Role of microglia in cerebral blood flow modulation

Ph.D. thesis

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1. Introduction

Microglia are the main immune cells of the brain, which play an important role in various physiological and pathological processes. Microglia are involved in regulation of brain development, synaptic pruning, synaptic plasticity, learning and memory processes, and key players in the regulation of central inflammatory responses to acute and chronic brain disorders. Microglia-mediated actions are highly implicated in the regulation of vascular inflammation and blood-brain barrier (BBB) function, which occur in common brain diseases, such as stroke, acute brain injury and chronic neurodegenerative diseases. Beside inflammation, impairment in blood supply of the brain is considered as a key driver of common brain diseases. The underlying mechanisms, which contribute to the development and progression of brain disorders, are still poorly understood. Therefore, it is crucial to explore the mechanisms, which regulate cerebral blood flow (CBF) under physiological and pathological conditions. Thus, the main goal of my work is to elucidate the importance of microglia in CBF regulation under physiological conditions and in the context of hypoperfusion.

It has long been recognized that microglia interact with the cerebral vasculature. Previous findings have shown that dynamic microglial processes come into close proximity with blood vessels both in the intact and the injured brain, however the precise function of these interactions has remained vaguely defined. Most research has focused on understanding these interactions in the developing brain and in the context of different brain diseases such as stroke, Alzheimer's disease and Multiple Sclerosis. During development, microglia are involved in angiogenesis, and it has been noticed that microglial processes are closely associated with developing blood vessels and take part in the formation of vascular branching. Microglia are known to play an important role in the regulation of vascular inflammation and BBB function, actively communicate with the endothelium, affect BBB permeability and leukocyte infiltration. Besides, microglia produce various inflammatory mediators, including interleukin-1β, tumour necrosis factor α, nitric oxide, prostaglandin E2 or reactive oxygen species (ROS), some of which are known as vasoactive mediators. There is growing evidence for interactions between microglia and the cells of neurovascular unit (NVU) including astrocytes, pericytes, endothelial cells and neurons, which cells are critical regulators of CBF. However, previous research focused on the significance of these interactions during development and neuroinflammation and the potential contribution of microglia to CBF has been largely neglected to date.

Impairment in blood supply of the brain is considered to be a key contributor to development of various brain pathologies such as ischemic stroke and neurodegenerative brain diseases. Thus, understanding the mechanisms which modulate CBF under physiological conditions and the processes that contribute to the progression of brain diseases is essential to develop appropriate therapies. Here, we identify

microglia as important modulators of CBF both under physiological conditions and during hypoperfusion.

2. Objectives

1) To identify the role of microglia in activity-dependent CBF regulation.

2) To study the role of microglia in the regulation of CBF response to hypercapnia.

3) To explore the role of microglial actions in cerebrovascular adaptation to reduced cortical perfusion after common carotid artery occlusion.

4) To identify the mechanisms through which microglia mediate alterations in CBF.

3. Materials and methods

3.1. Mice

Experiments were performed on 11-17 weeks old C57BL/6J, P2Y12R$^{-/-}$, CX3CR1$^{GFP/+}$, CX3CR1$^{GFP/+}$/P2Y12$^{-/-}$, CX3CR1$^{GFP/GFP}$, CX3CR1^{tdTomato}, Thy1-GCaMP6s, MicroDREADD^{Dq}, CX3CR1$^{CGaMP5g\text{-}tdTomato}$, MicroDREADD^{Dq} x CGaMP5g-tdTomato mice, IL-1R1$^{fl/fl}$ and IL-1R1$^{fl/fl\ \Delta\ Slco1c1}$ (all on C57BL/6J background). Mice were kept in a 12h dark/light cycle environment, under controlled temperature and humidity with food and water ad libitum. All experimental procedures were in accordance with the guidelines set by the European Communities Council Directive (86/609 EEC) and the Hungarian Act of Animal Care and Experimentation (1998; XXVIII, Sect. 243/1998).

3.2. Generation of CX3CR1^{tdTomato}, MicroDREADD^{Dq}, MicroDREADD^{Dq} × CGaMP5g–tdTomato, and CX3CR1$^{CGaMP5g–tdTomato}$ mice

CX3CR1^{tdTomato}, MicroDREADD^{Dq}, CX3CR1$^{CGaMP5g–tdTomato}$, and MicroDREADD^{Dq} × CGaMP5g–tdTomato mice were generated by crossing tamoxifen (TMX)-inducible CX3CR1^{CreERT2} mice with a mouse line expressing Cre-dependent tdTomato, hM3Dq DREADD or CGaMP5g–tdTomato. To induce tdTomato, hM3Dq DREADD, or CGaMP5g–tdTomato expression in microglia, Cre recombinase activity was induced by two intraperitoneal injections of TMX (2 mg/100 µl, dissolved in corn oil; Sigma-Aldrich), 48 h apart in 3–4-week-old male mice, shortly after weaning. 4 week after

TMX induction, 95.3% of microglia expressed hM3Dq receptors, as confirmed by anti-GFP (goat anti-GFP antibody, 1:300; Rockland) and anti-P2Y12R immunostaining, to detect mCitrine and microglia, respectively. Using CX3CR1^{CreERT2} mice, microglia show constant Cre-dependent expression, while most peripheral macrophages/monocytes expressing CX3CR1 are replaced by the end of the fourth week after TMX induction, owing to their rapid turnover. Therefore, all experiments were carried out at 11 and 12 wk of age. Microglial responses were modulated in real time either by i.p. CNO (0.5 mg/kg; Bio-Techne Corp.) or intraperitoneal deschloroclozapine (1 μg/kg; HelloBio) administration, via the activation of hM3Dq DREADD.

