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Sickness Behavior: Immune System Influences on Brain and Behavior

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IMMUNE SYSTEM INFLUENCES ON BRAIN  
AND BEHAVIOR**

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# Sickness Behavior: Immune System Influences on Brain and Behavior

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*Till Aleksander*



# ABSTRACT

Sickness behavior is a motivational state that redirects the needs and priorities of the organism during infection to aid recovery. The behavioral changes include fatigue, lowered mood and aches. Peripheral cytokines signal to the brain via autonomic nerves and the blood-brain interface and change the inflammatory status of the brain, a mechanism that in recent years has been implied in complex syndromes like long-term pain, depression, fatigue and overall poor well-being. Epidemiological studies also suggest that chronic inflammatory disease like allergy increases the risk of developing Alzheimer disease (AD) later in life. In this thesis we explored how acute experimental immune activation affects pain sensitivity and self-rated general health. We also investigated inflammatory and degenerative effects in the brain following chronic allergic inflammation in a mouse model.

In **Paper I**, eight healthy participants (1 woman) were injected with 0.8 ng/kg body weight lipopolysaccharide (LPS) and with saline 28 days apart in a balanced double-blind within-subject design. Subsequently, 52 subjects were injected with 0.6 ng/kg LPS (31, 18 women) or saline (21, 11 women) in a double-blind between-subject design (data from this protocol was also used for **Paper II and IV**). Pro-inflammatory peripheral cytokine (TNF- $\alpha$ , IL-6 and IL-8) levels increased significantly in the LPS group. We demonstrated that in particular deep pain sensitivity increased during immune activation. Women were more affected than men by the inflammatory signals with regard to pain, as women also demonstrated increased cutaneous pain sensitivity and impaired descending pain inhibition during LPS provocation, whereas men did not. Pain sensitivity was associated with peripheral IL-6 and IL-8 levels for both men and women.

In **Paper II** (second sample in **Paper I**), we investigated the neural correlates underlying these findings, using functional magnetic resonance imaging. LPS attenuated descending endogenous pain inhibition reflected as decreased activity in the rostral anterior cingulate (rACC) and lateral prefrontal cortices. Also, the LPS group demonstrated increased insular activity, which may reflect amplified interoceptive and/or affective processing. An overall weaker pain regulation (lower rACC activity) and an association between insular activation and peripheral pro-inflammatory cytokines were found in women, which may explain the sex differences found in pain sensitivity. The higher susceptibility to inflammation-driven pain sensitivity in women may be one of the mechanisms behind more women suffering from pain conditions.

In **Paper III** we studied the impact of long-term peripheral inflammation on inflammatory and neurodegenerative processes in the brain. We used a murine model for chronic allergic inflammation by ovalbumin provocation and assessed AD and inflammation relevant markers. We showed that chronic allergic inflammation induces *tau*-phosphorylation in mice,



a hallmark of AD. Also, chronic inflammation resulted in antibody increases (IgG and IgE) in the mouse brain, which in turn could lead to neuroinflammation over time.

In **Paper IV** (same sample as **Paper II**) we assessed self-rated general health (SRH) and subjective sickness behavior during the peak of the peripheral inflammatory response. We showed that SRH ratings worsened markedly during experimental inflammation, and that these effects were statistically mediated by the symptoms of sickness behavior perceived by the subjects.

In conclusion, our findings corroborate related clinical research findings, suggesting that the inflammatory models used in this thesis may serve as useful tools for studying neuroimmune mechanisms relevant for chronic pain, neurodegeneration and states characterized by poor subjective health. A better understanding of sickness-induced brain changes may aid future treatment strategies for such complex diseases that currently often lack successful treatment.

# POPULÄRVETENSKAPLIG ÖVERSÄTTNING

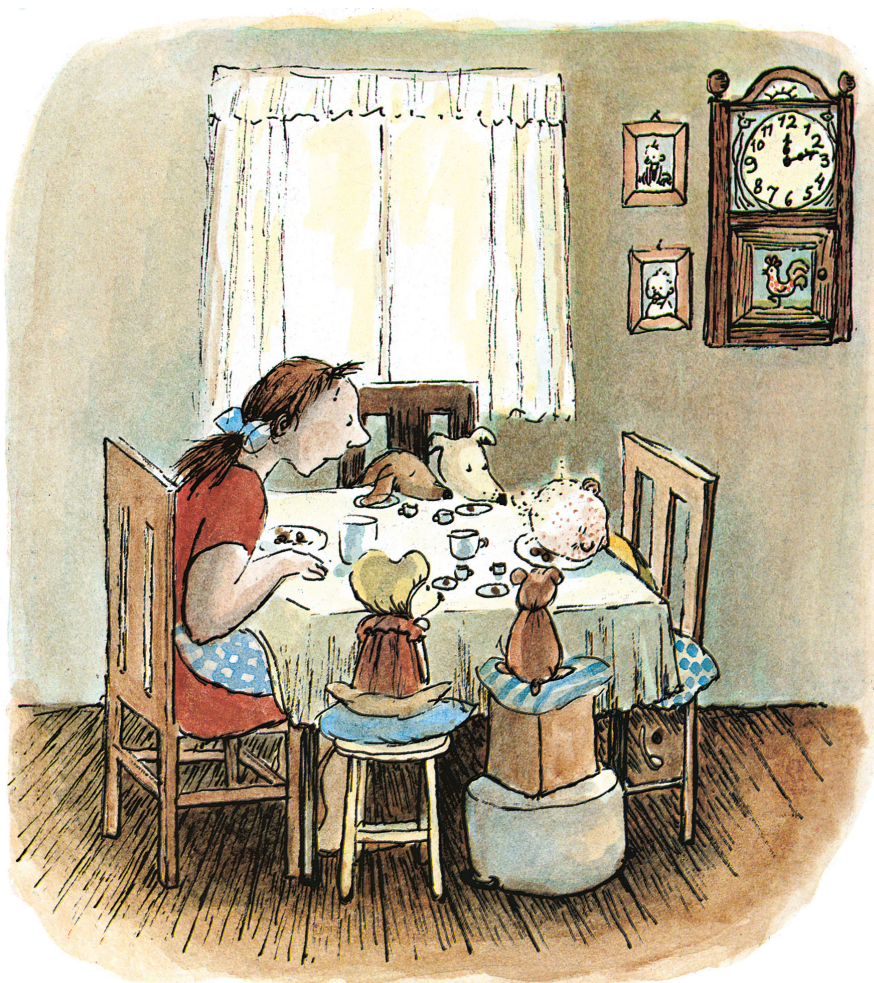
De flesta vet precis hur det känns att vara sjuk. Kroppen värker, man känner olust och trötthet, och en annars företagsam person kan vara helt nöjd med att ligga ihoprullad i sängen och göra mest ingenting medan hen är sjuk. Man kan tro att det är bakterier eller virus i sig som påverkar oss, men i själva verket utgör reaktionen en välorganiserad beteendeförändring som drivs av våra egna immunceller och kallas *sjukdomsbeteende*. Normalt sett återställs beteendet när man blivit frisk, men i vissa fall tros de mekanismer som reglerar sjukdomsbeteende vara satta ur spel, t.ex. om den inflammatoriska signalen från kroppen blir långvarig eller uppkommer för ofta eller för kraftigt. Då tycks ett funktionellt beteende tippa över i sjukliga förändringar. Man tror t.ex. att vissa patienter med kronisk smärta, depression, långvarig trötthet eller försämrat allmäntillstånd även kan lida av en lågradig inflammation, i kroppen eller i hjärnan, som inte kan uppmätas kliniskt. Studier visar även på att kronisk inflammatorisk sjukdom kan öka risken för att utveckla demens senare i livet, vilket kan bero på ökad och ackumulativ inflammatorisk aktivitet i hjärnan.

Vi har studerat immunsystemets förmåga att förändra beteende och orsaka hjärnförändringar i experimentella human- och djurmodeller. Vi har använt en vanlig modell för experimentell sjukdom, där friska försökspersoner injiceras med låga doser lipopolysackarider (LPS). LPS är ett bakterieämne som startar en inflammationsreaktion i kroppen som varar i 4-5 timmar. Vi försökspersonernas smärtkänslighet under den experimentella inflammationen, samt deras smärtrelaterade hjärnaktivitet med funktionell magnetkamerateknik (fMRI). Vi undersökte även deras subjektivt skattade hälsa och sjukdomsbeteende. För att studera effekten på hjärnan vid långvarig inflammatorisk aktivitet använde vi en musmodell för kronisk allergisk astma.

**I den första studien** kunde vi visa att friska försökspersoner blir mer smärtkänsliga när deras immunsystem är aktiverat, och intressant nog drabbas kvinnor starkare än män. Vi såg även att inflammation försvagade kvinnors smärthämningssystem, vilket inte verkade vara fallet för männen. Smärtkänsligheten var relaterad till styrkan i den perifera inflammationen för både män och kvinnor. **I den andra studien** visade vi med fMRI en lägre aktivitet i frontalloberna, i områden som bland annat har smärthämmande funktioner. Vi såg även en ökad aktivitet i främre insula, en del i hjärnan som bearbetar inre signaler från kroppen och känslomässiga aspekter av smärta. Dessa fynd ger en möjlig förklaring till i den ökade smärtkänsligheten vi fann i första studien. Vidare såg vi att kvinnor generellt hade mindre aktivitet i ett område som är involverat i smärthämning. Sammantaget kan dessa fynd vara en orsak till de könsskillnader vi såg i smärtkänslighet första studien, samt varför kvinnor oftare drabbas av kroniska smärttillstånd. **I avhandlingens tredje studie** visade vi att långvarig allergisk inflammation ökar markörer relevanta för Alzheimers demenssjukdom i hjärnan på möss och även förekomsten av antikroppar i hjärnan. Dessa förändringar skulle på sikt kunna leda till större inflammatorisk aktivitet i hjärnan. **I den fjärde studien** visade vi att

människor bedömer sitt allmänna hälsotillstånd som sämre när deras immunsystem är aktiverat, och att den självskattade hälsan beror på hur starkt sjukdomsbeteende de upplever.

Våra resultat passar väl in på kliniska fynd inom forskning på smärtsyndrom och demenssjukdomar. Experimentella modeller som de vi använt kan därför vara användbara redskap för att förstå inflammationsbetingade mekanismer i kroniska sjukdomar som i nuläget inte kan behandlas tillfredsställande, såsom oförklarlig smärta, trötthet och nedstämdhet, samt demens.



*Illustration by Eva Eriksson.  
From "Mamman och den vilda Bebin" by Barbro Lindgren.*

# LIST OF SCIENTIFIC PAPERS INCLUDED IN THE THESIS

- I. Modality and sex differences in pain sensitivity during human endotoxemia.

**Karshikoff B**, Lekander M, Soop A, Lindstedt F, Ingvar M, Kosek E, Olgart Höglund C, Axelsson J. Brain Behav Immun. 2014 Dec 5. pii: S0889-1591(14)00558-3.

- II. Why sickness hurts: Systemic inflammation decreases pain inhibition and increases interoceptive processing.

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- III. Allergy influences the inflammatory status of the brain and enhances tau-phosphorylation.

Sarlus H, Höglund CO, **Karshikoff B**, Wang X, Lekander M, Schultzberg M, Oprica M. J Cell Mol Med. 2012 Oct;16(10):2401-12.

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- V. Sick man walking: Perception of health status from body motion.

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Olsson MJ, Lundström JN, Kimball BA, Gordon AR, **Karshikoff B**, Hosseini N, Sorjonen K, Olgart Höglund C, Solares C, Soop A, Axelsson J, Lekander M. Psychol Sci. 2014 Mar;25(3):817-23.

