Role of microglia in neuronal health, inflammation and brain injury

Doctor of Science Theses

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Introduction

Brain disorders affect millions of people worlwide and emerge as one of the leading causes of morbidity and mortality. Despite all medical advances made so far, treatment opportunities for most brain dieases remain limited. In line with this, accumulating data indicate that targeting the mechanisms of neuronal injury alone may turn out to be ineffective. This is also indicated by the failure of numerous clinical trials in stroke, Alzheimer's disease (AD), Parkinson's disease (PD) and in other conditions. Importantly, recent research has highlighted the prominent role of brain-immune interactions and inflammatory processes in the pathophysiology of most common brain diseases, which had been largely neglected in previous studies. Neurons in the central nervous system (CNS) not only require precisely regulated blood supply and support from glial cells, but the mechanisms of neuronal injury (both acute and chronic) are also profoundly influenced by complex neuro-vascular-glial interactions, which are highly sensitive to inflammation. Of note, the overall burden of inflammation increases by ageing alonsgside with the development of immune dysfunction, while common chronic disorders represent key risk factors for brain diseases. Therefore, the development of novel diagnostic and therapeutic tools is strongly needed, which relies on better understanding of complex biological processes and mechanisms of disease pathophysiology through rapidly evolving multidisciplinary research technologies.

Microglia are the main immune cells of the CNS and are key regulators of central inflammatory processes. They are derived from the extra-embryonic yolk sac, colonize the developing CNS in the first trimester and comprise a self-renewing population throughout life. Tissue disturbance, injury, inflammatory stimulus or infection not only results in rapid changes microglial reactivity, transcriptional fingerprins or inflammatory responses, but altered microglial phenotypes are also maintained for prolonged periods. Therefore, alterations in this long-lived cell population are expected to impact on CNS function from early CNS development into old age. Importantly, recent data indicate that beyond their immune functions, microglia also play instrumental housekeeping roles during brain development and in adulthood. In line with this, altered activity of microglia and microglial dysfunction emerge as a major contributors to almost all brain diseases, including neurodevelopmental disorders, acute brain injury, neuronal hyperactivity, psychiatric disorders, and age-related conditions, such as stroke, AD, PD and other forms of neurodegeneration.

Microglia are considered the most versatile inhabitants in the mammalian CNS with a multitude of broad functions, dynamically changing at cellular, subcellular and molecular levels to adapt to their ever-changing surroundings. However, the molecular mechanisms

through which microglia communicate with other cells in the brain, have remained vaguely defined to date. Microglia are known to express numerous receptors to communicate with neurons and other cells and respond to a broad range of neurontransmitters, purinergic mediators, or metabolites. In line with this, numerous microglia-derived substances, with particular respect to inflammatory mediators, have long been known to markedly influence the activity of neurons, glial cells or cells of the neurovascular unit in the CNS. For example, interleukin-1 (IL-1), a key proinflammatory cytokine produced by microglia and other cells has been identified as an important contributor to neuronal injury, glial activation or vascular inflammation. In addition, IL-1 together with other inflammatory mediators have been identified as major drivers of chronic vascular diseases, such as hyperlipidaemia, atherosclerosis, diabetes, hypertension, or obesity, which are important risk factors for stroke, neurodegeneraion and other brain disorders. Systemic inflammation is also known to be associated with markeldy altered microglial phenotypes and responses. It is likely that experimental studies of brain inflammation and injury underestimate the impact of inflammatory mechanisms and in particular, the effect of microglial dysfunction on neuronal responses and fate considering the heterogeneity of the human population and the broad range of age-related conditions and associated comorbidities. Thus, understanding how microglial mechanisms contribute to different disease states is of utmost importance.

