Talking to the Brain at the Blood-Brain Barrier through Inflammation-Induced Prostaglandin E₂

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After all, we *know* nothing more or less than what we *can* reveal.

Adaptation from
House of Cards, character Francis Underwood

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Abstract

The immune-to-brain signaling is a critical survival factor when the body is confronted by pathogens, and in particular by microorganisms. During infections, the ability of the immune system to engage the central nervous system (CNS) in the management of the inflammatory response is just as important as its ability to mount a specific immune response against the pathogen, since the CNS can provide a systemic negative feed-back to the immune activation by release of stress hormones and also can prioritize the usage of the energy resources by the vital organs. Prostaglandin E₂ (PGE₂) and pro-inflammatory cytokines were among the first mediators to be identified to participate in the immune-to-brain signaling, a process that is clinically recognized by the development of manifestations of common illness such as fever, anorexia, decreased social interactions, lethargy, sleepiness, and hyperalgesia.

In this thesis the contribution of PGE₂ to the immune-to-brain signaling was further characterized at the blood-brain-barrier (BBB) and in the anterior preoptic area (POA) of the hypothalamus (i.e. the thermoregulatory region or, in sickness, the fever generating region).

BBB is the major interface region between peripheral circulating cytokines and the neuronal parenchyma and a critical source of PGE₂. Using chimeric mice lacking the inducible enzyme for PGE₂ synthesis, microsomal PGE synthase-1 (mPGES-1), in either hematopoietic or non-hematopoietic cells, we demonstrate in paper I that brain endothelial cells are the critical source of PGE₂ in BBB during peripheral inflammation. For the demonstration of the mPGES-1 expression in the BBB cells we developed in paper I a method for enzymatic dissociation of these cells, followed by fluorescence activated cell sorting (FACS). Using the same method, we show in paper II that the BBB response to immune stimuli is towards an increased production of PGE₂ in endothelial cells and an increased sensitivity of these cells for pro-inflammatory cytokines. These changes are supported by decreased PGE₂ degradation and decreased synthesis of other prostanoids in perivascular macrophages, which hence respond in concordance with the endothelial cells in enhancing PGE₂ signaling.

Once released in the neuronal tissue, PGE₂ has been shown to be critical for the fever response by acting on the type 3 PGE₂ receptors (EP3) within POA. By laser capture microdissection (LCM) we extracted the EP3 receptor expressing region in POA, defined by *in situ* hybridization histochemistry, from mouse brain sections. We demonstrate in paper III that the predominant subtypes of the EP3 receptor in POA are EP3 α and EP3 γ . In paper IV we further analyze the effect of PGE₂ on the LCM dissected EP-rich POA using gene expression microarrays. We demonstrate that PGE₂ has a limited effect on the gene expression changes within POA, suggesting that the neuronal activity is modulated by PGE₂ in a transcription-independent manner and that the profound gene expression changes that are seen in the CNS during inflammation are accordingly PGE₂-independent.

Abbreviations

15-PGDH	15-hydroxy-prostaglandin dehydrogenase
Abcc4	ATP-binding cassette transporter 4 or Mrp4
ADAM	A disintegrin and metalloproteinase (10,17)
Anpep	Alanyl membrane aminopeptidase
AP1	Activating protein-1
BBB	Blood-brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
COX-(1-2)	Cyclooxygenase (1-2)
cPGES	Cytosolic PGE synthase
CR1	Carbonyl reductase-1
CSF	Cerebrospinal fluid
CVO	Circumventricular organ
CXCL10	C-X-C motif chemokine 10
DAMPs	Danger-associated molecular patterns
EP1-4	Prostaglandin E receptor 1-4
FACS	Fluorescence activated cell sorting
FC	Fold change
FMO	Fluorescence minus one
FSC	Forward-scatter light
gp130	Glycoprotein 130
HPA	Hypothalamic-pituitary adrenal
H-PGDS	PGD ₂ synthases, hematopoietic
HSP	Heat shock protein
Iba1	Ionized calcium binding adapter molecule 1
icv	Intra-cerebroventricular
IFN	Interferon
IkB α	Inhibitor of kappa B, subtype alpha
IKK	I κ B kinase complex
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	IL-1R accessory protein
IL-6R	IL-6 receptor
ip	Intraperitoneal
iPGE ₂	Inflammation-induced PGE ₂
IRFs	Interferon transcription factors
iv	Intravenous
KO	Knock-out transgenic mouse
LCM	Laser capture microdissection
L-PGDS	PGD ₂ synthases lipocalin-type
LPS	Lipopolysaccharide

MAPKs	Mitogen activated-protein kinases
MMR	Macrophage mannose receptor or CD206
mPGES-1	Microsomal PGE synthase-1
mPGES-2	Microsomal PGE synthase-2
Mrp4	Multi drug resistance-associated protein 4 or Abcc4
MyD88	Myeloid differentiation factor 88
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTS	Nucleus of the solitary tract
OVLT	Organum vasculosum of lamina terminalis
PAMPs	Pathogen-associated molecular patterns
Pdgfrb	Platelet-derived growth factor receptor β (CD140b)
PECAM1	Platelet endothelial cell adhesion molecule-1 or CD31
PGE ₂ (D ₂ -I ₂)	Prostaglandin E2 (D2, E2, F2, G2, H2, I2)
PLA ₂	Phospholipase A2
POA	Anterior preoptic area of the hypothalamus
PRRs	Pathogen recognition receptors
PVN	Paraventricular nucleus of the hypothalamus
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
Slc	Solute carrier transporter family (organic cation)
Slco	Solute carrier organic anion transporter family
SOCS3	Suppressor of cytokine signaling 3
SSC	Side-scatter light
STAT	Signal transducer and activator of transcription
TAK1	Tumor growth factor β -Activated Kinase-1
TICAM-1	TIR domain-containing adaptor-inducing IFN-beta (or TRIF)
TIR	Toll/IL-1Receptor
TLDA	TaqMan Low Density Array
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRIF	TIR domain-containing adaptor-inducing IFN-beta (or TICAM-1)
vWF	Von Willebrand factor
WT	Wilde type mouse

Background

Introduction

Feeling sick is the lesser of the two evils when we are confronted with disease. While alarming us of an impending need for medical attention, the sickness behavior has helped researchers to reveal an intimate, critical relationship between the central nervous system and the immune system. Fever and release of stress hormones were the first sickness manifestations demonstrated to be brain-mediated responses triggered by the activation of the immune system when confronting pathogens, and not by pathogens alone. This direct relationship between the immune system and the central nervous system has given the mechanisms of immune-to-brain signaling a permanent place in the pathophysiology of inflammation, thus opening up for new therapeutic strategies in several inflammatory and inflammation-related diseases (Couzin-Frankel, 2010; Haroon et al., 2012).

Homeostasis in health and in sickness

The central nervous system (CNS) and the immune system are indispensable in complex organisms where several organs and systems have to interact. Through intricate feedback mechanisms, morphological, functional and metabolic demands are supported for each organ and system to create a stable unity, i.e. homeostasis (Figure 1).

To achieve homeostasis, there is a need to uninterruptedly monitor the integrity of tissues by removing senescent cells, apoptotic debris and pre-malign cells (immunological surveillance), as well as to coordinate the organs functionality (by the autonomic nervous system), and to adjust their metabolic demands and resources and regulate the body core temperature (through the neuroendocrine system, behavioral changes and thermoregulation).

Moreover, whenever the organism is challenged by invasive pathogens not only does the necessity of having the two systems as such become clear, but also the necessity of an immune-CNS collaboration. Systemic acute phase responses like fever, metabolic changes with release of stress hormones, as well as behavioral changes like anorexia, fatigue, increased slow wave sleep, warm-seeking behavior, and decreased social interaction (i.e. sickness behavior) (Hart, 1988) (Figure 2) are common cerebral-mediated manifestations in diseases associated with systemic inflammation.

Figure 1. Homeostasis requires tissue integrity, functional coordination, and metabolic and thermal regulation, which can be reinforced by behavioral changes. These processes are controlled by the CNS (left side) or the immune system (right side) through feedback mechanisms (colored circles with directional arrowheads; *orange triangle* shows the approximate position of the thermoregulatory region and *green line* marks of the pituitary gland in a sagittal brain section through the mouse brain).

Figure 2. Acute phase responses in systemic inflammation and the PGE_2 contribution in the CNS. Typical changes representing the sickness syndrome are presented in red text. Inflammation-induced prostaglandin E2 (PGE_2), released in the brain from the blood-brain barrier (BBB), is participating and contributing in varying degrees to the CNS responses. While it is critical for the fever response (whole red feedback circle), PGE_2 is only partly regulating (partially red feedback circles) the stress hormone release (cortisol) and sickness behavioral changes like anorexia.

Acute systemic inflammation

Inflammation is a central event in the pathogenesis of many diseases. It can express itself in various ways, playing a role in infections as well as in allergies, tissue damage, chronic rheumatic diseases and cancers. One of the most complex examples of molecular networking is seen in infections associated with acute systemic inflammation. While supporting the immune system in its fight to eliminate the intruder, the acute inflammation with its fast developing, simultaneous processes engaging several systems and organs can run the risk of progressing towards the worst scenario of septic shock and eventually death (Deutschman and Tracey, 2014).

Inflammation *per se* is a protective process against microbial intruders or endogenous danger molecules, aimed at minimizing both the invasion of pathogens and resulting tissue damage, and at restoring the internal homeostasis. It encompasses well-orchestrated induction and resolution processes that during a limited time frame, as long as the pathogen is recognized, allow defense mechanisms to identify and specifically eradicate intruders while it also initiates the healing process and tissue remodeling. The main conductor of inflammation is the immune system with its effector cells, leucocytes, macrophages, dendritic cells and mast cells, and its miscellaneous molecules, most importantly cytokines [interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), growth factors and chemokines], immunoglobulins and complement.

The intensity of the inflammatory process varies depending on the immune response and extent of the tissue damage. Scavenging is an *inflammation-free* function of immunity that takes place continuously in all tissues and helps maintaining a normal histology by eliminating senescent cells and apoptotic debris through phagocytosis (a physiological process of the innate immunity, i.e. immune surveillance) (Kerr et al., 1972). As soon as danger molecules are identified, the immune cells in the tissues release pro-inflammatory cytokines to initiate immune responses as well as *local inflammation*, clinically recognized by the cardinal signs of *calor, rubor, tumor, dolor* and *functio laesa* (Celsus A.C. 25 BC - ca AD 50, Galenus C AD 129 - ca 210, Virchow R *Cellular Pathology* 1858) (Ryan and Majno, 1977). If this process cannot be restrained or if the pathogens spread out, the immune system intensifies its effects by increasing leucocyte trafficking and cytokine release leading to *systemic inflammation* and clinical onset of systemic acute phase responses (Figure 2).

With an escalating severity of the infection there is a need for the immune system to amplify, renew and specialize the molecular and cellular immune components, leading to synthesis and release of acute phase proteins from the liver, enhanced myelo- and lymphopoiesis in the bone marrow, and selection and proliferation of pathogen-specific B and T-lymphocytes (adaptive immune response). Hence, the metabolic demands will increase, placing a strain on the

available energy resources. Together with other potentially aggravating factors like hydro-electrolytic and cardiovascular imbalances, organ functionality can deteriorate leading to organ/system failure and life-threatening conditions such as septic shock. Critical survival factors in acute systemic inflammation are the ability of the organism to prioritize the functions of the vital organs by maintaining the supply of blood and energy to these organs through the contribution of the CNS and the ability to sustain but also at the same time to restrain the immune response and minimize the risk of extreme immune activation/ immune overshooting (Besedovsky and Sorkin, 1977; Sternberg, 2006).

Immune-to-brain signaling

The collaboration between the immune system and CNS was long underestimated due to the dogmatic view of the brain as an immune privileged organ. The seclusiveness of the CNS was demonstrated more than a century ago by Paul Ehrlich (Ehrlich, 1885) and Edwin Goldman (Goldmann, 1913) using trypan blue stains (Hawkins and Davis, 2005; Siso et al., 2010). In various animals they noticed that when the dye was injected into the circulation, the brain and spinal cord remained “*white as snow*” in contrast to all other tissues that turned blue. While Ehrlich believed that this phenomenon depended on the brain’s low affinity for the dyes, Goldman demonstrated that the brain microvasculature functions as a barrier, since intracerebroventricularly (icv) administered dyes could stain the neuronal tissue but did not pass through it, to the outside the CNS (Bentivoglio and Kristensson, 2014; Goldmann, 1913). The barriers enclosing the CNS were later shown to display lower permeability and transcytosis than capillaries in other tissues, as well as limited trafficking of immune cells, properties that pointed out the brain as a tissue escaping the normal immune surveillance and which, as a consequence, became to be considered an “immune privileged organ”.

The immune-to-brain collaboration was revealed along with the discovery and characterization of pro-inflammatory cytokines as inducers of fever, a well-known cerebral (hypothalamic)-mediated response to infections. The first recognition of this collaboration resulted from the identification of *pyrogens* (molecules inducing fever), like *pyrexin*, in sterile extracts of damaged tissue (Menkin, 1944), later on to be named *endogenous pyrogens* (body’s own produced pyrogens) of sterile tissue extracts and exudates, and of neutrophils (Bennett and Beeson, 1953). Subsequently, Atkins and Wood independently could show that upon stimulation, not only neutrophils but several types of immune cells released different molecules that induced fever (Atkins and Bodel, 1971; Moore et al., 1970; Wood, 1962). These *endogenous pyrogens* proved to be rather large proteins of 15-40 kDa (Bernheim et al., 1979; Dinarello et al., 1974). Despite being unable to pass the blood-brain barrier (BBB), the cytokines evoked cerebral-mediated responses like fever and sickness behavior even if

administered peripherally and in the absence of a viral or bacterial infection that might affect the integrity of the CNS-barriers. Thus, early studies of cytokines (reviewed in Dinarello, 1996; Kluger, 1991 and briefly annotated in the cytokine section) revealed not only their functions but also demonstrated that sickness symptoms are not primarily due to infection-induced organ loss-of-function, but instead are early brain-coordinated adaptive responses evoked by peripheral immune signals (Dantzer et al., 1998; Hart, 1988), anticipating and preparing the organism for the septic-shock hazard. Hence, the question was raised on how this immune-to-brain signaling (or cytokine-to-brain signaling) takes place.

In addition to cytokines, another major mediator of inflammation was acknowledged due to its connection to an ancient remedy. Used as extracts of willow tree bark by Assyrians, Egyptians, and Greek and Roman physicians like Hippocrates, Celsus and Galen, the active substance salicylate was purified in the early 1800s and refined to the modern non-steroidal anti-inflammatory drugs (NSAIDs) to become the most common antipyretic, anti-analgesic and anti-inflammatory drugs used worldwide (Levesque and Lafont, 2000). Despite centuries of use, the action mechanism of acetylsalicylic acid (Aspirin) was not described until 1971 by Sir John Vane¹ who showed that acetylsalicylic acid was an inhibitor of prostaglandin synthesis (Vane, 1971). At this time, the prostaglandins² had already been structurally characterized by Sune Bergström and Bengt Samuelsson (Bergström, 1967; Bergström and Samuelsson, 1965; Samuelsson, 2012) making it possible to identify PGE as an inflammatory prostaglandin. Reports were soon published showing that immune-challenged animals displayed increased levels of PGE in the cerebrospinal fluid. In addition, PGE₂ injected into the cerebral ventricles (icv) was pyrogenic at low doses (Feldberg and Gupta, 1973; Feldberg and Saxena, 1971a; Feldberg and Saxena, 1971b; Milton and Wendlandt, 1971; Philipp-Dormston and Siegert, 1974). And additionally, if injected intradermally or intravascularly, PGE₂ decreased the pain threshold to induce hyperalgesia (Ferreira, 1972; Ferreira et al., 1973).

As a result of these early observations, pro-inflammatory cytokines and prostaglandins, particularly PGE₂, were acknowledged as key mediators in immune-to-brain signaling. Therefore, before describing the paradigms of immune-to-brain signaling that are central for the fever response and sickness behavior in general, each of the cytokines and prostaglandins relevant for the research for this thesis are introduced individually, addressing their roles in inflammation and sickness behavior.

¹ Sir John Vane, Sune K. Bergström and Bengt I. Samuelsson were awarded the Nobel Prize in Medicine in 1982.

² The name prostaglandin was given by Ulf von Euler after the prostatic gland, since prostaglandin was first identified in seminal fluid by Kurzrok, Lieb and Goldblatt during the 1930's.

Cytokines, PRRs, PAMPs, alarmins and DAMPs

The foundation for cytokine research was set almost 60 years ago by studies of endogenous pyrogens (Atkins and Wood, 1955; Bennett and Beeson, 1953) and the contemporary identification of interferon (IFN) (Isaacs and Lindenmann, 1957; Lindenmann, 2007). Since then, researchers have identified more than 300 cytokines (ILs, IFNs, TNFs, growth factors and chemokines) (Turner et al., 2014). These extracellular molecules are primarily produced to support the functions of the immune cells, and trafficking and communication between them, but also with and between somatic cells (from Greek, *cyto*-, cell and *-kinos*, movement), in health as well as during pathological processes.

Danger recognition

Immune and inflammatory responses are initiated by danger molecules (Matzinger, 2002). Exogenous, microbial-specific antigens expressing conserved *pathogen-associated molecular patterns* (PAMPs) and endogenous danger molecules i.e. *alarmins* (antigens that normally are not exposed to the immune surveillance unless there is a pathological accumulation or a tissue injury), are referred to together as *danger-associated molecular patterns*: DAMPs. They bind to *pathogen recognition receptors* (PRRs) expressed on cells at the front line of defense, namely macrophages, monocytes, dendritic cells, neutrophils and epithelial cells. PRRs are grouped into Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Kumar et al., 2011). Depending on the subsets of PRRs and cellular specialization/machinery of the receiver cell (e.g. macrophages, dendritic cells) the message will be translated into various degrees of innate (mostly inflammatory) and/or adaptive immune responses (antigen presentation and lymphocyte proliferation). While macrophages play a significant role in initiating the inflammatory response by an *inflammasome*³-dependent maturation and release of IL-1 β and IL-18 (Schroder and Tschopp, 2010), the dendritic cells are critical for initiating adaptive immune responses and further selection of pathogen-specific B and T-cells (Medzhitov and Janeway, 2002). PRRs respond to DAMPs most commonly by activating pro-inflammatory transcription factors like nuclear transcription factor beta (NF- κ B) and activating protein-1 (AP1), and interferon transcription factors (IRFs) for type I interferon production (Figure 3).

