Multi-modal imaging of anoxic and peri-infarct depolarizations

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- Farkas E., Obrenovitch T., Bari F. (2009) Aging and chronic cerebral hypoperfusion alters the evolution of potassium-induced cortical spreading depression (CSD) in rats, Acta Physiologica Hungarica, 97(1), 100-101.
- Farkas E., Obrenovitch T., Institóris Á., Bari F.(2009) Effects of early aging and cerebral hypoperfusion on spreading depression in rats, Neurobiology of Aging, available online, doi:10.1016/j.neurobiolaging.2009.10.002
- Farkas, E., Bari, F., Obrenovitch, T.P. (2010) Multi-modal imaging of anoxic depolarization and hemodynamic changes induced by cardiac arrest in the rat cerebral cortex. Neuroimage, 51(2), 734-742.
- Farkas, E., Obrenovitch, T.P., Bari, F. (2010) Az agytraumával vagy stroke-kal járó agykérgi kúszó depolarizáció és peri-infarktus depolarizáció pathofiziológiai jelentősége. Vascularis Neurologia. 2(3), 62-67.

A KUTATÁS SORÁN ELÉRT EREDMÉNYEK NYILVÁNOSSÁGA, KONFERENCIA-RÉSZVÉTELEK:

Konferencia-előadások:

- Farkas, E., Obrenovitch, T.P., Bari, F. Effect of aging, with or without chronic hypoperfusion, on potassium-induced cortical spreading depression (CSD) in rats. 11th Meeting of the Co-operative Study of Brain Injury Depolarisations (COSBID), Heidelberg, Germany, April 22-24, 2009.
- Obrenovitch, T.P., Chen, S., Farkas, E. Dual imaging of cortical spreading depression and associated cerebral blood flow changes by combining voltage-sensitive dye and laser speckle contrast methods. 11th Meeting of the Co-operative Study of Brain Injury Depolarisations (COSBID), Heidelberg, Germany, April 22-24, 2009.
- Farkas, E., Obrenovitch, T.P., Bari, F. Az öregedés hatása a kísérletesen kiváltott tovaterjedő agykérgi depolarizáció (CSD) kinetikájára. A Magyar Élettani Társaság LXXIII. Vándorgyűlése, Budapest, Hungary. August 27-29, 2009.
- Farkas, E., Bari, F., Obrenovitch, T.P. Multi-modal imaging of anoxic depolarization and hemodynamic changes induced by cardiac arrest in the rat cerebral cortex. 12th Meeting of the Co-operative Study of Brain Injury Depolarisations (COSBID), Barcelona, Spain, May 5-8, 2010.
- Bere, Z., Kulmány, A., Obrenovitch, T.P., Bari, F., Farkas, E. Peri-infarktus depolarizáció vizsgálata többkomponensű képalkotó eljárással patkányban. A Magyar Élettani Társaság LXXIV. Vándorgyűlése, Szeged, Hungary. June 16-18, 2010.

Konferencia-poszter:

- Farkas, E., Bari, F., Obrenovitch, T.P. multi-modal imaging of anoxic depolarization and hemodynamic variables during forebrain ischemia in rats. 9th World Congress for Microcirculation, Paris, France; September 26-28, 2010.
- Farkas, E, Bari, F., Obrenovitch, T.P. Multi-modal imaging of membrane potential and hemodynamic changes induced by cardiac arrest and subsequent anoxic depolarization in the rat cerebral cortex. IBRO International Workshop, Pécs, Hungary; January 21-23, 2010.
- Bere, Z., Kulmány, A., Obrenovitch, TP., Bari, F., Farkas, E. Characterization of periinfarct depolarisation (PID) with multimodal imaging method during global forebrain ischemia in rat

cerebrocortex. The 13th Annual Meeting of the Hungarian Neuroscience Society (MITT), Budapest, Hungary; January 20-22, 2011.
- Bere, Z., Kulmány, A., Obrenovitch, TP., Bari, F., Farkas, E. Characterization of global cerebral ischemia-induced peri-infarct depolarization (PID) with multimodal imaging in the rat brain. XXVth International Symposium on Cerebral Blood Flow, Metabolism and Function (Brain '11), Barcelona, Spain; May 24-28, 2011.

