MULTIMODAL IMAGING OF ISCHEMIA INDUCED BRAIN DEPOLARIZATIONS AND ASSOCIATED FLOW TRANSIENTS IN RAT GLOBAL FOREBRAIN AND MULTIFOCAL ISCHEMIA MODEL

Zsófia Bere MD

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University of Szeged, Department of Medical Physics and Informatics

PhD Program:

Neuroscience

Program director: Prof. Gábor Jancsó D.Sc.

Supervisor: Eszter Farkas PhD

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1. Introduction

Stroke is the third most common cause of death in Hungary and Europe, coming only after heart disease and cancer. Much of the primary damage in the acute phase of ischemic stroke may prove irreversible despite prompt intervention, yet the management of secondary pathophysiological processes is more feasible and of fundamental importance to improve the prospect of successful recovery.

The neurological deficit caused by stroke, thus, obviously depends upon the severity and nature of the initial injury, but also upon secondary and progressive deleterious events, such as spreading depolarization (SD). Research on SD considerably accelerated in the last fifteen years due to the highly significant observation that spontaneous SDs occur consistently and repeatedly in the injured human brain, and contribute to lesion progression. This justifies research efforts to understand the pathophysiological role of SD in ischemic brain injury.

In 1944, A.P. Leao described that direct electrical stimulus of cerebral cortex causes transitional suppression of spontaneous cortical activity seen as transient depression of the electrocorticogram (ECoG). The phenomenon was detected first at the recording site nearest the stimulus, and spread with 3-6 mm/min to the more distant electrodes. Based on these observations, the phenomenon was named “spreading depression of cortical activity”. Subsequent electrophysiological studies that recorded direct current (DC) potential demonstrated, that suppression of the ECoG coincided with a near-complete, sustained depolarization of a critical population of cortical neurons. The depolarization is caused by the breakdown of ion equilibrium across neuronal membranes, propagates across the cortex, considerable osmotic changes follow the ionic redistribution, which manifests in swelling of neurons and dendritic spines. Due to sustained depolarization, neuronal membrane channels producing action potential are inactivated.

Since restoration of transmembrane potential after SD relies on ATP-dependent ion pumps, the obvious metabolic demand requires rapid increases in oxygen and glucose delivery, which is mediated by neurovascular coupling. Neurovascular coupling is a vital feed-forward control mechanism, which adjusts local cerebral blood flow (CBF) to the energy requirements of activated neurons. The resulting functional hyperemia increases the delivery of oxygen and essential nutrients, affords effective washout of waste-products, and maintains an optimal homeostatic balance in the local neural microenvironment. SDs are coupled with typical changes in local CBF. In the rat - and

most probably in human - the physiological pattern of the SD-associated CBF response includes four sequential components: (i)an initial, brief hypoperfusion; (ii)a marked, transient peak hyperemia; (iii) a less obvious late hyperemia; and (iv)a sustained hypoperfusion also known as spreading oligemia or post-SD oligemia. After peak hyperemia, CBF stabilizes below baseline level and persists at oligemic values for 30-60 min.

For decades, SD was speculated to develop only under experimental conditions. Although, Leao specifically linked SD to migraine aura and cerebral ischemia, further studies proved pathophysiological relevance of SD in patients of malignant stroke, subarachnoid hemorrhage and traumatic brain injury too. SDs have long been suspected to aggravate ischemic injury. In particular, SDs have been considered to recruit electrically silent but viable penumbra tissue into the non-viable core region, and expanding the injury, moreover a linear correlation between the total number or cumulative duration of recurrent SDs and the infarct volume was also proved. Under ischemic conditions, spontaneous SDs occur within minutes. However, the duration of depolarization may vary greatly, dependent on the metabolic status of the tissue. If oxygen and nutrient supply is undisturbed, the depolarization is short transient, and repolarization is complete within a minute. In case a mismatch between energy demand and supply evolves (i.e. due to perfusion deficit), the efficacy of the sodium pump will decrease due to the restricted availability of ATP, and repolarization will be delayed, coincident with a persistent but reversible depression of cortical activity. This is typical of the ischemic penumbra. Finally, severe shortage of oxygen and energy substrates as it occurs after cardiac arrest or in the core region of focal ischemia impedes repolarization, and leads to terminal depolarization. The ability of neurons to repolarize indicates viability, while terminal depolarization is a clear sign of neuronal death. Taken together, the duration of depolarization can be indicative of the metabolic crisis, and is attributed a predictive value as to injury outcome.

