NMDA- and asphyxia induced alterations of neurovascular responses in the cerebral cortex of newborn pigs, implications for the pathomechanism of neonatal hypoxic-ischemic encephalopathy

Ph.D. Thesis

Gábor Remzső, MS

Supervisor:
Ferenc Domoki, MD, Ph.D.

University of Szeged, Faculty of Medicine, Department of Physiology
Doctoral School of Theoretical Medicine

2021
Szeged

INTRODUCTION

Perinatal asphyxia (PA) is a major cause of neonatal mortality and it can also lead to hypoxic-ischemic encephalopathy (HIE) in the surviving newborns. Despite the availability of therapeutic hypothermia, HIE still results in long term disability in many infants, as hypothermia alone is often not sufficient to fully mitigate the HIE caused neuronal damage. To find new adjunct therapies, further investigation of the neuronal and vascular pathomechanisms underlying HIE development is warranted, and the newborn pig used in our studies is one of the best large animal model for the preclinical translational study of PA/HIE. In the present thesis, we focused on two important issues, namely the role of brain interstitial pH (pH_{brain}) alterations and neurovascular unit dysfunction in the mechanism of neuronal injury during PA and HIE development.

Primary and secondary pH_{brain} alterations are known to contribute to neuronal injury in the newborn neocortex during PA/HIE. Despite tight pH_{brain} regulation under physiological conditions, pH_{brain} disturbances can highly affect the neuronal excitability. It is well documented in rodents that pH_{brain} alterations modulate various types of ion channels. For instance, acidosis suppresses, while alkalosis facilitates the opening of N-methyl-D-aspartate (NMDA) receptors (NMDAR) in rat hippocampal neurons. Furthermore, changes in pH_{brain} can also modify the currents through voltage-gated Na^+, K^+ and Ca^{2+} channels in rat hippocampus. pH_{brain} measurements in rat pups showed that PA-induced acidosis was reversed to an alkalosis upon reventilation highly associated with seizure burden. Furthermore, intermittent asphyxia was also shown to trigger hyperexcitability (lowered seizure threshold) resulting in spontaneously occurring seizures. Seizures were suppressed by 5% CO_2 slowing down pH_{brain} recovery. This so-called graded restoration of normocapnia was also beneficial on cerebral metabolic acidosis, oxygen and lactate levels in guinea pigs as well. However, we had so far very few information about pH_{brain} alterations in large animal models under the acute and subacute phase of PA/HIE.

The neurovascular unit is a conceptual framework describing all the structural and functional connections among neuronal and vascular elements that assure the continuous adequate tissue perfusion to supply the metabolic demands of neuronal activity. Neurovascular unit dysfunction can be caused by hypoxic-ischemic (HI) injury including PA demonstrated by altered microvascular reactivity to hypercapnia or NMDA contributing to neuronal injury. Glutamatergic mechanisms are known to play an important role in the neurovascular coupling matching local perfusion with neuronal activity. Previously, many studies elaborated on the mechanism of NMDA-induced pial arteriolar dilation and increase in cortical blood flow, and also the attenuation

of this neurovascular response after PA/HIE or other types of hypoxic/ischemic stress in the piglet. The vascular response to NMDA appears to be dependent on neuronal nitric oxide synthase (nNOS) activity, while the attenuation of the response seems to be dependent on the action of reactive oxygen species (ROS) produced in the reoxygenation/reventilation period in this species. Interestingly, NMDARs can be not only a target but also a mediator of neurovascular unit dysfunction. Spreading depolarization (SD), a continuous wave of almost complete neuronal depolarization accompanied by spreading depression of neuronal activity, can also induce neurovascular unit dysfunction and alter the microvascular reactivity. Even a single SD can abolish the normal microvascular response to hypercapnia. NMDA can trigger an SD when topically applied onto the surface of the cortex in rodents. In neonates including newborn pigs, SD cannot be yet elicited, despite the presence of functional NMDAR. The potential interaction of NMDAR activation and neurovascular dysfunction has been unexplored in neonates.

