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DISSERTATION

Role of lactate for synaptic transmission, recovery of ion gradients and maintenance of epileptiform activity in rat hippocampal and human neocortical slices

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1. Zusamenfassung

Die Energieversorgung des Gehirns beruht unter physiologischen Bedingungen beinahe ausschließlich auf oxidativem Metabolismus von Glukose. Laktat, aus dem anaeroben Metabolismus der Gliazellen, kann unter speziellen Umständen als alternatives Substrat dienen. Tatsächlich wurde nachgewiesen, dass Laktat als Substrat des Energiemetabolismus, die synaptische Übertragung stützen kann, und zudem an neuronaler Plastizität beteiligt und für die Konsolidierung von Gedächtnisinhalten notwendig ist. Zusätzlich kann Laktat durch Aktivierung eines G-Protein gekoppelten Hydroxykarbonsäure-Rezeptors (HCA1) direkt Wirkung auf die neuronale Funktion ausüben. Obwohl der aktivitätsbedingte Anstieg der parenchymalen Laktatkonzentration schon sehr lange bekannt ist, ist die Rolle von Laktat für neuronale Erregbarkeit, sowie für die Wiederherstellung der transmembranären Ionengradienten weitestgehend unerforscht geblieben. Der Laktataustausch zwischen Gehirnzellen und der Blut-Hirn Schranke wird durch Ionenkanäle, Hemikanäle und Monokarboxysäuretransporters (MCT1-4) getragen. In chronisch epileptischem Gewebe wird MCT1 an Endothelzellen vermindert und an Astrozyten vermehrt exprimiert, was wiederum eine Laktatakkumulation im Gehirngewebe mit verringerter Abgabe in die Blutzirkulation zufolge hat. Ob diese Prozesse pro oder antiiktogen wirken (jeweils durch metabolische Unterstützung oder Laktacidose), ist zur Zeit noch nicht bekannt.

In dieser Studie habe ich den Beitrag des Laktat-abhängigen Anteils der ATP Synthese zum Energiebedarf der synaptischer Übertragung, zur Aufrechterhaltung von transmembranären Ionengradienten und an pathologischer Netzwerkaktivität in hippokampalen Gehirnschnitten der Ratte und in chronisch epileptischen, kortikalen Gehirnschnitten von Temporallapenepilepsiepatienten untersucht. Stimulus induzierte Änderungen des Feldpotentials, der extrazellulären K^+, Na^+, Ca^{2+}, H^+ Konzentrationen und des Sauerstoffpartialdrucks wurden mit oder ohne Blockade des MCTs durch alpha-Cyano-4-hydroxyzimtsäure (4-CIN) oder D-Laktat gemessen.

Die Gabe von 4-CIN führte zur Reduktion des Sauerstoffverbrauchs, zur Verringerung synaptischer Übertragung und Verlangsamung von extrazellulären Ionenänderungen. Der Effekt auf die synaptische Übertragung wurde zum Teil durch die Aktivierung der K_{ATP} Kanäle getragen, welche im Falle eines ATP-Mangels die Nervenzellen hyperpolarisieren, während der Beitrag von HCA1 Rezeptoren ausgeschlossen werden konnte. Im Gegensatz dazu, hat die Blockade der Laktat-

aufnahme in chronisch epileptischem Gewebe weder die synaptische Übertragung noch die Wiederherstellung transmembranärer Ionengradienten beeinflusst, obwohl die Zellatmung eindeutig durch 4-CIN gehemmt wurde. Gleichzeitig haben die MCT Inhibitoren 4-CIN und D-Laktat jedoch die Inzidenz, Dauer und Amplitude der evozierten anfallsartigen Ereignisse in den kortikalen Gehirnschnitten der Ratte und der Epilepsiepatienten verringert. Diese antiiktogene Wirkung wurde durch die Aktivierung von Adenosinrezeptoren (A1) in Folge der verminderten ATP-Verfügbarkeit und nicht durch Laktazidose ausgelöst. Zusammenfassend konnte ich feststellen, dass Laktat einen wichtigen Beitrag zum Energiebedarf der synaptischen Übertragung und zur Wiederherstellung der Ionengradienten und zum Aufrechterhalten pathologischer anfallsartiger Aktivität leistet.

Abstract

Under normal conditions brain energy metabolism relies almost exclusively on the oxidative metabolism of glucose. Lactate, derived from glycolytic metabolism of glial cells, has been speculated to serve as an alternative substrate under specific circumstances. Indeed, lactate was shown to support synaptic transmission, neuronal plasticity and memory consolidation even in the presence of ample glucose. In addition, lactate may also modulate neuronal activity through its own hydroxycarboxylic acid receptor 1 (HCA1). Even though activity dependent extracellular lactate increases are long known, their contribution to neuronal excitability and maintenance of transmembrane ion gradients has barely been studied. Lactate exchange between brain cells and across the blood-brain-barrier is mediated via hemichannels, ion channels and monocarboxylate transporters (MCT 1-4). In chronic epileptic tissue, the expression of MCT1 is reduced on endothelial cells and upregulated on astrocytes, which is in favor of increased extracellular lactate accumulation and impaired clearance to the circulation. Whether this process is pro or anti-ictogenic (due to metabolic support or lactic acidosis, respectively) is currently not known.

Here, I determined the contribution of lactate derived ATP to synaptic signaling, ion recovery kinetics and maintenance of pathological network activity in rat hippocampal slices as well as in chronic epileptic tissue resected from patients with temporal lobe epilepsy (TLE). Stimulus induced changes in field potential, extracellular K^+, Na^+, Ca^{2+}, H^+ concentration as well as tissue oxygen level (pO_2) were determined in layer V/VI of human neocortex, and rat medial entorhinal cortex (MEC) and hippocampus slices in the presence or absence of the MCT blockers alpha-cyano-4-hydroxycinnamic acid (4-CIN) or D-lactate.

Application of 4-CIN reduced stimulus induced oxygen consumption and synaptic transmission and prolonged ion transient recovery time. The effect on synaptic transmission was partly mediated by activation of K_{ATP} channels which hyperpolarize neurons during state of ATP depletion, whereas the contribution of HCA1 receptors could be excluded. On the contrary, despite supporting oxidative energy metabolism, lactate uptake inhibitors did not affect field potential responses or decay kinetics of extracellular K^+ in chronic epileptic tissue. MCT inhibitors reduced the incidence and duration/amplitude of seizure-like events (SLEs) in rat MEC and in human neocortical slices. The anti-seizure effect of lactate uptake inhibitors was mediated by adenosine A1 receptor and not by changes in pH. In conclusion, lactate contributes to oxidative metabolism and supports synaptic transmission and recovery of ion gradients changes in healthy tissue while it maintains SLEs in pathologic states.

