Aus der Klinik für Neurologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Electrocorticographic characterisation of clusters of spreading depolarisations in a rat model and in humans - a translational approach from bench to bedside

zur Erlangung des akademischen Grades
Doctor of Philosophy in Medical Neurosciences
(PhD in Medical Neurosciences)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Ana Isabel Oliveira Ferreira
aus Braga/Portugal

Gutachter: 1. Prof. Dr. med. J. Dreier

2. Prof. Dr. Alon Friedman

3. Prof. MD, PhD R. M. Pluta

Datum der Promotion: 24 February 2012

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Preliminary Remarks

This synopsis comprises the three peer-reviewed publications that I contributed to within the scope of my PhD thesis. The structure and extent of the synopsis follows the PhD guidelines of the International Graduate Program "Medical Neurosciences". In the following the three publications are abbreviated as Study 1 (Oliveira-Ferreira *et al*., 2010), Study 2 (Dreier *et al*., 2009) and Study 3 (Jorks *et al.*, 2011), respectively. For further details of each study: introduction, methods, results and discussion, please, see the complete publications in section "Publications" starting on page 25.

Zusammenfassung

Die verzögerte zerebrale Ischämie gilt als eine der wichtigsten und schwerwiegendsten Komplikationen nach aneurysmatischer Subarachnoidalblutung. Neue Erkenntnisse deuten auf einen Zusammenhang von Spreading Depolarisation und der Entwicklung verzögerter zerebraler Ischämien hin.

In der vorliegenden Dissertation werden drei Studien präsentiert, an denen ich im Rahmen eines translationalen Forschungsprojektes beteiligt war.

In der ersten Arbeit werden die Charakteristika von Serien (sogenannten Clustern) von Spreading Depolarisations in einem Tiermodell und bei Patienten mit aneurysmatischer Subarachnoidalblutung beschrieben. Für die Tierexperimente wurde hier ein Endothelin-1-Modell entwickelt. Dieses erlaubt, Cluster von Spreading Depolarisations in einem sich konzentrationsabhängig entwickelnden fokalen Ischämiegebiet auszulösen. In diesem experimentell erzeugten Ischämiegebiet und im gesunden umliegenden Gewebe wurden daraufhin Messungen des DC-Elektrokortikogramms und des zerebralen Blutflusses durchgeführt. Dabei wurden zwei unterschiedliche Cluster-Muster beobachtet. Die gleichen Muster konnten parallel bei Patienten mit aneurysmatischer Subarachnoidalblutung beobachtet werden.

Die Ergebnisse dieser Arbeit deuten sowohl im Tierexperiment, als auch im menschlichen Kortex auf die Existenz eines „elektrisch stummen“ Gewebesaumes um das Ischämiegebiet hin.

In einem weiteren Teil der Arbeit wurde das Spektrum hämodynamischer Reaktionen auf Spreading Depolarisation bei Patienten untersucht. Hierbei wurde die Möglichkeit eines vaskulären Signals als nicht-invasiver Biomarker für Spreading Depolariation diskutiert.

Das stark vasokonstriktiv wirkende Endothelin-1 spielt eine bedeutende Rolle bei der Auslösung von Spreading Depolarisation und damit möglicherweise der Entwicklung verzögerter zerebraler Ischämien. Wir untersuchten daher im Tierexperiment, ob ET-1 $_{(1\text{-}31)}$, ein Zwischenprodukt bei der Endothelin-1-Synthese, ebenfalls Spreading Depolarisation auslösen kann. Dies war nur in geringerem Ausmaß möglich. Diese Erkenntnis könnte therapeutische Konsequenzen bei der Behandlung von Patienten mit aneurysmatischer Subarachnoidalblutung haben.

Abstract

Delayed cerebral ischaemia (DCI) cannot be dissociated from aneurismal subarachnoid haemorrhage (aSAH) and, despite all efforts to reduce its negative impact on the patients' outcome, it remains a heavy burden. Among the new strategies evolving in the last years, spreading depolarisation arose as one plausible mechanism to help understanding the development of delayed infarcts following aSAH.

In this synopsis I present three studies that emerged from a translational project and to which I contributed as part of my PhD thesis.

The first study deals with the characterisation of clusters of spreading depolarisations in a rat model and human subjects. In the rat, an endothelin-1 (ET-1) model of focal ischaemia was developed to induce clusters of spreading depolarisations in a concentration-controlled manner. Direct current electrocorticography (DC - ECoG) and regional cerebral blood flow (CBF) were recorded in ET-1-exposed ischemic and normally perfused surrounding cortex. We discovered two distinct patterns of clusters of spreading depolarisations in those two regions. In aSAH patients with clusters of spreading depolarisations, similar patterns were observed, suggesting that in both rat and human cortex a belt of electrical silence surrounds the ischaemic area. Possibly this has implications for protection and repair.

Another part of this project focused on the characterisation of the full spectrum of haemodynamic responses to spreading depolarisations in aSAH patients. These recordings revealed a suppression of low frequency vascular fluctuations (LF-VF) during spreading depolarisation, regardless of the polarity of the slow perfusion change coupled to spreading depolarisation. Such vascular signal can be monitored with available neuroimaging technology, and may serve as a biomarker to monitor spreading depolarisations non-invasively.

An important player in the mechanism of DCI and inducer of spreading depolarisation is the potent vasoconstrictor ET-1. We further studied ET-$1_{(1\text{-}31)}$, an alternate intermediate that can be produced during the synthesis of ET-1. Our data showed a lower ability of ET-$1_{(1\text{-}31)}$ to induce spreading depolarisation in rats. Future studies could address whether shifting the metabolism towards ET-$1_{(1\text{-}31)}$ production is beneficial in aSAH patients.

