

# **Involvement of Free Radicals in Epilepsy Workshop**



25<sup>th</sup> – 28<sup>th</sup> September

Budapest

Center of Excellence on Biomolecular Chemistry

Chemical Research Centre

Hungarian Academy of Sciences

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## Schedule

25<sup>th</sup> September, **Thursday**

Arrival in Budapest: visit to the Chemical Research Centre (optional)

26 <sup>th</sup> September, <b>Friday</b> Chairperson Prof. Julianna Kardos	27 <sup>th</sup> September, <b>Saturday</b> Chairperson Prof. Uwe Heinemann
<b>10.00</b> Opening words by Prof. György Hajós on behalf of the Center of Excellence on Biomolecular Chemistry	<b>9.30 – 10.30</b> Discussion of the Scientific part of the Proposal on the basis of the planned Work Packages
<b>10.15– 12.00</b> Morning Session	<b>10.30 –11.00</b> Coffee break
<b>12.00–13.30</b> Lunch	<b>11.00 – 12.00</b> Discussion of the financial part of the Proposal
<b>13.30– 15.00</b> Afternoon Session I:	<b>12.00 –13.30</b> Lunch
<b>15.00–15.30</b> Coffee break	<b>13.30 –15.00</b> Administrative Questions of the Proposal
<b>15.30– 17.00</b> Afternoon Session II.	
<b>17.00</b> Closing words by Prof. Heinemann Strategic considerations/ discussion	
<b>19.00</b> Dinner	<b>19.00</b> Farewell Party

28<sup>th</sup> September, **Sunday**

Departure

## Scientific Programme/ Abstracts

### Friday, Morning Session

10.00 Opening words by Prof. György Hajós

10.15 – 10.45

Sylvia Pietri

#### **Some ESR Methods Used for Free Radical Detection in Several Biological Models**

*SREP-CNRS UMR 6517, Centre Saint-Jérôme, POB 521, F-13397 Marseille, France*

The formation of highly reactive species deriving from molecular oxygen, including free radicals, has been implicated in a large number of pathologies, including ischemia/ reoxygenation injury, neurodegenerative diseases, atherosclerosis or rheumatoid arthritis. In the course of the disease processes, these deleterious species can cause the disruption of the mitochondrial respiratory chains, the release of iron ions from various cellular sites, or some modifications in the activities of key enzymes such as xanthine oxidase. Experimental or clinical studies have focused on the possible contribution of activated oxygen species to tissue injury by measuring the occurrence of membrane peroxidation and destruction, and DNA or proteins oxidation.

However, in situations where the initial formation of oxygen-derived free radicals is postulated, analytical methods based on ESR spectroscopy can be decisive. In the present communication, some applications of ESR in *in vitro* (cerebellar granule cells, isolated perfused organs) or *in vivo* (myocardial surgery, retinal ischemia reperfusion) models will be presented.

*Acknowledgement.* This work was supported by the grants from the CNRS (UMR 6517), Ipsen-Beaufour-Pharma, and the S.A.R.L. Oxylab

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1. Pietri S, Mercier A, Mathieu C, Caffaratti S, Culcasi M. Hemodynamic and metabolic effects of the beta-phosphorylated nitroxide 2-diethoxyphosphoryl-2,5,5-trimethylpyrrolidinoxyl during myocardial ischemia and reperfusion. *Free Radic Biol Med.* (2003) 34:1167-77.
2. Muller A, Pietri S, Villain M, Frejaville C, Bonne C, Culcas M. Free radicals in rabbit retina under ocular hyperpressure and functional consequences. *Exp Eye Res.* (1997) 64:637-43.
3. Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J. NMDA-dependent superoxide production and neurotoxicity. *Nature.* (1993) 364:535-7

10.45 - 11.15

Antal Rockenbauer

#### **ESR Investigation of Dynamic Phenomena for Free Radicals in Biological Systems**

*ESR Laboratory, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, POB 17, Hungary*

The ESR time scale, where the spectra show dynamic effects is in the order of  $10^5$ - $10^9$  Hz, which range is important for the molecular motions in biological systems. The dynamic information can be extracted by computer simulation of the ESR spectra. Different applications of the method are demonstrated.

