Study of the early, intracellular events of brain ischemia by fluorescent imaging techniques and *in vitro* ischemia models

Doctoral Theses

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1. Introduction

Here, I summarize the results of my doctoral dissertation which are related to the early, intracellular events of brain ischemia. Clinical name of the disease is stroke. During stroke, blood flow of the brain stops abruptly causing a cessation of supply of O_2, glucose and other nutrients. The high metabolic activity of the brain aggravates the damaging effect of stroke. According to the WHO (World Health Organization) definition, stroke is a cerebrovascular disturbance that causes neurological deficits at least for 24 hours or death in 24 hours.

Increased metabolic activity of the human brain, as compared to the other organs, is shown by the fact that the mass of human brain is only 2.5 % of whole body mass while it receives 15 % from the ejection fraction of the heart, 20 % and 25 % from the O_2 and glucose supply of the whole body.

Sudden stop of blood supply damages the brain tissue owing special biochemical properties (high energy consumption, low anaerob capacity, minimal glucose stores, glucose is the main energy source). Network of neurons and their intracellular signaling pathways also contribute significantly to the injury. Thus stroke may induce transient or permanent neurological deficits, disablement, or even death (mortality:~30 %). Stroke is the second most common cause of death and one of the most common causes of disablements (10-12 %) and is responsible for the 4 % of medical costs in the developed countries. Risk factors are endemic diseases like high blood pressure, diabetes mellitus, high cholesterol level, obesity, alcoholism, smoking, atrial fibrillation.

2. Aims

Aim of my work was the more accurate understanding of the cellular mechanism of ischemic cell damage. I investigated the initiator, activator and perpetrator factors participating in the cellular damage, namely the spatial and temporal changes in intracellular Na^+ ($[Na^+]_i$) and Ca^{2+} ($[Ca^{2+}]_i$) concentrations and reactive oxygen species (ROS) level. We performed our experiments on rat hippocampal slices because the *in vitro* model allows experiments with high spatial and temporal resolution, even at cellular level. Advantage of hippocampal investigations is that it constitutes in the vicinity a region highly vulnerable to ischemia (CA1) and a region relatively resistant to ischemia (dentate gyrus – DG). This allows us to examine the pathological factors of ischemia synchronously and to understand the mechanism of ischemia more accurately.

2.1. Investigation of the interaction of persistent Na^+ influx and the $[Ca^{2+}]_i$

Changes in $[Na^+]_i$ and $[Ca^{2+}]_i$ are important factors of the ischemia pathomechanism resulting in neuronal cell death. Since the homeostasis of both ions are tightly interconnected (voltage-gated ion channels, ligand-gated ion channels, Na^+-Ca^{2+} exchanger (NCX)), their changes influence the level of the other and the damaging effect of ischemia. However, the specific and multiple connection between the persistent, noninactivating Na^+ current ($I_{Na,P}$) and Ca^{2+} homeostasis, and the

details of the mechanisms and dynamics of $[Ca^{2+}]_i$ changes have not been well explored. Since the interconnection between neuronal $I_{Na,P}$ and Ca^{2+} homeostasis is complicated (determined by both electrical and ion concentration gradients), it is necessary to measure membrane potential (E_m), $[Na^+]_i$ and $[Ca^{2+}]_i$ in the same experimental paradigm that has never done before.

To examine and simulate the effect on $[Ca^{2+}]_i$ of the ischemia/hypoxia evoked $I_{Na,P}$, we perfused our slices with the $I_{Na,P}$ activator alcaloid, veratridine. Veratridine inhibits inactivation of voltage-gated Na^+ channels through their neurotoxin receptor binding site 2. Some antiepileptics (carbamazepine, phenytoin) with a protective effect in *in vitro* and *in vivo* ischemia have an identical binding site. Veratridine evokes $[Na^+]_i$ elevation and neuronal death both in neuronal cultures and hippocampal slices.

Aims:

1. Setting up intracellular Na^+ concentration measurement in acute brain slices.
2. Verification of veratridine-evoked persistent Na^+ influx in CA1 pyramidal cells of acute hippocampal slices.
3. Investigation of the mechanisms and dynamics of the persistent Na^+ influx evoked Ca^{2+} response.

2.2. Investigation of the spatial and temporal distribution of ischemic excitotoxicity-evoked ROS production

More and more data have accumulated about the ramifying damaging effect of oxidative/nitrosative stress (ROS/RNS; reactive oxygen and nitrogen species) but their distinct role in the pathomechanism of neurodegenerative diseases has not been well clarified yet. Understanding of the effects is often difficult because they are often summarized as oxidative/nitrosative stress or ROS/RNS while they differ significantly in their reactions (reaction kinetics, substrate availability), typical production places, timings and mechanisms.

Role of ROS/RNS is suggested in the ischemic damage of the ischemia vulnerable brain regions like hippocampal CA1 subfield. However, conclusion was based on indirect results as differences in ROS scavenging capacity or damaging effect of exogenous ROS application. A study that directly and simultaneously follows the endogenous ROS level changes with high spatial and temporal resolution in an ischemia vulnerable and relatively resistant brain region has not been done yet thus our main purpose was to do that. (Furthermore, we also aimed to investigate the type of ROS/RNS produced in another type of the neurodegenerativ diseases (Parkinson's disease) and in another brain region.)