In addition to their complex roles in disease, numerous physiological roles for microglia have also been recently indentified, which indicates the importance of studying microglial interactions with other cells under physiological conditions. At present, the molecular anatomy and operation of these complex intercellular interactions at different temporal and spatial scales is largely unclear, which greatly limits our understanding of how these cell-cell interactions change in different forms of CNS disorders. In fact, appropriate tools to study microglial actions by *in vivo* imaging, transcriptomics, proteomics, immunophenotyping, genetics and other cross-disciplinary research approaches have become available in the last two decades, while advanced tools to selectively manipulate microglia have emerged only very recently. Beyond recent advances in microglia research, the integration of information from studies using different approaches has not yet been sufficient to allow translation of experimental data into clinical benefit concerning the role of inflammatory mechanisms in common brain disorders. Thus, understanding microglial intercellular interactions and their role in health and disease appears to be increasingly important to identify specific targets for intervention in inflammation-related CNS pathologies.

Summary of research and specific aims

Since the discovery of microglia, these cells have been linked with pathophysiological changes in different disease states, which has also been supported by later observations concerning inflammation-mediated injury in the CNS. Because microglia are an important souce of inflammatory mediators, earlier studies linking changes in microglial phenotypes with diverse neuropathologies have strenghtened the view of their detrimental roles. However, in the absence of appropriate research tools to selectively target microglia, their contribution to CNS injury has remained highly controversial. Therefore, the key aim for my research in the last 20 years was to understand the mechanisms through which inflammation contributes to pathological processes in the brain, by using the constantly improving methodologies in neuroscience, immunology, genetics, molecular biology, imaging and other related fields of science. Our research could benefit from some of the latest research tools to manipulate inflammatory pathways and microglial actions in different models of brain injury. For appropriate translation of research findings into possible clinical benefit, we have devoted increasing attention in recent years to also study microglia in the human brain.

First, I will discuss some of the mechanisms through which microglia may contribute to, or alter inflammation-related brain injury (**Chapter 1**). Then, I will elaborate upon the effects of systemic inflammation on microglial responses and brain injury (**Chapter 2**). Finally, I will detail our recent findings concerning the molecular mechanisms that govern fine-tuned microglia-neuron interactions, which appear to be critical contributors to neuronal health and neuronal injury (**Chapter 3**). I will intentionally devote slightly more room to discuss Chapter 3, as this part contains our latest results with some of the most advanced tools and approaches, but felt important to also include some related earlier studies to put these recent discoveries into a broader context.

- *Chapter 1*. **Inflammation and microglial actions modulate injury in the brain**

- *Chapter 2*. **Systemic inflammation promotes CNS inflammation, alters microglial function and augments brain injury**

- *Chapter 3*. **The role of compartment-specific microglia-neuron interactions in shaping neuronal activity and neuronal injury**

Summary of methods

We have established and developed several state-of-the-art approaches aiming to investigate the mechanisms of inflammatory processes and microglia-mediated effects in the brain. To visualize these processes at high spatial and temporal resolution in real time, we used *in vivo two-photon imaging* for monitoring neuronal and microglial calcium responses or microglial interactions with neurons, astrocytes and blood vessels under physiological conditions and after brain injury. Visualization of cells and their activity changes was performed by cell-specific labelling, biosensors, AAV-mediated transfer of genetically encoded calcium indicators, mouse lines expressing neuronal- or microglial reporter proteins or calcium indicators, and by using in utero electroporation to express reporter proteins in neurons or in neuronal mitochondria.

We have used and developed different pharmacological and genetic approaches for *targeted manipulation of microglia* including blockade of microglial P2Y12 receptors by PSB-0739 injected into the cisterna magna; microglia depletion by CSF1R blockade; or deletion of key microglial receptors such as CX3CR1 or P2Y12; to alter microglial function and their interactions with other cells. We also developed a novel mouse model allowing selective chemogenetic targeting of microglia (MicroDREADD^{Dq} mice).

The *effects of microglia manipulation* on neuronal responses and fate have been studied under physiological conditions and after different forms of brain injury such as experimental stroke, cerebral hypoperfusion, brain trauma, neuronal hyperactivity or neurotropic virus infection. We have studied the effects of microglia manipulation or actions of inflammatory mediators on changes of cerebral blood flow (CBF), as assessed by laser speckle contrast imaging, laser Doppler flowmetry, and *in vivo* two-photon imaging. SPECT, MR and PET imaging have also been used to study BBB function, inflammation and metabolism in the brain after experimental stroke and/or systemic inflammation.