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# TABLE OF CONTENTS

<b>1</b>	<b>INTRODUCTION</b>	<b>15</b>
<b>2</b>	<b>UNDERSTANDING BODY FUNCTION IN TERMS OF SYSTEMS</b>	<b>15</b>
<b>2.1</b>	<b>THE IMMUNE SYSTEM</b>	<b>16</b>
2.1.1	BASIC IMMUNOLOGY	16
2.1.2	CYTOKINES AND INFLAMMATION	17
2.1.3	IMMUNE SYSTEM-TO-BRAIN COMMUNICATION	18
<b>2.2</b>	<b>THE NERVOUS SYSTEM</b>	<b>19</b>
2.2.1	BRAIN-TO-IMMUNE SYSTEM COMMUNICATION	19
2.2.2	THE IMMUNE SYSTEM OF THE CENTRAL NERVOUS SYSTEM	20
<b>2.3</b>	<b>THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS</b>	<b>20</b>
<b>2.4</b>	<b>THE INTEROCEPTIVE SYSTEM</b>	<b>21</b>
<b>2.5</b>	<b>THE PAIN SYSTEM</b>	<b>22</b>
2.5.1	CHRONIC PAIN	24
<b>3</b>	<b>SICKNESS BEHAVIOR</b>	<b>25</b>
<b>3.1</b>	<b>ANIMAL STUDIES</b>	<b>26</b>
<b>3.2</b>	<b>HUMAN STUDIES</b>	<b>27</b>
<b>4</b>	<b>CHRONIC INFLAMMATORY DISEASE AND THE BRAIN</b>	<b>29</b>
<b>5</b>	<b>SUBJECTIVE HEALTH PERCEPTION</b>	<b>30</b>
<b>6</b>	<b>AIM OF THE THESIS</b>	<b>31</b>
<b>7</b>	<b>METHODS</b>	<b>32</b>
<b>7.1</b>	<b>HUMAN STUDIES (PAPERS I, II, IV)</b>	<b>32</b>
7.1.1	PARTICIPANTS AND STUDY DESIGN	32
7.1.2	LPS-STIMULATION AND INFLAMMATORY PARAMETERS	33
7.1.3	QUESTIONNAIRES	35
7.1.4	PAIN SENSITIVITY MEASURES	37
7.1.5	BRAIN IMAGING	39
7.1.6	STATISTICAL METHODS	40
<b>7.2</b>	<b>MURINE STUDY (PAPER III)</b>	<b>42</b>
7.2.1	MURINE INFLAMMATORY MODEL	42
7.2.2	ANIMALS AND DATA COLLECTION	43
<b>8</b>	<b>SUMMARY OF STUDY I-IV</b>	<b>44</b>
<b>8.1</b>	<b>PAPER I</b>	<b>44</b>
<b>8.2</b>	<b>PAPER II</b>	<b>45</b>
<b>8.3</b>	<b>PAPER III</b>	<b>46</b>
<b>8.4</b>	<b>PAPER IV</b>	<b>47</b>
<b>9</b>	<b>OVERALL DISCUSSION</b>	<b>49</b>

<u>10</u>	<u>CONCLUDING REMARKS</u>	<u>51</u>
<u>11</u>	<u>FUTURE DIRECTIONS</u>	<u>51</u>
<u>12</u>	<u>ACKNOWLEDGEMENTS</u>	<u>53</u>
<u>13</u>	<u>REFERENCES</u>	<u>55</u>

## LIST OF ABBREVIATIONS

ACC	Anterior cingulate cortex
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer disease
APP	Amyloid precursor protein
BAL	Bronchoalveolar lavage
BBB	Blood-brain-barrier
BOLD	Blood-oxygen-level dependent
CNS	Central nervous system
CPM	Conditioned pain modulation
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CVO	Circumventricular organ
DNIC	Diffuse noxious inhibitory control
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
LMM	Linear mixed model
IPFC	Lateral prefrontal cortex
LPS	Lipopolysaccharide
OVA	Ovalbumin
PAG	Periaqueductal gray
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PFC	Prefrontal cortex
PPT	Pressure pain threshold



PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
rACC	Rostral anterior cingulate cortex
RVM	Rostral ventromedial medulla
SRH	Self-rated health
T <sub>c</sub> -cell	Cytotoxic T-cell
T <sub>h</sub> -cell	Helper T-cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T <sub>reg</sub> -cell	Regulatory T-cell
VAS	Visual analogue scale
vIPFC	Ventrolateral prefrontal cortex

# 1 INTRODUCTION

With all due respect to saber-toothed tigers, they were probably not the main threat to group living predators like human beings – pathogens were (*I*). Quiet, invisible, odorless threats that could hardly be avoided, and had to be dealt with when already present, *after* the attack had already occurred.

This thesis concerns one of the most important systems of a living organism – the immune system. The main focus will not be on the intricate ways in which the immune system fights pathogens, but on how the immune system tells the brain that an infection has occurred. I will present the immune system as a form of sensory system and describe its communication with the nervous system and its effect on behavior. The key term of this thesis is **sickness behavior** – how a sick individual adapts behavior during illness to promote recovery. In the studies of this thesis, we have explored how the immune system affects the brain and behavior from different angles, and the main concern is what happens when this powerful system is pushed too far.

## 2 UNDERSTANDING BODY FUNCTION IN TERMS OF SYSTEMS

We tend to describe the different functions of the body in terms of systems to aid our understanding of the complicated inner workings of a living organism. I will here describe a few of the biological and behavioral systems that play a part in sickness behavior. All descriptions of systems will be brief and tinged by my sickness-perspective.

Although I will use the term “system” throughout this thesis, I would like to point out the obvious: The systems do not actually exist, they are simply constructs of ours to help us understand and conceptualize biological function. The body does not know systems, the body merely knows homeostasis<sup>1</sup>.

In order to maintain homeostasis, the organisms uses whatever biological functions it has at hand to protect itself from damage and maintain internal stability – whether it is releasing or binding insulin to maintain blood sugar balance, emitting toxic substances to kill intruding parasites, or alter behavior so that the individual bundles up to warm a too cold body. This is why every biological system described overlaps with other biological systems and why some

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<sup>1</sup> A self-regulating process by which the biological system maintains equilibrium and stability despite changing conditions.

biological substances or brain areas appear as core players in seemingly unrelated systems. Also, the systems are intertwined and communicate reciprocally.

## **2.1 THE IMMUNE SYSTEM**

The immune system constitutes a diverse and widespread organization of organs, cells and soluble substances throughout the body. It is a system that is built, learns and adapts during a lifetime and it has a memory of what the body has been through. The importance of having a functioning immune system is quite obvious, as individuals who are born without core parts of the immune system have poor survival prospects if they do not receive extensive immunotherapy (2). Disorders like rheumatoid arthritis (RA), allergies and sepsis demonstrate the power and toxicity of the immune system. In RA, the immune system turns against one's own healthy body cells, causing extensive damage in the joints and much pain and suffering. In allergies, the immune system turns towards innocuous substances, causing tissue damage and in some cases death. In sepsis, the uncontrolled immunological response itself causes organ failure and often death.

If not fiercely controlled our own gatekeepers, the immune cells, may cause our death. What most people do not realize is that these gate keepers of ours do not only fight outwards killing pathogens – they also report back to central headquarters continuously, informing the brain about the health status of the body.

### **2.1.1 Basic immunology**

The immune system handles pathogens in two steps. The initial immunological reaction is called the innate immune response. It is fast, non-specific and gives the organism a chance to stay alive while the adaptive immune develops, which generally takes 1-2 weeks (3).

The innate immune system comprises phagocytic immune cells such as macrophages and dendritic cells in the tissue, monocytes and neutrophils in the blood and specific peptides. Cells in the body's first line of defense are sometimes called sentinel cells, and may also comprise mast cells and epithelial cells. Sentinel cells carry receptors on the cell surface, so called pattern recognition receptors (PRRs), which recognize and react to a broad array of pathogen-associated molecular patterns (PAMPs) (4). One of the most studied PRRs in innate immunity is the toll-like receptor (TLR) 4, an unspecific receptor that binds components of gram-negative bacteria called lipopolysaccharides<sup>2</sup> (LPS). The innate immune system is thus efficient towards a broad array of infections and has no memory. The activated immune cells ignite an inflammatory cascade for the protection against pathogens, which also triggers adaptive immunity - the part of our immune system that remembers prior encounters.

---

<sup>2</sup> Also known as endotoxin, a bacterial substance that elicits strong immune responses.

The adaptive immunity needs some time to reach full reactivity at the first encounter with a pathogen, but once initiated it is highly efficient. It comprises two main branches. A humoral (blood-born) part mainly combats bacteria, which are self-sustained living organisms in our tissue or our blood, and a cellular part protects against e.g. viruses, which reside inside cells needing cellular gene transcription mechanism to proliferate. The main cells of the humoral defense are B-cells and their antibodies, the immunoglobulins (Ig). Antibodies appear in different structures depending on where and how in the body they are to be used. IgG is the most common type of antibody in the circulation, while IgE is produced in allergies. The B-cells are stored as memory cells for years after having fought a pathogen, and the next time the individual encounters the same pathogens the immune response is so fast and efficient that the infection can go by undetected by the individual. Vaccines are based on this part of the immune system, as the vaccine tricks the adaptive immune system into thinking it has been infected by a disease and producing antibodies for protection that can last for years, in some cases throughout life. The cellular protection is handled by the T-cells, which comprise several subclasses. Cytotoxic T-cells ( $T_c$ -cells) kill infected and malfunctioning cells, carry receptors with a similar structure as antibodies and form memory cells. Helper T-cells ( $T_h$ ) and regulatory T-cells ( $T_{reg}$ ) (5) have regulatory function and  $T_h$ -cells activate both  $T_c$ -cells and B-cells, so the two branches of the adaptive immune system are tightly intertwined (3).

For especially difficult infections caused by parasites, the immune system has a particularly toxic branch. Here mast cells and eosinophils, immune cells carrying corrosive toxins and high amounts of histamines, play an important role. Severe parasitic infections are luckily uncommon in this part of the world, but the parasitic immune system remains. This is the part of the immune system that is wrongfully activated by innocuous substances during allergies.

### **2.1.2 Cytokines and inflammation**

Cytokines are the signaling molecules of the immune system and include chemokines<sup>3</sup>, interferons (IFN), interleukins (IL) and tumor necrosis factor (TNF). Different immune cells express characteristic patterns of cytokines specific for their function and role in the immune system. Other cell types than white blood cells that are involved in an immune response also express cytokines, like endothelial and epithelial cells. Cytokines can act on neighboring cells (paracrine functions), on the cells that expresses the cytokine itself (autocrine functions) or on cells further away by following the blood stream (endocrine functions). Cytokines work in cascades and have redundant (overlapping) functions. Particularly macrophages produce high amounts of cytokines in the first phase of an immune response.  $T_h$ -cells also produce a wide range of cytokines, the combination of which is often used to characterize them as Type 1 or Type 2 cells (6). A Type 1 cytokine response includes  $INF-\gamma$  and IL-2 and promotes cellular

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<sup>3</sup> Chemotactic cytokines.

protection. The Type 2 profile instead comprises IL-4, IL-5, IL-10 and IL-13, cytokines essential for antibody production and extracellular parasitic protection. For this reason, different inflammatory processes have specific cytokine profiles. Acute inflammation driven by sentinel cells is characterized by elevated levels of IL-1 $\beta$  and TNF, the first two cytokines to be induced by the innate immune system, followed by IFNs (during viral infections mainly), IL-6, IL-8 and IL-10 secretion. All these cytokines are mainly considered pro-inflammatory, except IL-10, which is an anti-inflammatory cytokine that down-regulates TNF and other pro-inflammatory cytokines and is thus part of the regulatory feedback loop of the immune system that keeps the immune response within boundaries. Allergic inflammation on the other hand has a Type 2 profile that drives mast cell and eosinophil recruitment.

Most commonly, the term inflammation is described in terms of disease. Inflammation is however essential for survival (7). Local inflammation is a local reaction to infection or tissue damage and is characterized by the five cardinal signs of inflammation: *dolor* (pain), *calor* (heat), *rubor* (redness), *tumor* (swelling) and *functio laesa* (disturbance of function). The inflammatory process confines the damage to avoid spreading to the blood and increases blood flow and extravasation, which is the migration of immune cells from the blood stream across the endothelial cell layer. The infiltrating immune cells at the site of inflammation ultimately kill the pathogens and eliminate damaged tissue. Macrophages and neutrophils are the first cells on site, and pro-inflammatory cytokines and other peptides such as substance P (causes pain) and histamines (swelling) help orchestrate the inflammatory process. Systemic inflammation is an inflammatory process that has spread into blood. When it is low-grade, it can be resolved through the feedback processes within the immune system described previously, but when it is too intense and control functions are inadequate, sepsis may follow. Systemic inflammation does not show the five classical signs of inflammation, neither does inflammation within the CNS (see chapter 2.2.2).

### **2.1.3 Immune system-to-brain communication**

The central nervous system is much more sensitive to inflammation than the peripheral body and is thus diligently protected from the circulation by the blood-brain-barrier (BBB). The BBB is comprised of endothelial cells that are firmly connected to one another by tight junctions. Tight junctions enable the cells to communicate, but stop cellular migration across the endothelium that occurs during a local inflammation. When the BBB malfunctions and becomes leaky, particularly difficult infections arise (8).

There are controlled ways around the BBB, so that the immune signal can reach the brain during sickness. Cytokines can diffuse or be transported across specialized areas without a tight BBB, called the circumventricular organs (CVOs). Furthermore, a systemic inflammation uses active transport mechanisms to signal across the BBB. Finally, the immune system signals to the brain via vagal and trigeminal nerves. The neural route is

particularly important for local inflammations, which never reach the blood. Nerves, glia and other cell types within the CNS have cytokine receptors which can bind diffusing cytokines across the CVOs (9). Furthermore, the inflammatory signal induces prostaglandin secretion from cells of the brain parenchyma, which in turn acts as sickness signals within the brain (9, 10). The peripheral inflammatory signal is hence reproduced within the brain (10, 11), where cytokines are produced de novo from microglia and astrocytes (12-14) but at much lower concentrations.

## **2.2 THE NERVOUS SYSTEM**

Compared to the immune system, the nervous system has a more hardwired structure with signaling happening in the immediate vicinity of the nerve cells. The nervous system comprises a central part that is the brain and the spinal cord, and a peripheral part that are the nerve fibers outside the brain and spinal cord. The efferent nerves send information out to the body and the afferents send information in the other direction, up into the brain. Some parts of the nervous system are controllable by the individual itself, like the nerve impulses to the muscles for voluntary movements. The sensory part of the nervous system is also readily and consciously experienced, like touch to the skin. Other parts are mostly not perceived consciously, like the signals from inner organs (viscera). These inner areas of the body are controlled by the "involuntary" nervous system - the autonomic nervous system. The autonomic nervous system makes sure that the core bodily functions are maintained during our daily life, without conscious cognitive effort: heart beat, breathing, bowel movements, etc. In short, the autonomic nervous system is an essential component for the maintenance of homeostasis.