The mechanisms behind the injurious potential of SD are still being investigated. At present, the deficiency of the SD-associated CBF response is accepted to mediate neurodegeneration, because the insufficient CBF response sustains the state of depolarization and delays repolarization by depriving the tissue at risk of essential nutrients required to maintain ionic balance across neuronal cell membranes. In contrast to the hyperemic response related to SD in healthy tissue, impaired neurovascular coupling under ischemic conditions induces severe vasoconstriction, and leads to marked,

prolonged hypoperfusion, which spreads together with SD. Such events will deepen the perfusion deficit, which will give rise to further depolarization events. Finally prolonged depolarization with spreading ischemia extends cortical necrosis, and enlarges the ischemic core.

The characterisation of SD waves traditionally relies on the recording of extracellular DC potential from the cerebral cortex, which is an accurate and suitable approach, but lacks spatiotemporal resolution. Our group has developed and validated a method for direct, live imaging of K^+-induced SD in a rat closed cranial window preparation, using a voltage-sensitive (VS) dye. VS dyes are chemicals that bind to cellular membranes and whose fluorescence increases with reduced cellular transmembrane. The two-dimensional spatial resolution feature of VS dye imaging is uniquely appropriate to monitor the site of initiation of SDs, and their subsequent route of propagation, which cannot be predicted in the ischemic cortex. Next, the VS dye method was extended by coupling it to laser speckle contrast (LSC) blood flow imaging. LSC imaging provides information on the velocity of blood cells, and thereby data on relative blood flow changes. Finally, the multimodal imaging approach has been completed by the analysis of intrinsic optical signals (IOS). IOS evoked with 540 to 550 nm green light illumination allows the estimation of local cerebral blood volume (CBV): decreasing green IOS intensity (i.e., decreasing intensity of reflected light) is accepted to correlate with increasing CBV. IOS under 620 to 640nm red light illumination is indicative of the oxygen saturation of hemoglobin: decreasing red IOS intensity represents decreasing hemoglobin saturation; in case hemoglobin is totally desaturated, red IOS intensity changes follow that of CBV.

The rat MCAO model for stroke is a widely used method to study the pathogenesis of focal brain infarcts. As an alternative to MCAO, the microsphere-induced, multifocal stroke model in rats has been utilized to mimic the formation of small, permanent cerebral emboli and multiple infarct areas. The model is relevant for small embolic infarcts in patients, complications with cardiac or pulmonary arteriovenous shunts (e.g., cerebral emboli), and possibly multiinfarct dementia.

SDs are generally considered to be initiated at sharply demarcated ischemic border zones in focal ischemia. However, it remains unclear whether depolarizations may also occur in non-terminal global ischemia, i.e. conditions in which no distinct ischemic core and penumbra can be delineated, and thereby no marked heterogeneous ischemia be anticipated. The investigation of depolarizations in global ischemia may be relevant to human conditions such as hypovolemic shock, cardiac arrest or complex cardiac surgery,

which involve a risk of reduced CBF and related poor neurologic outcome.

2. Aims

Because the insufficiency of the CBF response to SD has been suspected to mediate SD-related injury, our major goal was to identify various types of hemodynamic responses to SD with relation to the severity of cerebral ischemia, and to determine a possible association between depolarization types and associated hemodynamic responses. Further objectives included the characterization of specific conditions that favor SD elicitation and propagation and of patterns of SD propagation over cortical areas with various severity of ischemia.

3. Materials and methods

Inhalation anesthesia was performed on adult male Sprague-Dawley rats. Body temperature was kept between 37.1- 37.4^{o}C using a heating pad feedback-controlled by a rectal probe.

Preparations for the induction of incomplete global forebrain ischemia: rats (n=13, 300-450 g) were anaesthetized. The left femoral vein and artery were cannulated for the monitoring of mean arterial pressure (MAP), and latter induction of hypovolemic hypotension. Surgical sutures were loosely looped around the common carotid arteries (CCA) for permanent bilateral occlusion (2VO) during image acquisition.

Preparations for the induction of microembolic stroke: rats (n=13, 300-450 g) were anaesthetized. The right femoral artery was cannulated for the monitoring of MAP. The external and internal carotid arteries (ECA, ICA) were ligated proximally. A polyethylene tube was inserted into the right common carotid artery and was advanced gently into the ICA. The catheter was secured to the vessel with surgical sutures.