List of Abbreviations

(a)CSF – (artificial) Cerebrospinal fluid

AgCl – Silver chloride

ANOVA – Analysis of variance

(a)SAH – (aneurismal) Subarachnoid haemorrhage

ATP – Adenosine triphosphate

CBF – Cerebral blood flow

COSBID – Co-Operative Study on Brain Injury Depolarizations

DC – Direct current

DCI – Delayed cerebral ischaemia

DIND - Delayed ischaemic neurological deficit

ET-1 – Endothelin - 1

ECoG – Electrocorticography

H&E – Haematoxylin and eosin

KCl – Potassium chloride

LDF – Laser - Doppler flowmetry

LF-VF – Low frequency vascular fluctuation

NO – Nitric oxide

ROC – Receiver-operating characteristic

ROI – Region of interest

Introduction

Subarachnoid haemorrhage

Aneurismal subarachnoid haemorrhage (aSAH) is a less frequent type of stroke but has a high impact on public health. Aneurismal SAH accounts for only 5% of all strokes nevertheless it is responsible for one fourth of productive life years lost due to stroke (1) as it affects a relatively young population and shows a high morbidity and mortality (2). These statistics incited extensive research over the past decades in order to obtain a better understanding of the pathophysiological mechanisms of cerebral damage and to improve therapeutic options. SAH occurs when there is bleeding into the subarachnoid space. In 85% of the cases SAH aetiology consists of the rupture of an incracranial aneurysm. The treatment of a ruptured aneurysm will depend mainly on its location. The two current treatment options are "coiling" (platinum coils are deployed through a catheter to cause a blood clot in the aneurysm and obliterate it) and "clipping" (direct placement of a clip around the neck of the aneurysm following craniotomy). In another 10% the haemorrhage is restricted to the pretruncal area with no apparent malformation and with relatively little damage. The remaining 5% correspond to all other rare triggers of SAH (2).

Delayed cerebral ischaemia

Clinical evidence suggests that the initial brain damage is the most proeminent causative factor for death and disability after aSAH (3, 4). Delayed cerebral ischaemia (DCI) is nevertheless the most important in-hospital complication and a specific syndrome of aSAH developing in about 35% of aSAH patients mostly between 5-14 days after the initial bleeding. Increased headache and body temperature typically accompany decreased consciousness and focal neurological deficits such as hemiparesis or aphasia (4, 5). The clinical symptoms of DCI are usually referred to as delayed ischaemic neurological deficits (DIND). DCI coincides with the peak of subarachnoid haemolysis after aneurismal rupture (6). Clinical features of DCI constitute a diagnosis *per exclusionem* and are especially difficult to diagnose in patients who are comatose or sedated (7). Although these deficits often occur in conjunction with angiographic evidence of vessel narrowing, they can also occur in absence of angiographic vasospasm (8, 9).

For several decades vasospasm of basal cerebral arteries has been considered the prime mechanism underlying DCI. Early observations on aSAH patients linked angiographic/proximal vasospasm to DCI. In recent years this relationship has been questioned based on a set of observations: a) only weak correlations were found between sites and severity of vasospasm and perfusion changes (10); b) the positive predictive value of proximal vasospasm is low for DCI or local development of infarcts (11, 5); c) large arteries play a limited role in the control of cerebral blood flow (CBF) (12) and d) some patients can have clinical features of DCI in the absence of

radiologically confirmed vasospasm (8, 9). Meanwhile other phenomena have been suggested providing alternative foci of research on the aetiology of DCI. Among those are: chronic vasospasm in the cortical microcirculation (13); microthrombosis (14, 15); spreading depolarisation and spreading ischaemia (16 - 18).

Spreading depolarisation

Spreading depolarisation describes a depolarisation wave of neurons and astroglial cells propagating in grey matter at a rate of ~3 mm/min. This phenomenon is associated with a large disturbance in ion homeostasis between the intra- and extracellular compartments. These exchanges are not one for one but there is a net gain of solutes in the neurons. As a result cells draw water by osmosis and swell at the expense of the interstitial space (19, 20).

An important trait of spreading depolarisation is the negative extracellular slow voltage variation that presumably results from intraneuronal potential gradients (21, 22). The slow voltage variation is visible in the low-frequency band of the electrocorticogram. Furthermore, spreading depolarisation is accompanied by a depolarisation block of neuronal activity. This depolarisation block is measured as spreading depression of electrocorticographic (ECoG) activity in the high-frequency band of the electrocorticogram (23). Even under normal conditions, spreading depolarisation is responsible for an immediate reduction of ATP to ~50% (24). The increased energy demand is compensated by a transient increase in regional CBF in healthy naïve tissue, which also contributes to the clearance of the tissue.

Focal ischaemia and penumbra

Focal ischaemia is a condition characterized by gradients of perfusion, oxygen and glucose between the ischaemic core and the healthy surrounding tissues with normal blood supply and energy metabolism. According to Hossmann (25) the ischaemic penumbra is defined as a region of constrained blood supply in which the energy metabolism is preserved (25, 26). This definition largely replaced the earlier definition of ischaemic penumbra by Astrup and colleagues (27) that focused on electrocorticographic features of the tissue. According to the earlier definition, the penumbra was defined as the region where no terminal near-complete sustained depolarisation had occurred yet but the flow level was below the threshold for spontaneous arrest of the neuronal activity. In other words, Astrup and co-workers described the penumbra as tissue with cerebral blood flow between two critical flow rates, the threshold of electrical failure and the threshold of membrane failure. In contrast, Hossmann (25) added changes in metabolism including changes in protein synthesis to the penumbra definition. Thereby, he defined a larger tissue volume as being part of the penumbra compared to Astrup et al. (27). The key feature of Hossmann's concept of penumbra has been that these areas can be rescued if reestablishment of blood flow happens

before the neurons run out of their energy reserves and spreading depolarisation becomes terminal initiating cascades leading to cell death (25 -27).

After middle cerebral occlusion, spreading depolarisation starts in the centre of a low flow region as a terminal, harmful wave. From there, it propagates through the penumbra where it progressively becomes shorter-lasting and eventually more benign as it reaches the healthy surroundings where it is recorded as a harmless wave (28, 29). Additional spreading depolarisations subsequently arise at the border zone of the permanently depolarised core and seem to recruit further tissue into necrosis.