1. Superoxy radicals can be trapped by DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) yielding to persistent nitroxide radicals. The hindered rotation of OOH group results chemical exchange in the ESR spectra [1].

2. By chemical damage of proteins free radicals are produced and the radicals are observed by spin trapping. To characterise the motion of proteins anisotropic line width tensor is introduced and effective g- and hyperfine tensors are applied. The impact of denaturation is also demonstrated [2, 3].

*Acknowledgment.* This work was supported by the Centre of Excellence on Biomolecular Chemistry (Project CBCH) QLK2-CT-2002-90436.

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1. Rockenbauer: Determination of chemical exchange parameters in ESR spectroscopy. *Molecular Physics Reports* (1999) 26, 117-127.
2. J-L Clément , B.C. Gilbert , A Rockenbauer and P. Tordo. Radical damage to proteins studied by EPR spin-trapping techniques. *J. Chem. Soc. Perkins Trans.* (2001) 2, 1463-1470.
3. J-L Clément, B.C. Gilbert, A. Rockenbauer, P. Tordo, A.C. Whitwood: Observation of protein-derived (BSA) oxygen-centered radicals by EPR spin-trapping techniques. *Free Radical Res.* (2002) 36, 883-891

### **11.15 – 11.45**

Éva Szárics, Richard Kovács, Julianna Kardos

#### **Subsecond Time-Scale Formation of Free Radicals in Isolated Mitochondria under Epileptic Condition**

*Department of Neurochemistry, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, POB 17, Hungary*

Here we report on a fast kinetics method to monitor the formation of free radicals under epileptic condition. Free radical formation in rat mitochondria was followed by the application of the technique of rapid mixing in combination with fluorescence detection. Aliquots (75 µl) of rat brain mitochondrial suspensions, isolated from sucrose-gradient purified rat cerebrocortical synaptosomes, were rapidly mixed with equal amount of buffer A (containing in millimoles: KCl 140, NaCl 3, CaCl<sub>2</sub> 0,05 , EGTA 1, HEPES 10, pH=7.4; control condition) or buffer B (containing, in millimoles: KCl 140, NaCl 30, CaCl<sub>2</sub> 1 , EGTA 1, HEPES 10, pH=7.4; epileptic condition) in the presence of 300 µM dihydroethidium. Dihydroethidium is oxidized by hydroxyl and superoxide radicals to ethidium, showing red fluorescence, monitored at 610 nm using an 530 nm excitation filter [1]. Under these conditions, a two-phased increase in ethidium fluorescence is observed between 0.001-0.01 s and 0.05-0.1 s time intervals, respectively. Fluorescence in both phases decreased in the presence of the radical-scavenger, TEMPO-9AC (0.5 mM).

*Acknowledgment.* This work was supported by grants 1/ 047 NKFP MediChem, OTKA T 035225, (Hungary), the Centre of Excellence on Biomolecular Chemistry (Project CBCH) QLK2-CT-2002-90436 and Training and Excellence ICA1-CT-2002-70008.

*Reference*

Haugland, R. P. 1996. Handbook of fluorescent probes and research chemicals. Leiden: Molecular Probes Inc. pp 483-502.

### **12.00 - 13.30 Lunch**

**13.30– 14.00**

Uwe Heinemann and Oliver Kann

**Mechanisms of Coupling of Neuronal and Metabolic Activity**

*Johannes Müller Institute for Physiology, Charité, Humboldt University, Berlin, Germany*

Increased neuronal activity causes transmembrane ionic shifts. Active and secondary active transport processes are required to restore transmembrane ionic gradients, which depends on delivery of ATP. In neurones most ATP generation depends on oxidative phosphorylation requiring well functioning mitochondria. Previously it was assumed that adaptation of ATP generation to cellular needs is regulated by the ratio of ATP/ADP. However, some enzymes of the tricarboxylic acid cycle are  $\text{Ca}^{2+}$  sensitive. We present evidence that  $\text{Ca}^{2+}$  entry through the plasma membrane leads to mitochondrial  $\text{Ca}^{2+}$  uptake and increased generation of NADH. We also show that  $\text{Ca}^{2+}$  release from the endoplasmic reticulum mediated through metabotropic receptors can also trigger NADH generation. Both mechanisms contribute to seizure-associated increased production of NADH which leads to increased ATP production.

