Brain inflammation following stroke and cardiac arrest
-Restraining neuroinflammation and subsequent psychiatric disorders

Purpose and aims
We hypothesize that inflammatory targets and manipulation of the neuroinflammation will be important for future stroke therapies. Our aims are to find such targets/elucidating neuroinflammatory mechanisms that can limit the infarct size and support neurological/psychiatric recovery after brain ischemia, i.e. stroke and cardiac arrest.

General hypothesis
Microglia in the injured brain comprise a family of cells with different phenotypes and origin; some which are beneficial for the injured CNS and others that are more of a destructive nature. The different phases of inflammation need to be controlled, from the acute, subacute phase of the injury in the brain. The basic questions we ask are:
- How can destructive microglia/neuroinflammation be regulated into a protective mode?
- Can modulation of neuroinflammation mitigate post-stroke depression/anxiety.
- What instructs microglia to acquire a particular phenotype and is the phenotype of resident microglia different from the phenotype of blood-derived microglia?

Specific hypothesis
1) Galectin-3: Galactin-3 is a key regulator of the pro-/anti-inflammatory and phagocytotic phenotype of microglia via its ability to modify caspase activity. Elucidating the role of galectin-3 in microglia activation and manipulation of its function in the ischemic brain will be important for neurological and psychiatric recovery.
2) Macrophage migration inhibitory factor (MIF): We believe this pleiotropic cytokine to be a “hub” in the inflammatory cascade. Blockage/genetic knockout of MIF should inhibit the pro-inflammatory microglia, inhibit monocyte infiltration and provide neuroprotection.

Survey of the field
Microglia as a key mediator in injury-induced inflammation
Ischemic stroke is the third leading cause of death and is the largest cause of long-term disability in the western world. Modulation of the innate microglia/cytokine inflammatory response can provide neuroprotection/neurological recovery when instituted late after the onset (Wang et al. 2007). The innate immunity, including microglial response and TLR4 signalling (activated by LPS or modified host molecules in injured tissue) is known to be central in ischemic brain injury. Microglia are the most abundant inflammatory players in acute brain injury as well as in the subacute/chronic phase where they are involved in reconciling the injury and reorganisation of the injured brain. Recent data demonstrate that microglia are not one uniform proinflammatory cell population. Microglia phenotype can be changed, i.e. bad microglia (pro-inflammatory) can be directed to become good (growth promoting, anti-inflammatory). For example, the endotoxin LPS or the inflammatory mediator IFN-gamma can induce a proinflammatory phenotype of microglial cells (producing IL-1 and IL-6) whereas anti-inflammatory IL-4 will initiate an anti-inflammatory activation of microglia (producing TGF-beta, IL-10, IL-14 and growth factors) which will promote neuronal survival and plasticity (Butovsky et al. 2006). We believe that manipulation of the microglial response will be a future target in stroke therapy.
Microglia involvement in post-stroke anxiety and depression

Cerebrovascular injuries, such as ischemic stroke and cardiac arrest/cardiopulmonary resuscitation can lead to several neuropsychiatric disorders. Post-stroke depression and anxiety is the most common psychiatric disorders and is also known to hamper the cognitive and physical recovery (Lenzi et al. 2008). The underlying cause seem not to be related to sensory-motor impairment after brain injury since physically ill patients with similar levels of disability do not experience mood disorders in the same extent (Lenzi et al. 2008). Post-stroke depression affects almost half of the stroke patients within the first two months after stroke and is major problem for the rehabilitation. Increased anxiety has been able to be mimicked in relevant animal models of cardiac arrest/global ischemia (Neigh et al. 2004) and in experimental stroke (Winter et al. 2005). Proinflammatory cytokines have been suggested to play a key role in the pathophysiology of depression where reactive microglia is recognized to be important (Gonzalez-Perez et al. 2001). For example, interferon (IFN)gamma and tumor necrosis factor (TNF)alpha production by microglia was found to be essential in the induction of inflammation-induced depression (O'Connor et al. 2009).

