Thesis of PhD Dissertation

Synaptic Reorganization as the Mechanism of the Development of Epilepsy – Electrophysiological and Photostimulation Measurements in a Rat Model of Epilepsy

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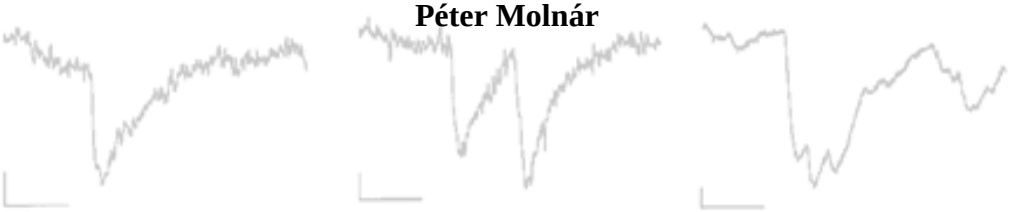

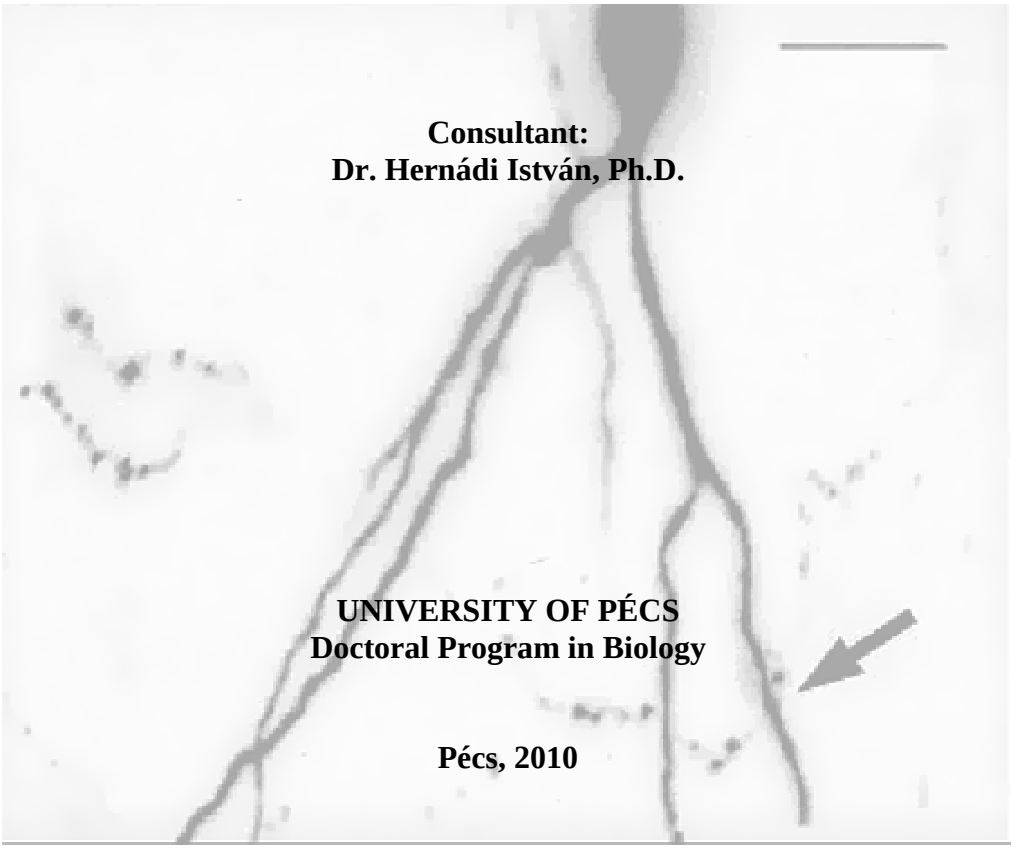

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Introduction

Temporal lobe epilepsy (TLE) is the most common epileptic syndrome in adults (Halász et al. 2006). In most of TLE patients epilepsy can be traced back to some form of initial brain damage, which, after a long latent period, leads to functional reorganization in the brain resulting in the emergence of spontaneous seizures (Sharma et al. 2007). Hippocampal lesion is present in about 70-80% of the cases, and it characterized by neuronal degeneration, astroglyosis, dispersion of granule cells and aberrant mossy fiber sprouting. Mossy fiber sprouting is the reorganization of the axons of the granule cells (these are the mossy fibers). During this process mossy fibers appear in the dendritic layer of the granule cells and form excitatory synaptic connections with granule cells (Sloviter 1994). It is widely accepted, that this positive feedback, created by mossy fiber sprouting, could play a role in the development of epileptic seizures.

