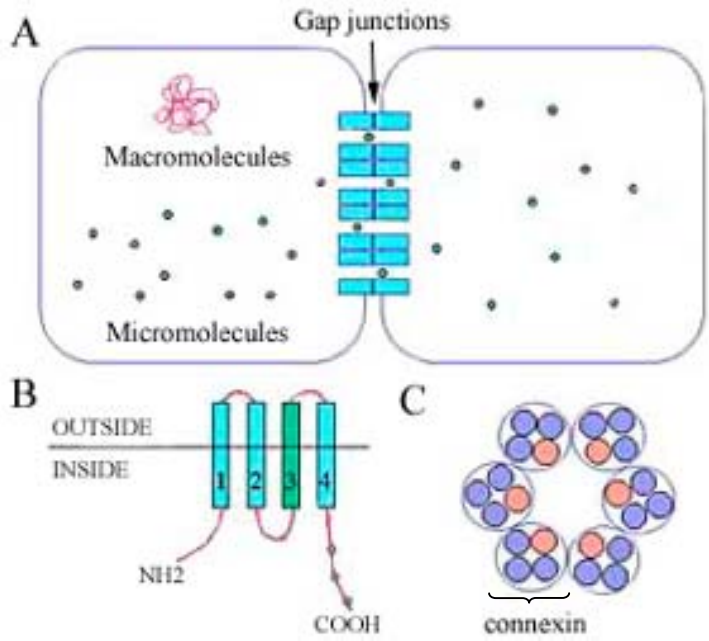


Figure 1. Schematic view of gap junction channels between adjacent cells (A), and of its hexameric connexin subunits called connexon (C) and of one connexin protein composed by four transmembrane spanning domains, two extracellular and one intracellular loop and citoplasmic amino and carboxyl tails (B).

The GJ coupling was for a long time regarded as a static way of communication before it appeared to be finely regulated. GJs can be regulated at the level of acute opening or closure as well as at the level of expression including synthesis, protein trafficking and degradation.

Table I. *Connexin subtypes and cellular expression in the central nervous system*

Connexin subtype	Cell type (expressed stage)
26	neuron (developing) astrocyte ependyma
29	oligodendrocyte
30	astrocyte (matured)
32	neuron (matured) oligodendrocyte
36	neuron
37	motor neuron
40	neuron (developing) astrocyte
43	neuron (mainly developing) astrocyte microglia (activated)
45	neuron astrocyte
46	astrocyte
47	neuron astrocyte oligodendrocyte

The subset and the expression of Cxs vary depending on the type of cell and the stage of development.

The degree of coupling is sensitive to a variety of stimuli, including neurotransmitters and changes in the intracellular/extracellular Ca^{2+}, pH, in transjunctional applied voltage and in phosphorylation/dephosphorylation processes (Rouach et al., 2000; Hervé, 2004).

In consequence of the restricted coupling between neurons the role of electrotonic coupling through GJ channels in the generation of epileptiform activity in the adult nervous system was not considered to be important (Haas and Jefferys, 1984; Bennett and Spray, 1985). The hypothesis of strengthened interconnections between neurons through electrical synapses, as a possible mechanism underlying neuronal synchronization and epileptogenesis, was introduced about some twenty years ago (Taylor and Dudek, 1982). Recent, theoretical and experimental work, mostly on brain slices and in particular on preparations of the hippocampus, offers support for this concept (Michelson and Wong, 1994; Perez Velazquez et al., 1994; Benardo, 1997; Bennett, 1997; Draguhn et al., 1998; Naus, et al., 1991; Bikson et al., 1999; Carlen et al., 2000; Perez Velazquez and Carlen, 2000; Tamás et al., 2000; Li et al., 2001; Szabadics et al., 2001). It has been shown that GJs can contribute to sharpened neuronal activity by synchronizing large neuronal ensembles (including their oscillatory activity) at different frequency bands, which have been proposed to underlie different cognitive processes, such as perception, memory and learning (Söhl et al., 2005). On the other hand, several studies indicate that GJs may be involved in pathology several instances implicating GJs in neurological disorders. The relevance of gap junction-mediated coupling in human seizure disorders is reported in the literature (Lee et al., 1995; Mas et al., 2004). One form of intractable human epilepsy is associated with alterations in glial gap junction coupling (Lee et al., 1995). Blockade of GJ communication has been shown to reduce seizures in different epilepsy models (De Curtis et al., 1998; Ross et al., 2000; Jahromi et al., 2001; Traub et al., 2001a,b). Treatment that favors GJ channels opening has been found to promote seizure-like activity in *in vitro* (Khöling et al., 2001). Nevertheless, to date, very little evidence has been published showing an involvement of GJs in seizures under *in vivo* conditions.

Intercellular coupling via GJs has been reported between neurons, astrocytes and oligodendrocytes, and also between different cell types, such as neurons-astrocytes (Nedergaard, 1994; Alvarez-Maubecin et al., 2002) and astrocytes-oligodendrocytes (Nedergaard, 1994; Nagy and Rash, 2000). Neuronal synchronization can be mediated by GJ channels, mostly between dendrites (Traub et al., 1995, 2001a; Fukuda and Kosaka, 2003) or allegedly between axons of pyramidal neurons (Draguhn et al., 1998; Schmitz et

al., 2001; Traub, 2003) or interneurons (Belluardo et al., 2000; Traub et al., 2001a; Deans et al., 2001; Hormuzdi et al., 2001; Simon et al. 2005).

Among the 11 GJ proteins in the nervous system, the Cx36 appears to be expressed exclusively in neurons (Table I) and to form electrical synapses at the different interneurons in the adult brain (Belluardo et al., 2000; Deans et al., 2001; Perez Velazquez et al., 1994; Sohl et al., 1994; Condorelli et al., 1998; Rash et al., 2000). More recently, targeted deletion and LacZ reporter gen expression have also shown the expression of Cx45 and Cx47 in adult neurons, which would otherwise, remained undiscovered (Söhl et al., 2005). GJs between developing neurons can be composed of Cx26, although the expression of Cx26 restricted to ependymal and astroglial cells in the adult brain. Beside Cx26, Cxs 30, 40, 43 are expressed mainly in astrocytes, while Cxs 29, 32, 47 are present mostly in oligodendrocytes in the adult brain (Table I).

Recently, there has become an increasing awareness that glial cells are actually an integral part of the electrical circuitry of the brain. The realization that glia signal back to neurons to directly modulate synaptic activity has led to a re-definition of the synapse (Nedergaard et al., 2003; Newman, 2003; Stevens, 2003). Astrocytes, acting as a single cell or syncytium may control and synchronize the firing patterns of synapses of a great many neurons (Eduardo et al., 2005; Tian, et al., 2005). Indeed, reactive astrogliosis is a prominent pathological feature of the epileptic brain (D'Ambrosio, 2004). Several studies indicate that glial cells in epileptic tissue undergo not only morphological but also physiological alterations. It is supposed that GJ communication plays an intrinsic role in the associated neuronal/astrocytic networks that are involved in the generation and propagation of epileptiform activity. However, to date, very little work has been done showing the function and involvement of the specific neuronal or glial Cxs in seizures.

Clinical experience and various experimental data indicate that the developing nervous system is more sensitive than the mature one to different convulsive effects (Moshe, 1983, 1987; Holmes and Ben-Ari, 1998; Johnston, 1996; Swann and Hablitz, 2000). 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. The developing brain exhibits a high metabolic rate, abundant neuronal and synaptic networks, the overexpression of receptors and enzymes, the depolarizing effect of gamma-amino-acid, the hypersynchrony of neuronal circuits and enhanced synaptic plasticity (Avoli, 1990; Ben-Ari et al., 2004). In addition, the immature cerebral cortex and hippocampus have higher densities of excitatory

amino acid receptors and GJ channels as compared with the adult organs (Johnston, 1996; Nadarjah et al., 1997).

Intercellular communication via GJ channels is an important form of cell-to-cell communication in early brain development (Nadarjah et al., 1997; Prime et al., 2000; Venance et al., 2000; Peinado, 2001; Bittman et al., 2002; Condorelli et al., 2003; Long et al., 2005). Electrical coupling via GJ channels has been reported both between pairs of inhibitory neurons and among inhibitory and excitatory neurons during the early postnatal days in the rat cortex (Venance et al., 2000). Moreover, the incidence of coupling between neurons and glia has been observed at this early age in rats (Nadarjah et al., 1997; Nagy and Rash, 2000; Nedergaard, 1994; Bittman et al., 2002). 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 (Johnston, 1996). However, the role of GJal coupling in epilepsy in the developing nervous system is still not fully understood.

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 (the process of development of epilepsy in normal brain tissue) and seizure susceptibility,
    - induction, manifestation and propagation of seizures at already established epileptic foci (ictogenesis),
    - control of the duration of seizures,

- the selective contribution of neuronal GJs via Cx36 to epileptogenesis and ictogenesis in the adult brain,

- the functional significance of GJs in the epileptogenicity and seizure susceptibility of the immature mammalian brain,

- the basal expression levels and the plastic changes induced by epileptiform activity in the mRNA levels for Cx 26, 32, 36 and 43 in the developing and adult rat neocortex.

3. Materials and methods

3.1 Animals

For electrophysiological and reverse-transcriptase polymerase chain reaction (RT-PCR) studies, P9-28 (18-50g) and adult (>P28, 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. The pups were housed together with their mothers in individual cages until chosen for study or until weaning on day P24 (the day of birth was taken as day P0). For the assessment of seizure susceptibility, animals were selected randomly from a litter of the appropriate age. To avoid inter-litter variations in susceptibility to seizures, the animals for tests of the same kind of treatment were chosen from different litters.

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.

3.2 Electrophysiology and induction of seizure activity

Under general anaesthesia (sodium pentobarbital, 50 mg/kg, i.p.), the heads of the animals were secured in a stereotaxic instrument. For the recording of ECoGs, four holes (2-3 mm wide) were drilled in the skull, and the dura mater was carefully removed at the prospective site of the Pf.

The Pf was induced by the local application of crystalline 4-AP (on a piece of filter paper soaked with saline) to the somatosensory cortical surface. The A-type K^+ channel blocker, 4-AP induces periodic, tonic-clonic ictal epileptiform activity, resembling human temporal lobe epilepsy. This acute model of experimental seizures provides opportunity for studying the genesis of 4-AP-induced seizure potentials and underlying intracellular events. On the other hand, the model also can be considered as a semichronic one because of the speeded-up appearance of the consecutive spontaneous seizures (23-30 seizures/60 min), allowing the study of the induction, maintenance and propagation of seizures at already established epileptic foci. The 4-AP model is appropriate for studying the effects of various pharmacological interventions on the electrophysiological manifestation of

seizures by combining molecular biological techniques. In addition, the development of Mf provides good opportunity for studying secondary epileptogenezis.

Four silver ball electrodes were used to record the ECoG from the Pf, from the secondarily induced Mf, 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.

Somatosensory responses were evoked by electrical stimulation of the whiskers field through a bipolar needle electrode (3 V, 0.3 ms, 0.3 Hz square pulses) and recorded from the punctum maximum of the cortex.

During the experiments, the general state of the animal (level of anaesthesia and pupil size) was regularly checked, the body temperature was maintained at 37-39 °C by a heating lamp, and the exposed cortical surface was kept wet with 39 °C saline. Adequate measures were taken to minimize unnecessary pain and discomfort to the animals and to minimize animal use. At the end of the experiments, the animals were given a lethal dose of sodium pentobarbital.

3.3 Pharmacological manipulation of GJal communication

Two types of experimental protocols (Figs. 2 and 3) were used in order to investigate the possible contribution of GJal communication to epileptogenesis and seizure susceptibility in normal brain tissue (*pretreatment*) and to the manifestation of seizures at already established epileptic foci as well as the propagation of seizure discharges to other cortical areas and to the contralateral hemisphere (*treatment*).

In the pretreatment and treatment experiments, carbenoxolone (Gareri et al., 2004) and octanol (Dermietzel, 1993; Deans, 2001) 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 (Srinivas, 2001; Uusisaari, 2002). TMA, an intracellular alkalinizing agent was used for opening of the GJ channels (Köhling, 2001).

Pretreatment

In adult animals, the intact cortex was pretreated locally with either quinine (35 μM, dissolved in saline; n=6); carbenoxolone (10 mM, dissolved in saline; n=6); octanol (1 mM, dissolved in saline; n=6) or TMA (100 mM dissolved in saline; n=6), respectively (Fig. 2). In developing animals carbenoxolone (10 mM, dissolved in saline), and TMA

(100 mM dissolved in saline) were used (n=5 animals for each drug, at the five developmental stages, see details later).

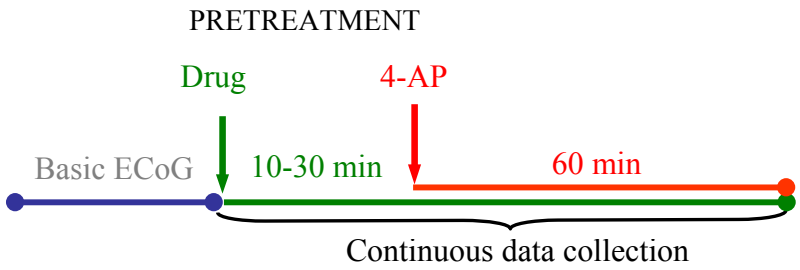


Figure 2. Experimental protocol applied in pretreatment experiments.

The durations of pretreatment were 5 min in the cases of quinine and carbenoxolone, 25 min in the case of octanol and 10 min in the case of TMA. The time between the application of the drugs and of 4-AP was chosen in accordance with the time of the peak effect of the drug observed in the preliminary experiments. In pretreatment experiments, the filter paper containing the 4-AP was applied onto the top of the filter paper soaked with quinine, carbenoxolone, octanol or TMA solution, respectively that covered the cortical surface.

In pretreatment experiments, data were collected for 20 min following the occurrence of the first seizure. The control values for these experiments were collected from different animals (n=5) of the same litter submitted to an identical experimental paradigm, but pretreatment was carried out with a piece of filter paper soaked with saline.

Treatment

In these experiments, the already active Pf was treated locally with quinine, carbenoxolone, octanol or TMA (n=6 adults for each drug and n=5 animals for carbenoxolone and TMA of the five developmental stages), respectively, 60 min after the appearance of the first seizure (Fig. 3). In another group of adult animals the already active Mf (n=6) was treated locally with carbenoxolone, 60 min after the induction of epileptiform activity. A piece of filter paper was soaked with a solution of the appropriate drug, applied on top of the filter paper containing crystalline 4-AP, and left in place until the end of the experiment in order to diffuse into the cortex.

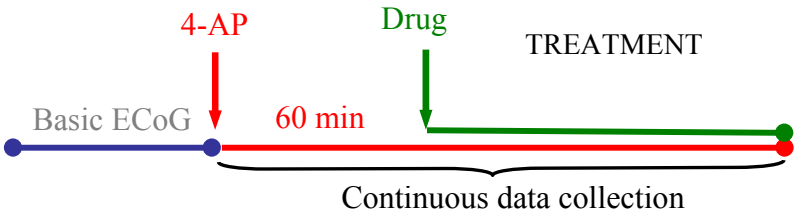


Figure 3. Experimental protocol applied in treatment experiments.

In treatment experiments, data were collected for 20 min after the development of the peak effects for each drug (based on the observations of our preliminary experiments) and compared to the data that were collected from the same animals before the application of the drugs.

To test whether carbenoxolone and TMA converge to the same target (i.e. GJs), TMA was applied 10 min after a previous application of carbenoxolone in adult animals (n=6), and data were then collected for an additional 60 min in the presence of the two drugs.

To unmask and compare the electrophysiological consequence of the selective or global blockade of the different Cxs, after the completion of data collection during the action of quinine, carbenoxolone was additionally applied at the Pf, and measurements were made in the joint presence of the two blockers. In these experiments, the data collected from the same animal for 20 min before the application of the blocker served as the control values.

3.4 Tissue isolation and RT-PCR

In adult animals samples were taken from the areas of the Pf and Mf of 6 rats, 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 (n=4 animals from each developmental time point). For control values, the identical cortical areas of animals without induced epileptic activity were used both in adults (n=6) and developing animals (n=4 from each developmental time point). Tissue samples were frozen immediately in liquid nitrogen, and stored at –80 ^{o}C.

An RT-PCR-based strategy was employed to quantify the expression levels of the Cx43, Cx36, Cx32 and Cx26 genes and their inducibility by cortical seizure discharges.

These measurements were carried out in collaboration with Edit Hermesz (Department of Biochemistry, University of Szeged). About 3 μg total RNA, prepared from the epileptic foci and the identical area of the control animals was used as template for first-strand cDNA synthesis. PCR amplification was performed on 3 μl RT product, using 33 cycles of 95 ^{o}C for 40 s, 55 ^{o}C for 40 s, and 72 ^{o}C for 60 s for the Cxs, and 26 cycles for β-actin. For the normalization of Cx mRNAs, the level of β-actin mRNA was utilized as an internal standard. The relative levels of Cx mRNAs are expressed as ratios (Cx/β-actin x 100). Images of ethidium bromide-stained agarose gels were digitized with a GDS 7500 Gel Documentation System and analyzed with GelBase/GelBlot^{TM} Pro Gel Analysis Software (UVP).

Primers:

Cx43F: 5' TACCACGCCACCACCGGCCCA 3'

Cx43R: 5' GGCATTTTGGCTGTCGTCAGGGAA 3'

Cx36F: 5' GCAGAGAGAACGCCGGTACT 3'

Cx36R: 5' CTTGGACCTTGCTGCTGTGC 3'

Cx32F: 5' CTGCTCTACCCGGGCTATGC 3'

Cx32R: 5' CAGGCTGAGCATCGGTCGCTCTT 3'

Cx26F: 5' CGGAAGTTCATGAAGGGAGAGAT 3'

Cx26R: 5' GGTCTTTTGGACTTCCCTGAGCA 3'

β-actin3: 5' GCAAGAGAGGTATCCTGACC 3'

β-actin4: 5' CCCTCGTAGATGGGCACAGT 3'

3.5 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 stored in a computer memory with the aid of Digidata 1200B (BD, BNC, Axon Instruments, Inc.) in parallel with the electroencephalograph, and analyzed after the experiments. Results are given as means±S.D. 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 $P \leq 0.05$.

RT-PCR reactions for each sample were performed in triplicate to increase the reliability of the measurements. Data were analyzed by averaging across animals, and expressed as means$\pm$SD. The statistical analysis of the data was done by a two-way ANOVA (Jandel SigmaStat Statistical Software, Version 2). When significant differences were found with the overall analysis, the Tukey HSD test was used for post hoc comparisons between groups (significance criterion: $P \leq 0.05$).

3.6 Drugs

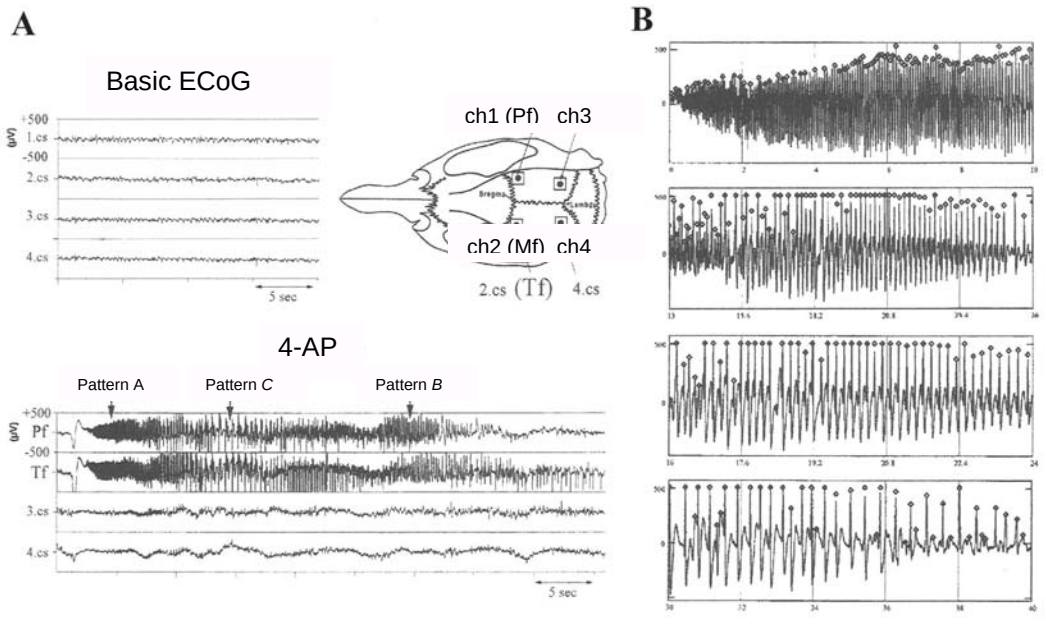
For electrophysiological and pharmacological experiments: 4-AP, carbenoxolone, quinine, octanol and trimethylamine (TMA) were purchased from Sigma, St.Louis, MO, USA.

For molecular biological measurements: 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, MO, USA.

4. Results

4.1 Electrophysiological and pharmacological experiments on adult animals

The electrocorticographic characteristics of aminopyridine-induced *in vivo* seizure activity have already been described, together with the accompanying intracellular events at both the Pf and the Mf in anesthetized cats and rats (Szente et al., 1979, 1987). Briefly, the first ictal episode develops in the Pf about one min after the application of 4-AP, and then recurs spontaneously (23-30 seizures/h). With a short latency, similar periodic paroxysmal activity occurs at the Mf in most of the animals (Fig. 4*A*). Three different patterns of seizure discharges are distinguished, depending on their frequencies, amplitudes and waveforms (Fig. 4*A, B*). Most commonly, the seizures begin with repetitive biphasic spikes of relatively low, but gradually increasing voltage, with frequencies of 11-14 Hz (pattern *A*); followed by spikes of high voltage with frequencies of 4-10 Hz (pattern *B*) or by the third pattern (pattern *C*), with lowest frequency (1-3 Hz), represented either by a spike-wave complex, or by a fast single spike of high amplitude accompanied by a burst of high frequency. In 30% of the control animals, after 8-10 repetitions of the ictal periods, the original focal seizure activity progressed to the entire cortex and became generalized.



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4.1.1 Opening or closing of GJ channels by pharmacological tools

The aim of the pretreatment experiments was to examine how the functional state of neuronal and/or glial GJ channels before the induction of epileptiform activity influences the epileptogenesis and the generation of seizures. In the treatment experiments we examined how the functional state of neuronal and/or glial GJ channels at an already active epileptic focus influences the ictogenesis and propagation of seizures.

In general, pretreatment of the cortical surface either with the blockers or opener prior to the application of 4-AP had a weak influence on the induction and maintenance of epileptiform activity. In contrary, pharmacological manipulation of the GJs after 25-30 repetitions of seizures spectacularly modified the manifestation and propagation of epileptiform discharges.