AIMS

The major aim of our studies was to explore pathophysiological mechanisms in our translational large animal HIE model that may be of relevance for the development of adjunct neuroprotective therapies in the clinical management of HIE. The specific goals of our studies were the following:

1. We wanted to quantitatively determine the magnitude and the temporal dynamics of PA on the brain interstitial pH both during asphyxia and throughout the subacute phase of HIE development to be able assess the significance of pH alterations in our PA/HIE model and to allow comparison data from studies on rodents. We also wanted to determine the selective contribution of the respiratory component (the hypercapnia) in the PA-induced acidosis.
2. We wanted to determine if NMDA application alone (simulating generalized glutamatergic activation in the absence of PA) would affect the neurovascular unit function using hypercapnia-induced vasodilation as a test of cerebrovascular reactivity. As we proved NMDA-induced neurovascular dysfunction, we assessed if neuronal NO synthase activity plays a role in the mechanism.
3. Emanating from our results of specific goal #2, we wanted to start the electrophysiological exploration of cerebrocortical neuronal activity and connectivity in the piglet. Specifically, we studied if NMDA could alter both the spontaneous and also the hypercapnia-induced changes in electrical activity coinciding with its effect on cerebrovascular reactivity.

MATERIALS AND METHODS

1. Animals and surgery

The newborn pigs were restrained and anesthetized with intraperitoneal sodium thiopental injection. The animals were placed on a servo-controlled heating pad, keeping their core temperature in the physiological range (38.5±0.5 °C). The piglets were intubated through a tracheostomy then mechanically ventilated with humidified medical air occasionally supplemented with oxygen (FiO_2: 0.21-0.25) with the following ventilation parameters: respiration rate (RR): 30-35/min; peak inspiratory pressure (PIP): 12-14 cmH_2O. A catheter was inserted into the right femoral vein under aseptic conditions and anesthesia/analgesia was switched to intravenous morphine and midazolam as used previously along with supportive fluid therapy. A second catheter was inserted into the right carotid artery for taking blood samples, monitoring the mean arterial blood pressure (MABP) and heart rate (HR). Blood samples were analyzed for pH, gases, electrolytes and metabolites with an epoc® Blood Analysis System. We monitored the peripheral saturation (SpO_2) using pulse oximetry.

After instrumentation, the heads of the animals were fixed into a stainless steel stereotactic frame. For the Laser Speckle Contrast Imaging (LSCI) studies, we implanted a stainless steel closed cranial window over the parietal cortex. For the electrophysiology and the interstitial pH measurement studies, we obtained an open cranial window over the left parietal cortex for electrode insertion. The subarachnoidal space was filled with warmed (37 °C) artificial cerebrospinal fluid (aCSF) and was equilibrated with a gas mixture containing 6.3% O_2, 6.2% CO_2, and 87.5% N_2, respectively. According to the study protocols, we divided the animals into 2 and 4 groups, respectively:

1.1 GrHyp group (n=3): graded normoxic hypercapnia was elicited with 5% step-wise increase in CO_2 from 0% to 20%

1.2 Asph group (n=13): we induced asphyxia with hypercapnic-hypoxic gas mixture (20% CO_2 and 6% O_2, RR=15/min) and recorded the cortical pH in different time windows (baseline, PA and the first 4 hours of reventilation (n=6), 8^{th}-14^{th} hours (n=8) and 20^{th}-24^{th} hours (n=3) of reventilation). HIE development was confirmed with previously established neuropathology assessment.

2.1 Control group (n=6): we used repeated graded hypercapnia (5% and 10% CO_2) without NMDA stimulations

2.2 NMDA group (n=14): between the two graded hypercapnia increasing concentrations (0.1 and 1 mM) of NMDA dissolved in aCSF were topically washed onto the cortex under the closed cranial window (n=7) to study cortical blood flow (CoBF) or the open cranial window (n=7) to study local field potentials (LFP)

2.3 MK-801 + NMDA group (n=4): we used the same stimuli as in the NMDA group, except we used NMDA-receptor antagonist MK-801 (0.1 mM dissolved in aCSF) to pretreat the cortex and co-applying with NMDA

2.4 AAAN + NMDA group (n=7): selective nNOS inhibitor (N-(4S)-4-amino-5-[aminoethyl]aminopentyl-N'-nitroguanidin; AAAN; dissolved in saline, 0.4 mg/kg iv) were given then we used the same stimuli as in the NMDA group