2. Introduction

Electrical signals underlying brain function prominently rely on oxidative energy metabolism [1]. In fact, despite accounting only for 2% of our body weight, the brain consumes 20% of oxygen and 25% of glucose of the resting body uptake [2]. Recovery of ion gradients associated with action potentials and synaptic currents, neurotransmitter recycling, presynaptic calcium clearance and vesicular turnover utilize the majority of brain signaling energy budget. And maintenance of transmembrane ion gradients during synaptic transmission alone accounts for 50% of the energy expenditure [3–5].

This energy is mainly derived from oxidative metabolism of glucose, whereas the contribution of alternative substrates, such as ketone bodies, is rather restricted to specific conditions [2]. However, observation of uncoupling between glucose utilization and oxygen use gave a notion that the brain undertakes non-oxidative metabolism as well [6]. Indeed, an increase in lactate, the glycolytic byproduct of glucose metabolism, has been observed with increased neuronal activity and exercise [2,7,8]. Glycogen is exclusively stored in glial cells and it can be immediately mobilized upon neuronal activation [9]. Lactate gradient has been demonstrated between astrocytes and neurons *in vivo*, by using fluorescent lactate probes [10]. Lactate release into the extracellular space is mediated by monocarboxylate transporters (MCT1-4), hemichannels and ion channels [11–13], whereas its uptake is favored by the high affinity MCT2 isoform (Km 0.74 mM) selectively expressed on neurons [14,15]. MCT4 is exclusively expressed on astrocytes while MCT1 is expressed in both astrocytes and endothelial cells [11]. The astrocytic-neuronal-lactate shuttle hypothesis (ANLS) claims that neuronal oxidative metabolism relies on glial lactate [14,16]. In spite of the original description of ANLS about twenty years ago, it is still unclear to what extent lactate serves as a substrate for neuronal oxidative energy metabolism [7,17,18]. Nevertheless, recent studies attributed crucial role to lactate in different physiological functions such as: synaptic plasticity, memory formation, gamma oscillations, cAMP mediated signaling [19–21]. However, much less is known about the mechanisms by which lactate or lactate derived ATP is able to modify these functions.

Once lactate is taken up, it is converted to pyruvate which creates a cytosolic reducing shift of the $NADH/NAD^+$ ratio, whereas pyruvate is further metabolized in the Krebs cycle finally resulting in ATP synthesis. Brain tissue is highly compartmentalized with respect to oxidative energy metabolism [2]. Thus different aspects of neuronal and glial functions, such as generation of action

potentials, synaptic transmission, transmitter recycling and vesicle filling might unevenly depend on lactate as metabolic substrate.

Here, I investigated the dependence of synaptic transmission and recovery kinetics of stimulus induced changes in extracellular K^+, Na^+ and Ca^{2+} concentrations on endogenously generated lactate in area CA3 of rat hippocampus and neocortical tissue from patients with temporal lobe epilepsy (TLE). Area CA3 is known for its recurrent collateral connection allowing simple differentiation between presynaptic and postsynaptic processes. It is also more vulnerable to hypoxia and spreading depolarization as compared to other hippocampal regions; differences in neurometabolic coupling and energy metabolism could attribute for that [22–24].

Immediate contribution of lactate to oxidative metabolism was monitored by measuring changes in tissue pO_2, which are exclusively determined by the rate of respiration in the absence of blood flow [5,25]. As metabolism of lactate exerts immediate effects on intracellular redox potential, I also recorded changes in auto-fluorescence of the intracellular redox couples flavin adenine dinucleotide ($FADH_2$/FAD) and nicotinamide adenine dinucleotide ($NADH/NAD^+$). Instead of applying exogenous lactate at non-physiological concentrations, I have chosen to counteract lactate transport by using MCT inhibitors, α-cyano-4- hydroxycynamate (4-CIN) and D-lactate.

Extracellular lactate accumulation with blockade of MCT might alter intra- and extracellular pH, which by itself could affect synaptic transmission [26,27]. Hence, I assessed possible contribution of a pH drift on the aforementioned parameters. Lactate also could serve as a signaling molecule by acting through its recently described G-protein coupled hydroxycarboxylic acid receptor 1, (HCA1) and induce effects irrespective of its role as metabolic substrate [20]. This possibility was tested by comparing the effects of lactate accumulation with the effects of a HCA1 receptor agonist.

After determining the contribution of lactate to synaptic transmission and maintenance of transmembrane ion gradients under physiological conditions, I investigated the changes which may occur in chronic epileptic tissue obtained from patients with TLE. Epileptic seizures have extremely high energy demand and are associated with increased extracellular lactate concentration and decreased high energy phosphates [28]. However, it is not known whether the increased lactate would contribute to energy metabolism during seizures, especially as histological studies revealed altered expression patterns of MCTs in chronic epileptic tissue [29–31]. Hence, I investigated the

effect of lactate shuttle on seizure activities in healthy rodents and resected chronic epileptic tissue from patients with TLE (Angamo et al. 2017, manuscript in preparation).

3. Aims

I determined the effects of neuronal lactate uptake inhibition on: 1) mitochondrial oxygen consumption, 2) intracellular flavin redox potential 3) extracellular pH 4) pre- and postsynaptic components of synaptic transmission as well as on 5) recovery kinetics of stimulus induced extracellular ion transients (K^+, Ca^{2+} and Na^+) in the CA3 region of hippocampus and neocortical tissue from patients with TLE. Then, I tested whether the observed effects were mediated by activation of the HCA1 receptor, changes in pH or by activation of K_{ATP} channels due to depletion of lactate derived ATP in the neuronal compartment. Finally, I compared the effects of lactate uptake inhibitors both on stimulus induced as well as on spontaneous epileptiform activity in healthy rat slices and resected chronic human epileptic tissue.