4.1.1.1 Closing of GJ channels by carbenoxolone

Pretreatment

Local pretreatment of the cortical surface of adult rats with carbenoxolone did not noticeably influence the basic cortical activity (not shown), and did not induce changes in the configuration of the somatosensory evoked responses (Fig. 5).

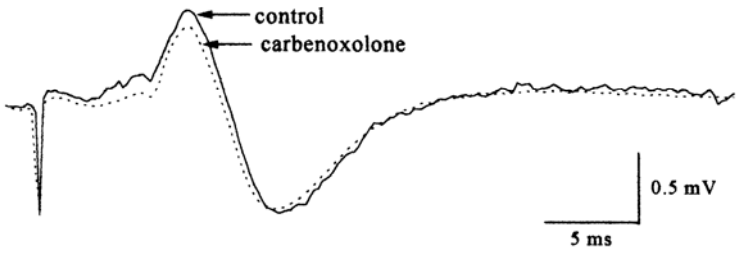


Figure 5. Effects of local application of carbenoxolone on the somatosensory evoked responses. Somatosensory evoked responses (average of 20 individual traces) recorded before (*control*) or after pretreatment (*carbenoxolone*) are superimposed for comparison.

The duration of the ictal periods fell slightly but not significantly in the first 20 min in comparison with the control value (Figs. 6 and Table II); then, after a small increase, it approached the control value by the end of the 60-min recording.

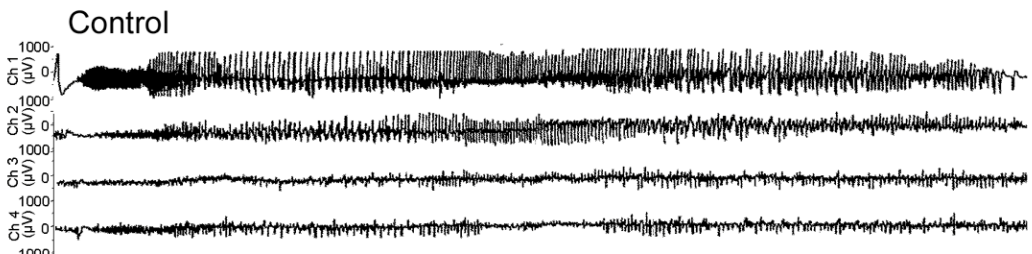


Figure 6. Representative EEG samples showing the effects of carbenoxolone in the pretreatment experiments.

The average latency for the first seizure after 4-AP application, in the presence of carbenoxolone, was in the same range as for the control animals (Table II). The number of ictal periods did not differ significantly from controls throughout the 60-min recording (Table II).

Table II. *Modifications in epileptonesis following selective or global blockade of the gap junctions*

	Primary focus				Mirror focus			
Pretreatment	Control n=5	Quinine n=6	Control n=5	Carb n=6	Control n=5	Quinine n=6	Control n=5	Carb n=6
Latency (s)	122±10	127±9	58.8±8	59.6±9	123±9	128±6.8	59.66±7	59.9±6
Number of seizures/20 min	12±2.3	11.33±0.8	13.1±2.8	11.75±2.8	11±2.3	10.33±0.8	12±2.8	10.7±1.5
Duration of seizures (s)	55.8±15	52.7±10	47.3±14.9	43.98±13	38.06±5.8	35±7	41.85±8	37.74±6.1
Amplitude A (μV)	517±38	423±54	529±56	457±49	337±44	299±31	398±26	358±19
Amplitude B (μV)	976±68	805±71	913±98	776±65	573±31	550±29	502±38	488±25
Amplitude C (μV)	1272±80	1017±98	1128±93	1026±89	741±66	711±58	733±38	689±26

The data illustrate the effects of quinine and of carbenoxolone on the different parameters of epileptiform activity in the *pretreatment* experiments. The site of the pretreatment was the Pf. Data are expressed as mean ± SD. Significance criterion: $P \leq 0.05$.

The differences in duration and numbers resulted in a lower summated ictal activity by the end of the observation period (Fig. 7*A*) in the carbenoxolone-pretreated animals.

The average amplitude of the typical epileptiform discharges was slightly, but not significantly smaller in the carbenoxolone-treated animals in comparison with the control value (Figs. 6 and 7*B*).

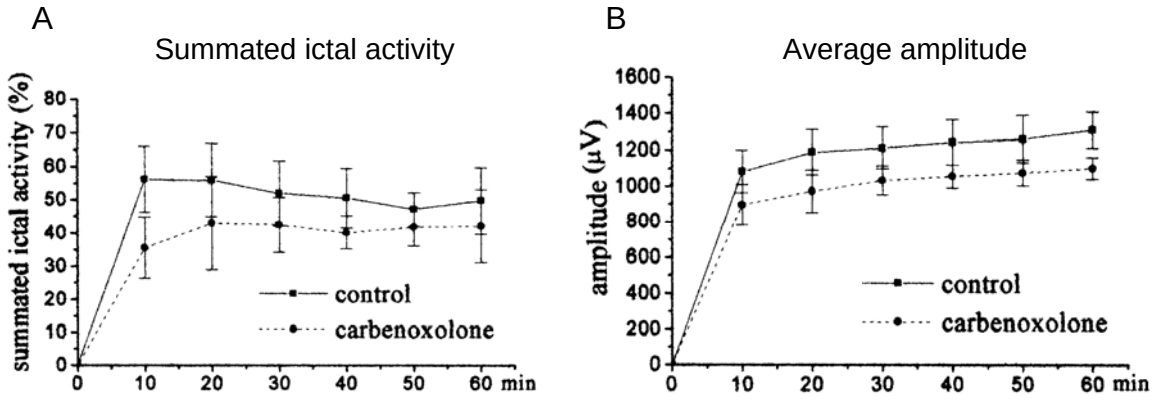


Figure 7. Effects of local pretreatment of carbenoxolone on the summated ictal activity (A) of cortical seizure activity induced by 4-AP (considered as control) and on the amplitude of seizure discharges (B). Data were collected at each consecutive10 min time window. Data are expressed as mean $\pm$ SD. Significance criterion: $p \leq 0.05$.

The propagation of epileptiform activity to other cortical areas was apparently not modified by carbenoxolone (Fig. 6); the original focal seizure became secondarily generalized in the same proportion (30%) in the pretreated animals as in the controls.

Treatment

The electrocorticographic manifestation of ictal discharges was significantly depressed after the local application of carbenoxolone both at the already active Pf and at the Mf, relative to the seizure activity in the previous 60 min (Fig. 8). Although the anticonvulsant effect of carbenoxolone was stronger and there was a delay at the Mf as compared with the effects at the Pf, the modifications induced in the various parameters of the seizures were in the same directions for both foci.

When the Pf was treated with carbenoxolone, the maximal effect of the blocker was detected at 10 min after its application and then there was a tendency for recovery in all measured parameters of epileptiform activity (Figs. 9 and 10).

Figure 8. Representative EEG samples showing the anticonvulsive effects of carbenoxolone in the treatment experiments.

The average duration of the ictal periods decreased significantly (Figs. 8 and 9*A,* Table III) in the first 10-40 min in the Pf, followed by a gradual recovery.

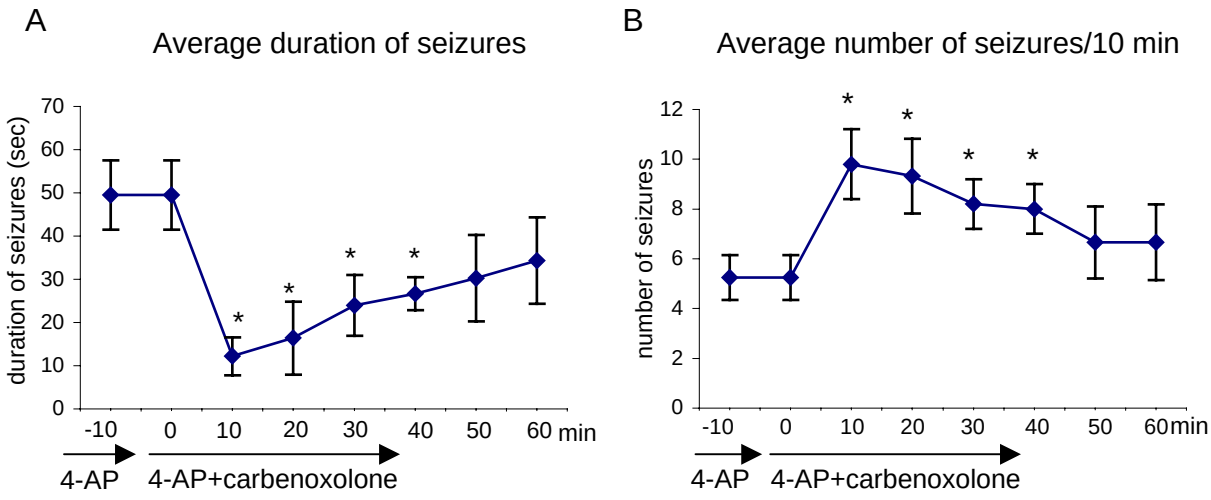


Figure 9. Effects of treatment with carbenoxolone after 60 min of repeated seizures at the site of the active Pf on the average duration of seizures (*A*) and on the average number of seizures (*B*). The ongoing treatment is indicated below each vertical arrow. *–10* on the time scale represents the last 10 min of the preceding 60-min seizure activity, just before the application of carbenoxolone. Stars indicate significant changes. Data were collected at each consecutive10 min. Data are expressed as mean $\pm$ SD. Significance criterion: $P \leq 0.02$.

Although the number of seizures was significantly above the control value when the Pf was treated with carbenoxolone (Fig. 9*B,* Table III), the summated ictal activity

displayed a significant decrease in the first 40 min (Figs. 8 and 10*A*), due to the very short seizures, followed by a slow recovery.

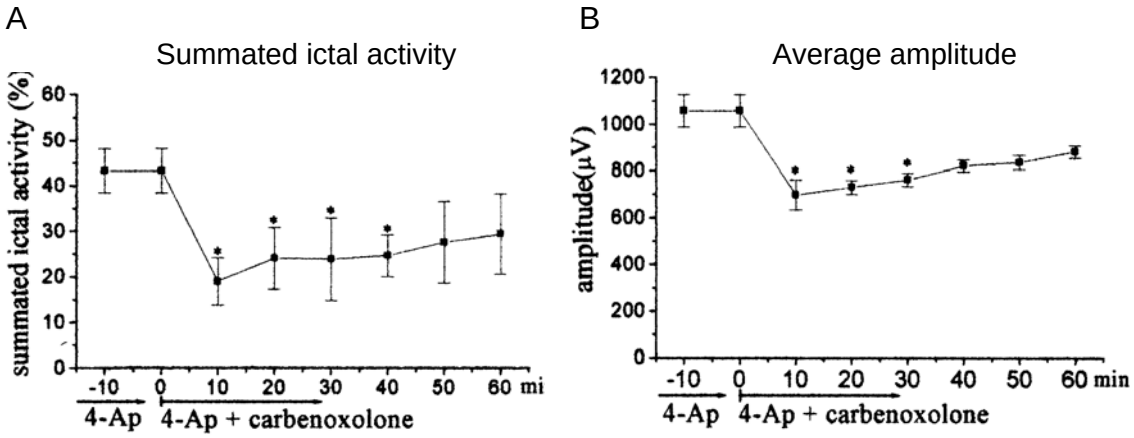


Figure 10. Effects of treatment with carbenoxolone after 60 min of repeated seizures at the site of the active Pf on the summated ictal activity (*A*) of cortical seizure activity induced by 4-AP (considered as control) and on the average amplitude of seizure discharges (*B*). The ongoing treatment is indicated below each vertical arrow. *–10* on the time scale represents the last 10 min of the preceding 60-min seizure activity, just before the application of carbenoxolone. Stars indicate significant changes. Data were collected at each consecutive 10 min time window. Data are expressed as mean ± SD. Significance criterion: P≤0.02.

The amplitudes of all characteristic seizure discharges decreased significantly in the presence of carbenoxolone (Figs. 8 and 10*B,* Table III), followed by a gradual recovery. Both the summated ictal activity and the amplitude of seizure discharges were still considerably reduced at 60 min of recording (Fig. 10).

Table III. *Modifications in ictogenesis following selective or global blockade of the gap junctions*

	Primary focus					Mirror focus				
Treatment	Control n=5	Quinine n=6	Quin+Carb n=6	Control n=5	Carb n=6	Control n=5	Quinine n=6	Quin+Carb n=6	Control n=5	Carb n=6
Number of seizures/20 min	13±0.9	18±0.9*	13±0.8#	10.5±0.8	19.6±1.8*	13±0.9	18+0.9*	13+0.8#	10.5+0.8	19.6+1.8*
Duration of seizures (s)	52.8±5	20.8±2.5*	16.1±2#	49.5±5.3	11.38±1.8*	40±5.1	21±2.8*	19±1.7*	47±8	18.8±2*
Amplitude B (μV)	998±65	914±58	683±34*#	985±24.6	714±31*	628±41	368±26*	375±22.5*	529±31	241±18*
Amplitude C (μV)	1117±89	1055±71	828±43*#	1135±58	749±29*	754±32.8	473±29*	434±23.6*	708±39	302±21*

The data illustrate the effects of quinine applied alone or together with carbenoxolone and of carbenoxolone on the different parameters of epileptiform activity in the *treatment* experiments. The sites of the treatments were the Pf. Data are expressed as mean ± SD. Significance criterion: P≤ 0.05. * Significant difference in comparison with the control. # Significant difference in comparison with quinine.

Following carbenoxolone treatment, a new component (A0) occurred at the very beginning of the individual seizures, with the highest frequency (19.6±0.3) and lowest

amplitudes (269±29 μV) (Fig. 11). This A0 section was not observed in the controls. The appearance of the new A0 section at the initiations of seizures was not noticeable in most cases at the Mf in the carbenoxolone-treated animals (Fig. 11). The new discharge pattern also appeared in the quinine treated animals (see further details later).

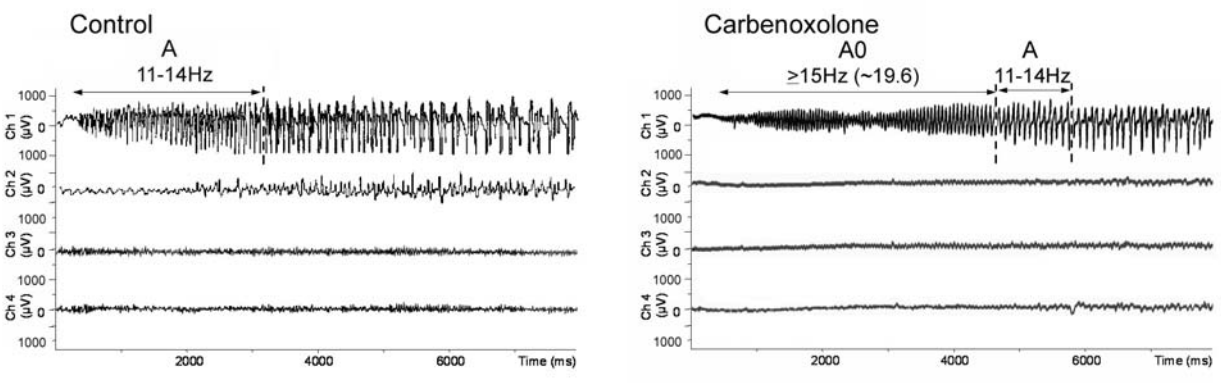


Figure 11. EEG samples representing the effects of carbenoxolone on the manifestation of the new discharge pattern (A0) at the initiation of seizures in the *treatment* experiments.

When the Pf was treated with carbenoxolone, as a consequence of the appearance of the new discharge pattern, the ratio of higher frequency seizure discharges (above 11 Hz) increased significantly in the individual ictal periods (Figs. 8 and 12). On the other hand the ratio of discharges of 4-10 Hz and 1-3 Hz was reduced significantly during carbenoxolone treatment (Figs. 8 and 12) in comparison to control.

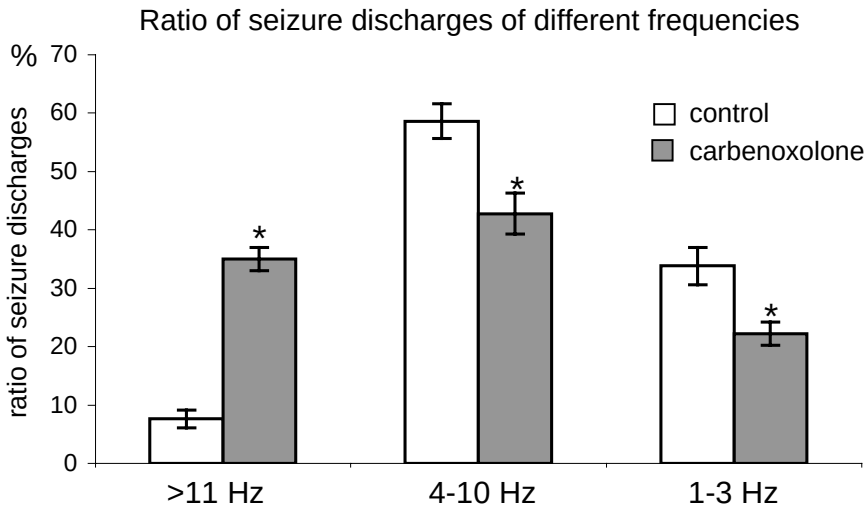


Figure 12. Effects of treatment with carbenoxolone after 60 min of repeated seizures at the site of the active Pf on the ratio of seizure discharges of different frequencies. Stars indicate significant changes. Data are expressed as mean ± SD. Significance criterion: P≤0.02

When carbenoxolone was applied at the active Mf, the maximal effect of the blocker was detected 30 min after its application (Fig. 13). Similar changes occurred in the

duration and number of seizures (to those detected when the treatment was done at the active Pf). These changes resulted in a significantly reduced summated ictal activity (Fig. 13*A*). The shortest average duration (12.3±3.8 s, as compared to the control value of 49.4±9.6 s) was measured 20-30 min after the application of carbenoxolone. The average amplitude of the typical seizure discharges was also significantly reduced (Fig. 13*B*). However, there was some delay in the peak effect, and the depression was more durable when treatment was applied to the Mf rather than to the Pf.

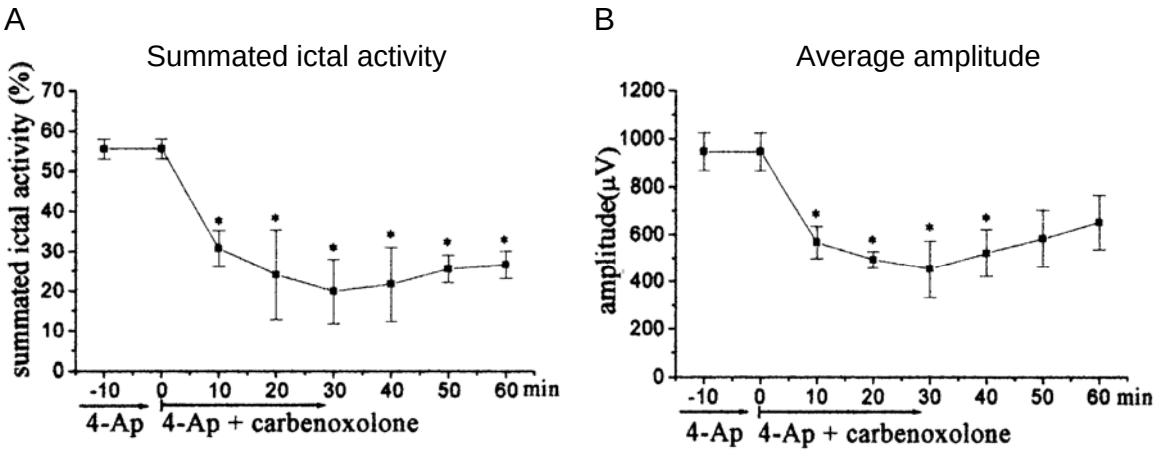


Figure 13. Effects of treatment with carbenoxolone after 60 min of repeated seizures at the site of the active Mf on the summated ictal activity (*A*) of cortical seizure activity induced by 4-AP (considered as control) and on the average amplitude of seizure discharges (*B*). The ongoing treatment is indicated below each vertical arrow. *–10* on the time scale represents the last 10 min of the preceding 60-min seizure activity, just before the application of carbenoxolone. Stars indicate significant changes. Data were collected at each consecutive 10 min time window. Data are expressed as mean ± SD. Significance criterion: $P \leq 0.02$

The differences between the time courses of the effects of carbenoxolone in the active Pf and Mf could be explained by the differences in induction mechanism and dynamics of the primary and secondarily induced epileptic foci.

4.1.1.2 Closing of GJ channels by octanol

In order to verify whether the anticonvulsive effects of carbenoxolone, described previously, is attributable to the blockade of the GJs, the effects of carbenoxolone were compared with those of octanol (Dermietzel, 1993; Deans, 2001), another widely used GJ blocker (Fig. 14). The maximal anticonvulsive effect of octanol developed slower (in about 30 min after the application), but lasted longer than the effect of carbenoxolone. The seizure intensity gradually returned to the control value by 150 min after the application of octanol.

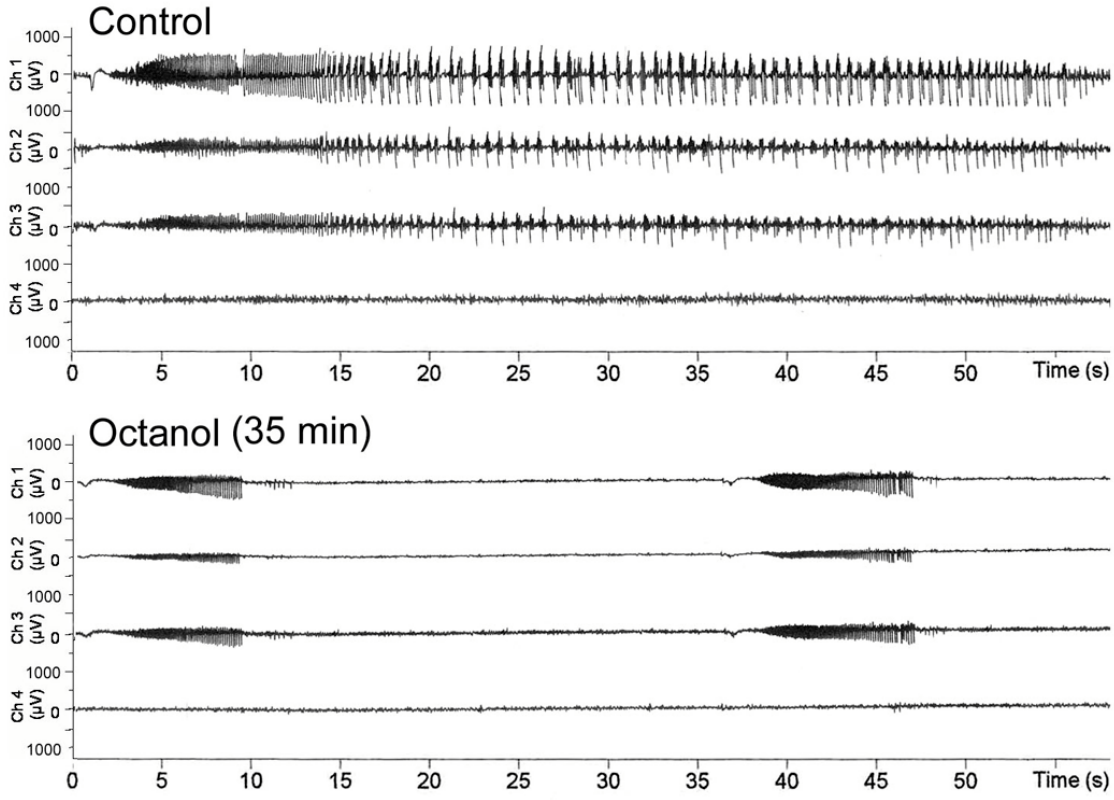


Figure 14. Representative EEG samples showing the anticonvulsive effects of octanol in the treatment experiments.