2. pH_{brain} measurements

Measurements were performed inside a self-made Faraday cage with 4 Hz sampling rate with pH-selective microelectrodes. The electrodes were mounted on stereotaxic manipulators for calibration in 3 different warmed (38°C) buffer solutions (pH: 6.10, 7.10, and 8.10) before each experiment. The tips of the pH and reference microelectrodes were installed ~1-2 mm deep into the exposed cortex, and a Ag/AgCl ground electrode was placed under the scalp. The electrode signals were recorded, digitized and stored either using a custom-built differential electrometer, a 16-bit analog-to-digital converter and WinEDR software, or using a Microsensor Multimeter and SensorTrace Logger software. Evaluation of the recordings was performed offline: by applying linear regression analysis, the signals from the calibration solutions were fitted with a curve and the data were converted to pH values using linear interpolation. As the technique allows stable continuous pH_{brain} measurements reliably only for 3-4 hours, in different animals, different time windows were chosen to be assessed.

3. LSCI measurements and analysis

The brain was illuminated with near infrared polarized light (λ=808 nm, 200 mW) with a laser diode. The images were recorded (1 Hz, 1 ms) with a monochrome camera which is using a

polarizer and a color filter. The LSCI analysis was performed offline in LabVIEW. The contrast maps were calculated from the raw speckle images using a 5x5 pixel window (~1200 μm^2). For each image, the calculated $1/\tau$ values were normalized and expressed as percentages of the baseline. Pial arteriolar diameters were determined at selected time points where peak changes in CoBF to the applied stimuli were detected. To obtain better resolution, 30 images were averaged then the Otsu filtering method was applied to reduce noise. For determining the internal diameter of the arterioles were used edge detection and Euclidean distance measurement in MATLAB. The arteriolar diameter data were normalized and expressed as percentages of the baseline, and values from all 4 arterioles were averaged in each animal.

4. Neurophysiological recordings

Neurophysiological recordings were taken with 16-channel, acute single shank planar silicone electrodes. Data acquisition was performed with RHD2000 Evaluation System. All recorded data were sampled at 20 kHz, the broad band signals were filtered with a 1-9000 Hz bandpass filter. All data were analyzed off-line in MATLAB environment with implemented toolboxes (Chronux, FMAtoolbox) and custom written scripts. We decomposed the signal to calculate the power spectral density (PSD), using Fast Fourier Transform (FFT). The calculated PSDs were summed for each frequency bands. All the PSDs were averaged and normalized to the baseline activity. Addressing the 'inverse problem' of LFP, we computed the second spatial derivative, the current source density (CSD) to reveal how the different sources contribute to the mixed signal. We used the standard CSD method for the computation. Spike sorting was done with the Klusta package. Single units were detected by a threshold crossing-based algorithm. The spikes were automatically clustered, which was followed by the manual adjustment of the clusters using phy KwikGUI software. The putative interneurons and pyramidal cells were identified by their waveform characteristics and auto-correlograms (ACG) with the further examination of their cross-correlograms (CCG) to reveal the monosynaptic interactions with other single units.

5. Statistical analysis

The LSCI and neurophysiological statistical analyses were performed in IBM SPSS 22 using one- and two- way ANOVA with the appropriate *post hoc* tests (Tukey and Bonferroni). All results show mean±SD, respective to the baseline. For the Z-score computation we used MATLAB's

statistics toolbox. Relative PSD changes were determined as significant above/below $Z \geq \pm 2$* and $Z \geq \pm 4$**. pH, hemodynamic and physiological parameters were analysed in SigmaPlot or MATLAB environment. Data are expressed as mean±SEM. We performed one-way ANOVA with repeated measures (SNK *post hoc* test). The correlation between P_aCO_2 and pH_{brain} data were calculated with MATLAB's polynomial curve fitting ($p<0.05$* and $p<0.01$**).

RESULTS

1. Effects of PA/HIE and graded hypercapnia on physiological parameters

Ventilation with 5-10% CO_2 resulted in graded hypercapnia that was similar in all experimental groups both for LSCI and for electrophysiology experiments. Graded elevations in arterial pCO_2 were accompanied by the expected development of marked respiratory acidosis and a slight increase in plasma HCO_3^- levels, however, arterial pO_2, blood oxygen saturation, MABP and HR were all maintained during graded hypercapnia. The stimulus was highly repeatable, repeated ventilation with 5-10% CO_2 resulted in virtually identical changes in blood gases compared to the first application.