³ Large intracellular protein assembly occurring after NLRs recognition of DAMPs. NLRs cleave pro-caspase-1 to its active form caspase-1 which subsequently cleaves pro-IL-1 β and pro-IL-8

Endotoxin

Lipopolysaccharide (LPS) is an endotoxin, a heat-stable component of the cell-wall in gram-negative bacteria. It is the most widely used PAMP in animal models of inflammation as it can induce a septic-like syndrome in the absence of bacterial infection (Dantzer et al., 1998; Kluger, 1991) (and Beutler and Rietschel, 2003, with historical notes on endotoxin). Its effects have been shown to be transduced through Toll-like receptor 4 (TLR4) (Poltorak et al., 1998a)⁴ and its co-receptor myeloid differentiation factor-2 (MD2). On the cell membrane LPS binds to CD14 and is subsequently loaded on MD2 and presented for TLR4. This process induces oligomerization of TLR4 and signal transduction (Figure 3) (Kumar et al., 2011; Shimazu et al., 1999).

TLRs and Interleukin-1 receptors

The interleukin-1 receptor (IL-1R)/Toll-like receptor superfamily is characterized by the common, well conserved intracellular signaling TIR domain (Toll/IL-1Receptor; earlier coming from resistance). Activated TIR domains bind to two main adaptor proteins, myeloid differentiation factor 88 (MyD88, adaptor for all IL-1Rs and TLRs except TLR3) and TIR domain-containing adaptor-inducing IFN-beta (TRIF also known as TICAM-1, specific for TLRs) (Figure 3) (Casanova et al., 2011).

TLR4 is mostly expressed on myeloid cells (monocytes, macrophages, and glial cells) but also on non-hematopoietic cells like fibroblasts, endothelial cells and epithelial cells (Akira et al., 2006; Zhang et al., 1999). Although studied mostly for its LPS recognition, TLR4 has several other ligands of various origins. In addition to bacterial LPS, TLR4 can bind DAMPs of parasite (glycoinositolphospholipids), fungus (mannan), viral (envelope proteins RSV) and host origin (heat-shock proteins, fibrinogen) (Akira et al., 2006). Upon TLR4 activation, adapter proteins are recruited to TIR-domains and initiate complex downstream signaling pathways to activate several transcription factors like IRF3, NF- κ B and AP1. Of these, NF- κ B is activated by two pathways: an *early, MyD88-dependent* canonical pathway and a *late, MyD88-independent, TRIF-dependent* alternative pathway (Figure 3) (Akira et al., 2006; Palsson-McDermott and O'Neill, 2004).

Interleukin-1 receptors (IL-1Rs) binding IL-1 α and IL-1 β are type I and type II IL-1R (IL-1R1, IL-1R2), and IL-1R accessory protein (IL-1RAcP). Typical for IL-1Rs is the extracellular chain with 3 immunoglobulin-like domains in addition to the intracellular TIR domain shared with TLRs (Figure 3). IL-1R2 is different from the other IL-Rs by lacking the TIR-domain.

⁴ Bruce A Beutler and Jules A Hoffmann, finders of TLR4, were awarded the Nobel Prize in 2011 together with Ralph A Steinman, for his discovery of the dendritic cell.

Without TIR-domain IL-1R2 lacks the possibility of downstream signal transduction and thus acts as a decoy receptor trapping IL-1. IL-1R2 exists both as a membrane receptor and a soluble receptor in the extracellular space.

IL-1R1 and IL-1RAcP form the active IL-1 receptor by heterodimerization and intracellular TIR-dimerization. They are found prominently on endothelial cell, smooth muscle cells, epithelial cell, hepatocytes, fibroblasts, keratinocytes, epidermal dendritic cells, monocytes and T-lymphocytes (Dinarello, 1996). IL-1Rs signal through a *MyD88 canonical pathway* to activate NF- κ B and/or AP1 (Figure 3) (Casanova et al., 2011; O'Neill, 2008).

Pro-inflammatory cytokines

IL-1 β , IL-6 and TNF α as the main pro-inflammatory cytokines have been extensively studied in different animal inflammatory models (acute, chronic, local or systemic) and more recently as mediators of autoinflammation.

Autoinflammation as a pathological process of the phagocytic cells was described a century ago by Elie Metchnikoff (1845-1916) but was then neglected until the late 1990s. The new term *autoinflammation* was defined by McDermott along with the discovery of the cause of the **hereditary periodic fevers**, a set of distinct clinical entities that could not be attributed to “*infectious, allergic, endocrinologic, epileptic or migrainous influences*” [as reviewed by (Reimann, 1949)]. Hereditary periodic fevers proved to be monogenic syndromes, with mutations limited to the genes of the innate immunity such as TNF-receptor 1 gene (McDermott et al., 1999) or IL-1/inflammasome-related genes (Mariathasan et al., 2004). These discoveries led to viewing *autoinflammation* (McDermott et al., 1999) as a new immunopathogenesis distinct from *autoimmunity* (driven by adaptive immune effectors: autoantibodies and auto-reactive B and T-lymphocytes). It came to confirm that cytokine imbalances leading to “sterile” systemic inflammation can evoke cerebral responses like fever. The distinction between autoinflammation and autoimmunity has led to revision of the pathogenesis of a long list of chronic diseases, not only monogenic but also polygenic, like Crohn’s disease, diabetes mellitus and most of rheumatic diseases (McGonagle and McDermott, 2006) and even depressive disorders and neurodegenerative diseases like Alzheimer’s disease, Parkinson’s disease, and amyotrophic lateral sclerosis. As a result, anti-proinflammatory cytokine therapy trials (anti-TNF α , anti-IL-6 and anti-IL-1 β) have been started for several of these entities (Bradley, 2008; Chou, 2013; Dinarello et al., 2012; Rothwell and Luheshi, 2000).

Interleukin-1

IL-1 β and IL-1 α (together referred to as IL-1) were the first cytokines to be sequenced and cloned in the IL-1 family (Auron et al., 1984; March et al., 1985), a family of 11 known members that are ligands for receptors within the IL-1R-family (Garlanda et al., 2013). Although sharing only 24% protein sequence identity and acting in different pathological conditions, IL-1 α and IL-1 β induce similar responses as they bind to the same receptors. IL-1 α is expressed in epithelial cells, endothelial cells and astroglia. It is considered an *alarmin* as it can bind to IL-1R1 only during tissue stress when IL-1 α is exposed on the cell membrane or released in the extracellular space from necrotic cells. IL-1 α is active both as precursor and in a cleaved form. In contrast, IL-1 β is mainly produced by monocytes and phagocytes in response to DAMPs, immune complexes, and pro-inflammatory cytokines (IL-1, TNF α , IL-6). Its activity is controlled at several levels, enhancing the possibility to restrain the powerful inflammatory effect of IL-1 β . Firstly, in unstimulated cells, IL-1 β is similar to IL-1 α expressed as a pro-peptide, but at very low levels and as an inactive form. For an IL-1 β activation to occur two processes are needed, first an up-regulation of the pro-IL-1 β transcript, through TLRs or cytokine receptor signaling, and the second a cleavage activation of pro-IL-1 β peptide by caspase-1 (IL-1-converting enzyme) that is also cleaved from its pro-form by NLRs activated *inflammasomes* (Schroder and Tschopp, 2010). Hence, the production and release of IL-1 β is depending on a double PRR-signaling. Secondly, extracellular IL-1 β acts mostly in an auto- and paracrine way as IL-1R1 and IL-1R2 are richly expressed on most cells and even as soluble receptors (IL-1sRII). The blood levels of IL-1 β are accordingly lower than what could be expected, even in septic patients. Thirdly, specific negative regulators like IL-1 receptor antagonist (IL-1Ra) are released in response to inflammation. IL-1Ra binds and blocks IL-1Rs, although with a lower affinity than IL-1. It is estimated that up to 1000-times more IL-1Ra than IL-1 is needed to block IL-1 signaling, because of the lower affinity of IL-1Ra compared to that of IL-1. It is well-known that low doses of IL-1 β are enough to induce specific responses. On a cellular level, it was shown that as few as 2% of all IL-1R1 need to be activated to induce an effect (Arend, 2002; Dinarello et al., 2012; Garlanda et al., 2013; Sims and Smith, 2010; Turner et al., 2014).

IL-1 β in immune-to-brain signaling

Discovered as a macrophage-released factor that can potentiate T-lymphocytes, IL-1 was first named *lymphocyte-activating factor* (LAF) and studied for its role in the adaptive immune system (Gery et al., 1972; Mizel et al., 1978). Soon after it was acknowledged that IL-1 is a “pleiotropic, nonspecific hormone-like cytokine” that can induce various cells to differentiate and proliferate (Oppenheim et al., 1986). For its capacity to potentiate T-cells and induce neutrophilia (or neutrophil leukocytosis) at low doses, intravenous (iv) injection of IL-1 β was even tested on human volunteers and cancer patients but with unsatisfactory results since it was

found to be a strong inducer of unspecific sickness-related symptoms like fever, anorexia, fatigue, and gastrointestinal and sleep disturbances (Dinarello, 1996). Also during the 1980s it was shown in animal models that IL-1 β , when injected intravenously, gives rise to complex cerebral responses as it induces not only fever and sickness behavior, but also regulates the HPA-axis (hypothalamic-pituitary adrenal axis), increasing the blood levels of ACTH and glucocorticoids (Besedovsky et al., 1986) via central release of corticotropin- releasing factor (Berkenbosch et al., 1987; Sapolsky et al., 1987). Still, during systemic inflammation these properties proved not to be specific for IL-1 β as first hypothesized (Atkins, 1984). Several other pyrogens were soon identified like TNF α and interferons (Atkins, 1988; Dinarello et al., 1988), and it was also shown in animal models that blocking IL-1 signaling would not abrogate fever, sickness behavior or HPA-axis activation. These studies led to various results due to their methodological complexity. Hence, in LPS-treated animals, impeding the IL-1 signaling either by exogenous IL-Ra, by blocking antibodies for IL-1 or IL-1R1, or by deleting genes critical for the IL-1 signaling (genes for IL-1 β and IL-1R1, caspase-1) led to divergent results (Alheim and Bartfai, 1998; Dinarello, 1996; Fantuzzi and Dinarello, 1996). The fever response was either attenuated (Kozak et al., 1998), normal (Leon et al., 1996) or exacerbated (Alheim et al., 1997). As for the HPA-axis activation by IL-1 β , corticosteroid levels were reported to be either unchanged (Kozak et al., 1998) or increased (Alheim et al., 1997). These studies and many other related studies came to reveal what is widely accepted today, namely that the cytokine networking in systemic inflammation is complex as pro-inflammatory cytokines are pleiotropic and redundant. They have many different target cells, and the individual cytokines interact in complex ways by inducing each other or by subserving similar functions which makes it possible for them to compensate for each other (Zetterstrom et al., 1998). An elegant study on cytokine redundancy was carried out on mice lacking the gene for IL-1R1. It showed that in the absence of IL-1 signaling, the LPS-induced sickness behavior is controlled by TNF α , compensating for IL-1 β , and that it can be abrogated by TNF-binding protein (Bluthe et al., 2000).

Interleukin-6

Interleukin 6 is the main member of the IL-6 family, a family characterized by the common plasma membrane glycoprotein 130 (gp130, also known as CD130) needed as signal transducer subunit in their receptor complexes (Figure 3). IL-6 blood levels were found to be increased in septic patients and animal models of inflammation, and it is hence well-known for its immune and pro-inflammatory properties. It is a strong inducer of acute phase proteins in the liver, it activates the HPA-axis, and it modulates lymphocyte differentiation and proliferation. It also activates endothelial cell to express adhesion molecules and chemokines and it increases vascular permeability. Recent advances [reviewed in (Scheller et al., 2011)] assign IL-6 newer

functions in e.g. metabolic regulation and neuronal plasticity (which fall outside the aim of this thesis). It has also been shown that IL-6 has both pro- and anti-inflammatory properties, mostly as a result of its intricate receptor-complex building.

IL-6 receptor alpha (IL-6R, alternative names gp80, CD126) and gp130 exist as preformed receptors on the plasma membrane and as soluble receptors in the extracellular space. While gp130 is ubiquitously expressed on cells, IL-6R is constitutively expressed on macrophages, neutrophils, glial cells, hepatocytes and some types of T-lymphocytes, and after immune challenges also in the brain blood vessels (Vallieres and Rivest, 1997). After IL-6 ligand binding, IL-6R associates with two gp130 subunits resulting in dimerization of the intracellular signaling domains and downstream signal transduction (Figure 3). This is the IL-6 *classic-signaling* in cells that express membrane IL-6R (mbIL-6R). Cells lacking mbIL-6R can still respond to IL-6 by attaching soluble IL-6R (sIL-6R). IL-6/sIL-6R complexes can bind to membrane gp130, and induce an IL-6 *trans-signaling*. The soluble IL-6R is hypothesized to be generated by a proteolytic and/or transcriptional mechanism. Shedding or proteolytic cleavage of existing membrane IL-6R was demonstrated to be done by membrane metalloproteases ADAM17 and ADAM10, whereas the transcription mechanism is dependent on generation of an alternatively spliced IL-6R mRNA lacking the transmembrane and cytosolic domains (Scheller et al., 2011). *Trans-signaling* has been shown to be a central mechanism for monocyte recruitment during the non-phlogistic removal of apoptotic neutrophils (scavenging). Senescent neutrophils programmed to die (apoptotic neutrophils) shed their membrane IL-6Rs which instead activate endothelial cells through *trans-signaling*, thus inducing release of monocyte-specific chemokines (Chalaris et al., 2007). This process was proved to be relevant also in the resolution phase of acute inflammation, thus identifying IL-6 as an anti-inflammatory cytokine. The initial neutrophil tissue infiltration during the induction phase of inflammation is followed by apoptotic neutrophil IL-6R-shedding and IL-6 trans-signaling on endothelial cells. This process leads to a switch in the endothelial cells from release of neutrophil specific chemokines to release of monocyte and T-lymphocyte specific chemokines and subsequent monocyte infiltration in the surrounding tissue (Chen et al., 2006; Hurst et al., 2001). Proteolytic shedding is seen also for the membrane gp130, but unlike sIL-6R, soluble gp130 is inactive and blocks sIL-6/IL-6R trans-signaling by complex sequestration.

IL-6 and immune-to-brain signaling

IL-6 has an indirect, critical role for fever generation. IL-6 fails to elicit fever by itself, unless given together with a low-dose of IL-1 β (Cartmell et al., 2000). Nonetheless, in mice lacking IL-6 (IL-6 knock-out/KO mice or mice pretreated with IL-6 antiserum) the fever response and other components of sickness behavior are blunted both to LPS (Chai et al., 1996; Nilsberth et al., 2009a), though not to a high dose of LPS (Kozak et al., 1998), and to turpentine (Kozak et al., 1998; Kozak et al., 1997). Thus circulating IL-6 is necessary but not sufficient for fever

and other sickness-behavior responses. LPS-treated IL-6 KO mice were shown to release more TNF α , and have lower circulating levels of PGE₂ (Kozak et al., 1998). Contrasting findings were shown by our group. Thus, PGE₂ levels in the cerebrospinal fluid and hypothalamic transcripts for cytokines and PGE₂-synthesizing enzymes were similar between LPS treated WT and IL-6 KO mice (Nilsberth et al., 2009a), suggesting that IL-6 is not directly involved in the production of PGE₂ but that it might modulate the PGE₂ release on its targets, i.e. neuronal cells. However, in a subsequent study on IL-6R α transgenic mice, deletion of the IL-6 receptor on brain endothelial cells resulted in decreased COX-2 levels and blunted fevers, implying that COX-2 is downstream of and partly regulated by IL-6 (Eskilsson A 2014).

Tumor necrosis factor

TNF α was first considered to be a component of endotoxins and was studied as early as 1891 by Coley (Coley, 1893) for its property of being able to induce tumor regression (Nauts et al., 1946). As bacterial toxins could not directly induce necrosis in tumor cultures, an indirect endotoxin action mechanism was suggested by Algire (Algire et al., 1952). This was proved to be true in 1975, when a macrophage-derived serum factor was shown to retain the tumor necrosis effect of endotoxin (thus called tumor necrosis factor) (Carswell et al., 1975). Ten years later, at the time of its isolation and characterization (Aggarwal et al., 1985), it became clear that TNF is a pleiotropic cytokine, proving to be the same molecule as cachectin (Beutler et al., 1985) that induced anorexia and cachexia, and in high doses septic shock (Tracey et al., 1986). This effect was not due to an LPS contamination, as was first hypothesized, since it was shown also in endotoxin-resistant mice (Cerami et al., 1985), which were later demonstrated to be lacking a functional TLR4 (Poltorak et al., 1998a; Poltorak et al., 1998b).

Today the TNF superfamily includes 30 receptors and 19 associated ligands [reviewed in (Aggarwal, 2003; Tracey et al., 2008; Turner et al., 2014)]. TNF α plays important roles in cell differentiation, inflammation, immunity, metabolism and apoptosis. It is produced by activated immune cells, monocytes, macrophages, T-cells, mast cells, and NK cells, but has also been shown to be expressed in keratinocytes, fibroblasts, and neurons. It has a membrane precursor (mTNF α) that once present on the plasma membrane is assembled non-covalently in trimers. The soluble TNF α (sTNF α) is the cleaved ectodomain of mTNF α released by metalloproteases like ADAM17 (Figure 3).