Aims:

1. Setting up the *in vitro* ischemia model (OGD).
2. Setting up ROS measurement in acute hippocampal slices.
3. Demonstration of the layer-specific ROS response and its dynamics.
4. Determination of cellular damage.
5. Evaluation of the role of NMDA-R and NOS.
6. Determination of the ROS type produced in the *in vitro* model of Parkinson's disease.

3. Methods

3.1. Acute hippocampal and striatal slice preparation

16-21 days old or 280-320 g male Wistar rats were used to prepare acute hippocampal or striatal slices, respectively. Following decapitation, the brain was removed and placed in ice cold cutting solution (composition in mM: NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 0.5; $MgCl_2$, 5; NaH_2PO_4, 1.25; glucose, 10 pH = 7.4) that was continuously bubbled with 95% O_2 + 5% CO_2. 250-300 µm thick coronal slices were cut with a vibratome (Vibratome 1000). Slices were kept in artificial cerebrospinal fluid (ACSF; in mM: NaCl, 126; KCl, 2.5; $NaHCO_3$, 26; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.25; glucose, 10) bubbled with a mixture of 95% O_2 + 5% CO_2 (pH 7.4).

3.2. *In vitro* ischemia models

We applied two different models: 1. 10 µM veratridine perfusion for 1 min, at room temperature (2 ml/min), and 2. 10 min oxygen and glucose deprivation (OGD) at 36°C (3,5 ml/min) bubbled with 95 % N_2 + 5 % CO_2. In the OGD, glucose was replaced by saccharose (10 mM). O_2 tension of the ACSF was 722 Hgmm at 36°C in the perfusion chamber. O_2 tension of the OGD decreased to 52 Hgmm (ISO2; Oxygen Meter; World Precision Instruments).

3.3. Measurement of intracellular Ca^{2+} concentration

Hippocampal slices were loaded in ACSF supplemented with fura2/AM (5 µM, 50 min) and 0.025% (w/v) of Pluronic F-127 at room temperature .

Slices were continuously superfused with ACSF (2 ml/min; NaH_2PO_4 was omitted in experiments with $CdCl_2$) in an experimental chamber mounted on a Gibraltar BX1 platform (Burleigh) and viewed with a 40X water immersion objective (Olympus) on an Olympus BX50WI upright microscope. The dye was alternately illuminated (340 ± 5 nm and 380 ± 5 nm; Polychrome II monochromator, T.I.L.L. Photonics). The emitted light (510 ± 20 nm) was detected by a cooled CCD camera (Photometrics Quantix). Cell image intensities were background-corrected. Values of the $[Ca^{2+}]_i$ were calculated off-line: $[Ca^{2+}]_i = K_d \times F_{max380} / F_{min380} \times (R - R_{min}) / (R_{max} - R)$, where R is the actual ratio of emission intensity at 340 nm excitation to emission intensity at 380 nm excitation, R_{min} and R_{max} are the same ratios at 0 mM or saturating $[Ca^{2+}]$, respectively and F_{max380} and F_{min380} are the fluorescence intensities for 0 mM or saturating $[Ca^{2+}]$ at 380 nm excitation, respectively. The parameters K_d, F_{max380} / F_{min380}, $R_{min,}$ and R_{max} were determined experimentally.

To load the CA1 pyramidal cells by fura2 dextran, we pulled glass micropipettes (Sutter Instrument, P-87). Fura2 dextran was dissolved in BSA (1.5 %) then desiccated. On the day of loading, we dipped the tip of the micropipettes into the mixture of fura2 dextran and BSA dissolved in distilled water. We pickled the tip of the micropipettes into the stratum radiatum of CA1. We kept the slices at room temperature for 60 min to ensure dye loading. The dye was excited at 340 ± 5 and 380 ± 5 nm, the emitted light was detected at 510 ± 20 nm.