During the 20 min asphyxia period, O_2 saturation fell to below 30% and HR increased from about 140 1/min to nearly 200 1/min within the first 2 min, after which they showed no major changes, whereas the response in MABP was biphasic with a transient rise to about 90 mmHg followed by a slower decrease to below baseline. A rapid recovery of O_2 saturation to above 80% was seen during the first 2-3 min of reventilation, paralleled by transient increases in HR and MABP to above 240 1/min and 80 mmHg, respectively, after which the signals gradually recovered towards their normal values.

Longer-term monitoring of the physiological parameters reflected well the expected effects of PA and reventilation. Blood haemoglobin concentration, core body temperature, O_2 saturation, MABP and HR were within the normal range at baseline before PA and the values were not significantly different from baseline throughout the survival. In addition to the pulse oxymetry data, arterial blood gas analysis at the end of PA also confirmed the development of central haemoglobin desaturation (from 94±4 to 13±4%) along with severe acidosis, hypoxia and hypercapnia. Indeed, the fall in the arterial blood pH (pH_a) from 7.53±0.03 to 6.79±0.02 was substantial and paralleled by a rise in P_aCO_2 to 160±6 mmHg. Blood glucose and lactate levels were also profoundly raised, indicating the metabolic response to PA. The large drop in base

excess by 17.4±1.5 mmol/L and reduction in bicarbonate concentration together with the low pH_a (6.79) at the end of PA indicate that asphyxia developed much beyond the key clinical criteria of severe BA in human neonates (pH <7.0 and base deficit ≥12 to 16 mmol/L). Reventilation quickly restored normoxia in arterial blood, but P_aCO_2 levels remained slightly elevated, although the change was statistically significant only at 4 hours. Base excess was already normalized by this time point, and pH_a returned to 7.39±0.02, normal for piglets. In a similar fashion, blood glucose and lactate levels also returned to baseline by 4 hours, although both were still significantly elevated at 1 hour after PA.

2. pH_{brain} changes during HIE development and graded normoxic hypercapnia

Graded hypercapnia experiments were performed to evaluate the contribution of the respiratory component to the pH_{brain} changes recorded during PA. Step-wise, 5% increases in inhaled CO_2 resulted in proportional step-wise reductions in pH_{brain}. Arterial blood gas analysis revealed similar graded reductions in pH_a, thus the difference between pH_{brain} and pH_a remained unchanged during hypercapnia. Maximal changes were observed during the inhalation of 20% CO_2 when pH_a dropped from 7.52±0.06 to 6.98±0.02, whereas pH_{brain} from 7.32±0.01 to 6.77±0.02. The pH_{brain} and pH_a changes were fully reversed by restoration of normocapnia.

Induction of PA elicited a reduction in pH_{brain} that continued without levelling off over the 20 min insult, during which pH_{brain} dropped from the baseline value of 7.21±0.03 to 5.94±0.11 by the end of asphyxia. Upon reventilation, pH_{brain} was restored to 7.0 in 29.4±5.5 minutes with subsequent stabilization at a level that was virtually indistinguishable from the original baseline. Thereafter, from 2 h onwards pH_{brain} remained slightly below baseline (on average, by 0.10±0.02 pH units) without showing any marked alterations at any observed time point within the 24-hour follow-up period. Neuropathology analysis confirmed the development of HIE by revealing medium/severe neuronal damage in all examined neocortical regions, and also in the hippocampal CA1 region, the thalamus and the putamen. These findings are in accordance with previously reported neuronal injury using the same PA stress.