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Konferencia: 7th Forum of European Neuroscience, July 3-7, 2010, Amsterdam, The Netherlands
A szimpózium szervezői, címe: Bari, F., Farkas E.: Emerging mechanisms in neurodegenerative disorders - The role of spreading depression (CSD).
A meghívott előadás szerzője, címe: Farkas, E.: Aging and chronic cerebral hypoperfusion alter the pattern of CSD in the rat.

Felkérés könyvfejezet írására:
Editors: Lauritzen, M., and Bari, F., (2011), Springer, New York.

A pályázatban előirányzott nemzetközi együttműködés:
Tiho Obrenovitch Professzor (University of Bradford, Bradford, United Kingdom) három alkalommal látogatott Szegedre (2009. április: 3 hét, 2009. május: 1 hét, 2010. szeptember: 2 hét). A módszertani fejlesztések mellett az SZTE ÁOK Élettani Intézet szervezésében szemináriumot tartott a "Why did I get away from neurotoxicity?" címmel (2009. április 9., SZAB Székház, Szeged).

Habilitációs eljárás:
A pályázatban szereplő munka részét képezte a témavezető nyilvános, tudományos habilitációs előadásának (2010. március 23.)

AZ ELÉRT TUDOMÁNYOS EREDMÉNYEK RÖVID ISMERTETÉSE:

Studies with stroke models showed that recurrent spreading depression (waves of cellular depolarization that propagate slowly across grey matter regions) occur in the vicinity of the ischemic core and possibly contribute to lesion progression. Peri-infarct depolarization (PID) evolves also in patients suffering from stroke or traumatic brain injury.

Our overall aim is to improve our understanding of the genesis and propagation of stroke-related PID, and of their contribution to infarct expansion and maturation. The proposed research relies on novel imaging techniques applied to relevant rat models.

Phase I

We have reported previously that, in otherwise physiological conditions, spreading depression (SD) induced experimentally with the topical application of KCl solution in a rat closed cranial window preparation can be visualized directly by using a fluorescent, voltage sensitive (VS) dye. The synchronous monitoring of cerebral blood flow (CBF) changes is essential for the study of PID, because the degree and heterogeneity of the ischemic insult, and PID-related CBF responses can be accurately followed. Therefore, we developed the coupling of the VS dye method to laser speckle contrast (LSC) imaging, a well validated method to image local CBF changes with a high temporal and spatial resolution.

In stroke models, where PID occur spontaneously near the ischemic core, marked hemodynamic variables - such as changes in cerebral blood volume (CBV) and hemoglobin (Hb) deoxygenation - are expected to interfere significantly with VS dye imaging. In the first

phase of the proposed research, we conducted studies that provide the scientific basis necessary for accurate interpretation of VS dye images captured from ischemic rat brains. Building on our previously established technology (i.e. dual imaging of membrane potential and CBF), additional images of cerebral cortex reflectance under green (540-550 nm) and red (620-640 nm) illumination were captured. The former was expected to reflect changes in local CBV, and the latter the level of Hb oxygenation, within the tissue under study (Fig. 1).

