Aus dem Institut für Neurophysiologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

"Disturbance of blood brain barrier in the hippocampus and spreading depolarisation in a model of cortical stroke"

zur Erlangung des akademischen Grades

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von

Ezequiel Gustavo Lapilover

aus Buenos Aires

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1. Zusammenfassung

Um ein spezifisches neuronales Milieu zu ermögliche bilden zerebrale Gefäße eine Bluthirnschranke aus an der Endothelzellen, Astrozyten-Endfüße und Neurone beteiligt sind (Zlokovic et. al. 2008). Die Zusammensetzung der zerebrospinalen Flüssigkeit unterscheidet sich von der des Serums und es ist vorgeschlagen worden, dass die Zusammensetzung der zerebrospinalen Flüssigkeit um die synaptischen Kontakte kritisch für die Regulation normaler Signalprozesse im Gehirn ist (Abbot, Rönnbäk, Hansson 2006). Damit ist die Bluthirnschranke hauptsächlich für Austauschprozesse verantwortlich (Abbot, Rönnbäk, Hansson 2006). Schlaganfall und andere neurologische Erkrankungen sind häufig durch ein Öffnen der Bluthirnschranke gekennzeichnet, dessen Konsequenzen noch nicht vollständig verstanden sind (Stoll et. al. 2009, Friedman A & Heinemann U). Der Schlaganfall ist häufig tödlich und Überlebende weisen oft erhebliche Einschränkungen auf, wozu auch epileptische Anfälle nach dem Schlaganfall und eventuell eine spätere Epilepsie gehören. In meiner Doktorarbeit interessiere ich mich für die Störungen des ionalen Mileus nach Öffnung der Bluthirnschranke, wobei ich Messungen mit ionenselektiven Mikroelektroden und hippokampale Hirnschnittpräparate verwende. Um resultierende Veränderungen zu erfassen, verfolgte ich zwei Ansätze. Zum einen stellte ich Hirnschnitte her, nachdem die Tiere vor 24 Stunden eine intraventrikuläre Applikation von bovinen Serumalbumin erhalten hatten, zum anderen wurde akute Hirnschnittpräparate von naiven Ratten mit Albumin behandelt. Um die ionalen Veränderungen nachzuahmen wurden die Hirnschnitte mit einer Lösung behandelt, deren ionale Zusammensetzung der des Serums der Ratte entsprach. Ich habe diese Daten mit denen verglichen die an Hirnschnitten erhoben worden waren, die von Tieren gewonnen wurden, an denen 24 Stunden zuvor durch eine Photothrombose ein kortikaler Schlaganfall induziert wurde.

Wir beobachteten eine ausgeprägte Öffnung der BHS an der Seite der photothrombotisch-induzierten Läsion. Die Öffnung der BHS war am stärksten 12 Stunden nach der Induktion des Schlaganfalles, welche den Neokortex sowie subkortikale Hirnstrukturen wie den Hippokampus mitbetroffen hat. Spontan auftretende SDs wurden oft (n=8/10) in hippokampalen Hirnschnittpräparaten der Läsionsseite beobachtet, die 24 Stunden nach der Induktion des Schlaganfall untersucht wurden, wobei eine künstliche Serumartige Lösung ohne Albuminzusatz verwendet wurde, deren Zusammensetzung der des Serums der Ratte entsprach. In Hirnschnitten die 24 h nach intraventrikulärer Albuminapplikation untersucht wurden traten keine spontanen SDs auf unabhängig davon, ob die Hirnschnitte mit künstlicher albumin-freien serumartigen Lösung oder mit künstlicher zerebrospinalen Lösung behandelt wurden.

Es wurde hingegen keine spontane SDs bei hippokampalen Hirnschnitpräparaten beobachtet, die aus Tieren stammten, die mit einer intrazerebroventrikulären Applikation von Albumin 24 Stunden zuvor behandelt worden sind, gleichgültig ob die hippokampalen Hirnschnittpräparate mit künstlichem Liquor oder mit künstlichem ionalen Serum der Ratte behandelt wurden. Allerdings wurde eine abgesenkte Reizschwelle für die Induktion von SDs beobachtet, wenn die Hirnschnittpräparate mit künstlichem Liquor (n=3/29) sowie mit künstlichem Serum (n=8/10) behandelt wurden. Die direkte Applikation von Albumin in Hirnschnitpräparaten des Hippokampus *in-vitro* konnte eine erniedrigte Reizschwelle für die Induktion von SDs bestätigen, wenn Reizfrequenzen von 10 Hz und mehr

verwendet wurden. In den aus vorbehandelten Tieren stammenden Hirnschnittpräparaten wurde oft nach repetitiver Reizung prägnante epileptische Nachentladung beobachtet aus denen die SD entstanden. Die epileptische Aktivität war mit abnorm großen Anstiegen der extrazellulären Kaliumkonzentration assoziiert. Unsere Ergebnisse zeigen, dass die Öffnung der BHS und die Diffusion vom Albumin in das peri-infarktöse Hirngewebe eine wichtige Rolle bei der Entstehung von Peri-infarkt Depolarisationen spielt.