3.4. Measurement of intracellular Na^+ concentration

Acute hippocampal slices were loaded with SBFI/AM (10 µM, 60 min) at room temperature and then deesterified for 90 min. We added 0.05% (w/v) Pluronic F-127 to the loading solution. We illuminated our slices at 365 ± 5 nm where we could measure the highest ratio of Na^+-free (0 mM) to Na^+-bound (130 mM) fluorescence (intensity decreases in the entire spectrum as $[Na^+]$ increases). The emitted light was detected (510 ± 20 nm) by a cooled CCD camera as described above. Image intensities were exponentially-corrected (bleaching) after background subtraction. The $[Na^+]_i$ concentrations was calculated off-line: $[Na^+]_i = K_d \times (F - F_{min}) / (F_{max} - F)$, where F is the actual emission intensity at 365 nm excitation, F_{min} is the fluorescence intensity at the lowest $[Na^+]_i$ at 365 nm excitation and F_{max} is the fluorescence intensity at saturating $[Na^+]_i$ at 365 nm excitation. F_{max} and F_{min} values were determined *in situ* at the end of every individual experiment. We measured F_{min} after at least 15 min application of calibration media (0 mM Na^+, 155 mM K^+ and 10 µM gramicidin D (monovalent cation ionophore)). F_{max} was measured at the beginning of the perfusion of the same calibration media, as it depolarizes the cells and induces a massive Na^+ influx attributable to its high potassium content and the still high extracellular $[Na^+]$ that needs time to be washed out. K_d was calculated from nine-point *in situ* calibrations ($[Na^+]$ in mM: 0, 3, 6, 10, 20, 40, 80, 130, 155) made in CA1 pyramidal cells of the acute hippocampal slices. Calibration media contained (composition in mM) 0.6 $MgCl_2$, 0.5 $CaCl_2$, 10 HEPES, 10 glucose and 0.01 gramicidin D. The $[Na^+ + K^+]$ was equal to 155 mM, Cl^- was 30 mM and gluconate was 125 mM. Electrical field stimulations were delivered through two platinum wires (10 Hz, 50 shocks, 45 V/cm).

3.5. Measurement of electrophysiology

For whole-cell current-clamp recordings of CA1 pyramidal neurons 5-9 MΩ borosilicate based glass electrodes (1,2 mm OD; Harvard Instruments, March-Hugstetten, Germany) were filled with intracellular solution (composition in mM: K-gluconate, 125; KCl, 10; HEPES, 10; Di-Tris-salt phosphocreatine, 10; Na-GTP, 0,3; Mg-ATP, 4; NaCl, 10). Data acquisition and analysis were performed using a pClamp8 (Axon Instruments, Foster City, CA, USA).

3.6. Measurement of intracellular ROS

CM-H_2DCFDA (50 µg) was dissolved in 10 µl DMSO. Slices were loaded with CM-H_2DCFDA (45 µM, 45 min; 25°C; 0.005 % [w/v] Pluronic F127) in ACSF then deacetylated for 30 min. 5 mM probenecid (inhibitor of organic anion transporter) was added to improve dye retention. Slices were submerged and superfused with ACSF (3.5 ml/min) in an experimental chamber. Slices were viewed with a 10X objective (Olympus) excited at 488 ± 5 nm by the imaging set up described above. The emitted light was detected at 535 ± 25 nm. We set excitation intensity as low as possible and a red filter (RG665; Schott Filter Glass) was put over the illuminator. The image frame rate was always 6/min and exposure time was held constant for all experiments. This gave a reasonable time resolution with a reduced level of photooxidation. We studied these layers of the hippocampus: CA1 stratum pyramidale (SP), stratum radiatum (SR), stratum lacunosum (SL) and dentate gyrus (DG) stratum moleculare (SM), stratum granulosum (SG). Autofluorescence correction in every examined region of every individual hippocampal slice was made by point by point

subtraction of the respective average intensity values of identical experiments performed on unloaded slices (OGD, n=6 and OGD+ATZ+MS, n=5).

We measured intracellular superoxide ($O_2^{\bullet-}$) production by the $O_2^{\bullet-}$-selective dye hydroethidine (HEt). Acute striatal slices were loaded at room temperature (25 min, 60 μM). Slices were excited at 495 ± 5 nm, emitted light was detected at 605 ± 37,5 nm by a 10X objective mounted on the imaging set up described above.

3.7. Measurement of swelling

Ischemia induces cell swelling which dilutes the dye and decreases the intensity of fluorescence signals. In order to determine volume changes of the hippocampal layers directly we have developed a method to measure it in the same slices which were used for ROS experiments. Enlargement of layers in the x (direction of apical dendrites of pyramidal cells) and y (parallel to the pyramidal layer) axes on the transversal hippocampal slices were measured directly on the fluorescent images off-line. We drew rectangular regions of interest (ROIs) over the layers and enlarged them synchronously with swelling. Side-lengths of the rectangular ROIs were expressed in image pixel numbers. Based on the anatomical structure of CA1 and DG, i.e., shape and orientation of principal cells, the change of the "ROI body" on third axis (z) was assumed to be the same as on the y axis. Dividing the volume measured at the peak of the reoxygenation response (30^{th} min of the traces; $x_{30\ min}\ y_{30\ min}\ z_{30\ min} = V_{30\ min}$) with the volume before OGD (10^{th} min of the traces; $x_{10\ min}\ y_{10\ min}\ z_{10\ min} = V_{10\ min}$) gave the OGD- and reoxygenation-evoked relative swelling between these time points in each layer ($V_{30\ min}/V_{10\ min}$). Multiplication of the actual normalized fluorescence values with the average relative swelling of the same layer was used to correct the swelling-evoked dilution of CM-H_2DCFDA.