We aimed to understand the mechanisms through which *acute brain injury induces inflammatory changes in peripheral organs*, including the bone marrow, the lung, the gut, or the liver, by using *in vivo* imaging, flow cytometry, metagenome sequencing, anatomical approaches and assessed of inflammatory mediators by cytometric bead array or ELISA. To understand how brain injury and central inflammatory changes are influenced by systemic inflammatory actions outside the CNS, we used different models of *systemic inflammation* (induced by bacterial lipopolysaccharide, acute infection or chronic immunopolarization, etc.) in combination with models of experimental stroke.

We have studied the contribution of *proinflammatory mediators,* which are produced by microglia and other cell types, to neuronal- and vascular injury. In many of these studies, we investigated the mechanisms of production and sites of actions of the *key proinflammatory mediator, IL-1*, to assess how IL-1-mediated changes impact on acute brain injury and to understand, how systemic inflammation contributes to brain injury via IL-1, using different experimental models of acute brain injury and acute or chronic inflammation. To this end, we have performed bone marrow transplantation to study the role of haematopoietic-derived IL-1α/β, generated mice for conditional deletion of neuronal or brain endothelial IL-1 receptor 1 (IL-1R1, the main signaling receptor for IL-1 isoforms IL-1α and IL-1β), or used pharmacological blockade of IL-1R1 by IL-1 receptor antagonist (IL-1Ra) to assess changes in inflammation and functional outcome among other approaches. Using different transgenic mouse lines, we have also assessed how *inflammasomes* – intracellular molecular complexes regulating the production of IL-1β in response to infection, injury or cellular stress – contribute to microglial activation, inflammation and brain injury.

To study *compartment-specific interactions of microglia* with other cells in the brain with the latest technologies available, we have developed a top-down methodological approach spanning from the millimeter to the nanometer scale linking *in vivo* imaging findings with specific anatomical features of these cell-cell interactions. To this end, a comprehensive *molecular anatomy* platform including 3D confocal microscopy, superresolution microscopy, immunoelectron microscopy, correlated confocal- and electonmicroscopy, array tomography and electron tomography has been developed. *For clinical relevance*, experimental models of brain injury or microglia manipulation have been complemented with high resolution molecular anatomy studies using *post-mortem human brain tissues,* including those from patients without neurological disorders and those with brain inflammatory conditions, viral infection or stroke.

In several studies, we have performed detailed *phenotyping of inflammatory cells and mediators* by using immunofluorence, flow cytometry, cytometric bead array, HPLC and other approaches. To further study the cellular/molecular mechanisms of microglial inflammatory mediator production, intercellular interactions, purinergic mechanisms of microglia-neuron interactions or intracellular mechanims related to injury or inflammation, we have established different *ex vivo models,* including neuronal and glial cell cultures for high resolution imaging using specific biosensors and a broad array of cell biology assays.

Combination of the tools and approaches outlined above has greatly supported the execution of these complex studies aiming to understand novel aspects of microglia biology and neuro-immune interactions that emerge as important contributors to common brain disorders.

Results and summary of novel findings

(Only selected, key findings have been summarized below with minimal mechanistic detail for clarity)

Chapter 1. **Inflammation and microglial actions modulate injury in the brain**

1. Microglial response to brain injury induced by cerebral ischemia in mice precedes substantial invasion of peripheral macrophages, which includes rapid phenotypic transformation, expression of activation markers and proliferation 24h onwards. Microglial cell death also occurs at sites of severe injury. Proliferating microglia phagocytose invading neutrophil granulocytes.

2. Acute brain induced by cerebral ischemia leads to rapid production of both isoforms of the key proinflammatory protein, IL-1, in microglia. Brain IL-1α mRNA levels and production of microglial IL-1α protein occurs within 4h in the core of the infarct and in the peri-infarct tissues showing BBB injury or focal neuronal loss, followed by microglial IL-1β expression within 24h post-injury. Nuclear retention of IL-1α in microglia also occurs, a possible mechanism to prevent release of this highly proinflammatory cytokine to the injured tissue.

