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# Inflammation-Induced Gene Expression in Brain and Adrenal Gland

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The cover illustration depicts endotoxin-induced expression of preproenkephalin heteronuclear RNA in the paraventricular hypothalamic nucleus, detected with *in situ* hybridization.

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“My precious”

*Gollum*  
(J.R.R. Tolkien, *The Fellowship of the Ring*, 1954)

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

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## ABSTRACT

The autonomic nervous system serves to maintain a constant inner environment, a process termed homeostasis. Thus, in response to the homeostatic challenge posed by infectious agents, the autonomic nervous system answers to signals from the immune system and elicits adaptive physiological and behavioral reactions. These so called sickness responses include fever, anorexia, hyperalgesia, social avoidance, and the release of stress hormones.

Neuropeptides, used in the communication between neurons, are because of their release properties and sustained actions likely mediators of homeostatic responses. The enkephalineric system constitutes one of the largest neuropeptidergic systems in the brain, but its involvement in inflammatory conditions has been little studied. We first examined the immune-induced activation of the parabrachial nucleus (paper I), an enkephalineric autonomic relay center in the brain stem. We found that intravenous injection of bacterial endotoxin, lipopolysaccharide (LPS), activated the external lateral parabrachial subnucleus, as measured in terms of Fos expression, but that the enkephalineric cell population in this subnucleus was largely separated from the LPS-activated neurons. Because Fos may not always be a reliable activity marker, we next examined by *in situ* hybridization the immune-induced expression of newly transcribed preproenkephalin (ppENK) heteronuclear RNA (hnRNA), which gives a direct indication of the utilization of enkephalin in a particular neuron (paper II). We detected induced expression of ppENK hnRNA in several autonomic structures in the brain, including the paraventricular hypothalamic nucleus (PVH) but not the parabrachial nucleus, indicating increased enkephalineric signaling activity in the positively labeled structures during inflammatory condition. We then examined the projections of the immune-induced ppENK transcribing PVH neurons by injecting rats intraperitoneally with the retrograde tracer substance Fluoro-Gold, hence labeling neurons with axonal projections outside the blood-brain barrier, followed by systemic injection of LPS (paper III). Dual-labeling histochemical and hybridization techniques showed that the vast majority of the ppENK hnRNA expressing cells were hypophysiotropic cells, hence being involved in neuroendocrine regulation. These findings suggest that centrally produced enkephalin is involved in the coordination of the sickness responses during systemic immune challenge, including the modulation of the release of stress hormones or other hypothalamic hormones during inflammatory conditions.

We next turned to the role of prostaglandins in the hypothalamic-pituitary-adrenal (HPA) axis response to inflammation. We injected mice deficient for the terminal prostaglandin (PG) E<sub>2</sub> synthesizing enzyme mPGES-1 with LPS and studied their stress hormone release (paper IV). The genetically modified mice displayed attenuated plasma levels of adrenocorticotrophic hormone (ACTH) and corticosterone during the later phases of the HPA-axis response compared with wild type mice, and this impairment did not depend on a changed activation pattern in the brain, but instead correlated to an early decrease in corticotropin-releasing hormone mRNA

expression in the PVH, hence being the likely cause of the blunted ACTH and corticosterone responses at later time-points. Based on these findings we suggest that a neural, mPGES-1-independent pathway, and a humoral, mPGES-1-dependent pathway act in concert but in distinct temporal patterns to initiate and maintain the HPA-axis response during immune challenge.

In addition to activating the central limb of the HPA-axis, inflammatory mediators have been suggested to act directly on the adrenal gland to induce the release of corticosterone, but little is known about the underlying mechanisms. We examined adrenal tissue isolated from rats injected with LPS or interleukin-1 $\beta$  (IL-1 $\beta$ ) (paper V), and found that immune stimulation resulted in dynamic changes in the adrenal immune cell population, implying a rapid depletion of dendritic cells in the inner cortical layer and the recruitment of immature cells to the outer layers. These changes were accompanied by an induced production of IL-1 $\beta$  and IL-1 receptor type 1, as well as of cyclooxygenase-2 and mPGES-1 in these cells, implying local cytokine-mediated PGE<sub>2</sub> production in the adrenals, which also displayed EP<sub>1</sub> and EP<sub>3</sub> receptors in the cortex and medulla. Additional mechanistic studies using an IL-1 receptor antagonist showed that IL-1 $\beta$  acts locally to affect its own synthesis, as well as that of cyclooxygenase-2. Taken together these data demonstrate a mechanism by which systemic inflammatory agents activate an intrinsically regulated local signaling circuit that may influence the adrenals' response to immune stress and may help explain the dissociation between plasma levels of ACTH and corticosteroids during chronic immune perturbations.

## ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AP	area postrema
AP-1	activated protein-1
BNST(ov)	bed nucleus of the stria terminalis (oval part)
CeA(l)	central nucleus of the amygdala (lateral part)
CGRP	calcitonin gene-related peptide
CRH	corticotropin-releasing hormone
Cox	cyclooxygenase
CVO	circumventricular organ
cPGES	cytosolic prostaglandin E synthase
cPLA <sub>2</sub>	cytosolic phospholipase A <sub>2</sub>
CRE	cAMP-responsive element
CREB	cAMP-responsive element-binding protein
CREM/ICER	cAMP-responsive modulator/inducible cAMP early repressor
FG	Fluoro-Gold
EP	prostaglandin E <sub>2</sub> receptor
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
GR	glucocorticoid receptor
hnRNA	heteronuclear RNA
HPA	hypothalamic-pituitary-adrenal
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.v.	intravenous
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IL-1R1	interleukin-1 receptor type 1
IL-1R2	interleukin-1 receptor type 2
IRAK	interleukin-1 receptor associated kinase
LH	luteinizing hormone
LPS	lipopolysaccharide
MHC	major histocompatibility complex
mPGES-1	microsomal prostaglandin E synthase-1
mPGES-2	microsomal prostaglandin E synthase-2
MR	mineralocorticoid receptor
MyD88	myeloid differentiation factor 88
NTS	nucleus of the solitary tract
PB	parabrachial nucleus
elo	outer part of external lateral subnucleus
eli	inner part of external lateral subnucleus
PGE <sub>2</sub>	prostaglandin E <sub>2</sub>

PoA	preoptic area
ppENK	preproenkephalin
PVH	paraventricular hypothalamic nucleus
dp	dorsal parvocellular subdivision
mp <sub>dd</sub>	dorsal part of the dorsal medial parvocellular subdivision
mp <sub>dv</sub>	ventral part of the dorsal medial parvocellular subdivision
mp <sub>v</sub>	ventral part of the medial parvocellular subdivision
pm	magnocellular subdivision
sPLA <sub>2</sub>	secretory phospholipase A <sub>2</sub>
TLR	Toll-like receptor
TNF- $\alpha$	tumor necrosis factor- $\alpha$
TRAF6	TNF-receptor-associated factor 6
TSA	tyramide signal amplification
VLM	ventrolateral medulla

## INTRODUCTION

Our body is constantly exposed to a variety of environmental challenges that pose potential threats to our survival. Already in the middle of the 19<sup>th</sup> century, Claude Bernard recognized that the maintenance of a stable inner milieu is essential for life, a process that Walter B. Cannon later termed “homeostasis”. The autonomic nervous system plays a crucial role in maintaining the steady-state. Thus, when we are hungry, thirsty, too cold or too warm, central autonomic circuits are activated and initiate appropriate behavioral and physiological responses, which serve to fulfill our basic, life-promoting needs. Perhaps even more obvious as a threat, humankind has during all times been tormented by unpredictable and deadly infectious illnesses caused by surrounding bacteria and viruses. By means of our immune system, these intruders can in many cases be recognized and eliminated. As a result of an intricate interplay between the immune system and the brain, central nervous structures elicit a set of finely tuned sickness responses that serve to facilitate, regulate and balance the inflammatory process. These include an elevated body temperature, loss of appetite, increased pain sensitivity, sleepiness, and social avoidance. The sickness responses serve to prioritize the use of energy for the most relevant life-promoting processes, to enhance the efficacy of the immune system (while compromising that of the pathogens), and to reduce the dissipation of pathogens to other individuals. However, an uncontrolled immune response can turn against the body and cause tissue damage, respiratory and cardiovascular shock and even death. Thus, it is of major importance that it be properly balanced. The centrally activated hypothalamic-pituitary-adrenal (HPA) axis, controlling the release of anti-inflammatory glucocorticoids, plays a crucial role in this process.

### **Mediators of sickness responses – endotoxin and cytokines**

#### *Endotoxin (lipopolysaccharide)*

The term ‘microbiology’ was created by Louis Pasteur (1822-1895), who proved that microorganisms were necessary and sufficient to cause an infectious disease. Another important discovery was made by Richard Pfeiffer, who in 1886 showed that dead cholera bacteria retained their toxic potential. On the basis of this and other observations he coined the term ‘endotoxin’, referring to a heat-stable toxic substance associated to the bacterial cell (reviewed by Beutler and Rietschel, 2003). Endotoxin was discovered to be part of the outer membrane of Gram-negative bacteria and examination of its structure revealed that it was composed of polysaccharide and lipid components, hence termed lipopolysaccharide (LPS). The lipid component, named lipid A, is a glycopospholipid that holds the pyrogenic and toxic properties of endotoxin and that is essential for bacterial viability. The basic structure of lipid A is similar for all Gram-negative bacteria. Attached to the lipid A is a branched core polysaccharide of 9-12 sugar molecules. The core polysaccharide is in turn linked to the O-antigen, which is a long linear polysaccharide consisting of 50-100 repeating

saccharide units of 4-7 residues each. The O-antigen is present on the bacterial surface and confers strain specificity of a bacterial species. In patients with Gram-negative bacterial sepsis, LPS elicits fever, anorexia, hyperalgesia, hypotension, shock due to vasodilatation and capillary leakage, and intravascular coagulation (Murray, 1997).

### Toll-like receptors and intracellular signaling

Once pathogens have penetrated our protective barriers, the body turns all efforts towards their swift removal. Thus, the immune system must be able to recognize microbial presence. By its non-specific and general nature of action, the innate immune system provides an immediate defense against infectious disease and is found in all classes of plant and animal life (for review, see Kimbrell and Beutler, 2001). The mechanisms for microbial recognition were long obscure, but in 1996 it became clear that a Toll receptor in the adult *Drosophila* was required for a functional anti-fungal response in this species (Lemaitre et al., 1996). The following year, the first human Toll homologue was cloned, showing preserved signaling ability promoting cytokine synthesis (Medzhitov et al., 1997). However, the agonist was unknown. A major leap in the understanding of microbial recognition mechanisms was taken with the discovery that the Toll-like receptor (TLR) 4 was required for LPS signaling in mice (Poltorak et al., 1998). Thus, the TLRs, first cloned and shown to be involved in dorsoventral patterning of the *Drosophila* embryo (Anderson et al., 1985), were proposed to collectively be the sensors of invading microbes. To date, there are altogether 11 TLRs cloned in human and mouse and their natural ligands are becoming unraveled. They have been shown to bind conserved microbial elements that are essential for the survival of the microbe. Thus, it is now known that the TLR2 recognizes lipoproteins, TLR3 double-stranded viral RNA, TLR4 LPS, TLR5 flagellin, TLR7 and 8 single-stranded viral RNA, TLR9 CpG DNA, and TLR11 profilin (Poltorak et al., 1998; Aliprantis et al., 1999; Hemmi et al., 2000; Alexopoulou et al., 2001; Hayashi et al., 2001; Heil et al., 2004; Yarovinsky et al., 2005). TLR1 and 6 form heteromers with TLR2 (Buwitt-Beckmann et al., 2006). The natural ligand of TLR10 remains unknown.

In 1990, it was discovered that LPS binds to an acute phase protein (LPS binding protein; LBP) and interacts with the plasma membrane-bound CD14 receptor (Wright et al., 1990). However, this complex lacks an intracellular domain and it remained obscure as to how the signal could be propagated into the cell. Later on, it was shown that the role of CD14 was to load LPS on the glycoprotein MD-2, which then could form a complex with TLR4 (Shimazu et al., 1999). TLR4 shares, with all other TLRs and the interleukin-1 $\beta$  (IL-1 $\beta$ ) receptor (IL-1R) family (see below), a common signaling pathway (Fig. 1). A conserved protein-protein interaction module, namely the intracellular domain called the Toll/IL-1R (TIR) domain, interacts with myeloid differentiation factor 88 (MyD88), which in turn recruits kinases from the interleukin-1 receptor associated kinase (IRAK) family to the receptor complex (Cao et al., 1996a; Muzio et al., 1997; Medzhitov et al., 1998). IRAK-1 then interacts with TNF-receptor-associated factor 6 (TRAF6), which in turn activates kinases that subsequently

phosphorylate I $\kappa$ B kinase, thereby activating the nuclear factor NF- $\kappa$ B transcriptional pathway (Cao et al., 1996b) or the p38/JNK pathway that leads to the formation of the activator protein-1 (AP-1) complex (Krappmann et al., 2004). Additionally, interactions between TRAF6 and the adaptor protein ECSIT (evolutionary conserved signaling intermediate in Toll pathways), can, via MEKK1, trigger the AP-1 and the NF- $\kappa$ B transcriptional pathways (Kopp et al., 1999). The result of LPS signaling is the induced synthesis of a number of inflammatory mediators, such as the cytokines IL-1 $\beta$ , IL-6 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), proteins of the complement system, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) producing enzymes, adhesion molecules, and immune response receptors (reviewed by Palsson-McDermott and O'Neill, 2004). Additionally, it should be mentioned that TLR4 can signal via a MyD88 independent pathway, regulating the expression of major histocompatibility complex II (MHC class II) and dendritic cell maturation (Kaisho et al., 2001).

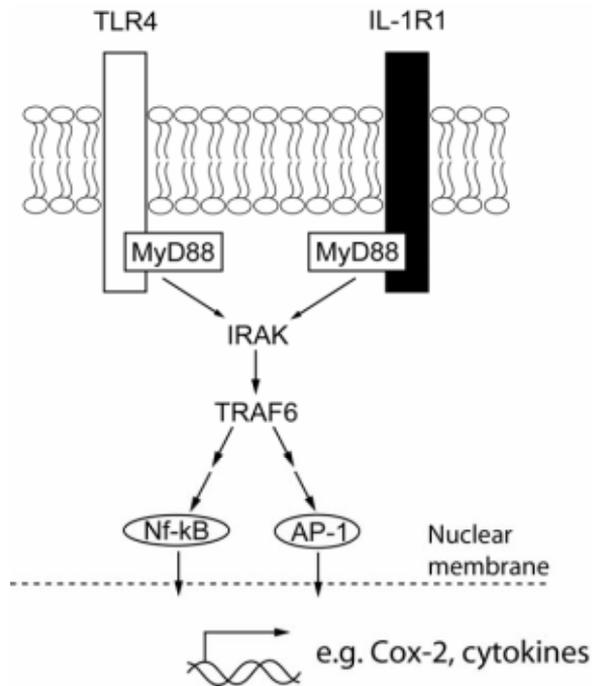


Fig. 1. Simplified signaling pathways for LPS (via TLR4) and IL-1 $\beta$  (via IL-1R1), which share the same intracellular signaling mechanisms. Ligand-binding leads to the recruitment of the adaptor protein MyD88 to a conserved element of the receptors. Ultimately, the transcription factors NF- $\kappa$ B or AP-1 are activated and translocated to the nucleus where they can influence the transcription of cytokines and enzymes involved in prostaglandin E<sub>2</sub> synthesis.

## *Cytokines*

Cytokines are large proteins produced by immune cells. They deal with regulation of growth and the differentiation and function of cells, and are most commonly associated with immune/inflammatory processes, in which they mainly work in an autocrine or paracrine manner. However, they can also regulate processes such as sleep, fever, anorexia, malaise, and alterations in neuroendocrine functions. Thus, not only do they regulate local processes, but also more systemic homeostatic mechanisms in response to trauma and infection (reviewed by Turnbull and Rivier, 1999). The main cytokine studied in relation to sickness responses and immune-to-brain signaling is IL-1 $\beta$ , which was used as an immune stimulus in this thesis work. Therefore, a summary of its properties will be given below.

### Interleukin-1 $\beta$

The IL-1 family is constituted by several different gene products, namely IL-1 $\alpha$ , IL-1 $\beta$ , IL-1 receptor antagonist (IL-1ra), and IL-18 (March et al., 1985; Carter et al., 1990; Eisenberg et al., 1990; Eisenberg et al., 1991; Okamura et al., 1995; Bazan et al., 1996). Two receptors that bind the agonists IL-1 $\alpha$  and IL-1 $\beta$  have been cloned, and are named the IL-1R1 and 2 (Sims et al., 1988), respectively. Both IL-1 $\alpha$  and IL-1 $\beta$  signal via the IL-1R1, whereas the IL-1R2 is a decoy receptor that does not produce an intracellular signal upon ligand binding (Sims et al., 1993). While IL-1 $\alpha$  seems to be involved in close intercellular signaling (Auron et al., 1987), IL-1 $\beta$  mainly mediates paracrine and systemic responses. The IL-1ra has been shown to block the IL-1R1 and thereby antagonize the actions of IL-1 without producing an intracellular signal by itself (Carter et al., 1990), although exceptions seem to exist (Cole et al., 1993; Mitchell et al., 1993; Brown et al., 1998). IL-1 $\beta$  is synthesized as a large pro-peptide, which is cleaved by IL-1 $\beta$  converting enzyme to yield the final product of 17 kDa (Cerretti et al., 1992; Thornberry et al., 1992).

As mentioned above, the IL-1R1 and TLRs share the intracellular TIR domain, and hence, signaling via the IL-1R1 functions in a similar way as for TLRs (see Fig. 1). However, it has been shown that the IL-1R1 first has to dimerize with an IL-1 receptor accessory protein (IRAP) in order to propagate the signal (Greenfeder et al., 1995).

IL-1 $\beta$  is present in the blood of septic patients, and can also be detected in plasma of animals that have been challenged with LPS (Givalois et al., 1994; Ronco et al., 2003). In experimental setups, IL-1 $\beta$ , injected directly into the blood stream or peritoneal cavity of animals, is a potent elicitor of the acute-phase response. Thus, upon peripheral administration, symptoms like fever and anorexia are manifest within short (Dinarello et al., 1986; Saha et al., 2005; Elander et al., 2006), as well as an increased release of adrenocorticotrophic hormone (ACTH) and corticosterone, the hallmarks of HPA-axis activation (Sapolsky et al., 1987). Indeed, during experimental conditions, IL-1 $\beta$  is more potent in activating the HPA-axis than e.g. IL-6 and TNF- $\alpha$ , two other cytokines closely associated with the acute-phase response (Besedovsky et al., 1991; Dunn, 2000a).

Despite its frequent usage as an immune stimulus and its presence in the circulation during systemic inflammatory conditions both in humans and rodents (Cannon et al., 1990; Givalois et al., 1994), it remains unclear to what extent IL-1 $\beta$  is critical for the different sickness responses. In the case of LPS-induced fever, the concomitant injection of a recombinant IL-1ra has been shown to attenuate the febrile response (Luheshi et al., 1996), suggesting that endotoxin-elicited pyresis is dependent on endogenously produced IL-1 $\beta$ . However, in studies examining knockout mice that lack either the IL-1R1 or the ability to produce IL-1 $\beta$ , the febrile response has been reported either unaffected or even enhanced after peripheral injections of low doses of LPS (Leon et al., 1996; Alheim et al., 1997; Kozak et al., 1998), and only slightly attenuated after high doses of the endotoxin (Kozak et al., 1998). Studies on the role of IL-1 $\beta$  in the LPS-elicited HPA-axis response have also produced contradictory results. Thus, while IL-1 $\beta$  itself elicits an HPA-axis response, some studies have suggested that injection of an IL-1ra is ineffective in attenuating the HPA-axis response (Hadid et al., 1999; Dunn, 2000b), while other studies report the opposite (Rivier et al., 1989; Ebisui et al., 1994). Furthermore, research on IL-1 $\beta$  knockout mice has shown that these mice respond with a normal HPA-axis activation to LPS-injections (Fantuzzi et al., 1996; Kozak et al., 1998). Thus, the role of circulating IL-1 $\beta$  during systemic inflammation remains unclear. Functional redundancy in the cytokine response may be a likely explanation for the above-mentioned observations.

In contrast to what is the case for systemic inflammatory conditions, the role of IL-1 $\beta$  during local inflammatory responses is well established. Thus, studies in IL-1 $\beta$  and IL-1R1 knockout mice have shown that fever, anorexia and HPA-axis activation in a model of local inflammation induced by turpentine, are completely dependent upon functional IL-1 $\beta$  signaling (Zheng et al., 1995; Leon et al., 1996). Studies in which LPS was injected into a subcutaneous pouch have further substantiated the role for IL-1 $\beta$  as an indispensable mediator of sickness responses during local inflammation (Miller et al., 1997a; Miller et al., 1997b).

### **Signaling of pro-inflammatory mediators to the brain**

In order to activate the central component of the acute-phase response during infection/inflammation, peripherally produced cytokines – or LPS itself – have to communicate with the central nervous system. During the past 10-15 years, intense research has put forward several hypotheses for how this communication can take place. An obvious conceptual problem has been the existence of the blood-brain barrier, partly made up by tightly coupled endothelial cells lining the cerebral blood vessels. This barrier protects the brain against harmful circulating substances. Only small lipophilic molecules are allowed to pass freely, while cytokines, which are large and hydrophilic molecules, are not permitted entrance. The presence of an easily saturated transport system for cytokines has been suggested, but if it exists it is unlikely to permit enough cytokines to pass through the barrier to cause any relevant

effects during inflammation (Banks et al., 1995). Thus, other mechanisms must be at work.