4. Material and methods

4.1 Slice preparation

Experiments were performed on combined horizontal entorhinal- hippocampus slices from Wistar rats and neocortical slices from resected tissue of TLE patients (approved by the Ethics Committee of Charité-Universitätsmedizin Berlin; EA2/111/14). Rats were sacrificed in accordance with the Helsinki declaration and institutional guidelines (as approved by the State Office of Health and Social Affairs Berlin LaGeSo, T0096/02 and the animal welfare regulations of Charité) with deep anesthesia using isoflurane (3% vol/vol) and laughing gas (70% N_2O, 30% O_2). After removing the brain from the calvarium, I prepared 400 µm thick combined horizontal hippocampal-entorhinal slices in cold aCSF using Leica VT 1200 S vibratome (Wetzlar, Germany). The aCSF solution is composed of NaCl (129 mM), $NaHCO_3$ (21 mM), glucose (10 mM), KCl (3 mM), NaH_2PO_4 (1.25 mM), $CaCl_2$ (1.6 mM), and $MgCl_2$ (1.8 mM) with an osmolarity of 295–305 mosM and pH 7.35 to 7.45. Slices were kept in interface chamber at a flow rate of 2 ml/min and temperature of 34 – 36 °C. NAD(P)H and $FADH_2$ experiments were done in submerged chamber with a flow rate of 8 ml/min at room T^o.

The resected neocortex from TLE patients was transported with Na^+ free sucrose containing aCSF

solution consisting of KCl (3 mM), NaH_2PO_4 (1.25 mM), glucose (10 mM), $MgSO_4$ (2 mM), $MgCl_2$ (2 mM), $CaCl_2$ (1.6 mM), $NaHCO_3$ (21 mM), sucrose (200 mM), and (±) α-tocopherol (0.1 mM, dissolved in ethanol; pH 7.4, osmolality 303 mOsmol/kg, 0.005 v% ethanol). Na^+ was decreased to inhibit hypoxia induced neuronal Na^+ influx. α-Tocopherol was added to the transport and aCSF solution to scavenge reactive oxygen species [32]. The slices were 500 µM thick and were kept in interface chamber for 4-5 hrs for recovery before the start of experiments. The chamber was continuously perfused with carbogenated (95% O_2, 5% CO_2) aCSF at a rate of 2 ml/min and temperature was maintained at 34-36 ^oC.

4.2 Seizure Induction

In order to induce epileptiform activity in resected material from TLE patients, elevated potassium aCSF (8 mM) and bicuculline methiodide (50 µM) was used. Alteration of aCSF electrolyte composition (Mg^{2+} (0 mM), Ca^{2+} (0 mM), elevated K^+ (8 mM)) by itself was able to induce epileptiform discharges in neocortical slices from TLE patients and slices from wistar rats [33]. Seizures like events in acute rat slices were induced using 4-aminopyridine (50 µM).

4.3 Electrophysiology and oxygen recordings

DC coupled extracellular field potential and K^+, Ca^{2+}, Na^+, H^+ ion recordings were done in rat CA3 pyramidal layer and human neocortex layer V/VI using ion sensitive microelectrodes. For stimulus induced responses, I recorded in rat CA3 pyramidal layer and human neocortex layer V/VI while stimulating Schaffer collateral fibers and white matter respectively with a 2 sec train stimuli of 20 Hz, 0.1 ms inducing 80% of the maximal response. This stimulation protocol is used as it does not induce synaptic potentiation or depression [5]. In area CA3, the field potential response consists of two components; the first component results from direct antidromic axonal stimulation and the second orthodromic response is synaptically mediated due to recurrent excitatory interactions. Amplitude, 1^{st} and 2^{nd} half decay time of the ion transients were assessed [34].

Na^+, K^+, Ca^{2+} sensitive electrodes were built using double barreled theta glasses as specified in Liotta 2012. For pH sensitive electrode, a filling solution with a pH of 7 consisting KCl (500 mM), NaH_2PO_4 (64.7 mM) and Na_2HPO_4 (85.3 mM) was used in the ion sensitive barrel and KCl (500 mM) was used on the reference side. Hydrogen ionophore II cocktail A (Flucha, Buchs, Switzerland) - H^+ binding cocktail - was used to fill the tip of the ion sensitive side

[35]. Extracellular O_2 recordings were done using the Clark-style oxygen sensor microelectrodes (tip: 10 µm; Unisense, Aarhus, Denmark). The electrode was polarized for 2 h and calibrated in aCSF solution saturated with 0, 50, and 95% O_2 before each experiment.

4.4 Fluorescence imaging and spectrophotometry

The electron carriers, nicotinamide- and flavine-adenine-dinucleotides (NADPH, NADH and $FADH_2$), shuttle reducing equivalents from glycolysis and Krebs cycle to electron transport chain (ETC). As the fluorescence spectra of NAD(P)H and NADH are virtually indistinguishable with our methods, I refer to them as NAD(P)H. FAD reduction exclusively occurs in the mitochondria while NAD(P)H reduction can occur either in mitochondria or cytoplasm. The oxidized FAD and reduced NAD(P)H are intrinsically fluorescent which makes them a relevant tool to monitor living tissue metabolic state using fluorescence imaging at appropriate wavelengths (excitation 360/450 nm, emission 450/515 nm, for NADH and FAD, respectively). I recorded FAD fluorescence using a custom-built equipment with a light emitting diode (LED, 460 nm, Lumen, Prior Scientific, Rockland, MA, USA) and a photomultiplier tube (PMT, Seefelder, Messtechnik, Germany) with a filter set (exciation BP 475/50 nm; dichroic mirror HC BS 506, emission BP 540/50, AHF Tübingen, Germany). The data was acquired and digitized using CED 1401 interface (Cambridge electronic design, Cambridge, UK). The recorded trace of $FADH_2$ has a sharp overshoot, indicative of increased oxidation, followed by a plateau like undershoot (reductive phase) while NAD(P)H is a biphasic mirror image of FAD fluorescence [36]. I recorded stimulus induced FAD and NAD(P)H responses in CA3 pyramidal layer by stimulating Schaffer collateral fibers as specified above. The absorption spectra of 4-CIN, NADPH, FAD was evaluated using a Nanodrop 1000 (Thermo Scientific) spectrophotometry [34].