In both models, after the surgical steps above, animals were placed in a stereotactic frame in the prone position. A cranial window was prepared at the center of the right parietal bone. A doughnut shape ring of methylmethacrylate resin was built around the craniotomy, incorporating a perfusion inlet and outlet, the inlet connected to a peristaltic pump. The chamber was filled with artificial cerebrospinal fluid, and the dura carefully dissected before sealing the cranial window with a glass coverslip. Unless otherwise

specified, the closed chamber was continuously perfused with aCSF at a rate of 25μL/min. The pressure in the closed cranial window was kept near the physiologic intracranial pressure. In each experiment, the VS dye was dissolved in aCSF and circulated over the exposed cortex, than surplus dye was washed out. Subsequently, data acquisition was initiated and a 10-min baseline was acquired in both models.

Incomplete global forebrain ischemia model: 2VO was produced by pulling and fixing the surgical thread looped around the common carotid arteries at both sides. The successful induction of 2VO was confirmed by an increase in MAP due to baroreceptor reflex. Cerebral ischemia was enhanced 10 min later by hypovolemic hypotension, produced by the gradual withdrawal of blood via either the femoral venous or arterial catheters, until MAP decreased to 40 mmHg, then stabilized between 35-45 mmHg by mobilizing reserve blood. Each experiment was terminated 20 min later by either induction of sudden cardiac arrest (i.v. injection of 0.5 ml of 1M KCl), or halothane overdose. Recording was pursued 10 min after the induction of cardiac arrest.

Microembolic stroke model: Multifocal ischemia was induced by injecting 45-53 um diameter microspheres (2000 particles/ 0.6 ml vehicle) via the ICA cannule. Optical signals were continuously captured for 60 min after ischemia induction. Each experiment was terminated by halothane overdose, and 10 min biological zero was recorded after cardiac arrest.

A multi-modal imaging system, designed previously in our laboratory was applied to reveal SD-related changes in transmembrane potential and the coupled hemodynamic response in the cortical area under study. At carefully selected illumination and strict timing of triggering-image optical signals related to changes in membrane potential and hemodynamic variables were visualized with a good spatiotemporal resolution. To detect membrane potential changes, a VS dye that binds to cell membranes, and whose fluorescence increases with reduced cellular membrane potential was used. To capture VS fluorescence images, the cortical surface was illuminated with a flashing red high-power light emitting diode (LED). Images were captured with a monochrome CCD camera, attached to a stereomicroscope. Cerebral blood flow (CBF) was monitored at the exposed cortex with laser speckle contrast (LSC) imaging. The raw laser speckle images were captured by a second CCD camera, identical to that used for VS dye imaging, and attached to the same stereomicroscope. CBF maps were calculated with Image-Pro-Plus from the obtained raw speckle images. To reveal information about cerebral blood volume (CBV) and hemoglobin (Hb) deoxygenation in the observed area, intrinsic optical

signals (IOS) were captured with Camera 2. CBV in the cortex was assessed using IOS evoked at 540 to 550 nm green light illumination (100 ms illumination and 100 ms exposure): decreasing green IOS intensity (i.e., decreasing intensity of reflected light) was expected to correlate with increasing CBV. In addition, changes in extracellular volume associated with SD were expected to contribute to variations in green IOS signal intensity. Hemoglobin deoxygenation was recorded with the help of IOS under 620 to 640 nm red light illumination (100 ms illumination, 10 ms exposure): decreasing red IOS intensity represented decreasing Hb saturation; in case Hb was totally desaturated, red IOS intensity changes followed that of CBV.

Pial arteriolar diameter was measured on selected arteriolar segments in the incomplete global forebrain ischemia model. Measurements were carried out on green ISO images.

Local changes in VS dye fluorescence, CBF and IOS at red and green illumination with time were determined by positioning selected areas of interest (AOI) on the image sequences in such a way that visible blood vessels were avoided. The position of AOI was random for general data assessment, and carefully selected when the focus of an SD or distinct SD evolution was analyzed (microembolic stroke model). The average gray level within an AOI was measured in individual frames constituting each image sequence, and plotted against the corresponding time of data acquisition.

To confirm ischemic lesion evolution, 7 male Sprague-Dawley rats (360 to 430 g) were anesthetized 24 hours after microsphere embolism, and decapitated. Brains prepared for slicing and were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) than stored in 4% paraformaldehyde. Because TTC staining may not be sensitive enough to detect microinfarcts, stained sections were latter embedded in paraffin, sectioned to 5 μm-thick slices, stained with hematoxilyyn/eosine (HE), and examined with optical microscopy.