Recently, patients with depressive disorders were found to have increased levels of proinflammatory cytokines (Lindqvist et al. 2009). Anti-depressants have been shown to alter the cytokine network and decrease production of proinflammatory cytokines and increase production of anti-inflammatory cytokines (Camacho-Arroyo et al. 2009). The ongoing brain inflammation, that occurs for several months after stroke, will directly affect neural circuitry (e.g. MIF can affect neuronal activity (Sun et al. 2004)) and most likely important for mood regulation and the depression/anxiety following stroke.

**Novel role of caspases in microglial inflammation:** Caspases, a family of cysteinyl aspartate-specific proteases, are executioners of apoptotic cell death and their activation is considered a commitment to cell death. Inhibition of caspase activation protects against neuronal loss in several animal models of brain diseases involving activated microglia, including experimental stroke. Recent data obtained from our laboratory demonstrate for the first time a direct regulation of the inflammatory response in the microglia after various inflammatory stimuli through a new pathway that involves orderly caspase 8, 3/7 activation, processing of PKCδ, and translocation of p65 subunit of NF-κβ into the nucleus (Burguillos M and Deierborg T et al. resubmitted to Nature). The involvement of microglia in these inflammation-inducing pathways shed a completely new light on microglia activation. What was believed to be only signs of apoptosis (such as cleaved Caspase-3) turns out to be signalling pathways to induce microglial inflammation. We have confirmed Caspase 8, 3/7 activation related to microglial activity in microglial cell cultures, in mouse models (MPTP) and rat models (6-OHDA) of Parkinson’s disease as well as in brains of Parkinson’s disease and Alzheimer’s disease patients.

We will now further elucidate the role of caspases in the acute brain inflammation following experimental stroke and in cell cultures after in vitro ischemia to clarify the role of caspases in the acute neuroinflammation and microglial activation following stroke.

**Galectin-3 as modulator of caspases in microglial activation:** Galectin-3 is only expressed in the brain after a brain injury and is suggested to amplify or modulate the inflammatory response. Extracellularly, galectin-3 can function in a para/autocrine fashion and function as an opsonin, facilitating phagocytosis. Typically galectin-3 is found in microglia at later/chronic stages in brain inflammation where it is believed to have a central role in the clearance of damaged tissue. Galectin-3 was recently suggested to be a key factor in alternative M2 activation of macrophages (MacKinnon et al. 2008). Interestingly, a proliferative population of resident microglia, with galectin-3 immunoreactivity, could provide neuroprotection after stroke (Lalancette-Hebert et al. 2007).
We believe that our target galectin-3 is a regulator of brain inflammation following stroke and we have preliminary *in vivo* and *in vitro* data supporting this hypothesis.

We think that the role of galectin-3 and its suggested role to change the microglia phenotype can be explained partly by galectin-3’s well-known function to modify caspases. Both the extrinsic pathway, caspase 8 signaling pathway by intracellular interaction with CD95, (Fukumori et al. 2004) and the intrinsic caspase-dependent pathway, stabilizing the mitochondria and inhibition of caspase-3 activation (Fukumori et al. 2006) can be governed by galectin-3.

The regulation of caspases by galectin-3 was recently reported following murine neonatal hypoxic-ischemic brain injury, where galectin-3 contributed to hypoxic ischemic brain injury (Doverhag et al. 2010). Galectin-3 deficient mice had reduced caspase 3 activity in the injured brain, reduced microglial accumulation and basically no microglial cells with immunoreactivity for cleaved caspase 3. These data suggest an altered inflammatory status in the microglial cells in view of our recent findings of caspase-dependent inflammation. The findings of Doverhag et al further suggest that galctin-3 is involved in the shift between neuroprotective and neurotoxic phenotype, which could explain the contradictory results of the protective role of microglial cells in adult (Imai et al. 2007) and detrimental role in immature brain injury (Arvin et al. 2002).