3.8. The pH-sensitivity of the fluorescence of the dye CM-H_2DCFDA

CM-H_2DCFDA was deacetylated by NaOH and the fluorescence of the dye was examined in buffers with different pH (MOPS: 7,1; 6,7; 6,4; acetate: 4,8; composition of buffers in mM: NaCl: 142; KCl: 2.5; $CaCl_2$: 2; $MgCl_2$: 2; NaH_2PO4: 1.25; glucose: 10; MOPS or acetate, 10).

3.9. Measurement of cell viability

Following 10 min OGD and 10 min reoxygenation, slices were fixed (4 % paraformaldehyde in 0.1 M PBS) and stained with propidium iodide (PI, 1.5 μM, 30 min). Slices were excited at 535 ± 25 nm. Images were acquired with a SPOT RT Color Camera (Diagnostic Instruments) viewed with a 2X objective (Nikon) on a Nikon Eclipse E600 microscope. Then the slices were permeabilized by 5 min incubation in 99.8 % methanol and reloaded with PI (maximal damage). Control slices were handled in parallel.

3.10. Data analysis and statistics

Veratridine: In the measurements of $[Ca^{2+}]_i$, $[Na^+]_i$ and E_m the "n" values show the number of cells, slices and animals (e.g., n of cells/slices/animals).

OGD-Ca^{2+}: $[Ca^{2+}]_i$ was characterized by fura2 ratio (I_{340nm} / I_{380nm}). The "n" value shows the number of cells. In every treatment group the results come from at least four animals.

OGD-ROS: Following autofluorescence correction, the baselines of the experimental traces were fitted with an exponential and then corrected. The "n" values show both the number of slices and animals. Statistical analysis were performed in the 7th min of OGD (OGD phase) and in the 10th min of reoxygenation (reoxygenation phase).

Data are presented as means ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's post-hoc test unless otherwise noted. Differences were considered significant at p<0,05. (* p<0,05; ** p<0,01; *** p<0,001).

4. Results

4.1. Interaction of the persistent Na^+ influx and the $[Ca^{2+}]_i$ elevation

4.1.1. Veratridine evokes $[Ca^{2+}]_i$ elevation in CA1 pyramidal neurons

Administration of veratridine (10 μM, 1 min) to CA1 pyramidal cells of acute hippocampus slices generated a biphasic Ca^{2+} response. The first peak was smaller ($peak_1$ = 241.5 ± 24.9 nM, $peak_2$ = 616.2 ± 68.9 nM; n = 38/19/18). The first and second peaks came 10.4 ± 1.0 and 80.3 ± 5.2 sec later than the beginning of the veratridine response, respectively. Resting $[Ca^{2+}]_i$ was 56.8 ± 3.9 nM (n = 182/71/54). Ca^{2+}-free media abolished $[Ca^{2+}]_i$ peaks, suggesting an extracellular source of $[Ca^{2+}]_i$ elevation (n = 8/5/5; $p < 0.05$, unpaired t-test).

We previously showed that 1 μM TTX (selective inhibitor of VGSCs) abolishes the $[Ca^{2+}]_i$ response. Since TTX interferes with both the direct (i.e., on the imaged cell) and indirect (through other connected neurons) action of veratridine, in order to determine the involvement of the indirect stimulatory effect, we inhibited the excitatory glutamate inputs. Combined application of the NMDA and AMPA/Kainate receptor inhibitors AP-5 (50 μM) and CNQX (30 μM) did not significantly influence the $[Ca^{2+}]_i$ peaks, indicating the dominance of the direct action of veratridine on the neurons ($peak_1$ = 319.5 ± 56.6 nM; $p > 0.05$; $peak_2$ = 894.6 ± 189.5 nM; $p > 0.05$; n = 15/6/4).

4.1.2. Veratridine induces depolarization and firing of neurons

Resting membrane potential of the CA1 pyramidal cells was -64.0 ± 3.9 mV (n = 7/7/5). Veratridine application (10 μM, 1 min) induced a spontaneous membrane depolarization (-56.6 ± 2.8 mV) and a high frequency action potential firing (33.0 ± 4.9 Hz in the 1st sec). This burst of APs was correlated with the appearance of the first $[Ca^{2+}]_i$ peak. Further depolarization (-39.9 ± 4.1 mV) caused a rapid decrease in AP frequency (9.0 ± 2.4 Hz; P1, 10th s, average time of the first $[Ca^{2+}]_i$ peak). Plateau potential reached -36.6 ± 3.7 mV (firing rate: 1.6 ± 0.7 Hz) at 80 s into the veratridine response (P2; average time of the second $[Ca^{2+}]_i$ peak). The depolarization plateau always finished abruptly.

4.1.3. Veratridine evokes a persistent Na^+ influx

Electric field stimulation (fs) evoked a TTX- and stimulus strength-dependent Na^+ spike in CA1 pyramidal cells. Compared to the fs-evoked response, veratridine treatment (10 μM, 1 min) induced a long-lasting, massive and monophasic $[Na^+]_i$

increase (persistent Na^+ influx; peak: 39.7 ± 8.6 mM, n = 14/6/4) that returned slowly to the baseline. The resting $[Na^+]_i$ was 7.3 ± 1.3 mM.