4. Results

Changes in the acquired variables with ischemia induction

After 2VO onset, baseline MAP suddenly dropped to 70±4 mmHg and then immediately increased to 107±6 mmHg, which is considered to be the sign of intact baroreceptor reflex. MAP then persisted at a higher level until the initiation of hypovolemic hypotension. With gradual blood withdrawal, MAP reduced progressively, then persisted at 40±4 mmHg until the termination of the experiment CBF varied in a

similar fashion and with a minimal latency with respect to MAP. Following a brief perfusion drop immediately after 2VO onset, CBF increased parallel with MAP and persisted at around 78%, then reduced with blood withdrawal (39±5 %) and settled on near this value.

Transient drop of pial arteriolar diameter of selected vessels was detected due to 2VO onset; from a baseline of 80±5 μm, arteriolar diameter reduced to 60±7 μm, and subsequently dilated to 77±6 μm.

Green IOS suddenly increased (7.50±0.7 gl%) immediately after 2VO onset, in accordance with the marked reduction of CBF. A similar positive shift in green IOS occurred in the course of the development of hypovolemic hypotension (6.87±1.4 gl %). Red IOS dropped with 2VO onset (-5.86±0.8 gl%), and also with the initiation of hypovolemic hypotension (-4,58±0.9 gl%).

Embolisation (i.e. ischemia induction) was considered successful, if the presence of microsphere could be confirmed in pial arterioles revealed by the cranial window. Immediately after microsphere infusion, CBF dropped distal to the occlusion in selected pial arterioles. CBF in the cortical parenchyma revealed by the cranial window showed a heterogenous reduction. CBF dropped below 50% in 8% of the cortical area; was maintained between 50-90% about ~ 61% of the cortex, and stayed above 90% in around 30% of the visualized area. In order to confirm that microembolisation did cause brain infarction, TTC stained brain slices prepared 24 h after ischemia induction were investigated.

Characterization of spreading depolarizations and associated hemodynamic responses

In global model, experiments fell into 3 categories based on whether depolarization was elicited successfully, and if so, during which phase of the experimental procedures SD appeared; (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=3) and (iii) depolarization occurred after forebrain ischemia had been completed by hypovolemic hypotension (n=4). No SD emerged under 2VO alone, or during the 10 min between 2VO and blood withdrawal. Average time to SD appearance with respect to the initiation of hypovolemic hypotension was 4.6±0.8 min.

A single SD was detected in each experiment, which invaded the cranial window predominantly from the fronto-lateral corner of the observed area and progressed to caudo-medial direction (n=8 of 9 experiment), in one case, propagation was retrograde (i.e caudo-lateral fronto-medial). Average propagation rate was 2.8±0.2 mm/min.

Based on the kinetics of the wave front and recovery of transmembrane potential, three SD types were established:

Persistent/terminal SDs (n=4): sudden, profound depolarization, without recovery of transmembrane potential during the observation period typical of the ischemic core, or depolarization seen after cardiac arrest in the rat. CBF decrease was sustained in these cases. Intermediate/prolonged SD waves (n=3): sudden depolarization, and delayed recovery of transmembrane potential coupled with transient CBF reduction. Transient SD waves (n=2): transient depolarization, with immediate recovery of transmembrane potential. This SD type was typically associated with no flow response i.e. CBF stabilized at the pre-event level. IOS changes occurred simultaneously with SD waves. The intensity of both red and green IOS was suddenly increased with transient and intermediate SD occurrence

In multifocal ischemia study, 31 SDs were recorded in 7 experiments. All the observed SDs appeared to be related to ischemia, since they occurred ischemia induction. The number of SDs during the period of observation (i.e., 1 hour) ranged from 2 to 12 events per experiment. The foci of five SDs were seen within the cortical area revealed by the cranial window; all the other SDs invaded the area from a distant origin.

