Combined neuroprotective strategies on global ischemic models

(Closing report)

The main goal of this project was to carry on basic research on the field of neuroprotection on brain ischemic models.

During the whole period, our research activity was carried out according to the research plan of project proposal. Studies of neuroprotective agents and interventions were planned in the course of *in vivo* and *in vitro* electrophysiological experiments, histological, immunohistochemical, molecular biological and behavioral methods.
In addition to the planned experiments, the research has brought many new problems and some of unexpected results.

According to our research plan, the postconditioning (a special approach of neuroprotection) has been planned to study during the first quarter of the project period. Our own earlier results have been supported by these studies: postconditioning with kainic acid (KA) on two-vessel occlusion (2VO) models were carried out. It was found that administration of KA in subconvulsive dosage (5 mg/kg, i.p.) resulted in maximal protective effect if the second pathophysiologival stress (KA) is given 1 day (or longer) delay.

In addition to global ischemia, there was a need to study focal ischemia, in particular the effects of Transient Ischemic Attack (TIA). However, in the literature, there was no appropriate rat brain model to study the TIA. We have developed a well-controllable TIA model, in which controlled hypoperfusion resulted in well-reproducible cellular changes without massive tissue damage as it is for TIA in human cases (***Knapp et al., Neuropathol. Appl. Neurobiol.,*** published in ***2014***).
With the newly developed TIA model, we have shown that L-kinurenine sulfate (L-KYN) treatment might also be neurotoxic, while in most cases it is neuroprotective (functioal and histological studies) (***Gellért et al., Neuroscience, 2013***).

In the course of study of neuroprotection it turned out that not only the ischemic model (global ischemia (4VO, 2 VO) vs focal ischemia) but choose of inbred lines of the studied animal is extremely important. Two lines (Wistar and Spregue_Dawley) were studied in 4VO models; electrophysiological properties (ECoG-power spectrum, burst suppression ratio) and neuronal degeneration were studied. We have found that Wistar rats were much more sensitive to hypoperfusion (***Fuzik et al., Neuroscience, 2013).***

Our main activity was to investigate the protective effect of kynurenic acid and its derivatives. In some cases we used not only ischemia models for testing of neuroprotection, but other models (e.g. cortical spreading depression: CSD) as well. We have found that during CSD (a model for a kind of migraine), the permeability of the blood-brain barrier (BBB) increased, therefore, the systemically administered KYNA might also be neuroprotective (.(***Olah et al., Drug Design, Development and Therap, 2013***).

According to our work plan, during the second year of the project period, we tested such kind of endogenous protective compounds like KYNA (and its derivatives), oxaloacetate (OxAc) and acetyl-L-carnitine ALC).

Since many years, we work on the problem of hyperexcitability resulted by the high glutamate (Glu) level following brain ischemia. One of the ways of our research strategy is the so called "Glu-scavenging" from the brain by the intravenously administered OxAc. The essence of the method is that intravenous Glu administration increases the brain-to-blood Glu efflux (see Teichberg 2011). With the aid of our previously detailed focal ischemic model (Knapp et. al., 2014) we have shown that in case of repeated TIAs, the amplitudes of cortical somatosensory evoked responses decreased, but that can be prevented by OxAc treatment. Histological studies (Fluoro Jade C) supported the electrophysiological results, namely, the protective effect of Glu scavenging. (***Knapp et al., Cell. Mol. Neurobiol. 2014***).

In another serial of experiments, the neuroprotective property of ALC was studied, specially focused to the after-treatment. ALC is an endogenous compound and can be used at high dose without toxicity. Its neuroprotective properties have been reported in many studies, but its potential action on long-term potentiation (LTP) and dendritic spine density has not been described to date. The aim of the present study was an evaluation of the possible protective effect of ALC after ischemic insults inflicted on hippocampal synaptic plasticity in a 2-vessel occlusion (2VO) model in rats. For electrophysiological measurements, LTP was tested on hippocampal slices. The Golgi-Cox staining technique was used to determine spine density. 2VO resulted in a decreased, unstable LTP and a significant loss of dendritic spines. ALC administered after 2VO was not protective, but pretreatment with ALC (prior to 2VO) restored the LTP nearly to the control level. This finding paralleled the histological analysis: ALC pretreatment resulted in the reappearance of dendritic spines on the CA1 pyramidal cells. Our data demonstrate that ALC administration can restore hippocampal function and spine density. ALC probably acts by enhancing the aerobic metabolic pathway, which is inhibited during and following ischemic attacks (***Kocsis et al., Neuroscience, 2014***).

In this project, special attention was paid to the endogenous compounds and to the procedure of after-treatment. Earlier it has been shown that ALC after-treatment did not resulted in good recovery in LTP function of the ischemic hippocampus, but in the dendritic spine density it proved to be protective significantly. In a serial of experiments, combined ALC and OxAc treatment was given. It was found that ALC (100 mg/kg) combined with OxAc after-treatment, resulted in significant protective effect both in LTP function and dendritic spine density (***Kocsis et al., J. Neural Transm., 2014***).

In histological studies with KYNA, we found that KYNA in pre-treatment decreases the sensitization and nitroglycerine (NO)-induced activation of the cervical part of trigemino-cervical complex in dose-dependent way (***Fejes-Szabó et al., J. Neural Transm., 2014***).