The signaling molecules of the nervous system are neurotransmitters like GABA, acetylcholine, noradrenaline, serotonin and dopamine, and neuropeptides like substance P as well as endogenous opioids. The latter two are of great importance for pain perception, as are serotonin and noradrenaline (15). The neuropeptide circuits form systems of their own within the nervous system, but their functional specificities overlap. An in depth discussion of neural functions is beyond the scope of this thesis, but of importance for sickness-related actions is that both dopamine, serotonin and noradrenaline pathways appear to be sensitive to peripheral inflammatory signals (16).

### **2.2.1 Brain-to-immune system communication**

For sickness research, the division of immune system and nervous system presents a problem given their mutual dependence. In this field, the notion of a *neuroimmune* system makes more sense (17). Nerve cells interact with the immune system both in the periphery, in the spinal cord and in the brain. This is called neuroimmune communication, and when behavioral aspects are involved, the term *psychoneuroimmunology* applies. The autonomic nervous system innervates the main sites of the immune system; the liver, spleen, bone marrow,

thymus lymph nodes, skin and gastrointestinal system (18, 19). The “anti-inflammatory reflex”, an anti-inflammatory neuroimmune loop described by Tracy and co-workers (20, 21), illustrates these hardwired neuroimmune communication routes. Cytokines stimulate the afferent component in the vagus nerve reaching the brain stem. There, efferent vagal neurons induce acetylcholine production from T-cells in the spleen, which in turn inhibits pro-inflammatory cytokine production in macrophages, hence forming a negative feedback loop for inflammation. The brain also aids and controls immune function via fever induction (22), cortisol (see section 2.3), epinephrine and norepinephrine secretion, and through the action of immune cells and cytokines centrally (see section 2.2.2).

## **2.2.2 The immune system of the central nervous system**

Within the immunologically protected space of the CNS, microglia constitute about 10% of all cells (23) and astrocytes outnumber neurons (24). Under basal conditions, microglia engage in immune surveillance (23) and astrocytes regulate normal function of neurons and synapses (25). Astrocytes also form a border layer at the BBB (8). However, both cell types react readily to inflammatory signals and release inflammatory products within the CNS. Microglia are a down-scaled version of macrophages; whereas macrophages are highly reactive cells in the periphery with an aggressive and diverse inflammatory repertoire, microglia have a more uniform reaction pattern and expresses cytokines in much lower concentrations (26) due to the sensitivity of the brain to inflammatory processes. Microglia do however react to the slightest neuronal or inflammatory change within the CNS and have the ability to become primed. The priming can be induced by peripheral neural damage (27), peripheral inflammation (28) and central neuronal and inflammatory processes (26). Primed microglia react much stronger to a consecutive provocation and may maintain the inflammatory reactivity for a longer time (29, 30). These actions may cause *neuroinflammation*<sup>4</sup> that is initially a protective function, but dysregulated or chronic inflammation has been implicated in several chronic diseases (see chapters 2.5.1 and 4). Astrocytes are activated in a similar manner as microglia, but with a delay in time (31) and the two cell types co-operate in neuroinflammatory function.

## **2.3 THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS**

Inflammatory activation of the hypothalamus induces corticotropin-releasing hormone (CRH) secretion, which in turn induces adrenocorticotrophic hormone (ACTH) release from the anterior pituitary and ultimately leads to cortisol secretion from the adrenal cortex. This pathway is called the HPA axis and represents a part of the stress system, and is per definition part of the neuroendocrine system. Cortisol is essential for the regulation of the immune system, having both enhancing and mitigating effects depending on the timing of stress

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<sup>4</sup> Inflammation in the nervous system.

exposure in the inflammatory cascade, concentrations and duration (32). A detailed discussion on stress and endocrine functions is beyond the scope of this thesis, although both stress and hormones have profound effects on neuroimmune function (reviewed in e.g. (32-34)). A few short facts are however worthwhile mentioning in this section. Inflammation is in itself a stressor, but is also strongly modulated by other stressors (35). Particularly, chronic stress has been shown to have long-term negative effects on immune function, while acute stressors stimulate innate immunity (32, 35). Stress may also prime microglia in a similar fashion as inflammatory stimuli does (36), and interacts with inflammation to exacerbate chronic disease (37). Furthermore, sex hormones are always implicated when discussing sex differences in all physiological systems, including the HPA axis (38), the nervous system (39) and immune function (40, 41).

## 2.4 THE INTEROCEPTIVE SYSTEM

The traditional perspective of interoception has been limited to visceral processing, i.e. senses from deep within the body that are diffuse, often subconscious and integral to autonomic function and homeostatic regulation (42). These sensations are contrasted to the five classical senses of sight, sound, touch, smell and taste that animals and humans use to monitor their environment. A.D. Craig has been very influential in expanding the concept of interoception to include sensations and feelings like pain, temperature, itch, sensual touch, flushing, hunger and thirst (43). Craig defines interoception as *the sense of the physiological condition of the body*. The term interoception is not to be confused with introspection (reflection of one's own thoughts and feelings) or introception (a term that has an ambiguous meaning). The neural systems underlying an interoceptive system are believed to involve homeostatic control regions in the brainstem, the anterior cingulate cortex (ACC) and the insula. In this neural circuit, the (right) anterior insula has been deemed a neural hub for interoceptive awareness (44, 45). Craig contrasts the interoceptive system to cutaneous mechanoreception and proprioception. However, the importance of cutaneous sensations is complex as information about the thermal, chemical, metabolic and hormonal status of the skin, as well as light (sensual) touch, is believed to be part of this system (43). The importance of somatosensory afferent from the skin was also highlighted in a case study with a patient with ACC and insular lesions (46). The authors argue that the ACC and insula are in fact not critical for interoceptive awareness as measured by the heartbeat detection task – one of the few experimental tests available to measure interoceptive ability (47, 48). Instead, information about the heartbeat from the skin around the heart was processed in parallel to the proposed interoceptive ACC-insula network. In reference to the multifaceted senses of the inner body, one should mention the immune system and its ample ability to inform the brain about the inflammatory condition of the body. The immune system has been described as both a sensory organ (49) and a sixth sense (50), hence an inner sense that can be used for perceiving bacterial and viral pathogens which clearly cannot be seen, heard, smelled or tasted by the individual (4). This perspective, along with the vagal and trigeminal communication routes of neuroimmune afferent communication, couples the immune system



to the interoceptive system. The ability to perceive inner bodily states is proposed to be of importance for several disorders, for example chronic pain (51), anxiety (52), anorexia (53), depression (54) and autism (55). Practically however, interoceptive ability is difficult to measure. The most commonly used behavioral test is the heartbeat detection task, i.e. how well a person can count their own heartbeats (56) or match cardiac activity with external stimuli (44). From a sickness perspective, it is however questionable how the ability to feel ones heart is of importance during inflammatory provocation, and the development of alternative interoceptive behavioral measurements is needed.

## 2.5 THE PAIN SYSTEM

The definition of pain according the International Association for the Study of Pain (IASP) is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage.” This definition states that there are two layers to the experience of pain. One is the nociception, the actual physical sensation that occurs when pain fibers are stimulated (57). For nociception to become pain however, the “emotional experience” of nociception is also required. The second layer of pain is thus the subjective experience of the same. Crucial in this definition is also that actual tissue damage is not required. Pain can be perceived even when nothing appears to be wrong at all physically. Clinically, pain is a common symptom, which is costly for society and causes much suffering. From a basic sickness research perspective however, pain is not only the perception of a hurting body. Pain is an interoceptive signal, a strong behavioral motivator and has a core function in sickness behavior.

Pain is essential for survival. This becomes particularly obvious in individuals who cannot feel pain due to heritable diseases (58). These individuals suffer from multiple inner bleedings and fractures and they can even get seriously hurt while sleeping, as they do not move a limb when it becomes ischemic. Pain is thus the main warning signal of the body to signal tissue damage of some kind. The sensation of pain is for this reason also closely linked to emotion, learning and memory (59). The first sharp pain of a knife cut is relayed by A $\delta$ -fibers, which are myelinated and carry the ascending nociceptive information fast to the brain. The secondary, dull ache that occurs after a cut is transmitted by c-fibers, which are unmyelinated and the sensation thus needs more time to reach the brain. A $\delta$  fibers are mainly activated during cutaneous<sup>5</sup> pain, while deep muscular pain is a more c-fiber driven type of pain. A nociceptive signal reaches the first main relay station of pain in a single neuron, terminating in the dorsal horn of the spinal cord (60, 61). Here, the neurons connect to ascending projection neurons that transfer the nociceptive information to the brain, as well as to descending neurons that regulate the neuronal signaling. The pain signal is also divided anatomically to convey the many aspects of pain. Most ascending neurons cross to the

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<sup>5</sup> Cutaneous nerves innervate the skin, so this means superficial pain.

contralateral side and carry the nociceptive information along the spinothalamic tract, which terminates in the thalamus, the main relay area for nociceptive input to cortical and subcortical structures (62). There are however alternative neuronal passages of nociception in the spinal cord, which convey neural input to homeostatic control regions in the brainstem and to the hypothalamus (63). The pain system is thus physically designed to relay different types of information from the body and eventually integrate this information in the brain.

The most common areas activated in the brain during painful stimuli are the primary and secondary somatosensory cortices, the insula, the ACC, the prefrontal cortex (PFC) and the thalamus (64). A term that is sometimes used to describe this pattern of neuronal activation is the “pain matrix” (65). However, the concept of a “pain matrix” has been criticized as the pattern of brain activation varies with the surrounding circumstances (62) and can be activated by non-painful stimuli as well (66-68). An alternative way of looking at this cortical network is in terms of a *salience system*. A salience system would detect, process and react to salient<sup>6</sup> sensory events of all sensory channels used to convey such information (69). As such, the pain network described in literature is actually a basic network by which the brain detects any stimuli that can represent a potential threat for the integrity of the body. Similarly, the broader function of pain signaling has been elaborated by Craig (70), who describes pain as a *homeostatic emotion*. In this perspective, the autonomic and homeostatic neural projections are of particular importance, which occur simultaneously with the major sensory, cognitive and emotional processing that accompanies nociceptive stimulus. Pain hence serves as an interoceptive signal and motivational drive for behavioral adaptation.

Whenever pain is perceived, pain regulation ensues through descending control (62, 71). Descending pain regulation is an integrative part of the normal pain response, and while it was originally seen as an analgesic system, it has now been shown that descending control encompasses both inhibition and facilitation of noxious input in a dynamic relationship (72). Through this ongoing regulation the “pain input is prioritized relative to other competing behavioral needs and homeostatic demands”, as expressed by Heinricher et al. 2009 (72). The descending pain system involves serotonergic, noradrenergic and opioidergic inhibitory pathways (73) that can be more or less activated, both reflexively as soon as pain occurs and tonically so that innocuous sensations are not perceived as painful (72). In humans, the descending pain inhibitory circuit mainly involves the lateral PFC (IPFC), rostral ACC (rACC), periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) (62, 71). The main regulation occurs in the dorsal horn of the spinal cord, where the released peptides create an appropriate level of signal transduction in the ascending nerve cells. More tissue damage generally means more pain and after healing, the pain stops. As long as the system is healthy, that is.

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<sup>6</sup> Striking or exceptional sensory events that stand out from the background noise, so to speak.

### 2.5.1 Chronic pain

Chronic pain is an enigma. Even after surgically cutting tracts that convey pain, the pain may persist (74). When pain has become chronic, it appears to have a life of its own within the nervous system of the affected individual, and there is surprisingly little agreement between the physical changes and damage that ought to cause pain and the perceived pain intensity. Long-term pain affects about 20% of the adult population, particularly women and the elderly (75). The cost of chronic pain is estimated to €200 billion in Europe and over \$150 billion in the USA per year (62) and includes many types of long-term pain; localized pain such as back pain, widespread general pain such as fibromyalgia, inflammatory pain such as rheumatoid arthritis, pain from nerve damage (neuropathic pain) or with unknown origin (idiopathic pain). Chronic pain can also arise in diseases like cancer, HIV, dementia and diabetes.

Several mechanisms underlying chronic pain have been identified. Peripherally, the area around the nerve endings and the nerve endings themselves can have changed morphologically, resulting in augmented activation. This amplification of pain is called *peripheral sensitization* (76). Reichling and Levine (77) describe a process of peripheral priming, highlighting that repeated inflammatory provocation to nociceptors increase the risk of developing long-term pain. Sustained or prolonged nociceptive stimulation due to peripheral tissue or nerve damage may initiate and maintain central neuronal hyperexcitability (78, 79). This may be enhanced by reduced inhibition of nociceptive neurons on both the spinal and supraspinal level, resulting in a *central sensitization* (78, 80). Because central sensitization is an enhancement of neuronal functions within the CNS, the pain is no longer dependent on the intensity or even presence of a peripheral noxious stimulus (80). Common for several chronic pain disorders is also poor descending pain regulation (81), as well as elevated peripheral (82) and central cytokine levels (83). Furthermore, the nerve pathways in the brain appear to restructure when the brain is subjected to long-term pain and even a decrease in gray matter occurs, which has prompted the theory of chronic pain being a type of neurodegenerative disease (84). This also highlights the fact that chronic pain is a disease in itself (62, 85), changing the central nervous system structurally.