### *Circumventricular organs*

Circumventricular organs (CVOs) are specialized structures in the brain that lack a functional blood-brain barrier. Thus, via fenestrated capillaries, the neurons of the CVOs are exposed to the blood circulation and can hence, via their efferent connections, transmit the presence of circulating stimuli to relevant brain regions. The CVOs in mammals include the area postrema (AP; situated in the bottom of the fourth ventricle, and involved in e.g. satiety signaling and in initiating the vomiting-reflex), the median eminence (situated at the base of the pituitary stalk, and conveying signals from the neuroendocrine hypothalamus to the pituitary gland), the subfornical organ (located in the roof of the third ventricle, and involved in maintaining fluid balance of the body), and the organum vasculosum of the lamina terminalis (surrounding the anterior part of the third ventricle, and implicated in e.g. body temperature regulation) (Fry and Ferguson, 2007). Messenger RNA for TLR4, CD14, and cytokine receptors (the IL-1R1, the IL-6 receptor, and the TNF- $\alpha$  receptor p55) have been found to be constitutively expressed in CVOs (Ericsson et al., 1995; Vallieres and Rivest, 1997; Nadeau and Rivest, 1999; Laflamme and Rivest, 2001; Konsman et al., 2004) and immune stimulation with IL-1 $\beta$  causes activation of the CVOs in terms of Fos expression (an activity marker; see Methodology) (Ericsson et al., 1994; Herkenham et al., 1998). Lesion studies have implicated the organum vasculosum of the lamina terminalis and the AP in some of the centrally elicited inflammatory responses, such as fever (Blatteis, 1992) and activation of the HPA-axis (Lee et al., 1998), respectively. However, these studies should be interpreted with caution, since closely situated structures such as the preoptic area (critical for the febrile response (Lazarus et al., 2007)), and the nucleus of the solitary tract (NTS; important for activation of the HPA-axis (Ericsson et al., 1994; Buller et al., 2001)) may have been affected during the lesioning procedures. Accordingly, others have failed to show any effect of surgical removal of the AP on the activation of the HPA-axis (Ericsson et al., 1997).

### *The vagus nerve*

An alternative pathway for pro-inflammatory mediators to activate central neuronal circuits is through interaction with peripheral nerve fibers that convey sensory information to the central nervous system. The vagus nerve has emerged as the most important candidate for such an interaction. The vagus nerve is the tenth cranial nerve and belongs to the parasympathetic part of the autonomic nervous system. It innervates the organs of the thoracic and abdominal cavities and contains both afferent and efferent fibers. The termination area for the afferent fibers is situated in the caudal brain stem and is called the nucleus of the solitary tract (NTS). From here, the visceral information is transmitted to other brain stem and forebrain structures (Kandel et al., 2000). Research has shown that mRNA for TLR4, IL-1R1, and PGE<sub>2</sub> receptors are expressed by the sensory pseudo-unipolar cells of the vagal nodose ganglion (Ek et al.,

1998; Hosoi et al., 2005). Being consistent with these observations, immune stimulation with IL-1 $\beta$  and LPS has been shown to activate vagal afferents (Ek et al., 1998; Gaykema et al., 1998; Goehler et al., 1998).

Despite the presence of obvious prerequisites for a vagus-dependent inflammatory signaling to the brain, studies that have employed vagotomy in inflammatory models have not been able to provide conclusive data regarding the role of the vagus nerve in eliciting the centrally coordinated sickness responses. This probably depends on the usage of different doses of inflammatory substances in different studies, as well as on the side-effects associated with vagus transection, such as gastrointestinal stasis and concomitant leakage of intestinal contents to the blood stream. However, the general view seems to be that afferent activation of the vagus nerve is important during intraperitoneal injections of low doses of pro-inflammatory substances, whereas high doses of IL-1 $\beta$  or LPS supposedly cause spill-over into the general circulation and hence also affect other immune-to-brain signaling pathways. Thus, the febrile response to a low dose of IL-1 $\beta$  can be attenuated by subdiaphragmatic vagotomy, while that to higher doses cannot (Konsman et al., 2000; Hansen et al., 2001). Similar results have been obtained when studying the HPA-axis response to intraperitoneally injected LPS and IL-1 $\beta$  (Gaykema et al., 1995; Kapcala et al., 1996). Furthermore, when the inflammatory substance is injected into the blood stream, vagotomy has no effect on social exploration (Bluthe et al., 1996) or on the activation of autonomic relay nuclei in the brain (Ericsson et al., 1997). Thus, the vagus nerve may be relevant during conditions of restricted inflammation/infection in e.g. the abdominal cavity, whereas systemic conditions seem to rely on multiple pathways for conveying immune signals to the brain.

### *Signaling across the blood-brain barrier*

The blood-brain barrier is impermeable to cytokines but it is nevertheless critically involved in the relay of circulating inflammatory signals to the brain. Thus, receptors for IL-1 $\beta$  (Ericsson et al., 1995; Ek et al., 2001; Konsman et al., 2004), IL-6 (Vallieres and Rivest, 1997), and TNF- $\alpha$  (Nadeau and Rivest, 1999) are present in the brain vasculature, and injection of either LPS, IL-1 $\beta$ , or TNF- $\alpha$  induces rapid expression of I $\kappa$ B $\alpha$  mRNA (an index for NF- $\kappa$ B activity) in brain blood vessels (Laflamme and Rivest, 1999; Konsman et al., 2004). This suggests that circulating cytokines can bind to brain endothelial cells, and, via a messenger produced within the barrier itself, affect neuronal structures involved in coordinating the sickness response. Convincing evidence now exist that PGE<sub>2</sub> is the likely candidate for this task (for review, see Engblom et al., 2002b).

### Prostaglandin E<sub>2</sub>

PGE<sub>2</sub>, which is a small and lipophilic molecule, is an important mediator of inflammation. Prostaglandins were first recognized in the 1930's, by Von Euler and Goldblatt who demonstrated that semen and extracts from prostates contained a factor

that stimulated smooth muscle contraction and reduced blood pressure. In the 50's and 60's, Sune Bergström purified and structurally determined the first prostaglandins, and the work by Bengt Samuelsson characterized the pathways by which PGs are formed and metabolized (see e.g. Samuelsson, 1983). In 1982, Sune Bergström and Bengt Samuelsson were awarded the Nobe prize for their discoveries, together with John Vane who discovered that aspirin exerts its effects by interfering with the prostaglandin synthesis cascade (Vane, 1971; Vane, 1983).

Prostaglandins are synthesized via several enzymatic steps and depending on the context, distinct enzymes are involved in the synthesis cascade (Fig. 2). The first steps are the same for all prostaglandins, while the last step that involves the conversion of PGH<sub>2</sub> to the final prostaglandin species requires distinct terminal synthases. Briefly, phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is recruited in the first step that requires synthesis of arachidonic acid from cell membrane phospholipids. There are several different forms of PLA<sub>2</sub>s, two of which, cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) and secretory PLA<sub>2</sub> Iia (sPLA<sub>2</sub>-Iia), are induced during inflammatory conditions (Ivanov et al., 2002). Thereafter, arachidonic acid is converted to PGH<sub>2</sub> via two enzymatic steps, both catalyzed either by cyclooxygenase (Cox)-1 or 2. Cox-1 is a constitutively expressed enzyme, present in virtually all tissues of the body and typically not related to inflammatory conditions. In contrast, Cox-2 is tightly coupled to inflammation, and is therefore inducible in its nature, exhibiting an otherwise low basal expression (see e.g. Crofford, 1997). PGH<sub>2</sub> is converted to PGE<sub>2</sub> by a terminal prostaglandin E synthase (PGES). Three PGESs have to date been identified, namely cytosolic PGES (cPGES) and microsomal prostaglandin E synthase (mPGES)-1 and 2 (Jakobsson et al., 1999; Tanioka et al., 2000; Tanikawa et al., 2002).

Microsomal PGES-2, which is functionally coupled to Cox-1, is constitutively expressed and appears to be unrelated to inflammation. The same seems to apply to cPGES (Tanioka et al., 2000), although it has been reported that inflammation induces cPGES mRNA expression in the brain (Ivanov et al., 2002). Microsomal PGES-1 has typically very low constitutive expression but is readily induced by e.g. IL-1 $\beta$  and LPS (Jakobsson et al., 1999; Ek et al., 2001; Uematsu et al., 2002). Hence, it is closely associated to inflammation and is considered functionally coupled to Cox-2, but can, in the case of strong cPLA<sub>2</sub> induction, also couple to Cox-1 (Murakami et al., 2000).

Once synthesized, PGE<sub>2</sub> exerts its effect via four distinct G-protein coupled receptors, termed EP<sub>1-4</sub>. The EP<sub>1</sub> receptor is stimulatory and elevates the intracellular level of calcium (Kato et al., 1995; Negishi et al., 1995). The EP<sub>2</sub> and EP<sub>4</sub> receptors both increase the intracellular level of cAMP (Honda et al., 1993; Negishi et al., 1995), while the EP<sub>3</sub> receptor, which exists in several splice variants, mainly mediates inhibitory effects via coupling to Gi proteins and hence lowering the level of cAMP in the cell. However, in rat and mouse, the EP<sub>3B</sub> and EP<sub>3 $\gamma$</sub>  isoforms increase the level of intracellular Ca<sup>2+</sup> and cAMP, respectively (Takeuchi et al., 1994; Negishi et al., 1996).

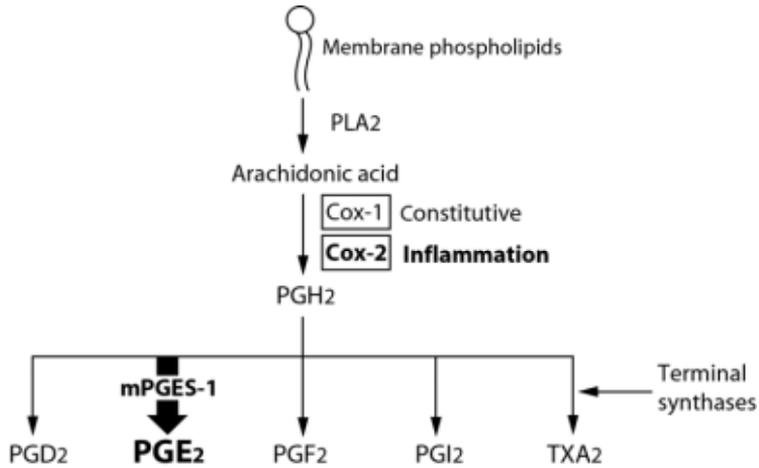


Fig. 2. Synthesis cascade for prostaglandins. During normal conditions, Cox-1 is coupled to constitutively expressed PGESs (not shown) for the production of PGE<sub>2</sub>. During inflammation, Cox-2 is heavily induced, as well as the terminal isomerase mPGES-1, leading to increased production of PGE<sub>2</sub>.

### Prostaglandin E<sub>2</sub> production in the blood-brain barrier

Studies have shown that injection of PGE<sub>2</sub> directly into the brain ventricles rapidly causes fever (Feldberg and Saxena, 1971) and activation of the HPA-axis (Rassnick et al., 1995), as well as recruitment of autonomic relay structures throughout the neuroaxis (Lacroix et al., 1996), hence suggesting that centrally produced PGE<sub>2</sub> participates in the activation of sickness responses. An indication of PGE<sub>2</sub> synthesis in the blood-brain barrier arose when it was discovered that Cox-2 expression is induced in the cerebral vasculature following immune challenge with LPS and IL-1 $\beta$  (Cao et al., 1995; Cao et al., 1997; Lacroix and Rivest, 1998; Quan et al., 1998). Subsequent studies have shown that not only Cox-2, but also mPGES-1, is present in endothelial cells of brain vessels after injection of IL-1 $\beta$ , as well as during more chronic inflammatory conditions, such as adjuvant-induced arthritis (Ek et al., 2001; Engblom et al., 2002a). Thus, PGE<sub>2</sub> produced at the blood-brain barrier is the likely source of brain PGE<sub>2</sub> during inflammatory conditions. In contrast, neuronally derived PGE<sub>2</sub> has been shown to be unlikely to contribute to the sickness symptoms seen during peripheral inflammation (Cao et al., 1997). In good accord with the induction of PGE<sub>2</sub>-synthesising enzymes along the blood-brain barrier, mice lacking the ability to synthesize Cox-2 or mPGES-1, and that hence cannot produce PGE<sub>2</sub> in response to inflammatory stimuli, fail to mount a febrile response to LPS, IL-1 $\beta$ , or turpentine, and show attenuated anorexia after IL-1 $\beta$  injection (Li et al., 1999; Engblom et al., 2003; Saha et al., 2005; Elander et al., 2006; Pecchi et al., 2006). Evidence also suggests that

PGE<sub>2</sub> may be involved in the activation of the HPA-axis response, since Cox-inhibitors can attenuate ACTH and corticosterone release after immune challenge with IL-1 $\beta$  (Rivier and Vale, 1991; Parsadaniantz et al., 2000). Additionally, EP<sub>1</sub> and EP<sub>3</sub> receptor knockout mice show an attenuated ACTH response after injection with LPS (Matsuoka et al., 2003).

## **Integration of afferent signals – role of neuropeptides**

### *Neuropeptide signaling during the acute-phase response*

When the brain is contacted by the immune system, independent of route, the information has to be properly processed and integrated in order to achieve appropriate efferent responses for the maintenance of homeostasis. The central structures involved are parts of the brain stem, diencephalon, and the limbic forebrain that are interconnected in a highly intricate manner. These neuronal structures signal to each other via the release of both classical neurotransmitters (such as amines and amino acids) and neuropeptides, commonly co-stored in the same axon terminal, as first demonstrated in the 70's (Hökfelt et al., 1977). While the classical transmitters, stored in small clear vesicles and often being in close apposition to the active zone of the synapse, are readily released upon neuronal firing and exhibit immediate and short-term effects on the post-synaptic neuron, neuropeptides differ in their storage and kinetics of release. Thus, they are stored in large dense-core vesicles (which however also can contain classical transmitters) in the cytoplasm and generally require stronger signaling, in terms of increased frequency/bursts of action potentials, in order to be released into the synaptic cleft (Bartfai et al., 1988; Whim and Lloyd, 1989; Karhunen et al., 2001). Once released, they cause long-term changes of the excitability of the post-synaptic neuron. Thus, while classical transmitters are released and signal under “normal” circumstances, it is believed that neuropeptides are recruited as signaling molecules during more demanding situations (e.g. stress) that require long-term modulation of signaling circuits (Hökfelt, 1991). Accordingly, neuropeptides emerge as highly suitable for modulating signaling in autonomic circuits during homeostatic challenges, such as during inflammatory conditions.

A large number of neuropeptides have so far been identified, but there is limited knowledge about which neuropeptidergic systems that are involved in coordinating the sickness responses during systemic inflammation. Thus, even though the structures that are activated during immune challenge have been identified (Ericsson et al., 1994; Rivest and Laflamme, 1995; Elmquist et al., 1996; Lacroix et al., 1996; Herkenham et al., 1998), in general there is a lack of data regarding the neuropeptidergic identity of the immune-activated neurons. However, it has e.g. been demonstrated that anorexigenic and orexigenic neuropeptides involved in the general regulation of feeding behavior and metabolism (Schwartz et al., 2000; Cone, 2005), also are implicated in the LPS-induced changes in food intake, since the expression of such neuropeptides is affected in the arcuate nucleus and the lateral hypothalamus during

inflammatory conditions (Huang et al., 1999; Sergeev et al., 2001). Furthermore, the expression of peptides related to the HPA-axis response, such as corticotropin-releasing hormone (CRH) and vasopressin, have been examined in the hypothalamus after stimulation with LPS and IL-1 $\beta$  (Rivest et al., 1992; Lee and Rivier, 1994; Rivest and Laflamme, 1995; Lacroix and Rivest, 1998). In addition, Day and collaborators studied the expression of CRH, enkephalin and neurotensin in neurons in the lateral part of the central amygdala (CeAl) and bed nucleus of the stria terminalis (BNST), and could show that these neuropeptides were expressed by inhibitory GABAergic neurons and that they to different extents co-localized with Fos after immune stimulation (Day et al., 1999). Studies from this laboratory have examined activated brain stem circuits with focus on an autonomic relay nucleus in the dorsolateral pons, named the parabrachial nucleus (PB). This nucleus is divided into several subnuclei, which express an array of neuropeptides (e.g. enkephalin, dynorphin, calcitonin gene-related peptide, cholecystokinin, and substance P) (Hermanson, 1997). The expression of some of these neuropeptides have been related to Fos and EP-receptor expression after immune and/or aversive stimuli (Paues et al., 2001; Engblom et al., 2004; Paues et al., 2006) (see Results and General Discussion for more information).

The opioids, and the enkephalins in particular, constitute the most abundant neuropeptidergic system in the mammalian brain. Part of this thesis work has focused on the enkephalinergic system in the rat brain during systemic inflammatory conditions.

### *Endogenous opioids – focus on enkephalins*

In 1975, the first endogenous opioids were identified by Hughes and Kosterlitz, who demonstrated that brain extracts could mimic the effects of morphine on vas deferens contractility (Hughes, 1975; Hughes et al., 1975). Shortly thereafter, two enkephalin penta-peptides, named met-enkephalin (Tyr-Gly-Gly-Phe-Met) and leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), were isolated from brain tissue (Hughes et al., 1975; Simantov and Snyder, 1976). Subsequently,  $\beta$ -endorphin and the dynorphins were isolated (Li et al., 1976; Goldstein et al., 1979). All these opioids are derived from larger propeptides, namely proenkephalin, pro-opiomelanocortin, and prodynorphin. In more recent years, other endogenously produced opioids have been discovered, including nociceptin/orphanin FQ (derived from pronociceptin/orphanin FQ) and endomorphin-1 and 2. The two latter opioids are structurally unrelated to the other opioids, which all contains the core opioid sequence Tyr-Gly-Gly-Phe-Met/Leu. No precursor has yet been identified for endomorphin-1 and 2 (Waldhoer et al., 2004). Because the experiments in this thesis focus on enkephalins, the characteristics of these peptides will be the main topic dealt with here.

The human, bovine and rat cDNAs encoding the enkephalin peptides were cloned and sequenced in the early 80's (Comb et al., 1982; Gubler et al., 1982; Legon et al., 1982; Noda et al., 1982a; Yoshikawa et al., 1984), as well as the preproenkephalin (ppENK) gene (Noda et al., 1982b; Comb et al., 1983; Rosen et al., 1984). The rat ppENK gene

is ~5.3 kb long and comprises three exons and two introns. The nucleotide sequence encoding the mature proenkephalin precursor peptide is located on exons 2 and 3, while the 5' untranslated region is encoded by exons 1 and part of exon 2. The 3' untranslated region is found in exon 3 (Rosen et al., 1984). The spliced brain ppENK mRNA is ~1.4 kb and is translated to the ppENK precursor peptide (Yoshikawa et al., 1984), which, after further processing that involves removal of the NH<sub>2</sub>-terminal signal peptide, yields the proenkephalin peptide. This peptide contains four copies of met-enkephalin and one copy of leu-enkephalin, as well as one hepta- and one octapeptide containing the met-enkephalin sequence (Fig. 3). The cleavage of proenkephalin to the biologically active peptides is made at dibasic residues flanking the individual peptide sequences (Hook et al., 2008). In addition to the short enkephalin peptides derived from proenkephalin, larger peptides have been isolated and demonstrated to contain the opioid core sequence, among them enkelytin (Goumon et al., 1996). The latter has received attention due to its highly conserved sequence (from invertebrates to mammals) and its antibacterial properties.

### *Opioid receptors*

Before the endogenous opioids were identified, pharmacological studies with e.g. morphine and naloxone suggested the existence of opioid receptors (for review, see Snyder and Pasternak, 2003). In 1973, the first receptor binding studies were made that identified the presence of opiate receptors in the mammalian brain (Pert and Snyder, 1973; Terenius, 1973). In the 90's, a decade after the opioid peptide genes were identified, the  $\mu$ -,  $\delta$ - and  $\kappa$ -receptors were cloned (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Thompson et al., 1993; Wang et al., 1993), as well as the receptor for nociceptin/orphanin FQ (Bunzow et al., 1994). These receptors are all G-protein coupled receptors that, upon ligand binding, hyperpolarize the neuron by increasing potassium conductance and by inhibiting voltage-gated calcium channels. Also, they couple negatively to adenylate cyclase, thus inhibiting formation of cAMP (Waldhoer et al., 2004). Met-enkephalin preferentially binds to the  $\delta$ -receptor, but also possesses high affinity for the  $\mu$ -receptor. Leu-enkephalin has higher affinity for the  $\delta$ -receptor than met-enkephalin, but instead binds to the  $\mu$ -receptor with lower affinity. Both peptides have poor affinity for the  $\kappa$ -receptor (Mansour et al., 1995c).