Like carbenoxolone, octanol reduced the seizure induction only slightly, when applied 25 min before seizure initiation, and noticeably and reversibly reduced the generation and propagation of seizure discharges when applied at the already active epileptic focus (data not shown). These results confirm our earlier conclusion that the reduction in seizure activity after the application of carbenoxolone is probably due to the blockade of GJ communication.

Since no significant differences were detected between the anticonvulsive effects of carbenoxolone and of octanol (see Figs. 8 and 14 for comparison), below we use the data obtained with carbenoxolone as a broad-spectrum GJ blocker for comparison with the data obtained by using quinine as a selective blocker of neuronal Cx36.

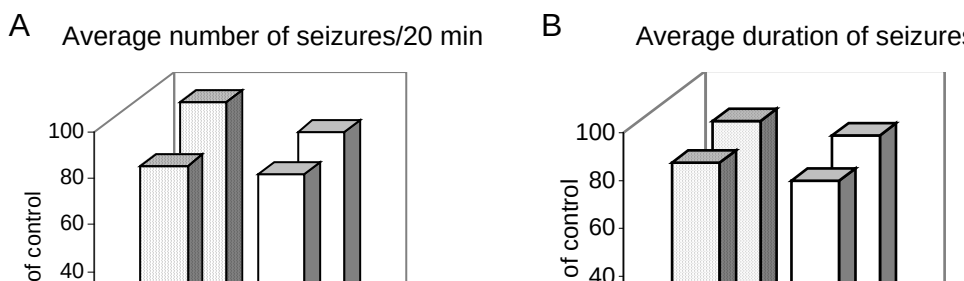
4.1.1.3 Closing of neuron specific Cx36 by quinine

Quinine has been reported to selectively block Cx36, without any significant effect on several other Cxs, including astrocytic Cx43 (Srinivas et al., 2001; Uusisaari et al., 2002; Cruikshank et al., 2004). The major drawback of quinine is that at relatively high concentration it is not specific for the GJs, but also affects a number of channels (Srinivas et al., 2001). Nevertheless, it has been demonstrated in *in vitro* conditions that below a critical concentration threshold quinine does not influence basic electrophysiological parameters and neuronal excitability (Smirnov et al., 1999; Bikson et al., 2002).Our preliminary experiments revealed a concentration dependence of the effect of quinine on the intensity of the epileptiform activity. At low concentrations (20-40 μM), quinine suppressed the expression of seizures, but at higher concentrations (>80 μM) its effect was rather proconvulsive (not shown). In both cases, the effects were manifested rather quickly; in 5-6 min. We attempted to minimize the potential for non-specific effects of quinine by choosing a low concentration demonstrated to be more selective for Cx36 with little or no effects on nonjunctional parameters. Because of the concentration dependence, in all experiments quinine was applied locally only one occasion, in order to avoid the possible accumulation of the drug. However, it must be borne in mind that the drug diffuses in the brain and can be metabolized in time, and therefore we can not claim an exact concentration of quinine in the treated tissue.

Pretreatment

Pretreatment with quinine, starting 5 min before the application of 4-AP did not noticeably influence the basic cortical activity, and it only slightly reduced the induction of seizure discharges (not shown). In general, all the changes induced by local pretreatment with quinine were similar to those induced by local pretreatment with carbenoxolone, both in the Pf and in the Mf (Table II).

The average latency for the first ictal periods was in the same range in the presence of quinine as in the presence of carbenoxolone (Table II). Both values were close to those measured in the control animals. The number of ictal periods did not differ significantly from controls (Fig. 15*A* and Table II). The moderate decrease in the average duration of the seizures resulted in a somewhat shorter summated ictal activity in the quinine-pretreated animals in comparison with the control values (Fig. 15*B, C* and Table II).



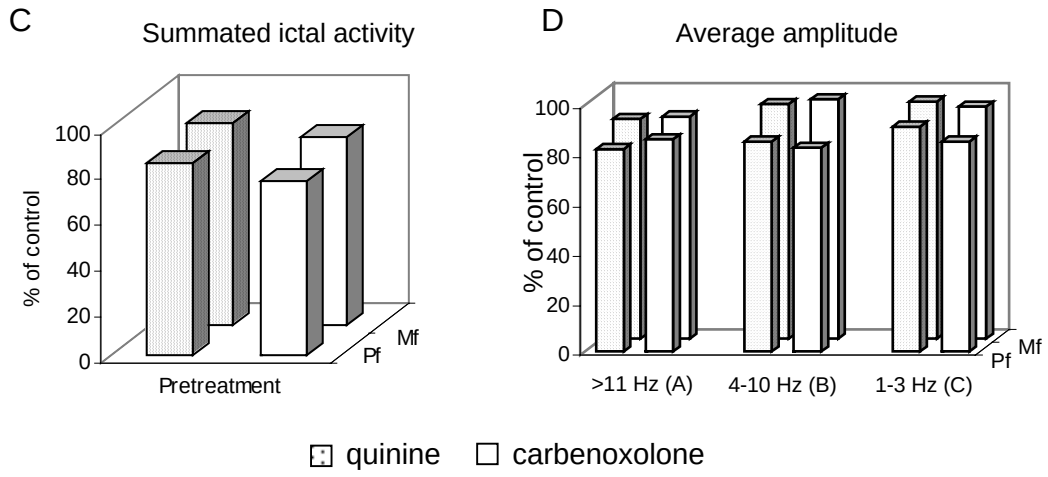


Figure 15. Effects of quinine and of carbenoxolone on the average number of seizures (A), on the average durations of seizures (B), on the summated ictal activity (C) of cortical seizure activity induced by 4-AP (considered as control) and on the average amplitudes of seizure discharges (D) in the pretreatment experiments. The data indicated for quinine and carbenoxolone are taken from different groups of animals (n=6); for control data n=5 animals. Data were collected at 20 min time window. Data are expressed as % of control. Significance criterion $P<0.05$.

The average amplitude of the characteristic seizure discharges was smaller, but not significantly in the quinine-pretreated animals in comparison with the control value (Fig. 15*D* and Table II). The propagation of epileptiform activity to other cortical areas was apparently not modified by quinine (not shown); the original focal seizure became secondarily generalized in the same proportion (30%) in the pretreated animals as in the controls.

Treatment

The peak effect of quinine developed 10-11 min after its application (Fig. 16*B*), then the values of the various parameters of the seizure activity gradually returned to the control levels by about 90-100 min after quinine application (not shown). The main observation of these experiments was that seizure discharges of various frequencies and

configurations displayed qualitatively different modifications, depending on the GJ pool, which was blocked either by quinine or by carbenoxolone (see details below).

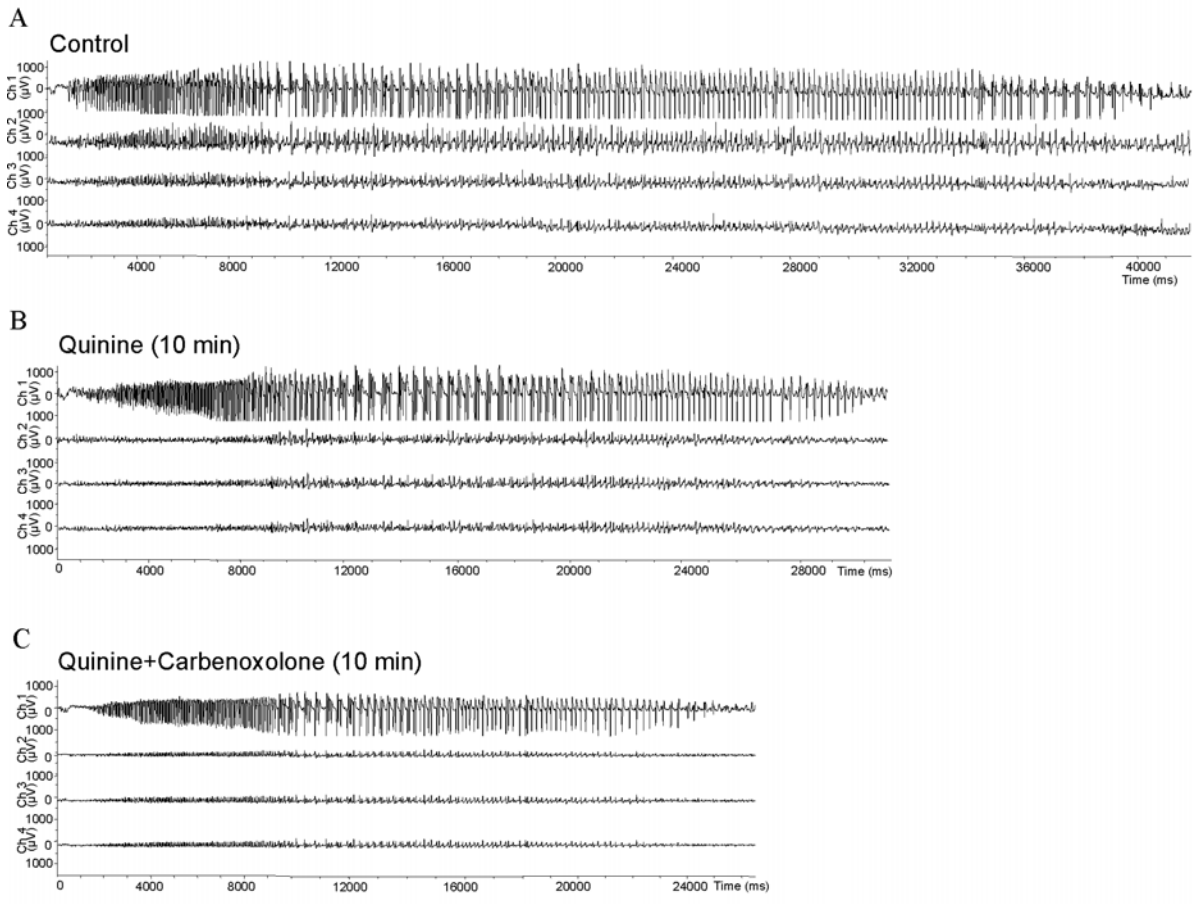


Figure 16. Representative EEG samples showing the effects of quinine applied alone or together with carbenoxolone on the manifestation of the individual seizures at the already active epileptic focus from the same animal.

Durations of seizures

Treatment with quinine significantly decreased the average duration of the seizures (to 39% of the control value) (Figs. 16*B* and 17*A*, Table III). When carbenoxolone was added to quinine, the duration of the seizures shortened further, to a value (29% of the control) (Figs. 16*C* and 17*A,* Table III), which was comparable to that measured when carbenoxolone was applied alone (Figs 17*A,* Table III).

Numbers of seizures

When quinine was applied at the already active focus, the average number of seizures increased significantly (up to 138% of the control value) (Fig. 17*B* and Table III). However, when carbenoxolone was added to quinine, the number of seizures fell back almost to the control value (Fig. 17*B* and Table III). As in the case of quinine, when

carbenoxolone was applied alone, the number of seizures increased significantly (up to 186% of the control value) (Fig. 17*B* and Table III).

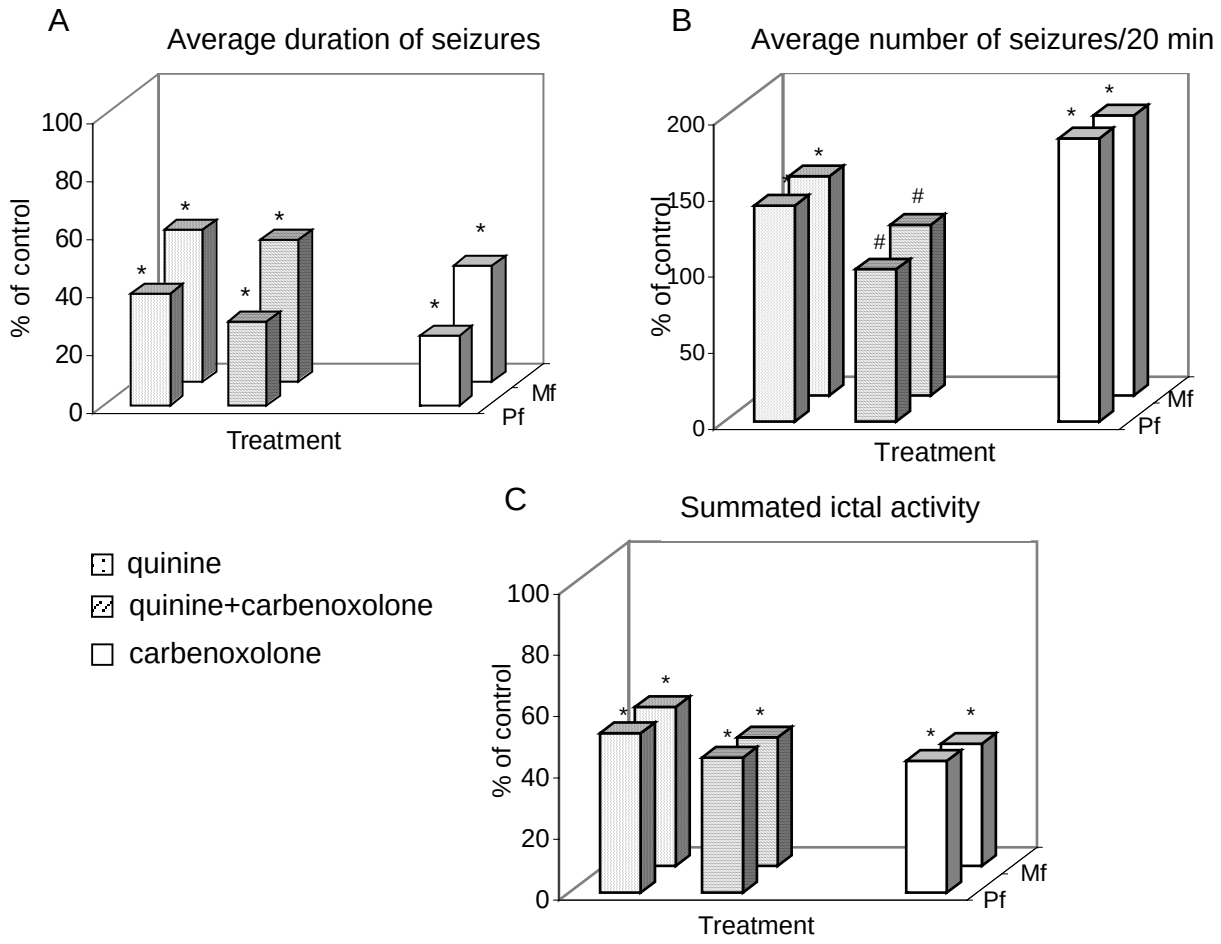


Figure 17. Effects of quinine applied alone or together with carbenoxolone and of carbenoxolone on the average durations of seizures (A), on the average number of seizures (B), and on the summated ictal activity (C) of cortical seizure activity induced by 4-AP (considered as control). The data indicated for quinine and quinine+carbenoxolone are taken from the same animals (n=6). The data indicated for carbenoxolone are taken from the other group of animals (n=6); for control data n=5 animals. * Significant difference in comparison with the control. # Significant difference in comparison with quinine. Data were collected at 20 min time window. Data are expressed as % of control. Significance criterion $P<0.05$.

Summated ictal activity

Although the number of seizures was significantly above the control value when the Pf was treated with quinine, the summated ictal activity underwent a significant decrease (Fig. 17*C*), due to the significant reduction in the durations of the individual seizures. When carbenoxolone was added to quinine, the summated ictal activity decreased further to a value similar to that observed when carbenoxolone was applied alone (Fig. 17*C*).

Manifestation of a new discharge pattern due to treatment with quinine

Following quinine treatment, as it was shown already in the case of treatment with carbenoxolone (see earlier Fig. 11), a new component (A0) occurred at the very beginning of the individual seizures, with the highest frequency (17±0.4 Hz) and lowest amplitudes (251±23 μV) (see Fig. 18/*2, 4*). This A0 section was not observed in the controls.

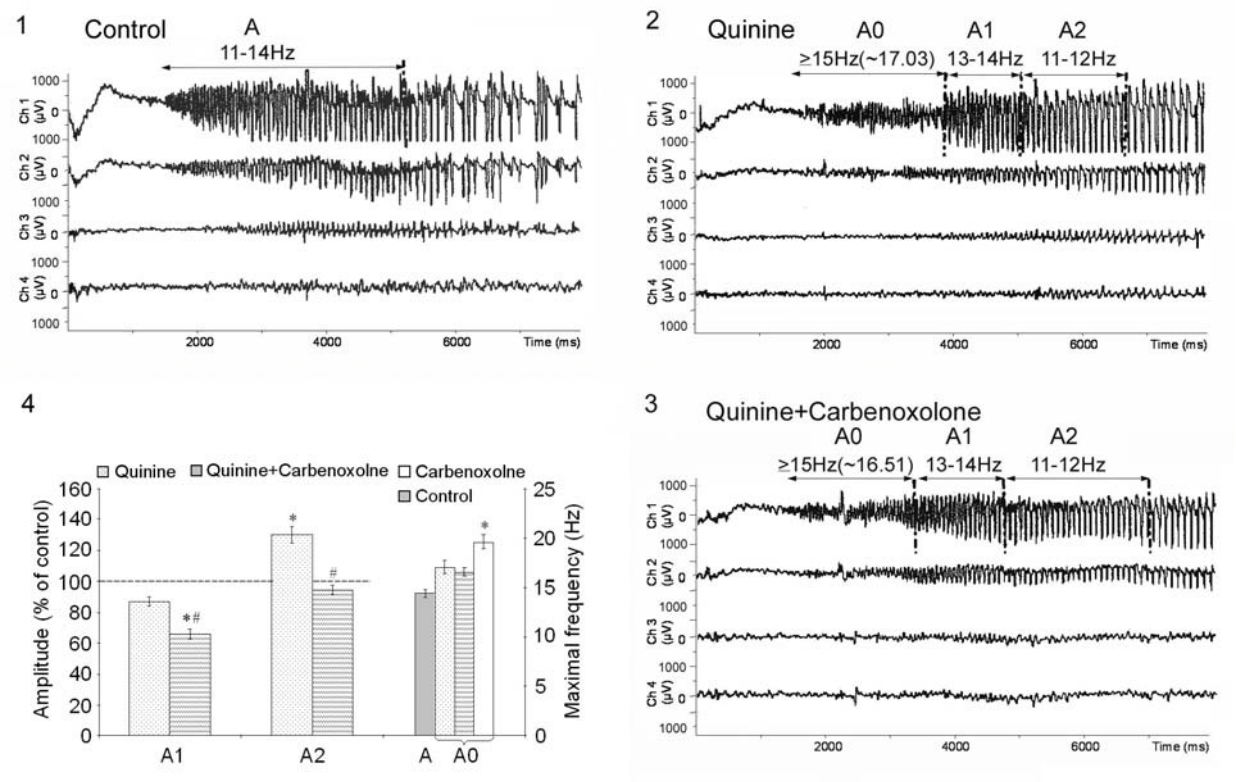


Figure 18. EEG samples representing the effects of quinine applied alone or together with carbenoxolone on the manifestation of the new discharge pattern at the initiation of seizures in the *treatment* experiments. The summarizing diagram (A/4) demonstrates the modifications in the average amplitudes of pattern A discharges and in the maximal frequency of seizure discharges. * Significant difference in comparison with the control. # Significant difference in comparison with quinine. Significance criterion $P<0.05$.

However, the frequency of A0 discharges was higher (19.6±0.3) (Fig. 18/*4*) and the duration of A0 was longer under the influence of carbenoxolone than during quinine treatment (see Figs. 11 and 18/2 for comparison). The amplitude of the A0 discharges (269±29 μV) was in the same range as for the quinine-treated animals.

When carbenoxolone was added to quinine, the frequency and amplitudes of the A0 discharges and also the duration of the A0 section decreased slightly as compared with the values observed when quinine was applied alone previously (see Fig. 18/*3, 4*).

Pattern A discharges

In response to quinine treatment, the amplitudes of the pattern A discharges did not behave uniformly. Their characteristic changes allowed the distinction of two sections (A1 and A2) immediately following A0 (Fig. 18/*2*). The amplitudes of the A1 (13-14 Hz) discharges (which correspond to the first part of pattern A) decreased slightly, while the amplitudes of the A2 (11-12 Hz) discharges increased significantly (Fig. 18/*3, 4*).

When carbenoxolone was added to quinine, the average amplitudes of the seizure discharges in both the A1 and A2 sections decreased significantly as compared with the values measured during the previous quinine treatment (Fig. 18/*3,4*).

However, in those experiments when carbenoxolone was applied alone, the A section behaved rather uniformly and did not segregate into A1 and A2 (see earlier Fig. 11). The duration of the A section became considerably shorter in comparison with the control (Fig. 11).

Pattern B and C discharges

While the selective blockade of Cx36 by quinine differently modified the amplitudes of the pattern A1 and A2 discharges, the average amplitudes of both the pattern B and C discharges decreased slightly during quinine treatment (Figs. 16*B* and 19, Table III) in comparison with the control.