4.1.4. Components of the veratridine-evoked $[Ca^{2+}]_i$ elevation

We suggested that the depolarization and action potential firing opened the VGCCs. $CdCl_2$ (100 μM), the non-selective blocker of all types of VGCCs, abolished the first peak of $[Ca^{2+}]_i$ response ($peak_1$ = 28.8 ± 6.6 nM vs. veratridine alone; $p < 0.001$; n = 26/9/4). The effect of $CdCl_2$ on the second peak cannot be measured in our experimental model, because Cd^{2+} enters the cells through the opened VGCCs and intracellular Cd^{2+} increases fura2 fluorescence ratio. With the use of a cocktail of VGCC inhibitors that do not interfere with fura2 fluorescence we showed VGCCs role also in the second peak. The cocktail contained nifedipine (10 μM), an L-type Ca^{2+} inhibitor, the R- and T-type Ca^{2+} inhibitor $NiCl_2$ (100 μM) and ω-conotoxin-MVIIC (ω-CTx-MVIIC; 100 nM), the N- and P/Q-type VGCC inhibitor. The cocktail significantly reduced both peaks ($peak_1$ = 141.6 ± 18.5 nM vs. veratridine alone; $p < 0.05$; $peak_2$ = 212.2 ± 25.3 nM vs. veratridine alone; $p < 0.01$; n = 21/8/7).

The increase in $[Na^+]_i$ in our experiments (32.5 ± 8.57 mM) during veratridine application raised the possibility of $[Ca^{2+}]_i$ elevation resulting from the reversal of plasma membrane NCX. KB-R7943 (10 μM), an inhibitor of the plasma membrane NCX did not affect the first peak ($peak_1$ = 169.8 ± 17.2 nM vs. veratridine alone; $p>0.05$; n = 28/8/6) but significantly reduced the second peak ($peak_2$ = 265.0 ± 38.4 nM vs. veratridine alone; $p < 0.01$; n = 28/8/6), showing the role of NCX in the $[Ca^{2+}]_i$ elevation. Furthermore, we calculated the electrochemical driving force for Ca^{2+} movement through NCX (E_{NCX}) with high temporal resolution to see the dynamics of NCX activity. Resting E_{NCX} was -30.22 mV and remained in the negative range until the 5th s after the beginning of veratridine response, indicating a forward operation. At the 5th s, E_{NCX} exceeded zero and reached 0.99 mV. After the 5th s of veratridine response, i.e., during both the first and the second $[Ca^{2+}]_i$ peaks, E_{NCX} permanently resided in the positive range (NCX were operating in the reverse mode). Our result shows that a 5 s of pyramidal neuron firing is able to reverse the electrochemical driving force and the NCX activity. Operation of NCX in both forward and reverse mode during the first $[Ca^{2+}]_i$ peak may explain why KB-R7943 had no significant effect on this peak. Conversely, KB-R7943 significantly inhibited the second peak, when NCX was operating exclusively in the reverse mode and had a higher driving force.

We tested the role of mitochondria in two different ways. First we inhibited mitochondrial function by the application of CCCP (1 μM). As a protonophore, CCCP dissipates mitochondrial membrane potentials and decreases the electrochemical gradient, thus preventing Ca^{2+} influx (Ca^{2+} sequestration). When applied prior to veratridine, it altered the biphasic characteristic of the $[Ca^{2+}]_i$ elevation to a monophasic plateau and caused a significant increase of $[Ca^{2+}]_i$ during both phases ($peak_1$ = 544.8 ± 61.0 nM vs. veratridine alone; $p<0.001$; $peak_2$ = 1039.0 ± 154.06 nM vs. veratridine alone; $p<0.05$; n = 11/5/5). Second, we inhibited mitochondrial Ca^{2+} release via the combined administration of CGP-37157 (10 μM) and CsA (1 μM), specific inhibitors of the mitochondrial NCX and the mitochondrial permeability transition pore (mPTP), respectively. These inhibitors did not influence the first peak ($peak_1$ = 220.9 ± 27.3 nM vs. veratridine alone; $p>0.05$; n = 25/11/6) but significantly inhibited the second ($peak_2$ = 286.6 ± 43.1 nM vs. veratridine alone; $p < 0.05$; n = 25/11/6). Our results suggest that during the first peak, Ca^{2+} sequestration is

the dominant action, but mitochondrial Ca^{2+} release subsequently becomes relevant (second peak). Effect of CCCP during the second peak indicates that mitochondrial sequestration still exceeds mitochondrial Ca^{2+} release in our hands.

The ER, another important regulator of $[Ca^{2+}]_i$, may also influence the veratridine-evoked $[Ca^{2+}]_i$ elevation through Ca^{2+}-induced Ca^{2+} release (CICR). A major determinant of CICR is the filling state of ER Ca^{2+} stores. Empty ER Ca^{2+} stores prevent CICR. A simple way to empty ER Ca^{2+} stores involves the inhibition of ER Ca^{2+}-ATPase. We inhibited ER Ca^{2+}-ATPase by 1 μM thapsigargin. Thapsigargin did not affect the peaks of veratridine-evoked $[Ca^{2+}]_i$ elevation ($peak_1$ = 268.4 ± 38.2 nM; $p>0.05$; $peak_2$ = 768.5 ± 153.7 nM, both vs. veratridine alone; $p>0.05$; n = 18/5/3), suggesting the lack of contribution of ER Ca^{2+} stores.

