The role of microglia in central and systemic inflammation after cerebral ischemia

Final report

Our research aims to investigate the role of microglia and central inflammatory processes in brain injury following cerebral ischemia. In accordance with the objectives and the research plan set out in the proposal, we have established novel surgical approaches combined with *in vivo* two-photon imaging that allow the investigation of neuronal excititoxic processes and inflammation in real time after cerebral ischemia. We have introduced selective microglia manipulation techniques and assessed the functional contribution of microglia to neuronal activity changes, excitotoxic processes, blood brain barrier (BBB) injury and inflammation. We have further explored the role of microglia in controlling neuronal activity in experimental models of spreading depolarization – a key pathophysiological event of mass neuronal depolarization that takes place after cerebral ischemia - and identified some of the mechanisms mediating these actions. We have also investigated microglia-neuron interactions under both physiological and pathophysiological conditions at the nanoscale level using advanced microscopy and explored how these interactions are influenced by inflammation. During the lifetime of the project, no major deviation from the initial project objectives has occurred; only novel research approaches and models have been introduced or fine-tuned in light of the results to facilitate a more complete understanding of the microglial mechanisms involved in neuronal injury.

Main results:

- **Microglia-neuron interactions and their role in neuronal injury**. Using genetically encoded fluorescent calcium indicators and microglia reporter (Cx3Cr1 +/GFP) mice, we investigated neuronal calcium changes before, during and after cerebral ischemia, in real time with *in vivo* two-photon imaging, which has not been performed in previous studies. In contrast to most of the available literature data, we found that excitotoxic processes develop with delay after the onset of ischemia in the brain, and microglial responses rapidly follow neuronal activity changes. Neurons showing increasing intracellular calcium levels in response to

ischemia are surrounded by microglial processes within minutes. We have also shown that microglial processes rapidly respond to spreading depolarization (SD) in the injured brain, independently of previous calcium changes in individual cells. SD is a major neuropathological phenomenon characterized by a mass wave of neuronal depolarization, which is a hallmark of pathology in several brain diseases such as stroke, epilepsy or migraine. To investigate the functional contribution of microglial actions to cerebral ischemia and changes in neuronal activity, we have tested several models of microglia depletion. We found the pharmacological inhibition of CSF1R – an essential signalling pathway for self-maintaining microglia - being the most efficient, with high level of selectivity for microglia and no visible effect on peripheral macrophages or resident brain cells. Selective depletion of microglia in the brain (loss of over 95% of the cells) resulted in dysregulated neuronal network activity during and after cerebral ischemia, which was associated with increased neuronal injury. In addition, a marked reduction in the incidence of SD was observed. To our knowledge, no haematopoietic cell type has been previously implicated in changes in neuronal network activity and SD in the injured brain.

We have also shown that regulation of neuronal activity by microglia is a key protective response after acute brain injury. An absence of microglia results in a markedly increased and accelerated neuronal death, yielding 60% larger infarct size and profoundly impaired neurological outcome in mice after experimental stroke. This effect can be fully reversed by microglial repopulation. We have also shown that although microglia quickly react to changes in BBB permeability after ischemia and are recruited to sites of vascular injury, increased neuronal death in the absence of microglia is not due to changes in BBB injury. We have also identified P2Y12 as a key receptor mediating the recruitment of microglial processes to neurons in the injured brain. STORM super-resolution microscopy has identified the clustering of P2Y12 specifically at sites of microglia-neuron interactions. Collectively, these results have been published in Nature Communications (Szalay et al., 2016).

- **The role of inflammation in microglial activation and brain injury**. To understand how inflammatory pathways activated by sterile tissue injury and

systemic inflammatory stimuli interact in processes of brain injury, we have studied the role of inflammasomes after cerebral ischemia. Inflammasomes are intracellular molecular complexes that sense diverse molecular patterns released by injured cells or pathogens, and regulate the processing and release of the key proinflammatory cytokine, interleukin-1β (IL-1β). We found that inflammasomes are critically important in ischemic injury, since genetic deletion of inflammasome components led to markedly reduced neuronal death after stroke. Inflammasome-related pathways were also found crucial in shaping microglial activation after cerebral ischemia. In collaboration with researchers at the University of Manchester, we showed that inflammasomes sensing DNA and flagellin (namely AIM2 and NLRC4 inflammasomes) contribute to brain injury via their common adaptor protein, ASC (Denes et al., PNAS 2015). Surprisingly, inflammasome-related pathways were also found to influence brain injury independently of the production of IL-1 in the brain.