3.3. In vivo experiments

3.3.1. In vivo two-photon imaging

CX3CR1$^{GFP/+}$, CX3CR1^{tdTomato}, CX3CR1$^{GFP/+}$ x P2Y12$^{-/-}$ or Thy1-GCaMP6s mice were anaesthesized using 1,8% isoflurane or fentanyl (0.05mg/kg) and a 3 mm diameter cranial window was opened on the left hemisphere, above the primary somatosensory or the barrel cortex without damaging the dura mater. Three weeks after cranial window surgery, microglia-vascular interactions in response to 3x CCAo or hypercapnia (ketamine-medetomidine anesthesia, i.p. 30mg/kg-0,1mg/kg), and neuronal $[Ca^{2+}]_i$ in response to whisker stimulations (in ketamine-medetomidine anaesthesia) were imaged in body-temperature controlled animals. To image microglia vascular interaction, blood vessels were labeled either with Rhodamine B-Dextran or with FITC-Dextran injected into the retro-orbital sinus or into to the tail vein. Two-photon imaging was performed with a Femto2D-DualScanhead microscope coupled with Chameleon Discovery laser (Femtonics Ltd.). Data acquisition was performed by MESc software and data analysed by MES software (Femtonics Ltd.).

3.3.2. Tissue processing and immunostaining

Under terminal (ketamine-xylazine) anaesthesia mice were transcardially perfused with 4% paraformaldehyde (PFA) and brains were dissected. Brain samples were post-fixed, cryoprotected for 24h and 25 μm thick coronal sections were cut, using a sledge microtome. Immunostaining was performed on free-floating brain sections, blocked with 5% normal donkey serum. For high resolution confocal laser scanning microscopy and electron microscopic assessments, 50 μm thick vibratome sections were washed in phosphate buffer (PB) and tris-buffered saline (TBS), followed by blocking with 1% human serum albumin (HSA). Then sections were incubated with a mixture of primary antibodies overnight at 4 °C. Next day after washing in TBS, sections were incubated in the corresponding mixtures of secondary antibodies. Incubation was followed by washing in TBS and PB, then sections were mounted on glass slides with Aqua-

Poly/Mount (Polysciences Europe). Immunofluorescence was analyzed using a Nikon Eclipse Ti-E inverted microscope.

3.3.3. Pre-embedding immunoelectron microscopy

After extensive washes in PB and TBS (pH 7.4), vibratome sections were blocked in 1% (human serum albumin) HSA. Then, they were incubated with primary antibodies dissolved in TBS for 2-3 days. After several washes, sections were incubated in blocking solution (Gel-BS) containing 0.2% cold water fish skin gelatine and 0.5 % HSA for 1 h. Next, sections were incubated with 1.4 nm gold-conjugated goat anti-rabbit Fab-fragment alone or mixed with biotinylated donkey anti-mouse antibodies diluted in Gel-BS overnight. After extensive washes, sections were treated with 2 % glutaraldehyde for 15 min to fix the gold particles into the tissue. For the combined immunogold-immunoperoxidease reactions, this was followed by an incubation in avidin–biotinylated horseradish peroxidase complex for 3 h at room temperature or overnight at 4°C. The immunoperoxidase reaction was developed using 3,3-diaminobenzidine (DAB) as chromogen. To develop the immunogold reaction, sections were incubated in silver enhancement solution for 40-60 min at RT. The sections were then treated with 0.5 % OsO_4 in PB at RT, dehydrated in ascending alcohol series and in acetonitrile, and were embedded in Durcupan. During dehydration, sections were treated with 1 % uranyl acetate in 70 % ethanol for 20 min. Ultrathin sections for conventional electron microscopic analysis were examined in a Hitachi H-7100 electron microscope and electron tomography sections were examined in FEI Tecnai Spirit G2 BioTwin TEM equipped with an Eagle 4k camera.

3.3.4. Post mortem human brain samples

Post mortem human brain tissue was obtained from one 60-years-old female, one 73-years-old male and one 27-years-old male patient without any known neurological disease as also confirmed by neuropathological examination (ETT TUKEB 31443/2011/EKU [518/PI/11]). Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki. Brains of patients who died of nonneurological diseases were removed 4–5 h after death. The internal carotid and the vertebral arteries were cannulated, and the brain was perfused first with heparin containing physiological saline, followed by a fixative solution containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid (vol/vol) in PB. The hippocampus was removed from the brain after perfusion and was postfixed overnight in the same fixative solution, except that glutaraldehyde was excluded. Blocks were dissected, and 50-μm-thick sections were prepared on a vibratome (VT1200S; Leica).