4.2. Interaction between the intracellular ROS and ischemic excitotoxicity

4.2.1. OGD as an *in vitro* model of ischemia

We set up OGD, an *in vitro* ischemia model that mimics well the deficit of energy supply characteristic of ischemia *in vivo*. We tested the model by studying the changes in $[Ca^{2+}]_i$ because it has been well known that ischemia elevates its level. 10 min OGD evoked a biphasic $[Ca^{2+}]_i$ elevation in CA1 pyramidal cells at 36°C. The Ca^{2+} response was inhibited significantly by hypothermia (32°C), perfusion of Ca^{2+}-free solution or TTX (0,3 μM). This proves that OGD is an effective *in vitro* ischemia model in our system.

4.2.2. ROS measurement in acute brain slices with high spatial and temporal resolution

Measurement of ischemic ROS level changes with similar spatial and temporal resolution in acute brain slices has never been examined thus our task was setting up the method, testing the properties of CM-H_2DCFDA.

CM-H_2DCFDA can be easily oxidized by illumination thus we set excitation intensity as low as possible and used a red filter during visualization of the slices. The image frame rate was always 6/min and exposure time was held constant for all experiments. This gave a reasonable time resolution with a reduced level of photooxidation.

Fluorescence of the fluorescein-based compounds (e.g. CM-H_2DCFDA) may show a pH-dependent quenching (fluorescein's pK_a=6,4). Thus we measured the pH-dependent fluorescence change of CM-DCF in buffers having different pH. We did not find any quenching in the pH range 7,1-6,4 (pH 7.1, 100 ± 1.7 %, n=12; pH 6.7, 96 ± 4.4 %, n=4; pH 6.4, 97 ± 1.8 %, n=4; pH 4.8, 69 ± 1.4 %, n=4). This result is in accordance with the lower pK_a (4.8) value of the chlorinated fluorescein-based compounds (e.g. CM-H_2DCFDA).

CM-H_2DCFDA loaded acute hippocampal slices performed a decrease in fluorescence at 36°C. Inhibition of the organic anion transporters by probenecid (5 mM) successfully inhibited the decrease in fluorescence resulting from lower dye retention.

We administered H_2O_2 and sodium nitroprusside (SNP) to test the sufficiency of loading and sensitivity of CM-H_2DCFDA in our preparation at 36°C. Both drugs increased fluorescence in all layers which showed a proper dye loading. The effect of SNP was much larger; 10 mM H_2O_2 was needed to produce a response comparable

with that of 200 μM SNP. H_2O_2 oxidizes the dye directly. SNP, considered to be a nitric oxide ($NO^{\bullet}$) donor that also generates superoxide ($O_2^{\bullet-}$) in biological systems and forms peroxynitrite ($ONOO^-$), which makes the $NO^{\bullet}$ and $O_2^{\bullet-}$ insensitive dye, CM-H_2DCFDA, fluorescent more efficiently than H_2O_2.

All of these careful procedures by that we set up and validated our method ensure that our measurements are reliable and artifact-free and we can make the correct conclusions.

4.2.3. OGD-evoked increase in ROS level is layer-specific in acute hippocampal slices

We measured the fluorescence of all layers of CA1 and DG in CM-H_2DCFDA-loaded hippocampal slices. We applied 10 min OGD as an *in vitro* ischemia model. After about 4 min of OGD, the fluorescence started to decrease in all layers of both regions. When oxygen and glucose supplies were restored (reoxygenation), the fluorescence signal started to increase in all CA1 and the DG, SM layers. In contrast, signal intensity over DG, SG continued to decline. In the 10^{th} min of reoxygenation the difference between the fluorescence intensities of DG, SG vs. any other layers was significant ($p<0.001$, $n=9$). When no OGD treatment was applied (control; $n=7$), fluorescence remained unchanged in all layers during the entire length of experiments.

What may be the explanation for the decrease in fluorescence of the layers?

Ischemic insults cause swelling of cells, which results in the dilution of intracellular dye and a decline in fluorescence signal intensity. We investigated how OGD influences the volume (V) of the hippocampal layers to exclude false interpretation of the region-specific ROS results. In the 10^{th} min of reoxygenation ($V_{30\ min}$) and at the beginning of OGD ($V_{10\ min}$) treatment on four slices (out of nine) we measured off-line the volume of the layers of hippocampal slice. Taking the ratio of the two values ($V_{30\ min}$ /$V_{10\ min}$) we could calculate a relative volume enlargement, and from that the swelling (ΔV) of the layers. Magnitude of swellings were in the 10-17 % range in the SR and SL layers of CA1 and DG layers and could reach the 30 % in the CA1, SP layer. Multiplying the normalized fluorescence values in the 10^{th} min of each layer with the average relative volume enlargement of the corresponding layer gives the swelling corrected ROS level changes. These results clearly showed that reoxygenation induced a significant increase in ROS levels in CA1 and DG, SM layers while in DG, SG there was no change compared to the control conditions. Swelling-corrected ROS level changes during reoxygenation and their comparisons to control experiments confirmed that the region specific differences can not be attributed to swelling. They are real ROS level differences.

