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DISSERTATION

Experimental and clinical studies of the extracellular direct current (DC) potential: implications for (i) the assessment of electrical blood-brain barrier integrity and (ii) detection of spreading depolarization

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Preliminary remarks

This thesis consists of the three peer-reviewed publications to which I contributed. The three publications are referred to as Study 1 (Kang *et al*., 2013), Study 2 (Drenckhahn *et al*., 2012) and Study 3 (Winkler *et al*., 2012), respectively. In each section the main aspects of the three publications are summarized. For further details, please see the complete publications in the part "Publications" starting on page 24.

Abbreviations

AC	Alternating current
aCSF	Artificial cerebrospinal fluid
aSAH	Aneurysmal subarachnoid hemorrhage
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BK	Big conductance Ca^{2+}-sensitive K^+ channel
$[Ca^{2+}]_o$	Extracellular calcium concentration
COSBID	Co-Operative Studies on Brain Injury Depolarization
DC	Direct current
DHC	Sodium dehydrocholate
DIDS	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid: inhibitor of astrocytic Cl^- channel
ECoG	Electrocorticography
EEG	Electroencephalography
Gd-DTPA	Gadolinium-diethylene-triamine-pentaacetic acid
IEE	Ictal epileptic activity
$[K^+]_o$	Extracellular potassium concentration
KCl	Potassium chloride
LaGetSi	Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin
MHS	Malignant hemispheric stroke
MRI	Magnetic resonance imaging
p_aCO_2	Arterial partial pressure of carbon dioxide
$p_{ti}O_2$	Tissue partial pressure of oxygen
rCBF	Regional cerebral blood flow
SD	Spreading depolarization
SPC	Slow potential change
SK	Small conductance Ca^{2+}-sensitive K^+ channel
TBI	Traumatic brain injury
TBR	Target to background ratio
TEA	Tetraethylammonium

Zusammenfassung

Direct current (DC) Potentiale sind langsame elektrische Potentiale im Extrazellulärraum, die neuronal/astrozytäre Phänomene wie spreading depolarization (SD) oder ictal epileptic events (IEE) anzeigen. DC Potentiale könnten aber auch an der Bluthirnschranke (BBB) entstehen. Dies wird z.B. für das CO_2-abhängige DC Potential diskutiert. In Studie 1 habe ich diese Hypothese weiter untersucht. Zunächst musste ich feststellen, dass ein wichtiges Argument dafür, nämlich die DC Potentialänderung nach intrakarotidealer Gabe von Dehydrocholat (DHC), nicht stichhaltig ist, weil keine isolierte Öffnung der BBB sondern eine fokale zerebrale Ischämie durch DHC induziert wird. Im Anschluss applizierte ich jedoch eine Reihe von neuronalen/astrozytären Kanalinhibitoren *in vivo*, die das CO_2-abhängige DC Potential beeinflussen sollten, würde es in Neuronen oder Astrozyten generiert werden. Dies war jedoch nicht der Fall. Außerdem zeigen Hirnschnitte, denen eine intakte BBB fehlt, die typischen DC Potentiale von SD und IEEs, das typische CO_2-abhängige DC Potential konnten wir jedoch nicht nachweisen. Auch dies unterstützt seine Entstehung an der BBB. Messungen mit pH- und K^+-sensitiven Mikroelektroden *in vivo* unterstützten zudem die Annahme, dass das CO_2-abhängige DC Potential durch den Protonengradienten an der BBB entsteht. Somit wäre es ein Marker für den physiologischerweise geschlossenen, parazellulären Passageweg, der für die Aufrechterhaltung der Ionengradienten über die BBB verantwortlich ist. Mit diesem Marker konnten wir dann zum ersten Mal ein funktionelles Argument für Befunde früherer elektronenmikroskopischer Studien liefern, dass sich die BBB unter pathologischen Bedingungen in hierarchischer Weise öffnet. Zuerst öffnet sich der transzelluläre Passageweg für Makromoleküle wie Albumin, erst später der parazelluläre Passageweg für kleine Moleküle wie Protonen oder Kalium.

In der zweiten Studie untersuchten wir die Detektion von SDs mittels DC/alternating current (AC)-scalp-Elektroenzephalografie (EEG) parallel zur invasiven DC/AC-Elektrokortikografie (ECoG) in Patienten mit aneurysmatischer Subarachnoidalblutung (aSAH). Die DC/AC-ECoG ist der Goldstandard zur SD-Messung. Jedoch fanden wir auch Korrelate der DC- und AC-Veränderung in der scalp-EEG. Dies könnte in Zukunft zu einer nicht-invasiven, klinischen Methode der SD-Detektion weiterentwickelt werden.

In der dritten Studie analysierten wir hämodynamische Antworten auf SDs und IEEs in einem Patienten mit aSAH anhand DC/AC-ECoG und Laser-Doppler Flussmessung. Nicht nur bei SDs sondern auch bei IEEs traten hypoämische Antworten auf, die einen räumlichen Zusammenhang mit erhöhter BBB-Permeabilität aufwiesen. Dies deutet möglicherweise auf eine Beziehung zwischen BBB-Störung und abnormalen hämodynamischen Antworten auf SDs und IEEs hin.