3. The cerebrocortical microvascular response to graded hypercapnia and NMDA

LSCI provided two-dimensional maps of cortical perfusion that served to determine changes in parenchymal perfusion and pial arteriolar diameters. The first exposure to graded hypercapnia

resulted in similar, CO_2 concentration-dependent increases in CoBF in all groups, the peak CoBF values, the integrated hyperemic CoBF response, and the pial arteriolar diameter changes were all similar. Topical application of 0.1mM NMDA resulted in a significant increase in CoBF (peak CoBF was 151±14% of baseline) and pial arteriolar diameters (138±31% of baseline) that peaked within 3-4 min, the elevated CoBF returned to baseline levels after perfusing the cranial window with aCSF. Repeating the stimulation with 1mM NMDA resulted only in slightly higher elevations both in the peak (172±23%) and the total CoBF responses and the arteriolar diameters (145±20%), and all these changes were reversible upon removal of NMDA. Topical application of the NMDA receptor inhibitor MK-801 did not affect CoBF, however, coapplication of MK-801 with either NMDA doses completely abolished the hyperemic and the pial arteriolar response to NMDA. Systemic administration of the selective nNOS inhibitor AAAN did not affect the CoBF response to NMDA, however, it caused a significant reduction in the pial arteriolar dilation to NMDA. In the control group, the CoBF response to the second exposure to graded hypercapnia was virtually identical to the first stimulation, the peak and the integrated CoBF values were very similar, and the cerebrovascular reactivity to either CO_2 concentration was fully preserved. In sharp contrast, cerebrovascular reactivity to the second exposure to graded hypercapnia was abolished in the NMDA-treated group. Pre- and coapplication of MK-801 with NMDA prevented the attenuation of the CoBF response to graded hypercapnia, cerebrovascular reactivity was preserved. In the nNOS inhibitor treated group, the CoBF response to graded hypercapnia was attenuated but not abolished after NMDA, thus cerebrovascular reactivity was partially (68±35%) preserved in this group.

4. Neurophysiological changes under graded hypercapnia and NMDA

Induction of hypercapnia with 5% CO_2 elicited first increases then decreases in LFP power, especially in the delta (δ) and theta (θ) ranges. Highest increases in δ were observed at cortical depths 100-400 μm and in θ at 100-600 and at 1000-1200 μm. The subsequent reduction in power started in the deeper cortical layers (below 900 μm) gradually shifting upward. Switching to 10% CO_2 further reduced LFP power both in the δ and θ ranges. These depressions were largely reversed upon restoration of normocapnia. LFP changes to graded hypercapnia following the stimulation with NMDA were markedly different, most strikingly the early increases in θ LFP

power did not develop. δ LFP powers were also significantly altered, and the pattern of LFP changes appeared to have disorganized.

NMDA (1 mM) selectively increased δ LFP power only in the upper layers (100-700 μm) however, activity in the θ range were simultaneously suppressed. This characteristic increase in the δ was identified as a ~2.5Hz δ oscillation down to 600 μm. CSD analysis showed that NMDA (1 mM) altered significantly the size of the sinks and sources causing the activation first in layer I. then layer II/III and IV. This NMDA-induced activation was δ band-limited. All recorded neurons fired with low frequency (~0.3-3 Hz). The ACGs identified major neuronal types of the parietal cortex such as interneurons, bursting and regular spiking pyramidal cells. CCGs helped to identify the most characteristic cell connections that were typically excitatory with 3-4 ms latency. We observed only a few inhibitory connections or reciprocal excitation/inhibition between the cells. Graded hypercapnia and NMDA increased spiking activity mainly in the II/III. and IV. layers down to 900 μm. From the total 164 connections 71% have been associated with layer II/III including intralaminar connections as well.

DISCUSSION

The major findings of the present study are:

1. Our translational PA/HIE model shows the major hallmarks of the moderate/severe injury. Normoxic hypercapnia provided the opportunity to evaluate the respiratory component, which was responsible for ≅40% of the developing acidosis. Cerebrocortical pH dropped below 6.0 due to PA and the acidosis exceeded ≅0.8 pH unit lower in the brain, compared to the blood. Cerebrocortical pH remained stable after the reoxygenation throughout the 24h observation period, without observing any pH shift.
2. Graded hypercapnia caused concentration-dependent increase in CoBF which was attenuated by topical NMDA application. MK-801 fully-, while AAAN partially prevented the attenuation of the hemodynamic response which proves the involvement of the NMDA receptors and their mediation by nNOS activation.
3. Attenuation of the LFP's δ and θ bands under the repetition of hypercapnia was associated with the attenuated cerebrovascular response. NMDA evoked ~2.5 Hz δ oscillations 500-700 μm deep from the cortical surface, indicating the location of the major targets of NMDA.

