Perinatal asphyxia (PA) is a major cause of neonatal mortality and it can also lead to hypoxic-ischemic encephalopathy (HIE) in the surviving newborns. HIE is a devastating condition that occurs in 3 out of every 1,000 full-term newborns in developed countries, and the incidence is even higher in developing countries. Its diagnosis relies on the detection of signs of PA and the subsequent development of encephalopathy marked by altered consciousness, abnormal/absent reflexes, often epileptiform seizures and alterations in brain electrical activity[1]. HIE causes 15-20% of affected infants to die in the neonatal period, and another 30% suffer from severe long-term neurodevelopmental deficits amounting to over a million of neonates yearly all over the world. In fact, HIE represents 2.4% of the total burden of disease amounting to 50.2 million disability-adjusted life years world-wide[2] [3]. HIE imposes an enormous burden on the affected families, health care systems, and economics. Our long-term goal is to help developing treatment strategies that can mitigate brain injury, decrease mortality and the occurrence of motor and cognitive disabilities to alleviate this socioeconomic burden.

The current clinical management of HIE employs therapeutic hypothermia (moderate whole-body cooling; TH) as the only clinically effective neuroprotective therapy of HIE. However, TH alone is clearly insufficient for the successful treatment of all HIE patients; on average, seven infants affected with severe HIE must be treated with TH to reduce by one the number of deaths or major disabilities [4]. Therefore, further preclinical research is warranted to identify additional treatment options that can complement the neuroprotection afforded by TH. Unfortunately, drug development for HIE and drug development for neonates as a patient group in general is not yet among the top priorities of the pharmaceutical industry for obvious concerns of high risks and moderate expected benefits, therefore, public funded research is of utmost importance in the field.

In preclinical research the animal models are mandatory to reveal the mechanisms of different pathophysiological processes - including PA/HIE as well - and to test the potential neuroprotective strategies. In the recent years, the domestic piglet has emerged as an excellent model animal for studying neurobehavior and neurodevelopment. Notably, the major brain growth spurt in pigs and as well as in humans, extends from the late prenatal to the postnatal period [5]. Other commonly used animal models have differences in growth spurt timing, in most the brain growth spurt can be observed postnatally. The pig is a gyrencephalic species and the cortical surface is similar to the human gyrencephalic neocortex. The neonatal pig brain is very similar to the human neonate brain in anatomical, topological, developmental, cytoarchitectonical features as well. Furthermore, the maturation, myelination and electrical activity are also comparable to that of humans [6]. Perhaps not surprisingly, PA results in similar derangement of physiological parameters and results in similar pattern of selective neuronal injury in piglets and humans[7]. TH was also shown to be effective in a piglet HIE model as well, lending support to the model's translational value [8].
In the last 5 years, research efforts in our laboratory focused on translational research studying both HIE pathomechanisms and the efficacy of putative neuroprotective treatments in a subacute newborn pig HIE model. Our group established the human/instrumental

resources necessary to perform translational HIE studies: we employ truly newborn (<1 day old) piglets, induce asphyxia that results in alterations complying with the human diagnostic criterias of severe PA (blood pH <7.0, ΔBE > 15 mmol/L, low amplitude EEG etc.), and provide the supportive care to maintain physiological parameters in the normal ranges (using controlled mechanical ventilation, fluid therapy, morphine analgesia, antibiotic prophylaxis etc.) thus, the neuroprotective effects of new interventions can be meaningfully tested. We can monitor and control core temperature, blood pressure, blood gases, glucose and lactate levels. Continuously monitoring the EEG can help intervening in the occurring seizures as well.

The major aims of the present proposal **in the first year** were the following: (1) to extend the translational value of our piglet HIE model by introducing hypothermia treatment to better reflect current state-of-the-art HIE management, (2) to test the neuroprotective potential of novel treatments in our model, and (3) to investigate the molecular mechanisms of brain injury, more specifically, we would like to investigate expression changes for BDNF, its downstream signal transduction elements, as well as Caspase- 3 and its upstream elements.

In the second research period, we continued our research effort according to the work plan, although the number of new animal experiments were limited by the COVID-19 pandemic situation. However, we successfully completed the major study started in the first year and made significant progress with another one as well. Three *in extenso* research papers (all Q1) from the project have been published. The detailed results are the following:

We completed a study in which we established a piglet HIE model using asphyxia followed by state-of-the-art supportive therapy and TH. Against this background, we tested two purported neuroprotective gases: molecular hydrogen and carbon dioxide, the first is thought to reduce postasphyxial oxidative stress and neuroinflammation, the second can be used to achieve the so called “graded restoration of normocapnia” that was shown to aid brain pH regulation in the postasphyxial period to limit brain damage in rodent models.[9] Using neuropathology examinations, we demonstrated that TH elicited limited neuroprotective effect that was not significantly improved by co-treatment with either hydrogen or carbon dioxide. The EEG shows the recovery of neuronal activity after asphyxia, but it may also yield important information about abnormal activities. We have recently proposed that the determination of instantaneous spectral entropy may be a useful tool to detect the loss of EEG signal complexity, lower entropy values may point toward a more deterministic activity that may signal the onset of electrographic seizures affecting neuronal survival. Importantly we determined that BDNF expression changes were not correlated with TH induced neuroprotection in any studied brain areas, whereas AIF and Caspase- 3 levels were induced in areas where TH induced neuroprotection was absent or no significant. We published these results in the following paper: **V. Kovács, G. Remzső, V. Tóth-Szűki, V. Varga, J. Németh, and F. Domoki- Inhaled H2 or CO2 Do Not Augment the Neuroprotective Effect of Therapeutic Hypothermia in a Severe Neonatal Hypoxic-Ischemic Encephalopathy Piglet Model –Int J Mol Sci. 2020 Sep; 21(18): 6801. (IF:4,556).[10]**