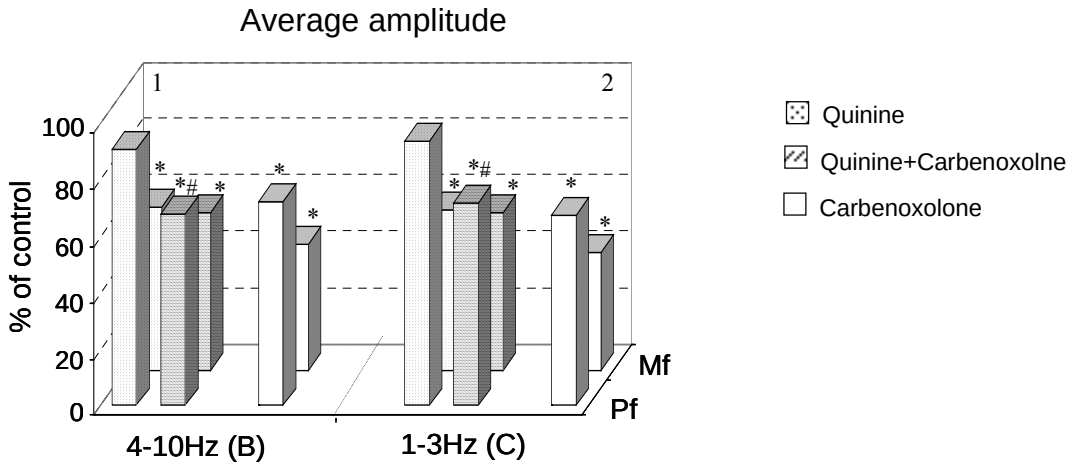


Figure 19. Effects of quinine applied alone or together with carbenoxolone and of carbenoxolone on the amplitudes of typical seizure discharges patterns in *treatment* (n=6 animal) experiments, for control data n=5 animals. * Significant difference in comparison with the control. # Significant difference in comparison with quinine. Data were collected at 20 min time window. Data are expressed as % of control. Significance criterion $P<0.05$.

However, when carbenoxolone was added to quinine, the average amplitudes of the two patterns decreased significantly, to values which were comparable with that observed when carbenoxolone was applied alone (Figs. 16*C* and 19, Table III).

Modifications in the seizure activity at the mirror focus under quinine treatment

These data were collected in parallel with those at the Pf in the same animals. In the *pretreatment* experiments, when either quinine or carbenoxolone was applied, all of the measured parameters of the seizure activity changed in the same direction at the Mf as at the locally treated Pf, though to slightly lower extents (Fig. 15 and Table III). We must draw attention to those parameters of the seizure activity, which behaved differently at the Mf than at the locally treated Pf in the *treatment* experiments.

The appearance of the new A0 section at the initiations of seizures, described earlier in connection with the Pf, was not noticeable in most cases at the Mf in the quinine-treated animals (as it was in the case of carbenoxolone treatment) (Figs. 11, 16*B,C* and 18). The average amplitude of the pattern A discharges decreased noticeably and did not exhibit the characteristic segregation under the influence of quinine at the Mf, as was observed at the Pf (Figs. 16*B, C* and 18).

However, the average amplitudes of the seizure discharges of both patterns B and C displayed a larger reduction at the Mf than at the Pf under quinine treatment (Fig. 19 and Table III). The average amplitudes of discharges were the smallest when carbenoxolone was applied alone (Fig. 19 and Table III).

Propagation of seizure discharges under the influence of quinine

The propagation of seizure discharges to the Mf and other cortical areas was apparently not modified following quinine *pretreatment* (not shown). However, in the *treatment* experiments the propagation of seizure discharges both to the contralateral hemisphere and to the ipsilateral cortical areas was noticeably depressed in comparison with the controls when quinine was applied (Figs. 16*B* and 18*/2*). We observed a larger depression in the spread of the seizure discharges when the carbenoxolone was applied either alone or together with quinine (Figs. 8, 11, 16*C* and 18*/3*).

4.1.1.4 Opening of GJ channels by TMA

Pretreatment

Local pretreatment of the cortical surface with TMA did not noticeably influence the basic cortical activity in the majority of animals, although in some cases, interictal-like discharges appeared on the EcoG activity after TMA pretreatment (not shown).

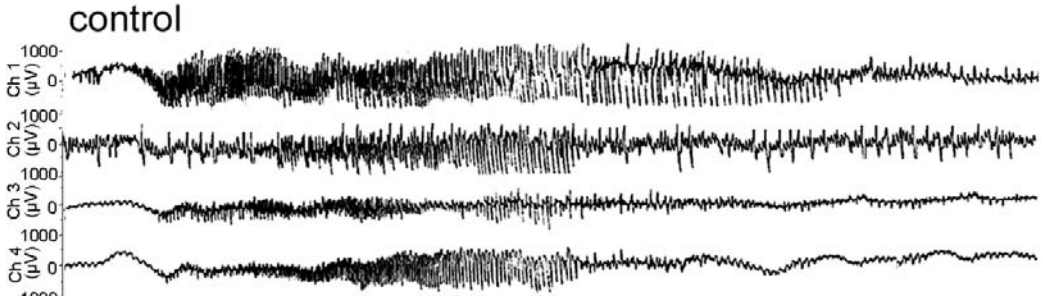


Figure 20. Representative EEG samples showing the effect of TMA in the pretreatment experiments.

When the Pf was treated with TMA, the average latency for the first seizure (58.6±9 s) after 4-AP application was in the same range as for the control animals (61.8±8 s). The increase in the average duration of the seizures (Figs. 20 and 21*A*) resulted in a somewhat longer summated ictal activity (Fig. 22*A*) in the TMA-pretreated animals in comparison with the control values. The number of ictal periods did not differ significantly from controls (Fig. 21*B*).

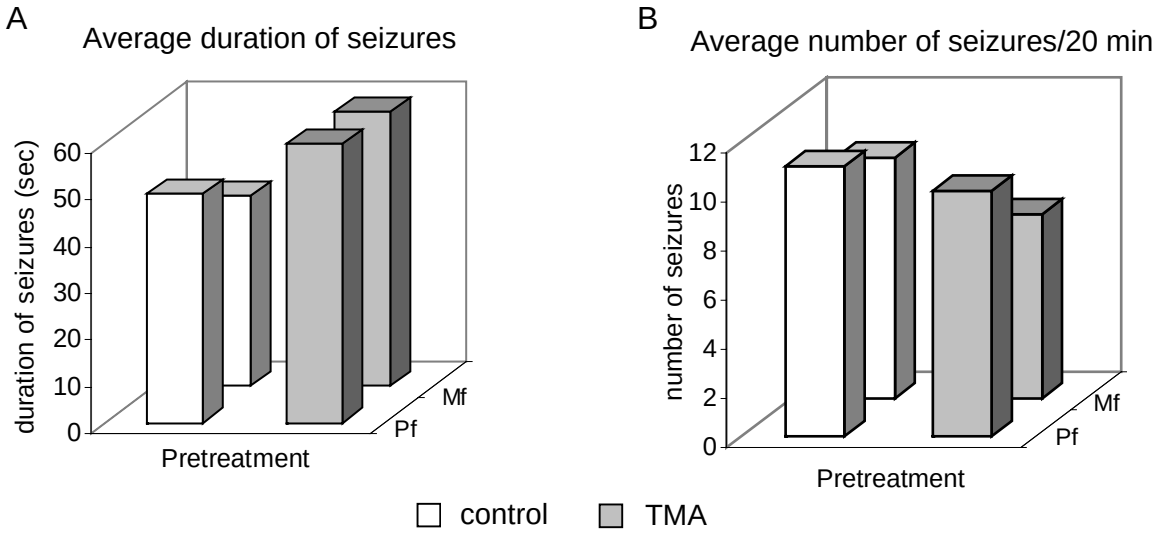


Figure 21. Graphs illustrate the effects of TMA on the average duration of seizures (A), on the average number of seizures (B), in the pretreatment experiments. Data were collected at 20 min time window. Data are expressed as mean ± SD. Significance criterion: $p \leq 0.05$.

Average amplitude

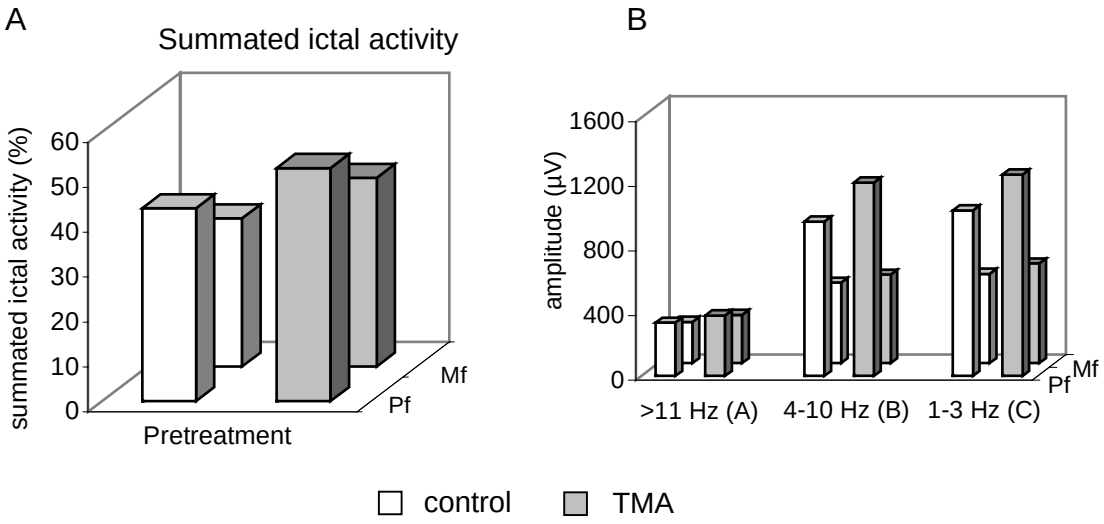


Figure 22. Graphs illustrate the effects of trimethylamine (TMA) on summated ictal activity (A) of cortical seizure activity induced by 4-AP (considered as control), and on the average amplitudes of seizure discharges (B), in the pretreatment experiments. Data were collected at 20 min time window. Data are expressed as mean $\pm$ SD. Significance criterion: $p \leq 0.05$.

The average amplitude of the seizure discharges was larger, but not significantly in the TMA-pretreated animals in comparison with the control value (Fig. 22*B*). There was a tendency to the generalized appearance of epileptiform activity in the pretreated animals compared to controls (not shown).

Treatment

The peak effect of TMA was detected 40-50 min after its application, with a still noteworthy facilitation at the end of the recording period. The first sign of the effect of TMA was the appearance of giant spike-and-wave discharges in the interictal periods, with gradually increasing frequency (Fig. 23*B*).

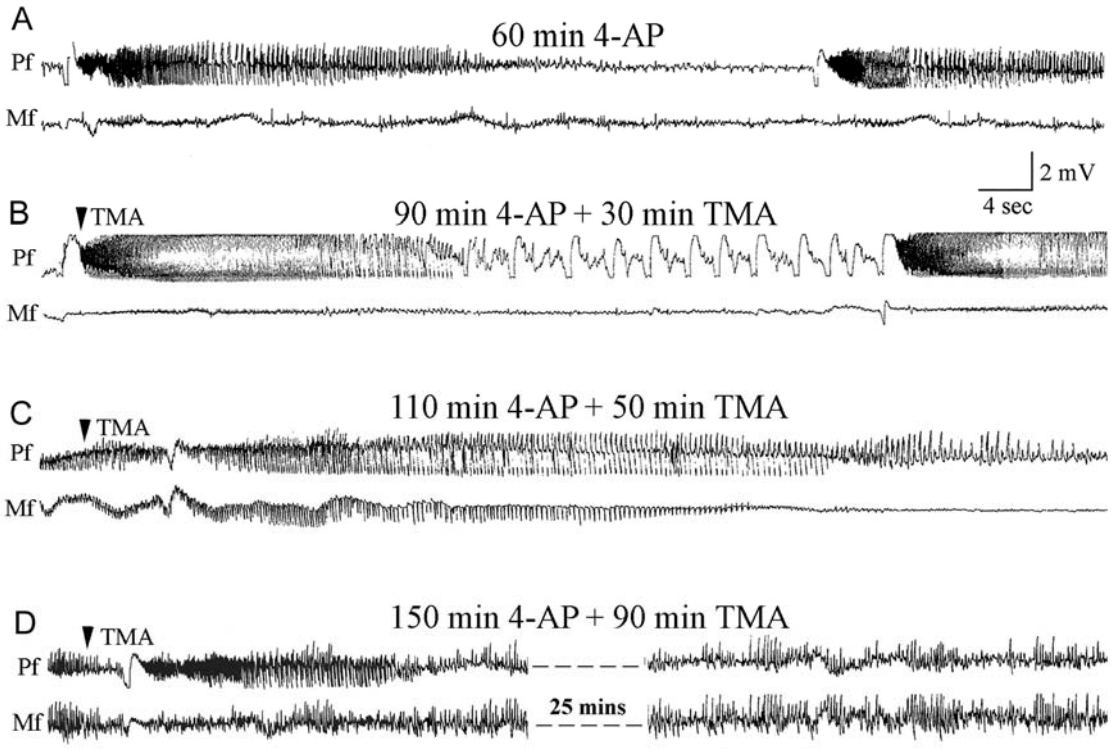


Figure 23. Effects of gap junction opening with TMA on the epileptiform activity in the treatment experiments. 4-AP- induced seizure activity before (*A*) and (B, C, D) 30, 50 and 90 min, respectively, after the application of TMA. Arrowheads indicate the application of drugs.

The average duration of the seizures increased significantly (Figs. 23*C, D* and 24*A*) after TMA treatment. On the other hand, the average number of seizures decreased significantly under the influence of TMA (Fig. 24*B*).

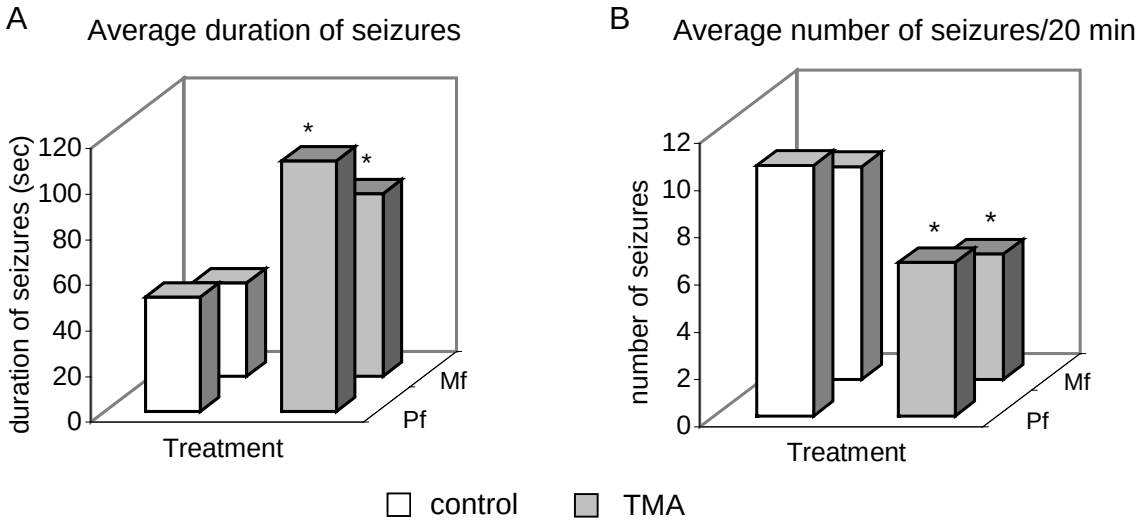


Figure 24. Graphs illustrate the effects of TMA on the average duration of seizures (A), and on the average number of seizures (B), in the treatment experiments. * Significant difference in comparison with the control. Data were collected at 20 min time window. Data are expressed as mean $\pm$ SD. Significance criterion: $p \leq 0.05$.

The modification in the numbers and durations of the seizures resulted in significant increase in the summated ictal activity (Fig. 25*A*) in comparison with the control.

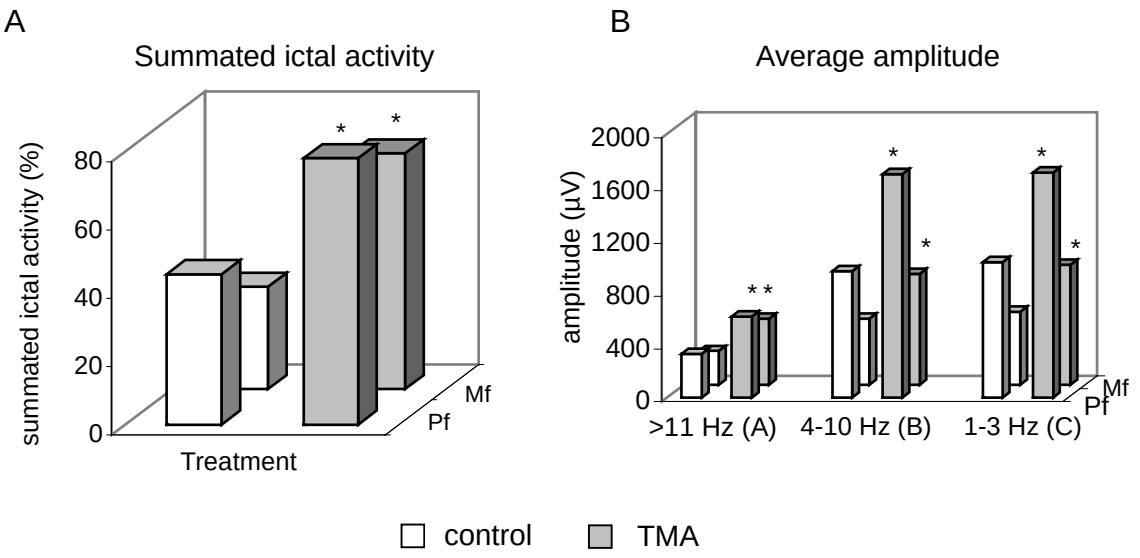


Figure 25. Graphs illustrate the effects of trimethylamine (TMA) on summated ictal activity (A) of cortical seizure activity induced by 4-AP (considered as control), and on the amplitudes of seizure discharges (B), in the treatment experiments. * Significant difference in comparison with the control. Data were collected at 20 min time window. Data are expressed as mean $\pm$ SD. Significance criterion: $p \leq 0.05$.

In some cases, the alternating ictal and interictal periods, which were seen before TMA were not separated anymore under TMA treatment, the seizures lasted for 20-40 min (Fig. 23*D*) resembling status epilepticus. The average amplitude of the epileptiform discharges in the presence of TMA also increased significantly (Fig. 25*B*).

Table IV. *Probability of mirror focus activation and generalisation in controls and under the influence of TMA.*

	Mf	Generalisation
Control	3	2
After opening of gap junctions	6*	6*

Numerals show the number of animals. Maximal n=6. * indicates significant changes. Significance criterion: $p \leq 0.05$.

The propagation of the seizure discharges to the homolateral and contralateral areas was highly facilitated due to TMA treatment in comparison with the control (Table IV).

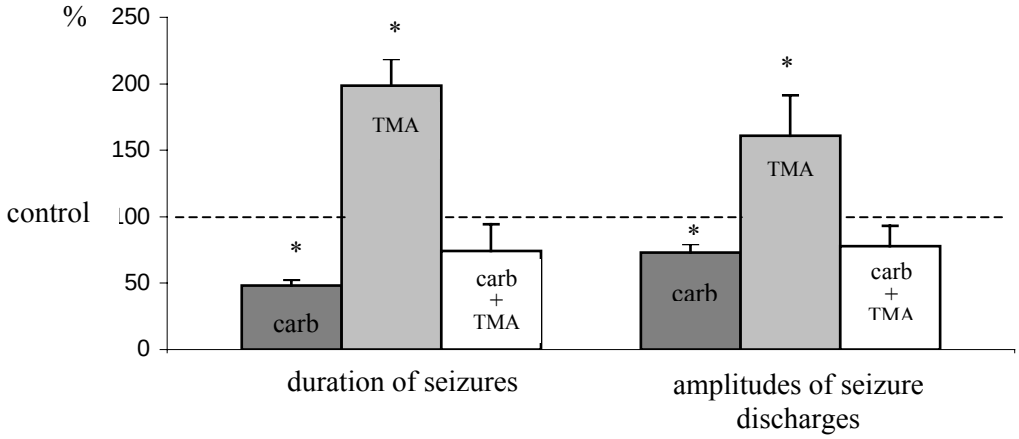


Figure 26. Effects of carbenoxolone and TMA applied either separately or together on the average duration of seizures and on the average amplitude of seizure discharges, as percentages of control values. Measurements were taken for 60 min at the site of Pf. Significance criterion: $P \leq 0.05$. * indicates significant changes compared to controls.

When TMA was applied after carbenoxolone, the average duration of the seizures (26.61±3.2 s) and the average amplitude of the seizure discharges (733±240 μV) were noticeably below (Fig. 26) those values that were measured in the case when TMA was applied alone (average duration: 110±20 s, average amplitudes: 1663±340 μV).

4.2 Electrophysiological and pharmacological experiments on developing animals

During the development of the nervous system, both the basic ECoG and the Cx mRNA expression patterns exhibited a characteristic maturation; the 4-AP-induced epileptiform activity correlated well with these changes.

4.2.1 Basic electrocortical activity

The structure of the basic ECoG progressively changed with age, with the appearance of new, faster components with higher frequencies (Fig. 27). The basic ECoG on P9-P12 was rather simple, characterized by slow sinusoid-like waves of amplitude 0.1-0.2 mV and frequency 1-3 Hz. By the age of P28, the amplitudes and frequency configurations were almost identical to those for the adult form.

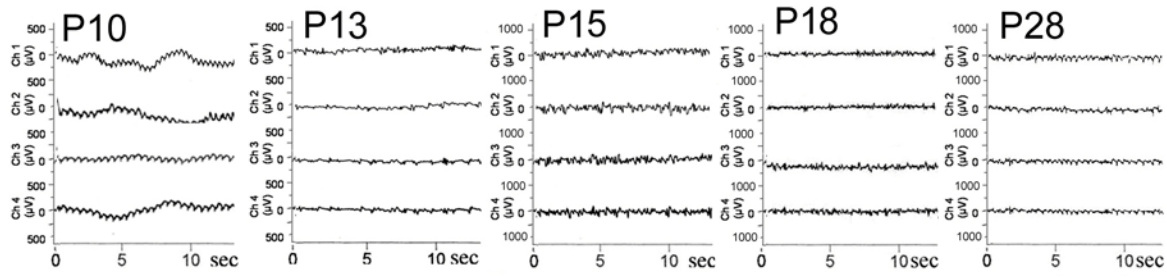


Figure 27. Representative samples showing the basic ECoG at different ages. P: here and in the following Figures means postnatal day.