### *Transcriptional regulation of the preproenkephalin gene*

A variety of neuronal stimuli have been shown to induce ppENK gene transcription (location written within parenthesis). These include splanchnic nerve stimulation (adrenal gland) (Kanamatsu et al., 1986), seizures (hippocampus) (Pennypacker et al., 1993), estrogen treatment and nociceptive stimuli (dorsal horn) (Draisci and Iadarola, 1989; Amandusson et al., 1999), stressors including hypertonic saline, opiate

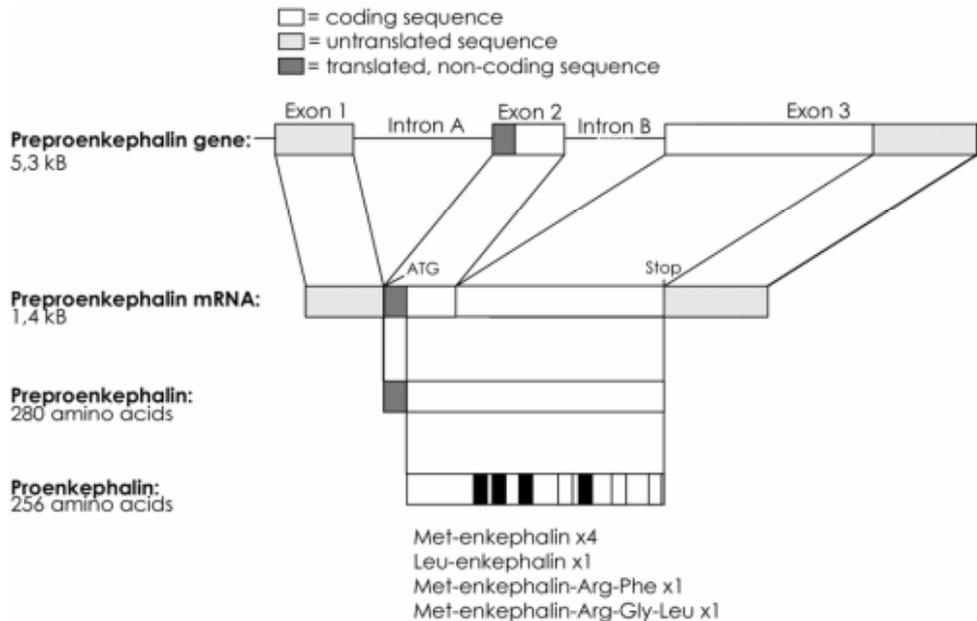


Fig. 3. Basic structure of the rat ppENK gene, mRNA and propeptides. All neuropeptides are produced as propeptides, which are cleaved to yield one or several active neuropeptide transmitter molecules. Met-enkephalin is the most abundant enkephalin. Each proenkephalin peptide contains four copies of met-enkephalin and one copy of leu-enkephalin. Additional enkephalins, such as met-enk-arg-phe and met-enk-arg-gly-leu are also present, as well as larger intermediates (not shown here). Differential processing of the propeptide can be seen during different conditions, probably depending on which proteases that are present. Note that the mRNA species in the figure is the one present in brain. An alternative transcription start site has been identified in intron A, which leads to a spermatogenic specific transcript.

withdrawal, and ether (hypothalamus) (Lightman and Young, 1987; Harbuz et al., 1991; Ceccatelli and Orazzo, 1993), and treatment with D2-dopamine receptor antagonist (striatum) (Tang et al., 1983; Romano et al., 1987).

The necessary regulatory sequences for cAMP and  $Ca^{2+}$  dependent ppENK transcription are located -65 to -104 bp upstream from the ppENK transcription start site and consist of three regulatory elements, ENKCRE-1 (CRE stands for cAMP response element), ENKCRE-2, and AP-2. ENKCRE-2 has been shown to be sufficient for both basal and inducible ppENK transcription, and is also called the AP-1/CRE-sequence, suggesting by its name that it can bind AP-1 proteins (such as Fos/Jun heterodimers) and cAMP-response element-binding proteins (CREB) (Comb et al., 1988; Hyman et al., 1989; Kobierski et al., 1991). The ppENK gene is also regulated by glucocorticoids via a glucocorticoid response element (GRE) (Weisinger, 1995). Thus, studies have shown that glucocorticoids are important for the basal

expression of the ppENK gene in several regions in the forebrain, and that treatment with glucocorticoids differentially affects the expression of ppENK mRNA in those regions (Chao and McEwen, 1990; Ahima et al., 1992). Additionally, ppENK mRNA expression in the PVH, induced by stress from injection of hypertonic saline, was shown to be completely blocked by the concomitant intracerebroventricular administration of a glucocorticoid receptor antagonist (Garcia-Garcia et al., 1998). In contrast, this antagonist did not affect the basal levels of ppENK mRNA in the PVH.

As mentioned above, both AP-1 proteins and CREB can bind to the ENKCRE-2 regulatory sequence. However, it has not been easy to establish whether it is predominantly Fos or CREB that regulate the ppENK gene in the brain, and it is of course possible that both candidates can do so during specific physiological conditions or in distinct brain regions. Co-localization studies have shown that both Fos and pCREB can be expressed in enkephalinergic neurons (Borsook et al., 1994; Hermanson and Blomqvist, 1997a), but provide only indirect evidence for an interaction between a certain transcription factor and its regulatory sequence. However, gel-shift assays have suggested CREB to be a likely regulator of the ppENK gene in the hypothalamus during stress posed by injections of hypertonic saline (Borsook et al., 1994). In contrast, seizure experiments showed that the ppENK gene expression in the hippocampus was associated with AP-1 binding to the ENKCRE-2 element (Sonnenberg et al., 1989).

In a study by Van Koughnet and collaborators (Van Koughnet et al., 1999), transgenic mice expressing  $\beta$ -galactosidase controlled by the human ppENK promoter were challenged with systemic injections of LPS or IL-1 $\beta$ , resulting in elevated levels of  $\beta$ -galactosidase in the PVH. This expression was sensitive to muscimol, a GABA-A receptor agonist (Van Koughnet et al., 1999). Some of the same investigators also showed that pCREB was elevated in the PVH after LPS-injection, and that muscimol induced the expression of a negative regulator of pCREB transcription, namely cAMP-responsive modulator/inducible cAMP early repressor (CREM/ICER) in the same structure (Borsook et al., 1999). Co-transfection studies in primary hypothalamic cell cultures showed that CREM/ICER inhibited LPS-induced ppENK gene transcription, thus suggesting that pCREB may regulate the ppENK gene in the PVH during such conditions (Borsook et al., 1999). On a further note regarding inflammation and ppENK gene expression, induced ppENK mRNA synthesis in T-lymphocytes has been shown to depend on a more upstream situated BETA2 element, which binds NF- $\kappa$ B (Rattner et al., 1991).

### *Physiological functions of enkephalins*

Enkephalins are involved in e.g. pain inhibition, morphine tolerance, reward behavior, anxiolytic effects, and neuroendocrine responses (Akil et al., 1984; Howlett and Rees, 1986; Pechnick, 1993; Konig et al., 1996; Ragnauth et al., 2001; Hayward et al., 2002; Nitsche et al., 2002), but they have also potent immune modulatory functions, as well as effects on chemotaxis, cytotoxicity, and immunoglobulin production (see review by

Salzet et al., 2000). The latter observations are consistent with the presence of opioid receptors on immune cells (Sharp et al., 1998). In response to LPS-injection, ppENK mRNA is upregulated in lymphocytes and macrophages of the spleen, lymph nodes, and adrenal glands (Rosen et al., 1989; Behar et al., 1994; Nobel and Schultzberg, 1995). High levels of the antibacterial proenkephalin A-derived peptide enkelytin have also been detected in e.g. the adrenal glands and in abscess fluid (Goumon et al., 1998). It is likely that enkephalin and enkelytin are concomitantly released from immune cells and adrenal gland as part of the innate immune response.

The expression of enkephalin in the brain has been investigated both at the protein and at the mRNA level (Hökfelt et al., 1977; Williams and Dockray, 1983; Harlan et al., 1987). Preproenkephalin mRNA is widely expressed in the rat brain and present in many of the autonomic structures that are activated by immune challenge (Harlan et al., 1987), such as the BNST, the CeA, several hypothalamic nuclei such as the paraventricular hypothalamic nucleus (PVH), the PB, the ventrolateral medulla (VLM), and the NTS. Considering the involvement of these structures in a variety of homeostatic functions, the brain enkephalinergic system emerges as potentially interesting in modulating the acute-phase response.

### **The hypothalamic-pituitary-adrenal axis**

In addition to the studies of the enkephalinergic system during systemic inflammatory conditions, I have in this thesis also examined the inflammation-induced activation of the HPA-axis and the involvement of mPGES-1 derived PGE<sub>2</sub> in this process. Furthermore, possible direct effects of inflammatory mediators on the adrenal gland have been addressed. Therefore, a general description of the hypothalamic control of pituitary hormone release, and the principal anatomy and the function of the HPA-axis (with focus on inflammatory stress), will be given below.

#### *Historical note*

The hypothalamus and the pituitary gland were first described in the 2<sup>nd</sup> century AD by Galen of Pergamon. While he described the hypothalamic-infundibular system as a way of draining impurities from the ventricles to the nasopharynx, the first reference to the function of the region of the third ventricle as a regulator of homeostasis stems from the 14<sup>th</sup> century anatomist Mondino de'Liuzzi, who stated in his "Anothomia" that this region was involved in the "entire animal behavior" and that it was specifically related to the thyroid gland (see review by Toni, 2000). The term hypothalamus was first introduced in 1893 by the Swiss anatomist Wilhem His, and while it was early recognized that the pituitary gland was involved in the secretory control of e.g. the gonads and the thyroid and adrenal glands, as well as in the general somatic growth of the individual, it was not until the 1940's and 50's that Green and Harris established the vascular connection between the hypothalamus and the pituitary gland (Green and Harris, 1949). In 1955, Saffran and Schally could prove by co-

incubating anterior pituitary fragments with hypothalamic extracts, that hypothalamic factors were responsible for releasing a number of hormones from the anterior pituitary (Saffran and Schally, 1955). In 1977, the Nobel prize in medicine was awarded Drs. Roger Guillemin and Andrew Schally for having established the concept of hypothalamic control of pituitary secretion via releasing factors, and for having isolated and characterized several of these small peptides (see review by Guillemin, 2005). Their efforts established neuroendocrinology as a science.

### *The hypothalamic-infundibular-pituitary system*

The hypothalamus is situated directly above the pituitary gland and consists of several different subdivisions and fiber tracts, symmetrically located around the third ventricle. In its rostro-caudal extent it encompasses the region from the optic chiasm, lamina terminalis and anterior commissure to the cerebral peduncles and interpeduncular fossa. While much can be written about the different hypothalamic subdivisions, the main focus of this thesis concerns the neuroendocrine role of the hypothalamus, and the paraventricular nucleus in particular, and hence, these topics will be dealt with in more detail. In general, the neuroendocrine hypothalamus exerts its effects through axonal projections to the capillary network situated at the base of the pituitary stalk: the median eminence. There, releasing or inhibiting hormones are secreted, and they reach the anterior pituitary via portal vessels. In the anterior pituitary, they exert their effect on hormone-secreting cells. The neuroendocrine hypothalamus can also release hormones via direct axonal projections to capillary networks in the posterior pituitary gland. The hormones released from the pituitary gland stimulate the release of other hormones from ductless glands in the body, such as the gonads, and the thyroid and adrenal glands.

The hypothalamic subdivisions that are involved in neuroendocrine regulation are the medial preoptic, the periventricular, the paraventricular, the supraoptic, and the arcuate nuclei. The neuroendocrine role of the medial preoptic nucleus is to control the release of reproductive hormones via the secretion of gonadotropin-releasing hormone (GnRH) that, in the pituitary, in turn controls the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The supraoptic nucleus, as well as part of the PVH, contains magnocellular neurons that project to the posterior pituitary and that express vasopressin and oxytocin. Vasopressin has impact on water balance, while oxytocin is involved in natriuresis (Haanwinckel et al., 1995), the milk-ejection reflex and general contractility of reproductive organs, such as that of the uterus during partus. The periventricular hypothalamus releases e.g. somatostatin that inhibits the release of growth hormone from the pituitary gland. The arcuate nucleus is involved in feeding regulation and metabolism, but its direct role in neuroendocrine regulation concerns its production of growth hormone releasing hormone and dopaminergic projections to the pituitary stalk that inhibit the release of prolactin. The PVH controls, via projections to the median eminence, the release of ACTH via the release of CRH and vasopressin. Circulating ACTH then causes the release of glucocorticoids from the adrenal gland, forming the HPA-axis (Kandel et al., 2000).

### *The paraventricular hypothalamic nucleus*

The PVH is the main coordinator of a variety of neuroendocrine responses to internal and external stressors and other physiological demands, and is comprised of several different subnuclei, each with distinct neuropeptidergic profiles and efferent and afferent connections. In this thesis, I have followed the subnuclear delineation defined by Swanson and collaborators (Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983). The PVH plays several important roles in regulating homeostasis. Via descending projections to preganglionic sympathetic neurons in the brain stem, medulla and spinal cord, the neurons of the ventral ( $mp_v$ ), lateral ( $lp$ ) and dorsal parvocellular ( $dp$ ) subnuclei control a variety of autonomic functions (Hallbeck, 2000). The magnocellular division ( $pm$ ) of the PVH is responsible for the secretion of vasopressin and oxytocin in the posterior pituitary (Kandel et al., 2000). The neurons of the dorsal and ventral divisions of the dorsal medial parvocellular subnucleus ( $mp_{dd}$  and  $mp_{dv}$ ) send their axons to the median eminence (Fig. 4). They are thus involved in the control of hormone release from the anterior pituitary and produce several neuropeptides (see e.g. Swanson and Sawchenko, 1983). The ones with clearly established neuroendocrine functions are thyrotropin-releasing hormone (TRH) that controls the release of thyrotropin (Burgus et al., 1969), CRH (Saffran and Schally, 1955; Vale et al., 1981) and vasopressin (Guillemin and Hearn, 1955) that together control the release of ACTH from the anterior pituitary gland, and hence the secretion of glucocorticoids from the adrenals. Thus, the PVH constitutes the central limb of the HPA-axis.

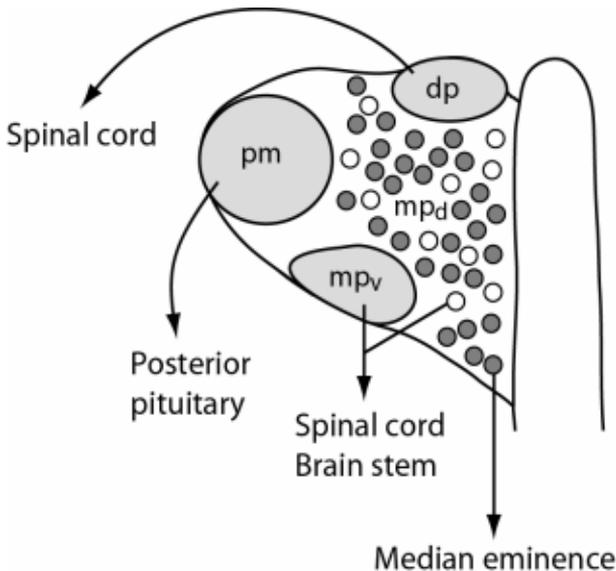


Fig. 4. Efferent projections from the different subdivisions of the PVH. The neurons projecting to the brain stem and spinal cord are mainly found in the dorsal parvocellular subdivision ( $dp$ ) and in the ventral part of the medial parvocellular subdivision ( $mp_v$ ). Neuroendocrine neurons are found in the magnocellular subdivision ( $pm$ ) (projecting to the posterior pituitary) and in the dorsal part of the medial parvocellular subdivision ( $mp_d$ ; projecting mainly to the median eminence).

### *Corticotropin-releasing hormone*

The CRH peptide, named for its effect on corticotropin (ACTH) release, was isolated and completely characterized in 1981 from ovine hypothalamus, and was found to consist of 41 amino acids (Vale et al., 1981). The gene was later cloned in several species (Thompson et al., 1987a; Thompson et al., 1987b). It contains two exons and a single intron, with the coding sequences and the promoter region being highly conserved among mammalian species. CRH is not only expressed in the hypophysiotropic part of the hypothalamus, but also in many other brain regions, where it e.g. functions as a modulator of behavioral and autonomic responses to stress (Cummings et al., 1983; Swanson et al., 1983) and has been isolated in diverse species representing each vertebrate. Illuminating its importance in the maintenance of homeostasis, the expression of CRH is increased in the PVH (or in neurons corresponding to the PVH) of both mammalian and non-mammalian species in response to stress (Lovejoy and Balment, 1999). For example, in rats, increases in CRH mRNA or heteronuclear RNA (hnRNA) in the PVH have been observed after a variety of physical and psychological stimuli, such as footshock, restraint, dehydration, ether stress, hypovolemia, and immune stress (Herman et al., 1989; Rivest and Laflamme, 1995; Watts and Sanchez-Watts, 1995; Kovacs and Sawchenko, 1996a; Ma et al., 1997; Ma and Aguilera, 1999). CRH in the PVH and median eminence is to some extent co-expressed with other neuropeptides, such as e.g. vasopressin. Vasopressin has been shown to potentiate the ACTH-releasing properties of CRH (Rivier and Vale, 1983) and the expression of vasopressin is also induced after a variety of stressors, including immune stress (Rivest and Laflamme, 1995). It is believed that vasopressin is the main ACTH secretagogue during chronic stress (de Goeij et al., 1992; Harbuz et al., 1992; Chowdrey et al., 1995).

### Negative feedback by glucocorticoids and transcriptional regulation of the CRH gene

Several factors influence the basal expression of the CRH gene. The levels of ACTH and glucocorticoids fluctuate during the day, reaching a nadir at the end of the waking cycle, while peaking at the end of the sleeping period. The levels of CRH mRNA and heteronuclear RNA are inversely correlated to these events (Watts et al., 2004), and are believed to be regulated by input from the suprachiasmatic nucleus (Szafarczyk et al., 1979). Additionally, the CRH expression is influenced by glucocorticoids. Thus, as the main ACTH secretagogue, the expression of CRH is controlled by an inhibitory feedback system posed by the end products of the HPA-axis response. Hence, adrenalectomy increases the levels of CRH transcript in the PVH. There are two types of glucocorticoid receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). High density of GR in the brain is found in the PVH and pituitary gland, while the MR is mostly expressed by cells of the hippocampus. These hippocampal neurons exert a basal tonic inhibitory input on the CRHergic neurons. The GR in the PVH is instead thought to mediate a negative feedback from glucocorticoids during stressful situations that serve to switch off the HPA-response. However, this classical view of feedback regulation may be oversimplistic. For

example, studies by Watts and collaborators have shown that low levels of glucocorticoids are necessary for proper transcriptional activation of the CRH gene to occur and for the transcription to be sustained during certain kinds of stress (Tanimura and Watts, 1998). Thus, perhaps high circulating levels of glucocorticoids, such as those obtained during stressful situations, exert a negative feedback on CRH mRNA levels, while lower plasma levels of glucocorticoids exert a permissive action on stressor-dependent CRH gene activation. Additionally, the ability of glucocorticoids to suppress the expression of CRH also depends on the type of stressor (Watts, 2005).

The promoter region of the CRH gene contains several different regulatory elements, including a CRE-site and a region where AP-1 sites overlap with GRE-sites, called GRE/AP-1 (Yao and Denver, 2007). *In vitro* studies have shown depolarisation-dependent CREB-binding to the CRH gene promoter (Guardiola-Diaz et al., 1994), and several *in vivo* studies suggest that the protein kinase A/pCREB pathway, rather than the protein kinase C/AP-1 pathway, regulates the CRH gene in the PVH during stress. For example, during ether stress, CREB is phosphorylated rapidly, paralleling an increase in CRH hnRNA expression, while Fos protein appears long after CRH transcription is initiated (Kovacs and Sawchenko, 1996b). Furthermore, antisense oligonucleotides against the mRNA encoding CREB, but not against that encoding Fos, block the CRH gene expression in the PVH during hypoglycemic stress (Itoi et al., 1996). Thus, despite the facts that *in vitro* studies have shown that Fos can bind to the GRE/AP-1 region (Malkoski and Dorin, 1999), and that *in vivo* studies consistently show that *c-fos* is induced in CRHergic neurons during stress, it has so far been difficult to find evidence for a role for this transcription factor in CRH gene regulation.

After synthesis in the hypophysiotropic neurons of the PVH, the CRH peptide is transported to the axon terminals located in the median eminence. Upon bursts of neuronal firing, the CRH peptide is released into the capillary plexus and, via the portal vessels, reaches the corticotrops of the anterior pituitary. Two G-protein coupled CRH receptors have to date been cloned and characterized, the CRFR1 and CRFR2. The CRFR1 is mainly present in the pituitary gland and in neocortical areas of the brain (Potter et al., 1994; Chalmers et al., 1995), while CRFR2 is mainly expressed in peripheral tissues and e.g. in certain hypothalamic areas (Chalmers et al., 1995). The CRFR1 is believed to be the more important of the two in initiating the stress-induced release of ACTH from the corticotrops (Rivier et al., 2003).

### *Bi-directional cross-talk between the brain and the immune system*

Corticosterone (cortisol in humans), the major glucocorticoid that is synthesized and released by the adrenal glands, is a pluripotent hormone that regulates many aspects of physiological functions, such as glucose metabolism, and the maintenance of vascular tone and permeability, and it also potentiates the effect of catecholamines on vasoconstriction. During systemic inflammatory conditions, it plays a life-protecting role as an immunosuppressant, balancing and limiting the extent of the inflammatory response. Thus, adrenalectomized animals display an increased mortality rate after

injection of LPS or cytokines (Bertini et al., 1988). Corticosterone lowers the levels of e.g. IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , while it elevates the levels of anti-inflammatory cytokines, such as IL-10. The mechanisms behind the anti-inflammatory effects of glucocorticoids can involve repression of transcription via GRE sites, induction of I $\kappa$ B $\alpha$  (that negatively regulates the NF- $\kappa$ B pathway; see above), competition with other transcription factors (AP-1 and NF- $\kappa$ B) for nuclear co-activators, or be exerted via post-transcriptional effects, such as destabilization of proinflammatory mRNAs (De Bosscher et al., 2003).

While the impact of the adrenal gland on the immune response was acknowledged already in the middle of the 19<sup>th</sup> century by Thomas Addison, it was not until much later that the reverse relationship was shown, i.e. that immune stimuli could affect glucocorticoid secretion (Turnbull and Rivier, 1999). In the mid 1980's the first experiments were done that showed that IL-1 $\beta$  induced the release of ACTH from a corticotropic tumor cell line, and that it elevated the plasma levels of ACTH and corticosterone in mice (Woloski et al., 1985; Besedovsky et al., 1986). Furthermore, it could be demonstrated that the IL-1 $\beta$ -induced release of ACTH was dependent on the hypothalamus and CRH (Berkenbosch et al., 1987; Sapolsky et al., 1987). With the findings that IL-1 $\beta$  could activate the HPA-axis, the bi-directional communication between the immune system and the brain was clearly established.