pH_{brain} acidosis under hypercapnia, asphyxia and HIE development

We can conclude from the previously observed studies that higher levels of hypercapnia, blood glucose and cerebral blood flow can all promote the development of cerebral acidosis during PA stress. The increased P_aCO_2 to 140-160 mmHg, which was observed in human and piglet PA as well, is able to reduce alone the pH_i to 6.5-6.6 under normoxic conditions. In our present study, we provide evidence that the inhalation of 20% CO_2 alone (P_aCO_2:120 mmHg) under normoxic conditions can result in the drop of the pH_{brain} to 6.8. Applying linear regression, we calculated that the developing hypercapnia (at P_aCO_2: 160 mmHg) resulted in the drop of the pH_{brain} to 6.50 in our PA model, which is in a full agreement with the previous results. Further acidification during PA will be dominantly determined by the increases in the rate of anaerobic glycolysis fueling the subsequent lactic acid production. The glucose delivery to the hypoxic brain limits the rate of glycolysis, as its reduction by either reducing cerebral blood flow or blood glucose levels attenuated the development of acidosis. We showed previously that significant cerebral ischemia did not develop in our PA model as in most of the animals the MABP did not drop to the lower limit of blood flow autoregulation. Thus, our model represents the acute phase of PA phase before the cardiovascular adaptation mechanisms are exhausted and likely the most significant pH_{brain} alterations occur.

pH_{brain} alterations have important pathophysiological roles both during PA and also during reventilation/reoxygenation. In piglets, pH_{brain} was described earlier to remain stable after restoration from PA, but the observation lasted only for four hours after the recovery. In contrast, we extended the post-asphyxia observation period to 24 hours. In our piglet PA/HIE model, we didn't find any significant secondary alterations in pH_{brain} during the 24-hour period after PA. Our results are in agreement with the previously reported piglet and human studies reporting intracellular brain pH data using magnetic resonance spectroscopy over the first 24 hours after HI stress or PA. However, we would like to emphasize that our piglet model represents only those babies with HIE who remain normocapnic due to mechanical ventilation. Spontaneously breathing babies can hyperventilate and subsequently develop hypocapnia elevating pH_{brain}, and hypocapnia has been indeed documented as an independent risk factor for adverse neurological outcome. In piglets, moderate hypocapnia alone reduced the cerebral perfusion with an increase in pH and lactate levels even in the absence of asphyxia. Our findings suggest that maintaining the normocapnia/ slight hypercapnia may have a role to prevent secondary pH_{brain} alterations. It

remains to be studied if graded restoration of normocapnia after PA would confer similar beneficial effects in piglets as reported in rat pups and guinea pigs.

The cortical microvascular response to NMDA

The cortical microvascular response to graded hypercapnia and NMDA has been extensively studied in the piglet, however just a few experiments have data about the pial arteriolar diameter and CoBF changes simultaneously. The NMDA induced pial arteriolar vasodilation was first described in newborn pigs, and later it was confirmed in different species. The NMDA-induced pial arteriolar dilation is mediated by neuronal NO production in most of the animal models studied. In the present study we found similar attenuation (~30-40%) of pial arteriolar dilation to NMDA by nNOS inhibition as Bari *et al.* using 7-nitroindazole. However, we now presented that the attenuated arteriolar vasodilation to NMDA did not result in attenuated blood flow response in the underlying parenchyma, indeed the parenchymal flow response remained unchanged. Our results suggest that dilation of intraparenchymal arterioles plays a more decisive role in determining the CoBF response and can compensate for the somewhat smaller pial arteriolar dilation in response to NMDA when nNOS activity is compromised. Additional vasodilatory mechanisms may act predominantly on intraparenchymal vessels and must play an important role in mediating the observed increases in CoBF to NMDA in the piglet, which can be our further research subject.