3.3.5. Laser Speckle Contrast Imaging (LSCI)

CBF was measured by a PeriCam PSI High Resolution Laser Speckle Contrast Imaging (LSCI) system. Before CBF measurements, the head of the mouse was secured in a stereotaxic head holder, and after a midline incision, the skull was exposed by retracting the scalp. Imaging was performed through the intact skull bone. CCA occlusion experiments were performed under ketamine-xylazine (i.p. 100mg/kg - 10mg/kg) anaesthesia. The whisker stimulation protocol and the hypercapnic challenge was performed under mild ketamine-medetomidine (i.p. 30mg/kg – 0.1mg/kg) sedation.

3.3.6. Selective elimination of microglia from the brain

C57BL/6J mice were fed a chow diet containing the CSF1R inhibitor, PLX5622 (Plexxikon Inc., 1200 mg PLX5622 in 1 kg chow) to eliminate microglia from the brain, or with control diet for 3 weeks.

3.3.7. Cisterna magna injection for drug delivery into the brain and intraperitoneal drug administration

To block P2Y12 receptor mediated microglial actions, a P2Y12R antagonist, PSB0739 (dissolved in 0.9% saline, 40 mg/kg in 5 µl volume) was injected into the cisterna magna 35 min prior imaging, while vehicle (0.9% saline) injection was used as control. Cisterna magna injections were done under 1-1.5% isoflurane anaesthesia. L-NAME, a non-selective Nitric Oxide Synthase (NOS) inhibitor was injected intraperitoneally (30 mg/kg dissolved in 0,9% saline) 5 minutes before imaging.

3.3.8. SPECT and PET imaging

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) studies were carried out on mice anaesthetized with 2% isoflurane. SPECT measurements were performed using the [^{99m}Tc]-HMPAO ligand. The acquisition started 3 minutes after the i.v. injection of the radiotracer via the tail vein (injected activity: 99.22 ± 9.33 MBq). The measurements were performed on a NanoSPECT/CT PLUS device (Mediso Ltd, Hungary) equipped with multi-pinhole mouse collimators. After SPECT acquisition, [^{18}F]-FDG (2-deoxy-2-(18F)fluoro-D-glucose) PET measurements were performed. PET acquisition started 20 minutes after i.v. [^{18}F]-FDG injection with an acquisition time of 10 minutes using a microPET P4. After reconstruction, manual coregistration and atlas-based region of interest (ROI) measurements were done using VivoQuant software (InviCRO, USA) in the cerebellum, cerebral cortex and the whole brain.

3.3.9. Whisker stimulation protocol

Whisker stimulation was performed manually and electromechanically. For manual stimulation an earpick was used (at 4-5 Hz frequency) according to the following

protocol: left whiskers were stimulated for 30 sec, repeated 6 times, 60 sec apart. During electromechanically controlled stimulation (5 Hz) whiskers were stimulated for 15 sec, repeated 10 times with 40 sec intervals. CBF measurements were carried out under ketamine-medetomidine sedation (30 mg/kg - 0.1 mg/kg dissolved in 0.9% saline, i.p.).

3.3.10. Functional ultrasound (fUS)

The acquisition was done with a 15 MHz probe of 128 elements connected to a prototype ultrafast research ultrasound scanner (Iconeus One, Iconeus France, Paris). Recordings were performed through the skull while the animal was anaesthetised with ketamine-medetomidine (i.p. 30mg/kg - 0.1mg/kg). The head of the animal was shaven and fixed into a stereotactic frame. The probe was positioned using a built-in software based registration to the 3D Allen Brain Atlas.

3.3.11. In vivo electrophysiology

Briefly, custom-built microdrives with eight nichrome tetrodes (diameter, 12.7 μm) and a 50-μm core optic fiber were implanted into the right barrel cortex AP: -1.4; ML: 3.0 DV 0.75−2.0 mm. The custom-built microdrives were implanted under deep anaesthesia using an intraperitoneal injection of ketamine - xylazine (125 mg/kg - 25 mg/kg in 0.9% NaCl). The stereotaxical surgery was followed by a 3 day-long resting period. During electrophysiological recordings animals were anesthetized using an intraperitoneal injection of a ketamine – medetomidine (3mg/kg – 0.1 mg/kg). Data analysis was performed in Matlab R2016a.

3.3.12. Induction of hypercapnia in vivo

Under mild ketamine-medetomidine (i.p. 30 mg/kg - 0.1 mg/kg) sedation, baseline cortical blood flow was recorded with LSCI for 5 minutes, then hypercapnia was induced by inhalation of a 10% CO_2-containing air mixture (21.1% O_2 and N_2 ad 100%) for 2 minutes under normoxic conditions. In a group of control and microglia-depleted mice, before the hypercapnic challenge, 0.01 μg/g atipamezole was administered i.p. to withdraw α-2-agonistic effects of medetomidine.

3.3.13. Blood gas analysis

Under ketamine-medetomidine anaesthesia, the left femoral artery was exposed and cannulated for arterial puncture. Arterial blood was sampled to glass capillaries before and after 2 min hypercapnic challenge and samples were measured with a blood gas analyzer (ABL90 Flex Plus, Radiometer Medical ApS, Denmark) to determine arterial blood gas tensions (pO_2, pCO_2) and pH.