About 20-50% of patients with a certain disease (like diabetes) or lesion (e.g. an operation) will develop chronic pain (86), which means that at least half of the patients in fact do not. Likewise, localized long-term pain (e.g. lower back pain) spreads to other body parts only in about 10-25% of pain patients, resulting in chronic widespread pain (85). The reason for some individuals being spared while others suffer gravely appears to be a combination of genetics, age, sex, co-morbidity with other pain syndromes, and outside stressors like infections, stressful events and mood disorders (86). Studies show that common brain areas are activated in pain disorders and depression, which might suggest that once one disorder is in place, the subsequent changes in the CNS facilitate the development of the other (87). Also, a common denominator for chronic pain and depression has been suggested, namely inflammation (88).

The mentioned mechanisms will most likely have different weight in different pain syndromes and even differ amongst individuals with the same pain diagnosis. Any pain state could thus for any particular individual be due to a varied combination of peripheral and central pain enhancing mechanisms (89), which complicates pain treatment severely. In addition, a dysfunction of the autonomic nervous system and of the HPA axis has been implicated in chronic pain, systems which in turn also will exhibit individual differences in function and susceptibility to disruptions (89). Finally, a source of individual differences in the development and maintenance of chronic pain can be the overall stimuli processing sensitivity, as formulated by Phillips and Clauw (89): "All individuals (with and without pain) have different 'volume control' settings on their pain and sensory processing. As such, their position on this bell-shaped curve of pain or sensory sensitivity determines to a large part whether they will have pain or other sensory symptoms over the course of their lifetime and how severe these symptoms will be." Understanding individual differences in the underlying mechanisms leading to long-term pain is of utmost importance for better and individualized treatment strategies. On this note, I believe that determining the potential impact of a peripheral/central inflammation and interindividual sensitivity in (psycho)neuroimmune function is of essence for chronic pain treatment.

### 3 SICKNESS BEHAVIOR

Fighting pathogens is an energy consuming activity and a successful immunological protection is crucial for survival. Through evolution, animals and humans have therefore developed a behavioral repertoire to accompany and strengthen the chemical and cellular protection provided by the components of the immune system (9). The behavioral changes are elicited by cytokines during immune activation and are very similar in humans and animals. These behaviors are collectively called sickness behavior, or the generalized sickness response. The typical behavioral changes include fever, fatigue, nausea, malaise, anhedonia<sup>7</sup>, lethargy, depressed mood, increased anxiety, changed sleep patterns, anorexia, increased pain sensitivity, decreased sexual activity and decreased movement. The changes are not simply a side-effect of sleepiness or weakened muscles, but are a targeted set of behaviors that help recovery by conserving energy and promoting rest, thus reorganizing the priorities of the sick individual (9, 90). The sickness response is a *motivational state* (91-94) defined as a central state that reorganizes perception and action (9, 95). This concept postulates a flexible behavioral output that enables a selection of appropriate actions depending on the current situation. According to this theory, the motivational state of sickness would furthermore compete with other motivational states, like fear.

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<sup>7</sup> Decreased reactivity to reward.

The behavioral reactions to infection are the same for a wide range of different pathogens, such as bacteria, virus, fungal infections and parasites. This is achieved through the sentinel cells of the innate immune system carrying PRRs that perceive the sickness signal of PAMPs (see chapter 2.1.1). The common output from these cells are pro-inflammatory cytokines IL-1 $\beta$ , TNF and IL-6, cytokines that have the ability to signal to the brain via humoral (BBB and CVOs) and neural routes (vagus and trigeminal nerves) (see chapter 2.1.3) and induce a corresponding inflammatory pattern centrally. The cytokine profile of the brain mimics that of the periphery, and the level of infection is roughly proportionate to the level of cytokines produced in the CNS and to the induced behavioral changes (4). The behavioral changes are elicited through specific changes in neural activity, although the precise areas in the brain that deal with sickness behavior are still not defined (4). One may think of the overall sickness response, with its many subparts and diverse mechanisms, as a *sickness system* that is activated during illness. Normally, when the infection resolves, this presumed sickness system is turned off and physiology and behavior are restored. However, if the inflammatory input is extremely high or prolonged, or if the neuroinflammation in the CNS is not resolved, behavioral dysfunction may follow and eventually even cell death (4, 26). Some of the chronic diseases where a sickness response driven too far is implicated are chronic pain (chapter 2.5.1), depression (88) and fatigue (16).

### 3.1 ANIMAL STUDIES

Sickness behavior is provoked in research animals by injections of pro-inflammatory cytokines directly or by substances that elicit an innate immune response, such as LPS. Injections have been administered locally, systemically and centrally and elicit sickness behavior regardless of injection site. Of note is that aged mice show more severe sickness behavior after LPS provocation than younger mice (96). Physiologically, the sickness response is commonly quantified by a body temperature rise, loss of body mass and decreased water and food intake compared to control animals. Changes in sickness-induced motivation are for example assessed with social explorations tests, which are behaviors with a strong motivational drive in rodents, motivated by curiosity and sexual drive. These types of tests are also used to assess anxiety levels. Consumption of sweetened liquid or food also has a strong motivational drive for animals and is used as measures of anhedonia and anorexia. Mood changes and anhedonia, i.e. “depressive-like” behaviors, are often quantified by the forced swim test and tail suspension tests, where rodents are placed in an inescapable situation like in a bucket of water or suspended by the tail, and the increase in helpless behavior i.e. the lack of trying to escape is studied (4).

The humoral and neuronal pathways have additive effects. Blocking the neural pathway through vagotomy drastically dampens the sickness behavior elicited by systemic and locally injected LPS (97-100), but does not completely block sickness behavior or the increase of cytokines in the brain (101). This highlights the independence and significance of the humoral signaling across BBB and CVOs (102). What has also been shown is that sickness

behavior is cytokine dependent, whether transmitted by the blood or by nerves (103). The current literature suggests that IL-1 $\beta$  and TNF can induce sickness behavior alone, but that IL-6, IFN and prostaglandins cannot in the absence of IL-1 and TNF. IL-6, IFN and prostaglandins are however required for full-scale sickness behavior and may affect different parts of the sickness behavior specifically. For example, IFN enhances depressive symptoms in the presence of an inflammatory cytokine cascade, but cannot elicit depressive behavior in an isolated situation. IL-6 has a wide range of effects and complements IL-1 $\beta$  and TNF signaling (4).

Pro-inflammatory cytokines modulate memory and learning in rodents (104, 105), mainly impairing these functions. Nonetheless, some types of learning are directly related to immune functioning, such as conditioned taste aversion (106), where specific tastes have been linked to sickness inducing agents. Interestingly, immune reactions are also conditioned in such a set-up. Cytokines also make animals tired and sleepy (107). Anorexia is manifested as eating less if food is offered ad libitum (108, 109), and rather refraining from eating if the animals have to make a physical effort to gain the food (110). Furthermore, rodents tend to ingest a higher proportion of carbohydrates and less protein during sickness, while fat intake remains unaffected (94). Depression-like behaviors are induced, reflected as decreased social interaction, withdrawal and lethargy (111). Depression-like behaviors have been given ample attention as their underlying mechanisms may underlie inflammation driven depression in humans (112). For instance, Dantzer and colleagues (113) argue that depression-like behaviors may in part be driven by inflammatory changes in the tryptophan synthesis pathway, possibly affecting the serotonin levels in the brain. Finally, pain sensitivity increases in animal models of experimental sickness (25, 114, 115), making hyperalgesia<sup>8</sup> one of the core symptoms of sickness behavior. Hyperalgesia appears to be particularly dependent on glial activation, as the effect on pain is blocked with drugs that selectively block glial function (25), a mechanism that is believed to partake in the transition from acute to chronic pain (116). Interestingly, glia cells are more sensitive to deep tissue injury than skin lesions, when such models are used as noxious stimuli (117).

### 3.2 HUMAN STUDIES

One incentive to study the mechanisms of sickness behavior came from clinical observations of immunotherapy eliciting side effects that resemble sickness behavior, like depressive symptoms, fatigue and aches (121). In for example hepatitis C patients undergoing INF- $\alpha$  therapy, up to 45% of the patients develop depression (122). It is estimated that in a subgroup of clinically depressed patients, the symptoms include low-grade systemic inflammation (37), as in some chronic pain patients (see chapter 2.5.1). This knowledge may be crucial for the

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<sup>8</sup> Increased pain sensitivity.

treatment outcome in these patient groups, as inflammation driven syndromes may need a different medication profile than the generic medicines currently used.

There are some methodological concerns when comparing experimental sickness behavior in laboratory animals with human subjects. Firstly, the LPS doses used in rodents are strikingly much higher than in humans (1.000-10.000 times higher) (26). This is partly because the rodents can tolerate much higher doses of LPS, but also, the behavior recorded may in fact rather resemble symptoms of septic chock (10) when considering the strength of the immunological reaction in response to these doses. Teeling et al. (10) have demonstrated the behavioral difference between high and low dose LPS injections in rodents in an elegant study, and the behavior elicited by the low dose is in fact more subtle and more like those seen in human experimental subjects. For example, while the behavioral effects were not as pronounced in the low-dose groups they were undoubtedly present – but without the changes in appearance like hunched posture and piloerection, and the animals only developed fever acutely and transiently. In human low-dose LPS studies the effects can in fact be so subtle that blinding can be maintained. Furthermore, it may pose a health risk to inject humans with pro-inflammatory cytokines for pure experimental purposes, although some studies have used this model (118). However, human studies can benefit from vaccinations as an inflammatory model and patients undergoing immunotherapy can be studied. The second methodological concern regards the nature of the animals studied. Rodents are prey animals, and prey refrain from showing sickness, weakness or pain as far as possible as such display renders them targets for predators. Thirdly, a rodent brain is of course quite different from a human brain. Despite these obstacles, animal research has been translated to human experiments in virtually all aspects of sickness behavior, as the behavior is highly evolutionary conserved (119, 120).

Low-dose LPS injections as well as vaccinations decrease the subjectively rated mood and increase anxiety in human subjects (123-126). Memory functions worsen (125-129), but the findings are inconclusive and appear to depend on the LPS-dose used. Conditioned smell aversion has also been demonstrated in humans, using just one pairing of sickness inducing agents with a novel taste/smell (130). Sleepiness and sleep patters are also affected (131-134), although the effects depend on the dose used. Appetite is reduced (135) and fatigue and anhedonia increase parallel to decreased social interest (136, 137). Interestingly, the effects on fatigue are ameliorated by pre-treatment of serotonin reuptake inhibitors, but not by dopamine and noradrenaline reuptake inhibitors (28). As we have shown, experimentally sick individuals are also readily identified as sick by observers through odor (138) and movement cues (139). Moreover, several studies demonstrating LPS-induced pain sensitivity in humans have been published recently (124, 140-143). These data were however not published when the human studies in the thesis were planned and executed.

Several studies have attempted to elucidate the neural correlates of sickness behavior in the human brain. The main methodological limitation for this type of research is the fact that only

the lower LPS doses used in humans (those that do induce nausea or shivering) are compatible with a brain scanning protocol. Secondly, in the studies using task-dependent brain activity, the behavioral output of the tests often do not differ between the treatment group and the placebo group, which makes interpretations of observed brain activities more difficult. Most studies have used functional magnetic resonance imaging (fMRI) (see chapter 7.1.5) with cognitive (144-147) and emotional (148-150) paradigms, but none have employed pain paradigms at this point in time. Overall, the ACC and insula are implicated in sickness behavior (145, 147-150) and their activity appears to correlate with peripheral IL-6 levels. Also, inflammation induced psychomotor retardation measured as slower reaction time, is associated with substantia nigra activity (146). Furthermore, our group has shown that brain areas involved in pain regulation show decreased activity during LPS-provocation (**Paper II** in this dissertation).

## 4 CHRONIC INFLAMMATORY DISEASE AND THE BRAIN

Inflammatory components clearly have a profound effect on the brain molecularly, immunologically and behaviorally, potentially leading to chronic syndromes as described in the previous chapters. So what happens to the brain after months or years of inflammatory push, as for individuals with chronic inflammatory diseases? Most chronic diseases, like RA or allergies, do not form a straight inflammatory line but result in repeated bursts of inflammation even with successful medication. Furthermore, even localized inflammation, like allergic asthma spreads into the blood stream (151), or affects the brain via the vagus nerve (152). Also, even in symptom-free allergy patients a minimal persistent inflammation is seen in the mucosa (153). Thus, the brain will receive long-term and repeated inflammatory provocation that may result in brain effects that accumulate over a lifetime.

Peripheral chronic inflammation may become chronic within the CNS via neuroinflammation. Transient peripheral infections and inflammations or chronic exposure to low level (sub-clinical) inflammations (154) can both activate microglia directly (155) or prime the cells so that a recurrent inflammatory provocation becomes more severe (156). In rodents, a systemic inflammatory challenge leads to an exaggerated fever response and sickness behavior in the presence of primed microglia (29, 30). The underlying mechanisms appear to be an increased synthesis of pro-inflammatory cytokines and nitric oxide in the brain. At chronic activation of microglia, a function that is initially protective becomes detrimental and causes atrophy of nerve cells (157). The endpoint of excessive neuroinflammation is hence neurodegeneration (30). More than 35 million people live with dementia in the world and the number is expected to double every 20 years (158). Of particular interest in this perspective is Alzheimer Disease (AD), the most common type of dementia, as AD has the inflammatory profile of an innate immune reaction. It is known that mutations in the amyloid precursor protein (APP) is a risk factor for sporadic (spontaneous, not hereditary) AD, an enzyme involved in the development of amyloid plaques that signify



AD along with neurofibrillary tangles composed of hyperphosphorylated tau protein. Importantly, most remaining identified genetic risk factors do not have associations with CNS inflammation *per se*, but rather play a role in innate immunity and systemic inflammation (26).