Because the effect of drugs on OGD-evoked ROS production was always compared to the OGD effect alone in the same hippocampal layer and none of the drugs influenced swelling significantly during the length of the experiments ($p>0.05$, $n=2-5$), correction of fluorescence with swelling was not performed for the rest of the experiments.

Intracellular acidification could not be the reason of the fluorescence decrease, because, as mentioned above, in the 7.1-6.4 pH range that is the range of pH_i (intracellular pH) changes in response to OGD in acute hippocampal slices, there is no significant quenching.

4.2.4. OGD does not cause detectable cell death during the experiments

We tested the potential changes in the viability of the hippocampal layers attributable to OGD and reoxygenation during the experiments. 10 min OGD and reoxygenation (time-window of our experiments; n=4) did not cause any detectable cell damage as compared to the controls (n=4).

4.2.5. Antioxidant enzymes scavenge ROS during the OGD phase

To test the significance of the antioxidant enzyme system and explore the production of ROS attributable to OGD, we inhibited catalase and glutathione peroxidase. In the presence of enzyme inhibitors (ATZ, 10 mM and MS, 10 mM, respectively), the fluorescence started to rise right after the onset of OGD in every layer, however, the inhibitors were ineffective during the reoxygenation phase (n=6).

4.2.6. OGD-evoked ROS production is NMDA-R- and NOS-dependent

Excitotoxicity participates in the initiation of the cascade of ischemia which includes excess release of Glu, generation ROS through NMDA-R, release of $O_2^{\bullet-}$ from the mitochondria, activation of nitric oxide synthase (NOS). $O_2^{\bullet-}$ and $NO^{\bullet}$ produced form $ONOO^-$ which is highly capable to oxidize CM-H_2DCFDA.

In our hippocampal slices, the NMDA-R antagonist (AP-5; 50 µM; n=6) and the non-specific NOS inhibitor (L-NAME; 100 µM; n=7) significantly decreased the OGD-evoked fluorescence rise during the reoxygenation phase in all CA1 layers and DG, SM, while they did not have effects in the DG, SG. Increase in ROS production during OGD, revealed by ATZ+MS was also inhibited by AP-5 (n=5) and L-NAME (n=5) in the principal cell layer of the CA1 but not of the DG. During reoxygenation AP-5 and L-NAME similarly showed an inhibitory action in CA1, SP and had no significant effect in DG, SG in the presence of ATZ+MS. Perfusion of D-NAME (100 µM; inefficient L-NAME isomer; n=4) was inefficient.

For selective inhibition of neuronal NOS, we administered TRIM (100 µM n=4) to the slices. TRIM inhibits neuronal NOS with an IC50 of 28.2 µM and endothelial NOS with an IC50 of 1057.5 µM [53]. TRIM only had a significant inhibitory effect in CA1, SR and SL.

4.2.7. Rotenon produces $O_2^{\bullet-}$ but not $ONOO^-$ in the striatum

We examined the ROS producing effect of rotenone (5 µM; an *in vitro* model of Parkinson's disease), a mitochondrial complex I. inhibitor in acute striatal slices. Rotenone (n=3) and its solvent (DMSO, 0,01 %; n=3) had no effect on the fluorescence of the dye, CM-H_2DCFDA which is mainly sensitive to $ONOO^-$. However, rotenone increased the fluorescence of Het (n=3), the $O_2^{\bullet-}$-selective dye. DMSO had no effect (n=2). Our results show that $O_2^{\bullet-}$ production occurs in the rotenone model but we were unable to confirm $ONOO^-$ production.

5. Conclusions

I. Investigation of the interaction between persistent Na^+ influx, important in the early phase of ischemia, and $[Ca^{2+}]_i$ elevation by the veratridine model:

1. We successfully set up the $[Na^+]_i$ measurement in CA1 pyramidal cells of acute hippocampal slices.
2. We showed first in CA1 pyramidal cells of acute brain slices that the inhibition of voltage-gated Na^+ channel inactivation by veratridine evokes a persistent Na^+ influx. Veratridine also evoked firing of action potentials and sustained depolarization.
3. The interaction between persistent Na^+ current activated by noninactivating voltage-gated Na^+ channels and $[Ca^{2+}]_i$ has not been examined in such a complex way yet. We have discovered the sources of persistent Na^+ influx-evoked biphasic $[Ca^{2+}]_i$ elevation and described their activation as a function of time. The components of Ca^{2+} response are the voltage-gated Ca^{2+} channels, plasma membrane Na^+-Ca^{2+} exchangers and mitochondria. The Ca^{2+} stores of the endoplasmic reticulum do not contribute to the response. At beginning of the Ca^{2+} response the Na^+ influx depolarizes the cell membrane, the voltage-gated Ca^{2+} channels are activated, and the Ca^{2+} entering the cell is sequestered by the mitochondria (first peak). Parallel with further $[Na^+]_i$ elevation Ca^{2+} enters the cell also through the reversal of the plasma membrane Na^+-Ca^{2+} exchangers and is both sequestered to and release from the mitochondria (second peak).
4. Mechanisms that interconnect persistent Na^+ influx, an important initiator of the early phase of ischemia, and $[Ca^{2+}]_i$ elevation may play a role in the ischemic neuronal damage. This allows us to indicate new, neuroprotective drug targets.