3. Damage associated molecular patterns (DAMPs) released from injured cells stimulate an inflammatory response in microglia and astrocytes even in the absence of a priming stimulus, leading to the production of cytokines and chemokines such as IL-6 or CXCL1, which is IL-1 dependent (significantly reduced in IL-1αβ KO mice). In line with this, serum proteins, specifically the acute phase protein serum amyloid A (SAA) can act as a priming stimulus in glial cells, resulting in the release of IL-1β after stimulation with ATP.

4. Both elimination of IL-1αβ from haematopoietic cells by bone marrow transplantation and the absence of IL-1αβ from the brain result in significanlty reduced neuronal death and BBB injury after experimental stroke.

5. Inflammasomes – intracellular molecular complexes regulating the production and release of IL-1β – contribute to the pathophysiology of acute brain injury. Mice deficient for ASC, a common adaptor protein for the assembly of several inflammasomes display significantly reduced brain injury after experimental stroke, similarly to mice deficient for NLRC4 (recongizes bacterial flagellin) and AIM2 (recognizes DNA) inflammasomes, independently of the NLRP3 inflammasome.

6. Elimination of IL-1R1 (the main signaling receptor for IL-1α and IL-1β) from the cerebrovascular endothelium results in reduced vascular inflammation, recruitment of

neutrophil granuloctyes and brain injury. Neuronal IL-1R1 deletion, in particular deletion from cholinergic neurons also protects against brain injury after experimental stroke.

7. Mice deficient for IL-1αβ display reduced production of CXCL1, a potent chemokine for neutrophil recruitment and impaired granulocyte mobilisation into the circulation, which is also apparent in mice lacking haematopoietic IL-1αβ. Neutrophils transmigrating IL-1-stimulated endothelial cells become highly neurotoxic by producing proteases and de-condensed DNA, known as neutrophil extracellular traps (NETs). In turn, pharmacological depletion of microglia by the CSF1R inhibitor, PLX5622 leads to increased neutrophil numbers in the brain after experimental stroke.

8. The Na^+-K^+-$2Cl^-$ cotransporter, NKCC1, is highly expressed in microglia, regulates both baseline and reactive microglia morphology, process recruitment to the site of injury, and adaptation to changes in cellular volume in a cell-autonomous manner via regulating membrane conductance. Conditional NKCC1 deletion in microglia results microglial dysfunction, characterized by NLRP3 inflammasome priming and increased production of IL-1β, which is associated with increased brain injury and impaired functional outcome after experimental stroke. Central and systemic administration of the specific NKCC1 blocker, bumetanide, have opposite effects on the production of inflammatory mediators in the brain.

Chapter 2. **Systemic inflammation promotes CNS inflammation, alters microglial function and augments brain injury**

9. Systemic inflammation exacerbates brain injury induced by cerebral ischemia via proinflammatory mechanisms. Chronic T helper 1 (Th1)-polarized immune response to a nematode, Trichuris muris, prior to experimental stroke augments BBB injury and neuronal death alongside with increased production of CCL5 (RANTES), which is markedly induced by IL-1. Systemic inflammation mediates its detrimental effects in part through actions of leukocyte-derived proteases in the brain and increased platelet aggregation in brain microvessels. Selective blockade of RANTES by a neutralizing antibody reversed systemic inflammation-induced increases in brain injury, suggesting a functional role for RANTES. Using the same infection model, a Th2-polarized immune response is not associated with increased RANTES production and has no significant effect on inflammation or brain injury after experimental stroke.

10. Systemic inflammation induced by intraperitoneal lipopolysaccharide (LPS) or anaphylaxis prior to experimental stroke results in altered microglial phenotypes, higher level

of microglial IL-1 production, increased mortality, increased leukocyte recruitment and more severe BBB injury. The detrimental effect of systemic inflammation on BBB injury and brain oedema are still present even if infarct size is effectively reduced by hypothermia.