Diabetes, atherosclerosis, periodontitis and age (26) are risk factors for AD. The immune system of humans turn towards a chronic low-grade pro-inflammatory state with age, which has been termed “inflammaging” (159). Studies have also established a connection between asthma and AD (160-162), a finding that formed the basis of **Paper III** in this thesis. Whereas the peripheral inflammation may lead to neuroinflammation, so do also the actual neuronal damages that accompany AD. Conversely, this neuroinflammatory state renders the brain more sensitive to subsequent infections and inflammations, which have been shown to exaggerate neurodegeneration (163, 164) – and even more so if inflammation occurs repeatedly (165). Perry and his research group have shown that systemic inflammation and acute infections are associated with increased cognitive decline (166) and exacerbation of sickness symptoms (167) in AD patients. Of importance here is the fact that a significant proportion of the aging population has more than one systemic disease (168), so systemic inflammation is of high clinical importance as a risk factor for e.g. AD.

## 5 SUBJECTIVE HEALTH PERCEPTION

Self-rated health (SRH) is the most commonly used health question in social sciences and has proved useful as an independent factor predicting future co-morbidity, mortality and health care usage (169-171). The question is asked in many ways, and somewhat surprisingly it does not seem to matter much how the question is posed (171, 172). The most commonly used version is phrased as “How would you rate your general health?” requiring a response on a five-point grading scale ranging from ‘very good’ to ‘very bad’ (SRH-5) (172). Although SRH has been extensively investigated, the actual mechanisms leading to individual health perceptions remain elusive (170, 173). Clearly, the mechanisms are complex and situation sensitive, but the last few years a connection with the inflammatory status of the own body has emerged as a probable biological mechanism underlying subjective health perception (170, 174-179). Several studies have shown a relationship between poor SRH and higher levels of systemic inflammatory markers, and our group and others have suggested sickness behavior and the interoceptive components of immunological communication as a theoretical basis for how a person forms an opinion about his or her general health status (170, 177, 179). This proposal is further substantiated by the fact that the three primary health symptoms that have been shown to drive SRH are pain, fatigue and low mood (172, 173) – core symptoms of sickness behavior. Cross-sectional and longitudinal studies can however only point to associations between inflammatory activity or sickness related symptoms and health appraisals and do not confirm causality. At present, experimental studies that aim at teasing out mechanisms that drive subjective health appraisals are lacking.

## 6 AIM OF THE THESIS

The aim of this thesis was to explore immune-to-brain communication using a human model for acute systemic inflammation and a murine model for chronic inflammation. The human model was used to investigate experimentally induced sickness behavior and its effect on pain perception and subjective general health. The murine model was used to investigate central inflammation resulting from an experimental model for chronic allergic inflammation.

The specific aims of each paper were:

- **Paper I (published):** To investigate modality and sex differences in LPS-induced pain sensitivity.
- **Paper II (manuscript):** To investigate pain-related brain activity during LPS-induced immune activation.
- **Paper III (published):** To investigate the effects of allergic inflammation on inflammatory and neurodegenerative markers in the brain.
- **Paper IV (manuscript):** To investigate if self-rated general health is affected by acute experimental inflammation, and if a potential effect is mediated by the inflammation itself or by sickness behavior.

## 7 METHODS

### 7.1 HUMAN STUDIES (PAPERS I, II, IV)

#### 7.1.1 Participants and study design

The first human study (**Paper I**) was performed in 2010 on eight healthy participants (1 woman), in a randomized and balanced double blind cross-over design. They were injected two times, once with an intravenous (i.v.) injection of 0.8 ng/kg body weight LPS (*Escherichia Coli*, Lot nr G3E0609, United States Pharmacopeia Rockville, MD) and once with saline injection, 28 days apart. The female participant was studied during her follicular phase, but was excluded from all pain analyses due to equipment failure during the first experimental day. This study was performed to validate the LPS effects, the cytokine profiles and time contingency of pain sensitivity (Figure 1B).

One year later, 52 healthy subjects were recruited (**Paper I, II and IV**). 31 (18 women) subjects were injected with 0.6 ng/kg LPS intravenously, and 21 (11 women) subjects were injected with saline in a double-blind randomized order. Appart for a slight change of timing of pain tests and blood samples, the second study also used a dose lowered to 0.6 ng/kg and a between-subject design to improve blinding and to facilitate brain scanning of subjects with magnetic resonance. Women were tested in the early follicular phase, except when employing contraceptives abrogating menses (7 subjects) and none had reached menopause. The follicular phase was chosen as prior research has shown the smallest sex differences in pain sensitivity during this hormonal phase (180) and the largest during the luteal phase. Furthermore, the follicular phase can be predicted fairly well from the first day of menstruation. The LPS group was designed to be slightly larger to account for a larger variation in this group as compared to placebo injected subjects as well as to provide better statistical power for correlation analyses performed in this group (Figure 1A).

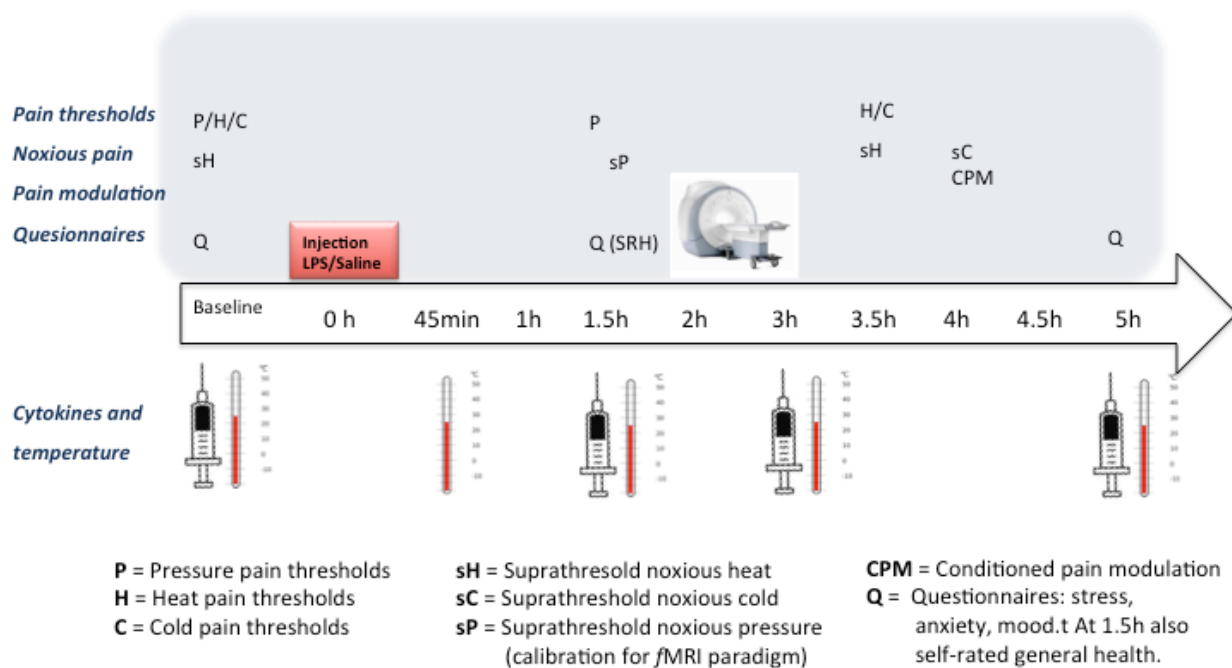
For inclusion, subjects had to be 18–50 years old, right-handed, medication free, non-smokers without a history of drug abuse, inflammatory-, psychiatric- or sleep disorders, or chronic pain, and with a normal body mass index. Particular rigid exclusion criteria were needed to ensure safety during scanning (**Paper II**), so in the second study furthermore no metal was allowed in the body, no prior operations to avoid the risk of inserted metal during a prior operation, and no claustrophobic tendencies. Participants were recruited by advertising and screened through questionnaires and a health examination by a physician. They were asked not to engage in strenuous physical activities, sleep regular hours and refrain from alcohol the day before the experiment. If the participants felt ill, e.g. coming down with a cold, they were instructed to call and were rescheduled for a later appointment. C-reactive protein (CRP) was assessed to exclude participants having an ongoing infection on the experimental day. Pregnancy was also an exclusion criteria and a pregnancy test was administered for all female participants on arrival

at the laboratory. As the test may be insensitive to very early pregnancies, we informed the female participants thoroughly about the risks LPS-injections could potentially pose very early in pregnancy and asked them to take particular care before participation.

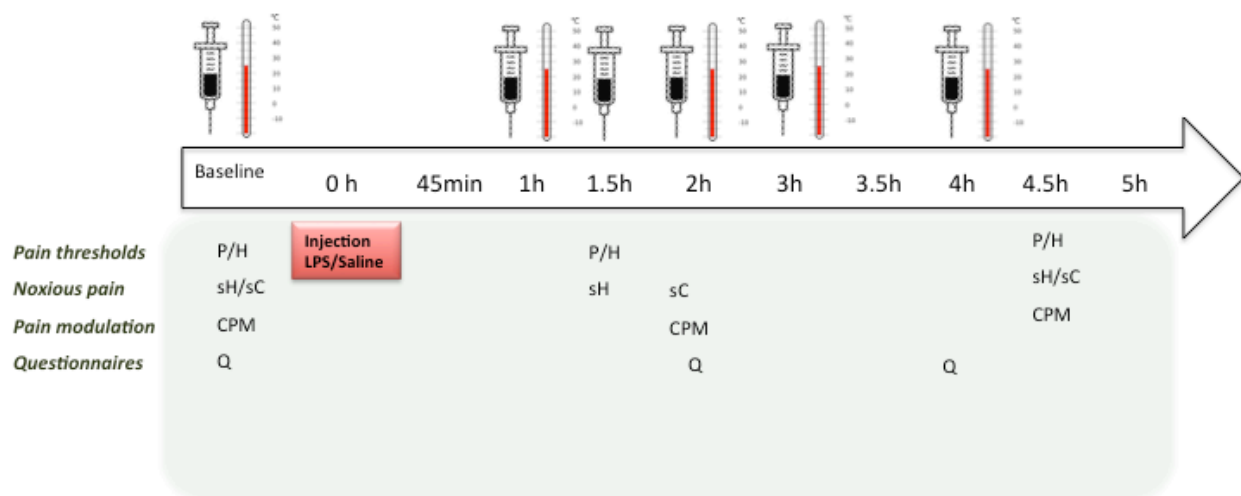
### **7.1.2 LPS-stimulation and inflammatory parameters**

An innate immune reaction, resulting in an acute systemic inflammation, was provoked by low doses of lipopolysaccharide (LPS) injections. This method has been used in humans since the 1960s using doses between 0.2-4ng/kg with LPS from e.g *Escherichia Coli*, and is very well documented (181-184). The typical immunological effect is an increase in pro-inflammatory cytokines such as IL-1, TNF, IL-6 and IL-8. Anti-inflammatory cytokine IL-10 and cortisol levels also increase and the reaction is subsequently quenched by endogenous control mechanisms within 4-6 hours. The acute symptoms are highly dose-dependent, but also depend on what kind of LPS is used and what batch. The stimulation has no negative long-term health effects.

In the present study, blood was drawn before and several times after injection. Blood samples were collected in 10 ml EDTA plasma tubes (BD Vacutainer) and cold centrifuged at 1500 g for 20 min. Plasma was frozen at -70 °C and later thawed for analysis with a Luminex assay Millipore's MILLIPLEX MAP high sensitivity human cytokine kit (Millipore Corporation, Billerica, MA, USA). TNFa, IL-6, IL-8 and IL-10 were measured according to the manufacturer's instructions.



**Figure 1:** A) Above are the timings of tests over the experimental day in the 0.6 ng/kg LPS dose cross-over study with 52 participants (men and women) depicted (**Paper I, II and IV**). B) Below are the timings in the smaller study with only men and 0.8 ng/kg LPS-stimulations (**Paper I**). For further details see **Paper I**.



### 7.1.3 Questionnaires

#### *Screening*

The screening questionnaires included the Swedish versions of the Hospital Anxiety and Depression Scale (HADS) (185) and the trait-part of the State-Trait Anxiety Inventory (STAI) (186). We also asked for the common sleeping pattern with selected items from the Karolinska Sleep Questionnaire (KSQ) (187) and stress level with the Perceived Stress Scale (PSS-10) (188).

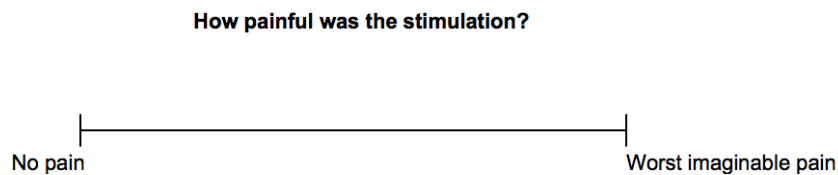
#### *Experimental day*

Upon arrival, the participants rated the state-part of the STAI, their subjective stress levels (Visual Analogue Scale (VAS) 1–100mm) (189), and completed the Positive and Negative Affect Schedule (PANAS-X) (190) and self-rated general health with SRH-5 (172), using the wording “How would you rate your general state of health?”. Ratings were then repeated throughout the day. An extended version of the newly developed questionnaire SicknessQ (Andreasson et al, submitted) for perceived sickness behavior was also used. The scale has shown a good internal consistency and criteria validity (Figure 2).