Foci of five SDs were identified within the cranial window in four different rats. All the other depolarization waves originated from further cortical areas. The VS dye signal extracted at the proper SD focus indicated three different SD types similar to those observed in the global forebrain ischemia model above short, transient SD (n=3), intermediate SD (n=1) and persistent SD (n=1). Intermediate and persistent waves converted to short transient waves and propagated in radial manner to further cortical regions. The CBF signal taken at carefully selected AOIs positioned at the SD focus and at increasing distances from the focus indicated a gradual conversion of the SD-associated CBF response as SD propagated. Local CBF measured shortly before SD evolution was the lowest at the focus of the latter SD and increased with the distance. At the SD focus, classic hyperemic response failed to evolve; either minor hyperemia (n=2), or no flow response (n=3) were detected with depolarization events, irrespective of the type of the SD. The magnitude of SD-related hyperemia increased with the distance from the SD

focus. Cerebral blood flow returned to the pre-SD level after the passage of the hyperemic response.

The majority of the detected SDs displayed short, transient changes of membrane potential and invaded the area from a distant origin. Most of the SD waves originated from the frontal-frontolateral site (n=19), 5 SD events originated from lateral edge of the window, and 2 SD waves travelled from the caudal direction. The site of SD entry to the cranial window was not conserved within individual experiments, implying multifocal SD origin in the ischemia model used.

Several SDs gradually extinguished over their course of propagation through the observed area. In these cases, CBF was relatively homogeneous within the field of view at the time of SD elicitation. Typically, hyperemic CBF response was coupled with SD waves. Peak hyperemia was the highest at the site where the wave front entered the visualized area, and became gradually less pronounced. The changes in green and red IOS evolved parallel with the shifts in VS dye fluorescence and CBF: the SD-related decrease in green and red IOS became gradually smaller over the course of SD propagation.

In 2 experiments, it was clearly seen in videos that several SDs curved around patches of the cortex as they propagated. Terminal depolarization was not detected in the regions avoided by SDs during image acquisition. In these particular experiments, CBF in the cortex estimated right before SD elicitation showed areas of higher and lower values ranging between 40% and 100% of baseline

Hyperemic CBF response to SD-s was typically accompanied by a decrease in green IOS as the sign of increase in CBV (increasing amount of hemoglobin absorbs more green light). Red IOS changes related to SD appeared to be biphasic; the following phases were established: (i) an early increase in signal intensity (Hb saturation) coincident with the initial phase of CBF elevation, (ii) a latter, sharp decrease in red IOS below baseline (Hb desaturation), the start of which preceding the peak of hyperemia. Based on the dominance of either of the two phases, the following types of red IOS signatures were defined: Type 1 dominant initial phase (n=5) Type 2 equal magnitude of both phases (n=7) Type 3 dominant later phase (n=12). In the single case when hypoperfusion rather than hyperemia evolved with SD, red IOS showed a monophasic, marked reduction (i.e. Hb desaturation)

5. Discussion

Ischemia-induced SD waves in hypoxic-ischemic brain injury contribute to the extension of tissue damage and predict worse neurological outcome. The complexity of the phenomenon still require appropriate animal models and sophisticated methods to provide a comprehensive picture about the evolution of depolarization waves and neurovascular coupling in cerebral ischemia.

In our experiments, we characterized the evolution, propagation and kinetics of spontaneous depolarization waves and the coupled hemodynamic variables in the ischemic rat cerebral cortex. Further we identified ischemic perfusion patterns that favor SD occurrence. Finally, we recognized various types of neurovascular coupling with SD in global forebrain and multifocal cerebral ischemia. Going beyond classic one point electrophysiological measurements and perfusion monitoring our multimodal imaging technique enabled the simultaneous detection and visualization of ischemia- and SD-related changes in transmembrane potential and the coupled hemodynamic response (i.e. local cerebral blood flow, blood volume, hemoglobin saturation) in a cortical area at high spatiotemporal resolution.

Our global forebrain ischemia model was used to show the effect of prolonged, stepwise ischemia development achieved by carotid occlusion in combination with persistent hypotension. This approach enabled the examination of the compensatory capacity of the circulation and exhaustion of these mechanisms: shortly after 2VO onset, transient elevation of MAP was detected, which was accepted as a sign of intact baroreceptor reflex. The sharp drop in CBF after 2VO onset confirmed the evident cessation of the anterior blood supply to the forebrain. The initiation of systemic hypotension caused a further decrease of CBF which followed MAP reduction. This, and the low level of CBF after completion of hypovolemic hypotension demonstrated, that the autoregulatory capacity of the cerebral circulation had been exhausted. Increasing green IOS intensity with ischemia onset indicated mass hemoglobin amount/CBV reduction in the examined cortical tissue, while the simultaneous decrease in red IOS intensity with 2VO and the later induction of hypovolemic hypotension reflected hemoglobin desaturation.