2. Abstract

To maintain a specific neuronal environment mammalian brain vessels form a blood-brain-barrier (BBB) which comprises endothelial cells, pericytes, astrocytic endfeets, and neurons (Zlokovic et. al. 2008). The composition of the cerebrospinal fluid differs from that of blood and it has been argued that the precise regulation of the local ionic microenvironment around synapses is critical for the regulation of neuronal signalling (Abbot, Rönnbäk, Hansson 2006). The BBB is the major site for blood-cerebrospinal fluid exchange (Abbot, Rönnbäk, Hansson 2006). Stroke and other neurological diseases are associated with opening of the BBB, and its consequences are not yet known (Stoll et. al. 2009, Friedman A & Heinemann U). Stroke is a mortal disease and the survivors can show profound functional disabilities including post-stroke seizures and epilepsy. Thus, in this thesis I focus on the disturbance of the ionic microenvironment following BBB opening using ion-sensitive electrodes and acute hippocampal slice preparations obtained from rodents to study alterations in neuronal signalling. I used two approaches for mimicking the consequences of the opening of the BBB in the hippocampus. First approach was to expose *in-vitro* hippocampal slices to bovine serum albumin. Because I intended to mimic the ionic alteration occurring in the extracellular space during BBB opening, hippocampal slices were exposed to a solution containing the ionic concentration of serum ions of rodents. The delivery and exposure of albumin to the hippocampus *in-vivo* was also achieved using a single injection of bovine serum albumin into the brain ventricles. I compared these data to that obtained from acute hippocampal slices from animals which were sacrificed 24 hours after inducing a focal neocortical stroke using a photothombotic stroke model. These animals showed a massive BBB opening on the lesion side that affected also subcortical structures such as the hippocampus (opening BBB peak 12 hours after stroke). We observed often spontaneous SDs in acute hippocampal slices (n=8/10) prepared from the lesional side which were exposed to serum electrolytes 24 hours after photothrombotic stroke. Acute hippocampal slices prepared from naive animals 24 hours after intraventricular injection of albumin showed no spontaneous SDs, but a reeduced threshold for SDs using recurrent repetitive stimulation during serum electrolyte exposure (n=8/10) or normal cerebrospinal fluid (n=3/29). *In-vitro* exposure of albumin confirmed a reduced threshold for stimulus-induced SDs (≥ 10 Hz). Acute hippocampal slices after stroke or albumin exposure show that seizure like events are readily evoked by repetitive stimulation which transform

into spreading depolarizations due to abnormal potassium accumulation. I conclude that opening of the blood brain barrier and the diffusion of albumin into non-necrotic tissue might play an important factor in the generation of peri-infarct depolarisations.

3. Introduction

Recent studies showed that spreading depolarizations (SDs) are common in experimental stroke models and in stroke patients. Stroke affects a large part of the population in an age dependent manner with 6 per 100,000 individuals in infants, 42 per 100,000 individuals in the age of 15 to 45 years, with 310 per 100 000 in the age group of 45 -65 years and with 1.612 per 100.000 in the age group f more than 65 years) [Data obtained from Diagnosis data of German Hospitals ICD-10:I60-I69 for the year 2010, *Gesundheitsberichtserstattung des Bundes*]. SDs are characterized by a rapid and strong neuronal and glial depolarization and near-complete breakdown of the ionic gradients between the extracellular and the intracellular compartment due to a net influx of sodium and calcium into the cells, release of potassium and cellular edema (Kraig and Nicholson, 1978; Somjen 2001). Experimental evidence in rodents showed that SDs can be recorded in cortex around the infarct focus and that frequent occurrence is related to a secondary increase in infarct volume (Nakamura et. al. 2010). It was suggested that the SDs in peri-ischemic cortical tissue are due to diffusion of potassium and glutamate from the ischemic core into its surround (Dirnagl 1999, for review). Whether opening of the blood brain barrier and diffusion of blood electrolytes and proteins contributes to generation of SDs is unknown.

Rodents are a suitable model for studying BBB opening occurring in human brain diseases. Indeed, recent studies conducted in the neocortex of rodents have shown that BBB opening with extravasation of serum albumin mediates an astrocyte transcriptional response with consequent disturbance of potassium and glutamate homeostasis (Seiffet et.al. 2004, Ivens et.al. 2007, David et. al. 2009). It was recently shown that induction of a cortical stroke through the intact skull using the Rose Bengal method resulted in blood brain barrier opening also in subcortical structures such as hippocampus. We used this approach and studied the alterations in neuronal function and potassium accumulation in acute hippocampal slices prepared from the lesion hemisphere 24 hours after focal neocortical stroke and compared these data to data obtained from naiv animals exposed for 24 hours to albumin through intraventricular application of albumin. Acute exposure of hippocampal slices to albuminsupported our hypothesis.

Aims

The principal aim of this project was to understand which processes occur during the opening of the BBB which affects subcortical anatomical structures in the first 24 hours after stroke (Stoll et. al. 2009). For this purpose we used (a) the first 6 hours after a direct *in-vitro* exposure of hippocampal slices to a serum-like solution containing albumin, and (b) hippocampal slices obtained from animals that were exposed for 24 hours to an *in-vivo* intraventricular injection of bovine serum albumin.

4 Methods

4.1 *In-vivo* application of albumin

We exposed the brain of rats to bovine serum albumin (BSA) for 24 hours using a single intraventricular application. For intraventricular application of BSA, animals were subjected to a ketamine–xylazin anesthesia and placed in a stereotactic frame. Local anesthetic Xylocain (1 %) was injected subcutaneously before opening the skin over the skull. After exposing the calvarium a burr hole (1.5 mm diameter) was drilled into the bone at 0.9 mm posterior, 1.6 mm lateral to bregma. BSA (0.4 mM, purity ≥96 %) dissolved in 10 µl of artificial cerebrospinal fluid (ACSF) was injected at a depth of 3.5 mm from the surface of the skull with a speed of about 1–2 µl/min. Sham operated animals were treated with intraventricular injection of ACSF. Following the procedure, the wound was sutured and treated with xylocain gel (2%). Buprenorphin subcutaneously (0.05 mg/kg body weight) was also applied. To localize the distribution of albumin in the tissue, fluorescein isothiocyanate conjugated bovine albumin in ACSF was used in a few experiments (concentration used 0.4 mM).