Abstract

Direct current (DC) potentials are slow electrical potentials measured in the extracellular space which reflect biophysicochemical phenomena in neurons and astrocytes such as spreading depolarization (SD) or ictal epileptic events (IEE). It is nevertheless assumed that DC potentials can also arise at the blood-brain barrier (BBB). For example, the latter may apply to CO_2-dependent DC shifts, which I further investigated in rats in study 1. To start with, I found that one of the major supporting arguments for this hypothesis is invalid, namely the DC change following intracarotideal dehydrocholate (DHC) application, because intracarotideal DHC causes not only BBB opening but also middle cerebral artery thrombosis with focal cerebral ischemia. Intracarotideal DHC is thus not a suitable model to study isolated BBB opening. Nevertheless, I then applied a number of neuronal/astrocytic channel blockers topically to the brain. They should have altered the CO_2-dependent DC shift if it were of neuronal/astrocytic origin, but they failed to do so. Moreover, the typical CO_2-dependent DC shift was lacking in brain slices, which lack an intact BBB, whereas DC shifts of SDs or IEEs are preserved. This further supported the origin of the CO_2-dependent DC shift at the BBB. Using pH- and K^+-selective microelectrodes *in vivo*, we then found further evidence that the CO_2-dependent DC shift is specifically generated by the proton gradient across the BBB. Thus, it seems to be a marker for the closed paracellular pathway, which maintains the ion gradients across the BBB under physiological conditions. Using this tool, we then provided functional evidence for previous results with electron microscopy for the first time that the BBB opens in a hierarchical manner under pathological conditions. First, the transcellular pathway allows macromolecules such as albumin to pass. Only later, the paracellular pathway opens for small molecules such as protons or potassium.

In the second study, we investigated the detection of SDs using DC/alternating current (AC)-scalp-electroencephalography (EEG) simultaneously with invasive DC/AC-electrocorticography (ECoG) in patients with aneurysmal subarachnoid hemorrhage (aSAH). DC/AC-ECoG is the gold standard to measure SDs. However, we also found correlates of both DC and AC changes of SDs in the scalp-EEG. This may offer a non-invasive approach to detect SDs in patients in the future, but the tool requires further refinement.

In the third study, we investigated hemodynamic responses to SDs and IEEs in an aSAH patient using DC/AC-ECoG and laser-Doppler flowmetry. We found that hypoemic responses occurred not only during SDs but also during IEEs. Interestingly, these hypoemic responses showed a spatial association with increased BBB permeability, indicating a possible relationship between BBB dysfunction and abnormal hemodynamic responses to SDs and IEEs.

Introduction

Blood brain barrier and generation of CO_2-dependent intracortical DC deflection

The blood brain barrier (BBB) consists of endothelial cells, surrounded by astrocytic endfeet and pericytes. It is a highly selective barrier that maintains the homeostasis of the brain microenvironment because it limits the permeability of chemical factors between blood and interstitial compartment and controls the disposal of waste products (1). High selectivity of the BBB is attributed to continuous belt-like cell-cell complexes of adherens and tight junctions, which restrict the paracellular pathway. The transcellular pathway by passive diffusion, receptor-mediated and adsorptive transcytosis as well as carrier-mediated transport is an alternative route.

Notably, it is often believed that the paracellular pathway opens before the transcellular one when the BBB is disrupted under pathological conditions (2). This would mean that BBB opening to macromolecules implies BBB opening to small molecules such as protons, sodium and potassium ions. As their concentration gradients across the BBB establish the electrical potential difference between intravascular and interstitial compartment, the potential difference should disappear whenever the BBB opens to macromolecules. By contrast, permeability to macromolecules while the electrical barrier is preserved would suggest opening of the transcellular before the paracellular pathway. Unfortunately, functional investigation of this question is not trivial because the potential difference across the BBB is difficult to measure. Possibly, an alternative approach are recordings of the CO_2-dependent DC deflection based on the hypothesis that it is generated by the proton gradient across the BBB.

In the literature, the concept that the paracellular opens before the transcellular pathway was, for example, seemingly supported in a cat model showing that sodium dehydrocholate (DHC) caused both extravasation of Evans blue and disappearance of the CO_2-dependent DC shift (2). In contrast, earlier electron microscopic studies had indicated that the transcellular pathway would display increased vesicular transcytosis and formation of transendothelial channels (3, 4), long before tight junctions are impaired (5).

On this basis, we investigated in study 1 in rats: 1) the electrophysiological responses following intracarotideal DHC application, 2) whether the CO_2-dependent intracortical DC shift is a diffusion potential across the BBB driven by the proton gradient (6), and 3) whether BBB opening to Evans blue by either topical DHC application to the brain or intracarotideal mannitol administration is associated or not with changes in the extracellular potassium concentration ($[K^+]_o$) and/or the CO_2-dependent intracortical DC shift indicating opening of the electrical barrier.

Spreading depolarization and its correlates in scalp electroencephalographic (EEG) recordings

Spreading depolarization (SD) describes a propagating wave of near-complete breakdown of the ion gradients across the neuronal membrane. SD can be induced experimentally by various noxious conditions including chemicals such as potassium, glutamate, sodium pump inhibitors, status epilepticus, hypoxia and ischemia (7). The electrophysiological characteristics of SD are (i) an abrupt large negative slow potential change (SPC) in the low-frequency or direct current range of the electrocorticogram (DC-ECoG) (8-10) and (ii) silencing of brain electrical activity (spreading depression) in the high-frequency or alternating current range of the ECoG (AC-ECoG). The massive disturbance of ion homeostasis between intra- and extracellular compartment is due to a persistent influx of small cations such as sodium and calcium. As a result, neurons swell because water follows the cation influx (11). The recovery from SD is energy-dependent. Without recovery or when the recovery takes too long such as in ischemia, neurons die from SD e.g. due to the intracellular calcium surge (12).