	Disagree	Agree somewhat	Mostly agree	Agree
1. I want to keep still	1	2	3	4
2. My body feels sore	1	2	3	4
3. I wish to be alone	1	2	3	4
4. I don't wish to do anything at all	1	2	3	4
5. I feel depressed	1	2	3	4
6. I feel drained	1	2	3	4
7. I feel nauseous	1	2	3	4
8. I feel shaky	1	2	3	4
9. I feel tired	1	2	3	4
10. I have a headache	1	2	3	4

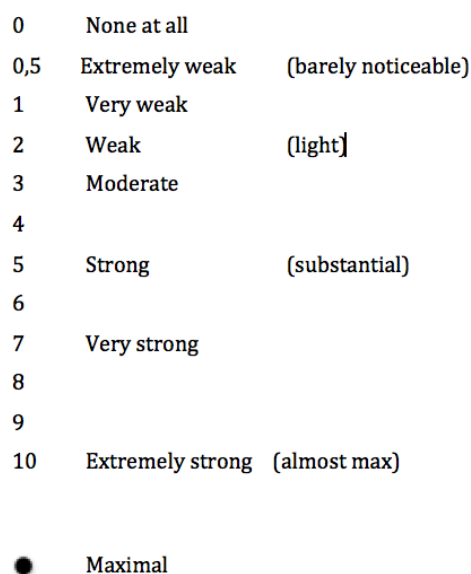
**Figure 2.** The final items measuring subjective sickness behavior (SicknessQ) used in the analysis of **Paper IV**.

For the pain ratings, two scales were used. Firstly, the common VAS scale (189), similar to the stress question, but phrased for pain testing (Figure 3).



**Figure 3.** The Visual Analogue Scale (VAS) for pain ratings.

The second pain rating scale was the Borg Scale<sup>®</sup> (191), which uses verbal descriptions of pain as reference for the number the participant chooses. The scale is non-linear and has no defined end point. The instructions are crucial for this scale and are worded as such that the participant should use the words as descriptors of their pain sensation, rather than the accompanying number. It is explained that 10 is an “extremely strong painful sensation, almost max” and it is suggested it may correspond to “e.g. the strongest pain you have previously experienced yourself”. As it is possible that the pain experienced in the experiment is stronger than anything previously experienced, the absolute maximum value is positioned beyond the end of the scale and the scale after 10 is open. From piloting with this scale we know that without the proper instructions, participants may rate their pain as more than 10 in some instances, whereas none of our participants chose a number higher than 10 after the proper instructions. The Borg scale is thus somewhat more defined than the VAS scale, where “worst imaginable pain” is left to be freely interpreted. Both scales (as all other pain scales) have advantages and disadvantages, but generally appear to work quite well in pain research.



**Figure 4.** The Borg Scale<sup>®</sup> for pain ratings.

In the scanner, the subjects did not have to rate the pain, but after scanning we asked for the overall pain and the most painful stimulation on a scale from 1-10, to assure that the calibration was successful and the pain was rated about 5.

#### **7.1.4 Pain sensitivity measures**

Experimental pain research is often contradictory, partly because people differ greatly in pain sensitivity so the results of the experiment naturally varies in different samples. But also, the results are highly dependent on both the pain modality used and the pain intensity applied. Particularly, deep and cutaneous noxious stimuli have slightly differing ascending tracts and are differently affected by descending pain modulation (192, 193). We therefore chose to test threshold and suprathreshold intensities, as well as deep and cutaneous stimulation in our human studies.

Stress may affect pain perception (194), so the baseline measurements were performed after an hour of rest and filling out questionnaires and before blood sampling or injection. Overall, stress levels remained low throughout the day, despite this heavy experimental setup. It was of great importance for us to create a calm and stable environment during our experiments, having both experienced nurses and doctors on site, so that the sickness effects would not be overridden by stress or fear. We appear to have succeeded, also judging from the terminative short interview with the subjects before leaving the laboratories.

##### *Pressure pain thresholds*

Pressure algometry (Algometer<sup>®</sup>, Somedic Sales, Sweden) was used to assess pressure pain thresholds (PPT) on different sites of the body, as a measure of deep pain sensitivity. The technique is used clinically to diagnose fibromyalgia and both muscles, tendons and nerves can be used to quantify pressure pain sensitivity (195, 196). To facilitate the experimental procedure, only 3 or 4 points were used, as this has been shown to suffice for an individual pressure sensitivity assessment (197). The points chosen on the body were slightly varied between study 1 and study 2 (**Paper I**), which strengthens the notion of a general effect on pressure sensitivity that is not dependent on the specific location of the pressure point used. The algometer is a hand held pistol-like apparatus with a 1 cm<sup>2</sup> probe, that is held at a 90° angle and pushed against the skin with muscle force at a steady pace (195). No sensitive body points were singled out as has been done in previous studies (124), but the average averaged over the three/four respective pressure points was calculated as the individuals' overall PPT (196, 198) and was used in statistical analysis.

##### *Heat and cold pain thresholds*

This pain test uses cutaneous pain stimulations in contrast to deep muscle stimulation of the PPT. Heat pain was induced on the inside forearm using a 3 x 3 cm computer controlled Peltier-type heat probe (Pathway model ATS, Medoc, Israel). The probe works like a tiny



flat-iron, but the temperature change is instantaneous and well-controlled. For threshold pain sensitivity, the temperature of the surface of the thermode rises continuously from 32 °C at a constant rate and participants press a stop-button when their individual pain threshold is reached. The stimulated skin area was alternated to avoid sensitization.

#### *Suprathreshold pressure pain*

Suprathreshold pressure pain was used for the fMRI pain test (**Paper II**), which provokes deep pain sensation like the PPT. Stimulations were applied to the thumbnail using an automated, pneumatic, computer-controlled stimulator with a plastic piston that applies a pressure via a 1 cm<sup>2</sup> hard rubber probe. The thumb was positioned so that the force was applied to the middle of the nail bed, each pressure lasting for 2.5 seconds with a minimum 30 second intervals between pressures.

#### *Suprathreshold heat pain*

Five-second heat stimulations of 45, 46, 47, 48 and 49 °C were applied in a randomized order to the inside of the lower arm using a heat 3 x 3 cm heat probe. Pain was rated directly after each heat stimulation using a 100 mm VAS (Figure 3) with the 0.6 ng/kg dose (both men and women). The individual VAS ratings were used for statistical analysis. At the 0.8 ng/kg LPS dose (men only), the temperature rose continually, until the subject rated the pain as “very painful” (7 on the Borg CR Scale<sup>®</sup>, Figure 4) (191, 193). The individual temperature perceived as “very painful” was used for analysis.

#### *Suprathreshold cold pain*

Suprathreshold cold pain sensitivity was assessed by the cold pressor test. A pre-defined amount of cold water and ice were mixed in a water bath prior to testing and the temperature of 2 °C was ensured by an attached thermometer. The participants submerged their right forearm and elbow into the ice water and pain was rated on the Borg CR Scale<sup>®</sup> at specified time intervals. The subjects were instructed to endure as long as they could, but were free to interrupt the test if they found it intolerable. As this test strongly activates the descending inhibitory pain pathways, it was only performed once at the end of the day (at 4 h) so that the other pain tests were not compromised in the 0.6 ng/kg LPS study. At the 0.8 ng/kg LPS dose, baseline pain measurements were performed several hours before the LPS/placebo injection, so we were confident that the effect would have worn off until further pain ratings. Here, assessments were thus made both at baseline and at 2 h.



#### *Conditioned pain modulation*

The CPM, previously also referred to as diffuse noxious inhibitory control (DNIC) (Yarnitsky, 2010), reflects endogenous pain inhibition. In short, a strong noxious stimulation

(in this case the cold pressor test described above) is used to trigger descending pain inhibitory circuits, and the strength of inhibition is tested using a secondary pain test (in this case PPT on the contralateral trapezius muscle). The suprathreshold cold pain test and conditioned pain modulatory test were thus integrated. PPTs were assessed before submersion, after 15 s and then at intervals between the Borg ratings. This may sound complicated for the subject, but with proper instructions and prior familiarization with the PPT test and Borg scale, this test works quite well.

### 7.1.5 Brain imaging

In fMRI, changes in the so called blood oxygen level dependent (BOLD) signal is used to obtain information about neural activity in the brain, using the increase of blood flow in a given brain region is an indirect measure of increased neural activity. The BOLD effect relies on the difference in magnetic properties of oxygenated and deoxygenated hemoglobin in the blood. Since the oxygen consumption of an active brain region is coupled with an increase of fully oxygenated blood, active regions will generate a relative increase of BOLD signal. The subjects were placed in the scanner with the thumb pressure device attached to their right thumb. No visual or auditory presentation was given during the experiment and subjects were instructed to focus on the pressures on the thumb. Two different types of stimulations were used during scans: individually calibrated painful pressure representing each subject's 50 mm VAS, and a non painful pressure perceived as light touch, representing 0 mm VAS. All stimulations were randomly jittered over the scanning time, preventing subjects from anticipating the onset time and event type. Two semi-randomized sequences of jitter were created and each subject received both sequences in a randomized order. The total duration of the pain scans was 16 minutes (199). The participants were subjected to two more fMRI test after the pain test, which are not included in this thesis.

The fMRI images always include noise such as head movement and image artifacts and therefore the images need to be preprocessed before the statistical analyses. The usual steps include motion correction (called realignment), slice-time correction (one brain volume is captured within 3 seconds, so each brain-slice has slight but systematic different timings), spatial normalizing (as to normalize for brain anatomical variability) and spatial smoothing (increasing the signal to noise ratio of data). The details of the scanner settings and statistical analysis can be found in the method section of **Paper II**. The actual clusters that eventually appear after statistical analysis are the difference in BOLD signal between the respective two groups we are contrasting. Increased neural activity can be due to the neurons sending more output to other brain areas or receiving more input, or it is due to increased "internal" processing within that area.

### 7.1.6 Statistical methods

The details of the statistical measures are found in each respective paper, so this section will describe some of the statistical considerations made choosing the respective statistical analysis:

All repeated measures in **Paper I** were analyzed using a linear mixed-model (LMM) repeated-measures regression analysis. There are several advantages of linear mixed-model analysis compared to e.g. repeated-measures ANOVA. Most importantly, ANOVAs assume balanced data, which are rare in real life longitudinal data. The group sizes may differ, as in our design, there will be some missing data, and the time points of measurements are perhaps not evenly distributed, as in our studies. Also, repeated measures may violate the assumption of independence between the different measurements over time, which is a problem in generalized linear models but is accounted for in LMMs. Most importantly, the LMM gives a measure for both intra- and inter-individual differences and accounts for individual differences in baseline value and regression slope over time. Also, if the changes in the data of interest are not linear over time, a better estimation of the regression slope can be made and thus improving the model fit to the real data at hand significantly. LMM is overall a more sensitive tool for analyzing repeated measures and makes more realistic assumptions of the data at hand, which results in better statistical estimations (200, 201) (**Paper I**).

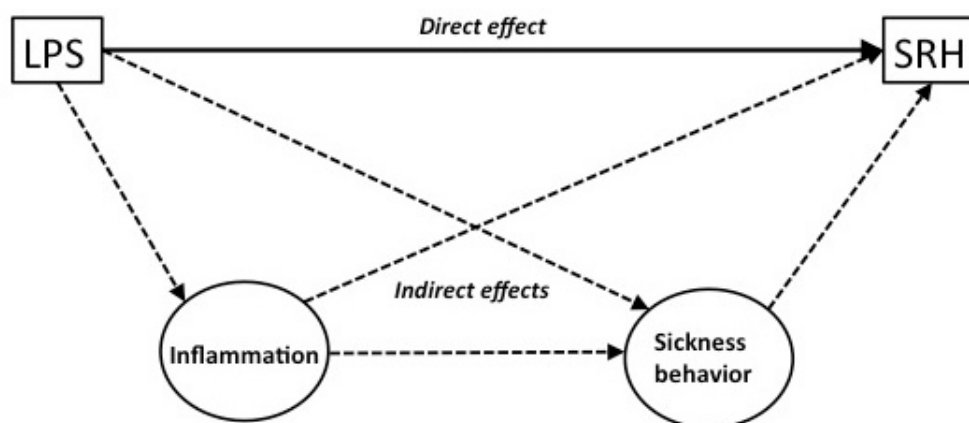
For the correlation analyses in **Paper I and II**, Spearman correlation was used due to non-normal distribution of cytokines when injected with LPS. In all, there is a lack of clear guidelines of how to perform correlations and the literature includes a wide range of different methods performing such analyses, e.g. using the percentage change (124), absolute changes or without transforming the data first. This makes direct comparison between studies difficult. Therefore, we decided on a fairly strict approach. The cytokines analyzed were chosen specifically based on prior sickness research at the time of analysis (TNF, IL-6), and IL-8 was chosen as this cytokine has emerged as important in chronic pain (202, 203). For the analysis, the following decisions were made in advance:

- a) For **Paper I**, we decided to use all PPT points as one global measurement as has been defined in previous literature, although the effects were stronger in some points than in others.
- b) To avoid the problem of normality and outliers, which varied for different measurements, we chose Spearman correlation, which was also suitable as we performed correlations with ordinal data as well (anxiety, stress and mood).
- c) In **Paper I** we decided to use after-injection measurements, not delta measurements, so that data could somewhat compare to clinical data, where baseline values are not available. In **Paper II** the delta values were used to be comparable to previous similar research.