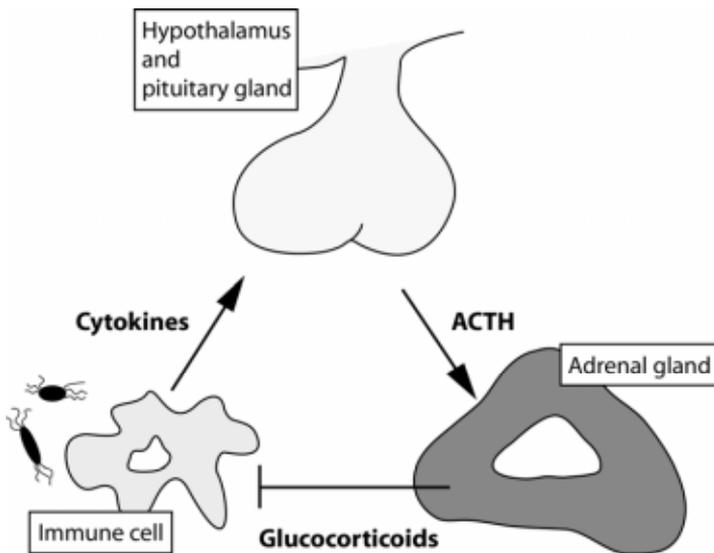


Fig. 5. Bi-directional cross-talk between the brain and the immune system. During infection, immune cells such as macrophages release a number of cytokines (e.g. IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ). By activating afferent nerves or through signaling via humoral pathways across the blood-brain barrier, these cytokines ultimately activate the PVH, which causes the release of CRH to the anterior pituitary, which in turn releases ACTH that acts on the adrenal cortex to increase plasma levels of glucocorticoids. These steroids act as immunosuppressors, balancing the immune response.

### *The hypothalamic-pituitary-adrenal axis and immune challenge*

The HPA-axis response to immune challenge is not exclusive to vertebrates; rudimentary stress systems are also present in invertebrates, such as snails, in which cytokines seem to play a similar role as in rodents and humans. LPS-injection rapidly stimulates the HPA-axis response and this response is thought to be mediated by cytokines such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . Exogenous administration of a number of cytokines activates the HPA-axis, not only the cytokines of myeloid origin, but also cytokines produced by e.g. lymphocytes (Turnbull and Rivier, 1999). However, the responses to injections of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are the most well-studied. Upon i.v., i.p. and i.c.v. injections, these cytokines stimulate the release of ACTH and corticosterone, but with different potency and temporal patterns that also differ depending on injection route and species involved (for review see Turnbull and Rivier, 1999; Dunn, 2000a). For example, in mice, i.v. injections of IL-1 $\beta$  produce a very rapid increase in ACTH (starting within 5-10 minutes), but that dissipates already after 1 h. The response after i.p. injections is instead slower in onset (appear within 30 minutes), but generally lasts longer (Dunn and Chuluyan, 1992). The response to IL-6 and TNF- $\alpha$  are generally weaker (Besedovsky et al., 1991). However, one has to keep in mind that during more physiological circumstances, the concentration of the different cytokines in plasma may be the main determinant of which cytokine that has the largest influence on the HPA-axis. In general, the literature is contradictory regarding the exact role of the distinct cytokines in the HPA-axis response to LPS (Turnbull and Rivier, 1999). As pointed out earlier, the inconsistent results obtained may partly be due to redundancy in the system, as well as to synergistic effects between cytokines on ACTH release (Perlstein et al., 1993; Van der Meer et al., 1995).

It is not entirely clear which level(s) of the HPA-axis that is activated during immune challenge. Measurements of CRH release into the median eminence after LPS-injection have shown that CRH levels rise in conjunction with ACTH (Givalois et al., 1995). In accordance with such data, pharmacological blockade of CRH has been shown to inhibit the HPA-axis response to a low dose of LPS, however it had less effect after a high dose, suggesting an additional, independent activation downstream of the pituitary gland (Schotanus et al., 1994). On the other hand, the effects of circulating IL-1 $\beta$  seem to be exclusively dependent on the hypothalamus, since lesions of the PVH and neutralizing antibodies towards CRH completely blocked the ACTH-response to IL-1 $\beta$  in rats and mice (Berkenbosch et al., 1987; Sapolsky et al., 1987; Rivest and Rivier, 1991). Being in line with these observations, injections of IL-1 $\beta$  or LPS have been shown to activate CRHergic neurons in the PVH and have also been shown to increase the expression of CRH mRNA and hnRNA in this nucleus (see e.g. Ericsson et al., 1994; Rivest and Laflamme, 1995; Ericsson et al., 1997).

The large body of evidence thus suggests that the stimulatory effect on the HPA-axis during immune challenge is mediated via the PVH, even though some data point toward direct effects of inflammatory stimuli on the adrenal gland (which will be

discussed below). But first, what are the mechanisms behind the activation of the CRHergic neurons in the PVH?

### Immune-activated afferent pathways to the paraventricular hypothalamic nucleus

When the body is exposed to different kinds of stressors, neuronal circuits in the brain are recruited in order for the organism to answer to the demands in an adaptive way. Evidence suggests that different kinds of stressors, which can be divided into psychogenic and physical, may recruit distinct pathways. However, they all activate the HPA-axis, and therefore converge on the PVH. While psychogenic stressors require higher-order sensory processing and are perceived as stressors only in a contextual sense (stressors such as foot-shock, restraint and immobilization would fit into this group), physical stressors constitute a direct threat to survival. Thus, physical stressors (e.g. inflammation and hypoglycemia) can bypass cognitive processing and rapidly transmit signals to the PVH e.g. via visceral afferent pathways through the brain stem (see review by Herman and Cullinan, 1997).

Studies have shown that the main HPA-axis activating input to the PVH is constituted by catecholaminergic projections from the A1/C1 and A2 cell groups in the VLM and the NTS, respectively (Sawchenko and Swanson, 1982; Cunningham and Sawchenko, 1988). Accordingly, injection of norepinephrine directly into the PVH causes increased synthesis and release of CRH and concomitant release of ACTH and corticosterone via the activation of  $\alpha$ -adrenergic receptors (Plotsky, 1987; Itoi et al., 1994; Cole and Sawchenko, 2002). Additionally, it has been shown in rats that the norepinephrine concentration in the PVH rises soon after an i.p. injection of IL-1 $\beta$  (Wieczorek and Dunn, 2006), and, in accordance with IL-1 $\beta$  being a physical stressor, its activation of the PVH (using Fos-expression, CRH-gene activation, ACTH and corticosterone release as read-outs) is attenuated if the catecholaminergic afferents are ablated (see e.g. Chuluyan et al., 1992; Ericsson et al., 1994; Buller et al., 2001).

In addition to a catecholaminergic input to the PVH during immune stress, signaling via the CeAl and BNST also seem to play a role. Thus, lesions of the CeA attenuate the HPA-axis response to i.v. injections of IL-1 $\beta$  (Xu et al., 1999). However, no direct projections from the CeA to the PVH exist. Instead, retrograde tract tracing experiments in combination with IL-1 $\beta$  injections have suggested that GABAergic projections from the CeA to the BNST affect the HPA-axis response via disinhibitory mechanisms (Crane et al., 2003).

Other possible mechanisms for the central activation of the HPA-axis involve the direct action of IL-1 $\beta$  or PGE<sub>2</sub> on the PVH, or on nerve terminals of the median eminence. The IL-1R1 is sparsely expressed in the brain parenchyma. Studies on rats have shown that its expression mainly occurs in brain vessels, the meninges, and the choroid plexus. However, it is also present in a restricted number of neuronal structures, although not in the PVH (Ericsson et al., 1995; Konsman et al., 2004). Expression of IL-1 $\beta$  protein in the brain is seen during immune stress. Subseptic doses (<500  $\mu$ g/kg) of LPS lead to a quick rise of IL-1 $\beta$  expression in the choroid plexus, the

meninges, and circumventricular organs (Quan et al., 1999), while septic doses additionally induce expression in the brain parenchyma (Eriksson et al., 2000). It is possible that brain-derived IL-1 $\beta$  contribute to several of the sickness responses seen after subseptic doses of LPS. However, in the case of the HPA-axis, i.c.v. injection of IL-1ra failed to inhibit the ACTH release after i.p. or i.v. injection of LPS and IL-1 $\beta$ , respectively (Dunn, 2000b). Thus, taken together with the findings that CRHergic neurons seemingly are devoid of IL-1R1 (Ericsson et al., 1995; Konsman et al., 2004), it seems unlikely that IL-1 $\beta$  would bind to and affect the CRHergic neuronal cell bodies directly.

Because Cox-inhibitors attenuate the HPA-axis response after injections of IL-1 $\beta$  and because i.c.v. immunoneutralization of PGE<sub>2</sub> also reduces the ACTH plasma levels (Watanobe et al., 1995; Parsadaniantz et al., 2000; Matsuoka et al., 2003), it is likely that PGE<sub>2</sub> is a mediator of the cytokine-induced HPA-axis response. Since the IL-1R1 is enriched in the vasculature of the PVH, it is conceivable that signaling across the blood-brain barrier results in increased production of PGE<sub>2</sub> in this area, and that PGE<sub>2</sub> here could affect EP-receptors on CRHergic neurons (or on local interneurons) (Zhang and Rivest, 1999; Matsuoka et al., 2003).

#### Direct effects of inflammatory mediators on the adrenal gland?

While activation of the central limb of the HPA-axis probably constitutes the dominating pathway during immune stress, there is also evidence for direct effects of LPS, cytokines, and PGE<sub>2</sub> on the adrenal gland. Both *in vivo* and *in vitro* evidence exist that support such interactions. Thus, the corticosterone response to i.p. injections of IL-1 $\beta$  or LPS was not completely attenuated in CRH-deficient mice (Dunn and Swiergiel, 1999; Bethin et al., 2000). Furthermore, Schotanus and collaborators showed that hypothalamic lesions or antiserum against CRH abolished the ACTH response to i.p. injection of LPS, while only attenuating the corticosterone response (Schotanus et al., 1994). Additionally, LPS-treated hypophysectomized rats were found to still respond with elevated corticosterone levels in plasma, although the responses were attenuated compared with sham-operated animals (Mazzocchi et al., 1995). Thus, the available evidence suggests that inflammatory mediators may exert direct effects on the adrenal gland. In support of this idea, *in vitro* experiments have shown stimulatory effects on corticosterone release after application of IL-1 $\beta$  and LPS to adrenal cell cultures, with the possible involvement of PGE<sub>2</sub> as a mediator of these effects (Whitcomb et al., 1988; Winter et al., 1990; Andreis et al., 1991a; Tominaga et al., 1991). Studies have also shown the presence of TLR2 and 4 in the human adrenal gland (Bornstein et al., 2004; Vakharia and Hinson, 2005). Thus, taken together with data on intra-adrenal production of cytokines after immune stimulation (Schobitz et al., 1992; Muramami et al., 1993; Nobel and Schultzberg, 1995; Pournajafi Nazarloo et al., 2003), these observations suggest that LPS-induced intra-adrenal inflammatory mechanisms may contribute to the release of corticosterone during immune challenge.



## AIMS

The overall aim of this thesis was to extend our knowledge about how the brain coordinates sickness responses to inflammatory homeostatic challenge. Focus has been on neuropeptidergic gene expression in autonomic structures of the brain, and on the role of prostaglandin E<sub>2</sub> as an inflammatory mediator of the stress hormone response, both in the brain and adrenal gland.

The specific aims were:

- To analyze the activation pattern in the parabrachial nucleus after immune challenge with particular reference to its enkephalinergic cells.
- To examine the presence of preproenkephalin gene transcription in autonomic structures of the brain following immune challenge.
- To determine the efferent projections of immune-sensitive paraventricular hypothalamic neurons displaying an induced preproenkephalin gene transcription.
- To examine the role of microsomal prostaglandin E synthase-1 dependent prostaglandin E<sub>2</sub> synthesis in the hypothalamic-pituitary-adrenal axis response.
- To examine possible direct effects of circulating inflammatory substances on the adrenal gland, with regard to its local expression of proinflammatory mediators.



## METHODOLOGY

In this thesis, the following methods and animal models were used:

- In vivo models of systemic inflammation
  - a) Intravenous injections of IL-1 $\beta$  and LPS in rats (**paper I-III and V**)
  - b) Intraperitoneal injections of LPS in mice (**paper IV**)
- Fos as a marker for neuronal activity
- Immunohistochemistry (**papers I, III-V**)
  - a) The avidin-biotin complex (ABC) method
  - b) Indirect fluorescence
  - c) The peroxidase anti-peroxidase (PAP) method
- *In situ* hybridization
  - a) Single-labeling (radioactive; **paper II and V**)
  - b) Combined with immunohistochemistry (radioactive; **papers I and III**)
  - c) Dual-labeling (radioactive + digoxigenin; **papers II and III**)
- Retrograde tract tracing (**paper III**)
- Quantitative real-time RT-PCR (**papers IV and V**)
- Immunoassays (**paper IV**)

The detailed protocols are described in the individual papers. Here I will mainly deal with the general nature of the respective techniques, their associated advantages/limitations and the choice of appropriate controls.

### Models of inflammation

In order to elucidate the neural structures in the central nervous system underlying the inflammatory-elicited acute phase response, one must rely on animal models mimicking such conditions. The methods most commonly used in order to elicit inflammatory responses are injection of LPS or single cytokines. One should remember that these models are more or less artificial regarding e.g. kinetics and resulting cytokine profiles. However, although they probably only give us a simplified picture of the situation during more natural disease states, their ability to induce many of the sickness responses associated with common illness suggests that they are valuable tools with several advantages. They provide great means of control, are well-defined and highly reproducible.

In this thesis, I have used two different stimuli to elicit the acute phase response. In **papers I and III**, we injected LPS intravenously to rats. In **paper II** we used the same route to administer recombinant IL-1 $\beta$ , and in **paper V** both stimuli were applied. Intravenous injections were made through intrajugular siliastic catheters that were exteriorized at the animal's neck. The catheter allows the animal to move freely in its cage and permits the investigator to perform the injections without any handling of the animal. This greatly reduces any stress otherwise posed from the restraint during the injection procedure, which is known to cause widespread *c-fos* expression throughout

the brain (Cullinan et al., 1995). In addition, injection via a catheter provides a much more reproducible way to administer the inflammatory agent than intraperitoneal injections, in which the injection-site in the peritoneal cavity may differ from animal to animal. If unsuccessful, the injection may end up in the intestine. In **paper IV** we utilized mice instead of rats. The size and general behavior of mice (smaller and more active than rats) make it difficult to implant catheters and hence to give intravenous injections. Therefore, we were reduced to give intraperitoneal injections of LPS.

A more detailed description regarding the nature of LPS and IL-1 $\beta$  as inflammatory stimuli is given below.

### *Lipopolysaccharide*

In **papers I, III and V**, rats were injected intravenously with LPS. LPS dose-dependently activates a series of autonomic structures in the brain (Rivest and Laflamme, 1995; Elmquist et al., 1996) and it also causes a wide array of sickness responses such as fever, anorexia, lethargy and HPA-axis activation (see e.g. Hart, 1988). LPS may, due to its ability to initiate the release of a cascade of cytokines upon administration in both humans and animals, be regarded as a more physiological immune stimulus than IL-1 $\beta$  (Givalois et al., 1994). Accordingly, LPS better mimics a natural disease state (Hennet et al., 1992; Ronco et al., 2003), although the duration of disease symptoms, and accordingly, the dynamics of the cytokine profiles, differ.

The difference in Fos labeling after low and high LPS doses are mainly reflected in the number of activated neurons in the responding neuronal groups. A higher dose (e.g. 125  $\mu\text{g}/\text{kg}$ ) recruits more neurons in a certain area than a low dose (e.g. 5  $\mu\text{g}/\text{kg}$ ), but the anatomical structures that respond are virtually the same (Elmquist et al., 1996). The dose we used, 25  $\mu\text{g}/\text{kg}$ , considered to be in the lower range, was chosen since it reliably causes expression of Fos while not triggering hypotension (Tkacs et al., 1997), which by itself may cause Fos expression in certain nuclei (Li and Dampney, 1994).

In **paper IV**, the dose used was 2  $\mu\text{g}/\text{mouse}$  (i.e.  $\sim 100 \mu\text{g}/\text{kg}$ ), which reliably has been shown to produce fever and anorexia in this species (Engblom et al., 2003; Elander et al., 2006).

### *Interleukin-1 $\beta$*

Soon after peripheral injection of IL-1 $\beta$ , sickness responses are manifest such as fever (Duff and Durum, 1983; Busbridge et al., 1989), anorexia (Hellerstein et al., 1989; Mrosovsky et al., 1989), decreased social exploration, and HPA-axis activation (Besedovsky et al., 1986; Berkenbosch et al., 1987; Sapolsky et al., 1987). In **papers II and V**, human recombinant IL-1 $\beta$  (2  $\mu\text{g}/\text{kg}$ ) was given intravenously to rats. The same dose is known to cause activation, in terms of *c-fos* expression, of a number of autonomic structures in the rat brain (Ericsson et al., 1994) and to give rise to fever and HPA axis activation (Watanabe et al., 1991). The half-life of intravenously injected recombinant IL-1 $\beta$  is 2.9 minutes in rat plasma (Reimers et al., 1991). Thus,

immediately upon administration, a high but rapidly falling plasma concentration is achieved. For this reason, IL-1 $\beta$  must be regarded as an artificial immune stimulus, since more prolonged responses are seen after e.g. injection of LPS, in other inflammatory models, and during clinical sepsis (Givalois et al., 1994; Ronco et al., 2003; Lustig et al., 2007). On the other hand, it offers an advantage, because it is well-defined (as compared to e.g. LPS that cause production of an array of cytokines (Givalois et al., 1994)), and because the physiological effects can be monitored with a more exact time resolution.

### *Interleukin-1 receptor antagonist*

In **paper V** we wanted to investigate the effect of locally produced IL-1 $\beta$  on the intradrenal inflammatory response. For this purpose we utilized a commercially available IL-1R antagonist (IL-1ra). The IL-1ra was injected intravenously 30 minutes prior to the LPS- or IL-1 $\beta$  injection. The dose used (16 mg/kg) has previously been shown to reduce fever (Luheshi et al., 1996). In a series of control experiments, we could show that this dose of IL-1ra was able to completely block the effect of intravenously injected rIL-1 $\beta$  on Cox-2 mRNA expression in both the hypothalamus and the adrenal gland, demonstrating that this dose was effective in blocking IL-1 $\beta$  activity. Furthermore, the IL-1ra itself had no significant impact on the basal levels of any of the transcripts investigated.

### **Fos as a marker for neuronal activation**

The gene *c-fos*, encoding the protein Fos, is one of several so called immediate-early genes whose transcription does not require *de novo* protein synthesis (Greenberg and Ziff, 1984). Various second messenger systems have been shown to regulate *c-fos* transcription, for which Ca<sup>2+</sup> influx and increased intracellular levels of cAMP constitute key regulatory pathways (Kruijer et al., 1985; Morgan and Curran, 1986; Morgan and Curran, 1989; Sheng et al., 1990). Upon synthesis, Fos dimerizes with different proteins from the Jun family, creating the activator protein-1 (AP-1) complex that is translocated to the nucleus where it binds to the AP1 or AP1/CRE regulatory sequences genes (Morgan and Curran, 1989).

While the basal levels of Fos protein is generally very low in neurons, it is rapidly synthesized, and degraded, upon neuronal depolarisation (Hunt et al., 1987; Sagar et al., 1988). These features make Fos very suitable as a marker for neuronal activity and has been widely used as such in various fields of neuroscience, such as studies of anorexia (Li and Rowland, 1993), restraint and swim stress (Cullinan et al., 1995), and seizures (Morgan et al., 1987). In **papers I, II and IV** we detected Fos protein and *c-fos* mRNA expression with immunohistochemistry and *in situ* hybridization, respectively, in order to identify immune-activated neurons. Previous studies have characterized the labeling pattern in the brain after stimulation with either IL-1 $\beta$  (Ericsson et al., 1994) or LPS (Elmqvist et al., 1996; Tkacs et al., 1997) and found a

similar pattern after both stimuli. Although Fos-patterns have proven to be useful for identifying depolarized neurons, there are however several aspects to consider when interpreting the results. For example, as shown by Luckman et al., it is changes in signal transduction that causes *c-fos* expression, not necessarily depolarization *per se* (Luckman et al., 1994). Also, there can be changes in second messenger signaling without any depolarization taking place. Growth factors and mitogens can cause *c-fos* expression via serum response element (SRE) without any link to neuronal firing, and finally, inhibition of neurons does not lead to *c-fos* transcription (Hoffman and Lyo, 2002). Hence, during inflammatory conditions, cells negative for Fos could still be part of stimulus-significant and actively participating neuronal circuitries, and conversely, Fos expressing cells may not have been depolarized at all.

### **Immunohistochemistry**

Immunohistochemistry is a widely used method employing antibodies to detect, visualize and localize different target molecules at the cellular level. A crucial aspect in immunohistochemistry is the specificity of the antibodies employed. This can be controlled for in several different ways (Saper and Sawchenko, 2003). An efficient control is to apply the antibody of interest on tissue that is known to lack the epitope (e.g. from knockout animals). If the antibody is specific, this should render no labeling. Unfortunately, such animals may not always be available and the species (mouse) may not be suitable when dealing with antibodies directed against e.g. rat or human epitopes that are not conserved between species. Instead, if possible, one can use negative and positive tissue controls from the appropriate animal species (tissues in which the antigen is known to be lacking or present, respectively) processed in parallel.

Another way to control specificity is to incubate the primary antiserum with an excess amount of antigen before applying it to the tissue (pre-absorption), a procedure which should extinguish labeling. If labeling persists, the antiserum contains a clone that recognizes epitopes in the tissue that are unrelated to the immunogen. It should be remembered that this method has its limitations. Extinguished labeling does not necessarily mean that the antibody is 100% specific. It could still detect antigens that are closely related to the immunogen (regarding e.g. peptide sequence). If this is a potential problem, one can perform pre-absorption tests with these related antigens. In those cases, labeling should persist. Finally, the antibody should yield a band of correct size in Western blot. It is desirable to get a single band, but one should remember that the conditions in Western blot differ from the conditions in the tissue (e.g. non-fixed vs. formalin-treated material).