Spreading depolarization is not only an experimental phenomenon at the bench. Since the last decade SD has been abundantly recorded in patients with aneurysmal subarachnoid hemorrhage (aSAH), delayed ischemic stroke after aSAH, malignant hemispheric stroke (MHS), spontaneous intracerebral hemorrhage or traumatic brain injury (TBI) (10, 13-17) and is associated with worse outcome (12, 18). However, the current technology to record SDs at the bedside has the limitation that it requires invasive neurosurgical interventions to place a subdural electrode strip. Thus, in study 2, we investigated whether and how reliably the non-invasive scalp DC/AC-EEG reflects SDs which were simultaneously recorded using invasive near DC/AC- ECoG or DC/AC-ECoG.

Hemodynamic responses to SD and ictal epileptic activity (IEE)

Under physiological conditions, regional cerebral blood flow (rCBF) increases in response to both SD and epileptic activity to match the elevated energy demand. In contrast, under pathological conditions, SD can lead to severe hypoperfusion, which in turn leads to prolonged depolarization, contributing to lesion progression (19). However, it remains unknown whether this abnormal hemodynamic response can also occur during IEEs. In study 3, we focused on hemodynamic responses to SD and IEE in a patient with aSAH.

Material and methods

Animal preparation

All animal experiments were approved by the Governmental Animal Care and Use Committee (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin (LAGetSi)). Male Wistar rats (n=119, 250-400g; Charles river Laboratories, Wilmington, MA, USA) were anesthetized with 100 mg/kg thiopental-sodium intraperitoneally, thracheotomized, and artificially ventilated. Another ten male Wistar rats underwent isoflurane anesthesia (1.5% to 2.0% in 30% O_2 and 70% N_2O) and were allowed to breathe spontaneously. The left femoral artery and vein were cannulated for monitoring of the systemic arterial pressure and administration of Evans blue, respectively. Vital parameters, including end-expiratory CO_2 pressure and body temperature, were monitored during the entire experimental period.

Cranial window preparation

In 120 animals an open cranial window was implanted over the somatosensory cortex to place ion-selective microelectrodes, whereas a closed window covered with a glass slip was built in 9 animals to image the pial arterioles using a CCD camera. The dura mater was removed in 95 animals with an open window and 9 animals with a closed window (type 2 preparation). In the remaining 25 animals with an open window, only two slits were made in the dura for the placement of the microelectrodes (type 1 preparation). In both preparation types, the cortex was continuously superfused with physiological artificial cerebrospinal fluid (aCSF). A burr hole was rostrally implanted to measure epidural potential changes and a lateral burr hole to elicit SD by either pin prick (n=4) or a drop of 3M KCl solution (n=5).

Cannulation of external carotid artery

The right external carotid artery was cannulated in a retrograde manner as described by Bullard et al. (20) to administer either DHC (n=16) or mannitol (n=16).

Animal recording techniques

Both the subdural DC/AC-ECoG (bandpass: 0-45 Hz) at the closed cranial window and the epidural DC/AC-ECoG at the rostral burr hole were measured with Ag/AgCl electrodes. The intracortical DC/AC-ECoG as well as extracellular changes in potassium ($[K^+]_o$), and pH were recorded in a cortical depth of 300 µm using potassium- and pH-selective microelectrodes. Regional CBF was continuously monitored with laser-Doppler flow probes. Systemic arterial blood pressure and

changes in cerebral ion concentration, voltage and rCBF were continuously recorded using a personal computer and Spike 2 software.

Experimental paradigms

Hypercapnic episodes were induced by ventilation with a gas mixture containing 21% O_2, 10% CO_2, and 69% N_2 for 5 min. Serial blood gas analyses were performed before, during, and after hypercapnia.

Brain slices

Six male Wistar rats (250-400g) were sacrificed by decapitation under deep isoflurane anesthesia for in vitro electrophysiological recordings. Brains were quickly extracted and cut. Coronal slices of 400 µm thickness from the somatosensory cortex were perfused with prewarmed aCSF in an interface-type recording chamber. Extracellular field potential (DC/AC-ECoG), $[K^+]_o$, $[Ca^{2+}]_o$, and pH were measured by ion- and pH-selective microelectrodes. Hypercapnic episodes were induced by switching the gas mixture from 80% O_2, 5% CO_2, and 15% N_2 to 80% O_2, 20% CO_2, and 0% N_2 every 5 min.

Patient recruitment

The research protocol was approved by the local ethics committees. Clinical and research consents were obtained according to the Declaration of Helsinki. Patients were recruited at two centers of the Charité University Medicine Berlin, Campus Virchow Klinikum (n=6) and Campus Benjamin Franklin (n=4), as part of the Co-Operative Studies on Brain Injury Depolarization (COSBID, www.cosbid.org). Study 2: aSAH n=5, MHS n=4. Study 3: aSAH n=1.