- **Mechanisms through which systemic inflammation leads to microglial activation and brain injury**. We have also investigated the role of systemic inflammation preceding cerebral ischemia in brain inflammation, BBB injury and neuronal death. To this end, we have used different models of systemic inflammation. In one set of these studies a non-resolving lung infection / inflammation model was developed using a human *Streptococcus pneumoniae* (*S. pneumoniae*) strain with researchers at the University of Manchester (UK). Infection-induced systemic inflammation preceding cerebral ischemia resulted in a significantly larger brain injury and BBB breakdown after experimental stroke. This was associated with increased brain inflammation, microglial IL-1α production and platelet activation. Blockade of IL-1 actions by IL-1 receptor antagonist (IL-1Ra) or blocking platelet-endothelial interactions by an anti-GPIbα Fab fragment, reversed increased brain injury, BBB breakdown and impaired functional outcome in infected mice, implying the important role of these pathways in brain injury preceded by systemic inflammation (Denes et al., Ann Neurol, 2014).

  In collaboration with researchers at the Semmelweis University, we developed a novel SPECT (singlephoton emission computed tomography)-based approach that

enables very early (within 1-2 hours) detection of BBB injury and perfusion changes following cerebral ischemia. We have shown that preceding systemic inflammation – induced by administration of bacterial lipopolysaccharide (LPS) - leads to augmented BBB injury, and prolonged reduction in cerebral blood flow even beyond the occlusion period (1-2-3 hours after reperfusion induced following cerebral ischemia). These research tools also revealed that after brain injury, inflammatory changes in the gut and the lungs take place within two hours, followed by the development of infectious complications later (Szigeti et al., JCBFM, 2015). The main novelty of these results is indicated by the fact that currently no experimental or clinical imaging tools are available to reveal the earliest signs of lung or gut inflammation / infection, which are key factors for prolonged hospitalization and early death of stroke patients via the development of pneumonia, urinary tract infection or paralytic ileus. We have also shown that systemic inflammation results in increased microglial activation, augmented BBB injury and increased leukocyte infiltration after cerebral ischemia (Szigeti et al., JCBFM, 2015). As specified in the proposal, we have also planned to investigate whether the effect of preceding systemic inflammation is mediated via altered microglial function in the brain by using selective microglia manipulation / depletion approaches. However, the unexpected discovery that elimination of microglia results in markedly augmented brain injury (Szalay et al., Nature Communications 2016) made us reconsider some of these studies. Our present results show that the lack of the major proinflammatory cytokine, IL-1 (mediating both central and systemic inflammation and produced by both peripheral immune cells and microglia) results in markedly delayed neuronal excitotoxic responses as assessed by *in vivo* two-photon imaging after brain injury. In contrast, IL-1 stimulation of neurons results in increased excitability and altered neuronal network activity, mechanisms of which are currently being investigated (unpublished). Transgenic mice allowing microglia-specific gene manipulation (CX3CR1-creER) that had not been available at the beginning of the present NKFIH project, have recently been established at IEM HAS and are being crossed with IL-1$\alpha\beta$ fl/fl mice to investigate the role of microglial IL-1 vs systemic IL-1 in furher studies.

We have also shown that systemic inflammatory changes induced by gut bacteria could in part be responsible for inflammasome activation in the brain and hence augmented brain injury. We found that acute brain injury results in profound and specific changes in the gut microbiota and these are in part mediated by altered autonomic nervous system activity in the gut. Changes in microbiota and autonomic activity showed a good correlation with brain injury in individual mice (Szigeti et al., Brain Behav Immun, 2016). Thus, bacterial products could shape systemic immune responses locally in the gut as well as leak into the circulation after brain injury resulting in inflammasome activation in microglia and other cells that may lead to altered brain injury.

In summary, research performed during the lifetime of the project has revealed novel mechanisms through which microglia control neuronal activity and injury. Microglial responses, brain perfusion and neuronal injury after cerebral ischemia are profoundly influenced by systemic inflammatory responses and specifically, by the microbiota. Understanding microglial actions that are important for the regulation of neuronal responses after both acute neuronal injury and different forms of neurodegeneration could pave the way of novel treatment opportunities in brain diseases. Similarly, targeted blockade of inflammatory pathways, specifically those linking inflammasome activation and IL-1 production with microglial responses and neuronal injury could be therapeutically effective in cerebrovascular diseases to maintain the regulation of central inflammatory responses and limit neuronal injury. Recent advances in this field including the above research findings have been summarized in our latest review article (Lénárt et al., JCBFM 2016).