3.3.14. Acute slice hypercapnia experiment and cyclic guanosine monophosphate (cGMP) immunolabeling

Mice were deeply anesthetized with isoflurane and decapitated, the brains were removed and cut on vibratome. Slices were preincubated for 20 minutes with 1 ml of modified artificial cerebrospinal fluid (mACSF). After preincubation in mACSF or mACSF+PSB0739, slices were gradually subjected to hypercapnia by elevating the CO_2 level from 5% to 14.6% with bubbling. After 15 minutes hypercapnia, the slices were immediately fixed with ice-cold 4% paraformaldehyde for 48 h at 4°C. After washing with 0.1 M PB, slice were embedded to 4% agar and 50 μm thick vibratome sections were cut. Then sections were incubated in primary antibody mixture diluted in PBS for 48h at 4°C. After subsequent washes in PBS, sections were incubated in a mixture of corresponding secondary antibodies diluted in PBS. Sections were mounted onto glass slides. Fluorescent images were acquired using a Nikon Eclipse Ti-E inverted microscope.

3.3.15. Simultaneous measurement of CBF and brain pH during hypercapnia

Electrophysiological variables (DC potential, brain pH) and local CBF (by lased Doppler) were simulatenously monitored after craniotomy using ion-sensitive microelectrodes connected to a custom-made dual-channel high input impedance electrometer via Ag/AgCl leads and associated filter modules. In each experiment, a pH-sensitive microelectrode was lowered into the cortex with a micromanipulator, together with another glass capillary microelectrode (tip diameter = 20 μm) filled with saline to serve as reference. The reference electrode acquired slow cortical or DC potential. An Ag/AgCl electrode was implanted under the skin of the animal's neck to be used as common ground. Surgical preparations were done under 1.5–2% isoflurane, while pH and LDF measurements were performed under medetomidine anesthesia (initiation: i.p. 0.5 mg/kg, repeated 5 min later for maintenance) in a Faraday cage. The experiments were done in the University of Szeged, Cerebral Blood Flow and Metabolism Research Group.

3.3.16. Repeated, transient CCA occlusion

A transient, repeated unilateral common carotid artery (CCA) occlusion model was developed to induce hypoperfusion without causing ischemia or cellular injury to the brain. The CCA was temporarily pulled away with a silk suture for 5 min, followed by a 5 min-long reperfusion period. The protocol consisted of repeating these steps three times (3x CCAo) on anesthetised (ketamine-xylazine, i.p. 100mg/kg - 10mg/kg dissolved in 0.9% saline) mice. During CBF measurements, the core temperature of mice was maintained at 37 ± 0.5°C using a homeothermic blanket (Harvard Apparatus).

3.3.17. Elimination of perivascular macrophages

Perivascular macrophages were depleted by a single dose of clodronate-containing liposomes (70µg/mouse in 10µl volume) injected into the left ventricle (ICV) as described earlier. Three days later, at maximal efficacy of depletion, laser speckle contrast imaging was carried out.

3.3.18. Focal cerebral ischemia

Anaesthesia was induced by inhalation of 4% isoflurane and was maintained at 1.75%. Body temperature was monitored throughout surgery and maintained at 37 °C ± 0.5 °C using a heating blanket (Harvard Apparatus). A laser Doppler blood flow monitor was used to monitor cerebral blood flow (Moor Instruments Ltd.). A midline incision was made on the ventral surface of the neck and the right common carotid artery isolated and ligated. The internal carotid artery and the pterygopalatine artery were temporarily ligated. A 6-0 monofilament was introduced into the internal carotid artery via an incision in the common carotid artery. The filament was advanced approximately 10 mm distal to the carotid bifurcation, beyond the origin of the middle cerebral artery. After 45 min of occlusion the filament was withdrawn back into the common carotid artery to allow reperfusion to take place.

3.4. *In vitro* experiments

3.4.1. Primary endothelial cells

Primary endothelial cultures were prepared from 6-8 weeks old C57BL/6J mouse brains. In brief, mouse forebrains were collected to PBS and the meninges were removed using sterile chromatography paper. The tissue was cut into small pieces by scalpels and was enzymatically digested in a mixture of Collagenase II and deoxyribonuclease I (DNase I) in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12) for 55 min at 37C°. Using a 20% BSA gradient (1000x g, 20 min, three times), microvessels were separated from the myelin. The collected microvessels were further digested using a mixture of Collagenase/Dispase and DNase I for 35 min at 37C°. Digested cerebral microvessels were washed three times with DMEM-F12, then seeded to Collagen type I coated plates. During the first four days, puromycin selection was applied in the primary medium for seeding, 10% for cultivation, 1ng/ml basic fibroblast growth factor (bFGF), 100µg/ml heparin, 100x insulin-transferrin-selenium (ITS), 4µg/ml puromycin in DMEM-F12 to selectively eliminate P-Glycoprotein (P-gp) non-expressing cells. After reaching confluency in 5-6 days, the cells were passaged to Collagen type IV and fibronectin coated 48-well plates at a cell density of 15.000 cells/well, and used for *in vitro* hypoxia or hypercapnia experiments in P1.