4.2 *In-vitro* application of albumin

For the *in-vitro* application of albumin, brain slices obtained from naive rats were left to recover for 60–120 min after the slicing procedure with carbogenated ACSF, then incubated for one h in carbogenated albumin-containing serum electrolyte solution (ASERUM) that contained in mM 0.4 BSA, 129 NaCl, 21 NaHCO3, 1.25 NaH2PO4, 0.8 MgSO4, 1.3 $CaCl_2$, 5.7 KCl, 10 glucose, 1 glutamine, pH 7.4 at 34-36°C osmolarity 305–312 mOsmol/l (Seiffert et al., 2004). Recordings were performed in the presence of the aforementioned solution: Carbogenated albumin free serum electrolyte solution (afSERUM) but without BSA, pH 7.4 at 34–36 °C, osmolarity 295–300 mOsmol/l.

4.3 Morphological Analysis

For morphological analysis histological standard methods were used (Seiffert et al., 2004). Tissue was fixed with 4% paraformaldehyde in 0.1 M phosphate buffered saline overnight, followed by cryoprotection in 30% saccharose in 0.1 M phosphate buffer solution (PBS) overnight and sliced on a cryostat. 25 µm thick sections were used for immune fluorescence staining and incubated with rabbit anti-NeuN (1:500, Sigma-Aldrich, Germany). Signal detection was achieved by incubation with Alexa Fluor 568 goat anti-rabbit secondary antibody (1:200, MoBiTec, Göttingen, Germany) for two hours at room temperature. Sections were mounted on gelatine-coated slides and cover slipped using Fluoromount.

4.4 Extracellular recordings

For electrophysiology *in-vitro* recordings in hippocampal slices were performed in stratum pyramidale of area CA1 and occasionally also in area CA3 using an interface chamber and extracellular glass electrodes (154 mM NaCl, tip diameter 2–3 µM, resistance 2 to 4 MΩ). Stimulation frequencies were between 1 and 100 Hz (applied in stratum radiatum) or 20 or 200 Hz (applied in stratum lacunosum moleculare). Evoked signals were obtained using 50% of the intensity required to induce maximal responses. Two protocols were used for recurrent repetitive stimulation: 10 trains of 10 stimuli each with an inter-train interval of 1 s, or 3 trains of 40 stimuli each with an inter-train interval of 40 s (Ul Haq et al., 2011; Wöhrl et al., 2007).

4.5 Substances

For pharmacological experiments Ifenprodil (3 µM), a NMDA-2B receptor antagonist, was added to afSERUM. We used BSA (modified method by Cohn also called 'Cohn fraction V'), since BSA differs slightly from the rat serum albumin (Bos Taurus vs. Rattus Norvergicus shows 70 % of identity by protein analysis) and is also biologically active in rodents (Seiffert et.al. 2004, Ivens et.al. 2007). The osmolarity of the solutions (ACSF and afSERUM) after adding albumin showed a physiological range (308-312 mOsmol, Seiffert et. al 2004, Ivens et. al. 2007).

4.6 Ion-sensitive electrodes

Extracellular Potassium was measured using double barreled ion-sensitive microelectrodes. The ion-sensitive barrel was filled with potassium chloride (100 mM) and the tip (2–3 µM) was filled with a K+-sensitive valinomycin based resin (Fluka 60031 ionophore,Sigma-Aldrich, Switzerland). The reference barrel was filled with sodium chloride (154 mM, 10 MΩ).

4.7 Statistics

Statistical differences between control and treated slices were proven using Mann Whitney Rank Sum Test and the chi-square test. When the total number of each group was less than 10 experiments, Fisher's exact test was employed. Statistical significance was assumed with p values <0.05. Values are presented as mean± standard error of the mean (SEM).

5 Results

Peri-infarct hippocampus affected by BBB opening 24 hours after photothrombosis shows higher incidence of SD

It was previously described (Stoll et al. 2009) that exposing the skull to halogen light immediately after intravenous injection of Rose Bengal results in a focal neocortical infarct (Watson et.al 1985) surrounded by a cortical and subcortical peri infarct zone which display permeable BBB (Stoll et. al 2009). Our group could reproduce this result (Lapilover et. al. 2012) and found that those hippocampal slices obtained from the peri infarct hippocampus 24 hours after photothrombosis (4 animals) showed spontaneous SD (8 out of 10 slices, Figure 1) when exposed *in-vitro* to the concentration of serum electrolytes (afSERUM). In those two peri infarct hippocampal slices obtained 24 hours after photothrombosis, which did not show spontaneous SDs, recurrent repetitive stimulation of stratum radiatum using half-maximal intensity (at 0.05 Hz and 100 Hz) could readily induce SD. SDs could also be induced in almost 80 % of peri infarct hippocampal slices (n=9) obtained 24 hours after photothrombosis exposed to ACSF.