Human recording techniques

A single, linear, six-contact platinum ECoG recording strip with a diameter of 5 mm was placed on the cortex at the end of surgery as described previously (14) to continuously record the subdural ECoG for up to 15 days after aSAH and 8 days after MHS. Subdural electrodes were connected in sequential bipolar fashion as well as in unipolar fashion to a GT205 amplifier to record the near-DC/AC-ECoG (bandpass: 0.01-45 Hz) and each electrode was referenced to an ipsilateral subdermal platinum electrode. In some patients, the DC/AC-ECoG was recorded using a BrainAmp amplifier (bandpass: 0-45 Hz) in parallel to the near-DC/AC-ECoG. The scalp DC/AC-EEG was recorded by sintered Ag/AgCl-electrodes (bandpass: 0-1000 Hz), positioned according to the international 10-20 system. Six to 8 electrodes were placed ipsilateral to the ECoG recording strip

and 2-5 electrodes contralaterally. The reference electrode was positioned on the mastoid ipsilateral to the ECoG recording strip. In study 3, rCBF was measured by four optodes integrated in the subdural recording strip adjacent to the electrodes 3, 4, 5, and 6 as well as the tissue partial pressure of oxygen ($p_{ti}O_2$) by a Clark-type intraparenchymal oxygen sensor. Data were recorded and analyzed with a Powerlab 16/SP analog/digital converter, LabChart 7 software and BrainVision Recorder software.

Analysis of human recording data

SD was defined in the subdural recordings by the simultaneous onset of an SPC in the DC or near-DC frequency range (<0.05 Hz), and depression of spontaneous activity in the AC frequency range (~0.5-45 Hz) in individual channels as well as the sequential onset of SPC and spreading depression on adjacent channels, indicating the propagation of SD (14, 16). The duration of the depression period was determined using the integral of power of the AC-ECoG, starting with the initial decrease and ending with the onset of the recovery.

In the scalp recordings, the amplitude of the SPC was measured from the baseline to the peak negativity. Duration of EEG depression was scored in a similar fashion to the subdural recordings. EEG depressions were accepted in a range of ± 15 min around time points of SD occurrence in the subdural recordings because of the spatial distance between subdural and scalp electrodes. Ictal epileptic activity, rCBF, and $p_{ti}O_2$ in study 3 were analyzed as reported previously (12, 15, 21, 22).

Assessment of BBB dysfunction

Evaluation of BBB dysfunction in study 3 was performed using a quantitative analysis of gadolinium-diethylene-triamine-pentaacetic acid (Gd-DTPA)-enhanced MRI based on Tomkins et al. (23).

Statistical analysis

DC potentials were analyzed as absolute changes. Regional CBF and integral of the power of ECoG/EEG measurements were calculated as percentage changes from baseline. In all studies data are given as median (first, third quartile).

Results

Study 1: Generation of CO_2-dependent intracortical DC potential and BBB

Intracarotideal DHC application causes focal cerebral ischemia

An open cranial window was implanted to measure changes in the intracortical DC potential, $[K^+]_o$ and rCBF in response to hypercapnia. First, in six ventilated rats under thiopental anesthesia, DHC was applied intracarotideally (17.5%, 1ml) to disrupt BBB after two control hypercapnic episodes. Interestingly, the intracarotideal application of DHC led to a drastic decrease in rCBF to 27 (25, 41) % after a short increase to 365 (274, 413) %. In parallel with this rCBF decrease, baseline $[K^+]_o$ increased slowly from 3 to 8.5 (5.6, 11.9) mM, the ECoG recordings displayed ictal epileptic activities succeeded by a reduction of spontaneous activity. This rCBF decrease was followed by a sharp saddle-shaped negative intracortical DC shift with an amplitude of -20.5 (-18.9, -22.0) mV and a steep increase in $[K^+]_o$ to 53.5 (40.9, 57.4) mM, typical of SD in ischemic tissue (7, 24). Epidural recordings, obtained from the frontal burr hole, showed initially a positive DC shift of 0.6 (0.2, 1.0) mV at the onset of the rCBF decrease, and then a persistent negativity of -3.2 (-7.2, -2.6) mV. These features of ischemic SD were clearly different from those obtained from pinprick-induced SD (n=4). Furthermore, all animals, in which DHC was intracarotideally administered, showed post mortem pronounced Evans blue extravasation in the whole brain.

In the further experiments various concentrations of DHC in a lower volume were tested [0.5 ml: 17.5% (n=4); 15% (n=5); 10% (n=1)]. The animals were anesthetized with isoflurane to let them survive for 24 hours for histological assessment of ischemic injury afterwards. Unfortunately, they died shortly after the intracarotideal DHC application. However, they showed the characteristics of typical ischemic SD before they died.

In 9 animals, a closed cranial window was implanted to observe pial vascular changes. The intracarotideal DHC application caused an obstruction of this circulation imaged by videomicroscopy. Moreover, post mortem inspection of the brains demonstrated a thrombosis of the ipsilateral middle cerebral artery and its branches.

In stark contrast to the view in the literature, these experiments provided us with the important information that intracarotideal DHC application is not a good model to study a selective BBB disruption because it produces intraarterial thrombosis and severe ischemia.