The chronic epileptic state evoked by a single injection of pilocarpine in rats considered as one of the best model of human TLE, because it is reproducing not only the physiological and histopathological symptoms, but also the mechanism of the progression of the disease (Curia et al. 2008). Acute pilocarpine treatment causes status epilepticus (SE) in rats and the prolonged generalized seizure causes permanent damages in the brain resulting in the development of chronic epilepsy. The pattern of neurodegeneration throughout the brain in this model is very similar to that described in human TLE initiated by brain injury (Cavalheiro 1995). The mossy cells in the dentate gyrus almost completely disappear in this model, which increases the excitability of the dentate gyrus, because the mossy cells play an important role in the feed-back and feed-forward inhibition in this brain area (Lothman et al. 1992; Ratzliff et al. 2004). The main input of the mossy cells is the mossy fibers, but they might also receive some innervations from the perforant path (Amaral et al. 2007). The axons of mossy cells are forming excitatory synapses mostly with inhibitory interneurons.

Neurogenesis is drastically speeding up in the dentate gyrus after status epilepticus in the pilocarpine model (Parent et al. 2006). The newly born granule cells could play a major role in the development of seizure activity, because they are hyperexcitable and located deep in the hilus, thus they could receive aberrant synaptic connections from granule cells. According to other theories, they could increase inhibition by taking over the role of degenerated mossy cells in feed-back and feed-forward inhibition (Scharfman et al. 2007). According to other researchers the role of the newly born granule cells is minimal in TLE (Haas et al. 2009).

Mossy fiber sprouting occurs during the ‘quiet period’ after status epilepticus in the pilocarpine model (Tauck et al. 1985; Okazaki et al. 1995; Scharfman et al. 2003). Similar synaptic reorganization has already been described in other brain areas, but there is no simple and specific staining method exists for those pathways. It is not clear, what is the role of synaptic reorganization in epilepsy, because its effect could be either epileptic or anti-epileptic depending on the type of cells the pathways are innervating.

Based on its electrophysiological properties the dentate gyrus is considered as the ‘doorkeeper’ of the hippocampus (Lothman et al. 1992). *In vivo* and *in vitro* experiments showed that it is very difficult to evoke epileptic activity in the dentate gyrus, because of the strong feed-back and feed-forward inhibition and because of the lack of excitatory positive feedback (Ribak et al. 1991; Acsady et al. 1998). After status epilepticus there is

a drastic change in the dentate gyrus, the degeneration of mossy cells causes a significant change in the feed-back and feed-forward inhibition (Magloczky et al. 2005) and mossy fiber sprouting creates a positive feed-back which could explain the decreased resistance of the dentate gyrus against epileptic synchronized activity in the pilocarpine TLE model (Cronin et al. 1992; Hardison et al. 2000). There is experimental evidence that sprouted mossy fibers do form synapses on granule cells, but also on inhibitory interneurons (Ribak 1985; Sharma et al. 2007). Electrophysiological experiments also showed that the newly developed granule cell – granule synapses are functional and excitatory (Molnar et al. 1999; Scharfman et al. 2003).

Mossy fiber sprouting could contribute to the increased excitability of the dentate gyrus not only by the creation of the positive feedback but by the release of zinc, a compound mossy fibers contain at high concentrations. Zinc is activity dependently released from mossy fibers and could inhibit postsynaptic $GABA_A$ receptors. It was reported that zinc sensitivity of $GABA_A$ receptors drastically increased in epilepsy (Buhl et al. 1996). In contrast, zinc also has anti-epileptic effects; zinc released together with glutamate from synaptic terminals inhibits postsynaptic N-methyl-D-aspartate (NMDA) receptors (Vogt et al. 2000). The role of zinc in TLE is still not clear, because both epileptic and anti-epileptic effects of zinc have been reported (Timofeeva et al. 2003; Noyan et al. 2007; Foresti et al. 2008).

The exact cause of mossy fiber sprouting is not known (Curia et al. 2008). According to the leading theory, with the degeneration of the mossy cells the mossy fibers are losing their postsynaptic target cells and they are trying to find new targets. A contradictory observation is that mossy fiber sprouting could occur without mossy cell degeneration in the kindling model of TLE. Other theories explain mossy fiber sprouting with changed expression of growth factors and axon guidenance molecules which could cause enhancement of mossy fiber growth (Morimoto et al. 2004).

The role of mossy fiber sprouting in the development of temporal lobe epilepsy is still controversial (Morimoto et al. 2004). It was shown that if mossy fiber sprouting was prevented with cyclohexamid epileptic seizures still develop (Longo et al. 1998). Also, even in the epileptic hippocampus, where mossy fiber sprouting is significant, it is very difficult to evoke epileptic synchronized activity *in vitro*. Most possibly, mossy fiber sprouting is only one (although significant) mechanism among many which promote the development and spread of epileptic activity in temporal lobe epilepsy.

Aims

(1) Functional characterization of granule cell – granule cell synaptic connections formed during the development of spontaneous seizures in the pilocarpine model of temporal lobe epilepsy.

(2) Pharmacological characterization of granule cell – granule cell synaptic connections especially focusing on the NMDA component of the synaptic current.

(3) Characterization of the effects of zinc, which is synaptically released from the mossy fibers, on the postsynaptic $GABA_A$ and NMDA receptors in epileptic rats.