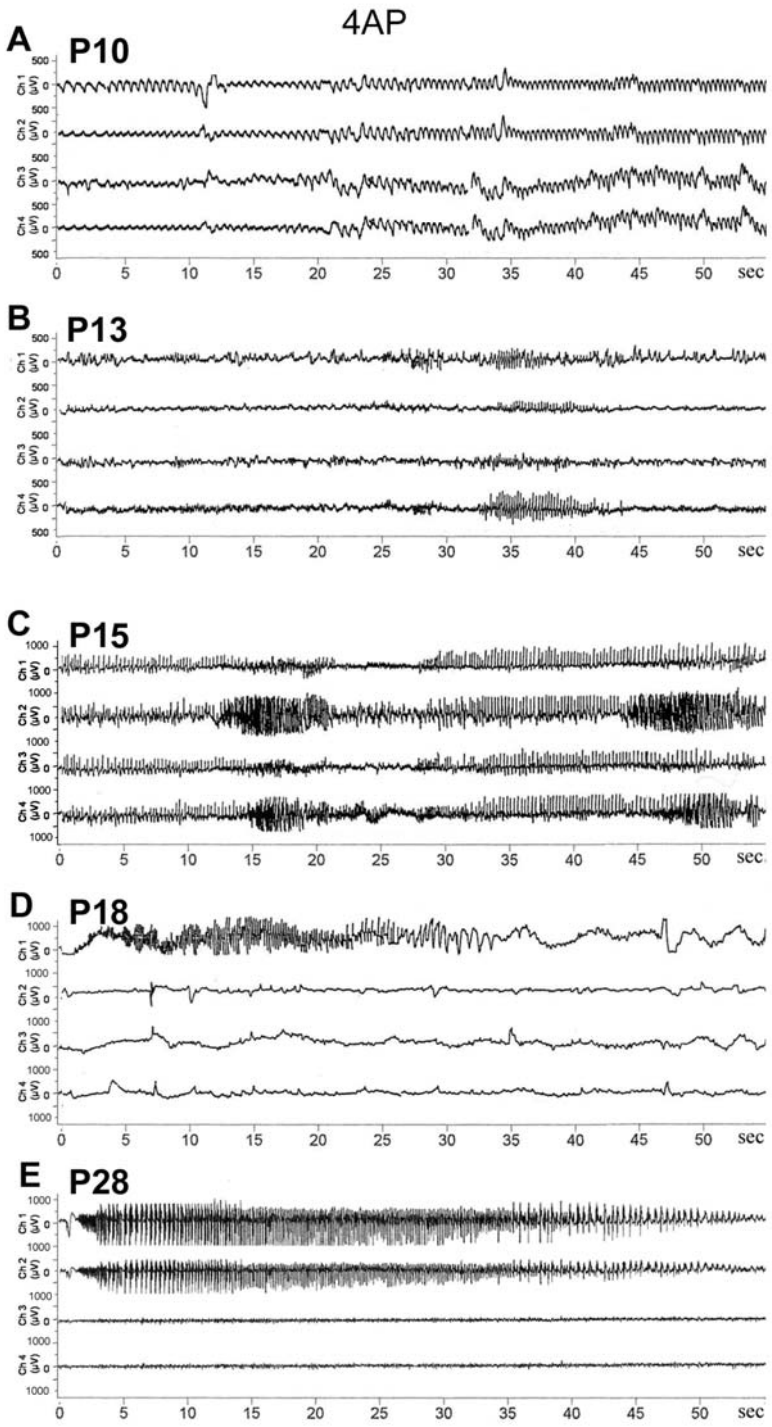
4.2.2 4-AP-induced epileptiform activity

On the basis of the pattern of 4-AP-induced ECoG activity, the animals from P9 to P28 could be divided roughly into 5 age groups (n=5 animals were studied in each group), without a sharp border between them (Fig. 28).

P9-12: After 4-AP application, the rats of this age exhibited a sustained, generalized rhythmic activity with relatively slow waves of amplitudes between 0.2 and 0.6mV, and frequencies between 0.8 and 3Hz. In some cases, either the frequency or the amplitude of these potentials fluctuated (Figs. 28*A* and 29). The alternation of ictal and interictal-like periods, characteristic of the epileptic activity of adults could not be recognized at this age. Paradoxically, in some cases we observed a depressed activity at the area of 4-AP application, probably due to a possible transfer inhibition originating from the contralateral side, through the rostral corpus callosum and/or the commissura anterior.

P13-14: A transient reduction in seizure susceptibility occurred at the age of P13-14 (Figs. 28*B* and 29). The 4-AP-induced activity was mostly restricted to the periodic appearance of groups of roughly synchronous ictal-like discharges. These potentials, however, were shorter in duration and often higher in amplitude (0.6-0.8 mV) and frequency (6-10 Hz) than in the younger animals.

P15-16: At this age, there were profound changes in the manifestation of the 4-AP-induced activity (Fig. 28*C*). The elevated seizure susceptibility was characterized by the generalized occurrence of synchronized rhythmic seizure discharges in the ECoG (with amplitudes of 0.8-1.2 mV and frequencies of 4-6 Hz), superimposed with randomly repeated and generalized seizures. The amplitudes and the frequencies of the seizure discharges gradually became higher (0.8-1.2 mV and 8-12 Hz) in comparison with the younger animals, although the discharges of highest frequency (9-15 Hz) appearing on the



initiation of the seizures in adult rats were missing (Figs. 28*E* and 29). Although the number of the seizures basically did not change, the duration of the individual seizures increased significantly (from 5-8 sec to 60-90 sec) in comparison to the P13-14 animals, resulting in a remarkable increase of the summated ictal activity (Figs. 28*C* and 29). In this age group, there were noticeable differences between the activity patterns of the two hemispheres in most animals.

Figure 28. Representative samples showing the cortical activity induced by 4-AP at different ages (from A to E).

On the primary side (the side of 4-AP application), there was an obvious tendency to the separation of periodic ictal-like and interictal-like activity patterns.

P17-22: In the P17-P22 animals, the seizure susceptibility decreased in parallel with a strong tendency to focalization and periodicity. The appearance of more typical ictal-like periods was followed by interictal phases resembling the basic ECoG activity (Figs. 28*D*

and 29). Typical synchronous epileptiform activity occurred only at the site of 4-AP application and was not manifested at the identical point on the contralateral side.

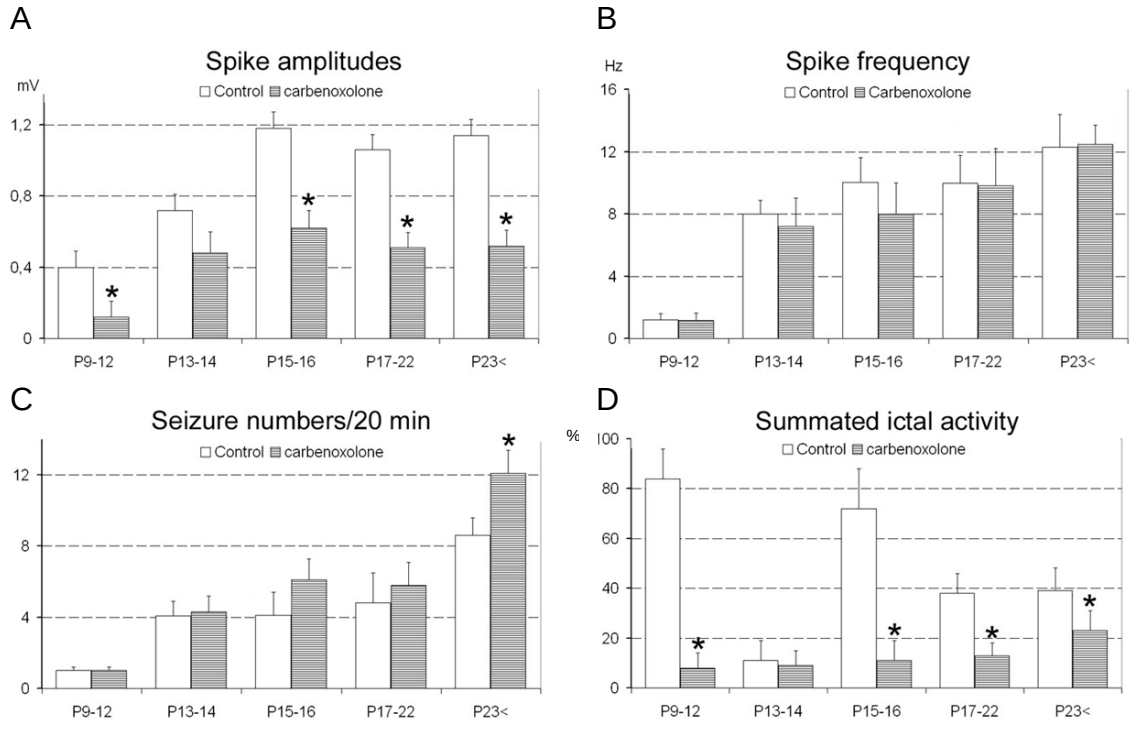
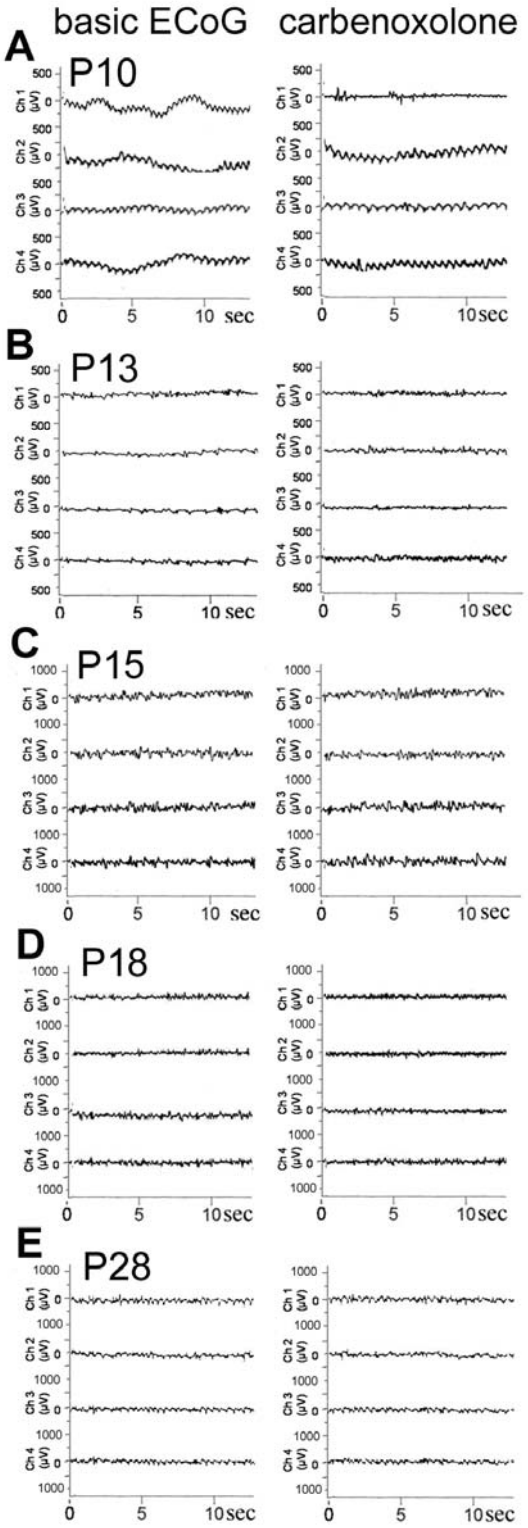


Figure 29. Graphs illustrate the effects of carbenoxolone on the spike amplitude (A), spike frequency (B), seizure numbers (C) and summated ictal activity (D) of cortical seizure activity induced by 4-AP (considered as control, Co) at several developmental time points. The drug was applied at the already active Pf 60 min after repeated seizures. Data were collected for 20 min between the 10^{th} and 30^{th} min of the presence of the drug. Each bar represents the mean values $\pm$ S.D of five animals per group. Asterisk (*) indicates statistically significant differences in comparison with the control. Significance criterion: $p \leq 0.05$.

P23 and later: The 4-AP-induced epileptiform activity was focal (restricted to the Pf and the Mf) and periodic (15-25 seizures/h) with the highest-frequency (9-15 Hz) discharges at the initiation of the tonic phase and spike-wave complexes of 1-3 Hz (clonic phase) at the end (Figs. 28*E* and 29). The ictal periods were 50-80 s long, followed by interictal periods. In most animals, after a few repetitions of the seizures, a secondary Mf developed at the identical point on the side contra lateral to 4-AP application, with activity synchronous with that of the Pf, this being characteristic for 90% of the animals after P23. In about 30% of the animals, the epileptiform activity became secondarily generalized.

4.2.3 Opening or closing of GJ channels by pharmacological tools

We observed a dynamic fluctuation in the effects of carbenoxolone and TMA on the basic ECoG, the induction and maintenance as well as the propagation of seizures at



the different age groups (between P9 and P28), both in the pretreatment and treatment experiments, supposedly depending on the actual composition and size of the GJ pool. The effect of *pretreatment* with both carbenoxolone and TMA was quite obvious at P9-12, while at P13-14 pretreatment was apparently not effective. From the age of P13, pretreatment with carbenoxolone did not noticeably modify either the basic ECoG, or the induction of seizures by 4-AP. At P15-16 TMA pretreatment slightly enhanced both the ECoG and the epileptiform activity, while in older animals only the propagation of seizures was facilitated. On the other hand, either a proconvulsive or anticonvulsive effect of *treatment* at the already active epileptic focus with TMA or carbenoxolone respectively was much more obvious at any age studied as it was seen in adult animals too.

Figure 30. Representative samples showing the basic ECoG (right column) and the effects of carbenoxolone (right column), recorded from the same animal at different ages (from A to E).

Treatment with TMA transformed the 4-AP-induced periodic seizure activity to a continuous and generalized rhythm (like status epilepticus). See details below.

4.2.3.1 Closing of GJ channels by carbenoxolone

P9-12: In these young animals, pretreatment with carbenoxolone locally eliminated the rhythmic oscillations in the basic ECoG (Fig. 30*A*), and suppressed the induction of synchronous activity induced by 4-AP (not shown). Treatment with carbenoxolone strongly depressed the maintenance of epileptiform activity indicated by a significantly smaller spike amplitudes and reduced summated ictal activity (Figs. 29 and 31*A*). However, this effect was restricted to the site of application of carbenoxolone.

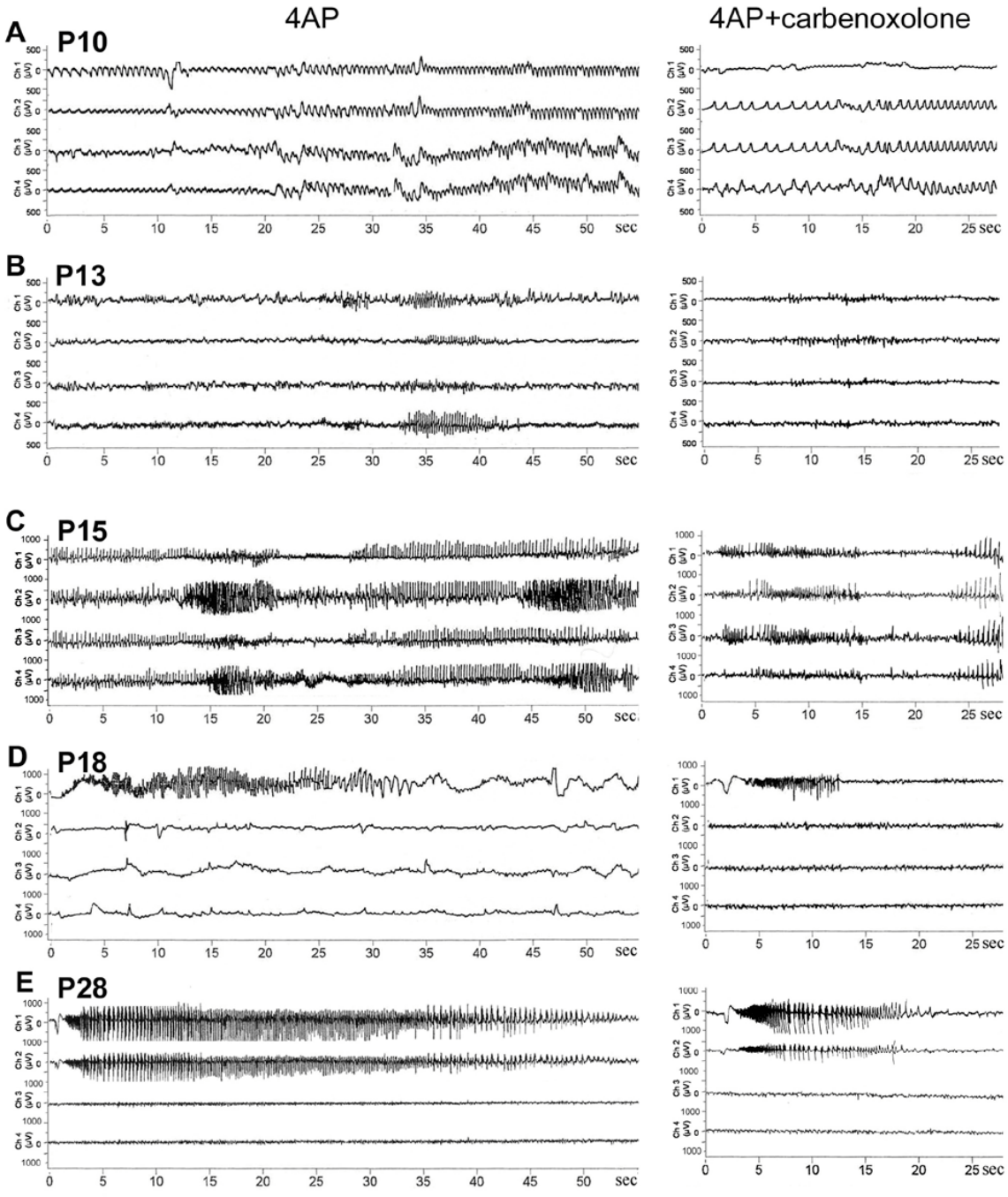


Figure 31. Representative samples showing the cortical activity induced by 4-AP (left column) and 10 min after the application of carbenoxolone (right column), recorded from the same animal at different ages (from A to E).

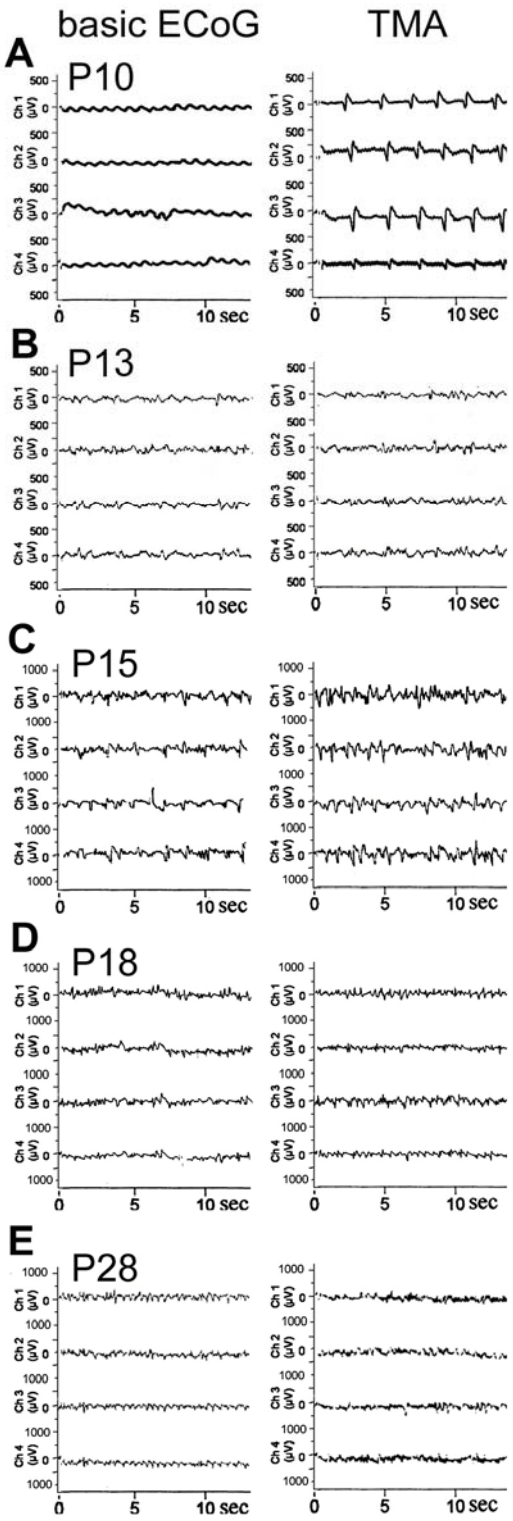
P13-14: From the age of P13, pretreatment with carbenoxolone did not noticeably modify the basic ECoG (Fig. 30*B*), nor the induction of the first seizures after *pretreatment* with carbenoxolone (not shown). However, when the already active epileptic focus was *treated* with carbenoxolone, it noticeably depressed the manifestation and propagation of the 4-AP-induced synchronous activity (Figs. 29 and 31*B*).

P15-16: Carbenoxolone *pretreatment* did not apparently modify either the basic ECoG (Fig. 30*C*) or the induction of 4-AP epileptiform activity (not shown). Conversely, the seizures became shorter, the amplitudes of the discharges became smaller and their frequency became lower, with the obvious separation of ictal and interictal periods, when carbenoxolone was applied 60 min after the appearance of the first ictal periods (Figs. 29 and 31C). Although, the numbers of the seizures slightly increased, the summated ictal activity considerably decreased, due to the short seizures. The seizures occurred in a generalized manner in most of the animals of this age, in spite of the *treatment* with carbenoxolone.

P17 and later: Local *pretreatment* of the cortical surface with carbenoxolone did not noticeably influence the basic ECoG in the animals older than 18 days (Fig. 30*D*, *E*). When the electrical synaptic transmission was depressed relative to the initial baseline prior to the induction of an epileptic focus, there was only a mild influence on the induction of seizure discharges (not shown). By contrast, in another series of experiments, when carbenoxolone was applied at the already active epileptic focus, a significant decrease in the intensity of seizure activity and a considerable increase in the duration of the silent ‘‘seizure-free’’ period were detected both at the Pf and at the Mf (Figs. 29 and 31*D*,*E*).

4.2.3.2 Opening of GJ channels by TMA

P9-12: At this age pretreatment with TMA, even in the absence of the convulsant 4-AP, noticeably enhanced the neuronal synchrony, resulting in the generalized appearance of rhythmic potentials (0.5-0.8 mV and 0.5-0.7 Hz) resembling seizure discharges (Fig. 32*A*). These discharges were rather uniform in their pattern, and often occurred in 25-30 s periods followed by a silent phase. The subsequent application of 4-AP did not noticeably modify the TMA-induced activity pattern (Fig. 33*A*, first column). Similarly, *treatment* with TMA did not obviously influence the 4-AP-induced activity pattern (Fig. 33*A*, second column).



P13-14: In these animals, TMA failed to induce the synchronous, rhythmic activity in the basic ECoG described for the P9-12 animals (Fig. 32*B*). The 4-AP-induced activity following TMA *pretreatment* did not differ considerably from that without TMA pretreatment (Fig. 33*B*, first column). On the other hand, *treatment* with TMA 60-65 min following 4-AP application perceptibly altered the activity pattern (Figs. 33*B,* second column and 34). Although, the number of the seizures decreased significantly, the summated ictal activity increased considerably due to the long seizures (Figs. 33*B*, second column and 34). In some animals, the amplitudes of the discharges at the site of the Mf were higher in comparison with those appearing at other cortical areas (not shown).