The possibility of regaining full activity of endangered brain tissue has attracted an immense interest over the last 30 years ranging from bench to bedside research which ultimately strives to improve the outcome of stroke patients (26). In this context spreading depolarisation became in the last years a plausible mechanism to explain the recruitment of endangered, but still viable, tissue into necrosis. This suggestion gained further momentum after clusters of spreading depolarisations had been observed in patients with traumatic brain injury (30, 31), as well as in patients with aSAH time locked with DCI (17, 18), and patients with malignant hemispheric infarcts (32).

Endothelin-1 (ET-1)

Discovered in 1985 and referred to as endothelium-derived constricting factor (33), it was identified as the endothelin peptide by Yanagisawa (34).

ET-1 is the predominant isoform of a family composed of four members: ET-1, ET-2, ET-3, ET-4 (35, 36). ET-1 is not only one of the most potent vasoconstrictors in the cerebral circulation but also induces spreading depolarisation (8). The spreading depolarisations presumably result from ET-1 inducing vasoconstriction and ischaemia (37).

ET-1's synthesis starts with the translation of preproendothelin mRNA into a 203-amino acid preproendothelin peptide which is cleaved to an inactive 38-amino acid peptide big-ET-1 by furin convertase and a carboxypeptidase (38, 39). The bond Trp_{21}-Val_{22} is then cleaved to form ET-1 mainly by an endothelin-converting enzyme 1. Alternatively, big-ET-1 can be cleaved by the mast-cell derived chymase at the bond Tyr_{31}-Gly_{32} originating $ET\text{-}1_{1\text{-}31}$ (40).

In the past years different studies investigated ET-1's role in the pathophysiology of cerebral vasospasm following aSAH. Macdonald and colleagues showed that the ET_A receptor antagonist clazosentan caused a 65% relative risk reduction of angiographic vasospasm. Nevertheless, overall outcome did not improve (41, 42), enhancing the controversy of whether or not angiographic vasospasm plays the central role in the development of DCI.

Aims

Previous work suggested that clusters of spreading depolarisations with persistent depression of high-frequency ECoG activity indicate tissue at risk for necrosis.

In the first part of this thesis I aimed to characterise the electrophysiological signature of clusters of recurrent spreading depolarisations. The central questions included whether persistent ECoG depression between spreading depolarisations also occurs in well perfused tissue surrounding the zone of injury and how ECoG depression periods relate to prolonged negative direct current (DC) shifts of the spreading depolarisations. Firstly, I applied a two-cranial window model of focal ischaemia in rats in which the potent vasoconstrictor ET-1 induces focal ischemia in one window and surviving, normally perfused cortex can be studied in the second window. Subsequently, DC-ECoG recordings of spreading depolarisation clusters with persistent depression from patients with aSAH were analysed for comparison with the animal data.

In the second part of the thesis the goal was to determine whether spreading depolarisations could be identified by a vascular signal that is independent of the magnitude and polarity of the slow perfusion change coupled to spreading depolarisation. Such a vascular signal could provide the option to distinguish spreading depolarisation from other neuronal and non-neuronal processes associated with slow perfusion changes such as hypercapnia using non-invasive recordings with near-infrared spectroscopy or functional magnetic resonance imaging. Previous studies suggested that low frequency vascular fluctuations (LF-VF) arise from the resting neuronal activity which is reflected in the high-frequency ECoG (43, 44) and can possibly be tracked with neuroimaging technology. For that purpose we analysed recordings of LF-VF, electrocorticographic and perfusion data obtained from aSAH patients as part of a prospective, multicentre study. The overall aim of this study was to investigate whether the full spectrum of haemodynamic responses to spreading depolarisations occurs in the diseased human brain in a similar manner to that seen in animals. All patients included in the study were implanted with subdural opto-electrode technology for simultaneous laser-Doppler flowmetry (LDF) and DC-ECoG recordings. In parallel we observed the occurrence of clusters of prolonged spreading depolarisations time-locked with structural brain damage monitored by neuroimaging.

In the third part we investigated the ability of the metabolic alternate of ET-1 synthesis ET-$1_{(1-31)}$ to induce spreading depolarisation in a similar fashion to ET-1. For this purpose increasing concentrations of either ET-1, ET-$1_{(1-31)}$ or vehicle were topically applied to the brain using an open cranial window model in rat. Each concentration was superfused for 60 minutes while regional CBF and DC-ECoG were acquired.

Methods

Elaborate methods are inserted in their complete form in section "Publications" (Oliveira-Ferreira *et al*., (2010), Dreier *et al*., (2009), Jorks *et al.*, (2011)) starting on page 24. Here, main features underlying the methods' choice in the different studies are briefly explained.

Animals

The animal experiments were performed in conformity with the Governmental Animal Care and Use Committee (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin (LAGetSi)).

Study 1: Male Wistar rats (250 to380 g; Charles River Laboratories, Wilmington, MA, USA) (n = 47). Study 3: Male Wistar rats (220 to 410 g; Charles River Laboratories, Wilmington, MA, USA) (n = 29). All animals were housed under standard conditions.

Cranial window preparation

In Studies 1 and 3, rats were anesthetised intraperitoneally with thiopental sodium, tracheotomised and artificially ventilated. A craniotomy was performed in all animals. One cranial window was established over the somatosensory cortex (Study 1, groups 1-3; Study 3, groups 1-3) and a second smaller window was established over the frontal cortex (Study 1, groups 4 and 5) of the same hemisphere. In Study 1, group 4, the caudal window was closed to allow imaging of the pial arterioles using a CCD camera. Otherwise, the windows were not covered.

ET-1-induced focal ischemia

Different degrees of focal ischemia were induced by brain topical application of aCSF containing ET-1 at the following concentrations: 10 nmol/L, 100 nmol/L and 1µmol/L.

The solutions were superfused in a stepwise manner ranging from the highest concentration alone (Study 1, group 1) to the two highest (Study 1, groups 4 and 5) to all three concentrations in a rising manner (Study 1, group 2). Each solution was superfused for 60 minutes.