We could also successfully complete two more studies that help understanding the HIE developing in our experimental model. Asphyxia results in severe acidosis in the brain extracellular fluid, surprisingly the interstitial brain pH determined with pH sensitive

microelectrodes may drop 1.0 pH unit below the pH value in the blood (to 5.8-6.0!). However, during reventilation, the recovery of brain pH does not overshoot to alkalosis like shown previously in rodents, but remains stable for at least 24 hours. These results correspond well with the lack of neuroprotection by carbon dioxide. These results were published in the following paper: - **G. Remzső, J. Németh, V. Varga, V. Kovács, V. Tóth-Szűki, K. Kaila, J. Voipio, and F. Domoki- Brain interstitial pH changes in the subacute phase of hypoxic-ischemic encephalopathy in newborn pigs- PLoS One. 2020; 15(5): e0233851. (IF:2,74)**[11]

In adult cerebral hypoxic-ischemic stress, spreading depolarization induced damage to the neurovascular unit is thought to contribute to more severe neuronal damage. We investigated if stimulation of the NMDA receptors – an important component of spreading depolarizations – would affect neurovascular unit function, and we found that NMDA can abolish cerebrovascular reactivity to carbon dioxide. Local application of NMDA even in the absence of asphyxia triggers neurovascular dysfunction: the neuronal response was altered, and the blood flow response was virtually abolished in the cerebral cortex when applying graded hypercapnia as a stimulus to test cerebrovascular reactivity. This effect of NMDA is qualitatively identical to that of spreading depression that has a well established role in ischemia induced neuronal injury and is critically dependent on NMDA receptor activation. Importantly, spreading depression is yet absent (cannot be triggered) in the neonatal brain. We published these results in the following paper: –**G. Remzső, J. Németh, V. Tóth-Szűki, V. Varga, V. Kovács, and F. Domoki- NMDA attenuates the neurovascular response to hypercapnia in the neonatal cerebral cortex- Sci Rep. 2019; 9: 18900. (IF:3,998).**[12]

We continued the study on the potential neuroprotective effect of the kynurenic-acid analog SZR72 in our experimental model. Kynurenic acid (KYNA), an endogenous product of tryptophan metabolism, was previously shown to be beneficial in rat HIE models. We sought to determine if the KYNA analog SZR72 would afford neuroprotection in piglets. Based on our preliminary results, we decided to use a continuous infusion administration regimen (170 mg/kg bodyweight bolus followed by continuous infusion of 170 mg/kg b.w./12h). Based on the results of the above detailed study, we modified our asphyxia protocol to reduce the severity of the model (increased FiO_2, from 4% to 6% O_2). In addition, we included a TH group to compare it to the effect of SZR72. At the end of the second year the study groups have been completed, and the evaluation of the major electrophysiological and neuropathological measures were underway.

In the third research period we finished our research effort according to the work plan. However, we successfully completed the major study, the applied methodology were modified based on the results obtained in previous years. After our published research papers in the last year, in this year we published another study (Q1).

The latest detailed results were the following:

SZR72 administration did not affect significantly on the monitored physiological parameters but resulted in the expected large increase in serum SZR72 levels, the continuous SZR72 infusion maintained serum SZR72 concentrations in the ~50–100 µmol/L range. SZR72 treatment did not affect serum kynurenine but significantly increased serum KYNA levels.

Asphyxia resulted in an isoelectric EEG that recovered gradually over the observation period. The return of a continuous high-amplitude EEG was markedly present in all SZR72 animals, and it was completed in 4 h after asphyxia clearly indicating blood–brain barrier penetration and direct neuronal effects of the drug. Furthermore, at 24 h after asphyxia, EEG power spectral density (PSD) analysis revealed that PSD-s virtually in all leads and in all frequency ranges were significantly higher in the SZR72 group, compared to either the vehicle-treated (VEH) or the TH groups. However, instantaneous spectral entropy (InstSpEnt) values reflecting EEG signal complexity showed that, unlike the higher PSD values, the InstSpEnt values in the SZR72 group were quite similar to the VEH group and lower than in the TH group. Concerning VEP, the latency of the P100 component was unaffected by asphyxia, but its amplitude was significantly reduced in the SZR72 and VEH groups but not in the TH group, suggesting a lack of SZR72-induced neuroprotection.

Despite its marked electrophysiological effects, SZR72 did not prevent selective neuronal damage in the subacute phase of our HIE model.

Our results suggest that the use of exogenous KYNA analogs with higher neuroprotective/less atypical neuronal actions may be feasible in the management of HIE, warranting further preclinical research.

We published these results in the following paper: **Viktória Kovács, Gábor Remzső, Tímea Körmöczi, Róbert Berkecz, Valéria Tóth-Szűki, Andrea Pénzes, László Vécsei and Ferenc Domoki- The kynurenic acid analog SZR72 enhances neuronal activity after asphyxia but is not neuroprotective in a translational model of neonatal hypoxic ischemic encephalopathy- Int J Mol Sci. 2021 May; 22(9): 4822. (IF:5,923)[13]**

Completion of the specific aims achieved three major goals. First, the results were considerably increase our understanding of HIE pathophysiology in this important preclinical large animal model. Second, the results may identify targets of intervention that can significantly enhance the neuroprotective efficacy of therapeutic hypothermia. Third, we were able to increase the translational value of our HIE model that is critical for the meaningful testing of further neuroprotective interventions.

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