Figure 32. Representative samples showing the basic ECoG (right column) and the effects of TMA (right column), recorded from the same animal at different ages (from A to E).

This could indicate a transient desynchronizing role of GJal communication locally at an already active epileptic focus, and a facilitating effect on the propagation of seizure discharges to the identical point of the contralateral hemisphere.

15-16: In these animals, *pretreatment* with TMA slightly enhanced the ECoG activity (Fig. 32*C*), and increased the amplitudes (from 1,2 $\pm$0,11 mV to 1,4$\pm$0,13 mV) of the seizure

discharges induced by 4-AP following TMA application (Fig. 33*C,* first column). *Treatment* with TMA at the Pf 60 min after the appearance of the first ictal period induced by 4-AP somewhat increased the manifestation of epileptiform activity. Seizure discharges occurred continuously without the separation of ictal and interictal periods, but, the discharges became slower, and their frequency decreased from 8-12 to 4-6 Hz (Figs. 33*C*, second column and 34).

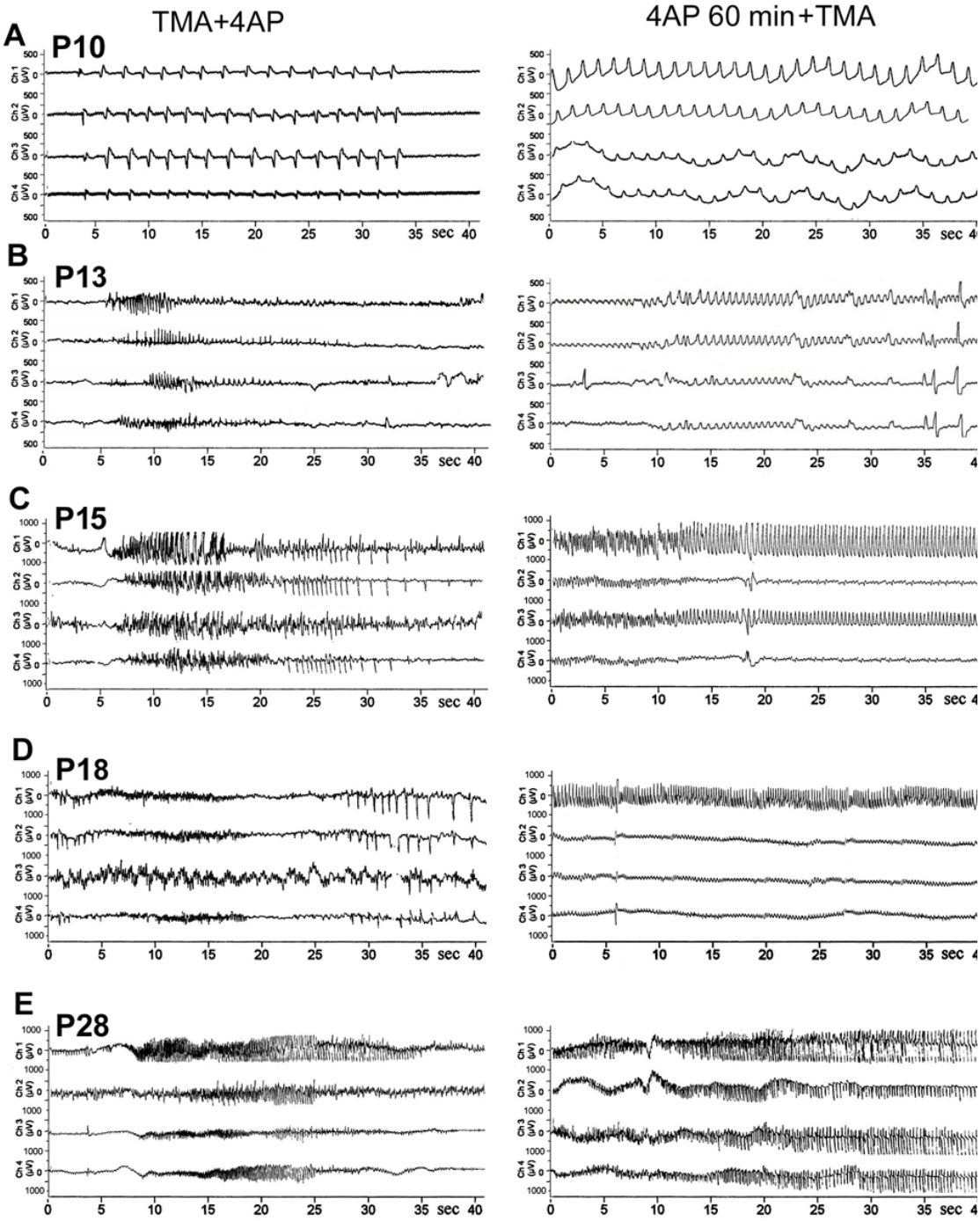


Figure 33. Comparison of the effects of pretreatment with the gap junction opener TMA on the manifestation of 4-AP-induced activity (left column) and the effect of TMA treatment at the already active epileptic focus (right column) at different developmental time points (from A to E).

P17-22: TMA *pretreatment* did not visibly modify the basic ECoG at this age (Fig. 32*D*, second column), though it influenced the seizure activity induced following TMA pretreatment (Fig. 33*D*, first column). High-frequency spikes (12-15 Hz) with small amplitudes (0.3-0,4 mV) were accompanied by discharges of high amplitude (1,0-1,5 mV) but lower frequency (3-8 Hz) (Fig. 33*D*, first column; compare with Fig. 31*D*, first column). This activity pattern seemed to be generalized, and was interrupted only by short, interictal like periods. When TMA was applied 60 min following the induction of seizure activity, almost permanent rhythmic spiking developed, with frequencies of 5-8 Hz, and usually higher amplitudes (0,8-1,6 mV) at the Pf than in other cortical areas (0,1-0,2 mV, Figs. 33*D*, second column and 34). The increase of the duration of the seizures resulted in significant increase of the summated ictal activity (Fig. 34).

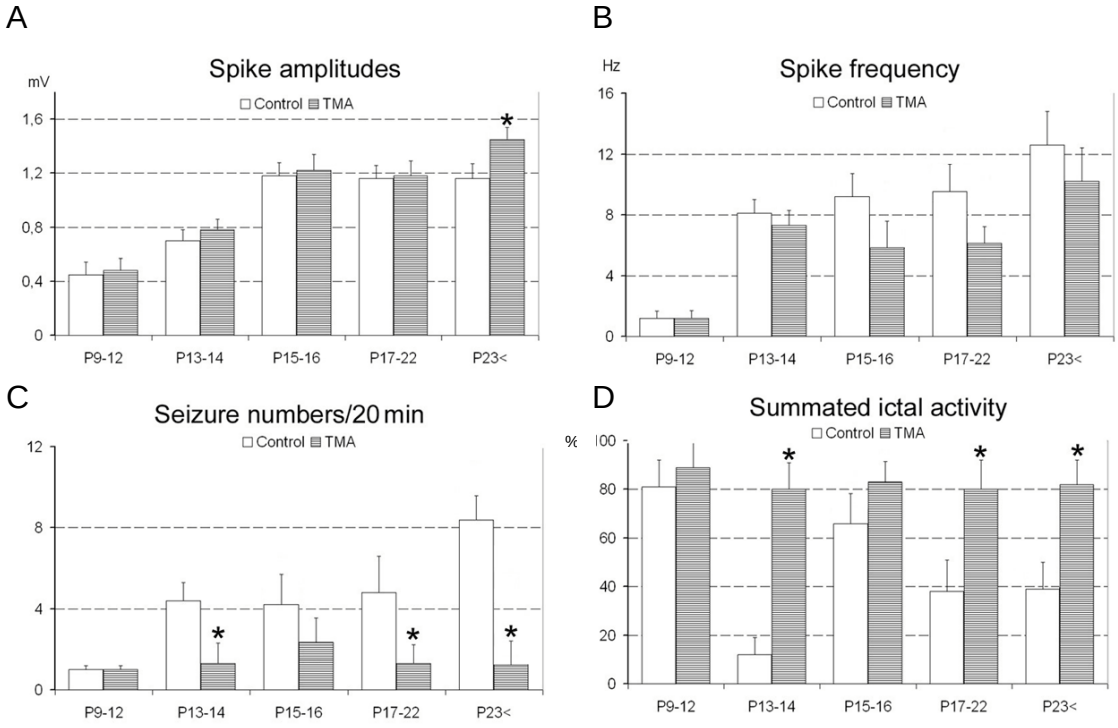


Figure 34. Graphs illustrate the effects of trimethylamine (TMA) on the spike amplitude (A), spike frequency (B), seizure numbers (C) and summated ictal activity (D) of cortical seizure activity induced by 4-AP (considered as control, Co) at several developmental time points. The drug was applied at the already active primary focus 60 min after repeated seizures. Data were collected for 20 min between the 10^{th} and 30^{th} min of the presence of the drug. Each bar represents the mean values $\pm$ S.D of five animals per group. Asterisk (*) indicates statistically significant differences in comparison with the control. Significance criterion: $p \leq 0.05$.

P23 and later: Pretreatment with TMA in P23 and older animals did not qualitatively influence the basic ECoG, similarly as in adults. The seizures induced by 4-AP after TMA pretreatment were generalized in most animals, and separated by interictal periods (Fig. 33*E*, first column). However, when TMA was applied at the already active Pf after 60 min

period of repeated seizures, the amplitudes of the discharges increased significantly, the seizures gradually became longer resulting in significant increase in the summated ictal activity, and finally no interictal periods could be detected. This kind of epileptiform activity appeared in a rather uniform manner throughout the cortex (like generalized status epilepticus) (Figs. 33*E*, second column and 34).

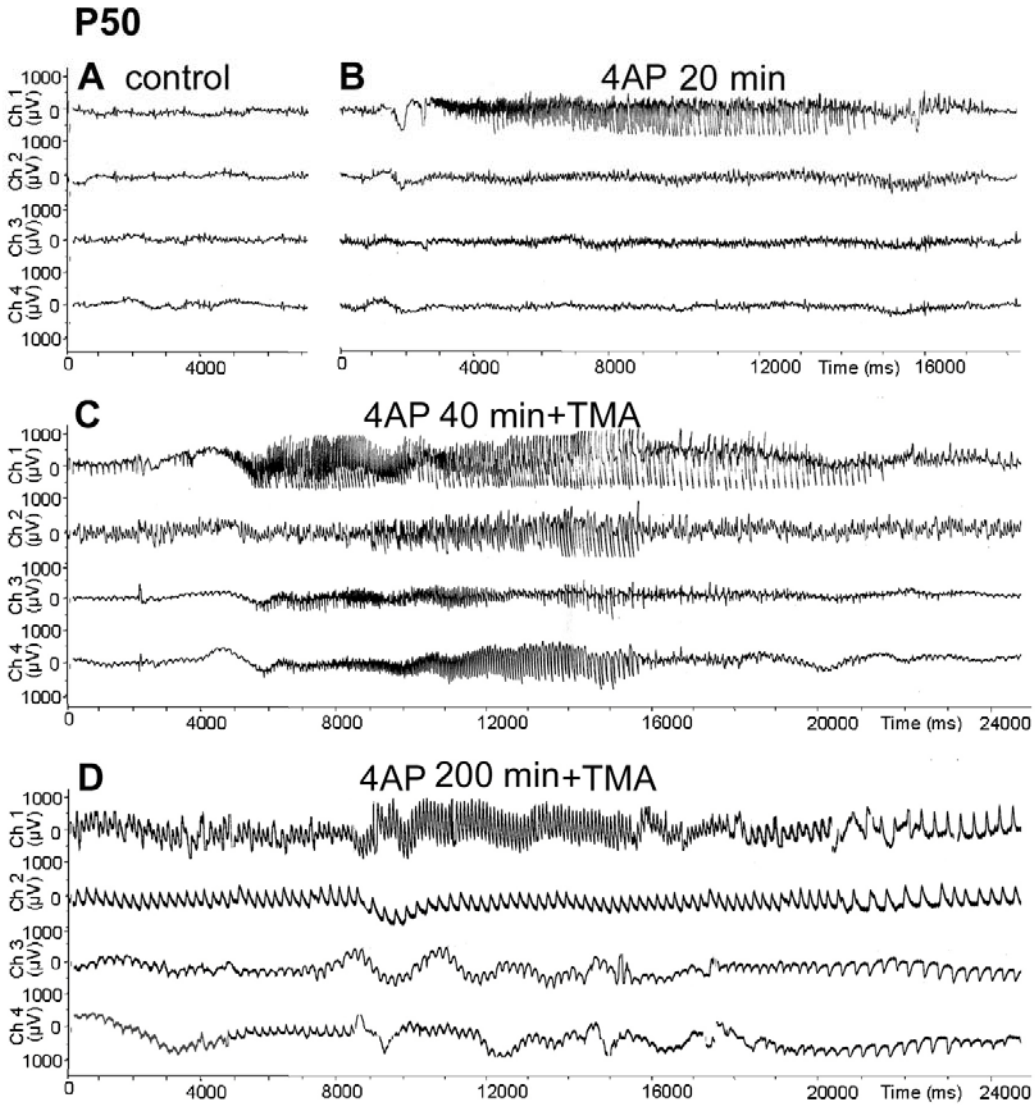


Figure 35. The cortical activity pattern of an adult animal after a long period (200 min, approximately 120 seizures) of repeated seizure activity in the presence of the gap junction opener TMA became similar to that seen in the young animals. *A*: control ECoG; *B*: representative samples showing the cortical activity induced by 4-AP applied alone; C and D: effects of TMA on the 4-AP-induced cortical activity when applied 40 and 200, respectively, min after 4-AP.

It is worthwhile to mention here that, in adult animals, TMA application after a longer period of repeated seizures (200 min, approximately 120 seizures) converted the cortical activity into a synchronous rhythmic pattern, lacking the highest frequencies, resembling the cortical activity in younger animals (Fig. 35).

4.3 Basal expression levels and the inducibility of Cx mRNAs

The basal expression levels and the plastic changes induced by epileptiform activity in the mRNA levels for Cx32, Cx36 and Cx43 in the adult and for Cx 26, Cx32, Cx36 and Cx43 in the developing rat neocortex were investigated by semiquantitative RT-PCR amplification. In adult animals, samples were taken from the areas of the Pf and Mf. In developing animals due to the generalized appearance of epileptiform activity the cortical tissues of the Pf area were isolated. The same animals were used for electrophysiological experiments and molecular biological measurements. The β-actin/connexin mRNA ratio was used as a measure of the relative abundance.

4.3.1 The basal expression of connexin mRNAs

In adult animals, the Cx32 mRNA showed the lowest basal expression level (it was about 15% of the β-actin mRNA level). The abundance of Cx36 mRNA was about 39% of the β-actin mRNA level. The relative level of Cx43 mRNA was the highest between the examined Cxs; it was about 84% of the β-actin mRNA level (not shown).

In developing animals, the expressions of Cx26, 36 and 43 did not display significant alterations during the first two weeks under physiological conditions (Fig. 36). The abundances of Cx26 and 43 mRNA were about the same, while the relative level of Cx36 mRNA was somewhat lower. However, after the first two weeks, the expression patterns of these three Cx genes exhibited marked differences. The level of Cx26 mRNA had declined by about 50% at P16, and remained around this low level till P23. In contrast, the Cx36 expression was significantly upregulated (175%) by P16. At P23 a somewhat lower specific mRNA level was detected (140%), but still significantly higher than that of measured at P10-P14. A significantly elevated level of Cx43 expression was detected only by P23, when it reached 230% of the P10-14 value. The Cx32-specific mRNA could not be detected on P10, but it then gradually increased to about 15% of the β-actin mRNA level by P23, this being the least expressed Cx gene examined in this study.

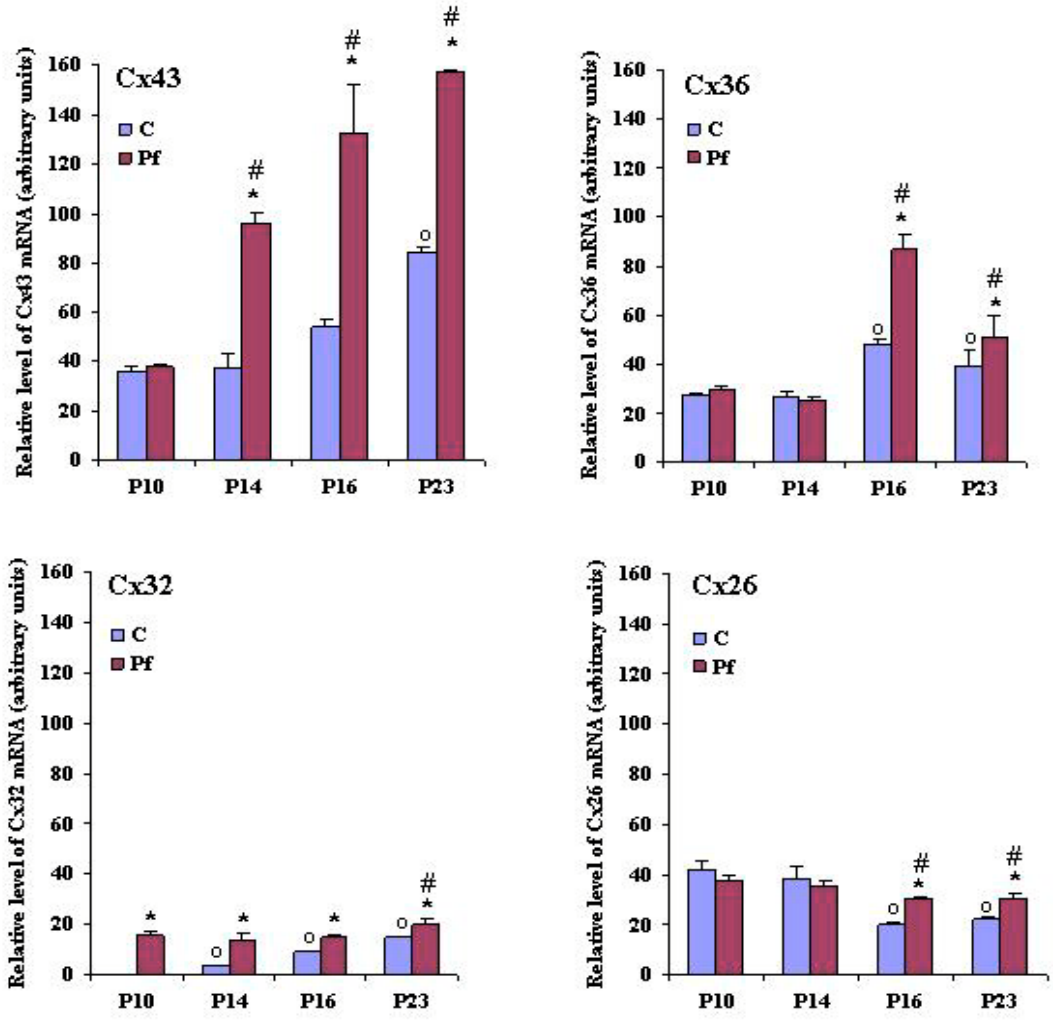


Figure 36. Expression levels of Cx43, Cx36, Cx32 and CX26 mRNAs in the cortical region of control and 4-AP-treated animals, at several developmental time points. Cx mRNA levels were normalized to that of β-actin mRNA. Data are means ± S.D. from measurements on 4 animals at each time point. Each PCR was performed in triplicate in order to increase the reliability of the measurements. C: control; Pf: primary focus. * indicates significant difference between control and Pf values at a given time point; o and # indicates values significantly different from that of at P10 within the basic and induced data set, respectively.

4.3.2 Expression levels of Cx mRNAs after repeated seizure activity

In adult animals, as the consequence of the repeated seizures the mRNA levels for Cx36, Cx32 and Cx43 increased significantly both at the Pf and at secondary induced Mf (Fig.37).

In developing animals, the seizure activity did not change the expression of Cx26 mRNA markedly on P10 and P14, but on P16 and P23 we observed a significant increase (50% and 37%, respectively) in the mRNA level as compared with the basic expression

(Fig. 36). This inducibility could be a consequence of the decreased basic expression at these ages, since the induced transcript levels are slightly but significantly lower than at P10.

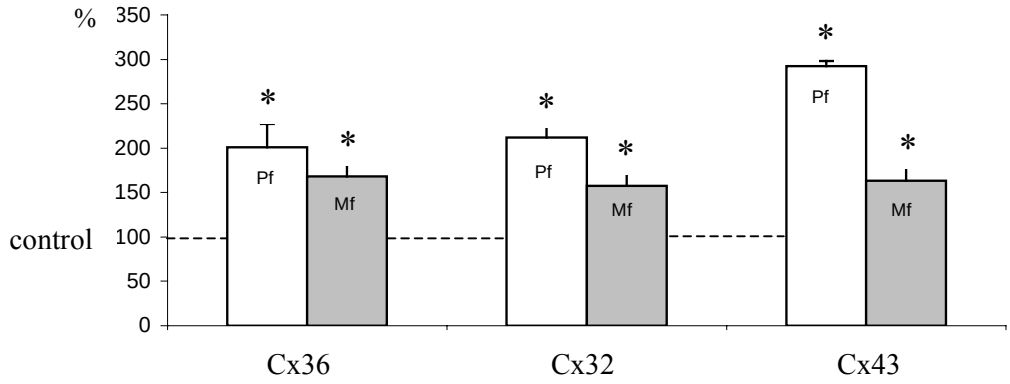


Figure 37. Expressions of Cx36, Cx32 and Cx43 mRNAs in the Pf and in the homotopic area contralateral to the primary focus (Mf), as percentages of control values. Data are means $\pm$ SD from measurements on 6 animals. Significance criterion: $P \leq 0.05$. * indicates significant changes.

The inducibility of the Cx36 gene after repeated seizures was not detected on P10-14, but the mRNA level was notably higher around P16, when the gene-specific transcript was about 180% of the control value (Fig. 36). By P23, the Cx36 expression was still affected in the epileptic foci; the mRNA level was about 150% of the control.

We found the highest induced mRNA level for Cx43. This gene was not inducible by seizure activity on P10, but its expression was highly upregulated in later stages (P14-23). The specific mRNA level reached 250% of the control level on P14-16, but was somewhat decreased by P23, when the transcript level was 185% of the control value.
Although Cx32 mRNA could not being detected on P10 in the controls, the seizure activity considerably induced its expression level at this age. The induced levels were about the same at the first three time points, differing significantly from the highest induced level measured onP23.

5. Discussion

In the present thesis, the assumption that gap junctional communication may contribute to epileptogenesis and manifestation of cortical ictal discharges was tested on the 4-aminopyridine acute epilepsy model in in vivo experiments both at the primary focus and at the mirror focus in anaesthetized adult rats. In addition the possible roles of GJ channels in the epileptogenicity and seizure susceptibility of the immature mammalian brain were also investigated.