*A acknowledgement:* This work was supported by the grants SFB 507

*References*

1. Kann O, Kovacs R, Heinemann U. Metabotropic receptor-mediated  $\text{Ca}^{2+}$  signaling elevates mitochondrial  $\text{Ca}^{2+}$  and stimulates oxidative metabolism in hippocampal slice cultures. *J Neurophysiol* (2003) 90:613-21.
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3. Schuchmann S, Kovacs R, Kann O, Heinemann U, Buchheim K. Monitoring NAD(P)H autofluorescence to assess mitochondrial metabolic functions in rat hippocampal-entorhinal cortex slices. *Brain Res Brain Res Protoc*. (2001) 7:267-76.

**14.00 – 14.30**

Richard Kovács<sup>1,2</sup>, Oliver Kann<sup>2</sup>, Julianna Kardos,<sup>1</sup> Uwe Heinemann<sup>2</sup>

**Changes of Mitochondrial Membrane Potential during Seizure-like Events in Hippocampal Slice Cultures**

*<sup>1</sup>Department of Neurochemistry, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, POB 17, Hungary; <sup>2</sup>Johannes Müller Institute for Physiology, Charité, Humboldt University, Berlin, Germany*

The maintenance of transmembrane ion gradients during epileptic activity represents an extraordinary stress for neurones. Here we addressed the question, whether mitochondria are able to keep up the large ATP demand during seizures. Since the largest part of the proton motive force is represented by the mitochondrial membrane potential ( $\Delta\Psi$ ), we monitored its changes by fluorescence means at multicellular, single cell and mitochondrial levels during epileptiform activity induced by application of  $\text{Mg}^{2+}$ -free ACSF in hippocampal slice cultures. Rhodamine-123 (Rh-123) application through the patch pipette in the whole cell mode allowed measurement of changes of  $\Delta\Psi$  in mitochondria from the soma and dendrites of a single CA3 pyramidal cell by using confocal laser scanning microscopy. Rh-123 staining showed a complex

network of tubular and granular mitochondria with different  $\Delta\Psi$ . Upon interictal activity, localised mitochondrial depolarisation occurred in small dendritic branches, often restricted to a single mitochondria. In contrast, seizure-like events (SLEs) were associated with a homogeneous, overall increase of cytosolic Rh-123 fluorescence, indicating simultaneous depolarisation in a large population of mitochondria. However, signs of mitochondrial dysfunction (i.e. sudden, large depolarisation with apparent swelling), known from excitotoxic glutamate application, could be only seen after a sequence of several (>5) recurring SLEs. Mitochondrial depolarisation depended on  $\text{Ca}^{2+}$  influx, as blockade of the mitochondrial  $\text{Ca}^{2+}$ -uniporter by Ru360 inhibited SLE-associated Rh-123 fluorescence rise. Selective blockade of the electrophoretic mitochondrial  $\text{Ca}^{2+}/\text{Na}^{+}$ -exchanger by CGP-37157 also inhibited fast Rh-123 fluorescence changes, suggesting that enhanced mitochondrial  $\text{Ca}^{2+}$ -cycling, rather than  $\text{Ca}^{2+}$ -uptake alone, is responsible for the mitochondrial membrane potential changes.

*Acknowledgement.* This work was supported by grants 1/ 047 NKFP MediChem, OTKA T 035225, (Hungary), the Centre of Excellence on Biomolecular Chemistry (Project CBCH) QLK2-CT-2002-90436 and Training and Excellence ICA1-CT-2002-70008 and SFB 507.