3.4.2. Primary astroglia and microglia cells

Primary cultures of astroglial cells were prepared from neonatal mouse brains. Meninges were removed from P0-P2 whole brains and tissues were chopped. The tissue pieces were digested with 0.05% w/v trypsin and 0.5mg/ml DNAse I in phosphate-buffered saline for 10min, at RT. Cells were then plated onto poly-L-lysine coated plastic surfaces at a cell density of 3–4 × 10^5 cell/cm^2. The cultures were grown in Minimal Essential Medium supplemented with 10% fetal bovine serum, 4 mM glutamine, and 40 μg/ml gentamycin. Primary microglia cells were isolated from astroglia/microglia mixed cultures derived from the whole brains of C57BL/6J newborn mouse pups. Microglia isolation was performed between days 21 and 28 of culture maintenance, by mild trypsinization. For the *in vitro* hypoxia and hypercapnia experiments the isolated cells were seeded in a 1,5 × 10^5 cell/cm^2 density into poly-L-lysine coated 48 well plates and used within 48hrs.

3.4.3. In vitro hypoxia and hypercapnia

Cytation 5 Cell Imaging Multi-Mode Reader (BioTek Instruments) equipped with $O2/CO_2$ gas controllers was used to maintain 1% O2/5% CO2/94% N2 (Hypoxia) or 15% CO2/85% air (Hypercapnia) levels at 37ºC. Endothelial or astroglial cells grown in 48 well plates to confluency, or microglia were placed into the reading chamber of the instrument for 5 (Hypercapnia) or 10 minutes (Hypoxia), after taking the lids off. To follow the build-up of hypoxia at the cellular level some cultures were loaded with 5uM Image-iT™ Green Hypoxia Reagent for 30min at 37ºC. The Hypoxia Reagent begins to fluoresce when atmospheric oxygen levels drop below 5%. Fluorescent images taken with Cytation5 (10x magnification) at 0/10minutes were analyzed with FIJI software (v1.53, NIH). Changes in medium pH during hypercapnia were measured by Phenol Red absorbance at 415 and 560 nm using the Cytation 5 Multi-Mode Reader. Changes in the intracellular pH during hypercapnia were determined by fluorescence intensity readings of pHrodo Green AM labeled glial cells with Cytation 5 Multi-Mode Reader (BioTek Instruments).

3.4.4. Primary microglia cell cultures and calcium imaging

Primary microglia cells were isolated from astroglia/microglia mixed derived from the whole brains of MicroDREADD^{Dq} newborn mouse pups. Half of the mixed cultures were treated repeatedly with 2 μM 4-hydroxy TMX for 1 week before microglia isolation, which was performed between days 21 and 28 of culture maintenance, by mild trypsinization. The cells were seeded onto poly-L-lysine–coated glass coverslips at cell density of 40,000 cell/cm2 and used for imaging within 2–3 days. Phase-contrast time-lapse images to assess microglia process motility were captured on a Zeiss Axiovert 200M microscope at 20× magnification (20× Plan-Neofluar Ph2 objective), with a frame rate of 0.2 fps. For calcium imaging, the cells were loaded with 1 μM

Oregon Green 488 BAPTA-1 AM (Invitrogen) or 5 μM Calbryte 590 AM (AAT Bioquest) dyes in the presence of 2,000× Pluronic F-127 (Invitrogen) or 100× PowerLoad Concentrate for 30 min at room temperature (RT). During imaging, the cells were perfused with ACSF (125 mM NaCl, 2.5 mM KCl, 8 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM Hepes acid, and 10 mM glucose) with a flow rate of 1 ml/min at RT. In some experiments, no perfusion was used. The cells were treated by DREADD agonists CNO (1 μM or 100 nM; Bio-Techne Corp) or C21 (1 μM; HelloBio). In experiments related to Fig. S2, the cultures were repeatedly treated with 1 μM C21 for 1 min, 10 min apart, followed by a single application of 10 μM ATP. Calcium imaging was performed either on a Nikon A1R confocal laser-scanning system built on a Ti-E inverted microscope, at 60× magnification (60× Plan Apo VC WI objective, NA = 1.2), with a frame rate of 20 fps, or on a Nikon Ti2 microscope equipped with a CoolLed pE-4000 illumination system and a Hamamatsu ORCA-Flash 4.0 camera, at 40× magnification (40× Apo WI λS objective, NA = 1.25), with a frame rate of 2 fps. Signal extraction from the time-lapse series was computed in Fiji (ImageJ; National Institutes of Health [NIH]), and the data were analyzed on Clampfit (pClamp10 suite; Molecular Devices). Statistics were calculated with GraphPad Prism 8.4.3.

3.4.5. Quantification of nucleotides and nucleoside

Released concentrations of adenine nucleotides (adenosine triphosphate - ATP, adenosine diphosphate - ADP, adenosine monophosphate - AMP) and adenosine (Ado) from culture media and tissue homogenates were determined using high-performance liquid chromatography (HPLC) by Shimadzu LC-20 AD Analytical System using UV (Agilent 1100 VW set at 253 nm) detection. Concentrations of the homogenates were calculated by a two-point calibration curve using internal standard method. The data (n=4 or 5 in each group) are expressed as pmol per mg protein or nmol per mg.