Animal recording techniques

The subdural DC-ECoG (lowpass filter with upper frequency limit of 45 Hz) was measured with Ag/AgCl electrodes. Changes in the extracellular K^+ concentration ($[K^+]_o$) (Study 1, groups 1, 3), the extracellular pH (Study 1, groups 2, 3), and the intracortical DC-ECoG (lowpass filter with upper frequency limit of 45 Hz) (Study 1, groups 1 – 3) were recorded in a cortical depth of 300 µm with ion-sensitive microelectrodes. Systemic arterial blood pressure as well as local cerebral ion, voltage and blood flow changes were continuously recorded using a personal computer and Spike 2 software.

Spreading depolarisation was defined by the sequential onset in adjacent channels of a propagating, polyphasic slow potential change (32) that corresponds to the negative slow voltage variation described by Leão (22) and Hartings et al. (45). The parallel electrocorticographic depression was defined by a rapidly developing reduction of the power of the electrocorticographic amplitude by at least 50 % (31, 32). The duration of the depression period of electrocorticographic activity (> 0.5 Hz) was used as an indirect indicator of tissue energy supply since restoration of this activity after spreading depolarization is energy dependent (28, 46). This duration, used here to assess the spatial and temporal relation between compromised energy supply and the development of brain infarcts, was measured as the interval between depression onset and onset of restoration of activity using the integral of power of the highpass filtered activity (lower frequency limit, 0.5 Hz; time constant decay, 60 s).

Pial arteriolar diameter calculation

To calculate the changes in arteriolar diameter in the cranial window (Study 1, group 4), a custom made Matlab program was developed to adjust to the particular requirements of the experimental data. Regions of Interest (ROI), representing lines across a given arteriole were selected and defined as an index profile of light absorbance (x = length in pixels and y= axis brightness). The curve valley of absorbance in the green channel was used to detect the two edges of the vessel by applying a second derivative of the curve representing the slope. The length between the two edges was the Euclidean distance and the real diameter in µm was obtained after correction for the manually determined pixel length. Changes in the intrinsic optical signal or illumination artifacts were compensated by the algorithm. The lower diameter limit for detection was set to 5 µm.

Histology and microscopy

To study the ET-1-induced lesion a two open window model was used. Five male Wistar rats (Study 1, group 5) were anesthetised with isoflurane and allowed to wake up after the surgery and recording procedures. Sixty hours after the experiment the animals were sacrificed and 20-µm slices of the sagittal plane obtained serially at 800µm intervals were stained with hematoxylin-eosin (H&E). The following parameters were calculated: the diameter of the caudal window as assessed by the brain's herniation, the largest rostral extent of the ET-1-induced lesion beyond the border of the caudal window, and the distance between the rostral border of the ET-1-induced necrosis and the rostral window.

LDF

LDF is a blood flow measurement technique based on the Doppler shift of coherent light induced by moving blood cells. Its limited spatial resolution and no absolute blood flow measurement are compensated by high temporal resolution and non-invasiveness, making it a technique suitable for both animal and human subjects.

Patients

Clinical and research consents were obtained according to the Declaration of Helsinki after a clinical decision had been taken to offer surgical treatment after aneurysmal SAH and approved by the local ethics committees. Patients with major aSAH were recruited by different centres of the Cooperative Study on Brain Injury Depolarizations (COSBID, www.cosbid.org). Study 1: Campus Charité Virchow Berlin (n = 4) and Campus Benjamin Franklin Berlin (n = 2). Study 2: Campus Charité Virchow Berlin (n = 9), Campus Benjamin Franklin Berlin (n = 2), King's College London (n = 1), and Glostrup Hospital Copenhagen (n = 1).

Human recording techniques

ASAH patients eligible for surgery had a single, linear, 6-contact (platinum) electrocorticography recording strip (5 mm diameter) placed on the cortex accessible through the craniotomy or via an extended burr-hole as described (17, 31).

The first electrode in the strip was used as ground and the others (2-6) were connected in a unipolar fashion to the amplifiers, each referenced to an ipsilateral subgaleal platinum electrode. Using a BrainAmp amplifier (bandpass filter: 0–50 Hz) it was possible to record DC changes in parallel with high-frequency ECoG changes. Regional CBF was acquired through four optodes neighbouring electrodes 3–6. After a maximum of 14 days of continuous data acquisition the probes were withdrawn at the bedside. Data were sampled at 200 Hz, recorded and reviewed with the use of a Powerlab 16/SP analog/digital converter, Chart-5 software and BrainVision Recorder software.

Statistical analysis

Regional CBF and integral of the power of ECoG measurements were expressed as % changes of baseline values in all studies. Further data processing and statistical analyses are described in more detail in the publications.

Results

The results of this thesis have been included in the publications listed in section "Publications". Here, the main findings of each study are summarised.

Study 1: Electrical silence around the penumbra

Electrocorticographic signatures of spreading depolarisation in the Endothelin-1-exposed cortex in rats

To find out whether the patterns of negative DC shifts in the ET-1-exposed cortex depended on the dynamics of application, immediate application of 1µmol/L (group 1, n = 9) and a two-step increase of ET-1 concentration from 10 to 100 nmol/L to 1 µmol/L (group 2, n = 7) were compared. Most importantly, in group 1, ET-1 led to a decrease of regional CBF to 68 ± 18 % of the baseline at the rostral and to 58 ± 12 % at the caudal laser probe before the first spreading depolarisation. In group 2 this parameter did not change (97±33 % of baseline). This was significantly different from group 1 (t-test, P = 0.007). Very prolonged negative DC shifts were significantly more frequent when the exposure to ET-1 started with the high concentration (group 1) and was not preceded by lower concentrations as in group 2 (two-tailed Fisher's exact test, P = 0.009).