Methods

Status epilepticus evoked by pilocarpine injection

Male Sprague-Dawley rats (150–200 g) received a single injection of pilocarpine hydrochloride (330–360 mg/kg i.p.). The animals were pretreated 30 min. earlier with scopolamine methyl bromide (2 mg/kg i.p.) to block peripheral side effects and maintain respiration. If status epilepticus developed, it was terminated 3–4 h after onset with a single injection of phenobarbital sodium (50 mg/kg i.p.).

Histology

10 weeks, or in some experiments 4-6 days, after pilocarpine treatment transverse 400-µm-thick slices of the caudal hippocampal formation were cut with a vibratome. Slices set aside for histology were incubated in Na_2S solution for 90 min and then were fixed in phosphate buffered 0.9% (wt/vol) saline that contained 10% formalin. After storage in 10% formalin at 4°C for 1–2 days, the slices were embedded in albumin-gelatin and cut into 30-µm-thick sections. Alternate sections were stained for the presence of heavy metals. The remaining sections were stained with cresyl violet. A separate group of rats was used to determine the extent of neuronal degeneration. The animals were perfused transcardially with 4% (wt/vol) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After 3–20 days of postfixation at 4°C, the brains were cut into 40-µm-thick frozen coronal or horizontal sections. Alternate sections were stained with cresyl violet or impregnated with silver to visualize degenerating neuronal somata and terminals.

Extracellular electrophysiological measurements

Transverse 400-µm-thick slices of the caudal hippocampus were used for electrophysiological recordings. The artificial cerebrospinal fluid (ACSF) contained (in mM) 122 NaCl, 25 $NaHCO_3$, 3.1 KCl, 1.8 $CaCl_2$, 1.2 $MgSO_4$, 0.4 KH_2PO_4, and 10 D-glucose, pH 7.4. The 25-µm nichrome monopolar stimulating electrode was placed in the stratum lucidum of area CA3b, 100 µm from the opening of the dentate hilus. Then a glass extracellular electrode (filled with 1 M NaCl; resistance of 2–6 MΩ) was placed in the granule cell body layer. The stimulus current was set to a near-maximal value and rectangular pulses of 100-ms duration were applied in every 30 s.

In other experiments the stimulating electrode was placed in the perforant path where it enters into the hippocampus from the direction of the entorhinal cortex. In those experiments when only the glutamaterg synaptic transmission was measured, 30 µM bicuculline (a $GABA_A$ antagonist) was added to the extracellular solution. Glutamatergic synaptic transmission was validated by inhibition by NBQX (AMPA antagonist) and D-AP5 (NMDA antagonist).

Whole-cell patch clamp measurement with electric stimulation

Traditional whole cell patch-clamp recordings were made from dentate granule cells located close to the extracellular recording electrode with an Axon Axopatch 200A amplifier. The intracellular solution consisted of (in mM) 120 cesium gluconate, 10 HEPES, 2 MgATP, and 10 QX-314 (N-ethyl lidocaine) chloride, pH 7.2; 276–277 mOsm and 5-8 MΩ. Series resistance was compensated in ~75%. Signals were filtered at 2 kHz, digitized at 10 kHz, and stored and analyzed with Axon's PClamp 6 software. The antidromically evoked EPSC was studied at a holding potential of -80 mV in the presence

of 30 µM bicuculline methiodide or 50 µM picrotoxin and was defined as the inward current abolished by 50 µM D-AP5 and either by 20 µM DNQX or 5 µM NBQX. The antidromically evoked $GABA_A$ IPSC was studied at a holding potential of 0 mV and was defined as the outward current abolished by bicuculline methiodide or picrotoxin. The AMPA and NMDA components of the EPSC was separated based on their voltage dependence and pharmacology. AMPA mediated currents were measured at -80 mV holding in the presence of bicuculline and D-AP5 and was defined as the current component blocked by DNQX. NMDA mediated currents were measured at -22 mV holding in the presence of bicuculline and was defined as the current component blocked by D-AP5.

Photostimulation
An 80-mW Coherent Enterprise 653 argon ion UV laser was used for photostimulation. The laser was coupled to the epifluorescence input of the Nikon Optiphot-2 microscope by a fiber optic cable. The microscope was equipped with a Noran Odyssey confocal imaging system. The laser beam was focused on the tissue with a 40x Olympus water-immersion objective. To locate a presynaptic granule cell, 200 µM γ-(CNB-caged) L-glutamate was added to the extracellular solution. First, we performed a patch clamp recording on a granule cells at -80 or -20 mV holding. Then we focused the laser beam in the granule cell body layer; 75 µm from the recorded cell. The photostimulation consisted of 4 ms, 50 mW pulses of UV light repeated in every 15 s. In attempts to study mossy cell– granule cell pairs, the laser beam was focused at sites within the dentate hilus where mossy cells were most likely located. In other experiments the NMDA and AMPA components of the EPSC were selected with 50 µM D-AP5.

Visualization of the location of photostimulation and mossy fiber – granule cell synapses
In some experiments, DiI was applied to the uncaging spot. DiI was dissolved in hot cod liver oil and pressure ejected through a glass micropipette. The recorded cell was filled with Lucifer yellow. The slice was then fixed, was embedded in an albumin-gelatin mixture and sections of 50-µm thickness were cut with a vibratome. Fluorescent dyes were visualized under the confocal microscope by excitation at 488 nm and use of a 510-nm barrier filter.