11. Systemic inflammation induced by subchronic, lung-restricted *Streptococcus pneumoniae* infection results in higher levels of proinflammatory cytokines/chemokines in peripheral organs including the liver and the spleen, and drives vascular inflammation in the brain prior to any experimentally induced injury. Infection preceding experimental stroke leads to increased production of proinflammatory cytokines/chemokines, elevated microglial IL-1 production, as well as increased BBB injury and neuronal death in both mice and rats. Systemic inflammation-induced pathologies are largely reversed by blockade of IL-1 actions by IL-1 receptor antagonist (IL-1Ra) or by blocking platelet-endothelial interactions using a selective anti-GPIbα Fab fragment. In separate studies we have revealed that platelet-derived IL-1 promotes cerebrovascular inflammation alone, or via interactions with the lectin complement pathway.

12. Lung-restricted subchronic *Streptococcus pneumoniae* infection augments vascular pathology in different vascular beds, including the brain and the heart, resulting in increased number of plaques in the aorta of atherosclerosis-prone mice. Systemic inflammation induced by an atherogenic diet also parallels profoundly altered microglial phenotypes in the brain similarly to that seen after different models of infection. Vascular inflammation and microglial alterations in atherogenic mice were largely reversed by blockade of IL-1 actions using IL-1R1 KO mice. In turn, only vascular inflammation, but not microglial reactivity was reversed by a specific neutralizing anti-IL-1β antibody.

13. Patients with multiple risk factors for stroke and chronic inflammation (as shown by chronically elevated CRP levels) display increased microglial activity based on TSPO-PET measurements, in the absence of any obvious neurological disease (negative MR). In line with this, obese, atherosclerotic and insulin resistant Corpulant rats, showed increased TSPO radioligand binding compared to lean rats, with increasing difference by aging. Corpulant rats also present more severe BBB injury after experimental stroke, which is partially reversed by blockade of IL-1 with IL-Ra.

14. Acute brain injury induces central and peripheral changes within hours, including increased production of inflammatory mediators and mobilisation of different immune cell populations. Experimental stroke leads to the release of CXCR2-positive granulocytes from the bone marrow, which is associated with rapid systemic upregulation of CXCL1 (a ligand for CXCR2) and granulocyte-colony-stimulating factor, a key cytokine involved in the mobilisation of bone marrow leukocytes. Stroke-induced laterality was also observed in brain

stem autonomic nuclei and in the bone marrow indicating the involvement of the autonomic nervous system in stroke-induced cell mobilisation. In line with this, experimental stroke and traumatic brain injury alter the composition of caecal microbiota in mice, and these effects were mediated by noradrenaline release from the autonomic nervous system with altered caecal mucoprotein production and goblet cell numbers.

15. We have developed novel approaches to investigate the earliest inflammatory changes in peripheral organs after stroke. SPECT imaging based on lipophilic 99mTc-hexamethylpropylene amine oxime (HMPAO) and 99mTc-DTPA (diethylene triamine pentaacetic acid) signal changes revealed that acute brain injury leads to perfusion- and barrier function changes in various peripheral organs including the lung and the gut within hours. SPECT imaging also revealed that preceding systemic inflammation induced by bacterial LPS prior to experimental stroke alters microglial activity, augments BBB injury, and reduces cerebral blood flow in the affected neocortex, leading to poor functional outcome.

16. Changes in microglial phenotypes and altered neuroimmune interactions contibute to CNS pathology even in the absence of direct CNS injury. IL-1 and microglial IL-1β play a key role in the maintenance of chronic pain in a translational passive transfer trauma mouse model of complex regional pain syndrome (CRPS). This is confirmed by blockade of IL-1 actions using IL-1αβ KO mice, or perioperative IL-1R1 blockade by anakinra, which almost completely prevented lasting CRPS symptoms, while reduced pathology was observed in microglial IL-1β KO mice, with no effect seen after treatment with the glucocorticoid prednisolone.

Chapter 3. **The role of compartment-specific microglia-neuron interactions in shaping neuronal activity and neuronal injury**

17. Mice deficient for the fractalkine receptor, CX3CR1, which is expressed exclusively by microglia in the brain parenchyma, show altered microglia-neuron interactions in the injured brain. CX3CR1 KO mice display reduced IL-1β production, brain injury and neuronal death and after experimental stroke.