Significant constriction of pial arterioles precedes endothelin-1-induced spreading depolarisation

Arteriolar diameters of 102 arterioles of eight experiments (group 4, n = 11) were analysed. In each experiment, the median diameter of large, medium, and small arterioles was determined before wash-in of ET-1 and during the last 60 s before the first spreading depolarisation. Large arteriole diameters changed insignificantly from 81 ± 24 to 69 ± 31 µm, whereas medium and small arteriole diameters changed significantly from 58 ± 18 to 38 ± 20 µm and from 37 ± 14 to 27 ± 15 µm, respectively (paired t-test, P = 0.013 and P = 0.012, respectively).

During this period, regional CBF significantly decreased to 63 ± 26% in the ET-1-exposed cortex whereas it remained unchanged in the control window (98 ± 13%, P = 0.004, paired t-test).

Persistent depression of high-frequency electrocorticographic activity in both the Endothelin-1-exposed and surrounding cortex

In the ET-1-exposed cortex, the ET-1-induced cluster of spreading depolarisations was associated with a subdural DC negativity of -2.6 ± 2.2 mV (ultraslow negative potential component), on which sharp transient negative DC shifts typical of spreading depolarisations were riding (amplitude: -4.0 ± 1.9 mV). This was accompanied by a shallow DC positivity of 0.9 ± 0.9 mV (ultraslow positive potential component) superimposed with spreading depolarisations (amplitude: -1.2 ± 1.0 mV) in the control window.

The mean integral of power of the high-frequency ECoG activity was similar in the ET-1-exposed and remote cortex before the cluster of recurrent spreading depolarisations (0.52 ± 0.22 versus 0.46 ± 0.18 mV^2s (bandpass: 0.5 – 45 Hz)).

The cluster of recurrent spreading depolarisations was associated with a persistent depression of high-frequency ECoG activity in both the ET-1-exposed and remote cortex, but the depression was significantly more pronounced in the ET-1-exposed cortex (mean reduction to 27 ± 13% under ET-1 (100 nmol/L) versus 55 ± 15% in the remote cortex ($P < 0.001$) and to 16 ± 7% under ET-1 (1 mmol/L) versus 55 ± 18% in the remote cortex ($P < 0.001$, paired t-tests)). Moreover, after wash-out of ET-1 the integral of high-frequency power returned to 71 ± 19 % in the remote, normally perfused cortex whereas it only returned to 39 ± 16 % in the ET-1-exposed cortex ($P = 0.004$, paired t-test).

The Endothelin-1-induced lesion does not include the rostral recording site

Based on the brain's herniation, the caudal window had a diameter of 4310 ± 960 µm. The sagittal diameter of the lesion of the ET-1-exposed cortex was 5140 ± 1060 µm. The distance between the rostral border of the ET-1-induced lesion and the caudal border of the rostral control window was 4570 ± 840 µm. The ET-1-induced lesion did not include the rostral control window in any of the experiments.

Evidence of two distinct zones associated with persistent electrocorticographic depression in patients with aSAH

We analyzed six clusters of six different patients with recurrent spreading depolarisations and persistent depression of high-frequency ECoG activity between spreading depolarisations.

Clusters were accompanied by significant depression of high-frequency ECoG activity from 0.162 ± 0.141 to 0.016 ± 0.012 mV^2s (integral of power, $P < 0.05$, one way repeated-measures analysis of variance) with a partial recovery to 0.095 ± 0.081 mV^2s after the cluster. The depression lasted for 18.0 ± 21.0 hours.

We then investigated whether less recovery of high-frequency ECoG activity after the cluster would be associated with longer negative DC shifts in patients similar to the animal experiments where ET-1-exposed ischaemic cortex showed longer-lasting negative DC shifts and less recovery of activity compared to normally perfused surrounding cortex. Would it be possible to use those electrophysiological parameters to distinguish in patients between penumbra and normally perfused surrounding zones in a similar fashion to that in animals? For that purpose we determined a cut-off value with a receiver-operating characteristic (ROC) analysis using the empirical data of group 4 of the animal experiments and a model-based approach. The best cut-off value for the recovery of high-frequency ECoG activity after the cluster was 55 %, with a sensitivity of 80 % and a specificity of 85 %. Consistently with the animal experiments, longer-

lasting negative DC shifts were found in the recording group with < 55 % recovery of high-frequency ECoG activity after the cluster (duration of negative DC shift: 36.1 ± 32.5 versus 6.3 ± 5.3 minutes, P < 0.001, Mann–Whitney rank sum test).

Study 2: Low-Frequency Vascular Fluctuations

In this study 603 spreading depolarisations were recorded in 2467 h recording time in 13 patients. Pooling of all spreading depolarisations with simultaneous recordings of regional CBF and ECoG (1953 h recording time) resulted in 295 of 417 spreading depolarisations with no initial hypoperfusion > 30 s, whereas 78 spreading depolarisations showed an initial hypoperfusion between 30 s and 2 min, 16 between 2 and 5 min and 28 of 45 min up to 144 min in at least one opto-electrode pair.

Spreading suppressions of low-frequency vascular fluctuations accompany spreading depolarisation

We observed that LF-VF were suppressed during spreading depolarisation regardless of the haemodynamic response. LF-VF (0.05 – 0.1 Hz) were observed in 10 of 12 cases. The median frequency of the LF-VF was 0.079 (0.070, 0.091) Hz. During spreading depolarisation, LF-VF became significantly suppressed (Repeated measures ANOVA on ranks with Dunn's post hoc test, n = 10, P < 0.05). The power of the LF-VF robustly and significantly decreased from 128.3 (88.4, 199.2) to 2.8 (2.0, 7.8) LDF units^2 followed by a significant recovery to 99.7 (47.3, 206.2) LDF units^2 after spreading depolarisation (Repeated measures ANOVA on ranks, n = 10, P50.05). The degree of LF-VF suppression was not significantly different between spreading depolarisations with different haemodynamic responses (spreading hyperaemia versus spreading hypoperfusion). Also the duration of the spreading suppression of LF-VF and spreading depression of high-frequency ECoG activity correlated significantly.