**Macrophage migration inhibitory factor (MIF) in stroke:** MIF is a protein that is essential in both innate and acquired immunity and activates monocytes/macrophages, lymphocytes and granulocytes. MIF plays a detrimental role in inflammatory conditions such as rheumatoid arthritis, atherosclerosis and in cancer. MIF can counteract the physiological function of steroids, thus playing a role in immune system regulation. MIF is increased in peripheral blood mononuclear cells in stroke patients and in the rat brain following experimental stroke which negatively correlated with functional recovery, suggesting a detrimental function of MIF (Wang et al. 2009). MIF is found in astrocytic end feet, sealing the blood brain barrier (BBB) and MIF has been suggested to have a role in monocyte infiltration.

**Preliminary data**

1) **New models of anxiety after cardiac arrest and post-stroke depression**

Psychiatric disorders following stroke/cardiac arrest are a neglected research area. This is from a clinical perspective very unfortunate since these conditions cause a lot of suffering for the patients. We have set up several behavioural models for psychiatric conditions, e.g. anxiety (Elevated Plus Maze and Open Field) and depression (Tail Suspension Test and Forced Swim Test). Fortunately, we have been able to mimic the anxiety that cardiac arrest patients suffer from in our mouse model of global ischemia when mice are observed in Elevated Plus Maze where injured mice show an anxiety behavior (sham, n=10; ischemia, n=14; p=0.02).

We have also induced experimental stroke, pMCAO, in left and right hemisphere of mice and discovered a very strong depressive-like behaviour in the Tail Suspension Test (sham, n=4; ischemia, n-left, 6; n-right 5; p=0.005). Interestingly, this lateralisation shows that mice are similar to human in that sense that depression often occur following stroke in the dominant (left) hemisphere. We are very happy to have mouse models that are highly relevant for psychiatric diseases following stroke/cardiac arrest in patients and will use these models to elucidate possible inflammatory mechanisms.

2) **Galectin-3, post-stroke anxiety and the origin of microglia**

We have induced experimental stroke (permanent middle cerebral artery occlusion, pMCAO) that resembles human stroke) in galectin-3 ko (n=18) and wild-type littermates (n=13) and used an advanced behavioural software SMART (PanLab, Spain) to be able to detect several
aspects of neurological and psychiatric animal behaviour. We found that mice without galectin-3 have worse neurological functions and psychiatric behaviour compared to their wt littermates. This could be seen in contralateral circling behavior (P<0.05), sensory motor function in the contralateral hind limb (P=0.01) and their ability to stand on an inclined plane with a rough surface (P<0.01, fig. 1). In the Open Field test we detected an anxiety related phenotype in stroke-injured mice lacking galectin-3 (P<0.01) as well as a strong trend (P=0.059) in the time spent in the opened arms in the anxiety test Elevated Plus Maze.

We have further studied the microglia production of nitric oxide (NO), which has been implicated in the stroke-injury. A model of LPS-induced nitric oxide synthase (iNOS) in mouse microglial cells (BV2) was used to study the effect of galectin-3. We found a more than 3-fold protein increase in iNOS when selective galectin-3 inhibitor (collaboration with Hakon Leffler, Lund University) was used suggesting again a beneficial effect of galectin-3 to restrain inflammation (fig. 1b). We have also used primary mouse microglia to study the secretion of Th1/Th2 cytokines (Mesoscale platform) at 24 h after LPS challenge. We found a several-fold increased production of the inflammatory cytokines, e.g. IFN-γ, IL-1β, IL-2 and TNF-α after galectin-3 inhibition supporting a positive role of galectin-3 in the acute inflammatory phase (fig. 1c). These findings confirm our hypothesis that galectin-3 control an anti-inflammatory response coordinated by microglial cells in the acute inflammation after stroke.

Housing rodents in an enriched environment is the most effective therapy following stroke to restore lost functions. We have used four weeks of enriched environment following experimental stroke (transient middle cerebral artery occlusion) in mice to look for differences in brain plasticity (Nygren et al. 2006). Galectin-3 antibody was used to visualize the activated microglial population, suggested to preferentially recognize the proliferative population of activated microglia (Lalancette-Hebert et al. 2007). In mice kept in an enriched environment (n=14), we found a robust decrease in galectin-3 immunoreactivity in the peri-infarct area that correlates with functional recovery, compared to standard housed mice (n=13), suggesting a beneficial effect of galectin-3 downregulation (fig. 2) in the late ischemic phase. These data is line with the function of galectin-3 in chronic inflammation where galectin-3 is involved in fibrosis (Henderson and Sethi 2009) and where an alternative M2 microglial activation is detrimental. The beneficial effect of galectin-3 positive microglia
early following stroke (Lalancette-Hebert et al. 2007) is not advantageous at later time points when the injury is reconciled and the brain is undergoing reorganisation.