We carried out electrophysiological experiments, combined with pharmacological manipulations of the GJ channels, and measurements of developmental and 4-AP-induced synchronous activity-dependent plastic changes in the Cx26, 32, 36 and C43 mRNA levels in the rat neocortex in different postnatal developmental stages were also carried out.

Our results have provided direct evidence that experimental manipulations of the functional state of GJs under *in vivo* conditions significantly modify the expression, the duration and the propagation of seizures to other cortical areas. The epileptic activity induced by 4-AP is accompanied by specific quantitative alterations in the different Cx mRNA levels and suggests a cause-effect relationship between the electrical communication pathway and epileptiform synchronization. Our observations also suggest that GJal communication is involved in rhythm genesis and synchronization of both basic electrocortical and epileptiform activity, and may enhance the epileptogenicity of the developing brain.

5.1 The possible role of gap junctional communication in epileptogenesis and manifestation of epileptiform activity after repeated seizures in adult neocortex

The blocking of GJs in a normal physiological state of the brain apparently influenced neither the basic cortical electric activity nor the configuration of somatosensory evoked responses (Fig. 5); nor did it prevent the induction of epileptiform discharges. Treatment with carbenoxolone or octanol prior to the application of 4-AP exerted a weak influence on the induction and maintenance of epileptiform activity (Fig. 6 and 7). Similarly, opening of the GJs with TMA in physiological state of the brain has no significant effect either on the basic ECoG or on the induction and maintenance of seizure discharges (Figs. 20, 21 and 22). These observations are probably related with the results of our molecular biological measurements. In the control animals, we measured relatively low basal Cx32, Cx43 and Cx36 mRNA levels in comparison with the β-actin expression.

These observations are in harmony with the findings from earlier studies, which indicated that the proportion of functionally active electrotonic coupling between cells of the adult nervous system under physiological conditions is relatively low (Dudek et al., 1983). This has been confirmed indirectly by dye-filling experiments, which demonstrated coupling between small groups of neurons in the mature brain rather than extensive neuronal aggregates (Perez Velazquez and Carlen, 2000).

In the contrary, we observed more expressive changes in the seizure activity when the GJs were blocked in the already active epileptic foci, either at the Pf or at the Mf, in comparison with the pretreatment situation (Figs. 10 and 13). These observations can suggest that the intensity of GJal communication in an epileptic focus with a history of earlier recurring seizure activity could be higher in comparison to an intact cortex. Indeed, we measured significant increases in expression of three connexin genes, Cx32, Cx36 and Cx43, both at the Pf and at the Mf after 30-40 repeated seizures (Fig. 37). The upregulation of Cx32, Cx43 and Cx36 after repeated seizures both at the Pf and at the Mf could be an indication of the increased gap junctional coupling between different cells (glia/glia, glia/neurons and neurons/neurons) in the epileptic foci.

These observations indicate that the consequence of the functional manipulation of the GJs on the cortical epileptogenicity depends on the initial size and composition of the GJ population. We suppose that GJ activity/expression is probably increased relative to the baseline level during the initial seizure activity, which can further amplify the expression of epileptiform discharges. An altered composition, number or function of the GJs could be involved in epileptogenesis and could result in increased seizure susceptibility.

The involvement of gap junctions could be crucial in sustaining neuronal synchronisation in the already active epileptic foci, since their opening or blocking significantly increased or decreased respectively the duration of the seizures. Notably decreased amplitudes when the gap junctions were in the closed state and significantly increased amplitudes when they were in the opened state, could testify to decreased or increased numbers of cells, respectively, firing synchronously during seizure discharges. The facilitated secondary epileptogenesis occurring when the gap junctions were in the opened state could indicate increased afferent inputs to the Mf, and the more frequent antidromic spike generation of pyramidal cells located in the Mf (Szente and Boda, 1994), as well as elevated horizontal propagation ipsilaterally through electrical communication between the neurons.

The fact that the blocking effect of carbenoxolone prevented the full manifestation of the facilitatory effect of TMA favors the possibility that these two drugs converge to the same target, i.e. conformational changes in the gap junctions. Nevertheless, carbenoxolone did not completely prevent the effects of TMA. This can be explained by the different mechanisms of action of these two drugs. Carbenoxolone is thought to be related to glycyrrhetinic acid, which binds directly to the Cx molecule, causing a conformational change and leading to closure of the gap junctions (Gladwell et al., 2001). TMA causes intracellular alkalinization, which not only leads to opening of the gap junctions, but also induces intracellular Ca^{2+} mobilisation from the endoplasmic reticulum Ca^{2+} stores (Willoughby et al. 2001). Although carbenoxolone is also a mineralocorticoid agonist, Ross et al. (2000) suggested that such receptors are not involved in the induction or maintenance of 0 Mg^{2+}/4-AP-induced spontaneous activity. In addition, we compared the effects of carbenoxolone with those of octanol, another widely used GJ blocker. Since no significant differences were detected between the anticonvulsive effects of carbenoxolone and of octanol, these observations confirm our conclusion that the reduction in seizure activity after the application of carbenoxolone is probably due to the blockade of GJ communication. The significant decreases in the seizure activity at the already active foci during by blocking gap junctions with carbenoxolone treatment could be a consequence of the reduction of the synchronization of the neurons contributing to the generation of seizure discharges.

In vitro studies have demonstrated that the gap junctional conductance can be dynamically modulated by alteration of the intracellular pH and Ca^{2+} level (Chesler et al., 1992; Perez-Velazquez et al., 1994; Jefferys 1995; Curtis et al., 1998; Rouach et al., 2000; Schmitz et al., 2001.). Indeed, during individual seizures a gradual intracellular acidification (Perez Velazquez et al., 2000; Xiong et al., 2000; Jahromi et al., 2001; Köhling et al., 2001) and a decrease in the intracellular Ca^{2+} level (Perez-Velazquez et al., 1994; Jefferys 1995) have been reported under *in vitro* circumstances. In the course of earlier intracellular study on the 4-AP *in vivo* epilepsy model, in some cells the transient appearance of spikelets, followed by their disappearance, in parallel with the initiation and termination of a seizure was detected, giving the impression that they effectively contribute to and amplify synchronization between cells. As an explanation for this observation, it might be more relevant to consider short-term, transient functional changes in GJal communication between ictal and interictal periods induced by seizure activity than to consider their absolute numbers. Our observations with the GJ opener TMA study have

provided direct evidence that experimental manipulations of the functional state of gap junctions under *in vivo* conditions significantly modify the expression, the duration and the propagation of seizures.

The investigations carried out in the frame of this thesis on the Mf, appear to provide support for the idea that long-lasting plastic functional and structural changes can occur locally not only in the Pf, but also in the homotopic area contralateral to the Pf contributing to the development of an independent secondary epileptic focus (Szente and Boda, 1994).

We are aware of that conclusive evidence of a link between the observed increases in connexin mRNA levels and the proposed GJ-mediated effects can be obtained only by specifically preventing the transcription of these Cx genes and demonstrating that this has a significant effect on the epileptiform activity. However, this was beyond the scope of the present study.

The possible role of GJs in seizures has been proposed already by several studies on *in vitro* seizure models (McVicar and Dudek, 1981; Perez Velaquez and Carlen, 2000; Perez Velaquez et al., 1994; Jensen and Yaari, 1997; De Curtis et al., 1998; Draguhn et al., 1998; Dudek et al., 1999; Traub et al., 1999; Carlen et al., 2000; Perez Velazquez and Carlen, 2000; Traub and Bibbig, 2000; Traub et al., 2001a,b;), and from human epilepsies, where the levels of connexin mRNAs were elevated in tissues from epileptic patients (Naus et al., 1991). Electrophysiological and anatomical data revealed that the GJ coupling was more pronounced and the expressions of Cx32 and Cx43 were increased in cells isolated from epileptic tissue relative to those from normal tissue (Naus et al., 1991). The upregulation of Cx32 was also detected in the isolated mouse hippocampus with epileptiform activity (Li et al., 2001). On the other hand, significant changes in Cx43 mRNA level were not found in hippocampal tissues either resected from patients with partial seizure disorder (Elisevich et al., 1997) or from kainate-treated and kindled rats (Shol et al., 2000). mRNA expression for Cx36 was not affected in mouse hippocampus exposed to bicuculline (Li et al., 2001). However, another study of hippocampus in kindled and kainate-treated rats (Shol et al., 2000) reported a decrease in Cx36 mRNA and protein expression. The differences between our own and previous findings might be explained by the differences in the experimental set-ups. Firstly, we measured the Cxs expression 20 min after the last seizure, while the earlier workers studied the expression after a recovery period of 2-4 weeks, during which time the increased expression might have returned to the basal level. Secondly, the experimental models of epilepsy differed in the various

studies, and it is conceivable that the Cxs expression differs in epileptic models induced in different ways. Although Cx43 expression is best documented in astrocytes (Yamamoto et al., 1990; Dermietzel, 1996; Rush, et al., 2001), it has also been described in adult neocortical pyramidal cells (Simbürger et al., 1997). It is possible that the increased electrical activity due to seizures in our epilepsy model upregulates the expression of Cx43 either in neurons or in astrocytes, and the expression of Cx32 in neurons or oligodendrocytes or in both. The increase in the mRNA level of Cx32 after the induction of epileptiform activity may be an indication either of a functional involvement of Cx32 in synchronization between neurons (Li et al., 2001), or of an increased GJal coupling in olgodendrocytes/astrocytes (Rash et al., 2001) as a result of increased axon firing due to synchronized neuronal firing during epileptiform activity (Li et al., 2001). The upregulation of Cx36 mRNA after repeated seizures could be an indication of the increased GJal coupling between neurons in the epileptic foci. Recent publications have explored the functional electrical coupling between neurons and glia that can effectively modulate neuronal excitability (Emmi et al., 2000; Noctor et al., 2001; Alvarez-Maubecin et al., 2002; Amzica et al., 2002). The present study does not pinpoint the exact locations of the expressions of the connexins, but either of the above variations may be of importance.

5.2 Selective contribution of neuronal gap junctions via Cx36 to epileptogenesis and ictogenesis in adult animals

This *in vivo* study has revealed that the application of quinine a selective pharmacological blocker of Cx36 in the physiological state of the brain did not noticeably influence the basic electrocortical activity and did not prevent the induction of seizures. *Pretreatment* with quinine slightly reduced the epileptogenesis and the expression of seizure discharges. In contrast, more obvious changes in ictogenesis were detected when quinine was applied after 25-30 spontaneously occurring seizures. These observations suggest that the neurons that are involved in the mechanisms of epileptogenesis are probably rarely interconnected by GJ channels, or the function of these channels may be less effective under basic, physiological circumstances. On the other hand, plastic modifications in Cx36 expression during an acute episode of repeated seizures might underlie the pathophysiological mechanism involved in ictogenesis and the propagation of seizures. These assumptions are supported by our earlier observations of a relatively low

level of expression of Cx36 mRNAs in the neocortex of control animals, which displayed a significant increase following 25-30 spontaneously occurring seizures.

The main findings of the *treatment* experiments are as follows: (i) a new discharge pattern (A0) appeared at the initiation of seizures when quinine was applied to block Cx36 channels; (ii) although the number of seizures increased significantly when Cx36 channels were uncoupled, because of the significant reduction in the durations of the individual seizures, the overall effect of the blockade of Cx36 channels is anticonvulsive; (iii) the amplitudes of the seizure discharges of all the patterns decreased, with the exception of those with frequencies of 11-12 Hz; and (iv) the selective blockade of Cx36 and the global blockade of the GJs resulted in qualitatively different modifications in ictogenesis.

The uncoupling of Cx36 channels provoked the appearance of a new discharge pattern (A0) at the initiation of seizures with frequencies above 15 Hz and relatively low amplitudes (Fig. 18/*2*). This pattern was not observed in the absence of quinine or carbenoxolone. Although the A0 pattern was manifested both in the presence of quinine and in that of carbenoxolone, since quinine selectively blocks Cx36, we presume that the generation of this discharge pattern is related mostly to Cx36. The assumption that GJ communication via Cx36 is attributable to the generation of A0 discharges is supported by the observation that the occurrence of this pattern was restricted to the Pf treated with quinine or carbenoxolone, and it was not detected at the Mf, which was out of reach of the blockers. The manifestation of A0 and the small amplitudes of the discharges suggest synchronization induced by GJ blockade between nearby cells in a small interconnected network restricted to a micro domain in the cortex. Such small neuronal networks may play a key role in initiating seizures and are probably under control by interneuronal communication when Cx36 channels are not blocked. This could be the reason why we observed a higher number of individual seizures when either quinine or carbenoxolone was used in order to block GJ channels (Fig. 17*B* and Table III). It has recently been reported that inspiratory motoneurons may display more robustly synchronized activity in the presence of carbenoxolone than under control circumstances (Bou-Flores and Berger, 2001). This observation also suggests a desynchronizing role for electrical synapses on certain neuronal networks. A theoretical paper provides a possible explanation for the surprising experimental observation that the blockade of electrical synapses may increase the synchrony of neuronal activity (Pfeuty et al., 2003).

The number of seizures was the highest when carbenoxolone was applied alone. However, when carbenoxolone was applied following quinine, the number of seizures fell

back almost to the control value (Fig. 17*B* and Table III). A possible explanation for this could be that the organization and co-operation inside or between neuronal networks could be altered in a plastic way, depending on whether only Cx36 channels is blocked, whether both neuronal and glial Cxs are blocked in parallel, or whether the glial GJ communication is blocked following an earlier, separate blockade of neuronal Cx36 channels. Unfortunately, no data are available so far to show whether quinine and carbenoxolone inhibit the Cx36 channels in the same way or with the same intensity. Accordingly, we can not exclude the possibility that, because of the different blocking efficacies of the two drugs on Cx36 channels, they provoke different changes in seizure activity.

Interestingly, application of quinine decreased the amplitudes of the discharges at all frequencies in the interval 1-15 Hz, with the exception of 11-12 Hz, which changed in the opposite direction (Figs. 18, 19 and Table III). A possible explanation for this observation could be that, at the network level, the sites of origin of the seizure discharges of various frequencies are probably different. The reduction observed in the amplitudes of discharges in the frequency ranges 13-14 Hz and 1-10 Hz could indicate that the blockade of Cx36 channels decreases the numbers of cells firing synchronously that are involved in the generation of seizure discharges at these frequencies. On the other hand, the significantly increased amplitudes of the 11-12 Hz discharges could indicate that Cx36 coupling between certain particular neurons reduces the synchrony in the network involved in the generation of seizure discharges in this frequency range. The fact that carbenoxolone treatment following quinine further decreased the amplitudes of all patterns (Figs. 16, 18, 19 and Table III) could be the sign that the glial syncytium might also control the numbers of cells which are activated synchronously during seizure discharges.

Cx36, the major neuronal Cx, is present predominantly in GABAergic interneurons (Belluardo et al., 2000; Deans et al., 2001). In mice lacking Cx36, the gamma rhythms are desynchronized both in the cortex and in the hippocampus (Deans et al., 2001; Hormuzdi et al., 2001). Furthermore, a synchronizing role for electrical coupling in the spindle frequency (around 10 Hz) has been described in the cat reticular neurons *in vivo* (Fuentealba et al., 2004), and it has been suggested that, due to the low-pass properties of GJs (Landisman et al., 2002), they can transmit spikes that can activate neighbouring cells and thus contribute to the propagation and synchronization at low frequencies (Landisman et al., 2002). In this *in vivo* study, we have observed modifications of the neocortical seizure discharges at lower frequencies around the beta and theta ranges. It has been demonstrated that pharmacological blockade of the GJ channels shortened the duration of

seizures at an already active epileptic focus, while their opening lengthened it. These findings are supported by the observation in the present study that the uncoupling of Cx36 channels significantly shortened the duration of seizures (Fig. 17*A* and Table III). This may suggest that the closed state of Cx36 channels reduces the effective level of hyperactivity in the seizure-prone tissue and contributes to the control of the seizure duration.

Nevertheless, it must be remembered that, in spite of the clear and characteristic effects of separate blockade of the different Cxs on the seizure activity, these results do not provide an exact insight into the functional role of GJ communication (i.e. metabolic and/or electrical coupling) in the generation and modulation of the rhythm and synchronization of the output of the neuronal networks.

Our results also revealed noticeable effects of Cx36 blocking on the activity at the Mf and on the propagation of seizure discharges to other parts of the cortex situated out of reach of the blockers (Figs. 16*B* and 18*/2*). In connection with this finding, we refer to a computational modelling study suggesting that GJ channels can mediate the spread of activity not only between electrically connected neurons but also to a great distance (Landisman et al., 2002). The depressed seizure activity at the Mf during quinine treatment could indicate that the GJ communication, including that through Cx36 channels, effectively contributes to the propagation of seizure discharges to the contralateral hemisphere. The depressed propagation of seizure discharges to the ipsilateral cortical areas may be explained by the possible involvement of electrical communication between neurons in the horizontal propagation of ictal discharges. GJ blockers have been shown to abolish horizontal wave propagation and also the epileptiform activity induced by picrotoxin (Peinado, 2001).

The selective or global blockade of both neuronal and glial GJ channels results to some extent in different modifications in ictogenesis. In earlier studies, significant increases were detected in the levels of expression of the major astroglial Cx43 and oligodendroglial Cx32 mRNAs at the already active epileptic foci. The spontaneous activity in the astrocytes and neurons is patterned into correlated neuronal/astrocytic networks (Spray et al., 1999). Dual intracellular recordings from neuron-glia pairs in the cat neocortex *in vivo* suggest that cortical network oscillations of the slow sleep or paroxysmal type could result from complex glial-neuronal interactions (Amzica and Massimini, 2002). In addition, it has been shown recently that astrocytic glutamate release induced epileptiform discharges in hippocampal CA1 pyramidal neurons (Kang et al., 2005). These observations might indicate that the glial GJ communication efficiently

modifies the neuronal network activity and participates in the generation of the rhythmic and synchronous activity of the neocortex.

The fact that the modifications in seizure activity differ in the presence of quinine and when quinine and carbenoxolone are present together raises an unresolved issue: are the differences attributable only to the contribution of the glial GJ communication? Recent evidence indicates that GJ-blocking agents such as carbenoxolone and octanol also inhibit hemichannels (Contreras et al., 2002). It is tempted to speculate that at an epileptic focus the activation of Cx43 hemichannels can compromise ionic homeostasis, and contribute to maintenance of epileptic activity. Moreover, the pannexins are present in both interneurons and pyramidal cells in the rodent brain (Connors and Long, 2004). Although no data are available as yet, they may serve as putative targets for GJ blockers. Thus, the possibility can not be excluded that the modifications induced by broad-spectrum GJ blockers could be mediated in part by the inhibition of Cx43 hemichannels and/or pannexins.

5.3 The functional significance of gap junction channels in the epileptogenicity and seizure susceptibility of the immature mammalian brain

With regard to recent findings that implicate a role for GJ channels in the synchronization of neuronal activity, in the present study we have focused on possible roles of GJ channels in the epileptogenicity and seizure susceptibility of the immature mammalian brain. We carried out electrophysiological experiments, combined with pharmacological manipulations of the GJ channels, and measured developmental and 4-AP-induced synchronous activity-dependent plastic changes in the Cx26, 32, 36 and C43 mRNA levels in the rat neocortex in different postnatal developmental stages. The epileptic cortical activity induced by 4-AP is accompanied by specific quantitative alterations in the different Cx mRNA levels and suggests a cause-effect relationship between the electrical communication pathway and epileptiform synchronization. Our RT-PCR results revealed a highly diverse basic expression pattern for the Cx26, 32, 36 and 43 genes, and showed quantitative alterations in their expression during the epileptogenic process in an age-related manner. Each of the Cxs examined displayed a unique basic pattern of postnatal development with a common feature: there was a marked alteration in their expression levels at around P16. Expressions were significantly upregulated at around this age for all the followed genes except Cx26. The Cx26 mRNA level was characterized by a marked decrease after the first two weeks.

During the postnatal development, the epileptiform activity induced strong, subtype-specific changes in the levels of expression of all the examined Cx genes: the transcript level of Cx43 was gradually elevated up to P23, the mRNA level of Cx36 showed a transient increase with a marked peak expression around P16, while the mRNA levels of Cx32 and 26 demonstrate only a very modest age-dependent changes after repeated seizures. The inducibility of the Cx26, 36 and 43 genes was highest at around P16 and had significantly declined by P23. Up-regulation of Cx26 and Cx45 mRNA was detected in neuronal cells undergoing apoptotic cell death in vulnerable regions such as hippocampus, amygdala and some thalamic nuclei, whereas Cx36 was down-regulated after kainate-induced *in vivo* model of status epilepticus (Condorelli et al,. 2003). In our 4-Ap-induced seizure model, characterized by isolated, repeating seizures, although some kind of neuronal and astroglial damage was detected (Mihály et al., 1985) approximately at the time point of the sample collection for mRNA analysis, apoptotic cell death is not characteristic in the neocortex.

Pharmacological manipulation of the GJ channels differentially affects the basic ECoG, the induction and maintenance of seizures in different postnatal developmental stages. In the basic ECoG we found a characteristic inherent sinusoid-like, generalized pattern, restricted until P12. The ability of the cortex to express sustained wave activity around these postnatal days has already been reported (Peinado, 2001). The widespread, synchronous rhythmic activity in the basic ECoG was markedly enhanced by 4-AP (Fig. 28) and strongly abolished by carbenoxolone (Fig. 30). GJ blockers were shown earlier to abolish horizontal wave propagation and to eliminate epileptiform waves completely in young animals (Peinado, 2001). In contrast, the GJ opener TMA alone induced considerable rhythmic, highly synchronous seizure-like activity in the P9-13 animals, which was apparently not modified by 4-AP (Figs. 32, 33). The failure of 4-AP to modify the TMA-induced cortical activity suggests that the targets of the two drugs may overlap each other, and supports GJ involvement in the widespread, synchronous rhythmic activity at this age. 4-AP in P9-12 animals induced a rhythmic activity, characterized by the generalized occurrence of sustained seizure-like, relatively slow potentials, with variable amplitudes and low frequencies. On maturation, the seizure discharges became faster, with increasing amplitude and frequency.

The onset of spontaneous, and the 4-AP- and/or TMA-induced generalized synchronous activity could be correlated in part with the extensive cell coupling by the GJ channels (Bittman et al., 2002; Peinado et al., 1993), overwhelming the poorly developed

chemical synaptic systems (Swann and Hablitz, 2000) in the first two weeks in the rat neocortex. Under physiological conditions, we measured relatively high levels of Cx26 and 43 mRNA at P10-14, and the presence of Cx36 mRNA was also detected, although at a lower level (Fig. 36). In time, the level of Cx26 mRNA somewhat declined, while the Cx43 expression progressively increased up to P23, and the Cx36 transcript level increased transiently, with peak expression at around P16 (Fig. 36).