Both sTNF α and mTNF α bind to the same receptors TNFR1 (TNFRSF1a, CD120a) and TNFR2 (TNFRSF1b, CD120b). TNFR1 and TNFR2 are homo-trimers formed on the plasma membrane independently of ligand-receptor binding. While TNFR1 is ubiquitously expressed on all cells, TNFR2 is considered an inducible receptor present mostly on leukocytes and

endothelial cells (Bradley, 2008). TNFR1 differs from TNFR2 by presenting an intracellular death-domain (TNF receptor associated death domain, TRADD) that can induce either apoptosis or promote cell survival and inflammation through its two adaptor molecules FADD (Fas-Associated Death Domain) and TRAF (TNF Receptor Associated Factor), respectively (Figure 3). The pathway-selection strategy is as yet poorly understood. Of the two receptors TNFR1 has proved to be a stronger NF- κ B activator and to have higher affinity for TNF α than TNFR2, thus being considered the most important pro-inflammatory TNF α receptor. TNFR2 is less well characterized but was shown to promote tissue repair and angiogenesis (Bradley, 2008). Like gp130, TNFRs are shed by metalloproteases (e.g. ADAM17) stopping the intracellular TNF α signaling and eventually promoting the resolution phase of inflammation.

TNF α and immune-to-brain signaling

TNF α administered to cancer patients induced sickness symptoms (Creagan et al., 1988; Steinmetz et al., 1988), similarly to IL-1 β . In contrast to IL-1 β , TNF α is not a direct pyrogen. When administered to humans or animals it induced fever (Dinarello et al., 1986; Morimoto et al., 1989b) probably through production of IL-6 (Sundgren-Andersson et al., 1998). However, physiologically it is considered an endogenous antipyretic (a cryogen) opposing the pyrogenic effect of IL-1 β , as animals with TNF-blockade, by TNF-blocking antibodies (Long et al., 1990; Mathison et al., 1988) or lacking the TNFR-genes (Leon et al., 1997), responded with exacerbated fever to LPS treatment.

General remarks on the main transcription factors of inflammation

The signaling pathways downstream from the cytokine receptors are too complex to be addressed in detail, but some important characteristics deserve to be highlighted (Figure 3).

The cellular responses depend not only on the presence of a certain individual receptor, but on the cellular receptor profile and on the specific intracellular machinery. Cytokine signals are integrated and several transcription factors can be induced to translocate simultaneously to the nucleus. Inducible genes have complex promoter regions where several transcription factors (e.g. AP-1, NF- κ B, STAT3, IRF3) and nuclear receptors (e.g. glucocorticoid receptors) interact to switch on/off the gene transcription (De Bosscher et al., 2003; McKay and Cidlowski, 1999). Both TLR4, IL-1R1, and TNFR converge on the NF- κ B and AP-1 transcription factors but through different intermediate steps (Aggarwal, 2003; O'Neill et al., 2013).

For **NF- κ B** (Figure 3) two main pathways have been described: a canonical pathway for TLR4 and IL-1R1 (TIR-receptors) and a non-canonical pathway for TNFRs. In the *canonical pathway* IKK (I κ B kinase complex) is phosphorylated by TAK1 (TGF β -Activated Kinase-1) with its

associated binding proteins TAB1 and TAB2. As mentioned above, TLR4 can bind two different receptor adaptors MyD88 and TRIF that activate NF- κ B in an early and a late manner, respectively (Casanova et al., 2011; Hayden and Ghosh, 2008). In the *non-canonical pathway* IKK is phosphorylated directly by RIP1 (Receptor-Interacting Protein-1), a protein often associated to TRAF proteins. The activation of the IKK complex leads to phosphorylation and degradation of I κ B α with subsequent release of the NF- κ B heterodimers p65/p50 to translocate to the nucleus. NF- κ B activation induces the expression of well over 150 genes, for example inflammation-related genes like IL-1, IL-6, TNF- α , cell adhesion molecules (E-selectin, ICAM-1, VCAM-1, fibronectin), and prostaglandin synthases (phospholipase A2, cyclooxygenase-2) (comprehensive list in Table 1 of Kumar et al., 2004). Interestingly, recent reports have found a TAK1 independent NF- κ B activation diverging at TRAF6 (Yamazaki et al., 2009) as well as a predominant NF- κ B independent TAK1-dependent COX-2 up-regulation, through p38 MAPK and c-Jun activation, in brain endothelial cells (Ridder et al., 2011). These later findings raise questions about the complexity and degree of specialization of the brain endothelial cells.

IL-6 communicates through a **JAK/STAT** (JAnus Kinase/ Signal Transducer and Activator of Transcription) signaling pathway but can also activate MAPKs (mitogen activated-protein kinases) and eventually AP-1 (Heinrich et al., 2003) through its receptor adaptor JAK. IL-6-JAK/STAT signaling transmission takes place through nuclear translocation of STAT3 (most commonly) (Figure 3) or STAT1, as phosphorylated homo-dimers.

There are complex, not fully elucidated crosstalk communications between the signaling pathways. While several pathway regulators await characterization, I κ B α , a typical NF- κ B binding inhibitor (Kumar et al., 2004) and SOCS3 (suppressor of cytokine signaling 3) blocking the JAK kinases from phosphorylating STAT proteins (Heinrich et al., 2003), are generally accepted as markers for pathway activation of NF- κ B and STAT3, respectively.

Figure 3. IRF3, NF-κB, and STAT3 signaling. Upon activation, both IRF3, NF-κB and STAT3 move to the nucleus to contribute to the gene transcription regulation together with nuclear hormone receptors like glucocorticoid receptors (GR) bound to glucocorticoid hormones. IRF3 is activated by LPS at TLR4 through TRIF-TRAF3 IKKε phosphorylation. NF-κB is activated by IKKαβ either by LPS (at TLR4) or IL-1 (at IL-1R1/IL-1RacP) through a canonical TAK1-pathway, or by TNF (at TNFR1 and TNFR2) through a non-canonical RIP mediated pathway. Both RIP and TAK1 kinases are activated by specific TRAF proteins recruited and phosphorylated by receptor adaptor kinases, i.e. IRAK2 (interleukin-1 receptor associated kinase-like 2) for MyD88, TRADD for TNFR1 or directly by TNFR2. TNFR1 can induce either inflammation/cell survival by NF-κB activation or apoptosis by the FADD-caspases pathway. IL-6 induces phosphorylation and dimerization of STAT3 by the IL-6R adaptor protein JAK. The IL-6 receptor can be formed either by IL-6R and gp130, for classic signaling, or by soluble IL-6R (sIL-6R) and gp130 for trans-signaling (yellow box). TNFα and several receptors (TNFR1, TNFR2, gp130 and IL-6R) can be cleaved from membranes by proteases (represented by scissors) like ADAMTS17. IκBα and SOCS3 are inhibitors for NF-κB and STAT3, respectively.

Prostaglandins

The prostaglandin cascade

Prostaglandins are cyclic, oxygenated eicosanoids (Greek *eicos*-, 20) originating from twenty-carbon, polyunsaturated fatty acids like arachidonic acid from membrane phospholipids. The prostaglandins (as all eicosanoids) are highly bioactive lipids, produced in minute volumes throughout the body. In general, they act locally fulfilling both physiological and pathological functions, playing a role in inflammation, immunity, cardiovascular and renal homeostasis, bone metabolism, reproduction, and sickness behavior. Several stimuli can modulate the prostaglandin production at three different enzymatic levels (Figure 4).

The first level of regulation is the cytosolic release of arachidonic acid from the *sn*2 position of the membrane phospholipids. This step is enzymatically catalyzed by phospholipase A₂ (PLA₂)

Figure 4. The Prostaglandin Cascade. In red inflammation-induced enzymes, COX-2 and mPGES-1

a heterogeneous enzyme superfamily that may also be associated with other enzymatic activities unrelated to the eicosanoids metabolism (Six and Dennis, 2000). The PLA₂s can roughly be classified into cytosolic and secreted forms, with tissue specificities that await characterization. The cytosolic PLA₂s are intracellular membrane associated enzymes and have been shown to have a high arachidonate-substrate preference and therefore to be the dominant but not exclusive PLA₂ regulating the eicosanoid metabolism (Farooqui et al., 1997). In a rat model of acute inflammation, both secretory PLA₂–IIA and cytosolic PLA₂– α were upregulated by LPS in the brain as well as in the peripheral organs, connecting them to the fever response and immune-to-brain signaling (Ivanov et al., 2002).

The second level of regulation is a prostaglandin specific step. Arachidonate, a substrate for all eicosanoids, once mobilized from the membrane is committed towards prostaglandin production by cyclooxygenases (also known as prostaglandin endoperoxide H synthases, PTGS). The first enzyme to be identified was cyclooxygenase 1 (COX-1 or PTGS-1) (Hamberg et al., 1974), followed in 1991 by the identification and cloning of cyclooxygenase 2 (COX-2 or PTGS-2) (Kujubu et al., 1991; Xie et al., 1991). Although catalyzing the same steps, converting arachidonic acid to PGG₂ and finally to PGH₂, the COX enzymes are distinct in several aspects. They are encoded by genes on different chromosomes (in mouse on chromosome 2 and 1, respectively; gene ID for *Ptgs1*:

19224 and for *Ptgs2*: 19225), and share only 60-65% amino acid sequence identity (Smith et al., 2011). They also show different patterns of expression. COX-1 is expressed constitutively in all tissues but by fewer cell types (e.g. macrophages, fibroblasts), estimated to be limited to approximately 10% of all mammalian cells, while COX-2 is transiently expressed, probably in all cells during replication/differentiation (Smith et al., 2011) and also during inflammation/cellular stress (Dubois et al., 1998). Of the two enzymes, inducible COX-2 was demonstrated to be necessary for the sickness syndrome (Li et al., 1999; Lugarini et al., 2002). Brain COX-2 was found to be constitutively expressed in neurons (Breder et al., 1995; Yamagata et al., 1993) and reported to be induced by immune stimuli in vascular cells (Cao et al., 1995), and PVCs (Breder and Saper, 1996) but also in neurons by e.g. peripheral local inflammation, mechanical pain, seizures or brain tissue injury (An et al., 2014; Samad et al., 2001; Serrano et al., 2011; Vardeh et al., 2009; Yamagata et al., 1993). While neuronal COX-2 is seen in neuronal insults or neuronal progenitor cells, proposed to be participating in cellular survival, development and proliferation, vascular COX-2 is central for the immune-to-brain signaling.

The third level of regulation is specific for each terminal prostaglandin. From the common precursor PGH₂, prostaglandin products are formed through specific synthases such as hematopoietic and lipocalin-type PGD₂ synthases (H-PGDS, L-PGDS), PGE₂ synthases (to be presented separately), PGF₂ synthase (PGFS), prostacyclin synthase (PGIS) and thromboxane A synthase 1 (TXAS).

Prostaglandins exert their functions through specific membrane G-protein coupled receptors (GPCRs) with seven transmembrane domains, i.e. heptahelical or serpentine receptors: DP1, EP1-4, FP, IP and TP, and in some cases even nuclear peroxisome proliferator activated receptors (PPARs). In addition, a second PGD₂ receptor was identified which is not a GPCR but a chemoattractant receptor expressed on some leucocytes (Abe et al., 1999; Hirai et al., 2001).

PGE₂ biosynthesis

Three PGE₂ specific enzymes have been identified and characterized, two microsomal (membrane-bound) and one cytosolic enzyme. Microsomal prostaglandin E synthase-1 (mPGES-1) is an inducible enzyme (Ek et al., 2001; Jakobsson et al., 1999), with low constitutive levels in most tissues (Jakobsson et al., 1999) and is often co-expressed and functionally coupled with COX-2 (Murakami et al., 2000; Samuelsson et al., 2007; Thoren and Jakobsson, 2000). Being inducible enzymes, the COX-2 and mPGES-1 genes have complex promoter regions with binding motifs for several transcription factors like NF- κ B (Crofford et

al., 1997; Diaz-Munoz et al., 2010) and early growth response-1 (Egr-1) (Diaz-Munoz et al., 2010). Cytosolic prostaglandin E synthase, the second enzyme to be discovered, is constitutively expressed and functionally coupled with cPLA2 and COX-1 (Tanioka et al., 2000). The third enzyme is microsomal prostaglandin E synthase-2 (mPGES-2), also a constitutively expressed enzyme that was found to couple unselectively to both COX-1 and COX-2 (Murakami et al., 2003).

PGE₂ degradation

Prostaglandins are short-lived lipid mediators, with an estimated half-life in blood plasma of 30 seconds to a few minutes (Samuelsson et al., 1975). The 15-hydroxy-prostaglandin dehydrogenase (15-PGDH or PGDH type I) and carbonyl reductase-1 (CR1 or PGDH type II) are key enzymes to inactivate prostaglandins (Ensor and Tai, 1995) as they mediate the first catabolic step, the oxidation of the 15(S)-hydroxyl group with subsequent high reduction of the prostaglandin activity (Anggard, 1966). 15-PGDH is a prostaglandin specific enzyme while CR1 has a low prostaglandin turn-down kinetics and a larger substrate profile, extending outside the eicosanoid family (Tai et al., 2002). 15-PGDH is an ubiquitous enzyme, but with its highest activity levels measured in the lungs (Piper et al., 1970). For the PGE₂ metabolism, there are some reports showing a reciprocal regulation between COX-2 and 15-PGDH in inflammation with increased PGE₂ production due to a coordinated up-regulation of COX-2 and down-regulation of 15-PGDH (Hahn et al., 1998; Ivanov et al., 2003; Tai et al., 2006).

PGE₂ transporters

Prostaglandins are known to have a low membrane permeability capacity. Newly synthesized prostaglandins, protonated in the acidic cellular environment, are released by passive diffusion to become anionic at the higher extracellular pH levels thus losing their ability to readily cross membranes (reviewed in Schuster, 1998 and Schuster, 2002). These findings, originating during the 1960s and early 1970s, led to the search for prostaglandin transporters that would allow cellular influx of PGs and enzymatic degradation by PGDHs. In the CNS, the necessity of such transporters was inferred to be even greater than in other organs and tissues, as the brain was shown to have a low capacity to metabolize prostaglandins (Nakano et al., 1972), and also to have a high sensitivity to icv-injected PGE₂ affecting body temperature (Feldberg and Saxena, 1971b), release of neurotransmitters (Bergström et al., 1973) and, at high doses, causing sedation and catatonia among other behavioral changes (Horton, 1964). Thus it was hypothesized that the CNS prostaglandin clearance was mainly achieved by barrier-transporters that can act against an increased blood-brain prostaglandin gradient (active or

facilitated transport) to protect the brain against the deleterious effects of high PG levels during conditions like infections or pregnancies (Bito et al., 1976). Influx/efflux prostaglandin transporters have been characterized in an ever growing number. The transporters were shown to allow prostaglandin outflow from the brain tissue by exerting their actions at the polarized CNS-blood barrier. It was also demonstrated that this process is slowed at the transporter level by β -lactam antibiotics like cefmetazole and cefazolin or during inflammation (Akanuma et al., 2010; Akanuma et al., 2011; Tachikawa et al., 2012). Prostaglandin influx transporters are found in both the solute carrier organic anion (Slco) transporter family and the cation (Slc) transporter family. **Slco2a1**, the very first one to be identified and thus also known as prostaglandin transporter (Slco2a1/PGT) (Kanai et al., 1995) and **Slco2b1** (or multispecific organic anion transporter, moat1) (Nishio et al., 2000) are known to be widely expressed in almost every tissue, while **Slco3a1**, also an ubiquitous transporter (or subtype D organic anion transporter OATP-D) (Adachi et al., 2003), **Slco1a2** (Oatp3) and **Slco1b2** (Oatp4) (Cattori et al., 2001), are transporters with very broad substrate specificity (Huber et al., 2007). Cation influx transporters shown so far to have prostaglandin affinity are **Slc22a8** (Oat3) (Kobayashi et al., 2004) and **Slc22a22**, (Shiraya et al., 2010), with Slc22a22 mostly expressed in the kidneys. The best characterized efflux transporter is a membrane transport pump, Multi drug resistance-associated protein 4 or ATP-binding cassette transporter 4 (**Mrp4/Abcc4**) (Akanuma et al., 2010; Reid et al., 2003), but still more transporters might be expected to exist.

Another transportation modality, through nanovesicles also called exosomes, was recently shown functional for eicosanoids and their synthesizing enzymes. Exosomes are involved in cell-cell communication and transportation of several different RNAs and lipid molecules. For prostaglandins they were shown to allow transcellular prostaglandin synthesis through exosomes originating from mast cells (Record et al., 2014). The importance of the exosomes, believed to originate from all living cells, has not yet been tested in immune-to-brain signaling.

PGE₂ receptors

Among prostanoids, PGE₂ has the widest receptor profile with four subtypes EP1, EP2, EP3 and EP4 and several EP3 splicing isoforms at the C-terminal tail, allowing binding to different G-proteins. The number of EP3 splice isoforms is species specific, with 3 identified in mouse (EP3 α , EP3 β and EP3 γ) (Irie et al., 1993; Sugimoto et al., 1993), 4 in rat (Neuschaferrube et al., 1994; Oldfield et al., 2001; Takeuchi et al., 1993) and 9 isoforms (out of which 8 are functional) in humans (Kotani et al., 1997). The EP receptors bind PGE₂ with varying affinities. In Chinese hamster ovary cells expressing stable levels of prostanoid receptors (Kiriya et al., 1997) or in HEK cells (human embryonic kidney cells) expressing recombinant prostanoid receptors (Abramovitz et al., 2000) the highest affinity was shown by EP3 followed by EP4

and then by low affinity receptors EP2 > EP1. In addition to its high affinity for PGE₂, EP3 showed also a broad ligand binding profile not only among EP ligands but also for other prostanoids as e.g. the thromboxane analog STA₂ or the prostacyclin analog iloprost (Kiriya et al., 1997).