Publications[#]:

[#]Peer-reviewed papers only, no conference posters included

Lénárt N, Brough D, Dénes Á* (2016). Inflammasomes link vascular disease with neuroinflammation and brain disorders. J Cereb Blood Flow Metab. 36:1668-1685.

Szalay G, Martinecz B, Lénárt N, Környei Z, Orsolits B, Judák L, Császár E, Fekete R, West B.L., Katona G, Rózsa B and Dénes Á* (2016). Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. Nat Commun, 7:11499.

Bekő K, Koványi B, Gölöncsér F, Horváth G, Dénes Á, Környei Z, Botz B, Helyes Z, Müller CE, Sperlágh B (2017). Contribution of platelet P2Y12 receptors to chronic Complete Freund's adjuvant-induced inflammatory pain. J Thromb Haemost. 15:1223-1235.

Houlden A, Goldrick M, Brough D, Vizi ES, Lénárt N, Martinecz B, Roberts IS, Denes A* (2016). Brain injury induces specific changes in the caecal microbiota of mice via altered autonomic activity and mucoprotein production. Brain Behav Immun 57:10-20.

Szigeti K, Horváth I, Veres DS, Martinecz B, Lénárt N, Kovács N, Bakcsa E, Márta A, Semjéni M, Máthé D, Dénes Á* (2015). A novel SPECT-based approach reveals early mechanisms of central and peripheral inflammation after cerebral ischemia. J Cereb Blood Flow Metab., 35(12):1921-9.

Denes A*, Coutts G, Lénárt N, Cruickshank SM, Pelegrin P, Skinner J, Rothwell N, Allan SM, Brough D (2015). AIM2 and NLRC4 inflammasomes contribute with ASC to acute brain injury independently of NLRP3. Proc Natl Acad Sci U S A 112(13):4050-5.

Brough D, Denes A*. Interleukin-1α and brain inflammation (2015). IUBMB Life 67(5):323-30.

Smith CJ, Denes A, Tyrrell PJ, Di Napoli M. Phase II anti-inflammatory and immune-modulating drugs for acute ischaemic stroke (2015). Expert Opin Investig Drugs 24(5):623-43.

Polyák A, Ferenczi S, Dénes A, Winkler Z, Kriszt R, Pintér-Kübler B, Kovács KJ (2014). The fractalkine/Cx3CR1 system is implicated in the development of metabolic visceral adipose tissue inflammation in obesity. Brain Behav Immun 38:25-35.

Denes A*, Pradillo, JM, Drake, C, Sharp A, Warn P, Murray KN, Rohit B, Dockrell C, Chamberlain J, Casbolt C, Francis S, Martinecz B, Nieswandt B, Rothwell NJ, and Allan SM (2014). Streptococcus pneumoniae worsens cerebral ischaemia via IL-1 and platelet GPIbα. ANNALS OF NEUROLOGY 75(5):670-83.

** Corresponding author*

Selected invited presentations[#]:

[#]Invited conference talks by AD only

- ***Centrális és perifériás gyulladásos folyamatok szerepe az agyi károsodás kialakulásában.*** Szimpózium: A gyulladásos folyamatok és a glia szerepe az idegrendszeri betegségekben. A Magyar Élettani Társaság LXXXI. Vándorgyűlése, 2017. jún. 13-16., Debrecen (Presenter, Symposium organizer).

- ***Microglia: a grumpy maid in the brain***. BRAIN 2017, Berlin, Germany 01-04.04.2017 (Invited Symposium Lecture).

- ***Mikroglia-neuron interakciók és szisztémás gyulladás szerepe az idegrendszeri betegségekben.*** A Magyar Immunológiai Társaság és a Magyar Allergológiai és Klinikai Immunológiai Társaság 2017. évi Vezetőségi Továbbképzése: IMMUN-MEDIÁLT NEUROLÓGIAI KÓRKÉPEK. 2017. április 7. BUDAPEST (Invited Lecture).

- ***Microglial control of neuronal activity and injury: recent lessons and future perspectives.*** Achucarro - Basque Center for Neuroscience. Bilbao, Spain, 10.03.2017 (Invited Lecture).