Effect of externally applied Zinc on $GABA_A$ and NMDA receptors
Zinc was added to the extracellular mediun at a concentration of 200 µM. The $GABA_A$ agonist (muscimol, 400 µM) was pressure ejected from a glass pipette (5-10 µm opening) directly to the dendrite of the recorded granule cell. NMDA-receptor mediated currents were evoked by electrical stimulation of the perforant path and were recorded at -20 mV holding in the presence of 5 µM NBQX and 30 µM bicuculline.

Effect of Zinc on spontaneous miniature inhibitory postsynaptic currents (mIPSC)
Zinc was added to the extracellular mediun at a concentration of 200 µM. In these experiments the intracellular solution contained CsCl. The extracellular solution contained 5 µM NBQX, 50 µM D-AP5 and 1 µM tetrodotoxin. The experiments were performed in PO_4/SO_4-free ACSF.

Effect of mossy fiber released zinc on $GABA_A$ receptor mediated currents
Mossy fibers were antidromically stimulated in the presence of NBQX and D-AP5 in PO_4/SO_4 -free ACSF. $GABA_A$ receptors were tested by UV activation of γ-aminobutyrate, α-carboxy-2-nitrobenzyl ester-t (cGABA; 200 µM). The laser was focused to the soma of the recorded and fluorescently labeled (Alexa 488) granule cell, then to different spots along the labeled dendrite, 50, 75 and 100 µm distance from the soma. Zinc was released by high-frequency antidromic stimulation (10 or 100 Hz for 1 s or 10 Hz for 5 min) of the mossy fibers. At the same time $GABA_A$ currents were recorded in every 10 s. In some experiments we attempted to increase the probability of Zinc release by removing Mg from the extracellular solution or by increasing the extracellular potassium concentrations to 6 mM or by increasing the temperature to 33°C.

Effect of cGABA on monosynaptic IPSCs
A bipolar stimulating electrode was placed in the granule cell layer of the dentate gyrus, 200–500 µm distance away from the recorded cell. For the stimulation 100 µs long square impulses were used in every 30s. The stimulation current was selected to evoke a near-maximal IPSC in the recorded granule cell. The patch clamp recordings were performed at 0 mV membrane potential in the presence of 5 µM NBQX and 50 µM D-AP5. In some experiments the membrane potential was changed in 10 mV steps.

Effect of CaEDTA (a zinc chelator) on the NMDA receptor mediated component of the EPSCs, evoked by mossy fiber or perforant path stimulation
EPSCs were evoked by antidromic mossy fiber or perforant path stimulation. The NMDA component of the EPSC was separated with NBQX and inhibited by D-AP5. In these experiments the effect of 1 mM CaEDTA, a high-affinity and specific zinc chelator was tested on the mossy fiber (which contains zinc at high concentrations) and on the perforant path (which does not contain zinc) evoked NMDA mediated currents in granule cells in the dentate gyrus in epileptic animals.

Results

Cell death, mossy fiber sprouting and spontaneous epileptic seizures after pilocarpine induced status epilepticus[1]
Single injection of pilocarpine (330 mg/kg, i.p.) evoked status epilepticus (SE) in about 2/3 of the treated rats. About 2/3 of the rats survived the 4 h of status epilepticus. Those animals which did not show the signs of SE were treated as controls, because there was no measurable difference between the pilocarpine treated, vehicle treated or non-treated control groups based on the performed tests. The first spontaneous seizures were observed about 3 weeks after status epilepticus. The frequency of the seizures varied widely (between 1/week to 3-4/day). The frequency of the spontaneous seizures stabilized by the end of the 5th week and remained stable until the end of the experiments (10 weeks after pilocarpine injection). Histological and electrophysiological experiments were performed

[1] Okazaki, M., **P. Molnar** and J. V. Nadler, "Recurrent mossy fiber pathway in the rat dentate gyrus: synaptic currents evoked in the presence and absence of seizure-induced growth," J. Neurophysiol. 81:1645-1660 (1999).

either 4-6 days (by this time the rats had recovered from the acute effects of pilocarpine, but neither spontaneous seizures nor mossy fiber sprouting could be observed) or 10 weeks (when the frequency of spontaneous seizures and mossy fiber sprouting reached maximum) after pilocarpine treatment.

In every epileptic rat Timm's staining showed strong 'mossy fiber – like' staining in the inner molecular layer of the dentate gyrus 10 weeks after status epilepticus, whereas there was no significant staining in any control animal, except some very weak sporadic labeling. Mossy fiber sprouting could not be observed in any groups 4-6 days after status epilepticus. There was extensive cellular degeneration observed 1-3 days after status epilepticus. In the hippocampus there was sporadic neuronal degeneration in areas CA1, CA3 and the dentate gyrus. The strongest degeneration was observed in the hilus, where about 50% of the cells died. There was a strong axonal degeneration in the inner molecular layer of the dentate gyrus, in the terminal zone of the mossy cells, but there was no degeneration in the outer molecular layer, in the terminal zone of the perforant path. These data suggest that mostly the mossy cells in the hilus died in consequence of SE. There was no observable neurodegeneration in control animals.