The CO_2-dependent intracortical DC deflection in the rat does not depend on changes in rCBF

Interestingly, hypercapnia induced two different types of rCBF responses, namely, the normal hyperemic (n=53, type 1-rCBF) and an unusual hypoemic response (n=42, type 2-rCBF). Systemic parameters did not differ between the two groups, indicating that the different rCBF responses might have been attributed to a local factor at the window site, e.g., the two different preparation types used in the study (s. Materials and methods). The distribution analysis showed a significant association of the type 1-rCBF response with the type 1 preparation and of the type 2-rCBF response with the type 2 preparation, respectively (P=0.001, n=95, Chi-Square test with Yates correction for continuity). However, the intracortical DC potential always displayed a steady positive shift in response to hypercapnia. In contrast, the CO_2-dependent epidural DC shift was clearly affected by the rCBF response type, showing a significant relation of a positive polarity with the type 1-rCBF response and of a negative polarity with the type 2-rCBF response.

Evidence in vivo against neurons/astrocytes as generators of the intracortical CO_2-dependent DC deflection (preparation type 2)

To exclude the role of neurons/astrocytes in the generation of the positive CO_2-dependent intracortical DC deflection, various inhibitors of neuronal K^+-channels were topically superfused; amiloride (acid-sensing ion channel inhibitor, n=6), tolbutamide (inhibitor of ATP-sensitive K^+ channels, n=5), apamin (inhibitor of small conductance Ca^{2+}-sensitive K^+ channels (SK), n=5) and tetraethylammonium (TEA; inhibitor of big conductance Ca^{2+}-sensitive K^+ channels (BK), n=5). To block potassium and chloride uptake by astrocytes, barium (inhibitor of inwardly rectifying K^+ channels, n=6), and DIDS (inhibitor of astrocytic Cl^- channels, n=5) were applied. However, none of these inhibitors did affect the hypercapnia-induced positive intracortical DC shift. Ouabain (n=6), a Na,K-ATPase inhibitor, did not alter the positive CO_2-dependent intracortical DC shift, either, when it was applied at a concentration of 10 µM in which the neuronal α2 and the astrocytic α3 isoforms are selectively inhibited in rats (25).

Indirect evidence in vitro that the CO_2-dependent intracortical DC shift is generated at the BBB

To verify the findings of the *in vivo* experiments, changes in the DC potential, $[K^+]_o$, $[Ca^{2+}]_o$, and pH were measured in six neocortical slices from six different rats. Hypercapnia caused a significant decrease in the intracortical pH from 7.398 (7.398, 7.423) to 6.982 (6.970, 7.106) pH units (P<0.001, n=6, Paired t-test), and a significant increase in $[K^+]_o$ from 3.0 (3.0, 3.1) to 8.9 (8.7, 11.0) mM (P=0.042, n=3, Paired t-test). $[Ca^{2+}]_o$ increased insignificantly from 1.57 (1.56, 1.58) to 1.87 (1.72, 2.44) mM (n=3). In stark contrast to the positive DC shift *in vivo*, hypercapnia

always induced a small and steady negative intracortical DC shift of -0.61 (-0.54, -0.71) (P<0.001, n=6, Paired t-test) in the brain slices.

Evidence that CO_2-dependent intracortical DC deflection represents a diffusion potential related to the proton gradient across the BBB in rats

To confirm the hypothesis that the CO_2-dependent intracortical DC shift is a diffusion potential driven by the proton gradient at the BBB (6, 26, 27), intracortical pH changes during hypercapnia were measured in 16 animals and arterial pH changes were analyzed (n=80). Then, the CO_2-dependent intracortical DC shift was calculated using these pH changes according to the formula of Tschirgi and Taylor (6), which describes the potential difference dependent on the proton gradient between intravascular and interstitial compartment. The calculated value included the measured value of the positive CO_2-dependent intracortical DC shift, confirming the findings by Tschirgi and Taylor [the calculated vs. the empirical value; 2.9 (2.4, 3.4) mV vs. 3.1 (2.6, 3.4) mV (n=95)].

BBB opening to albumin is neither associated with an increase of baseline $[K^+]_o$ nor a change in the CO_2-dependent intracortical DC deflection

To investigate whether BBB opening alters the positive CO_2-dependent intracortical DC shift, DHC was topically applied to the brain in 9 rats at a concentration of 2 mM for 90 min. At this concentration, DHC opens the BBB without obvious acute neurotoxic effects (28). BBB opening to albumin was evaluated using the target to background ratio (TBR) of relative Evans blue extravasation in the window area compared to the contralateral corresponding area, as described previously (29). During DHC application neither the intracortical DC potential nor the baseline $[K^+]_o$ changed. The positive CO_2-dependent intracortical DC shift did not show any differences between the three hypercapnic episodes, either [1st, 2nd control hypercapnia and 3rd hypercapnia after DHC application: 2.9 (2.7, 3.4) mV, 2.8 (2.5, 3.1) mV, and 2.9 (2.8, 3.3) mV, n=9, one way repeated measures analysis of variance], although Evans blue extravasation was significantly larger in the DHC group than the sham control [TBR DHC vs. sham: 1.44 (1.37, 1.49), n=9 vs. 1.12 (1.11, 1.14), n=6; P=0.002, Mann-Whitney Rank Sum test].