- ***Microglia: a grumpy maid regulating neuronal activity and injury in the brain.*** Marie Curie Initial Training Network „nEUROinflammation“ Final symposium

„Neuroinflammation: A common denominator for stroke, multiple sclerosis and Alzheimer´s disease“ 23rd to 25th February 2017 Lübeck, Germany (Invited Lecture).

- ***Microglial control of neuronal activity and its role in brain disease.***University of Manchester, 01.18.2017 (Invited Lecture).

- ***Microglial control of neuronal activity and its role in brain disease.*** University of Helsinki, 01.12.2016 (Invited Lecture).

- ***MIKROGLIA-NEURON INTERAKCIÓK NEUROINFLAMMATORIKUS KÓRFOLYAMATOKBAN.*** Magyar Neurológiai Társaság Kongresszusa, Eger, 2016. október 20-22 (Invited Lecture).

- ***Central and systemic inflammatory mechanisms in cerebral ischemia.*** 2nd Central European Biomedical Congress, Krakow, Poland, 17.06.2016 (Invited Lecture).

- ***Systemic immune activation shapes stroke outcome.*** 7th Kuopio Stroke Symposium "From bench to bedside and back", Kuopio, Tietoteknia, Finland, 08.06. 2016. (Invited Lecture).

- ***Microglia-neuron interactions in stroke.*** MIGRAINE AND STROKE: COMMON AND SPECIFIC MECHANISMS, Kuopio, Tietoteknia, Finland, 07.06. 2016. (Invited Lecture).

- ***Mikroglia-neuron interakciók szerepe a neuronális károsodás kialakulásában.*** 46. Membrán - Transzport Konferencia Sümeg, 18.05.2016. (Invited Lecture).

- ***SPECT and two-photon imaging to reveal early central and peripheral inflammatory changes after stroke.*** 9th International Symposium on Neuroprotection and Neurorepair, Lepizig, Germany, 21.04. 2016. (Invited Lecture).

- ***Microglia control neuronal excitability in vivo.*** 3rd European Stroke Science Workshop, Garmisch, Germany, 21.11.2015. (Invited Lecture).

- ***The role of microglia in integrating central and systemic inflammatory responses in the brain.*** Roche, Basel, Switzerland, 30.09.2015. (Invited Lecture).

- ***Centrális és szisztémás gyulladásos folyamatok szerepe a stroke patofiziológiájában.*** Magyar Stroke Társaság XII. konferenciája, Sopron, 2015.09.15-17. (Invited Lecture).

- ***Modeling common stroke comorbidities and their impact on stroke outcome.*** BRAIN 2015, Vacouver, Canada, 27-30.06.2015. (Invited Lecture).

- ***Inflammation and brain injury – how far are we from understanding the mechanisms?*** II. FAMÉ, Pécs, 02.06.2015. (Invited Lecture).

- ***The role of neutrophils and NETs in neuroinflammation.*** Nothing but NETs: Cross-Disciplinary Symposium on Neutrophil Extracellular Traps, Biogen Idec, 31.10.2014, Cambridge, US (Invited Lecture).

- ***Brain-Gut-Microbe Interactions in CNS Injuries.*** Symposium: Systemic Responses in Stroke Injury and Recovery. International Stroke Conference, Nasville, US, 10-12.02.2015 (Invited Lecture).

- ***A centrális és szisztémás gyulladás szerepe a cerebrális ischaemia patofiziológiájában.*** Magyar Klinikai Neurogenetikai Társaság XIII. konferenciája, GrandHotel Galya, Galyatető, 2014.12.05-06 (Invited Lecture).

- ***Centrális és perifériás gyulladásos folyamatok szerepe az iszkémiás stroke után kialakuló agyi károsodásban.*** A MAGYAR ÉLETTANI TÁRSASÁG 79. VÁNDORGYŰLÉSE ÉS A MAGYAR MIKROCIRKULÁCIÓS ÉS VASZKULÁRIS BIOLÓGIAI TÁRSASÁG 2015. ÉVI KONFERENCIÁJA, Szeged, 2015.05.27-29 (Invited Lecture).

- ***Full transcriptome sequencing for unbiased discovery of common inflammatory pathways mediating brain injury.*** Bis2014, Rome, Italy, 11-12.12.2014 (Invited Lecture).