Functional characterization of mossy fiber – granule cell synapses developed during mossy fiber sprouting

The major problems hindering the characterization of granule cell – granule cell synapses were: 1) The probability of finding two connected granule cells is extremely low 2) Selective stimulation of the mossy fibers is difficult because of the many celltypes in the hilus.

For the solution of these problems we used three different methods which gave indirect partial information concerning functionality and characteristics of mossy fiber – granule cell synapses. These methods were: 1) Activation of mossy fibers with antidromic stimulation and record extracellular field responses in the granule cell layer 2) Antidromic mossy fiber stimulation combined with whole-cell patch clamp recordings of synaptic currents 3) Specific and local stimulation of presynaptic granule cells with photostimulation combined with patch clamp recordings from postsynaptic cells.

Antidromic mossy fiber stimulation evoked an antidromic population spike in the granule cell layer and a synaptically mediated field potential in the terminal zone of the stimulated mossy fibers. Using *extracellular recordings* in the granule cell layer we could always observe the antidromic population spike, but we could see the synaptically mediated field potential component only when the mossy fibers monosynaptically or polysynaptically innervated the granule cells. We found synaptically mediated field potentials evoked by antidromic mossy fiber stimulation only in about half of the slices obtained from epileptic rats, although we performed these experiments when mossy fiber sprouting was maximal (10 weeks after SE). This component could be blocked with NBQX. Surprisingly, in a small percentage of the treated and untreated controls we could measure synaptically mediated field potentials evoked by antidromic mossy fiber stimulation. This component was also blocked by NBQX.

When we used whole cell *patch clamp recordings* from granule cells and antidromic mossy fiber stimulation we always found a postsynaptic $GABA_A$ receptor mediated inhibitory current component in the epileptic and also in the control groups. 4-6 days after SE the GABAergic feed-back inhibition decreased significantly in the

hippocampus compared to the control group. Interestingly, by the 10th week GABAerg IPSCs evoked by antidromic mossy fiber stimulation did not change in the epileptic group, but significantly decreased in the control group. Thus, comparing epileptic rats to age-matched controls, at 10 weeks the GABAergic inhibition in epileptic rats was stronger. Excitatory postsynaptic currents (EPSC) evoked by antidromic mossy fiber stimulation were observed not only in the epileptic, but also in control rats. EPSC could be measured most frequently in epileptic rats, 10 weeks after SE, in about 74% of the measured slices. In the pilocarpine treated control this percentage was 38%, whereas in the untreated group it was 26%. The maximal amplitude of the EPSC was also significantly higher in the epileptic group. In the epileptic rats delayed EPSCs could also be observed (several 100s of ms delay) with amplitudes above 700 pA. There was no obvious correlation between the amplitude of the EPSC and mossy fiber sprouting visualized by Timm's staining. We did not find differences in any other parameters (membrane potential, membrane resistance) between the measured groups. We did not find any difference between the groups 4-6 days after pilocarpine treatment.

The NMDA receptor mediated component of the EPSC evoked by antidromic mossy fiber stimulation was determined by the application of NBQX and later by D-AP5. There were larger NMDA currents observed in the epileptic group compared to the control 10 weeks after SE. The NMDA current was larger not only in absolute value but also as the percentage of the AMPA component of the EPSC. We did not find any difference in the voltage dependence of the NMDA component in any of the measured groups.

In contrast to mossy fibers, there was no reorganization reported in connection with the perforant path in epilepsy. We did not find any difference in the perforant path evoked responses (GABA, AMPA or NMDA receptor mediated currents) between the epileptic and the control groups. Similarly to the mossy fiber evoked responses, the amplitude of the $GABA_A$ receptor mediated IPSC component evoked by perforant path stimulation showed an age-dependent decline.

Laser *photostimulation* was introduced to enable the local and specific stimulation of a small group of granule cells[2]. In these experiments we used an inactive glutamate analogue in the extracellular solution which activation required an UV pulse. We have determined that we could evoke an action potential from a granule cell from a distance of 12 µm. Stimulation of the granule cell layer with UV pulses evoked EPSC most frequently in the epileptic group. We had to try in average of 7 stimulation sites before EPSC were observed in the recorded granule cell. This means, that we had to stimulate about 176-266 granule cells before we could find two connected ones. Photostimulation evoked different types of EPSCs in the recorded postsynaptic cell. In optimal case we could observe a single EPSC with a fast rise time, short (10-20 ms) delay, mono-exponential decay and an amplitude of about 30 – 100 pA. Not all of the stimuluses generated an EPSC; the error rate was about 60-70%. In other cases we could observe complex EPSCs evoked by the photostimulation consisting of multiple superpositioned inward currents or complex, polysynaptic EPSCs. We did not find any

[2] **Molnar P.** and J. V. Nadler, "Mossy fiber - granule cell synapses studied with whole cell patch clamp recording and laser photostimulation," J. Neurophysiol. 82:1883-1894 (1999).

difference in the properties of EPSCs between the epileptic and control groups, only in the probability of finding an EPSC.