3.5. Statistical Analysis

Data were assessed for normal distribution using the D'Agostino-Pearson normality test or the Shapiro-Wilk W-test in order to determine parametric or non-parametric analysis. For comparing two or more groups with normal distribution the unpaired t-test with Welch's correction either one-way ANOVA with Dunett's multiple comparison test or two-way ANOVA with Tukey's or Sidak's multiple comparison test was used. For unevenly distributed data, the Mann-Whitney test either one-way ANOVA with Dunett's multiple comparison test or two-way ANOVA with Tukey's or Sidak's multiple comparison test was used.

4. Results

4.1. Microglia establish direct, purinergic contacts with cells of the neurovascular unit that shape CBF at all levels of cerebrovascular tree

- *In vivo* two-photon imaging and confocal laser scanning microscopy (CLSM) revealed that microglia cover arterial bifurcations at the level of first, second and third order vessels and identified contacting microglial processes at all levels of the cerebrovascular tree.
- CLSM imaging demonstrated that processes of parenchymal microglia extend beyond glial fibrillary acidic protein (GFAP)-positive/ aquaporine-4 (AQP4)-posotive astrocytic endfeet at the level of penetrating arterioles directly contacting smooth muscle actin (SMA)-positive VSMCs and endothelial cells in both large vessels and microvessels.
- Our CLSM and 3D electron tomography analysis revealed that contacting P2Y12 receptor (P2Y12R)-positive microglial processes are close apposition with endothelial mitochondria while immunogold particles were enriched at the interface. Besides, unbiased immunfluorescent analysis showed that the immunfluorescence intensity of TOM20 (mitochondrial marker) is about 214% higher in endothelial cells at microglial contact sites than at the non-contact sites.
- CLSM and electron microscopy imaging showed that individual microglia cells contact multiple microvessels and nearby neurons simultaneously in the brain. Microglial processes not only directly contact all cells of the NVU along the cerebrovascular tree, which cells are highly implicated in modulation of cerebral blood flow (CBF), but simultaneously contact neurons and vascular structures.
- CLSM and electron microscopy imaging showed similar contacts in the human cerebral cortex as it was seen in the mouse brain: P2Y12R-positive microglial processes directly contact both perivascular astrocytic endfeet and endothelial monolayer of small arterioles and capillaries.

4.2. Microglia modulate neurovascular coupling via P2Y12R-mediated processes

- *In vivo* HMPAO-SPECT and FDG-PET measurements revealed that the elimination of microglia from the brain by using CSF1 receptor inhibitor, PLX5622 do not influence vascular architecture and cerebral metabolism. We did not find significant difference between control and microglia-depleted mice regarding [^{99m}Tc]-HMPAO and [^{18}F]-FDG uptake in any brain areas.
- *In vivo* Laser Speckle Contrast Imaging (LSCI) measurements demonstrated that in the absence of microglia NVC is impaired, as evidenced by that CBF responses to whisker stimulations are significantly decreased in depleted-mice compared to that seen in controls. *In vivo* functional ultrasound imaging confirmed that in microglia-depleted mice CBV increases in response to whisker stimulation in the contralateral barrel cortex are significantly smaller compared to that seen in control mice.

- *In vivo* LSCI revealed that in P2Y12R KO mice CBF response to whisker stimulation is significantly decreased compared to control mice. Selective, acute blockade of microglial P2Y12R using PSB0739 injected to cisterna magna leads to markedly smaller CBF response to whisker stimulation compared to control mice.
- Using *in vivo* LSCI we studied the relationship between microglia depletion and nitric-oxide synthase blockade by L-NAME (N omega-Nitro-L-arginine methyl ester hydrochloride). Both microglia depletion and L-NAME markedly reduced the CBF response to whisker stimulation compared to control mice, whereas L-NAME administration into microglia-depleted mice resulted in additional CBF decrease compared to microglia-depleted or L-NAME injected mice.

4.3. Increased neuronal activity in the barrel cortex induced by whisker stimulation does not explain altered CBF responses after microglia manipulation in vivo

- *In vivo* measurements using chronically implanted tetrode electrodes revealed that baseline firing rates of barrel cortex neurons are markedly increased in both microglia-depleted and P2Y12R KO mice compared to control ones. We did not find significant differences in the extent of stimulus-evoked neuronal responses between experimental groups.
- *In vivo* two-photon imaging demonstrated that somatosensory stimulus-induced increases in the neuronal GCaMP6s signal in Thy1-GCaMP6s mice do not show significant differences between control and microglia-depleted mice. While the absence (PLX5622 depleted) or dysfunction (P2Y12R KO) of microglia may shift baseline neuronal activity, stimulus-evoked neuronal responses do not explain the marked differences in CBF changes observed after microglia manipulation.

4.4. Real-time chemogenetic activation of microglia leads to impaired neurovascular coupling response

- *In vitro* administration of CNO or C21 induced rapid increases in intracellular calcium levels in microglia cells derived from MicroDREADD^{Dq} mice, which was completely absent in TMX-untreated cells.
- *In vitro* measurements demonstrated that microglia show reduced calcium responses to repeated C21 stimulations and decreased responsiveness to single administration of ATP after repeated chemogenetic stimulations.
- Both *in vivo* and *in vitro* measurements showed that single chemogenetic activation led to the blockade of microglial process motility within a few minutes and altered microglial morphology.