*References:*

1. Kovacs R, Schuchmann S, Gabriel S, Kann O, Kardos J, Heinemann U. Free radical-mediated cell damage after experimental status epilepticus in hippocampal slice cultures. *J Neurophysiol.* (2002) 88:2909-18.
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3. Schuchmann S, Luckermann M, Kulik A, Heinemann U, Ballanyi K.  $\text{Ca}^{2+}$ - and metabolism-related changes of mitochondrial potential in voltage-clamped CA1 pyramidal neurons in situ. *J Neurophysiol.* (2000) 83:1710-21.

**14.30 – 15.00**

Alexei P. Kudin, Nana-Yaw Bimpong-Buta and Wolfram S. Kunz

**Localization of ROS Producing Sites in Rat Brain Mitochondria**

*Department of Epileptology, University Bonn Medical Center, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany*

ROS (reactive oxygen species)-induced alterations of brain function play an important role under many pathological conditions of the central nervous system including epilepsy. Mitochondrial respiratory chain complexes I and III have been shown to be the main contributors to neuronal ROS production since the superoxide anion, a product of one-electron reduction of oxygen, is the byproduct of normal functioning of mitochondrial respiratory chain. While the superoxide producing site at respiratory chain complex III (b-c<sub>1</sub> complex) has been determined to be the bound semiquinone at center 'o' of Q-cycle the ROS producing site at respiratory chain complex I has not been identified so far. In the present study we approached this question investigating the effects of inhibitors and of redox potential of the NAD system on  $\text{H}_2\text{O}_2$  production of isolated rat brain mitochondria applying *p*-hydrophenylacetate as fluorescent probe. While mitochondria in the presence of glutamate+malate alone do generate only small amounts of hydrogen peroxide a high production is observed after the addition of the complex I inhibitor rotenone or in the presence of the respiratory substrate succinate alone. This is an indication that most of the superoxide radicals are produced at complex I and that a high production of reactive oxygen species is a feature of respiratory chain-inhibited mitochondria or of reversed electron flow. Very importantly, the rate of hydrogen peroxide generation by respiratory chain complex

III which can be observed in presence of the complex III inhibitor antimycin A is substantially lower. Furthermore, we determined the redox potential of the superoxide production site at complex I to be -290 mV. This result suggests that the site of superoxide generation at complex I is FMN. Since short term incubation of rat brain mitochondria with H<sub>2</sub>O<sub>2</sub> in the lower mM range induced increased ROS production at this site we propose that reactive oxygen species activate a self-accelerating vicious cycle leading to mitochondrial damage and neuronal cell death in epilepsy.

*Acknowledgement:* This study was supported by grants of the University of Bonn (BONFOR) and of the Deutsche Forschungsgemeinschaft (Ku 911/ 11-1) to WSK.

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1. Kudin AP, Kudina TA, Seyfried J, Vielhaber S, Beck H, Elger CE, Kunz WS. Seizure-dependent modulation of mitochondrial oxidative phosphorylation in rat hippocampus. *Eur J Neurosci.* (2002) 15:1105-14.
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3. Kunz WS, Goussakov IV, Beck H, Elger CE. Altered mitochondrial oxidative phosphorylation in hippocampal slices of kainate-treated rats. *Brain Res.* (1999) 826:236-42.

#### **15.00 – 15.30 Coffe Break**

#### **Friday, Afternoon Session II.**

#### **15.30 – 16.00**

Paul J. E. Smith<sup>1</sup>, Toju Lori<sup>2</sup>, Armando A. Gennazzani<sup>2</sup> and Ruth M. Empson<sup>1</sup>

#### **Damage or Protection Arising from Interictal type Epileptiform Activity in Organotypic Hippocampal Slice Cultures from Rat?**

<sup>1</sup>*School of Biological Sciences, Royal Holloway University of London,* <sup>2</sup>*Department of Pharmacology, University of Cambridge, UK.*

In experimental animal models and in human surgical tissue from intractable epileptic patients, there is often sclerosis of the hippocampus that may exacerbate the patient's seizures. A better understanding of how damage is initiated, how it progresses and what intrinsic neuroprotective mechanisms may be at play, are necessary if we are to develop new therapies to counteract intractable epilepsy.