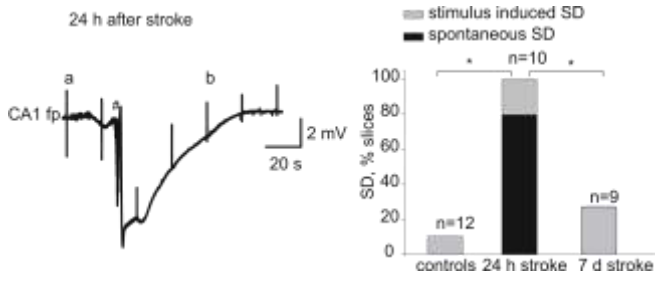

Figure 1 modified from (Lapilover et.al. 2012) (left) Hippocampal field potential recording 24 h post-stroke showing an SD during very low frequency (0.05 Hz) stimulation of the stratum radiatum in presence of afSERUM. (right) Incidence of spontaneous and stimulus-induced SDs.

In-vivo application of albumin into the ventricles for 24 hours affects both hippocampi and results in low threshold for stimulus-induced SDs

After applying fluorescent albumin (fluorescein isothyiocyanate conjugated albumin) into the ventricles in a few experiments, we observed a homogeneous diffusion of albumin to the

homolateral and contralateral hippocampi 24 hours after injection (Figure 2). The histological study suggested that the uptake of albumin occurs in non-neuronal cells, since FITC-albumin positive cells stained negative with anti-NeuN antibody and we observed that the distribution of these cells was outside the pyramidal cell layer (Figure 2).

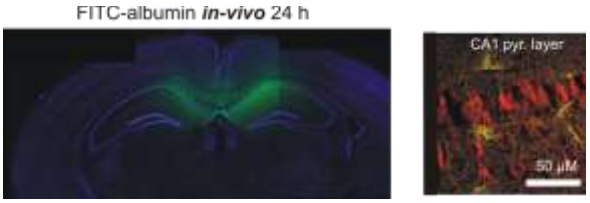

Figure 2 modified from Lapilover et.al.

(left) Coronal brain section showing the distribution of fluorescein isothiocyanate conjugate bovine (FITC)-albumin (green) 24 h after intraventricular injection. Cells were stained with DAPI (blue). (right) FITC-albumin positive cells and Neu-N staining of CA1 pyramidal cell layer

When hippocampal slices were studied *in-vitro* 24 hours after *in-vivo* BSA injection (11 animals) we could not find spontaneous SDs neither with the exposure to afSERUM (10 slices) nor with ACSF (29 slices), in contrast to observations in peri infarct hippocampal slices treated with afSERUM. However, when repetitive recurrent stimulation was applied in stratum radiatum of hippocampal slices from *in-vivo* albumin treated animals exposed to afSERUM (8 animals) SD incidence was 80 % (10 slices). Repetitive recurrent stimulation under ACSF of hippocampal slices 24 hours after *in-vivo* albumin treatment (11 animals) show an SD incidence of 10 % (29 slices). Control slices under ACSF (n=15) or under afSERUM (n=6) from sham-operated animals (n=6) did not show stimulus induced SDs (Figure 3).

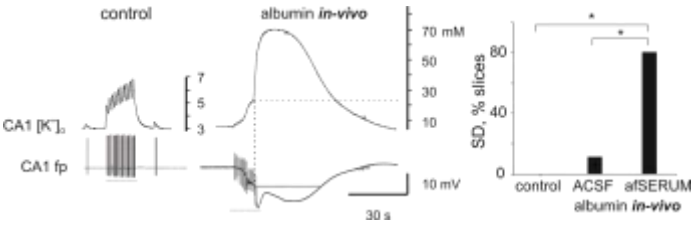

Figure 3 modified from Lapilover et.al.

Stimulus induced changes in $[K+]_o$ and field potential recording in slices obtained from a sham-operated animal (control, left) and a 24 h albumin-treated animal (middle). Incidence of stimulus-induced SDs in all conditions (right)

The direct cortical input to CA1 was also investigated with respect to induction of SDs. Stimulus-dependent generation of SDs using recurrent repetitive stimulation was observed under afSERUM perfusion in all slices (Figure 4, n=10) obtained from *in-vivo* albumin treated animals (n=4), but never

in slices perfused with ACSF (n=10 slices, 7 animals) nor in slices from sham-operated animals (Figure 4, under afSERUM n=5 slices, 4 animals).

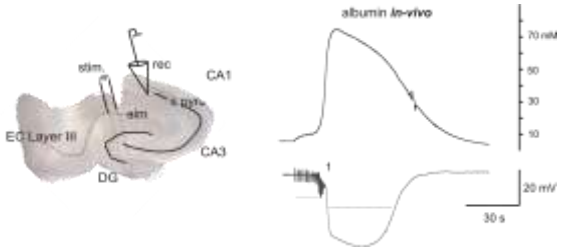

Figure 4 modified from Lapilover et.al.

Photograph of a slice for representing the stimulation positions (left). Repetitive recurrent stimulation-induced elevated $[K^+]_o$ and SD in a slice perfused with afSERUM from an animal treated with intraventricularly albumin 24 h earlier (right).