In subsequent experiments we have determined that NMDA receptors did participate in the synaptic transmission between granule cells. Some other experiments the site of a successful photostimulation was labeled with a hydrophobic dye (DiI), whereas the recorded cell was filled up with Lucifer Yellow. The DiI labeled, most possibly granule cell axons traveled parallel with the granule cell layer in the inner molecular layer of the dentate gyrus and formed synapse-like structures with the dendrites of the measured granule cell. The DiI labeled axons had large varicosities (~2 µm) which are characteristic for mossy fibers.

Effect of Zinc, synaptically released from mossy fibers, on the excitability of granule cells in epileptic rats[3]

Externally applied zinc inhibited muscimol-evoked $GABA_A$ currents and NMDA currents evoked by perforant path stimulation only in *PO_4/SO_4*-free ACSF. Externally applied zinc also inhibited spontaneous miniature inhibitory postsynaptic currents (mIPSC) in *PO_4/SO_4*-free ACSF.

The effect of mossy fiber – released zinc on postsynaptic $GABA_A$ receptors was studied with high frequency (10 and 100 Hz) antidromic stimulation of mossy fibers, whereas the postsynaptic $GABA_A$ receptors were tested with UV activation of a GABA precursor. The site of the photostimulation of $GABA_A$ receptors were located in the terminal zone of the sprouted mossy fibers, in the inner molecular layer of the dentate gyrus, closer than 100 µm to the cell body of the recorded and fluorescent dye - filled granule cell, along the labeled dendrite. Because we have observed that cGABA inhibited spontaneous GABAergic IPSCs, thus we continued these experiments only after we have verified that this feature of cGABA would not affect the result of our experiments[4,5]. We have determined that 160-200 µM cGABA in the extracellular solution almost completely blocked the IPSCs, but did not significantly affect the EPSCs. In contrast, 200 µM cGABA only marginally affected $GABA_A$ receptor mediated currents. We concluded that these effects of cGABA could not significantly influence the results of our original experiment.

In order to maximize the probability of zinc release, we have tried different stimulation protocols (10 Hz, 100 Hz, zero magnesium, high potassium, continuous stimulation, high temperature), but we could not be able to observe any cases when antidromic mossy fiber stimulation affected $GABA_A$ receptor mediated currents in granule cells.

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[3] **Molnar P.** and J. V. Nadler, "Lack of effect of mossy fiber-released zinc on postsynaptic $GABA_A$ receptors in the pilocarpine model of epilepsy," J. Neurophysiol. 85:1932-40 (2001).

[4] **Molnar P.** and J. V. Nadler, "O-(CNB-caged) GABA selectively blocks inhibitory synaptic transmission in rat hippocampal slices," Eur. J. Pharmacol. 391:255-262 (2000).

[5] **Molnar P.** and J. V. Nadler, "O-(CNB-caged) GABA selectively blocks inhibitory synaptic transmission in rat hippocampal slices," Eur. J. Pharmacol. 391:255-262 (2000).

In contrast, we have observed inhibitory effect of mossy fiber – released zinc on postsynaptic NMDA receptors[6]. In these experiments the effect of a high affinity and specific zinc chelator, CaEDTA was studied, whereas NMDA receptors were activated by antidromic mossy fiber stimulation (mossy fibers contain zinc) or perforant path stimulation (they do not contain zinc). 1 mM CaEDTA in the extracellular solution increased the amplitude of NMDA currents evoked by mossy fiber stimulation, but did not affect NMDA currents evoked by perforant path stimulation.

Discussion

In this work the functional consequences of mossy fiber sprouting was studied using histological, electrophysiological, photostimulation and pharmacological methods in the pilocarpine rat model of temporal lobe epilepsy. We showed that functional granule cell - granule cell synaptic connections were present in the caudal part of the hippocampus not only in epileptic, but also in control rats, only with a much smaller probability. This observation is in contradiction with earlier data; other researches could not find monosynaptic granule cell – granule cell synaptic connections in healthy normal rats (Wuarin et al. 1996). One possibility is, that because of technical difficulties, we stimulated not only mossy fibers but also some mossy cells in the dentate hilus. We excluded this possibility, because in our experiments all EPSC, evoked by antidromic mossy fiber stimulation or photostimulation, had an NMDA receptor mediated component in the presence of NBQX, thus, most possibly they were monosynaptic. Moreover, in our photostimulation experiments we observed monosynaptic granule cell - granule cell synaptic connections in normal rats. Based on these facts we had to accept that, at least in the caudal hippocampus, with low probability, functional granule cell – granule cell synaptic connections are present in normal animals.