18. CSF1R inhibitors such as PLX3397 and PLX5622 allow effective pharmacological depletion of microglia without inducing CNS inflammation or toxicity to neurons, astrocytes, endothelial cells, pericytes, oligodendrocytes or smooth muscle cells. Elimination of microglia by PLX3397 results in significantly (by 60%) increased brain injury following experimental stroke, whereas repopulation of microglia reverses this effect.

19. *In vivo* two-photon calcium imaging, using genetically encoded calcium indicators revealed activity-dependent interactions between microglial processes and compromised neurons in the boundary zone of the evolving infarct. Elimination of microglia by PLX3397 is associated with markedly dysregulated neuronal network activity patterns and increased neuronal calcium load, leading to augmented neuronal death. These experiments revealed that activity-depented formation of contacts between microglia and neurons represents an essential protective mechanism for controlling pathological neuronal activity by microglia in the injured brain.

20. We have discovered an entirely novel form of interaction between microglia and neurons through which microglia sense and influence neuronal activity and neuronal injury. Using *in vivo* two-photon imaging and 3D confocal microscopy we revealed that microglial processes repeatedly contact specific somatic sites on neurons (termed somatic purinergic junctions), with the average lifetime being 3-4 times longer (25 min) than those made with dendrites (7.5 min). This novel structure turned out to be one of the main sites of microglia-neuron interactions in the brain: both in mice and in post-mortem human brain tissues, more than 90% of pyramidal cells and interneurons receive somatic microglial contact, whereas only around 10% of GABA-ergic and glutamatergic synapses are contatced by microglia in perfusion-fixed tissues at given time points.

21. Microglial processes preferentially contact Kv2.1 and Kv2.2 channel clusters on neuronal somata, which are key sites for somatic exocytosis. Transfection of HEK cells with Kv2.1 (which do not express Kv2.1 or Kv2.2. channels) recruit microglial processes *in vitro*, which effect is dependent on clustering of Kv2.1 in the cell membrane as confirmed by a dominant-negative mutant Kv2.1 construct.

22. Superresolution microscopy and 3D electron tomography revealed that enrichment of the main microglial ADP receptor, P2Y12, occurs specifically at sites of somatic somatic purinergic junctions in apposition with Kv2.1 clusters in the neuronal membrane. EM tomography revealed a higly specialized ultrastructure in neurons at these sites characterized by closely localized mitochondria, mitochondria-derived and other vesicles, mitochondria-associated membranes and ER-like structures. Functional *in vivo* and *ex vivo* studies also revealed that microglia sense changes in neuronal mitochondrial function at these somatic sites, which was dependent on P2Y12R-mediated actions. Vesicular nucleotid transporter (vNUT) is enriched in neurons at the junctions where vNUT-dependent somatic ATP release takes place in response to changes in neuronal activity. In turn, the formation and lifetime of somatic junctions is promoted by neuronal activity *in vivo*, in a P2Y12R-dependent manner.

23. Establishment of somatic junctions is associated with increases in NADH fluorescence in neuronal mitochondria, which process is P2Y12R-dependent, confirming interactions between contacting microglial processes and neuronal mitochondrial function.

24. Microglia sense neuronal injury via somatic purinergic junctions. Compromised, but viable neurons at the boundary zones of the infarct after experimental stroke display fragmentation of neuronal mitochondria and declustering of Kv2.1, which is associated with P2Y12R-dependent increases in microglial process coverage at somatic sites. Blockade of microglial P2Y12 by injection of a selective inhibitor, PSB-0739 into the cisterna magna increased neuronal calcium load, dysregulated functional connectivity and led to increased brain injury and worse functional outcome in mice after stroke (similar effects to those seen after depletion of microglia).