The origin of microglia: We are currently looking into the origin of the microglia in the strong microgliosis following ischemic brain injury (collaboration with B. Finsen/K. Lambertsen in Odense). It is still a question of debate, from where the microglia are derived (blood vs brain) and their specific function after brain damage (Soulet and Rivest 2008). We have used my own established model of global brain ischemia (Olsson et al. 2003) that leaves BBB intact with its selective and delayed cell death in the hippocampal subregion CA1. By the use of bone-marrow (BM) chimeric mice and rats we found a very surprising result. About 40% of the activated microglia in CA1 of the BM-chimeric mice had been derived from the blood (fig. 3), whereas in the injured rat hippocampus, not one single microglia were derived from the blood. We are now elucidating the phenotype of the blood-derived microglial cells.

3) Macrophage migration inhibitory factor (MIF)
We have analysed the transcription level of more than 2 000 different genes. Out of many genes related to inflammation, one gene, MIF, was significantly changed at several time points during the first 24 h following stroke. Our studies of the MIF protein levels confirm a robust upregulation after stroke (fig. 4). This response could be a detrimental overactivation of MIF. Preliminary in vivo data, where MCAO have been induced in MIF ko mice revealed a diminished brain injury compared to control mice (29% reduction; \( n_{wt}=10 \) \( n_{ko}=7 \); \( p<0.05 \)). Behavioural data confirmed the histology: 25% lower grip strength (\( p<0.05 \)) and lower Neuroscore in wt mice (fig. 4). The detrimental effect of MIF shows a concentration-related effect where data from hz mice were in between ko and wt mice (fig. 4). This was further confirmed in cultured cortical neurons, exposed to sub-lethal oxygen and glucose deprivation where MIF levels increase and where the pharmacological inhibition of MIF by ISO-1 that protects neurons against cell death. Also, housing rats in an enriched environment reduced levels of MIF in the brain after permanent MCAO, compared to animals housed in standard

Figure 2. Immunoreactivity of microglia, Galectin-3 in ischemic mouse brain (A). Mice housed in an enriched environment for four weeks following stroke showed a reduction in immunoreactivity of Galectin-3 (B). Bars denote mean value, significance \( P<0.05 \) (t-test).

Figure 3. Microglial cells in ischemia-injured mouse brain. A) Confocal microscopy identifying infiltrating microglial cells/ macrophages by their immunoreactivity for GFP (infiltrating cells from the blood) and CD11b (marker for microglia), arrow. Resident microglial cell is only positive for CD11b, arrow head. B) Merged picture of 25 confocal series showing a 30 \( \mu \)m projection of the microglial cells in A.
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cages. The mechanisms of action are under investigation using primary cultures of microglia, astrocytes and neurons.

Project description

Experimental stroke models. My PhD student, Ana Rita Inacio, and I are able to do both the transient (Nygren et al. 2006) and the permanent (Gregersen et al. 2000) MCAO model in mice. In addition, we have a model of global brain ischemia (brain damage after heart arrest) that I developed during my doctoral thesis (Olsson et al. 2003).

Multi-photon microscopy. Within the research network, NeuroFortis (VR, Starka Forskningsmiljöer), we have recently purchased a Multi-photon microscopy (Zeiss LSM710 NLO). Collaborators within our EU-funded stroke network ARISE, have extensive knowledge of using multi-photon microscopy in vivo. This cutting-edge technology enables us to study neuron, astrocytes and microglia within the living brain in relation to their origin, function for infarct size evolution, glial scarse formation and brain parenchymal reorganisation. Using the multi-photon microscopy we (collaboration with Håkan Toresson, LU) have been able to visualize neuronal changes after mouse global ischemia and how this correlates to a reduction in endoplasmic reticulum ER assembly and cell death (Fig. 5).