The versatile actions of PGE₂, widely produced in the body, are controlled by the specific cellular EP profiles with their dynamic changes through receptor up-regulation (for EP2, EP4) and/or desensitization (for EP4, EP3), transactivation or cross-desensitization, and finally by the different signal transducing properties of each EP receptor (Sugimoto and Narumiya, 2007; Woodward et al., 2011). Thus, EP1 is a “contractile” receptor coupled to G_q that by its activation increases intracellular Ca²⁺ (Coleman and Kennedy, 1985; Coleman et al., 1985; Watabe et al., 1993). EP2 and EP4 are “relaxant” receptors coupled to stimulatory G proteins (G_s) mediating increases in intracellular cAMP (An et al., 1993; Coleman et al., 1994; Honda et al., 1993; Narumiya and FitzGerald, 2001). Reports from 2000s show that they were also able to mediate transcriptional activation through e. g. phosphokinase A-dependent pathway for EP2 or phosphatidylinositol 3-kinase-dependent pathway and other pathways for EP4 that in the latter case was shown to bind to a G_i protein (Fujino and Regan, 2006; Fujino et al., 2005; Fujino et al., 2002) (reviewed in Yokoyama et al., 2013). EP3, the only constitutive “inhibitory” receptor in the prostanoid receptor family (Narumiya and FitzGerald, 2001), is the most abundant PGE₂ receptor subtype in the brain (Sugimoto and Narumiya, 2007). Its isoforms are constitutively coupled to inhibitory G-protein (G_i) decreasing cAMP but they can also activate other second messenger systems like inositol triphosphate with subsequent increase of intracellular calcium (Namba et al., 1993; Negishi et al., 1989), Rho to mediate cytoskeleton formation (Hasegawa et al., 1997), or, as shown for EP3γ, by dual coupling to both G_i and G_s to modulate cAMP levels in an agonist-dependent manner (Irie et al., 1993; Negishi et al., 1996).

In the brain, EPs were mostly found to be associated with neuronal and glial cells (Batshake et al., 1995; Ek et al., 2000; Nakamura et al., 2000; Oka et al., 2000; Sugimoto et al., 1993; Zhang and Rivest, 1999). However, EP2 and EP4 were but recently reported to be expressed in the endothelium, and to be induced after stroke (Li et al., 2008). Interestingly, the endothelial EP4 receptor was also reported together with the EP3 receptor to have perinuclear localization (Bhattacharya et al., 1999). In the latter case, the perinuclear EP3 receptors seemed to have an atypical downstream signaling that by PGE₂ binding at the nuclear membrane could induce the expression of endothelial nitric oxide synthase (eNOS) (Gobeil et al., 2002).

Pathways for cytokine-to-brain signaling

Several paradigms for immune-to-brain signaling have been tested and the number of substances suggested to participate in the process has increased tremendously. To the pro-inflammatory cytokines and prostaglandins have been added other cytokines (e.g. interferons) and eicosanoids, complement factors (e.g. C5a), chemokines (IL-8, CCL5, CXCL10, MIP-1), adhesion molecules, intracellular signaling molecules and many more (Rivest, 2009). Nonetheless, for the purpose of present thesis, only general issues will be addressed regarding the cytokine-to-brain signaling pathways, followed by a detailed characterization of PGE₂ at the BBB in immune-to-brain signaling, particularly in the fever response.

The cytokine-to-brain signaling pathways can be divided into neuronal and humoral pathways. Depending on the extension of the inflammatory process these pathways may supplement each other. Thus with the knowledge we have today it can be hypothesized that tissue-released cytokines are primarily sensed by the afferent neurons while circulating cytokines activate the CNS through barrier-specific, humoral pathways (Quan, 2014). Evidence for the importance of both routes could be shown concurrently already during the dawn of this field (reviewed in Elmquist et al., 1997b; Hopkins and Rothwell, 1995; Rivest et al., 2000; Rothwell and Hopkins, 1995; Watkins et al., 1995b). However, the first assumptions were biased towards the neuronal autonomic pathways due to the classical view of the BBB as a strict barrier and further strengthened by the diffused distribution of BBB in the brain parenchyma, not being restricted to the immune responsive regions within the CNS (Quan, 2008; Watkins et al., 1995b). More recently, this view has been revised since BBB activation has been demonstrated by several convergent findings presented below.

Afferent neuronal pathways

The afferent neurons are the major input pathway for information from the peripheral tissues to the CNS. Acute phase responses could be elicited by stimulating both afferent fibers bundled with autonomic nerves that innervate the internal organs, and somatic afferents innervating the skin and the locomotor system.

The importance of the vagus nerve, conveying the autonomic afferents from the majority of the internal organs, emerged during the characterization of the brain immune responsive regions. The nucleus of the solitary tract (NTS), the central terminal nucleus of the vagal afferents, was activated showing cFOS labeling both after LPS (Wan et al., 1993) and iv IL-1 β (Ericsson et al., 1994). These results led to the first vagotomy studies showing decreased cFOS labeling in NTS (Wan et al., 1994) as well as physiological effects like decreased hyperalgesia

(Watkins et al., 1994) and fever responses (Watkins et al., 1995a) to ip IL-1 β or LPS, hence confirming the vagal afferents as a major pathway for immune-to-brain signaling. Several other studies on vagotomized rats followed, dominating the field at the turn of the century (reviewed by Romanovsky, 2004). However, with our present knowledge on the importance of the humoral pathways, the conclusions of these studies have been given reduced importance. Nevertheless the contribution of the autonomic afferents cannot be dismissed completely as some critical findings have not been refuted, one example being immune activation of the NTS and of the vagal afferents (Ek et al., 1998) and the presence of TLR4, IL-1R1 and PGE₂ receptors in the vagal nodose ganglion (Ek et al., 1998; Hosoi et al., 2005) or on the glomus cell of the vagal paraganglia (Goehler et al., 1997). The importance of the vagal signaling was furthermore verified by intraperitoneal (ip) administration of low doses of IL-1 β , reducing the risk for cytokine spill-over in the circulation, (Hansen et al., 2001; Konsman et al., 2000) and in a model of gut bacterial infection, with absence of circulating cytokines (Goehler et al., 2005).

The somatic afferents, particularly unmyelinated C-fibers, were shown to mediate the induction of acute phase responses like fever and hypermetabolism in a model of localized inflammation induced by subcutaneous turpentine (Cooper and Rothwell, 1991). While largely neglected at that time this study gained more attention when the antipyretic effect of local anesthetics blocking C-fiber-signaling was verified by Roth's group (Ross et al., 2000; Roth and De Souza, 2001). Several models for local inflammation have been used, including subcutaneous turpentine (Cooper and Rothwell, 1991; Fantuzzi et al., 1997; Horai et al., 1998; Kozak et al., 1998; Lacroix and Rivest, 1998; Laflamme and Rivest, 1999; Leon et al., 1996), carrageenan-induced paw edema (Guay et al., 2004; Ibuki et al., 2003; Oka et al., 2007; Posadas et al., 2004; Prajapati et al., 2014), or immune stimuli administrated in artificial subcutaneous chambers (Cartmell et al., 2000; Miller et al., 1997; Rummel et al., 2005; Rummel et al., 2011; Zhang et al., 2008). However, most studies used local inflammation-models primarily to demonstrate an endotoxin-free cytokine-induced sickness syndrome (Horai et al., 1998; Kozak et al., 1998; Leon et al., 1996; Miller et al., 1997; Rivest et al., 2000) and not to investigate the role played by the afferent neurons. Thus, a humoral component was present in these models for local inflammation with measurable circulating cytokines (Horai et al., 1998; Kozak et al., 1998; Miller et al., 1997; Oka et al., 2007; Rummel et al., 2011) and brain expression of IkB α (Laflamme and Rivest, 1999), COX-2 (Horai et al., 1998; Ibuki et al., 2003; Lacroix and Rivest, 1998; Oka et al., 2007), and PGE₂ (Guay et al., 2004; Ibuki et al., 2003; Oka et al., 2007). Consequently, to address the role of the afferent neurons in immune-to-brain signaling (Cooper and Rothwell, 1991; Ross et al., 2000), the study designs ought to limit the cytokine released into the bloodstream and better discriminate between local (tissue) or central (brain) cytokine and/or prostaglandin action sites. Accordingly, using LPS air-pouch as a model-system, it was shown that local IL-1 β at low levels was critical for fever induction (Cartmell et al., 2000;

Miller et al., 1997), although the response was also shown to be dependent on circulating IL-6 (Cartmell et al., 2000). Better models have been developed of so-called restricted local inflammation that do not induce brain COX-2 and have low to undetectable circulating cytokines. In these studies the fever response could be attenuated by very low doses of locally administered diclofenac (Rummel et al., 2005) and could be shown to be dependent on the presence of TLR4, IL-1R1, IL-6 and COX-2 (Zhang et al., 2008).

The somatic and autonomic neuronal afferents, being able to gather information from any affected tissue, are certainly appealing signaling pathways. However, few older studies investigated the role of the afferent neurons in immune-to-brain signaling in animal models lacking a humoral component upon peripheral immune activation, and newer studies even dismiss the vagal contribution (Ootsuka et al., 2008) or its responsiveness to the pro-inflammatory cytokines (O'Connor et al., 2012). Still, in an endothelial-specific IL-1R1 KO mouse model impairing the immune signal transmission at the BBB, the discrepancy seen between iv and ip IL-1 β treatments (no fever if iv, fever if ip) can best be explained by activation of the afferent somatic and/or autonomic neurons (Ching et al., 2007). Through their fast impulse signaling to the CNS the afferent neurons were also proposed to be responsible for the early phase of the sickness syndrome (Blatteis, 2006; Dantzer et al., 2008; Konsman et al., 2002; Quan, 2008; Romanovsky, 2004). Nevertheless, as NSAIDs are effective also in local inflammation, the neural pathway is still prostaglandin-dependent and thus dependent on *de novo* cytokine and prostaglandin enzyme production, i.e. time-consuming processes. Furthermore, little is known today about the central neuronal pathways activated by the somatic afferents. An immunological homunculus was proposed (Oke and Tracey, 2008) and recently partly demonstrated (Belevych et al., 2010) in a unique study, at least to the time of publication of this thesis. Hence, some topographical representation could be seen in the paraventricular nucleus (PVN) of the hypothalamus in the casein model for localized peripheral inflammation but not in subcutaneous or intramuscular LPS models that supposedly give rise to humoral responses (Belevych et al., 2010).

While peripheral mediators might signal to the brain through afferent neurons, the neuronal ascending pathways, which in the periphery translate the cytokine/prostaglandin signal into neuronal impulses within CNS, could still not account for the direct effect of the cytokines and prostaglandins injected icv or into the brain parenchyma, as shown already during the 1970^s. This discrepancy made the humoral signaling pathway a critical subject for research very early in the development of this field.

Humoral pathways

Circulating cytokines engage the brain at the blood-brain interface regions: the BBB, the blood-cerebrospinal fluid barrier (BCSFB) and the arachnoid barrier. The latter two are believed to have a low impact on the central neuronal circuits in comparison with the large microvascular bed of the BBB. The calculated, total interface area of the BBB is 12-18 m² for an average human adult brain, with neurons being rarely more than 8-20 µm away from the BBB (Abbott et al., 2010). The main function of the BBB is to maintain a stable ion and protein milieu in the brain parenchyma. This is achieved by a high impenetrability of the endothelial lining within the BBB, with tight junctions cementing the cells, and lack of intracellular fenestrations that, combined with low endocytic levels, limit both the paracellular and transcellular molecular and cellular trafficking. Peripheral episodic ion and/or protein fluctuations given by food ingestion are thus prevented from disturbing the neuronal transmission. For influx of nutrients and elimination of potentially harmful compounds, compensatory mechanisms act in form of specific transporters like solute carrier transporters, ATP-binding cassette transporters, and receptor-mediated transcytosis (Abbott et al., 2006).

Figure 5. Structure of the Blood-Brain Barrier.

Apart from endothelial cells (ECs) with their tight junctions (TJ) (Figure 5), other BBB cellular constituents are pericytes (Pc) scattered along the ECs on the basal lamina (BL), astrocytic end-feet (AEF) bound by TJs or gap-junctions forming a second barrier (glia limitans), and perivascular cells (PVC) which are tissue macrophages at the vessel-parenchyma interface (the perivascular space).

The BBB and surrounding neuronal cells are also named the neurovascular unit (NVU) (Cohen et al., 1996). The BBB within the NVU adjusts to signals from both the abluminal (glial and neuronal) and luminal (blood) sides, and it plays a critical role in bidirectional humoral-signal transduction, which makes the BBB a dynamic barrier (Banks, 2009; Muldoon et al., 2013). Hence, while the BBB is mediating the immune signals in systemic inflammation, its characteristics are also changed by inflammation so that it becomes a more leaky barrier with decreased integrity of tight junctions (Hawkins and Davis, 2005; Huber et al., 2001) and increased endocytic processes (Banks and Erickson, 2010; Kastin and Pan, 2003).

The cytokine-to-brain signaling at the BBB has been described as taking place by four potential mechanisms: *i*) direct diffusion and/or signal transduction at circumventricular organs (CVOs), *ii*) active cytokine transport, *iii*) increased immune cell trafficking, and *iv*) direct binding at the

cognate receptors on the BBB with release of *de novo* cytokines or other central mediators such as prostaglandins (Haroon et al., 2012; Quan, 2014; Rivest, 2010; Turrin and Rivest, 2004). These mechanisms are validated to be functional and they are not mutually exclusive, but their individual importance might be correlated to the type and strength of the immunological signal. Accordingly, low levels of immune signals (cytokines or DAMPs) might be more easily detected and amplified by perivascular macrophages at CVOs, while higher levels can activate the endothelial cells and might result in a general BBB activation.

The circumventricular organs (CVOs) are leaky capillary structures within the BBB, already discovered by Paul Ehrlich (1885) as they allowed some trypan blue staining of the neighboring neuronal tissue. They were later shown to display looser endothelial lining and astroglial apposition than the general brain parenchyma, which creates a large and easily accessible perivascular space for blood macromolecules. The name CVOs denotes their periventricular positioning (Hofer, 1958), hence being tri-partner-meeting structures for blood, neuronal/glial and cerebrospinal fluid (CSF) signals (Johnson and Gross, 1993). Presenting fenestrated endothelial cells they were even called “windows of the brain” (Gross and Weindl, 1987), not only for their structural openings in the otherwise strict BBB, allowing the brain to “glimpse” into the periphery, but also metaphorically as “windows of opportunity”, opening up new research avenues to study the neuro-humoral interaction. Thus, CVOs were a given target in cytokine-to-brain signaling research and (among) the first humoral pathways to be investigated (Roth et al., 2004). Of particular interest became the sensory CVOs, organum vasculosum of lamina terminalis (OVLT), subfornical organ and area postrema, located near the hypothalamic and brain stem structures regulating neurovegetative functions (Johnson and Gross, 1993). OVLT, situated in the anterior hypothalamus within the preoptic region that is the central thermoregulatory region, has been studied in the fever paradigm (Blatteis, 1992), starting with lesion studies initiated by Blatteis (Blatteis et al., 1983). Area postrema, situated at the bottom of the fourth ventricle near the NTS, the autonomic afferent nucleus relaying onto the PVN (Ericsson et al., 1994), was mostly investigated for its contribution to the activation of the HPA-axis (Ericsson et al., 1997; Lee et al., 1998). Several lesion studies were performed on the CVOs but they failed to provide any conclusive data as both the nearby BBB integrity and adjacent neuronal tissues could be damaged (Roth et al., 2004). Still, these studies contributed to giving increased attention to the CVOs as possible immune signal gateways and thus to their subsequently thorough characterization in terms of immune receptiveness. Accordingly, CVOs present LPS and cytokine receptors as shown for CD14 (Lacroix et al., 1998), TLR4 (Laflamme and Rivest, 2001), IL-1R1 (Cao et al., 1996; Cunningham et al., 1992; Ericsson et al., 1995), TNFRs (Nadeau and Rivest, 1999a) and IL-6R (Vallieres and Rivest, 1997). Also, in response to various immune stimuli, local *de novo* cytokine production was demonstrated as shown by up-regulated mRNA transcripts for TNF (Breder et al., 1994; Nadeau and Rivest, 1999b), IL-6 (Vallieres and Rivest, 1997) and IL-1 β (Eriksson et al., 2000; Quan et al., 1998) with IL-1 β

being expressed in immune cells apposing neuronal terminals (Goehler et al., 2006). Additionally, both vascular and neuronal activation was shown, such as induced vascular STAT3 (Harre et al., 2002; Konsman et al., 2000) and neuronal cFOS labeling, respectively (Ericsson et al., 1994; Herkenham et al., 1998; Nadeau and Rivest, 1999a). The cytokine-induced neuronal activity was also shown to be mediated by local COX-2 induction (Konsman et al., 2000) and PGE₂ release (Matsuda et al., 1992; Ota et al., 1997).

Cytokine transporters, characterized by Banks and collaborators, are cytokine-binding sites at the BBB, often different from the specific cytokine receptors but in some cases, such as for TNFR1, even believed to be a product of the same receptor gene (Banks and Erickson, 2010). Specific cytokine transporters were found for several cytokines, including IL-1, TNF α , IL-6 (reviewed in Banks, 2005) but their contribution to the immune-to-brain signaling is believed to be limited as they show an easily saturated capacity, with an inflammation-insensitive, low rate kinetics (Banks and Erickson, 2010).

Increased trafficking of peripherally primed immune cells has recently been shown to contribute to the brain parenchymal release of cytokines (Aguliar-Valles et al., 2014; D'Mello et al., 2009; Wohleb et al., 2013), although not during the initial phase of the sickness syndrome. As cellular trafficking requires that there be pre-activated microglial cells after strong systemic immune challenge (Aguliar-Valles et al., 2014; Qin et al., 2007; Quan et al., 1998), long lasting liver inflammation (D'Mello et al., 2009), or social stress such as repeated social defeat (Wohleb et al., 2013), this pathway is considered a maladaptive response of the brain to chronic inflammation that by reinforcing an initial neuroinflammatory process (microglial activation) might eventually lead to behavioral dysfunctions (Dantzer et al., 2008; Quan, 2014).