4.5 Pharmacology

I investigated the metabolic role of lactate by looking at the effect of MCT inhibitor, 4-CIN (200 µM), on stimulus induced changes in FAD fluorescence (FAD redox state) and extracellular oxygen tension. 4-CIN has an IC_{50} of 24 ± 4 and 425 ± 155 (in µM) for MCT2 and MCT1 expressed in Xenopus laevis oocytes respectively [37] and it has been used at a concentration of 150 – 250 µM in rat slices [38]. The role of lactate shuttle fo**r** synaptic transmission and ionic homeostasis was also evaluated by applying 4-CIN. In rat slices, the role of lactate uptake for antidromic and orthodromic responses as well as ion recovery kinetics, respiration and redox status was assessed. In order to

isolate the energetic contribution of lactate for the presynaptic component (antidromic response), I coadministered 4-CIN and a cocktail of synaptic transmission inhibitors: the AMPA-kainate receptor blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 50 μM), the NMDA receptor blocker (2*R*)-amino-5-phosphonopentanoate (APV, 50 μM), and the GABA A blocker bicuculline methiodide (5 μM), the metabotropic glutamate receptor blocker (RS)-*α*-methyl-4-carboxyphenylglycine (MCPG, 150 μM) and presynaptic Ca^{2+} uptake inhibitor Nickel (2 mM). As lactate has been shown to exert immediate effects through HCA1 receptors [20], the effect of 3,5-DHBA (1.5 mM), HCA1 receptor agonist, on field potential responses and ion transients was assessed. After looking at the metabolic effect, I hypothesized that K_{ATP} channel could play a role in hyperpolarizing neurons due to decreased availability of ATP. Glibenclamide (50 μM), a K_{ATP} channel blocker, was used to reverse the observed effect on field potential responses [34]. The role of lactate shuttle for seizure activities in acute rat slices (entorhinal cortex) and resected chronic epileptic tissue from patients with TLE (layer V/VI) was evaluated by looking at the effect of D-lactate (20 mM) and 4-CIN (200 μM) on seizure frequency, amplitude and duration (Angamo et al. 2017, manuscript in preparation).

4.6 Data acquisition and analysis

Field potential responses and ion concentration changes were recorded using ion sensitive microelectrodes, tip size of 2-3 μM, and were low pass filtered with custom made amplifiers at 3 and 0.3 KHz respectively. The data got digitized and stored on computer disk using CED 1401 interface (Cambridge electronic design, Cambridge, UK). Extracellular pO_2 tension was measured using the Clark-style oxygen sensor microelectrodes (tip: 10 μm; Unisense, Aarhus, Denmark) and amplified with Unisense microsensor amplifier. Then, the data got digitized and stored on computer disk using CED 1401 interface. For all experiments, data visualization and analysis were done using spike 2 software scripts (Cambridge electronic design, Cambridge, UK) and Graphpad prism (Graphpad software inc. CA; USA). For stimulus induced responses, 3 data points were used from each slice for stastical analysis. In case of SLEs, the last 10 minutes of recording during control, drug perfusion and wash out phase were used to compare amplitude, incidence and event durations. All data are presented as mean ± standard error of the mean (SEM) and in the figures as box plots where the median, 1^{st} and 3^{rd} quartile, minimum and maximum values were displayed. The distribution of the data was tested for normality using Kolmogorov-Smirnoff and D'Agostino and

Pearson omnibus normality test and subsequently, group effects were compared using *t*-test for data with normal distribution and Wilcoxon signed rank test for data with non-normal distribution.

5. Results

5.1 Optimization of FAD measurement protocol and the effect of 4-CIN on energy metabolism

Complex neuronal activities critically depend on oxygen and substrate availability [25]. Apart from glucose, brain can utilize other energy substrates like glutamate, ketone bodies, and lactate during state of increased neuronal activity or starvation [2]. However, it is not clear to what extent lactate serves as energy substrate for oxidative metabolism. Thus, effect of 4-CIN on stimulus induced changes in NAD(P)H and FAD autofluorescence as well extracellular tissue oxygen tension were evaluated. Since the absorption spectra of 4-CIN and NAD(P)H overlapped at 360 nm, I used FAD autofluorescence to assess intracellular energetic state.

We initially used continuous LED illumination to measure FAD autofluorescence which led to rapid fluorescence decay and decreased tissue viability. Hence, optimization of FAD measurement protocol became mandatory [36]. Therefore, I looked for an optimal LED stimulation protocol by evaluating the effect of different pulse durations (1, 5, 10 and 50 ms) at constant frequency (5 Hz) or different frequencies (1, 5, 10 and 50 Hz) at fixed pulse duration (5 ms) on relative fluorescence decline, decay time constant as well as on other physiological parameters [39].

Continuous illumination resulted in increased photobleaching (88.2 ± 2.1%; n=7, 3 rats, $p < 0.001$, independent t-test) unlike a 5 Hz, 5 ms pulsed illumination (57.3 ± 3.0%; n=6, 3 rats, $p < 0.001$, independent t-test); the decay time constant was also shorter for continuous illumination 338.3 ± 17.8 vs. 467.8 ± 22.3 s (n = 6 and 7, 3 rats, $p = 0.002$, independent t-test). Upon evaluation of other physiological parameters, a 25 minute continuous illumination led to increased baseline oxygen level which is indicator of decreased respiration likely reflecting impaired oxidative metabolism (119.7 ± 10.2 to 147.7 ± 16.8 mmHg, $n = 7$, 3 rats, $p = 0.029$, paired t-test). Furthermore, I compared stimulation induced ΔpO_2, field potential responses and $\Delta[K^+]_o$ in both conditions by applying 2 s, 20 Hz train stimuli at 20 minute interval. When comparing 2nd stimulation induced responses with 1st baseline recording, continuous illumination decreased spike amplitude from 3.08 ± 0.4 to 2.35 ± 0.3 mV (n = 10, 4 rats, $p = 0.004$, paired t-test), the rise in $[K^+]_o$ from 0.87 ± 0.1 to

0.75 ± 0.1 mM (n = 11, 4 rats, p = 0.002, , paired t-test) and ΔpO_2 from 25.54 ± 6.8 to 20.32 ± 6.2 mmHg (n = 9, 4 rats, p = 0.005, Wilcoxon signed rank test) . On the contrary, pulsed illumination did not show a deleterious effect on these parameters. Therefore, I used 5 Hz, 5 ms pulsed illumination for the forthcoming experiments [39].