10 weeks (but not 4-6 days) after status epilepticus the number of functional granule cell – granule cell synapses increased drastically. Based on the photostimulation experiments we showed that the number of functional mossy fiber – granule cell synapses increased 6-fold after SE. The probability of finding two synaptically connected granule cells were about 0.5% in the epileptic rats. The observed changes were specific to mossy fibers, because we did not find any difference in perforant path mediated synaptic transmission between the epileptic and control groups. These results are in good agreement with earlier observations of other researchers concerning functional consequences of mossy fiber sprouting. Using a similar arrangement as our photostimulation setup Wuarin and coworkers have shown that local injection of glutamate to the granule cell layer increased the frequency of spontaneous EPSCs in granule cells in epileptic rats (Wuarin et al. 1996). Our photostimulation experiments showed first and unambiguously the formation and physiological properties of monosynaptic granule cell – granule cell synapses. But our experiments demonstrated also, that functional granule cell – granule cell synapses are very rare even in epileptic rats, where histological methods show very strong mossy fiber sprouting. Our experiments

[6] **Molnar P.** and J. V. Nadler, “Synaptically-released zinc inhibits N-methyl-D-aspartate receptor activation at recurrent mossy fiber synapses,” Brain Res. 910:205-207 (2001).

also showed a high failure rate in these newly formed synapses. Thus, it is not very likely that mossy fiber sprouting and the newly formed granule cell – granule cell synapses are the major / only cause of the development of spontaneous epileptic seizures in temporal lobe epilepsy.

Characteristics of monosynaptic granule cell – granule cell synapses

The average amplitude of the monosynaptic EPSCs evoked in granule cells by photostimulation in the granule cell layer was smaller (~30 pA) than it was reported in granule cell – CA3 pyramidal cell synapses (~70 pA) (Jonas et al. 1993), but larger than monosynaptic EPSCs described in other areas in the hippocampus (Allen et al. 1994; Kneisler et al. 1995). This observation is in good agreement with earlier data because the axon terminals of the mossy fibers in the molecular layer of the dentate gyrus are smaller than in the CA3, but definitely larger than an average axon terminal in the hippocampus (Okazaki et al. 1995). In our experiments the time course of the EPSCs were slower than it was reported in CA3 pyramid cells (Jonas et al. 1993). One possible explanation is that we used old animals for the experiments and electrophysiological properties of granule cells could depend on the age of the animals. The relatively high synaptic failure rate, we observed in these experiments, is in good agreement with earlier results concerning mossy fiber – CA3 pyramidal cell (Jonas et al. 1993) and Schaffer collateral – CA1 pyramidal cell (Allen et al. 1994) synapses. The failure rate for the mossy fiber – basket cell synapses was found to be about 33% in experimental conditions which were similar to ours (Kneisler et al. 1995).

Characteristics of AMPA and NMDA components of granule cell – granule cell synaptic transmission

All of our experiments unambiguously showed that both AMPA and NMDA receptors participated in the mossy fiber – granule cell synaptic transmission in epileptic and also in control animals. The role of NMDA receptors has already been described in the mossy fiber – CA3 pyramidal cell (Weisskopf et al. 1995) and also in the mossy fiber – basket cell (Kneisler et al. 1995) synaptic transmission. According to our data, the amplitude of the NMDA current was larger in the epileptic group than in the control group when the mossy fibers were stimulated. During epileptic synchronized activity this could be especially important, because during epileptic seizures the granule cells are depolarized and increased activation of the slowly inactivating NMDA receptors could promote the spread of epileptic synchronized activity through the dentate gyrus (Dingledine et al. 1990; Traub et al. 1994). Our data is supported by earlier observations, namely that D-AP5, an antagonist of NMDA receptors, inhibited the development of epileptic activity evoked by mossy fiber stimulation in the dentate gyrus (Patrylo et al. 1998). The mechanism by which the NMDA component of the EPSCs evoked by mossy fiber stimulation increased by several fold, whereas the NMDA component of the EPSCs evoked by perforant path stimulation did not change in epileptic animals, is not known. Interestingly, in the photostimulation experiments we did not see this increase. A possible explanation is that with the stronger antidromic mossy fiber stimulation we have activated not only synaptic, but also extrasynaptic NMDA receptors, whereas during the weaker photostimulation the extrasynaptic NMDA receptors played a much smaller role.

GABAergic inhibition in epilepsy

The amplitude of the inhibitory postsynaptic currents evoked by antidromic mossy fiber stimulation was about 40% smaller in epileptic rats than in the control group 4-6 days after status epilepticus. This is most possibly the result of the degeneration of mossy cells, which play a significant role in the stimulation of inhibitory interneurons (Obenaus et al. 1993). The most surprising result was that the mossy fiber – dependent GABAergic inhibition significantly decreased in control, but not in epileptic rats by 10 weeks after pilocarpine treatment. That means, there is some mossy fiber sprouting – dependent mechanism by which the GABAergic inhibition is increased in the dentate gyrus in epileptic animals. In contrast, although the perforant path – dependent inhibition also decreased with the age of the animals, we did not find any difference in perforant path evoked responses between epileptic and control animals. Besides the degeneration of mossy cells, the selective death of inhibitory interneurons could also explain the selective decrease of mossy fiber dependent GABAergic inhibition in the dentate gyrus after SE (Buckmaster et al. 1997; Lurton et al. 1997). Earlier results showed a decrease in the number of inhibitory interneurons as the age of the animals advanced (Shetty et al. 1998). If the neuronal population which degenerates with the age of the animals is the same which dies after status epilepticus it could explain that after the initial decrease the GABAergic inhibition did not decreased further with the age in epileptic rats (the age-sensitive cells have already been degenerated after SE).