The BBB as an interface for immune signal-transduction

The importance of the BBB for immune-to-brain signaling was acknowledged as the PGE₂ synthesizing enzymes COX-2 and mPGES-1 were shown to be induced in the BBB by varying peripheral and central immune stimuli (Cao et al., 1995; Cao et al., 1996; Cao et al., 1998; Ek et al., 2001; Engblom et al., 2002), corroborating previous studies which suggested that BBB was a source of PGE₂ (Bishai et al., 1987; Devries et al., 1995; Van Dam et al., 1993). Subsequently it was also demonstrated that the BBB is a cytokine target, presenting receptors for IL-1 β (Ek et al., 2001; Ericsson et al., 1995; Konsman et al., 2004), IL-6 (Vallieres and Rivest, 1997) and TNF α (Nadeau and Rivest, 1999a) as well as intracellular cytokine activation markers like I κ B α (Laflamme and Rivest, 1999; Quan et al., 1997) and SOCS3 (Lebel et al., 2000). Hence, the BBB displays all the elements needed for prostaglandin release in response to cytokine induction (Figure 6) leaving no doubt about its importance. Still, due to its cellular complexity, discrepant findings and hypotheses have been presented regarding the cell type

Figure 6. PGE₂ production in cerebral endothelial cells. NF- κ B activation by e.g. IL-1 β up-regulates COX-2 and mPGES-1 allowing an increased production of PGE₂. (AA arachidonic acid)

critical for the inflammation-induced PGE₂ production. Two main opposing views emerged, one supporting the endothelial cells (Cao et al., 1995; Ek et al., 2001; Matsumura et al., 1998; Yamagata et al., 2001) and the other favoring the perivascular macrophages (Breder and Saper, 1996; Elmquist et al., 1997a; Schiltz and Sawchenko, 2002). To address this question, we (**paper I**) (Eskilsson A 2014; Hamzic et al., 2013b; Matsuaki et al., 2014; Wilhelms et al., 2014) and other groups (Chakravarty and Herkenham, 2005; Ching et al., 2007; Gosselin and Rivest, 2008; Ridder et al., 2011) have developed complex transgenic mice models that allowed *in vivo* analysis of one gene in a BBB cell population at a time (reviewed with **paper I**).

The humoral pathways described above thus provide several routes for the peripheral cytokines to pass the BBB and act centrally within the CNS. Accordingly, the proinflammatory cytokines can either be transported through or act on the cells of BBB and/or CVOs to induce local production of prostaglandins and/or cytokines, which eventually pass into the brain parenchyma. In the CNS, cytokine receptors were demonstrated to be present on the endothelial linings, glial cells and also neurons. IL-1R1, for example, was first reported to be expressed mostly on neurons and choroid plexus by affinity studies (Breder et al., 1988; Farrar et al., 1987; Takao et al., 1990 and several other studies). However, subsequent studies using *in situ* mRNA hybridization as well as immunohistological labeling with well characterized antibodies pointed out the brain microvessels as a major binding site for IL-1 β (Ericsson et al., 1995; Konsman et al., 2004), but other studies reported parenchymal labeling in e.g. hypothalamus, hippocampus, anterior pituitary and cerebellum with inferred neuronal and/or glial representations (Cunningham et al., 1992; Wong and Licinio, 1994). Whether or not neurons express cytokine receptors is still a matter of debate (Dantzer et al., 2008; Quan, 2008). Several reports though, indicate that neuronal receptors contribute to normal neurophysiological processes like learning and memory at the hippocampal-amygdaloidal level, neuronal development and survival, or neuronal synchronization in specific cortical columns, reviewed in e.g. (Quan, 2014; Schultzberg et al., 2007). An *in vitro* model on microvessels showed that IL-1 β binding to the endothelial lining induced release of IL-6 and PGE₂ (Van Dam et al., 1996), leading to the hypothesis that the neuronal IL-1R1 was not participating in mediating the sickness behavior. A contemporary study approached this issue in an *in vivo* model by looking at the downstream I κ B α mRNA up-regulation to trace the NF- κ B induction in the brain after ip LPS. This study was able to reveal a spatio-temporal pattern

of cytokine-induced activation, which is restricted to non-neuronal elements, starting at 30 minutes in the endothelial linings of the large vessels, choroid plexus and CVOs, followed at 1-2 h by small vessels (BBB) and astroglial cells throughout the entire brain, and then after 2 h by meningeal cells that finally reach ependymal cells after 12 h (Quan et al., 1997). Thus, for the sickness behavior the neuronal activation seen by means of cFOS labeling (Ericsson et al., 1994; Herkenham et al., 1998) is achieved indirectly by other molecules, like prostaglandin E (Ferri and Ferguson, 2005), a hypothesis that is also supported by the lack of double-labeling for cFOS and I κ B α in neurons (Ericsson et al., 1995).

In contrast to the cytokine receptors, EP receptors repeatedly have been reported on neurons and glia although vascular representation of EP receptors was also observed in a few studies as described in detail in the PGE₂ receptor section.

Prostaglandin E₂ as a critical mediator in immune-to-brain signaling

PGE₂-sensitive CNS regions mediating sickness behavior

EP receptors in the CNS are associated with both neuroprotective and toxic roles (Andreasson, 2010), and pathological and physiological processes, and are reported to play a role in sickness-induced acute phase responses as well as in neural plasticity, learning and memory formation, and impulsive behavior (Furuyashiki and Narumiya, 2011). Accordingly, the four EPs have distinct distribution patterns (Batshake et al., 1995; Ek et al., 2000; Nakamura et al., 2000; Oka et al., 2000; Sugimoto et al., 1993; Zhang and Rivest, 1999). Hence they are not restricted to the typical CNS immune-responsive regions (Elmqvist et al., 1996; Ericsson et al., 1994; Herkenham et al., 1998) but are also present in structures like the cerebral cortex, hippocampus, thalamus or cerebellar nuclei.

In immune-to-brain signaling, the representation of EP receptors in regions like the hypothalamic preoptic area (POA) and PVN, and in brain stem nuclei like NTS and the ventrolateral medulla (VLM) was in line with results from studies pointing out the same regions as highly responsive to locally administrated PGE₂ (Ericsson et al., 1997; Oka et al., 1997; Scammell et al., 1996; Scammell et al., 1998). Hence, PGE₂ is a central (brain) mediator of cytokine-induced sickness syndrome, although not the sole mediator. Inflammation-induced PGE₂ was shown to be critical for the fever response (Engblom et al., 2003), but PGE₂ was also shown to be one of several mediators contributing to the HPA-axis activation (Elander et al., 2009; Morimoto et al., 1989a), aversive behavioral changes (Fritz, 2012), hyperalgesia (Ferreira, 1972; Ferreira et al., 1973; Kamei et al., 2004; Trebino et al., 2003), and wakefulness (Huang et al., 2003). However, it was found to have only a limited role in the anorectic response (Elander et al., 2007; Ruud et al., 2013a).

Fever

Fever is the prototypical PGE₂ mediated response, demonstrated to be highly dependent on the inducible synthesizing enzymes COX-2 (Li et al., 1999) and mPGES-1 (Engblom et al., 2003) as well as on EP3 receptors (Ushikubi et al., 1998), particularly in the thermoregulatory region, the preoptic area (POA) (Lazarus et al., 2007) (Figure 7). Initially, other EP receptors were also considered febrigenic, such as EP1 and EP4. The idea of EP1 mediated fever was based on pharmacological tests using icv or intra-POA administered agonists and antagonists with low specificity for EP1 (Oka et al., 1997; Oka and Hori, 1994). The EP4 receptor was shown on neurons activated by immune challenges, an activation that also decreased in NSAIDs pretreated animals and contrasting to the non-activated EP3 neurons (Oka et al., 2000; Turrin

and Rivest, 2004; Zhang and Rivest, 1999; Zhang and Rivest, 2000). Subsequent studies using highly selective agonists to each receptor (Oka et al., 2003b) as well as the introduction of transgenic mice lacking one of the EP1-3 receptors (Ushikubi et al., 1998) brought some clarity in this matter. Thus, EP3 was identified as the major fever mediating receptor (Ushikubi et al., 1998), whereas EP1 had but a minor contribution in the late phase of the fever response after intermediate doses of LPS (Oka et al., 2003a). EP4 was suggested to have a hypothermic effect (i.e. cryogenic receptor) upon agonist activation (Oka et al., 2003b).

Stress-hormone release

The finding that immune stimuli induced the activation of the hypothalamic-pituitary adrenal axis (HPA axis) with resulting stress hormone release was a major step in the development of the immune-to-brain signaling research field (Besedovsky et al., 1986; Sapolsky et al., 1987). Cortisol release from the adrenal glands is triggered by ACTH produced by the anterior adrenal gland, which in turn is released upon CRH stimulation from neurons of PVN. The prostaglandin contribution to the central activation of the HPA axis is complex. The initial phase seems to be dependent on COX-1 while the late phase is COX-2 driven (Elander et al., 2009; Elander et al., 2010; Garcia-Bueno et al., 2009). PGE₂ has a direct central effect on the HPA-axis as seen from local administration of PGE₂ in the brain parenchyma (Morimoto et al., 1989a). Its effect is mediated by EP1 and EP3, particularly in the central nucleus of amygdala (EP1) and POA (EP1 and EP3), but also in other regions projecting to PVN (Matsuoka et al., 2003; Saper et al., 2012; Ulrich-Lai and Herman, 2009). In contrast, EP2 and EP4 do not seem to be involved, since EP2 and EP4 KO mice display no disturbances in the activation of HPA axis (Matsuoka et al., 2003).

Hyperalgesia

Hyperalgesia represents an increased sensitivity to pain, due to sensitization of peripheral receptors or central neurons. Several inflammatory factors are involved in the pain modulation at the peripheral/tissue level and at central levels within the spinal cord, brain stem and forebrain. The PGE₂ contribution was demonstrated both peripherally (Ferreira, 1972; Ferreira et al., 1973; Kamei et al., 2004; Trebino et al., 2003) and centrally at POA (Abe et al., 2001). The hypothesized central pathway for the regulation of pain sensitivity that involves prostaglandins originates in POA with EP3 expressing neurons (Hori et al., 2000; Oka et al., 2000; Ueno et al., 2001) that presumably project to the periaqueductal gray (Rizvi et al., 1996) to further reach medullary and spinal nociceptive-related structures.

Anorexia

Sickness-induced anorexia has also been shown to be mediated by PGE₂ (Pecchi et al., 2006), although not exclusively. While being reversed by COX-2 inhibitors (Johnson et al., 2002; Lugarini et al., 2002), the anorectic response driven by inflammation-induced PGE₂ is dependent on the type of immune stimuli. Hence, IL-1 β initiated a PGE₂-dependent anorexia in contrast to LPS, which seems to be PGE₂-independent (Elander et al., 2007; Pecchi et al., 2006). Among EPs, the strongest candidate is EP4 as demonstrated by the use of specific agonists and antagonists (Ohinata et al., 2006). EP4 is well represented in the hypothalamic and brain stem structures regulating feeding behavior (Williams and Elmquist, 2012; Zhang and Rivest, 1999) and an anorexic response was obtained when PGE₂ was injected intraventricular (V3) or intra-PVN (Skibicka et al., 2011). However, contrasting results are seen in a tumor model for anorexia-cachexia where neither the neuronal EP4 nor global mPGES-1 deletions could rescue the tumor-induced appetite loss (Ruud et al., 2013a).

Sleepiness

Sleepiness, another typical component of the sickness behavior, was shown to be driven by PGD₂ originating mostly from meninges and choroid plexus where its synthesizing enzyme L-PGDS is expressed (Urade et al., 1993). PGD₂ in the subarachnoid space can then act on its DP1 receptors enriched in meninges ventral to ventrolateral preoptic region (VLPO) and induce large and sustained increases in NREM (non-rapid eye movement) sleep (Matsumura et al., 1994). In contrast to PGD₂, PGE₂ has been suggested to be a waking factor through its EP4 receptors in the tuberomammillary nucleus (TMN), activating histaminergic neurons (Huang et al., 2003; Onoe et al., 1992).

Fever in acute systemic inflammation

General considerations

From ancient times to modern medicine, fever as a sign of disease has always fascinated physicians and scientists in search of cures. In immune-to-brain signaling, fever experiments are the prevailing models. The main inducers used are LPS, followed by different cytokines and other molecules, prostaglandins or DAMPs (Kluger, 1991).

Central neuronal pathways for fever regulation

Mammals maintain their body temperature within a strictly regulated range (36.5 to 37.5°C in humans) through feedback mechanisms, collectively called thermoregulation. Thermoregulation encompasses neuronal circuits within the CNS that integrate central body temperature signals from brain warm-sensing neurons and peripheral temperature signals from cutaneous thermal receptors, and aims at restoring normal temperature through thermogenic (temperature increasing) or thermolytic (temperature decreasing) processes. Temperature changes are generated by cortical, conscious mechanisms generating adaptive behavioral actions (e.g. in rodents by changing environment), and by subcortical, diencephalic regions, i.e. unconscious mechanisms, that eventually employ the autonomic efferent pathways for temperature regulation (Morrison and Nakamura, 2011; Nakamura, 2011). In the latter case thermolysis is achieved in rodents by peripheral (cutaneous, tail) vasodilation, panting and saliva spreading, whereas thermogenesis is achieved by peripheral vasoconstriction (reducing heat loss through the skin) and non-shivering and shivering metabolic thermogenesis in brown adipose tissue (BAT) and skeletal muscle, respectively.

In fever, the thermoregulation process is switched by PGE₂ towards an increased central body temperature by engaging thermogenic mechanisms. The anterior preoptic area (POA) of the hypothalamus was early identified to be highly sensitive to locally administrated PGE₂ (Williams et al., 1977) and later on to express the febrigenic EP3 receptors (Ushikubi et al., 1998) in the median preoptic nucleus (MnPO) and the medial preoptic area (MPO) (Ek et al., 2000; Nakamura et al., 2000; Sugimoto et al., 1994) (**paper III**). Deletion of EP3 receptors specifically in MnPO has been shown to attenuate the febrile response (Lazarus et al., 2007). EP3 neurons within the POA are hypothesized to be GABAergic (Nakamura et al., 2002), which during normal conditions inhibit downstream neurons in the dorsomedial hypothalamic nucleus (DMH) (Nakamura et al., 2005) and raphe pallidus nucleus (RPa) (Nakamura et al., 2002). DMH neurons (glutamatergic) project as well to the RPa. Upon PGE₂ binding to the EP3 receptor-bearing POA neurons, the GABAergic tonus in RPa is decreased allowing

Aims

Inflammation-induced PGE₂ has been shown to be a main mediator of the sickness syndrome, particularly of the fever response. The starting point for the research leading to this thesis was to further characterize the BBB contribution to the PGE₂ production in acute peripheral inflammation and also to identify the PGE₂ effect driving the fever response from the thermoregulation area.

Specific aims:

- . To identify the cell type within the BBB critical for the fever response (**paper I**)
- . To characterize the inflammatory profiles of the individual cell populations within the BBB, in response to acute peripheral inflammation (**paper II**)
- . To identify the EP3 receptor expressing region in the mouse preoptic area and to characterize the expression of the EP3 receptor isoforms in this region (**paper III**)
- . To examine the gene regulatory effects of PGE₂ in the preoptic area, during acute peripheral inflammation (**paper IV**)

Methodology

Animal models

Mice strains

All experiments leading to the present thesis were conducted on mice, often transgenic mice (**papers I, III and IV**) but also chimeric mice (**paper I**) or wild-type mice (WT) (**paper II**), on a C57Bl/6 (**papers I-III**) or DBA1/lacJ background (**paper IV**).

Transgenic mice have allowed scientists to test *in vivo* the functions of many different proteins by manipulating the genes of the mice. The attractiveness of such an approach explains the driving force behind the development of more and more refined transgenic mice. The first largely used models were the *global transgenic mice* that have either a knocked-out (KO, i.e. deleted gene) or a knocked-in gene (overexpressed gene). The drawbacks of such a model are that the modified gene is expressed life-long, and in all cells (globally). In some cases this is incompatible with life, which renders this strategy useless for the genes in question. For other genes, the physiological implications of a global transgene risk appear to be too complex to comprehend. Newer developments have allowed the introduction of *conditional transgenic mice*. By using a versatile Cre-loxP recombination technology (Nagy, 2000), gene manipulations can be obtained in mice at a desired age, globally or in a tissue-restricted manner.

In this thesis only global transgenic mice were used (Table 1); however several important studies in immune-to-brain signaling have used conditional transgenic mice (Eskilsson A 2014; Ridder et al., 2011; Wilhelms et al., 2014).

Table 1. Mice strains used in the present thesis

Type		Target Protein	Paper	Reference	Effect
Ptges ^{-/-}	transgenic	mPGES-1	I-IV	(Trebino et al., 2003)	Global
Ptgs2 ^{-/-}	transgenic	COX-2	I	(Morham et al., 1995)	Global
EP3 ^{-/-}	transgenic	EP3	III	(Fleming et al., 1998)	Global
GFP ⁺	transgenic	GFP	I	(Okabe et al., 1997)	Global*
Ptges ^{+/+} ↔ Ptges ^{-/-}	chimeric	mPGES-1	I	(Turrin et al., 2007)	Hematopoietic
				Paper I	vs. somatic

*Bone marrow cells from GFP⁺ (green fluorescent protein) WT or mPGES-1 KO mice were used for the generation of chimeric mice

Chimeric mice were generated to differentiate between hematopoietic and non-hematopoietic production of PGE₂. A chimeric organism is characterized by having genetically distinct cells either occurring spontaneously (extremely rare), or generated artificially during embryogenesis or induced by transplantations or blood transfusions at later time points.