Schaffer collateral stimulation has been shown to increase tissue oxygen consumption in CA1 hippocampus along with a proportional extracellular ion concentration changes [5]. Hence, I evaluated the effect of 4-CIN (200 µM applied for 20 minutes) on stimulus induced changes in ΔpO_2 and baseline respiration in CA3 pyramidal layer of rat hippocampus and layer V/VI of human neocortex. In acute rat slices, 4-CIN decreased ΔpO_2 by 20% from −26.7 ± 7.9 to −22.4 ± 7.5 mmHg, (n = 9 (27 data points), 3 rats, P < 0.0001, Wilcoxon signed rank test), indicating direct contribution of intrinsic lactate to oxidative metabolism. Moreover, it increased baseline pO_2 by 12.7% (64 mmHg) which is suggestive of reduced tissue oxygen consumption [34]. Likewise, 200 µM 4-CIN reduced ΔpO_2 from -21.8 ± 1.2 to -15.3 ± 0.7 mmHg in human neocortical slices (n=9 (27 data points), 5 patients, p< 0.001, paired t-test) (Angamo et al. 2017, manuscript in preparation).

Presynaptic processes - presynaptic action potentials and transmitter release - also depend on oxidative metabolism [5]. Inhibiting postsynaptic AMPA and NMDA receptors reduced oxygen consumption and NAD(P)H transients by about 50%; adding blocker of transmitter release further decreased oxygen transients by ∼30% and the rest was abolished with tetrodotoxin; these experiments demonstrate that about 20% of the consumed oxygen is used to maintain presynaptic action potential [3,5]. Thus, by applying a cocktail of synaptic transmission and vesicular release inhibitors (see methods), I was able to isolate the level of oxygen consumed by mere axonal stimulation in acute rat slices. The cocktail decreased stimulus induced ΔpO_2 from -34.8 ± 8.8 to -12.2 ± 6.8 mmHg (a decrease of 65.9% relative to the baseline). Then, the effect of 4-CIN on stimulus induced presynaptic oxygen consumption was assessed; 4-CIN further reduced ΔpO_2 to -8.7 ± 4.3 mmHg (decrease by 75.6% relative to baseline; n = 7 (21 data points), 4 rats, P < 0.0001, paired t-test) [34].

The effect of 4-CIN (200 µm) on stimulus induced FAD transients was evaluated using pulsed illumination. 4-CIN increased the oxidative peak of the biphasic FAD transients from 3.5 ± 2.2 to 4.9 ± 3.8 (n = 9, 5 rats, P < 0.05, Wilcoxon signed rank test) while it slightly decreased the undershoot, reductive phase, from −4.4 ± 2.9 to −3.5 ± 2.5% (n = 9, 5 rats, P = 0.09, Wilcoxon

signed rank test). It also increased FAD baseline fluorescence, indicative of massive oxidation which could be due to compensatory activation of complex II [34].

5.2 Effect of 4-CIN on field potential responses

I evaluated the role of lactate shuttle for synaptic transmission in CA3 region-pyramidal layer- of rat hippocampus and layer V/VI of resected human neocortex. DC coupled field potential and ion transient recordings were done in these layers by stimulating Schaffer collateral fibers and the white matter in rat and resected human tissue slices, respectively [34].

Application of the high affinity MCT blocker, 4-CIN (200 µM), significantly decreased the orthodromic population spike from 3.7 ± 1.3 to 2.3 ± 1.3 mV (n = 10 slices (30 data points), 5 rats, $P < 0.0001$, Wilcoxon signed rank test), while it did not have any effect on the antidromic response in rat slices [34]. On the contrary, similar concentration of 4-CIN did not affect the field potential response, (2.9 ± 0.4 mV versus 2.8 ± 0.4 mV), in human neocortical slices ($p>0.5$, n=9, 5 patients, Wilcoxon signed rank test). Taking into account the longer diffusion distance of the human slices (slice thickness ~ 500 µm) and possible pathological alteration of MCT expression in chronic epileptic tissue, in a subsequent set of experiments the concentration of 4-CIN was increased to 500 µM, but even at this concentration 4-CIN did not affect the field potential response either (Angamo et al. 2017, manuscript in preparation)..

In acute rat slices, the effect on synaptic transmission could result from decreased availability of lactate derived ATP. The K_{ATP} channel links the metabolic state of a cell to its excitability. Decreased cytosolic ATP favors channel opening and allows efflux of potassium leading to hyperpolarization [40]. Hence, I investigated if the decrease in orthodromic population spike is due to activation of K_{ATP} channels. Application of glibenclamide (50µm), K_{ATP} channel blocker, together with 4-CIN, partially rescued the orthodromic population spike by reducing the effect of 4-CIN from 38.3% to 13.4% (n=6 slices, 3 rats) [34]. Thus, the effect of lactate uptake blockade might be partly mediated by activation of K_{ATP} channels due to decreased availability of lactate derived ATP.

Since lactate transport is coupled with H^+ ion, lactate uptake inhibitor could lead to extracellular acidosis. Indeed, 4-CIN induced a decrease in extracellular pH by 0.093 ± 0.034 pH units (n = 8; 3 rats). I mimicked this pH change by adjusting bicarbonate level and checked if it has any effect on

field potential responses and I did not observe any [34].

5.3 Effect of 4-CIN on extracellular ion transients

Neuronal activity leads to accumulation of extracellular K^+ and decreases in Na^+ and Ca^{2+} concentrations. The clearance of accumulated extracellular potassium ($[K^+]_o$) is mediated by Kir4.1-mediated spatial buffering by glial cells, the $Na^+/K^+/2Cl^-$ cotransporter 1 (NKCC1), and/or Na^+/K^+-ATPase activity [41]. Since postsynaptic transmembrane ionic gradient consumes about 50% of brain signaling energy budget [4,5], I looked at the effect of blocking lactate shuttle on the amplitude and recovery half decay time of extracellular K^+, Na^+ and Ca^{2+} transients [34].

In acute rat slices, a peak concentration of $[K^+]_o$, 1.7 ± 0.4 mM (n = 7, 5 rats), was measured with 2 sec, 20 Hz repetitive stimulation of the Schaffer collateral fibers. Application of 4-CIN (200 µm) for 20 minutes did not affect the peak concentration of $[K^+]_o$, however , it significantly prolonged the 1st half decay time from 2.2 ± 0.6 to 3.2 ± 1.4 s (n = 7 slices(21 data points), 5 rats, $P < 0.001$, Wilcoxon signed rank test) and the second half decay time from 6.3 ± 1.4 to 15.3 ± 5.6 s (n = 7 slices (21 data points), 5 rats, $P < 0.001$, Wilcoxon signed rank test) [34].