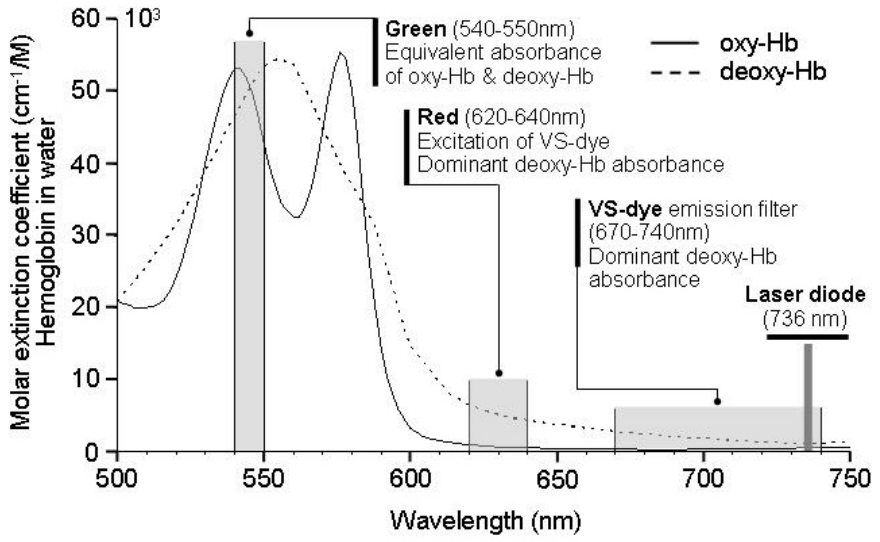

Figure 1. Specifications of the different illuminations used in these experiments (green and red LED; laser diode), of the VS dye fluorescence emission filter, and their relation to the 'absorbance' spectra of oxy-Hb and deoxy-Hb. Values for deoxy-Hb and oxy-Hb molar extinction coefficients were taken from, http://omlc.ogi.edu/spectra/haemoglobin (i.e. 'compiled by Scott Prahl using data from: W. B. Gratzer, Med. Res. Council Labs, Holly Hill, London; and N. Kollias, Wellman Laboratories, Harvard Medical School, Boston').

Using two cameras and carefully selected illuminations, multiple image sequences of the rat cortex were captured through a cranial window during cardiac arrest and subsequent anoxic depolarization (AD) (n=10). This terminal cerebral ischemia model was chosen for two reasons: (i) the induction of cardiac arrest and subsequent AD is highly reproducible and leads to uniform, comparable changes over individual experiments; and (ii) the considerable hemodynamic changes offer the possibility to detect potential artifacts of hemodynamic origin on the VS dye signal with high confidence.

Four synchronous image sequences were obtained from each experiment to reveal the changes in cellular membrane potential (i.e. AD) and associated hemodynamic responses in the cortical region under study, and to identify potential inter-relationship(s) between optical signals. The different image sequences, each acquired under carefully selected illumination and image capture conditions, allowed us to collect information on changes in the following variables: (i) cellular membrane potential, using a VS dye whose fluorescence increases with decreasing membrane potential ; (ii) CBV in the microvascular bed of the brain parenchyma using the intrinsic optical signal (IOS) evoked with 540-550 nm green light illumination (i.e. isosbestic point of optical hemoglobin absorption; illumination wavelength at which absorbance is identical for both deoxygenated and oxygenated Hb – deoxy-Hb and oxy-Hb, respectively; (iii) Hb deoxygenation in the microvascular bed of the brain parenchyma based on the IOS recorded under 620-640 nm red light (identical to the VS dye excitation wavelength) illumination at which deoxy-Hb absorbs much more than oxy-Hb, and (iv) cortical cerebral blood flow (CBF) with laser speckle contrast (LSC) imaging (Fig 1).