25. Microglia respond to and influence spreading depolarization (SD) in both the injured and in the otherwise intact (uninjured) brain. SD incidence and kinetics are markedly altered in mice without functional microglia after experimental stroke. SD induced in the normally perfused and metabolically intact brain tissue is associated with larger latency to depolarization and reduced intracellular calcium load. Induction of SDs induce rapid morphological changes in microglia, facilitate microglial process recruitment to neurons, while depolarization and hyperpolarization during SD are microglia- and P2Y12R-dependent. The absence of microglia was associated with altered potassium uptake after SD, independently of P2Y12R.

26. Microglia-neuron interactions at neuronal somata are essential for the recognition/discrimination of healthy, injured but salvageable, as well as terminally injured neurons by microglia. A specific aspect of these interactions concerns the instrumental role of microglia and microglial P2Y12R in recogntition, isolation and elimination of virally infected neurons from the brain, through which actions microglia limit the spread of neurotropic virus infection. *In vivo* two-photon imaging revaled the recruitment of microglial processes and isolation of neurons infected transsynaptically by a recombinant pseudorabies virus strain (member of the alpha herpesvirus family) within 1-3 hours. Elimination of microglia by PLX5622 or genetic deletion of P2Y12R resulted in markedly increased viral spread and deficient phagocytosis of infected cells due to impaired responses to ATP released from compomised neurons. P2Y12R positive microglia are also recruited to and phagocytose infected neurons in the human brain as demonstrated by using post-mortem brain tissues of patients with HSV encephalitis.

27. Microglia establish direct, dynamic purinergic contacts with cells in the neurovascular unit that shape cerebral blood flow (CBF), namely with endothelial cells, astrocytes, smooth muscle cells and pericytes in both mice and humans. Superresolution microscopy and electron tomography revealed accumulation of microglial P2Y12R at contact sites with endothelial cells near endothelial mitochondria. Microglia depletion by PLX5622 or blockade of microglial P2Y12R substantially impairs neurovascular coupling induced by whisker stimulation, which effect is confirmed by using chemogenetically induced microglia dysfunction and impaired ATP sensitivity in MicroDREADD[Dq] mice. Altered CBF responses after microglia manipulation are not explained by altered neuronal activity.

28. Hypercapnia induces rapid microglial calcium changes, P2Y12R-mediated formation of perivascular phylopodia, and microglial adenosine production, while depletion of microglia reduces brain pH and impairs hypercapnia-induced vasodilation.

29. Microglial effects on CBF regulation are partially independent of nitric oxide, while after hypercapnia, microglial actions modulate vascular cyclic GMP levels.

30. Microglial dysfunction (depletion of microglia, or both genetic and pharmacological blockade of P2Y12R) markedly impairs cerebrovascular adaptation to common carotid artery occlusion resulting in cerebral hypoperfusion.

The dissertation is based on the following original publications:

Chapter 1

1. **Denes A***,#, Vidyasagar R, Feng J, Narvainen J, McColl BW, Kauppinen RA, Allan SM# (2007) Proliferating resident microglia after focal cerebral ischaemia in mice. *Journal of cerebral blood flow and* metabolism: official journal of the International Society of Cerebral Blood Flow and Metabolism 27 (12):1941-1953. doi:10.1038/sj.jcbfm.9600495

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2. Luheshi NM, Kovacs KJ, Lopez-Castejon G, Brough D*,#, **Denes A***,# (2011) Interleukin-1alpha expression precedes IL-1beta after ischemic brain injury and is localised to areas of focal neuronal loss and penumbral tissues. *Journal of neuroinflammation* 8:186. doi:10.1186/1742-2094-8-186

IF: 4.35   *Independent citations:* 94

3. Savage CD, Lopez-Castejon G, **Denes A**#, Brough D*,# (2012) NLRP3-Inflammasome Activating DAMPs Stimulate an Inflammatory Response in Glia in the Absence of Priming Which Contributes to Brain Inflammation after Injury. *Frontiers in immunology* 3:288. doi:10.3389/fimmu.2012.00288

IF: 1.552   *Independent citations:* 152

4. **Denes A**#, Wilkinson F, Bigger B, Chu M, Rothwell NJ, Allan SM*,# (2013) Central and haematopoietic interleukin-1 both contribute to ischaemic brain injury in mice. *Disease models & mechanisms* 6 (4):1043-1048. doi:10.1242/dmm.011601