The role of mossy fiber released zinc in epilepsy

Zinc, added to the extracellular solution, decreased $GABA_A$ and NMDA receptors mediated responses evoked by externally applied muscimol or perforant path stimulation, but only when polyvalent anions were removed from the extracellular medium. Zinc also decreased the amplitude of spontaneous inhibitory postsynaptic currents. Zinc in our experiments had a significantly weaker inhibitory effect on $GABA_A$ receptors than it was described earlier (Gibbs et al. 1997). One possible explanation for this is that we measured GABAA receptor mediated responses on the dendrite of granule cells, in the inner molecular layer of the dentate gyrus in brain slices, whereas earlier studies were performed on the soma of isolated cells.

Antidromic mossy fiber stimulation in the presence of NBQX and D-AP5 had no effect on the $GABA_A$ receptor mediated responses evoked by photostimulation on the dendrites of granule cells in epileptic rats, even in those conditions when we have increased the probability of zinc release. Because of technical difficulties, this does not mean with 100% probability that we excluded the possibility of any effect of mossy fiber released zinc on $GABA_A$ receptors in epilepsy, but, based on our experiments, it is not likely. For the inhibition of postsynaptic $GABA_A$ receptors zinc should be released from the presynaptic mossy fiber terminals in a high – enough concentration to overflow the synaptic cleft and reach the neighboring $GABA_A$ receptors and block them. Free diffusion of zinc from the synaptic cleft is inhibited by two factors. First, there is a transporter in the presynaptic membrane which role is the uptake of zinc and its removal from the synaptic cleft, thus, after presynaptic activity the extracellular concentration of zinc decreases very fast with non-diffusion based mechanism (Howell et al. 1984). Second, the extracellular fluid always contains polyvalent anions, which bind zinc and hindering it ineffective. These properties of zinc could explain our negative result.

In other experiments we have shown that mossy fiber released zinc slightly inhibited postsynaptic NMDA receptors, thus, zinc could play (based on this effect – an inhibitory) role in the development of epileptic seizures. Other studies and our subsequent experiments showed that the role of zinc in epilepsy is very complex, but usually weak and, thus, difficult to interpret. According to latest observations, zinc is an epileptogenic agent in the kindling model (Foresti et al. 2008), having no effect on seizures evoked by pilocarpine injection (Noyan et al. 2007), but facilitating the epileptic activity of the granule cells of the dentate gyrus in fully developed epilepsy in the pilocarpine model (Timofeeva et al. 2006).

According to earlier measurements, the GABA precursor we used in these experiments was inactive on GABA receptors (Gee et al. 1994). In our experimental system 200 µM of cGABA showed weak $GABA_A$ antagonist properties. In contrast, it completely inhibited spontaneous GABA mediated IPSCs in granule cells. cGABA had no measurable effect on the glutamatergic synaptic transmission in the dentate gyrus. Based on our experiments we could not decide whether the effects of cGABA on the GABAergic transmission are mediated by presynaptic or postsynaptic mechanism, but we found cGABA effects very similar to the actions of some phenothiazine-type antipsychotics (described earlier in Zorumski et al. 1988). Based on these data we concluded that the weak, $GABA_A$ receptor-mediated inhibitory effect of cGABA could not influence our results concerning the role of mossy fiber released zinc in epilepsy. Especially, that cGABA had no effect on the excitatory synaptic transmission and, most possibly, on zinc release from mossy fibers.

Summary

In these experiments we showed that functional granule cell – granule cell synaptic connections are formed in normal, non-epileptic animals in the caudal hippocampus. As the consequence of status epilepticus, during mossy fiber sprouting, the number of functional granule cell – granule cell synaptic connections increased drastically. The physiological and pharmacological properties of these newly formed synapses were not different than that observed in control animals. Not only AMPA but also NMDA receptors participated in the granule cell granule cell synaptic transmission. Zinc, released upon synaptic activity from mossy fibers, continuously and (probably) frequency dependently[7] inhibited postsynaptic NMDA receptors, but most possibly it has no or only minimal effect on the neighboring GABAergic synapses. These results are supporting the idea that during mossy fiber sprouting a functional positive feedback is developing in the dentate gyrus, which consequently increase the excitability of the dentate gyrus and could promote the development / spread of epileptic activity. But the probability of functioning granule cell – granule cell synapses are relatively low even in epileptic animals, thus, mossy fiber sprouting most possibly cannot be the primary / only mechanism of epileptogenesis in temporal lobe epilepsy.

[7] Feng, L., **P. Molnar** and J. V. Nadler: Short-Term Frequency-Dependent Plasticity at Recurrent Mossy Fiber Synapses of the Epileptic Brain. J. Neurosci. 23. 5381-5390 (2003).

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