As far as we know, it is not clear if the change in cytokine expression is a determinant of the sickness response, or the actual concentration in the blood/CNS during sickness.

Furthermore, as cytokines are expressed in cascades, it is impossible to separate their specific effects on the brain in this experimental model.

In **Paper IV** we used a path model analysis. Our hypothesis for this study was, for reasons discussed earlier, that LPS stimulations will worsen general subjective health and that the effect will depend on sickness behavior. We hence chose a statistical model to test this specific hypothesis using a formal mediation analysis with serial mediators. Mediation analysis allows inference of more than a merely descriptive relationship between variables, but also gives a functional understanding of the relationship between the measured variables (204, 205). Other components than sickness behavior that could mediate a potential effect of LPS on SRH were the induces inflammation directly, or none of the components that we measure in this study. These possibilities were thus included in the statistical design as depicted in Figure 5. The analysis describes the direct relationship between LPS and SRH (the relationship between the two variables independent of mediators), the indirect relationship (relationship dependent on mediators) and the total effect (direct + indirect effects).



**Figure 5:** The path model used for mediation analysis in **Paper IV**.

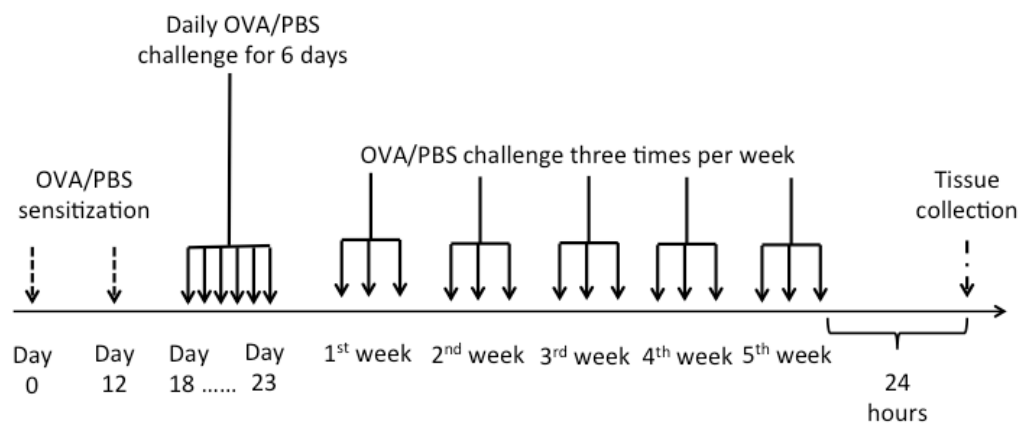
For the fMRI analysis of **Paper II**, the areas included in our hypothesis were based on previous studies on interoceptive ability and affective pain processing and the pain research conducted in fibromyalgia patients by parts of our group, Dr. K. Jensen, Prof. E. Kosek and Prof. M. Ingvar. This study is thus translational in its design and attempts to bridge experimental sickness-induced pain research with clinical research. We did a whole brain analysis as described in the **Paper II** and ROI analysis on the relevant clusters that appeared in this analysis. ROI coordinates were chosen from unrelated research to maintain analytical stringency, as the generic areas in brain atlases were too large for our purposes. Of note is that the areas of interest were the main clusters in the analysis.

## 7.2 MURINE STUDY (PAPER III)

### 7.2.1 Murine inflammatory model

This study was a cooperation between a research group studying AD and our research group studying psychoneuroimmune mechanisms. Allergic inflammation was the chosen chronic model partly to explore the longitudinal findings of Erikson et al. that allergic inflammation is associated with increased prevalence of AD and also a shortened lifespan after AD was diagnosed (160). In addition, allergic inflammation poses a good model disease for chronic inflammation from a translational perspective. The inflammation is mainly treated locally in clinical populations, which may pose an advantage since systemic anti-inflammatory treatment that is often used for autoimmune diseases could be a confounder in related human research. AD is the most common neurodegenerative disease and has an interesting inflammatory profile of innate immunity, thus also serving as a suitable model disease for neurodegeneration from a psychoneuroimmunological perspective.

Mice are commonly used in allergy research, but usually an acute allergic reaction is modeled. The most common substance used to induce allergic inflammation is ovalbumin (OVA<sup>9</sup>) with intraperitoneal (i.p.) injection<sup>10</sup> and/or inhaled. In our protocol, the animals were initially challenged with OVA that causes hypersensitivity. To mimic chronicity, the allergic inflammation was maintained with repeated challenges during an extended time period (Figure 6).



**Figure 6.** The experimental protocol for the murine model of chronic allergic inflammation.

<sup>9</sup> Ovalbumin is the main protein found in egg white and is highly allergenic.

<sup>10</sup> Injections in the abdominal cavity.

Briefly, mice were sensitized by receiving an i.p. injection of OVA suspension on day 0 and 12. The animals were challenged daily from day 18 to day 23, and then 3 times per week for another 5-week period by intranasal instillation<sup>11</sup> of OVA (Fig. 1). Intranasal instillation was performed under light anesthesia. Control animals underwent the same treatment but received PBS<sup>12</sup> instead of OVA. The animals were sacrificed 24 h after the last antigen challenge and brain tissue was collected for subsequent analysis of inflammatory and degenerative markers. Methodological details can be found in the method section of **Paper III**.

### 7.2.2 Animals and data collection

Balb/c mice are prone to develop allergic reactions with high numbers of eosinophils and high IgE expression, and it is the most widely used strain in experimental allergy models (206). Moreover, the C57B6 mouse strain was chosen because it is frequently used as background strain for transgenic mouse models of AD. We thus aimed to validate the chronic allergic model on both strains, as the team interested in AD was planning to subsequently conduct similar experiments on transgenic mice (207).

Male mice were housed under controlled conditions of light-dark cycle, temperature, relative humidity (60–65%) and food and water ad libitum. Upon arrival, the animals were habituated to the environment for two weeks before the start of experiments and handled daily to minimize the stress during subsequent experimental handling. Male mice were used in all studies, to avoid hormonal variation due to ovulation. However, variation is unavoidable because male mice, by nature, will establish hierarchy and the less dominant mice will be more stressed (207).

The brains were quickly dissected and either frozen or pre-treated by perfusion<sup>13</sup> for later analysis. Allergic inflammation was confirmed by analysing the cell content of the bronchoalveolar lavage (BAL)<sup>14</sup>. Next, the lungs were hence dissected and carefully flushed, thus recovering the cells present in the lungs. The number and type of immune cells were counted under microscopy based on their morphology after a standardized staining protocol. For details please see **Paper III**. We were interested in whether inflammatory markers increased in the allergic mouse brain and if AD-related changes increased. We thus used different techniques of quantifying and localizing increased immunoglobulin expression (IgG for general inflammation, IgE for allergic inflammation), glia activation, cytokine expression and increases in tau-phosphorylation and APP expression (the hallmarks for AD). With Western blot we could detect specific proteins in a sample of tissue homogenate, which was

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<sup>11</sup> The animals inhaled OVA solution through the nose, to cause allergic inflammation in the lungs.

<sup>12</sup> PBS: Phosphate buffered saline, a balanced salt solution for laboratory use.

<sup>13</sup> Washing out the blood and running fixative through the vascular system.

<sup>14</sup> In simple terms this means a lung wash.

prepared from the mouse brain by sonication<sup>15</sup>. The different proteins in the sample are separated with regard to their molecular mass using gel electrophoresis, transferred to a membrane and visualized as bands with the aid of target-specific antibodies. Using immunohistochemistry, the localisation of the proteins of interest in the brain can be determined. Slices of the brain are stained with target-specific antibodies, in our case for antibodies binding to IgG, IgE, a marker for IgG-secreting plasma cells (CD138) and markers for glia activation (GFAP for astrocytes and F4/80 and Iba1 for microglia). Of note is that the glia markers bind to activated cells, but not to primed cells or to cells that have an altered function but no change in morphology. The levels of pro- and anti-inflammatory cytokines in mouse brain homogenates were analysed with multiplex assays developed by Meso Scale Discovery (MSD) technology.

## 8 SUMMARY OF STUDY I-IV

### 8.1 PAPER I

#### *Background and objectives*

Inflammatory processes both peripherally and centrally have been implicated in chronic pain, as well as impaired descending pain inhibition. When the study was designed, animal studies had shown that pain sensitivity increases as part of a sickness response. In the last couple of years several studies have shown that previous animal findings of increased pain sensitivity during systemic inflammation hold true for humans as well. The aim of our study was to explore how pain sensitivity was effected in humans during experimental immune activation.

#### *Methods*

We conducted two consecutive studies with LPS injections to provoke an innate immune response. Eight healthy participants (1 woman, who was excluded from analysis due to experimental failure) were recruited to a randomized and balanced double blind within-subject design. They were injected twice, once with 0.8 ng/kg body weight LPS and once with saline injection, 28 days apart. One year later, 52 healthy subjects were recruited (29 women). 31 (18 women) subjects were injected with 0.6 ng/kg LPS and 21 (11 women) subjects were injected with saline in a double blind randomized order in a between-subject design. As experimental results in pain studies often depend on the pain modality used, we included modalities measuring both deep and cutaneous pain at threshold and suprathreshold noxious levels. Heat- and cold (cutaneous) pain sensitivity was assessed for threshold stimuli and intense noxious stimuli, as well as pressure (deep) pain thresholds and CPM (descending pain inhibition). The study design, pain tests, timing and LPS dose were slightly varied in the two studies.

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<sup>15</sup> Ultrasonic frequencies are used to essentially mash the tissue.

### *Results and conclusions*

We could show that primarily deep pain is affected by experimental sickness in healthy men and women. Furthermore, this is the first study with both men and women in this pain model. In congruence with clinical findings, women became more pain sensitive during immune activation than men, as cutaneous pain perception was also affected for women, but not for men. The results also imply that descending pain inhibition may be of importance for this sex difference, as the inflammatory provocation attenuated CPM in women, whereas male CPM remained unchanged in the LPS group. Pain sensitivity correlated with circulating pro-inflammatory cytokines IL-6 and IL-8, and particularly strongly with IL-8, which is of interest as IL-8 has previously been identified as a central component in fibromyalgia patients by Prof. E. Kosek and her group. These results fit well with the current knowledge of inflammatory mechanisms driving long-term pain, and we argue that low-grade inflammation may be one of the mechanisms involved in the development of chronic pain.

## **8.2 PAPER II**

### *Background and objectives*

In this study we attempted to identify neural correlates for the behavioral findings in **Paper I**. Previous research by our group has shown decreased rACC activity in fibromyalgia patients as a neural correlate for impaired pain inhibition, and the involvement of the lateral prefrontal cortex in descending pain inhibition in placebo studies. Imaging studies using experimental inflammatory designs have also implicated ACC and insula as important areas affected by peripheral inflammation.

### *Methods*

52 healthy subjects were recruited (29 women). 31 (18 women) subjects were injected with 0.6 ng/kg LPS and 21 (11 women) subjects were injected with saline in a double blind randomized order, i.e. the same sample as the second study in **Paper I**. 51 subjects were scanned and 48 were used in the analysis. A deep pain model with individually calibrated pain levels previously developed for a clinical sample was used (208). We hypothesized that the anterior insula would be involved in sickness-driven pain as interoceptive and/or affective pain processing should increase during a sickness response. We also hypothesized that areas involved in pain inhibition should be affected, which would thus result in increased pain sensitivity during experimental inflammation.

### *Results and conclusions*

Deep pain sensitivity in the LPS group increased, for both men and women, as was shown in the previous study. This behavioral outcome was paralleled by decreased endogenous pain inhibition as reflected by weaker rACC and ventrolateral PFC (vlPFC) activity in the LPS group. This finding corroborates previous findings demonstrated in fibromyalgia patients. Furthermore, increased anterior insula activation was found in the LPS group, which we



interpret as increased interoceptive and/or affective pain processing during experimental sickness. The LPS provocation appeared to affect men and women equally, but a functional sex difference was found that may explain the behavioral sex differences. The men had a significantly stronger pain regulation to start with. As the inflammatory provocation affects this system, a stronger initial activity in rACC ought to render men more resilient to the pain-inducing effects of sickness. This is also an explanation that fits very well with current clinical findings of both sex differences in chronic pain and individual differences in the development of long-term pain. The sex differences were further substantiated by the fact that insular activity correlated with pro-inflammatory circulating cytokines for women, but not for men. Together, **Paper I and II** signify the importance of the acute LPS-model for studying mechanisms underlying chronic pain (209).

### 8.3 PAPER III

#### *Background and objectives*

Chronic inflammatory disease has been shown to increase the risk of developing dementia, and as this study was planned one epidemiological longitudinal study showed a connection between asthma and the occurrence of AD later in life, as well as a shortened life span when AD had developed. These findings have been corroborated by two later studies. The aim of this study was to investigate the connection between chronic allergic inflammation and AD in a mouse model.