Furthermore, mannitol (1.6 M, 1ml, n=16) was intracarotideally administered. The mannitol application led to a decrease in the systemic pH to ~7.1 as measured ex vivo and a transient but significant rCBF increase from 110 (96, 138) to 544 (277, 655) % (P<0.001, Mann-Whitney Rank Sum test). This was accompanied by a significant, short-lasting positive DC shift of 2.1 (1.7, 2.8) mV (P<0.001, Paired t-test), but no change in the baseline $[K^+]_o$. Similar to the topical DHC

application, the hypercapnia-induced positive intracortical DC shifts were not influenced by mannitol application which however caused a pronounced Evans blue extravasation as assessed by the TBR between ipsi- and contralateral hemisphere.

Study 2: Correlates of SD in human scalp EEG

Slow potential changes in scalp and subdural recordings in patients with aSAH

The simultaneous recording time of the near-DC/AC-ECoG and the scalp DC/AC-EEG was 694.0 h during which 275 SDs were identified in the near-DC/AC-ECoG. Of these, 193 SDs were detected in the scalp DC/AC-EEG, which occurred with a delay of 1.8 (0.8, 3.5) min. Spreading depolarizations displaying an SPC correlate in the scalp recordings showed a shorter interval between successive SDs compared to SDs, which were not reflected in the scalp recordings [33.0 (27.0, 76.5) vs. 50.0 (28.0, 130.5) min, P=0.009, n=273, Mann-Whitney Rank Sum Test].

Thirty six of the 275 SDs were silent, which means that brain electrical activity had been already suppressed before the onset of SD. These silent SDs with the persistent suppression of brain activity were well displayed in the scalp recordings. Moreover, the scalp EEG detected slow potential changes riding on a negative ultraslow potential, which indicates infarct development in animals (9, 30, 31). MRI scans during the monitoring period showed an association between the clustered silent SDs and a new delayed ischemic infarct in the ipsilateral hemisphere, as described previously (14).

The remaining 239 SDs identified in the near-DC/AC-ECoG were accompanied with spreading depression of brain spontaneous activity. Unfortunately, 34 of these were excluded for further analysis since the scalp AG-EEG could not detect depression of spontaneous activity due to an artifact caused by the automatic drift correction of the BrainAmp amplifier. Of the remaining 205 SDs, only 96 SDs displayed spreading depression of spontaneous activity in the scalp AC-EEG. Spreading depolarizations accompanied with a depression in the scalp AC-EEG showed a significantly longer interval between successive SDs [44.0 (28.0, 132.0) vs. 30.0 (26.5, 51.5) min, P=0.001, n=205, Mann-Whitney Rank Sum Test] as well as a significantly higher reduction of spontaneous activity in the integral of power of AC-ECoG. Also, both the depression durations and the depression levels of subdural AC-ECoG activity were significantly correlated with those in the scalp AC-EEG [n=96, correlation coefficient for depression duration/ for depression level; shortest depression: 0.301 (P=0.003)/ 0.287 (P=0.005), longest depression: 0.233 (P=0.023)/ 0.435 (P<0.001), Spearman's Rank Oder Correlation].

In contrast to the subdural electrodes, no spread either of slow potential change or of AC-EEG depression was observed between the scalp electrodes.

Patients with MHS

Due to insufficient statistical power, correlations between ECoG and EEG parameters were not analyzed.

Study 3: hemodynamic response to SD and IEE and BBB dysfunction

Both spreading depolarization and ictal epileptic event are energy-consuming processes that normally lead to an increase in rCBF to compensate the elevated energy demand. In contrast to such a normal hemodynamic response to SD, the inverse hemodynamic response describes an SD-induced severe hypoperfusion in injured tissue resulting in cell death through prolonged SD and persistently insufficient energy supply. Whether the inverse neurovascular coupling occurs in response to IEE is largely unknown. Here, hemodynamic responses to SD and IEE were analyzed in a patient with aSAH.

The total recording time was 248.4 h, during which 118 SDs occurred. Regional CBF measured by at least one of the four optodes (3-6) in 76 SDs did not show a uniform response to SDs. Thus, while at optode 3 and 6, hyperemic responses were observed [rCBF increase by 40.0 (22.3, 54.8) %, n=27; 31.0 (28.8, 39.5) %, n=13, respectively], optode 4 and 5 displayed both hyperemic [rCBF increase by 43.5 (38.0, 51.0) %, n=10; 33.0 (25.8, 40.3) %, n=13, respectively] and hypoemic responses [rCBF decrease by 15.0 (24.3, 8.5) %, n=5; 12.0 (16.0, 8.8) %, n=41, respectively]. Furthermore, 31 events of isolated IEE were observed. In 22 IEEs, rCBF was measured with the optode 3, 5, or both. Optode 3 recorded a hyperemic response (rCBF increase by 57.0 (30.0, 82.0) %, n=18), whereas optode 5 displayed a hypoemic response (rCBF decrease by 12.0 (13.0, 9.0) %, n=10). Interestingly, a higher BBB permeability was observed by a quantitative analysis of the Gd-DTPA enhanced MRI in the tissue neighboring optoelectrodes 4 and 5, which showed the hypoemic responses.