In-vitro pretreatment of hippocampal slices with albumin shows also reduced threshold for stimulus-induced SDs

Stimulation at low frequencies revealed altered potassium homeostasis after BBB dysfunction (Ivens et.al. 2007, David et.al. 2009). Extracellular potassium accumulation depends strongly on the frequency of stimulation (Heinemann and Lux 1977; Nixdorf-Bergweiler et. al. 1994, David et. al. 2009). To study the threshold frequency for SD induction we exposed naive slices to a serum-like solution containing 0.4 mM albumin (ASERUM) for 1 h to allow albumin uptake into cellular compartments (Ivens et al., 2007) and subsequently 1 h to afSERUM, which contains only the electrolyte composition of serum. Under these experimental conditions (afSERUM perfusion after BSA exposure), SDs were induced in 12.5% of slices when stimulation frequency was < 10 Hz (n=24 slices, 8 animals). SDs were significantly more likely to be induced (53% of slices) when stimulation frequency was between 10 and 100 Hz (n=32 slices, chi-square test, $p< 0.01$; Table 1 in Lapilover et.al. 2012). In slices perfused with afSERUM without prior exposure to BSA, stimulus-induced SDs occurred in 12.8% (47 slices, 16 animals). In contrast, SDs could be induced overall in 44.6% of the slices (n=65) that were pretreated with BSA and recorded only in the presence of afSERUM, showing a significant higher incidence of stimulus-induced SDs than in naïve slices exposed only to afSERUM (Figure 5, chi-square test, $p < 0.01$).

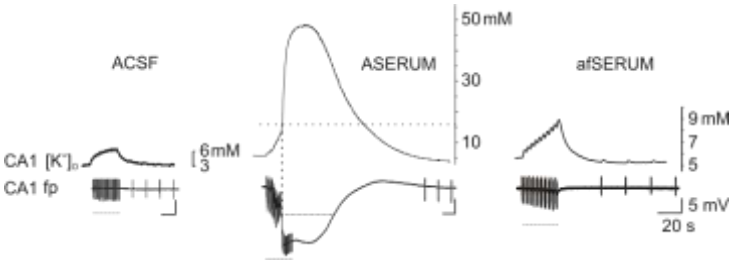

Figure 5 modified from Lapilover et.al.

Representative traces showing simultaneous $[K^+]_o$ and field potential recorded during stratum radiatum repetitive recurrent stimulation (10 Hz) in naiv slices perfused with ACSF (left), pretreated with BSA and recorded only in the presence of afSERUM (middle), and perfused only with afSERUM (without BSA pretreatment, right)

In-vivo or in-vitro exposure of hippocampal tissue to albumin leads to alteration of stimulus-induced potassium clearance

We studied potassium clearance by measuring $[K^+]_o$ changes evoked by stratum radiatum paired-pulse stimulation (50 ms inter-pulse interval) recorded after exposure to albumin *in-vivo* or *in-vitro*. Treated slices included those pre-exposed to ASERUM *ex-vivo* for 1 hour (n=5 slices/5 animals) or slices from albumin-treated animals *in-vivo* for 24 hours (n=5 slices/5 animals). Recordings were obtained using afSERUM solution. Control slices were from naive animals perfused with ACSF (n=5 slices/4 animals) or afSERUM (n=4 slices/3 animals). Stimulation parameters were adjusted to yield similar field potential responses in all experimental conditions (see Lapilover et. al.2012). Changes in amplitude, half time of rise and half time of decay of changes in $[K^+]_o$ measured as well as the slope of the fexcitatory postsynaptic portential (fEPSP20–80) recorded in the stratum radiatum and the population spike amplitude in CA1 stratum pyramidale. Comparison the epsp-coupling (slope of the fEPSP20–80 versus the amplitude of the population spike) between experimental conditions indicated a similar neuronal activation in all groups (see Lapilover et. al., 2012). No differences were found between treatments in the mean rise and half time of rise in $[K^+]_o$ (Figure 6, Lapilover et. al. 2012). In contrast, the half time of decay in $[K^+]_o$ was significantly longer in slices exposed to ASERUM *in-vitro* (1.6 ± s) or 'pre-treated' with BSA *in-vivo* (1.75±0.32 s) compared to control slices treated with either ACSF or with afSERUM (0.95±0.3 s and 0.55±0.1 s, respectively, one-way ANOVA, p=0.0037).

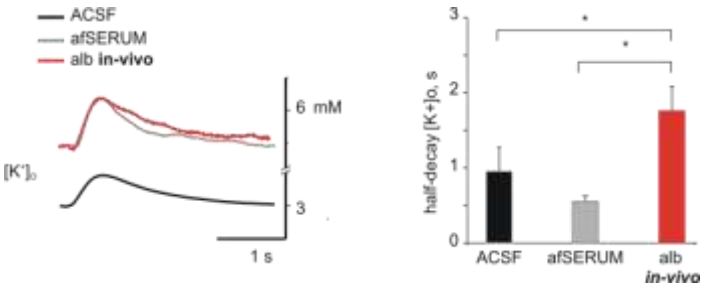

Figure 6 modified from Lapilover et.al. Representative $[K^+]_o$ signals in response to a paired-pulse stimulus under the different experimental conditions (left). Averaged and standard error of the mean of the half time of $[K^+]_o$ decay (right)

In-vivo exposure of neocortex to albumin is also associated with disturbance of K^+ accumulation

Treatment of neocortex for 24-48 hours with the direct application of albumin was shown to downregulate transcripts of inward-rectifying K^+ channel specific from astrocytes (David et. al. 2009).

Our group also reported slower decay kinetics of extracellular levels of K^+ in response to pressure application in BBB-treated animals (Ivens et al., 2007). The repetitive stimulation at the border between white and gray matter led to abnormal extracellular potassium accumulation in the same cortical column (neocortical slice layer IV) already at low frequencies of stimulation (Figure 7, ≥ 10 Hz).