In **paper I** chimeras were created by bone marrow transplantation. Bone marrow transplantations in laboratory mice are in general uncomplicated procedures as tissue-typing is not required in the very near-related mice. Also the risk of infections is limited in the pathogen-free environments the mice are kept in. However some complications have been reported related to the whole-body irradiation treatments, which can increase vascular permeability of the BBB the first days post-exposure (Diserbo et al., 2002). There may also be white-blood cells infiltration into the brain (Simard et al., 2006) and hippocampal dysfunction (Monje et al., 2002). Nonetheless, we and others have shown that the immune response both in the periphery and centrally (in the brain) are not compromised in chimeric mice, as measured by the release of cytokines like IL-1 β (paper I) and IL-6 (Hamzic et al., 2013b) or by absence of white blood cell infiltration of the brain tissue (Turrin et al., 2007) (paper I).

The principles of creating mPGES-1 chimeric KO mice are described here, while the complete protocol for bone marrow transplantation in mice is given in **paper I**. Mice of both genotypes Ptges^{+/+} (WT) and Ptges^{-/-} (KO) underwent a myeloablative whole-body γ -irradiation to deplete the hematopoietic cells in the recipient mice prior to transplantation with donor bone marrow. The degree of hematopoietic reconstitution was easily analyzed as bone marrow was harvested from GFP⁺ (green fluorescent protein) mice of both mPGES-1 genotypes. At the time for LPS challenge, five months after transplantation, the hematopoietic reconstitution was around 90% in peripheral blood and brain, 67% in liver, and 78% in lung. There was little infiltration of GFP⁺ cells in the brain parenchyma (Figure 1, A-C in paper I). Combining the two mPGES-1 genotypes between the recipient and donor mouse, four chimeric groups were generated, namely WT or KO bone marrow to WT mice (WT $\rightarrow$ WT and KO $\rightarrow$ WT) and WT or KO bone marrow to KO mice (KO $\rightarrow$ KO and WT $\rightarrow$ KO). At the BBB the chimeras may produce PGE₂ either in perivascular macrophages (WT $\rightarrow$ KO) or in the endothelium (KO $\rightarrow$ WT). WT $\rightarrow$ WT and KO $\rightarrow$ KO correspond genetically to the non-transplanted WT and KO littermates and were included as controls.

The same strategy has been used by others to investigate the contribution to the sickness syndrome of hematopoietic vs. non-hematopoietic expression of TLR4 (Chakravarty and Herkenham, 2005; Steiner et al., 2006a), MyD88 (Gosselin and Rivest, 2008; Ruud et al., 2013b), and IL-1R1 (Matsuwaki et al., 2014).

Induction stimuli for inflammation and stress hyperthermia

The aim of the studies was to further characterize the role of PGE₂ in acute systemic inflammation and thus the standard model for this thesis is *intraperitoneal injection of LPS* (**papers I-IV**) at a moderate dose of 120µg/kg body weight. This model has been extensively used in our laboratory for the study of fever (Engblom et al., 2003; Hamzic et al., 2013b; Nilsberth et al., 2009a; Nilsberth et al., 2009b; Saha et al., 2005), HPA-activation (Elander et al., 2009; Elander et al., 2010), anorexia (Elander et al., 2007), and conditioned place aversion (Fritz, 2012). Other complementary models used in the present thesis were *intravenous injections of either LPS or IL-1β* (**paper I**) and *cage exchange-induced stress hyperthermia* (**paper IV**). The intravenous models were selected to test different stimuli-based hypothesis but also to allow investigation of the first phase of fever as the mice were implanted with catheters that allowed administration of the pyrogen without handling the animals, hence minimizing the initial injection-induced hyperthermia. The cage-exchange model was used to test the thermoregulatory capacity of mPGES-1 KO mice that do not mount a febrile response to LPS administration. The cage-exchange model induces a strong emotional stress as mice have an urge to mark and defend their territory. In the experiment, two adult male mice are removed from their littered home cages (marked cages) and placed in the cage of others (cage exchange). On detecting the smell from the other male mouse, in the home cage of the other mouse, the visiting mouse becomes agitated and starts exploring the new environment. Telemetric recordings indicate both a high locomotor activity and increased body core temperature during the first 2 h (Figure 2 in paper IV).

Telemetric recordings are done through intraperitoneally implanted transmitters, which permit long time measurements of both body core temperature and locomotor activity without disturbing the mice (**papers I and IV**).

Intraperitoneal injection of the LPS dose used in this research generates a typical tri-phasic fever that can be measured up to 7-8 h during the light diurnal period (Figure 8) (Romanovsky et al., 1998). The first phase of fever, up to 1-1.5 h after injection, coincides with the stress-induced hyperthermic peak. This temperature peak is followed by a slight hypothermic response that later evolves into the second phase of fever. In unstressed animals, for example animals injected through an indwelling iv catheter, the first fever response could be measured between 16 to 70 minutes after LPS injection (Romanovsky et al., 1998). This response is believed to be governed by mechanisms other than phase 2 and phase 3 fevers (Blatteis, 2006; Ivanov and Romanovsky, 2004). The second phase from 1.5 to 3 h, peaking at approximately 2 h, and the third phase are believed to depend on the same mechanisms but with the third phase being highly dependent on the LPS dose (Romanovsky, 2004).

Figure 8. Fever curves after ip LPS or saline in chimeric mice. Saline treated mice are represented as an average for all transgenes.

Laser capture microdissection and enzymatic cell dissociation

Analysis of homogeneous cell populations or small regions from complex, solid tissues such as the brain is possible by using either laser microdissections methods or enzymatic tissue dissociation with subsequent magnetic cell isolation or fluorescent activated cell sorting (FACS) flow cytometry.

Laser capture microdissection

Two similar ***laser microdissection*** systems are commercially available. The one used in **papers III and IV**, Laser Capture Microdissection (LCM; Arcturus/Applied Biosystems) is based on a low energy laser that melts a plastic film above the desired region (*capture* method). The other method, Laser Microdissection (LMD; Leica Microsystems) uses a higher energy laser that causes lysis of the tissue around the desired region which then falls into the collecting vessel. There are advantages and disadvantages with each system. LCM is an easy and fast method for a manual tissue dissection (for each laser impulse one cell or a small region can be extracted), but it bears the risk of sample contamination. LMD on the other hand provides a contact and contamination-free technology but with a drawback of needing an automatized

method that allows faster dissections as each target cell/region has to be encircled by the laser beam to detach from the section; this would be a terribly time-consuming and skill-demanding process with an operator-controlled laser.

Both systems can be used to dissect immunolabeled cells. Our initial aim for paper III was to label and analyze EP3-positive neurons. Lack of specificity of the commercially available EP3-antibodies, tested on EP3 WT and KO mice, led us to approach another strategy and to investigate the general effect of PGE₂ on the thermoregulation area by dissecting the whole anterior preoptic area of the hypothalamus (discussed with paper IV) instead of a specific PGE₂ effect on the EP3 neurons.

Enzymatic cell dissociation and magnetic isolation

Tissue cell dissociation and magnetic isolation can also be used to separate homogenous cell groups, as shown in **paper I** for the CD45⁺ bone marrow cells. Magnetic isolation offers the advantage of simultaneous isolation of all specifically labeled cells from cell suspensions, like blood or cell suspensions generated after enzymatic cell dissociation of tissues. Magnetic isolation eliminates the need for microscopy and individual cell identification such as that needed by LCM, and is conveniently used for cell populations that can be characterized by one antigen, against which specific antibodies can be raised. There is a risk of antigen degradation by the enzymatic treatment, which should be considered whenever troubleshooting for lack of labeling by a specific antibody. The cell isolation depends on the intensity of the magnetic field, the size of the magnetic bead and the cellular abundance and availability of the antigen. Different strategies of negative and positive selections can be used to sort the cells. Future perspectives are to allow labeling for several consecutive positive selections, by enzymatically detaching the magnetic beads from the previous antibodies. However, for cells needing multiple positive labeling it is preferable to use FACS isolation, unless sterile conditions are desired. For the brain tissue this methodology cannot be applied for the branched and/or myelinated neurons, which are mechanically torn apart. On the other hand, enzymatic cell dissociation allows separation of the BBB cells, particularly endothelial cells and pericytes, which are otherwise technically difficult to dissect by LCM due to their near contact to each other and elongated morphology. Kits are provided by e.g. Miltenyi Biotec for easy and fast magnetic separation of endothelial cells, astroglia, neuronal progenitor cells or brain cancer cells. With an *in house* protocol developed by us, microglia (unpublished) and the BBB cells (**papers I and II**) were isolated by FACS flow cytometry.

Flow cytometry

Flow cytometry offers many possibilities to analyze individual cells or particles in heterogeneous suspensions. Cellular characteristics of different types of cells and structures that can be labeled with fluorochromes are analyzed simultaneously and sorted accordingly into desired populations for further experiments. This versatility is given by the lasers and optical capacity of the instrument. The efficacy of such instruments is so high today that several 1000 cells/particles can be analyzed per second with up to 5 lasers, including measurement of 20 parameters per particle: 18 colors and 2 morphologic parameters (e.g. with BD LSRFortessa™ X-20).

In the flow cytometer the stained cells are first lined-up into a single-cell stream by hydrodynamic focusing, allowing each cell to pass individually through several consecutive laser beams. Fluorochromes in the laser beam absorb light energy of a specific wavelength increasing their energy state in a process called excitation. The excited fluorochromes are unstable and emit light (emission) of longer wavelengths than the wavelength of the exciting laser. Light scatter (forward and side) and fluorochrome emission signals are gathered and analyzed in the collection optics and then individually converted from electrical impulses into digital data. Thus, each cell is eventually represented by two light scatter parameters (forward and side) and multiple color parameters, depending on the number of fluorochrome-labeled structures. Forward-scatter light (FSC) is proportional to the cell size and side-scatter light (SSC) to the intracellular complexity. Multiple staining, although appealing, increases the risk of spillover of the emission signals into several detectors, as the fluorochromes have rather broad emission spectra. To overcome this problem careful compensation programs are run, extracting for each fluorochrome used the percentage of the leaky signal from the wrong detectors.

In **papers I and II**, the cell sorters used were FACSAria III or II (BD), which, in addition to flow cytometry, can separate cells from the flow into single cell-droplets and deflect the desired cells/droplets from the stream into the collection devices.

In **paper I** white blood cells from chimeric mice were analyzed by flow cytometry for their GFP expression. Brain and liver tissues from LPS and saline treated WT animals were enzymatically dissociated and BBB cells and liver endothelial cells and macrophages were sorted and further analyzed by RT-qPCR for their mPGES-1 expression.

In **paper II** BBB cells from LPS and saline treated WT mice, were sorted into pericytes (CD45⁻CD31⁻CD140b⁺), endothelial cells (CD45⁻CD31⁺), and perivascular macrophages (CD45⁺CD206⁺) (Table 2, Cellular markers) and further analyzed by RT-qPCR on a 48-format TaqMan Low Density Arrays (48-TLDA).

Protein methods

Proteins can be isolated, identified, quantified, and morphologically localized in tissues and cells with a tremendous variety of methods. In this thesis though few measurements were done at protein level as the LPS mouse model used (paper I-IV) has been extensively characterized in our laboratory for e.g. COX-2, lipocaline-2, cytokines and PGE₂ expression, and cortisol release (Elander et al., 2009; Nilsberth et al., 2009a), and recently also for IL-1R1 (Matsuwaki et al., 2014) and mPGES-1 (Eskilsson et al., 2014).

The chimeric mice were characterized by *in house immunohistochemistry* methods for COX-2 and cellular markers (Table 2), and by several commercially available *immunoassays* for PGE₂ and IL-1 β in plasma and CSF (methods presented in **paper I**).

Table 2. Cellular markers (papers I and II)

Name	Antigen	Cell type	Method
CD45	LCA	All hematopoietic cells	FACS
CD206	MMR	Perivascular cells	FACS and immunohistochemistry
CD140b	PDGFR β	Pericytes	FACS
CD31	PECAM1	Endothelial cells	FACS
vWF	vWF	Endothelial cells	Immunohistochemistry
Iba1	Iba1	Microglia	Immunohistochemistry
CD68	Macrosialin	Lung macrophage	Immunohistochemistry
F4/80	-	Pan-macrophage marker	FACS and immunohistochemistry

LCA: leukocyte common antigen; MMR: macrophage mannose receptor; PDGFR β : platelet derived growth factor receptor, beta polypeptide; PECAM1: platelet endothelial cell adhesion molecule 1; vWF: von Willebrand Factor; Iba1: ionized calcium binding adapter molecule 1

Messenger-RNA methods

Molecular biology is another field that has experienced an extraordinary development in the past three decades. The advantages of detecting messenger-RNA molecules have turned the molecular biology in a good complementary methodology of characterizing proteins, allowing the detection of the cellular distribution of e.g. signaling molecules for which it has been difficult to develop good detection antibodies or that are expressed on cellular membranes or at long-distance from the cell body (as membrane receptors or neurotransmitters and synaptic proteins). Other advantages are given by the unlimited possibilities of developing different nucleotide sequences allowing the generation of any kind of probes for *in situ hybridization* (**paper III**), as well as assays for isolated RNA for further applications, here used with *gene*

expression microarray (**paper IV**) and *RT-qPCR* (**papers I-IV**). The disadvantages coupled with an mRNA detection concern the low stability of the molecules and the relatively high abundance of degrading enzymes (RNases) that should be considered at any time. Another disadvantage is that the physiological impact of a detected transcript is uncertain, as the mRNA level is not directly proportional to the protein level due to post-translational regulating factors like RNA interference, which are gene/protein specific.

In situ hybridization

In *situ* hybridization was used in **paper III** for localization of the EP3 receptor in the anterior hypothalamus. The method employed is based on a radioisotope-labeled complementary RNA riboprobe (905 bp) for the EP3 mRNA that is hybridized on tissue slices (“in situ”). The probe is synthesized in plasmids in the presence of nucleotides labeled with an isotope (in paper III ³³P). Tissue sections on slides are first fixed with paraformaldehyde and treated with proteinase K that by removing proteins increases tissue permeability for the probes (pre-hybridization). The probes are then hybridized at 58°C in a special hybridization solution that facilitates nucleotide binding. In the post-hybridization step, single-stranded RNAs are disintegrated by RNase treatment and unspecific probe-binding by rinses in a series of low salt buffers at high temperature (72°C). Finally, the slides are prepared for autoradiography. In *situ* hybridization was run both on WT mice showing a robust EP3 labeling in POA (Figure 1 A in paper III), and on EP3 KO mice that showed no signal in the corresponding area (Figure 1C in paper III). For regional localization, the sections adjacent to the *in situ* hybridized sections were stained with thionin (Figures 1 B and D in paper III).

Gene expression microarray

The technological progress and our increased knowledge of molecular biology have led researchers from the US and elsewhere around the world to the most ambitious project of our times, The Human Genome Project (1990-2003), aimed at mapping the whole human genome (Collins et al., 2003). Most of the mouse genome (one of the other species sequenced in the project) had already been mapped by the mid-1990s and at that time the first microarrays were already commercially available. This enabled the manufacturing of whole genome arrays which “*catapulted many laboratories from studying the expression of one or two genes in a month to studying the expression of tens of thousands of genes in a single afternoon.*” (Collins et al., 2003; Nature, 2002)

In **paper IV** a microarray methodology was used to analyze the effect of PGE₂ on the gene expression changes within the neuronal tissue of POA. The study was designed to analyze RNA

samples from LCM dissected POAs (as in paper III) from both WT (n=5) and KO (n=5) genotypes, treated with LPS. The RNA samples were individually prepared and hybridized on the arrays.

For this study the oligonucleotide microarray GeneChip Mouse Genome 430 2.0 Array was used, the array being the first whole transcriptome chip for the mouse developed by Affymetrix. In the workflow the isolated RNA was amplified, biotin-labeled, purified and fragmented in accordance with the instructions provided by the manufacturers of the kits (Affymetrix and Applied Biosystems; presented in paper IV). The resulting biotin-labeled complementary RNA (cRNA) was hybridized on the chip. The chip covers the whole mouse transcriptome, 39000 mouse transcripts, and has the advantage of having several probes (oligonucleotides) for one transcript (a probe-set per transcript), with a total of 45000 probe-sets for mouse and control transcripts on each chip. The oligonucleotides are 25-base long probes synthesized on the chip through photolithography during the chip manufacturing process. The probes are designed to bind with similar affinities to the cRNA fragments and to either match perfectly (perfect-match probes: PM) or to differ at one base (miss-match probes: MM), which can be used to estimate the tendency for cross-hybridization. Both PM and MM probes are distributed along the whole length of the transcript from the 5'-end to 3'-end allowing estimations of the mRNA integrity (3'/5' signal ratio should be less than 3). The chip is also provided with probes for chip intensity controls ("control spikes") and external controls (transcripts of prokaryotic origin). The control spikes (*bioB*, *bioC*, *bioD*, *cre*) provide chip-specific quality control measurements. The external controls (*dap*, *lys*, *phe*, *thr*) are standardized poly-A RNAs that are added to the sample RNA to be treated together in the pre-hybridization process. In this way the controls become an effective tool for the normalization process within and between the arrays. After hybridization, the samples are stained on the chip, scanned and analyzed. The raw data of each probe are analyzed with the GenChip Operating Software (GCOS), normalized within the project, and saved in CEL files for further functional genomics data analysis. In paper IV GeneSpring (Agilent) or Ingenuity Pathway Analysis System (Sigma) were used.