Clusters without recovery of high-frequency ECoG activity between the recurrent spreading depolarisations were identified in 5 of the 13 analysed patients. LF-VF preceded the clusters in only three of five cases. The dominant frequency was 0.063 (0.021, 0.072) Hz. The power of the LF-VF (bandpass: 0.05 – 0.1 Hz) decreased from 84.4 (70.9, 1370.3) to 3.4 (2.1, 6.5) LDF units^2 followed by a recovery to 63.4 (32.1, 71.1) LDF units^2 after the cluster.

The duration of the depression period of the LF-VF ranged from 211 min to at least 60 h.

Study 3: Endothelin-1$_{(1-31)}$ induces spreading depolarisation in rats

ET-1$_{(1-31)}$ induces spreading depolarisations at higher concentration than ET-1

All physiological variables remained within the normal range in the three groups. In group 1, administration of ET-1 led to spreading depolarisations in all 19 animals. Of these, three animals

developed spreading depolarisations in response to 10 nmol/L (15.8 %), eleven animals in response to 100 nmol/L (57.9 %) and five in response to 1 µmol/L (26.3 %). All animals of group 2 showed spreading depolarisations after administration of ET-$1_{(1-31)}$. However, in contrast to group 1, ET-$1_{(1-31)}$ only induced spreading depolarisations at a concentration of 1 µmol/L. This difference was statistically significant ($P < 0.001$, two-tailed Fisher's Exact Test). No significant differences were observed in other parameters between ET-1 and ET-$1_{(1-31)}$ induced spreading depolarisations. Vehicle controls (n = 3) did not show spreading depolarisations.

Discussion

The occurrence of spreading depolarisations when ECoG activity was already depressed has been previously shown in patients suffering from traumatic brain injury (31). Moreover such spreading depolarisations were shown to occur in clusters time locked with the development of DCI/delayed ischaemic stroke after aSAH (17) and malignant hemispheric stroke (32).

Earlier experimental work had led to the speculation that clusters with persistent depression of high-frequency ECoG activity might indicate tissue at risk (25). In the framework of my PhD thesis this concept was further investigated in an animal model of ischemia and by use of state of the art neuromonitoring techniques in aSAH patients.

In the experimental part of the first study full band DC–ECoG recordings were obtained using a two-cranial window model in rats in which ET-1 was topically applied to the brain in one window with the second window serving as control. Our results suggested the existence of two distinct zones with persistent depression of high-frequency ECoG activity between recurrent spreading depolarisations. In an outer zone of normally perfused cortex, the clusters were characterised by stereotypical and short-lasting spreading depolarisations with good recovery of the high-frequency ECoG activity after the cluster while an endangered inner zone revealed spreading depolarisations with longer-lasting, more variable negative DC shifts and a more pronounced depression of high-frequency ECoG activity with limited recovery. The human recordings suggested that similar zones also exist in the injured human brain after aSAH.

Such aspects can now be studied in parallel in animals and humans thanks to the novel neuromonitoring technology as applied in studies 1 and 2. This neuromonitoring technology could be very helpful in the future to perform treatment stratification in patients with acute cerebral injury. Thus, aggressive treatment would be restricted to those patients who develop clusters of spreading depolarisations indicating progressive neuronal injury.

In the second study presented here, a combination of invasive recordings of regional CBF and regional DC-ECoG allowed the observation that spreading suppressions of LF-VF was associated with spreading depressions of high-frequency ECoG activity, irrespectively of the polarity of the haemodynamic response to the spreading depolarisation. The discovery of the spreading suppression of LF-VF could be a useful identifier since it should be possible to also record this phenomenon with non-invasive recording tools such as near-infrared spectroscopy or functional magnetic resonance imaging for example. The development of non-invasive recording technology could be useful to widen the application spectrum of the monitoring to all patients with acute cerebral injury, including those who do not require neurosurgical procedures that allow for placement of a subdural electrode strip. This will, of course, require further clinical studies.

The Endothelin-1 Model of Focal Ischemia

Well established models of focal ischaemia are designed to replicate severe sudden onset ischaemia in humans, which is not typical of ischaemia in aSAH for example that often shows a waxing and waning clinical picture (47). We sought to develop a model of slowly developing focal ischaemia to better understand what might happen in conditions of slowly developing ischemia in the human brain such as after aSAH. The ET-1 model allows the ischaemic condition to be graded according to the applied concentration. In addition, it has been suggested that ET-1 is directly involved in the induction of cerebral vasospasm following aSAH although its role is controversial (48). Increased levels of ET-1, ET-3 and big-ET-1 in both plasma and CSF of aSAH patients have been detected accompanying the development of vasospasm but the cerebrospinal concentrations of ET-1 are lower than the concentrations necessary for induction of ischemia. However, the sensitivity of vascular smooth muscle to ET-1 significantly increases after aSAH (48, 49) and ET-1 concentrations in the relevant abluminal compartment for vasoconstriction are possibly higher than those detected in the cerebrospinal fluid (50). Moreover, cerebrospinal ET-1 may not reflect the increased abluminal ET-1 release of endothelium stimulated by subarachnoid clot, but the release by astrocytes in response to oxidative substrate depletion (51). Thus, elevation of cerebrospinal ET-1 occurred time-locked to the neuronal damage after aSAH rather than to the development of angiographic vasospasm (52).

An important aspect of this model is that both the regional CBF decrease and the neuronal response to the ischemic condition depended not only on the absolute ET-1 concentration but also on the rate of concentration increase.

Thus, in contrast to a 2-step increase of ET-1, an immediate application of 1 µmol/L induced a more pronounced decline of regional CBF before the first spreading depolarisation. In addition, very prolonged negative DC shifts (≈20 minutes duration) were only observed with immediate application of 1 µmol/L, but not when lower concentrations preceded the application of 1 µmol/L. One explanation for this is tachyphylaxis to the vasoconstrictor effect of ET-1 (53) and possible counterregulatory mechanisms (e.g. release of vasodilators like NO) (54). These results indicate that a comparison between the effects of ET-1 in different models and pathological conditions should consider not only the absolute concentrations but also the time course of exposure to ET-1.