II: Investigation of the interaction between ischemic excitotoxicity and ROS production by the OGD model:

1. We set up the 10 min OGD in acute brain slices which is an appropriate *in vitro* ischemia model because the evoked $[Ca^{2+}]_i$ elevation is inhibited by the decrease in temperature and voltage-gated Na^+ channel activity.
2. We set up first in the literature ROS measurement with high spatial and temporal resolution in acute brain slices with CM-H_2DCFDA and hydroethidine.
3. We demonstrated by the new method that the *in vitro* ischemia evokes ROS level increase in the ischemia vulnerable CA1 region but not in the relatively resistant dentate gyrus. At the border of the vulnerable and resistant regions (dentate gyrus, molecular layer), ROS level also elevates. Our results support the possible role of ROS in the ischemic vulnerability of CA1 region.
4. ROS production starts early at the very beginning of the *in vitro* ischemia.
5. ROS level increase during *in vitro* ischemia is scavenged by the antioxidant enzymes, the catalase and the glutathione peroxidase, but they are inefficient during reoxygenation. It means that the protective effect of the antioxidant enzymes prevail only at the very beginning of ischemia, later it is exhausted.
6. NMDA receptor and nitric oxide synthase-dependence of ROS production, together with the ROS-sensitivity profile of CM-H_2DCFDA known from the literature and proven by us, shows that peroxynitrite is certainly produced in response to *in vitro* ischemia.
7. In the time-window of our experiments, we could not detect cell death, thus our *in vitro* ischemia model is rather a model of delayed cell damage, the region of penumbra. The typical experimental time-window of acute brain slices does not allow us to verify delayed cell damage.

8. ROS profile of distinct neurodegenerative diseases is diverse: superoxide is produced in the *in vitro* model of Parkinson's disease, but we were unable to detect peroxynitrite production. Contrary to this, in the *in vitro* model of ischemia peroxynitrite is certainly produced. The differences in ROS profile may influence essentially the course of the disorders.

III: Components of ischemia have multiple, sometimes even indirect connections with each other, they can interact through different pathways as a function of the space and the time thus we may imagine the patomechanism of ischemia as a network of the components. If we interpret the patomechanism of ischemia as a network then we can conclude to the followings from our direct results:

1. Patomechanism of ischemia is multifactorial, we also investigated some of the factors including $[Na^+]_i$, $[Ca^{2+}]_i$, ROS level, membrane potential changes and swelling, thus we can not expect significant neuroprotective effect from single-target treatments. New way of research may be the simultaneous treatment of the critical points of the network.
2. Significance of the separate, cellular components may vary with the progression of ischemia, namely as a function of temporal changes, thus new approaches fitting to the phases of ischemia should be found.
3. Ischemia vulnerability of some brain regions and relative resistance of others, namely the spatial differences, reveal that the ischemia network itself may also be divided to parallel subnetworks which may differ in their critical points and dynamics. This may hamper the finding of a uniform neuroprotective treatment.
4. Simultaneous investigation of the cellular parameters with high spatial and temporal resolution is necessary for the more accurate understanding of the ischemia patomechanism, for the mapping of the network.

6. List of Own Publications

6.1. List of Publications Connected to the Dissertation

1. **Fekete A.**, Vizi E. S., Kovacs K. J., Lendvai B. and Zelles T. (2008) Layer-specific differences in reactive oxygen species levels after oxygen-glucose deprivation in acute hippocampal slices. Free Radic Biol Med 44, 1010-1022.
2. Milusheva E., Baranyi M., Kittel A., **Fekete A.**, Zelles T., Vizi E. S. and Sperlagh B. (2008) Modulation of dopaminergic neurotransmission in rat striatum upon in vitro and in vivo diclofenac treatment. J Neurochem 105, 360-368.
3. **Fekete A.**, Franklin L., IkemotoT., Rózsa B., Lendvai B., Vizi E. S., Zelles T. Mechanism of the persistent sodium current activator veratridine-evoked Ca2+ elevation: implication for epilepsy. (under revision)

6.2. List of Publications Not Connected to the Dissertation

1. Halmos G., Horvath T., Polony G., **Fekete A.**, Kittel A., Vizi E. S., van der Laan B. F., Zelles T. and Lendvai B. (2008) The role of N-methyl-d-aspartate receptors and nitric oxide in cochlear dopamine release. Neuroscience 154, 796-803

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