IF: 5.537   *Independent citations:* 20

5. **Denes A***,#, Coutts G, Lenart 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. *Proceedings of the National Academy of* Sciences of the United States of America 112 (13):4050-4055. doi:10.1073/pnas.1419090112

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6. 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 SM*,# (2019) Interleukin-1 mediates ischaemic brain injury via distinct actions on endothelial cells and cholinergic neurons. *Brain, behavior, and immunity* 76:126-138. doi:10.1016/j.bbi.2018.11.012

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7. Allen C#, Thornton P, **Denes A***, McColl BW, Pierozynski A, Monestier M, Pinteaux E, Rothwell NJ, Allan SM# (2012) Neutrophil cerebrovascular transmigration triggers rapid neurotoxicity through release of proteases associated with decondensed DNA. *J Immunol* 189 (1):381-392. doi:10.4049/jimmunol.1200409

IF: 5.788   *Independent citations:* 116

8. Toth K, Lenart N, Berki P, Fekete R, Szabadits E, Posfai B, Cserep C, Alatshan A, Benko S, Kiss D, Hubner CA, Gulyas A, Kaila K, Kornyei Z, **Denes A***,# (2022) The NKCC1 ion transporter modulates

microglial phenotype and inflammatory response to brain injury in a cell-autonomous manner. *PLoS biology* 20 (1):e3001526. doi:10.1371/journal.pbio.3001526

IF: 8.029   *Independent citations:* 1

Chapter 2

1. **Denes A**[#], Humphreys N, Lane TE, Grencis R, Rothwell N*[,#] (2010) Chronic systemic infection exacerbates ischemic brain damage via a CCL5 (regulated on activation, normal T-cell expressed and secreted)-mediated proinflammatory response in mice. *The Journal of neuroscience*: the official journal of the Society for Neuroscience 30 (30):10086-10095. doi:10.1523/JNEUROSCI.1227-10.2010

IF: 7.271   *Independent citations: 73*

2. **Denes A**[*,#], Ferenczi S, Kovacs KJ (2011) Systemic inflammatory challenges compromise survival after experimental stroke via augmenting brain inflammation, blood- brain barrier damage and brain oedema independently of infarct size. *Journal of neuroinflammation* 8:164. doi:10.1186/1742-2094-8-164

IF: 4.35   *Independent citations: 114*

3. **Denes A**[*,#], Pradillo JM, Drake C, Sharp A, Warn P, Murray KN, Rohit B, Dockrell DH, Chamberlain J, Casbolt H, Francis S, Martinecz B, Nieswandt B, Rothwell NJ, Allan SM[#] (2014) Streptococcus pneumoniae worsens cerebral ischemia via interleukin 1 and platelet glycoprotein Ibalpha. *Annals of neurology* 75 (5):670-683. doi:10.1002/ana.24146

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4. Drake C, Boutin H, Jones MS, **Denes A**, McColl BW, Selvarajah JR, Hulme S, Georgiou RF, Hinz R, Gerhard A, Vail A, Prenant C, Julyan P, Maroy R, Brown G, Smigova A, Herholz K, Kassiou M, Crossman D, Francis S, Proctor SD, Russell JC, Hopkins SJ, Tyrrell PJ, Rothwell NJ, Allan SM*[,#] (2011) Brain inflammation is induced by co-morbidities and risk factors for stroke. *Brain, behavior, and immunity* 25 (6):1113-1122. doi:10.1016/j.bbi.2011.02.008

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5. **Denes A**[#], Drake C[#], Stordy J[#], Chamberlain J, McColl BW, Gram H, Crossman D, Francis S, Allan SM, Rothwell NJ*[,#] (2012) Interleukin-1 mediates neuroinflammatory changes associated with diet-induced atherosclerosis. *Journal of the American Heart Association* 1 (3):e002006. doi:10.1161/JAHA.112.002006

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IF: 5.008   *Independent citations:* 47

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IF: 5.964   *Independent citations:* 180

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