The ischaemic origin of ET-1-induced spreading depolarisations is supported by several arguments: (a) the receptor profile of ET-1-induced spreading depolarisation is consistent with vasoconstriction, since ET_A receptors only mediated ET-1-induced spreading depolarisation (55); (b) a slow rise of the extracellular K^+ concentration typical of ischemia preceded spreading depolarisation under ET-1; (c) ET-1 failed to elicit spreading depolarisation in brain slices which lack a blood circulation (8); and (d) an area with selective neuronal necrosis was found in the ET-1-exposed cortex whenever ET-1 elicited, but not when ET-1 failed to trigger spreading

depolarisation (56). In this study, we quantified the vasoconstriction in response to increasing concentrations of ET-1 and found a marked decrease of medium and small arteriolar diameters in the ET-1-exposed cortex before the first SD. Also, the pattern of pH changes further supported the ischemic origin of the ET-1-induced SDs. Thus, a significant acidification preceded the alkaline shift at SD onset, contrasting with the healthy surrounding tissue.

Doubts have persisted in previous work regarding the fact that slightly prolonged spreading depolarisations were associated with neuronal necrosis (56). It was a main goal of this project to characterise those depolarisations in more detail. We found in group 4 that mildly prolonged spreading depolarisations typically occur superimposed on a more shallow subdural negativity in the ET-1-exposed cortex while shorter-lasting spreading depolarisations rode on a shallow DC positivity in the healthy surrounding cortex. These data were recorded with subdural Ag/AgCl electrodes which record from a larger volume than the microelectrodes used in other groups in this study. The shallow subdural negativity between spreading depolarisations could reflect the fraction of dying neurons that fail to repolarise whereas the persistent positivity observed in the healthy cortex represents the current source of the circuit established between ET-1-exposed and naïve cortex.

A remarkable observation in this study was that also in the normally perfused surrounding tissue, spreading depression of high-frequency ECoG activity can persist between recurrent spreading depolarisations. It has been suggested that persistent depression between spreading depolarisations indicates neuronal injury (18, 31), but the actual neuronal injury may occur in a remote region. Yet to be clarified is whether the prolonged suppression of synaptic activity in a belt around the ischemic penumbra may be important for the induction of repair and regeneration in this area (26, 57, 58). Possibly, prolonged synaptic depression between short-lasting spreading depolarisations provides a stimulus in this zone to establish novel synaptic contacts. Furthermore, acutely, it may save energy that could be beneficial for the survival of the neighbouring penumbra (59, 60). In light of this set of results in both animal and human recordings it is tempting to relate the role of spreading depolarisations to the very recent concept that the early death signals confer repair and regeneration later (26).

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Declaration of own contribution to the submitted publications

The contributions of the doctoral student Ana Isabel Oliveira Ferreira to the here submitted publications present as follows.

Prof. Dr. Jens Dreier

Ana Isabel Oliveira Ferreira

Publication 1: Oliveira-Ferreira AI, Milakara D, Alam M, Jorks D, Major S, Hartings JA, Lückl J, Martus P, Graf R, Dohmen C, Bohner G, Woitzik J, Dreier JP; COSBID study group, Experimental and preliminary clinical evidence of an ischemic zone with prolonged negative DC shifts surrounded by a normally perfused tissue belt with persistent electrocorticographic depression. J Cereb Blood Flow Metab 30(8): 1504-19, (2010)

Contribution in percent: 70; *Detailed contribution:* Participation in designing and planning of the experiments, developed ET-1 model of focal ischaemia, conducted the animal preparation and data acquisition, performed the histological and imaging analysis, participated in the analysis of patients data, performed statistical analysis, co-drafted the manuscript, designed all figures, took part in the process of the peer review, presented the data in conferences.

Publication 2: Dreier JP, Major S, Manning A, Woitzik J, Drenckhahn C, Steinbrink J, Tolias C, Oliveira-Ferreira AI, Fabricius M, Hartings JA, Vajkoczy P, Lauritzen M, Dirnagl U, Bohner G, Strong AJ; COSBID study group, Cortical spreading ischaemia is a novel process involved in ischaemic damage in patients with aneurysmal subarachnoid haemorrhage, Brain 132(Pt 7):1866-81, 2009

Contribution in percent: 20; *Detailed contribution:* Accompanied the patient recording process, participated in the analysis of patient's data and statistical analysis, collaborated in figure selection and preparation, made critical review of the manuscript.

Publication 3: Jorks D, Major S, Oliveira-Ferreira AI, Kleeberg J, Dreier JP (2011). Endothelin-1(1-31) induces spreading depolarization in rats. Acta Neurochir. Suppl. 110:111-7
Contribution in percent: 40; *Detailed contribution:* Participation in planning the study, analysed data and performed statistical analysis, made critical review of the manuscript.

Selected Publications

The following three peer-reviewed publications are submitted within the scope of this PhD thesis.

Publication 1

Oliveira-Ferreira AI, Milakara D, Alam M, Jorks D, Major S, Hartings JA, Lückl J, Martus P, Graf R, Dohmen C, Bohner G, Woitzik J, Dreier JP; COSBID study group, Experimental and preliminary clinical evidence of an ischemic zone with prolonged negative DC shifts surrounded by a normally perfused tissue belt with persistent electrocorticographic depression. J Cereb Blood Flow Metab 30(8): 1504-19, (2010)

Impact factor (2009): 5.457

Publication 2

Dreier JP, Major S, Manning A, Woitzik J, Drenckhahn C, Steinbrink J, Tolias C, Oliveira-Ferreira AI, Fabricius M, Hartings JA, Vajkoczy P, Lauritzen M, Dirnagl U, Bohner G, Strong AJ; COSBID study group, Cortical spreading ischaemia is a novel process involved in ischaemic damage in patients with aneurysmal subarachnoid haemorrhage, Brain 132(Pt 7):1866-81, 2009

Impact factor (2009): 9.490

Publication 3

Jorks D, Major S, Oliveira-Ferreira AI, Kleeberg J, Dreier JP (2011). Endothelin-1(1-31) induces spreading depolarization in rats. Acta Neurochir. Suppl. 110:111-7

Impact factor (2009): 1.472

Publications

In the following, the complete published versions of Oliveira-Ferreira *et al.* (2010), Dreier *et al*. (2009) and Jorks *et al.* (2011) are inserted.