RT-qPCR,

Gene expression changes were quantified with a two-step RT-qPCR method. For this purpose commercially available kits were used both for RNA extractions (Arcturus or Qiagen), cDNA synthesis (Applied Biosystems) and TaqMan RT-qPCR chemistry (Applied Biosystems). The kits were chosen on the basis of their recommended usage, in that several different RNeasy kits were used for the RNA purification depending on the type and amount of tissue used. A DNA elimination step was employed on all occasions. The purified RNA was reversely transcribed with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems), a kit

recommended for the pre-amplification step and TLDAs. Several check-points were used, such as mRNA integrity controls with 2100 Bioanalyser, Agilent RNA 6000 Pico, or Nano Assays (**papers III and IV**), and RNA quantitative measurements with NanoDrop spectrophotometer (**papers III and IV**). The RNA levels were expected to correlate with the amount of tissue used in the RNA extraction step, when comparing similar tissues. The RNA values were used to synthesize cDNA with similar concentrations, which in the RT-qPCR also resulted in similar Cq values for GAPDH, the control (housekeeping) gene used in the present studies. In **papers I and II** neither 2100 Bioanalyser nor NanoDrop could be used for the BBB cells due to their low numbers. As a quality control the GAPDH Cq values were expected to be similar when starting with cDNA loading volumes that were calculated based on the cell counts used in the RNA extraction step and aimed to yield same “cell loading counts” in the RT-qPCR reaction. Most of the assays (except those used in paper III, see below) used in RT-qPCR were inventoried TaqMan assays, and, whenever possible, assays were chosen that span exons to eliminate the risk of interference from genomic DNA. Linearity and efficiency were controlled for several genes and also for the pre-amplification step. Quantitative measurements were done by calculating $\Delta\Delta Cq$, comparing the degree of gene expression variation for each gene against the reference gene and between treated and control animals.

In **paper III** PCR primers and RT-qPCR assays were designed *in house*, with the exception of the GAPDH assay. To test the specificity of our assays, specific transcripts were reversely transcribed, amplified and purified to prepare individual standard curves for each EP3 isoform and GAPDH. The assays were then tested individually against each standard curve. They showed high efficacy with correlation coefficient over 0.99 for each assay, and displayed no cross-reactivity.

Results and Discussions

Lipopolysaccharide-induced fever depends on prostaglandin E2 production specifically in brain endothelial cells

In **paper I** the cellular origin of the inflammation-induced PGE₂ at the BBB was analyzed in chimeric mice that lacked mPGES-1 either in non-hematopoietic cells, i.e. endothelial cells within BBB (KO→WT), or in hematopoietic cells, i.e. perivascular macrophages within BBB (WT→KO). Non-irradiated/non-transplanted mice and WT→WT and KO→KO mice were used as controls. The febrile response to ip LPS was similar throughout to that displayed by the background genotype, irrespective of whether the mice were transplanted with KO or WT bone marrow (Figure 8). Thus, WT→WT and KO→WT mice had a fever curve characteristic for the WT genotype, demonstrating that the ablation of mPGES-1 in the hematopoietic cells (KO→WT) is not critical for the pyrogenic response to peripheral immune challenge. Similarly, KO→KO and WT→KO mice showed a temperature response characteristic for the KO genotype, implying that mPGES-1 in the transplanted perivascular cells and hematopoietic cells was not rescuing the KO genotype either by local PGE₂ production in the BBB or by peripheral release of PGE₂. Similar patterns were seen also after iv LPS injections, a treatment that by minimizing the handling-induced stress allows analysis of the first phase of fever. The telemetric findings were largely consistent with the PGE₂ levels in CSF, but not with the plasma levels of PGE₂. Hence, hematopoietic mPGES-1 (WT→KO) could rescue the peripheral production of PGE₂, but not the fever response. Immunohistochemical staining of the liver for COX-2 showed immune-induced labeling of immune cells like Kupffer cells, and this was also seen in mice in which these cells derived from the transplanted cells. In contrast, staining of lung and brain tissue showed immune-induced COX-2 labeling in endothelial cells. Apart from the standard LPS dose of 120 µg/kg ip, a low dose of LPS (1µg/kg iv as in Schiltz and Sawchenko, 2002) or a moderate dose of IL-1β (30µg/kg iv as in Givalois et al., 1994) were also tested, since these stimuli have been reported to induce COX-2 expression preferentially in perivascular macrophages. However, these stimuli also resulted in COX-2 labeling exclusively in endothelial cells. A critical issue for determining in which cells PGE₂ production takes place was the identification of mPGES-1 expressing cells in the mouse. At the time when this study was performed, no specific antibody for mouse mPGES-1 was available, and in *situ* hybridization did not yield any specific signal (unpublished). Therefore, we instead developed a method to dissociate and isolate endothelial cells and macrophages from the brain and the liver, and subsequently analyze their gene expression by RT-qPCR. The induction patterns in the BBB were distinct and opposite from those seen in the liver cells. While endothelial cells within the BBB expressed much higher levels of mPGES-1 transcripts than perivascular

macrophages, and also showed a prominent upregulation after immune stimulation (9 times; $P < 0.05$), the PGE₂ production in the liver took place in Kupffer cells, whereas liver endothelial cells had no detectable mPGES-1 transcripts.

Discussion:

The data shown in **paper I** demonstrate that PGE₂ synthesis by non-hematopoietic cell is critical for the febrile response to LPS, whereas PGE₂ synthesis in hematopoietic cells does not seem to be involved. These functional data, when taken together with our demonstration of mPGES-1 expression in brain endothelial cells but not in perivascular macrophages, identify the brain endothelial cells as the cells responsible for the PGE₂ production that underlies the febrile response.

Figure 9. Studies on the TLR4 and IL-1R1 signaling.

(Ching et al., 2007), brain endothelial TAK1 KO mice, i.e. specific brain endothelial IL-1 signaling blockage (Ridder et al., 2011), and MyD88 chimeric mice (Gosselin and Rivest, 2008) (Figure 9).

While some controversies exist for the first phase of fever, particularly regarding the importance of TLR4 on hematopoietic cells (see below) (Steiner et al., 2006a), data from the abovementioned studies regarding IL-1-signaling are concordant. Hence, endothelial cells are strongly responsive to IL-1 β , while IL-1 β signaling in perivascular macrophages does not rescue the IL-1 KO genotype (Ching et al., 2007; Gosselin and Rivest, 2008). Blood-borne IL-1 β signaling to CNS is completely dependent on endothelial IL-1R1 (Ching et al., 2007), which at BBB was also shown to be highly expressed on endothelial cells and not on perivascular cells (Konsman et al., 2004). A later study in which the brain endothelial IL- 1 β signaling was specifically targeted by TAK1 deletion in brain endothelial cells resulted in blunted fever

While **paper I** is the first study that identifies the PGE₂ producing cells critical for fever, several other studies have addressed the contribution of the BBB cells in immune-to-brain signaling by examining other levels of the signal transduction pathway. These studies include work on TLR4 chimeric mice (Chakravarty and Herkenham, 2005; Steiner et al., 2006b), endothelial specific IL-1R1 KO mice

response and decreased locomotion (lethargy) (Ridder et al., 2011). However, intraperitoneally injected IL-1 β may signal to CNS through other pathways, probably by activating peripheral macrophages (Ching et al., 2007).

In the study by Steiner et al., (2006) it was reported that the presence of functional TLR4 in hematopoietic cells in mice with a TLR4 KO background rescued the initial febrile response. Their data thus suggested a critical role for hematopoietic cells for the initiation of fever which may seem to be in contrast to our finding, which showed intact initial fever in mice lacking PGE₂ production in hematopoietic cells. However, the obvious reconciling interpretation is that intact TLR4 in hematopoietic cells foster the release of proinflammatory cytokines which in turn act on endothelial cells in the brain to induce PGE₂ release and subsequent fever.

The critical role of endothelial cells for the PGE₂ production that elicits fever has recently also been demonstrated by targeted deletion of COX-2 and mPGES-1 in brain endothelial cells (Wilhelms et al., 2014). Taken together these studies should end a long controversy concerning this issue (Saper et al., 2012).

Immune challenge by intraperitoneal administration of lipopolysaccharide directs gene expression in distinct blood–brain barrier cells towards an enhanced prostaglandin E₂ signaling

In **paper II**, with the method developed in paper I, FACS isolated BBB cells (Figure 10) and subsequent TLDA RT-qPCR analysis, we aimed at analyzing the effect of peripheral immune stimulation on gene transcripts related to inflammatory signaling in each BBB cell population in WT mice. We thus analyzed 48 gene-transcripts representing enzymes and proteins for prostaglandin metabolism and transport, cytokines, chemokines and lipocalin-2 with associated receptors, cytokine signaling molecules, peroxidase proliferator activated receptors, and cell-type specific molecules (Tables 1 and 2 in paper II). The emerging picture (Figure 11) was that each cell population had a distinct transcriptional profile at base line and after ip LPS. The investigated endothelial and perivascular cell-type markers showed high specificity, but not the pericyte markers, discussed below. Down-regulation of endothelial and pericyte molecules known to participate in cell-to-cell contact suggested a decreased integrity of the barrier in response to LPS. Among prostaglandins, all the production at the BBB was switched towards an increase of PGE₂ both by increased synthesis through inducible COX-2 and mPGES-1 in endothelial cells but also by decreased degradation in endothelial cells and perivascular macrophages, coupled with decreased production in the latter for other prostanoids. An interesting finding was the high, LPS-stable level of L-PGDS in the pericytes as the PGD₂

synthesis previously has been assigned to the leptomeninges, choroid plexus and oligodendrocytes (Urade et al., 1993) but not to BBB.

Figure 10. FACS sorting of endothelial cells (CD45-CD31+), perivascular macrophages (CD45+CD206+), and pericytes (CD45-CD31-PDGFRb+). (A) The cell populations were gated based on fluorescent minus one (FMO) controls in which the antibodies against CD31, CD206, and PDGFRb (CD140b) were omitted (FMO CD31, FMO CD206, and FMO PDGFRb, respectively). The left column shows gating for CD31 and CD45, and identifies three populations (red boxes): CD45-CD31+ cells, CD45+CD31- cells, and CD45-CD31- cells. Note that the gate used for CD31+ cells is defined according to FMO CD31 control (top panel) where no signal was observed in the gate. The middle column shows gating of CD206+ cells within the CD45+CD31- population. Note that the gate used for CD206+ cells contains only a single background event (accounting for 0.05% of total CD45+ cells) in the FMO CD206 control (third panel from top). The right column shows gating for PDGFRb+ cells among the CD45-CD31- population. Note that the gate used for the PDGFRb+ cells is devoid of any signal in the FMO PDGFRb control (second panel from top). (B) Purity analysis of the sorted cell populations CD45+CD206+, CD45-CD31+, and CD140b/PDGFRb+. Note that the sorted CD31+ cells (ECs) were negative for PDGFRb and that the pericytes (PDGFRb+) were negative for CD31 (right).

Some of the prostaglandin transporters were up-regulated in endothelial cells (*Abcc4* and *Slco2b1*) and down-regulated in perivascular cells (*Slco2b1*). As regards cytokine signaling, there was a clear up-regulation in the endothelial cells of IL-1R1, TLR4, IL-6R α , whereas the perivascular cells responded with up-regulation of IL-6 and the pericytes with increased TNF expression, although the latter at low levels. Both endothelial cells and perivascular cells showed signs of cytokine activation as seen by induced expression of downstream signaling molecules such as I κ B α , MyD88, STAT3, Akt1 in endothelial cells and Myd88 or SOCS3 in perivascular cells, or of immediate early genes like lipocalin-2 and CXCL10, in both endothelial cells and perivascular cells.

Discussion:

Because the method used to examine the inflammatory transcriptome in BBB cell is novel, it is of interest to note that several of the transcripts now studied by RT-qPCR were previously shown in the same BBB cell populations by *in situ* hybridization or at protein level by immunohistochemistry. Some examples are COX-2 (Cao et al., 1995; Engström et al., 2012;

Figure 11. Gene expression changes in the BBB. Significant changes are represented for each cell type. Up-regulated genes in red (high expression) or pink text (low expression), and down-regulated genes in blue. Genes are group by dashed line into functional groups: BBB integrity, cytokine signaling in endothelial cells (ECs), prostaglandins synthesis and transport in ECs and perivascular cells (PVCs), chemoattraction and IL-6 signaling in PVCs.

Inoue et al., 2002), mPGES-1 (Ek et al., 2001; Eskilsson et al., 2014), IL-1R1 (Cao et al., 2001; Ek et al., 2001; Konsman et al., 2004; Matsuwaki et al., 2014), IkB α (Laflamme et al., 1999; Quan et al., 1997), and inducible lipocalin-2 (Hamzic et al., 2013a) in endothelial cells, and thromboxane-A synthases-1 (Tbxas1) in perivascular macrophages (Cyrus et al., 2010; Gabrielsen et al., 2010). Furthermore, functional coupling of increased production (COX-2, mPGES-1) and decreased degradation (15-PGDH) as revealed here was also previously reported to occur for PGE₂ (Hahn et al., 1998; Tai et al., 2006). Thus, while providing a novel and

comprehensive view of the inflammatory transcriptome in the BBB, our data corroborate several observations in earlier studies underlining the feasibility of the present method. Still, several considerations ought to be addressed regarding the methodology and present results.

Methodological limitations.

The pericyte-specific markers *Anpep* (CD13) and *Pdgfrb* (Armulik et al., 2010; Daneman et al., 2010; Eskilsson et al., 2014) showed in our RT-qPCR similar expression levels in endothelial cells as in pericytes suggesting a pericyte contamination of the endothelial cell population. However, this possibility can be dismissed by the flow cytometric controls such as CD31/PGDFRb gated FMO (fluorescence minus one) controls and purity controls (Figure 10) or SSC-H/SSC-W and FSC-H/FSC-W gating strategy (Figure 1 in paper II) to eliminate doublets in case of an incomplete dissociation of pericytes from endothelial cells. Moreover, a pericyte contamination would have generated a generalized effect on RT-qPCR data from endothelial cells and that was not seen for the highly expressed pericyte transcripts, *Slc22a17* and *L-PGDS*. Thus, the expression of the pericyte markers by the endothelial cells is rather a phenomenon limited to the transcript-assay interaction depending either on a cross-reactivity

of the used assays (*Anpep* and *Pdgfrb*) with other transcripts expressed in endothelial cells or on that the transcripts detected are largely un-translated in endothelial cells.

A potential limitation in the present study is the risk of activating the cells during the long processing time needed for enzymatic dissociation and FACS isolation. Although the cells were processed at 4°C to minimize this risk, several results that differ from those reported in previous studies were noted particularly as regards the perivascular cells, such as high COX-2, IL-1 β and TNF α mRNAs levels both in saline and LPS treated samples. These results are in strong contrast to those obtained in morphological studies, pointing out endothelial cells as the cells expressing immune-induced COX-2 (both as protein and mRNA) (Engblom et al., 2002; Engström et al., 2012). They also differ from results from studies showing absence of cytokine expression in perivascular cells in naïve animals (Nadeau and Rivest, 1999b; Quan et al., 1998). Hence, some *in vitro* activation of transcripts cannot be excluded. However, this potential limitation does not invalidate the general conclusions of the study, which was based on the comparison of saline and LPS treated animals, since any *in vitro* effect should have affected both groups equally.

Another limitation of our study is analytical. The analyzed cells were shown to present different GAPDH levels (Table 1 in paper II), which was expected due to their different sizes and structural characteristics (Schmidt and Schibler, 1995). However, this implies that direct quantitative or qualitative comparisons of target genes between different cell-types may be uncertain. The present results are accordingly presented only as relative expressions in each cell population and treatment, and differences were calculated only between saline and LPS samples within the same cell-type (Table 1 in paper II). Nevertheless, for each cell-type, transcripts belonging to a protein family (as prostaglandin enzymes or cytokine receptors) are expected to reflect the relative abundance of the corresponding protein. Taking as an example IL-1R1 and TLR4, respectively, it may be inferred from comparing the relative mRNA expressions that endothelial cells express more IL-1R1 protein than TLR4, and furthermore, that perivascular cells express more TLR4 than endothelial cells. This inference is supported by the expression in these cells of their adaptor molecules TRIF and MyD88. Thus, TRIF (Ticam1) is much more abundantly expressed in perivascular cells than in endothelial cells, whereas Myd88 is about equally abundant in both cell types, which is what is to be expected by the canonical signaling pathways of TLR4 and IL-1R1, respectively (Figure 3).

Regarding *results that conflict with results from previous reports*, our data do not support the production of IL-6 and TNF α in endothelial cells (Verma et al., 2006). Instead we found an upregulation of IL-6 and TNF α in PVCs and pericytes, respectively. Another finding contrasting to those previously reported in the literature is the responsiveness of the different BBB cell populations to immune stimuli. It has been proposed that perivascular cells are the cells sensing low levels of IL-1 β , whereas endothelial cells have been proposed to respond

mostly to LPS (Saper et al., 2012; Schiltz and Sawchenko, 2002; Serrats et al., 2010). These proposals contrasts strongly with our interpretations based on our data, which demonstrate that endothelial cells have high expression of IL-1R1, in agreement with other studies on the endothelial IL-1 signaling (Ching et al., 2007; Ek et al., 2001; Gosselin and Rivest, 2008) and IL-1R1 representation (Konsman et al., 2004; Matsuwaki et al., 2014). Perivascular cells, in contrast, displayed very little IL-1R1, compared to their high levels of TNFRs, IL-6R α and TLR4.

In conclusion, our study demonstrates that both endothelial cells and perivascular cells are responsive to immune stimuli, but that the two cell types respond in often opposing, yet complementary ways. Endothelial cells increased their cytokine sensitivity (receptor expression) while perivascular cells were contributing through an *in situ* cytokine production of IL-6. There was also increased prostaglandin transport and production that was switched towards more PGE₂ through increased synthesis in endothelial cells, decreased degradation in endothelial cells and perivascular cells, and decreased synthesis of other prostanoids in perivascular cells.

Expression of PGE₂ EP3 receptor subtypes in the mouse preoptic region.

In **paper III** we aimed at characterizing the constitutive and LPS-induced expressions of the mouse EP3 isoforms (EP3 α , EP3 β , and EP3 γ) in the preoptic region. For this purpose we first identified the EP3 expressing region in the anterior hypothalamus by *in situ* hybridization (Figure 1 A, C in paper III). This region was mapped relative to the third ventricle, anterior commissure and optic nerve structures. With the anatomical landmarks we obtained we could specifically extract the EP3-rich preoptic region by laser capture microdissection and use it for RT-qPCR analysis of EP3 isoforms. We found that EP3 α was the most abundant isoform, followed by EP3 γ , and very low levels of EP3 β . None of the isoforms changed its expression 5 h after ip LPS (Figure 3 in paper III).