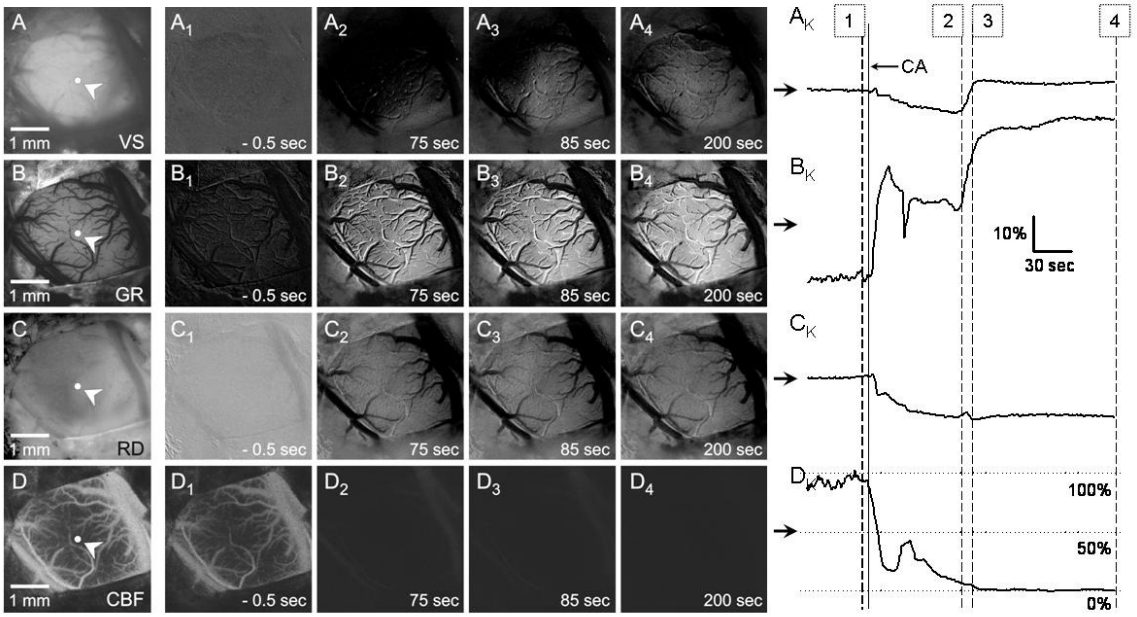

Figure 2. Representative image sequences, and kinetics of changes in the 4 variables under study, recorded early after cardiac arrest (CA). A1-A4, A_K, Voltage sensitive (VS) dye fluorescence intensity; B1-B4, B_K, change in intrinsic optical signal (IOS) acquired under green illumination (GR, 540-550 nm) expected to reflect, primarily, changes in cerebral blood volume (CBV); C1-C4, C_K, change in IOS under red illumination (RD, 620-640 nm), for which hemoglobin oxygenation level appears to be the main contributor; and D1-D4, D_K, laser speckle contrast (LSC) imaging of cerebral blood flow (CBF). In the left pictures, the small circles (pointed to by arrowheads) show the position of a representative area of interest, used to extract the synchronous kinetics of changes from each of the four image sequences. The numbers on dotted vertical lines of the kinetics (A_K, B_K, C_K, D_K) correspond with the index of the presented images. The solid vertical line over the kinetics plots indicates when cardiac arrest was induced.

Careful analysis of the obtained image sequences (Fig. 2) has revealed that, with acute ischemia, the associated dramatic changes in brain hemodynamics become significant interferents for VS dye signals. More specifically, we propose that decreasing blood oxygen tension (i.e. increasing deoxy-Hb concentration within the cerebrovascular space) can result in an additional, progressive absorption of both VS dye excitation light and resulting emitted fluorescence, leading to a reduction of the recorded VS dye signal intensity. The nature of these interferences would not impair the identification of evolving PID, neither the characterization of their temporal distribution after ischemia induction, but would make the interpretation of the kinetics of individual PID-related VS dye signatures more difficult. Through this study, we have also found that VS dye fluorescence is not markedly altered by CBV changes, as long as there is not a marked depletion of oxy-Hb (i.e. marked conversion to deoxy-Hb that absorbs much more of the red light used for VS dye excitation).
In summary, we have shown that imaging of VS dye fluorescence can be combined – in addition to LSC imaging of CBF - with that of CBV and Hb oxygenation. Such a multi-parametric strategy favors the analysis and interpretation of the VS dye signature of any depolarization recorded during experimental ischemic insults, and the subsequent evolution of tissue damage. In addition to a better understanding of the acquired signals, this technology makes it possible to examine the coupling between membrane potential changes and hemodynamic variables (i.e. CBF, CBV and Hb deoxygenation) with high spatial and temporal resolution.

Potential applications of this new technology are multiple and important. In addition to the genesis of cellular damage after an acute insult to the brain, it should help advance our knowledge in migraine, epilepsy, cerebral blood flow pharmacology, and neurovascular

coupling (currently the fundamental basis of neuroimaging in humans) under both normal and pathological conditions.

(*Reference for more details:* Farkas, E., Bari, F., Obrenovitch, T.P. (2010) Multi-modal imaging of anoxic depolarization and hemodynamic changes induced by cardiac arrest in the rat cerebral cortex. Neuroimage, in press. IF: 5.694; JCR 2008.)

Phase II

The established technology presented above was applied for the imaging of PID in the second phase of the proposed research, making use of a rat global cerebral ischemia model. Global cerebral ischemia was induced in halothane-anesthetized rats by the combination of permanent, bilateral occlusion of the common carotid arteries and hypovolemic hypotension (i.e. withdrawal of 5-10 ml blood to lower mean arterial pressure to 40 mmHg). Image sequences were captured through a closed cranial window created on the parietal bone, above the frontoparietal cortex. As described above, image sequences of the 4 synchronous optical signals representing membrane potential (VS dye method), CBV (green reflectance), Hb oxygen saturation (red reflectance) and CBF (LSC imaging) were obtained (Fig. 3).