#### *Method*

Male 12–14 weeks old Balb/c (n = 30) and C57B6 (n = 15) mice were subjected to an experimental protocol for chronic allergic inflammation raging over two months, with recurrent provocations with OVA or placebo. Brain and lung tissue was dissected and analyzed for AD markers, cytokine expression, antibody occurrence and inflammatory cells levels. Cytokine levels in the brain were analyzed with a multiplex cytokine assay, and cell infiltrates in BAL were counted in a Bürker chamber and morphologically defined with May-Grünwald/Giemsa staining. Antibodies, APP, tau-protein and tau-phosphatases were analyzed with immunoblotting and immunohistochemistry.

#### *Results and conclusions*

The allergic inflammation increased tau-phosphorylation in the mouse brain as well as IgG and IgE levels. APP did not increase. No microglia activation was seen, but microglial priming or a switch in the inflammatory profile to a more pro-inflammatory mode cannot be excluded due to methodological constraints. The BBB appeared to be intact (unpublished data) and no secretory B-cells were present in the brain, so the antibodies must have been actively transported into the brain or produced centrally. The mode of entry to the CNS is currently unknown to us. However, diffusion across the choroid plexus or CVOs is unlikely, as the distribution was even throughout the brain. These findings have been replicated by Sarlus et al. in subsequent studies (207) and the same pattern has been found after LPS-

stimulation in a model for prion disease (210), which suggests that IgG increases are not unique to allergy. Both tau-phosphorylation, which is a hallmark of AD, and antibody increases may be signs of protective functions initially, but IgG binds to both microglia and nerve cells and excess hyperphosphorylated tau protein disrupts neural function. The long-term consequences of these changes may thus be those of increased neuroinflammation. The fact that IgE was present is of particular interest, as these antibodies are normally not present in the organisms unless allergies or protozoa infections are present, so this finding strengthens the notion of the central nervous system forming a kind of mirror image of the peripheral inflammation as part of neuroimmune signaling.

## 8.4 PAPER IV

### *Background and objectives*

The general question “How do you rate your health?” encompasses many facets of life and health that have not been entirely defined. Despite the ambiguity of the question and the subjective interpretation of it, this simple measure has been very useful in social and health sciences, as it is an independent predictor of future morbidity and mortality. Previous research by our group established a connection of subjective health ratings to the inflammatory status of the body, and it has been proposed that the immune system serves as an interoceptive cue for a person when rating his/her subjective well-being. To test this hypothesis, we investigated whether SRH would be rated as worse in an acute experimental sickness model. This is the first study to our knowledge that attempted to manipulate general SRH experimentally using an inflammatory agent.

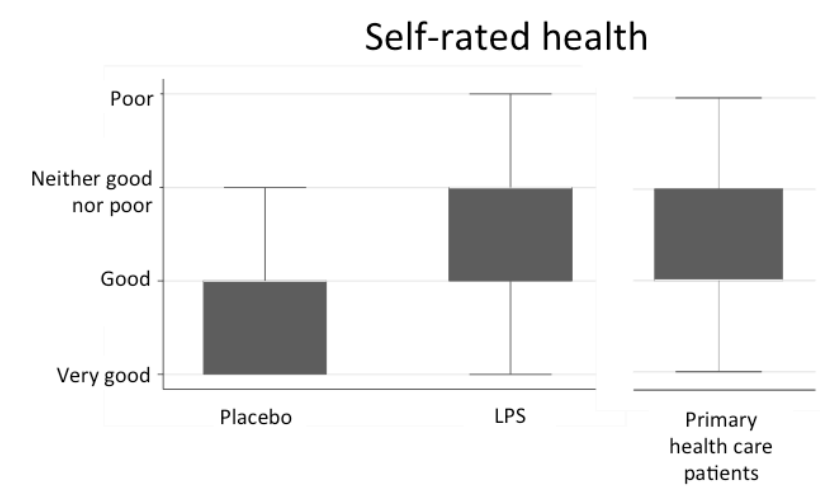
### *Method*

52 healthy subjects were recruited (29 women). 31 (18 women) subjects were injected with 0.6 ng/kg LPS and 21 (11 women) subjects were injected with saline in a double blind randomized order, i.e. the same sample as the second study in **Paper I**. 47 participants without missing data were included in the analysis. SRH was tested at peak cytokine levels in the blood. A formal mediation analysis using path analysis was used to test a model which hypothesizes that LPS-stimulation increases inflammatory cytokines which in turn increases sickness behavior, that then causes a worsening of self-rated health.

### *Results and conclusions*

We found that experimental inflammation did indeed affect subjective overall health ratings. LPS injected subject rated their general health as significantly worse than the placebo group, despite the question specifically asking for the *overall* health. Our statistical model also implies that it is not the level of inflammation that predicts SRH, but the subjectively perceived sickness behavior. Interestingly, although the participants are well aware that the current illness they are perceiving is only transient (this fact is carefully explained to the participants before study participation), the individuals appear to operate in a “sickness mode” during systemic inflammation that affects the way they feel about their overall health

status. This fact highlights the motivational aspect of sickness behavior. Of note is that the subjects were also asked about their momentary health status in this study, so the difference between general SRH and current health should have been adequately clear for the subjects. Furthermore, the decrease in SRH during immune activation corresponds to the SRH-ratings in a clinical primary health care population investigated by Dr. A. Andréasson and Prof. M. Lekander previously (176) (Fig. 7). This finding supports the generalization of the findings in this study.



**Figure 7.** Box plot diagram of ratings of general self-rated health (SRH) in our experimental study sample compared to the ratings in a clinical population from an earlier study by our group. The changes during experimental manipulation are similar to those experienced by primary health care patients.

## 9 OVERALL DISCUSSION

This thesis has explored the acute systemic inflammatory effects on pain and subjective health in humans, and the effects of long-term inflammation on the brain in mice. Our findings indicate that deep pain is more affected during sickness than cutaneous pain, and that the pain system of women is more easily modulated by inflammation than that of men. One mechanism for this appears to be a greater attenuation of descending pain regulation in women during experimental sickness. We have also shown that general self-rated health can be manipulated in an experimental sickness setting and that perceived sickness behavior is determinant for the subjective perception of health. Furthermore, we have shown that long-term peripheral inflammation leads to changes in the brain that may exasperate neuroinflammation. Taken together, I argue that our findings and those of others in this field of research point to a possible general underlying mechanism for chronic diseases like chronic pain, fatigue, depression and neurodegeneration; namely, a sickness response pushed too far.

The sickness models used in this thesis (LPS stimulations in humans and long-term allergic provocation in mice) represent a snapshot of an immunological cascade. However, the inference made in this thesis is of chronic disorders, which require months or even years to develop. This may seem unreasonable, but both models do in fact seem useful for understanding both short-term and long-term mechanisms of inflammatory effects on the brain and behavior. With regard to the human LPS-model, we have been able to make a translational connection to previous clinical research on fibromyalgia patients (208). Our findings in **Paper I and II** converge with clinical data in a manner that provides tentative support for the validity of experimental inflammation to understand mechanisms involved in chronic pain. The usefulness of the acute LPS-model for studying chronic diseases has been stated before (112, 211). It is however possible that our pain findings are more relevant for some chronic pain disorders than others. Fibromyalgia may be a particularly suitable clinical pain model for bridging experimental sickness behavior with chronic pain mechanisms, and perhaps we would not have been as successful in repeating a paradigm of e.g. lower back pain or neuropathic pain using LPS-provocation. We suggest mechanisms to why pain problems are more prevalent in women and propose that this experimental model may be helpful in understanding some of the mechanisms leading to chronic pain. With regard to the murine model, the findings in **Paper III** of antibody increases centrally fit well into the framework of understanding neurodegenerative diseases from the perspective of neuroinflammation and an excessive sickness response (26). Although the inflammatory changes may be of a protective nature initially, they can potentially increase the risk of neuroinflammation in the long run. Interestingly, while allergic provocation induces inflammatory responses in wild type mice, Sarlus et al. recently have found that allergic inflammation appears to have a rather anti-inflammatory effect in the brain of transgenic AD

mice (207). This fact highlights the importance of the underlying phenotype on the end effect of the peripheral inflammation. Finally, the findings in **Paper IV** points to two interesting facts. Firstly, humans translate very subtle sickness cues to the subjective statement “I do not feel so great”, which in turns affect the general health appraisal we make. This may seem trivial, but diffuse complaints of ill-health accompanying disease are often disregarded in clinical practice, although SRH is in fact, as stated above, one of the best known predictors of future morbidity and mortality (169). The main purpose in the clinic is naturally to define the disease and treat it specifically. General well-being may however be a very important cue to inflammatory activity that cannot be identified with objective measures and should not be overlooked. This is of particular importance for syndromes with seemingly no medical grounds. Secondly, it may in some instances be beneficial to study sickness behavior as a unified system, instead of separate parts of it or levels of particular cytokines. The SicknessQ scale will hopefully be of value for sickness research in this respect.

Our findings of sex differences in LPS-induced pain sensitivity exemplify the absolute necessity of using both male and female subjects in human and animal research. Using female mice may in fact be advantageous, as male mice can become territorial and aggressive when housed together (207), which causes high stress levels in the subordinate males. This type of stress is often overlooked although it surely has profound effects on physiological systems, not the least those pertaining to sickness behavior. The female estrous cycle is often used as an explanation for using only male subjects, as the hormonal changes of the menstrual cycle are perceived as a scientific vexation. This is of course an untenable approach, particularly for human research where the phases of the menstrual cycle can be estimated fairly easily. The menstrual cycle is not a research problem but a part of human (mammalian) physiology. Current scientific knowledge concludes that sex hormones matter for the female predominance in pain disorders, but not *how* (209, 212). For example, oestradiol and progesterone have both pro- and anti-nociceptive effects (212), and immune reactions show complicated patterns of sexual dimorphism (40, 41, 213-215). Our findings indicate that women have basic neurological prerequisites in terms of weaker descending pain control that may render them more sensitive to inflammatory provocation. A potentially more responsive immune system of women may add to the effect (41). Future research will have to elucidate the exact underlying mechanisms of sex differences in pain prevalence (209). Hopefully, our findings will add to the understanding of both sex differences in chronic pain and interindividual predispositions to inflammation driven pain, regardless of sex.

To sum up, the endpoint of medical research must surely be to improve health, which requires a good understanding of disease etiology and appropriate experimental models. The difficulties that can arise in this regard can be exemplified by some attempts to tackle AD. Traditionally, AD etiology is described as an excess of amyloid plaques and neurofibrillary tangles. Consequently, immunotherapy using antibodies that bind to amyloid plaques to increase their removal has been attempted. However, the human experiments were terminated due to side effect in the patients (216). In fact, in neurodegenerative disease microglia express

increased levels of Fc-type Ig receptors, which drive the pro-inflammatory microglial state (26). The Ig levels in the healthy brain are very low, but the antibody therapy appeared to have the long-term effect of causing more damage than benefits by exacerbating neuroinflammation through increased microglia activation (216), paralleling our reasoning regarding the elevated IgG and IgE levels in **Paper III**. Taken together, one can conclude that neuroscientific research cannot afford to overlook immunological aspects.

## 10 CONCLUDING REMARKS

Exploring the sickness response as a system and understanding its mechanisms, sub-components and neuronal network, may lead to a better understanding of currently untreatable syndromes like deliberating pain and fatigue, and common co-morbidity between such syndromes. As suggested by others (217), a brain activation pattern of one complaint may facilitate for a second disorder to take hold, if the two use overlapping brain networks/areas. One could think of the immune system as “nudging” this theoretical sickness system. If the nudging becomes a constant push, such as during peripheral low-grade ongoing inflammation or neuroinflammation, the system becomes imbalanced and loses its homeostatic ability. The effect will depend on the type, strength, length and reoccurrence of the immunological nudges, as well as the phenotype on which the immunological push imposes its actions.

## 11 FUTURE DIRECTIONS

Proceeding from the notion that a dysregulated sickness behavior may underlie several complex syndromes, therapeutic approaches should focus on restoring the balance in the system, while allowing for individual differences on many levels. Future sickness research would thus benefit from elucidating individual differences in immunological reactivity, in neuronal sensitivity, and in the interaction patterns between the two. A newly developed sickness questionnaire, SicknessQ (Andreasson et al, submitted) may prove helpful when studying sickness behavior as an entity, hopefully aiding the understanding of individual differences in its expression. It is also of importance to explore the disparate effects of different levels of inflammation (doses of LPS e.g.) in both human and animal studies, as well as effects of timing, duration and reoccurrence.

Overall, better behavioral measures are required for fMRI studies of sickness, such as e.g. LPS-sensitive tests of interoceptive abilities and focus. The many aspects of pain, other than the purely sensory/noxious quality, could also be exploited further in related fMRI research. Resting state paradigms, i.e. the study of brain activity during rest and thus not task-dependent, may constitute promising approaches for studying a potential neuronal network

activated during sickness. Furthermore, positron emission tomography may help elucidate central inflammatory processes in humans by e.g. imaging microglial activation in the brain (28).

Personally, I think chronic pain is one of the most important medical predicaments at present. The individual sensitivity that determines if a patient will recover pain-free after trauma or not will most likely include several elusive components, like personality factors, lifestyle factors and neuroimmune predisposition, and certainly ample interactions of the same. It is my hope that a better understanding of a sickness network and underlying phenotypes can aid the understanding of how to help patients suffering from inexplicable and incurable pain.

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