Na^+ extrusion also depends on Na^+/K^+ ATPase pump [42]; hence, I evaluated the effect of 4-CIN on the amplitude and recovery kinetics of stimulus induced extracellular Na^+ concentration changes ($\Delta[Na^+]_o$) in acute rat slices. The peak Na^+ concentration change was reduced from -5.9 ± 1.9 to −5.3 ± 1.9 mM (n = 8 (24 data points), 5 rats, $P < 0.05$, Wilcoxon signed rank test) with 4-CIN application while it increased the 1st half recovery time from 1.2 ± 0.4 to 1.3 ± 0.4 s (n = 8, 5 rats (24 data points), P = 0.01, paired t-test) and second half recovery time from 1.7 ± 0.9 to 2.7 ± 1.6 s (n = 8 (24 data points), 5 rats, $P < 0.001$, Wilcoxon signed rank test) [34].

Intracellular Ca^{2+} concentration is tightly regulated as it is involved in different signaling pathways. Ca^{2+} influx during synaptic transmission occurs through ligand and voltage gated calcium channels. Its level is regulated by Ca^{2+} binding proteins and $HCO3^-$ buffers, Na^+/Ca^{2+}exchanger, plasma membrane Ca^{2+} ATPase etc. As these processes consume ATP, the contribution of lactate metabolism to extracellular Ca^{2+} ($[Ca^{2+}]_o$) homeostasis is evaluated in acute rat slices [43]. Stimulus trains induced decrease in extracellular Ca^{2+} (−0.2 ± 0.1 mM). 4-CIN did not affect the amplitude but it slowed down the recovery time. The first half recovery time changed from 2.2 ± 0.9 to 2.6 ± 1.1 s (n = 9 (27 data points), 6 rats, $P < 0.05$, paired t-test) and the second half recovery time

increased from 4.9 ± 2.6 to 6.1 ± 3.3 (n = 9 (27 data points), 6 rats, $P < 0.001$, paired t-test) [34].

In human neocortical slices, 1.7 ± 0.1 mM of $[K^+]_o$ was measured in layer V/VI with repetitive stimulation of the white matter. 4-CIN did not affect either the amplitude or the 1st half decay time at 200 and 500 µM concentrations The effect on $\Delta[Na^+]_o$ and $\Delta[Ca^{2+}]$ has not been evaluated due to restricted number of patients and slices available for experiments (Angamo et al. 2017, manuscript in preparation).

5.4 Effect of monocarboxylate transporter inhibitors on seizure like events

Spontaneous seizures increase lactate level while depleting locally available glucose and glycogen [28,44]. Lactate could either decrease seizure duration by reducing tissue pH or maintain seizure activities by serving as energy substrate. I entertained these possibilities and investigated the role of lactate shuttle for seizure like events in rat entorhinal cortex and resected chronic epileptic tissue from TLE patients by applying the MCT inhibitors, D-lactate and 4-CIN (Angamo et al. 2017, manuscript in preparation).

In rat entorhinal cortex, 4-CIN (200 µM) reduced the incidence of 4-AP induced SLEs from 2.1 ± 0.1 to 1.1 ± 0.1/ 10 min ($p < 0.05$, n=8, 4 rats, Wilcoxon signed rank test) and duration from 1.8 ± 0.4 to 0.9 ± 0.3 sec ($p < 0.01$, n=8, 4 rats, paired t-test) without affecting the amplitude of the events. Increasing the concentration of 4-CIN to 500 µM abolished seizure-like events completely in all slices investigated and the activity recovered upon washout of 4-CIN (n=8, 4 rats). Likewise, application of D-lactate decreased 4-AP induced SLE incidence from 3.6 ± 0.6 to 2.1 ± 0.4/ 10 min ($p<0.05$, n=8, 4 rats, paired t-test) and duration from 46.6 ± 11.4 to 14.2 ± 4.6 sec ($p \sim 0.05$, n=8, 4 rats, Wilcoxon signed rank test). Unlike 4-CIN, D-lactate did not induce extracellular acidosis and this could be due to its action on all MCT subtypes blocking both uptake and release of lactate. The reduced incidence of SLEs with MCT inhibitors is not due to extracellular acidosis as both 4-CIN and D-lactate displayed similar anti-seizure effect despite the fact that D-lactate had no effect on extracellular pH (Angamo et al. 2017, manuscript in preparation).

Reduced oxidative metabolism leads to net hydrolysis of ATP increasing the level of adenosine which plays a neuroprotective role by suppressing excitatory neurotransmission through adenosine A1 receptor [45]. As MCT inhibitors decreased oxidative metabolism, they will presumably increase the level of adenosine. Thus, I evaluated if the anti-seizure effect of 4-CIN is mediated through

adenosine A1 receptor by co-administering DPCPX, A1 receptor antagonist and 4-CIN. Indeed, seizure incidence was reduced from 3.3 ± 0.98 to 2.0 ± 0.4 (p= 0.09, n=9, 3 rats, Wilcoxon signed rank test) with concomitant application of 4-CIN and DPCPX (Angamo et al. 2017, manuscript in preparation).

In human neocortical slices, 4-CIN reduced SLE incidence from 5.9 ± 0.9 to 2 ± 0.4/ 10 min ($p< 0.01$, n=9 slices, 4 patients, paired t-test) and amplitude from 1.0 ± 0.1 to 0.9 ± 0.1 mV ($p<0.01$, n=9 slices, 4 patients, paired t-test) without altering duration (Angamo et al. 2017, manuscript in preparation).

5.5 Energy metabolism-independent effects of lactate transport blockade

In recent studies, lactate has also been shown to act through G-protein coupled receptor independent of its metabolic effect [20]. As blockade of lactate uptake via MCT might result in extracellular accumulation of lactate and subsequent activation of HCA1, in the next set of experiments, I investigated if 3,5-DHBA (HCA1 receptor agonist) could mimic the effect of 4-CIN. It increased the antidromic population spike by 14.6% (n = 5 (15 data points), 3 rats, $P < 0.001$, paired t-test) and the orthodromic population spike by 18.3% (n = 5 (15 data points), 3 rats, $P < 0.01$, paired t-test), unlike 4-CIN, it also increased stimulus induced ΔpO_2. The reduction of the orthodromic population spike observed with 4-CIN alone was partially prevented by combined application of 4-CIN and 3,5-DHBA (from 69 ± 3.8 to 80 ± 6.9%, n = 7, 4 rats, $P < 0.05$, paired t-tes). However, concomitant administration of 4-CIN and 3,5-DHBA did not significantly improve the effect of 4-CIN on $[K^+]_o$ half decay time and ΔpO_2. Hence, I concluded that the observed effect of 4-CIN is not receptor mediated [34].