The pial arteriolar response to graded hypercapnia is well known to be vulnerable to hypoxic/ischemic stress, endothelial injury, or seizures, which are good markers for identifying neurovascular unit dysfunction. SDs are also known to attenuate the microvascular response to hypercapnia in adult brains. In the present study, we demonstrated that NMDA attenuated the response to graded hypercapnia in the newborn cerebral cortex in the absence of HI stress, which is in fact very similar to the effect of SD in the adult cortex. In the piglet cortex *bona fide* SDs are unable to be generated. But cortical depolarization may not be of central importance in the mechanism of the attenuation, as cortical depolarization by KCl did not affect the microvascular response to hypercapnia and other assessed stimuli previously. Our current study suggests that the NMDA-induced change in microvascular reactivity appears to involve nNOS activity in its mechanism, but seems to be independent of the direct hemodynamic effect of NMDA-receptor activation. Naturally, we cannot prove that NMDAR activation is the key to the observed

neurovascular dysfunction after SDs (as SDs cannot be studied in newborns), but this remains an intriguing possibility.

The cortical neuronal response to NMDA

In our study, we just started to explore the connections between the well-known cerebrovascular effects of hypercapnia and NMDA and the virtually uncharted neuronal effects of these stimuli in the cerebral cortex. Our present study is the first that studied the electrophysiological properties in all cortical layers to graded hypercapnia and to NMDA. Our electrophysiology data show layer-specific and concentration-dependent effects of hypercapnia on both the unit activity (spiking) and the LFP as well. Our present findings also identified the superficial, and also the much deeper cortical structures to be affected by topical NMDA. Thus, the NMDA-induced robust 2.5 Hz δ-oscillation have been found most prominent at 600-800 μm from the cortical surface suppressing LFP power in virtually all other frequency bands. NMDA also had opposing effects on neuronal firing, it suppressed or stimulated spiking in different neuronal populations. After NMDA stimulation, the LFP powers were restored to baseline levels, and also no abnormal spikes were recorded during/after NMDA application, suggesting that the cortex was not affected by significant excitotoxic stress during NMDA stimulation. This notion is in accordance with previous findings showing that the neurovascular response to topical NMDA is preserved in piglets (four applications in a 5 hours period).

We showed that the marked electrophysiological response to graded hypercapnia was altered after NMDA application. Currently we cannot make causative statements that the alterations in the neuronal response trigger the changes in microvascular reactivity or perhaps vice versa, however, our findings provide strong evidence that the mechanism of NMDA-induced attenuation of the microvascular response is not only limited to the cerebral vasculature. We can hypothesize that some aspects of the electrophysiological response to NMDA could participate in the attenuation of microvascular reactivity to hypercapnia and in the developing CoBF response. Clearly, further studies are needed to identify the specific and selective roles of the topically applied NMDA on the neuronal-vascular mechanisms.

ACKNOWLEDGEMENT

I would like to thank to Prof. Dr. Gábor Jancsó, the formal leader of the Theoretical Medicine Doctoral School, for accepting my application. I would like to thank to Prof. Dr. Ferenc Bari, the leader of the Theoretical Medicine Doctoral School, for providing the circumstances to finish my PhD studies. I would like to thank to Prof. Dr. Gyula Sáry for enabling my work in the Department of Physiology. My eternal gratitude goes to my supervisor, Dr. Ferenc Domoki for giving me the opportunity to work in his lab, teaching me all the surgical and medical knowledge and helping me to improve my english skills. I would like to thank to all my current and formal labmates Dr. Viktória Kovács, Dr. János Németh, Dr. Viktória Varga and Valéria Tóth-Szűki for their help in performing the experiments and giving advices. I would like to express my thanks to all colleagues in the Department of Physiology for their support and kindness. I am grateful to my family for their support and love. I am grateful to my mother, Éva Vass, who always stands beside me, no matter where the life drifts me away. I am the most grateful to my father, Attila Remzső who always inspired me for precise and hard work. Without them I couldn't reach my goals in life. I am grateful to all my friends, Zoltán Molnár, Ádám Sulumán, Tamás Puskás, Henrik Somodi, Dr. Fanni Kelemen who always cheered me up when I needed them the most.

FUNDING

We are grateful for the financial funding of the following grants:

- EU-funded Hungarian grant GINOP (GINOP 2.3.2 15 2016 00034)
- EU-funded Hungarian grant EFOP (EFOP-3.6.1-16-2016-00008)
- Hungarian Brain Research Program 2.0 (2017-2.1 NKP 2017 00002)

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Cumulative impact factor: 22.649