The coupling of combinations of pyramidal and non-pyramidal cells and between neurons and glia, mediated by Cx26, 36, and 43, has been reported at this early age in rats (Nadarajah et al., 1997; Venance et al., 2000; Bittman et al., 2002). Besides the dominating homotypic coupling, a heterotypic form of coupling is also involved in connecting pyramidal and non-pyramidal neurons, or neurons and astrocytes at P7 and P14 (Bittman et al., 2002). In networks where large numbers of neurons transmit electrical signals directly through GJ channels, the temporal heterogeneity of the discharges decreases, the synchrony thereby being enhanced (White et al., 1998).

The generalized synchronized, rhythmic pattern of both the spontaneous and the epileptiform activity, and their sensitivity to pharmacological manipulation of the GJ channels on P9-12, may indicate that GJ channels in the immature cortex link neurons and glia cells into extensive networks that may allow electrical activity to spread over long distances. The horizontal propagation of waves has been correlated with the presence of dendrodendritic GJ channels during the first 10–12 postnatal days in rats (Peinado et al., 1993, 2001; Rorig et al., 1996). Furthermore, GJ channels are also reported to be capable of producing large functional clusters of coupled neurons in vertical columns, serving to synchronize the activity of several cortical layers (Peinado et al., 1993; Connors and Long, 2004).

The complex role of GJ channels at this early age could additionally be related to the relatively low number of chemical synapses (Swann and Hablitz, 2000). Neurons with intrinsic bursting properties that are frequently presumed to be involved in synchronous, rhythmic activity are not present in the sensorimotor cortex of rats in the first two weeks (Hoffman and Prince, 1995).

In the first two weeks after birth, pyramidal cells have been shown to use predominantly Cx26, while the non-pyramidal cells may equally use both Cx26 and 43 for the formation of GJ channels (Bittman et al., 2002). Cx26 has been reported both in the astrocytes and in the neurons of the developing brain and spinal cord (Alvarez-Maubecin et al., 2000; Nagy et al., 2001), with the highest expression prenatally and during the first

three weeks of postnatal life (Peinado et al., 1993¸ Nadarajah et al., 1997; Bittman et al., 2002). In addition more recently Cx45 gene expression has also been reported in neurons with genetic approach during embryogenesis with a peak of expression at P1 and declined subsequently during brain development (Condorelli et al., 2003; Sohl et al., 2005). However, Cx45 protein is widely expressed in many developing and mature non-neural cell types beside selected subsets of neurons. On the other hand Cx36 may be of particular interest, as it is expressed exclusively in neurons, and is present both in the young neurons and also in adult cortex (Sohl et al., 2005). Nevertheless it is possible, that Cx 45 might play certain role in some of the epilepileptiform events of the young animals described in this paper, either participating in neuronal-neuronal or in neuronal-glia communication mediated by GJs, what should be the subject of further analysis.

The mRNA level of Cx36 underwent a transient increase, with a marked peak expression at around P16 in our study. The level of developmental change and its time course for Cx36 exhibits region-specificity (Belluardo et al., 2000). In the cerebral cortex, a progressive increase was observed, with peaks between 7 and 16 days (Prime et al., 2000; Srinivas et al, 1999; Belluardo et al., 2000; Condorelli et al., 2000; Rash et al, 2001; Hormuzdi et al., 2004). In the early stages of postnatal development, Cx36 is detectable even in neuronal populations that are devoid of Cx36 mRNA in the adult stage (Belluardo et al., 2000). Up-regulation of Cx36 has been linked to juvenile myoclonic epilepsy (Mas et al., 2004).

The astrocytic Cx43 is present in the cortex throughout the period of development. (Giaume and Vennance, 1995; Dermietzel et al., 1989; Nagy et al., 2000, 2001; Condorelli et al., 2000). Astrocytes in adults compose an astrocytic syncytium which gives physical and metabolic support to the neurons (Walz and Hertz, 1983; Jefferys, 1995). In the first two postnatal weeks, astrocytes are more likely to couple to neurons than to other astrocytes (Bittman et al., 2000) through GJ channels, and they may promote the synchrony of spontaneously active neural networks (Alvarez-Maubecin et al., 2000).

Recent findings have lent support to the concept that the Cxs, although redundant, may play a significant role in unstable, transient cell-cell contacts (Segretain and Falk, 2004). With regard to the relatively high level and unspecific occurrence of the Cxs, it seems justified to presume that the extensive communication through the GJ channels in the first two weeks may play a key role in the enhanced seizure susceptibility, and mediate not only the induction, but also the propagation of rhythmic synchronous activity. Consequently, the decline of this feature later could be explained by the decreased

frequency of GJal coupling between the neurons as the cells mature, and in parallel by the formation of more specific neuronal networks involving excitatory and inhibitory chemical synapses (Peinado et al., 1993; Swann and Hablitz, 2000, Bittman et al, 2002).

The spontaneous rhythmic pattern that was described at P9-12 was not manifested in the basic ECoG (Fig 32), and it was not induced by TMA pretreatment in the animals after (Fig. 32). In addition, we observed a transient reduction in the epileptogenicity of the animals during a brief developmental window at around P13-14 (Fig. 28). In fact, the sudden disappearance of the rhythmic features of the basic electrocortical activity and the inability of carbenoxolone and TMA to influence the basic cortical activity (Figs. 30, 32) suggest a weaker GJ involvement in this developmental stage. We detected a decreased expression of Cx26 by P15-23 (Fig. 36), while the level of expression of Cx43 gradually increased. The expressions of these Cxs probably became restricted to specific cell types after P16, contributing to more specific neuronal networks. These observations confirm the findings of earlier experiments, where the glutamate-independent coordinated activity decreased and a neurobiotin tracer indicated a low incidence of neuronal coupling at around P15 (Peinado, 2001, Bittman et al., 2002). Although the basal level of Cx43 mRNA was increased in the P17 and P23 animals, it was markedly elevated by the epileptiform activity (Fig. 36). This increment of Cx43 mRNA could be an indication of a more specific astrocytic activity and adjustability to the elevated neuronal firing activity. Although the oligodendrocyte-specific Cx32 mRNA (Belliveau and Naus, 1995) was not detected at P10, it was dramatically induced by the convulsant 4-AP at this age (Fig. 36, P10). At later ages (P14-23), the basic level became detectable and gradually increased with age. As a consequence, the relative inducibility of this gene progressively decreased (Fig. 36).

The lack of manifestation of the fastest seizure discharges in animals before P13-14 could be related to the relative scarcity of chemical synaptic connections and the slow nature of synaptic potentials at this age (Swann and Hablitz, 2000). Accordingly, the increase in the frequency and the appearance of a faster discharge in older animals could be explained in terms of the observation that the number of excitatory synapses significantly increased during the second postnatal week and these synapses are able to follow activation faithfully at high frequencies (Swann and Hablitz, 2000). In addition, the actual number and ratio of the open versus closed GJ channels can modulate the frequency of the discharges. The application of the GJ opener TMA at already active epileptic focus at most developmental points enhanced generalized synchronization and increased the duration of ictal events, resulting in the cortical activity pattern that is characteristic of

young animals with abundant GJal communication (Figs. 33, 34 and 35). These observations confirm our earlier findings in adult animals showing, that epileptiform activity can up-regulate the expression of Cxs 26, 32, 36 and 43 mRNAs, indicated also by the strong efficacy of pharmacological manipulation of GJal communication by carbenoxolone and TMA. However, the inducibility of the Cx mRNAs examined in the present paper revealed some subtype specificity at the different developmental time points.

One possible explanation of the transiently elevated epileptogenicity of young animals at around P16-17 (Fig. 28) could be the appearance of the first intrinsic bursting neurons during the third postnatal week (Franceschetti et al., 1993; Swann and Hablitz, 2000) and/or the transient hyperactivity of the N-methyl-D-aspartate-mediated neurotransmission and/or the immature stage of GABAergic inhibition (Ben-Ari et al., 2004). Since the percentage of neurons with intrinsic bursting capacity is high in early postnatal life (Wellmer et al, 2002; Yaari, 2005), these cells can act as cellular pacemakers coupled by GJ channels (Ben-Ari et al., 2004).

In addition, although the basic level of neuron-specific Cx26 mRNA gradually decreased with age, the other neuron-specific Cx36 expression was significantly increased and approached the highest level at around P16 (Fig. 36). The elevated level of GJal communication in scattered subpopulations of cells express Cx36 mRNA can substantially contribute to the elevated seizure activity observed at this age. A recent *in vitro* study based on abrupt maturation at the end of the second postnatal week of synchronous activity among electrically coupled low-threshold spiking inhibitory interneurons revealed, that such activity was absent on earlier postnatal days (Long et al., 2005). The strong synchronizing ability of this inhibitory cell network may contribute to the increased seizure susceptibility of rats after the second postnatal week.

All measured Cx mRNA levels exhibited an obvious increase after 60 min periods of seizure activity, indicating that, besides the increased efficacy of excitatory chemical neurotransmission, electrical coupling through the GJ channels may also contribute to the transiently elevated level of epileptogenicity of P15-16 animals. After this age, the progressive decline in seizure susceptibility could be an indication of the fine-tuning of local synaptic connectivity, and the pruning back of some excess of the chemical and electrical synapses, in parallel with the development of the fully active inhibitory $GABA_A$ prune-back receptor system (Swann and Hablitz, 2000; Ben-Ari, 2004*)*. The incidence of coupling between excitatory and inhibitory cells declines with age (Venance et al., 2000; Naus and Bani-Yaghoub, 1998; Meyer et al., 2002) and in the adult most electrical

coupling exists between homogeneous cell types, a condition that may diminish the susceptibility of the neural networks to synchronization (Connors and Lomg, 2004).

The findings of the present study suggest that GJal communication is not only involved in rhythm genesis and the synchronization of cortical activity, but may be responsible part for the elevated epileptogenicity of the developing brain. In addition, repeated seizures can induce changes in expression of the different Cx genes and the remodelling of GJal communication. If these plastic modifications after early epileptiform disorders are persistent, they may facilitate epileptogenesis and ictogenesis at a later developmental point without any discrete morphological alterations.

Further studies are needed in order to analyze in detail the balance between excitatory and inhibitory functions, and the establishment of chemical and electrical synaptic connections in the network and cellular levels.

6. Summary

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: (i) the functional involvement of neuronal and/or glial GJal communication in epileptogenesis and manifestation of epileptiform activity of adult neocortex; (ii) the selective contribution of neuronal GJs via Cx36 to epileptogenesis, ictogenesis and propagation of seizures in adults; (iii) the functional significance of GJs in the epileptogenicity and seizure susceptibility of the

immature mammalian brain; (iv) the basal expression levels and the plastic changes induced by epileptiform activity in the mRNA levels for Cx26, 32, 36 and 43 in the developing and for Cx32, 36 and 43 in the adult rat neocortex.

For this purposes, *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. In adult animals carbenoxolone and octanol, as broad-spectrum GJ blockers; quinine, a selective blocker of Cx36; and TMA, a GJ opener were applied locally, prior to the induction (*pretreatment*) or at already active epileptic foci (*treatment*). In developing animals, GJs were manipulated with carbenoxolone or TMA before the induction or at the active epileptic foci between postnatal days 9 and 28. 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.

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.

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, Cx26, Cx43 and Cx36 mRNAs increased significantly in the active epileptic foci.

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. 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.

Our observations indicate that in physiological circumstances the GJs have weak involvement in epileptogenesis and seizure susceptibility in the adult neocortex. On the other hand, plastic modifications in the Cx32, 36, 26 and 43 expressions 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 Our findings also 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. We also suggest that both neuronal and glial GJ communication contribute to the manifestation and propagation of seizures in the adult rat neocortex, although with different ways and degrees.

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. 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. 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

7. Összefoglalás

Az epilepszia spontán visszatérő rohamokkal jellemzett agyi működészavar, melynek hátterében az agyi neuronok excesszív, hiperszinkron kisülése áll. Az utóbbi évtizedekben egyre inkább előtérbe került az a hipotézis, mely szerint a sejtek közötti gap junction (GJ) kapcsolatok (elektromos szinapszisok) fontos szerepet játszhatnak az epileptiform tevékenység hátterében álló fokozott neurális szinkronizáció kialakulásában. A feltevés helyességét ma már számos elektrofiziológiai és anatómiai adat támasztja alá. Az utóbbi időkben *in vitro* epilepszia modellekben kimutatták, hogy a GJ-ok funkcionális állapotának megváltoztatása hatással van a görcstevékenység különböző paramétereire, azonban hiányoztak a bizonyíték a GJ-ok epilepsziás működészavarban betöltött szerepéről *in vivo* körülmények között.

A klinikai tapasztalatok és kísérletes eredmények azt mutatják, hogy a fejlődő idegrendszer fokozottabban érzékeny a görcsindukáló behatásokra, mint a felnőtt idegrendszer. A jelenség hátterében álló neurológiai tényezők összességében még nem teljesen ismertek, mégis van néhány jellegzetes neurobiológiai tulajdonság, amely magyarázatul szolgálhat. Számos közlemény számol be arról, hogy a fejlődő idegrendszerben lényegesen nagyobb számban vannak jelen GJ-ok és, hogy a GJ-kon keresztül megvalósuló intercelluláris kommunikáció elterjedtebb formája a sejtek közötti párbeszédnek az agyfejlődés korai szakaszában, mint a felnőtt idegrendszerben. Ezért adódik a feltételezés, hogy összefüggés lehet a fejlődő idegrendszer fokozott görcskészsége és a GJ-okon keresztül megvalósuló kommunikáció magas szintje között. Azonban ez a terület még számos nyitott és megválaszolásra váró kérdést vet fel.

Napjainkban egyre inkább egyértelművé válik, hogy a glia sejtek nélkülözhetetlen részei az agy működő hálózatainak. A neuronok és asztrocyták sokrétű kapcsolatában, pl. a neuronok szabályozzák az asztrocyták hálózati tulajdonságait, ugyanakkor az asztrocyták glutamátot és egyéb neuroaktív anyagokat bocsátanak ki, amelyekkel befolyásolják a szomszédos neuronok ingerelhetőségét. Bár számos adat támasztja alá a GJ-okon megvalósuló interneuronális kommunikáció fontosságát a szinkronizációs mechanizmusokban, a glia sejtek közötti GJ-ok szerepét az epileptiform tevékenység indukciójában, fenntartásában és terjedésében eddig még nem tanulmányozták.

A Szegedi Tudományegyetem Összehasonlító Élettani Tanszéke Epilepszia laboratóriumában több évtizede folyik epilepszia alapkutatás a 4-aminopyridine (4-AP) *in*

vivo epilepszia modellen. Így kínálkozik a lehetőség, hogy a fent említett izgalmas témákat megvizsgáljuk ezen a modellen. Kísérleti munkánk során a következő kérdésekre próbáltunk választ kapni: (i) az ideg- és/vagy glia sejtek közötti GJ kommunikáció részt vesz-e az epileptogenezisben és az epileptiorm tevékenység manifesztációjában a felnőtt neocortexben; (ii) a neuron specifikus Cx36 GJ kommunikáció milyen mértékben járul hozzá az epileptogenezis és ictogenezis folyamataihoz, valamint a görcspotenciálok terjedéséhez a felnőtt állatokban; (iii) milyen funkcionális jelentőséggel bírnak a sejtek közötti GJ kapcsolatok a fejlődő idegrendszer fokozott görcskészségében és epileptogenitásában; (iv) hogyan változnak a Cx26 (fiatalkori, neuron specifikus), 32 (oligodendrocyta specifikus), 36 (felnőttkori, neuron specifikus), és 43 (astrocyta specifikus) mRNS-ek alap expressziós szintjei a fejlődés során, valamint az ismételt rohamtevékenység okoz-e változást ezen mRNS szintekben a fejődő és felnőtt agykéregben.

A feltett kérdések megválaszolása érdekében, elektrofiziológiai és farmakológiai kísérleteket végeztünk altatott patkányokon *in vivo* körülmények között, az iktális epileptiform tevékenységet az agykéreg felszínére helyezett 4-AP-el indukáltuk. A GJ csatornák funkcionális állapotát különböző, lokálisan alkalmazott farmakonok segítségével manipuláltuk a görcstevékenység kialakítása előtt (*előkezelés*) illetve a már aktív epilepsziás fókuszokban (*közben-kezelés*). Carbenoxolont és octanolt használtunk a GJ-k széles spektrumú blokkolására. Kinint alkalmaztunk a neuronális Cx36 fehérje szelektív blokkolására, illetve TMA-t használtunk a GJ-k nyitott állapotban tartására felnőtt állatok esetében. A fejlődő (9-28 napos) állatoknál carbenoxolont és TMA-t alkalmaztunk a GJ-k blokkolására és nyitására. A különböző Cx mRNS expressziós szinteket a kontroll és az epilepsziás kéregben szemikvantitatív RT-PCR technikával követtük nyomon.

Eredményeink azt mutatják, hogy a GJ-ok funkcionális állapotának módosítása a felnőtt agyban, kontroll, fiziológiás körülmények között (*előkezelés*) látszólag nem változtatta meg az alap ECoG-t, és nem befolyásolta jelentősen a görcspotenciálok indukcióját és manifesztálódását. Eredményeink arra utalnak, hogy fiziológiás körülmények között a GJ kommunikáció valószínűleg csak kismértékben befolyásolja a felnőtt neocortex görcskészségét és az epileptogenezis mechanizmusait.

Ezzel szemben, a GJ-ok blokkolása az aktív epilepsziás fókuszban (*közben-kezelés*) markáns antikonvulzív, míg nyitásuk erőteljes prokonvulzív hatással volt az epileptiform tevékenységre. Carbenoxolon illetve octanol közbenkezelés jelentősen csökkentette a rohamok hosszát, a görcspotenciálok amplitúdóját és az epileptiform tevékenység

terjedését más kérgi területekre. Nyitott állapotban tartásuk TMA-al ugyanakkor, a GJ-ok blokkolásával ellentétes hatást fejtett ki, és szignifikánsan megnövelte az előbb említett paraméterek értékeit. Az aktív epilepsziás fókuszokban, az előzőeken kívül, szignifikáns növekedést figyeltünk meg a Cx32, Cx26, Cx36 és Cx43 mRNS-ek expressziójában. A neuron specifikus Cx36 szelektív blokkolása kininnel 25-30 ismételt rohamtevékenység után, egy új görcspotenciál mintázat megjelenését eredményezte a rohamok kezdetén, jellemzően 15Hz feletti frekvenciával, amely együtt járt a rohamok számának növekedésével is. Annak ellenére azonban, hogy a rohamok száma megnőtt, a kinin közbenkezelés antikonvulzív hatást fejtett ki a rohamok hosszának jelentős rövidülése következtében, amely az össziktális tevékenység szignifikáns csökkenését eredményezte. A görcspotenciálok amplitúdói többnyire csökkentek, a 11-12 Hz frekvenciájú görcspotenciálok kivételével, melyek amplitúdói nőttek. A globális connexin blokkolás (glia is) a neuronális Cx36 blokkolását követően tovább csökkentette az össziktális tevékenységet, a görcspotenciálok amplitúdóit és további rövidülést eredményezett a rohamok hosszának alakulásában.

Eredményeink arra utalnak, hogy fiziológiás körülmények között a GJ kommunikáció valószínűleg csak kismértékben befolyásolja a felnőtt neocortex görcskészségét és az epileptogenezis mechanizmusait. Más részről viszont, ismételt rohamtevékenység következtében felerősödik a GJ kommunikáció az idegsejtek és/vagy glia sejtek között fokozva a sejtek abnormális szinkron tevékenységét, amely hatékonyan hozzájárul a további görcstevékenység fenntartásához és terjedéséhez. Ezen kívül, a GJ-ok átjárhatósági állapotának dinamikus változása fontos szerepet játszik a rohamok hosszának szabályozásában.

A neuron specifikus Cx36 GJ-ok szelektív blokkolása az aktív epilepsziás fókuszban antikonvulzív hatást fejtett ki, és jellegzetesen befolyásolta a görcspotenciálok manifesztációját. Eredményeink arra utalnak, hogy az epileptiform tevékenységet indukáló és fenntartó mechanizmusok eltérő módon érintettek a GJ kommunikáció intenzitásának változásakor. Azt feltételezzük, hogy a Cx36 GJ kommunikáció kontroll alatt tartja azon neuronhálózat(ok) működését, amely(ek) involvált(ak) a rohamok indukálásában. Továbbá, mind a neuronok közötti, mind pedig a glia sejtek közötti GJ kommunikáció hozzájárul a felnőtt neocortex epileptiform tevékenységének manifesztációjához és terjedéséhez, azonban, valószínű eltérő mértékben és módon.

A fejlődő állatokban mind az alap ECoG mind a 4-AP-el indukált tevékenység jellegzetes változásokat mutatott a kor előrehaladtával: magasabb frekvenciájú és gyorsabb

komponensek jelentek meg az alap ECoG-s tevékenységben. A fiatal állatoknál jellemzően generalizált, és többnyire folyamatos görcspotenciálok egyre inkább az elsődleges- és a tükörfókusz helyére korlátozódtak, és időben iktális és interiktális szakaszokra tagolódtak. E folyamatokkal párhuzamosan gyorsabb és magasabb frekvenciájú görcspotenciálok jelentek meg, illetve a görcspotenciálok amplitúdói frekvencia szerint differenciálódtak. A fejlődő idegrendszer görcskészségét a korai fokozott epileptogén állapot után egy átmeneti csökkenés (P13-14) majd növekedés (P15-16) és végül ismételt csökkenés után (P23 körül) a felnőtt korra jellemző állapot jellemezte. A Cx36, 32, 26 és 43 mRNS expressziója a fiziológiás és epilepsziás kéregben Cx altípustól és életkortól függő változásokat mutatott. A Cx mRNS alap szint szignifikánsan megnövekedett P16 körül (a Cx26 kivételével). A Cx26, 36 és 43 gén indukálhatósága P16 körül volt a legmagasabb, ezután pedig szignifikánsan csökkent. A GJ-ok nyitása görcspotenciálokra emlékezető ritmikus, szinkron tevékenységet indukált a P9-12 napos korú állatokban. Ellentétben a felnőttekkel, a GJ-ok funkcionális állapotának módosítása hatékonyan befolyásolta az epileptogenezist is és az epileptiform tevékenység fenntartását is a fiatal állatokban. Elektrofiziológiai, farmakológiai és molekuláris biológiai eredményeink arra utalnak, hogy a GJ populáció jellegzetes mennyiségi és minőségi összetétele alapját képezheti a fejlődő idegrendszer fokozottabb görcskészségének és epileptogenitásának.

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10. List of publications

Papers

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I. **Gajda Z,** Szupera Z, Szente M. Involvement of electical synapses in the epileptiform activity induced in vivo by 4-aminopyridine. VIII. Annual Congress of the Hungarian Society for Neuroscience, 2001

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VI. **Gajda Z,** Gyengési E, Presztóczki B, Hermesz E, Said Ali, Szente M. Involvement of electrical synapses in the manifestation and duration of seizures. IBRO International Congress, Prague, 2003

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