There are numerous detection systems available on the market. In this thesis we have employed the avidin-biotin-complex (ABC) system with peroxidase as detection enzyme (**papers III-V**), the peroxidase-anti-peroxidase (PAP) system (**papers I and IV**), and indirect fluorescence (with and without tyramide amplification; **paper V**).

The ABC method utilizes the high affinity between avidin and biotin and provides greater sensitivity than the PAP method. However, in contrast to the PAP method, it does not as well differentiate between high and low concentrations of antigen in the tissue (Sternberger and Sternberger, 1986). We used in both cases 3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen, which polymerizes upon oxidation and causes a visible precipitate. The staining intensity can be further enhanced by adding substances such as ammonium nickel sulphate, resulting in binding of the metal ion to the precipitate. It is insoluble in alcohol and other organic solvents.

The ABC and the PAP methods were combined in dual-labeling experiments in **paper IV** for the simultaneous detection of Fos and tyrosine hydroxylase in mouse brain stem. This was possible due to the nuclear and cytoplasmatic localization of the Fos and tyrosine hydroxylase proteins, respectively. In this case, the tyrosine hydroxylase was first demonstrated using DAB, yielding a brown cytoplasmatic reaction product. Thereafter, the Fos protein was detected the same way, but in the presence of ammonium nickel sulphate, which yielded a black reaction product.

In **paper V**, we performed dual-labeling experiments for antigens present in overlapping cellular compartments. For this purpose, we turned to fluorescent-based detection systems. Fluorescent labeling is generally less sensitive than chromogen-based ABC and PAP methods. Therefore, we developed a protocol for dual-labeling using Tyramide Signal Amplification (TSA) technology. TSA is a sensitive technique because it amplifies the signal via deposition of tyramide molecules in the tissue, in this case conjugated to fluorophores. The enzyme that catalyzes the reaction is horseradish peroxidase and it causes the tyramide molecules to be covalently linked to tyrosine residues. The deposition of tyramide takes place in close proximity to the enzyme and hence, a high resolution is maintained.

We combined several different antibodies in the dual-labeling protocol. When using the TSA system, labeling was greatly enhanced for all antibodies as compared to when applying only e.g. Alexa-labeled secondary antibodies for detection. Since the technique depends on peroxidase activity and because of its great sensitivity, a more efficient quenching of endogenous peroxidases was required than that for the ABC or PAP protocols. Hence, we incubated the sections in 100% methanol + 1% hydrogen peroxide for 90 minutes, both before applying the primary antibodies and before applying the second secondary antibody (i.e. after the first TSA reaction). This last step efficiently quenched any residual horseradish peroxidase activity derived from the first secondary antibody. For each experiments, control sections were always processed in parallel with other sections – some without primary antibodies (hence controlling for proper quenching of endogenous peroxidases and for non-specific interactions of the secondary antibodies or tyramide with the tissue) and some without the second secondary antibody (controlling for complete quenching of the peroxidase activity from the first secondary antibody).

## ***In situ* hybridization histochemistry**

*In situ* hybridization is an important technique for the visualization of gene expression, in this case in the brain. The basis for *in situ* hybridization is the usage of e.g. radiolabeled or digoxigenin-labeled oligonucleotide- or riboprobes that target specific mRNAs in the tissue of interest. The former are detected by autoradiography, preferably using liquid photographic emulsion, the latter by an immunochemical reaction using antibodies against the digoxigenin molecule. Other detection methods are available (e.g. fluorescent probes), but will be omitted from this discussion since they were not employed in this thesis.

In contrast to immunohistochemistry, *in situ* hybridization detects the gene product upstream of the protein synthesis machinery. Therefore it constitutes, compared to immunohistochemistry, a helpful tool in characterizing the neuropeptidergic phenotype of neuronal cell groups, since these signaling molecules are rapidly packed in vesicles and transported to the nerve terminal upon synthesis, making their detection in the cell body difficult. Of the three different detection systems, the radiolabeled probes provide the most sensitive system and can during optimal conditions detect as little as a few copies of mRNA in a cell (Simmons et al., 1989). The general limitation of *in situ* hybridization, apart from being a time-consuming and difficult technique to master, mainly is associated with the fact that one are studying gene expression at the mRNA level, assuming that it is translated into protein, something that may not always be the case.

Oligonucleotide probes are short (~50 nucleotides) DNA probes and provide less sensitivity than riboprobes, which are longer RNA probes (up to ~2 kb). The oligonucleotides are typically labeled at the 3'-end, whereas approximately every fourth nucleotide in the riboprobes is labeled with a radioactive isotope/digoxigenin. This is accomplished by adding e.g. labeled dUTPs in the probe synthesis reaction, together with unlabeled dATP, dCTP and dGTP. In addition, the usage of riboprobes usually gives rise to less non-specific background, since the longer and more stable RNA:RNA hybrids withstand higher stringency washes than do the shorter DNA:RNA hybrids. The isotopes used for labeling of riboprobes are either  $^{35}\text{S}$  or  $^{33}\text{P}$ .  $^{33}\text{P}$  is often utilized when the RNA target is in low abundance, since the higher energy emitted from this isotope facilitates detection. On the other hand, the lower energy emitted from  $^{35}\text{S}$  permits a better cellular resolution, since the spreading of signal in the overlying photographic emulsion is more restricted. In this thesis I employed radiolabeled and digoxigenin-labeled riboprobes (ranging from ~0.4 to 2.1 kb in length) labeled with either  $^{35}\text{S}$  or  $^{33}\text{P}$ . The protocol used originally derives from that of Simmons et al. (1989).

The specificity of the probes utilized in *in situ* hybridization can be tested in several different ways. Most importantly, the obtained sequence of the cloned fragment (called template) should always be run against a sequence data base to confirm its unique identity. Additional ways of checking specificity are to mix labeled and unlabeled probe in different proportions and see if the signal is attenuated in a linear manner, and

to use sense probes (identical, not complementary, to the mRNA in the tissue), which should not give any labeling. Also, if available, immunohistochemical data using antibodies against the target protein can be compared with the *in situ* hybridization signal.

*In situ* hybridization can be performed either as single-labeling or dual-labeling (often combining radiolabeled probes with digoxigenin-labeled probes or immunohistochemistry). This approach has been utilized e.g. in order to map and characterize activated neurons (often detected by probes or antibodies against the immediate-early gene *c-fos*/Fos protein) regarding neuropeptide expression or receptor-expression.

#### *Messenger RNA probes versus intronic probes*

Probes complementary to mRNA in tissue are most commonly used for *in situ* hybridization. However, while working very well for phenotyping neurons e.g. regarding their peptidergic or receptor-expressing profile, they are often unsatisfactory when it comes to detecting differences in gene expression after some kind of intervention. This is due to an often pre-existing sizeable pool of mRNA in the cells, which renders any further increase hard to detect. A solution to this problem is the use of probes complementary to intronic elements of the gene, hence recognizing the primary transcript instead of the processed mRNA (Fremeau et al., 1989; Herman et al., 1991; Herman et al., 1992; Kovacs and Sawchenko, 1996b). This approach offers a more reliable way to study changes in gene expression, since the basal levels of the primary transcript mostly are very low or non-detectable. In addition, the technique offers a more precise way of coupling *de novo* synthesis to physiological changes, since the time-resolution is very high. It also provides a stronger indication that the neuron actually has released the neuropeptide in question, compared with using dual-labeling of neuropeptide mRNA with e.g. an activity marker such as *c-fos*. In fact, *de novo* synthesis of transcripts and release of peptide has been tightly linked in studies on CRH primary transcript during ether stress (Kovacs and Sawchenko, 1996a; Kovacs and Sawchenko, 1996b). However, it should be recalled that gene expression and protein release are two different aspects of neuronal physiology and even though increased transcription from a neuropeptide gene very well could reflect a neurons way of replenishing its store of the neuropeptide in question, it cannot be seen as solid proof for secretion (Watts, 2005). Another limitation of the technique is that neurons may not necessarily utilize *de novo* synthesis upon increased demands for a certain peptide. Messenger RNA stabilization may also be important, a phenomenon that will not be detected by using intronic probes (Bolognani and Perrone-Bizzozero, 2007).

In this thesis, I utilized radioactive riboprobes (targeting either mRNA or primary transcripts) in single-labeling (**papers II and V**) and in combination with digoxigenin-labeled probes (**papers II and III**) or immunohistochemistry (**papers I and III**).

### *Single-labeling in situ hybridization*

In **paper II** we studied the expression of preproenkephalin in the brain of rats challenged with i.v. IL-1 $\beta$ . Preproenkephalin mRNA is constitutively expressed throughout the rat brain and therefore, to be able to study eventual increases in transcription, we used a probe that recognizes 423 bp of intron A in the rat preproenkephalin gene. The template was cloned by conventional PCR and the fragment ligated into a pDP18CU- vector before being sequenced. In experiments controlling the probe specificity, sense probes yielded no labeling, and, using dual-labeling *in situ* hybridization, we could also confirm that virtually all neurons that expressed the ppENK primary transcript also expressed ppENK mRNA. In line with the nuclear localization of primary transcripts, the ppENK hnRNA labeling was more localized/restricted in the cell body than the labeling seen with conventional mRNA probes. In contrast to the heavy basal ppENK mRNA expression, very little constitutive expression was seen using the ppENK hnRNA probe, with a few exceptions. For example, in both the striatum and the central nucleus of the amygdala, strong labeling was seen in all animals. Such constitutive expression probably reflects an ongoing release of enkephalin.

In **papers I and III**, radiolabeled probes for ppENK mRNA or ppENK hnRNA were used on tissue from LPS-stimulated rats that prior to the *in situ* hybridization had been processed with immunohistochemistry for the detection of Fos or the retrograde tract tracer Fluoro-Gold, respectively. In these cases, the immunohistochemistry was performed under clean, RNase free conditions. The caveat with this procedure is that RNases could, despite all precautions taken, be introduced during the immunohistochemistry and attenuate the *in situ* hybridization signal. This could cause underestimation of the proportion of dual-labeled cells, especially if the RNA target is in low abundance. Dual-labeled cells were defined as cells expressing a brown immunohistochemical reaction product with an accumulation of black silver grains in the overlying emulsion layer.

### *Dual-labeling in situ hybridization*

In **papers II and III**, radiolabeled probes (ppENK hnRNA) were combined with digoxigenin-labeled probes (in **paper II**, complementary to *c-fos* or ppENK; in **paper III**, complementary to CRH mRNA) in dual-labeling *in situ* hybridization. RNases are less likely to be introduced during this procedure, but instead, the lower sensitivity of the digoxigenin based detection system could, in a similar way as mentioned above, result in underestimation of the number of digoxigenin-labeled cells. Dual-labeled cells were identified as cells expressing a purple/brown cytoplasmatic reaction product (representing digoxigenin-labeling) in combination with an accumulation of black silver grains in the overlying photographic emulsion layer (representing the radiolabeled probe).

## Retrograde tract tracing with Fluoro-Gold

In **paper III** we explored the identity of the paraventricular neurons that expressed ppENK hnRNA during systemic inflammation. For this purpose, we used the retrograde tracer Fluoro-Gold (FG; hydroxystilbamidine, MW 532.59). It was first used as a neuronal tracer in the mid 1980's (Schmued and Fallon, 1986) and proved to exhibit some excellent qualities as such. For example, it is not taken up by intact axons of passage, it does not diffuse out of the retrogradely labeled neuron, and it is a stable fluorochrome both *in vivo* and *in vitro*, which allows for a wide range of survival times and which makes it compatible with many kinds of neurohistological techniques, e.g. *in situ* hybridization and immunohistochemistry (Burgunder and Young, 1988; Ju et al., 1989).

When injected peripherally, Fluoro-Gold is unable to cross the blood-brain barrier and is only taken up by nerve terminals of peripheral nerves and in areas with fenestrated capillaries. Hence, uptake of peripherally injected Fluoro-Gold is confined to motor-end plates, autonomic ganglia, the pituitary and circumventricular organs (such as the median eminence) (Ambalavanar and Morris, 1989; Merchenthaler, 1991b). Previous studies have successfully used Fluoro-Gold in order to identify hypophysiotropic neurons of the paraventricular hypothalamus (Merchenthaler, 1991a; Merchenthaler, 1992; Loum-Ribot et al., 2004). We wanted to investigate if the LPS-responsive ppENK hnRNA expressing neurons were hypophysiotropic neurons and chose to detect the labeled cells with a polyclonal antibody against Fluoro-Gold using the ABC method, in combination with single-labeling *in situ* hybridization for ppENK hnRNA. The labeling pattern for Fluoro-Gold immunoreactivity observed in the forebrain was in good accord with previous observations (see above).

## Real-time RT-PCR

Real-time RT-PCR is a powerful technique for the quantification of RNA in biological samples. In **papers IV and V**, we quantified immune-induced gene expression in the mouse hypothalamus and the rat adrenal gland, respectively. For this purpose we utilized TaqMan®-based real-time RT-PCR, which has several advantages compared with quantification via conventional PCR and ethidium bromide-stained gels. Most importantly, and in contrast to conventional PCR, the quantification takes place in the exponential phase of the PCR reaction, during which a doubling of target RNA occurs with each cycle (assuming 100% assay efficiency). Accordingly, it allows for more accurate comparison between samples. In addition, it provides a higher sensitivity and can detect as little as 2-fold changes in gene expression.

TaqMan-based assays rely on the 5'-3' exonuclease activity of the Taq-polymerase and short specific oligonucleotide probes targeting a sequence between the two primer pairs (Holland et al., 1991; Heid et al., 1996). The probes are labeled with a fluorescent reporter dye in the 5'-end and a fluorescent or non-fluorescent quencher in the 3'-end, which greatly reduces the emission of fluorescence from intact probes

(Livak et al., 1995). When the probe binds to its specific site, the polymerase cleaves off the 5'-end and separates the fluorescent dye from the quencher. This procedure unmasks the fluorescent reporter dye signal, which then can be detected. The procedure removes the probe from the target strand and therefore does not inhibit the PCR process. During each cycle, additional reporter dyes are cleaved off, causing an accumulation of fluorescent signal intensity which is proportional to the amount of produced amplicon.

The reliability of the real-time PCR technique is highly dependent on factors such as RNA quality, cDNA synthesis and assay performance, (see e.g. Bustin and Nolan, 2004; Nolan et al., 2006). We consistently used the same RNA preparation kits (Qiagen) which we validated by Agilent Bioanalyzer 2100 regarding RNA integrity. We used NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) on each sample for measurement of RNA purity (260/280 ratio) and concentration. Contamination of gDNA was not present in the RNA samples.

For cDNA synthesis, we always processed all samples to be compared simultaneously, used the same amount of RNA as input and the same priming strategy (random hexamers in **paper IV**, oligo dT primers in **paper V**) with Superscript III as reverse transcriptase (Invitrogen). As the name implies, random priming targets the different RNA species in a sample randomly. Thus, one RNA sequence is primed at multiple sites, rendering more than one cDNA molecule per RNA sequence. This gives a high yield and is good for samples with low amounts of RNA, for targets with complicated secondary structure or when the RNA integrity is compromised. However, random priming may cause problems if the target sequence is in low abundance, in which case the target sequence may not be primed proportionately due to the high abundance of ribosomal RNA. During such conditions, no reliable quantitative data can be achieved. The latter problem is avoided by instead using oligo dT primers, which offers more specificity since these primers only targets the polyA tail. Thus, only mRNA molecules are reversely transcribed and therefore, the oligo dT priming method is considered to give the most reliable representation of the isolated mRNA pool. However, this method instead suffers when significant secondary structure is present in the target mRNA or when RNA integrity is poor. We evaluated, for both priming methods, the linearity of the cDNA synthesis reactions with real-time PCR using serial dilutions of input RNA. The lack of co-eluted PCR inhibitors in the RNA preparation was similarly examined by using serial dilutions of the synthesized cDNA.

In this thesis, I used pre-designed TaqMan® assays from Applied Biosystems and the reactions were performed using their 7700 ABI Prism Sequence Detection System (**paper V**) and 7500 Fast Real-time PCR System (**paper IV and V**). The assays utilized MGB (Minor Groove Binder) probes which have the advantage of a non-fluorescent quencher that limits background fluorescence and thus allows for more precise measurements of reporter dye signal. Also, the MGB-molecule stabilizes the hybridization to the target, allowing for shorter probes, and increased design flexibility. In order to get reliable quantitative data (especially when using the ddCt

method), assay performance must be 100%, or very close to 100%. Applied Biosystem claims that each TaqMan® assay can be used immediately with this method without any pre-validation. In order to confirm this, we ran initial experiments with serial dilutions of cDNA for some of our assays and got a slope of our standard curve of -3.2 - -3.5, with an  $R^2$ -value of  $>0.98$  (showing the precision of the sample duplicates/triplicates), which is considered sufficient (Nolan et al., 2006). The endogenous controls (GAPDH and  $\beta$ -actin in **paper IV**,  $\beta$ -actin in **paper V**) did not significantly differ between genotypes or experimental groups.

## **Immunoassays**

Immunoassays are helpful and extensively used tools for measurements of target molecules in e.g. plasma, serum and urine. We have in **paper IV** utilized two different types of commercially available assays, i.e. enzyme immunoassay (EIA), for the measurement of corticosterone, and Luminex xMAP® technology, for measurement of ACTH, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . All experiments were carried out on mouse plasma (collected in EDTA-coated tubes).

### *Enzyme-based immunoassay (singleplex)*

The technique using enzyme-based immunoassays was first published in 1971 by two independent research groups (Engvall et al., 1971; Van Weemen and Schuurs, 1971). The basis for competitive EIA, which we have used in **paper IV**, is the binding of target substance (in this case corticosterone) in the unknown sample to a primary antibody (in this case, rabbit anti-corticosterone) coated onto the surface of microtitre wells. Enzyme (horseradish peroxidase) labeled target substance is added and competes for binding. After washing and developing with a chromogenic substrate (tetramethylbenzidine, TMB), the reaction is stopped and absorbance is read in a spectrophotometer. Color intensity is inversely proportional to the concentration of the target substance in the unknown samples. The individual concentrations are calculated from a standard curve. The limitations with enzyme immunoassays are similar to those of other techniques relying on antibodies, e.g. immunohistochemistry (see above). Thus, antibody specificity is of crucial importance. In the specific case of corticosterone, cross-reactivity of the antibody had previously been tested by the supplier.

Enzyme immunoassays are easy to perform but one general downside is that one can only analyze one target molecule at a time. Therefore, if it is desirable to perform multiple analyses on a single sample, the method becomes relatively labor-intensive and requires large sample sizes.

### *Fluorescence-based immunoassay (multiplex)*

Multiplex immunoassays were first developed some 10 years ago (Fulton et al., 1997; Oliver et al., 1998; Swartzman et al., 1999). The advantage with these assays is the

ability to analyze several different targets simultaneously in a single sample, hence being more cost-effective and less labor-intensive, and also permitting smaller sample size. We utilized the Luminex xMAP<sup>®</sup> technology with commercially available assays in order to simultaneously detect ACTH, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ . The Luminex xMAP<sup>®</sup> technology system uses the principles of flow-cytometry and was first utilized for multiplexing cytokines by Pickering et al. (Pickering et al., 2002). The technology relies on 5.6  $\mu$ m microspheres internally labeled with graded proportions of red and near-infrared fluorophore (658-712 nm) and has the capacity to process and classify 100 discrete beads. Thus, at least theoretically, up to 100 different analytes can be detected simultaneously in a single sample. A specific antibody that recognizes the analyte is covalently linked to the bead and a second antibody is used to quantify the amount of analyte captured on the bead. The secondary antibody is coupled to a fluorophore. The analyzer uses laser to excite the internal dyes of the microspheres and also any captured secondary antibody. Several readings are made on each bead set. The individual concentrations are then deduced from a standard curve.

## RESULTS

The results of each study are summarized and commented below. For a broader discussion of the data, see General Discussion.

### **Systemic immune challenge activates only a small proportion of the enkephalinergic neurons in the parabrachial nucleus (paper I)**

In **paper I**, we challenged rats with different doses of intravenously injected LPS. Survival time was 2 hours. Subsequently, Fos expression and ppENK mRNA expression were examined in the pontine parabrachial nucleus (PB). *The key finding in this study was that the immune-activated neurons in the PB are largely distinct from its enkephalinergic cell population.*

#### *Fos expression*

All three doses of LPS injected (25, 50 and 100 µg/kg) caused dense Fos expression in the PB with a similar distribution pattern. The immunohistochemical staining showed that the Fos expressing neurons were located preferentially in the outer part of the PBel, but scattered cells were also found in the PBcl, PBdl, PBvl and Kölliker Fuse (KF). Control animals injected with saline showed none, or very few, Fos labeled cells (preferentially localized to the PBcl).

#### *Preproenkephalin mRNA expression and dual-labeling*

*In situ* hybridization for ppENK mRNA demonstrated a strong signal in several different subnuclei. Labeling was seen preferentially in the PBel, but also in the PBcl, PBvl, PBil, PBem and KF. In contrast to the Fos labeled cells, the ppENK mRNA expressing neurons in the PBel were largely localized to the inner part of this subnucleus. Consequently, in the dual-labeling experiment, only a very small overlap between the Fos labeled and ppENK mRNA expressing cell populations was found, and only a small proportion of the neurons were positive for both Fos and ppENK mRNA (approximately 13% and 12% of the Fos and ppENK mRNA populations in the PBel, respectively). Virtually no dual-labeled cells were seen in other subnuclei than PBel.