6. Discussion

Hereby, I conclude that lactate shuttle supports synaptic transmission and recovery of transmembrane ionic gradient in rat CA3 pyramidal layer but not in chronic epileptic tissue from patients with TLE despite contributing for oxidative metabolism in both species. Lactate uptake inhibitors reduced tissue oxygen consumption, synaptic transmission and slowed recovery of extracellular K^+, Na^+ and Ca^{2+} concentration changes in rat hippocampal slices. Moreover, increased stimulus induced and baseline $FADH_2$ oxidation was observed likely from compensatory overactivation of complex II. I was partly able to reverse the effect on synaptic transmission with

glibenclamide, the K_{ATP} channel blocker. Together with the observed redox changes, this supports the contribution of lactate for cellular oxidative metabolism [34]. Remarkably, lactate uptake inhibitors, 4-CIN and D-lactate, reduced the incidence of SLEs in both acute rat slices and human chronic epileptic tissue. This suggests that extracellular lactate accumulated during seizures is rather pro-epileptic and meets the high energy demand to maintain seizure like activities (Angamo et al. 2017, manuscript in preparation). The pH change observed with extracellular lactate accumulation was not significant enough to affect synaptic transmission or SLEs while HCA1 receptor activation was rather excitatory [34].

The majority of ATP is believed to be synthesized from oxidative metabolism of glucose [2]. In my thesis, I confirm that lactate shuttle also supports oxidative metabolism and other energy dependent processes. Blocking neuronal lactate uptake decreased stimulus induced oxygen consumption in rat CA3 hippocampus [34] and similar results have been reported in CA1 region as well [38]; whereas it increased the oxidative peak of stimulus induced FAD autofluorescence and shifted baseline FAD towards increased oxidation [34]. One explanation for this apparent controversy could be that decreased lactate shuttle also reduces pyruvate availability in mitochondria leading to decreased production of reducing equivalents in the Krebs cycle causing a shift in FAD/$FADH_2$ ratio. With respect to this, 50% of the mitochondrial FAD fluorescence originates from the NAD-linked alpha-lipoamide dehydrogenase enzyme of the pyruvate dehydrogenase complex [46]. Moreover, restricted availability of NADH-linked substrates could facilitate electron flux via complex II likely contributing to enhanced $FADH_2$ oxidation [47].

Lactate uptake inhibitor decreased orthodromic pop-spike without affecting the antidromic response. Nagase et al also reported that lactate shuttle supports excitatory synaptic transmission in rat solitary nucleus. Moreover, they addressed the controversy that 4-CIN might have inhibitory effect on mitochondrial MCT using intracellular 4-CIN during whole cell patch clamp recordings. Intracellular application of 4-CIN did not affect excitatory postsynaptic current which indicated that the observed effect is mediated by inhibition of lactate transport through cell membrane MCTs [48]. The effect that I observed is due to depletion of ATP as it is partly reversed by blocking K_{ATP} channel with glibenclamide. These are inward rectifier potassium channels (Kir 6.2) which open during ATP depletion leading to K^+ efflux and hyperpolarization [40]. Lactate has been shown to modulate neuronal activity through HCA1 receptors [49,50]. As 4-CIN led to accumulation of

extracellular lactate, the observed effect could also be due to HCA1 receptor activation. But, using 3,5-DHBA (lactate receptor agonist) did not reverse the effect that I observed with 4-CIN. On the contrary, it increased the orthodromic and antidromic pop-spikes followed by increased oxygen consumption suggesting that lactate receptor mediated effect could be excitatory in this region. Alternatively, extracellular lactate accumulation results in pH change which might also influence synaptic transmission [26,51]. However, adjusting bicarbonate level to mimic the extracellular acidosis induced by 4-CIN did not change the orthodromic and antidromic pop-spikes [34].

Ion concentration changes accompany physiologic or pathologic neuronal activity. On the other hand, alteration of extracellular ion concentration could lead to seizure development and propagation; increasing aCSF K^+ concentration from 3 to 8.5 mM induced seizure in rat CA3 hippocampus and resected human tissue [5,33]. Hence, maintenance of stable transmembrane ion gradient is a crucial and high energy consuming process in the brain. Na^+/K^+ ATPase, Na^+/Ca^{2+} exchanger, plasma membrane and sarcoplasmic reticulum Ca^{2+} ATPase are all involved in regulating extracellular K^+, Na^+, and Ca^{2+} at the expense of ATP. Application of 4-CIN prolonged recovery time of $[K^+]_o$, $[Na^+]_o$, and $[Ca^{2+}]_o$; it also resulted in an increase in baseline extracellular K^+ which indicates impaired activity of the Na^+/K^+ ATPase and other energy dependent pumps [34,52]. This proves the importance of lactate shuttle for maintenance of ionic homeostasis.

Hippocampal MCT1 expression is reduced on endothelial cells and upregulated on astrocytes in different animal models and patients with temporal lobe epilepsy which will possibly impair uptake of lactate, ketone bodies and drugs like valproic acid as well as extracellular lactate clearance across the blood brain barrier [29,31]. Likewise, MCT2 level is also reduced on perivascular astrocytic endfeet and increased on astrocytic membrane facing synapses which could indicate enhanced anaerobic metabolism and substrate exchange between astrocytes and neurons [30]. These changes facilitate accumulation of extracellular lactate which might exert either, anti- or pro-ictogenic effects, by causing acidosis or by supplementing substrate for energy metabolism, respectively. Here, I demonstrated that blocking lactate shuttle has anti-seizure effect which is partly mediated by adenosine A1 receptor (A_1R). Noxious stimuli increase adenosine level which acts through A_1R and reduce presynaptic glutamate release subsequently altering neuronal excitability [45]. Inhibition of lactate dehydrogenase has also been shown to have anti-epileptic effect in animal models of chronic epilepsy [53]. Reversing MCT expression changes can have a neuroprotective role. Ketogenic diet

has been shown to upregulate endothelial MCT1 expression and similar pharmacologic manipulations could have clinical relevance for drug resistant epilepsy [54,55].