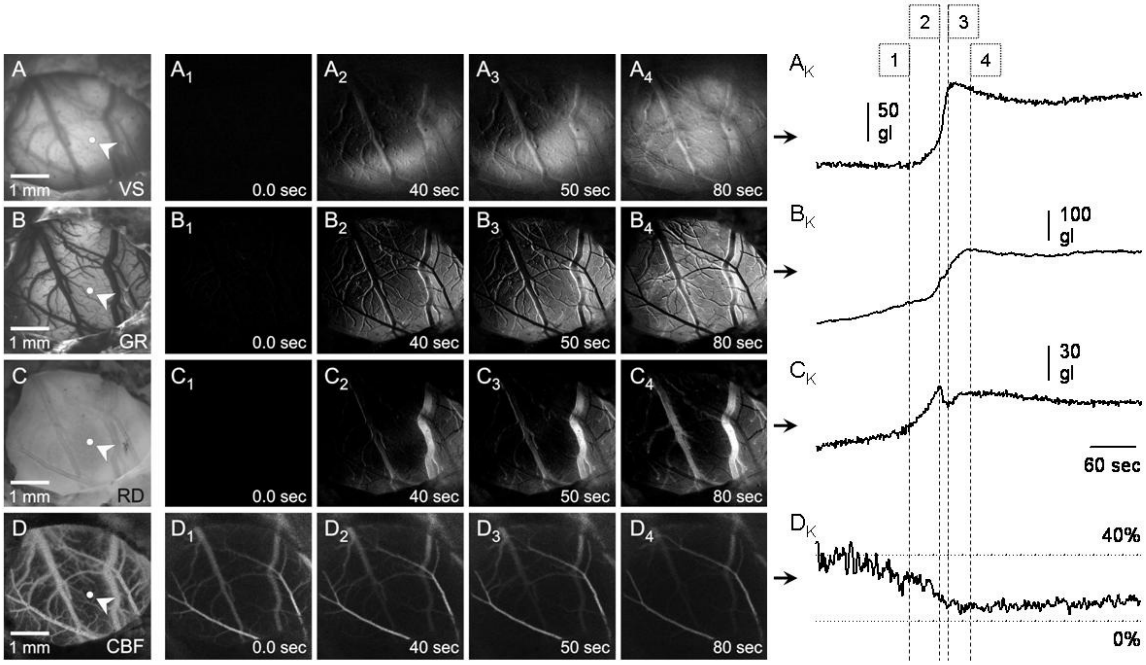

Figure 3. Representative image sequences, and kinetics of changes in the 4 variables under study, recorded during the evolution of a spontaneously occurring PID after global cerebral ischemia induction. A1-A4, A_K, Voltage sensitive (VS) dye fluorescence intensity; B1-B4, B_K, change in intrinsic optical signal (IOS) acquired under green illumination (GR, 540-550 nm) expected to reflect, primarily, changes in cerebral blood volume (CBV); C1-C4, C_K, change in IOS under red illumination (RD, 620-640 nm), for which hemoglobin oxygenation level appears to be the main contributor; and D1-D4, D_K, laser speckle contrast (LSC) imaging of cerebral blood flow (CBF). In the left pictures, the small circles (pointed to by arrowheads) show the position of a representative area of interest, used to extract the synchronous kinetics of changes from each of the four image sequences. The numbers on dotted vertical lines of the kinetics (A_K, B_K, C_K, D_K) correspond with the index of the presented images.

The experiments fell into 3 categories based on whether depolarization was elicited successfully, and if so, during which phase of the experimental procedures depolarization evolved. The following experimental groups were thus established: (i) depolarization did not evolve during the period of recording (n=4), (ii) depolarization emerged at a time during blood withdrawal carried out to enhance forebrain ischemia by hypovolemic hypotension (n=6), and (iii) depolarization occurred after forebrain ischemia had been completed by

hypovolemic hypotension (n=3). The characteristics of the observed PID (n=9) are presented in Table 1.

Table 1. Features of PID events observed in our study.

Feature	Observation	Number of cases
Occurrence of depolarization in the course of an experiment	No	4
	Yes	9
Direction of propagation	From frontolateral to caudomedial	7
	From caudolateral to frontomedial	1
	Double wavefront (i.e. mixed of the above)	1
Rate of propagation	2.8±0.22 mm/min	9
Recovery of membrane potential (repolarization)	No (AD-like)	7
	Yes (CSD-like)	2
Mean arterial pressure at the onset of depolarization	41.2±3.7 mmHg	6
Local cerebral blood flow at the onset of depolarization	43.4±4.9 %	9

Abbreviations: AD: anoxic depolarization , CSD: cortical spreading depression.