#### Comments on paper I:

Although previous studies have shown that the PB is activated by immune challenge (Elmqvist et al., 1996; Tkacs and Li, 1999), the present results are the first to describe the detailed Fos expression pattern in the PB during such conditions. We demonstrate that the Fos labeling preferentially is localized to the outer region of the PBel. Subsequent studies have shown that approximately 50% of these neurons project to the CeAl (Richard et al., 2005). The ppENK mRNA distribution in the PB was demonstrated in a previous study from this laboratory, but no distinction between the

outer and the inner subdivision of PBel was done at that time (Hermanson and Blomqvist, 1997b). The present study confirms and extends those results by showing that the ppENK expressing population is largely confined to the inner portion of PBel. Hence, the inflammatory-activated projections from the PBel to its target structures (among them, the CeAl) are most likely not enkephalinergic. Indeed, studies from this laboratory have shown that most of the immune-activated neurons in the PBel instead express CGRP (Paues et al., 2001).

The absence of Fos expression in the inner part of the PBel does not necessary mean that these neurons are unaffected by the immune stimulus (see discussion in the Methodology section). Firstly, an inhibitory input would not result in Fos expression. Secondly, it is also possible that the neurons express other transcription factors than Fos upon synaptic activation. For example, it has been shown that certain stimuli result in phosphorylation of CREB in select populations of PB neurons, while not giving rise to Fos expression in those neurons (Hermanson and Blomqvist, 1997a; Hermanson and Blomqvist, 1997c). Because of these caveats, monitoring the intronic expression of ppENK could give more direct insight into an eventual transcriptional activation of the enkephalinergic cells in the PB (see **paper II**).

### **Systemic immune challenge activates transcription of the ppENK gene in autonomic structures throughout the rat brain (paper II)**

In **paper II**, we examined the transcriptional activation of the ppENK gene throughout the rat brain after intravenous injection of rIL-1 $\beta$  (2  $\mu$ g/kg). Control animals received saline only. Survival times were 0.5, 1 and 3 hours. A fragment corresponding to 423 bp of the ppENK gene intron A was cloned and used as a template for probe synthesis and subsequent *in situ* hybridization analysis. *The key finding in this study was that immune stimulation increases the transcription of the ppENK gene in several autonomic structures of the brain.* Since these structures previously have been identified as main coordinators of the cerebral component of the acute-phase response (Ericsson et al., 1994; Rivest and Laflamme, 1995; Elmquist et al., 1997), the present findings suggest that centrally released enkephalin may modulate a variety of sickness responses. For example, one of the structures that displayed immune-induced ppENK transcription was the neuroendocrine subdivision of the PVH, indicating an involvement of enkephalin in the neuroendocrine responses during inflammation. Notably, virtually no ppENK hnRNA expression was seen in the PB, being consonant with the data reported in **paper I**. In all, the strategy of using an intronic probe for the detection of LPS-activated enkephalinergic neurons proved to be useful in this experimental setup.

#### *Constitutive expression of ppENK hnRNA*

Most enkephalinergic structures showed no constitutive labeling for ppENK hnRNA. However, some structures displayed labeling of varying magnitude. Structures

displaying weak and diffuse labeling but without any discernable cells were the piriform cortex, the central part of the anterior hypothalamic nucleus, the intercalated nuclei of the amygdala and the PBcl, PBil and PBvl. Structures with somewhat stronger labeling with a tendency to discernable cells were the oval part of the bed nucleus of the stria terminalis (BNSTov), the medial perifornical region of the lateral hypothalamic area, the nucleus prepositus hypoglossi, nucleus incertus, the area postrema and the trigeminal dorsal horn. Finally, structures displaying strong labeling with clearly discernable cells comprised the striatum, the olfactory tubercle, the islands of Calleja, the caudolateral region of the lateral hypothalamic area and the lateral and capsular parts of the CeA.

#### *Induction of ppENK hnRNA after injection of rIL-1 $\beta$*

Already at 30 min post-injection, induction of ppENK transcription could be seen in the BNSTov and in the parvocellular PVH. After 1 h, even stronger expression, engaging a large number of distinctly labeled cells, was in addition to the BNSTov and the PVH also present in the area postrema and in the NTS, preferably its commissural, medial and dorsomedial parts. Weaker labeling could also be seen in the A1/C1 region of the ventrolateral medulla, although this labeling was somewhat inconsistent. At 3 h post-injection, the labeling had returned to control levels.

A detailed cytoarchitectonical analysis of the ppENK hnRNA labeling was performed for the paraventricular hypothalamus. While ppENK mRNA was expressed in most parvocellular subnuclei of the PVH, the IL-1 $\beta$  induced ppENK hnRNA was almost exclusively restricted to the dorsal part of the dorsal medial parvocellular subdivision (mp<sub>ad</sub>).

#### *Dual-labeling for ppENK hnRNA and c-fos mRNA*

Expression of *c-fos* mRNA was seen in all structures that displayed labeling for ppENK hnRNA, but generally engaged more cells than the latter population. It was, for example, evident that while the ppENK hnRNA expressing cells had a very restricted distribution in the PVH, *c-fos* mRNA expressing cells were seen in all of its subnuclei. The degree of dual-labeling between the two transcripts was determined in the PVH and BNSTov. The analysis showed that about 85% and 60%, respectively, of the ppENK hnRNA expressing cells were positive for *c-fos* mRNA. Co-localization of *c-fos* mRNA and ppENK hnRNA expressing cells was also present in the NTS. In the lateral part of the CeA, strong *c-fos* expression and extensive dual-labeling were seen, but due to the high constitutive expression of ppENK hnRNA, any additional upregulation could not be determined with certainty. In contrast, in the PBel dense *c-fos* mRNA expression was seen but, as mentioned above, virtually no ppENK hnRNA was detected.

### *Dual-labeling for ppENK hnRNA and ppENK mRNA*

Dual-labeling for ppENK hnRNA and ppENK mRNA showed that ppENK hnRNA expression occurred exclusively in enkephalinergic cells, thus confirming the specificity of the probe. This was true both for structures showing induced expression of hnRNA, but also for those displaying a high constitutive expression of the transcript, such as the lateral part of the CeA and the striatum. In contrast, many structures displayed labeling for ppENK mRNA but lacked labeling for ppENK hnRNA. An example of this was the PBel, suggesting low transcriptional activity of the ppENK gene in this structure during immune challenge.

### **Immune-sensitive neurons expressing ppENK hnRNA in the paraventricular hypothalamus project to the median eminence (paper III)**

In **paper II** we showed that it was only a subpopulation of the enkephalinergic neurons in the PVH that responded to rIL-1 $\beta$ . These neurons were mainly localized to the hypophysiotropic part of the PVH. However, since hypophysiotropic neurons to some extent are intermingled with presympathetic neurons in this location (Hallbeck et al., 2001; Buller et al., 2003; Reyes et al., 2005), we next sought to clarify the projections of the neurons that induced their enkephalin-expression during inflammation.

In **paper III** we hence investigated if the paraventricular neurons that responded with increased ppENK hnRNA expression to immune challenge were hypophysiotropic neurons. *The key finding in this study was that the vast majority of the neurons that showed an LPS-induced increase of transcription of the ppENK gene in the PVH also projected to the median eminence and that a large proportion of the same cells also expressed CRH mRNA.*

We gave i.v. injections of LPS (25  $\mu$ g/kg) to rats that had previously been injected intraperitoneally with the retrograde tracer Fluoro-Gold (FG; 15 mg/kg). The paraventricular hypothalamus was then examined for dual-labeling between ppENK hnRNA (detected by radioactive *in situ* hybridization) and FG (detected by immunohistochemistry) and between ppENK hnRNA and CRH mRNA, respectively. However as a first step, a time series for the immune-induced ppENK hnRNA expression was conducted. The strongest labeling was seen 2 h after LPS-injection and hence, this time-point was chosen for the subsequent experiments.

### *Labeling for Fluoro-Gold*

Following i.p. injection of FG, the magnocellular subdivision (pm) and the dorsal and ventral part of the dorsal medial parvocellular (mp<sub>dd</sub> and mp<sub>dv</sub>) subdivisions of the PVH, projecting to the pituitary and median eminence, respectively, were heavily labeled with tracer substance, as expected. No labeling was seen in the subdivisions known to project to the brain stem and spinal cord. The specificity of the labeling was

further demonstrated by the absence of immunohistochemical reaction product in animals injected with NaCl instead of FG.

#### *Dual-labeling for ppENK hnRNA and Fluoro-Gold*

The labeling pattern for ppENK hnRNA followed that previous seen after injections of rIL-1 $\beta$ , although the number of labeled cells appeared slightly larger. Cell counts showed that ~93% of the ppENK hnRNA expressing neurons were co-labeled with FG. No ppENK hnRNA was seen in control animals injected with saline instead of LPS.

#### *Dual-labeling for ppENK hnRNA and CRH mRNA*

Dual-labeling *in situ* hybridization showed that approximately 40% of the neurons in the PVH that showed immune-induced expression of ppENK hnRNA co-expressed CRH mRNA. The large majority of the dual-labeled neurons were located to the mp<sub>dd</sub>.

#### Comments on papers II and III:

The main conclusion that can be drawn from **papers II and III** is that transcription from the ppENK gene is induced in subsets of ppENK expressing cells in autonomic structures of the rat brain during systemic inflammation. The finding suggests that centrally produced enkephalin is involved in the coordination of the acute-phase response. Furthermore, we showed that the immune-responsive neurons in the PVH that displayed induced ppENK transcription were exclusively hypophysiotropic, implying a neuroendocrine role for enkephalin during inflammation.

The structures that displayed induced ppENK hnRNA expression have previously been shown to exhibit Fos/*c-fos* expression (Ericsson et al., 1994; Rivest and Laflamme, 1995; Elmquist et al., 1996). However, while we found that the majority of the ppENK hnRNA expressing neurons also expressed *c-fos* (two structures examined in detail), a considerable proportion of these neurons did not. Hence, examining the expression of heteronuclear RNA proved to be a successful way to detect immune-responsive enkephalinergic neurons, and can give a more comprehensive view of activity in certain neuropeptidergic circuitries than what is provided by examining *c-fos* transcription in combination with neuropeptide mRNA expression. It should be remembered, however, that regulation of neuropeptidergic gene expression could also take place on other levels, e.g. through mRNA stabilization (DeCristofaro et al., 1993; Ma and Aguilera, 1999; Staton et al., 2000; Bolognani and Perrone-Bizzozero, 2007). This phenomenon is not detected when relying on the intron-approach.

Could the probe have detected transcriptional activity in non-enkephalinergic cells? In the early 90's, it was demonstrated that an alternative transcription start site was present in the preproenkephalin gene intron A (Kilpatrick et al., 1987; Kilpatrick et al., 1990). Additionally, a short transcript named PPEIA-3', encompassing a part of this intron downstream of the alternative transcription site, was found to be expressed in

the rat brain (Brooks et al., 1993). In the rat forebrain, PPEIA-3' was found in the reticular thalamic nucleus, the medial septum and the diagonal band of Broca. No signal was seen in these areas using exonic probes and the conclusion was drawn that the alternative transcription occurred exclusively in non-enkephalinergetic neurons. In another study, the hindbrain was analyzed and PPEIA-3' was found to be expressed in the deep cerebellar nuclei, the vestibular and trapezoid complexes, the ventral lateral lemniscus, the anterior tegmental nucleus, the nucleus abducens, the cochlear nucleus, in scattered cells in the ventral pontine and medullary reticular formation, and in the gray matter of the spinal cord. In addition, PPEIA-3' was induced in the ventromedial hypothalamus in response to estrogen treatment (Brooks et al., 1997).

The probe we used might recognize PPEIA-3'. However, we found no labeling in the structures mentioned above, and, more importantly, we only found labeling for ppENK hnRNA in ppENK mRNA expressing neurons. Hence, it is unlikely that the signal obtained in the present study was due to the detection of PPEIA-3'. PPEIA-3' is probably a short transcript and the lack of labeling in the above-mentioned structures in the present study could be explained by our high washing temperature during the *in situ* hybridization. In fact, it was 30°C higher than that used by Brooks et al. (Brooks et al., 1997), and is likely to have resulted in the removal of short RNA:RNA hybrids.

Our results on ppENK hnRNA induction in the rat PVH is in good accord with what previously has been shown in transgenic mice, in which the human ppENK fragment (including 3 kb of the 5'-flanking sequence, the first exon and intron and 1.2 kb of the 3'-flanking sequence) was fused with the gene for  $\beta$ -galactosidase (Borsook et al., 1992; Van Koughnet et al., 1999). Thus, i.p. administration of various doses of LPS and rIL-1 $\beta$  caused a delayed expression of  $\beta$ -galactosidase in the mouse PVH. Our finding that the ppENK hnRNA expressing neurons almost exclusively project to the median eminence and the considerable co-localization between ppENK hnRNA and CRH mRNA, strongly supports a role for enkephalin in neuroendocrine regulation during immune stress, and suggests that enkephalin could be co-released with CRH. This is in agreement with previous studies showing that enkephalin to some extent is co-localized with CRH and vasopressin in the PVH and median eminence (Hökfelt et al., 1983; Hisano et al., 1986; Hisano et al., 1987; Ceccatelli et al., 1989a; Pretel and Piekut, 1990). As to date, the physiological function of released enkephalin from these neurons remains to be clarified.

### **Microsomal PGES-1 deficiency causes impairment of inflammatory-induced HPA-axis activity (paper IV)**

In **paper IV** we investigated the role of mPGES-1 in the regulation of the HPA axis response during immune challenge. We examined mice deficient for mPGES-1 that were given intraperitoneal injections of LPS (2  $\mu$ g/mouse) or saline and sacrificed 1, 3 or 6 hours post-injection.

*The key finding in this study was a phenotypic difference between mPGES-1 knockout mice compared with wild type mice regarding their HPA axis response to immune stress (impaired in the knockout mice), which was associated with an impairment of CRH mRNA transcription in the PVH of the knockout mice.* Hence, these data suggest that production of PGE<sub>2</sub> via mPGES-1 is crucial for a proper HPA axis response after injection of LPS. In contrast, the LPS-induced Fos expression in the PVH and several other autonomic relay structures was found to be unrelated to the mPGES-1 gene deletion.

#### *Hormone analysis*

Plasma analysis showed a strong increase in ACTH levels at 1 h post-injection, and there was no significant difference between genotypes. In contrast, at 3 hours the ACTH levels in the knockout mice were attenuated compared with those seen in wild type mice. At 6 h, the ACTH concentrations were back to basal level in both genotypes. Corticosterone levels were elevated at all time points investigated. A difference between genotypes was observed at 6 h post-injection, at which time the corticosterone levels were significantly attenuated in the knockout animals.

#### *Cytokine analysis*

In order to track down the mechanism behind the impaired stress hormone release in the knockout animals, we first examined if the mPGES-1 gene deletion affected the plasma levels of pro-inflammatory cytokines. We found that IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in naïve animals were low or below detection limit, and did not differ between genotypes. After LPS injection, the plasma levels of these cytokines were elevated, and again, no significant difference could be seen between genotypes, except for a small elevation of the TNF- $\alpha$  concentration in the knockout mice at 6 h. However, at this time-point, the plasma levels of this cytokine were low in both knockout and wild type mice.

#### *Quantification of the expression of CRH mRNA in the hypothalamus*

Next, we examined the expression of the CRH gene in the hypothalamus. We collected fresh hypothalamic tissue from animals sacrificed 1 and 3 hours after LPS injection. RNA was purified and cDNA was synthesized and used as template in quantitative real-time RT-PCR. Basal levels of CRH mRNA did not differ between genotypes. However, while the wild type animals expressed twice as much CRH mRNA at 1 h after LPS compared with their saline-injected controls, the mRNA levels in knockout animals showed no increase. The difference between genotypes was statistically significant. At 3 h, both genotypes displayed similarly elevated levels of CRH mRNA.

### *Expression of Fos protein and c-fos mRNA in the hypothalamus and in hypothalamus-projecting cell groups*

As a last step, we examined the neuronal activation (in terms of Fos protein expression) in autonomic relay nuclei that control HPA-axis activity. Examination of the PVH showed that there was a strong Fos induction in both genotypes. However, the number of cells appeared more numerous in the knockout animals than in the wild type animals, an observation that was supported by quantitative real-time RT-PCR. Hence, while the wild type mice displayed a 2.6 times upregulation of *c-fos* mRNA in the hypothalamus compared with their saline-injected controls, the *c-fos* mRNA levels in the knockout mice were 4.5 times higher than in their controls. This difference between genotypes was statistically significant.

Since catecholaminergic cell groups in the VLM are important for the activation of PVH during stress, we utilized dual-labeling immunohistochemistry in order to examine the co-localization between tyrosine hydroxylase (the first enzyme in the noradrenalin/adrenalin synthesis pathway) and Fos protein. The results showed that the percentage of dual-labeled cells did not differ between genotypes. Additionally, induced Fos expression in the CeA, the AP, the NTS and the PB was seen in both knockout and wild type mice, with a tendency to stronger expression in the knockout animals.

### Comments on paper IV:

Previous studies have suggested an involvement of prostaglandins in the regulation of stress hormone release during immune challenge (Katsuura et al., 1988; Dunn and Chuluyan, 1992; Watanobe et al., 1998; Parsadaniantz et al., 2000; Matsuoka et al., 2003; Gadek-Michalska and Bugajski, 2004; Wieczorek and Dunn, 2006). However, our study is the first to identify a terminal isomerase that is responsible for this effect.

While CRH mRNA levels at 1 h were increased in the wild type mice, this response was blunted in the knockout animals. Considering the time delay between mRNA synthesis and peptide release (approximately 60-90 minutes) (Watts, 2005), such a blunted response would not be reflected in the ACTH level at this early time-point. Accordingly, the ACTH levels were similar in both genotypes at 1 h post-injection. However, being consistent with the time delay between mRNA synthesis and CRH peptide available for release, at 3 h post-injection the ACTH levels were blunted in the knockout mice.

The early effect of the mPGES-1 gene deletion on the CRH mRNA expression suggests that PGE<sub>2</sub> is rapidly synthesized after LPS-injection. This is somewhat inconsistent with what is known for mPGES-1 induction, based on studies in rat. Thus, with RT-PCR mPGES-1 mRNA expression in the brain was detected at 90 minutes, but not at 30 minutes, after LPS-injection (Ivanov et al., 2002), and *in situ* hybridization experiments have shown that mPGES-1 mRNA expression in brain endothelial cells starts to appear at 1 h after injection of IL-1 $\beta$  (Ek et al., 2001).

However, in mice, the inducibility of mPGES-1 mRNA is not reliably detected with real-time RT-PCR after immune challenge in e.g. the brain or the adrenal gland (unpublished observations). We can therefore not exclude that the effects of mPGES-1 on the CRH mRNA synthesis at 1 h could rely on the existence of a certain basal expression of mPGES-1 protein in the brain/endothelial cells, consistent with the observation by Matsuoka and collaborators of a constitutive mPGES-1 immunoreactivity in the PVH of mice (Matsuoka et al., 2003).

Our data clearly show a dissociation between Fos expression in the PVH and the induction of CRH mRNA, in line with the findings in previous studies using ether stress, in which the CRH induction preceded the Fos induction by 1-2 hours (Kovacs and Sawchenko, 1996b). Thus, other transcription factors than Fos are more likely to regulate the CRH gene during these conditions, such as pCREB, e.g. via the ERK1/2 MAP kinase pathway (Khan et al., 2007; Singru et al., 2008). Fos activation in the PVH has previously been used as an indirect marker for HPA axis activation (Ericsson et al., 1994; Ericsson et al., 1997) and the number of Fos labeled CRH-expressing cells in the PVH have been positively correlated to ACTH release after injection of rIL-1 $\beta$  (Buller et al., 1998). Furthermore, the administration of indomethacin has been shown to attenuate the Fos response in CRH-expressing cells of the PVH (Ericsson et al., 1997). Our data contrast with these observations since the mPGES-1 knockout mice, despite a blunted ACTH response at 3 h, showed even more Fos immunoreactivity and *c-fos* mRNA expression in the PVH than wild type mice at this time point. The choice of immune stimulus as well as route of administration may help explain this discrepancy. A recent study using endothelial-specific knockdown of the IL-1R1 points toward the differential involvement of neural vs. endothelial pathways for CNS activation depending on through which route the inflammatory cytokine is administered (Ching et al., 2007). The animals lacking the IL-1R1 in their endothelial cells showed an extinguished Fos expression in the PVH after i.v. injection of rIL-1 $\beta$ , but not after i.p. injection, suggesting that only the i.v. route is dependent on the inflammatory pathway across the blood-brain barrier. This pathway is known to involve the induction of mPGES-1, and hence, induced PGE<sub>2</sub> synthesis (Ek et al., 2001; Engblom et al., 2003). Thus, the dependence of the Fos expression on an intact Cox-activity that was reported in the above-mentioned studies may be relevant only during conditions posed by i.v. injection of the inflammatory substance and not after i.p. injection. Our data are consistent with this idea and suggest that i.p. injected LPS acts on the vagus nerve in an mPGES-1 independent manner to elicit the central Fos response. On a further note regarding our finding that the Fos expression is dissociated from the ACTH response at 3 h, it can be mentioned that polymicrobial sepsis induced by cecal ligation and puncture is associated with elevated levels of ACTH, corticosterone and CRH mRNA but with very little Fos staining in the PVH (Carlson et al., 2007), further supporting the suggestion that Fos may not be a reliable HPA axis activity marker during endotoxemia.

Fos expression has been associated with EP<sub>3</sub> receptor expressing neurons in the brain (Ek et al., 2000; Engblom et al., 2001) and EP<sub>3</sub> receptors have been interpreted to

confer PGE<sub>2</sub> mediated Fos expression (Ericsson et al., 1997). However, since the EP<sub>3</sub> receptor mainly is linked to inhibitory G-proteins (Hasegawa et al., 1996), ligand-binding to this receptor is more likely to result in decreased excitability. Accordingly, the absence of inducible PGE<sub>2</sub> production would augment, rather than attenuate the Fos expression, which in fact is what was found in the present study, both in the PVH as well as in several other autonomic structures. It should also be noted that examination of the LPS-induced Fos expression in catecholaminergic neurons in the VLM did not reveal any reduction of the degree of dual-labeling in knockout mice. Catecholaminergic projections are important for the rIL-1 $\beta$ -induced Fos and CRH mRNA expression in the PVH (Ericsson et al., 1994); however, our data suggest that the LPS-induced activation of these neurons is not dependent upon mPGES-1 mediated PGE<sub>2</sub> synthesis, or at least, that such a dependence is not reflected in their Fos expression.