We are embryotransferring in transgenic mice (Cx3cr1-GFP) where microglia can be visualised. By monitoring microglia in these mice we will study dynamical changes related to early (neuroprotection), late (reorganisation/cleaning up) and intracranial drug manipulation (e.g. galectin-3 inhibitor) in view of protrusion changes, phagocytosis etc.
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Behavioural tests. We have established a panel of several behavioural tests to study functional recovery and psychiatric alterations (Lambertsen et al. 2009). We obtained funding for a SMART video tracking system to monitor complex behavioural deficits such as depression, anxiety, curiosity and social interaction. These parameters are also of importance for the clinical assessment of stroke treatment. I am currently training an undergraduate student, Totte Stankovich, who will start working specifically with post-stroke behaviour.

Mesoscale Assays. Up to ten analytes (cytokines or growth factors) can be measured in blood, culture medium or tissue simultaneously at one occasion with high accuracy. This set up allows us to study the complex inflammatory regulation.

Primary cultures and cell lines of neurons, microglial cells and astrocytes harvested from embryonic and postnatal tissue are used frequently.

Immunohistochemistry and molecular biology. The activity of caspase 3, 7 and 8 will be measured in collaboration with (Bertrand Joseph, KI). Histology, FACS, immunohistochemistry, western blotting, PCR and other molecular biology techniques such as gene subcloning, gene transfection and short interference RNA techniques are routinely run in our lab.

A vivid and talented postdoc, Miguel Burguillos Garcia, will be running cell culture experiments and molecular biology techniques.

Experimental plan: Galectin-3, origin of microglia and MIF

Neuroprotection and the specific function of microglia phenotype and the mechanisms responsible for the invading monocytes are related to both MIF and galectin-3. Experimentally, the galectin-3 ko (Colnot et al. 1998) and MIF ko (Leech et al. 2003), that we now have in big breeding colonies, will be studied in a similar way based on our hypothesis.

- Neuroprotection and functional behaviour including psychiatric behaviour after MCAO will be studied in ko/wt mice and newly developed inhibitors for MIF (Al-Abed et al. 2005) and Gal-3 (Tejler et al. 2007). These experiments will be combined with the following approaches to answer our hypothesis:
  - Monocytes in the blood will be depleted using i.v. injections of clodronate-encapsulated liposomes that, when engulfed, will kill the monocytes/macrophages in the blood; alternatively intrasplenically injection of cell tracker CFDA, allowing for detection of blood-derived monocytes within the CNS.
  - GFP-Bone marrow chimeric mice of MIF and/or galectin-3 ko mice will be generated as we have done before (Lambertsen et al. 2009) to study the infiltration/contribution of blood-derived microglia in the injured brain.
  - Bone-marrow chimeric naïve mice grafted with bone marrow from MIF ko or galectin-3 ko mice will be lesioned to study the effect of the injury if the blood-derived cells lack MIF or galectin-3. Spleen samples will be used to verify chimerism.
  - The microglia in the injured brain will be analysed according to their specific phenotypic markers by FACS and immunohistochemistry in regards to their pro-/anti-inflammatory phenotype (eg. IL-1B/IGF-1 resp.), pro-/anti-inflammatory phagocytosis (eg. TLR4/TREM2 resp.) and immunogenic response (Meso-Scale for Th1/Th2 cytokine profile in brain/blood).
  - Multiphoton microscopy: Phagocytosis real-time in vivo following stroke using the, which will be achieved by intracerebral injections of LDL/HSP70 (macrophage food) connected to a fluorophore. This will be achieved in galectin-3 ko mice that has been crossed with the microglia transgenic mouse Cx3cr −GFP.
  - Analysis of stroke patient blood. Single-nucleotide polymorphism analysis: Galectin-3 polymorphism H64 polymorphism is associated with 100 fold higher odds for breast cancer.