The CBF responses associated with the various types of depolarization were not uniform across cases. In most experiments, CBF decreased further with the onset of a wave of depolarization (inverse neurovascular coupling); in a few other experiments, a slow, long lasting hyperemia was observed (Table 2).

Table 2. Features of cerebral blood flow (CBF) responses associated with various PID events observed in our study.

Recovery of membrane potential (repolarization)	CBF response	Magnitude of CBF response (% of baseline)	Number of cases
No (AD-like)	No change	-	1
	CBF decrease	-16.0±3.7	6
Yes (CSD-like)	CBF increase	7.1±2.6	2

Abbreviations: AD: anoxic depolarization , CBF: cerebral blood flow, CSD: cortical spreading depression.

In summary, we have applied our technology to visualize PID in a rat model of global cerebral ischemia successfully. With this, experimental approach, we can identify the focus, rate and direction of propagation, and the kinetics of PID, as well as the ischemic threshold for spontaneous PID elicitation. In global cerebral ischemia, PID appears to generate at the lower limit of the autoregulatory range of CBF. PID originates at a focus probably located in an area with high vulnerability to ischemia, and propagates similar to that known for cortical spreading depression, whether or not the PID involves the recovery of membrane potential. CBF responses associated with PID most often display inverse neurovascular coupling.

Phase III

In the third phase of the research, a focal cerebral ischemia model was established to detect PID generation. Focal cerebral ischemia was induced in halothane-anesthetized rats by infusing polyethylene fluorescent microspheres (d=45-53 µm, dose: about 2000 in 0.6 ml vehicle) over 2 min into the left common carotid artery. In a few experiments, the development of ischemic infarct was confirmed 24h later by 2,3,4-triphenyltetrazolium chloride (TTC) staining of 1mm thick brain slices. In other experiments, image sequences were captured through a closed cranial window created on the parietal bone, above the frontoparietal cortex. As described above, image sequences of the 4 synchronous optical signals representing membrane potential (VS dye method), CBV (green reflectance), Hb

oxygen saturation (red reflectance) and CBF (LSC imaging) were obtained for 60 min after ischemia induction.

TTC staining has demonstrated that the infusion of microspheres successfully induced ischemia in the parietal cortex and the striatum, which is in agreement with previous reports applying the same method.

Multi-modal imaging revealed that 3 PID generated typically within 60 min following the onset of microsphere-induced ischemia. Ischemia was less severe than in case of the global ischemia model applied in Phase II of the project. All the observed PID involved the rapid recovery of membrane potential, reminiscent of cortical spreading depression (CSD). The CBF responses associated with PID were transient hyperemia, also typical of CSD. Green reflectance demonstrated transiently increased CBV with each PID, which is in agreement with the CBF signal (i.e. functional hyperemia). Red reflectance displayed inconsistent variances with the observed PID: with the first PID in each experiment, the signal dropped in a transient fashion, which suggests Hb desaturation, while increased red reflectance was coupled with later PID, which indicates Hb saturation with oxygen.

In summary, microsphere infusion-induced focal ischemia that was monitored for 60 min after ischemia onset produced PID that showed features typical of CSD. This is in contrast with what we have observed during global ischemia, during which PID occurred without repolarization. The kinetics of early PID and associated CBF responses after focal ischemia induction, therefore, seem not to worsen the hemodynamic status of the ischemic tissue, and appears not to contribute to tissue damage.

The research has obtained 3 goals:

- First, a multi-modal, live imaging strategy was established to monitor membrane potential variations and associated hemodynamic changes directly.
- Second, the technology designed and developed in our lab was applied in a global ischemia model, in which PID proved to be deleterious to the tissue and are proposed to contribute to infarct evolution.
- Third, our method was used to detect PID in a focal ischemia model, in which early PID appeared to be harmless to the brain, and are suggested not to worsen ischemia outcome.

Conclusion:

Our observations modify the view held so far, that PID are invariably damaging to the brain tissue. Instead, PID that are not followed by the recovery of membrane potential and involve inverse neurovascular coupling (i.e. decreased CBF) are suggested to be destructive, while PID with repolarization and associated transient functional hyperemia appear not to be harmful to the nervous tissue.