Discussion:

EP3 receptors within the preoptic area are critical for the fever response (Engblom et al., 2003; Lazarus et al., 2007; Ushikubi et al., 1998). Our findings suggest that the predominant effect of PGE₂ on the EP3 isoforms would be an increased activity at Gi, through the dominant EP3 α isoform, but, in an agonist-dependent manner, there could also be an activation of Gs through the EP3 γ , with opposing effect on the cellular cAMP levels. Other processes governing fever regulation downstream of the EP receptors within the preoptic region are yet little elucidated. The EP4 receptors, also highly expressed in POA, are constitutively coupled to Gs and

recognized as a hypothermic receptor (Oka et al., 2003b), thus opposing the general EP3 effect. However, the interaction between EP3 isoforms and EP4 in POA or their possible co-localization at neuronal level are issues that have not yet been addressed.

Minor changes in gene expression in the mouse preoptic hypothalamic region by inflammation-induced prostaglandin E2

In **paper IV** the gene regulatory effect of inflammation-induced PGE₂ was investigated in the EP rich thermoregulatory region of the hypothalamus. LPS treated animals that were able to (WT) or unable to (KO) produce inflammation-induced PGE₂ (iPGE₂) were compared with respect to their gene expression changes within the mouse preoptic area. The time point for analyzing the iPGE₂ effect was set at 5 h, that is about 2 h after the PGE₂ peak in the CSF, which corresponded to a robust transcriptional up-regulation of the PGE₂ synthesizing enzymes, COX-2 and mPGES-1 (Figure 1 in paper IV). The microarray analysis yielded a list of 253 unique genes that displayed significantly changed transcripts between genotypes. The genes could be grouped into neural-specific and cAMP dependent pathways, yet, no pathway was significantly changed in an integrated pathway analysis (Ingenuity Pathway Analysis System). As the changes in general were small, and no particular process was identified by pathway analysis, a smaller number of genes were selected for further analysis, based on the transcript's fold change (FC) and significance levels, or on previous studies. Hence, 47 genes and 1 reference gene (Gapdh) that displayed a FC of at least 2 ($P < 0.05$) or a FC between 1.5 and 2 ($P < 0.01$), as well as some other genes of interest, were analyzed by RT-qPCR. The validation step was run on new LPS or saline treated WT and KO mice, but following the same LCM and RNA-extraction protocols as the samples used for microarray analyses. Of the 47 genes examined, we could validate significant differences between genotypes only for *HSPA1b* and *Hspa1a* (together HSPs) encoding inducible forms of heat-shock proteins (HSP) of the HSP70 family (Tables 2 and 3 in paper IV). Analyses of cDNA from our time-course study on WT mice (Figure 1 in paper IV) suggested that the expression of the HSPs was dependent on PGE₂ since they were induced at a later time point than the PGE₂ synthesizing enzymes (COX-2, mPGES-1). Furthermore, they were either more abundantly expressed in the hypothalamus (an EP rich region) than in striatum (an EP low expressing region), or they showed a stronger up-regulation in hypothalamus than in striatum. Nonetheless, the fever response is a major confounder in our model, as LPS-treated KO animals produce neither iPGE₂ nor mount a fever response. Accordingly, the expression of HSPs was investigated in a cage exchange-induced hyperthermia paradigm. In response to emotional stress and in the absence of iPGE₂ (Figure 2 in paper IV), both WT and KO mice displayed increased core body temperature and an up-

regulation of HSPs. In conclusion, HSPs were independent of iPGE₂, but dependent on the increased body temperature, which implied that inflammation-induced PGE₂ regulates few, if any, of the gene expression changes that are seen in the CNS during systemic inflammation (Hamzic et al., 2013a; Mastronardi et al., 2007; Reyes et al., 2003).

Discussion:

In the model used, both WT and KO mice display most of the inflammation-induced characteristics upstream of PGE₂ (i.e. cytokine production, BBB activation) (Nilsberth et al., 2009b). The critical difference is that KO mice lack iPGE₂ and thus display no fever (Engblom et al., 2003) and they also show attenuated HPA activation (Elander et al., 2009), and abolished conditioned place aversion (Fritz, 2012). The very low fold changes that was seen in induced gene expression between the two genotypes differ from the strong LPS effect seen in the mouse brain (Hamzic et al., 2013a; Mastronardi et al., 2007; Reyes et al., 2003). To control that the small differences seen in induced gene expression between genotypes were not due to a general lack of responsiveness to the LPS treatment, we also examined the LPS-induced induction by comparing gene expression in LPS-treated WT mice with that in saline injected WT mice. Genes known to be strongly induced by LPS such as CXCL-10, COX-2 and lipocaline-2 (Hamzic et al., 2013a; Reyes et al., 2003) were all strongly up-regulated also in the present material (Table 3 in paper IV). Thus, as we could not find any PGE₂-dependent genes, despite a strong LPS effect as such, the present data imply that the gene expression changes that take place in the hypothalamus/brain in systemic inflammation are not necessary for the PGE₂ dependent sickness behavior, as they are not regulated by PGE₂ but by some other, unknown pathway.

A potential limitation of our study concerns the chosen time point that could be set too late to allow detection of immediate-early genes like c-FOS, shown to be highly expressed in POA after ip LPS (Elander et al., 2009; Elmquist et al., 1996). According to some previous studies c-FOS expression in several autonomic relay regions of the brain is dependent on induced PGE₂ production (Dallaporta et al., 2007; Zhang et al., 2003), however this was not confirmed in a subsequent study from our laboratory (Elander et al., 2009). While gene expression changes may also occur at time points later than 5 h post LPS injection, these are unlikely to be PGE₂ driven since PGE₂ levels in the CSF peak at 3 h post LPS injection to subsequently fall back and reach baseline levels at 12 h (Inoue et al., 2002).

Another limitation is the heterogeneous cell population analyzed from POA, a highly vascularized region encompassing the OVLT. The consequence of extracting the entire POA might be that any effect of PGE₂ on gene expression in neurons, if present, could be diluted and hence undetected due the presence of a large number of vascular and glial cells. Another approach would have been to dissect individual cells expressing EP receptors like shown for EP3 positive neurons (Tsuchiya et al., 2008). Nonetheless, even in that model, in which the

gene regulatory effects were examined briefly after icv injection of PGE₂, few genes were identified that were changed by the stimulus, and these genes generally displayed low fold changes (between 1.5 and 2). However, in that study (Tsuchiya et al., 2008) it was reported that the GABA_A transcript was attenuated by PGE₂, a finding that could not be verified in our present work.

In conclusion, very few, if any, gene-expression changes can be attributed to mPGES-1 induced PGE₂. This implies that the centrally-elicited disease symptoms controlled by PGE₂ are mostly generated by regulating the neuronal excitability through intracellular transcriptional-independent cascades affecting the membrane potential.

Concluding remarks and future perspectives

The search for immune-to-brain signaling pathways started more than 40 years ago as the *endogenous pyrogens* (Atkins and Bodel, 1971; Moore et al., 1970; Wood, 1962) proved to be too large to penetrate the BBB (Dinarello et al., 1974). Complementary to the pro-inflammatory cytokines, PGE₂ emerged at the same time as a critical mediator in inflammation as the mechanism behind aspirin was revealed (Vane, 1971). Since that time, the missing links between cytokines and prostaglandins have been filled in by the identification of cytokine receptors and down-stream intracellular signaling molecules (e.g. NF- κ B, STAT3, AP1) as well as by the identification of prostaglandin synthesizing enzymes (COX-2, mPGES-1) and PGE₂ receptors (EPs). As a result, the sites for cytokine-prostaglandin interactions could be mapped in the periphery and in the brain with strongly compelling data pointing out the BBB as an active immune-to-brain interface region.

Inflammation-induced PGE₂ produced by the endothelial cells of the BBB, a critical mechanism in immune-to-brain signaling

From the idea of an immune privileged brain with its impenetrable barriers, through the discovery of cytokines and prostaglandins, newer functions of the BBB were revealed that changed our view on the interactions between the immune system and the central nervous system. Today the immune privilege is considered *relative* and *specialized* (Muldoon et al., 2013). Thus, leukocytes have been shown to pass back and forth across the barriers much more easily than was initially considered possible, and the brain barriers, particularly the BBB, have been shown to be sensitive to circulating cytokines. Hence, in light of the data we present, the BBB production of inflammation-induced intermediates, like PGE₂, can be added to the classical barrier properties.

Conflicting data have pointed out different cells as PGE₂ producers within the BBB, i.e. endothelial cells (Cao et al., 1995; Ek et al., 2001; Matsumura et al., 1998; Yamagata et al., 2001) vs. perivascular cells (Breder and Saper, 1996; Elmquist et al., 1997b; Schiltz and Sawchenko, 2002). In **paper I** we demonstrate for the first time that endothelial cells are critical for the inflammation-induced PGE₂ and the febrile response. Our findings are in agreement with other similar functional studies showing that the brain endothelial lining is highly responsive to IL-1 and that the IL-1 signaling cascade in the endothelium is critical for the brain-mediated acute phase responses (Ching et al., 2007; Gosselin and Rivest, 2008; Matsuwaki et al., 2014; Ridder et al., 2011) or microglia activation (Wohleb et al., 2014; Wohleb et al., 2013). These data corroborate the hypothesis of an IL-1-induced PGE₂ production in endothelial cells that was previously inferred from morphological studies

localizing COX-2, mPGES-1 and IL-1 signaling elements to the endothelial cells (Cao et al., 2001; Cao et al., 1995; Ek et al., 2001) (Figure 6), and refute the idea of critical PGE2 production in perivascular cells (Breder and Saper, 1996; Elmquist et al., 1997b; Schiltz and Sawchenko, 2002). However, the perivascular cells are not completely inert to peripheral inflammation as shown in **paper II**. We found them to respond to inflammation by CXCL10, lipocalin-2 and cytokine production (IL6) and decreased synthesis of other prostanoids. We also demonstrated that the perivascular cells express high levels of TNFRs and TLR4 but moderate levels of IL6R, and very little mRNA for IL-1R1. On the other hand they express high levels of cytokine mRNAs and can probably provide an amplification loop within the BBB upon activation. Several functional studies analyzing the role of the barrier TLR4-signaling might be worth undertaking here.

In the TLR4 chimeric models discussed in paper I, brain cytokine and peripheral cortisol levels after LPS challenge were more dependent on a TLR4⁺endothelial cell activation (KO→WT mice) than on a TLR4⁺perivascular cell activation (WT→KO mice). Still, TLR4⁺ in perivascular cells or peripheral LPS-sensitized macrophages was found to be contributing to the stress hormone release response (Chakravarty and Herkenham, 2005). The fever response in the same chimeric mice lacking TLR4 on hematopoietic cells (Steiner et al., 2006a) showed a 30 minute delay in its development, but the mice displayed normal fevers at later phases. From this study it could be similarly inferred that endothelial cell activation without activation of hematopoietic cells gives rise to a fever response, though at a later time point. Thus, TLR4⁺ non-hematopoietic cells alone can mount acute phase responses and cytokine release, but at a slower pace. An important observation was that the endothelial TLR4 seemed to be redundant, since mice lacking TLR4 on endothelial cells displayed a fever response that was not significantly different from that of WT mice (Figure 2 in Steiner et al., 2006a)].

In conclusion, **paper I and II** and the above mentioned studies reveal the endothelial immune activation at BBB to be critical for the brain's adaptive responses like fever and release of stress hormones. The endothelial cells are highly responsive to IL-1 β , while the TLR4 signaling has but a low impact on the endothelial activation. Clinically this can give an answer to why simple bacteremia (i.e. circulating LPS) does not induce sickness symptoms. Bacteremia, in contrast to sepsis, is an inflammation-free bacterial passage in the blood stream, i.e. no IL-1 β is released and thus there is no activation of the brain endothelial cells. In our LPS model, TNF α , IL-1 β and IL-6 were detected in plasma at distinct time points, each with its own specific kinetics (Elander et al., 2009). Thus, in our present studies the BBB was activated by several circulating cytokines which resulted in an up-regulation of the cytokine receptors and several cytokine intracellular signaling pathways in endothelial cells. Nonetheless, there are also indications of an activation of perivascular cells, either by LPS at TLR4 or by other cytokines, resulting in an IL-6 *in-situ* production that might provide an intra-barrier regulatory loop. Perivascular cells

might be directly activated by LPS at CVOs, regions known to have a more accessible perivascular region and shown earlier to express immune-induced IL-1 β in its perivascular cells and to be among the first structures activated by immune stimuli (Goehler et al., 2006; Quan et al., 1998). This activation of perivascular cell within CVOs could thus be the first event at the BBB until an IL-1 β response is raised to engage the endothelial cells and induce a generalized BBB activation. Such an activation, as shown above in paper II, would bring about several functional changes like an increased cytokine sensitivity in endothelial cells and at the prostaglandin level a switch towards an increased production of PGE₂ in endothelial cells and decreased synthesis of other prostanoids both in endothelial cells and perivascular cells.

Brain PGE₂ as a mediator of fever at the preoptic region

Inflammation-induced PGE₂ levels in CSF after ip LPS were significantly increased already at 40 minutes (**paper I**), even though at that time point only COX-2 mRNA, but not mPGES-1, was up-regulated in the brain (**paper IV**) or specifically in dissociated barrier cells (unpublished data). This discrepancy was seen also in rats (Ivanov et al., 2002) but in those studies no clear PGE₂ increase in CSF was seen 45 minutes after LPS injection (Inoue et al., 2002). As peripheral organs like liver and lungs displayed an earlier up-regulation of the enzyme transcripts (Ivanov et al., 2002) than the brain, it was suggested that peripheral PGE₂ was responsible for the first phase of fever (Ivanov and Romanovsky, 2004). This idea was further supported by a study from the same group showing that the fever response was suppressed after administration of blocking antibodies against PGE₂ (Blatteis, 2006; Steiner et al., 2006b). In mice, however, we found that non-hematopoietic (i.e. endothelial in CNS) production of PGE₂, which was associated with an early rise of the PGE₂ level in CSF, is needed also for the first phase of fever as demonstrated in **paper I** (Figure 2B in paper I). Hence, at least in mice we could dismiss a peripheral hematopoietic origin of PGE₂ as responsible for fever. Nonetheless, the PGE₂ elevation in CSF precedes the up-regulation of the mPGES-1 transcript. Several explanations for this apparent contradiction could be considered, such as the presence of a preexisting PGE₂ pool in, for example, the cell components of the neuro-vascular unit (Tachikawa et al., 2012), or an initial effect mediated by constitutively expressed enzymes coupled transiently to rapidly produce and release PGE₂ (Turrin and Rivest, 2004). The latter hypothesis is supported by studies identifying a COX-1 dependency of the early behavior changes (Swiergiel and Dunn, 2002), the initiation of fever (Zhang et al., 2003), the hypothermic response after the first phase of fever (Steiner et al., 2009), and the HPA-activation (Elander et al., 2009; Elander et al., 2010). It is also supported in mouse by the presence of constitutive expression of mPGES-1 in the brain endothelial cells (Eskilsson et al., 2014b). For the inconsistency between the rapid releases of PGE₂ in the absence of induced mPGES-1 expression at the same time point, there are also methodological considerations to be addressed, such as different sensitivity levels for methods in general and

for protein vs mRNA methods in particular. As an example, measuring PGE₂ levels and mPGES-1 immunoreactivity, Inoue et al. (2002) reported an earlier increase of PGE₂ than of mPGES-1. In the same study, another contradiction discussed by the authors was the early drop of PGE₂ in CSF despite remaining high levels of COX-2 and mPGES-1. This as yet unsolved problem might have been answered in **paper II** by the up-regulation of the prostaglandin transporters in the barrier that by their suggested functions and cellular membrane localizations would contribute to the brain PGE₂ clearance (Akanuma et al., 2010; Tachikawa et al., 2012).

Once released in the neuronal parenchyma PGE₂ binds to its receptors and initiates different acute phase responses. The preoptic region of the anterior hypothalamus was shown to mediate the fever response and to participate to the HPA-activation (Saper et al., 2012; Ulrich-Lai and Herman, 2009) and hyperalgesia (Abe et al., 2001). Despite a high representation of EPs in this region we could not identify any of the inflammation-induced gene expression changes to be regulated by PGE₂ (**paper IV**), which seems to be in line with the sparse evidence in the literature (Tsuchiya et al., 2008), and which suggests that most of the effects of PGE₂ are non-transcriptional.

Future perspectives

The methodological arsenal available together with the multitude of transgenic animal models that exist today offer hope to elucidate the complex interactions between the BBB cells and their specific contribution in both physiological and pathological conditions, e.g. immune-to-brain signaling and sepsis, depressive disorders, multiple sclerosis and Alzheimer's disease, to name a few. By dissociating vascular cells from the brain and sorting them in specific populations we could analyze cellular specific characteristics and responses to immune stimuli. In immune-to-brain signaling most of the focus of our work was towards the PGE₂ production in the barrier and the roles played by IL-1 β or IL-6 but the contributions of several other cytokines await characterization, as well as the effect of the cytokines on other properties of the BBB, such as the role of IL-6 in barrier integrity, or the effect of different cytokines on the prostaglandin transporters.

We here examined 48 transcripts (**paper II**) but the sensitivity of the methods has increased and whole transcriptome analyses are already provided even for low cell counts, at more accessible prices. Enzymatic cell dissociation and cell isolation by FACS flow cytometry might be the way to go to characterize cells like pericytes that are otherwise difficult to isolate by e.g. LCM due to their localization, apposing endothelial cells. Dissociation and FACS-sorting is also possible for glial cells, although not for neurons. Analyzing glial cells would provide much information on the parenchymal response to peripheral inflammation and could help to elucidate the mechanisms underlying a persistent activation and neuroinflammation.

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