Using organotypic hippocampal slice cultures and acutely prepared hippocampal slices, we have combined a model of interictal type epileptiform activity with semi quantitative imaging and RT-PCR to identify the extent of epilepsy induced damage and immediate early gene transcription. Our aim is to understand how mild epileptiform activity can initiate immediate early gene expression that either damages or protects the hippocampal neurones.

Our model of focal epilepsy uses bicuculline, a GABA<sub>A</sub> receptor antagonist, that elicits short regular interictal type epileptiform activity in hippocampal subregions. To determine the extent of damage elicited within this model we have compared the damage it causes with that caused by two other agents reported to induce seizure-like activity: 3-nitropropionic acid, an inhibitor of succinate dehydrogenase (3-NPA); and kainic acid (KA), a known excitotoxin. To determine the extent of the damage caused, slices were exposed to the three different agents for 4hrs, followed by 15hrs recovery. A dual-stain fluorescent assay assessed viable (calcein-AM,

4 $\mu$ M) and damaged cells (propidium iodide, 20 $\mu$ M). Confocal images were collected (BioRad) from four randomly selected areas within hippocampal subregions CA1 and CA3, using a 60x water-immersion objective. Fluorescence area was determined using Simple-PCI (C-Imaging). Electrophysiological field potential recordings determined, in separate slices, the extent of epileptiform activity under the three conditions. We have also determined in separate experiments, using RT-PCR, the activation by the mild form of bicuculline induced epileptiform activity of a number of potential immediate early gene candidates.

The results show how the damage caused by interictal type epileptiform activity in the hippocampus is very mild in comparison with the other models, but in all models the damage starts early and progresses. The discussion will explore some of our results that support the idea that ongoing interictal type activity may actually be protective, perhaps via activation of the novel immediate early genes we have identified.

*Acknowledgement:* We acknowledge the support of the Epilepsy Research Foundation UK and Action Research UK.

#### *References*

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2. Empson RM, Gee VJ, Sheardown MJ, Newberry NR. Chlormethiazole inhibits epileptiform activity by potentiating GABA(A) receptor function. *Brain Res*. (2000) 884:31-4.
3. Schmitz D, Empson RM, Gloveli T, Heinemann U. Serotonin blocks different patterns of low Mg<sup>2+</sup>-induced epileptiform activity in rat entorhinal cortex, but not hippocampus. *Neuroscience*. 76:449-58 (1997)

#### **16.00 – 16.30**

Bálint Lasztóczy, Lajos Nyikos, Károly Antal, Richárd Kovács and Julianna Kardos

#### **High Frequency Oscillation and Millisecond Time-Scale Synchrony of Synaptic Activity During Experimental Seizures.**

*Department of Neurochemistry, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, POB 17, Hungary*

Seizure-like events (SLE) induced by low-[Mg<sup>2+</sup>] ACSF perfusion of transverse hippocampal slices from young rats were characterised by a wide range of frequencies from <1 Hz up to 800 Hz. Wavelet transform of CA3 field potential recordings, disclosed a single rhythm declining from about 200 Hz at the onset of SLE to below 1 Hz in the clonic phase with a discontinuity at the tonic-clonic border [1].

SLEs were preceded by a few paroxysmal spikes (PS). These, along with the onset of SLE frequently started with a fast negative deflection increasing in amplitude as approaching the SLE onset. Both PSs and SLE onsets were associated with high frequency oscillations (HFO, > 300 Hz), that often shown up at the initiation of these events, disappeared in the tonic phase and reappeared in the clonic phase. HFO energy dropped at the SLE onset and remained low during the clonic phase. Single CA3 neurones expressed action potential firing at substantially lower frequencies (40-160 Hz).

Composite postsynaptic currents, associated with PSs and SLE onsets, arose from a flat baseline and frequently showed a smooth rising phase of 2-10 ms. The risetime of composite postsynaptic currents associated with SLE discharges showed that the synchrony increased with PSs, peaked around the SLE onset, decreased with the evolution of tonic phase discharges, and after a slight increase, remained stable for the clonic phase. These data indicate, that multiple

subgroups of synchronously firing neuronal aggregates coexist, and the number of these decreases, while the size increases as the system approaches the SLE onset.