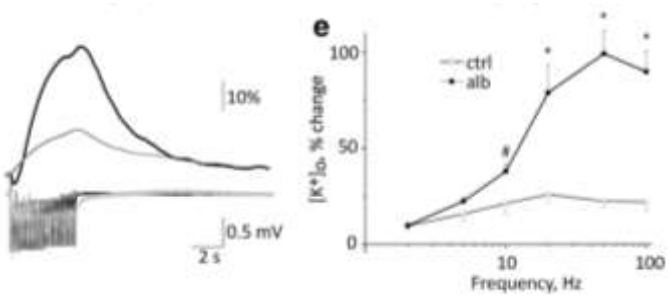

Figure 7 modified from David et. al. 2009

$[K^+]$-levels recorded in layer IV of neocortex in controls and 24 h albumin treated slices during stimulation at 20 Hz (left). Mean $[K^+]$ levels in control and treated slices (24 h albumin) during stimulation 2-100 Hz.

SDs in slices exposed to albumin are associated with epileptiform activity

In slices from animals 24 h after photothrombosis epileptiform activity preceded spontaneous SDs in 6 out of 8 slices. In the other cases we noted stimulus induced epileptiform discharges preceding onset of SDs. Epileptiform activity appeared during the recovery phase of the SDs as well (Dreier et al., 2012; Leão, 1944; Pomper et al., 2006; Van Harreveld and Stamm, 1953), when $[K^+]_o$ declined to 19.5±1.6 mM, and it ceased when $[K^+]_o$ declined to less than 5.7 mM. This seizure-like activity during SD recovery lasted 38.7±8.6 s and was composed of clonic like after discharges which lasted between 170 and 320 ms at a frequency of 1–2 Hz (Figure 8). The frequency content of the field potential fluctuations during a single clonic like after-discharges was usually not higher than 60 Hz.

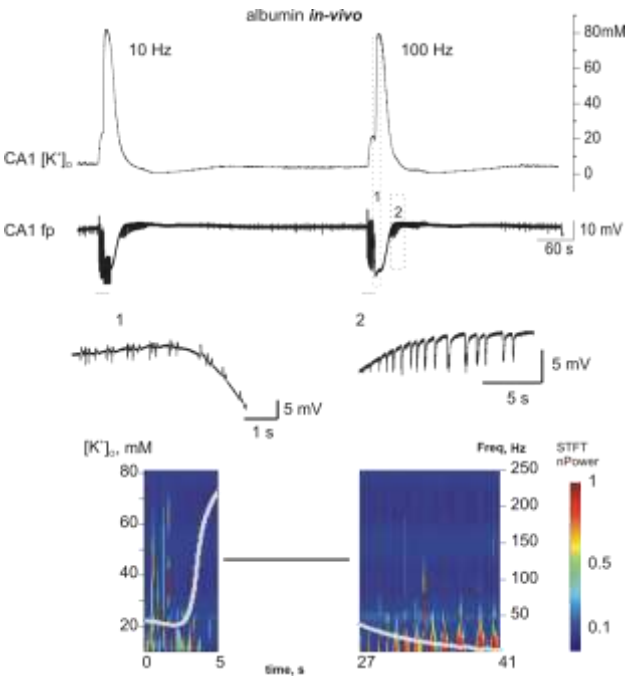

Figure 8 modified from Lapilover et. al.

Slices were obtained from animals treated with intraventricularly albumin 24 h earlier and perfused with afSERUM. Representative traces of two consecutive SDs showing simultaneous $[K^+]_o$ and field recordings in the stratum pyramidale of area CA1. First and second SDs were induced by recurrent repetitive stimulation of stratum radiatum at 10 and 100 Hz, respectively (Top). Numbers 1 and 2 indicate the epileptiform activity observed during the recovery of the field potential. The representation of the frequency of epileptiform activity and the $[K^+]_o$ (Bottom).

Blockage of the specific N-methyl-D-aspartate receptor type 2 B prevents stimulus induced epileptiform discharges and SDs

In the presence of ifenprodil in afSERUM, recurrent repetitive stimulation of stratum radiatum or stratum lacunosum moleculare only rarely (1 out of 6 slices and 1 out of 5 slices, respectively) resulted in generation of SD after *in-vivo* albumin exposure for 24 hours (stratum radiatum and lacunosum moleculare without ifenprodil: 90%; with ifenprodil: 16.7%, $p<0.05$ Fischer's exact test). Ifenprodil also prevented generation of epileptiform discharges which were usually induced during recurrent repetitive stimulation (n=6). Only in the slice in which ifenprodil did not prevent induction of SDs, ifenprodil also failed to prevent induction of epileptiform discharges.

6. Discussion

In this thesis I focused on the potential role of BBB dysfunction in the susceptibility for SDs and found that (1) hippocampal tissue exposure to serum albumin *in-vivo* or *in-vitro* is sufficient to reduce the threshold for induction of stimulus-induced SD; (2) serum levels of electrolytes further increases the likelihood for the generation of SD; (3) hippocampus slices affected by albumin show reduced clearance of $[K^+]_o$ following repetitive stimulation; (4) SD is usually triggered when $[K^+]_o$ rises above 17 mM, potentially due to disturbed $[K^+]_o$ homeostasis; (5) SD is often associated with epileptiform activity preceding the SD and re-appearing during its recovery. These findings are in contrast to a previous study in which susceptibility for generation of SDs in the chronic epileptic tissue was found to be reduced (Tomkins et al., 2007). In that study, experiments were done one month after blood

brain barrier damage, in a time window when epileptogenesis had already occured. Moreover, that tissue was not exposed to a solution with the electrolyte composition of serum. This suggests that the alterations we describe might apply to an early situation after stroke, after which serum albumin and serum electrolytes affect naive hippocampal tissue in the first 24 hours. In the chronically epileptic tissue the threshold of SD is increased (Tomkins 2007 et. al). Accordingly, in the paper of Maslarova et. al. 2011, it was shown that the SD threshold is altered by a number of factors. The age of the animals seems to play a role: The younger the animals, the lower the threshold for SD. This was measured by using the latency and extracellular potassium levels needed for inducing a SD. The chronically epileptic tissue show higher levels of extracellular potassium for inducing an SD.