- *In vivo* two-photon imaging study revealed that microglial processes interacting with arterioles and microvessels in the cerebral cortex showed dynamic calcium fluctuations in MicroDREADD^{Dq} × CGaMP5g–tdTomato mice.
- *In vivo* LSCI measurements showed that that chemogenetic activation of microglia leads to a similar degree of CBF reduction to whisker stimulation as seen after microglia depletion.

4.5. Microglia modulate hypercapnia-induced vasodilation via P2Y12R signaling

- *In vivo* two-photon imaging revealed that a population of dynamic microglial processes readily changed their morphology both at arterioles and microvessels in response to vasodilation induced by hypercapnia. Besides, *in vivo* two-photon imaging showed that around arterioles, microglial processes dynamically contact SR101-labeled perivascular astrocytic endfeet and the number of contacting phylopodia at the end of microglial processes markedly increased in response to hypercapnic challenge. *In vivo* two-photon imaging study demonstrated that in CX3CR1$^{CGaMP5g\text{-}tdTomato}$ mice perivascular microglia respond rapidly (within 1-2 min) to hypercapnia with calcium pulses, which was observed in both large processes and phylopodia.
- *In vivo* two-photon imaging revealed that hypercapnia-induced vasodilation is significantly impaired in meningeal and penetrating arteries in microglia-depleted mice, which paralleled markedly decreased CBF response to hypercapnia as measured by *in vivo* LSCI.
- *In vivo* LSCI showed that in P2Y12R KO mice hypercapnia-induced CBF response markedly decreased compared to controls. Besides, *in vivo* two-photon microscopy measurements demonstrated that hypercapnia-induced vasodilation is impaired in the absence of microglial P2Y12R (using CX3CR1$^{GFP/+}$ x P2Y12R KO mice) compared to control (CX3CR1$^{GFP/+}$) ones and formation of perivascular phylopodia was also significantly reduced after hypercapnia in P2Y12R KO mice compared to that seen in controls.
- *In vivo* electrophysiology studies revealed that there are no significant differences between control, microglia-depleted and P2Y12R KO mice in neuronal activity during hypercapnia.
- *Ex vivo* hypercapnia-induced cGMP increases were markedly inhibited by the blockade of microglial P2Y12R with PSB0739 in neocortical acute slices.

4.6. Hypercapnia and hypoxia induce rapid stimulus-dependent release of purinergic metabolites by NVU cells and microglia in the brain

- *In vivo* measurements with pH-selective electrode showed that baseline brain pH is significantly lower in the absence of microglia but the relative amplitude of the hypercapnia-induced negative pH shift was not different in control vs microglia-depleted mice.
- Our *in vitro* experiments revealed that hypercapnic challenge decreased both extracellular and intracellular pH. Besides, hypercapnic challenge triggered rapid ATP and ADP release from cultured endothelial cells, whereas astrocytes produced mainly ATP and adenosine, while cultured microglia released ADP and adenosine in response to hypercapnia. *In vivo* hypercapnia-induced adenosine levels were attenuated in the absence of microglia.
- *In vitro* hypoxic challenge leads to production of ATP and AMP by cultured endothelial cells, while astrocytes and microglia released mainly ADP.

4.7. Microglia sense and modulate cerebral blood flow changes during hypoperfusion induced by repeated common carotid artery occlusion (CCAo)

- *In vivo* two-photon imaging showed that microglial processes rapidly respond to CBF reduction, as shown by increased process motility of blood vessel-associated microglia immediately after CCAo.
- *In vivo* LSCI measurements revealed that adaptation to decreased cortical perfusion after CCAo is significantly impaired in microglia-depleted mice compared to control ones. Impaired CBF recovery was also evident in both hemispheres in the absence of microglia.
- *In vivo* LSCI study demonstrated that perivascular macrophages (PVMs) are involved in modulation of CBF during repeated CCAo. We did not find significant difference in CBF changes after CCAo between control and PVM-depleted mice.
- *In vivo* LSCI measurements showed that the genetic deletion or acute pharmacological blockade of microglial P2Y12R using PSB0739 injected into the cisterna magna leads to altered CBF responses after repeated CCAo. CBF recovery was significantly impaired after both genetic and pharmacological P2Y12R blockade in both ipsilateral and contralateral hemispheres, similarly to that seen in microglia-depleted mice.

4.8. Deletion of brain endothelial IL-1R1 improves early cortical perfusion deficits after cerebral ischemia

- We have also studied the inflammatory mechanisms through which microglia may interfere with vascular responses. *In vivo* LSCI measurements revealed that interleukin-1 signaling mediates cerebral perfusion changes following cerebral ischemia through endothelial IL-1R1. We found that deletion of endothelial

interleukin-1 receptor 1 (IL-1R1) leads to a significantly smaller perfusion deficit in the ipsilateral hemisphere at the MCA area compared to controls.