### **Systemic inflammation activates a local inflammatory circuit in the rat adrenal gland involving IL-1 $\beta$ and PGE<sub>2</sub> (paper V)**

In **paper V**, we examined the local inflammatory response in the rat adrenal gland at various time-points after i.v. injection of either LPS or rIL-1 $\beta$ . The expression of protein and/or mRNA was examined for the non-inducible and inducible Cox enzymes (Cox-1 and Cox-2, respectively), mPGES-1, IL-1 $\beta$  and IL-1R1 by immunohistochemistry and real-time RT-PCR. The time-points examined were 1, 3, 5 and 12 h after injection of the inflammatory stimulus. In addition, changes in the immune cell population were examined with single- and dual-labeling immunohistochemistry, and related to the above-mentioned proteins. By using an IL-1ra we investigated the potential role for locally produced IL-1 $\beta$  in regulating the intra-adrenal inflammatory response. Finally, we studied the adrenal expression of PGE<sub>2</sub> receptors EP<sub>1</sub> and EP<sub>3</sub>.

*The key finding in this paper is that the immune cell population of the adrenal gland participates in a local inflammatory circuit that is activated during systemic immune challenge, involving the production of IL-1 $\beta$ , its receptor and PGE<sub>2</sub>-synthesizing enzymes. These pro-inflammatory products may help regulate the release of corticosterone (and/or catecholamines) from the adrenal gland independently of the centrally elicited HPA-axis response.*

#### *Changes in the immune cell population during LPS challenge*

The antibodies that we used in order to detect immune cells labeled either the MHC class II molecule on professional antigen presenting cells (OX6), or a lysosomal protein present in monocytes, macrophages and dendritic cells (ED1). The OX6-labeled cells were numerous in the control animals, exhibiting a dendritic appearance. The highest density was seen in the zona reticularis, radiating into the zona fasciculata, while the ED1-labeled cells, also dendritic in their appearance, were less abundant. Injection of LPS (25  $\mu$ g/kg) resulted in profound changes in OX6- and ED1-labeling in

the adrenal gland. The most conspicuous finding was the appearance of large OX6-labeled oval/round cells in the fasciculate layer 3-5 h after immune stimulation, and a strong reduction of dendritic-looking cells, particularly in the innermost layer. Similarly, preferentially large ED1-labeled cells appeared in the zona fasciculata and these cells were the dominating ED1-labeled cell type at 5 h post-injection.

#### *Constitutive and LPS-induced expression of pro-inflammatory mediators*

We first investigated the constitutive expression of IL-1 $\beta$ , the IL-1R1, Cox-1, Cox-2 and mPGES-1 and the PGE<sub>2</sub> receptors EP<sub>1</sub> and EP<sub>3</sub> in NaCl-injected rats. We found limited constitutive expression in the adrenal cortex, largely restricted to the part of the zona reticularis adjacent to the medulla. The labeled cells resembled immune cells. Expression of pro-inflammatory mediators was also seen in the medulla, both in chromaffin-like cells and in scattered immune cell-like cells. Messenger RNA for the EP<sub>1</sub> and EP<sub>3</sub> receptors was densely expressed in the medulla, but weak and somewhat inconsistent labeling was also seen in the zona reticularis and fasciculata.

Intravenous injection of LPS resulted in induced expression of IL-1 $\beta$ , IL-1R1, Cox-2 and mPGES-1 in the adrenal cortex along a time course that differed between these substances. Prominent induced labeling was seen for IL-1 $\beta$  already at 1 h, whereas IL-1R1, Cox-2, and mPGES-1 started to show strongly induced labeling at somewhat later time points. The appearance of the cells labeled for IL-1 $\beta$  followed during the time course studied that demonstrated for the cells labeled with OX6 and ED1. The cells expressing IL-1R1 and Cox-2 consisted mainly of small and large round cells in the zona reticularis and the inner part of zona fasciculata, as well as by thickened dendritic-type cells in the inner part of the reticulate zone. In contrast, induced mPGES-1 expression occurred almost exclusively in large round cells in the fasciculate zone, in addition to being displayed by thickened fusiform cells in the inner part of the reticular zone.

#### *Dual-labeling for pro-inflammatory mediators and immune cell markers*

Since the cells expressing IL-1 $\beta$ , IL-1R1, Cox-2 and mPGES-1 resembled immune cells, we next performed dual-labeling fluorescent immunohistochemistry to see if the cells expressing the pro-inflammatory mediators also expressed ED1 and OX6. These experiments demonstrated that the vast majority of the cells expressing pro-inflammatory mediators were ED1 and/or OX6 labeled cells. Furthermore, while some ED1/OX6 labeled cells only expressed one or a few pro-inflammatory mediators, there was a considerable population of the immune cells that expressed all inflammatory mediators examined. This was the case for both the fasciculate and the reticulate layers.

#### *Quantification of the LPS-induced transcripts for IL-1 $\beta$ , IL-1R1, Cox-2 and mPGES-1*

Real-time RT-PCR showed, consistent with the immunohistochemistry, elevated levels of all the pro-inflammatory mediators examined. However, the magnitude of induction

varied greatly between transcripts. Thus, both IL-1 $\beta$  and Cox-2 were heavily induced (180-190 fold compared with controls, peaking at 1 h), while IL-1R1 and mPGES-1 showed much more modest inductions (4-7 fold compared to controls, peaking at 3-5 h).

*Recombinant IL-1 $\beta$ -induced transcripts for IL-1 $\beta$ , IL-1R1, Cox-2 and mPGES-1 and their intrinsic regulation by IL-1ra*

The expression of pro-inflammatory mediators after injection of rIL-1 $\beta$  showed a similar time-course of induction as after injection of LPS. However, the responses were weaker, and, in the cases of IL-1 $\beta$  and Cox-2, disappeared earlier. Thus, the levels of IL-1 $\beta$  and Cox-2 mRNA rose to approximately one tenth and one fourth, respectively, of that seen after injection of LPS. The levels of the IL-1R1 and mPGES-1 transcript were about 50% lower than what was seen after LPS injection.

Injection of IL-1ra (16 mg/kg; see Methodology for further information regarding dosage and preceding control experiments) in conjunction with LPS resulted in an attenuation of the IL-1 $\beta$  mRNA induction at 3 h, but not at 1 h. In contrast, the expression of Cox-2 mRNA was at 3 h further enhanced by the IL-1ra, while the mPGES-1 induction remained unchanged.

Comments on paper V:

Previous studies have only examined the adrenal immune cell population in naïve rats (Sato, 1998; Schober et al., 1998). In the present study, we have expanded these data to encompass the distribution and phenotypic characteristics of that population during immune challenge posed by LPS. We found a profound change in distribution and morphological appearances of OX6 and ED1-labeled cells in the adrenal gland that was closely related to the expression of both mRNA and protein for IL-1 $\beta$ , IL-1R1, Cox-2 and mPGES-1. The expression of IL-1 $\beta$  and Cox-2 have previously been shown to be induced in the rat adrenal gland after injection of LPS (Nobel and Schultzberg, 1995; Ichitani et al., 2001), but so far, the identity of the IL-1 $\beta$  and Cox-2 expressing cells has been unknown. We show that a considerable proportion of the OX6 and ED1 labeled cells in both the fasciculate and the reticulate zones express all the above-mentioned proteins. In addition, we show that there is a constitutive expression of mPGES-1 and Cox-1 in a discrete immune cell population at the border of the medulla, cells that seem to be well-positioned for potential cross-talk with adrenal chromaffin cells and that are responsive to IL-1 $\beta$  during immune stress.

Based on our dual-labeling data showing that IL-1R1 was present on IL-1 $\beta$ , Cox-2 and mPGES-1 expressing immune cells, we hypothesized that these cells, via the prominent induction of IL-1 $\beta$ , could regulate their own expression of IL-1 $\beta$ , as well as their PGE<sub>2</sub> synthesis. The attenuation of the LPS-induced IL-1 $\beta$  induction seen after concomitant injection of IL-1ra supports this idea. A contrasting finding was that IL-1ra further increased the expression of Cox-2, which may suggest that IL-1 $\beta$  downregulates prostaglandin synthesis in the adrenal gland. This finding was

unexpected, particularly since IL-1 $\beta$  is known to induce the expression of Cox-2, for example in the blood-brain barrier (Ek et al., 2001). Alternatively, it could suggest a mechanism by which locally produced IL-1 $\beta$  and IL-1ra act synergistically to further potentiate the prostaglandinergic pathway in the adrenal gland, something that has been shown to occur in e.g. decidual cells (Brown et al., 1998), or it could imply that IL-1 $\beta$  induces negative feedback on Cox-2 expression via the production of anti-inflammatory cytokines. Lack of such negative feedback after IL-1ra administration would then enhance the Cox-2 expression (Harizi and Gualde, 2006). Previous studies have shown that IL-1ra is locally produced in the adrenal medulla and cortex and that its expression is induced after injection of LPS (Schultzberg et al., 1995), supporting a physiological role for locally produced IL-1ra during inflammation.



## GENERAL DISCUSSION

The first part of the General Discussion will deal with the potential implications of our findings regarding the enkephalinergic system in the brain during inflammatory challenge (**papers I-III**). The second part will discuss the role of prostaglandin E<sub>2</sub>-signaling in the inflammatory-induced stress hormone response (**papers IV-V**).

### **Role of the central enkephalinergic system during immune stress**

#### *Enkephalin in the parabrachial nucleus and the extended amygdala – influence on hedonic state and anxiety*

The brain enkephalinergic system has been implicated in basal hedonic homeostasis, a term used to describe the maintenance of a balanced emotional, affective or motivational state of the organism (Koob and Le Moal, 2001). This involvement was first attributed to the rewarding effects of morphine and to the ability of naloxone and naltrexone to produce conditioned aversive responses (Bals-Kubik et al., 1989; Parker and Rennie, 1992; Gerrits et al., 2003). Recent research has extended such findings by showing that both enkephalin and  $\mu$ -opioid receptor knockout mice fail to display conditioned aversion to naloxone (Skoubis et al., 2001; Skoubis et al., 2005). Additionally, studies of  $\mu$ -opioid receptor knockout mice show that the  $\mu$ -receptor is crucial for the analgesic and rewarding effects of morphine (Matthes et al., 1996). The enkephalinergic system also serves to dampen anxiety. Thus, enkephalin and  $\delta$ -opioid receptor knockout mice show elevated levels of anxiety (Konig et al., 1996; Filliol et al., 2000), and, conversely, overexpression of enkephalin in the CeA potentiates the anxiolytic effects of benzodiazepine (Kang et al., 2000). Enkephalin in the CeA has also been coupled to the attenuation of anxiety-related behavior and of conditioned fear responses associated with stress (Gallagher et al., 1982; Ray and Henke, 1990; Siegel et al., 1997).

The CeAl and its efferent target, the BNSTov, are part of the extended amygdala. They are central structures for the integration of behavioral and emotional responses to fear, anxiety and stress and have reciprocal connections with for example the PB (Davis, 1992; Maren and Fanselow, 1996; Alheid, 2003). Previous research has shown that systemic administration of IL-1 $\beta$  induces expression of *c-fos* mRNA in the CeAl and BNSTov (Day and Akil, 1996; Day et al., 1999; Buller et al., 2001; Crane et al., 2003; Buller et al., 2005), and that the Fos-expressing cells co-express ppENK and GAD65/67 mRNA (Day et al., 1999). Additionally, administration of naloxone in conjunction with IL-1 $\beta$  results in enhanced Fos-expression, suggesting that central opioids dampen the neuronal activation in the CeAl and dorsal BNST during inflammatory conditions (Buller et al., 2005). Our finding in **paper II** of a high basal constitutive ppENK transcription in these structures is consonant with the presence of a tonic basal enkephalinergic influence on the hedonic state. Furthermore, following immune challenge with IL-1 $\beta$ , we could readily detect an upregulation of ppENK

hnRNA in the BNSTov, and we found that the ppENK hnRNA transcript to a large extent co-localized with *c-fos* mRNA. Due to the high basal expression of ppENK hnRNA in the CeAl, it was difficult to ascertain any changes in expression levels in this structure. However, similar to the ppENK hnRNA expressing cells in the BNSTov, the cells in the CeAl that expressed the heteronuclear transcript co-expressed *c-fos* mRNA, suggesting that they also were activated by the immune stimulus. Our data hence indicate that systemic inflammation results in increased enkephalinergic activity in the extended amygdala. It is conceivable that this activity counterbalances the negative influence of the inflammatory condition on the hedonic state.

The PB is an important autonomic relay center in the rostral brain stem that integrates and conveys somatosensory and visceral information to e.g. the extended amygdala, a connection that is believed to be important for the acquisition of conditioned taste aversion (i.e. the ability to associate a negative experience after ingesting a certain substance to its smell and/or taste). Fos-expression in the PBel is elicited by anorexigenic and aversive stimuli, such as injections of dexfenfluramine and LiCl (Yamamoto et al., 1992; Li and Rowland, 1993; Paues et al., 2006). Furthermore, PBel also expresses Fos in a rat model of cancer-induced anorexia-cachexia (Ruud and Blomqvist, 2007). We demonstrated in **paper I** that the PBel, which contains a large population of enkephalinergic cells (Hermanson and Blomqvist, 1997b), was activated during immune challenge posed by LPS-injection, as determined by its induced Fos expression. However, the Fos expressing neurons were localized to PBelo, whereas the ppENK mRNA expressing cells were localized to the PBeli. The PBelo cells, including those that display Fos in response to immune stimulation, instead have been shown to express CGRP as well as the melanocortin receptor 4 (Paues et al., 2001; Paues et al., 2006). The efferent projections from the PB to the CeAl have been shown to originate preferentially in the PBelo, and encompass about 50% of its LPS-activated cells (Tkacs and Li, 1999; Richard et al., 2005). Evidence thus suggests that the Fos-labeled cells in the PBelo transmit aversive visceral information to the extended amygdala via a CGRP-ergic, non-enkephalinergic, projection. These data fit well with the anorexigenic and aversive properties of CGRP (Krahn et al., 1984; Krahn et al., 1986), as opposed to the orexigenic properties of opioids, as well as their ability to antagonize e.g. conditioned taste aversion (Olszewski et al., 2000).

What is then the possible role of the enkephalinergic neurons in the PBel? While this at present only can be speculated about, it is of interest to note that there is a dense expression of  $\mu$ -opioid receptors in this subnucleus (Chamberlin et al., 1999). The possibility hence exists that the enkephalinergic cells in the PBeli, via local projections within the PBel, under certain conditions may exert an inhibitory effect on the cells of the PBelo. Alternatively, they could provide inhibitory input to some of its efferent forebrain targets, such as the zona incerta and substantia innominata (Fulwiler and Saper, 1984). So far, only few studies have been able to provide evidence for the activation of PBeli by natural stimuli, with the possible exception of sodium deprivation (Geerling and Loewy, 2007) and oral infusion of a bitter substance, quinine (Travers et al., 1999; Yamamoto and Sawa, 2000; King et al., 2003), leaving

its functional role obscure. In any case, its enkephalinergic population does not seem to play a role for the central processing underlying the inflammatory response.

### *Enkephalinergic influence on neuroendocrine responses during inflammatory challenge*

The literature on the influence of opioids on the HPA-axis is somewhat contradictory (for review see Pechnick, 1993). However, the general view seems to be that opioids pose a stimulatory effect on the HPA-axis in rodents. Thus, injection of morphine peripherally or i.c.v. increases the release of ACTH and corticosterone, and this response can be inhibited by naloxone (Gibson et al., 1979; Buckingham and Cooper, 1984; Pechnick et al., 1985; Buckingham and Cooper, 1986). The stimulatory effects are believed to be centrally mediated, since neither enkephalin nor morphine has any effect on ACTH release when applied directly to pituitary glands *in vitro* (Buckingham, 1982). Additionally, i.c.v. injections of morphine was shown to stimulate the release of ACTH (Eisenberg, 1985; Suemaru et al., 1986), whereas peripheral injection of a morphine-analogue that does not cross the blood-brain barrier, failed to induce an HPA-axis response (Pechnick et al., 1987).

Recent studies have shown that naloxone attenuates the IL-1 $\beta$ -induced Fos expression in the PVH and in the A1/C1 and A2 catecholaminergic cells of the brain stem (Buller et al., 2005). This was interpreted to suggest that centrally produced opioids possess stimulatory effects on the HPA-axis during immune stress. The main brain stem enkephalinergic input to the PVH arises from the A1/C1 and A2 regions (Beaulieu et al., 1996), and enkephalin-like immunoreactivity has been reported to be expressed preferentially in noradrenergic cells in these regions (Ceccatelli et al., 1989b). Thus, taken together with these observations, our findings in **paper II** that ppENK hnRNA expression is induced in the ventrolateral medulla and the catecholaminergic part of NTS following injection of IL-1 $\beta$ , open the possibility that ppENK hnRNA expressing neurons are involved in modulating the HPA-axis response to IL-1 $\beta$ . However, this is unlikely to be exerted through a direct opioidergic effect on CRH neurons, since opioid-receptor stimulation is inhibitory in nature. Accordingly, if enkephalin released in the PVH during immune stress would bind to opioid receptors situated on CRH-ergic neurons, these neurons would be less likely to release CRH to the median eminence. Thus, if an enkephalinergic stimulatory effect on the HPA-axis is exerted at the level of the PVH, enkephalin would bind to opioid receptors situated either on local inhibitory interneurons or presynaptically on inhibitory neurons projecting to the PVH. In fact, it has been shown that mRNA for  $\mu$ -opioid receptors is sparse in the PVH (Mansour et al., 1994), while there is a positive labeling of immunoreactive fibers (Mansour et al., 1995b), hence supporting the latter hypothesis.

In addition to the possible effects of enkephalin on PVH neuronal activity, our data also show immune-stress induced enkephalinergic activity in the hypophysiotropic neurons *per se*. Thus, in line with the above-mentioned finding of an immune-induced ppENK- $\beta$ -galactosidase transgene upregulation in the PVH (Van Koughnet et al.,

1999), our results show that systemic injection of both IL-1 $\beta$  and LPS causes a rapid increase of ppENK hnRNA in the PVH (**papers II and III**, respectively). Taken together with several previous observations on ppENK mRNA expression in the PVH by different types of stressors (see Introduction), our findings thus suggest that the recruitment of enkephalinergic cells in the PVH constitutes a robust part of the general adaptive response to stress.

Previous studies have demonstrated immunolabeling for enkephalin in the external layer of the median eminence (Hisano et al., 1986; Hisano et al., 1987). Co-localization between enkephalin and CRH has also been demonstrated in the PVH (Hökfelt et al., 1983; Ceccatelli et al., 1989a; Pretel and Piekut, 1990), suggesting that the PVH could be the origin of the enkephalinergic terminals in the median eminence. In 1992, Merchentaler showed via i.p. injections of Fluoro-Gold that approximately 50% of the enkephalin-immunoreactive neurons of the PVHmp projected to the median eminence. Thus, a considerable proportion did not. In contrast, our results in **paper III**, using the same tracing technique as Merchentaler, but combining it with *in situ* hybridization against LPS-induced ppENK hnRNA, showed that the specific subpopulation of enkephalinergic cells that induced their expression of ppENK hnRNA synthesis upon immune challenge almost exclusively projected to the median eminence. Because only 40% of the ppENK hnRNA expressing neurons co-expressed CRH mRNA, other neuropeptides are likely to be co-released with enkephalin during immune stress, such as neurotensin, which has been shown to be induced in hypophysiotropic neurons following systemic LPS-injection (Loum-Ribot et al., 2004). Neurotensin has previously also been shown to co-localize with enkephalin during e.g. hypovolemic stress (Watts and Sanchez-Watts, 1995).

The physiological significance of enkephalins released in the median eminence remains to be clarified. The stimulatory effect of enkephalin on the HPA-axis is probably not exerted at the level of the pituitary gland (see above), and, due to the inhibitory nature of the opioid receptors, the median eminence also seems unlikely as the major level of interaction between enkephalin and CRH. Diverse actions of enkephalin on peptide hormone secretion have been described. For example, enkephalin has been shown to stimulate the release of GH and prolactin, while inhibiting the release of GnRH (Bruni et al., 1977; Buckingham and Cooper, 1984). Thus, it is well-known that the reproductive axis is inhibited during immune challenge and other stressful conditions, and that  $\mu$ -receptors play a role in this process (Kalra et al., 1990; Rivest et al., 1993). In sheep, infusion of met-enkephalin antiserum but not of serum against  $\beta$ -endorphin into the mediobasal hypothalamus (in close vicinity of the ME), increased the plasma levels of LH (Weesner and Malven, 1990). Interestingly, an intravenous injection of LPS that is known to inhibit the release of LH was unable to affect the GnRH-induced release of LH if GnRH was injected intravenously (He et al., 2003). Since circulating GnRH can directly influence the release of LH from the pituitary, this observation suggests that LPS exerts its inhibitory effect on the HPG-axis upstream of the pituitary. This is consistent with the present demonstration of an immune-induced increase in ppENK transcription in

hypophysiotropic PVH neurons, which would permit a local action of enkephalin on GnRH release in the median eminence. The rapid time course of inhibition of LH release in plasma after intravenous LPS, occurring as early as 60 minutes after injection (Refojo et al., 1998), is consistent with rapid induction of preproenkephalin transcription that we have reported in **papers II** and **III**. Consistent with the idea of local effects of enkephalin in the median eminence and not directly on the pituitary gland, it has been shown that  $\mu$ -opioid receptors are present on both nerve endings and tanycytes in the external layer of the ME (Beauvillain et al., 1992; Mansour et al., 1995b), where enkephalin-, CRH- and GnRH-containing terminals from the PVH and the medial preoptic nucleus can be found (Naik, 1975; Hisano et al., 1986). In contrast, *in situ* hybridizations and receptor-binding studies have failed to show any evidence for the presence of  $\mu$ - or  $\delta$ -opioid receptors in the anterior pituitary gland (Herkenham et al., 1986; Mansour et al., 1986; Mansour et al., 1995a; however, see Carretero et al., 2004). Recently,  $\delta$ -receptors were found on GnRH-containing nerve endings in the median eminence (Pimpinelli et al., 2006), a finding that further strengthens the hypothesis that an inflammation-associated inhibitory action of enkephalin on the HPG axis may be exerted at this level.