Publication 1

Publication 2

Publication 3

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gruenden in der elektronischen Version meiner Arbeit nicht veroeffentlicht.

Complete list of publications

Publications in peer-reviewed journals

Oliveira-Ferreira AI, Milakara D, Alam M, Jorks D, Major S, Hartings JA, Lückl J, Martus P, Graf R, Dohmen C, Bohner G, Woitzik J, Dreier JP; COSBID study group (2010). Experimental and preliminary clinical evidence of an ischemic zone with prolonged negative DC shifts surrounded by a normally perfused tissue belt with persistent electrocorticographic depression. J Cereb Blood Flow Metab 30(8): 1504-19.

Oliveira-Ferreira AI, Winkler MKL, Reiffurth C, Milakara D, Woitzik J, Dreier JP (2011). Spreading depolarization, a pathophysiological mechanism of stroke and migraine aura. Future Neurology (in press).

Lee E, Oliveira-Ferreira AI, de Water E, Gerritsen H, Bakker MC, Kalwij JA, van Goudoever T, Buster WH, Pennartz CM (2009). Ensemble recordings in awake rats: achieving behavioral regularity during multimodal stimulus processing and discriminative learning. J Exp Anal Behav 92(1):113-29.

Dreier JP, Major S, Manning A, Woitzik J, Drenckhahn C, Steinbrink J, Tolias C, Oliveira-Ferreira AI, Fabricius M, Hartings JA, Vajkoczy P, Lauritzen M, Dirnagl U, Bohner G, Strong AJ; COSBID study group (2009). Cortical spreading ischaemia is a novel process involved in ischaemic damage in patients with aneurismal subarachnoid haemorrhage. Brain 132(Pt 7):1866-81.

Jorks D, Major S, Oliveira-Ferreira AI, Kleeberg J, Dreier JP.(2011) Endothelin-1(1-31) induces spreading depolarization in rats. Acta Neurochir. Suppl. 110:111-7

Conference proceedings, selected abstracts

Oliveira-Ferreira AI, Alam M, Major S, Milakara D, Dreier JP (2009). Endothelin related pathophysiology in cerebral vasospasm. What happens to the brain tissue? 10th Int. Conf. On Cerebral Vasospasm, Chong Qing, China

Oliveira-Ferreira AI, Alam M, Major S, Milakara D, Dreier JP (2009). Does Endothelin-1 induce cortical spreading depolarization (CSD) via a direct effect on the vasculature? XXIVth Int. Sym. on Cerebral Blood Flow, Metabolism, & Function, Chicago IL, USA

Oliveira-Ferreira AI, Alam M, Major S, Milakara D, Dreier JP (2008). Does Endothelin-1 induce cortical spreading depolarization (CSD) via a microvascular spasm or a direct effect on the neurons? 38th Annual Meeting Neuroscience, Washington DC, USA

Oliveira-Ferreira AI, Alam M, Major S, Dreier JP (2007). Migraine Aura, Cortical Spreading Depression and Endothelin-1: a unifying approach. Berlin Brain Days 2007, Berlin, Germany

Erklärung

Ich, Ana Isabel Oliveira Ferreira, erkläre, dass ich die vorgelegte Dissertation mit dem Thema:

"Electrocorticographic characterisation of clusters of spreading depolarisations in a rat model and human subjects - a translational approach from bench to bedside"

selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

Berlin, 24 February 2012

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Ana Isabel Oliveira Ferreira

Acknowledgements

Foremost, I would like to thank Prof. Dr. Jens Dreier for his constant motivation and patience to help me developing the concept of this project. Since the first day, Prof. Dreier has been a source of advice and encouragement to get out of dead-ends at times.

I am especially fortunate to have worked with Dr. Alam Mesbah, Dr. Sebastian Major, Mag. rer nat**.** Denny Milakara, Dr. Nikolas Offenhauser, DeVi Jorks, Dr. Janos Lückl and Maren Winkler, whose precious help in solving technical questions, useful suggestions and support was fundamental in the earlier and later steps of this work.

To Dr. Eunjeong Lee with whom I have learnt that science is so target oriented and precise as captivating and passionate, I wish to thank her unique friendship, advice and vivid discussions since the first day we worked together. Ao Dr. Armindo Salvador, agradeço a dedicação e profissionalismo no ensino da Biologia que fizeram desta a minha vocação.

To the friends from everywhere, without whom Berlin would probably not be called home: Ferah, Ceci, Anna, Euni, Ryan, Uldus, Migas, Barbara, 2 sis Jo & Yla, Matt, Björn, Andrea and Bruno. Ao Júlio César. To Huguinho and Keesha who helped revising the text I owe special thanks.

Maninhos Johnny, Rita e Mília, partilhámos sobressaltos juvenis, à descoberta de um mundo que se tornou muito maior mas onde o coração tem um berço feito de vós. E a toda uma trupe que cresceu com os anos. Aos biólogos mais ou menos aplicados que são, hoje, a melhor memória dos tempos académicos e de amizades verdadeiras lá forjadas. À Xinha e ao Jojó devo a contribuição gigante para a minha sanidade mental ao longo destes anos.

MDMV, irmã cujo sangue corre no meu. Os primos Tiago, Filipe e Taninho que sempre estiveram com abraços e mensagens reconfortantes anos a fio. À tia João, que tem a melhor gargalhada do mundo e me ajudou a abrir a porta ao agora e apreciar o Universo tal como é. Ao Mi(guel), por ter estado presente na hora certa e ter sido um porto de abrigo em tantos momentos. Por toda uma aprendizagem paralela de melodias.

Aos meus pais e mano devo tudo, o amor incondicional, o apoio e entusiasmo desde sempre. É a eles que dedico o culminar de toda uma aventura em terras outrora desconhecidas (e frias). O que de melhor existe em mim, deriva deles.