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*References:*

1. Nyikos, L., Lasztóczy, B., Antal, K., Kovács, R., and Kardos, J. Desynchronisation of spontaneously recurrent experimental seizures proceeds with a single rhythm. *Neurosci.* (2003) in press.
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**16.30 – 17.00**

Lajos Nyikos, Károly Antal, Bálint Lasztóczy, Zsuzsa Emri and Julianna Kardos

**Detection of Phase Synchrony in a Low-[Mg<sup>2+</sup>] Model of Epilepsy**

*Department of Neurochemistry, Chemical Research Centre, Hungarian Academy of Sciences, H-1525 Budapest, POB 17, Hungary*

Pathological synchronisation is generally believed to be associated with epileptiform activity. Due to the nonstationary and noisy character of signals, however, traditional Fourier-based spectral and coherence methods are not well suited to study epileptiform activity, because they assume stationarity. In contrast, complex wavelet transform (CWT) methods are suitable for nonstationary signals and offer the additional advantage of fine-tuning the analysis by selecting the best wavelet from a number of wavelet families. Wavelets were shown to be useful to characterise the instant frequency components in seizure like events (SLE) induced by low extracellular [Mg<sup>2+</sup>] in transversal hippocampal slices from young rats [1].

To follow the temporal variations in synchrony of extra- and intracellular signals, the Hilbert transform (HT) method was shown [2] to be suitable to quantify time-dependent phase locking of field potential and either membrane potential or membrane current recorded during and between SLEs. In addition to the HT method, we also use the CWT method to calculate phase synchrony during interictal activity and SLEs (Fig. 1). The two methods are theoretically equivalent, but CWT is preferred in practice since bandpass filtering required by the HT method is unnecessary. Between 0.25-2 Hz, the mean phase coherence is found to be high in the interictal period (not shown) with occasional losses of coherence as the SLE approaches – a phenomena which might be utilised for seizure prediction or anticipation.

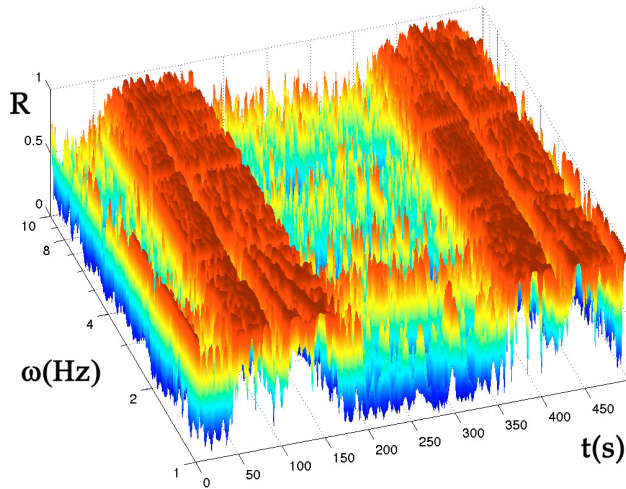


Fig. 1. Mean phase coherence ( $R$ , vertical axis), calculated by the CWT method, of the field potential and the membrane potential for two consecutive SLEs recorded in the low- $[Mg^{2+}]$  model, as a function of time ( $t$ ) and frequency ( $\omega$ ). The  $R$  values are close to unity during the SLEs, with a transient decoherence period in the tonic phase appearing at lower frequencies.

*Acknowledgement.* This work was supported by grants 1/047 NKFP MediChem, OTKA T 035225, (Hungary), and the Centre of Excellence on Biomolecular Chemistry (Project CBCH) QLK2-CT-2002-90436.

#### *References*

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2. Lasztóczy, B., Nyikos, L., Antal, K., Emri, Zs. and Kardos, J. Synaptic transmission shapes dynamics of nonlinearly coupled neuronal oscillators in low- $[Mg^{2+}]$  induced seizures. *J. Neurophysiol.* submitted.

**17.00** Closing words by Prof. Heinemann

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