Data from animal models of epilepsy indicate a key role for vascular angiogenesis and leak of serum proteins through a dysfunctional BBB in initiating specific signalling cascades within different elements of the neurovascular unit (Rigau V et. al. 2007; Aronica E. et. al. 2004; Friedman A & Heinemann U, book chapter). Transformation of astroglia cell populations, a robust inflammatory response, and angiogenesis are associated with impaired homeostasis of the extracellular milieu, release of cytokines and continuous increase in vessels permeability (Friedman A, Heinemann U, book chapter). Dysfunction of the BBB is a hallmark of brain insults and is usually surrounding the core lesion (Heinemann, Kaufer, Friedman, 2012). In animal studies, a role for BBB opening was suggested in the progression of temporal lobe epilepsy based on the finding of serum albumin presence in brain parenchyma following status epilepticus, and a positive correlation between the extent of BBB opening and the number of seizures (Heinemann, Kaufer, Friedman 2012; van Vliet et al., 2007). Experimental focal opening of the BBB

in the rat neocortex has been shown to result in epileptogenesis, evident by the delayed development of paroxysmal hypersynchronous activity recorded *ex vivo* (acute

slice preparation) and *in vivo* (Ivens et al., 2007; Seiffert et al., 2004; Tomkins et al., 2007). This epileptogenesis was recapitulated by exposure of brain cortex to serum albumin.

Extravasation of serum albumin into the cerebral cortex microenvironment activates a transforming growth factor beta (TGF-ß) receptor-mediated signaling cascade in astrocytes (Cacheaux et al., 2009; David et al., 2009; Ivens et al., 2007). In our study we also found alterations in the homeostasis of $[K^+]_O$ after exposing hippocampal tissue to albumin *in-vivo* for 24 hours. The affected hippocampus showed uptake of fluorescent albumin in hippocampal glia constituents but not in neurons. These observations might explain the fact why potassium homeostasis is affected after BBB dysfunction, since glia cells are key elements in potassium spatial buffering (Dietzel, Heinemann, Lux, 1989).

The alteration of potassium homeostasis led to a reduce threshold for the generation of epileptiform activity together with SDs by applying a broad range of frequencies of stimulation on CA3-CA1 Schaffer-collateral input. The CA3-CA1 Schaffer-collateral pathway belonging to the stratum radiatum was not exclusively affected, since stimulation of the direct cortical input, which is relevant in hippocampal synaptic plasticity and temporal lobe epilepsy (Empson and Heinemann 1995; Wöhrl et. al. 2007; Fidzinski et. al. 2012), showed a massive epileptiform activity followed by SD. These stimulation frequencies applied in our protocols are within the range of physiological hippocampal oscillatory activity (Boehlen, Kunert, Heinemann, 2009; Huchzermeyer et. al. 2008; Fano et. al., 2007; Fano et. al. 2012; Schulz et. al. 2012) and are frequently employed for inducing long-term-potentiation changes in the CA1 or CA3 area of hippocampus (Behrens et. al. 2005; Wöhrl et. al. 2007; Ul Haq et. al. 2011).

Our recordings from peri-infarct hippocampus slices demonstrated increased incidence of SD, consistent with a previous study that shows SD in cortical regions that were not part of the infarct core (Dietrich et al., 1994). Human studies in traumatic brain injury, subarachnoidal hemorrhage and stroke patients confirm high incidence of SD in peri-lesional brain tissue (Dreier, 2011), at a similar time window in which BBB opening in the cortical phothrombosis model was observed. In contrast to peri-infarct hippocampal tissue, which shows a massive BBB dysfunction after few hours of stroke and spontaneous induction of SD after serum electrolyte exposure, hippocampal tissue affected by albumin exposure *in-vivo* for 24 hours did not show spontaneous SD. This point toward other mechanisms involved in the reduction of epileptiform activity and SD after BBB dysfunction, which are beyond the albumin-induced glial response or affect the glia reaction additionally, like TNF-alpha (Cipolla et. al. 2012). Epileptiform activity in peri-infarct hippocampal tissue 24 hours after cortical photothrombosis was consistent and observed at the front of the SD. However, when SDs were stimulus induced in naive hippocampal tissue exposed to albumin for 24 hours, epileptiform activity was observed at the front and at the recovery phase of SD. This difference might be explained by the fact that the function of sodium-potassium pump in the peri-infarct hippocampal cells might be affected (possibly increased), since SDs were only observed in albumin-affected hippocampal tissue after electrical stimulation, which was observed to reduce drastically the 0_2 tension of the tissue (Pomper et. al. 2006). However, the difference in oxygen consumption between peri-infarct hippocampal slices affected by BBB dysfunction and naive hippocampal slices affected by albumin needs further studies. The after-discharges at the front and at the recovery phase of SDs after BBB dysfunction in subcortical structures like the hippocampus might be relevant for ictogenesis and cell degeneration after stroke, since this epileptiform activity was already observed in other human and animal studies (Leao 1944, Van Harreveld and Stamm 1953, Pomper et. al. 2006, Dreier et. al. 2012).