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(Balan et al. 2008) (collaboration with Hakon Leffler, LU). MIF polymorphism (173 CC genotype) is involved susceptibility to multiple sclerosis (Akcali et al.) (Richard Bucala, Yale University). Inflammatory cytokines will be measured in blood. The neurological and psychiatric data will be analysed in patients with polymorphism and in age/gender matched stroke control patients. This is a collaborative project with Arne Lindgren at the Department of Neurology at Lund University Hospital who is in charge of Lund Stroke register, a biobank with blood samples from 3000 patients with a first ever stroke.

**Timeplan:** Up to year 1: We are now doing the last experiment for the first large MIF project where we will show the effect of MIF in stroke for the first time. We will start to characterize the molecular pathway of galectin-3 and its connection to caspases and microglial phenotype. Year 2-3; A focus will be on in vivo with cell infiltration, integrative cellular functions that will be studied with 2-photon microscopy. Year 4-5; confirming the in vivo data with patient material (brain sections and blood samples) and to study a possible effect of galectin-3/MIF polymorphism. Regression analysis of neurological and psychiatric patient data will be correlated with inflammatory molecules.

**Significance, clinical importance**

We believe our targets, Galectin-3 and MIF, will be of importance for the understanding of the stroke-injury, the microglia phenotype and response following stroke. The use of specific inhibitors, well tolerable *in vivo*, opens the possibility to even approach direct clinical importance. Psychiatric conditions in stroke/cardiac arrest patients have mostly been overlooked preclinically. With our broad knowledge in experimental stroke, inflammatory focus and behavioural testing system we believe that we can contribute to the understanding and underlying cause of these psychiatric conditions.

**Part of project cost**

The project described will depend on the funding. We have currently no VR funding and all experiments and salaries for coworkers have been paid by external grants from foundations and from the Portuguese research council. The proposed research program will only be possible if we are able to raise research money and funding from VR would make a big difference. In percentage, the estimated funding from VR would be between 40-60%.

**Facilities and equipment for the proposed projects**

BMC at Lund University is fully equipped for the performance of all the experiments described.

**International collaborations**

- Prof. Bente Finsen, Odense, University of Southern Denmark: Microglia origin
- Prof. R. Bucala, Yale University, USA: MIF project; ko mice, inhib, MIF-GFP, MIFab, etc.
- Dr. Kate Lambertsen, Miami University, USA: Microglia phenotype and neuroprotection
- Dr Denis Soulet, Quebec, Canada: Models of Inflammation, microglia
- Dr. Thomas Moeller, University of Washington, US: Microglia culturing
- Dr. Antje Diester, Charité Universitätsmedizin Berlin: Microglia culturing

**National collaborations**

- Dr. Thomas Areschough, Lund University Phagocytosis
- Dr. Karin Sävman, Gothenburg University: Galectin-3 ko mice
- Prof. Hakon Leffler, Lund University: Galectin-3 inhibitors and recombinant protein
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- Dr. Lena Brundin: Lund University: Clinical depression research
- Dr. Elisabeth Englund, Lund University: Human stroke brain pathology
- Dr. Bertrand Joseph, Karolinska Institute: Caspases and cell death
- Prof. Arne Lindgren, Lund University Hospital: Blood samples from stroke patients

Ethics and gender
All experiments are approved by an ethical committee. In my stroke research I will to some extent use both male and female animals as well as young and aged animals.

Independent research profile
In 2004, I became a postdoctoral fellow at the Neuronal Survival Unit at Lund University, where I was responsible for collaborative research on neurogenesis with the company NeuroNova AB. In 2007, my application “Inflammation in Stroke” was granted 4 years of salary from the Portuguese research council for my PhD student Ana Rita Inacio. She is now my PhD student. Inflammation in general and microglia in particular in ischemic brain damage has developed into our independent research field, specifically MIF.

I have just (April 2010) recruited a postdoc Miguel Burguillos Garcia that will work with galectin-3. I have a technician 20% (Elisbeta Bularca) and I am training a motivated undergraduate student (Totte Stankovich).

I am working within Neurofortis (VR starka forskningsmiljöer) and I am connected to both T. Wieloch’s and P. Brundin’s groups. I am having my own research focus and my own collaborators independently and without T. Wieloch’s and P. Brundin’s intellectual guidance. The proposed project has no overlap with their research projects.

References
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