In the second study, the multifocal microsphere-induced stroke model modified to our specific purposes was applied to induce multiple ischemic foci in the brain. In our experiments, the perfusion deficit immediately after embolization and during the early

phase (i.e. the first hour) of ischemia was similar to that previously described in MRI study. Clear signs of ischemia-related tissue damage, extended cortical and subcortical infarctions were identified in TTC stained brain slices 24 hours after ischemia induction. Flow distribution was irregular, heterogeneous due to the unpredictable pattern of microsphere capture in vessels. Whereas CBF reduction was immediate, the mean CBF drop in the field of view after embolization was not severe compared to global forebrain ischemia, where CBF decrease was homogenous, and more profound after ischemia completion.

In our global forebrain ischemia study, only one SD emerged 16-17 min after 2VO- during or after the completion of hypovolemic hypotension. No further SDs was detected during the observation period. The latency of depolarization development with respect to the onset of the ischemic insult in global forebrain ischemia as shown here is suggested to be the result of a gradual progression of ischemia, in contrast with a sudden insult (i.e. abrupt vascular occlusion).

In contrast, multifocal ischemia gave rise to numerous (2-12) SDs over the hour following ischemia induction. Higher CBF level at SD generation indicates, that the level of perfusion deficit alone does not predict SD elicitation. We suggest that conditions other than the degree of perfusion deficit must also be involved; especially when ischemia is heterogeneous, sharp gradients of extracellular K^+ may promote SD elicitation, even in the face of relatively moderate local CBF reduction.

In global forebrain ischemia, the focus of SD elicitation fell out of the cortical region covered by the cranial window, and depolarization events propagated without attenuation. Frontolateral propagation suggested that SDs were generated either in the frontolateral cortex, or arrived at the cortex from deeper structures more sensitive to ischemia. Alternatively, SD may originate in the striatum, and propagate to the cerebral cortex through an anterior route (i.e. claustrum and nucleus accumbens), as demonstrated earlier since the striatum contains a mixed pool of cells with different susceptibility to ischemia compared to the cortex with relative uniform cytoarchytecture.

In multifocal ischemia, the number of SDs recorded in each experiment and localization of SD focus showed quite some variation. The foci of five SDs were seen within the cortical area revealed by the cranial window, but the site of their origin was not a typical ischemic core region. Our observations confirm that the focus of SD does not necessary overlap with the ischemic core. It is also possible that non-terminal SD in ischemic tissue does not cause immediate injury; nonetheless, the reestablished

membrane potential may not guarantee ultimate neuronal survival. Indeed, the impact of recurrent SDs that appear harmless alone can be additive together, and their increasing total duration corresponds with worse neurological outcome. The present results also revealed that CBF before SD elicitation was the lowest, and the peak of SD-associated hyperemia was smallest at the focus of SDs, as compared with other sites more distant to the focus. These observations imply that in tissue with uniform structure (i.e., rodent cortex), SD is elicited at a site where the reduction in CBF reaches a critical threshold, and subsequently, neurovascular coupling is the least efficient to support functional hyperemia. Here, we show that the duration of SD (time between depolarization and repolarization) tends to be prolonged at the proper focus compared with sampling sites more distant to the focus, and the rate of SD decelerates with the increasing distance from the focus. The rate of SD propagation is predicted to increase when (1) extracellular K^+ removal is hampered, (2) the extracellular space is smaller (cell swelling), and (3) extracellular K^+ and/or glutamate concentrations are increased.

A major advantage of our experimental approach is that the kinetics of SD and the associated CBF response can be directly correlated. The duration of SD itself has been proposed to determine the duration of SD-related hyperemia, because longer duration of the DC shift coincided with longer hyperemia. Since the recovery of resting membrane potential and thus normal electrical activity of the nervous tissue requires the activity of the ATP-consuming Na^+ pump, the return of CBF to baseline after hyperemia may be postponed by the continuing energy need, reflected by the longer duration of the electric silence with SD. On the other hand, the longer duration of the SD-related spreading ischemia was suggested to delay repolarization, as nutrient supply necessary for repolarization does not match the need of the tissue.