### **Role of mPGES-1 in the HPA-axis response to immune stress**

Studies during the last few years have revealed that induced PGE<sub>2</sub> synthesis through mPGES-1 is crucial for several aspects of the sickness response. Thus, using mPGES-1 deficient mice (Trebino et al., 2003), it has been shown that the febrile response to LPS, IL-1 $\beta$  and turpentine is almost completely dependent on this enzyme (Engblom et al., 2003; Saha et al., 2005). Furthermore, the anorexia associated with IL-1 $\beta$  injection, as well as the anorexia seen in pre-starved LPS-treated mice, is partially dependent on mPGES-1 (Elander et al., 2006; Pecchi et al., 2006). Many of the sickness responses are mimicked by central injections of PGE<sub>2</sub>, including the activation of the HPA-axis (Feldberg and Saxena, 1971; Rassnick et al., 1995), hence suggesting that PGE<sub>2</sub> synthesis in the brain plays a critical role. The finding that PGE<sub>2</sub> synthesizing enzymes are induced in the brain endothelial cells upon immune stimulation, and that this induction is associated with increased intracerebral concentrations of PGE<sub>2</sub>, thus provides strong evidence that a humoral pathway across the blood-brain-barrier is critically involved in the immune-to-brain signaling (Cao et al., 1995; Cao et al., 1997; Lacroix and Rivest, 1998; Quan et al., 1998; Nadeau and Rivest, 1999; Ek et al., 2001; Engblom et al., 2002a; Gosselin and Rivest, 2008).

The findings in **paper IV** are consistent with a role of centrally produced PGE<sub>2</sub> in the HPA-response to immune challenge by showing that the delayed ACTH and corticosterone release was attenuated in mPGES-1 knockout mice. However, the immediate ACTH and corticosterone responses were unaffected, and there was no attenuation of the neuronal response in the PVH or in upstream relay structures such as catecholaminergic cells in the ventrolateral medulla. How do these data relate to the prevailing view on HPA-axis activation during immune stress? The established view

seems to be that such activation during immune stress is that it occurs via increased transcription of the CRH gene in hypophysiotropic PVH neurons, resulting in release of CRH peptide in the median eminence. Catecholaminergic projections from the A1/C1 and A2 neuronal cell groups are considered crucial in this process (Chuluyan et al., 1992; Ericsson et al., 1994; Buller et al., 2001), similar to what has been found for other physical stressors such as hypoglycemia, but in contrast to what is the case for psychogenic stressors, such as forced swim stress or foot-shock (Li et al., 1996; Ritter et al., 2003). Additionally, immune-induced PGE<sub>2</sub> has been suggested to play a role in the activation of the catecholaminergic neurons. This is based on the findings that systemic administration of IL-1 $\beta$  induces Fos-expression in EP<sub>3</sub>-receptor expressing cells of the A1/C1 area, and that local injections of PGE<sub>2</sub> in the same area induce Fos-expression in the catecholaminergic neurons, as well as in neurons of the PVH (Ericsson et al., 1997; Ek et al., 2000). Our data in **paper IV** contradict this hypothesis. The mPGES-1 knockout mice failed to show any attenuation of the LPS-induced Fos-expression in these areas, and furthermore, the number of dual-labeled cells expressing both Fos and tyrosine hydroxylase in the A1/C1 region did not differ from that in wild type mice. This finding is consistent with the observations by Matsuoka and collaborators, who showed a similar degree of LPS-induced Fos-activation in EP<sub>3</sub> deficient mice as in wild type mice (Matsuoka et al., 2003). Indeed, the inhibitory nature of the EP<sub>3</sub> receptor suggests that this receptor is unlikely to be responsible for the activation of the catecholaminergic neurons during immune challenge. Instead we hypothesize that the intact Fos-expression in the brain stem and the PVH reflects an early vagus-mediated response that is independent of mPGES-1. Recent findings in mice lacking the IL-1R1 specifically in endothelial cells have shed new light on the neural versus humoral activation pathways to the brain that supports our hypothesis. Experiments on these mice have shown that the Fos-expression in the PVH following i.p., but not i.v., administration of IL-1 $\beta$  was independent of the blood-brain barrier, whereas induced Cox-2 expression in the brain was extinguished in the transgenic mice irrespective of the route of cytokine administration, indicating the presence of a neuronal pathway for Fos activation which is not mediated by central prostaglandin synthesis (Ching et al., 2007). Our results in **paper IV** showing an intact Fos response in all structures investigated (thus being independent of mPGES-1), which contrasts to the decreased CRH mRNA expression in the PVH (an mPGES-1 dependent effect), suggests that the humoral signaling via the blood-brain barrier directly targets the PVH, and hence the regulation of CRH gene expression, or affects the PVH indirectly, via an influence from for example the preoptic area (see below).

Previous research has suggested the EP<sub>4</sub> receptor to be implicated in the inflammatory-elicited HPA-axis response, based on its immune-induced expression in CRH-ergic neurons of the PVH, as well as in the A1 catecholaminergic cell group (Zhang and Rivest, 1999). However, further studies have failed to support a role for EP<sub>4</sub>, but instead showed a blunted HPA-axis response in EP<sub>1</sub> and EP<sub>3</sub> deficient mice (Matsuoka et al., 2003). Because ligand-binding to the EP<sub>3</sub>-receptor is likely to cause inhibition of the target cell, an EP<sub>3</sub>-dependent activation of the HPA-axis should be exerted through

indirect, disinhibitory mechanisms. This is in agreement with the apparent lack of EP<sub>3</sub>-receptor mRNA in the PVH (Sugimoto et al., 1994). Indeed, studies have shown that injection of PGE<sub>2</sub> into the preoptic area where the EP<sub>3</sub> receptor is densely expressed (Sugimoto et al., 1994; Ek et al., 2000; Oka et al., 2000; Vasilache et al., 2007), caused immediate release of ACTH. This effect was mimicked by a similar injection of a GABA<sub>A</sub>-receptor agonist, suggesting that PGE<sub>2</sub> in the preoptic region could switch off an otherwise tonic inhibitory input to the PVH. Additionally, PGE<sub>2</sub> could act on local inhibitory interneurons in the PVH area (Bali and Kovacs, 2003), e.g. on presynaptically located EP<sub>3</sub> receptors to decrease the inhibitory input to the hypophysiotropic neurons. Similar mechanisms have been observed in the supraoptic nucleus (Shibuya et al., 2002). Additionally, weak and diffuse labeling for EP<sub>1</sub> mRNA is present in the PVH, suggesting that PGE<sub>2</sub> also may bind directly to hypophysiotropic neurons in order to activate CRH transcription (Oka et al., 2000). Our hypothesis for how the HPA-axis response is regulated during immune challenge, based on previous literature and our findings in **paper IV**, is summarized in figure 6.

In all, our data in **paper IV** suggest that the LPS-induced HPA-axis response in mice is initiated by non-mPGES-1-dependent mechanisms involving the vagus nerve and/or PGE<sub>2</sub> derived from another PGE<sub>2</sub> isomerase. However, the sustained response is dependent on mPGES-1-derived PGE<sub>2</sub>, probably at the level of the hypothalamus, either via direct action on EP-receptors in the PVH, or via indirect, disinhibitory mechanisms involving local inhibitory interneurons or projections from the preoptic area. These data thus suggest that humoral signaling via cytokine-induced PGE<sub>2</sub>-synthesis interacts in a time-dependent supplementary sequence with afferent neuronal signaling in creating the HPA-axis response to immune stress. This idea fits well with a more general concept of combined neural and humoral signaling in the temporal regulation of autonomic responses. For example, the short- and long-term regulation of feeding behavior and metabolism show striking similarities, with the vagus nerve being responsible for relaying immediate satiety signals from the internal organs to the brain stem, while humoral pathways, involving circulating adipocytic hormones, instead target primarily hypothalamic structures for the long-term regulation of food-intake (Cone, 2005).

#### *Dissociation between the ACTH and corticosterone responses – direct effects on the adrenal gland?*

While there is overwhelming evidence for a centrally elicited ACTH-regulated corticosterone release during acute immune challenge (see Introduction), conditions exist in which there is clear dissociation between the plasma levels of ACTH and corticosterone. Thus, patients with chronic sepsis and patients who have experienced multiple trauma, have been reported to show high corticosterone levels in the absence of a significant ACTH response (Vermes et al., 1995; Beishuizen et al., 2001). In a mouse model of graft-versus-host disease, a similar decoupling was seen between the late corticosterone response and the serum levels of ACTH (You-Ten et al., 1995), and

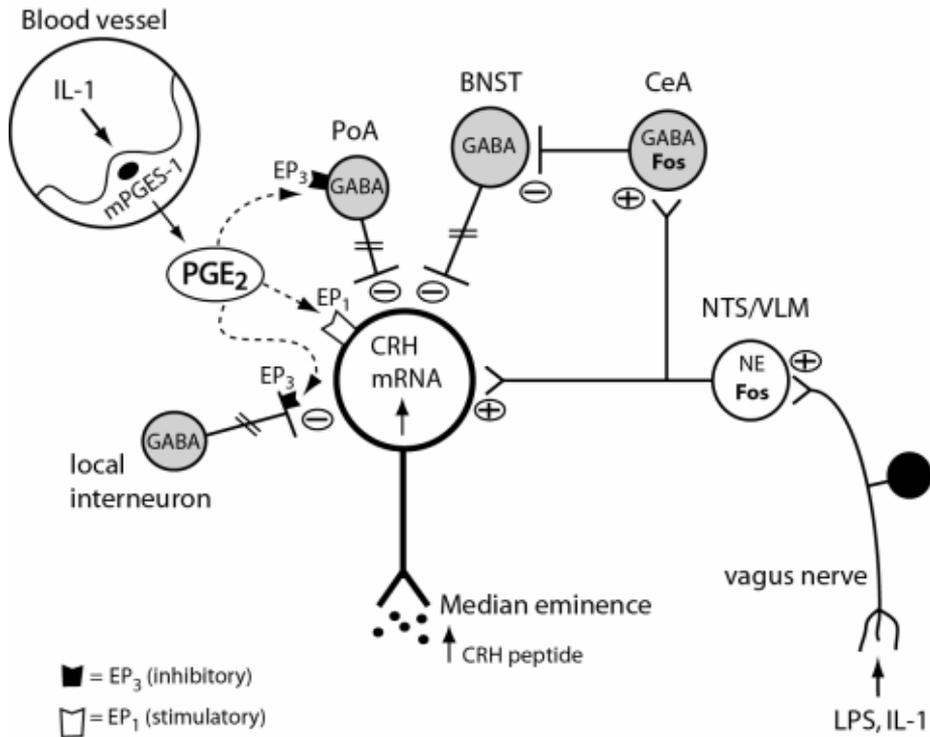


Fig. 6. Hypothetical scheme for the HPA-axis activation during immune challenge. The CRHergic hypophysiotropic neuron in the center of the picture is influenced by both neuronal and humoral signaling pathways. Norepinephrinergic input from brain stem regions activates the PVH during the early phase of the HPA-axis response, both directly but also indirectly via disinhibitory mechanisms that involve switching off tonic inhibitory input from the BNST. These pathways are independent of mPGES-1. The delayed phase of the HPA-axis response is in addition dependent on humoral signaling via the blood-brain barrier. Thus, PGE<sub>2</sub> released in the hypothalamic area may act directly on the PVH (e.g. on the EP<sub>1</sub> receptor, which would exert stimulatory effects on the CRHergic neuron) or indirectly, via disinhibitory mechanisms involving local GABAergic interneurons, or GABAergic neurons projecting from the preoptic area. By binding to the inhibitory EP<sub>3</sub> receptor situated either presynaptically or on the cell bodies of these GABAergic neurons, a tonic inhibitory input to the hypophysiotropic neurons would be switched off, allowing for increased CRH mRNA synthesis, CRH peptide release, and ultimately, the release of ACTH and corticosterone. This latter pathway is lacking in the mPGES-1 deficient mice, causing an impairment of the delayed HPA-axis response during immune challenge with LPS. Note that the neuronal network depicted is simplified and that the vagus nerve only terminates in the NTS, not in the VLM. However, catecholaminergic neurons projecting to the PVH arise from both these locations.

corticosterone. Thus, patients with chronic sepsis and patients who have experienced multiple trauma, have been reported to show high corticosterone levels in the absence of a significant ACTH response (Vermees et al., 1995; Beishuizen et al., 2001). In a mouse model of graft-versus-host disease, a similar decoupling was seen between the late corticosterone response and the serum levels of ACTH (You-Ten et al., 1995), and

in a model of colitis, the ACTH levels did not differ from controls at any time point, whereas the corticosterone levels were highly elevated from day one (Franchimont et al., 2000). There are also findings from experimental studies employing peripheral injections of LPS that support such dissociation between the corticosterone and ACTH levels. For example, the ACTH response to LPS could be completely blunted by using an antiserum towards CRH, whereas the corticosterone response only was attenuated (Schotanus et al., 1994). The same phenomenon was observed following injection of a low dose IL-1 $\beta$  in vagotomized animals (Kapcala et al., 1996). It should be remembered, however, that it is difficult to ascertain that CRH was completely neutralized or that the remaining low ACTH-levels were unable to elicit the corticosterone response in these studies. Perhaps more convincing are the results of studies on ACTH-supplemented hypophysectomized rats, which have demonstrated that such animals still can respond with corticosterone release to an LPS-injection, but not to psychological stress (Suzuki et al., 1986; Mazzocchi et al., 1995).

While it thus seems clear that during systemic inflammatory conditions, the adrenal gland itself possesses the ability to release corticosterone independent of influence from the brain, very few studies exist that have examined the local inflammatory response in the adrenal gland during such conditions (see Introduction). *In vitro* experiments have suggested that IL-1 $\beta$  has stimulatory effects on corticosterone release and that these effects are mediated by PGE<sub>2</sub> (Whitcomb et al., 1988; Winter et al., 1990; Andreis et al., 1991a; Tominaga et al., 1991; O'Connell et al., 1994), although contradictory data also exist (Gwosdow, 1995). The limitation with the *in vitro* studies is that the adrenal tissue integrity was disrupted to produce dispersed cell cultures. The tissue of the adrenal gland is highly organized and there seems to exist a great interdependence between the medulla and the cortex (see e.g. Andreis et al., 1991b; Andreis et al., 1992; Bornstein et al., 1997; Bornstein et al., 2000). Therefore, it is not unlikely that disruption of this organization will produce misleading results. However, supporting a role for IL-1 $\beta$  and prostaglandins in intra-adrenal signaling, high doses of LPS have *in vivo* been shown to induce Cox-2 and IL-1 $\beta$  expression in the rat adrenal gland, as seen with *in situ* hybridization (Nobel and Schultzberg, 1995; Ichitani et al., 2001).

In **paper V** we show for the first time that mPGES-1 is induced in immune cells situated in the corticosterone-producing layers of the adrenal gland during endotoxemia, suggesting that the local adrenal production of PGE<sub>2</sub> is increased during this condition. The synthesis of mPGES-1 followed that of Cox-2, IL-1 $\beta$  and the IL-1R1, which were induced in the same cells, as well as in other immune cells of the adrenal cortex. Because we found that the IL-1R1 was expressed exclusively on immune cells of the adrenal cortex, and not directly on steroid-producing cells, we provide support for the hypothesis that has emerged from *in vitro* studies, namely that IL-1 $\beta$  may affect the release of corticosterone via indirect mechanisms (Winter et al., 1990; Tominaga et al., 1991). Taken together, the data suggest that corticosterone release may be regulated locally in the adrenals via IL-1 $\beta$  induced synthesis of PGE<sub>2</sub>, similar to what has been demonstrated for the PGE<sub>2</sub> production in the blood-brain

barrier (Ek et al., 2001). Our demonstration that EP-receptors are expressed in the corticosterone-producing layers of the cortex is consistent with this idea.

In addition to the induced expression of prostaglandin synthesizing enzymes, we also demonstrated constitutive expression of mPGES-1, associated with Cox-1, in cells localized to the innermost part of the adrenal cortex. Immune cells in the same location that were positive for mPGES-1 showed reactive changes after LPS administration, with induced expression of Cox-2, as well as of IL-1 $\beta$  and IL-1R1, indicating that these cells produce PGE<sub>2</sub> both during constitutive conditions and as a response to immune challenge. Their juxtamedullary position and frequent association with blood vessels suggest that the PGE<sub>2</sub> released by these cells may influence medullary cells, being consistent with the strong EP<sub>1</sub> and EP<sub>3</sub> receptor expression that we could see in the adrenal medulla, in which the EP<sub>4</sub> receptor also has been shown to be expressed (Muro et al., 2000). These observations are in line with the above-mentioned functional and anatomical connections that have been demonstrated between the cortex and the medulla, and hence indicate a potential role for PGE<sub>2</sub> in the release of catecholamines (Yokohama et al., 1988) and co-stored neuropeptides such as ACTH, corticotropin releasing hormone and enkephalins (Whim, 2006) that in turn may influence corticosterone release (Andreis et al., 1992; Hinson et al., 1994; Kapas et al., 1995). For a summary of our findings in **paper V**, see figure 7.

Are these intra-adrenal mechanisms relevant during the acute exposure to inflammatory stimuli? The *in vitro* studies mentioned above suggest that prolonged exposure to IL-1 $\beta$  is required for a corticosterone response to occur. Indeed, Van der Meer and collaborators showed that short-term incubation of adrenal cells with IL-1 $\beta$  failed to cause corticosterone release, and similarly, a short perfusion session of adrenal tissue with IL-1 $\beta$  was insufficient. However, prolonged perfusion (6 h) did cause release of corticosterone (van der Meer et al., 1996). Thus, it seems that the acute corticosterone release during immune stress is unlikely to involve ACTH-independent effects in the adrenal gland. Our findings in **paper V** are consistent with these data. Thus, if intra-adrenal production of IL-1 $\beta$  and mPGES-1 is relevant for corticosterone release, the effect is unlikely to be present until at least 5 h after LPS-injection, the time span that was required to induce IL-1R1 and mPGES-1 mRNA and proteins. Thus, being in accordance with the conditions in which a dissociation between the ACTH and corticosterone response are present (see above), it is likely that the intra-adrenal pro-inflammatory circuit may be relevant during more prolonged inflammatory conditions. Future studies on the intra-adrenal pro-inflammatory circuit in chronic inflammatory disease models may help answer these questions.

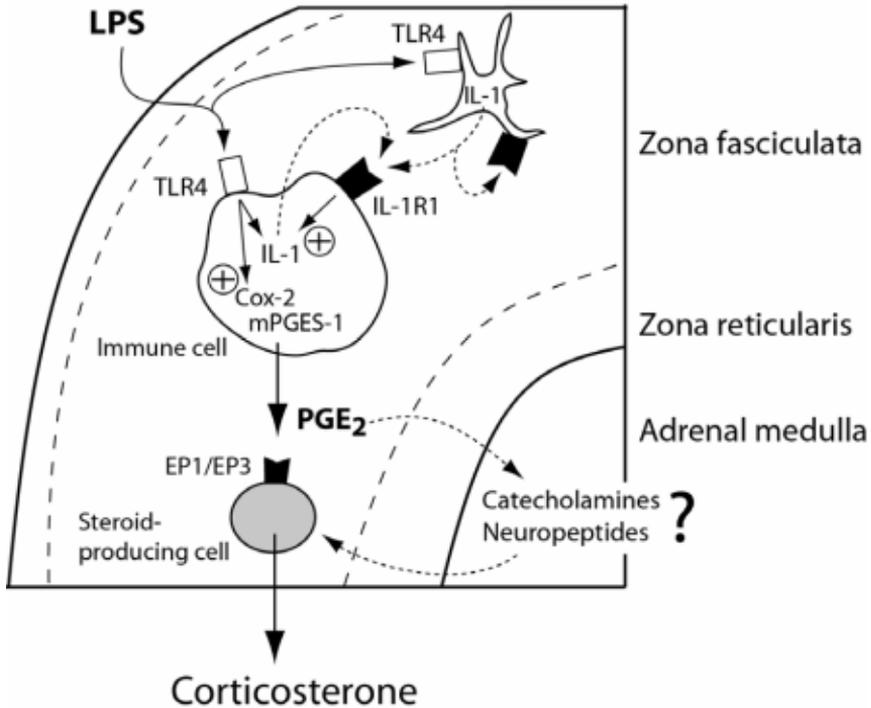


Fig. 7. Hypothetical scheme, based on the findings in paper V, for how circulating LPS may influence the synthesis and release of pro-inflammatory mediators in the adrenal cortex. LPS acts on TLR4 on immune cells of the adrenal gland. The large cell-type in the center of the figure is not present until 3-5 hours after injection of LPS, while the dendritic-looking cell-type is present also during the basal state. Both types of cells respond to LPS-challenge by expressing IL-1 $\beta$  and its receptor. The expression of IL-1 $\beta$  is positively regulated by autocrine or paracrine mechanisms. Cox-2 and mPGES-1 are also induced. The latter is largely confined to the large and immature immune cells. Its presence is presumed to lead to release of PGE<sub>2</sub>, which can bind to EP-receptors present in the cortex or the medulla (cell types have not been identified). This may, as suggested from *in vitro* data, ultimately cause increased release of corticosterone from the adrenal gland, either by direct actions on steroid-producing cells, or indirectly, via the release of mediators from the adrenal medulla. Note that mPGES-1-expressing immune cells also are situated in the innermost part of the zona reticularis, although not shown in this picture. These cells also have the ability to synthesize IL-1 $\beta$  and its receptor during immune challenge and are ideally situated for cross-talk with the medulla.



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