In conclusion, astrocytic lactate is important for oxidative metabolism even in the presence of ample glucose and it supports synaptic transmission and ionic homeostasis in healthy brain while it also maintains seizure like events under pathologic conditions rather than being antiepileptic as speculated previously [26,56].

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8. Affidavit-Declarations of own contributions to the selected publications

I, Eskedar Ayele Angamo, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “Role of lactate for synaptic transmission, recovery of ion gradient and maintenance of epileptiform activities in rat hippocampus and human neocortical slices” I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

I, Eskedar Ayele Angamo, had the following share in the following publications:

Publication 1:

Angamo EA, Rösner J, Liotta A, Kovacs R, Heinemann U. (2016). A neuronal lactate uptake inhibitor slows recovery of extracellular ion concentration changes in the hippocampal CA3 region by affecting energy metabolism. **J Neurphysiol.**

Planning of experiments: Conception of the project was done by Heinemann U and Kovacs R, detailed planning of the electrophysiology experiments was done by Angamo EA, planning of FAD imaging was done by Angamo EA and Rösner J.

Execution of experiments**:** All field potential, K^+, Ca^{2+}, Na^+, pH and pO_2 recordings in rat slices were done by Angamo EA, FAD fluorescence imaging was done by Angamo EA and Rösner J; absorption spectrophotometry was done by Rösner J.

Data analysis: All data analysis and preparation of figures (Fig 1-6, except fig 1C) were done by Angamo EA; figure 1C was prepared by Rösner J. Input on how to analyze the data was given by Liotta A and Heinemann U.

Manuscript preparation: First draft of the manuscript was written by Angamo EA; correction, proof reading and revision of the manuscript were done by Angamo EA, Heinemann U, Kovacs R, Liotta A and Rösner J.

Publication 2:

Rösner J, Liotta A, **Angamo EA,** Spies C, Heinemann U, Kovacs R. (2016). Minimizing photodecomposition of flavin adenine dinucleotide fluorescence by the use of pulsed LEDs.

J Micros.

Planning of Experiments: Planning for pulsed LED experiments- by Kovacs R and Rösner J.

Execution of experiments: I contributed field potential, K^+ recordings and FAD imaging for figure 1 and 4; majority of experiments were done by Liotta A and Rösner J.

Data analysis: Analysis was done by Angamo EA, Heinemann U, Kovacs R, Liotta A and Rösner J.

Manuscript preparation: Manuscript was written by Kovacs R; proof reading and revision was done by Angamo EA, Heinemann U, Kovacs R, Liotta A and Rösner J.

Publication 3:

Antonio LL, Anderson ML, **Angamo EA,** Gabriel S, Klaft ZJ, Salar S, Sandow N, Heinemann U. (2016). Invitro seizure like events and changes in ionic concentration. **J Neurosci Methods.**

Planning of Experiments: Concept was developed by Heinemann U.

Execution of experiments: Human resected epileptic neocortex and rat brain slices were prepared by Angamo EA, FP and K^+ recordings for figure 2 and 6 were done by Angamo EA.

Data analysis: Analysis and preparation of figure 2 was done by Angamo EA.

Manuscript preparation: I was involved in proof reading of the manuscript after the first draft was written by Heinemann U and Antonio LL and during the peer review.

Prof. Jörg Geiger Eskedar Ayele Angamo

9. Selected publications

Electronic version of the thesis does not contain original publications due to copy right restrictions. The selected publications can be accessed through the link given.

9.1 Publication 1

A neuronal lactate uptake inhibitor slows recovery of extracellular ion concentration changes in the hippocampal CA3 region by affecting energy metabolism.

Angamo EA, Rösner J, Liotta A, Kovacs R, Heinemann U.

J Neurphysiololgy (2016)

DOI: http://dx.doi.org/10.1152/jn.00327.2016

9.2 Publication 2

Minimizing photodecomposition of flavin adenine dinucleotide fluorescence by the use of pulsed LEDs.

Rösner J, Liotta A, **Angamo EA,** Spies C, Heinemann U, Kovacs R.

J Micros. (2016)

DOI: http://dx.doi.org/10.1111/jmi.12436

9.3 Publication 3

Invitro seizure like events and changes in ionic concentration.

Antonio LL, Anderson ML, **Angamo EA,** Gabriel S, Klaft ZJ, Salar S, Sandow N, Heinemann U

J Neurosci Methods. (2016)

Doi: https://doi.org/10.1016/j.jneumeth.2015.08.014

10. Curriculum Vitae

My curriculum vitae is not uploaded in the electronic version of this thesis due to data privacy regulations.

11. Complete list of own publications

Published

1. Heinemann U, **Angamo EA** and Liotta A. Non-synaptic mechanisms. (2017). Modulation of neuronal excitability by changes in extracellular ion composition.

   Reference module in Neuroscience and biobehavioural psychology,

   http://dx.doi.org/10.1016/B978-0-12-809324-5.00163-2

2. **Angamo EA,** RösnerJ, Liotta A, Kovacs R, Heinemann U. (2016). A neuronal lactate uptake inhibitor slows recovery of extracellular ion concentration changes in the hippocampal CA3 region by affecting energy metabolism.

   J Neurphysiol, 116 (5), 2420-2430.

   Impact factor (2016): 2.396

3. Rösner J, Liotta A, **Angamo EA,** Spies C, Heinemann U, Kovacs R. (2016). Minimizing photodecomposition of flavin adenine dinucleotide fluorescence by the use of pulsed LEDs.

   J Micros, 264 (2):215-223.

   Impact factor (2016): 1.692

4. Antonio LL, Anderson ML, **Angamo EA,** Gabriel S, Klaft ZJ, Salar S, Sandow N, Heinemann U. (2016). Invitro seizure like events and changes in ionic concentration.

   J Neurosci Methods, 260:33-44.

   Impact factor (2016): 2.554

5. Zehendner CM, Librizzi L, Hedrich J, Bauer NM, **Angamo EA,** de Curtis M, Luhmann HJ. (2013). Moderate hypoxia followed by reoxygenation results in blood-brain barrier breakdown via oxidative stress-dependent tight juction protein disruption.

   Plos One, 8 (12):e82823.

   Impact factor (2016): 2.806

Manuscript Under preparation

6. **Angamo EA**, Ul haq R, Rösner J, Gabriel S, Gerevic Z, Heinemann U, Kovacs R. (2017). Contribution of intrinsic lactate to maintenance of seizure activity in brain tissue of epilepsy patients.

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