In our global ischemia paradigm, repolarization after SD was either prolonged, or no recovery of transmembrane potential was visualized, and only few of the SD events proved to be short transient SDs. At the same time, none of the SDs were coupled with physiological, hyperemic flow response. In the ischemic brain, the CBF response to SD is more dominated by vasoconstrictive elements, leading to diminishing hyperemia and more prevalent hypoemia. In the most severe form, the hypoemic element completely outweighs hyperemia, and turns into spreading ischemia. In summary, delayed repolarization or longer SD duration, as well as the insufficiency of the associated CBF response are clear signs of energy crisis in the tissue, and reflect the inability of ion pumps to re-establish resting membrane potential. Moreover, persistent depolarization or

continuing spreading ischemia indicate irreversible energy crisis and predicts consequent neuronal damage. In our multifocal ischemia model, SDs were short transient, and the SD related CBF response was invariably hyperemic, albeit the hypoemic elements of the response (i.e. initial brief hypoperfusion and prolonged oligemia) remained undetectable. We speculate that both the initial, brief decrease in CBF and the final prolonged oligemic component remained obscure during ischemia, because CBF shortly before SD occurrence was already low due to the created perfusion deficit. The diverse kinetics of changes in red IOS intensity (representative of Hb saturation) with transient hyperemia denotes various metabolic consequences of SDs in the ischemic cortex. Because the Type 1 to 3 red IOS intensity changes were all coupled to transient hyperemia, and the types of red IOS signature were unrelated to the magnitude of hyperemia, the analysis of the kinetics of hyperemic CBF responses alone appears not to be sufficient to draw conclusions about the metabolic consequences of SD.

The multifocal ischemia study provided an excellent opportunity to follow SDs along their course of propagation over tissue of heterogeneous metabolic status. We observed that some propagating SDs gradually diminished over their course of traveling. We propose that a diminishing SD may propagate against an extracellular ionic gradient that does not support depolarization to evolve (possibly decreasing K^+ levels as a result of enhanced reabsorption, or increasingly higher Mg^{2+} concentrations), and brings the SD to a gradual halt. Aletrnatively, hampered water movement through glial aquaporin channels was proposed to counteract the propagation of SD, which may be another potential mechanism to bring SD to cessation.

A few SDs in our experiments avoided a distinct region of the visible cortex. In contrast with the diminishing waves presented above, these SDs were not extinguished gradually, but sharply curved around an area showing the lowest CBF in the field of view, and thus propagated with an irregular wave front. According to the best of our knowledge, an SD does not invade a bulk of tissue if (i) the area has depolarized terminally (therefore subsequent depolarization cannot occur) or (ii) the area is suffering of ongoing epileptic activity. Primary infarct evolution in the area avoided by SDs was not seen in our experiments (i.e., signature of potential terminal depolarization was not acquired), therefore infarct maturation cannot be responsible for the obstruction of SD propagation. Focal seizures are known as potential consequences of ischemic brain injury and microembolization in the rat has been shown to induce seizures, but the methods applied here did not allow seizure detection to justify or exclude seizure activity. All

things considered, presently we can only claim that the area not being able to substantiate SD propagation was of the lowest perfusion within the field of view, but was presumably not infarcted.

6. Conclusions

Simultaneous imaging of changes in membrane potential and hemodynamic variables during global forebrain ischemia revealed, that sustained and transient hypoperfusion occurred in response to persistent and intermediate depolarization, respectively, whereas short, transient depolarization produced no clear flow response. Under less severe perfusion deficit produced in a multi-focal cerebral ischemia model, hyperemic CBF responses were coupled to short, transient SDs. In addition, hyperemic responses to SDs were shown to be associated with various kinetics of Hb saturation during ischemia, despite the similar amplitude and duration of hyperemia. Accepting that inverse coupling (e.g. hypoperfusion evolving in response to SD) jeopardizes the integrity of the nervous tissue, hyperemic CBF responses alone may have low predictive value as to the metabolic crisis during SD propagation in the tissue. In order to evaluate the metabolic impact of SDs with hyperemic CBF responses, hemoglobin saturation, tissue lactate level or tissue pH must be assessed.

The results of the global forebrain ischemia experiments may be relevant to human conditions such as hypoxic–ischemic brain injury as a consequence of cardiac arrest or hypoxia during surgery or critical illness. At the same time, microsphere-induced recurrent SDs in the rat cerebral cortex indicate that SDs could evolve in small embolic infarcts in patients, complications with cardiac or pulmonary arteriovenous shunts (e.g., cerebral emboli), and possibly multi-infarct dementia. The potential pathogenic role of SDs in these conditions is still to be clarified.