Discussion

CO_2-dependent intracortical DC deflection and BBB

The underlying mechanism to generate CO_2-dependent DC shifts is not entirely clarified. One possibility is that neurons and/or glial cells could generate these potential shifts. On the other hand, it has been reported that the BBB might be the main contributor to the generation of CO_2-dependent DC shifts, showing a marked reduction of subsequent DC responses to respiratory maneuvers after BBB disruption by intracarotideal DHC application in the cat (2). We used a similar experimental protocol to find out whether BBB disruption following intracarotideal DHC application alters the CO_2-dependent intracortical DC shift in the rat. Surprisingly, the intracarotideal application of DHC led to focal ischemia, showing the characteristics of ischemic SD and a thrombosis in the ipsilateral middle cerebral artery. These findings suggest that intracarotideal DHC application is rather a model for focal cerebral ischemia than selective BBB opening. Presumably, this results from severe injury of the endothelium by DHC (5).

Next, we topically applied various channel inhibitors to exclude neuronal/astrocytic origins of the positive CO_2-dependent intracortical DC shift. This positive DC potential shift could result from uneven neuronal hyperpolarization, analogous to mechanisms underlying extracellular field postsynaptic potentials or the negative DC shift during SD (8, 32). Accordingly, the current would internally move towards the more hyperpolarized regions, leave cells and enter through the less hyperpolarized regions. Hyperpolarization could occur when potassium channels, such as ATP- and calcium-sensitive potassium channels, were activated by protons (33).

The positive CO_2-dependent intracortical DC shift could be also attributed to potassium regulation by astrocytes, which passively depolarize by potassium release from activated neurons due to changes in the potassium transmembrane gradient (34). Thus, excessive potassium ions are transported into the astrocytes, accompanied by both chloride influx and sodium efflux due to activation of the Na,K-ATPase. As a result, this inflow of positive charges leads to a negative extracellular DC shift in the activated region. According to the spatial buffering mechanism, potassium ions are internally transferred toward regions of lower concentration through electrically coupled astrocytes and leave the cells in a remote region, resulting in a positive extracellular DC shift in the remote region.

However, our results showed that none of the neuronal and astrocytic channel inhibitors, including the Na,K-APTase inhibitor ouabain, did alter the positive CO_2-dependent intracortical DC shift. Furthermore, our brain slice experiments showed an opposite polarity of the CO_2-dependent intracortical DC shift in contrast to the experiments *in vivo*, supporting the hypothesis

that the positive CO_2-dependent intracortical DC shift might be generated at the BBB, which is not functional in brain slices because there is no circulation in brain slices.

Consistent with this hypothesis, the previous finding by Lehmenkühler et al. (35) showed a polarity reversal of CO_2-dependent DC shifts between the scalp and the cortical surface, suggesting that these potentials could be generated by an electrochemical diffusion of ions across the BBB. Tschirgi and Taylor (6) suggested a formula to determine the potential difference due to the proton gradient between the intravasal and the interstitial space. We measured extracellular and intravascular pH and calculated a positive CO_2-dependent intracortical DC shift using this formula which corresponded well with the measured DC shift. This finding supports that the positive CO_2-dependent intracortical DC shift is a diffusion potential resulting from the proton gradient at the BBB, which shows a linear relationship between the positive CO_2-dependent intracortical DC shift and arterial pH (6, 26, 27) and can be useful for the BBB permeability assessment to protons.

Then, we applied DHC topically and mannitol intracarotideally to find out the effect of BBB disruption on the positive CO_2-dependent intracortical DC shift. Neither DHC nor mannitol had an influence on it, although BBB was apparently disrupted, showing a significant Evans blue extravasation in both cases. Moreover, there were no changes in $[K^+]_o$ after the application of both drugs. Our findings are consistent with the previous studies from electron microscopic observations, which showed that the transcellular pathway was first disrupted following hyperosmolar solutions while the endothelial tight junctions remained intact (3-5). This means that there is a hierarchical pattern of BBB damage, i.e., the transcellular pathway is involved in mild to moderate BBB dysfunction, whereas opening of the paracellular pathway, which implies electrical barrier disruption, only occurs with severe BBB damage. If the electrical barrier was disrupted by either DHC or mannitol simultaneously with the transcellular pathway, changes in either $[K^+]_o$ or the positive CO_2-dependent intracortical DC shift should have been observed, as tight junctions would have also been open to small ions, such as potassium and protons. The stability of the positive CO_2-dependent intracortical DC shift and $[K^+]_o$ in the presence of the prominent Evans blue dye extravasation, hence, confirm the hierarchical pattern of BBB damage and both parameters provide a functional tool to evaluate the electrical barrier integrity.

DC potentials at the scalp

In study 2, we aimed to find out whether complex potential changes in the brain cortex such as SD could be reflected in the scalp EEG recordings. For this purpose, the ECoG recordings were performed to detect SDs simultaneously with the scalp EEG recordings in patients with aSAH.

Both the negative DC shifts and the reductions of brain activity detected in the scalp EEG were well correlated with those in the subdural ECoG. No polarity reversal between the scalp and the cortical space was observed, indicating the cortical origin of these potentials similar to in experiments in rats (36). In contrast to the subdural recordings, there was no spread of either the negative DC shifts or the brain activity suppressions between the scalp electrodes, which could be attributed to the superposition of volume conducted EEG sources from cortical generators.
These findings are promising, since scalp EEG recordings are non-invasive and detection of harmful ischemic potentials could be performed in a larger patient population, which does not need a surgical procedure. On the other hand, interpretation of scalp EEG recordings should be performed with caution, since DC potentials can be confounded by many different factors, especially changes in p_aCO_2 (37).