I suggest that the diffusion of blood substances into the peri-necrotic tissue increase the susceptibility for Spreading Depolarizations and this might play a relevant factor during the generations of peri-infarct depolarizations. This conclusion is supported by (1) the evidence of altered stimulus-induced potassium accumulation in albumin-treated hippocampi and (2) an increased susceptibility for spontaneous spreading depolarization without the diffusion of high glutamate and potassium from the infarct zone in the peri-infarct hippocampus.

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8. Anteilserklärung/Eidesstattliche Versicherung

„Ich, Ezequiel Gustavo Lapilover, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „***Disturbance of blood brain barrier in the hippocampus and spreading depolarisation in a model of cortical stroke“*** 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.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen ich Autor bin, entsprechen den URM (s.o) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

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Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Ezequiel Gustavo Lapilover hatte folgenden Anteil an den folgenden Publikationen:

Publikation 1: [David Y, Cacheaux LP, Ivens S, **Lapilover** E, Heinemann U, Kaufer D, Friedman A], [Astrocytic dysfunction in epileptogenesis: consequence of altered potassium and glutamate homeostasis?], [J. Neursci.], [2009]

10 % Prozent

Beitrag im Einzelnen (bitte kurz ausführen): Diskussion über die Durchführung von Protokollen *in-vitro* nach *in-vivo* Tierversuch. Erstellung von Hirnschnittpräparaten *in-vitro*, Messung von Kaliumkonzentrationsänderungen mit Hilfe ion-sensitive Elektroden *in-vitro*, Diskussion über die Ergebnisse, Datenanalyse und Anfertigung von Abbildungen. Beteiligung am *Peer-Review* Vorfahren.

Publikation 2: [Maslarova A, Alam M, Reiffurth C, **Lapilover** E, Gorji A, Dreier JP.], [Chronically epileptic human and rat neocortex display a similar resistance against spreading depolarization in vitro], [Stroke], [2011]

10 % Prozent

Beitrag im Einzelnen: Diskussion über die Speicherung von Daten (Intrisic Optic Signals bei *in-vitro* Hirnschnittpräparaten). Diskussion über die angewandte Protokolle für den *in-vitro* Versuch. Datenanalyse und Anfertigung von Abbildungen. Diskussion über die Ergebnisse und Beteiligung an der Korrektur des Artikels.

Publikation 3: [**Lapilover E**, Lippman K, Salar S, Maslarova A, Dreier JP, Heinemann U and Friedman A.] [Periinfarct Blood-Brain Barrier Dysfunction Facilitates Induction of Spreading Depolarization Associated with Epileptiform Discharges], [Neurobiology of Disease], [2012]

80 % Prozent

Beitrag im Einzelnen (bitte kurz ausführen): Diskussion über das Krankheitsmodell (Bluthirnschranke Störung, BHS). Entwicklung zweier Protokolle zur Nachahmung einer BHS *in-vivo* und *in-vitro*. Durchführung von sowohl *in-vitro* als auch *in-vivo* Experimenten. Erstellung eines Antrages zur Zulassung eines *in-vivo* Versuches. Durchführung verschiedener Stimulationsprotokolle. Messung vom Feldpotential und Kaliumsignalen an hippokampalen Hirnschnittpräparaten mittels ion-sensitiver Mikroelektroden. Daten Auswertung mittels eigenentwickelter Computeralgorithmen. Diskussion über die Ergebnisse. Anfertigungen von Abbildungen. Schreiben und Korrektur des Artikels.

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

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Unterschrift des Doktoranden/der Doktorandin

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10. Publications

1: Lapilover EG, Lippmann K, Salar S, Maslarova A, Dreier JP, Heinemann U, Friedman A. Peri-infarct blood-brain barrier dysfunction facilitates induction of spreading depolarization associated with epileptiform discharges. Neurobiol Dis. 2012 Dec;48(3):495-506. doi: 10.1016/j.nbd.2012.06.024. Epub 2012 Jul 7. PubMed PMID: 22782081; PubMed Central PMCID: PMC3588590.

2: Maslarova A, Alam M, Reiffurth C, Lapilover E, Gorji A, Dreier JP. Chronically epileptic human and rat neocortex display a similar resistance against spreading depolarization in vitro. Stroke. 2011 Oct;42(10):2917-22. doi: 10.1161/STROKEAHA.111.621581. Epub 2011 Aug 11. PubMed PMID: 21836085.

3: David Y, Cacheaux LP, Ivens S, Lapilover E, Heinemann U, Kaufer D, Friedman A. Astrocytic dysfunction in epileptogenesis: consequence of altered potassium and glutamate homeostasis? J Neurosci. 2009 Aug 26;29(34):10588-99. doi: 10.1523/JNEUROSCI.2323-09.2009. PubMed PMID: 19710312; PubMed Central PMCID: PMC2875068.

10. Lebenslauf

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

11. Komplette Publikationsliste

- Maslarova A, Salar S, Lapilover E, Friedman A, Veh RW, Heinemann U. Increased susceptibility to acetylcholine in the entorhinal cortex of pilocarpine-treated rats involves alterations in KCNQ channels. Neurobiol Dis. 2013 Aug; 56:14-24
- Lapilover E, Lippman K, Salar S, Maslarova A, Dreier JP, Heinemann U and Friedman A. Peri-infarct Blood-Brain Barrier Dysfunction Facilitates Induction of Spreading Depolarization Associated with Epileptiform Discharges Neurobiol Dis. 2012 Dec;48(3):495-506
- Maslarova A, Alam M, Reiffurth C, Lapilover E, Gorji A, Dreier JP. Chronically epileptic human and rat neocortex display a similar resistance against spreading depolarization in vitro. Stroke. 2011 Oct;42(10):2917-22
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