5. Conclusions

Microglia, the resident immune cells of the central nervous system (CNS), play an important role in regulation of central inflammatory processes and BBB functions. It has been evidenced, that microglial dysfunction contributes to development of common brain diseases which are associated with impaired cerebral blood flow (CBF) and neurovascular coupling that often precede symptom onset in neurodegenerative diseases. Microglia are highly active in the healthy brain, motile microglial processes dynamically interact with the cells of the CNS and the cerebral vasculature and rapidly respond to changes in their enviroment mediated mainly by purinergic metabolits. Most previous research has focused on investigating microglia-neuron contacts, while the function of microglia-vascular interactions remained vaguely defined. We have discovered that individual microglial cells contact neurons and blood vessels simultaneously in the healthy brain, thus ideally positioned to sense and influence neurovascular responses.

My studies have identified microglia as a novel cell type modulating cerebral blood flow, which involves complex purinergic mechanisms. Using three different experimental models, we demonstrate that the presence of functional microglia is essential to maintain optimal CBF responses to physiological neuronal activity (neurovascular coupling), hypercapnia and during cerebrovascular adaptation to reduced cortical perfusion after common carotid artery occlusion (CCAo). These actions are dependent on microglial P2Y12R signaling, clearly discriminating microglial responses from those mediated by perivascular macrophages or other brain macrophages.

Our anatomical data show that microglia dynamically contact different levels of the vascular tree in vivo and form direct purinergic contacts with the cells of the neurovascular unit (NVU) including endothelial cells, astrocytes, pericytes and VSMCs in both the mouse and the human brain, which shape CBF. We found that through these interactions microglia modulate CBF during neurovascular coupling, hypercapnia-induced vasodilation and cerebrovascular adaptation to hypoperfusion. Our results revealed that microglia respond to purinergic signaling in the NVU and to produce adenosine and likely other vasoactive mediators. We demonstrate that the release of purinergic metabolites in response to different vascular stimuli is dependent on the cell type and also on the stimulus used. Microglia may be able to sense different perfusion-related changes in the NVU and interact with different cell types in a compartment-specific manner. Furthermore, we have identified the cerebrovascular endothelium as a

major target for IL-1 actions after ischaemic stroke. Brain endothelial IL-1 actions have a robust and early impact on cortical perfusion after acute brain injury. Thus, microglia-dependent effects and interleukin-1 (IL-1) actions on the cerebrovascular endothelium emerge as key events that may shape cerebral perfusion and contribute to the inflammatory conditions which may influence outcome in diverse cerebrovascular pathologies, such as cortical hypoperfusion or stroke.

Our findings demonstrate that microglia should be considered as an important modulatory cell type involved in physiological and pathological alterations of cerebral blood flow and understanding their actions may facilitate the discovery of novel treatment opportunities in common neurological disorders.

6. Bibliography of the candidate's publications

Publications related to this thesis:

Csaszar E., N. Lenart, C. Cserep, Z. Kornyei, R. Fekete, B. Posfai, D. Balazsfi, B. Hangya, A. D. Schwarcz, E. Szabadits, D. Szollosi, K. Szigeti, D. Mathe, B. L. West, K. Sviatko, A. R. Bras, J. C. Mariani, A. Kliewer, Z. Lenkei, L. Hricisak, Z. Benyo, M. Baranyi, B. Sperlagh, A. Menyhart, E. Farkas, and A. Denes. (2022) Microglia modulate blood flow, neurovascular coupling, and hypoperfusion via purinergic actions. **Journal of Experimental Medicine.** 219 (3): e20211071. doi: https://doi.org/10.1084/jem.20211071 **IF: 14,03**

Wong R., Lenart N., Hill L., Toms L., Coutts G., Martinecz B., **Csaszar E.**, Nyiri G., Papaemmanouil A., Waisman A., Muller W., Schwaninger M., Rothwell N., Francis S., Pinteaux E., Denes A., Allan S. M. (2019) Interleukin-1 mediates ischaemic brain injury via distinct actions on endothelial cells and cholinergic neurons. **Brain, Behavior, and Immunity.** 76:126-138. doi: https://doi.org/10.1016/j.bbi.2018.11.012 **IF: 7,21**

Other publications:

Varga D. P., Menyhart A., Posfai B., **Csaszar E.**, Lenart N., Cserep C., Orsolits B., Martinecz B., Szlepak T., Bari F., Farkas E., Denes A. (2020) Microglia alter the threshold of spreading depolarization and related potassium uptake in the mouse brain. **J Cereb Blood Flow Metab.** 40 (1_suppl):S67-S80. doi: 10.1177/0271678X19900097 **IF: 5,68**

Orsini F., Fumagalli S., **Csaszar E.**, Toth K., De Blasio D., Zangari R., Lenart N., Denes A., De Simoni M. G. (2018) Mannose-Binding Lectin Drives Platelet Inflammatory Phenotype and Vascular Damage After Cerebral Ischemia in Mice via IL (Interleukin)-1alpha. **Arterioscler Thromb Vasc Biol.** 38 (11):2678-2690. doi: https://doi.org/10.1161/ATVBAHA.118.311058 **IF: 8,31**

Szalay G., Martinecz B., Lenart N., Kornyei Z., Orsolits B., Judak L., **Csaszar E.**, Fekete R., West B. L., Katona G., Rozsa B., Denes A. (2016) Microglia protect against brain injury and their selective elimination dysregulates neuronal network activity after stroke. **Nature Commun.** 7:11499. doi: https://doi.org/10.1038/ncomms11499 **IF: 14,91**