Hemodynamic responses to SD and IEE and BBB dysfunction

In study 3, we analyzed hemodynamic responses to SD and IEE in a patient with aSAH. Not only hyperemic but also hypoemic responses were observed in association with both SD and subsequent IEE. This observation is interesting, since SDs might cause BBB dysfunction (38), which might in turn induce epilepsy (39-41). Consistently, the MRI scan demonstrated a BBB dysfunction, which was spatially restricted to the region, where the hypoemic responses were recorded, suggesting that there might be a relationship between the hypoemic responses and BBB dysfunction. However, we could not verify the temporal relationship between the onset of BBB dysfunction and of SD and epileptic activity, since the MRI scan was performed only after the rCBF measurements.

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Eidesstattliche Versicherung

„Ich, Eun Jeung Kang, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Experimental and clinical studies of the extracellular direct current (DC) potential: implications for (i) the assessment of electrical blood-brain barrier integrity and (ii) detection of spreading depolarization“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe. Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE -*www.**icmje**.org*) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o) und werden von mir verantwortet.
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Berlin, den 09.12.2016 Eun Jeung Kang

<u>Anteilserklärung an den erfolgten Publikationen</u>

Frau Eun Jeung Kang hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: <u>Kang EJ</u>, Major S, Jorks D, Reiffurth C, Offenhauser N, Friedman A, Dreier JP. Blood-brain barrier opening to large molecules does not imply blood-brain barrier opening to small ions. Neurobiol Dis. 52:204-18, 2013.
Beitrag im Einzelnen: Teilnahme an der Planung des Projektes, tierexperimentelle Präparation und Datengewinnung, Datenanalysen einschließlich Statistik, Entwurf/Erstellung der Abbildungen,

Präsentation der Daten auf Konferenzen.

Publikation 2: Drenckhahn C, Winkler MK, Major S, Scheel M, Kang EJ, Pinczolits A, Grozea C, Hartings JA, Woitzik J, Dreier JP; COSBID study group. Correlates of spreading depolarization in human scalp electroencephalography. Brain. 135(Pt 3):853-68, 2012.
Beitrag im Einzelnen: Aufnahme und klinisches Monitoring der Patienten einschließlich klinischer und transkranieller Doppler-Untersuchung sowie Dateneintragung in die Datenbank, Teilnahme an der elektrophysiologischen Datengewinnung, Verfassen der Case reports, Teilnahme an Fallkonferenzen und Präsentation der Fälle, Beteiligung an den Datenanalysen.

Publikation 3: Winkler MK, Chassidim Y, Lublinsky S, Revankar GS, Major S, Kang EJ, Oliveira-Ferreira AI, Woitzik J, Sandow N, Scheel M, Friedman A, Dreier JP. Impaired neurovascular coupling to ictal epileptic activity and spreading depolarization in a patient with subarachnoid hemorrhage: possible link to blood-brain barrier dysfunction. Epilepsia. 53 Suppl 6:22-30, 2012.
Beitrag im Einzelnen: Aufnahme und klinisches Monitoring des Patienten einschließlich klinischer und transkranieller Doppler-Untersuchung sowie Dateneintragung in die Datenbank, Teilnahme an der elektrophysiologischen Datengewinnung, Beteiligung an der Datenanalyse.

Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

Prof. Dr. Jens Dreier

Unterschrift des Doktoranden/der Doktorandin

Eun Jeung Kang

Publications

In this part the complete published version of the three publications, Kang et al., (2013; Blood–brain barrier opening to large molecules does not imply blood–brain barrier opening to small ions. http://dx.doi.org/10.1016/j.nbd.2012.12.007), Drenckhahn et al., (2012; Correlates of spreading depolarization in human scalp electroencephalography. http://dx.doi.org/10.1093/brain/aws010) and Winkler et al., (2012; Impaired neurovascular coupling to ictal epileptic activity and spreading depolarization in a patient with subarachnoid hemorrhage: Possible link to blood–brain barrier dysfunction. http://dx.doi.org/10.1111/j.1528-1167.2012.03699.x) are inserted.

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete list of publications

Kang EJ, Major S, Jorks D, Reiffurth C, Offenhauser N, Friedman A, Dreier JP. Blood-brain barrier opening to large molecules does not imply blood-brain barrier opening to small ions. Neurobiol Dis. 52:204-18, 2013.

Drenckhahn C, Winkler MK, Major S, Scheel M, Kang EJ, Pinczolits A, Grozea C, Hartings JA, Woitzik J, Dreier JP; COSBID study group. Correlates of spreading depolarization in human scalp electroencephalography. Brain. 135(Pt 3):853-68, 2012.

Winkler MK, Chassidim Y, Lublinsky S, Revankar GS, Major S, Kang EJ, Oliveira-Ferreira AI, Woitzik J, Sandow N, Scheel M, Friedman A, Dreier JP. Impaired neurovascular coupling to ictal epileptic activity and spreading depolarization in a patient with subarachnoid hemorrhage: possible link to blood-brain barrier dysfunction. Epilepsia. 53 Suppl 6:22-30, 2012.

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