Connecting to this theme; the histological and behavioral effects of probenecid was also studied in this period. (Probenecid is an organic acid transporter inhibitor, reduces the quick depletion of medicaments). We applied probenecid, to keep the level of KYNA in the CNS as long as possible. We found that after probenecid treatment, the formalin-induced biphasic behavioral effect and the c-Fos and nNOS immunoreactivity decreased. At the same time, we did not found any change in interleucin-1beta and NQO1 expression after formalin-treatment. At the same time, we did not found any change in interleukin-1beta and NQO1 expression after formalin-treatment (***Fejes-Szabó et al., CNS Neurol Disord Drug Targets, 2014***).

In the third year of our project, we continued the research according to the research plan. These studies based on the results detailed above. Namely, we continued the study of molecular mechanisms of protective processes. We have found that Akt/PI3K pathway has a role in post-conditionally induced neuroprotection, as well as in the protective effects of ALC. In our previous experiments post-conditioning resulted in the restoration of the functional (LTP) and structural (spine density) impairments caused by global hypoperfusion (Nagy et al., 2011). In the frame of this project, we investigated the possible molecular mechanisms that could be responsible for these results.
To determine the role of the Akt/GSK3b signaling pathway in mediating neuroprotection achieved by KA-post-conditioning (KA-PC), an inhibitor of the Akt upstream kinase PI3K (LY294002) was administered to the rats, and in vitro electrophysiological experiments and molecular analysis (qRT-PCR) was conducted. The LY294002 blocked the effect of KA-PC, decreased the LTP function, and the level of miR-132 (one of the main regulating element in the genesis and the plasticity of dendritic spines). So the PI3K/Akt/GSK3β signaling pathway probably involved in KA-PC.

ALC was effective against global hypoperfusion (2VO model) in different experimental paradigms (Kocsis et al., 2014; ***Kocsis et al., J. Neural Transm. 2015***). To investigate the potential neuroprotective effect of ALC against global ischemia an oxygen-glucose deprivation (OGD) model was established, where ALC was protective in a dose-dependent manner. Furthermore, since this OGD model provides a simple way for the measurement of the mechanisms underlying this neuroprotective effect, the role of the PI3K/Akt pathway was investigated (by the application of LY294002). Results showed that this signaling pathway is essential in the neuroprotection achieved by ALC.

One of the dedicated goals of this project was to develop neuroprotective strategies with kynurenines, to synthesize new KYNA analogues, which are neuroprotective. Within the frame of this project, we have developed more than 200 new compounds (we have 4 patents), and among these molecules 3 - 4 are promising (SZR-105, SZR-109, SZR-198). The result achieved by kynurenines and these new analogues were published in ***Varga et al., Front Behav. Neurosci., 2015.***

Parallel with experiences, based on our own results and the literature, we made some theoretical considerations on the utility of kynurenines in different neurological and

psychiatric diseases (***Török et al., Curr. Drog Metab., 2015; Bohár et al., Int. J. Mol. Sci., 2015***).

More than one and half decade, we study the role of kynurenines in the physiology and pathophysiology of the CNS. The causal role of altered KYNA production has been described in several neurodegenerative and neuropsychiatric disorders (e.g., Parkinson's disease, Huntington's disease, schizophrenia) and therefore kynurenergic manipulation with the aim of therapy has recently been proposed. Conventionally, KYNA is produced from its precursor L-KYN with the aid of the astrocytic kynurenine aminotransferase-2 (KAT-2) in the murine brain. Although the mouse is a standard therapeutic research organism, the presence of KAT-2 in mice has not been described in detail. Our study demonstrated the presence of kat-2 mRNA and protein throughout the adult C57Bl6 mouse brain. In addition to the former expression data from the rat, we found prominent KAT-2 expression not only in the astrocyte, but also in neurons in several brain regions (e.g., hippocampus, substantia nigra, striatum, and prefrontal cortex). A significant number of the KAT-2 positive neurons were positive for GAD67; the presence of the KAT-2 enzyme we could also demonstrate in mice brain homogenate and in cells overexpressing recombinant mouse KAT-2 protein. This new finding attributes a new role to interneuron-derived KYNA in neuronal network operation. Furthermore, our results suggest that the thorough investigation of the spatio-temporal expression pattern of the relevant enzymes of the KYN pathway is a prerequisite for developing and understanding the pharmacological and transgenic murine models of kynurenergic manipulation (***Heredi et al., Brain Structure and Function, 2016***).

As in previous years, in 2016 too, parallel with these experiments, based on our own experiences and the literature, we made some theoretical considerations on the utility of kynurenines in different neurological and psychiatric diseases. We reviewed the current neuropharmacological aspects of the topic, and estimated that the development of a well-tolerated NMDA antagonist (like KYNA or a KYNA derivative) may offer a novel therapeutic option for the treatment of e.g. Parkinson’s Disease (***Majláth et al., Curr Med Chem., 2016***), or in other different neurodegenerative diseases and pain syndromes (***Majláth et al., Current Neuropharmacology, 2016***), or in brain aging (***Török et al., Curr Drug Matab., 2016***).

This four-years-long project period has been extended with 6 months. During this time, we have completed a study on the effect of L-kynurenine sulfate on cerebral perfusion. The significance of this study is obvious: in our research projects we used kynurenines (KYN, KYNA, KYNA derivatives etc.) as neuroprotective compounds. Though, most of kynurenines are neuroactive molecules, we do not know much about their effects on brain circulation. This study showed that L-kynurenine sulfate induces cerebral hypoperfusion transients in mice (***Varga et al., Microvascular Research, 2017).***